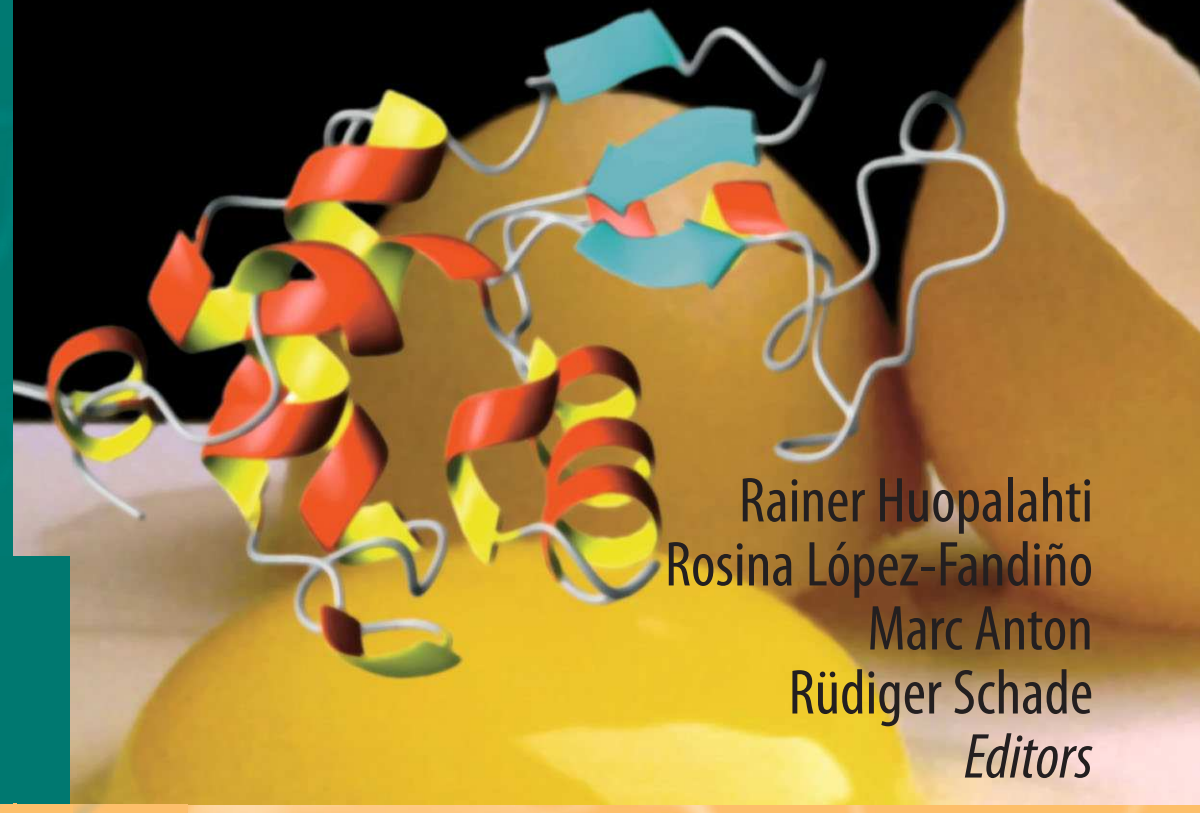

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Preface

The hen egg is one of the most versatile foods. It contains high-quality proteins and lipids, as well as valuable minerals, carbohydrates, and vitamins. Eggs are also widely used in the food industry due to their multifunctional properties (e.g., foaming, gelling, and emulsifying). Of course, the primary aim of the laying hen is not to produce high-value human food but to give rise to new life. Therefore, avian eggs contain the basic elements for life, and many of the egg compounds have so-called biological activity. For example, almost all the albumen proteins are antimicrobial, thus protecting the developing embryo. Consequently, hen eggs are very good potential sources of raw materials for health-promoting, so-called functional foods, as well as for the traditional food and pharmaceutical industries.

In 2002 a COST action called "Multidisciplinary Hen Egg Research" was founded that involves experts in different branches of egg research from 13 countries. The most important result of the COST action is the exchange of expert knowledge between the participating countries and research groups.

Since the COST action has joined together leading European scientists in egg research, the idea was born (in 2004) to write a book about latest results/concepts in bioactive egg compounds and their possible use in the food, pharmaceutical, and cosmetic industries.

The book comprises 33 chapter written by authors from 13 European and non-European countries and is divided in three parts.

Part I contains 17 chapters concerning the composition and the extraction of yolk, albumen, and shell compounds, respectively. We have particularly paid attention to updating the data, notably by the contribution of our own research results. We have opted to present, in the majority of cases, the structural aspect of the egg compounds, and of their interactions in relation to their function. Lastly, very recent data coming from our research regarding minor compounds are given exposure in the chapters dealing with proteases, antiproteases, lipocalins, clusterin, etc.

In summary, this part is not an "umpteenth" presentation of the composition of the egg, but an updated document, presenting original results seen from unique angles.

Part II, with 5 chapters, is concerned with the role of eggs in human nutrition. The nutritive properties of eggs are evaluated in detail, with discussions of the importance of the egg-contained macro and micronutrients, the

presence of functional substances, and the bioavailability of nutrients. Further chapters deal with improvements in the nutritive value of eggs, namely, by the enrichment in omega-3 fatty acids, vitamins, and selenium, and the implications embodied therein for daily nutrient intake and human health. The role of eggs as one of the main food allergens is also examined, including the factors that determine the allergenic properties of egg proteins and the possibilities for making hypoallergenic egg products.

Part III comprises 11 chapters divided in two subparts, and as in part 1 the contents of the chapters are mainly based on the experiences of the authors themselves. In particular, the use of specific bioactive egg compounds for human beings is introduced. For example, possible application of egg white compounds with antibacterial (and perhaps also antiviral) activity, or of egg-white-derived peptides with antihypertensive activity, with the prospect that a functional food fortified with these peptides may be produced. Further subjects are the application (at present and in the future) of specific chicken egg yolk antibodies in human and veterinary medicine, the potency of ion-binding proteins as nutraceutical (ovoceutical), and new and interesting fields of lecithin application.

The chapters of a second subpart are mainly focused on biotechnological aspects of egg use: for example, new methods for egg protein fractionation, the use of phospholipid-based liposomes/emulsions in pharmaceutics and cosmetics, the use of specific yolk components for cryoprotection of spermatozoa, and the usefulness of egg-protein-based films and coatings as biodegradable packaging material. Finally, results are presented on nanotechnology in egg research and news is given on avidin–biotin biotechnology.

The editors would like to express our thanks to all the authors who contributed their expertise and knowhow to the success of this book. Furthermore, we thank Springer for the patient and trustful cooperation during the processing and realization of the project. Finally, we hope to present an interesting and stimulating book that makes a contribution to understanding and disseminating the state of art in research on bioactive egg compounds.

Turku, Madrid,
Nantes, Berlin
September 2006

Rainer Huopalahti, Rosina López-Fandiño,
Marc Anton, Rüdiger Schade

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Chapter 1 Composition and Structure of Hen Egg Yolk

Marc Anton

1 Introduction

Unlike mammals, the embryos of birds are not fed by the mother during their development and have no possibility of elimination of metabolic waste. Consequently, the egg yolk provides vital nutrients (proteins, lipids, vitamins, and minerals) that are extremely well metabolized by the chicken embryo. Egg yolk is also a very attractive source of nutrients for humans: Its coefficient of digestive use is comparable to that of milk, and the biologic value of proteins in the egg is even superior to that of milk proteins (Bourgeois-Adragna 1994). Besides, hen egg yolk is a multifunctional ingredient widely used in many food products such as mayonnaise, salad dressings, cakes, pasta, creams, etc. Indeed, it possesses emulsifying, gelling, coloring, aromatic, and antioxidant properties. Each constituent of yolk possesses peculiar physical and chemical characteristics responsible for its own functional properties. Environmental conditions (pH, ionic strength, competition) and preservative treatment (heating, freezing, drying) can influence and modulate these functional properties. Finally, due to its original role as an embryonic chamber, yolk contains many constituents essential for life. Thus yolk represents a major source of active principles usable in medical, pharmaceutical, cosmetic, nutraceutical, and biotechnological industries.

2 Composition

Yolk makes up about 36% of the weight of the fresh whole hen egg. The dry matter of the freshly laid yolk varies from 50 to 52% according to the age of the laying hen and the duration of preservation. A transfer of water from the white to the yolk takes place during the storage of eggs (Kiosseoglou 1989; Thapon and Bourgeois 1994; Li-Chan et al. 1995). The compositions of fresh and dry yolks are presented in Table 1: the main components are lipids (about 65% of the dry matter) and the lipid to protein ratio is about 2:1.

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Table 1. Composition of hen egg yolk, from Powrie and Nakai (1986)

	Fresh yolk (%)	Dry yolk (%)
Water	51.1	–
Lipids	3.6	62.5
Proteins	16.0	33.0
Carbohydrates	0.6	1.2
Minerals	1.7	3.5

Lipids of yolk are exclusively associated with lipoprotein assemblies. They are made up of 62% triglycerides, 33% phospholipids, and less than 5% cholesterol. Carotenoids represent less than 1% of yolk lipids, and give it its color. Proteins are present as free proteins or apoproteins (included in lipoprotein assemblies). The interactions between lipids and proteins result in the formation of lipoproteins (low and high density), which represent the main constituents of yolk. So, on the basis of its dry matter, yolk is constituted of five major constituents: 68% low-density lipoproteins (LDL), 16 % high-density lipoproteins (HDL), 10% globular proteins (livetins), 4% phosphoprotein (phosvitin), and 2% minor proteins (Table 2; Powrie and Nakai 1986).

In the fatty acid composition of lipids, based on a standardized feed of hens, about 30–35% is of saturated fatty acids (SFA), 40–45% of monounsaturated fatty acids (MUFA), and 20–25% of polyunsaturated fatty acids (PUFA). The main fatty acids are oleic acid (C18:1, 40–45%), palmitic acid (C16:0, 20–25%), and linoleic acid (C18:2, 15–20%; Kuksis 1992). However, this composition is subject to strong variations, in particular according to the nature of fatty acids ingested by the hen (Posati et al. 1975; Anton and Gandemer 1997).

Table 2. Partition of hen egg yolk constituents, from Powrie and Nakai (1986)

	Yolk dry matter (%)	Yolk lipids (%)	Yolk proteins (%)	Lipids (%)	Proteins (%)
Yolk	100	100	100	64	32
Plasma	78	93	53	73	25
LDL	66	61	22	88	10
Livetins	10	–	30	–	96
Others	2	–	1	–	90
Granules	22	7	47	31	64
HDL	16	6	35	25	75
Phosvitin	4	–	11	–	95
LDLg	2	1	1	88	10

Dietary fatty acids particularly modify the proportions of PUFA and MUFA, whereas SFA proportions are slightly affected.

The major triglyceride fatty acids comprise most of the fatty acid total. The glycerol of the triglycerides is mainly esterified by palmitic acid in position 1, by oleic and linoleic acids in position 2, and by oleic, palmitic, and stearic acids in position 3 (Kuksis 1992).

Phospholipids are amphiphilic molecules that contain one hydrophilic head group: phosphoric acid + alcohol, amino acids or polyol, and one hydrophobic group: two fatty acids. Phospholipids of yolk are very rich in phosphatidylcholine (PC): 76% of total phospholipids (three-fold higher than natural soy phospholipids). Phosphatidylethanolamine represents 22% of the phospholipids. Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cardiolipins (CL), lysoPC, and lysoPE are present in very low amounts. PUFA represent 30–40% of the fatty acids phospholipids, whereas SFA account for 45%, and MUFA for 20–25% (Kuksis 1992). Phosphatidylcholine contains important quantities of ω3 fatty acids as the nonpolar part. Choline forms the polar part and is an important nutrient in brain development, liver function, and cancer prevention (Gutierrez et al. 1997), routinely added to commercial infant formulas as an essential nutrient. Consumption of phosphatidylcholine increases plasma and brain choline levels and accelerates neuronal acetylcholine synthesis. It has been demonstrated that consumption of yolk phospholipids tends to alleviate the symptoms of Alzheimer disease (Juneja 1997).

Cholesterol is the sterol found in egg yolk. It results partly from the hen feed and partly from synthesis in the liver during the elaboration of lipoproteins. It represents approximately 5% of total lipids in free (85–90%) or in esterified (10–15%) form (Bitman and Wood 1980). The free cholesterol participates in the structure of LDL. Cholesterol esters are present in the lipid core of LDL and contain 35% oleic acids, 33% palmitic acids, 12% linoleic acids and 11% stearic acid (Kuksis 1992).

Carotenoids are the natural pigments of hen egg yolk.. They confer it its yellow color, which can go from a very pale yellow to a dark brilliant orange. They are far from plentiful in eggs, but economically important because the color represents a quality criterion. They are mainly carotene and xanthophylls (lutein, cryptoxanthin, and zeaxanthin). The total concentration of lutein and zeaxanthin is ten times greater than that of cryptoxanthin and carotene, combined (Shenstone 1968). Carotene is the main carotenoid found in common hen feeds corn and alfalfa. After ingestion this compound is largely oxidized to form the xanthophylls.

Consequently yolk is a plentiful source of lipids, some of which are particularly suitable for nutrition and health. Particularly, omega 3 fatty acids (ω3), mainly found in phospholipids, are considered to be an essential nutrient for brain function and visual acuity in humans (Maki et al. 2003). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main ω3 fatty acids. They are particularly useful for pregnant and nursing mothers for optimizing

growth of their infants, especially as breast milk is currently low in ω3 due to the modern diet of most mothers.

3 Macrostructure

Yolk is a complex system with several particles in suspension in a clear yellow fluid (plasma) that contains proteins. The main types of particles are spheres, profiles, and granules. Spheres are minor components (1% of yolk dry matter) and have a diameter between 4 and 150 µm (Romanoff and Romanoff 1949). They appear as tightly packed drops of lipids and lipoproteins (Chang et al. 1977). Profiles are round particles of 12–48 nm diameter and are considered as low-density lipoproteins (Martin et al. 1964). Granules consist in circular complexes ranging in diameter from 0.3 µm to 2 µm (Chang et al. 1977). Consequently, yolk can be easily separated into two fractions after a dilution (two times) with 0.3 M NaCl and a centrifugation at 10,000 g (30 min) according to the method of McBee and Cotterill (1979): a dark orange supernatant called plasma and a pale pellet called granules are separated (Fig. 1). Yolk dilution helps to decrease its viscosity, thus permitting motion and separation of particles.

Granules represent 19–23% of yolk dry matter, accounting for about 50% of yolk proteins and 7% of yolk lipids. The dry matter content of granules is about 44%, and they contain about 64% proteins, 31% lipids and 5% ash (Dyer-Hurdon and Nnanna 1993 ; Anton and Gandemer 1997) Lipids of granules are 60% triglycerides, 35% phospholipids, and 5% cholesterol. They are mainly constituted by HDL (70%) and phosvitin (16%) linked by phosphocalcic bridges between the phosphate groups of their phosphoseryl residues

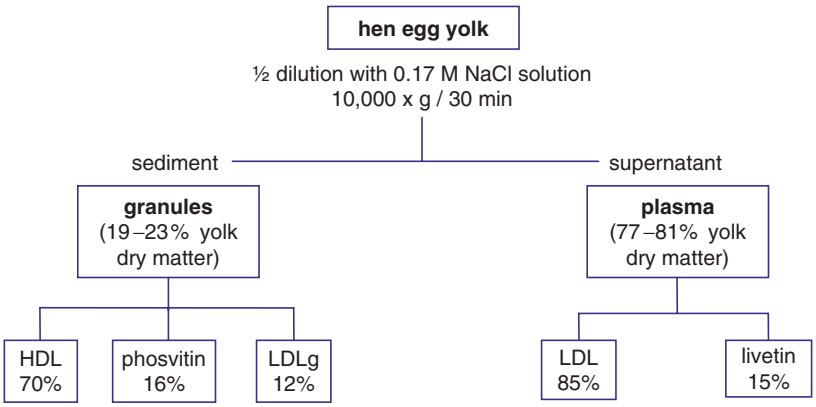

Fig. 1. Fractionation of yolk into granules and plasma

(Burley and Cook 1961 ; Saari et al. 1964). LDL (12%) are included in the granular structure (Table 2). At low ionic strength, granules mainly form insoluble HDL–phosvitin complexes linked by phosphocalcic bridges as HDL and phosvitin contain a high proportion of phosphoserin amino acids able to bind calcium (Causeret et al. 1991). The numerous phosphocalcic bridges make the granule structure very compact, poorly hydrated, weakly accessible to enzymes, and lead to an efficient protection against thermal denaturation and heat gelation.

At an ionic strength over 0.3 M NaCl, the phosphocalcic bridges, are disrupted because monovalent sodium replaces divalent calcium. In such conditions, the solubility of granules reaches 80% because phosvitin is a soluble protein and HDLs behave like soluble proteins (Cook and Martin 1969 ; Anton and Gandemer 1997). Complete disruption of granules occurs when ionic strength reaches 1.71 M NaCl. Acidification or alkalization likewise cause the disruption of granules and the solubilization of these constituents. At acidic pH the increase in number of the positive charges (NH_3^+) induces electrostatic repulsion and results in disruption of the granules constituents. At basic pH, carboxylate groups are deacidified leading to the repulsion of negative charges (COO^-), again causing disruption.

Plasma comprises 77–81% of yolk dry matter and is composed of 85% LDL and 15% livetins (Burley and Cook 1961; Table 2). It forms the aqueous phase where yolk particles are in suspension. It accounts for about 90% of yolk lipids (including nearly all the carotenoids), and 50% of yolk proteins. Plasma contains about 73% lipids, 25% proteins, and 2% ash. Lipids of plasma are distributed thus: 70% triglycerides, 25% phospholipids, and 5% cholesterol.

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Chapter 2 Low-density Lipoproteins (LDL) or Lipovitellenin Fraction

Marc Anton

1 Introduction

Low density lipoproteins are the main constituents of yolk: about 2/3 of the total yolk dry matter. They are mainly located in plasma but a small portion is included in granules (LDLg). LDLs of yolk are similar to very low-density lipoproteins of chicken blood (Nakamura et al. 1977) and are synthesized in the liver of the laying hen. They are transported with the blood to the ovary where they are transferred to the yolk without much change in structure and composition (Holdsworth and Finean 1972). LDLs constitute about two-thirds of the lipids present in hen egg yolk. The nomenclature of the LDL is complicated, some authors calling them lipovitellenin, while others use this latter term to designate only the protein part of LDL after extraction with ether. We prefer calling them low-density lipoproteins of hen egg yolk.

2 LDL Structure and Composition

LDLs are spherical particles (17–60 nm in diameter with a mean of about 35 nm) with a lipid core in a liquid state (triglycerides and cholesterol esters) surrounded by a monofilm of phospholipid and protein (in this circumstance called apoprotein; Cook and Martin 1969 ; Evans et al. 1973; Fig. 1). LDLs are soluble in aqueous solution (whatever the pH and ionic conditions) due to their low density (0.982). Phospholipids take an essential part in the stability of the LDL structure because association forces are essentially hydrophobic (Burley 1975). Some cholesterol is included in the phospholipid film, increasing its rigidity.

LDLs are composed of 11–17% protein and 83–89% lipid, out of which latter 74% is neutral lipid and 26% phospholipid (Martin et al. 1964). Their population is composed of two sub-groups: LDL_1 (10×10^6 Da) and LDL_2 (3×10^6 Da). LDL_1 represent 20% of total LDL and contain twice the amount of proteins as LDL_2 (Martin et al. 1964). Chemical compositions of both LDL fractions are

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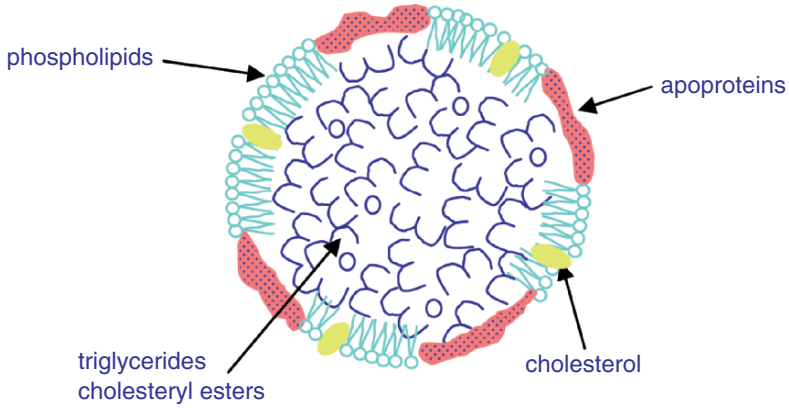

Fig. 1. Schematic representation of yolk LDL

similar but the proportions between apoproteins and lipids change, and size increases with lipid content. From a technofunctional point of view, it has been largely proved that LDLs are the main contributors to the exceptional emulsifying properties of egg yolk. This capacity is clearly due to the LDL structure, through interactions between amphiphilic apoproteins and phospholipids. This assembly of phospholipids and apoproteins allows transport of these insoluble amphiphilic species through the aqueous phase until the interface is reached. The lipoprotein disruption at the oil–water interface, already presumed in several studies concerning LDL emulsifying properties, is now confirmed. The interfacial films of LDL are constituted by a blend of apoproteins and phospholipids that assure both a decrease in interfacial tension and resistance to rupture. Formation and stability of food emulsions made with yolk is thus permitted.

An electron micrograph (TEM and Cryo-TEM) of LDL negatively stained with 2% sodium phosphotungstate is shown in Fig. 2. Particles of LDL appear spherical, with heterogeneous sizes between 20 and 60 nm diameter (average 35–40 nm). Contiguous particles have flattened edges; these polygonal shapes could be the result of particle aggregation due to dehydration on grids or to a vacuum in the electron microscope during analysis. The same sample of LDL was analyzed by photon-correlation spectroscopy (results not shown) and gave an average particle diameter of 35 nm, in agreement with the average value obtained from the electron micrograph. However, we observed some structures of about 200 nm diameter surrounded by aggregated LDL. It is possible that these structures are very low density lipoproteins as observed by Martin et al. (1964) or merged LDL as noted for human plasma LDL (Ala-Korpela et al. 1998).

Recently we have observed LDL by cryomicroscopy and confirmed the findings on the structure of LDL with a less denaturing microscopic technique.

LDL structure is sensitive to technological treatments (Anton et al. 2003). When LDLs are heated for 10 min at 75 °C, a disruption and a subsequent

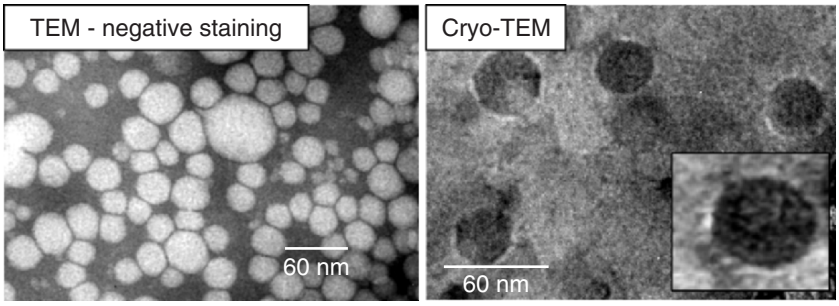

Fig. 2. Transmission electronic microscopy (TEM) and Cryo-TEM visualization of LDL

rearrangement of the fragments into big clusters occurs (average size about 300 nm). The treatment by high hydrostatic pressure (5,000 bars) also brought about a disruption followed by a rearrangement of the fragments into clusters, of about 90 nm diameter. Finally, the structure of LDL is not altered by passage through a high pressure homogenizer at up to 250 bars or through a rotor/stator homogenizer (Anton et al. 2003). This means that during the fabrication of mayonnaise, for example, the homogenization step did not disrupt its LDLs.

It has been suggested (Wakamatu et al. 1982) that LDLs form a gel during a freezing–thawing process due to the alteration of the interactions between phospholipids and apoproteins at their surface. Liberated apoproteins are thought to aggregate and form a gel (Kurisaki et al. 1981). However, temperature attained and medium conditions (LDL concentration, salts), as well as frozen and thawing kinetics, are important in this process. We have observed for LDL (1 mg protein/ml) frozen for 12 h at −80 °C and subsequently thawed at room temperature that this treatment did not visually change the structure of LDL. The time of storage at −80 °C was certainly too weak to induce destruction and gelation of LDL.

3 LDL Apoproteins

There are six major apoproteins of LDL. The main one accounts for more than 70% of the total; its molecular weight is estimated to be 130 kDa. The second apoprotein, with a molecular weight of 15 kDa, represents about 20% of the total. Four other minor apoproteins with molecular weights between 55 and 80 kDa have been identified (Fig. 3; Anton et al. 2003). The isoelectric point of all the apoproteins ranges from 6.3 to 7.5 (Kojima and Nakamura 1985). About 40% of the amino acids of apoproteins of LDL are hydrophobic (Tsutsui and Obara 1982). Consequently, these apoproteins are highly hydrophobic and flexible molecules. Recently, using circular dichroism,

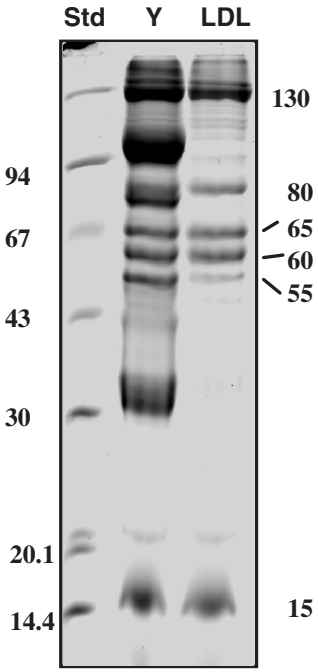

Fig. 3. SDS-polyacrylamide gel electrophoresis of yolk (*Y*) and *LDL* solution, gel with 10% acrylamide. Coomassie blue stain; low-molecular weight calibration kit was used as protein standard (*Std*)

Anton et al. (2003) have observed, for a blend of the main apoproteins a double minimum at 208 and 222 nm, and a maximum at 191–193 nm, consistent with a considerable proportion of α-helix chains. In this study sodium dodecyl sulphate (SDS) was used to solubilize apoproteins and it is suggested that SDS could lead to an induction of α-helix structures (Montserret et al. 2000). But, in the case of membrane proteins or amphiphilic proteins like LDL apoproteins, SDS is known to mimic the hydrophobic environment existing in biological membranes without changing protein conformation.

Apoproteins of LDL are glycosylated on asparaginyl residues: about 1.3% hexose, 0.7% hexosamine, and 0.4% sialic acid (Nakamura et al. 1977).

Apoproteins extracted from LDL with ether–ethanol are very difficult to dissolve in aqueous buffers. A basic pH (pH 12) associated with a low ionic strength helps to attain 30% solubility.

Very-low density lipoproteins (VLDL) of hen blood are the precursors of egg yolk LDL (Evans and Burley 1987). While VLDLs exist in the blood of immature pullets, their production in hen liver is considerably increased with sexual maturity due to estrogen secretion. VLDLs contain mainly two apoproteins, apo-VLDL II and apo-B. Apo-B is a 500 kDa protein constituted by only one subunit and highly similar to the human apolipoprotein B-100 precursor. During its transfer into the yolk, hen apo-B is enzymatically

cleaved, resulting in the production of apo-B fragments. The only apoprotein from blood lipoproteins to be transferred to yolk in large amount without any modification is apo-VLDL II, called apovitellenin I in the yolk. Apovitellenin I is a small homodimer with disulfide-linked subunits of 9 kDa. Apart from apovitellenin I, there is a lack of knowledge concerning the exact identification of the other apoproteins of LDL, and specially about the correspondence between LDLs and blood lipoproteins. Furthermore, gaps still exist in the knowledge of the exact maturation mechanism of apoprotein precursors. Recently, Jolivet et al. (2006) have confirmed the existence in egg yolk of apovitellenin I either as a monomer or a homodimer of disulfide-linked subunits, and this homodimer is resistant to SDS. The monomer has a molecular weight of 9.331 kDa in spite of a slight heterogeneity, and its amino acid sequence has been totally confirmed through the analysis of trypsin peptides. Hen apo-B is known to be cleaved into several protein fragments by cathepsin D. The structural homology of hen apo-B with human apoB-100 has been verified, and sequence alignment could be an interesting tool for further the characterization of hen apo-B.

4 LDL Extraction

Initially, techniques of ultracentrifugation have been favored because the low density of these assemblies (0.98) allowed an efficient separation. However, this type of technique is extremely time consuming (several days of centrifugation) and the yield of extraction is very low (only few grams of LDL), prohibiting a scale-up of the method. Recently a new method of separation of LDLs allowing an efficient extraction has been finalized that can be extrapolated to industrial dimensions (Moussa et al. 2002). The principle of separation is the addition of ammonium sulfate (40% saturation) to eliminate livetins from the solution of plasma extracted from egg yolk. After 1 hour under stirring at 4 °C, the blend is centrifuged at 10,000 g. The pellet obtained containing γ and β livetins is avoided. The supernatant is then dialyzed against distilled water for 20 hours at 4 °C with frequent changes of baths. The dialysis, desalting samples, provokes the aggregation of LDL, which precipitate. An orange-colored dough containing 97% LDL is obtained. The yield of extraction is about 60%. This process is protected by a patent (Anton et al. 2001). The purity of 97% obtained can be increased further by the use of gel filtration chromatography. Thirty grams of LDL extract are dispersed in 50 ml Tris-HCl 50 mM pH 7 buffer, and 12 ml of this dispersion are injected into the column of Ultrogel AcA 34 (Pall, East Hills, NY, USA). This chromatography permits separation of the LDL from contaminating livetins, hence to recovery of pure LDL. This last step is efficient for refining LDL purification in the laboratory, but scaling-up is not feasible because of the low quantities treatable by this technique.

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Chapter 3 High-density Lipoproteins (HDL) or Lipovitellin Fraction

Marc Anton

1 Introduction

HDLs are the second group of lipoproteins of yolk and account for about 1/6 of its dry matter and 36% of its proteins. They are specifically localized in granules and are designated as lipovitellin. HDLs are the result of the proteolytic cleavage of a precursor (vitellogenin), which is synthesized in the liver under the regulation of oestrogen. It can be noticed that phosvitin (see Chap. 4) comes from the same precursor. In native egg yolk, HDLs are complexed to phosvitins to form the granular structure through phosphocalcic bridges. HDLs are made up of 75–80% proteins and 20–25% lipids. So their density is close to that of proteins, 1.120 g/ml. Their lipids are composed of 65% phospholipids, 30% triglycerides, and 5% cholesterol (Cook and Martin 1969).

2 HDL Structure and Composition

HDLs have a molecular weight of about 400 kDa and a diameter from 7 to 20 nm (Burley and Cook 1961). Unlike LDLs, HDLs have no spherical micelle-like structure but rather a pseudo-molecular structure close to that of globular proteins. Consequently, we call them lipoproteins but they are not formally lipoproteins, bearing more of a resemblance to lipid transfer proteins. HDLs have in fact served as models for the study of proteins of lipid transfer and reserve, allowing more precise knowledge of the structure of these elements. HDLs present in the form of a dimer of 2 monomers of about 200 kDa each. Each monomer of HDL is a very structured globular protein containing a cavity in the form of a funnel composed essentially by two β-sheets predominantly constituted by hydrophobic amino acids (Anderson et al. 1998). The cavity has a volume of 68 nm^3 large enough to fix about 35 molecules of phospholipids concentrated on a monolayer interacting with the hydrophobic amino acids. Triglycerides can then be enclosed in this hydrophobic cavity through new hydrophobic zones created by phospholipids

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(Banaszak et al. 1990; Timmins et al. 1992). Thus phospholipids are entrapped and protected by the HDL structure, and phospholipase C can liberate only 6% of the lipid phosphorus of HDLs (Burley and Kushner 1963). Four other domains (three β-sheets and one helicoidal) form the rest of the polypeptide. The structure is maintained by five disulfides bridges but the different domains of HDL complex interact through numerous salt and ionic bridges; hydrophobic interactions also occur (Anderson et al. 1998).

Two sub-groups of HDL are separated by ion exchange chromatography: α- and β-HDL in a ratio of 1:1.5 (Radomski and Cook 1964). The α-HDLs contain 6 times more sialic acid and 2 times more phosphorus than β-HDL (0.5% versus 0.27%; Kurisaki et al. 1981). Consequently, α-HDL are more acidic than β-HDL while the latter are more water soluble. Except for these differences, the two types of HDL have a similar chemical composition. About 30% of amino acids of both types of HDL are hydrophobic.

At pH values below 7.0 a reversible dimerization of HDLs occurs. Dimers of β-HDL are dissociated more rapidly than those of α-HDL. The dissociation reaches 50% at pH 10.5 and 7.8 respectively for α- and β-HDL. The presence of 1 M NaCl hinders the dissociation in alkaline medium (Bernardi and Cook 1960), and temperature increase slows dissociation. Sulfhydryl and protein phosphorus groups are not directly involved in the dissociation reactions. All these data indicate that the dimerization proceeds by both electrostatic and hydrophobic interactions. The organization, hydration, and release of phospholipids in the cavity are unquestionably altered by the dissociation process. HDLs precipitate in water and become soluble when ionic strength is higher than 0.3 M NaCl.

Each monomer of HDL is composed of about 5 main apoproteins, with molecular weights ranging from 35 to 110 kDa (Fig. 1). Their respective quantities are 110 kDa: 21%, 100 kDa: 16%, 80 kDa: 20%, 50 kDa: 14%, and 35 kDa: 28%. Apoproteins of HDL are glycosylated (0.75% carbohydrate): mannose, galactose, glucosamine, and sialic acid.

HDLs are complexed to phosvitins to form the granular structure through phosphocalcic bridges. HDLs and phosvitins come from the same precursor, namely, vitellogenin (Wang et al. 1983). Vitellogenin is a dimeric protein of 480 kDa (Hermier 1990) synthesized under estrogenic control in liver, where it is phosphorylated and glycosylated. Vitellogenin interacts too with lipids and two calcium ions and one zinc ion (Montorzi et al. 1995). Vitellogenin is secreted into the blood circulation, from where it reaches the oocyte. During the transfer into oocyte by endocytosis, HDLs undergo a proteolytic cleavage generating several polypeptides that preserve its structure (Banaszak et al. 1990). Proteic fragments coming from this cleavage form the HDL–phosvitin complexes, basic element of granules. Enzymatic cleavage sites are identified in the sequence of vitellogenin II. From N-terminal extremity, the polypeptidic segment of 120 kDa (amino acids 16 to 1,111) corresponds to HDL1 whereas HDL2 is a proteic fragment of 32 kDa (amino acids 1,329 to 1,566). Amino

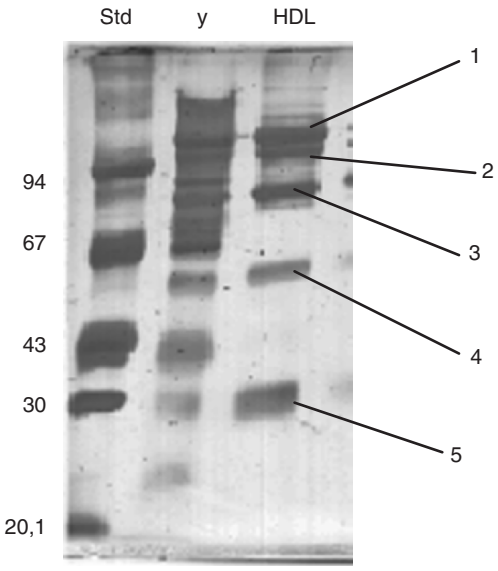

Fig. 1. SDS-polyacrylamide of a fraction enriched in *HDL* gel at 10% acrylamide. Coomassie blue stain; *Std*: standard molecular weights, *Y*: yolk

acids from 1,112 to 1,328 constitute phosvitin. The polypeptide YGP40 with a molecular mass of 40 kDa, corresponding to the C-terminal extremity (amino acids 1,567 to 1,650) is localized in the plasma (Yamamura et al. 1995).

3 HDL Extraction

Literature reports several methods of extraction based on salt precipitation. Joubert and Cook (1958) suggested that egg yolk be diluted in a solution of $MgSO_4$ 0.4 M in order to disrupt HDL–phosvitin complexes. The solution is then diluted 2 times and centrifuged at 10,000 g for 30 minutes. The pellet (principally phosvitin, because this protein forms aggregates in the presence of low $MgSO_4$ concentrations) is discarded, the supernatant diluted 2 times with distilled water, and centrifuged again as before. The supernatant is collected and diluted again with distilled water to attain a final concentration of $MgSO_4$ of 0.05 M. The pellet is dissolved in 50 ml of a 10% NaCl solution and then ultracentrifuged at 105,000g for 2 hours. The solution is then centrifuged again to obtain a pellet rich in HDL.

After this extraction, the HDL extract is purified on a hydroxylapatite column. The separation is based on the affinity of proteins for calcium ions.

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Chapter 4 Phosvitin

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1 Introduction

Hen egg yolk phosvitin is a phosphoglycoprotein that represents 4% of yolk dry matter. Of the known proteins, it is the most phosphorylated one; almost 50% of its amino acids are serine, out of which 90% are phosphorylated (Clark 1985). The high phosphorus content of phosvitin is responsible for its strong affinity for metal ions, particularly for iron; 95% of yolk iron is bound to phosvitin. Consequently, due to its peculiar physical and chemical characteristics, phosvitin possesses a variety of functional and biological properties. Particularly, its phospholipid antioxidant activity was demonstrated in both, egg yolk emulsion and meat model systems.

2 Phosvitin Composition and Structure

Hen egg yolk phosvitin represents about 11% of yolk proteins. It is a mixture of two polypeptides: α-phosvitin (160 kDa) and β-phosvitin (190 kDa; Ito et al. 1962). Both phosvitins are an aggregate of many different polypeptides: α-phosvitin contain three or four sub-units of 35 to 40 kDa, whereas β-phosvitin contain four or five sub-units of 45 kDa. Amino acids of these sub-units consist of about 50% serine. There are little or no sulphur-containing amino acids. Alpha-phosvitin is about 6% carbohydrate: 2.5% hexose, 1% hexosamine, and 2% sialic acid; whereas β-phosvitin is only 2% carbohydrate (mainly hexose; Itoh and Fuji 1983).

The two phosvitins contain 3% (α) and 10% (β) phosphorus, which accounts for about 80% of total protein phosphorus of yolk (Joubert and Cook 1958). Phosphorus is present as phosphoric acid bound to seryl residues; about 96% of seryl residues are phosphorylated. The phosphoserines are arranged in a singular way, forming blocks that can carry up to 15

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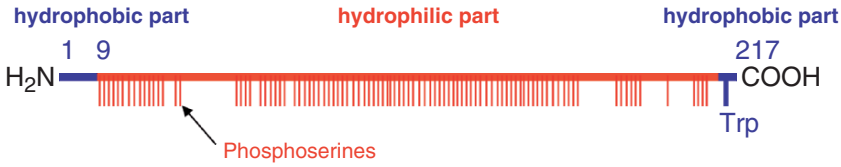

Fig. 1. Schematic representation of phosvitin

consecutive residues (Byrne et al. 1984; Fig. 1). This confers to the protein a very large and central hydrophilic area surrounded by two small hydrophobic areas at the N- (9 residues) and C- (3 residues) terminal parts.

Phosvitin is an elongated molecule 28 × 1.4 nm. At neutral pH, phosvitin predominantly exists in a random coil conformation, whereas a β-type conformation predominates at acidic pH (67% β-sheet; Taborski 1974; Damodaran and Xu 1996). Phosvitin is soluble in pure water and easily precipitated with 0.1 M magnesium sulphate. The isoelectric point of phosvitin is 4.0 (Ternes 1989), and at neutral pH it is highly charged: –179 mv. Phosvitin is synthesized from a precursor, vitellogenin, from which yolk HDL also comes (Taborsky 1983). After cleavage of the precursor, the two polypeptides are liberated, but they interact through phosphocalcic bridges to form the granular structure. Phosvitin is poorly modified by thermal treatment owing to its unordered structure; heating at 110 °C for 20 minutes does not cause its aggregation or insolubility (Albright et al. 1984).

3 Metal Chelating Properties

Phosvitin possess a very strong metal chelating property due to its polyanionic character. This unique primary structure makes this protein one of the strongest metal chelating agents (Hegenauer et al. 1979). Whole egg contains about 2 mg of iron per 100 g, and 95% of yolk iron is bound to phosvitin (Albright et al. 1984), which contains about 6mg of iron per gram. One phosvitin molecule can anchor 113 ions of Mn^{+2} or 120 of Co^{+2}, and the binding is not affected by temperature up to 60 °C (Grizzuti and Perlmann 1975). These authors have also described the interaction with Mg^{+2} and Ca^{+2}: at pH 6.5 phosvitin binds up to 103 and 127 ions/molecule, respectively. However, at pH 4.5 it can only bind 40 and 32 ions of Mg^{+2} and Ca^{+2}, respectively.

Iron is an important metal in food and health sciences (Gaucheron 2000). By equilibrium dialysis, Hegenauer et al. (1979) described a great affinity between ferric ions and phosvitin (Ka = 10^{18}). Furthermore, this protein can chelate ferric ions better than molecules like citrate or nitrilotriacetate. Phosvitin in egg yolk is not saturated with iron and additional iron can be

fixed. At saturation, two moles of organic phosphorus of phosvitin can bind one mole of iron (the maximum Fe/P ratio is then 0.5; Taborski 1974). Iron is bound in ferric form in a tetrahedral coordination structure with the oxygen of the phosphoseryl residues. Iron/phosvitin interaction depends on the method of ferric ion addition. If phosvitin is directly mixed with a ferric solution, the iron/phosvitin interaction is mainly intermolecular, and consequently the complexes are insoluble (McCollum et al. 1986; Taborsky 1991). However, when mixing phosvitin and ferrous iron solutions, iron will be oxidized into ferric iron by autoxidation (Grogan and Taborsky 1986), and in this case they form soluble complexes based on intramolecular interactions. The high iron binding capacity brings about the bactericidal properties of phosvitin (Sattar Khan et al. 2000) or its antioxidant activity (Lu and Baker 1986, 1987; Nakamura et al. 1998; Lee et al. 2002). It was shown that both pH and temperature affect the antioxidant activity. In general, decreasing pH values will decrease the antioxidant activity of phosvitin, and this potential is also affected by drastic thermal treatment like autoclaving, but not by mild conditions like those employed in pasteurization or similar treatment. Albright et al. (1984) have likewise studied the influence of heat on the release of iron from phosvitin, and they have determined that heating phosvitin solution at 110 °C for 40 min did not release iron.

Phosvitin structure is affected by pH because of modifications in electrostatic repulsion (Taborsky 1968; Yasui et al. 1990). Iron binding does not have the same effect on the structure, even if the interaction between iron and protein reduces electrostatic repulsion (Taborsky 1980).

The study of the influence of pH and ionic strength on the iron binding capacity of purified phosvitin indicates that the best conditions for iron fixation are almost neutral pH (6.5) and low ionic strength (0.15 M) (Castellani et al. 2004; Fig. 2). This is probably because at this pH phosphates are completely ionized, and there are not enough salt ions to prevent the iron binding by phosvitin. The maximum iron binding capacity was found to be 115 mg of iron/mg of phosvitin, which corresponds to one iron per two phosphate groups. In more acidic conditions (pH values lower than 3.5), there was no significant fixation (Castellani et al. 2004). It is likely that precipitation occurs at pH values lower than 2.9, where the secondary structure of phosvitin changes. The weak fixation obtained at acidic pH is not related to an inhibition of iron autoxidation because, at this pH value and for a time of 1 h, this process is largely allowed. Thus, the presence of partially protonated phosphates could inhibit the formation of an iron/phosvitin complex. A lower capacity of interaction with Ca+2 and Mg+2 ions when lowering the pH value has also been observed for phosvitin (Grizzuti and Perlmann 1973). These authors have described that metal binding capacity decreases to 40% and 25% for magnesium and calcium, respectively, when pH value is modified from 6.5 to 4.5. Another phosphoprotein, the β-casein of milk, behaves similarly even though it has a notably lower degree of phosphorylation (only between 4 and 5 phosphoserines per molecule). Calcium binding capacity of

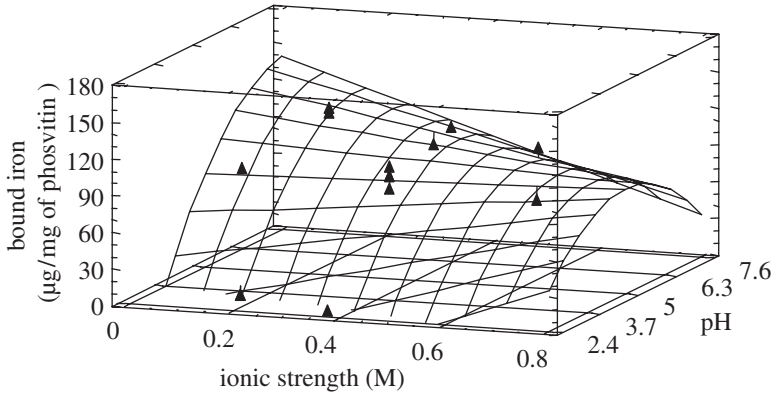

Fig. 2. Iron fixation by phosvitin as influenced by pH and ionic strength. The estimated response surface is represented in µg of bound iron per mg of phosvitin

this milk protein decreases by 70% when pH goes down from 7 to 5.5 (Baumy and Brulé 1988).

The relationship between the ionic strength and the iron binding capacity of phosvitin is linear between 0.1 to 0.6 M NaCl. The slopes at different pH values have opposite signs, and the critical pH value is 5.2. For higher pH values, increase in salt concentration leads to a decrease in iron binding capacity, and for lower pH values the opposite tendency is observed.

The pH value of 5.2 is suggestive because it is just below the pH of the second pKa of phosphoserines (5.8), and so the proportion of partially protonated phosphates begins to be important. Consequently, when phosvitin has a large proportion of completely dissociated phosphates, the presence of salt ions might have a screening effect between iron atoms and phosvitin when increasing NaCl concentration. Contrarily, when increasing the proportion of partially protonated phosphates, the addition of NaCl induces proton ionization by the reduction of it pKa value, as described by Baumy et al. (1989) for the β-casein system. This effect could improve the iron binding capacity of phosvitin. In spite of the fact that interactions at pH values under 5.2 are improved in the presence of NaCl, the iron binding capacity of phosvitin is lower than the optimal value, as it presents at pH 3.6, 40% of its maximal capacity.

Below about 90% of iron saturation the protein does not change its unordered structure (Castellani et al. 2004). But when phosvitin binds iron the complex has a more compact spatial shape, as seen by Taborsky (1980) and Castellani et al. (2004), by gel filtration chromatography and native electrophoresis, respectively. In the presence of iron, more compact molecules could elute at higher elution volumes, even if they have a higher molecular mass than free iron phosvitin. This conclusion does not disagree with structure results, because it is possible to have spatial changes that do not affect the main distribution of secondary protein structure.

Phosvitin initially treated by thermal or high pressure methods does not result in aggregation and its high iron binding capacity was found to be kept, even under the strongest treatment conditions (600 MPa or 90 °C; Castellani et al. 2004). These results correlate well with the fact that the protein has an unordered structure, so the influence of heat treatment that usually unfold proteins is not observed (Albright et al. 1984 ; Castellani et al. 2004). Grizzuti and Perlmann (1975) described that the interaction between phosvitin and metals (Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2}) are not modified below 60 °C. This heat resistance, particularly with respect to its iron binding capacity, is not only a property of the iron/phosvitin complex, as the protein by itself has a high thermal resistance. In the same way, no modification was detected from pressure treatment, again raising the possibility that its unordered structure and high negative charge prevents modification of the iron binding capacity of phosvitin.

Complexes formed under favorable conditions (pH 6.5 and ionic strength 0.15) are very stable. The decrease of pH to values where the formation of iron/phosvitin complex is normally prevented, does not release the iron bound by phosvitin (Castellani et al. 2004). Some modifications when the complex is formed are expected. Castellani et al. (2004) hypothesized that in an initial step the iron ions interact with only one phosphate group, and once their interaction is formed, another phosphate completes the process (tetrahedral stoichiometry), or in the case where the complex has an octahedral stoichiometry, two other molecules (i.e., water molecules) could complete the system. Once this condition is reached, the activation energy required to dissociate the complex could become high. Consequently, once the complex is formed, its dissociation by protons is not observed, even in the presence of a high proton activity and in conditions where the complex formation is prevented.

Only a chelating agent (o-phenanthroline or EDTA) with stronger iron binding capacity than phosvitin is able to release iron from the complex (Albright et al. 1984).

4 Phosvitin Extraction and Purification

The phosvitin purification methods described in the literature generally consist of two phases, first one of extraction and then one of chromatographic purification. Depending on the isolation method used, egg yolk phosvitin contains between three and six atoms of iron per molecule (Mecham and Olcott 1949; Albright et al. 1984). However, the study of iron binding activity of phosvitin requires that the protein at first be free of metal. This is usually achieved, after an isolation procedure, by the use of purification methods.

Wallace and Morgan (1986) have developed a method of extraction employing aqueous solutions. For further phosvitin purification these authors successfully used hydrophobic interaction chromatography. They described the presence of α and β-phosvitin polypeptides, and of three additional

phosphoproteins with high electrophoretic mobility: phosvettes. The phosvettes were not present in the more-often-used phosvitin preparations. In fact, the protein yield for their isolation method was only 0.96% of egg yolk dry matter, considerably less than the total phosvitin present in egg yolk.

A new purification method includes an extraction phase based on the insolubility of Mg^{+2}/phosvitin salt, and further ion-exchange chromatographic fractionation (Castellani et al. 2003). Yolk is fractionated into granules and plasma following the method of McBee and Cotterill (1979). Granules are diluted to a new concentraion of 10% with NaCl 1.74 M; this provokes the disruption of the phosphocalcic bridges binding phosvitin and HDL, and replacing them with sodium ions. The solution is then dialyzed against distilled water for 24 h. Calcium ions and excess sodium ions are eliminated by the dialysis. The pH of the solution is adjusted to 7.25 and the solution is centrifuged at 10,000 g for 30 min at 4 °C.

The resulting supernatant is then diluted with a solution of magnesium sulphate to obtain a final concentration of magnesium of 0.2 M. This provokes the precipitation of phosvitin after a centrifugation at 10,000 g for 30 min. The pellet enriched in phosvitin contains about 15% impurities (β-livetin, α-HDL et β-HDL; Fig. 3; Castellani et al. 2003). (We have also noted the presence of some additional polypeptides with high electrophoretic mobility called phosvettes.) This enriched pellet can be completely purified

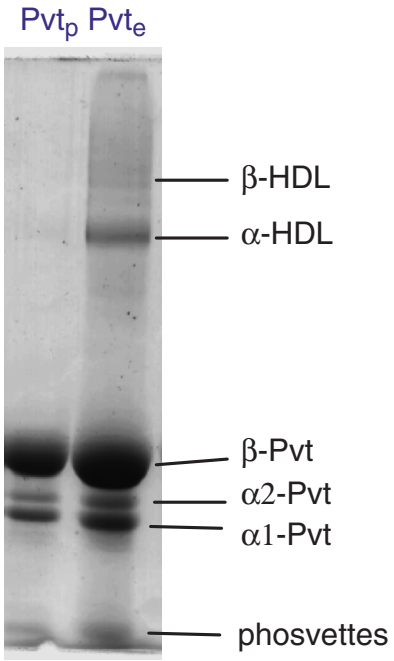

Fig. 3. Native polyacrylamide gel electrophoresis of extracted phosvitin (Pvt_e) and purified phosvitin (Pvt_p); gel at 6.5% acrylamide; Coomassie blue stain

by the use of anion exchange chromatography through MonoQ (Amersham Biosci., Little Chalfont, UK) resin (Castellani et al. 2003). We have been able to confirm by native electrophoresis that the purification through this resin allows one to obtain α- and β- phosvitins separated from contaminants. Furthermore, the phosvitins obtained are free of all multicharged metals because they are retained by the anionic resin. It is a necessary preliminary to the study of cation interactions with phosvitin.

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Chapter 5 Livetin Fractions (IgY)

Rüdiger Schade and Pablo Anibal Chacana

1 Introduction

The protein fraction of egg yolk represents approximately 16.6% of all its compounds. One relatively inhomogeneous fraction is the livetin fraction, including α-, β-, and γ-livetin. According to Bernardi and Cook (1960) the relative proportion of the three livetins in the yolk is 2:5:3, respectively. All livetins are water-soluble and they correspond to serum proteins (Williams 1962). The main component of α-livetin is an albumin whereas α-2-glycoprotein is the main component of β-livetin, and IgY (immunoglobulin Y) is the predominant fraction of γ-livetin (Kovacs-Nolan et al. 2005).

The following sections will discuss the molecular and physicochemical characteristics as well as the biological functions of IgY. The main focus of this contribution is on the characterization of IgY, since it is the most important component of the livetin fraction. Additionally, this chapter complements Chapter 25 "Use of IgY-Antibodies in Human and Veterinary Medicine" in this book.

2 Alpha-Livetin

Egg yolk alpha-livetin and chicken serum albumin are identical. According to sodiumdodecylsulfate polyacrylamide gelelectrophoresis (SDS PAGE) analysis the molecule has a molecular weight of 70 kDa and an isoelectric point (pI) between 4.3 and 5.7 (Williams 1962). Alpha livetin is also known as allergen Gal d5 due to its allergic properties, and this nomenclature has been approved by the WHO/IUS Allergen Nomenclature Subcommittee. It is partially heat labile since IgE reactivity to the protein was reduced by 88% after heating at 90 °C for 30 min (Quirce et al. 2001).

Alpha livetin, as well as egg white proteins transferrin and lysozyme, has been implicated in egg allergy. This association between egg proteins and

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

type I hypersensitivity with respiratory symptoms is known as bird-egg syndrome. It occurs after sensitization to these proteins and is characterized by the development of respiratory and gastrointestinal symptoms after egg intake or other contact with bird antigens. The initial symptoms are usually asthmatic with or without Rhinoconjunctivitis. Individuals become sensitized to bird proteins through contact with feathers, excrement, serum, and/or meat and subsequently develop egg allergy. (Nevot Falco et al. 2003). The sera of patients with bird-egg syndrome, pooled with budgerigar or hen feather extract and egg yolk extract, was found to lead to complete blocking of IgE binding to allergens in egg yolk and bird feather extract. However, IgE from patients with egg white allergy did not react with allergens in egg yolk and bird feather extract, despite strong IgE binding to egg white allergens. Researchers suggest that alpha-livetin leads to cross sensitization and consequently to bird-egg syndrome (Mandallaz et al. 1984, de Blay et al. 1994, Szepfalusi et al. 1994).

3 Beta-Livetin

Beta-livetin has been identified as a 45 kD α-2-glycoprotein. Chemically its composition includes 14.3% nitrogen and 7% hexose (Martin et al. 1957). Not much information is available about this protein and the data that do exist are ambiguous. For example, it has been reported to be very sensitive (Chang et al. 1970), moderately sensitive, (Dixon et al. 1981), and even highly stable to heat (Le Denmat et al. 1999).

4 Gamma-Livetin

4.1 Molecular Structure of Chicken IgY

The general structure of the IgY molecule, is of two light and two heavy chains and is comparable to the mammalian immunoglobulins. The heavy chains are indicated by the Greek letter Y (or ν), and contain one variable domain and four constant domains, in contrast to the three constant domains of the IgG heavy chain. The MW of IgY was found by mass spectrometry to be 167,250 Da (that of IgG is about 160,000 Da; Sun et al. 2001). Polyacrylamide gel electrophoresis gave somewhat different results (Sasse 1998; see Fig. 1). Each light chain consists of one variable and one constant domain (MW 18,660 Da): each heavy chain has a MW of 65,105 Da, and the Fab fragment has a MW of 45,359 Da (Sun et al. 2001). There is some homology between the Cν3 and Cν4 domains and the Cγ2 and Cγ3 domains of mammalian IgG, whereas the Cν2 domain is believed to represent the hinge region of IgG and is much less developed in IgY (Shimizu et al. 1992). The less developed hinge region of IgY may result in a reduced flexibility of the Fab moiety, which in turn may be the

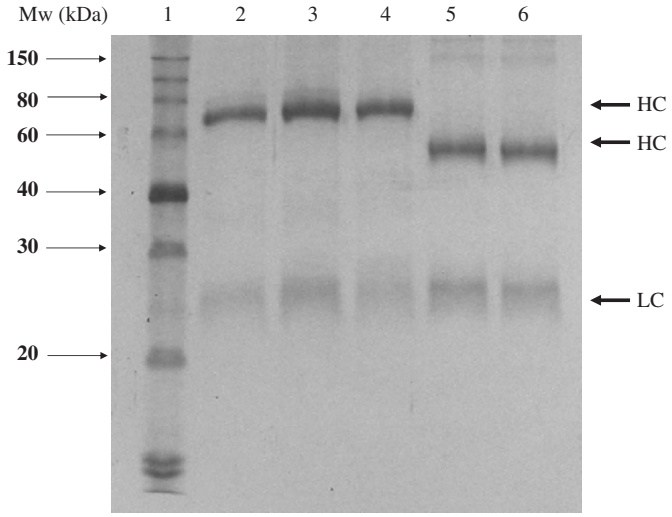

Fig. 1. Polyacrylamid gel electrophoresis (reducing conditions) of IgY (extracted by polyethyleneglycol precipitation) and IgG. *Lane 1* Mol weight marker (Roti-Mark 10-150, Roth, Karlsruhe, Germany); *Lane 2* IgY standard 5 µg (Sigma, Taufkirchen, Germany); *Lane 3* IgY standard 7.5 µg; *Lane 4* IgY extracted by PEG precipitation 2.6 µg (Polson et al. 1985); *Lane 5* IgG standard 2.5 µg (Sigma, Taufkirchen, Germany); *Lane 6* IgG standard 2.0 µg; *HC*(1) heavy chain of IgY; *HC*(2) heavy chain of IgG; *LC* light chain

reason for some differences between IgY and IgG concerning antigen epitope recognition (Warr et al. 1995). The Fc part of IgY is the site of most biological effector functions. It contains two carbohydrate side chains, in contrast to only one in IgG (see Fig. 2). The pI of IgY is in the range of 5.7 to 7.6, whereas that of IgG lies between 6.1 and 8.5 (Davalos-Pantoja et al. 2000, Sun et al. 2001). The most hydrophobic moiety of the antibody (Ab) molecule is the Fc fragment. Since the Fc fragment of the IgY is bigger than that of the IgG, the IgY molecule is more hydrophobic than the IgG molecule.

4.2 Biological Function of IgY

Similar to mammals, birds protect their offspring from infections by antibody transfer during early life. Thus, a passive immunity of the hatched chicken is ensured until its own immune system reaches full capacity. This process involves two steps: (1) IgY is transferred from the maternal blood stream to the egg yolk, mediated by oocyte membrane receptors; (2) IgY is transferred by special receptors from embryonic yolk sac to embryonic blood stream. The nature of these two sets of receptors is under discussion and it seems a difference exists, as discussed below.

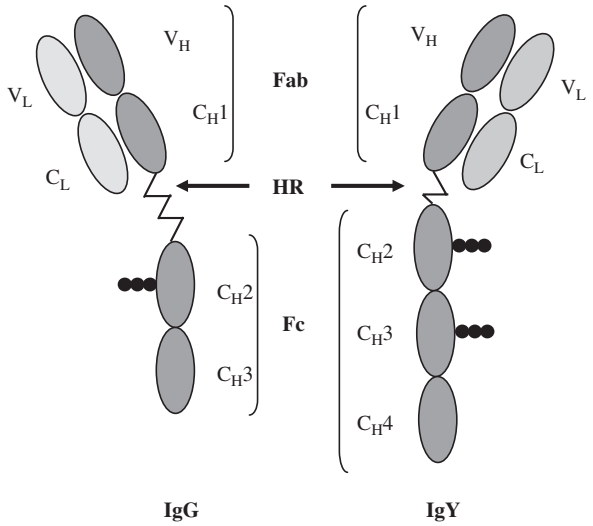

Fig. 2. Molecular structure of chicken IgY and rabbit IgG. *VL* variable domain of the light chain; *VH* variable domain of the heavy chain; *CL* constant domain of the light chain; *CH* constant domain of the heavy chain; *Fab* fragment antibody; *HR* hinge region; Fc fragment crystalline. The *joined black dots* symbolize carbohydrate chains

4.2.1 Transfer from Maternal Blood to Egg Yolk

According to Rose et al. (1974), minor quantities of IgA and IgM are transferred to the egg white from the plasma cells of the oviduct. Lösch et al. (1986) confirmed these results. Recent data show that IgY is exclusively transferred to the yolk by receptor-mediated processes (Mohammed et al. 1998, Morrison et al. 2001). The amount of IgY transferred is related to the IgY serum concentration, and it appears that all IgY populations are transferred (Morrison et al. 2001). Mohammed et al. (1998) and Morrison et al. (2001) showed that an intact Fc part and hinge region are both essential to accomplishing the transfer of IgY from serum to egg yolk. The Fc-associated carbohydrate side chains are not of special interest in this context. Apparently, two regions are of particular importance at the interface between Cν2 and Cν3, residues 251–254 (*Leu-Tyr-Ile-Ser* [LYIS]), and at positions 429–432 within Cν3 (*His-Glu-Ala-Leu* [HEAL]). All immunoglobulins transported to the yolk have the HEAL sequence. They found that murine IgG2b, with the sequence (*His-Glu-Gly-Leu* [HEGL]), and chicken IgA, with the sequence (*His-Asp-Gly-Ile* [HDGI]), were not transported to the yolk. In contrast, human IgA and IgG has the sequence HEAL. The transovarial passage of IgY takes about five days (Mohammed et al. 1998).

According to various authors, the specific serum Abs are transported to the yolk with a delay of 3–6 days (Patterson et al. 1962, Woolley and Landon 1995). The total IgY concentration is estimated to be 100–200 mg per egg. There is a controversy concerning yolk IgY and serum IgY concentrations. Some authors reported no differences between them, whereas others detected a higher IgY concentration in the yolk (for example, 1.23 times more in the yolk than in the serum; Woolley and Landon 1995). The yolk IgY concentration varies significantly among individuals (for example, 3–7 mg/ml; Carlander et al. 2001) and genetic lines or breeds (for example, Single-comb White Leghorn 2.2 ± 0.4 mg/ml, SLU-1329 2.0 ± 0.5 mg/ml, Rhode Island Red 1.7 ± 0.5 mg/ml; Carlander et al. 2002).

4.2.2 Transfer from Yolk Sac to Embryonic Circulation

The transport of maternal immunoglobulin to the offspring is a very important step to guarantee immunity during the early stages of life. In mammals the IgG transfer is realized by a MHC class I related receptor (FcRn; n = neonatal) being a heterodimer with two polypeptide chains, a membrane-bound heavy chain and a noncovalently attached light chain (β2-microglobulin; Ward 2004). FcRn is localized on different organs like placenta or intestine and reflects the pre- and postnatal passage of Ig either via bloodstream or milk (colostrum). However, according to recent studies (Ghetie and Ward 2000), the FcRn function is not restricted to Ig transfer during early stages of ontogenesis. It has been suggested that FcRn also plays a role in the Ig transport across cellular barriers, like lung epithelia, and in the control of serum half-life of IgG molecules by protection from lysosomal degradation (Spiekermann et al. 2002). The IgG binding is pH-dependent and strong under acid conditions (pH around 6) but not detectable at pH 7.4 (Raghavan et al. 1995). The mechanism of this pH dependence involves chemical rather than conformational changes, probably by switching from a protonated to a deprotonated form of FcRn (West et al. 2004).

The counterpart of the mammalian FcRn, called FcRY (West et al. 2004), is unrelated to a MHC-class-I-related receptor but is the avian homolog of the phospholipase A_2 receptor (PLA_2R; see Fig. 3). FcRY contains an N-terminal cystein-rich domain (CysR), a fibronectin type II repeat (FNII) and eight C-type, lectin-like domains (CTLDs; Fig. 3). IgY binding requires regions from CysR, FNII and CTLDs, and similar to FcRn the binding is pH dependent. It has been shown that IgY is bound at pH 6 but not at pH 8. In comparison, the FcRY IgY binding is ascribed as a conformational rather than a chemical change. At pH 6 FcRY takes a conformation allowing IgY binding, whereas at higher pH the binding affinity is lost (West et al. 2004). The binding site of the IgY molecule is the CH3 and CH4 domain of the Fc part. Interestingly, in contrast to the receptor involved in blood, yolk IgY transfer involves no binding and transport of mammalian immunoglobulins by FcRY. Taken together, the

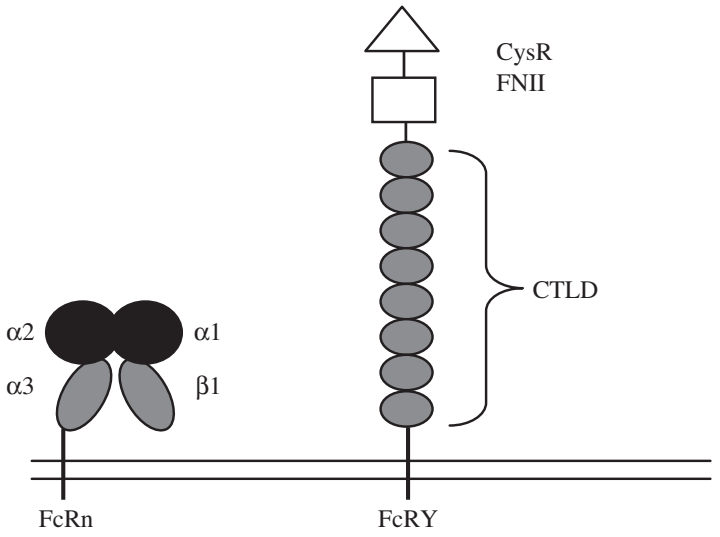

Fig. 3. Molecular structure of the mammalian neonatal Fc receptor (*FcRn*) and the chicken yolk sac IgY receptor (*FcRY*). Domains: *CysR* cysteine rich; *FNII* fibronectin type II; *CTLD* C-type lectin-like. Adapted from West et al. (2004)

two steps of IgY transfer involve two receptors that differ in function and in chemical constitution.

4.3 IgY Extraction

Various IgY extraction methods were reviewed in detail by De Meulenaer and Huyghebaert (2001) and Schade et al. (2005). In general, these methods can be divided into three groups:

1. *Precipitation methods*: most importantly precipitation by ammonium or sodium sulphate (e.g., Akita and Nakai 1993), and by polyethyleneglycol (PEG; Polson et al. 1985).
2. *Chromatographic methods:* most importantly affinity chromatography (e.g., Verdoliva et al. 2000).
3. *Ultrafiltration* (Kim and Nakai 1998).

In recent years, Polson's PEG precipitation method (Polson et al. 1985) has become the most commonly used and most effective protocol. A simple and frequently used procedure is the water dilution method, described by Akita and Nakai (1992). IgY purification by means of protein A (*Staphylococcus aureus*) or protein G (*Streptococcus* species, strains C and G) affinity columns is not possible, since these proteins have no binding site for IgY.

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Chapter 6 Lysozyme

Grzegorz Lesnierowski and Jacek Kijowski

1 Introduction

Lysozyme is a strongly basic protein, hen egg white its rich source. It can be obtained on a commercial scale as a preparation of biological activity. Lysozyme is known as a hydrolase that cuts the β-1-4 glycosidic bond, but its dimeric form has different and extremely valuable properties. It demonstrates bacteriostatic, bacteriolytic, and bacteriocidal activity, particularly against Gram-negative bacteria, among them a number of food pathogens. Egg lysozyme has desirable properties as a food preservative and is considered as a safe food ingredient. The modified lysozyme molecule is used in many areas, e.g., to prevent infections and simultaneously to act as a natural antibiotic or stimulant of the immune system. In the case of a lysozyme of higher content of the dimer form, effectiveness is substantially greater against, e.g., Gram-negative food spoilage microorganisms as well as pathogens. It has been demonstrated that the pure enzyme, well isolated, may be applied to extend the storage life of various kind of foods, among them poultry meat.

2 Characteristics of Lysozyme

Lysozyme (E.C.3.2.17), also known as muramidase or N-acetylmuramic-hydrolase, is a relatively small secretory enzyme that catalyses the hydrolysis of specific polysaccharides contained in cell walls of bacteria. Lysozyme from hen's egg white is a polypeptide of 129 amino acid residues having a molecular weight of 14.3 kDa. It is an elementary protein with the isoelectric point (pI) of 10–11. Basic chemical and physical properties of the enzyme from hen egg white are shown in Table 1.

Lysozyme was the first protein to be sequenced and whose three-dimensional structure was completely analyzed. It is a molecule consisting of two domains

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

Table 1. Chemical and physical properties of the enzyme from egg white

Property	Value
Molecular weight	14,400
No of subunits	1
Amino acids	129
pI	10.7
Disulfide bonds	4
% Carbohydrate	0
$E^{1\%}$ 280 nm	26.4
Thermal D at 93 °C (Time in min to destroy 90% of activity)	110
Assay of enzyme activity	*Micrococcus lysodeikticus* lysis by turbidity

linked by a long α-helix, between which lies the active site of the enzyme (Young et al. 1994). The N-terminal domain (residues 40~88) consists of some helices and is mostly the antiparallel β-sheet. The second domain is made up of residues 1~39 and 89~129 and its secondary structure is largely α-helical. The two domains of the molecule are separated by a helix-loop-helix motif (Asp 87–Arg 114), which has recently been found to play a key role in its antimicrobial function. The molecule conforms to the principle of being hydrophobic within and and hydrophilic without. All of its polar groups are on the surface and the majority of hydrophobic groups are buried in the interior of the enzyme particle. Conformational transition in lysozyme involves the relative movement of its two lobes toward each other in a manner allowing free access to the substrate and providing an appropriate environment for catalysis. The formation of the so-called "hinge-bending" structure is thought to play a critical role in the enzymatic (hydrolytic) action of lysozyme (Ibrahim 1997).

Lysozyme is an ubiquitous enzyme in nature, occurs in almost all secretions, body fluids, and tissues of human and animal organisms. It has also been isolated from some plants, bacteria, and bacteriophages (Table 2). Lysozyme is exceptionally abundant in poultry egg white (3.5% of the total egg white proteins), and hen egg albumen is the major commercial source of this enzyme. Its biological action in fowl eggs is unclear, but perhaps it serves as a defense mechanism before the embryo reaches the ability to produce immunoglobulins.

Its strong basic character has been utilized to develop the isolation procedure from egg albumen. Because of its high isoelectric point value the enzyme binds to the egg white ovomucin (pI 4.5–5.0) and negatively charged residues of sialic acid in glycoprotein, as well as ovotransferin (pI 6.0) and ovalbumin (pI 6.0); all these proteins have a carbohydrate moiety. Lysozyme is a very

Table 2. Selected sources of lysozyme

Source of lysozyme	Amount of lysozyme
Hen egg white	2500–3500 μg/ml
Duck egg white	1000–1300 μg/ml
Goose egg white	500–700 μg/ml
Tears	3000–5000 μg/ml
Human milk	55–75 μg/ml
Cow milk	10–15 μg/ml
Spleen	50–160 mg/kg
Thymus	60–80 mg/kg
Pancreas	20–35 mg/kg
Cauliflower juice	25–28 μg/ml
Papaya juice	8–9 μg/ml
Cabbage juice	7–8 μg/ml

stable enzyme. Four disulfide bonds of the single polypeptide chain of the enzyme make this small protein molecule unusually compact and highly stable. At least two of the -S–S- bonds must be intact to maintain its enzymatic activity. Two of the four disulfide bonds are also responsible for the thermal stability of the enzyme, its activity being retained even after 1–2 minutes at 100°C. Similar high stability is seen in acidic solution pH 3.0–4.0. However, thiol compounds rapidly inactivate lysozyme, and thus enzyme activity in hen egg white does not exist when heated at 60°C because of the presence of free SH groups of ovalbumin at that temperature (Tomizawa et al. 1994).

3 Antimicrobial Activity of Lysozyme

The lysozyme monomer demonstrates a strong antibacterial potential, mainly against Gram-positive bacteria. This phenomenon has found practical applications in the food and pharmaceutical industries as well as in medicine (Akashi 1972; Proctor and Cunningham 1988; Kijowski and Lesnierowski 1995). The enzyme hydrolyzes a number of structurally similar substrates, but the best known are the polysaccharide copolymers, i.e., N-acetyl glucosoamine (NAG) and N-acetyl muramic acid (NAM), which represent structural units of many bacterial cell walls. The action of the enzyme muramidase (hydrolase) in the cutting site breaks the β-1-4-linkage of the glycosidic bond between NAG and NAM. The method by which lysozyme lyses its substrate has been defined by Philips (1966). The active site of hen egg white lysozyme consists of six subsites, which together are sufficient to

bind six sugar residues. The six subsites along the active site cleft position the catalytic groups Glu-35 and Asp-52.

The antimicrobial activity of the lysozyme monomer is limited to Gram-positive bacteria. Cell walls of these microorganisms consist mainly of a peptidoglycan layer. The Gram-negative bacteria are less susceptible to the bacteriolytic action of this enzyme since they have a more complex envelope structure. The peptidoglycan layer, being the substrate of lysozyme, is covered by the outer membrane consisting of lipoproteins, lipopolysaccharides (LPS), and some hydrophobic peptides (Li-Chan and Nakai 1989). The outer membrane serves as a barrier to the access of enzyme to its site of action. It is most probable that lysozyme can be entrapped in the outer membrane by LPS of Gram-negative bacteria and inactivated (Ohno and Morrison 1989; Ibrahim et al. 1993). Generally, the limited action of lysozyme on Gram-negative bacteria seems to be influenced by both the composition and the sequence of N-acetylamino sugars in the bacterial cell wall. Moreover, there is evidence that lysozyme devoid of enzymatic activity kills different types of bacteria. It is also stressed that its bacteriostatic, bacteriolytic, and bactericidal activities seem to operate by different mechanisms. From extensive studies conducted on its binding capacity to different polysaccharides or lipids of bacteria, it is clear now that lysozyme exhibits both direct and indirect antimicrobial actions.

The bacteriostatic and bactericidal properties of lysozyme have been used to preserve various food items (Yashitake and Shinischiro 1977; Cunningham et al. 1991; Proctor and Cunningham 1988), as well as in pharmacy, medicine and veterinary medicine (Cunningham et al. 1991; Johnson 1994; Kiczka 1994). Lysozyme incorporated into food packaging materials has the potential to extend the shelf life of non-sterile or minimally processed foods by preventing the contamination by or growth of microorganisms. Edible films have also been investigated as potential antimicrobial packaging systems (Appendini and Hotchkiss 1997). The bactericidal and bacteriostatic properties of lysozyme have been examined against the saprophitic and pathogenic bacteria on poultry carcasses and chicken legs (with skin intact). The experimental results suggest that treatment with lysozyme solution could be used as an effective antimicrobial means in the extension of shelf life of poultry meat under refrigerated storage (Kijowski et al. 2005).

Of importance for pharmaceutical industry, hen egg white lysozyme can protect the body against bacterial or viral inflammatory diseases (Lacono et al. 1980). It has been used in aerosols for the treatment of bronchopulmonary diseases, prophylactically for dental caries, for nasal tissue protection, and is incorporated into various therapeutic creams for the protection against and topical reparation of certain dystrophic and inflammatory lesions of the skin and soft tissues.

In cheese making, lysozyme has been used to prevent growth of *Clostridium tyrobutyricum*, which causes undesirabel flavors and late "blowing" (unwanted fermentation) in some cheeses, in particular those made from pressed and

cooked curds (Stadhouders et al. 1987; Danyluk and Kijowski 2001). Another possible application of lysozyme may be in the acceleration of cheese ripening, because lysis of starter bacteria would cause the release of cytoplasmic enzymes, which play a key role in proteolysis during cheese ripening (De Roos et al. 1998). A well-known use of egg white lysozyme is as an antimicrobial agent in brewing for the control of lactic acid bacteria in beer (Daeschel et al. 1999). The control of lactic acid bacteria in wine making is essential in order to obtain wines of consistent high quality (Gerbaux et al. 1997).

4 Methods of Lysozyme Isolation

Several methods of lysozyme isolation from egg white have been developed so far. Most of them are used in laboratory practice to obtain the enzyme of high activity, but only some of these are feasible on a commercial scale. The most useful methods of extracting lysozyme from egg white are the conventional procedures of direct crystallisation and precipitation (Alderton and Fevold 1946), direct membrane filtration (Chang et al. 1986; Chiang et al. 1993; Lesnierowski 1997; Kijowski et al. 1998), affinity chromatography (Weaver et al. 1977; Muzzarelli et al. 1978; Yamada et al. 1985; Chiang et al. 1993) and ion exchange chromatography (Ahvenainen et al. 1979; Li-Chan et al. 1986; Banka et al. 1993; Weaver and Carta 1996; Lesnierowski 1997; Kijowski et al. 1998).

The crystallization method is a classical laboratory and commercial procedure of lysozyme separation from the egg white based on direct enzyme crystallization with 5% sodium chloride. Several re-solubilizations and re-crystallizations must be performed to obtain protein of high purity. In the remaining egg white after prior lysozyme separation it is possible to reduce salt by using ultrafiltration and diafiltration techniques. Desalted egg white has been found to demonstrate unchanged foaming ability and foam stability when compared with the native egg white (Lesnierowski 1997).

Taking into account the physical and chemical properties of lysozyme, especially its low molecular weight, it seems quite feasible to use membrane techniques, especially ultrafiltration (UF) to separate enzyme from the egg white. However, lysozyme ability to electrostatically bind ovomucin and the other negatively charged egg white proteins greatly reduces its diffusion through the membrane. More details on this subject are scarce.

The substrate recognition mechanism is the basis for the affinity chromatography method. The first adsorbent applied to bind lysozyme was chitin but soon new improved materials were developed. Glucochitin (deaminated chitin) or chitosan (deacylated chitin) also came under recommendation as affinity adsorbents for lysozyme recovery. Affinity methods have not been adopted for a large-scale isolation of lysozyme from egg white for several reasons, such as: high cost of affinity supports or ligands, poor flow rates of undiluted

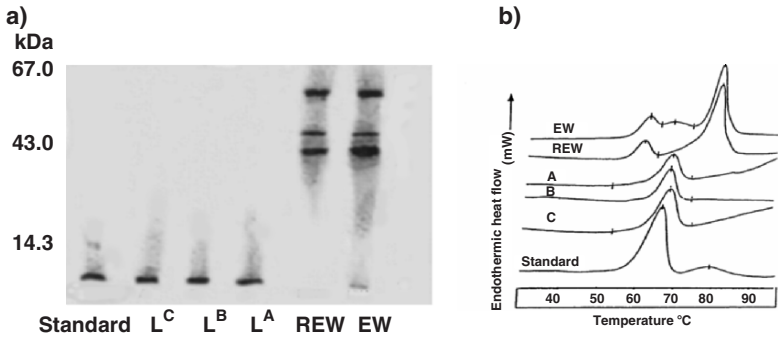

Fig. 1. SDS-PAGE **(a)** and calorimetric (DSC) **(b)** analysis of lysozyme (*L*), egg white (*EW*) and residual egg white (*REW*) obtained by ion-exchange chromatography. Lysozyme obtained by: *A*, sonication; *B*, shaking; *C*, stirring (Kijowski et al. 2000)

egg white, expensive and time-consuming elution procedures, and nonspecific adsorption of other egg white proteins.

The very high isoelectric pH value of lysozyme make it possible to separate the enzyme from other egg white proteins by ion exchange chromatography. The application of ion exchange resins proved to be a step forward in the effectiveness of enzyme separation. Resins such as amberlite, carboxymethyl cellulose (CMC), carboxymethyl-sephadex, and duolite are commercially available at moderate prices and can be used for such exchanges over extensive periods of time. Both column and batch techniques are used for larger scale operations. Among batch systems, sonication, mechanic shaking, and stirring techniques give good results in the sorption of lysozyme from egg white (Fig. 1).

Freeze drying and spray drying can be successfully used for dehydration of liquid lysozyme preparations (Lesnierowski and Kijowski 1997; Kijowski et al. 2000). Neither of these techniques reduces enzyme activity. Residual egg white may be decanted or filtered off and used for food.

5 Lysozyme Polymeric Forms

Lysozyme exists in two conformational states, between 20 and 30 °C with a transition point at 25 °C (Jolles and Jolles 1984). In nature the enzyme appears as a reversible dimer between pH 5.0 and 9.0. It is also known that hen egg white lysozyme tends to associate in an irreversible dimeric form (presumably through intermolecular disulfide exchange) when eggs are stored for long periods. The dimer retains enzymatic activity of the monomer, which means that this reaction is not responsible for the loss of hydrolytic activity. Dimerization, as well as any higher polymerization

process, depends on pH, enzyme concentration, and temperature. Modified lysozyme exhibits distinctly new properties and quite novel antimicrobial activity, which are being extensively examined nowadays.

A number of chemical modifications of lysozyme have been undertaken to increase its efficacy as an antimicrobial agent. The effect of lipophilization with long chain fatty acids (palmitic or stearic acid) and shorter chain saturated fatty acids (caproic, capric, or myristic acid) on the bactericidal action of lysozyme has been investigated (Ibrahim et al. 1991, 1993). Lipophilization was discovered to broaden the Gram-negative bactericidal action of lysozyme with little loss of enzymatic activity (Liu et al. 2000) ncs. Glycosylation, one of the most promising techniques, involves the attachment of carbohydrate chains to lysozyme and produces more stable proteins (Kato et al. 1988). A strong antimicrobial activity against *E. coli* is exhibited by the modified lysozyme by glycolization with palmitic acid (Liu et al. 2000). Extending the antimicrobial spectrum of lysozyme by conjugation without the use of chemical reagents yields a novel and potentially useful bifunctional food additive. Good emulsifying properties and heat stability found in a lysozyme-dextran conjugate (Nakamura et al. 1997) and lysozyme-xyloglucan hydrolysates (Nakamura et al. 2000). Conjugates show strong antimicrobial activity against *E. coli*.

Heat denaturation of lysozyme results in the progressive loss of enzymatic activity, but a greatly improved antimicrobial action towards Gram-negative bacteria. Partial unfolding of lysozyme can switch the antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive bacteria (Ibrahim 1994, 1998). The possibility to extend the range of lysozyme activity to include Gram-negative bacteria i.e. *E. coli*, is offered by the thermal and chemical-thermal modification (Figure 2), which leads to the formation of an enzyme preparation with increased content of polymeric forms (Lesnierowski et al. 2004).

An effective tool that can be used in the direct production of preparations with increased quantities of the polymeric forms of lysozyme is its modification by the membrane technique. It is showed that the temperature, pressure and time of modification as well as acidity of the medium significantly affected the quantity of polymers in the obtained preparations (Lesnierowski et al. 2003).

Nika Health Products Inc. has developed a system of preparing a highly purified lysozyme dimer product for use in treating viral and bacterial infections. The Nika method involves dimerizing the lysozyme monomer with a coupling reagent in a buffer solution adjusted to pH 10. Lowering the pH to seven by adding HCl or other suitable acid solutions stops the dimerization. The dimeric lysozyme is purified by a series of elution steps carried out using ion exchange resin column chromatography. Monomeric lysozyme remaining undimerized by the initial step is recycled into the process to increase yield. It is shown that lysozyme dimer stimulates the synthesis of some interleukins and interferon alpha and gamma. It also modulates the generation of TNF alpha, precluding all the negative effects associated with the excess level. The lysozyme dimer induces the activity of phagocytizing cells and at the same

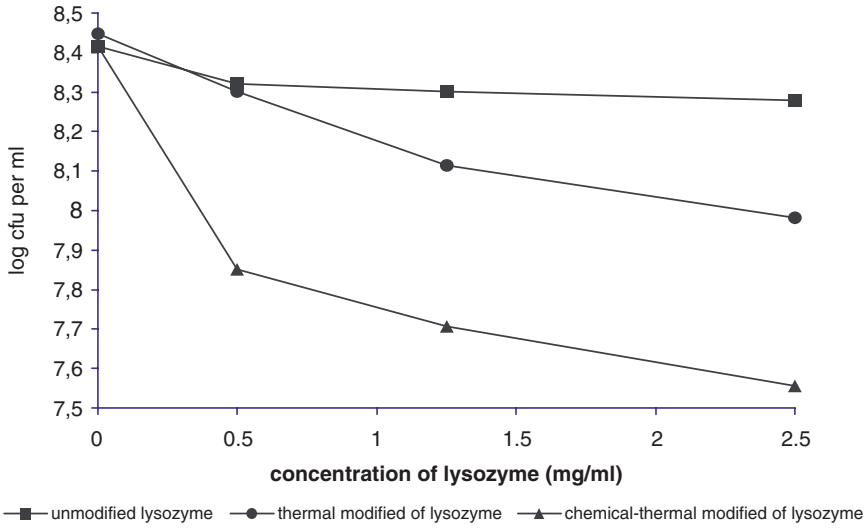

Fig. 2. The influence of concentration of modified lysozyme on its activity towards *Escherichia coli* (Lesnierowski et al. 2004)

time prevents excess generation of free radicals. It also demonstrates considerable antiviral activity against several viral strains and its antibacterial properties are similar to the antibacterial activity of some antibiotics. The lysozyme dimer is currently approved for use in veterinary medicine (Malinowski 2001).

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Chapter 7 Ovotransferrin

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1 Introduction

Under steroid control, the expression of the transferrin gene in the oviduct of birds leads to the synthesis of ovotransferrin (OTf). OTf (formerly conalbumin) is an egg white glycoprotein responsible for the transfer of ferric ions from the hen oviduct to the developing embryo. It belongs to the transferrin family and shows about 50% homology with mammalian transferrin and lactoferrin (Mazurier et al. 1983). This neutral glycoprotein is synthesized in the hen oviduct and deposited in the albumen fraction of eggs at a ratio of 12% of the total protein content. It has been demonstrated that OTf is capable of binding two Fe^{3+} ions per molecule with high affinity, thus delivering iron into host cells by membrane-bound specific receptors (Mason et al. 1996). There is also extensive evidence of an antimicrobial and antiviral effect of OTf (Antonini et al. 1977; Bullen et al. 1978; Valenti et al. 1981, 1982, 1983, 1985, 1986, 1987; Visca et al. 1990; Ibrahim et al. 1998, 2000; Giansanti et al. 2002, 2005) and its possession of therapeutic properties against acute enteritis in infants has also been demonstrated (Corda et al. 1983).

Moreover, OTf can be used as a nutritional ingredient in iron-fortified products such as iron supplements, iron-fortified mixes for instant drinks, sport bars, protein supplements, and iron-fortified beverages.

2 Composition

OTf is a glycoprotein consisting of a 686-residue single polypeptide chain with a molecular mass of about 78–80 kDa and a single glycan chain (composed of mannose and N-acetylglucosamine residues) in the C-terminal domain (Spik et al. 1988). OTf shows an isoelectric point of 6.0. Similarly to other transferrins, is a two lobed protein, and possesses the capability to reversibly

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bind two Fe^{3+} ions per molecule along with two CO_3^{2-} or HCO_3^- ions. OTf differs from transferrins only in the nature of the attached glycan and isoelectric point (Williams 1968; Thibodeau et al. 1978; Dorland et al. 1979; Lee et al. 1980). Each lobe (N lobe and C lobe) contains an iron binding site and is further divided into two domains (N1, N2, C1, and C2) of about 160 amino acid residues. OTf contains fifteen disulfide cross links and no free sulfhydryl groups (Williams et al. 1982). The sequences of cysteic-acid-containing peptides from digests of performic-acid-oxidized OTf have been determined and 34 unique cysteic acid residues were identified (Elleman and Williams 1970). A 102-residue sequence, which includes the point of attachment of the glycan, was also identified (Kingston and Williams 1975). Jeltsch and Chambon (1982) have determined the complete nucleotide sequence of hen transferrin cDNA transcribed from OTf mRNA.

The primary sequence of OTf shows 51% homology with human transferrin (hTf) and 49% homology with human lactoferrin (hLf; Jeltsch et al. 1987). Most of the homology is localized in the C-terminus region.

3 Purification and Structure

Many procedures to purify proteins belonging to the transferrin family have been developed, mostly making use of liquid chromatography, which avoids protein denaturation and possesses high selectivity. OTf has been purified either by cation exchange chromatography (Rhodes et al. 1958; Guerin-Dubiard et al. 2005) or anion exchange chromatography (Awade et al. 1994; Vachier et al. 1995). Recently, using three successive steps of ion exchange chromatography, a high yield (89%) of purified OTf was obtained (Guerin-Dubiard et al. 2005).

OTf shows structural characteristics similar to hen serum transferrin, since these proteins are derived from the same gene and differ only in their attached carbohydrate (Thibodeau et al. 1978). OTf is able to bind other transition metals very tightly (Valenti et al. 1987) and specifically with a binding log constant of about 15 at pH 7.0 and above. As already reported, OTf polypeptide chain is folded into two lobes and four domains. Each lobe comprises two distinct, similar-sized alpha/beta domains (N-terminal lobe, N1 and N2 domains; C-terminal lobe, C1 and C2 domains). The two domains are linked by two anti-parallel beta strands that permit them to open and close by a hinge. The iron-binding site is located in the inter-domain cleft in each lobe. Because of the sequence identity (37.4%), the two lobes show very comparable structures (Williams et al. 1982; Jeltsch et al. 1987). Most of the secondary structural elements are similar in the two lobes. The main differences between the two lobes are in loop regions, as expected by sequence insertions and deletions in the primary structure (Williams et al. 1982; Jeltsch et al. 1987). Comparison of the apo- (iron-deprived) and the holo-OTf (iron-saturated; Kurokawa et al. 1995) demonstrates that both the N- and C-lobes of OTf assume an open conformation upon iron release (Fig. 1). The results from both crystallographic

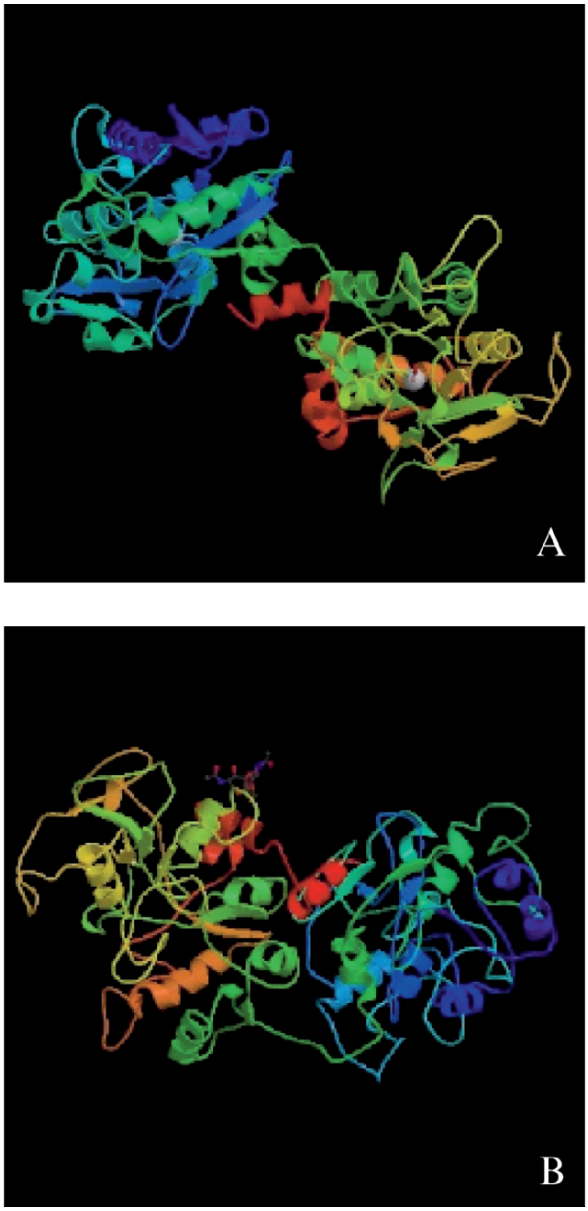

Fig. 1. Refined crystallographic structure of hen ovotransferrin, in **(a)** apo- form (Kurokawa et al. 1999; http://www.rcsb.org/pdb/explore.do?structureId=1AIV), and **(b)** holo- form (Kurokawa et al. 1995: http://www.rcsb.org/pdb/explore.do?structureId=1OVT)

analysis (Kurokawa et al. 1999) and x-ray solution scattering study (Grossmann et al. 1992) obtained on different transferrins demonstrate large scale conformational changes in both lobes upon Fe^{3+} binding or release (Fig. 1). OTf contains six disulfide bridges in the N lobe: two in the N1 domain, three in the

kringle of domain 2, and one in the N2 domain (Williams et al. 1982; Williams 1982). The N lobe does not contain inter-domain disulfide. This six-disulfide bridge motif is conserved in the OTf C lobe and also in the N and C lobes of serum transferrin and lactoferrin (Williams et al. 1982; Williams 1982; Crichton and Charloteaux-Wauters 1987). As observed in the C lobes of lactoferrin and serum transferrin, also OTf C lobe possesses three additional disulfides. Some of the three conserved disulfides appear to be related to the asymmetric structural and functional characteristics of the N and C lobes of the transferrins. Cys^{478}-Cys^{671} forms the only inter-domain cross-link in the C lobe of OTf. The C-terminal domain is glycosylated on Asn-473.

4 Antimicrobial and Antiviral Activity

OTf exerts in vitro an antimicrobial activity against different Gram-negative and Gram-positive bacteria. It has been reported that the most sensitive species are *Pseudomonas* spp., *Escherichia coli*, *Streptococcus mutans*, and the most resistant ones *Proteus* spp., and *Klebsiella* spp. (Valenti et al. 1983). OTf shows an antimicrobial activity mainly depending on its ability to sequester Fe^{3+}, essential for bacterial growth (Bullen et al. 1978). This action is largely bacteriostatic being reversed by the addition of ferric ions. Concerning the bacteriostatic activity of OTf, the presence of bicarbonate ions increased the effect of OTf, while citrate exerted an antagonistic effect toward this effect in bacteria possessing a receptor for iron–citrate complex (Valenti et al. 1983). Moreover, iron-related bacteriostatic activity of OTf is: higher at pH 8 than at pH 6 (Antonini et al. 1977); increased in the presence of bicarbonate (Valenti et al. 1981) or when OTf is immobilized by covalent linkage to Sepharose 4B (GE Healthcare, Amersham, Bucks., UK; Valenti et al. 1982); decreased when OTf is separated by a dialysis membrane from the bacteria, thus exerting only iron-chelating property (Valenti et al. 1985), and annulled by iron saturation (Antonini et al. 1977). Therefore, this function depends on iron concentration in the environment, on the degree of OTf iron saturation, and on the synthesis of bacterial siderophores, which compete with OTf in iron acquisition.

In other studies it has been suggested that the antibacterial activity of OTf is not due simply to the removal of iron from the medium, but involves a more complex mechanism. In fact, OTf or metal-ion-saturated OTfs, including Zn^{2+}OTf, show an antibacterial effect dependent on direct interaction with the bacterial surface (Valenti et al. 1987). It has been also demonstrated that OTf, in apo- or in holo- form, exerts a bactericidal effect against *Staphylococcus aureus*. Concerning Gram-negative bacteria such as *Escherichia coli* K12, however, it has been observed that the holo-OTf shows a more potent bactericidal action than the apo-form (Ibrahim et al. 1998). Therefore, in addition to the iron-related bacteriostatic activity, OTf shows a strong bactericidal activity not related to iron, but dependent instead on its capability to bind bacterial

membranes through its cationic domain (OTAP-92). The bactericidal peptide, OTAP-92, consists of 92 amino acid residues located within the 109–200 sequence at the lip of the N2 domain of the N lobe. OTAP-92 contains six cysteines engaged in three disulfide bridges, two of them forming a bilooped structural feature common in transferrin members (Ibrahim et al. 1998). This peptide is able of killing Gram-negative bacteria by crossing the outer membrane by a self-promoting uptake and causing damage to the biological function of the cytoplasmic membrane (Ibrahim et al. 2000).

Recent experimental data have shown, in reports similar to those regarding hLf (Visca et al. 1990), that the bacteriostatic and bactericidal action of OTf against *Salmonella* (*choleraesuis*) *enterica* can also depend on culture conditions that either favor or hinder binding OTf to the bacterial surface. For example, the binding of OTf to *S. enterica* occurs to a greater extent in saline solution than in complete medium, thus resulting in a bactericidal rather than the bacteriostatic effect of the complete medium (Table 1). As seen in Table 1, the bacteriostatic activity of OTf related to its iron-binding ability, is not exerted when the protein is completely iron-saturated. Conversely, the bactericidal activity of OTf that is unrelated to its iron-binding ability is exerted by both apo- and holo-OTf.

Concerning the antifungal activity of OTf, a direct interaction of iron-loaded protein with *Candida* cells has been reported (Valenti et al. 1985). In this study the inhibiting activity of OTf was tested toward one hundred strains belonging to *Candida* spp. A noticeable resistance was observed only by *C. krusei*, while, interestingly, the other *Candida* spp. appeared to be more sensitive than bacteria to the action of this protein. Moreover, it was demonstrated that apo- and holo-OTf exerted the same activity, suggesting that the antimycotic effect was not coupled to iron sequestration, but related rather to OTf binding on the *Candida* surface (Valenti et al. 1985; 1986).

The antiviral activity of OTf compared with that well known property of hLf and bovine Lf (bLf), has been reported for herpes simplex virus 1 (HSV-1)

Table 1. Bacteriostatic and bactericidal activity of OTf (1 mg/ml) against *Salmonella enterica*

Media	UFC/ml	
	Time 0	24 hours
BHI	$5.2 \pm 0.3 \times 10^5$	$3.0 \pm 0.2 \times 10^8$
BHI +apo-OTf	$5.0 \pm 0.4 \times 10^5$	$4.1 \pm 0.5 \times 10^6$
BHI +holo-OTf	$5.3 \pm 0.1 \times 10^5$	$1.0 \pm 0.2 \times 10^8$
PBS without Ca^{2+} and Mg^{2+}	$5.3 \pm 0.2 \times 10^5$	$1.0 \pm 0.1 \times 10^6$
PBS without Ca^{2+} and Mg^{2+} +apo-OTf	$5.1 \pm 0.3 \times 10^5$	$2.2 \pm 0.3 \times 10^3$
PBS without Ca^{2+} and Mg^{2+} +holo-OTf	$5.1 \pm 0.1 \times 10^5$	$2.8 \pm 0.3 \times 10^3$

BHI: brain Heath infusion broth; PBS: phosphate buffered saline

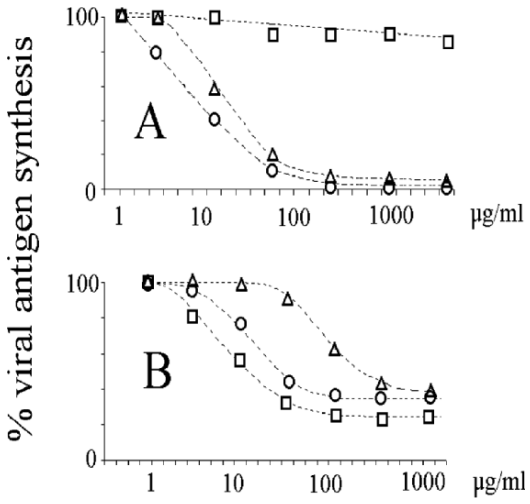

Fig. 2. Dose–effect relationship of the antiviral activities of human and bovine lactoferrin with ovotransferrin against (**a**) *Herpes simplex 1* virus and (**b**) Marek's disease virus. Symbols: ❑ = apo-OTf; *o* = apo-bLf; Δ = apo-hLf

and Marek's disease virus (MDV), an avian herpesvirus (Giansanti et al. 2002). OTf was more effective than hLf and bLf in inhibiting MDV infection (Fig. 2). In addition, no correlation between antiviral efficacy and iron saturation was found (Giansanti et al. 2002); this activity seems to be associated with two OTf fragments that have sequence homology with two bLf fragments effective toward HSV-1 (Siciliano et al. 1999), suggesting that these fragments could have a role in the anti-herpesvirus activity (Giansanti et al. 2005).

From an evolutionary point of view in birds, the defensive properties of OTf toward infections remain joined to iron transport functions, while in mammals iron transport functions is peculiar to serum transferrin, and the defensive properties are ascribed to lactoferrin.

Therefore, all these data suggest that OTf can be considered a protein of the non-immune innate system that resists pathogens and, like mammalian lactoferrins, plays a role in defense against pathogenic bacteria, fungi, and viruses.

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Chapter 8 Ovalbumin and Gene-Related Proteins

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and Catherine Guérin-Dubiard

1 Introduction

The ovalbumin gene family is composed of three genes: X, Y, and ovalbumin, which are expressed in the laying hen oviduct in response to steroid hormones (LeMeur et al. 1981; Stumph et al. 1983). They are found within a 40 kb region of the chicken genome in the order 5′-X-Y-ovalbumin-3′ (Royal et al. 1979) and share some sequence homologies establishing that they have evolved from a common ancestor gene by duplication events (Heilig et al. 1980). The relative extent of maximal expression of the three closely related genes is in the order of ovalbumin:Y:X 100:10:1 (Colbert et al. 1980). In databanks, ovalbumin gene X (Swiss-Prot accession number P01013) is referenced as a fragment of a larger protein. The whole chicken genome assembly by the genome sequencing center at Washington University Medical School in March 2004 gave the DNA sequence of the whole protein called ovalbumin-related Y protein (NCBI accession number XP_418984). The amino acid sequences of ovalbumin and gene-related proteins are given in Fig. 1, and their homology percentages for strict and non-strict identity are indicated in Table 1.

2 Ovalbumin

2.1 Amino Acid Sequence and Structure

Ovalbumin is the predominant protein in albumen with 54% of the egg white proteins by weight. It is a phosphoglycoprotein with a molecular mass of about 45 kDa (Warner 1954). The amino acid sequence of hen egg white ovalbumin, comprising 385 amino acids, was deduced from the mRNA sequence by McReynolds et al. (1978). It was in agreement with sequences of

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Fig. 1. Ovalbumin (P01012), ovalbumin gene Y (P01014), ovalbumin-related Y protein (XP_418984) and ovalbumin gene X (P01013) primary amino acid sequences. Protein sequence alignment was optimized using Clustal W multiple alignment procedure (Expasy web site using blosum 62 matrix; Thompson et al. 1994). *Italic bold characters* represent identical amino acids for the four proteins, *bold characters* identical amino acids for three proteins, and *italic characters* identical amino acids for two proteins

Table 1. Homology percentage for strict (*italicized characters*) and non-strict identity in amino acids between ovalbumin (P01012), ovalbumin gene Y (P01014) and ovalbumin-related Y protein (XP_418984) (Clustal W multiple alignment procedure, Expasy web site using blosum 62 matrix, Thompson et al. 1994)

	P01012	P01014	XP_418984
P01012	–	*57 %*	*61%*
P01014	76%	–	*73%*
XP_418984	77%	84%	–

the purified protein (Nisbet et al. 1981) and the cloned DNA (Woo et al. 1981). The N-terminal amino acid of the protein is an acetylated glycine (Narita and Ishii 1962). Thus ovalbumin does not have a classical N-terminal leader sequence, although it is a secretory protein (Huntington and Stein 2001). Instead, the hydrophobic sequence between residues 20 and 46 may act as an internal signal sequence involved in transmembrane location (Robinson at al. 1986).

Half of ovalbumin amino acids residues are hydrophobic and one third are charged, for the most part acidic, giving to the molecule an isoelectric point of 4.5 (Li-Chan and Nakai 1989). Two genetic polymorphisms of ovalbumin have been reported: a Glu→Gln substitution at residue 289 (Ishihara et al. 1981) and an Asn→Asp substitution at residue 311 (Wiseman et al. 1972). The sequence includes six cysteine residues (Cys^{11}, Cys^{30}, Cys^{73}, Cys^{120}, Cys^{367}, Cys^{382}), two of which (Cys^{73} and Cys^{120}) are involved in a disulfide bond (Fothergill and Fothergill 1970). Each ovalbumin molecule contains four sulfhydryl groups, three of which are weakly reactive ("masked") in native protein whereas the fourth is reactive only in the denatured protein (MacDonnell et al. 1951; Feeney 1964; Fernandez-Diez et al. 1964).

A single carbohydrate side chain is covalently linked to the amide nitrogen of Asn^{292} in a typical Asn-X-Thr sequence recognized by glycosyltransferases (Lee and Montgomery 1962; Nisbet et al. 1981; Li-Chan and Nakai 1989). A second potential recognition site, Asn-X-Ser at residues 316–318, is not glycosylated in the secreted form found in the egg white, but has been observed transiently in the oviduct (Suzuki et al. 1997). The carbohydrate chain is heterogeneous but the different ovalbumin glycopeptides have a molecular weight between 1560 and 1580 Da (Lee et al. 1964; Montgomery et al. 1965) and share a common core structure in which at least one mannose bonds to 2 N-acetylglucosamine by a β(1-4) bond (Tai et al. 1975; Conchie and Strachan 1978).

Ovalbumin has two potential phosphorylation sites at serines 68 and 344 (Nisbet et al. 1981), included in the phosphorylation recognition site Ser-X-Glu (Henderson et al. 1981). Ovalbumin A_1, A_2, and A_3 containing two, one, and zero phosphate groups per ovalbumin molecule, respectively, are present in the albumen in an approximate ratio of 81–84:14–16:2–4 (Longsworth et al.

1940; Cann 1949; Perlman 1952). A_1, A_2, and A_3 can be separated by ion-exchange chromatography, native polyacrylamide gel electrophoresis (PAGE), or isoelectrofocalization electrophoresis.

The crystal structure of ovalbumin was determined by Stein et al. (1990, 1991) using high resolution (1.95 Å) X-ray diffraction. They reported that native ovalbumin contained 30% α-helix and 32% β-sheet structures. These results are in agreement with Egelandsdal (1986) who found 30% α-helix and 40% β-sheet structures using circular dichroism measurements. However, they slightly differ from those obtained by Doi et al. (1987) also using circular dichroism, and Painter and Koenig (1976) using Raman spectroscopy who found 49% and 25% α-helix and 13% and 25% β-sheet structures, respectively. These differences may be explained by the experimental conditions, especially pH and ionic strength. General information can be obtained on ovalbumin tertiary structure: ovalbumin Stokes radius was found to be 27.3 Å by Nakamura and Ishimaru (1981) and 25.2 Å by Matsumoto and Chiba (1990); its intrinsic viscosity was measured between 3.9 and 4.5 ml g^{-1} (Ahmad cited from Ansari et al. 1975; Nakamura and Ishimaru 1981; Koseki et al. 1989); its hydrophobic coefficient was estimated at –0.06, 0.24, and 0.50 by Kato and Nakai (1980), Keshavarz and Nakai (1979) and Nakamura and Ishimaru (1981), respectively. Moreover, Ikura et al. (1992) drew the hydrophobicity profile of native ovalbumin that corroborated the fact that free sulfhydryl groups were buried in the heart of the molecule. Ovalbumin may be organized in a quaternary structure as shown by small angle X-ray diffusion (Matsumoto and Chiba 1990; Matsumoto et al. 1992). This molecular association is concentration dependent, ovalbumin being in monomeric form for concentrations lower than 0.1%, dimeric form for concentrations between 0.5 and 1%, and tri or tetrameric form for concentrations between 4 to 17%. The association rate would also increase with pH decrease: 2.1 molecules at pH 7.0 against 2.88 molecules at pH 4.01 for a concentration of 0.5%.

Ovalbumin in solution may be denatured and aggregated by thermal treatments or exposure to air–water interfaces (Chang et al. 1970; Matsuda et al. 1981; Kato et al. 1985; Kato and Takagi 1988; Koseki et al. 1989; Mine et al. 1990; Kitabatake and Doi 1987; Renault et al. 2002; Lechevalier et al. 2003).

2.2 Ovalbumin Function

Based on sequence homologies, ovalbumin is classified in the serpin (serine proteinase inhibitor) family although it lacks any protease inhibitory activity in its native form (Hunt and Dayhoff 1980; Wright 1984; Stein et al. 1990). However, Mellet et al. (1996) showed that heating ovalbumin 30 min at 97 °C transformed it into inhibitory ovalbumin, a potent reversible competitive inhibitor of different proteinases. But inhibitory ovalbumin differs from active serpins by its inability to form irreversible complexes with proteinases. The ovalbumin primary sequence thus contains the information required for enabling the first step of the serpin-proteinase interaction to occur, but does

not contain the information needed for stabilizing this initial complex. Some authors have thus said that ovalbumin could just be a storage protein (Whisstock et al. 1998). However, present in many embryonic organs under its S-form, egg white ovalbumin migrates into the developing embryo by changing its form to less ordered structures fitted to transportation. This result suggests that ovalbumin may have a dynamic function in developing organic cells (Sugimoto et al. 1999). Ovalbumin antigenicity and its role in the immunological and allergenic properties of egg white have been the subject of various investigations (Breton et al. 1988). Ovalbumin was classified in the major allergens in egg white (Langeland 1982; Langeland and Harbitz 1983; Hoffman 1983).

3 S-Ovalbumin

Smith (1964) and Smith and Back (1965) first reported that ovalbumin was converted to S-ovalbumin, a more heat-stable protein, during the storage of eggs. The denaturation temperature of ovalbumin was found to shift from 84.5 °C to 92.5 °C for S-ovalbumin, with an intermediate species denatured at 88.5 °C, as measured by differential scanning calorimetry (Donovan and Mapes 1976). The conversion was prevented by oiling the egg shell (Vadehra and Nath 1973). Present in up to 5% of ovalbumin in fresh laid egg white, more than half of the ovalbumin converts to the S form by the time eggs reach the supermarket and ultimately the consumer. The percentage of S-ovalbumin may reach 81% after 6 months storage at low temperature (Vadehra and Nath 1973). The appearance of S-ovalbumin coincides with the loss of the "food value" of eggs, since eggs with high S-ovalbumin content have runny whites and do not congeal as effectively on cooking (Shitamori et al. 1984). Most of the work on S-ovalbumin has been motivated by this loss in "food value" of stored eggs and not in relation to the potential function of ovalbumin in eggs (Huntington and Stein 2001).

S-ovalbumin is also easily formed in vitro by a 20 h incubation at 55 °C in 100 mM sodium phosphate, pH 10 (Donovan and Mapes 1976). Smith and Back (1965) estimated the activation energy for conversion to 105 ± 20 kJ/mole. The high pH and temperature increase the rate of conversion, the basis for which has been extensively studied. Many molecular models have been considered for years (Smith and Back 1965: Painter and Koenig 1976; Kint and Tomimatsu 1979; Nakamura and Ishimaru 1981; Nakamura et al. 1981; Kato et al. 1986; Castellano et al. 1996; Takahashi et al. 1996). The conversion of ovalbumin to the S form has also been addressed in light of its membership in the serpin superfamily by Huntington et al. (1995). These authors showed in biochemical and thermal stability studies that the conformational change in S-ovalbumin was an intramolecular insertion of the reactive center loop. But recently, Yamasaki et al. (2003) demonstrated

the crystal structure of S-ovalbumin at 1.9 Å resolution and this structure unequivocally excluded the partial-loop-insertion mechanism. The overall structure of S-ovalbumin, including the reactive center loop structure, is almost the same as that of native ovalbumin, except that Ser^{164}, Ser^{236}, and Ser^{320} take the D-amino acid residue configuration.

4 Ovalbumin Gene Y

Ovalbumin gene Y has been known as a member of the ovalbumin gene family since 1982, when Heilig et al. sequenced its encoding gene. The overall homology between the Y and the ovalbumin protein coding sequence was found to be 72.6%, resulting in a 58% homology for the amino acid sequences (Heilig et al. 1982). Ovalbumin gene Y has recently been identified for the first time in hen egg white by Nau et al. (2005) in a concentration ratio of ovalbumin gene Y:ovalbumin of about 13:100 which is in agreement with Colbert et al. (1980).

This protein comprises 388 amino acid residues for a theoretical molecular weight of 43.8 kDa and an isoelectric point of 5.2, whereas a higher molecular weight (53 kDa) and three isoforms differing in their isoelectric points (5.5, 5.4, and 5.3) were observed (Nau et al. 2005). This gap between measured and theoretical molecular masses could result from posttranslational modifications, such as glycosylation; like ovalbumin, ovalbumin gene Y is glycosylated (Nau et al. 2005), but the polymorphism observed on isoelectric focalization (IEF) gels is still unexplained. It can neither be attributed to phosphorylations since, unlike ovalbumin, and despite putative phosphorylation sites predicted from its gene sequence (NetPhos procedure at http://www.cbs.dtu.dk), ovalbumin gene Y is not phosphorylated, nor to genetic variations, since more than two isoforms were observed (Nau et al. 2005).

Like ovalbumin, the ovalbumin gene Y N-terminal residue seems to be acetylated (Nau et al. 2005).

Ovalbumin gene Y is also a member of the serpin family. However, it has not been determined yet if it is, unlike ovalbumin, a biologically active member of this family, it is a protease inhibitor.

5 Ovalbumin-Related Y Protein

The recent prediction of ovalbumin-related Y protein sequence by the genome sequencing center at Washington University Medical School showed that this protein included in its sequence ovalbumin gene X sequence. This prediction was confirmed by Guérin-Dubiard et al. (2005) who identified for the first time, ovalbumin gene X in egg white, but noticed a difference

between the experimental molecular weight [45 kDa determined by sodium dodecyl sulfate (SDS)-PAGE] and the theoretical value (26.3 kDa). More recently, Guérin-Dubiard et al. (2006) identified by bidimensional (2D)-PAGE analysis five spots containing ovalbumin-related Y protein, with an apparent molecular weight of 53 kDa. Actually, this protein comprises 496 amino acid residues for a theoretical molecular weight of 55.9 kDa and a theoretical isoelectric point of 6.47. No information is currently available to explain the ovalbumin-related Y protein polymorphism. However, as predicted from its gene sequence (NetPhos and NetNGlyc procedures at http://www.cbs.dtu.dk), this protein has 21 potential phosphorylation sites and 4 potential N-glycosylations sites. On the other hand, unlike ovalbumin and ovalbumin gene Y, ovalbumin-related Y protein N-terminal residue does not seem to be acetylated (NetAcet procedure at http://www.cbs.dtu.dk).

As ovalbumin and ovalbumin gene Y, ovalbumin-related Y protein belongs to the serpin family but its biological activity is still undetermined.

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Chapter 9 Ovomucin

JAAKKO HIIDENHOVI

1 Ovomucin – a Gelling Agent of Egg White

Ovomucin is a sulphated egg white glycoprotein, characterized by high molecular weight and a subunit structure. Besides in egg white, ovomucin is found in chalazaes (Sato and Kato 1980; Itoh et al. 1987) and in the outer layer of the vitelline membrane (Back et al. 1982; Debruyne and Stockx 1982). Ovomucin represents about 2–4% of the total egg albumen protein in avian eggs, its content in thick egg white being 2–4 times that in thin white (Brooks and Hale 1961; Kato et al. 1970; Adachi et al. 1973).

Ovomucin is responsible for the gel-like properties of thick egg albumen. Egg white thinning, the most important change in egg white during storage, is usually attributed to the degradation of the ovomucin complex (Kato et al. 1971; Robinson and Monsey 1972). Many theories have been suggested to explain egg white thinning, but there seems to be no general consensus among researchers about the causal factors that induce this phenomenon. Such theories have been reviewed, for example, by Vadehra and Nath (1973), Burley and Vadehra (1989), and Li-Chan and Nakai (1989).

2 Chemical and Physical Properties of Ovomucin

Crude ovomucin is composed of at least two subunits: α-ovomucin and β-ovomucin (Kato et al. 1971; Robinson and Monsey 1971). These subunits have different amino acid compositions and also differ in amounts of carbohydrate. The primary amino acid sequence of α-ovomucin was deduced from cloned cDNA (Watanabe et al. 2004a). The polypeptide consists of 2087 amino acids with a relative molecular mass of 230,900 Da. Referring to an earlier study (Tsuge et al. 1997a) indicating that α-ovomucin consists of 91% protein and 9% carbohydrate, Watanabe et al. (2004a) calculated the estimated size of α-ovomucin to be around 254,000 Da. This is higher than the

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previously reported molecular weights of 180,000 to 220,000 Da, obtained by using SDS-PAGE or ultracentrifugation (Robinson and Monsey 1971; Hayakawa and Sato 1978; Tsuge et al. 1997a).

Moreover, Itoh et al. (1987) reported that the α-ovomucin component of thick egg white ovomucin, which had been fractioned by gel filtration, separated into two bands on SDS-PAGE. Itoh et al. (1987) named the components as α1- and α2-ovomucin, estimating their molecular weights at 150,000 and 220,000 Da, respectively. Similar SDS-PAGE patterns for reduced crude ovomucin were also obtained by Guérin and Brulé (1992) and Toussant and Latshaw (1999). Further, ovomucin has been shown to eluate as three separate peaks by using a Superose 6 HR 10/30 (Amersham Biosciences, Uppsala, Sweden) dual-column gel filtration system in reducing conditions (Hiidenhovi et al. 2002).

The molecular weight of the β-ovomucin subunit has been estimated by SDS-PAGE or ultracentrifugation to be between 400,000 and 720,000 Da (Robinson and Monsey 1975; Hayakawa and Sato 1978; Itoh et al. 1987; Tsuge et al. 1997a; Hiidenhovi et al. 1999a; Toussant and Latshaw 1999). To the author's knowledge, the complete amino acid sequence of β-ovomucin has so far not been revealed. However, Watanabe et al. (2004b) have reported the amino acid sequence of a β-ovomucin fragment consisting of 827 amino acids and having molecular weight of 91,836 Da.

Egg white contains two forms of ovomucin: insoluble and soluble. Soluble ovomucin is presented both in thick and thin albumen, while insoluble ovomucin is found only in thick albumen (Sato and Hayakawa 1977). Insoluble and soluble ovomucin contain different proportions of ovomucin subunits. Hayakawa and Sato (1977) isolated both the insoluble and soluble ovomucin from thick egg white, reporting that α-ovomucin and β-ovomucin accounted for 67% and 33% of the insoluble and 87% and 13% of the soluble ovomucin, respectively. The molecular weight value of 23×10^6 Da for insoluble ovomucin in 6.5 M guanidine hydrochloride was estimated using light scattering (Tomimatsu and Donovan 1972). For soluble ovomucin, molecular weight values in the range of 5.6 to 8.3×10^6 Da have been reported (Lanni et al. 1949; Miller et al. 1981; Hayakawa and Sato 1976). These differences in molecular weights are presumably due to differences in measuring methods and conditions and the heterogeneity of ovomucin.

The carbohydrate contents of α-ovomucin and β-ovomucin are roughly 15% and 60%, respectively. Due to this large variation between different subunits, the carbohydrate contents of different ovomucin complexes also differ to some extent. According to Kato et al. (1970), thick white ovomucin contains 24% and thin white ovomucin 16% carbohydrate, while Adachi et al. (1973) reported the values 30.9% and 26.3%, respectively.

The carbohydrates in ovomucin occur as oligosaccharide moieties consisting of about 3–6 carbohydrate units with straight as well as branched chains (Strecker et al. 1987, 1989, 1992; Moreau 1996). Both *N*- and *O*-glycosidally linked oligosaccharides are found, the former linking to asparagine residues

of the polypeptide chain and the latter to serine and threonine residues. These oligosaccharide moieties consist of hexoses (mannose and galactose), hexosamines (*N*-acetylglucosamines and *N*-acetylgalactosamines), sialic acid, and ester sulphate.

3 Isolation and Purification of Ovomucin

3.1 Isoelectric Precipitation Method

Ovomucin is most often isolated from egg albumen by the isoelectric precipitation (IEP) method. Numerous variations of this method are found in the literature, but usually the IEP method can be said to comprise three steps as follows: a precipitation step (in which egg albumen is diluted with water, followed by acidification to or near to its isoelectric point), a collection step (usually done by centrifugation), and a washing step (ovomucin precipitate being washed several times with salt solutions, such as 2% KCl, to remove other egg white proteins, and finally with water to wash out salts). The method described by Nakamura et al. (1961) is given here as an example. Briefly, egg white is added slowly to three volumes of water. The suspension is adjusted to pH 6 and centrifuged. The precipitate is repeatedly washed with 2% KCl until the washings are free of protein. Finally, the precipitate is washed with water until the washings contain no chloride. The gelatinous ovomucin precipitate thus obtained is contaminated more or less with other albumen proteins as shown in Fig. 1. The obtained crude ovomucin precipitate is highly

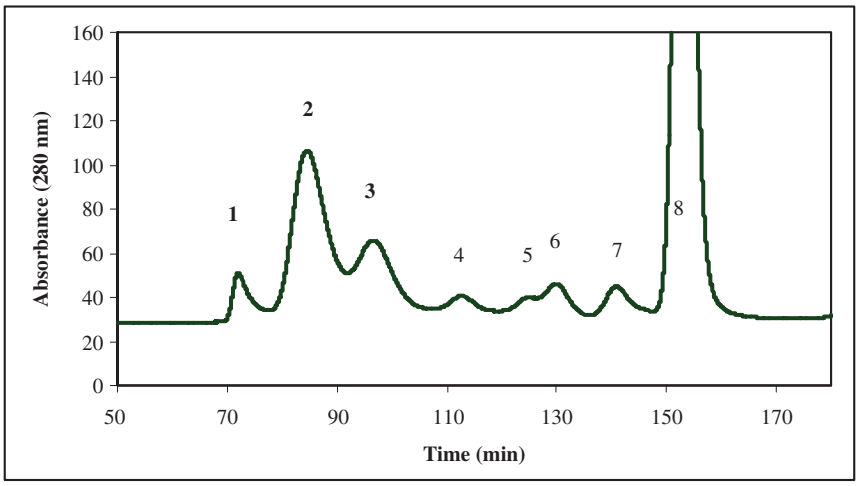

Fig. 1. Elution profile of crude ovomucin obtained by Superose 6 HR gel-filtration chromatography. *1* β-ovomucin; *2* α2-ovomucin; *3* α1-ovomucin; *4* ovotransferrin; *5* unknown (globulins?); *6* ovalbumin; *7* ovomucoid; *8* lysozyme

insoluble in common buffer solutions, and can be solubilized completely as subunits with buffers containing dissociating agents such as urea, guanidine hydrochloride, or SDS, and reducing agents such as b-mercaptoethanol or dithiotreitol (Robinson and Monsey 1971; Itoh et al. 1987), or by enzymatic hydrolysis to peptides (Guerin-Dubiard and Brule 1994; Moreau 1996; Moreau et al. 1997; Hiidenhovi et al. 2005). The advantage of the IEP method is that the method itself is very simple, cost-effective, and easy to up-scale.

3.2 Chromatographic Methods

Alternatively to precipitation methods, ovomucin has been isolated from egg albumen by gel filtration chromatography (GFC). The molecular weight of ovomucin is so substantial compared with other egg white proteins that by choosing appropriate gel filtration media, ovomucin will be eluated well ahead of other egg albumen proteins (Fig. 2). Ovomucin has been isolated from egg albumen in complex form using GFC both without (Young and Gardner 1970; Hayakawa and Sato 1976, 1977) and with detergents (Adachi et al. 1973; Hiidenhovi et al. 1999b). An advantage of the GFC method compared with IEP is that the separated ovomucin is usually very pure (free of other egg white proteins) and already in soluble form. The main disadvantage of GFC is a low yield of ovomucin; the reported values are quite modest: 5–10 mg per GFC run (Adachi et al. 1973; Awadé et al. 1994). Although this is also an upscaling issue, the main reason for the low yield is that samples for GFC are prepared by diluting egg white with buffer, thus leading to samples containing low amounts of ovomucin.

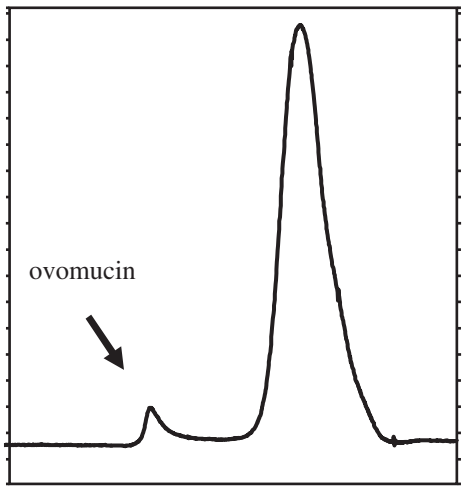

Fig. 2. Elution profile of whole egg white obtained by Sephacryl S-500 HR (Amersham) gel filtration chromatography

Ovomucin has also been separated into α- and β-subunits using different chromatographic methods, such as lysozyme-Sepharose 4B (Amersham) affinity chromatography (Kato el al. 1977), ion-exchange chromatography after sonication (Hayakawa and Sato 1976; Yokota et al. 1999a, 1999b) and GFC in reducing conditions (Itoh et al. 1987; Awadé et al. 1994; Tsuge et al. 1996; 1997a, 1997b; Hiidenhovi et al. 1999, 2002).

3.3 Other Methods

Sleigh et al. (1973) isolated ovomucin by high-speed centrifugation at high ionic strength. Ovomucin has additionally been separated into α- and β-subunits using density gradient ultracentrifugation (Robinson and Monsey 1971) and density gradient electrophoresis (Kato and Sato 1971).

4 Future Trends—Biological Roles of Ovomucin

In previous years ovomucin was studied mainly because of its role in egg white thinning. More recently, however, there has been a growing interest within the food industry toward health-promoting, "functional" foods. Today proteins are not valuated only by their nutritional value or functional properties, but also by their biological activity. Most ovomucin research has lately focused on its bioactive properties.

In 1993 Ohami et al. (1993) reported that the β-ovomucin subunit of ovomucin had a cytotoxic effect on cultured tumor cells. Later, β-ovomucin was shown to have growth-inhibiting and cell-damaging effects on sarcoma cells (Yokota et al. 1999a, 1999b). It was further demonstrated that certain fragments separated from pronase-treated hen egg white ovomucin—namely, 220 and 120 kDa, highly glycosylated peptides derived from the β-subunit, and 70 kDa, a highly glycosylated peptide derived from α-ovomucin—have antitumor effects in a double-grafted tumor system (Watanabe et al. 1998a; Oguro et al. 2000).

Antihemagglunation activity of ovomucin against swine influenza virus was reported in the late 1940s (Gottschalk and Lind 1949). Later, Tsuge et al. (1996) showed that ovomucin also has hemagglutination inhibition activity against bovine rotavirus (RV), hen Newcastle disease virus (NDV), and human influenza virus (IV). Moreover, it has been found that the sialic acid residues of β-ovomucin contribute to the binding of ovomucin to NDV (Tsuge et al. 1997b; Watanabe et al. 1998b).

Studies further indicate that ovomucin has immunopotentiator activity. Otani and Maenishi (1994) reported that ovomucin enhanced the proliferation of mouse spleen lymphocytes, stimulated by lipopolysaccharide. Tanizaki et al. (1997) found that the sulphated glycopeptides of ovomucin, chalazae, and yolk membrane revealed strong macrophage-stimulating activity.

Furthermore, there is some evidence that ovomucin can act as an antibacterial agent. Ovomucin has been shown to inhibit colonization of *Helicobacter pylori* (Kodama and Kimura 1999) and enzymatically hydrolyzed ovomucin to exhibit strong deactivation of food-poisoning bacteria (Ryoko et al. 2004).

According to Nagaoka et al. (2002), ovomucin attenuates hypocholesterolemia in rats and inhibits cholesterol absorption in Caco-2 cells.

In summary, ovomucin and ovomucin-derived peptides can be seen to possess different kinds of biological activities. Ovomucin, therefore, seems to be a highly potential source of bioactive ingredients for novel functional foods.

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Chapter 10 Riboflavin-Binding Protein (Flavoprotein)

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1 Introduction

Chicken riboflavin-binding/carrier protein (RfBP), also referred as flavoprotein or ovoflavoprotein, is a globular monomeric phosphoglycoprotein of approximate dimensions $50 \times 40 \times 35$ Å. It has a molecular weight 29.2 kDa and one high-affinity riboflavin (vitamin B2) binding site. Unlike most egg proteins, RfBP is found in egg white and egg yolk of laying hens (Monaco 1997). Egg white RfBP constitutes approximately 0.8% of the total egg white protein (Li-Chan and Nakai 1989).

2 Composition and Structure

Egg white RfBP is a single polypeptide chain of 219 amino acids exhibiting an unusual pyroglutamyl residue as the amino terminus (Hamazune et al. 1984). The polypeptide chain is modified post-traductionally by the phosphorylation of eight serine residues and glycosylation of two asparagine residues. Its high-resolution X-ray crystallographic structure has been reported (Monaco 1997). About 30% of the residues participate to the formation of 6 α-helix designated A to F and a little less than 15% are organized in β structures consisting of four series of discontinuous β sheets designated a to d (Monaco 1997). Four of the α-helix (A–D) and the four β sheets participate to the formation of the riboflavin-binding site while the two remaining α-helix (E and F) surround a flexible highly phosphorylated region that extends between amino acids 186 and 197. The polypeptide chain is organized in two domains: a larger, N-terminal (residues 1–170), containing the riboflavin-binding site and a smaller, C-terminal (residues 171–219), carrying many negatively charged amino acids including eight phosphoserine residues and ten glutamyl residues (Miller et al. 1984). Phosphoserine residues are organized as a dense cluster: Ser^{185}-Glu-Ser(P)-Ser(P)-Glu-Glu^{190}-Ser(P)-Ser(P)-Ser(P)-Met-$Ser(P)^{195}$-Ser(P)-Ser(P)-Ser(P)-Glu-Glu^{200} (Li-Chan et al. Nakai, 1989).

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

RfBP contains 18 cystein residues, all involved in nine disulfide bridges stabilizing the native protein structure (Hamazune et al. 1987). Of these nine bridges, eight are located in the riboflavin-binding domain and the last one links the two domains.

The carbohydrate in RfBP accounts for around 14% by weight and consists mainly of fucose, mannose, galactose, N-acetylglucosamine, and sialic acid. Although the two N-glycolyzation sites (Asn-36 and Asn-147) are contained in a conserved pentapeptide in which four out of five amino acids are identical, the carbohydrates show differences in the glycozylation pattern (type and distribution). Carbohydrates linked to Asn-36 have a lower degree of branching compared to those linked to Asn-147 in which triantennary, tetraantennary, and pentaantennary structures are present (Amoresano et al. 1999). Consequently, it is suggested that a site-specific glycosylation involving the local secondary or tertiary structure, or the order of glycosylation, influences the carbohydrate structure at the two N-glycozylation sites (Rohrer and White III 1992). Carbohydrates attached to egg white RfBP are different from those attached to egg yolk RfBP. These differences are due to the fact that the proteins are synthesized in different tissues and serve different functions.

RfBP is one of the most acidic proteins in egg white, with an isoelectric point close to 4.0. This characteristic is made use of for the extraction of RfBP by ion exchange chromatography (Rao et al. 1997), which offers an alternative to purification procedures by affinity chromatography (Froehlich et al. 1980). By displacement anion exchange chromatography on Q-Sepharose Fast Flow (GE Healthcare, Little Chalfont, UK), large amounts of RfBP are extracted from hen egg white (Guérin-Dubiard et al. 2005).

Riboflavin binds strongly to apo-RfBP at a 1:1 molar ratio; the dissociation constant is around 1.3×10^{-9} M (Becvar and Palmer 1982; Stevens 1996). Riboflavin is constituted of a ribityl chain and an isoalloxazine ring, which latter (responsible for the yellowish color of egg white) is amphypathic due to the hydrophobic properties of the xylene portion and the hydrophilic properties of the pyrimidine moiety (Massey 2000). Visible and ultraviolet irradiation causes the deterioration of free riboflavin into lumiflavin, especially in alkaline solutions. Once bound to the protein, riboflavin is stable to prolonged irradiation by laser pulse (Zhong and Zewail 2001). The riboflavin-binding domain of RfBP is a roughly 20 Å wide and 15 Å deep hydrophobic cleft containing five out of the six tryptophan residues present in the protein. The binding of riboflavin occurs in the cleft with the xylene moiety of the isoalloxazine ring buried most deeply in the protein. Riboflavin is stacked between the parallel plane of the Trp-156 and Tyr-75 of RfBP, inducing a quenching of the riboflavin fluorescence. Additionally, riboflavin interacts with a number of amino acid residues by forming hydrogen bonds and by hydrophobic interactions (Blankenhorn 1978; Monaco 1997).

The cleavage of a single disulfide bond results in a loss of the binding capacity of RfBP. Moreover, modification in one of the carbohydrates results in a decrease in the riboflavin-binding capacity (Miller et al. 1981). Contrarily, the riboflavin-binding capacity of completely dephosphorylated RfBP was undistinguishable from that of the native protein (Miller et al. 1982; Miller et al. 1984). Riboflavin is frequently found in nearly equal amounts in egg white and egg yolk, but the amounts are directly dependent on the dietary intake of the hen (Rhodes et al. 1959). The amount of riboflavin in an egg is limited by the amount of RfBP. Even at high riboflavin intake, little, if any, unbound riboflavin appears in the egg (White III et al. 1986). Usually, egg white RfBP is partially saturated (35–50%) with riboflavin as compared to the fully saturated egg yolk RfBP.

At pH values between about 4.5 and 9, riboflavin is tightly bound to the protein. At pH value below 4.0, the binding constant drops off rapidly: the riboflavin dissociates from RfBP, yielding the apoprotein and the free riboflavin (Rhodes et al. 1959; Kumosinski et al. 1982). The riboflavin-binding site exhibits a constant configuration at pH above 4.5, but at pH lower than 4.0 marked changes are observed in the mobility of aromatic side chains (as determined by circular dichroism), in the molecular volume, surface, and hydration of the protein, as well as in a decrease in anisotropy. Acidic pH values may induce the reorientation of the aromatic ring of tyrosine and tryptophan at the riboflavin binding site. This leads to the opening of the aromatic-rich cleft in the protein and the release of riboflavin (Blankenhorn 1978; Kumosinski et al. 1982). Increasing the ionic strength of RfBP solution produces a significant increase in the dissociation constant value at low pH values, but apparently not at neutral pH values (Becvar and Palmer 1982).

RfBP exhibits a high thermal stability and riboflavin binding increases this property. The thermal denaturation of RfBP is reversible and is described as a two-state model consisting of a sum of two independent processes (non-cooperative). This characteristic may be related to the protein structure and the existence of two distinct domains, which melt in relatively independent ways on heating (Wasylewsky 2000). Ligand binding greatly enhances the thermostability of the N-terminal domain, whereas the thermostability of the C-terminal domain is only slightly affected (Wasylewsky 2004).

Regarding chemical denaturants, native RfBP shows a two-phase unfolding process from 0 to 6 M GdnHCl (Zhong and Zewail 2001). RfBP loses some of its tertiary structure from 0 to 2 M GdnHCl, exists as a stable intermediate between 2 and 4 M GdnHCl and is fully denatured with a loss of its secondary structure from 4 to 6 M GdnHCl (McClelland et al. 1995). All riboflavin molecules are released from RfBP at 3 M GdnHCl as a result of the hydrophobic cleft collapse (Zhong and Zewail 2001). As long as the nine disulfide bonds remain intact, denatured RfBP will refold completely and rapidly after the removal of the denaturing agent. When RfBP is previously reduced, the correct disulfide bonds do not reform spontaneously and the protein loses its capacity to bind riboflavin (McClelland et al. 1995).

3 Synthesis

The biological function of RfBP is to store and transport the water-soluble vitamin riboflavin to the embryo, sustaining its growth and development until hatching. RfBP quantity in egg is relatively abundant among other nutrient-carrier protein because of the critical need for riboflavin for normal embryonic development. Fertilized eggs from laying hens deficient in RfBP are unable to develop into viable embryos. Embryos in these eggs die from riboflavin deficiency after around 13 days of development (White III et al. 1992). The 13th day of incubation coincides with a large increase in flavin kinase activity, an enzyme necessary for the biosynthesis of both FMN and FAD from riboflavin (Stevens 1996). RfBP in egg white serves as a scavenger of riboflavin, which facility may protect embryos from bacterial attack through depriving such micro-organisms of riboflavin.

RfBP in both egg white and yolk is the product of the same gene, but undergoes different post-transcription modifications, i.e., glycosylation patterns and length of polypeptide chains. The nucleotide sequence codes for a 238-amino-acid polypeptide chain, a 17-amino-acid signal peptide released post-transcriptionally, and 2 arginine residues that are not found at the carboxyl terminal extremity of the 219-amino-acid mature protein (Zheng et al. 1988). Egg white RfBP is synthesized in the oviduct, whereas yolk RfBP is synthesised in the liver of laying hens under estrogen control. Once secreted in the blood stream of laying hens, RfBP binds the riboflavin derived from the animal diet, and the holo-RfBP is transported to the chicken oocyte via the follicular circulatory system (MacLachlan et al. 1993). Deposition of the serum RfBP into the yolk of the developing egg is mediated by an oocyte-specific 95 kDa lipoprotein receptor. Located on the surface of the protein, the phosphopeptide is of critical importance for the binding of RfBP to the oocyte-receptor (MacLachlan et al. 1994). In vitro, the serum RfBP uptake by the oocyte decreases dramatically as soon as any phosphate residue is removed from phosphoserine by acid phosphatase. The glycosylation pattern, and particularly the presence of sialic acid, seem to be important for the transport of RfBP to egg yolk. Its transfer through the vitellogen membrane of the oocyte leads to the release of an acidic carboxyl terminal peptide of 11–13 residues. Such cleavage is not observed for egg white RfBP. Serum RfBP is concentrated about nine-fold when taken up into the yolk.

A search for sequence homology reveals that RfBP has significant similarity to the folate-binding proteins. In the N-terminal domain, the sequence identity is more than 30%, a value that exceeds the null hypothesis that the identities are due to chance. The presumption of homology is supported by structural and functional considerations: all but one of the pairs of cystein residues and all six of the tryptophan residues in RfBP are conserved in the folate-binding protein (Zheng et al. 1988). Moreover, the ligand-binding domain of the two proteins shows similar folding in relation to the fact that

folic acid contains a pterin ring structure, which is similar to the riboflavin structure (Foraker et al. 2003).

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Chapter 11 Avidin

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1 Introduction

Early scientific reports described the toxicity of uncooked egg white in rats when administered orally. The resulting syndrome, with peculiar anomalies such as dermatitis, indicated malnutrition and especially vitamin B deficiency (Boas 1927). Later, this malnutrition was attributed to a lack of available biotin (vitamin H or B8), because of the binding of this essential growth factor by the egg white glycoprotein avidin (Gyorgy et al. 1941). The biological function of avidin is still unknown, but because of its high affinity for biotin, it is thought to serve as a defensive protein against biotin-requiring micro-organisms (Korpela et al. 1983).

2 Physiochemical Characteristics

Avidin constitutes a maximum of 0.05% of the total protein content of egg white, i.e., about 50 mg/l (Green 1975). Avidin is an alkaline (pI about 10.5), highly stable, homotetrameric protein, binding up to four molecules of D-biotin with extremely high affinity. Each monomer contains 128 amino acid residues, for a theoretical molecular weight of 14,344 Da. The corresponding theoretical combined molecular weight is then about 57,400 Da, which is low compared to the experimental value (around 67,000 Da, Green 1975), since it does not take into account the glycosylation of avidin.

The carbohydrate moiety represents about 10% of the total molecular mass and is attached to the Asn 17 of each subunit (DeLange 1970). The heterogeneous structure of this oligosaccharide moiety has been determined by Bruch and White (1982); it mainly consists of mannose and N-acetylglucosamine, in a ratio ranging from 1.2 to 2.0. Nonglycosylated avidin was also found in commercial preparations of avidin. But it is not clear whether this form is artifactual and results from purification procedures or whether it reflects actual

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biological differences in the egg white. However, the biotin-binding properties of the nonglycosylated protein were equivalent to those obtained for the native glycosylated avidin molecule, indicating that the oligosaccharide moiety is not essential for the binding activity (Hiller et al. 1987).

The primary amino acid sequence of the monomer has been determined (DeLange 1970; DeLange and Huang 1971), as well as the nucleotide-coding sequence, which indicates a signal peptide of 24 amino acids (Gope et al. 1987). A single intramolecular disulfide bond between Cys4 and Cys83 has been identified (Livnah et al. 1993; Pugliese et al. 1994). This disulfide bridge can be reduced only after dissociation of the tetramer into subunits, but has not been identified as crucial for biotin binding (Green 1963). Fifty percent of the chains would correspond to a variant in which the residue 34 would be threonine instead of isoleucine. One conflict at position 53 (Glu versus Gln) is also mentioned (Chandra and Gray 1990; Wallen et al. 1995).

3 Structure and Interactions

The three-dimensional structure of hen egg white avidin has been investigated from different crystallographic experimentations (Livnah et al. 1993; Pugliese et al. 1994; Rosano et al. 1999). In all crystal structures, the four avidin subunits display an almost exact symmetry. Each avidin monomer is arranged in eight antiparallel β-strands which form a classical β-barrel, whose inner region defines the D-biotin binding site. Livnah et al. (1993) identified three regions involved in the monomer–monomer interactions, which contribute to the rigidity of the quaternary structure of avidin. These interactions involve van der Waals forces, hydrogen-bonding and hydrophobic interactions. The tetramer is dissociated into monomers in 6 M guanidine HCl, but this dissociation has been reported to be largely reversible (Green 1963; Green 1975).

The eight-stranded antiparallel β-barrel described for avidin is remarkably similar to that of the genetically distinct bacterial analogue streptavidin (Livnah et al. 1993), which is also a very stable tetramer with a twofold symmetry. Both proteins show an overall sequence homology of the order of 35%; nearly all of the conserved residues are located in six domains, in which over 60% of the amino acids are identical (Wilchek and Bayer 1989; Bayer and Wilchek 1990). This similarity explains why avidin and streptavidin have similar affinities for biotin, although they are vastly different in other respects. Indeed, outside of the functional domains, the respective sequences are very different. The two proteins have different molecular weights; sequencing of the native streptavidin gene gives a calculated subunit molecular weight of 16,500 and consists of 159 amino acids (Argaraiia et al. 1986). Moreover, streptavidin is not glycosylated and has a pI lower than 7, these differences explaining the lower nonspecific (biotin-independent) binding characteristics of streptavidin, in most instances, as compared to avidin (Bayer and Wilchek 1994). Additionally, the

biotin-binding site in streptavidin is "buried" compared to avidin, as demonstrated by X-ray crystallography studies (Hendrickson et al. 1989).

The avidin biotin-binding site is sterically complementary to the shape and polarity of the incoming vitamin, and is readily accessible in the apoprotein structure (Rosano et al. 1999). It is formed by several aromatic residues constituting a "hydrophobic box" in which the ureido ring of biotin is fixed to the side chains of polar amino acid residues through five crucial hydrogen-bond interactions. Finally, compared to streptavidin, avidin exhibits one additional aromatic residue participating to the stabilization of the "hydrophobic box," and three additional hydrogen bonds for biotin fixation. These two factors could explain why the binding of biotin to avidin is reportedly tighter than to streptavidin ($K_d = 6 \times 10^{-16}$ versus 4×10^{-14} M, respectively; Green 1990).

The avidin–biotin interaction exhibits the strongest noncovalent, biological dissociation constant ever reported for protein–ligand interaction. The avidin–biotin complex is much more heat stable than the apoprotein (Bayer et al. 1996; Gonzalez et al. 1999). It can withstand brief exposures to 132 °C (Donovan and Ross 1973), and the temperature resistance of the complex is increased in the presence of salts; in that case, a temperature of 120 °C for 15 minutes is required for the complete complex dissociation (Pai and Lichstein 1964; Wei and Wright 1964). At pH values between 2 and 13 no significant effect on the avidin–biotin complex is noted; and neither does guanidine HCl up to 8 M at neutral pH have an effect (Green 1975). Several experiments have indicated that detergents decrease the affinity of avidin for biotinylated molecules (Ross et al. 1986).

4 Extraction and Purification

Several methods for extracting and purifying avidin have appeared in the scientific literature, most of them involving multiple-step procedures. The earliest large-scale method was reported by Dhyse (1954). It consisted of six successive purification steps, based on differential protein precipitations. The resultant protein fraction contained about 50% active avidin (Green 1975). Later, several processes based on chromatographic separations have been proposed, involving either ion exchange resins (Melamed and Green 1963), or affinity columns (Cuatrecasas and Wilchek 1968; Heney and Orr 1981). With affinity chromatography, very high recovery yields (95%) and purity rate (99%) have been reported, but the materials are very expensive and their lifetimes are rather limited. This could explain why affinity chromatography is not classically used for large-scale avidin purification. However, an affinity precipitation process using iminobiotin-polymer was proposed by Garret-Flaudy and Freitag (2000/2001), enabling an avidin purity rate higher than 90%. And in 2003, Fan et al. developed a new affinity process using superparamagnetic and water-dispersible gamma-Fe_2O_3 nanoparticles,

functionalized with biotin, to achieve the affinity isolation of avidin with 96% efficiency. A novel ion exchange chromatography procedure described as "elution looping" was suggested and tested (Durance and Nakai 1988a; Durance et al. 1991). This was a single column cation exchange method which allowed the simultaneous recovery of lysozyme and avidin from undiluted egg white. Avidin recovery yield was around 75%, and the purity rate of the avidin fraction could reach 40% when 16 cycles of egg white application and lysozyme elution were achieved. To increase the purity rate of avidin fraction, subsequent chromatography procedures have been tested and compared: cation exchange, gel filtration, metal chelate interaction, and hydrophobic interaction chromatography have been applied on avidin and lysozyme rich egg white fractions (Durance and Nakai 1988b). Carboxymethyl cellulose exchange chromatography appeared superior to the other techniques, with a final avidin purity rate around 85%. A much higher purity rate (99%) was obtained by Piskarev et al. (1990) using ion exchange-HPLC after rough avidin isolation using successive differential $(NH_4)_2SO_4$ and organic solvent precipitations. However, the amount of avidin recovered in a single run was found to be low. Another original way was proposed by Rao et al. (2003) to obtain highly pure avidin fraction with cation exchange chromatography, using hydroxyazobenzene-2′-carboxylic acid (HABA) dye to selectively elute avidin from the exchanger. Preparative electrophoresis was also developed to purify avidin, through a sequential two-step protocol (Rothemund et al. 2002). The avidin recovery yield was around 65%, and 0.1% lysozyme was detected in the final avidin fraction. Both bacterial (Airenne et al. 1994; Hytönen et al. 2004) and eukariotic (Airenne et al. 1997; Kusnadi et al. 1998; Hood et al. 1999) expression systems for the production of recombinant avidin have been established, some of them including site-directed mutagenesis to modify the undesirable properties of avidin and to further expand the existing avidin–biotin technology (Marttila et al. 1998). Avidin-fusion proteins have also been developed with that aim (Airenne and Kulomaa 1995; Airenne et al. 1999).

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Chapter 12 Proteases

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1 Introduction

Proteases (also called peptidases, proteinases, or proteolytic enzymes) are enzymes that hydrolyze peptidyl bonds. They are of great importance in all physiological processes involving maturation and activation of proteins or their degradation (proteolysis) and are found in all biological kingdoms. Each protease is assigned to a family on the basis of a significant identity in amino-acid sequence, and to a clan which gathers families that display a common origin (Rawlings et al. 2006). Proteases are frequently classified according to their catalytic type, which is related to the chemical groups involved in the catalysis of the peptidyl bond. There are currently six clans of proteases. The better characterized clans are the serine proteases, the cysteine proteases, the aspartic proteases, and the metalloproteases. The "threonine proteases" and the "glutamyl proteases" clans contain only one family each.

Identification of proteases in egg white encounters several difficulties due the presence of numerous antiproteases (see Chapter 8) that inhibit their enzymatic activity. No protease could be identified among the major proteins of egg white that have been already identified and characterized, which means that proteases must be present at very low concentration, as minor components, and are likely to be inhibited by antiproteases. To our knowledge, only two reports describe the isolation and the biochemical characterization of two different proteases named "glutamyl aminopeptidase" (Petrovic and Vitale 1990) and "methionine-preferring broad specificity aminopeptidase" (Skrtic and Vitale 1994). Genomic and amino acid sequences are not known and no identification can be made at this time. The biological role of these two proteases has not been investigated yet and no data has been reported as to their isolation from other biological fluids. According to their enzymatic activity, they could be members of the metalloprotease family, in which some such enzymes are known to participate in several processes involving tissue remodeling, such as embryogenesis.

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2 Glutamyl Aminopeptidase (EAP)

EAP has an apparent molecular weight of 320 kDa as assessed by native PAGE. By SDS-PAGE on denatured protein, it appears as a single protein of 180 kDa. Both forms are active, as shown by zymography using α-Glu-4-methoxy-2naphthylamide as a substrate, which suggests that EAP is mainly present as a dimeric form in albumen. The protein seems to be glycosylated since it binds to Con A-Sepharose (GE Healthcare, Little Chalfont, UK); its pI has been determined to be 4.2.

Its enzymatic activity is stimulated by Ca^{2+}, Mn^{2+} ions and inhibited by Zn^{2+}, and Co^{2+}. Its optimal activity pH and temperature are pH 7.6 and 37 °C, respectively. Kinetic constants for the hydrolysis of α-Glu-2naphthylamide by EAP at 37 °C, pH 7.6 are $K_m = 3.1 \times 10^{-4}$ M and $k_{cat} = 191$ sec. This protease seems to be very specific for α-Glu-2naphthylamide and to a lesser extent for α-Asp-2naphthylamide substrates. Its activity can be inhibited by chelating agents such as ethylenedimethyl tetraacetate (EDTA), *N*-bromosuccinimide (NBS), and amastatin, but resists inhibition by serine and cysteine protease inhibitors (phenylmethylsulfonyl fluoride and 4-hydroxymercuricbenzoic acid) as well as bestatin and puromycin. The fact that EAP is activated by Ca^{2+} and inhibited by EDTA suggests that it belongs to the metalloprotease family (Petrovic and Vitale 1990).

The purification protocol described by Petrovic and Vitale (1990) from fresh egg whits involves precipitation of ovomucin in slightly acidic conditions, ammonium sulphate precipitation up to 35–60% saturation, chromatography onto a Sephacryl-200 column followed by a Sephacryl-300 column (both Pharmacia, Uppsala, Sweden) and further fractionation using hydroxylapatite chromatography. The last step of the purification was performed by FPLC on a Mono-Q (GE Healthcare) column (Petrovic and Vitale 1990). Fractions containing EAP were identified by following their ability to hydrolyze α-Glu-2naphthylamide substrate (Petrovic and Vitale 1990).

3 Methionine-Preferring Broad Specificity Aminopeptidase (MAP)

MAP appears as a glycoprotein of 180 kDa on native PAGE and of 120–130 kDa on SDS-PAGE. Its pI range is 4.15–4.40. Optimal pH activity was determined to be 7.0–7.5, obtained at 50 °C. Activity is drastically altered above 60 °C and MAP retains only 10% of its activity at room temperature. MAP preferentially hydrolyzes substrates having a methionine or alanine or leucine at the P1 position (according to Schechter and Berger (1967) nomenclature, where P1 is the residue after which the peptidyl bond is cleaved by the protease). It weakly cleaves substrates containing phenylalanine, tyrosine, tryptophan, arginine,

lysine, glycine, valine, isoleucine, threonine, or serine at this position. MAP has therefore a broad specificity with a marked preference for methionine residue at P1 position.

Bestatin and amastatin are potent inhibitors of this protease. MAP is also sensitive to puromycin whereas the serine protease inhibitor aprotinin is not inhibitory. Its activity is strongly reduced in presence of the chelating agent o-phenanthroline and to a lesser extent EDTA or iodoacetamide. Ca^{2+} and Mn^{2+} ions do not affect activity as opposed to Zn^{2+} (60% and 95% reduction at 0.1 mM and 1 mM, respectively), and Ni^{2+}, Hg^{2+}, Cd^{2+}, and Cu^{2+} weakly reduce it. As for EAP, these characteristics are consistent with a protease belonging to the metalloprotease family (Skrtic and Vitale 1994).

Purification of MAP is performed as described for EAP, except for some minor modifications. MAP purification was followed using leu-2naphthylamide as a substrate as opposed to α-glu-2naphthylamide for EAP. Similarly, homogenized albumen was fractionated by ammonium sulphate precipitation, and fractions corresponding to 45–60% saturation were dialyzed and loaded on consecutive Sephacryl S-200, Sephacryl S-300 (GE Healthcare) and hydroxylapatite columns. MAP was eluted at a low concentration of buffer, consisting of two fractions that were pooled prior to loading on the Mono-Q column. This protease does not have any activity in regard to EAP-specific substrates (Skrtic and Vitale 1994).

4 Others

Metalloprotease 2 (MMP-2) has been recently identified in uterine fluid, albumen, and yolk as an inactive pro- form (Rehault et al. unpublished results) and has been shown to be expressed all along the oviduct. Since MMP-2 is usually associated with tissue remodeling, these results suggest that egg MMP-2 may have a role during embryogenesis.

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Chapter 13 Antiproteases

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1 Introduction

As with proteases, antiproteases (also called protease inhibitors) are classified by families based on amino acid similarities, and families are grouped together in clans (Rawlings et al. 2006). Antiproteases inhibit protease activity through a mechanism that is usually characteristic for each family. Protease–antiprotease balance is known to be involved in major biological and pathological processes, and in egg white, antiproteases are thought to have a role in embryogenesis (Colella et al. 1989; Saxena and Tayyab, 1997) as well as in antimicrobial defense (Chap. 23). However the natural targeted proteases of these egg white inhibitors have not been identified yet and their physiological role remains unclear.

Egg white is a rich source of antiproteases that have been shown to inhibit cysteine, serine, aspartyl proteases, and metalloproteases. Five antiproteases have been discovered so far: ovostatin, ovomucoid, ovoinhibitor, cystatin C, and ovalbumin (Saxena and Tayyab, 1997). Ovalbumin belongs to the *ser*ine *p*rotease *in*hibitor family (serpins) but it does not have any inhibitory activity and is therefore not discussed in this section (see Chap. 8).

Ovostatin is related to the α_2-macroglobulins, which undergo a unique interaction with the four major classes of proteases (cysteine, serine, aspartic proteases and metalloproteases). Inhibition of proteases by macroglobulins involves the cleavage of the macroglobulin-susceptible "bait" region inducing a conformational change of the inhibitor entrapping the protease. The resulting complexes display an increased mobility on SDS-PAGE (Barrett et al. 1979). The macroglobulin scissile bond which is cleaved within the bait region during interaction depends specifically on the protease and on the α2-macroglobulin family's member that are involved (Sottrup-Jensen et al. 1989). One of the unusual features of the macroglobulin–protease interaction is that the thiol–ester bond created between the protease and the inhibitor does not involve the active site of the enzyme. Consequently, small synthetic substrates can still be hydrolyzed by the complexed protease in contrast to protein substrates (or antibodies) which cannot reach the protease active site because of steric hindrance (Barrett 1981).

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Ovomucoid and ovoinhibitor belong to the I1 family that groups together inhibitors that possess one or several Kazal-like units—defined as a consensus sequence with six conserved cysteines involved in disulfide bonds. Kazal-like inhibitors inhibit their targeted proteases by a "standard" mechanism (Laskowski and Kato, 1980). They possess on their surface a peptide bond called "reactive site" which specifically interacts with the active site of the cognate protease. This interaction results in the hydrolysis of the peptide bond and the formation of an enzyme–inhibitor complex. The reaction is reversible and the dissociation allows for the release of active protease and cleaved inactive inhibitor. Ovomucoid and ovoinhibitor possess three and seven Kazal domains, respectively.

Chicken cystatin (egg white cystatin) is the first identified member of the "cystatin" family (Barrett 1981), which is subdivided into four classes: A, B, C, and D. Chicken cystatin belongs to class B and inhibits cysteine proteases from the papain family. Like the other members of this subfamily, it contains two disulfide bonds. The inhibition mechanism of cystatins does not obey the "standard" mechanism described previously. The reactive site of chicken cystatin (and other members of this family) includes a peptide bond between Gly-9 and Ala-10, the QLVSG variation sequence of the QVVAG consensus sequence, and a region of the protein around the residue tryptophan at position 104 (Lindahl et al. 1988; Bode et al. 1988).

2 Ovostatin

Ovostatin belong to α_2-macroglobulin family. It is a very high molecular weight tetramer of subunits that are similar, and of which two pairs are bound by disulfide bonds (dimers) and assembled non-covalently. Ovostatin appears as a single band at the top of a 5% SDS-PAGE gel under non-reducing conditions with an apparent weight of 165 kDa under reducing conditions (Kitamoto et al. 1982; Nagase and Harris 1983a). Molecular weight of the tetramer could be estimated by ultracentrifugation studies, which gave a value of about 780 kDa. Ovostatin's pI is 4.9, and it is labile at acidic pH, relatively stable at alkaline pH (Nagase and Harris 1983a).

This antiprotease has been shown to inhibit several metalloproteases, such as collagenase, thermolysin, stromelysin (Nagase and Harris, 1983b, Enghild et al. 1989), and with a lesser efficiency, serine proteases including chymotrypsin, trypsin, neutrophil elastase, but also cysteine proteases (papain) and the aspartyl proteases, pepsin and renin (Nagase and Harris, 1983b, Ikai et al. 1989; Kato et al. 1991). Protease–antiprotease complexes display an increased electrophoretic mobility (Nagase and Harris, 1983b), which allows for the rapid discrimination between "reacted" and "unreacted" macroglobulins. As opposed to α_2-macroglobulin where a thiol-ester bond is formed upon interaction with proteases, interaction of proteases with ovostatin does not

involve this characteristic covalent bond (Nagase and Harris, 1983b; Nielsen and Sottrup-Jensen 1993). Ovostatin seems relatively specific to oviduct and egg white (Nagase et al. 1983a).

The purification of ovostatin described by Nagase and Harris (1983a) is the method most frequently used. It involves polyethylene glycol 8000 precipitation (GE Healthcare, Little Chalfont, UK), ACA 34 Ultrogel (Pall, East Hills, NY, USA) chromatography followed by anion-exchanger chromatography (diethyl-amino-ethyl (DEAE)-cellulose) in which ovostatin is eluted in a linear gradient of NaCl (0.02–0.4 M). Ovostatin is finally purified by Sephacryl S-300 (GE Healthcare) chromatography. Ovostatin purification can be monitored by analysis on SDS-PAGE and by assessment of its inhibitory activity towards thermolysin. Using this protocol, 50 mg of ovostatin can be recovered starting with 450 mL of albumen (Nagase and Harris 1983a).

3 Ovomucoid

Ovomucoid has a theoretical molecular weight and pI of 20.1 kDa and 4.82 respectively. This glycoprotein contains both sialyloligosaccharides and sulphated oligosaccharides (Yamashita et al. 1983) and is visualized by SDS-PAGE as a large band ranging from 30 to 40 kDa (Yousif and Kan 2002). Out of the six potential glycosylation sites, only five are actually glycosylated (Beeley 1976). It possesses nine disulfide bonds.

Ovomucoid contains three functional homologous Kazal-like domains characterized by different reactive sites (Kato et al. 1987). Ovomucoid seems to be a single-headed inhibitor that combines only one molecule of protease at once, in contrast to turkey ovomucoid and duck ovomucoid, which are respectively double-headed and triple-headed (Rhodes et al. 1960; Kato et al. 1987). Chicken ovomucoid has been shown to inhibit trypsin and chymotrypsin (Feeney et al. 1963; Kato et al. 1987; Kojima et al. 1999) but also locusta trypsin and chymotrypsin-like enzyme (CTLE) isolated from the digestive tract of the African migratory locust *Locusta migratoria* (Sakal 1988, 1989). This antiprotease is synthesized in the oviduct and its expression is regulated by the steroid hormones, estrogen and progesterone (Palmiter 1972a,b, Compere 1981).

Ovomucoid from fresh egg whites was shown to be chromatographically purified using a three-step method involving carboxymethyl (CM)-cellulose/DEAE-cellulose/CM-cellulose (Whatman, Brentford, Middlesex, UK; Rhodes et al. 1960) or a batch procedure including Bio-Gel CM (Bio-Rad, Hercules, CA, USA) and DEAE-cellulose (Davis et al. 1971).

However, as the first step of ovomucoid purification, the trichloracetic and acetone precipitation method is more routinely used (Lineweaver and Murray 1947). Ovomucoid can be further purified by several cold acetone purification steps (Lineweaver and Murray 1947; Bogard et al. 1980; Kato et al. 1987) and

chromatographied onto a PD-10 column (GE Healthcare; Bogard et al. 1980) followed by a carboxymethyl-Sepharose (GE Healthcare; Kato et al. 1987).

Fredericq and Deutsch (1949) reported purification of ovomucoid using a combination of trichloracetic acid precipitation and ethanol fractionation that was demonstrated to display the advantage of being less denaturing than the TCA–acetone precipitation described above. In this article, it is shown that the purity of the ovomucoid from egg white intimately depends on the pH chosen to perform trichloracetic precipitation. The best yields are obtained at pH 3.5 in which the supernatant contained 99 to 100% ovomucoid and only 0 to 1% ovalbumin contaminant. Ovomucoid purification is achieved by two other ethanol precipitation steps (Fredericq and Deutsch 1949). Alternatively, purified ovomucoid from crude ovomucoid can be obtained using a Bio-Scale Q5 column (Bio-Rad) (Kovacs-Nolan et al. 2000) or by a 90% ammonium sulfate precipitation followed by Sulfoethyl-Sephadex column (GE Healthcare; Waheed and Salhuddin 1975).

4 Ovoinhibitor

SDS-PAGE analysis indicates that ovoinhibitor is a 48 kDa protein (Liu et al. 1971). It's a glycoprotein, the sequence of which contains seven Kazal-like domains involving 21 disulfide bonds (Yet and Wold 1990; Rhodes et al. 1960; Scott et al. 1987). Ovoinhibitor inhibits serine proteases such as trypsin and chymotrypsin, but also elastase (Feeney et al. 1963; Gardi and Lungarella 1987; Vered et al. 1981; Gertler and Ben-Valid 1980), a collagenic serine protease from catfish (Yoshinaka et al. 1986) and the bacterial protease F (Birk et al. 1983). Oxidation of methionine residues of ovoinhibitor results in a loss of its inhibitory activity against trypsin, chymotrypsin, and elastase (Schechter et al. 1977). Domain I is a potent inhibitor of trypsin but is devoid of inhibitory activity against chymotrypsin, elastase or proteinase K (Galzie et al. 1996). Domains I to IV contain an arginine at the P1 position (according to Schechter and Berger nomenclature, 1967) and thus are thought to inhibit trypsin-like enzymes. Domain V has a phenylalanine at P1, which is consistent with antichymotrypsin activity, and domains VI and VII possess a methionine at P1, making them likely to inhibit chymotrypsin and elastase (Scott et al. 1987). Ovoinhibitor expression is under estrogen control (Zhu et al. 2001). It has been identified in liver, oviduct, and bursa of Fabricius (Scott et al. 1987; Moore et al. 2004).

Ovoinhibitor could be purified as described by Davis et al (1969). This protocol starts with several steps of ammonium sulphate precipitation. The fraction obtained is then passed through a P-150 column (Regent Medical, Irlam, UK) from which three peaks could be eluted, the second one containing ovoinhibitor and globulins. This latter fraction is concentrated, further precipitated with saturated ammonium sulphate and applied to a DEAE-cellulose column (Davis et al. 1969; Begum et al. 2003).

5 Chicken Cystatin

Secreted cystatin has a theoretical molecular weight of 13,147 Da. Two major isoelectric forms differing by the occurrence of phosphorylation on serine 80 have been identified (Laber et al. 1989). The nonphosphorylated and the phosphorylated forms display a pI of 6.5 and 5.6, respectively, and can be separated from each other by chromatofocusing (Anastasi et al. 1983). By SDS-PAGE, the two forms are indistinguishable, with an apparent molecular weight of 14.4 kDa (Anastasi et al. 1983). Cystatin does not possess any carbohydrates (Anastasi et al. 1983). Its X-ray crystal structure has been solved by Bode et al. (1988).

Cystatin inhibits most of the cysteine proteases with a 1:1 stoichiometry, including ficin, papain (Fossum and Whitaker 1968; Machleidt et al. 1989), cathepsin B, H, L, and papaya peptidase (Anastasi et al. 1983), chymopapain and actinidin (Bjork and Ylinenjärvi 1990). Interaction of human cathepsin C with chicken cystatin is characterized by a 4:1 stoichiometry in which one molecule of this oligomeric protease binds four molecules of cystatin (Dolenc et al. 1996). Inhibition of papain by cystatin forms a non-SDS-stable reversible complex (Anastasi et al. 1983; Bjork et al. 1989). Chicken cystatin is found in chicken egg and sera but also in lung, gizzard, brain, heart, oviduct, and is expressed by muscle cells and by the developing chicken embryo (Anastasi et al. 1983; Colella et al. 1989).

Anastasi et al. (1983) proposed a three-step method to purify cystatin, including precipitation of ovomucin, affinity chromatography on a carboxymethylated papain–Sepharose, and chromatofocusing, which latter can be replaced by DEAE chromatography (Lindahl et al. 1988). Trziszka et al. (2004) reported cystatin purification by compiling ovomucin precipitation, affinity chromatography on a papain-Sepharose, gel filtration on Sephadex G-100 (GE Healthcare), DEAE and reverse phase chromatography. Another method described by Turk et al. (1983) includes the adjustment of egg white pH to pH 12 and preheating at 62 °C. The resulting sample can be applied to a CM-Sepharose, loaded onto a phenyl-Sepharose, followed by a MonoS cation-exchange column (GE Healthcare; Gerhartz et al. 1997).

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Chapter 14 Minor Proteins

Catherine Guérin-Dubiard and Françoise Nau

1 Introduction

Egg white is generally presented as an aqueous solution of major and minor proteins. Structure and properties of major proteins are well known, whereas the minor proteins have been little studied, and all are not yet identified. Unfortunately, until recently no highly efficient methods were available to simultaneously analyze all the hen egg white proteins. Indeed, this biological fluid is unique and presents some difficulties for analysis.

The proteins have very different molecular weights (12.7 to 8,000 kDa) and pI values (4 to 11; Li-Chan and Nakai 1989). Concentrations of these minor proteins differ greatly, and complicated by the fact, for instance, that ovalbumin represents more than 50% of total proteins, their detection is often very difficult. These reasons probably explain why very little research has been devoted to the identification and characterization of the minor egg white proteins, even though they probably play essential roles with respect to the primary biological action of egg white, i.e., embryo protection and development. So, hen egg white remains surprisingly uncharacterized. The identification of these minor proteins necessitates high-resolution methods for protein separation, and two-dimensional electrophoresis has proved to be particularly efficient. This technique has highlighted the proposition that indeed many unidentified minor proteins are present in hen egg white. (Desert et al. 2001). Some of these minor proteins presented in this section were very recently identified.

2 Lipocalins

The lipocalin protein family is a large group of small extracellular proteins. Members of this family are characterized by several common molecular recognition properties: the ability to bind a range of small hydrophobic molecules (lipids, steroid hormones, retinoids) to specific cell-surface receptors,

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and to form complexes with soluble macromolecules (Flower 1996). In the past, the lipocalins have been classified as transport proteins, but it is now clear that lipocalins exhibit great functional diversity. This widely spread protein family is represented in hen egg white by three minor proteins, among them two were recently identified in hen egg white, and belong to the chondrogenesis-related lipocalin subfamily.

2.1 Extracellular Fatty Acid Binding Protein (Ex-FABP)

Ex-FABP, also called Ch21 protein or quiescence-specific protein, was observed for the first time in hen egg white by Larsen et al. (1999). This 21 kDa lipocalin is also present in chicken serum, and represents the first extracellular protein able to selectively bind and transport, in extracellular fluids and serum, fatty acid and in particular long chain-unsaturated fatty acid, (Cancedda et al. 1996). These authors purified the corresponding recombinant protein, and two isoforms have been separated (18 and 17.5 kDa) by ion-exchange chromatography. Guérin-Dubiard et al. (2006) detected a single form of Ch21 in egg white by 2D-electrophoresis; apparent molecular weight and pI were 21 kDa and 5.7, respectively. Ex-FABP is also expressed during chicken embryo development, during chondrocyte and myoblast differentiation, in hypertrophic cartilage, muscle fibers, and blood granulocytes (Descalzi-Cancedda et al. 2001). In adult cartilage, it is expressed only in pathological conditions such as in dyschondroplastic and osteoarthritic chickens (Cancedda et al. 2002). Gentili et al. (2005) also proved that Ex-FABP may act as a survival protein by playing a role as scavenger for fatty acids. By injecting antibody against Ex-FABP in chicken embryos, almost 70% of chicken embryos die; the target tissues is the heart. Qiu et al. (2005) proposed that this protein regulates the metabolism of fatty acid, muscle fiber, bone; they indicated that *Ex-FABP* is the main gene that regulates abdominal fat trait in chicken.

2.2 Chondrogenesis Associated Lipocalin Gamma (CAL gamma)

CAL gamma was first identified in egg white by Nau et al. (2003a); its encoding gene has been sequenced by Pagano et al. (2003). In fact, Pagano et al. (2002) already sequenced two other genes, Ex-FABP and CAL-beta. They suggest that the three genes may form a genomic cluster that can be coordinately regulated. Because of their similarity and peculiar developmental patterns of expression in chicken embryos, Pagano et al. (2003) suggested a synergistic action of CAL-gamma and Ex-FABP in the process of endochondral bone formation. Two isoforms of CAL-gamma can be separated in egg white, with apparent molecular weight and pI estimated by 2D-electrophoresis at 22 kDa, 5.6 and 6.0, respectively (Guérin-Dubiard et al. 2006).

2.3 Ovoglycoprotein

Ovoglycoprotein, a third protein of lipocalin family, was first detected in egg white many years ago (Ketterer 1962, 1965). Although it represents about 1% of egg white proteins, very little information is available about this protein. Currently, ovoglycoprotein is mainly used as a chiral selector to separate drug enantiomers by high performance liquid chromatography (HPLC) or capillary electrophoresis (Sadakane et al. 2002). Using ion-exchange chromatography, Guérin-Dubiard et al. (2005) produced fractions enriched in ovoglycoprotein. It is an acidic glycoprotein (pI 3.9) with a theoretical molecular weight of 20.3 kDa and a sugar content of 30% (Li-Chan and Nakai 1989).

3 Clusterin

Clusterin is a ubiquitous and highly conserved secreted glycoprotein. It is found in numerous biological fluids including semen, urine, and human plasma (Mahon et al. 1999). It was immunodetected in several chicken tissues including magnum, egg-shell, and egg white by Mann et al. (2003). Guérin-Dubiard et al. (2006) also confirmed clusterin presence in egg white by 2D-electrophoresis coupled with mass spectrometry (LC/MS/MS). Clusterin is a member of the chaperone proteins, which interact and stabilize unfolded or partly folded proteins, preventing their aggregation or precipitation. According to Poon et al. (2002), clusterin prevents the slow aggregation of proteins often associated with Alzheimer's, Creutzfeldt-Jakob, and Parkinson's diseases. Clusterin is characterized as α/β heterodimer, which is detected at 35 kDa under reducing conditions in electrophoresis (Mann et al. 2003). These authors determined the cleavage site, and the corresponding theoretical molecular weights: 24.5 kDa and 24.8 kDa for α and β clusterin, respectively. By 2D-PAGE analysis, Guérin-Dubiard et al. (2006) have revealed three clusterin isoforms (pIs 6.10, 6.30, and 6.60) with apparent molecular weight 33 kDa. They suggest that these isoforms could be attributed to the two clusterin monomers (α and β) more or less glycosylated.

4 HEP21

HEP21 is an egg white protein recently identified by Nau et al. (2003b). It is a new member of the multifunctional uPAR/CD59/Ly6 snake toxin protein superfamily. The uPAR protein (urokinase plasminogen activator receptor) is an important mediator in the cellular process of cancer development and invasion, angiogenesis, and metastasis (Andreasen et al. 2000; Mazar 2001). The Ly6-type antigens are involved in T-lymphocyte activation (Palfree et al. 1988;

Gumley et al. 1995). The CD59 proteins are the most potent inhibitors of complement-mediated lysis (Brooimans et al. 1992). Unlike most members of this protein family, HEP21 is glycosylphosphatidylinositol (GPI)-anchored and it is a secreted protein predominantly expressed in a tissue, e.g., the oviduct, and especially in the magnum where the egg white components are secreted. By 2D-electrophoresis, HEP21 is revealed as two isoforms with apparent molecular weights (17.1 and 18.1 kDa; Guérin-Dubiard et al. 2006) significantly different from each other and much higher than the theoretical value (10 kDa) according to encoding gene sequence. This gap could result from the glycosylation of this protein, as suggested by Nau et al. (2003b); indeed, most of the listed members of the uPAR/Ly6 family are glycosylated. The apparent pIs (6.3 and 6.4) were consistent with the theoretical value (6.7). It is still impossible to predict the biological activity of HEP21, because of the very wide spectrum of activities of the multifunctional uPAR/CD59/Ly6/snake toxin family.

5 Tenp

Tenp was revealed for the first time in unfertilized hen egg white by Guérin-Dubiard et al. (2006). Its gene was first identified and characterized by Yan and Wang (1998), who described it as a gene specifically and briefly expressed in developing neural tissues including the brain and the retina. A search of domain signature patterns clearly highlights a very strong homology between the end of the Tenp protein sequence and the C-terminal sequence domain of the BPI/LBP/CETP proteins (bactericidal permeability-increasing protein, lipopolysaccharide-binding protein, and cholesteryl ester transfer protein, respectively; Guérin-Dubiard et al. 2006). The biological activity assumed for such a BPI protein is to bind to the Lipid A component of lipopolysaccharide (LPS) in the outer envelope of Gram-negative bacteria (Bingle and Craven 2004). This binding causes immediate arrest of bacterial growth, linked to alterations in the outer membrane, followed later by bactericidal events coincident with damage to the inner membrane (Elsbach and Weiss 1998). Tenp could then be included as a participant in the anti-bacterial activity of hen egg white, but this assumption has to be confirmed. From a structural point of view, Tenp was revealed as three isoforms by 2D-electrophoresis, with an apparent molecular weight of 49 kDa and pIs of 5.9, 6.1, and 6.3 (Guérin-Dubiard et al. 2006).

6 Vitelline Membrane Outer Protein 1 (VMO1)

The VMO-1 protein is one of the proteins identified in the outer layer of egg vitelline membranes, and characterized by Back et al. (1982). Three other proteins were also found to compose this outer layer: ovomucin, lysozyme,

and a second vitelline membrane outer protein (VMO2), which was highlighted by Kido et al. (1992). VMO1, VMO2, and lysozyme bind tightly to ovomucin and participate in the vitelline membrane structure. Schäfer et al. (1998) showed that egg storage in non refrigerated conditions led to disintegration of VMO1 and VMO2 proteins from the vitelline membrane, leading to membrane deterioration. This could explain why Guerin-Dubiard et al. (2006) detected VMO1 for the first time among hen egg white proteins. They determined apparent molecular weight of VMO1 (17.6 kDa), which was consistent with the theoretical value (17.9 kDa) and with the apparent molecular weight (17 kDa) as determined in SDS-PAGE by Schäfer et al. (1998). Even though its pI could not be precisely determined, VMO1 clearly appeared in the alkaline area of 2-Dgel (pI near 10).

7 Conclusion

The list of egg white minor proteins presented here is certainly incomplete. Putative minor proteins currently masked under the major proteins are yet to be identified and analyzed. It is very important to emphasize that these proteins are involved with several protein families with various biological functions, among which some could be good subjects for further egg white evaluation.

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Chapter 15 Structure and Formation of the Eggshell

Yves Nys and Joël Gautron

1 Introduction

The avian eggshell is made of an organic matrix (3.5%), comprising the eggshell membranes and some constituents embedded in the layer of calcium carbonate (95%) in the form of calcite. The chicken eggshell is a natural porous bioceramic resulting from the sequential deposition of the different layers within the segments of the hen oviduct over a predetermined period. It is a perfectly ordered structure with a polycrystalline organization throughout the calcified shell described in many reviews (Hamilton, 1986; Tullet 1987; Nys et al. 1999, 2001, 2004).

2 Structure of the Eggshell

From inside to outside six different layers are observed (Fig. 1). The most internal layers are the shell membranes. The inner shell membrane (about 20 μm) is in direct contact with the albumen. The outer membrane is about 50 μm thick and is located between the inner membrane and the calcified part of the shell. Both inner and outer membranes are made up of organic fibers lying parallel to the egg surface and easily visible using scanning electron microscopy (SEM; Solomon 1991) (Fig. 1). This structure is important in acting as a barrier against microorganism penetration. Their organic composition has been subject to controversy. The identification of desmosine and isodesmosine suggested the presence of elastin-like proteins (Leach 1982) but did not fit with the low concentration of glycine. Presence of hydroxylysine revealed presence of collagen, which was identified by immunohistochemistry as collagen types I, V, and X (Wong et al. 1984; Arias et al. 1997; Wang et al. 2002). However, the bulk of the amino acid composition differs from collagen and suggests that collagen is not predominant but that a unique protein containing lysine-derived cross links may be present (Leach 1982).

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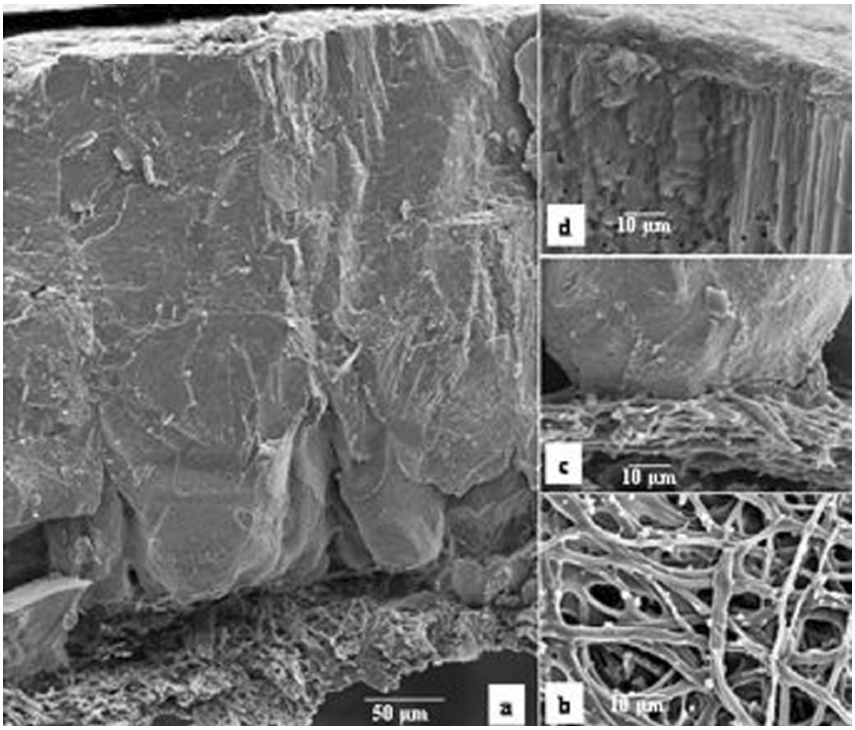

Fig. 1. Scanning electron micrographs illustrating the highly ordered calcareous structure of the chicken eggshell. **(a)** a cross-section through a fully formed eggshell that reveals the eggshell membranes, the cone mammillary layer, the palisade layer and cuticle; **(b)** the inner shell membranes showing the network of interlacing fibers; **(c)** a cross-section through the cone layer showing the insertion of fibers into the tips of the cone; **(d)** the vertical crystal layer at the upper part of the palisade layer and the cuticle overlying on the mineralized eggshell (Nys et al. 2001)

The inner calcified layer (cone or mammillary layer) is about 70 µm thick and is composed of basal parts of calcified columns and cones that penetrate the outer eggshell membranes (Fig. 1). On the surface of the outer eggshell membranes some organic cores (mammillary knobs) are deposited during the passage of the forming egg in the red isthmus (see below under "Eggshell Formation"). These organic cores are the seeding sites on which the calcium carbonate crystals deposition is initiated, beginning with a spherulitic deposition of microcrystals of calcite (the calcium carbonate polymorph of the shell) around the mammillary knobs resulting in hemispherical nucleation centers. The inward mineralization is, however, inhibited by the structure of the eggshell membranes and the presence of collagen X that facilitates this inhibition (Arias et al. 1997). In addition, the radial crystallites growing outward compete for growth space, and a columnar structure progressively rises. Only crystals oriented perpendicular to the outer eggshell membrane will grow, thus explaining the preferred orientation of the crystal in the palisade layer.

This palisade layer starts when the cones are cemented together at the top to form a compact shell. The palisade layer is made of calcite crystals in which an organic matrix (2–3%) is embedded. This layer (200 μm) represents two thirds of the total thickness of the shell. Pores are formed when adjacent columns are not amalgamated. These pores are essential to allow exchange of gas between outside and inside. The palisade layer extends beyond the bases of the cones and ends in the vertical crystal layer. It's a narrow band of vertically oriented crystals that are aligned perpendicular to the shell surface (Fig. 1).

The most external layer is the cuticle. It's a 10 μm organic layer that contains the majority of the eggshell pigments (Nys et al. 1991). It contains a thin film of hydroxyapatite crystals in the inner part of the cuticle (Dennis et al. 1996). The cuticle clothes the shell and plugs the pores, thus preventing microbial penetration. Using SEM, cuticle has a cracked appearance (Solomon 1991) that is a feature of drying. Cracks are essential for the exchange of gas via pores.

3 Eggshell Formation

The chicken eggshell is formed in the distal part of the oviduct. The forming egg with the egg white around the yolk, enters the white isthmus where eggshell membranes are deposited. In the red region of the isthmus (tubular shell gland), the mammillary knobs are deposited and the mineralization of the shell is initiated. In the uterus (shell gland pouch), the mineralization continues to form the compact shell. During its mineralization, the egg bathes in an acellular milieu (the uterine fluid) that contains all minerals and organic components necessary to its formation (Nys et al. 1991; Gautron et al. 1997). The eggshell calcification process can be divided into three stages: (1) the initial stage when the first crystals are deposited on the specific nucleation sites (mammillary knobs) on the surface of the outer eggshell membrane; (2) the growth phase when there is a rapid mineralization of the shell during the elaboration of the compact shell (palisade layer); and (3) the terminal phase when there is an arrest of the shell calcification during the cuticle deposition. Prerequisite to calcium carbonate precipitation is a hypersaturated milieu relative to the calcite solubility product, which depends on temperature and ionic constituents of the solution. In the chicken uterine fluid, bicarbonate concentrations are close to 100 mM and the ionic calcium concentration ranges from 5 to 10 mM. Therefore, the concentration of ionic elements is 60- to 100-fold hypersaturated relative to the solubility product of calcite. This is in agreement with the *in vitro* observation of spontaneous calcium carbonate precipitation in the uterine fluid just after collection (Nys et al. 1991; Gautron et al. 1997). The concentration of total phosphorus is higher in the uterine fluid collected at the final stages of shell calcification (Ogasawara et al. 1974; Murakami and Koga 1991; Nys et al. 1991; Gautron et al. 1997). This result is in agreement with the deposition and localization of

phosphorus in the upper part of the palisade layer and in the cuticle (Quintana and Sandoz 1978; Dennis et al. 1996). Phosphorus is well known to be poisonous for the calcium carbonate precipitation and it has been suggested that this element, secreted at the terminal phase, might inhibit the calcification process. The observation that uterine fluid collected at the end of shell calcification inhibits the calcium carbonate precipitation (Gautron et al. 1997) supports this hypothesis. This effect might be due to phosphorylated macromolecules rather than inorganic phosphorus, as suggested by the absence of inhibition of the ultrafiltrate (mineral) phase of the uterine fluid.

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Chapter 16 Eggshell Matrix Proteins

Joël Gautron and Yves Nys

1 Introduction

The chicken eggshell matrix is a complex mixture of proteins and polysaccharides (Tullet 1987; Nys et al. 2001, 2004). The total organic material is about 2.5% of the calcified layer (Panhéleux et al. 2000). At least 70% of the matrix is protein (Tullet 1987). About 11% is polysaccharide that contains chondroitin sulphate A and B, dermatan sulphate, keratin sulphate, and uronic and hyaluronic acids (Leach 1982; Tullet 1987; Nakano et al. 2001, 2002). Dermatan and keratan sulphate glycosaminoglycans have been biochemically and immunohistochemically demonstrated and localized (Arias et al. 1993; Carrino et al. 1997). The appearance of a keratan sulphate proteoglycan, secreted by the isthmus gland cells (Fernandez et al. 1997), coincides with the formation of the mammillae and its location corresponds with the site of nucleation of the first crystals.

Since 1990, numerous efforts have been carried out to identify and characterize the protein components of the calcified shell. Organic components can be extracted from the shell after demineralization using chelating agents. This method is limited by the low solubility and the high aggregation ability of the matrix extracted from the shell. An alternative source of matrix components is the uterine fluid. This acellular milieu bathes the egg during the shell deposition. It contains the precursors of the matrix in a soluble, functional, and native form prior to their incorporation in the shell (Gautron et al. 1997). It can be collected at the three stages of eggshell calcification (initial, growth, and terminal phases of shell calcification). Matrix proteins were identified using varied biochemical and molecular biological techniques (microsequencing, production of specific antibodies, and expression screening of cDNA libraries prepared from messenger RNA extracted from the uterus collected during the shell calcification). These approaches in combination with the recent development of the functional genomic tools and the sequence of the chicken genome led to the identification of a variety of eggshell matrix components that can be divided into three groups according to their origin.

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2 Egg White Proteins

This group is composed of egg proteins originally characterized in the egg albumen. Ovalbumin is the first egg white protein that was also observed in the shell (Hincke 1995). It is localized in the mammillae of the eggshell (Hincke 1995) and is secreted in abundance in the uterine fluid at the initial stage of eggshell formation (Gautron et al. 1997). Lysozyme and ovotransferrin, two other major egg white proteins, were also identified in the eggshell (Hincke et al. 2000; Gautron et al. 2001a). They were found to be present mainly in the basal parts of the shell (eggshell membranes, mammillae) in agreement with their secretion pattern during the initial phase of shell calcification in the uterine fluid. Their presence in the matrix is not only the consequence of a passive diffusion through the oviduct, but is also due to a local secretion of the tubular gland cells of the uterus, which has the ability to synthesize these proteins. In addition to their well known functions, lysozyme and ovotransferrin interact *in vitro* with the calcium carbonate polymorph (calcite) of the shell indicating that they could play a role in the mineralization process.

3 Ubiquitous Proteins

This group is made of proteins that are widely expressed in various organs and milieus. Osteopontin is a phosphorylated glycoprotein of bone, kidney, and various body secretions. The mRNAs coding for osteopontin were expressed in the epithelial cells of the uterus during the calcification of shell (Pines et al. 1994). Its expression is daily upregulated by mechanical strains upon the uterine wall when the egg enters the uterus (Lavelin et al. 1998).

Osteopontin is localized in the core of the non-mineralized shell membrane fibers, in the mammillae, and in the outermost part of the palisade layer of the shell (Fernandez et al. 2003). Chicken eggshell osteopontin is an inhibitor of calcium carbonate precipitation. Its inhibitory activity is removed when osteopontin is dephosphorylated using alkaline phosphatase (Hincke and St Maurice 2000), suggesting that osteopontin might act as a modulator of the calcium carbonate precipitation in the supersaturated uterine fluid, or even as an inhibitor at the end of calcification.

Clusterin a secretory disulphide-bonded heterodimeric glycoprotein was shown to be a component of the eggshell matrix (Mann et al. 2003). This protein is expressed in many tissues and is secreted by the tubular gland cells of the uterus in the uterine fluid, whatever the stage of shell calcification. Clusterin is detected in all calcified regions of the shell. Clusterin might act as an extracellular chaperone in the uterine fluid to prevent the premature aggregation and precipitation of eggshell matrix components before and during their assembly into the rigid protein scaffold necessary for ordered mineralization.

4 Proteins Unique to the Eggshell

This group is made of organic components that are novel and specific to the shell. They are only secreted by tissues where eggshell formation takes place and have only been identified in domestic hens.

Ovocleidin-17 (OC-17) was the first matrix protein purified to homogeneity (Hincke et al. 1995). It is synthesized in the tubular gland cells of the uterus and secreted into the uterine fluid in relative abundance during the calcification growth phase. It is present throughout the entire calcified part of the shell. OC-17 is a 142 amino acid phosphorylated protein with a C-type lectin domain (Mann and Siedler 1999; Reyes-Grajeda et al. 2004) also present in a minor form as a glycosylated 23 kDa protein (Mann 1999). *In vitro*, OC-17 modifies the shape of calcium carbonate crystals (Reyes-Grajeda et al. 2004). Of particular interest is the report of related proteins with a similar C-type lectin domain in other avian species. Ansocalcin is a goose protein of 40% identity with OC-17 (Lakshminarayanan et al. 2002, 2003). In ostrich, struthiocalcin I and II showed 65% identity with goose ansocalcin and 41% with OC-17 (Mann and Siedler 2004). Emu and rhea also exhibit C-type lectin-like proteins related to OC-17; they have been named dromaiocalcin-1 and 2 and rheacalcin-1 and 2 (Mann 2004, Mann and Siedler 2006).

Ovocleidin 116 (OC-116) was the first eggshell matrix protein that was cloned (Hincke et al. 1999). It's a 80 ka protein (742 amino acids) that contains two N-glycosylation and two disulphide bonds (Mann et al. 2002). This protein is secreted in the uterine fluid during the active calcification phase and is abundant in the palisade layer of the shell. OC-116 is the protein core of the major proteoglycan of the eggshell (Carrino et al. 1997); it was named ovoglycan (Fernandez et al. 2001, 2003). Its carbohydrates contain 17 different oligostructures (Nimtz et al. 2004). Four of them were of the high-mannose type, eight were hybrid types, and the remaining five were complex-type structures. This protein is present in the uterine fluid as a 116 kDa band that corresponds to the OC-116 modified by glycosylation, and also to an electrophoretic band of 190 kDa after glycanation. Eggshell dermatan sulphate proteoglycans are known to alter the morphology and to decrease the size of calcite crystals *in vitro* (Arias et al. 1992; Fernandez et al. 1997). Consequently, this eggshell matrix protein is thought to play an important role in the regulation of calcite growth.

Ovocalyxin-32 (OCX-32) is secreted by the surface epithelial cells of the uterus in the uterine fluid during the terminal phase of calcification and consequently is mainly localized in the most external part of the shell (palisade layer, vertical crystal layer and cuticle; Gautron et al. 2001b, 2003; Hincke et al. 2003). Therefore, its role has been associated with the termination of the shell calcification process. Database searching with the predicted protein sequence demonstrated homology to 2 distinct mammalian proteins, each with ~30% identity with OCX-32 after alignment. One is latexin, a carboxypeptidase inhibitor restricted to a subset of neurons and a few non-neural tissues (Hatanaya et al. 1994; Normant et al. 1995; Uratani et al. 2000). The other one is the putative

translation product of a tazarotene-induced gene 1 (*TIG1*), a retinoic-acid-receptor -responsive gene. (Duvic et al. 1997; Nagpal et al. 1996). The functional relationship of these mammalian proteins to OCX-32 is not clear.

Ovocalyxin-36 (OCX-36) has recently been cloned (Gautron et al. 2007). This protein is abundant in uterine fluid during the shell calcification. OCX-36 expression is strongly upregulated during eggshell calcification. OCX-36 is localized to the eggshell membranes and is most abundant near the calcified shell. OCX-36 is homologous to proteins associated with the innate immune response: lipopolysaccharide-binding proteins (LBP), bactericidal permeability-increasing proteins (BPI), and Plunc family proteins. OCX-36 may therefore participate in natural defense mechanisms that keep the egg free of pathogens.

Recently ovocalyxin-25 and -21 have been cloned in our laboratory (Gautron et al., unpublished data). Analysis of the ovocalyxin-21 protein sequence showed significant homologies with proteins containing the brichos domain. The brichos domain is about 100 amino acids long and based on its common properties, possible functions have been proposed including a chaperone-like function (Sanchez-Pulido et al. 2002). Ovocalyxin-25 contains 2 domains known to inhibit protease. One is a WAP type that is also present in lustrin A, a matrix protein from the nacreous layer of the shell and pearl of molluscs (Shen et al. 1997).

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Chapter 17 Function of Eggshell Matrix Proteins

Joël Gautron and Yves Nys

1 Introduction

The main function of the eggshell is to protect the embryo from external aggression during its development. Consequently, the eggshell as a physical barrier must have remarkable mechanical properties. It has to be solid but also easy breakable from inside to allow hatching. Furthermore, the egg content must remain sterile during the development of the embryo. This protective effect is due to antimicrobial molecules present in yolk, egg white, egg membranes, and also in the eggshell. The physical properties of the shell and the chemical antibacterial activities of the egg components constitute the natural defences of the egg that keep the egg free of pathogens and, therefore, suitable for human consumption. The eggshell matrix proteins are important components of these natural defences, firstly by their involvement in the eggshell calcification process and its resulting mechanical properties, and secondly because certain eggshell matrix proteins have a structure similar to antibacterial components.

2 Eggshell Matrix Proteins and Calcification Process

A number of experimental observations support the role of the eggshell matrix proteins in the fabric of the eggshell and its resulting mechanical properties. The first is relevant to the nature of chicken eggshell matrix in its content of specific components (ovocleidins and ovocalyxins) with nucleotides and proteins sequences that are novel and not yet reported in databases. These component proteins and mRNA are only expressed and synthesized in tissues where eggshell calcification takes place, namely, the uterus and red isthmus. These mRNAs expression are only detectable in the sexually mature oviduct (Gautron et al. 2005a) and some are upregulated by the mechanical distension exerted upon the uterine wall when the egg enters the uterus (Lavelin et al. 1998).

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Another piece of experimental evidence is the change in the organic composition of the uterine fluid during the fabrication of the eggshell. The egg is calcified in the uterus in a three phase process (initiation, active calcification, and termination of shell calcification). Each phase of shell mineralization is associated with a specific electrophoretic profile in the uterine fluid, suggesting specific roles for the organic contents during the calcification process (Gautron et al. 1997).

The nature of the interaction between organic matrix components and the mineral phase of the shell was carefully investigated.

2.1 Calcium Binding Proteins

Investigations have demonstrated that eggshell organic fractions exhibit calcium binding properties due to proteins (Abatangelo et al. 1978; Cortivo et al. 1982; Hincke et al. 1992) or keratan and dermatan sulphate proteoglycans (Arias et al. 1992). Similarly, in the uterine fluid, protein bands were found to correspond to ovalbumin and ovotransferrin at the initial stage, and abundantly to the 36 kDa band during the active phase of calcification, with a display of affinity for calcium (Gautron et al. 1997). This last band was further characterized as corresponding to clusterin (Mann et al. 2003) and ovocalyxin-36 (Gautron et al. 2007).

2.2 In vitro Interaction with Mineral Phase

One of the biological tests frequently used to analyze the involvement of matrix proteins in the calcification process is an *in vitro* measurement of the rate of calcium carbonate precipitation. Soluble eggshell extracts delay the precipitation of calcium carbonate in a dose-dependent manner (Arias et al. 1993; Gautron et al. 1996). A similar effect is observed with proteoglycan-rich fractions of the shell (Arias et al. 1992) and with the macromolecules of the uterine fluid (Gautron et al. 1997). The effect of the whole uterine fluid on the precipitation kinetics, on the size and the morphology of crystals grown *in vitro* was also investigated using a micromethod favoring a good reproducibility of crystal size and morphology (Dominguez-Vera et al. 2000; Jimenez-Lopez et al. 2003). The induction time which corresponded to the time elapsed to observe the first crystals, was reduced in presence of uterine fluid whatever the stage. The number of crystals was increased and their size decreased (Dominguez-Vera et al. 2000). In presence of uterine fluid, all *in vitro* crystals formed were found to be calcite demonstrating that this milieu promotes the calcium carbonate polymorph of the shell (Rodriguez-Navarro et al. in Gautron et al. 2005b). The morphology of calcite crystals is dramatically affected by soluble eggshell matrix proteins (Gautron et al. 1996) or uterine fluid proteins (Dominguez-Vera et al. 2000, Rodriguez-Navarro, et al. in Gautron et al. 2005b). The electrophoretic bands of uterine fluid were compared at collection

and after *in vitro* crystallization experiments or spontaneous precipitation (Gautron et al. 1997, 2005b). The bands that corresponded to ovocleidin-116 (Hincke et al. 1999) and ovocalyxin-32 (Gautron et al. 2001b) disappeared and were identified in the calcium carbonate after precipitation. Calcite crystals, grown *in vitro* in presence of purified lysozyme, ovotransferrin, and ovocleidin-17 showed large modifications of the calcite morphology (Hincke et al. 2000; Gautron et al. 2001a; Jimenez-Lopez et al. 2003; Reyes-Grajeda et al. 2004). The goose ansocalcin has been shown to nucleate calcite crystals *in vitro* (Lakshminarayanan et al. 2002, 2003). The polycrystalline aggregate obtained was shown to be similar to the crystal observed in the mammillary layer (Lakshminarayanan et al. 2002, 2003). This effect was related to multiplets of charged amino acids present in the central region of ansocalcin (Ajikumar et al. 2003, 2005). Despite their close similarity, OC-17 and ansocalcin may interact differently with the calcite suggesting that they could play different roles in eggshell calcification (Lakshminarayanan et al. 2005). Additionally, the dermatan sulphate glycosaminoglycan chain containing ovocleidin 116 as protein core, ovoglycan, is polyanionic and acidic with high calcium affinity and is likely to modulate crystal growth during palisade formation (Fernandez et al. 2001).

2.3 In vivo Relationships Between Eggshell Matrix and Eggshell Quality

If eggshell matrix participates in establishing the morphology of calcite crystals, it would affect the texture (crystal size and orientation) of the eggshell and influence its mechanical properties. This hypothesis was confirmed by quantifying components of matrix proteins in parallel with variations in eggshell mechanical properties (Panhéleux et al. 2000; Ahmed et al. 2005). Eggshell laid at the beginning of egg production was compared with that laid at the end of the laying period (Panhéleux et al. 2000). Eggshell mechanical properties are reduced in this last group even when modification in the eggshell weight is avoided by selection of egg samples. The concentrations of ovotransferrin and ovalbumin are lower in the shell extract collected on young hens when the shell solidity is higher. The total amount of matrix proteins was not modified suggesting that other matrix proteins were increased simultaneously with better shell mechanical properties (Panhéleux et al. 2000). The model of moult, which restores the decreased solidity of eggshell in aged hens, was also used to study changes in organic matrix composition and associated changes in eggshell microstructure and mechanical properties (Ahmed et al. 2005). Eggs from the same hens with similar weight of shell before and after moult were selected and their mechanical properties were measured. Moult was associated with an increase in shell breaking strength and toughness and a decrease in the average size of calcite crystals composing the eggshell. After moult, eggshell matrix proteins specific to the eggshell formation (OC-116 and OC-17) were at a higher concentration, while the nonspecific egg white proteins present in the shell (ovotransferrin, ovalbumin, and lysozyme) decreased (Ahmed et al. 2005). These observations suggest

that changes in organic matrix protein levels affect eggshell crystal size and provide mechanisms for improving the shell solidity.

2.4 Genomic Approach

A complementary avenue to establishing the role of matrix proteins in the variability of the eggshells physical and mechanical properties has been taken using genetic and genomic approaches (Dunn et al. 2007). These were aimed at determining the association between alleles of some eggshell matrix proteins (ovocleidin and ovocalyxins, osteopontin, ovalbumin) and measurements of eggshell solidity. This study reveals a number of significant associations between genotype (the marker) and phenotype (the trait e.g., acoustic resonance data, quasi-static compression test data, and the thickness of specific components of the shell; Bain 2005; Dunn et al. 2006). When expressed as allele substitution/standard deviation of the trait, the effect of a coding region polymorphism in ovocalyxin 32 was over 12% for breaking strength and 17% for deformation. The effect of a coding region polymorphism in ovocleidin 116 was 17% for shell stiffness, and polymorphism in the promoter of this gene accounted for around 10% for the thickness of the mammillary layer and its proportion in the shell (Dunn et al. 2007). This approach confirms *in vivo* the relationship between eggshell matrix and the properties of the eggshell.

3 Antibacterial Activities of Eggshell Matrix Proteins

The shell is a physically protective barrier against microbial assault against the developing embryo and may also contain proteins with antimicrobial properties. This function is most commonly ascribed to numerous egg-white proteins that possess antimicrobial properties (Nau et al. 2002). Eggshell is not a suitable medium for microbial growth, in part due to its physical characteristics (solid structure with low moisture content). However, some egg white proteins, well known for their antimicrobial properties, have been identified in eggshell, which may explain some of the antimicrobial activity observed in eggshell extracts (Mine et al. 2003).

Lysozyme is a key factor of the egg natural defense system against bacterial aggression in the egg white. It is well known for its antibacterial properties, due to its ability to hydrolyse 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycans of Gram-positive bacteria (Nau et al. 2002). Ovotransferrin, another major protein of egg white, is involved in iron binding and plays an important role in preventing growth of Gram-negative spoilage bacteria in egg white by reducing iron in the milieu (Garibaldi 1970). Ovotransferrin can also interact with the membrane of Gram-negative bacteria (Valenti et al. 1983) as well as some Gram-positive bacteria (Ibrahim et al. 1998). The presence of ovalbumin and ovotransferrin

in eggshell suggests that they may function as antibacterial agents in the eggshell in addition to their suspected role on eggshell mineralization. They also may be involved in protecting the egg in the uterus during the initial phase of eggshell mineralization.

Such a role can also be proposed for OCX-36 protein (Gautron et al. 2007). A database search for the ovocalyxin-36 protein sequence using the comparison algorithm Blastp showed significant identities with lipopolysaccharide binding proteins (LBP; Schumann et al. 1990), bactericidal permeability increasing proteins (BPI; Gray et al. 1989), and the PLUNC family of proteins (Bingle and Craven 2002). LBP, BPI, and PLUNC proteins are often described as "first-line host defense proteins," and it has been reported that they could be involved in the innate immune response. Members of the LBP/BPI family are proteins that bind to the lipid A portion of lipolysaccharide (LPS) cell wall in Gram negative bacteria, leading to death of the bacteria. Therefore, OCX-36 would contributes to natural egg defense by providing chemical protection of the egg contents, particularly in the lumen of the distal oviduct, in which the egg-information is bathed.

Analysis of ovocalyxin-25 (OCX-25) indicated the presence of two domain signatures that are found in serine protease inhibitors. These are of the WAP type, which contains eight cysteine residues involved in four disulfide bonds (Hennigahausen and Sippel 1982) and the Kunitz bovine pancreatic trypsin inhibitor motif (Laskowski and Kato 1980; Salier 1990). Protease inhibitors are known to regulate activities of serine proteases. Their role in natural defense against micro-organisms is unclear but their inhibitory properties suggest that they could interfere with the activity of bacterial proteases necessary for adhesion and infectivity of the pathogen (Molla et al. 1987).

Antibacterial proteins are only effective when they are in solution. Therefore, such eggshell matrix proteins would be effective in the uterine fluid during the period of eggshell calcification or, at oviposition, when the eggshell still contains moisture.

In conclusion, numerous major proteins have been identified as components of the eggshell matrix proteins. It is likely that the whole sequence of the hen genome associated with transcriptomic or proteomic approaches will complete the screening of the eggshell components in the near future. Further work will be needed to precisely identify the functional properties of these components and establish some hierarchy in their respective roles, either in the processes of the shell fabric or, as revealed by the recent data, as antimicrobial agents in this first efficient barrier against the aggressive milieu in which the embryo must grow.

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Chapter 18 Nutritional Evaluation of Egg Compounds

Ingrid Seuss-Baum

1 The Problem with the Egg

As a result of the changes in living conditions and progress in nutritional research we have come to expect something different from our diet in recent decades. Despite all the changes, a balanced diet still forms the basis of a healthy diet. But what is a balanced diet? It can be characterized as the intake of appropriate types and amounts of food and drink to supply nutrients and energy for the maintenance of our bodies' cells, tissues, and organs, and to support normal growth and development. It is simply a diet that meets nutritional needs adequately while not providing any macro- or micronutrients in excess.

The evaluation of eggs in human nutrition has typically been dominated by the high cholesterol content in eggs and the consequences of high cholesterol consumption. Recent studies, however, indicate that cholesterol from eggs does not have a negative effect on serum cholesterol levels (Hu et al. 1999, 2001). It is time, therefore, for a re-evaluation of eggs in nutrition, focusing on the nutritional content and extending to functional substances found naturally in eggs, or with which eggs might be fortified.

2 Nutritional Value of Eggs

There are different ways to illustrate the nutritional value of different foods. The simplest method is to list the content of different nutrients per 100 g or per 1 portion of selected foods. Other evaluation methods are the expression of nutrient content as a percentage of the recommended daily intake (RDI) and nutrient profiling.

2.1 Nutrient Content per 100 g or per Portion

The quantity of the most important nutrients in 100 g and in 1 portion of egg is presented in Table 1. This data comes from Germany's Federal Foodstuffs Database (BLS 1999). The general problem with such a study lies in identification

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Table 1. Nutrient and energy content of whole egg in 100 g and in one egg (~60 g, edible portion 53 g) and expressed as percentage of the Recommended (or Estimated) Daily Intake (RDI or EDI) or the Reference Labeling Value (RLV)

Nutritional analysis of whole egg without shell						
Constituents	Per 100 g[a]	Per egg (53 g) [a]	RDI or EDI[b]	One egg expressed as % of RDI or EDI[b]	Reference Labeling Value (RLV)[c]	One egg expressed as % of RLV
Protein (g)	12.9	6.8	55	12.4	–	–
Carbohydrates (g)	0.7	0.4	245	0.2	–	–
Fat (g)	11.1	5.9	85	6.9	–	–
Essent. fatty acids (g)	1.3	0.7	6.5	10.8	–	–
n-3 fatty acids (g)	0.4	0.2	1.6	12.5	–	–
Minerals						
Sodium (g)	0.14	0.08	0.55	14.5	0.6	13.3
Potassium (g)	0.15	0.08	3	2.6	2	4.0
Calcium (mg)	56	30	900	3.3	1,000	3.0
Phosphorus (mg)	216	115	700	16.4	700	16.4
Magnesium (mg)	12.1	6.4	350	1.8	375	1.7
Iron (mg)	2.1	1.1	12.5	8.8	14	7.9
Zinc (mg)	1.4	0.74	8.5	8.7	10	7.4
Fluoride (mg)	0.11	0.06	3.0	2.0	3.5	1.7
Iodine (µg)	11	6	200	3.0	150	4.0
Selenium (µg)	24.5[d]	13[d]	45	28,8	55	23.6
Vitamins						
Vitamin A[e] (mg)	0.28	0.15	1	15.0	0.8	18.8
Vitamin D (µg)	2.9	1.54	5	30.8	5	30.8
Vitamin E (mg)	2.0	1.1	13	8.5	12	9.2
Vitamin K (µg)	48	25	65	38.5	75	33.3
Vitamin B_1 (mg)	0.1	0.05	1.2	4.2	1.1	4.5
Vitamin B_2 (mg)	0.30	0.16	1.4	11.4	1.4	11.4
Vitamin B_6 (mg)	0.12	0.06	1.3	4.6	1.4	1.9
Vitamin B_{12} (µg)	2.0	1.06	3	35.3	2.5	42.4
Folic acid/ Folate (µg)	65	34	400	8.5	400	8.5
Niacin[f] (mg)	3.1	1.6	14.5	11.0	16	10.0
Biotin (µg)	25.0	13.25	45	29.4	50	26.5
Pantothenic acid (mg)	1.6	0.85	6	14.2	6	14.2

Table 1. Nutrient and energy content of whole egg in 100 g and in one egg (~60 g, edible portion 53 g) and expressed as percentage of the Recommended (or Estimated) Daily Intake (RDI or EDI) or the Reference Labeling Value (RLV) — Cont'd.

Nutritional analysis of whole egg without shell						
Constituents	Per 100 g[a]	Per egg (53 g) [a]	RDI or EDI[b]	One egg expressed as % of RDI or EDI[b]	Reference Labeling Value (RLV)[c]	One egg expressed as % of RLV
Others						
Cholesterol (mg)	396	210	300	70.0	–	–
Energy content (kcal/KJ)	154/646	82/342	2,500/ 10,450[g]	3.3	–	–

[a] BLS (Bundeslebensmittelschlüssel - Federal foodstuffs database (Germany)) - Version II.3 (1999)
[b] RDI: Recommended Dietary Intake and EDI: Estimated Dietary Intake for adults (mean of the values for men and women, 19–60 years) for Germany, Austria and Switzerland (D-A-CH 2000)
[c] SCF 2003a (Tables 7 and 8)
[d] Scherz et al. 2000
[e] Retinol equivalents
[f] Niacin equivalents (Niacin + Tryptophan)
[g] Assumed energy requirement of 2,500 kcal/day

of the best source of data. Various food composition databases and tables are maintained by different EU countries. An inventory of these databases is maintained by COST Action 99, which can be found on the website of the FAO (Schlotke and Møller 2000). Upon comparing the figures in the tables from different EU member countries and the USA, it is clear that values vary from country to country (Tables 2–4).

The differences could be due to the varying ages of the analytical data, the use of different analytical methods, or the normal range of the biological material. This last point is probably the most important as the differences in nutrient composition of the eggs could be caused by variations among the breeds of hens or the particulars of feeding and animal husbandry.

This is not a problem unique to data on eggs; the data for other foods indicate a similar variation in nutritional composition. For this reason, a Network of Excellence "European Food Information Resource" (EuroFIR) has been established and is funded by the EU (EuroFIR 2005). Over the next few years EuroFIR will work on building and disseminating a comprehensive, coherent and validated databank to provide a single, authoritative source of food composition data in Europe both for nutrients and newly emerging bioactive compounds with potential health benefits. This network will provide researchers with more uniform data in the future.

In addition to the national databases there are various other sources of analytical data on egg compounds. Tables 5 and 6 show the nutritional

Table 2. Nutrient content of whole egg from nutrient tables of different EU countries and the USA

Nutrients		Belgium (a)	Denmark (b)	France (c)	Finland (d)	Germany (e)	Italy (f)	Netherlands (g)	Spain (h)	UK (i)	USA (k)	Range
Energy content	kcal	152	152	146	143	154	128	138	160	147	147	128 –160
Water	g	74	74.6	75.8	–*	74	77.1	75.4	–	75.2	–	74 – 77.1
Protein	g	12.5	12.1	12.5	12.5	12.9	12.4	12.6	12.1	12.6	12.6	12,1 – 12.9
Carbohydrates	g	0.3	1.2	0.3	0.3	0.7	–	–	0.7	–	–	0.3 – 1.2
Fat	g	11.2	11.2	10.5	10.3	11.1	8.7	9.8	12.1	10.9	9.9	8.7 – 12.1
SFA	g	3.7	3.1	3.1	2.1	3.3	3.2	–	3.3	3.1	–	2.1 – 3.7
MUFA	g	5.1	4.3	4.2	3.3	4.5	2.6	–	4.9	4.7	–	2.6 – 5.1
PUFA	g	1.8	2	1.3	1.2	1.5	1.3	–	1.8	1.2	1.4	1.2 – 2
Linoleic acid	g	1.5	1.39	1*	0.89	1.02	1.06	–	–	–	1.15	0.89 – 1.5
Linolenic acid	g	–	0.12	0.02	0.06	0.27	0.04	–	–	–	0.03	0.02 – 0.27
EPA	mg	–	46	–	–	–	–	–	–	–	–	46
DHA	mg	–	180	–	107	–	–	–	–	–	–	107 – 180
Cholesterol	mg	352	548	380	360	396	371	333	410	–	423	333 – 548

SFA = Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, EPA = Eicosapentaenoic acid, DHA= Docosahexaenoic acid
*In some nutrient databases or tables not all nutrients have been listed or no value was presented.
(a) Belgische Voedingsmiddelentabel, NUBEL, Version 3 (2005)
(b) Danish Food Composition Databank, Version 6.0 (2005) - Danish Institute for Food and Veterinary Research
(c): Répertoire général des aliments, Table de composition. (1995), Lavoisier Technique & Documentation, Paris; INRA Editions
(d) Fineli - Finnish Food Composition Database, Release 5 (2004) - KTL National Public Health Institute
(e) BLS [Bundeslebensmittelschlüssel - Federal foodstuffs database (Germany)] - Version II.3 (1999)
(f) NEVO Nederlands Voedingsstoffenbestand (Dutch Food Composition Database) (2001). Netherlands Bureau for Nutrition Education, The Hague
(g) Tabelle di composizione degli alimenti - Istituto Nazionale della Nutrizione, Edra, Milan (1997)
(h) Tablas de composicion de alimentos espanoles. Ministerio de Sanidad y Consumo Espana (1999)
(i) The Composition of Foods, Sixth summary edition Food Standards Agency (2002) McCance and Widdowson's. Cambridge: Royal Society of Chemistry
(k) USDA - National Nutrient Database for Standard Reference - Release 18 (2005)

Table 3. Nutrient content (minerals) of whole egg from nutrient tables of different EU countries and the USA

Nutrients		Belgium (a)	Denmark (b)	France (c)	Finland (d)	Germany (e)	Italy (f)	Netherlands (g)	Spain (h)	UK (i)	USA (k)	Range
Sodium	mg	116	138	133	110	144	137	125	–*	140	140	110 – 144
Potassium	mg	125	130	125	130	147	133	131	–	130	134	125 – 147
Calcium	mg	91	40	55	57	56	48	50	56	56	53	40 – 91
Phosphorus	mg	312	210	188	210	216	210	179	–	200	191	179 – 312
Magnesium	mg	9	13	11	13	12	13	11	12	12	12	9 – 13
Iron	mg	2.9	2	1.8	2.5	2.1	1.5	–	2.2	1.9	1.8	1.5 – 2.9
Iodine	µg	–	21	–	44	10	–	–	12.7	52.3	–	10 – 52.3
Copper	mg	0.1	0.07	–	–	0.14	0.06	0.08	–	0.1	0.1	0.06 – 0.14
Zinc	mg	2.3	1.4	–	1.4	1.4	1.2	1.3	2.0	1.4	1.1	1.1 – 2.3
Selenium	µg	–	22.2	–	22.5	–	5.8	11	–	11.6	31.7	5.8 – 31.7
Fluoride	mg	–	–	–	–	0.11	–	–	–	–	–	0.11

*In some nutrient databases or tables not all nutrients have been listed or no value was presented.
See Table 2 for identifications (a)–(k)

Table 4. Nutrient content (vitamins) of whole egg from nutrient tables of different EU countries and the USA

Nutrients		Belgium (a)	Denmark (b)	France (c)	Finland (d)	Germany (e)	Italy (f)	Netherlands (g)	Spain (h)	UK (i)	USA (k)	Range
Vitamin A[a]	µg	192	208	240	262	278	225	192	227	190	140	140 – 278
Vitamin D	µg	–*	1.75	1.7	2.2	2.9	–	1.75	1.8	1.7	–	1.7– 2.9
Vitamin E (Total)	mg	–	1.9	1.2	2	2.0	–	2.8	2.0	1.1	1.5	1.1 – 2.8
Vitamin K	µg	–	50	–	0.8	48	–	–	–	–	0.3	0.3 – 50
Vitamin B_1	mg	0.1	0.07	0.08	0.12	0.1	0.09	0.1	0.11	0.1	0.07	0.07– 0.12
Vitamin B_2	mg	0.3	0.45	0.46	0.37	0.3	0.3	0.3	0.37	0.5	0.48	0.3 – 0.5
Vitamin B_6	mg	–	0.12	0.12	0.14	0.12	–	0.17	0.12	0.12	0.14	0.12– 0.17
Vitamin B_{12}	µg	2.3	2	1.6	2.5	2	–	2.3	2.1	2.5	1.3	1.3 – 2.5
Folic acid/ Folate	µg	–	21	60	58.3	65	–	57	51.2	50.4	47	21 – 65
Niacin	mg	–	0.1	0.07	3.4[b]	3.1[b]	0.1	0.1	0.08	3.8[b]	0.07	0.07– 3.8[b]
Biotin	µg	–	25	–	–	25	–	–	–	19.4	–	19.4 – 25
Pantothenic acid	µg	–	1.6	1.6	–	1.6	–	–	–	1.8	1.4	1.4 – 1.8
Lutein	µg	–	–	–	–	–	–	620	–	–	331	331 – 620

*In some nutrient databases or tables not all nutrients have been listed or no value was presented.
See Table 2 for identification of (a)–(k)
[a] Retinol equivalents
[b] Niacin equivalents (Niacin + Tryptophan)

Table 5. Composition of whole egg, egg yolk and white (in 100 g, without shell; adapted from Nys and Sauveur 2004)

Nutrients	Egg white	Egg yolk	Whole egg[a]	CV (%)[b]
Proportion[c]	60	30.7	90.7	–
Energy content (kcal)	47	364	154	–
Water (g)	88.6	49	74.4	1.2
Protein (g)	10.6	16.1	12.3	4.7
Carbohydrates (g)	0.8	0.5	0.7	–
Ash (g)	0.5	1.6	0.9	4.6
Fat (g)	0.1	34.5	11.9	6.9
Triglycerides (g)	–	22.9	7.7	–
Phospholipids (g)	–	10.0	3.4	–
Cholesterol (g)	0	1.2	0.42	9.5
Lecithin (g)	–	7.2	2.30	–
Saturated fatty acids (g)	–	13.0	4.4	–
16:0 palmitic acid	–	7.3	2.5	21.4
18:0 stearic acid	–	2.5	0.86	23
Unsaturated fatty acids (g)		20.7	7.0	–
16:1 palmitoleic acid	–	1.1	0.4	–
18:1 oleic acid	–	12	4.1	–
18:2 linoleic acid	–	3.6	1.25	30.4
18:3 linolenic acid (n-3)	–	0.12	0.04	18
20:4 arachidonic acid (n-6)	–	0.6	0.2	40
20:5 EPA[d] (n-3)	–	0	0	–
22:6 DHA[e] (n-3)	–	0.4	0.15	–
Essential amino acids (mg)				
Histidine	–	–	–	–
Isoleucine	240	410	290	–
Leucine	560	870	660	–
Lysine	880	1,390	1,040	–
Methionine + Cystine	660	1,170	820	–
Phenylalanine + Tyrosine	670	660	640	–
Threonine	1,020	1,420	1,150	–
Tryptophan	470	850	590	–
Valine	170	240	190	–

[a] Egg without shell
[b] Coefficient of variation (Gittins and Overfield 1991)
[c] Proportion of whole egg including shell
[d] EPA = Eicosapentanoic acid
[e] DHA= Docosahexaenoic acid

Table 6. Vitamin and mineral content of whole egg, egg yolk and egg white (in 100 g, without shell - from Nys and Sauveur 2004)

Nutrients	Egg white	Egg yolk	Whole egg[a]	CV (%)[b]
Minerals (mg/100g)				
Sodium	155	50	120	8–11
Chlorine	175	162	172	8–11
Potassium	140	100	125	8–11
Calcium	8	133	50	8–11
Phosphorus	18	530	193	8–11
Iron	0.1	4.8	1.7	12
Magnesium	10	15	12	–
Sulphur	163	165	164	–
Zinc	0.12	3.9	1.4	28
Copper	0.02	0.14	0.06	40
Manganese	0.007	0.11	0.04	28
Iodine	0.003	0.14	0.05	–
Vitamins (µg/100g)				
Ascorbic acid	0	0	0	–
Vitamin A, Retinol equivalents	0	450	150	37
Vitamin D	0	4.5	1.5	95
Vitamin E	0	3,600	1,200	46
Vitamin B_1	10	250	913	17
Vitamin B_2	430	480	447	21
Vitamin B_6	10	370	133	–
Vitamin B_{12}	0.1	2.8	1	45
Folic acid	12	140	56	35
Niacin	90	60	79	–
Biotin	7	60	25	–
Pantothenic acid	250	4,500	1,700	32

[a] Egg without shell
[b] Coefficient of variation (Gittins and Overfield 1991)

composition of eggs (whole egg, egg yolk, and egg white per 100 g) according to Nys and Sauveur (2004). The proportion of egg white to egg yolk is about 2:1. Although eggs contain 74% water they are potentially an important and balanced source of nutrients.

2.2 Nutrient Content Expressed as Percentage of RDI

More illustrative is the depiction of the nutrient content (per portion or per 100 g) in terms (percentage) of nutritional requirements of the various nutrients. Fig. 1 shows an example with data for raw whole egg from German nutrient tables (BLS 1999) expressed in percentage of the recommended daily intake of adults for Germany, Austria, and Switzerland. (D-A-CH 2000).

Particularly high values have been calculated for vitamin K, B_{12}, selenium, and cholesterol. But one egg also provides a lot of other nutrients with more than 10% of the recommended nutrient intake (protein, n-3-fatty acids, vitamins A, D, E, B_2, biotin, and pantothenic acid, plus minerals iron and zinc). The red line represents the proportion of the energy supply of one whole egg to a daily energy intake of 2,500 kcal. Most of the columns for the nutrients are higher than that line. This indicates a high nutrient density (proportion of nutrient content and energy content). The energy value of one egg would contribute only around 3% of the average energy requirement of an adult man or 4% for an adult woman (Table 1).

The nutritional evaluation of foods in terms of their nutritional contribution to the RDI is in some ways problematic because of the variance of values in the different databases available. The proportion of the RDI changes not only in accordance with the range of the values for the nutrient content but also with the defined recommendation of nutrient intake in the different countries. Tables 7 and 8 show a comparison of the RDI of minerals and vitamins for

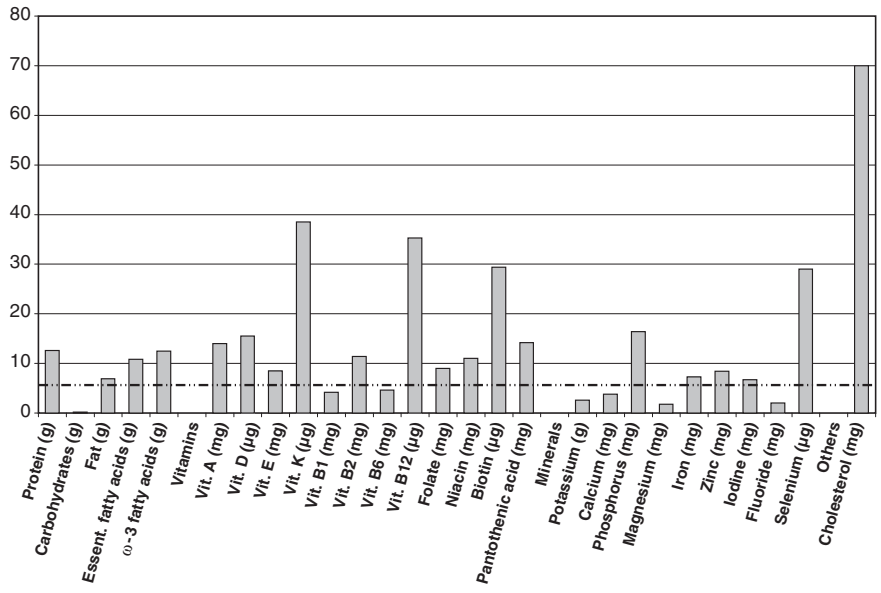

Fig. 1. Nutrient content (BLS 1999) in one egg (~ 60 g, edible portion 53 g) expressed as a percentage of RDI (D-A-CH 2000). The *broken line* represents the proportion of the energy supply of 1 portion of whole egg to a daily energy intake of 2,500 kcal

Table 7. Comparison of the Recommended Daily Intake of minerals for adults derived from different European governments and organizations (according to SCF 2003a)

Minerals	Sodium	Potassium	Calcium	Phosphorus	Magnesium	Iron	Iodine	Copper	Zinc	Selenium	Fluoride
	mg	mg	mg	mg	mg	mg	µg	mg	mg	µg	mg
European Union	575–3,500	3,100	700	550	150–500	9/20	130	1.1	9.5/7	55	–
Belgium	575–3,500	2,000–4,000	900	800	420/330	9/20	150	1.0	9.5/7	70	–
France	–	–	900	750	420/360	9/16	150	2.0/1.5	12/10	60/50	2.5/2.0
Germany, Austria, Switzerland	550	2,000	1,000	700	350/300	10/15	200	1.0/1.5	10/7	30–70	3.8/3.1
Italy	–	3.100	1,000	1,000	–	10/18	150	1.2	10/7	55	–
Netherlands	–	–	1,000	700/1,400	300–350/250–300	9/15	–	1.5/3.5	10/9	50–150	–
Nordic countries	–	3,500/3,100	800	600	350/280	10/18	150	–	9/7	50/40	–
Spain	–	–	600–850	–	350–400/330	10–15/18	140–145/110–115	–	15	–	–
UK	1600	3,500	700	550	300/270	8.7/14.8	140	1.2	9.5/7.0	75/60	3.7/3.0
US	500	2,000	1,000	700	400–420/310–320	8/18	150	0.9	11/8	55	4/3
FAO/WHO	–	–	1,000	–	260/220	9/20	130/110	–	7.0/4.9	34/26	–
Reference Labeling Value (RLV)	600	2,000	1,000	700	375	14	150	1.0	10	55	3.5

When there are 2 values separated by a slash (/), the value on the left is for men, the value on the right for women. An en dash(–) indicates a range of values.

Table 8. Comparison of the Recommended Daily Intake of vitamins for adults derived from different European governments and organizations (according to SCF 2003a)

Vitamins	Vitamin A (RAE)[a]	Vitamin D	Vitamin E (Total)	Vitamin K	Vitamin B_1	Vitamin B_2	Vitamin B_6	Vitamin B_{12}	Folic acid/ Folate	Niacin	Biotin	Pantothenic acid
	µg	µg	mg	µg	mg	mg	mg	µg	µg	mg	µg	µg
European Union	700/600	0–10	0.4[b]/ >4–>3	–	1.1/0.9	1.6/1.3	1.5/1.1	1.4	200	18/14	15–100	3–12
Belgium	700/600	2.5–10	10	–	1.1/0.9	1.6/1.3	1.7/1.2	1.4	200	18/14	15–100	3–12
France	800/600	5	12	45	1.3/1.1	1.6/1.5	1.8/1.5	2.4	330/300	14/11	50	5
Germany, Austria, Switzerland	1,000/ 800	5	15/12	70/60	1.2/1.0	1.4/1.2	1.5/1.2	3.0	400	16/13	30–60	6
Italy	700/600	0–10	>8	–	1.2/0.9	1.6/1.3	1.5/1.1	2.0	200	18/14	–	–
Netherlands	1,000/800	2.5–5	11.8/9.3	–	1.1	1.5/1.1	1.5	2.8	300	17/13	–	5
Nordic countries	900/800	5	10/8	–	1.4/1.1	1.8/1.3	1.5/1.2	2.0	300	19/15	–	–
Spain	750	2.5	12	–	1.2/0.9	1.8/1.4	1.8/1.6	2.0	200	20/15	–	–
UK	700/600	–	>4/>3	74/60	1.0/0.8	1.3/1.1	1.4/1.2	1.5	200	17/13	10–200	3–7
US	900/700	5	15	120/90	1.2/1.1	1.3/1.1	1.3	2.4	400	16/14	30	5
FAO/WHO	600/500	5	10/7.5	65/55	1.2/1.1	1.3/1.1	1.3	2.4	400	16/14	30	5
Reference Labeling Value (RLV)	800	5	12	75	1.1	1.4	1.4	2.5	400	16	50	6

When there are 2 values separated by a slash (/), the value on the left is for men, the value on the right for women. An en dash (–) indicates a range of values.

[a] Retinol equivalents

[b] mg/g PUFA

Table 9. Description of the model of a scoring system for the classification of nutrient profiles of foods (Rayner et al. 2004; Anonymous 2005)

Points are awarded for the content of eight nutrients in 100 g of the food (in the case of drinks the basis is 200 g).
A maximum of 10 points can be awarded for each nutrient.
For the calculation of the overall score three steps are necessary:

Step one: Calculation of 'A' points: Negative attributes Total 'A' points = (points for energy) + (points for saturated fat) + (points for NME sugars) + (points for sodium)											
'A' points	0	1	2	3	4	5	6	7	8	9	10
Energy (kJ)	≤335	≤670	≤1,005	≤1,340	≤1,675	≤2,010	≤2,345	≤2,680	≤3,015	≤3,350	>3,350
Saturated fat (g)	≤1	≤2	≤3	≤4	≤5	≤6	≤7	≤8	≤9	≤10	>10
NME- sugar[a] (g)	≤2.4	≤4.8	≤7.2	≤9.6	≤12	≤14.4	≤16.8	≤19.2	≤21.6	≤24	>24
Sodium (g)	≤90	≤180	≤270	≤360	≤450	≤540	≤630	≤720	≤810	≤900	>900
Step two: Calculation of 'C' points: Positive attributes Total 'C' points = (points for iron) + (points for calcium) + (points for n-3 PUFAs) + (points for fruit and vegetable content)											
'C' points	0	1	2	3	4	5	6	7	8	9	10
Iron (mg)	≤1.5	≤3	≤4.5	≤6	≤7.5	≤9	≤10.5	≤12	≤13.5	≤15	>15
Calcium (mg)	≤105	≤210	≤315	≤420	≤525	≤630	≤735	≤840	≤945	≤1,050	>1,050
n-3 PUFAs (g)	≤0.05	≤0.1	≤0.15	≤0.2	≤0.25	≤0.3	≤0.35	≤0.4	≤0.45	≤0.5	>0.5
Fruits + Vege tables(%)	≤30	–	≤50	–	≤70	–	–	–	–	–	100
Step three: Calculation of overall score Overall score = (total 'A' points) minus (total 'C' points)											
Overall score	0 or less	1	2	3	4	5	6	7	8	9 and more	
Category	Healthier			Intermediate						Less Healthy	

[a] NME-Sugars = non-milk extrinsic sugar

adults from various European organizations and governments. Deviations in RDI among the EU member states may result from different living conditions and eating habits. This fact could pose a problem for the labeling of products to be sold throughout the EU.

The so-called 'reference labeling value' (RLV) is important for labeling purposes. Information on vitamins and minerals must be expressed as a percentage of the RLV referred to as 'Recommended Daily Allowance' (RDA). The Scientific Committee on Food (SCF 2003a) has revised the RLVs for vitamins and minerals. The new values for adults are listed in Tables 7 and 8. Different values have been defined for children (SCF 2003a). An example for the calculation of the nutrient content of eggs (from German nutrient tables) expressed as a percentage of the RLV is shown in Table 1.

2.3 Nutrient Profile

The term 'nutrient profile' has been discussed in recent years in the search for a model for the nutritional evaluation of foods relevant to food promotion and health claims. Rayner et al. (2004; Anon. 2005) developed a scoring model for nutrient profiling in which points are awarded for the content of eight nutrients in 100 g of the food. A maximum of ten points can be awarded for each nutrient (Table 9).

According to the proposed nutrient scoring system, whole egg scores 5 'A' points and 8 'C' points for a total of –3, according to which egg would be classified as 'healthier', as described in Table 9. The overall scores for eggs and other selected foods are listed in Table 10 for comparison. Because of their high content of energy, fat, salt, and/or sugar, foods like salami, sausages, and cheese (with high fat content) are categorized as 'less healthy'.

The new model could be used as a tool to identify foods appropriate for enrichment or fortification. Foods categorized as 'less healthy' should not be modified to 'intermediate' or 'healthier' and it would not be appropriate to promote modified foods high in energy, fat, salt, or sugar with health claims.

3 Importance of Nutrients in Eggs for Human Nutrition

3.1 Macronutrients

3.1.1 Protein

The high content of highly bioavailable protein in eggs is of great benefit to human nutrition. It is notable that the quality of protein in eggs is used as the standard for measuring the quality of other food proteins. On the most common evaluation scale for protein quality, egg is defined as the highest value '100'. The high quality of the egg protein is the result of the high content of essential amino acids (Fig. 2 and Table 5).

Table 10. Nutrient Profiles[a] of various foods according to the proposed scoring system in Table 9

Scores	Whole egg raw	Milk (3.5 % fat)	Broccoli (fresh cooked)	Egg-based pasta (cooked)	Yogurt	Fruit yoghurt	Wheat roll	Whole-grain bread	Corn flakes	Camembert (50% fat in dm)	Frankfurter-type sausages	German Salami
'A' points	5	2	0	1	2	7	8	6	16	20	25	24
Energy (kJ)	1	0	0	1	0	1	3	2	4	3	3	4
Saturated fat (g)	3	2	0	0	2	1	0	0	0	10	9	10
NME- sugars (g)	0	0	0	0	0	5	0	0	3	0	3	0
Sodium (mg)	1	0	0	0	0	0	5	4	9	7	10	10
'C' points	8	1	11	0	1	1	0	1	1	4	7	0
Iron (mg)	1	0	0	0	0	0	0	1	1	0	1	0
Calcium (mg)	0	1	0	0	1	1	0	0	0	4	0	0
n-3-PUFAs (g)	7	0	1	0	0	0	0	0	0	0	6	0
Fruits + Vegetables (%)	0	0	10	0	0	0	0	0	0	0	0	0
Overall score (A – C)	–3	1	–11	1	1	6	8	5	15	16	18	24
Category	Heal-thier	Heal-thier	Heal-thier	Heal-thier	Heal-thier	Inter-mediate	Inter-mediate	Inter-mediate	Less healthy	Less healthy	Less healthy	Less healthy

[a] Nutrient contents from BLS (Bundeslebensmittelschlüssel - Federal foodstuffs database, Germany - version II.3, 1999)
For abbreviations, see Table 9

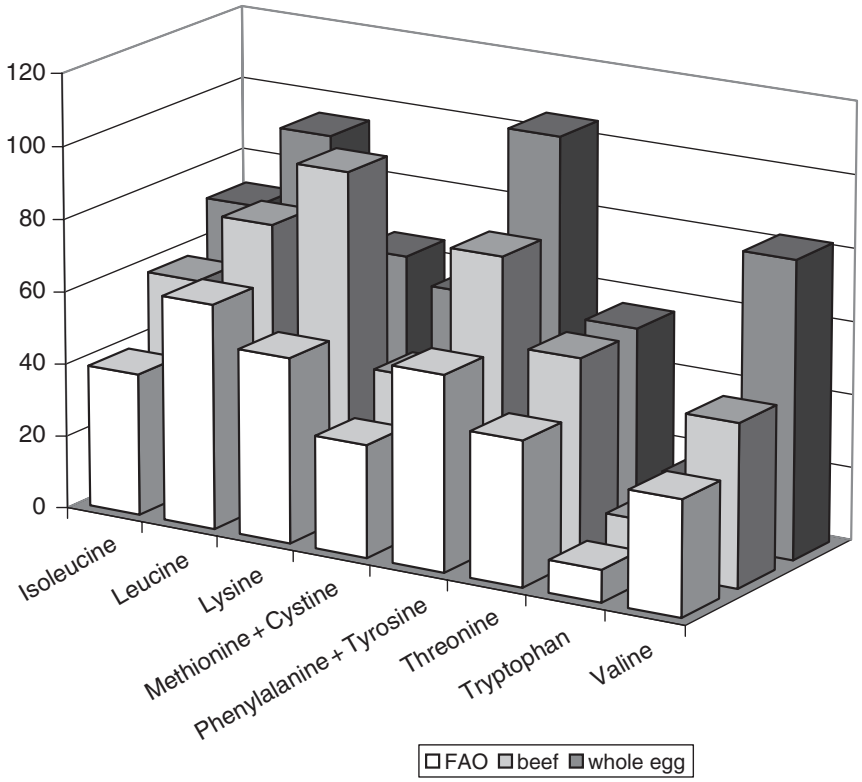

Fig. 2. Comparison of essential amino acid content of whole egg protein, FAO reference protein and beef protein (according to Ternes et al. 1994)

However, in most industrialized countries the supply of protein from eggs does not play an important role because of the high total protein intake (Elmadfa 2004). For example the recommended daily protein intake in Germany for male adults is about 55 g and the mean protein intake is about 76 g/day (DGE 2004).

Some proteins in eggs have been discovered to be suitable for commercial utilization. With reference to their properties they are called 'bioactive proteins'. For example the enzyme lysozyme, which is found in egg white, displays antibiotic characteristics and may be used to fight infections. Another important group of substances are immunoglobulin antibodies from egg yolk. Also displaying antibiotic characteristics, these antibodies are able to provide passive immunity against many intestinal pathogens in a wide variety of animals, including humans. Detailed information about these substances may be found in Part 1 of this book.

3.1.2 Fat

The total fat content of eggs is 10 to 12 percent on average (Table 2) and is found almost entirely in the yolk. Yolk contains nearly 33% fat. The fat consists of about 66% triglycerides, 28% phospholipids, and 5% cholesterol. How 'healthy' fats can be considered to be depends on the content of essential fatty acids, the ratio of polyunsaturated and saturated fatty acids, and the ratio of n-3 and n-6-polyunsaturated fatty acids.

Fatty Acids The content of essential fatty acids in eggs is not genetically encoded but rather reflects the hen's fatty acid diet. About 20% of total fat is comprised of essential fatty acids of which 90% are n-6 PUFA and 10% n-3 PUFA. However, this ratio depends significantly on the composition of the feed. The resulting n-6/n-3 ratio is about 9:1, which is about the same as in the average German diet (Table 11). In order to improve the healthiness of the diet, the aim is to achieve a ratio of 5:1, which would reflect the optimal balance based on the competition among the n-3 and n-6 fatty acids for storage in and release from tissue phospholipids. A proportion of 5:1 may reduce the risk of thrombosis, leucocyte adhesion, vascular wall inflammation, and myocardial arrhythmia (Lands 2000; Leaf et al. 2000).

To increase the concentration of n-3 fatty acids in eggs two feeding strategies are normally used. One is to feed the hens with an appropriate level of flaxseed, linseed or the oils from these seeds. A second is to add fish oil to the diet (Sparks 2005). In these ways the n-6 to n-3-ratio could be decreased to about 2:1 or less. A study by Sindelar et al. (2004) indicated that the consumption of one n-3 enriched egg has elevated the serum levels of linolenic acid and triglycerides in humans. More detailed information about fortified eggs can be found in Chapters 20 through 22.

Cholesterol Cholesterol in eggs plays an important role in the development of the embryo. It is a structural component of cell membranes and precursor for hormones, vitamin D, and bile acids. The amount of cholesterol in eggs and its contribution to the total human uptake of cholesterol is still a matter of contention among nutritionists. Recent reports have indicated that cholesterol from eggs does not have a negative effect on serum cholesterol levels (Hu et al. 1999; 2001; Kritchevsky and Kritchevsky 2000) and that there is no significant relationship between dietary cholesterol intake from eggs and coronary heart disease incidence. McNamara (2000) reached the conclusion that the increased risk associated with an extremely high dietary cholesterol intake as determined by epidemiological observations may not be due to the dietary cholesterol but rather to the absence of multiple nutrients (e.g., fiber, antioxidant vitamins, and polyunsaturated fatty acids) in diets high in animal products. These studies provide ample evidence for a revision of the issue of dietary cholesterol content of eggs and the effect on human health, so as to increase popular acceptance of eggs in diet. (Herron and Fernandez 2004). In order to make recommendations for diet, one must

Table 11. n-6/n-3-ratio: Recommendations and ratio in eggs (from Singer and Wirth 2003)

Recommendation for the human diet	5:1
German Society of Nutrition (D-A-CH 2000):	~ 5:1
Linoleic acid (n-6)	6–8 g/d
Linolenic acid (n-3)	1.5 g/d
Arachidonic acid (n-6)	?[a]
EPA and DHA (n-3)	?
European Commision (EURODIET 2001):	4 : 1
Linoleic acid (n-6)	6 g/d
Linolenic acid (n-3)	1.5 g/d
Arachidonic acid (n-6)	?
EPA and DHA (n-3)	?
International Society for the study of Fatty Acids and Lipids (ISSFAL): (Simopoulos 1995)	3 – 2 : 1
Linoleic acid (n-6)	6.7 g/d
Linolenic acid (n-3)	2.2 g/d
Arachidonic acid (n-6)	?
EPA and DHA (n-3)	0.65 g/d
Simopoulos et al. (2000):	~ 2 : 1
Linoleic acid (n-6)	4.44 g/d
Linolenic acid (n-3)	2.22 g/d
Arachidonic acid (n-6)	?
EPA and DHA (n-3)	0.65 g/d
Normal eggs[b]	~ 8 : 1
Enriched eggs[c]	~ 2 : 1

[a] ? = no recommendation
[b] Data from Table 5
[c] Caston and Leeson 1990, Botsoglou et al. 1998

take into consideration the total amount of foods containing fat and cholesterol (Wolfram 2001).

Attempts at modifying the cholesterol content in eggs have not been very successful in the past. The feeding of palm oil and amaranth (Punita and Chaturvedi 2000) or garlic (Chowdhury et al. 2002) to laying hens resulted in decreased cholesterol contents (15–25%) in the eggs. However, meaningful reduction in egg cholesterol content will most likely be achieved only through genetic manipulation of processes involved in lipoprotein synthesis and transport to the developing follicle (Leeson 2000) or through orally administered statins (Elkin et al. 1999). But with regard to negative effects caused by the treatments further studies are required. The reduction of cholesterol in

eggs is not effective in reducing the total dietary cholesterol intake; therefore it would be better to define an upper limit (recommendation) of the dietary cholesterol intake (e.g., 300 mg per day in the whole diet or no more than 1 egg per day) for reducing the cholesterol intake. For healthy men and women a moderate egg consumption can be part of a nutritious and balanced diet.

Phospholipids The lipid fraction of the egg consists of about 33% phospholipids. 70% of these are phosphatidylcholine, 15.5% phosphatidylethanolamine, 5.8% lysinphosphatidylcholine and about 2% each of sphingomyelin and lysophosphatidylcholine (Ternes et al. 1994). Phosphatidylcholine plays an important role in intestinal lipid absorption by enhancing micellar lipid solubility and formation of chylomicrons. Some authors (Jiang et al. 2001; Noh and Koo 2003) have shown that phosphatidylcholine and sphingomyelin from eggs lowers the lymphatic absorption of cholesterol. They have suggested that the intestinal absorption of egg cholesterol may be reduced by the presence of these substances in egg yolk.

Egg phospholipids are a good source of choline in the diet, and dietary choline is important for the synthesis of phospholipids in cell membranes, neurotransmission, transmembrane signaling, and lipid-cholesterol transport and metabolism. Studies have indicated that choline plays a role in liver function, prevention of cancer and development of memory. Choline intake during pregnancy may be important for brain development (Zeisel 1992, 2000).

3.1.3 Carbohydrates

Eggs contain no dietary fiber and only traces of carbohydrates, which have no significance in human diet. Recently, however, the carbohydrate moieties in egg yolk have been investigated and the results suggest that sialyloligosaccharides (present mainly in glycoprotein form) are likely to play an important role in the defense mechanism against pathogens, viruses, and toxins in humans and in improving infant learning ability (Juneja 2000).

3.2 Micronutrients

3.2.1 Vitamins

Eggs are recognized as a major source of vitamins in the diet. The fat-soluble vitamins are found almost exclusively in the egg yolk and most of the water-soluble vitamins as well. (Table 6) As mentioned above, one egg provides more than 10% of the recommended daily intake of various vitamins. (A; D, E, K, B_2, B_{12}, biotin, and pantothenic acid; Table 1 and Fig. 1) The vitamin concentration is influenced by genetics, rate of egg production and, just as in the case of fatty acids, it varies with the composition of the hen's diet (Naber 1993; Leeson and Caston 2003). Numerous publications have shown

successful ways of manipulating the concentration of these substances in eggs, e.g. of vitamin E (Flachowsky et al. 2000; Galobart et al. 2002; Jeroch et al. 2002); of vitamin D (Mattila et al. 2003), or of folic acid (Sherwood et al. 1993; House et al. 2002). The transfer efficiency of vitamins from the diet of the hens to the egg is very high for vitamin A; high for B_2, pantothenic acid, biotin, and vitamin B_{12}; medium for vitamin D_3 and E; and low for vitamin K_1, B_1 and folate (Naber 1993).

According to the first European Nutrition and Health Report (Elmadfa 2004), the intake of vitamins in European adults is generally relatively good. However, in some countries the vitamin D intake is low and the average folate intake is below the recommended level in adults of all participating countries. This outlines the significance of eggs as a source of vitamin D (~30% of RDI) and folate (~8.5% of RDI) in human diet. A low vitamin D intake may increase the risk of osteoporosis, diabetes, multiple sclerosis, cancer, and rheumatoid arthritis. An increase in folate intake may be beneficial for the population through reduction in the concentration of homocysteine in plasma, an independent risk factor for cardiovascular disease. The potential fortification of eggs with folic acid has been signaled by House et al. (2002).

Lutein and its stereoisomer zeaxanthin are not vitamin-A-active xanthophylls but are the most common ones found in egg yolk. The xanthophyll carotenoids accumulate in the macular region of the retina. It is stated that they may function to reduce the risk of development of age-related macular degeneration (AMD) and cataracts (Moeller et al. 2000; Olmedilla et al. 2001; Granado et al. 2003). Epidemiological data support the idea of the protective role played by lutein and zeaxanthin. Individuals with a higher consumption of foods rich in lutein and zeaxanthin (like spinach, broccoli, and eggs) have shown a lower risk for AMD (Goldberg et al. 1988). In addition to this function, studies have shown that xanthophylls also have antioxidant properties (Handelman 2001; Granado et al. 2003). Both the content of lutein in the egg and the color of the yolk are influenced by the concentration of lutein in the feed of the laying hen and could therefore depend on how hens are kept, in cages or outdoors. The lutein content in whole egg ranges from about 0.3 to 1 mg/100g (Leth et al. 2000; Hamulka et al. 2005). To achieve the protective effect of lutein within the context of AMD an estimated daily intake of 8 mg of lutein would be required (Leeson 2004), so that one egg with a content of about 0.8 mg of lutein provides 10% of the required intake. Along with vegetables, therefore, eggs must be recognized as an important source of lutein in human diet (Hamulka et al. 2005).

3.2.2 Minerals

Eggs are also a good source of minerals. Phosphorus, selenium, iron, and zinc in eggs show the highest proportion (~16, 29, 9 and 9%, respectively) of the recommended daily intake (Fig. 1 and Table 1). In addition to these important elements, eggs contain almost all minerals and trace elements in small amounts (Tables 1–6).

Because of its prominent presence in the average European diet, phosphorus intake is not a problem. Iron, zinc and selenium intake however are suboptimal (Flynn et al. 2003; Elmadfa 2004).

Again, as in the case of the fatty acids and vitamins, the content of minerals in eggs varies with the laying hen's diet (Stadelman and Pratt 1989). Therefore manipulation of the mineral concentration in eggs is possible and has already been applied in the production of fortified eggs, e.g. for selenium (Cantor 1997; Jeroch et al. 2002; Yaroshenko et al. 2003) or iodine (Jeroch et al. 2002). In a short controlled trial the consumption of selenium-fortified eggs did not change the selenium concentration in the plasma of healthy adults (Surai et al. 2000). However, a recent clinical trial conducted in Ukraine showed that the consumption of two selenium-fortified eggs per day for eight weeks significantly increased the selenium concentration in plasma (Surai et al. 2004).

4 Bioavailability of Nutrients from Eggs

It is not only the amount of beneficial components present in eggs, but also their bioavailability that plays a critical role in the supply of nutrients. The factors affecting bioavailability can be divided into two main groups—human factors and food component factors. Human factors are the digestive functions, the health and physiological status, and existent diseases. Food component factors influencing bioavailability are, for example, the presence of specific foods or food components in the diet which may inhibit or enhance absorption, such as enzyme inhibitors, fiber or vitamins, or the effect of food processing, e.g., improvement in the digestibility of proteins through mild heat treatment. Interactions between components during absorption and the nutritional state of the consuming individual may affect the bioavailability of eggs. In general most egg nutrients are highly bioavailable.

Studies with labeled egg protein allow digestibility and assimilation to be precisely determined. The results indicate a bioavailability of 65% for raw egg protein and 95% for cooked egg protein (Evenepoel et al. 1998). The digestibility of egg lipids is highly efficient (95–97%).

Bioavailability of preformed vitamin A is about 90%, but for vitamin-active carotenoids the assimilation is lower, depending on different factors (Castenmiller and West 1998; van het Hof et al. 2000). New studies have suggested that vitamin D in eggs is almost exclusively 25-hydroxyvitamin D, which is absorbed better and faster and has greater biological activity than cholecalciferol (Ovesen et al. 2003). The absorption rate of vitamin E varies widely between 15% and 65%, and the reason for this is not properly understood (Traber and Sies 1996). Lutein absorption is significantly higher from lutein-rich egg yolk than from vegetables or supplements (Handelman et al. 1999).

Decisive factors for the bioavailability of minerals are the form of mineral used (e.g., types of iron compounds), the use of food additives (e.g., chelating

agents), the presence of other minerals and other substances in foods (e.g., tannins, phytates, oxalates).

Iron is a problematic mineral in eggs by virtue of its particular bioavailability. The iron in eggs is present in ferric form (not in heme form like in other animal products) bound to the proteins ovotransferrin (egg white) and phosphovitin (egg yolk) with the consequence that the availability is relatively poor (~1–10%; Hallberg et al. 1997). Additionally, the presence of eggs in the diet reduces the bioavailability of iron from other food sources. Adding enhancing factors to the diet such as vitamin C, citric acid, cysteine-containing peptides or ethanol improves the iron bioavailability in the diet. In general iron bioavailability is determined by overall meal composition and under most conditions is not a unique property of the food source (Lynch 1997; Hallberg and Hulthén 2000). Selenium is found in eggs as selenomethionine and is absorbed and retained more efficiently (78–82%; King 2001) than inorganic selenate or selenite. A short overview of the bioavailability of nutrients (not exclusively from eggs) is presented in Table 12.

5 Enrichment and Fortification of Nutrients in Eggs

It is possible to increase the content of important egg components such as vitamins (and also functional plant components without vitamin activity), minerals, or specific fatty acids by suitably changing the composition of hen feed. From a nutritional point of view the amount of enriched nutrients in eggs should be high enough for beneficial effects to be detected and low enough that no adverse effects occur.

In recent years the tendency throughout Europe has been to restrict the nutrient enrichment of food, both in individual nations and EU-wide (BfR 2004, 2005; Flynn et al. 2003). Consideration must be given to the risks of both undersupply and oversupply of nutrients, as high doses of some nutrients can also cause adverse effects. For example, a published opinion from the FEEDAP Panel on the use of iodine in feedstuffs, which presents the worst-case-scenario model calculations with milk and eggs, based on the current approved maximum iodine level in feed, shows that the upper limit of iodine for adults and adolescents could be exceeded. Reducing iodine to a maximum of 4 mg/kg in complete feed for dairy cows and laying hens would result in a satisfactory margin of safety for the consumption of milk and eggs (EFSA - FEEDAP 2005).

The opinions of the Scientific Committee on Food (SCF) on the Tolerable Upper Intake Level in humans of beta carotene, vitamins A, B_1, B_2, B_6, B_{12}, D, E, K, folate, niacin, pantothenic acid, plus minerals calcium, chromium, iodine, iron, magnesium, manganese, molybdenum, selenium, and zinc provide the basis for the establishment of safety factors, where necessary, for individual vitamins and minerals that would ensure the safety of fortified foods and food supplements containing these nutrients (SCF 2003b).

Table 12. Bioavailability of egg components

Constituent	Bioavailability in humans		References
Protein	Raw eggs vs. Cooked eggs	~65% Raw ~95% Cooked	Enevepoel et al. 1998
n-3 Fatty Acids		60–70%[a]	Kinsella 1991
Vitamins			
Vitamin A	Preformed	>95%[a]	Borel et al. 2001
Carotenoids	Green leafy vegetables vs. Mixed vegetables	56–62% green leafy 10–22% mixed	van het Hof et al. 2000
Vitamin D	Whole eggs	Good	Ovesen et al. 2003
Vitamin E		15–65%[a]	Traber and Sies 1996
Vitamin B_2		~ 95%[a]	FAO and WHO 2002
Vitamin B_6		51–91%[a]	Roth-Maier et al. 2002; Gregory 1997
Vitamin B_{12}	Whole eggs	24–36%	Doscherholmen et al. 1975
Folate	Egg yolk	~70%	Seyoum and Selhub 1998
Biotin	Raw eggs vs. Cooked eggs	Low raw high cooked	Mock 1996
Minerals			
Iron	Eggs in meals	Less iron bio-availability	Hallberg and Hulthen 2000
Zinc	Habitual diet vs. Egg yolk powder	~30% diet ~76% powder	O'Dell et al. 1972
Iodine		High[a]	Stanbury 1996
Selenium	Whole eggs	78–82%	King 2001
others			
Lutein	Egg yolk	High	Handelman et al. 1999

[a] Bioavailability from a mixed diet or the diet was not defined

Studies have indicated that significant subgroups in most European populations have intakes below nationally recommended levels for several vitamins, minerals, and trace elements. Enrichment and fortification of food could be a solution to this problem. The nutrients which could be added safely can be divided in three groups. The first group—vitamins B_1, B_2, B_{12}, C, E, niacin, and

pantothenic acid–could be added at levels (per serving, e.g., 100 kcal) greater than the European Recommended Daily Intake (EC RDA). The second group—vitamins B_6, D, folic acid, and biotin, and minerals copper, iodine, and selenium—could be added at levels between 50 and 100% of the EC RDA. The third group—iron, zinc, calcium, phosphorus, and magnesium— could be added at levels between 10 and 40% of the EC RDA. Only the retinol (preformed vitamin A) intake is near the tolerable upper intake level and therefore problematic for enrichment or fortification of food (Flynn et al. 2003).

In Europe the enrichment or fortification of food, especially eggs, should be concentrated on substances recognized as deficient or as beneficial for the whole population or for special groups therein (e.g., pregnant women, elderly people, children) EU wide (EURODIET 2000a; BfR 2005). One of the problems in this field is that the range of intakes and EU member state recommendations for nutrients (e.g., for folate) vary considerably (EURODIET 2000b).

The report of the EURODIET project (EURODIET 2000a) suggests reference nutrient intakes (for some nutrients only) and goals for the adult population in the EU. For the evaluation of egg composition the recommendations on the content of folate, vitamin D, and iodine are the most interesting (Table 13).

The generally agreed-upon problem nutrients should receive the most attention. One of the next steps should be the assessment of the contribution of enriched eggs to the supply of such functional substances (e.g., folic acid). Similar studies have already been conducted on the contribution of folate fortification of foods (e.g., flour, salt, and milk) to the supply of folate (Oakley 2002; Quinlivan and Gregory 2003; Burger et al. 2004; Renwick et al. 2004).

As mentioned above, various egg components can be modified by changing the composition of the hen's diet. There is both promise and risk associated with the enrichment and fortification of eggs (Sparks 2005; Seuss-Baum 2005). A key factor in promotion of conventional or enriched eggs in the human diet and for success in the market is the consumers' understanding of the functional concept. Therefore education of the consumer and the nutrition and health sector on the beneficial effects of natural egg components or substances that could be enriched in eggs is necessary.

Table 13. Reference nutrient intakes and goals for the adult population (from EURO-DIET 2000a)

Component	Reference nutrient intakes and goals for the adult population
Folate from food (µg/d)	>400
Vitamin D (µg/d) for elderly	10
	150
Iodine (µg/d)	50 (infants)
	200 (pregnancy)

6 Conclusions

Eggs are a good source of nutrients such as protein, vitamins (such as B_2, B_{12}, A, D, K, and folic acid), minerals (such as selenium, iodine, and iron) and functional substances like lutein, bioactive proteins, and special fatty acids. Furthermore, these components from eggs are highly bioavailable. With a moderate enrichment of nutrients in accordance with the recommendations for fortification of foods, eggs could play an important role as a functional food. However, the most important aspect for the promotion of eggs in the human diet should be the education of the consumer and the nutrition and health sector on the beneficial effects of natural egg components or substances that could be enriched in eggs.

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Chapter 19 Concepts of Hypoallergenicity

Yoshinori Mine and Marie Yang

1 Introduction

It is now well-established that the prevalence and incidence of allergic diseases have significantly increased in the last two decades, particularly in industrialized countries. Food-induced allergies are estimated to occur in 6% of young children and 3–4% of adults according to a recent epidemiologic study completed in the US (Sicherer and Sampson 2006). Cow's milk and egg represent the most common causes of allergic reactions in young children, while adults are more likely to develop sensitivity to shellfish products (Sampson 2004).

A food allergic reaction is believed to result from an abnormal response of the mucosal immune system towards dietary antigens, mainly proteins, which would otherwise be harmless (Bischoff and Crowe 2005). Allergic reactions must be distinguished from food intolerances, which are non immunological in nature (Sampson and Cooke 1990). The best understood and most common form of food allergy is mediated by a class of antibodies called immunoglobulin E (IgE), and is classified as a type I hypersensitivity reaction (Ebo and Stevens 2001).

Currently, strict avoidance of the offending food is the only efficient prevention against or treatment of allergic reactions. However, complete avoidance is often difficult because many packaged or prepared foods contain minute amount of milk, egg, or nut proteins (also called "hidden allergens") with a dose sufficient to trigger allergic symptoms, and sometimes life-threatening reactions in sensitive individuals (Arshad 2001; Zeiger 2003). In addition, in some cases the elimination of essential foods for extended period of time may lead to malnutrition, especially in young children. There is therefore an urgent demand for the reduction or elimination of allergens from food products (Crevel 2001). The making of hypoallergenic food products represents one alternative and will be one of the main topics covered in the third section.

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2 Food Allergy Overview

2.1 Molecular Aspects of Food Allergy

The etiology of IgE-mediated food allergies involves two phases: (1) the sensitization phase, without symptoms, during which the immune system is primed, and (2) the elicitation phase with specific clinical responses (i.e., allergic symptoms), which occurs after subsequent contacts with the offending compound.

The acute symptoms of food allergies are due to the release of inflammatory mediators such as histamine, leukotrienes, and prostaglandins. These mediators are released when the allergen binds to IgE antibodies attached to high affinity receptors (FcεRI) present at the surface of mast cells and basophils, causing cell degranulation. At the cellular level, type 1-helper T cells (Th1), type 2-helper T cells (Th2), and regulatory T cells (Treg) are known to be involved in the pathogenesis of allergic disorders. A significant feature is that Th1 and Th2 are capable of cross-regulating each other. T regulatory cells include Th3 cells, T regulatory (Tr)-1 cells, CD4+CD25+ T cells, and natural killer T (NKT) cells (Taylor et al. 2004). They produce suppressive cytokines which keep inflammatory T cells (both Th1 and Th2) and their downstream effectors under control (Stock et al. 2006). It is suggested that the absence of Treg cells activity would result in a Th2-skewed immune response, with elevated levels of interleukin (IL)-4, IL-5, and IL-13. A Th2-skewed immune response is a hallmark of an allergic response and the generation of allergen-specific IgE by B cells precisely depends on the generation of IL-4-producing Th2 cells. In contrast, non-allergic individuals will show higher levels of Th1 cytokines characterized by interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and the regulatory cytokine IL-10 (Fig. 1).

2.2 Molecular Properties of the Major Egg Allergens

Egg proteins are part of the so-called big eight food allergens. They are responsible for one of the most common forms of food allergy, especially in children (Sampson 2004). In an effort to determine which egg proteins are major allergens, a recent study has investigated binding of specific IgE to eight purified egg white and yolk proteins by radioallergosorbent test (RAST) using sera from forty egg-sensitive children (Walsh et al. 2005). The study confirmed that the major egg allergens originate primarily from egg white, and include ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4). The same report confirmed previous results that egg yolk contains allergenic proteins as well, and are identified as apovitellenins I and VI and phosvitin (Anet et al. 1985; Walsh et al. 1987; Walsh et al. 1988). In previous studies, other proteins such as ovomucin, ovoflavoprotein, and ovoinhibitor were identified as antigenic, but lack allergenic activity (Langeland 1982; Hoffman 1983; Langeland 1983; Anet et al. 1985). The two major allergens ovomucoid and ovalbumin constitute about 11% and 54% of

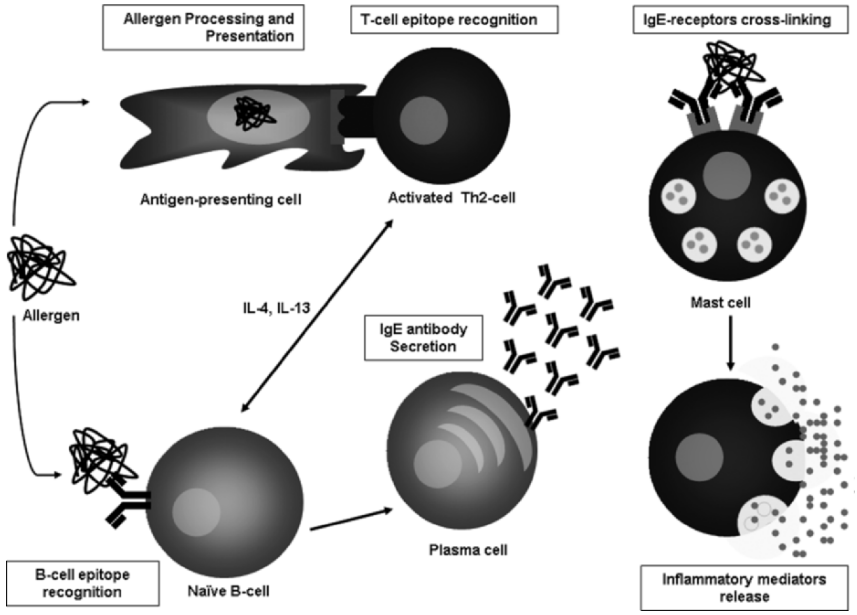

Fig. 1. Mechanisms of IgE-mediated allergic response. When food allergens are presented to T cells by antigen presenting cells (e.g. dendritic cells), the differentiation into Th2 cells will be induced. This phenomenon leads to the production of cytokines such as IL-4 and IL-13, which both promotes immunoglobulin E (IgE) production by plasma cells. The allergen specific-IgE will then bind to their high affinity receptor (FcϵRI) present at the surface of mast cells, triggering the cross-linking of the receptors, and the subsequent release of inflammatory mediators such as histamine, directly responsible for the clinical symptoms of food allergy

egg white proteins, respectively (Kovacs-Nolan et al. 2005). Both proteins are glycosylated, with as high as 25% of the mass of ovomucoid comprising carbohydrates. Debate flourished over the immunodominance of ovalbumin as the major egg allergen; however, studies showed that use of commercially purified ovalbumin led to an overestimation of its dominance as a major egg allergen in egg-sensitive patients (Bernhisel-Broadbent et al. 1994).

The glycosylated form of ovomucoid was shown to be highly resistant to tryptic digestion (Gu et al. 1989; Kovacs-Nolan et al. 2000). It retains its allergenicity after prolonged heating at high temperature (Gu et al. 1986), which stability is most likely due to the protein containing 4 to 5 carbohydrates chains and its 9 disulfide bonds (Hirose et al. 2004). Ovomucoid T cell and B cell epitopes have been reported in egg allergic patients and in murine models (Holen et al. 2001; Mine and Wei Zhang 2002; Mizumachi and Kurisaki 2003).

In its native form, ovalbumin is resistant to trypsin, while its heat-denatured form has shown an increased sensitivity to proteolysis (Kato et al. 1986). In one study, the allergenicity of ovalbumin was shown to be resistant to heat treatment (Elsayed et al. 1986), while another report indicated a 90% decrease in its allergenicity after exposure to a temperature of 80 °C for a duration of

three minutes (Honma et al. 1994). To explain these variations, it was suggested that sensitization to egg allergens was dependent upon variation inherent in the group of patients selected, and not solely upon the nature of the allergens. Ovalbumin B and T cell epitopes have also been reported in human and murine models (Besler 1999 and references therein).

The integrity of food allergen epitopes is mainly determined by their primary (B cell sequential epitope) structure, and sometimes tertiary (B cell conformational epitopes) structure. Presence of B cell linear epitopes explains why most food protein allergenicity can be quite resistant to the effects of food processing or gastrointestinal digestion. The concept of hypoallergenicity is based upon the knowledge of these epitope structures and the factors that determine their chemical and physical stability, as will be discussed in paragraphs below.

2.3 Role of Glycosylation in Protein Allergenicity

Many food proteins undergo post-translational modifications such as glycosylation during their passage through the endoplasmic reticulum. Since the existence of N-glycan-specific IgE has been reported (van Ree and Aalberse 1995; van Ree 2002), the allergenicity of carbohydrate moieties has been a matter of active debate. It is known that N-glycosylation can have a significant stabilizing or rigidifying effect on protein structure, conferring an enhanced resistance to denaturation (Pedrosa et al. 2000). In the particular case of highly glycosylated ovomucoid, it was initially reported that the carbohydrate moiety contributed to its IgE binding activity in human sera (Matsuda et al. 1985), but subsequent studies reported that it did not participate to the protein allergenicity (Besler et al. 1997). In fact, the carbohydrate chains may have an inhibitory effect on its IgG and IgE binding activities (Zhang and Mine 1998).

3 Concept of Hypoallergenicity

3.1 Development of Hypoallergenic Products via Food Processing

The use of food processing to develop hypoallergenic foods is a relatively novel and not very widely used approach (Wichers et al. 2003). The processing history of a protein allergen likely has an effect on its allergenicity as its native tertiary structure is changed.

The main challenge in the development of hypoallergenic food through elimination or denaturating of the food allergen is to maintain the nutritional and functional properties identical to the untreated food. Success in such efforts might allow allergic patients easy access to a rich and varied diet. Egg proteins constitute a unique model system for studying the effects of physical and chemical processing, as egg is used as an ingredient in a large variety of

processed foods (dry, heated, or pasteurized) with varying physicochemical states (from intact egg to an ingredient incorporated in a food product). There are only a few examples of actually marketed hypoallergenic foods, and proteolytically hydrolyzed milk proteins for infant formulations is probably the best known example (Hays and Wood 2005). A recent review described various processing technologies aiming at the reduction of allergenicity in food products (Wichers et al. 2003). These technologies include chemical, biochemical (using proteases or oxidases) and physical (such as heat or extraction) methods. Some of them are presented below.

3.1.1 Thermal Processing

Thermal processing is usually carried out to enhance texture and flavor, or to insure microbiological safety, but not to reduce allergenicity. Previous reports have described studies of the effect of thermal processing on the nutritional properties of egg-white proteins (Kato et al. 1986; Mine et al. 1990), but only a few studies have considered heat application as a fast and efficient procedure for the production of hypoallergenic foods. During heat denaturation, many of the sites recognized by IgE antibodies present on the native molecule can be destroyed; cooking can eliminate the allergenicity of some food proteins. The basis for this approach was demonstrated in a clinical study in which patients allergic to freeze-dried egg white did not react to cooked egg white (Urisu et al. 1997).

In contrast, other studies have shown that food proteins denatured by heat may reveal new antigenic sites or neoepitopes, due to the protein's unfolding process or to new chemical reactions occurring with other molecules present in the food (Davis and Williams 1998). One reaction in particular that occurs during heat processing has been the focus of several investigations, namely, the Maillard reaction. The reaction of protein amino groups with sugars leads to the production of so-called advanced-glycation end products. These protein derivatives have been shown to possess antigenic properties and are believed to contribute to the presence of neoantigens sometimes found in cooked foods (Davis et al. 2001).

In a recent paper, the allergenicity of the four major egg allergens (native vs denatured forms) were compared. It was shown that human anti-ovalbumin-specific IgE could recognize more sequential epitopes, and that anti-ovomucoid and anti-lysozyme antibodies could recognize both conformational and sequential epitopes (Mine and Zhang 2002). Similarly, the effects of thermal processing on peanut allergenicity have been the subject of numerous studies (e.g., Maleki et al. 2000; Maleki and Hurlburt 2004; Mondoulet et al. 2005). It was demonstrated that roasting (i.e., thermal processing) could enhance the allergenic properties of two peanut major allergens Ara h 1 and Ara h2, and that the structural modifications brought by the Maillard reaction had significantly contributed to this effect (Maleki et al.

2000, Mondoulet et al. 2005). For these reasons, thermal processing may not represent an adequate solution for decreasing the allergenicity of food products or ingredients, especially in cases such as peanut protein allergy where inappropriate food processing methods could lead to a fatal outcome. However, this phenomenon may be peculiar to peanut. Indeed, the roasting of hazelnuts, from a different botanical family, was shown to reduce its allergenicity, as shown by a double-blind placebo control food challenge (Hansen et al. 2003; Fiocchi et al. 2004).

Many food allergens are known to be resistant to heat; therefore it seems impractical to use heat as a way of producing hypoallergenic foods, unless it is combined with other approaches, such as for instance enzymatic processing.

3.1.2 Enzymatic Fragmentation

Enzymatic processing represents an approach more specific than thermal processing. However, epitopes present on most food allergens are known to be sequential, so that reduction in allergenicity would only be expected when allergenic epitopes are eliminated.

Proteinase treatment is a popular strategy for reducing the allergenicity of food products. One of the best known examples is derived from extensively hydrolyzed cow's milk-based formulas (Sampson et al. 1991; Hill et al. 1995; Hoffman and Sampson 1997). This approach was shown to be efficient in preventing sensitization to cow's milk proteins (Marini et al. 1996). In Japan, the enzymatic treatment of rice, which leads to the decomposition of globulin, a major rice allergen, has given birth to one of the first FOSHU, i.e., "Food for Specialized Health Uses", in 1993 (Arai 2000; Shimada et al. 2005). Further information on rice allergens and investigations of hypoallergenic rice products by enzymatic treatment is available from the internet Symposium on Food Allergens (Besler et al. 1999). Regarding staple foods such as wheat-containing products, preparation of hypoallergenic wheat flour has been investigated in a murine model using enzymatic fragmentation with cellulose and Actinase (Protica, Lafayette Hill, PA, USA) preparations (Watanabe et al. 2000, Watanabe et al. 2004).

Enzymatic hydrolysis may not represent a viable alternative for egg proteins, since egg proteins are used as an ingredient in food products for their unique functional properties, e.g. foaming and gelling.

3.1.3 Other Food Processing Methods

A recent review suggests that novel food processing methods, such as high-pressure or pulse-electric field processing, combined with physical and biochemical processing, hold great promise for the development of hypoallergenic food products (Wichers et al. 2003).

A summary of various technologies for production of hypoallergenic food products is found in Table 1.

Table 1. Current strategies for the production of hypoallergenic food products

Strategies	Allergen targets	Effects/End products	References
Thermal processing	Egg white	Reduced allegernicity	Urisu et al. 1997
Enzymatic fragmentation	Hazelnut	Reduced allergenicity	Fiocchi et al. 2004; Hansen et al. 2003
	Peanut	Increased allergenicity (*Maillard reaction*)	Maleki et al. 2000; Maleki and Hurlburt 2004; Mondoulet et al. 2005
	Food proteins (Review)	Increased or reduced allergenicity	Davis and Williams 1998; Davis et al. 2001
	Cow's milk proteins	Hypoallergenic infant formulas	Sampson et al. 1991; Hill et al. 1995; Hoffman and Sampson 1997
	Wheat	Allergy suppressive effects	Watanabe et al. 2000; Watanabe et al. 2004
	Rice	Potentially reduced allergenicity	Arai 2000; Besler et al. 1999; Shimada et al. 2005
Other processing technologies (e.g., high-pressure and pulse-electric field)	Plant food allergens (Review)	Potentially reduced allergenicity	Wichers et al. 2003

3.2 Development of Hypoallergenic Products for Immunotherapeutic Purposes

3.2.1 Full Recombinant Allergens

Clones of cDNA have been isolated for an increasing number of food allergens in recent years and have been expressed in prokaryotic and eukaryotic systems as recombinant allergens. The purpose of using recombinant DNA technology is to produce recombinant molecules that mimic the wild-type food protein but differ in their immunological properties (Chapman et al. 2002). A special issue of *Methods* (Valenta and Kraft 2004) contains thorough review articles dedicated to the potential use of recombinant allergens for clinical applications. For recombinant allergens to provide therapeutic value in a clinical setting, they need to display a reduced capacity to bind IgE bound to mast cells or basophils, thus ensuring a lower risk of IgE-mediated side-effects, while retaining their T-cell epitopes (Ferreira et al. 1998). In this approach, it is hypothesized that engineered proteins will not provoke any

allergic reactions when ingested by the allergic patient, and that they will be able to deviate the allergic response from a Th2- to a Th1-type response.

There are currently no recombinant allergen-based vaccines available on the market (Niederberger and Valenta 2004). However, results from a number of studies, including the first clinical trials using genetically modified grass pollen allergens (Niederberger et al. 2004; Jutel et al. 2005), suggest that it is feasible to engineer a hypoallergenic vaccine that fulfills these criteria.

It has been expressed that another advantage of using DNA technology was the possibility to combine multiple recombinant allergens in a single injection and bring a significant improvement for the treatment of multi-sensitized individuals (Wiedermann 2005).

3.2.2 Site-Directed Mutagenesis

Molecular biology tools today available have permitted the cloning of the most common food allergens and have allowed the production of recombinant molecules by site-directed mutagenesis. The manipulation of nucleotide bases allows the introduction of substitutions or deletions altering the coding sequences of allergens, resulting in a mutated sequence upon production of the protein (Prescott and Jones 2002).

Peanut allergens are probably the best known example, as they are responsible for the most common food allergies in children, and are also the most life-threatening reactions. The three major peanut allergens (Ara h1, Ara h2, Ara h3) were subjected to site-directed mutagenesis and successfully tested against patients' sera for their reduced IgE binding activity and their conserved lymphocyte proliferation activity. Since then, an increasing number of studies have used this technology. Site-directed mutants of egg-white proteins have been used in desensitization and tolerance induction studies. A very recent paper describes the desensitization study of mice where site-directed mutagenesis of the ovomucoid protein had a marked suppressive effects on the Th2 allergic response established in a murine model (Rupa and Mine 2003, 2006). The abolition of the IgE-binding epitopes is believed to be responsible for these anti-allergic effects.

3.2.3 Peptide-Based Immunotherapy

Peptide-based immunotherapy (PIT) is an elegant approach for the induction of peripheral T cell tolerance. Peptides have been proposed as a therapeutic alternative to specific immunotherapy (SIT) as they have the potential to inhibit T cell function but do not induce the adverse reactions often associated with SIT because of the loss of their IgE binding activity (Francis and Larche 2005). At least two distinct strategies have been investigated. The first approach mainly targets T lymphocytes and induces hyporesponsiveness though induction of regulatory T cells and cytokines, which are also known as tolerance-inducing peptides. The second approach mainly focuses on B lymphocytes. It consists in determining which "hypoallergenic" B cell epitopes are capable of

inducing inhibitory IgG antibodies. Immunodominant epitopes are generally selected as the best candidates for such approaches. Both these strategies are discussed below.

3.2.4 Production of Tolerance-Inducing Peptides

In the context of food allergy, the ingestion of dietary proteins is usually followed by an immune unresponsiveness to the ingested food. This vital phenomenon is also known as oral tolerance. Today, a number of studies have provided proof of the concept that food allergy results from an abrogation of oral tolerance. Ovalbumin egg white has been commonly used as an allergen model. Preliminary analyses have shown that orogastric administration of ovalbumin (OVA) peptidic fragments could induce hyporesponsiveness in neonatals rats (Brown et al. 1994). Subsequent reports have supported the use of peptides in specific immunotherapies (Briner et al. 1993; Alexander et al. 2005; Larche 2005).

Today, the approach using T cell epitope containing peptides to tolerize or anergize allergen-specific T lymphocytes has been taken up to clinical trials (Fellrath et al. 2003). A prerequisite in peptide-based immunotherapy using short peptides is information on patients' T cell epitopes. So far, two sources of non-dietary allergens, bee venom and cat dander, have been considered for peptide-based immunotherapy in clinical trials. Peptides derived from the major cat allergen Fel d 1 were evaluated for the treatment of cat dander-induced allergies, but results were inconclusive. The efficacy and safety of peptide-based immunotherapy is yet to be demonstrated (Simons et al. 1996; Maguire et al. 1999).

A major limitation in peptide-based immunotherapy is the problem caused by variations in HLA genotype among individuals. This means that the pool of tolerance-inducing peptides may need to be tailored to each individual (Prescott and Jones 2002). For these reasons, the use of longer and in some cases overlapping peptides has been suggested as an alternative (Fellrath et al. 2003).

The efficient dose, the route of administration, the frequency, and the amount of short or long peptides to be administered have yet to be optimized, and the mechanisms underlying oral tolerance induction by peptides still remain unclear. To the best of our knowledge, no report exists on tests in humans of peptide-based immunotherapy for food allergy.

3.2.5 Blocking IgG-Inducing Peptides [Mimotope (Mimotopes, Clayton, Australia) Immunotherapy]

Random-phage-display peptide technology represents a useful tool to identify the antibody-binding regions present on protein antigens, and is mainly applicable to conformational epitopes (Ganglberger et al. 2000). The strategy aims at inducing blocking IgG antibodies, which prevent the binding and the cross-linking of allergen-specific IgE antibodies at the surface of mast cells and basophils (Untersmayr et al. 2006). Further information can be found in a recent review paper (Riemer et al. 2004).

3.2.6 Chemically Modified Allergens (Allergoids)

Much effort has been made to chemically modify allergens. The resulting compounds have been termed allergoids. Their most critical features are: (1) their strongly reduced IgE-binding activity, and (2) their retained immunogenicity (T-cell reactivity) compared to their native counterpart. Formaldehyde and glutaraldehyde were among the first reagents to be used (HayGlass and Stefura 1990). The resulting compounds were found to have lower allergenicity but their molecular masses tended to be very high because of extensive cross-linking reactions. Carbamylation was then proposed as an alternative, modifying allergens in a monomeric fashion (Mistrello et al. 1996; Bagnasco et al. 2001). Hypoallergenicity of ovalbumin protein conjugated with the copolymer N-vinyl pyrrolidone and maleic anhydride has been demonstrated in a murine model (Babakhin et al. 1995). Allergoids have been established for allergen-specific immunotherapy against inhalant allergens for many years (Norman et al. 1982; Bousquet et al. 1987; von Baehr et al. 2005), but they have not been tested yet in clinical settings.

The summary of current strategies for producing hypoallergenic variants (dietary and non-dietary sources) for clinical purposes is highlighted in Table 2.

Table 2. Current strategies for the production of hypoallergenic formulas for clinical applications

Strategies	Allergen targets	Effects, Induced mechanisms	References
Full recombinant allergens	Grass allergens	Proven efficacy in clinical trials	Jutel et al. 2005; Niederberger et al. 2004; Wiedermann 2005
	Birch pollen, latex, venom	Mucosal tolerance induction	Wiedermann 2005
Site-directed mutagenesis	Egg ovomucoid	Allergy suppressive effects	Rupa and Mine 2006
Peptide-based immunotherapy	Egg ovalbumin	Allergy suppressive effects	Brown et al. 1994
	Cat dander (Fel d 1)	Allergy symptoms inhibition	Alexander et al. 2005; Briner et al. 1993; Larche 2005
	Bee venom	Hyporesponsiveness and immune deviation	Fellrath et al. 2003
Chemical modifications (allergoids)	Egg Ovalbumin	Hyporesponsiveness and reduced allergenicity	Babakhin et al. 1995
	Inhalant allergens	Hyporesponsiveness in allergic patients	Bousquet et al. 1987; Norman et al. 1982; von Baehr et al. 2005

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Chapter 20 Egg Enrichment in Omega-3 Fatty Acids

A.L. Yannakopoulos

1 Introduction

The role of food in human health is again in vogue, however, the link between what we eat and our health is a subject that was being written about long ago as 500 BC when Hippocrates wrote "let food be your medicine and medicine be your food." Eggs have been associated with creation, fertility, and new life since ancient times and are consumed by every generation from childhood to late life. Thus, compared with the hen's egg, no other single food of animal origin is eaten by so many people all over the world, and none is served in such a variety of ways. The egg is considered as nature's most complete food containing high quality proteins and a 2 to 1 ratio of unsaturated fats to saturated fats. It is an excellent source of iron, phosphorous, and other minerals, and contains all the vitamins with exception of vitamin C. The possibility of manipulating the nutrient composition of eggs was shown as long ago as 1934, and modification of the polyunsaturated fatty acid (PUFA) composition has been addressed since the early 1960s. Egg yolk composition depends on the dietary nutrient provision and information is accumulating suggesting that other nutrients in eggs can have their content manipulated.

Some egg producers today supply new-type or specialty eggs (organic eggs, free-range eggs, omega eggs, etc.). These eggs may be slightly different in nutrient value from regular eggs or they may come from hens housed or fed in a special way.

2 Fatty Acid Requirements in Humans

There are two families of PUFA, omega-6 (ω-6) and omega-3 (ω-3), defined by the position of the double bond close to the methyl end of the molecule (Simopoulos 2002).

The ω-3 fatty acids are long-chain PUFA (18–22 carbon atoms) with the first of many double bonds beginning from the third carbon atom when

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

counting from the methyl end (Holub 2002). Especially, the ω-3 can be categorized according to their chain length. The 18-carbon n (ω) linolenic acid (ALA18:3n3) is precursor to the longer ω-3 polyunsaturated fatty acids eicosapentaenoic acid or EPA (20 carbon atoms, 5 double bonds, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).

These ω-6 fatty acids are also long-chain PUFA with the first of many double bonds beginning at the sixth carbon atom from the methyl end. The parent acid of this family is linoleic acid (LA, 18:2, n-6) which is converted to the longer chain ω-6 fatty acid, arachidonic acid (AA, 20:4, n-6).

Of all fats found in food, ALA and LA cannot be synthesized in human body. These fats are called essential fatty acids because they are necessary for physiological functions.

2.1 Fatty Acid Metabolism in the Human Body

After ingestion, ALA and LA are converted into long chain PUFA. The liver hepatocyte is a main site for the biosynthesis of 20-and 22-carbon PUFA from ALA (e.g., readily to EPA and more slowly to DHA) and for the formulation of lipoproteins that transport fatty acids in the plasma (Pawlosky et al. 2001). The only known metabolic function of ALA is to be a dietary precursor of EPA and DHA. Conversely, supplementing the diet with ω-3 long chain PUFA (e.g., EPA and DHA) will not lead to increased tissue levels of ALA (Jones et al. 2001). Conversion of ALA to EPA and DHA occurs to a low degree (about 0–15 %) in the adult human body (Jiang and Sim 1993). This conversion is affected by several factors, including the concentration of ALA and ω-6 fatty acids, mainly LA, and it is not sufficient for optimal health (De Deckere et al. 1998). LA can also be elongated and desaturated into AA, but the conversion from LA to AA via γ-linolenic acid (20:3, n-6) appears to occur slowly in humans and there is a competition between ω-3 and ω-6 fatty acids for Δ-desaturase enzymes. Thus, a balance between ω-6 and ω-3 PUFA in the diet is important since both compete for the same enzymes and they have different biological functions. It appears that ω-3 fatty acids are preferred by 6-desaturase enzymes (Simopoulos 1988).

2.2 Effects of PUFA on Human Health

Researchers around the world have focused on the health effects of a dietary supply of ω-3 fatty acids, partly because those fatty acids have been reported to protect against cardiovascular and inflammatory diseases, as well as certain types of cancer, and partly because it has been shown that ω-3 fatty acids are essential nutrients for adults and children.

The potential health benefit of ω-3 fatty acids in the human diet has drawn attention since the original publication of Dyerberg and Bang (1974) who reported a link between dietary ω-3 fatty acid consumption and decreased

incidence of cardiovascular disease in Eskimos. The results of more recent studies have suggested that ω-3 PUFA, as EPA and DHA, are important for brain development in children, prevent platelet aggregation, reduce serum triglycerides and very low density lipoproteins that promote the clogging of arteries, etc. An increased intake of ω-3 fatty acids is now known to protect against heart disease, some inflammatory diseases, and certain autoimmune disorders.

While ω-3 PUFA play a major role in infant growth and development and have been implicated in many other protective roles, ω-6 PUFA are metabolically and functionally distinct and in many cases have opposing physiological effects. The absolute amount of PUFA in the diet is important to human health. Health authorities recommend, for all adults of 19 years and over, an intake of LA of 11-17 g/day and for ALA of 1.1–1.6 g/day (Holub 1998; Howe 1998). The balance between the two major types, ω-6 and ω-3 fatty acids, may also be crucial. The proper ratio of ω-6 to ω-3 PUFA in the human diet appears to be in the range of 1:1 to 4:1.The current ratio in the human diet in the developed world is about 10:1 and thus individuals are advised to consume more foods containing ω-3 fatty acids (green leafy vegetables, legumes, fish, etc.).

3 Lipid Metabolism in Avians and Lipid Composition of Eggs

The yolk of the average chicken egg contains 6 g of lipids mainly in the form of triacylglycerols, phospholipids, and free cholesterol (Noble and Cocchi 1990). Minor yolk components include cholesterol esters and free fatty acids. The phospholipid component exhibits levels of LA, AA and DHA as well as other PUFA (Noble and Cocchi 1990). Fatty acids destined for the yolk are synthesized in the hen's liver, which permits the manipulation of fatty acid components through dietary measures (Cherian et al. 1996). As Sim (1993) reported, the yolk lipid composition is the result of a combination of de novo lipogenesis and incorporation of lipid components from the hen's diet. Thus, egg yolks can be enriched with ω-3 PUFA by the incorporation of fats rich in these essential fatty acids.

Upon absorption from the intestinal lumen, hydrolyzed products of lipid digestion, including long chain fatty acids and monoacylglycerols, must be re-esterified within the endoplasmic reticulum of enterocytes prior to transport (Taylor 1998). The resultant triglycerides are packaged with cholesterol, phospholipids, and proteins to form lipoproteins. In human, these lipoproteins (chylomicrons) are transported within the lymphatic system (Bensadoun and Rothfeld 1972). In poultry, those lipoproteins that include long-chain fatty acids are referred to as portomicrons, because they are transferred to the hepatic portal circulation (Bensadoun and Rothfeld 1972). On the other hand, short-chain fatty acids and free glycerol are transported as such in poultry (also to the liver via the portal system). In the avian liver,

extensive reprocessing of glycerides and fatty acid residues from the portomicron remnants occurs. The lipids reform and are included in particles of very low density lipoprotein (VLDL), which then pass to the Golgi complex where they acquire phospholipids and further glycosylation. Finally, completed particles of VLDL concentrate in secretory vesicles and then discharge into the blood (Bensadoun and Rothfeld 1972).The VLDL are then carried by the blood to the ovarian follicles, where they diffuse through holes in the capillaries. The particles enter the yolk by receptor mediated endocytosis through the oolemma.

4 Omega-3 Enriched Eggs as a Means to Improve Human Health

Commercial table eggs contain a high proportion of ω-6 PUFA but they are a poor source of ω-3 fatty acids. There are two main approaches to increase the ω-3 content of eggs, either by the enrichment of the diet with ALA of a vegetable source (flaxseed, linseed, etc.) or by the addition of any source of EPA or DHA (fish meal, fish oil, algae, etc.).

Flaxseed is a rich source of unsaturated fats, it contains about 38% fat, and 50% of this fat is in the form of ALA. It appears that the concentration of ALA can be increased from a very low level of 21 mg/egg, when hens are fed diets without flaxseed, to a level of 580 mg/egg by the addition of flaxseed at levels of 20% (Table 1). ALA is the major ω-3 fatty acid that accumulates in the triglyceride fraction of yolk lipids due to the consumption of flaxseed, although research indicates that the concentration of EPA and DHA can also be increased to 8.5 and 87 mg/egg, respectively, for hens fed 10% flaxseed.

On the other hand, menhaden and herring oils contain about 1% ALA but a total 12–14% of combined EPA and DHA. Three percent dietary menhaden oil increased yolk DHA to 252 mg/egg (Van Elswyk et al. 1995; Table 1). Although the use fish oils has the advantage of enriching the eggs mainly with long ω-3 fatty acids that are specially involved in human health, the maximum level of fish oils that can be used in the layer diet is limited due to production of eggs with a fishy flavor. Van Elswyk et al 1995 reported that treatments with less than 3% oil did not significantly lower overall flavor quality. Another approach for the enrichment of eggs with long chain ω-3 is to use microalgae in the laying hen diet. Different kinds of microalgae may contain up to 50% of their total fatty acids in the form of EPA and DHA.

In general the ω-3 fatty acid profile of the egg is dependent on which supplements hens are fed (Van Elswyk 1997). The levels of ω-3 in the yolk of eggs achieved by the inclusion of different sources of ω-3 fatty acids in the diet varies considerably (Jiang and Sim 1994; Surai 2003). For example

Table 1. Enrichment of the ω-3 fatty acid content in eggs using different ω-3 sources (mg/egg) and ω-3 content in different egg types

Diet	ALA	EPA	DHA	Source
20% flaxseed	580	8	74	Caston and Leeson 1990
2% linseed + 2% mackerel oils	214	32	214	Farrell et al. 1991
3% menhaden oil	21	24	252	Van Elswyk et al. 1995
12% herring meal	66	8	100	Nash et al. 1995
Egg type				
Supermarket egg	8.5	0	19	Simopoulos 2000
Greek egg	117	20	112	Simopoulos and Salem 1992
Fish meal egg	70	3.5	110	Simopoulos 2000
Flaxseed egg	362	8.5	87	Simopoulos 2000

(Table 1), the inclusion of flaxseed rich in ALA increased the level of DHA in the egg up to 74–87 mg. Greek free range eggs prior to 1960 provided 112 mg DHA (Simopoulos and Salem 1992), while a similar amount of DHA, 100 mg, was accumulated in the egg yolk as a result of using 12% herring meal (Nash et al.1995) or 1.5% of menhaden oil (106 mg DHA) (Marshall et al. 1994).

4.1 Enriched ω3 Eggs in Practice

In the case of the ω-3 enrichment, eggs are naturally enhanced through programmed feeding of laying hens with PUFA, antioxidants, and vitamins which are transferred to the egg. This natural program of enrichment of eggs may be economically and socially beneficial for the egg industry. When considering options of enrichment of eggs by certain nutrients, e.g., ω-3 fatty acids, it is necessary to take into account several factors (Sparks 2005; Seuss-Baum 2005):

1. Form of nutrient in the diet and efficiency of the transfer from feed into the egg
2. Possible toxicity or adverse effects or interactions of the increased dietary supplementation on chicken health and productive characteristics
3. Total amount of the nutrient delivered with a single egg in comparison with the daily requirement of this nutrient
4. Availability and cost of commercial sources of effective feeds
5. Stability during cooking and shelf life
6. Effect on appearance and organoleptic characteristics (flavor, odor)
7. Nutrition claims and health benefits

Table 2. Omega-3 fatty acid content (EPA and DHA) of selected fish and ALA in other feeds

Product	Concentration of sum EPA and DHA mg/100g
Mackerel	2,500
Herring	1,700
Salmon	1,200
Trout	500
Tuna	400
Shrimp	300
Cod	300
	Concentration of ALA mg/g net weight
Purslane	4.05
Spinach	0.89
Flaxseed	23.4 g /100g of edible portion

Since enrichment is based only on the natural transformation of the substances from feed to egg, such eggs are positively accepted by consumers. It needs to be stressed that PUFA, so important for human life and health, are present in oils, fish, and flaxseed in the amount of 300–2,500 mg/100g (Table 2).

However, a higher PUFA content leads to an increase in lipid unsaturation, and thus to a greater susceptibility to oxidation and free radical production. Lipid oxidation is of major importance because it can adversely affect the overall quality of foods, including flavor, taste, and nutritional value. Also, secondary reaction products of the lipid oxidation process have been related to the development of cardiovascular and other diseases. Moreover, several human diseases at different stages of their development have been associated with free radical production and their metabolism, since normally there is a balance between the amount of the free radicals generated in the body and the antioxidant capacity of the tissues. In order to prevent lipid peroxidation, antioxidants (vitamin E, herbal mix, selenium, etc.) have been widely used by the food industry.

4.2 Health Benefits of ω-3 Enriched Eggs

The effect of consuming ω-3 enriched eggs has been the subject of many studies in healthy adults, the very young, and the aged (Sparks 2005).The major advantage of the consumption of ω-3 eggs is an enrichment of plasma lipids with these fatty acids (Farrell 1998). Sindelar et al (2004) reported that consumption of one ω-3 enriched egg results in elevated serum levels of ALA and triglycerides. Blood pressure was decreased and DHA concentration in the plasma increased as well (Farrell 1998). Consumption of four ω-3 eggs a

week by volunteers for 6 weeks caused a significant decrease in platelet aggregation (Van Elswyk et al. 1998). Therefore, depending on the amount of eggs consumed, ω-3 eggs are proved to have health-promoting properties by increasing ω-3 fatty acid levels in blood lipids and, in some cases, even reducing cholesterol and triglyceride levels in the plasma. Yannakopoulos et al. (1999a), reported that plasma total cholesterol was reduced ($P < 0.05$) in humans aged 41–50 years who consumed ω-3 eggs, while HDL cholesterol was raised. Narahari (2004) conducted an experiment with human volunteers to determine the effects of the consumption of designer eggs. He reported that the consumption of herbal enriched functional eggs not only reduced the serum triglycerides and LDL cholesterol levels, but also increased the HDL cholesterol level. The increasing awareness of these benefits may have helped the egg industry to rebound from a declining consumption over the past twenty years (Asselin 2005).

4.3 Organoleptic Characteristics

Eggs enriched with PUFA may be associated with off-odors and particular fishy taints. However, these can be minimized if the hens are fed 1.5% or less of a high quality fish oil, or 5% or less flaxseed, and if the PUFA are protected both in the diet and in the egg from oxidation (Surai and Sparks 2000).

The organoleptic quality of ω-3 eggs tends to be similar to regular table eggs although in some cases panelists are able to detect off flavors. A "fishy" or fish-product-related flavor was detected in eggs from hens fed on diets containing 15–20 % flaxseed.

Cooking characteristics of ω-3 eggs, including emulsification capacity, hardness, and springiness of sponge cakes prepared using these, were the same as in regular eggs.

Data on effects of ω-3 enrichment on egg quality during storage are limited and sometimes contradictory. Van Elswyk et al. (1992) reported that there is no alteration in the fatty acid profile of eggs enriched with ω-3 PUFA during cooking or during storage for 7 weeks at 25 °C (Oku et al. 1996).

4.4 The ω-3 Egg in the Market

Egg demand has been and continues to be affected by health information and nutrition concerns. The egg industry has been very responsive in seeking new technology to improve consumers' negative perception of the egg associated with cholesterol. Enriched eggs may constitute a valuable and safe supplement to the diet of children, smokers, people on unbalanced diets, people on slimming diets, vegetarians, and people engaged in intensive sports.

The early generation of modified eggs was enriched with ω-3 and vitamin E. For this purpose hens' diets were usually enriched with flaxseed, linseed, fish oil, algae, etc. Such eggs are now available in Europe, Asia, America, and

Australia, and constitute the most common type of enriched eggs. The predominance of these eggs in the marketplace is determined by two key characteristics. First, the general public is sensitized to the health benefits of consuming less saturated and more PUFA and, to a lesser extent, the need to adjust the fatty acid balance of the diet so that LA and ALA ratio falls between 5:1 and 10:1 (Anonymous 1998, Simopoulos 1998). Secondly, it is biologically possible to produce eggs with favorable fatty acid profiles. A new generation of ω-3 eggs has been enriched with ω-3 fatty acids and herbal mixtures or other antioxidants such as selenium, vitamin E, etc., simultaneously.

An additional requirement for enriched eggs should be a guaranteed and consistent product quality. The ω-3 egg occupies a niche market, but it is undoubtedly a niche that can be expanded, provided that the consumers remain confident in the product and in the claims made for it. In Greece, one of main reasons consumers buy ω-3 eggs is their "health value", since the 93.3% of the consumers believe that ω-3 eggs are healthier than the regular ones (Sosidou et al. 2005).

It would be very interesting to create a regulation for the EU-wide uniform addition of ω-3 to eggs. At present, such a lack has led to an obstruction in the free movement of these eggs because of the member states' separate regulations. Over the world, only Health Canada has made amendments to the regulation on ω-3 eggs in order to improve nutritional information. Regarding such regulations and labeling, since ω-3 eggs have a different nutrient profile, the fatty acid composition (e.g., content of EPA and DHA) should be declared on the package so that consumers are informed about the nutritional value of the food.

In general terms, labels on ω-3 eggs should bear the following information:

- National identification, name, and address of the organization that has packed the eggs
- Number, quality grade, and size of eggs
- "Best before" date
- Consumer advice, such as "keep refrigerated"
- Farming system for the eggs (battery, free range or barn, organic)

Of note is that the Joint Australia/New Zealand Food Standards Code now includes a nutrition claim for the ω-3 content of foods contain the following points:

- A food may be labeled as a "source" of ω-3 if it contains at least 200 mg ALA or 30 mg of combined EPA and DHA per serving.
- A food may be labeled as a "good source" of ω-3 if it contains at least 60 mg of combined EPA and DHA per serving.

Nutrient-enhanced eggs in the EU market comprise between 1and 5% and show an increasing trend yearly. The production of enhanced eggs involves more cost and they are usually sold at higher prices than regular eggs (2–3 times more).The prices are about to become similar to prices currently paid for free range and organically produced eggs. It is about 0.40 euro/egg.

5 Combined Enrichment with Omega-3 and Natural Antioxidants

Plants and their extracts have formed part of animal diets as preservatives, flavors, digestive enhancers, and remedies for millennia. Farmers in several countries use medicinal plants in the maintenance and conservation of the healthcare of the livestock. Medicinal plants are an integral component of ethnoveterinary medicine. Specific botanical components have been shown to be active against damaging compounds, such as mycotoxins, which they strongly bind and inhibit. A correct formulation of botanical supplements and ingredient activities is key for a product that delivers consistent benefits in vivo and optimizes the balance between cost of inclusion and overall efficacy (Botsoglou et al. 1997, Yannakopoulos et al. 2001). The herbs contain ingredients active in three main areas: gut microflora and environment, antioxidation, and liver function (Lange 1998). In Greece, Yannakopoulos et al. (1999b) have developed herbal enriched ω-3 eggs which are not only rich in ω-3 fatty acids and vitamin E, but also in active herbal principles such as natural antioxidants (thymol, carvacrol, and others). Since 1997, research has been undertaken to produce these herbal enriched ω-3 eggs (named Vi-ωmega-3; Table 3) (Yannakopoulos et al. 2004).

The Vi-ωmega-3 eggs were found to contain less saturated lipids and more PUFA compared to regular eggs. These eggs were also found to contain higher levels of ω-3 fatty acids, particularly of the DHA type, compared to regular eggs (120 mg vs 0 mg). This may be due to the diet supplementation with the herbal additive, which might have helped in a more efficient conversion of ALA to DHA. The Vi-ωmega-3 eggs have also been found to contain cholesterol at markedly lower levels than the control eggs, possibly due to the combined action of the herbal additive constituents. The higher levels of vitamin E compared to the control eggs, as well as the possible transfer of natural antioxidant compounds from the herbal additive, could justify the

Table 3. Comparison of Vi-ωmega 3 eggs with regular eggs of net weight of 53.6 g

Nutrients	Regular eggs	Vi-omega-3 eggs
Lipids per egg (g)	4.74	4.15
Saturated (g)	1.5	1.16
Monounsaturated (g)	2.1	1.62
Polyunsaturated (g)	0.9	1.19
N-3 fatty acids (mg)	40	350
DHA fatty acid (mg)	0.0	120
Cholesterol (mg per egg)	220	175
Vitamin E (mg per egg)	0.7	3.5

higher oxidative stability exhibited by the Vi-ωmega-3 eggs when all eggs were submitted to experimentally induced lipid oxidation (Botsoglou et al. 1997, Yannakopoulos et al.2004). Results from the taste panel showed that the organoleptic acceptability was higher in the ω-3 herbal enriched eggs compared to the control eggs (Tserveni-Gousi 2001). This could be due to the combined action of the herbs, vitamins, and minerals occurring in the herbal additive, since it is known that the ingredients used in layer diets to enrich eggs with polyunsaturated fatty acids often influence the sensory characteristics of the eggs (Van Elswyk 1997).

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Chapter 21 Enrichment in Vitamins

Federico Sirri and Ana Barroeta

1 Introduction

Egg is a natural food that contains a great number of essential nutrients, including all the vitamins with the exception of vitamin C. The vitamin concentration of the whole egg and the distribution in the yolk and albumen is described in Table 6 of Chapter 18 (Part II).

Nowadays, food is being looked at in a new way. More and more attention is being paid to the relationship of the diet to human health and well-being; as a animal product, eggs in particular represent an important part of human diet. Therefore, an improvement in the egg nutritive value might have direct positive implications for daily nutrient intake and consequently for human health.

In the attempt to achieve effective food enrichment, nutrients must have a proven efficiency and their amount must be modifiable in food, in this particular case in the whole shell egg. This enrichment in the nutritive content of the shell egg is achieved through the inclusion of appropriate nutrients in the feed of the laying hen. The best known and most clearly beneficial components for egg enrichment are unsaturated fatty acids (omega-3 and conjugated linoleic fatty acids), vitamins (above all fat-soluble ones and especially vitamin E), and a few minerals. Information related to egg enrichment in fatty acids and minerals is described in Chapters 20 and 22 of Part II.

Vitamin concentration in hen feed is the most important factor in determining vitamin content in the egg. This is particularly true for fat-soluble vitamins. The lipid components of the egg, including fat-soluble vitamins, are only present in the yolk. During the last 10 days before hen ovulation, the lipids of dietary origin are absorbed and deposited in the yolk in the last phase of its formation. As the fat-soluble vitamin concentration of the feed increases, so does the vitamin content of the egg yolk.

The most relevant data related to egg vitamin enrichment through nutrition are now presented, with special attention paid to vitamins E, A, and D. In particular the vitamin enrichment level reached in the egg and the implications

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

on daily vitamin intake and consequently on consumer health are discussed. More information about this subject can be found in the review articles of Stadelman and Pratt (1989) and Naber (1993).

2 Vitamin E Enrichment

It is widely recognized that vitamin E plays a fundamental role in the normal functioning of the organism. Moreover, there is an increasing evidence demonstrating that the inclusion of vitamin E in the human diet reduces the incidence of various serious pathologies. Of all the vitamin E active compounds, alpha-tocopherol (alpha-toc) is the most important in vivo antioxidant; its consumption has been linked to the prevention of several metabolic disorders (Traber and Sies 1996).

Supplementation of poultry diets with vitamin E can achieve different objectives. In the first place, it prevents nutritional deficiencies in poultry. Secondly, it improves the oxidative stability of broiler meat and egg products. Finally, it produces a highly nutritional food source of vitamin E for the human consumer.

The daily recommended intake (DRI) of vitamin E for humans is about 12 mg per day (Scientific Committee on Food 2003), but as the dietary polyunsaturated fatty acids (PUFA) content grows, the vitamin E requirements increase by 0.4–0.6 mg/g PUFA (Dutta-Roy et al. 1994; Muggli 1994). On this basis, a lot of research has been done in recent years on the enrichment of eggs with vitamin E (Frigg et al. 1992; Jiang et al. 1994; Grobas et al. 2002; Chen et al. 1998; Qi and Sim 1998; Pita et al. 2004). Dietary supplementation with DL-alpha-tocopheryl acetate (alpha-TA) has been tested successfully for alpha-toc enrichment of eggs, because a linear relationship exists between diet and vitamin E concentration in eggs. It has been observed that alpha-toc content in fresh egg increased with dietary alpha-TA supplementation up to doses of 20 g alpha-toc/kg feed (Frigg et al. 1992; Surai et al. 1995; Grobas et al. 2002; Flachowsky et al. 2002). The alpha-toc transfer efficiency decreases with increasing levels of alpha-toc in the diet. Galobart et al. (2001a) showed that the alpha-toc transfer efficiency from feed to egg ranged from 41.8%, when 50 mg/kg alpha-TA was added to the diet, to 26% with 200 mg/kg alpha-TA supplementation. In this case, for every 100 mg/kg of alpha-toc increase in the diet the transfer efficiency was reduced by 8.4 % (Fig. 1). The transfer efficiency calculated by Naber (1993) was found to be between 16 and 39% with dietary levels of alpha-toc ranging from 20 to 30 mg/kg; whereas Grobas et al. (2002), comparing diets with alpha-toc from 20 to 1,280 mg/kg, observed that the transfer efficiency of alpha-toc changed from 26.2 to 16.2%, respectively.

Another important aspect is the egg vitamin E deposition pattern. The most important studies in this field are those of Surai et al. (1995), Meluzzi et al. (1999, 2000), and Galobart et al. (2002). They all have studied the evolution of

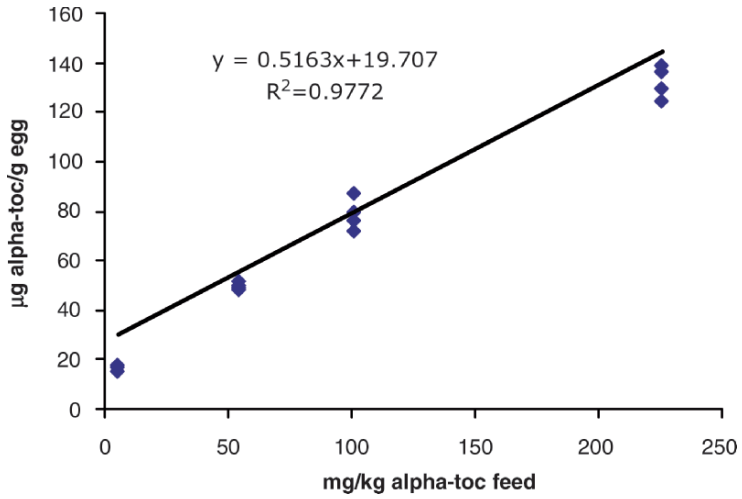

Fig. 1. Transfer efficiency of alpha-tocopherol from feed to egg

the egg alpha-toc content over an extended period of time. In general, it seems that the time needed to achieve the maximum level of alpha-toc in eggs is about 2 to 3 weeks, depending on the dietary supplementation. After reaching this maximum level, alpha-toc concentration remained constant for 2 weeks and then declined by approximately 50% over the following 4 weeks. Alpha-toc in egg then remained constant for as long as the dietary supplementation was maintained. Moreover, once the dietary vitamin E is reduced its content in egg is adjusted more quickly than when increasing the dietary supplement.

In Table 1 values of alpha-toc concentration in egg relative to dietary alpha-toc doses obtained in different studies are reported. By comparing the same dietary inclusions of the vitamin, huge differences in the egg vitamin concentration emerge. For instance, by using 200 mg/kg of alpha-TA supplementation, the alpha-toc concentration in egg ranges between 119 (Qi and Sim 1998) and 1,200 g egg yolk (Surai et al. 1995). This variation seems related to several factors, the most important being the lab methods and the instrumental conditions adopted by the different authors—but also to the hen diets. Indeed, differences in the alpha-toc content of the basal diets, the presence of antioxidants or particular feed ingredients, the interaction with nutrients during absorption (i.e., level of vitamin A; Grobas et al. 2002), and the type and amount of fat could be responsible for the differences observed in the egg vitamin E concentrations given in Table 1.

Regarding the type of fat, several authors have found that alpha-toc deposition in eggs was lower when higher levels of unsaturated fats were administered (Frigg et al. 1992; Meluzzi et al. 2000; Galobart et al. 2001a). A higher PUFA content produces a higher oxidative susceptibility in the animal body, which contributes to a greater utilization of alpha-toc, leading to a

Table 1. Effect of dietary DL-alpha tocopheryl acetate supplementation on alpha-tocopherol content of fresh egg yolk

Authors	WFS	α-tocopherol (mg/kg diet)	α-tocopherol (μg/g of yolk)
Jiang et al. 1994	3	BD = 27.5	135
		BD + 50	164
		BD + 100	235
		BD + 200	245
		BD + 400	390
Surai et al. 1995	3	BD (=14 – 18) + 200	1,200 approx
		BD + 2000	7,000 approx
		BD + 20000	15,000 approx
Chen et al. 1998	7	BD = 11	25
		BD + 15	35
		BD + 30	45
		BD + 60	50
		BD + 120	75
Gebert et al. 1998	4	0	120
		BD + 100	378
		BD + 200	606
Hossain et al. 1998	30	25	99
		50	169
		75	207
		100	203
Qi and Sim 1998	1–4	BD = 67	83.6
		BD + 200	119.0
		BD + 400	175.4
		BD + 800	234.9
Meluzzi et al. 2000	4	BD = 33.2 – 43.88	90.9
		BD + 50	136.1
		BD + 100	227.5
		BD + 200	313.8
Galobart et al. 2001a	4	0	50
		50	149
		100	237
		200	397
Grobas et al. 2002	6	0	28.1
		40	109.2
		160	353.9
		640	967.5
Flachowsky et al. 2002	10	BD = 15	7.82
		BD + 100	25.1
		BD + 1000	139.6
		BD + 10000	250.2
		BD + 20000	313.3
	44	BD = 19	10.4
		BD + 100	34.3
		BD + 1000	131.6
		BD + 10000	250.6
		BD + 20000	236.9

Table 1. Effect of dietary DL-alpha tocopheryl acetate supplementation on alpha-tocopherol content of fresh egg yolk — Cont'd.

Authors	WFS	α-tocopherol (mg/kg diet)	α-tocopherol (μg/g of yolk)
Franchini et al. 2002	5	BD < 10	47.2
		BD + 100	120.6
		BD + 200	219.1
Pita et al. 2004		BD = 36–38	34.0
		BD + 100	108.1
		BD + 200	177.4

WFS: weeks of feed supplementation
BD: basal diet

reduction in the alpha-toc available for the egg yolk formation. Therefore, the vitamin E requirements increase as the unsaturation level of the diet grows and, consequently, the efficiency of alpha-toc deposition in eggs diminishes when dietary unsaturation level increases.

Another important factor is the reduction of alpha-toc concentration in eggs during processing and storage. Some works have demonstrated that vitamin E content remained almost constant when eggs are stored (Cherian et al. 1996; Gebert et al. 1998; Meluzzi et al. 1999). To the contrary, the vitamin E content of the egg quickly declined with hard-boiled, scrambled, and spray-dry processing (Wahle et al. 1993; Galobart et al. 2001b; Cortinas et al. 2003; Murcia et al. 1999) or with high temperature during storage (Franchini et al. 2002).

3 Vitamin A Enrichment

Vitamin A occurs in animal tissues as retinol (alcohol), as retinal (aldehyde), as retinoic acid, or it is esterified with a fatty acid. Vitamin A itself is not present in plants, but only its precursors; the carotenes, defined as provitamins A, can be found in vegetables. Among the carotenes, a large group of orange-yellow pigments, the beta-carotene is the most active provitamin. In the intestinal mucosa of animals, the carotenes are converted into the different forms of vitamin A; among these , trans-retinol is considered to have 100% biological activity.

Vitamin A is involved in several processes such as vision, maintenance of integrity of the mucous membrane, reproduction, bone development, immune response, and cell differentiation and proliferation.

Vitamin A, being a component necessary for the embryo development, is stored in the egg yolk, and its concentration is closely related to the level of dietary inclusion. Egg content of vitamin A increases considerably in response to dietary level increases but not in direct proportions to the diet levels. Moreover some discrepancies among the different studies emerged (Table 2).

Table 2. Effect of dietary retinyl acetate on retinol content of fresh egg yolk

Authors	WFS	Retinyl acetate (IU/kg diet)	Retinol content (IU/g yolk)
Squires and Naber 1993	27–32	29	2.4
		4,029	10.3
		8,029	16.9
		16,029	24.0
Jiang et al. 1994	5	BD + 0[a]	38.7
		BD + 50[a]	38.3
		BD + 100[a]	40.1
		BD + 200[a]	46.4
		BD + 400[a]	42.9
Surai et al. 1998	12	0	13.4
		10,000	23.5
		100,000	68.4
		400,000	283.7
Mori et al. 2003	11	0	25.7
		15,000	35.0
		30,000	38.3

WFS: weeks of feed supplementation
BD: basal diet supplemented with 9,900 IU of retinyl acetate;
[a] β-carotene supplementation

Indeed, the intermediate storage of the vitamin in the liver alters the pattern of egg deposition in response to the increased concentration in hen feeding. For this reason, the egg vitamin A content in response to vitamin A level is delayed from 8 to 12 weeks as observed by Squires and Naber (1993). These authors, raising the retinyl acetate content of the diet from 4,000 to 16,000 IU/kg, were able to shift the egg vitamin A concentration from 10.3 to 24.0 IU/g, respectively. Jiang et al. (1994), feeding hens diets supplemented with 200 mg/kg of β-carotene, observed, in addition to its marked transfer into the yolk, a slightly but significant ($P < 0.05$) increase of retinol in egg in comparison with the control diet (13.93 μg/g; 46.43 IU/g vs 11.62 μg/g; 38.37 IU/g yolk). The authors concluded that the laying hen cannot only convert β-carotene to retinol, but also deposit limited amounts of it into the egg yolk.

Naber (1993) classified the vitamins in relation to the potential transfer efficiency from hen diet to the egg and grouped the vitamins into four categories (low, medium, high, very high) with efficiency ranging from 5 to 80%. Vitamin A was classified as "very high", with a transfer efficiency varying from 60 to 80%.

Several reports have demonstrated that a high intake of vitamin A may interfere negatively with other fat soluble vitamins, particularly with vitamin E. The yolk concentration of tocopherols was markedly reduced as supplemental dietary levels of either vitamin A or beta-carotene increased (Jiang et al. 1994; Surai et al. 1998; Grobas et al. 2002; Mori et al. 2003).

4 Vitamin D Enrichment

Vitamin D plays an important role in the formation and maintenance of bone. There are two sources that supply vitamin D: the action of sunlight on the skin and the diet. In some situations, dietary fortification could be necessary in people with restricted exposure to ultraviolet light. The DRI of vitamin D is from 5 μg/day in adults to 10 in elderly people (Scientific Committee on Food 2003).

Vitamin D is a generic term that refers to cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). The most normally recognized active vitamin D metabolite is 1,25-dihydroxycholecalciferol; its precursor is 25-hydroxycholecalciferol (25OHD), which has metabolic effects of its own in regulating calcium metabolism. It has been shown that 25OHD has from 1.5 to 5 times higher activity than standard vitamin D (Ovesen et al. 2003).

Eggs are among the limited number of natural foods that contain both vitamin D and 25OHD. According to the different studies of Mattila et al. (1999, 2003, 2004) the cholecalciferol content of eggs is proportional to the level of added cholecalciferol in hen feed (Table 3). When the feed cholecalciferol content was raised from the regular level (62.4 μg; 2,496 IU/kg feed) to a level of approximately 3.5 times higher (221.6 μg; 8,649 IU/kg feed), the

Table 3. Effect of dietary cholecalciferol supplementation on cholecalciferol and 25OHD content of fresh egg yolk

Authors	WFS	Cholecalciferol IU (μg/kg diet)	Cholecalciferol (μg/100 g yolk)	25OHD (μg/100 g yolk)
Mattila et al. 1999	4–6	1,064 (26.6)	1.2–1.5	0.5
		2,496 (62.4)	3.4–3.5	0.8–1.0
		8,640 (216.0)	21–23	1.4–1.5
Mattila et al. 2003	0	4,280 (107)	4.2	–
	2	12,000 (300)	30.2	–
	4	12,000 (300)	31.1	–
	16	12,000 (300)	26.6	–
	24	12,000 (300)	21.7	–
Mattila et al. 2003	0	1,720 (43)	4.2	1.1
	1	11,200 (280)	30.2	1.9
	2	11,200 (280)	31.1	–
	3	11,200 (280)	26.6	1.7
Mattila et al. 2004	4–44	2,500 (62.5)	2.5–5.0	–
		6,000 (150)	9.1–13.6	–
		15,000 (375)	25.3–33.7	–

WFS: weeks of feed supplementation

cholecalciferol content of egg yolk increased approximately 7-fold and the 25OHD content approximately 1.5-fold.

These authors also worked on the vitamin D deposition pattern in eggs (Mattila et al. 2003) and observed that 8 to 13 days of high dietary cholecalciferol supplementation (11,200 and 12,000 IU/kg feed) is enough to reach the top cholecalciferol content in egg (30 μg/100 g egg yolk). After 112 days feeding, the cholecalciferol content gradually decreased to approximately 22 μg/100 g yolk. Moreover, it has been reported that high doses of vitamin D (>15,000 IU/kg feed) were not toxic for hens and did not affect the sensory or functional properties, fatty acid composition, or the egg quality parameters such as egg shell strength (Mattila et al. 2003, 2004).

5 Enrichment of Other Vitamins

5.1 Vitamin K

In contrast to the other fat-soluble vitamins A, D, E. which are involved in several functions and of great biological importance, vitamin K is described with restricted function, mainly limited to the liver for the normal blood-clotting mechanism. Recently it has been suggested that vitamin K is involved also in bone mineralization and skin metabolism. Probably because of its restricted biological role, very few studies attempting to enhance its concentration in eggs have been carried out. Suzuki and Okamoto (1997), considering the increasing number of hemorrhagic diseases of newborn babies in relation to vitamin K deficiency of pregnant women, have demonstrated that feeding hens with high doses of either phylloquinone (vitamin K_1), menaquinone (vitamin K_2) or menadione (vitamin K_3), it is possible to increase the level of both K_1 and K_2 up to 1,908 and 240 μg/100 g egg yolk, respectively.

5.2 Folacin (Folic Acid)

The term folacin is the generic description for folic acid and related compounds that exhibit the biological activity of the vitamin. Much of the folacin in foodstuffs is conjugated with varying numbers of glutamic acid molecules. In the last decades there has been increasing interest toward these compounds particularly in pregnant women, since an appropriate pre-conceptional intake of folacin has been shown to reduce the occurrence of neural tube defects in children. Therefore, some attempts have been made to enrich the egg that naturally contains 34 μg/egg. Indeed, it has been recently confirmed that it is possible to enhance the folic acid content of eggs by 2 to 4 fold by feeding hens a folic acid supplemented diet. A linear increase in egg folacin content was described when crystalline folic acid was added from 0 to 2 mg/kg to hen diet. Then, egg folacin levels reached a

plateau at a concentration of approximately 45–50 μg/egg, with no additional increases until dietary concentrations reached 32 mg/kg. It has been also demonstrated that the folacin in enriched eggs is stable during storage at 4 °C for 4 weeks (House et al. 2002).

5.3 Other Water Soluble Vitamins

As for the other water soluble vitamins, there are very few indications from literature. Naber (1979) suggested by reviewing the literature that riboflavin and vitamin B_{12} could be markedly influenced by dietary changes and later demonstrated that egg concentration of vitamin B_{12} reflects the dietary level of this vitamin. Feeding hens diets with 0.5, 4, 8, and 16 μg/kg produced increases in the yolk vitamin of 0.4, 1.3, 2.6, and 4.8 μg/100 g of yolk, respectively (Squires and Naber 1992).

Some promising results have been highlighted by Leeson and Caston (2003) for the potential transfer efficiency of some vitamins from hen diet to the egg. These authors studied the effect of the supplementation of hen feeding with a vitamin premix containing supranutritional levels of the following vitamins: A (300% of the regular level), D_3 (143%), E (670%), K (166%), B_1 (500%), B_2 (420%), B_6 (200%), biotin (500%), folic acid (200%), niacin (1,000%), pantothenic acid (100%), and B_{12} (1,000%). Among the water soluble vitamins, an interesting response was observed for vitamin B_{12}, whose level in the egg increased nearly 4 times as a consequence of the 11-fold increase in diet vitamin supplementation, and for panthotenic acid, that doubled its level in egg in response to a 2-fold increase in hen diet.

6 Contribution by the Consumption of Enriched Eggs to the Recommended Daily Vitamin Intake

The potential health benefits of increasing the daily intake of some vitamins in humans are recognized, in particular with regard to those compounds involved in specific biological functions or having a clear role in the prevention of the onset of some diseases. Exploiting the great bioavailability of the nutrients contained in eggs, a new concept for delivering important class of nutrients, such as essential fatty acids, vitamins, and minerals to the human body has been studied in the last decade (Sim et al. 2000; Surai 2002). As previously described, several researches have confirmed that it is possible to produce novel eggs with an enhanced level of some important vitamins, in particular fat-soluble ones (vitamins E, A, and D), but also water-soluble (vitamin B_{12}, folic acid, etc.). Table 4 shows an example of how the vitamin content of an ordinary egg can be improved through the dietary supplementation of supranutritional doses of vitamins in hen feeding. These data were

Table 4. Vitamin content of ordinary egg (OE) and possible concentration in enriched egg (EE)

Vitamins		DRI[a]	OE[b] content	OE[b] (%DRI)	EE[b] content	EE[b] (%DRI)
Fat-soluble						
A (as retinol equivalent; RE)	µg	800	150	18.8	172 [e]	22
D	µg	5	1.54	30.8	4.67 [f]	93
E	mg	12	1.10	9.20	6.00 [g]	50
K	µg	75	25.0	33.3	287 [c, h]	383
					36.0 [d, h]	48
Water-soluble						
Cyanocobalamin (B_{12})	µg	2.50	1.06	42.4	3.35 [i]	134
Pantothenic acid	mg	6	0.85	14.2	1.20 [i]	20
Biotin	µg	50	13.3	26.5	18.0 [i]	36

[a] Daily Recommended Intake (Scientific Committee on Food 2003)
[b] (whole egg: 60 g; edible portion: 53 g; yolk: 15 g).
[c] phylloquinone
[d] menaquinone MK4
[e] Mori et al. (2003)
[f] Mattila et al. (2003)
[g] Galobart et al. (2001a)
[h] Suzuki and Okamoto (1997)
[i] Leeson and Caston (2003)

obtained by assembling different research findings on this topic. The criteria followed in the selection of the published data were not simply based on the choice of the highest vitamin increment. The values that, on the basis of the information available, would not compromise the productivity of the hens and could also be achieved at a reasonable increment of feed cost were chosen. Furthermore, the selection was based on those vitamin enrichments able to satisfy, at least 20% of DRI, with the consumption of one egg.

As shown in Table 4, by manipulating the feed of hens it is possible to enhance the egg content of the fat-soluble vitamins A, D, E, K by 1.15, 3.03, 5.45, and 4.31-fold, respectively, as well as of the water soluble vitamins B_{12}, panthotenic acid and biotin by 3.16, 1.41, and 1.36-fold, respectively. Therefore, concerning the DRI in each case, an enriched egg might supply 93% for vitamin D, 50% for vitamin E, 383% for vitamin K, 134% for B_{12}, and from 20 to 48% of the DRI for the remaining vitamins mentioned above.

This new approach in egg production might represent an outstanding way for improving the nutritional image of the egg, by positioning it as an important vehicle of nutrients for humans and useful for overcoming nutritional imbalances.

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Chapter 22 Enrichment in Selenium and Other Trace Elements

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1 Introduction

In addition to proteins, lipids, and various vitamins, eggs contain a wide range of minerals, including calcium, phosphorus, zinc, iron, magnesium, copper, and selenium (Se). However, egg consumption by humans accounts for only 1–3% of the dietary mineral intake. Further, manipulation of the egg's minerals is quite a difficult task since their delivery to the egg is physiologically regulated to ensure that the embryo is not exposed to toxic levels. Therefore, it is practically impossible to increase the level of zinc, copper, or iron in the egg to such an extent that they would be considered as an important source of these minerals in the human diet. Almost all the calcium, phosphorus, iron, magnesium, manganese, and many other trace elements of the yolk are found in the dense granules where they are bound to the protein phosvitin. This protein derives from the proteolytic cleavage of the much larger yolk precursor protein, vitellogenin (Griffin et al. 1984). Phosvitin is a highly unusual protein, with serine accounting for about 55% of its amino acid residues, almost all of the serines being phosphorylated. The negatively charged phosphate groups bind large amounts of positive ions, mainly calcium but with significant amounts of other minerals (White 1991). Since the phosvitin phosphate groups are normally saturated with the bound positive ions, there is little scope to further increase the concentrations of these elements in the yolk. These limitations do not, however, apply to Se since this element is incorporated into yolk proteins as a component of seleno-amino acids. Thus, the concentration of Se in the egg is a reflection of its concentration in the diet of the hen. Current diets for poultry are usually relatively low in Se. Moreover, inorganic forms of Se such as sodium selenite, which is widely used as a supplement in poultry diets, are relatively ineffective with regard to enhancing the Se concentration in the egg. Organic forms of Se are much more effective. Therefore, it is only the development of the technology

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

for production of Se yeast that has allowed an opportunity to substantially increase Se concentration in the egg to such an extent that a single egg can deliver 50% RDA in Se. Indeed, given the global scale of Se deficiency in humans (Table 1), production of Se-enriched eggs could be considered an important step towards solving this deficiency.

Table 1. Low daily selenium intakes in selected countries, μg/day (Adapted from Surai 2006 and references therein) Se requirement is 55 μg/day (USA) and 60 for women and 75 μg/day for men (UK)

Country	μg/day	Year reported
China, Keshan disease area	2–36	1985
China, Keshan disease area	7–11	2001
New Zealand, low-Se area	11	1984
Saudi Arabia	15	1997
Poland	11–40	2000, 2003
UK	12–43	1992, 1995, 1997, 1998, 2003
New Guinea	20	1992
Czech republic	15–50	2003
Nepal	23	1988
Finland before selenium fertilization	26	1987, 1984, 1985
India, vegan low income	27	1997
Egypt	29	1972, 1996
Serbia	30	2001
Slovenia	30	1998
China	26.0–37.2	2000
Croatia	27.3–33.9	1998, 2000
Slovakia	27–38.2	1996, 1998
Belgium	28.4–61.1	1989, 1994
Brazil	28.4–37.0	2004
New Zealand	29–38	1999, 2001, 2004
Sweden	29–44	1991, 2000, 2003
France	29–48	1994, 1994
Turkey	30–36.5	1996, 1997, 2004
UK 1994	32	1997
UK 1995	33	1997
England	35	2000
Spain	35	1996
Germany	35–48	1989, 2000
Portugal	37	1990

Table 1. Low daily selenium intakes in selected countries, μg/day (Adapted from Surai 2006 and references therein) Se requirement is 55 μg/day (USA) and 60 for women and 75 μg/day for men (UK) — Cont'd.

Country	μg/day	Year reported
Denmark	38–47	2000
Italy	43	1985
UK 1985	43	1997
India, conventional diet	48	1997
Austria	48	2001
Ireland	50	2002
UK 1974	60	1997

2 Selenium-Enriched Products

Since selenium content in plant-based food depends on its availability from soil, the level of this element in human (or food animal) foods varies among regions. In general, eggs and meat are considered to be good sources of Se in the human diet. When considering ways to improve human selenium intake, there are several potential options. These include:

- direct supplementation
- soil fertilisation
- supplementation of food staples such as flour
- production of Se-enriched functional foods.

It seems likely that the fourth strategy, production of 'functional foods' enriched with selenium, deserves more attention (Surai 2000a; Surai 2002).

Several important factors must be considered when choosing the best food supplementation strategy for a given population (Table 2). In general, main sources of dietary selenium may differ among different countries. For example, in the UK meat and meat products provide 32% of the daily Se consumption and dairy products and eggs are responsible for 22% (BNF 2001). In contrast, in Russia about 50% Se in the diet originates from bread and cereals whereas meat, milk and eggs provide about 20%, 10%, and 5%, respectively, of daily Se consumption (Golubkina et al. 2002). In the USA beef, white bread, pork, chicken, and eggs account for half of the Se in the diet (Schubert et al. 1987). In Ireland, meat and meat products (30%), bread and rolls (24%), fish/fish products (11%), and milk and yoghurt (9%) were found to be the main contributors to mean daily Se intake (Murphy et al. 2002).

Among animal-derived products, the egg is ideally suited to be an effective delivery system for improvement of human Se status. The egg is a traditional and affordable food in most countries and is consumed by people of all ages

Table 2. Some characteristics of food choice for Se-enrichment (adapted from Surai 2006)

The food should be	Comments
A part of traditional meals for the population	It would be counter-productive to attempt a change in culturally-based food habits by introducing a new type of food. Emphasis should be given to the possibilities of changing composition of existing foods such as by selenium enrichment.
Consumed regularly in a moderate amount	Since the objective is to deliver the amount of selenium needed to meet RDA, it is necessary to choose food that is consumed regularly in moderate amounts. Over-supplementation is unnecessary and undesirable.
Consumed by the majority of the population	This is particularly important given that immune function is more likely to be compromised in groups such as children and the elderly.
Affordable	Affordability of food would play an important role in the consumer choice.
Enriched with other health-promoting nutrients that are in short supply in the same population	Examples of minerals critical to health that are frequently deficient include iron and iodine. Vitamin E and lutein are also in short supply in the human diet. This plan can provide greater improvement in the diet.
Supplying a meaningful amount of the nutrient (e.g., at least 50% RDA)	This is an important point that distinguishes true functional foods from products that include 'tag-dressing' amounts of nutrients for advertising purposes.

more or less regularly and in moderation. It is also a very safe vehicle for supplementation given that a toxic dose of selenium from eggs would require consumption of 30 eggs per day over time, an impossible situation to imagine. There is an option of simultaneous enrichment of eggs with several important nutrients, including omega-3 fatty acids, vitamin E, carotenoids (Surai and Sparks 2002; Surai 2002; Surai 2006), and with a single egg it is possible to deliver at least 50% of the RDA for selenium. It seems likely that pork, beef, chicken, and milk can also be enriched with selenium.

3 Selenium-Enriched Eggs as a Route Toward Improving Human Selenium Status

Before the advent of commercially available organic selenium for food animal diets, the main problem as regards the enrichment of eggs with selenium was the low efficiency of transfer of inorganic selenium (selenite or selenate) to the egg. In fact, even high doses of selenite in the diet of laying hens were not able to substantially enrich eggs with this trace element (for review see Surai 2002, 2006).

Studies in our laboratory showed that egg selenium content can be easily increased when Sel-Plex (Se-enriched yeast; Alltech, Lexington, KY, USA) is included in the diet at a level to provide 0.4 ppm Se (Surai 2000a; 2000b; 2000c). In fact, Se content in the egg was increased from 7.1 μg up to 30.7 μg as a result of dietary supplementation with organic selenium. As a result, the technology for production of eggs delivering ~50% of selenium RDA was developed and successfully tested (Surai et al. 2000).

Our investigation of the commercially-relevant characteristics of Se enriched eggs (Surai 2006) indicate that:

- Inclusion of increased levels of organic Se, with or without supplementary vitamin E, for 12 weeks did not affect egg production, egg weight, ratio of yolk/white, feed consumption, FCR or body weight of laying hens.
- There was no significant difference in carotenoid and vitamin A levels in the egg yolk.
- Increasing dietary Se increased the concentration of vitamin E in the egg yolk.
- Total Se in a single egg reached 35–40 μg, providing 55–73% RDA.
- During storage of control eggs at 20 °C for 7–14 days, lipid peroxidation occurred in the egg yolk, as indicated by a significant increase in malondialdehyde (MDA) concentration.
- Egg yolk enrichment with Se was associated with a significant reduction in MDA accumulation, which was related to increased glutathione peroxidase (GSH-Px) activity in the yolk. When yolk was incubated at 37 °C, lipid peroxidation was enhanced and the protective effect of the elevated Se concentration was significant.

These data clearly indicate that enrichment of eggs with Se and vitamin E is beneficial not only from the point of view of nutritional value of the eggs but also as an important technological solution to decrease lipid peroxidation in eggs during storage.

It seems that Se in eggs is highly available for absorption by the human digestive tract. For example, a recent clinical trial conducted in the Ukraine showed that consumption of two Se-enriched eggs per day for eight weeks significantly increased the Se level of the plasma of volunteers (Surai et al. 2004). In this study, sixty volunteers (30 in control and 30 in experimental groups) successfully finished the trial. Eggs consumed in the control group contained 7–9 μg Se/egg and experimental eggs were enriched with selenium (28–32 μg Se/egg). Blood was collected before the beginning and at the end of experimental period and Se was determined in plasma by hydride generation atomic absorption spectrometry with fluorometric detection. The level of selenium in plasma of volunteers living in the Kiev area of Ukraine (0.055–0.081 μg /ml) was on the low side of the physiological range and was somewhat lower than we reported earlier in volunteers in Scotland (Surai et al. 2000). Consumption of commercially available control eggs for eight weeks only slightly increased Se in plasma, which reached physiological level

(0.075–0.085 μg/ml). In contrast, consumption of two enriched eggs daily, which together delivered the daily requirement of 55–65 μg Se, for eight weeks was associated with a significant increase in Se concentration in plasma. Plasma Se reached 0.09–0.14 μg/ml, indicating an improving Se status of the volunteers (Surai et al. 2004). This is the first clinical trial to prove that Se-enriched eggs could be used as an important vector to improve Se status in countries with low Se consumption, like Scotland or Ukraine. Se availability from eggs for humans needs further elucidation and the effect of different dietary sources of Se on its concentration in plasma probably depends on the Se status of the volunteer. For example, in our previous clinical study in Scotland, consumption of one Se-enriched egg per day for 8 weeks did not change the plasma Se concentration, which was in the physiological range (Surai et al. 2000).

After the successful clinical trial with Se-enriched eggs in Ukraine, the production of such eggs, enriched with Se and vitamin E, started commercially (1.2 million Se-eggs per day) and now the eggs called "Basket of life" can be found in supermarkets in Ukraine. This development is very important for this region. Selenium deficiency was documented in people working in the Chernobyl area (Tutelyan et al. 2002; Golubkina et al. 2002), and supplementation of Se and other antioxidants can be especially beneficial for people living in radionuclide-contaminated areas of the Ukraine. Currently various companies all over the world (more than 25 countries) market Se-enriched eggs including Columbus eggs in Belgium (Belovo, Bastogne), as well as Se-enriched eggs in Russia, Ukraine, Turkey, Thailand, and other countries. Price for those eggs vary from country to country and is similar to that for free-range eggs.

The next step in the development of nutritionally enhanced eggs is the combined enrichment of eggs with Se, omega-3-fatty acids, and various vitamins. In fact, the Columbus concept, which relates to returning egg composition to the natural situation as found in eggs of wild birds, already includes such a combination. Indeed, Columbus eggs enriched with omega-3 fatty acids, Se, and vitamin E are on the supermarket shelves in various countries all over the world. Therefore, simultaneous egg enrichment with omega-3 fatty acids and natural antioxidants has important benefits for egg quality (decreased lipid oxidation in the egg and increased egg storageability, improved absorption of vitamin E, etc.) and for consumers (delivery of daily requirement in vitamin E, more than 50% RDA in selenium and substantial amount of omega-3 polyunsaturated fatty acids (PUFA).

4 Conclusions

The analysis of current literature indicates that enrichment of eggs, meat, and milk with Se is a valuable option to improve Se status of the general population. Such eggs, currently being produced in more than 25 countries worldwide, deliver approximately 50% RDA Se with a single egg. There are

also various other combinations of egg enrichment, including enrichment with omega-3 PUFAs, vitamin E, carotenoids, iodine, etc. The most successful production and marketing programme of eggs enriched with Se, vitamin E, and omega-3 is that of Columbus eggs, which are sold in many countries worldwide. Commercial technologies for the production of Se-meat and Se-milk are under the development in various countries.

It has been suggested that for the past 150 years our diet has changed substantially, while our genes have not changed. In particular, animal-derived food composition has been dramatically changed as a result of using cheap feed ingredients. The meat from animals in the wild and chicken eggs produced under complete natural conditions contain higher amounts of omega-3 fatty acids compared to those from farmed livestock. Indeed, decreased Se levels in feeds and foods in many cases reflect consequences of our agricultural practices. Therefore, eggs or meat produced by free-range poultry/animals fed on natural feed sources grown on well-balanced soils 100–200 years ago would contain much higher Se concentrations than are currently found in many European and Asian countries. Again, by supplementing animal diets with natural organic sources of Se we are returning to nature. Our recent data on the Se profile of eggs from various avian species in the wild have confirmed this idea: Se concentrations in eggs collected in the wild are, in many species, much higher than those from commercial poultry. The Se level in chicken eggs even after organic Se supplementation (Surai 2000a) only raised the yolk Se level into the lower end of the range achieved by a range of avian species in the wild, suggesting that there may be scope for much higher levels of supplementation for poultry. It seems likely that the Se level considered to be the norm for current commercial eggs is in fact considerably lower than the natural physiological level; this should be studied in more detail in the future. Therefore, Se enrichment of eggs, meat, and milk is nothing but the production of naturally intended food ingredients. Indeed, production and commercialisation of such organic Se sources as selenized yeast (for example Sel-Plex) opened a new era in Se supplementation of animals and offered a real chance for producers to meet the nutritional requirements of consumers. What is more, production of these kinds of animal-derived foodstuffs is a natural way to health promotion.

Indeed, it is possible to provide consumers with a range of animal-derived products with nutritionally modified composition in such a way that they can deliver substantial amounts of health-promoting nutrients, such as Se, to improve the general diet and help in the maintenance of good health. Therefore, without changing habits and traditions of various populations ("habit is a second nature") it is possible to solve problems related to deficiency of various nutrients, in particular Se. The consumer will go to the same supermarket to buy the same animal-derived products (egg, milk, and meat), cook and consume them as usual. The only difference will be in the amounts of specific nutrients delivered with these foods.

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Chapter 23 Compounds with Antibacterial Activity

Florence Baron and Sophie Réhault

1 Introduction

In contrast to the immune system of animals, which produces antimicrobial polypeptides when needed, the avian egg white can efficiently resist microorganisms over a prolonged period in the absence of an innate systemic host defense. From its role in defense of the chicken embryo, one may infer that it contains anti-microbial compounds, and consequently that albumen may represents a major inexpensive source of antimicrobial molecules usable by pharmaceutical, cosmetic, and food industries. Some of these compounds are well studied and have already been used as preservatives; some others are the subject of advanced research and represent promising leads for the treatment of various diseases. Moreover, it appears quite likely that all antimicrobial potentialities of egg components have not been discovered. Beyond the evident advantage of these new molecules to human health, discovery of each new biologically active compound in the egg promises to be an opportunity for further development of the egg-product industry.

2 Lysozyme

Lysozyme, found in many biological fluids and tissues of a large number of living organisms, is an important component of nonspecific defense mechanisms, based on its ability to control the growth of susceptible bacteria. One of the antimicrobial mechanisms cited is the well known enzymatic activity: the degradation of the glycosidic (1-4) β-linkage between the N-acetylglucosamine and the N-acetylmuramic acid of the bacterial peptidoglycan. The peptidoglycan can thought to be a strong, woven mesh that allows solutes to pass through while maintaining the cell's shape and protecting it from osmotic lysis. Without such a barrier, or when attacked by lysozyme, the cell swells

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and bursts. Cell death thus occurs by the lytic action of lysozyme on the peptidoglycan only when in low-osmotic-strength media, or when the rate of the synthesis and polymerization process for new peptidoglycan formation is slower than the lysozyme-catalyzed degradation.

Unfortunately, the peptidoglycans of some Gram-positive bacteria are resistant to hydrolysis by lysozyme because of chemical modifications. This is the case for *Staphylococcus aureus*, which carries an O-acetylation on the C-6 hydroxyl group of the N-acetylmuramyl residues (Bera et al. 2005), and for *Bacillus subtilis*, in which there is a N-acetylation of the N-acetylglucosamine group (Atrih et al. 1999). Moreover, invasive Gram-negative bacteria are resistant to the lytic action of lysozyme. In contrast to Gram-positive bacteria, Gram-negative bacteria possess an additional membrane, namely, the "outer membrane." This is part of the cell wall, which also includes the lipoproteins, lipopolysaccharides, as well as peptidoglycan layers; and the peptidoglycan is protected from the lytic action of lysozyme because the outermost surface functions as a permeability barrier.

The antimicrobial spectrum of lysozyme can be broadened in a number of ways. Gram-negative bacteria can be sensitized to lysozyme by permeabilization of the outer membrane by chemicals such as EDTA (Samuelson et al. 1985). EDTA chelates the bivalent cations and thus disorganizes the lipopolysaccharide surface structure, allowing the penetration of lysozyme. Permeabilization can also be accomplished by physical stresses such as freeze-thawing, high-pressure treatment (Masschalck et al. 2002). Lysozyme could be modeled and converted into a membrane-penetrating form by adding a surface-exposed hydrophobic domain to mediate its fusion into the outer membrane and deliver it to the site of action, the peptidoglycan (Ibrahim et al. 2002). Moreover, the synergistic effect with lysozyme of other preservatives such as nisin, lactoferrin, glycine, organic acids, trypsin, aprotinin, and gelatin have been found to result in significant improvement of lysozyme activity against a wide range of bacteria.

In the last two decades, evidence has accumulated for the existence of alternative mechanisms of bactericidal action of lysozyme independent of its enzymatic activity. Further studies have shown that lysozyme denatured by heat, reduced by DTT (Düring et al. 1999; Ibrahim et al. 1996), or modified by site-directed mutagenesis (Ibrahim et al. 2001) and consequently devoid of enzymatic activity, has bactericidal properties against the Gram-negative and positive bacteria studied. Moreover, lysozyme possesses non-enzymatic bacteriostatic domains in its primary sequence, and these peptides which could be released from the natural lysozyme by protease digestion, exhibit bacteriostatic activity (Ibrahim et al. 2001; Düring et al. 1999; Mine et al. 2004). These studies on antimicrobial peptides derived from lysozyme provide evidence for membrane-perturbing activity of lysozyme. The correlation between membrane damage and cell death strongly suggests that the cytoplasmic membrane is a primary target for lysozyme for certain bacteria. Another mechanism has also been attributed to the induction of autolysins in

Bacillus subtilis (Ibrahim et al. 2001) and in oral streptococci (Laible and Germain 1985). Autolysins are peptidoglycan hydrolases that bacteria use to introduce breaks in the peptidoglycan chains at the point of cell wall growth but that, under particular conditions, are able to cause bacterial autolysis.

Depending on the type of bacteria and the environmental conditions (energizing status of the membrane, environmental pH, lysozyme structure), one of three mechanisms (enzymatic, membrane perturbation, or autolysin production) may predominate and cause cell death.

In vivo, lysozyme is particularly effective against oral affections due to the cariogenic bacteria *Streptococcus mutans* (Tenovuo et al. 1991), or against periodontitis-associated bacteria (Sava 1996). Lysozyme is also widely used as a food preservative. It is used in cheese to prevent contamination because of the absence of inhibition of Gram-positive bacteria which are used as "starters" in the cheese-making process. Lysozyme also prevents the growth of pathogenic bacteria on refrigerated foods: *Listeria monocytogenes*, *Yersinia enterocolitica*, and spore-forming bacteria such as *Bacillus staerothermophilus*, *Clostridium thermosaccharolyticum*, and *Clostridium tyrobutyricum* are susceptible to the lytic activity of lysozyme (Johnson 1994). Given orally, by local application, or when combined with specific immunotherapy, lysozyme is effective in a wide range of viral diseases (De Douder and Morias 1974; Asakura et al. 1990, Gavrilenko et al. 1992). The antiviral action of lysozyme has partly been explained by its role on the precipitation of viral particles and by its immune-enhancing action on the host, together with its interaction with the pathogens (Sava 1996). The modification of the lymphocytic responses suggests that lysozyme is of interest in cancer treatment and in recovery from immune suppression due to anticancer therapy or autoimmune disease (Sava 1996). These activities had been mainly attributed to human lysozyme, but hen egg white lysozyme has been shown to be effective on human immuno-competent cells. The majority of countries have acknowledged the non-toxicity of lysozyme and have approved its use in some foods and for pharmacological and therapeutic applications.

3 Ovotransferrin

Ovotransferrin is one of the transferrins, an extended family of metal-binding transport proteins with an in vivo preference for iron, and widely distributed in physiological fluids. Further studies (Garibaldi 1970; Baron et al. 1997) showed that the major role in preventing growth of Gram-negative spoilage bacteria in egg white is played by ovotransferrin, which produces an iron-deficient environment. This bacteriostatic effect on Gram-negative bacteria is overcome if the protein is saturated with iron.

Iron is an absolute necessity for life of all forms, participating in many major biological processes. Some bacteria deposit intracellular iron reserves that can then be used to enhance growth when external iron supplies are restricted.

Moreover, certain bacteria are able to counter the iron restriction imposed in biological fluids through the use of high-affinity extracellular ferric chelators called siderophores (Neilands 1995). These can compete with iron-binding protein for iron (Andrews et al. 2003).

Several lines of evidence have suggested that transferrin may have an effect on Gram-negative bacteria in addition to that resulting from iron deprivation. Valenti et al. (1986) suggest that ovotransferrin antimicrobial activity can result from a direct effect on the membranes: interaction of the ovotransferrin with the anionic outer membrane of Gram-negative bacteria can be hypothesized due to the remarkable cationic nature of ovotransferrin. Ibrahim et al. (2000) obtained a cationic peptidic fragment (OTAP-92) from hydrolysis of ovotransferrin that shows a wider antimicrobial spectrum than native protein and that kills bacteria by damaging the biological function of its cytoplasmic membrane. More recently Aguilera et al. (2003) showed that transferrins are able to permeate the outer membrane of *E. coli* to access the inner membrane where they cause permeation of ions in a selective manner. The consequence is an in vivo dissipation of delta-phi without a significant alteration of delta-pH and a decrease in the electrochemical potential from −198 mV to −56 mV. As a result of the uncoupling of the respiration-dependant energy production, bacteria enter stasis.

In conclusion, there is further evidence that ovotransferrin possesses distinct mechanisms of bacteriostatic action. One is based on the chelation of iron, which creates a deprived-iron environment for bacteria; and another is based on damage to biological functions of bacterial cytoplasmic membrane. The two mechanisms depend on the concentration of iron in the medium.

Ovotransferrin has been proposed for treatment of infants with acute diarrhea (Antonini 1977). Recently, the iron-chelating activity of ovotransferrin has been shown to increase the stimulation by an inhibitor of AMPc β-lactamase of some antibiotics that are efficient against most β-lactamase-producing bacteria (Babini and Livermore 2000). Ovotransferrin therefore appears as a key factor for drug associations able to overcome cephalosporin resistance. The strong bactericidal activity of OTAP-92 (Ibrahim et al. 2000) against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) strains brings these authors to envisage therapeutical applications for this natural peptide.

4 Proteinase Inhibitors

Antiproteases are thought to be involved in antibacterial defense mainly by inhibiting bacterial proteases that are secreted by pathogens during the host colonization process.

Ovostatin is a broad-spectrum antiprotease. It inhibits several pathogenic proteases such as 64K and 56K metalloproteases and 70K cysteine protease, which latter is expressed by Gram-negative *Serratia marcescens* and is responsi-

ble for corneal lesions (Matsumoto 2004). Ovostatin can prevent *Pseudomonas aeruginosa*- and *Bacillus stearothermophilus*-induced keratitis as a result of halting bacterial growth by inhibiting their respective proteases, the *P. aeruginosa* elastase and alkaline protease, and also the thermolysin from *B. stearothermophilus* (Molla et al. 1987; Miyagawa et al. 1991). Besides these activities, ovostatin has been shown to suppress *Vibrio vulnificus* dissemination in infected mice (Maruo et al. 1998) and have in vitro inhibitory effects on *Aspergillus fumigatus* upon inflammatory response to lung infection (Murayama et al. 1996). All together, these reports suggest that ovostatin has promise in the prevention of certain bacterial septicemias and local infections.

Ovomucoid and ovoinhibitor are serine protease inhibitors; however, their ability to inhibit microbial proteases has not been clearly established. But interestingly, turkey and pigeon ovomucoid homologues have been described to, respectively, inhibit *Streptomyces griseus* proteinase B (Bateman et al. 2001) and to bind to shiga-like toxin 1 of enterotoxic *Escherichia coli* (Miyake 2000). Chicken ovomucoid could have antiviral activities since it binds to hen Newcastle disease virus (Tsuge et al. 1996) and to viruses from aquatic birds (Matrosovich et al. 1999). As for ovoinhibitor, it can inactivate protease F from bacterial fibrinolysin (Birk et al. 1983). Ovoinhibitor can inhibit rotavirus replication through a mechanism that seems, however, independent of its antiprotease activity (Yolken et al. 1987).

Egg white cystatin inhibits a variety of cysteine proteases. Bacterial, viral, or protozoal cysteine proteases are involved in the mechanisms of penetration of normal tissues by the bacteria, in proteolytic cleavage of precursor proteins for virus replication, in facilitation of host invasion by parasites, in metabolism of host proteins, or in degradation of host immune molecules (Henskens et al. 1996). Thus these proteases constitute putative targets for chemotherapeutic inhibitors such as cystatin. Chicken cystatin has been described to reduce virus production of poliovirus-infected cells (Korant et al. 1986) and is effective against human rotavirus infection in mice and consecutive diarrhea in suckling mice (Ebina and Tsukada 1991). Cystatin and cystatin-derived peptides display antibacterial activity against *Porphyromonas gingivalis*, frequently associated with periodontitis (Blankenvoorde et al. 1998) and is also a potent inhibitor of cysteine proteases expressed by the *Trypanosoma* parasite responsible for bovine and human trypanosomiases (Serveau et al. 1996). Furthermore, cystatin has shown interesting effects in the treatment of *leishmaniasis* by favoring T cell response (Das et al. 2001), and it also increases interleukin production by human gingival fibroblast cell lines and murine splenocytes (Kato et al. 2002).

5 Other Proteins

Vitamin-binding proteins such as riboflavin-binding protein, avidin, and thiamin-binding protein have antimicrobial properties by chelating riboflavin, biotin, and thiamin respectively. But some bacteria are able to synthesize their vitamin needs. Other proteins not known for their antimicrobial properties

could exhibit such activity under certain conditions. Ovalbumin, one of the major proteins present in avian egg white, was digested by trypsin and chymotrypsin and the peptide fragments were investigated by Pellegrini (2003). The peptides were found to exert a strong activity against *Bacillus subtilis* and to a lesser extent against the other bacterial strains examined. Ovalbumin itself was not bactericidal against all the bacteria strains examined. Ovomucin, a fibriform glycoprotein, has been shown to exert an antihemagglutination activity against swine influenza virus, bovine rotavirus, and hen Newcastle disease virus (Tsuge et al. 1996). An active egg white product was obtained by egg white fermentation with *Saccharomyces cerevisiae* and spray drying. Its oral administration improves the nonspecific phagocytic activity of neutrophils in weanling piglets (Araki et al. 1992) and in calves (Nakagawa et al. 1993). It should therefore enhance the host defence mechanism against infectious diseases.

6 Conclusions

In addition to their individual antimicrobial mechanisms, egg white proteins may perhaps exhibit cooperative antimicrobial properties and act synergistically against bacteria in egg white. In egg white, all proteins can interact with each other and perhaps conformational change due to interaction or modification due to natural reaction would result in an antimicrobial mechanism—which is difficult to reproduce in vitro where protein activity is tested under experimental conditions. Among other factors, the activity of protein depends on the environmental conditions. For example, lysozyme is enzymatically inactive under the alkaline conditions encountered in egg white (Ibrahim et al. 1994). Bacteria are also sensitive to alkaline pH rarely encountered in the natural environment, but important for biological function, especially so on bacterial membrane status.

To conclude, we could say that egg white is a biological fluid that possesses many active antimicrobial molecules. Some of these molecules can be extracted for pharmaceutical or medical uses. Future research may well point the way to optimal targets in bacterial cells, to the modeling of active from inactive molecules, and to amplifying the activity of already known active molecules for food and other uses.

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Chapter 24 Egg-Protein-Derived Peptides with Antihypertensive Activity

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1 Introduction

Enzymatic hydrolysis of food proteins can release peptides able to exert different biological activities. Among the bioactive peptides known so far, those with blood-pressure-lowering effects are receiving special attention due to the prevalence and importance of hypertension in the Western population. Eggs are broadly recognised as a very valuable source of proteins for human nutrition and are now known to contain many substances with biological functions beyond basic nutrition. A review on biological activities of proteins and peptides derived from egg components was recently published (Kovacs-Nolan et al. 2005). This chapter discusses the production and effects of antihypertensive peptides described so far as arising from egg proteins.

2 Food Peptides with ACE-Inhibitory and Antihypertensive Effects

Hypertension is a risk factor for cardiovascular disease and stroke. As a result of an extensive research carried out during the past twenty years, a wide range of peptide sequences derived from food proteins, potentially useful in the prevention and/or treatment of hypertension, are known (Li et al. 2004). Most of these peptides act, at least in vitro, as inhibitors of angiotensin-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1), an exopeptidase that cleaves dipeptides from the C-terminal side of various oligopeptides. As part of the renin–angiotensin system, ACE hydrolyzes an inactive decapeptide, angiotensin I, to the potent vasoconstrictor angiotensin II. ACE is also part of the kinin-kallikrein system, as it hydrolyzes bradykinin, which has a vasodilator action (FitzGerald et al. 2004).

Some general features on the structure–activity relationship of ACE inhibitory peptides have been described (Meisel 1997a, b; FitzGerald et al. 2004).

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ACE appears to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions, and it is known that the presence of Pro as a C-terminal or antepenultimate residue enhances binding. On the other hand, ACE only binds weakly to competitive peptide inhibitors that have penultimate Pro residues. In addition, the presence of the positive charge of Lys (ε-amino group) or Arg (guanidino group) as the C-terminal residue may contribute to the inhibitory potency.

The most common strategy for the identification of novel antihypertensive food peptides is based on the preparation of protein hydrolysates and the search for fragments with in vitro ACE inhibitory activity. The in vivo effects are tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension. In general terms, the results of those tests have highlighted an important lack of correlation between the in vitro ACE inhibitory activity and the in vivo action. This poses doubts on the use of the in vitro ACE inhibitory activity as the exclusive selection criterion for potential antihypertensive substances, as it does not take into consideration the physiological transformations that determine the bioavailability of the peptides or the possibility of mechanisms of action other than ACE inhibition (López-Fandiño et al. 2006).

The physiological effects of ACE-inhibitory peptides depend on their ability to reach their target sites intact, which may involve survival of gastrointestinal digestion and absorption through the intestinal epithelium to get to the peripheral organs (Vermeirssen et al. 2004). The release of ACE-inhibitory peptides upon digestion of food proteins, as well as the resistance to digestion of known ACE-inhibitory sequences, have been tested in several in vitro studies showing that proteolysis by gastrointestinal enzymes is an essential factor in determining ACE-inhibitory activity (Vermeirssen et al. 2003; Gómez-Ruiz et al. 2004). In addition, the physiological effect is influenced by the action of brush-border peptidases, the recognition by intestinal peptide transporters, and the subsequent susceptibility to plasma peptidases (Pihlanto-Leppälä 2001). More research is needed in this respect, with the effort being concentrated in elucidating the pharmacokinetics and the distribution profile of ACE-inhibitory peptides in the different tissues.

Even if the hypotensive effects of food-derived ACE-inhibitory peptides have been demonstrated in SHR, only a few studies have been conducted to confirm the existence of an ACE-inhibitory mechanism in vivo (Fuglsang, et al. 2003). Furthermore, most food-derived peptides have lower ACE-inhibitory activity in vitro than the synthetic ACE inhibitor captopril, but they usually display higher in vivo activities than the efficacy levels extrapolated from the in vitro activities. This fact has been attributed to a higher affinity to the tissues and a slower elimination (Fujita and Yoshikawa 1999), but it may also be an indication of the existence of an additional mode of action, such as a direct (Kuono et al. 2005) or indirect action on vascular smooth muscle (Maes et al. 2004). It should also be mentioned that peptides

with antioxidant properties can be formed on hydrolysis of food proteins with different enzymes (Rival et al. 2001; Hernández-Ledesma et al. 2005), and strong experimental evidence indicates that oxidative stress and associated oxidative damage are mediators in cardiovascular pathologies.

Despite the higher doses needed in comparison to antihypertensive drugs, the consumption of food products containing antihypertensive peptides was shown to significantly reduce the blood pressure of moderately hypertensive patients. The most substantiated antihypertensive activity in humans has been obtained for the commercial fermented milks and milk protein hydrolysates that contain the ACE-inhibitory peptides IPP and VPP (FitzGerald et al. 2004). This is the case of a sour milk commercialized in Japan under the trade mark of Calpis (Calpis Ltd., Tokyo, Japan; Mizushima et al. 2004), a milk fermented with *Lb. helveticus LBK-16* (Evolus, Valio Ltd., Helsinki, Finland, and marketed as Kaiku Vitabrand in Spain; Seppo, et al. 2003; Tuomilehto et al. 2004) and a casein hydrolysate produced with *Aspergillus oryzae* (Mizuno et al. 2005), marketed by Calpis as AmealPeptide, that has been added to a new milk drink launched by Unilever (London) under the Flora/Becel proactive brand.

3 Antihypertensive Hydrolysates Obtained from Egg Proteins

For the production of ACE-inhibitory and antihypertensive peptides, a commonly used approach is to subject food protein preparations to the action of one or a combination of enzymes. Enzymes from various sources have been used, including animal digestive enzymes, and plant and microbial enzymes. Alternatively, the active peptides can be liberated during the fermentation of certain food products by the action of the proteolytic system of the fermenting organism, mainly lactic acid bacteria.

The hydrolysate of egg yolk with a crude enzyme from the genus *Rhizopus* exhibited ACE inhibitory action in vitro; and the fraction of the hydrolysate with molecular mass lower than 1 kDa suppressed the development of hypertension in SHR after oral administration for 12 weeks (Yoshii et al. 2001). Serum ACE activity of the hydrolysate-administered SHR groups was significantly lower than that of the control group in a dose-dependent manner, which implied the existence of an ACE-inhibitory mechanism in vivo (Yoshii et al. 2001).

ACE-inhibitory activities in vitro were also found in enzymatic hydrolysates of ovalbumin and crude egg white, while the non-hydrolyzed substrates did not show inhibitory properties (Fujita et al. 2000; Miguel et al. 2004). Maximum inhibition, expressed as the IC_{50}, or concentration required to inhibit 50% of the enzyme activity, was observed after hydrolysis with pepsin (IC_{50} = 45.3–55.3 μg/ml; Fujita et al. 2000; Miguel et al. 2004). Pepsin also exhibited the highest proteolytic activity toward ovalbumin, although

there was not a direct relationship between the level of intact protein and the resultant ACE inhibition. Treatment with trypsin and chymotrypsin did not produce ACE-inhibitory peptides of hydrolysis after 3 h of hydrolysis (Fujita et al. 2000). The IC_{50} of the hydrolysates with trypsin and chymotrypsin decreased with the incubation time, as the hydrolysis proceeded, but the resulting ACE inhibitory levels were much lower that those yielded by pepsin (Miguel et al. 2004). This indicates that the specificity of the proteinase used to release the peptides plays a very important role, so that even a low degree of hydrolysis can provide a significant ACE inhibition.

The hydrolysates may be used as such, or after enrichment, in the manufacture of food products. The enrichment of fractions or the isolation of specific peptides from the total peptide mixture is a technological challenge in the production of ACE-inhibitory and antihypertensive peptides. Since a common feature of ACE-inhibitory peptides is their relatively small size, fractionation using ultrafiltration and size exclusion chromatography constitutes an useful step for pre-concentration (Fujita et al. 2001). In fact, when the hydrolysate obtained from crude egg white with pepsin treatment for 3h (IC_{50} = 55.3 μg/ml) was filtered through a 3000 Da cut-off membrane, the permeate presented approximately ten times more inhibitory activity than the retentate (IC_{50} = 34.5 and IC_{50} = 298.4 μg/ml, respectively; Miguel et al. 2004).

The blood-pressure-lowering effects of the hydrolysate of egg white with pepsin and its fraction with molecular mass lower than 3000 Da were evaluated in vivo in experiments in which these products were acutely administered to SHR by gastric intubation (Miguel et al. 2005). Both caused a significant dose-dependent decrease in systolic blood pressure (SBP) and diastolic blood pressure (DBP), at lowest effective doses of 150 mg/kg and 50 mg/kg, respectively (Fig. 1). The maximal decreases in SBP and DBP were observed, respectively, 6 and 4 hours after the administration, and both variables had returned to baseline after 24 hours. It should be mentioned that in short term experiments non-hydrolyzed egg white did not show any blood-pressure-reducing activity. In fact, Matoba et al. (2001a) had reported that ovalbumin only exerted antihypertensive activity on SHR at a very high dose (2 g/kg). It is also important to highlight that the hydrolysates did not modify SBP and DBP in the WKY rats, which are the normotensive control used with SHR. This suggests that their effects are specific to the hypertensive state, that no blood pressure effects are likely to be produced in normotensive subjects (Miguel et al. 2005).

The in vivo antihypertensive activities of the hydrolysate and its low molecular mass fraction compare favorably with the activities of other food protein hydrolysates. In this context it can be mentioned that the thermolysin digest of dried bonito was active in SHR at a dose of 250 mg/kg, while its fraction with molecular mass lower than 3000 Da had approximately 2-fold higher activity (Fujita et al. 2001). Moreover, fermented caseinate-enriched milk decreased mean arterial blood pressure of SHR from −13 to −25 mm Hg at doses from 500 to 2,500 mg/kg (Leclerc et al. 2002).

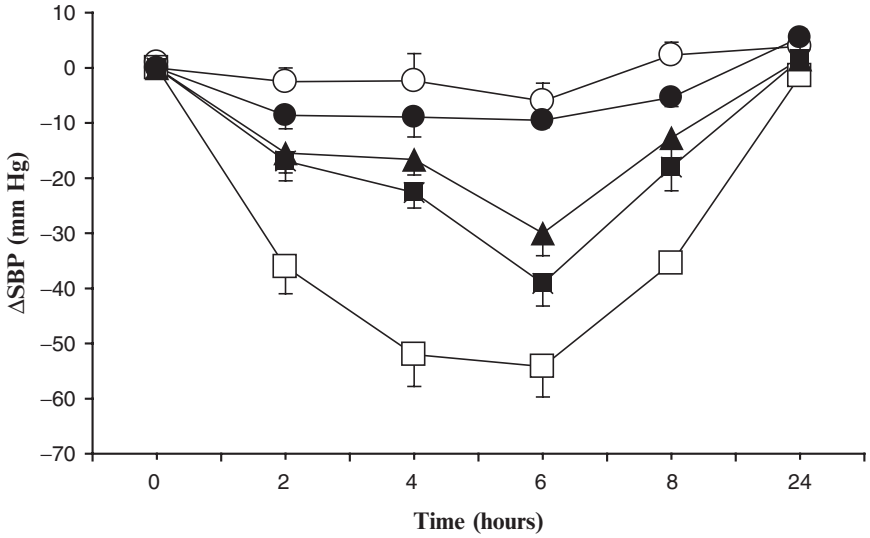

Fig. 1. Decreases in systolic blood pressure (*SBP*) caused in spontaneously hypertensive rats by different products: water (○), captoril [a synthetic ACE inhibitor, 50 mg/kg (□)], and the fraction of the hydrolysate of egg white with pepsin with molecular mass lower than 3000 DA [25 (●), 50 (▲), and 100 (■) mg/kg.] Data are expressed as mean ± SEM

The long term blood pressure effect of the hydrolysate was investigated in SHR that were given 0.5 or 1 g/kg/day from the 3rd to the 20th week of life (Miguel et al. 2006b). Both doses attenuated the development of the hypertension that occurs naturally early in the life of SHR. Moreover, the SBP of the SHR that drank a solution of non-hydrolyzed egg white was slightly, although not significantly, lower than the SBP in the SHR that drank tap water. Therefore, it cannot be discarded that antihypertensive peptides are produced in vivo during gastrointestinal digestion of egg white proteins. After withdrawal of the treatments, arterial blood pressure rose gradually in the rats that had taken the hydrolysate, although the SBP remained consistently lower than in the water control group.

4 Antihypertensive Egg Peptides: Bioavailability and Mechanism of Action

For purposes of identification of ACE-inhibitory and antihypertensive peptides, peptide enrichment and purification based on hydrophobicity, using C18 reversed-phase cartridges or reversed-phase chromatography, is common in the laboratory (Fujita et al. 2000). On-line coupling to tandem mass spectrometry allows simultaneous elucidation of the peptide sequence (Miguel et al. 2004). Amino acid sequence analysis is also used in the case of

peptides purified by consecutive chromatographic steps (Fujita et al. 1995b, 2000). Following those strategies, different peptides have been identified in enzymatic hydrolysates of egg white or ovalbumin. Chemical synthesis of these sequences and measurement of their ACE-inhibitory activities provided several potent ACE-inhibitory peptides in vitro (Table 1). However, it is important to test the in vivo effect of the ACE-inhibitory compounds to establish their possible usefulness. In fact, Fujita et al. (2000) isolated six ACE-inhibitory sequences from a pepsin digest of ovalbumin, with IC_{50} ranging from 0.4 to 15 μM, but all except for the dipeptide LW failed to show antihypertensive activities in SHR. This was attributed to those peptides being ACE substrates, and thus susceptible to being hydrolyzed by the enzyme, but not true inhibitors.

As shown in Table 1, among the peptide sequences identified in the hydrolysate of ovalbumin with pepsin, the octapeptide FRADHPFL, named ovokinin by Fujita et al. (1995b) possessed a high ACE-inhibitory activity (IC_{50} = 3.2 μM; Miguel et al. 2004) and exerted vasorelaxing effects in canine mesenteric arteries (Fujita et al. 1995b). Ovokinin significantly lowered the SBP in SHR, when orally administered in solution at a dose of 100 mg/kg or at a dose of 25 mg/kg, in the form of an emulsion in 30% egg yolk (Fujita et al. 1995a). Similarly, the peptide RADHPFL, also produced by pepsin, that resembles the sequence of ovokinin, but lacking the N terminus Phe residue, exhibited a high ACE-inhibitory activity (IC_{50} = 6.2 μM) (Miguel et al. 2004), as well as a significant antihypertensive effect, decreasing SBP and DBP in SHR in a dose-dependent manner, with minimum effective doses of around 2 mg/kg (Miguel et al., 2005).

A hexapeptide corresponding to the 2–7 fragment of ovokinin (RADHPF) was purified from a chymotrypsin digest of ovalbumin by Matoba et al. (1999). Ovokinin(2–7) exerted a dose-dependent vasodilation in mesenteric arteries from SHR as well as hypotensive activities after the oral administration of 10 mg/kg (Matoba et al. 1999). However, it should be noted that, contrary to most of the orally active antihypertensive food peptides described so far, ovokinin(2–7) was only a weak ACE inhibitor (IC_{50} > 400 μM; Miguel et al. 2004). In fact, when the strong ACE-inhibitory peptides FRADHPFL (ovokinin) and RADHPFL were subjected to a hydrolysis process mimicking gastrointestinal digestion, the main degradation products were RADHPF (ovokinin 2–7) and RADHP, and the ACE-inhibitory activity decreased considerably (Miguel et al. 2006a). RADHP, which seems to be the end product of the gastrointestinal digestion of the antihypertensive peptides FRADHPFL (ovokinin), RADHPFL, and RADHPF (ovokinin 2–7) significantly decreased blood pressure, 2 hours after administration to SHR, at doses of 2 mg/kg, but it is likely that its antihypertensive effect was not mediated through an ACE-inhibitory mechanism (Miguel et al. 2006a).

The peptide YAEERYPIL, a strong ACE inhibitor (IC_{50} = 4.7 μM) produced by pepsin on ovalbumin, also exhibited a significant antihypertensive effect in SHR at doses of around 2 mg/kg (Miguel et al. 2004, 2005; Table 1).

Table 1. Peptide sequences derived from egg proteins with biological activities related with the control of high pressure

Sequence	Origin	ACE inhibitory activity (IC_{50}, μM)	Antihypertensive activity in SHR (Δ mm Hg; time; and dose [a])	Other activities	Reference
ERKIKVYL	Peptic digest of ovalbumin	1.2	0; 10 mg/kg (iv) 0; 60 mg/kg (o)		Fujita et al. (2000)
FFGRCVSP	Peptic digest of ovalbumin	0.4	0; 10 mg/kg (iv) 0; 60 mg/kg (o)		Fujita et al. (2000)
LW	Peptic digest of ovalbumin	6.8	–45; 10 mg/kg (iv) –22; 2 h; 60 mg/kg (o)		Fujita et al. (2000)
SALAM	Peptic digest of ovalbumin	229	not determined	• Radical scavenging activity	Miguel et al. (2004)
				• Delays LDL lipid oxidation induced by Cu^{2+}	Dávalos et al. (2004)
				• Antimicrobial activity	Pellegrini et al. (2004)
FRADHPFL (ovokinin)	Peptic digest of ovalbumin	3.2	–18; 4h; 25 mg/kg[b] (o)	• Vasorelaxing activity	Fujita et al. (1995a, b)
				• Modest radical scavenging activity	Miguel et al. (2004)
					Dávalos et al. (2004)
RADHPFL	Peptic digest of ovalbumin	6.2	–34, 6h, 2 mg/kg (o)		Miguel et al. (2004, 2005)
RADHPF (ovokinin 2–7)	Chymotryptic digest of ovalbumin	> 400	–10, 6 h, 10mg/kg[b] (o)	• Vasorelaxing activity	Matoba et al. (1999)

(*Continued*)

Table 1. Peptide sequences derived from egg proteins with biological activities related with the control of high pressure—Cont'd

Sequence	Origin	ACE inhibitory activity (IC_{50}, μM)	Antihypertensive activity in SHR (Δ mm Hg; time; and dose [a])	Other activities	Reference
RADHP	Pancreatic digest of FRADHPFL, RADHPFL,	260	–25, 2h, 2 mg/kg (o)		Miguel et al. (2006a)
YAEERYPIL	Peptic digest of ovalbumin	4.7	–32; 6h, 2 mg/kg (o)	• Radical scavenging activity	Miguel et al. (2004, 2005)
				• Delays LDL lipid oxidation induced by Cu^{2+}	Dávalos et al. (2004)
				• Part of an antimicrobial sequence	Pellegrini et al. (2004)
YPI	Pancreatic digest of YAEERYPIL	> 1000	–37; 2h, 2 mg/kg (o)	• Radical scavenging activity	Miguel et al. (2006a)
					Dávalos et al. (2004)

[a] Maximum decrease in SBP of SHR after intravenous (iv) or oral (o) administration of the doses indicated.
[b] Administered as an emulsion in 30% egg yolk.

The maximum decrease in SBP and DBP was observed 6 hours after their administration and the effect lasted for at least 8 hours. The amount of this peptide required to show antihypertensive effects was relatively small, in line with the efficient doses of other ACE-inhibitory peptides. For instance, 6 hours after the administration of 5 mg/kg of the potent ACE inhibitors VPP and IPP to SHR, the SBP decreased 20.1 mm Hg and 18.3 mm Hg, respectively (Nakamura et al. 1995). YAEERYPIL was totally hydrolyzed after a simulated gastrointestinal digestion and its ACE-inhibitory activity decreased by approximately 100 fold. The main fragments released were YAEER and the tripeptide YPI. YAEER exhibited a slight, but not significant, antihypertensive effect in SHR, while YPI significantly decreased SBP when administered at doses of 2 mg/kg (Miguel et al. 2006a). The fact that the antihypertensive potency of YPI was very high in comparison with its in vitro efficacy to inhibit ACE (Table 1) might indicate that the inhibition of ACE is not its only mode of action. Alternatively, this peptide may need further activation into in vivo-active compounds by intestinal brush-border or plasma peptidases, such as the dipeptide YP that, as suggested by Yamamoto et al. (1999), significantly reduces blood pressure in SHR through an unknown mechanism.

As already explained, some evidence has been provided that a mechanism other than ACE inhibition is involved in the blood-pressure-lowering effect exerted by many food derived peptides In fact, ovokinin (FRADHPFL) and ovokinin 2–7 (RADHPF) were reported to lower blood pressure through different modes of vasorelaxing activity. Ovokinin showed a relaxing activity mediated by bradykinin B_1 receptors that was partially dependent on endothelium. Prostaglandin I_2 was released from the artery after ovokinin stimulation as relaxing factor (Fujita et al. 1995b). However, ovokinin (2–7) showed a completely endothelium-dependent vasorelaxation, and the relaxing factor was nitric oxide (Matoba et al. 1999). According to Scruggs et al. (2004), ovokinin(2–7) produces its effects by activation of bradykinin B_2 receptors.

Attempts were made to increase the hypotensive activity of ovokinin(2–7) by replacing certain amino acid residues. The synthetic analogues RPFHPF and RPLKPW significantly lowered blood pressure in SHR 2 hours after the oral administration of 1 and 0.1 mg/kg, respectively (Matoba et al. 2001b; Yamada et al. 2002). As was the case with ovokinin(2–7), none of these peptides inhibited ACE, but unlike ovokinin(2–7), RPFHPF did not show any vasorelaxing activity in isolated SHR mesenteric arteries, suggesting a different mode of action (Matoba et al. 2001b). Yamada et al. (2002) reported that these peptides had antihypertensive activities at lower doses after oral than after intravenous administration to Wistar SHR and that the stronger activity of RPLKPW was related to its higher resistance to proteases in the digestive tract. This suggested that they could lower blood pressure through the interaction with receptors expressed in the gastrointestinal tract, implying that no absorption was required. In contrast to these results, Scruggs et al. (2004) found that intravenous administration of ovokinin(2–7) lowered blood pressure in Sprague-Dawley rats at the low threshold dose of 0.1 mg/kg.

While further studies are necessary to clarify the mechanisms implicated in the antihypertensive activity of these products, the sequence encoding RPLKPW was introduced into three homologous sites in the gene for soybean β-conglycinin α' subunit that was expressed in *E. coli*. The modified subunit, given orally at a dose of 10 mg/kg, exerted antihypertensive effects in SHR, providing evidence that a bioactive peptide introduced in a food protein by site-directed mutagenesis could function practically in vivo at a low dose (Matoba et al. 2001a). In a further study, the amino acid residues surrounding the RPLKPW sites in the tailored α' subunit were modified to facilitate the release of this peptide by gastrointestinal proteases; and a fourth hexapeptide was introduced in the extension domain of the protein. This resulted in significant antihypertensive effects in SHR at doses of 2.5 mg/kg (Onishi et al. 2004).

Strong experimental evidence indicates that oxidative stress and associated oxidative damage are mediators in cardiovascular pathologies, and thus antioxidant-rich diets significantly reduce the arterial blood pressure in SHR (Rodriguez-Iturbe et al. 2003; Touyz 2004). The protective effect exerted by ACE inhibitors on the structure and functions of the cardiovascular system, the kidney, and the brain, also seems to be related to an antioxidant action and a reduced formation of reactive oxygen species (Basso et al. 2005). In this respect, and it is noteworthy that the hydrolysate of egg white with pepsin and some of its peptides have shown antioxidant effects in vitro (Table 1; Dávalos et al. 2004). Some of these peptides also exhibited other biological activities, such as antimicrobial properties (Pellegrini et al. 2004), which strongly suggests that proteolysis of ovalbumin can deliver multifunctional ingredients with therapeutic benefits in the prevention and treatment of hypertension.

5 Conclusions and Future Prospects

Research carried out to date has proven that antihypertensive peptides from food proteins, including egg proteins, are appropriate candidates for functional food products that can be used as part of diet-related measures for the prevention of hypertension. Designing new health-promoting food products containing these biologically active peptides looks promising and would be attractive to consumers and producers. However, numerous scientific and technological issues must be resolved. There is a need to develop strategies to produce and enrich fractions of active peptides. Further animal studies and detailed clinical trials must also be carried out in order to verify the efficacy of these peptides, to elucidate their molecular mechanisms, and to minimize possible adverse effects. In addition, the technological properties of these active peptide fractions should be studied in order to develop foods in which they retain their activity for a guaranteed period. On the marketing side, the cost effectiveness of the ingredient and its easy incorporation into a

good-tasting end product are important. Finally, human clinical data with the end product containing the bioactive ingredient are essential to formulate firm health claims. The development of these functional foods containing egg-derived ingredients might increase the applications of egg proteins and, from an economic point of view, favor more profitable uses.

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Chapter 25 Use of IgY Antibodies in Human and Veterinary Medicine

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1 Introduction

IgY technology, including the production and use of polyclonal IgY antibody (Ab), is a highly innovative and an expanding branch of biotechnology. In this chapter, we will discuss the advantages in the use of IgY Ab and the application of IgY in human and veterinary medicine (see also Kovacs-Nolan and Mine 2004). Other aspects such as immunization procedures, Ab titer development, IgY heat and acid stability and preservation of IgY solutions are reviewed in detail in Chacana et al. (2004), Hau and Hendriksen (2005), Leenars et al. (1999), Schade et al. (2000), and Schade et al. (2005). The usefulness of IgY Abs in biomedical diagnostic is well documented by an increasing quantity of literature and is therefore not included in this review.

2 Advantages of IgY Technology

The most important aim of animal welfare is to reduce painful manipulations. IgY technology fulfils this requirement in that chicken Abs can be easily sampled by a non-invasive method based on the simple act of collecting eggs. IgY technology also offers outstanding economical advantages because hens cost less to keep than rabbits. Furthermore, the Ab production of a hen roughly corresponds to that of a large mammal, such as a sheep or goat. Indeed, an extraordinary amount of Ab can be produced from only one hen—approximately 17–35 g of total IgY/chicken/year—of which 1–10% can be expected to be antigen-specific. This huge quantity of available Abs opens the door for new fields of IgY applications, such as immunotherapy and immunoprophylaxis for several viral and bacterial infections in veterinary and human medicine. In addition, IgY Abs have no cross reactivity with rheumatoid factors (Larsson et al. 1991) or human anti-mouse Ab (HAMA;

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

Carlander et al. 1999). IgY Abs are unable to activate the mammalian complement system (Larsson et al. 1992) and have no heteroagglutinins (Calzado et al. 2003). Furthermore, several authors have reported that chickens often produce Abs against phylogenetical highly conserved mammalian proteins or peptides more efficiently than do rabbits (Karlsson et al. 2004). As a consequence, a conserved antigen can remain "masked" to the rabbit immune system, and thus cause only a weak or a "silent" response. Furthermore, if chickens and rabbits are immunized with the same mammalian antigen, very often the chickens respond with an Ab specificity that can rarely be achieved in rabbits, as for instance, with the N-terminal collagen propeptide PIIINP (Gerl et al. 1996), parathyroid-hormone-related protein (Rosol et al. 1993), transforming growth factor β3 (TGF-β3; Danielpour and Roberts 1995), and YKL-40 glycoprotein (De Ceuninck et al. 2001).The advantages of using chicken Abs have been recognized by many authors (for example Lösch et al. 1986; Carlander 2002; Narat 2003; Karlsson et al. 2004).

3 Applications of IgY in Biomedical Research and in Human and Veterinary Medicine

3.1 General Applications

Several publications have described the successful use of IgY Abs in a variety of research fields (for a review, see Narat 2003). IgY-based immunoassays are being used to measure the concentration of proteins or peptides via ELISAs, RIAs or other assays in clinical chemistry and basic research. IgY Abs are successfully used in immunohistochemistry for detection of antigens of viral, bacterial, plant, and animal origin, to assess the incidence of intestinal parasites in domestic animals (Schniering et al. 1996) and the contamination of foods with toxins or drugs (Pichler et al. 1998). During the past decade, IgY Abs have increasingly been used in therapy or prophylaxis of disease as well as in the new context of the so-called functional foods.

3.2 IgY for Therapeutic or Prophylactic use in Veterinary Medicine

3.2.1 Treatment of Intestinal Infections

Powdered whole eggs or yolks have been used in veterinary medicine as an inexpensive IgY source for the treatment of enteric diseases. The most famous example of a successful therapeutic/prophylactic use of IgY is the treatment of calves and piglets with specific Abs against *Escherichia coli* (K88, K99, 987P), rotaviruses, and coronavirus (for review see Mine and Kovacs-Nolan 2002 and Kovacs-Nolan and Mine 2004). Studies using both animal models and trials in field herds have been carried out. The groups of Yolken

(Yolken et al. 1988), Lösch (Wiedemann et al. 1991), Erhard (Erhard et al. 1996), and Kuroki (Kuroki et al. 1997) have performed the studies mostly on the practical use of IgY (see also Bilbao et al. 2006 and Terzolo et al. 2003). These studies confirmed that the treatment of diarrhea in calves and piglets with specific egg yolk Abs has achieved significant prophylactic and therapeutic benefits. Pokorova et al. (2000) administered IgY to protect dogs against canine parvovirus, and supposed that the protection was due to interactions between IgY and viral surface components. Sunwoo et al. (2002) demonstrated in vitro a marked growth inhibiting effect of specific IgY on *E. coli* 0157:H7, showing that growth inhibition was actually caused by the binding of specific IgY to bacterial surface antigens, which caused significant changes in the bacterial surface structure. Another effect of the binding of IgY to bacterial surface antigens is a marked impairment of bacterial attachment to the intestinal mucosa (Marquardt et al. 1999; Lee et al. 2002). Therefore, therapeutic IgY administration might reduce the clinical use of antibiotics, and thus minimize the risk of bacteria developing antibiotic resistance.

3.2.2 IgY Application in Aquafarming

IgY Abs have been successfully applied in treatments of fish farming diseases, for example in salmon and trout. Researchers from Japan have extensive experience in this field. For example, Hatta and colleagues demonstrated a successful treatment of *Edwardsiella tarda*-infected Japanese eels with specific anti-*E. tarda* IgY and showed protective effects when the IgY extracts were administered directly into the aquarium water (Hatta et al. 1994). Other authors achieved protective effects of specific IgY Ab against infection with *Yersinia ruckeri* (Lee et al. 2000) or against *Vibrio anguillarum* (Arasteh et al. 2004) in rainbow trout. Mostly, the Ab was incorporated in commercial pellet food. Furthermore, protection of shrimp (*Penaeus chinensis*) from infection with white spot syndrome virus (WSSV) has been described. Kim et al. (2004) produced IgY Abs against four different dominant proteins of WSSV and studied the neutralizing activity of these Abs. The authors demonstrated that shrimps survived an injection of WSSV preincubated with anti-WSSV Ab.

3.3 IgY for Therapeutical or Prophylactic use in Human Medicine

3.3.1 Treatment of Intestinal Infections in Children

The adherence ability of many viral and bacterial pathogens is a major prerequisite for the successful colonization of a higher organism, especially with respect to the host's respiratory and intestinal mucosae. It has been shown that specific IgY Abs against *Salmonella* antigens are able to inhibit in vitro the adhesion of this bacterium to epithelial cells (Lee et al. 2002). Casswall (1999), Carlander et al. (2000), and Sarker et al. (2001) investigated the action

of hyperimmune bovine colostrum (HBC) and IgY against human rotavirus isolated from infected children. The oral administration of IgY Abs resulted in a significant protective effect (Sarker et al. 2001). An anti-human rotavirus (strains Wa, RV5, RV3, ST3) IgY Ab was also effective, although to a lower extent than with HBC.

3.3.2 Treatment of Helicobacter Pylori

Therapeutic protection through IgY anti-*Helicobacter pylori* Abs has also been investigated in animals (Nomura et al. 2005) and humans (Shimamoto et al. 2002; Suzuki et al. 2004). Shin et al. (2003) were able to identify the immunodominant proteins of *H. pylori*. Antibodies with specificity against these proteins were more effective as prophylactic reagents as compared to Abs directed against the whole bacterial lysate. Altogether, all studies demonstrated a curative effect of the anti-*H. pylori* Ab. In most cases no complete *H. pylori* eradication could be achieved. But in view of the increasing bacterial resistance, the use of specific IgY Ab minimizes the need for antibiotics. Horie et al. (2004) carried out a study with 42 volunteers to test the protective effect of drinking yogurt fortified with anti *H. pylori* urease IgY, obtaining a significant decrease in urea breath values of the treated group.

3.3.3 Use of IgY for Treatment of Colitis and Celiac Disease

Worledge et al. (2000) demonstrated significant protective effects after oral application of specific IgY against tumor necrosis factor (TNF) in an experimental rat model for colitis. TNF is implicated in the pathogenesis of inflammatory bowel disease. The oral use of such Abs is considered to have fewer systemic side-effects than the intravenous infusion of a humanised murine anti-TNF monoclonal Ab (Infliximab, Centocor, Malvern, PA, USA). Sunwoo and Sim (2004) reported on the use of IgY Ab against dietary gluten proteins that play a role in the autoimmune disorder of the celiac disease. The authors immunized chickens with gliadins and low- and high-molecular glutenin. The resulting Ab can be used in different forms, such as table eggs, liquid and powdered eggs, and encapsulated nutraceuticals for treatment of celiac disease.

3.3.4 Treatment of Cystic Fibrosis

Carlander et al. (2002) studied the benefits of IgY as a prophylactic tool against infectious diseases in patients with cystic fibrosis (CF), the most common fatal genetic disease of the Caucasian population in Europe and the USA. CF is caused by a mutation of the gene for a chloride channel protein, which results in the secretion of an abnormally thick mucus. This leads to secondary infections in the respiratory tract, caused by several bacterial species, one of which, *Pseudomonas aeruginosa*, infects virtually all CF patients. The

researchers treated CF patients orally with an aqueous IgY anti-*P. aeruginosa* solution (70 ml, 0.7 mg/ml IgY), given as a mouth rinse in the evening. A high level of the specific chicken Abs could be demonstrated in the saliva by an ELISA for approximately 8 hours after the treatment. The IgY concentration then gradually declined, and was completely undetectable in the saliva 16 hours after the treatment. These oral IgY treatments were successful in reducing chronic *P. aeruginosa* infections in CF patients, and thus resulted in a decrease in antibiotic prescriptions (Kollberg et al. 2003).

3.3.5 Prophylactic use of IgY in Dental Caries

An effective local protection against plaque formation related to dental caries was achieved with anti-*Streptococcus mutans* IgY (Otake et al. 1991; Hamada and Kodoma 1996; Hatta et al. 1997; Chang et al. 1999; Smith et al. 2001). This passive protection was clearly shown with both SPF rats and human volunteers, following the use of either purified IgY or whole-egg powder. Active immunization against *S. mutans* glucan-binding protein B (GBP-B), under experimental conditions, induces good protection against experimental dental caries. This protection results from the continuous secretion of salivary Abs against GBP-B, which prevents the accumulation of *S. mutans* on the dental biofilm. The passive protection achieved by IgY is based on the same principle. In fact, the administration of IgY anti-*S. mutans* GBP-B via the diet and drinking water of experimentally infected rats caused a significant decrease in *S. mutans* aggregation on dental biofilms. In all these trials, a direct correlation was found between a given IgY dose and a reduction in the incidence of dental caries (Smith et al. 2001). Furthermore, the decrease in the *S. mutans* infection rate did not require continuous IgY administration (Smith et al. 2001). Hatta et al. (1997) evaluated the efficacy of oral IgY anti-*S. mutans* rinses in human volunteers. This IgY inhibited *S. mutans* adherence to saliva-coated hydroxyapatite discs by 59%, while the control IgY from non-immunized hens only gave an 8% inhibition. All these results strongly support the efficacy of oral treatments with anti-*S. mutans* IgY as a new alternative for reducing dental plaque in humans. Zhou et al. (2003) investigated the protective effect of an anti *S. mutans* IgY spray in adult volunteers. There was no difference in dental plaque indexes between controls and IgY-spray group although a significant decrease in *S. mutans* colonies could be demonstrated in the test group after three weeks of IgY application.

3.3.6 Use of IgY for Treatment of Poisonings

The protective effects of anti-venom IgY against rattlesnake toxins and scorpion toxins has been shown in a mouse model (Thalley and Carroll 1990). Almeida et al. (1998) showed similar results, producing IgY against venom from Brazilian snakes of the *Bothrops* and *Crotalus* genera. Related investigations are being performed in Bangalore (Vittal Mallya Research Institute of

Bangalore, India [http://www.spiegel.de/spiegel/0,1518,231565,00.html]). A Chinese group (Yu et al. 2004) produced anti king cobra venom based on IgY Ab with neutralizing activity. The use of anti-venom IgY is advantageous, since fewer immunological side-effects may be expected (see Sect. 3.1 this chapter). Gomez and colleagues (2006) produced specific IgY Abs against botulinum toxin type A that have been successfully used for immunoneutralization in a mouse model. Further, Lemley et al. (1995) produced an avian anti-ricin Ab and proved a protective effect in a mouse bioassay.

3.3.7 Use of IgY as a Tool in the Context of Bioterrorism

To test the therapeutic use of IgY Abs, LeClaire and colleagues (2002; see Table 1) produced IgY Abs against the highly toxic staphylococcal enterotoxin B (SEB). SEB is considered to be a potential biological warfare agent. Therefore, an increasing necessity exists to develop vaccines and therapeutic approaches for intoxication with SEB. The authors demonstrated the prophylactic and therapeutic application of anti-SEB IgY. Complete protection of mice and rhesus monkeys against a lethal SEB aerosol challenge has been observed when applied twenty minutes before or four hours after challenge.

Table 1. Toxin neutralizing activity of specific IgY-Ab. Two studies from 1893 and 2002

Klemperer F (1893) Ueber natürliche Immunität und ihre Verwerthung für die Immunisierungstherapie. Archiv für Exp Pathol Pharmakol 31:356–382			
Repeated immunization (5 immunizations at intervals of 5–15 days each) of chickens with a tetanus culture (i.p. injection of increasing concentrations of virulent tetanus bouillon culture, from 5–30 ccm in steps of 5 ccm). After 4 weeks preparation of an egg yolk extract (11 ccm egg yolk mixed with 10 ccm saline). Treatment of mice with this extract (i.p.).			
4 mice	2 mice	2 mice	2 mice
1 ml extract	0.5 ml extract	0.25 ml extract	0.0 ml extract (saline)
Next day administration of a lethal dose of tetanus bouillon culture (lethal dose 0.001 ccm, administered 0.0015 ccm s.c.).			
live	live	dead	dead
LeClaire et al (2002) Protection against bacterial staphylococcal enterotoxin B by passive vaccination. Infect Immun 70:2278–2281			
Repeated immunization (4 immunizations at intervals of 2 weeks each) of chickens with bacterial staphylococcal enterotoxin B (250–500 μg SEB i.m.). After 8 weeks preparation of pure IgY (immunoaffinity chromatography). Administration of IgY 10 mg/kg or buffer (i.m. injection in monkeys), administration of toxin 5 × LD50 as aerosol (during the experiment the monkeys were anesthetized).			
4 monkeys	4 monkeys	1 monkey	1 monkey
IgY 20 min before toxin challenge	IgY 4 h after toxin challenge	buffer 20 min before toxin challenge	buffer 4 h after toxin challenge
live	live	dead	dead

3.3.8 IgY as a Tool in Proteomics

A new and an interesting field in the use of IgY technology is proteomic analysis. A problem in separation of complex protein mixtures by 2D-electrophoresis is the predominance of highly abundant proteins such as albumin, which disturbs the monitoring of low-incidence proteins. Low-incidence proteins can be of great importance for identification and monitoring of several human (and animal) diseases. Recently, it has been shown that IgY Abs directed against these high-incidence proteins are in fact useful tools for their removal. In addition, these Abs work more specifically than do matrices with affinity to albumin, as for example Blue Sepharose (GE Healthcare, Amersham, Bucks., UK; Hinerfeld et al. 2004; Ahmed and Rice 2005; Huang et al. 2005).

4 Conclusions and Future Prospects

Today, there is no doubt that chicken Abs can be produced and used, with minor modifications, in ways similar to the use of mammalian Abs. It can also be said that, depending on the circumstances, the use of IgY Abs often has significant advantages over the use of mammalian Abs. Chickens have the potential to be used to complete the spectrum of animals used for Ab production. The production of chicken monoclonal Abs (and also recombinant IgY or genetically engineered IgY; Nakamura et al. 2004; Tsurushita et al. 2004; Park et al. 2005; Finlay et al. 2005) would combine the advantages of monoclonal Abs with the advantages of chicken Abs. In addition, a further interesting aspect is the immunization of chickens or ducks using DNA constructs (Cova 2005). It is to be expected that studies on the therapeutic or prophylactic use of IgY Abs will be intensified in the future. In particular, due to the increasing resistance of microorganisms to antibiotics, research on all aspects related to the development of specific IgY Abs against pathogenic microorganisms will have to be stepped up. In the future, IgYs will be universally used in science, including both veterinary and human medicine. IgY technology is a fast developing field and in this concise review we have only described some of its uses. We are convinced that, once accepted and widely used, IgY technology will offer new alternatives and solutions for science, medicine, and society as a whole.

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Chapter 26 Egg Compounds with Antioxidant and Mineral Binding Properties

Catherine Guérin-Dubiard, Oscar Castellani and Marc Anton

1 Introduction

Among egg proteins, two are known for their excellent metal-ion-binding properties: ovotransferrin and phosvitin. The first exists in egg white, the second is a yolk protein. These binding properties allow certain biological processes to occur, particularly antioxidant and antibacterial activities.

2 Ovotransferrin

2.1 Ion-Binding Capacity

Ovotransferrin is a monomeric egg white glycoprotein, belonging to the transferrin family of proteins widely distributed in various biological fluids. It has the same structural characteristics as hen serum transferrin, because these proteins derive from the same gene and differ only by their attached carbohydrate (Mizutani et al. 2001). This protein has a molecular weight of 77 kDa and is made up of two domains, an N-domain and a C-domain, with a short linking region (Williams 1982). Each domain has a binding site for metal ion, tyrosyl, histidyl, arginyl residues being implicated as effective in this action; the site requires synergistic anion binding (Oe et al. 1989). Ovotransferrin binds iron very strongly, in particular Fe^{3+}, since its dissociation constant (K_{Diss}) is 10^{-24} M (Stevens 1991); it can bind two iron atoms per molecule. The order of iron binding is pH dependent; at pH 6.0 it binds first to the C-domain, but at pH 8.5 it first binds to the N-domain. The function of ovotransferrin is generally accepted as iron transport. It can bind other metal ions, including toxic ones. Mizutani et al. (2005) report that the aluminum-bound form is almost

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the same as iron-bound form; they suggest that ovotransferrin participates in the transport of Al^{3+} ions in vivo.

2.2 Metal-Binding Capacity and Biological Activities

Many studies have been focused on ovotransferrin iron-binding properties and to its biological activities; most of them concerned with antibacterial activities, based on the scavenging and the deprivation of iron as factors in microorganism development. This activity is more completely described Chapter 23. On the other hand, very few studies related to ovotransferrin antioxidant activity. However, Li-Chan and Nakai (1989) have reported that ovotransferrin's iron-binding ability may have an indirect role in prevention of lipid oxidation, and more particularly LDL oxidation. LDL is protected against oxidation by a wide variety of antioxidants. Several enzymes including superoxide dismutase, catalase, and ceruloplasmin are effective in vivo as reported by Roxborough et al. (1995). Ceruloplasmin is an enzyme that is more effective in the presence of transferrin. This major copper-containing protein of human serum has a an important role in oxidizing ferrous ions to the ferric state. Tests in vitro prove that this enzyme, by ferrous iron oxidation, facilitates iron binding by ovotransferrin, and thus prevents the ferrous-ion-catalyzed formation of hydroxyl radicals from superoxide (the Fenton reaction), thereby inhibiting iron-induced lipid peroxidation (Lovstad 1983). In fact, ovostransferrin is better known for its antibacterial, antiviral (Giansanti et al. 2002), antifungal (Valenti et al. 1985) activities, and immunomodulating effects (Otani and Odashima 1997) than for its antioxidant properties. However, this last property should spark great interest for its possible role in preventing diseases, including cancer.

3 Phosvitin

3.1 Ion-Binding Capacity

Phosvitin is the most highly phosphorylated protein known, containing 3–10% phosphorus and 6% carbohydrates (Taborsky and Mok 1967), and it represents 4% of hen egg yolk dry matter (Burley and Vadhera 1989). Phosvitin with a molecular weight of 35 kDa, has a very specific amino acid composition, presenting about 50% serine (Taborsky 1983). Practically all of the serines are phosphorylated (Clark 1985), moreover, the phosphoserines are arranged in clusters, forming blocks that can carry up 15 consecutive residues (Byrne et al. 1984); this unique primary sequence makes this protein one of the strongest metal chelating agents. Ninety-five percent of the iron in egg is present in the yolk and all is bound to phosvitin (Greengard et al. 1964).

Phosvitin contains 2 to 3 atoms of iron per molecule when isolated from hen egg, but its potential binding capacity is much higher (about 60 atoms per molecule; Taborsky 1983) with an affinity constant of (Ka) of 10^{18} M (Hegenauer et al. 1979). From a structural point of view, the iron ions are octahedrally coordinated by oxygen atoms of the serine-bound phosphate groups and by other ligands from either the protein or the solvent (Mangani et al. 1994).

3.2 Nutraceutical Applications

The extraordinary abundance of phosphate groups provides phosvitin with a strong binding ability for positive ions, as iron but also calcium and magnesium (Grizzuti and Perlmann 1973). Jiang and Mine (2000, 2001) have also proved that phosphopeptides, with molecular weights 1000–3000 Da, derived by phosvitin tryptic hydrolysis following partial dephosphorylation, enhance calcium-binding capacity and inhibit the formation of insoluble calcium phosphate. Similarly, Choi et al. (2005) have tested effectiveness of phosvitin peptides on enhancing bioavailability of calcium and its accumulation in bones. These results provide potential novel functional oligo-phosphopeptides as nutraceuticals for use in the prevention of osteoporosis.

3.3 Antibacterial and Emulsifying Properties

In addition, this multivalent metal binding capacity indicates other important attributes, including antibacterial, emulsifying, and antioxidant properties, the first two of which can be linked. In 2000, Khan et al. suggested that a significant part of the bactericidal activity of phosvitin against *E. coli* resides in the synergistic effect of the strong metal-chelating ability and the surface properties, such as interfacial tension and emulsification. In 2004, Choi et al. reported that phosvitin and its peptides exhibit clearly recognizable antibacterial and DNA leakage activities against *Escherichia coli*, under thermal stress at 50 °C, by cell disruption through chelation with metals in the outer cell membrane.

Despite a poor surfactant activity, phosvitin provides excellent properties of emulsion stabilization, and many studies have been focused on its emulsifying and adsorbing properties and the conditions affecting these capabilities (Chung and Ferrier 1991, 1992; Damodaran and Xu 1996; Castellani et al. 2004, 2005, 2006).

3.4 Antioxidant Activity

Lastly, excellent ion-binding capacity, in particular with iron, makes phosvitin a potential natural antioxidant, with application possible in both the medical domain and in the food industry.

3.4.1 Medical Domain

Iron is an essential element for all living organisms, but it can be toxic when not properly controlled by proteins. Under nonpathological conditions, iron levels are tightly contained by iron-binding proteins transferrin and ferritin, and iron-catalyzed free-radical reactions are kept to a minimum (Cairo et al. 2002). If iron balance is disturbed, either locally or systemically, excess iron saturates the binding sites of transferrin, allowing free iron to circulate and oxidize heart muscle cells, ultimately leading to heart failure (Nathan 1995). Fe^{2+} ions can react with H_2O_2, producing the hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction; this radical is known to induce oxidative damage to DNA leading to development of cancer (Toyokuni 1996; Kawanishi et al. 2002). Ishikawa et al. (2004) investigated the effectiveness of phosvitin in prevention of $^{\bullet}OH$ formation from H_2O_2 in the Fenton reaction system. Phosvitin can also be effective in prevention of UV-light-induced oxidative stress (Ishikawa et al. 2005). These authors exposed a mouse dorsal skin homogenate to ultraviolet light in the presence of ferric nitrilotriacetate (FeNTA). They reported that in vitro lipid peroxidation increases with increasing FeNTA concentration and UV-light exposure time, but in the presence of phosvitin, the formation of hydroxyl radicals is suppressed.

3.4.2 Food Industry

Lipid oxidation is of great concern to the food industry because of the many undesirable flavors, colors, and potentially toxic reaction products in many foodstuffs that it causes. So, it is absolutely necessary to limit oxidation in food products, and this can be done by using antioxidants. Synthetic antioxidants exist, but the demand for natural molecules has recently increased. Phosvitin, with its strong metal-ion-binding capacity, had already been tested as natural antioxidant. Lu and Baker (1986) report that this protein can inhibit Fe^{2+}-catalyzed phospholipid oxidation. But because food can have a wide range of pH, varying from 2.5 to 9, Lu and Baker (1987) studied the effect of pH on phosvitin effectiveness as an antioxidant. The ionization of the phosphate group in phosvitin is affected by the pH of the environment, and this could modify its conformation (Taborsky 1974), and indeed its metal-binding capacity, and thus its antioxidant activity. Lu and Baker (1987) found that at pH 6.1, phosvitin inhibits catalytic activity of Fe^{2+} and also that of Cu^{2+}. On the other hand, iron does not demonstrate any catalytic activity at pH 7.8, which is in accordance with findings of Castellani et al. (2004), who reported that phosvitin still strongly binds iron at pH 7.1 at low ionic strength (0.15 M). Lu and Baker (1987) reported that copper conserves a catalytic activity at this alkaline pH. They have followed the copper-binding capacity of phosvitin with increasing pH, and have observed considerable amount of free copper at pH 7.8, and so they suggested that unbound copper could still catalyze lipid oxidation. Moreover, the antioxidant activity of phosvitin can

be improved by chemical modification. Nakamura et al. (1998) have conjugated phosvitin with galactomannan through a controlled Maillard reaction at 60 °C in 79% relative humidity for one week. The conjugation reaction significantly enhances the antioxidant activity of phosvitin, and even improves emulsifying activity, emulsion, and heat stability.

Lastly, it is important to test phosvitin antioxidant activity not only at differing pH values, but also under the varying temperature and NaCl conditions to be expected in processed food. Lee et al. (2002) reported that phosvitin is capable of inhibiting lipid oxidation in phosphatidylcholine liposomes, muscle homogenates, and ground pork under the pH, temperature, and NaCl conditions expected in processed meat. However, it is not able to completely inhibit lipid oxidation in salted uncooked and cooked ground pork; in these cases, it is necessary to use phosvitin in combination with other antioxidant technologies to effectively increase the shelf-life of these meat foods.

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Chapter 27 Use of Lecithin and Lecithin Fractions

Margherita Rossi

1 Definition and Composition

Lecithin is the phosphatide fraction (phospholipid) of egg yolk lipid and of several oilseeds. Although it was first observed in egg yolk—its name comes from the Greek term for egg yolk "lekithos"—its impact became appreciable when people learned how to treat oilseeds in order to extract their phosphatide content. The first industrial lecithin appeared on the market in 1925 in Germany (Schäfer and Wywiol 1986).

Today, most of the lecithin produced is used in food applications (mainly as an emulsifier) and is derived from soybeans, although many non-food uses are feasible and other sources are available.

The term "lecithin" is commonly used as a synonym for phosphatidylcholine (PC), which is the major component of the phosphatide fraction isolated from either egg yolk or soybean and is commercially available in high purity (Wabel 1998). In commercial practice, however, the term "lecithin" refers to a mixture of different phospholipids (PLs), including "true lecithin" together with other substances such as triglycerides and minor constituents of the lipid fraction that are co-extracted with phosphatides.

Egg yolk is 32–35% lipid, made up of 66% triglyceride, 28% phospholipid, 5% cholesterol, and 1% other lipids (Mine and Kovacs-Nolan 2004).

Egg yolk lecithin is striking for its peculiar composition, which differs considerably from that of plant origin, with reference both to the kinds of phospholipids and of fatty acids esterified on the glycerol–phosphate backbone. Yolk lecithin is characterized by a higher abundance of phosphatidylcholine (Table 1), the major glycerophospholipid, and by the presence of long chain polyunsaturated fatty acids (LC-PUFAs) of the *n*-6 and *n*-3 series, mainly arachidonic (AA) and docosahexaenoic acids (DHA; Burley and Vadehra 1989), which are practically absent in soybean lecithin. Animal lecithins are also characterized by the presence of sphingomyelin (SM), a phospholipid with a sphingosine backbone.

Figure 1 and Table 2 display the general structures of the major egg yolk phospholipids and their fatty acid composition, respectively.

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Table 1. Phospholipid relative composition (%) in crude egg yolk and soybean lecithin

Component	Egg[a]	Soybean[b]
Phosphatidylcholine (PC)	66–76	33.0
Phosphatidylethanolamine (PE)	15–24	14.1
Phosphatidylserine (PS)	1	0.4
Phosphatidylinositol (PI)	–	16.8
Phosphatidic acid (PA)	–	6.4
Lysophosphatidylcholine (LPC)	3–6	0.9
Lysophosphatidylethanolamine (LPE)	3–6	0.2
Sphingomyelin (SM)	3–6	–

[a] Kuksis 1985
[b] Weber 1985

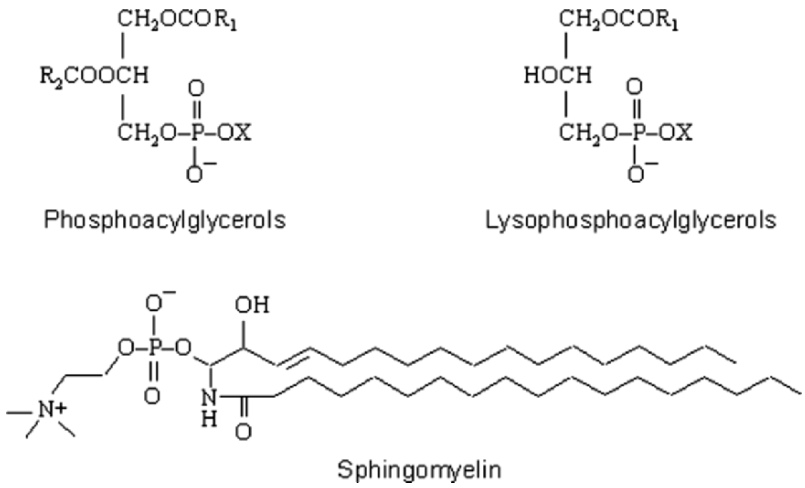

Fig. 1. Structures of the major egg yolk phospholipids. R_1 and R_2 are fatty acid residues. $X = -CH_2CH_2N^+(CH_3)_3$, phosphatidylcholine and lysophosphatidylcholine; $X = CH_2CH_2N^+H_3$, phosphatidylethanolamine and lysophosphatidylethanolamine; $X = CH_2CH(NH_2)COOH$, phosphatitylserine

Considering the fatty acid distributions in egg yolk total lipids and phospholipids, the dominant chain lengths are C16 and C18, with the longer chain PUFAs (C20, C22) more prevalent in phospholipids (Burley and Vadehra 1989). The fatty acid esterified in the *sn*-1 position of the glycerol–phosphate backbone is more likely to be a saturated C16 or C18, while an unsaturated C18 residue is more likely to be present on the *sn*-2 position. Phosphatidylethanolamine (PE) is different in that the second position usually has a longer chain PUFA (Burley and Vadehra 1989).

Table 2. Fatty acid relative composition (%) in egg yolk phospholipids and total lipid. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatitylserine; SM, sphingomyelin

Fatty acid	Total PCs[a]	Total PEs[a]	PS[a]	SM[a]	Total lipid[b]
Myristic (C14)	0.2	0.1–2.0	5.2	5.8	0.4
Palmitic (C16:0)	31.6–43.6	16.7–33.4	29.6	37.9	24.9–26.3
Palmitoleic (C16:1)	0.6–2.8	0.5–2.0	4.8	5.9	3.4–3.7[c]
Stearic (C18:0)	11.6–17.3	24.3–31.5	24.1	16.0	8.1–9.8
Oleic (C18:1)	27.5–31.4	15.0–27.6	14.0	21.5	35.9–39.6[c]
Linoleic (C18:2)	5.3–16.2	5.4–11.2	6.4	8.2	17–18.6
Linolenic (C18:3)	0.1–1.0	–	–	–	0.8–0.9
Arachidonic (C20:4)	2.7–4.3	12.5–15.8	8.4	–	2.2–2.5
Docosapentaenoic (C22:5)	0.1–0.8	0.3	–	–	0.6–0.7[c]
Docosahexaenoic (C22:6)	1.7–5.3	6.6–16.3	–	–	0.9–1.1

[a] elaborated from Burley and Vadehra 1989
[b] elaborated from Hidalgo et al. 2007
[c] as sum of the different *cis* isomers

2 Processes for Isolation, Purification and Modification

2.1 Extraction and Purification

Egg yolk lecithin is commercially available for use in the cosmetic, pharmaceutical, and nutritional fields. Commercial products are essentially composed of a mixture of phospholipids, triglycerides, and cholesterol at various levels to satisfy the requirements of the different application fields. Highly purified egg yolk lecithins with a defined fatty acid composition are also available, as well as solvent-fractionated or enzymatically/chemically modified products.

Phospholipids are soluble in hydrocarbons and other organic solvents such as chloroform and diethyl ether, while they are typically acetone insoluble. The latter characteristic allows the separation of the accompanying lipids in order to increase lecithin purity by a treatment with acetone, which dissolves triglycerides. Ethanol is also used to increase PC purity since PC is completely soluble in it, PE is partly soluble, and the other phospholipids are insoluble (Ziegelitz 1991). Lecithin is insoluble in water, but it forms so-called mesomorphic phases, which, when stirred, change from lamellar into globular forms, giving it a micellar structure (Ziegelitz 1991).

Industrial extraction methods for egg yolk lecithin are commonly based on organic solvents, whose safety has been questioned (Hartmann and Wilhelmson 2001). Thus, more environment- and health-friendly methods have been developed, based on physical treatments and solvents having less safety concern.

Sim (1994) described and patented (Sim 1991) a technology based on an ethanolic aqueous solvent system for the extraction and cold fractionation of lecithin and neutral oil from fresh or powdered egg yolk. A lecithin fraction of greater than 89% PL is obtained, together with neutral oil and a lipid-free yolk protein fraction.

Juneja et al. (1994) proposed a method for the large-scale preparation of high purity PLs, treating fresh egg yolk with ethanol and acetone and obtaining a > 95% PL lecithin fraction containing 80.8% PC. By treating the extracted lecithin on silica and ion exchange columns, higher purity PC fraction (> 98% PC) is obtained and the separation of individual PE, lysophosphatidylcholine (LPC) and SM fractions is accomplished.

An industrial method makes use of a combination of physical treatments such as electrophoresis, extraction with esterified alginate/carragenate resins, concentration by reverse osmosis, selective adsorption on silica gel or on aluminium oxide, and lyophilization with the aid of isopropanol and hexane or ethanol as solvent media (EU Commission 1999). Following this complex procedure, an intermediate lecithin product containing 85% PLs is obtained, as well as a final 100% pure PL fraction.

Another method to produce oil-free high-purity lecithin with reduced cholesterol content (< 1.5% by wt) is based on the extraction of lecithin-containing egg oil with a mixture of liquefied propane and diethyl ether in a separation column (Heidlas et al. 1998). Yolk lecithin containing more than 98% PLs and less than 0.5% cholesterol is also produced using multiple solvent extractions and phase separation by centrifugation (Palacios and Wang 2004). The almost complete purification of egg lecithin fraction from cholesterol by supercritical carbon dioxide extraction, avoiding the use of toxic/flammable organic solvents, is described as a tentative finding by Sim (1994).

2.2 Modification

The functional properties of PLs intended for specific applications can be improved by modifying the structure of the phosphoacylglycerol molecules through either physical, chemical, or enzymatic techniques. PLs' physical and chemical behavior is in fact dependent on the nature of their reactive groups and the polar characteristic of the molecule. A survey of the major practicable methods applied to alter egg yolk lecithin or fractions is presented in Table 3.

Purified lecithin fractions, enriched in the different PLs species, are obtainable by solvent fractionation, alcohols with 1 to 3 carbon atoms having excellent fractionating properties (Ziegelitz 1991). In order to increase the concentration of individual PLs, the fractionated extracts are then treated on silica, aluminum, or magnesium oxide, or ion exchange columns (Ziegelitz 1991; Juneja et al. 1994). This way an almost 100% pure PC fraction is achievable, presenting physiological and technological properties superior to those

Table 3. Methods industrially applied to modify egg yolk lecithin or fractions

Process	Action	Products	Application fields
Physical			
Solvent fractionation	Purification of the different PLs, relying on solubility differences in specific organic solvents or on different partition coefficients in diphasic organic solvent systems	Purified PL fractions rich in one or more PLs	Food, cosmetic, medical/pharmaceutical. (De Ferra et al. 2001)
Enzymatic			
Phospholipase A_1 and A_2	Specific hydrolysis of 1- and 2- acyl ester bonds in PLs, respectively. Insertion of defined acyl groups via esterification/transesterification	Lysophospholipids, glycerophosphorylcholine, PLs with different acyl groups	Food, medical/pharmaceutical, laboratory/diagnostic. (Kim et al. 2001, Pearce et al. 2002, Vijeeta et al. 2004, Tobolewski et al. 2005)
Lipase	Non specific or 1,3-specific hydrolysis of acyl ester bonds in PLs	Similar to those obtained with phospholipase A_1 and A_2	Food, medical/pharmaceutical. (Guo et al. 2005)
Phospholipase D	Hydrolysis of the phosphate ester group. Transphosphatidylation	Enrichment of particular PL species (e.g., PC, PS) by conversion of one PL species to another one. Synthesis of PL derivatives or novel compounds	Medical/pharmaceutical, cosmetic, food, laboratory/diagnostic. (Kirschner et al. 2002, Rutenberg 2005)
Chemical			
Hydrogenation	Conversion of PL unsaturated fatty acids to saturated ones	PLs bearing saturated fatty acids suitable for liposome preparation	Pharmaceutical, cosmetic, laboratory/diagnostic. (Nuhn et al. 1985, Anonymous 2004, Elzainy et al. 2004)

of plain egg lecithin and finding applications in the pharmaceutical, cosmetic, and nutritional fields (Ziegelitz 1991).

Many lipases/phospholipases from different sources (animal, vegetable, microbial) are also available for the selective and specific modification of PLs. The potentiality of enzymes as tools for the modification of PL molecules is thoroughly discussed by Guo et al. (2005) in an up-to-date review. Compared with physical or chemical methods, the biocatalytic approach is advantageous since

it greatly diminishes the use of toxic solvents, simplifies the complicated purification steps, and allows the use of mild reaction conditions, thus reducing heat or oxygen damage to PLs (Guo et al. 2005). Common applications include: (1) the production of lysophospholipids, in which an acyl residue is removed from the phosphoacylglycerol molecule in order to increase hydrophilicity, and (2) the production of PLs with changed acyl residues, relying on the esterification capability of phospholipases (Kim 2001; Vijeeta et al. 2004). Phospholipase D causes the hydrolysis of the phosphate ester group and/or the transphosphatidylation with new polar group. A common application is in the production of phosphatidylserine (PS), a PL widely used in the preparation of pharmaceutical compositions, liposome formulations, and food supplements (Kirschner et al. 2002). Using egg yolk lecithin as the substrate and by adding L-serine to the reaction mixture containing the enzyme phospholipase D, a PL fraction rich in PS plus phosphatidic acid is accomplished (Rutenberg 2005).

The most popular chemical modification applied to egg yolk lecithin is hydrogenation. Hydrogenation of PLs is a catalytic reaction which converts unsaturated acyl residues into saturated ones to increase product stability against oxidation. The degree of unsaturation is normally expressed as an iodine number, egg yolk lecithin typically having the value of 80, which drops down to 2–5 after saturation of the double bonds (Washington 1996). Hydrogenated PLs (especially PC) have found applications in lipid emulsions for drug delivery and are also used for the production of liposomes for pharmaceutical and cosmetic use.

3 Applications

3.1 Nutritional Field

Lecithin is ultimately used in the food industry as an emulsifier, viscosity reducer, lubricant, and as an antispattering, wetting, and release agent (Ziegelitz 1991). Its widespread uses are linked to its ability to act as a surface-active substance in multiphase systems, such as foodstuffs. A surface-active substance, also known as surfactant, is an amphiphilic molecule containing a hydrophobic part, represented by the fatty acid residues, and a hydrophilic head group. It has the ability to migrates to interfaces between immiscible phases, orienting itself so that the hydrophobic tail is in the nonpolar phase (such as lipid or air) and the hydrophilic head in the aqueous polar phase. The migration and concentration of surfactants at oil-water interfaces cause a reduction of the interfacial tension and allow the development of stable emulsions, when mechanical energy is applied to the system.

Lecithin is regarded as a well-tolerated nontoxic compound, whose generally recognized as safe (GRAS) status is approved by FDA (Wabel 1998). EU legislation includes lecithin (E322) in the list of allowed food additives (group of emulsifiers; EU Parliament and Council 1995). Having being considered safe,

lecithin addition to food intended for human consumption follows in general the "quantum satis" principle, meaning that the level of addition should be just enough to obtain the desired technological effect, within a general framework of good manufacturing practices. However, maximum levels of addition are defined (EU Parliament and Council 1995) for non-emulsified oils and fats (max 30 g/l), and for foods intended for infancy, such as infant formulae and follow-on formulae (max 1 g/l), and weaning foods (max 10 g/kg).

Lecithin used as a food additive is commonly of vegetable origin, but egg yolk lecithin is highly recommended for applications in infant formulae because of its relative high content both of arachidonic and docosahexaenoic acids, which play a significant role in early infant nutrition. In fact, dietary LC-PUFAs affect positively the growth and development of the infant and improve visual and cognitive functions, particularly in pre-term infants (Gil et al. 2003). Furthermore, it has been demonstrated that infant formula containing egg PLs fed to pre-term infants reduces the incidence of necrotizing enterocolitis, suggesting that one or more components of egg PLs may enhance, with a synergistic effect, the immature intestinal functions (Carlson et al. 1998).

As a dietary supplements of LC-PUFA and PC, egg yolk PLs have positive effects against age-related alterations of memory and learning, by restoring the proportion of LC-PUFA and enhancing the concentration of acetylcholine, an essential neurotransmitter that decreases in the cerebral cortex of Alzheimer's patients (Masuda et al. 1998; Favrelière et al. 2003). It has also been demonstrated that egg yolk PC decreases the lymphatic absorption of cholesterol in rats (Jiang et al. 2001).

The stabilizing effect of egg yolk lecithin in edible emulsions is a well known fact. Nevertheless, novel technological aspects regarding the utilization of plain or modified egg yolk lipid fractions in foods have been investigated. For instance, egg yolk containing modified lecithin has found specific application in the formulation of mayonnaise-like edible emulsions with reduced level of oil and substantially free of cholesterol and carbohydrate, but maintaining the viscosity and taste characteristics of a full-fat emulsion (Tobolewski et al. 2005). The egg yolk used for this invention has been treated with a phospholipase A_2 to convert lecithin to lysolecithin, a more hydrophilic emulsifier. Dickinson and Yamamoto (1996) observed a positive influence of pure egg lecithin on the elastic modulus of a whey protein concentrate emulsion gel and attributed it to the formation of a lecithin–protein complex, while pure soybean lecithin was not so effective in reinforcing the network. Likewise, Ikeda and Foegeding (1999) observed a positive effect of crude egg yolk lecithin on the rheological properties of whey protein isolate gels due to the acceleration of gelation rates during the heating process and also to the enhancement of the elastic nature of the networks during cooling. Egg yolk lecithin has been recently used in the preparation of haem liposome for flour fortification with iron in bread manufacturing, which has positive effects on the stability and rheological characteristics of the dough, and also improves loaf volume and crumb uniformity (Albaldawi et al. 2005).

3.2 Pharmaceutical Field

The pharmaceutical industry makes use of the emulsifying properties of egg yolk PLs in parenteral lipid emulsions and drug delivery systems (Hartmann and Wilhelmson 2001). Formulation examples are liposome and fluorocarbon emulsions as blood substitutes (Schneider 1999).

Egg has become the preferred lecithin source for parenteral use today, since some adverse reactions after parenteral application of soybean-lecithin-stabilized emulsions have been reported (Wabel 1998). Besides egg lecithin as the emulsifier, intravenously administered oil-in-water emulsions commonly contain triglycerides as the dispersed phase for providing calories and essential fatty acids to patients who cannot be nourished orally. Such emulsions are not only used as nutrients, but also as carriers of oil-soluble drugs (Wabel 1998).

Stable emulsions with droplet size 200–400 nm are suitable for intravenous administration, the physical behavior of the emulsifier playing a key role in shelf life and interaction of the colloidal system with the various additives that may be needed in the final clinical application (e.g., electrolytes; Washington 1996). The addition of drugs further complicates the system, frequently yielding emulsion stability or solubility problems (Triplett 2004). Fortunately, knowledge of the physicochemical properties of the drug (i.e., solubility and ionization properties) allows one to predict the effect of drug additions to emulsions (Washington 1996). From this point of view, the saturation and length of the acyl chains as well as the nature of the polar head group affect the physical behavior of PLs, particularly melting temperature (higher for saturated and longer chains) and the surface and ionization behaviors of the molecule. The predominant head groups choline and ethanolamine produce phosphatides (PC and PE) that are neutral at pH 7, while other minor PLs are negatively charged (Washington 1996). Lecithin containing 80% PC and a small amount of acidic PLs gives excellent emulsion stability. Contrarily, further purified lecithins (95–100% pure PC or PE) having low surface charge at pH 7 are not per se useful emulsifiers (Washington 1996). Hydrogenated PLs are also used, as well as individual PLs obtained via either enzymatic transesterification or transphosphatidylation (Table 3).

The use of solid lipid nanoparticles recently emerged as a novel approach to parenteral drug delivery systems, showing an improved therapeutic drug profile. These are spherical particles (50–500 nm diameter) possessing a solid lipid core matrix that can solubilize lipophilic molecules and is stabilized by surfactants (Triplett 2004). Egg yolk lecithin (> 60% PC) has also been used with success for the preparation of such delivery systems (Triplett 2004).

Other interesting therapeutic applications of egg yolk lecithin concerns the preparation of liposomes for topical and other medical uses. Liposomes are closed vesicles of one or more concentric phospholipid bilayers (lamellae)

with enclosed aqueous layers and cores (Elzainy et al. 2004). They are able to encapsulate both hydrophobic and hydrophilic ingredients within their structure and protect them until they are delivered to target cells or organs. Liposomes with hydrogenated egg yolk lecithin as phospholipid component exhibit a significantly increased encapsulation capacity and an improved stability (Nuhn et al. 1985). They have been shown to transport polar drugs into the skin yielding increased therapeutic effects and reduced adverse systemic reactions (Elzainy et al. 2004).

3.3 Cosmetic and Other Fields

Egg yolk lecithin and hydrogenated lecithin are advantageously used for the manufacturing of PC-rich liposomes intended for cosmetic formulas. By adhering to surfaces containing proteins like keratin, PC has conditioning and softening effects. Shampoos were thus formulated in the past with egg yolk to soften hair and prevent it from becoming charged with static electricity (Lautenschläger 2001). PC is known as a penetration enhancer and liposomes are the vesicles which are said to transport cosmetic agents more effectively into the horny layer (Lautenschläger 2001). In fact, when a liposome comes into contact with skin, it breaks and migrates into the stratum corneum forming a barrier that slows water loss through skin. In the meanwhile, the active agents contained in the liposome are released and sealed into the skin by the occlusive barrier (Seiller et al. 1994).

Egg yolk liposomes are also used as models to mimic cell membranes for studying the therapeutic potentiality of emerging drugs such as natural antioxidants (Sengupta et al. 2004), or the therapeutic efficacy of antifungal compounds for mammalian cells (Brajtburg et al. 1994), as well as lipid peroxidation induced by secondary products of the Maillard reaction (Nakayama et al. 1992).

Applications of egg yolk lecithin in the diagnostic and laboratory sectors are also reported such as in the formulation of selective culture media (Vasconcellos and de Rabinovitch 1995) or biomedical analysis. Fluorescent-labeled or enzyme-labeled liposomes prepared with egg lecithin, either hydrogenated or not, and cholesterol are available on the market, as well as customized liposomes with given size, phase transition temperature, surface charge, and conjugated compounds (e.g., antibodies; Anonymous 2004).

Egg yolk PLs have been proposed as natural antioxidants in squalene or highly unsaturated oils such as DHA-rich oils and borage oil (Sugino et al. 1997; Uk-Jung et al. 2001). It has been observed that the antioxidant activity of individual egg PLs is affected by the saturation level of acyl residues in PL molecules, decreasing with increasing saturation. Thus egg PE, PC, hydrogenated PE, and hydrogenated PC have decreasing antioxidant capacity (Sugino et al. 1997). Likewise, soybean lecithin is more efficient than egg yolk lecithin in reducing the oxidation rate in borage oil (Uk-Jung et al. 2001), probably due to a higher degree of unsaturation.

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Chapter 28 Extraction of Several Egg Compounds at a Pilot Scale

Heikki Aro

1 Introduction: Hen Egg is an Endless Reserve for Fractionation Processes

The largest quantity of hen eggs are consumed as shell eggs. As for their chemical composition, only a few egg compounds have been successfully utilized in the manufacture of food or non food products. Both egg albumen and egg yolk are rich sources of biologically active components. Egg albumen is protein solution of more than 25 different protein structures, and egg yolk is an excellent source of different lipid and protein components. All these constituents, individually or as complexes, have many important functional roles in foods (Alzagtat and Alli 2002), and their nutritional value is very high, too.

Even though many of the egg molecules have been studied, only a few egg compounds (eg., IgY, lysozyme, yolk phospholipids) are utilized in food, and the pharmaceutical and cosmeceutical industries. A good example is the need for suitable liposome structures, which has led to increasing use of egg yolk phospholipids. A number of different extraction methods for egg yolk lipids have been presented. Most of them are based on the use of organic solvents. However, especially in pharmaceutical and cosmeceutical applications, these methods are not completely innocuous due to the possible solvent residuals in the final products.

2 Supercritical Fluids - Sustainable Separation Technology

When a substance is compressed and heated above its critical pressure and temperature, it has liquid-like density and gas-like viscosity. When both of these parameters exceed the critical values, the substance in this state is called a supercritical fluid (SCF). Figure 1 presents this state schematically in pressure-temperature diagram. In the SCF region, a large variation in solvent properties is achieved with quite small changes in the temperature and pressure (Mukhopadhyay 2000).

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

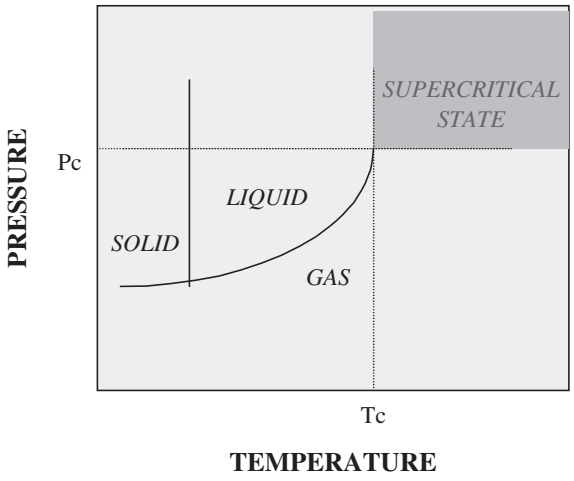

Fig. 1. Pressure–temperature diagram

The most common medium for the extraction of natural compounds in supercritical state is carbon dioxide (CO_2). The critical constants for supercritical carbon dioxide (SC-CO_2) are 73.8 bar and 31.1 °C. Supercritical carbon dioxide dissolves non-polar, lipophilic compounds such as oils and hydrocarbons. It does not dissolve most sugars, amino acids, or inorganic salts. At room temperatures CO_2 is in gas state, which means that no solvent residues are present in extracts after processing. Moreover, SC-CO_2 provides an inexpensive, non-toxic, recyclable and well-penetrating medium for many industrial processes. It has "generally regarded as safe" (GRAS) status and is therefore considered to be a quite suitable solvent for processing foodstuffs, cosmeceuticals, nutraceuticals, and pharmaceuticals.

Probably, the most common and well-known SCF application is the supercritical fluid extraction (SFE). Recent reviews concerning industrial applications of SFE techniques in food processes reveal that the industrial processes are concentrated on tobacco extraction, making of spice extracts, and extraction of fats and oils (Rozzi and Singh, 2001; Sihvonen et al. 1999). Recently, supercritical fluids have also begun to be utilized as an antisolvent. In these processes, the addition of SC-CO_2 to the liquid solution—or vice versa—results in the extraction of the compounds soluble in SC-CO_2 and precipitation of the compounds not soluble in SC-CO_2. Especially, micronization of pharmaceutical compounds is a continuously developing area of supercritical antisolvent processes (Järvenpää et al. 2007). A large group of acronyms for these techniques has been launched , and the techniques are reviewed recently by Harjo et al. (2005) and Yeo and Kiran (2005). Besides the micronization of pharmaceutical compounds, present industrial applications of these techniques include the production of microcellular polymer foams and fine chemicals. Some applications related to spray coating have been presented, too (Reverchon et al. 2003).

3 Solubility of Egg Compounds in SC-CO_2

The solubility of a chemical compound in SC-CO_2 depends mostly on the polarity and the number and the type of the individual functional groups of the compound. To some extent, the molecular weight also influences the solubility. Easily extractable compounds include nonpolar or slightly polar hydrocarbons, esters, ethers, ketones, lactones, and epoxides (Mukhopadhyay 2000). When there is a need to increase the solvent power of the SC-CO_2, a cosolvent or an entrainer is added to the supercritical medium. Ethanol is a typical entrainer used in food processes.

In eggs, the main chemical components are water (74%), lipids (12%), and proteins (12%). Small amounts of carbohydrates and minerals are found, too. In practice, the proteins of egg albumen are almost insoluble in SC-CO_2 and thus not extractable. Native egg yolk contains typically 50% water and 50% solid matter. From the structural point of view, yolk is classified as a dispersion containing different particles—spheres, granules, low-density lipids (LDL), and myelin figures—distributed uniformly in a protein solution (Powrie and Nakai 1985). LDL particles contain 90% of the yolk lipid, having a triacylglycerol (TAG) core (61.5%) and a coating of phospholipids (23%), cholesterol (3.5%), and a mixture of vitellenin apoproteins (Burley and Vadehra 1989). After spray-drying, the resulting powder contains about 60% lipids, which consist of neutral lipids (65%), phospholipids (31%), and cholesterol (4%). The main phospholipids are phosphatidylcholine (PC; 26%) and phosphatidylethanolamine (PE; 3.8%). Thus, both structurally and chemically, egg yolk powder (EYP) contains many suitable fractions to be extracted in SC-CO_2 processes.

4 SCF Applications and Egg Yolk

During the last two decades many SCF applications related to modification of different lipid compounds in egg yolk have been reported. Most of these applications are based on consumers' desires for healthier food. Partial delipidation or decholesterolification of EYP increases its nutritional value. However, it is important that the texture and the sensory properties of EYP are not altered substantially during the process. The potential food applications of SFC-extracted EYP include scrambled eggs, baked goods, egg noodles, ice cream, custard, and mayonnaise (Bringe and Chen 1995).

Froning et al (1990) extracted cholesterol and other lipids from dried egg yolk using SC-CO_2. In 1993, the NutraSweet Company (Chicago) introduced a low-fat, low-cholesterol (LFLC) egg yolk ingredient called Eggcellent. This ingredient was produced by SC-CO_2, and it contained 74% less fat and 90% less cholesterol than traditional egg yolk powder (Singer et al., 1993; Bringe and Chen, 1995). Bringe (1997) compared the properties of native and LFLC

egg yolk and noticed only minor differences in water-holding capacity, viscosity, and reactions with oxygen and glucose. The traditional functional properties of these products were also compared. Based on his results, Bringe (1997) concluded that LFLC egg yolk had valuable properties quite similar to native egg yolk but superior to many other ingredients. On the other hand, Paraskevepoulou et al. (1997) produced cholesterol-reduced egg yolk by SC-CO_2, and reported differences between the functional properties of SC-CO_2 treated and untreated dried egg yolk.

Recently, several studies have shown that not only the nonpolar lipids, but also the polar lipids can be processed using supercritical fluids if entrainers are added to the fluid. Ethanol is a typical entrainer used in food processes. The separation processes for polar egg yolk lipids have been presented by Sihvonen et al. (2000), Elst et al. (2003), and Shah et al. (2004). By these processes a mixture of phospholipids with various co-extractives solubilized in ethanol are obtained. Shah et al. (2004) separated a phospholipid-rich mixture from feed-grade dried egg yolk in a small scale instrument. They mixed the powder first with equal amount of sea sand, which makes the material inedible after the extraction process. The extraction recovery for phosphatidyl choline (PC) was 49 g/kg dried egg material, when the material was first extracted with pure SC-CO_2, then with SC-CO_2 mixed with 0.5 mol% ethanol.

4.1 The Pilot Scale Method for the Production of TAGs, Pure Phospholipids, and Lipid-Free Fractions From Egg Yolk Using Supercritical Fluids

Recently, Järvenpää et al. (2007) described a pilot scale method for the production of pure phospholipids from egg yolk using a technique mostly resembling the supercritical antisolvent technique (SAS). In this pilot scale process, commercial EYP was used as starting material. To avoid the "channeling phenomenon," EYP was mechanically pretreated (granulated) before the SFC processes. The pretreatment process is described in Aro et al. (2002).

A pilot-scale plant used in this study was equipped with two extraction chambers and two separators. One of the extractors was also utilized in an antisolvent process. The more technical description of the apparatus and its use for the supercritical antisolvent process is given in Adami et al. (2003).

4.2 Supercritical Fluid Extraction Steps

The pretreated EYP was extracted in two steps, first with SC-CO_2, then with a SC-CO_2–ethanol mixture. Extraction with pure CO_2 was carried out using supercritical conditions (70 °C, 450 bar, CO_2 flow 0.45 L/min measured as liquid). The total extraction time was 6 hours. The second step (SC-CO_2 with ethanol as co-solvent) was accomplished in altered but still supercritical conditions (70 °C, 400 bar, CO_2 flow 0.4 L/min measured as liquid) to extract

the slightly polar phospholipids from EYP, which had been partly delipidated in the first step. The ethanol solution of polar EYP lipids (PLS) was collected and condensed using a rotary evaporator.

4.3 Supercritical Antisolvent (SAS) Process

In the SAS procedure, the ethanol solution containing the PLS was pumped into an antisolvent precipitation chamber. The PLS were precipitated on the walls of the chamber, and the "waste" ethanol collected as liquid in the separator. After delivery of the ethanol solution, the SC-CO_2 was allowed to flow through the system to remove possible ethanol residues. Finally, CO_2 flow was stopped, the system depressurized down to atmospheric pressure, the precipitation chamber was opened, and the PLS precipitates were collected from the walls of the chamber.

4.4 Analysis of the Egg Yolk Fractions Produced in the Process

The pretreatment process did not influence the chemical composition of EYP. The extraction of EYP with pure SC-CO_2 produced an oily lipid mixture, strongly yellow in color. Using SC-CO_2, about 670–700 g of neutral lipids and 40 g of cholesterol would theoretically be extractable from 2 kg of starting material (Bringe 1997). About 960 g of product was obtained from granulated EYP. This value includes water, which makes up about 170 g of the 2 kg batch of granular EYP (Järvenpää et al. 2007).

As a product from the extraction of EYP with SC-CO_2 and entrainer, an ethanol solution, pale yellow in color, was obtained in the separator. About 50–55 g of PE and PC were recovered in the separator from 2,000 g of EYP. The ratio PC:PE in the product was 5.5–7.5:1, resembling that of the original materials. The ethanolic solution was subsequently utilized as feed in an antisolvent process described below. During extraction using SC-CO2 modified with ethanol, no essential differences in extraction efficiency of PC and PE was observed. The ratio of PC to PE has remained approximately the same through the extraction process (Järvenpää et al. 2007; Fig. 2).

The ethanol solution of phospholipids was utilized in the SAS process. The volume of the concentrate was 1,250 mL, and after the SAS precipitation the weights of the precipitates from different batches were 88–129 g. These precipitates were analyzed by HPLC for their lipid composition according to method of Kivini et al. (2004). Only phosphatidyl ethanolamine and phosphatidyl choline were quantitatively analyzed in samples, showing total values of PLS to be 720–980 mg/g, with a PC:PE relation 6–7.4 (Järvenpää et al. 2007).

After the extractions, a lipid-protein residual of EYP was collected from extraction chamber. This product is an interesting egg yolk preparation containing high-value egg proteins and phospholipids that may have potential for further applications (Hiidenhovi et al. 2005).

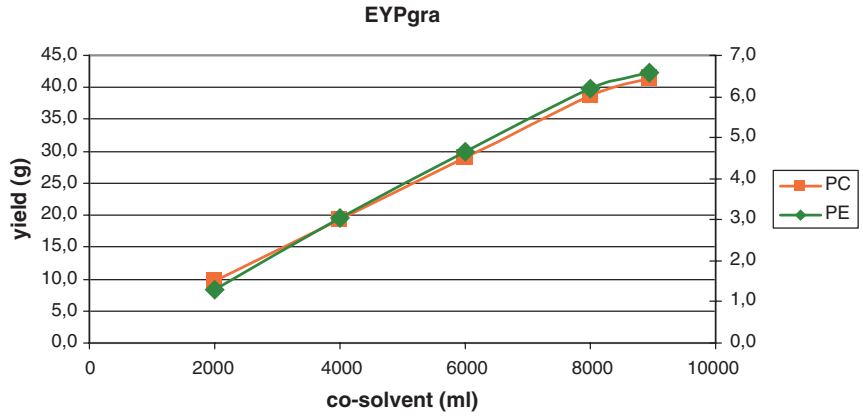

Fig. 2. Yields of PC and PE from 2 kg of granulated egg yolk powder in relation to cumulative co-solvent volume during SFE processing. The yield of PC (left) and PE (right) is given in grams

5 Utilization of the Resulting Fractions

All the fractions produced in the described process are food-grade products, and thus possible materials for nutraceutical, cosmeceutical, and food processes. The present results show that EYP fractions produced by supercritical fluid technologies are, as such, potential ingredients in many industrial applications, and provide a good basis for many new hen-egg-based biotechnological opportunities, too.

6 Conclusions

The need for new, biologically active ingredients for the development of novel functional foods is increasing. Egg yolk contains many biologically active compounds that can be separated while maintaining the activity of the molecule. Due to consumer demand for safe and healthier food, the need for the more sustainable separation technologies increases. Supercritical technologies offer an important possibility fo exploiting the great potential of the egg.

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Chapter 29 Use of Egg Compounds for Cosmetics and Pharmaceutics

Maud Cansell

1 Introduction

Egg yolk components have been increasingly recognized as valuable biochemicals in the field of pharmacy and cosmetics. Among the various components found in egg yolk, about two-thirds on a dry-weight basis consist of lipids. One-third of these lipids are phospholipid (PL) (Nielsen 2001), among which phosphatidylcholine (PC) is the most prevalent. Different commercial fractions of egg yolk lipids are available, from natural extracts of crude or purified lecithins to modified PL (partially or completely hydrogenated). Purified egg yolk PLs are needed for pharmaceutical applications while hydrogenated lecithins avoid fatty acid residue oxidation, a major issue for PL use in cosmetics. Moreover, owing to their natural origin, PLs are considered as biocompatible and biodegradable components. These properties may be exploited in pharmaceutics and cosmetics.

Based on their physico-chemical properties, PLs are applicable to various types of preparations. Since PLs contain hydrophilic and hydrophobic functional groups, they exhibit amphoteric characteristics, which makes them suitable agents for dispersion, emulsification, stabilization, and wetting. Moreover, some PLs (in particular PC) dispersed in water are organized in flexible bilayers that can form closed spherical structures, namely liposomes (Israelachvili 1985). Liposomes are used as model membrane systems or drug delivery vehicles of either hydrophilic drugs encapsulated within the aqueous volume or lipophilic drugs incorporated into the lipid bilayer (Lasic 1993).

This chapter is devoted to the use of egg yolk PLs as emulsifiers or liposome-forming agents. The applications of lecithins as dietetic and bioactive compounds is treated elsewhere in this volume (see Chap. 27). Because the literature and patent publications related to PLs and liposomes is extremely prolific (Wendel 2000; Gomez-Hens and Fernandez-Romero 2006), it is not possible to provide an overview of all the publications. Thus, only very recent (still not exhaustive) examples are given to illustrate the different uses of egg PLs in pharmaceutics and cosmetics.

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

2 Emulsions Based on Egg Phospholipids Used in Pharmaceutics and Cosmetics

Since PLs are nearly balanced surfactants, they may stabilize both oil-in-water and water-in-oil emulsions (Kabalnov et al. 1996). It is worth noting that the efficiency of an emulsion for pharmaceutical application depends on its physical characteristics. In particular, the fat droplet size is considered as a major issue for intravenously administered emulsions because it influences their half-lives. Thus, parameters like the nature and concentrations of the emulsion components (oil, water, and emulsifier) should be considered for optimal effectiveness (Chung et al. 2001; Nii and Ishii 2005a).

The main therapeutic applications of submicron emulsions should be sought in formulations designed for parenteral administration (Lundberg 1994; Sakaeda and Hirano 1998; Tamilvanan 2004). With this route, emulsions can be trapped by the reticuloendothelial system (i.e., circulating monocytes and tissue-resident macrophages) leading to their plasma clearance (Sakaeda and Hirano 1998; Weers and Arlauskas 2004) and can interact with erythrocytes causing hemolysis of the red cells (Ishii and Nagasaka 2004). The occurrence of hemolysis is dependent on the emulsion formulation and concentration used. There was no significant variation in the hematological and blood chemistry test values compared with normal values for both positively and negatively charged submicron emulsions containing egg yolk PL as a surface-active species, intravenously administrated to rats (Benita 1999). In contrast, hemolysis was actually observed and attributed to vesicle PL present in the water phase of the emulsions (Ishii and Nagasaka 2004). This result was interpreted in terms of an easier penetration and transport of vesicle PL into the erythrocyte membranes owing to a higher instability of the vesicle system compared with the emulsion (Ishii and Nagasaka 2004). Nevertheless, oil-in-water emulsions stabilized by egg yolk PL are well accepted as intravenous delivery systems for: (1) their ability to incorporate water insoluble drugs; (2) the possible improvement they bring to drug bioavailability; (3) their role in the stabilization of drugs. For instance, as to this last, the rate of hydrolysis of various parabens (preservatives) in intravenous emulsions was low when the partition coefficient of the solute was high (Pongcharoenkiat et al. 2002). Lipid emulsion of piperine for the treatment of visceral leishmaniasis was assessed in BALB/c mice. Piperine in emulsion was twice as effective in reducing the parasite burden in liver and spleen as was the free drug (Veerareddy et al. 2004). A fourth advantage of egg-yolk-stabilized emulsions for IV delivery lies in their ability to reduce side effects of various potent drugs. An emulsion formulation containing lorazepam, a benzodiazepine with antianxiety and sedative effects, decreased the hemolytic activity compared with the drug in organic solution, its usual form of administration (Yalin et al. 1997). Fifthly, such emulsions have the potential to control drug targeting and delivery to various organs (Seki et al. 2004). Finally, the development of perfluorocarbon emulsions is important to

note. These dispersions contain submicron fluoroorganic particles and are used for their capacity to dissolve large amounts of oxygen. By delivering oxygen at the tissue level, they ensure organ oxygenation during extreme hemodilution, thus, to cite just one consideration, reducing patient exposure to blood donors (Weers and Arlauskas 2004).

Emulsions are also used as non-parenteral delivery systems for oral, ocular, and topical drug administration (Tamilvanan 2004). Numerous studies have investigated the potential of submicron emulsion delivery systems to prolong the pharmacological effect of drugs with short biological half-lives or poor bioavailability following oral administration. Drug incorporation into oil-in-water emulsions significantly increases the drug absorption compared with that of equivalent aqueous solutions. Consequently, a greater biological effect of the emulsified drug can be observed. This is the case with ovokinin, a vasorelaxing peptide derived from ovalbumin, when dispersed in orally administered egg PL emulsion—although the biological effect was found to depend on the type of egg lipids used (Fujita et al. 1995).

When used in ocular delivery, oil-in-water emulsions increase local bioavailability of active ophthalmic compounds that generally have very low water solubility. Emulsions can improve drug absorption through the cornea and prolong the residence time of the drug in the eye, while minimizing potential local side effects. The effect of positively and negatively charged submicron emulsions containing egg yolk PL as a surface-active agent were compared for ocular tolerance in the rabbit eye (Benita 1999). Both emulsions were well tolerated without any toxic or inflammatory response to the ocular surface over 5 days (Benita 1999). Concerning topical application, many drugs exhibit low skin penetration, which results in poor efficiency. Lipid emulsions containing egg lecithin as emulsifier can be envisaged as improving drug delivery. For example, in vitro permeation and in vivo topical application of flurbiprofen, an anti-inflammatory drug used to treat rheumatoid arthritis and osteoarthritis, showed the potential of lipid emulsions although their efficiency depended on the oil type and the co-surfactant nature (Fang et al. 2004).

Concerning the use of egg-PL-stabilized emulsions in cosmetics, only few studies have been published. An example is found with α-tocopherol positively charged emulsions that prevented oxidative damage in rat skin subjected to UVA irradiation. This type of emulsion was a promising vehicle for the prevention of skin damage and aging following oxidative stress (Benita 1999).

3 Liposomes Based on Egg Phospholipids Used in Pharmaceutics and Cosmetics

Egg PLs are widely used in the preparation of liposomes in pharmaceutics and cosmetics applications. In pharmaceutics, an abundant literature deals with liposomes as model membrane systems. On one side, liposomes are used

to evaluate how drugs affect the integrity of phospholipid bilayers. For instance, the perturbations induced by a pyrimidine nucleoside on the structure and dynamics of egg yolk PC liposomes were characterized in order to validate the use of liposomes to amplify nucleoside delivery to tumor cells for boron neutron capture therapy. Both higher ordering and increased rigidity of the membrane lipids were the result of the constraints maintained by the embedded carboranyl-nucleoside (Rossi et al. 2005). Liposomes based on egg yolk PC also provide an effective model membrane system to study the antiperoxidative activity of carotenoids. As a consequence of their antioxidant properties, carotenoids are thought to exert a beneficial effect in preventing some types of cancer, and cardiovascular and degenerative diseases (e.g., cataracts or age-related macular degeneration). Liposomes were used to study the incorporation of carotenoids into the membranes (Balachandran and Rao 2003; Pintea et al. 2005) and the modulation of their antioxidant properties (Balachandran and Rao 2003; Bhosale and Bernstein 2005).

On the other side, liposomes are used to establish the possible effects of active compounds in biological membranes in relation to their pharmacological and therapeutic activities. Amphotericin B is an antibiotic used as a common antifungal agent for the treatment of deep-seated mycotic infections. Liposomes were applied to investigate how amphotericin B changed the permeability of membrane lipids, and especially how it disturbed ionic distribution leading to cell death (Hereć et al. 2005). The interaction of different uncharged local anesthetics with small vesicles of egg PC was studied (Fraceto et al. 2005). Depending on its hydrophobic and steric properties, each anesthetic was characterized by a positioning and orientation inside the bilayer. This preferential bilayer insertion could modulate, in the biological membranes, the access of the local anesthetic species to site(s) of action in the voltage-gated sodium channel (Fraceto et al. 2005).

Egg-PL-based liposomes can also constitute useful drug delivery systems of therapeutic agents. It is worth noting that for this purpose a preliminary study of the encapsulation efficiency of the liposomes should be required. This is important in that the encapsulation efficiency is affected by numerous parameters, such as the hydrophilic or lipophilic properties of the encapsulated drugs, the aqueous volume, the membrane rigidity, the surface area, and the preparation methods (Lasic 1993). A recent study explored the effect of the degree of unsaturation of different egg yolk lecithins used for liposome formulation on the encapsulation efficiency of various water-soluble, lipophilic and amphiphilic drugs (Nii and Ishii 2005b). Concerning the influence of the preparation method, a formulation called "liposomes in liposomes" based on egg PC has been developed for both internal and external liposomes. This system could provide new delivery characteristics, as shown with the encapsulation of leuprolide, an oligopeptide analogue of gonadotropin-releasing hormone used in the treatment of advanced prostate cancer, endometriosis, and precocious puberty (Saroglou et al. 2006).

The potential of liposomes as drug carriers in pharmacy lies in these properties and capabilities: (1) they allow the insolubility of hydrophobically

active compounds in aqueous media to be overcome; (2) they can improve drug stability or permeability; (3) they can modify the pharmacokinetic properties of encapsulated drugs due to their prolonged residence time; (4) they may allow the release of a drug at its target site or favor specific interaction (fusion) with its target; (5) they may reduce possible drug side effects. As a consequence, some drugs are able to show higher therapeutic effect when delivered by liposomes than can the free compounds. For example, anthraquinone-loaded egg yolk PC liposomes in vitro showed strong antiviral activity (inhibition of the infectivity of the hemorrhagic septicemia rhabdovirus) and antimicrobial activity (inhibition of *Escherichia coli* growth), whereas anthraquinones in their free form did not do so (Alves et al. 2004). Similar results were obtained with labdane-type diterpenes incorporated in egg-PC-based liposomes when compared with the free agents in activity against human cancer cell lines, although the results depended on the nature of the encapsulated terpene (Matsingou et al. 2005).

Like emulsions, drug loaded-liposomes can be envisaged in parenteral and non-parenteral delivery systems. Egg-PC-liposome entrapment enhanced the potency of parenterally administered calcitonin in rats. After intravenous administration, the hypocalcemic effect was dependent on the size and composition of the liposome preparations (Fukunaga et al. 1984). More recently, after intravenous injection in mice the liver uptake of encapsulated probucol, a lipophilic drug used to lower LDL and HDL cholesterol, was shown to depend on the liposome composition (Hattori et al. 2000). Liposomes containing egg PC and loaded with iron oxide particles have been designed for their application in magnetic resonance imaging (Martina et al. 2005). In biological media, the magnetic-fluid-loaded liposomes were highly stable while being nontoxic to the J774 macrophage cell line. Magnetophoresis demonstrated the possible guidance of magnetic-fluid-loaded liposomes by applying a magnetic field gradient. Liposome efficiency as contrast agents in vivo was proved through mouse magnetic resonance angiography (Martina et al. 2005). Studies are also being conducted in humans. For example, patients with resistant malignant tumors received 40 intravenous infusions of egg yolk lecithin-based liposomes entrapping a water-insoluble cytostatic agent. But while liposome therapy was very well tolerated, no objective regression of the tumors was observed—although the presence of the drug in the blood was prolonged for 120 hours after its administration (Sculier et al. 1986).

Liposomes are also envisaged for oral administration of pharmaceutical compounds. For instance, lactoferrin (which plays an important role in the immune system) was found to be more effective as an anti-inflammatory agent when encapsulated into egg yolk PC-based liposomes than when free. Moreover, lactoferrin-loaded liposomes exhibited more suppressive effects on CCl4-induced hepatic injury in rats as well as on lipopolysaccharide-induced tumor necrosis factor (TNF)-alpha production from mouse peripheral blood mononuclear leukocytes (Ishikado et al. 2005). In contrast, liposomes are of little value in transdermal drug delivery systems due to the

fact that they do not penetrate deeply into the skin (Cevc 2004). Only specially designed lipid vesicles were shown to penetrate into deeper layers of the skin, but they are based on soy rather than egg PL (Cevc 1996).

The poor ability of liposomes to cross the skin barrier makes them promising candidates for the cosmetic industry. Nowadays, there are several hundred commercially available products that include lipid vesicles. Liposomes are used as supplier systems of valuable raw material for skin regeneration. They supply lipids through the bilayers and water through both the internal aqueous volume of the vesicles and the hydration water driven by the lipid molecules. Thus, liposomes are often claimed to humidify the skin and, as a result, to improve skin elasticity and barrier function. However, only a few studies clearly demonstrate the effect of liposomes containing egg PL on skin water content and barrier function (Betz et al. 2005). Egg PL exhibited strong interactions with the skin. High hydration effects were measured during a 3.5 h liposome application on human skin. Long-term application (6 days) of a base cream containing liposomes showed that skin humidity was significantly increased using a formulation containing 20% egg PL (Betz et al. 2005).

4 Conclusion

Owing to their technological and biological properties, the demand for lecithins and purified PL is increasing in the fields of cosmetics and pharmaceutical industries. In this chapter, we have emphasized the uses of egg lecithin and egg PC as emulsifiers or liposome-forming agents. As emulsifiers, egg PLs are included in commercially available formulations for intravenous and other non-parenteral administrations designed for drug delivery (Tamilvanan 2004). It is worth noting that egg PLs are rarely used alone but rather in association with polymers (poloxamer, polysorbate, polyethyleneglycol) or charged lipids (phosphatidylglycerol, stearylamine) to modify their physico-chemical properties and, especially to increase their in vivo stability. The pharmaceutical industry also works on liposomes as drug delivery systems but, at present, only a few liposomal products exist on the market or are in advanced clinical studies (Lasic 1998; Wendel 2000). For instance, less than 10 liposome-based therapeutic products for dermal application have reached the market (Cevc 2004). In contrast, the cosmetics industry has adopted the liposome technology by developing many products, to a point that today most cosmetic companies propose at least one liposome product in their range. However, liposome properties regarding the skin are often claimed but more rarely scientifically proved. Many questions remain concerning the way liposomes increase the moisture of the top layers of the skin and the way they deliver active compounds to these layers. Moreover, in cosmetics formulations, liposomes are often mixed with emulsions, preservatives, and perfumes that may adversely affect their stability. Thus, final products should be carefully characterized in order to check that liposomes are still present after

formulation before claiming any liposome-based activity. Finally, it is worth noting that besides the classical use of PL as emulsifiers or liposome-forming agents, egg PL can also be formulated with hydrocolloids to prepare composite films for localized drug delivery (Grant et al. 2005); or egg PC-based liposomes can be incorporated into hydrogels to overcome the drawback of the liquid nature of the preparation, so to match a desirable application viscosity (Pavelić et al. 2005). These types of formulation broaden the spectrum of egg lipid applications in pharmaceutics and cosmetics even further.

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Chapter 30 Use of Egg Compounds for Cryoprotection of Spermatozoa

Lamia Briand-Amirat, Daniel Tainturier and Marc Anton

1 Introduction

For many years hen egg yolk has routinely been used in extenders to cryopreserve semen of domestic animals, including cattle, sheep, goats, dogs, horses, donkeys, rabbits and wild animals and fish as well (Trimèche et al.1998; Thun et al. 2002; Lahnsteiner et al. 2002; Silva et al. 2002; Garde et al. 2003; Marco-Jimenez et al. 2004; Eiman et al. 2004; Vidament 2005; Si et al. 2006). The goal of sperm conservation to produce a bank of sperm cells for artificial insemination (AI). However, various biochemical and anatomical compartments in the sperm cells (acrosome, nucleus, mitochondria, axoneme, plasma membrane) may be altered during freezing and thawing: what we call "cold shock." Consequently, the first aim of a sperm freezing protocol, including the use of extenders, is to prevent lethal intracellular ice crystal formation and to reduce damage to spermatozoa during and after cryopreservation.

Yolk is used in semen dilutions in association with other components, such as glycerol, glutamine, Tris buffer, citric acid, and fructose. It is generally used at the concentration of 20% (w/v) in classical extenders (for example, INRA 82 preservation medium). In the last few years there have been increasing demands to replace whole egg yolk in semen extenders because the presence of some substances in yolk could inhibit respiration of spermatozoa, diminish their motility, introduce a possible sanitary risk, and interfere with biochemical assays and metabolic investigations (Phillips 1939; Ahmad and Foote 1986; Soderquist et al. 1991; Andersson 1992; Bousseau et al. 1998; Wagtendonk-deLeeuw et al. 2000; Vishwanath and Shannon 2000). It would therefore be of great benefit to remove these detrimental substances from yolk, and after extraction add only the cryoprotectant agent to the extender, rather than complete egg yolk. This requires a better understanding of the cryoprotection mechanisms in order to determine and isolate the constituents in hen egg yolk that provide such protection.

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2 Low Density Fraction of Hen Egg Yolk

The precise mechanism by which egg yolk protects spermatozoa during the freeze-thaw process is not clearly understood. Kampschmidt et al (1953) suggested that egg yolk protects spermatozoa by two mechanisms: induction of a resistance to cold shock and improvement of spermatozoal survival during storage. Various yolk constituents have been investigated to identify the most cryoprotective constituent (Pace and Graham 1974; Watson 1976; Foulkes 1977; Quinn et al. 1980; Graham and Foote 1987; Demianowicz and Strezek 1996). Evidence indicates that the low-density lipoprotein fraction (LDL) gave, among yolk constituents, both the best resistance to cold shock and the best survival in storage.

In order to demonstrate that LDL offers good protection for spermatozoa, many experiments have been done. The use of egg yolk LDL in semen dilution was first reported for its beneficial effects as diluent for bull semen cryopreservation. Semen motility was evaluated after freezing in an LDL solution in comparison with bull semen frozen in egg yolk extender. Semen motility was twofold higher after freezing with LDL than after freezing with egg yolk (Moussa et al. 2002; Amirat et al. 2004). Consequently, LDL could easily replace egg yolk in extenders for bull semen freezing. Different concentrations of LDL were tested and 8% (w/v) was found to be the concentration of LDL that offers the best protection for spermatozoa (Moussa et al. 2002). At higher concentrations a decrease in semen motility was observed. This could be related to the osmotic pressure of the extender, which declined when the concentration of LDL increased.

Commercially, egg yolk extenders contain about 20 ml of egg yolk. If we consider that egg yolk possesses about 50% dry matter and that LDL represent about two thirds of yolk dry matter, egg yolk extenders contain 6–7% w/v of LDL. This result is surprising because this concentration of LDL is very near the optimal LDL concentration determined (8%). Pace and Graham (1974) have observed in bull semen extenders, using LDL, the water-soluble fraction, and granules of yolk, that granules had a detrimental effect on spermatozoal post-thaw motility. Watson and Martin (1975), in a study on ram spermatozoa, corroborated this assessment. Demianowicz and Strezek (1996) separated yolk into its two lipoproteins, LDL belonging to plasma and high density lipoprotein (HDL) belonging to granules, and they observed that HDL significantly decreased the motility of boar spermatozoa after freezing. It could be hypothesized that HDL in yolk has a detrimental effect on its cryoprotective properties. This reinforces the conviction that some constituents in egg yolk play a role antagonistic to the cryoprotective effect of LDL.

The effect of LDL on semen fertility has also been evaluated. The ability of spermatozoa to fertilize after cryopreservation is clearly a key factor in maintaining high pregnancy rates in cattle after insemination (De Leeuw et al. 1993). However, no correlation has been established between motility and fertility of mammalian semen. Measure of spermatozoal motility is therefore an insufficient

predictor of in vitro and in vivo fertility in many species (Comizzoli et al. 2001). Still, parameters such as in vitro and in vivo fertility could be associated with motility results. Amirat et al. (2004) have evaluated in vitro bull semen fertility after the freeze-thaw process with LDL compared to whole egg yolk extender. The experiments have shown that bull semen fertility was preserved after freezing in LDL extender and a high number of functional spermatozoa remained available for artificial insemination, which allows the number of spermatozoa in the straws to be reduced and the number of straws for sale to be increased.

3 Proposed Mechanisms of LDL Cryoprotection Capacities

The mechanisms of sperm protection induced by LDL are still unclear. Several hypotheses have been proposed. Watson (1976), Foulkes (1977), and Quinn et al. (1980) suggested that LDL could bind or adhere to spermatozoa and form a protective film at the surface of spermatozoa membranes. However, the respective roles of the protein and lipid components of LDL in interactions with this membrane are not clearly established. Graham and Foote (1987) thought that phospholipids are implicated by merging with the spermatozoa membranes and replacing some lipids, thus decreasing their phase transition temperature. Presently, questions exist about the respective role of lipids and apoproteins on the cryoprotective action of LDL.

This speculative association of LDL with membrane is a hypothesis widely espoused over the last few years. It is known that seminal plasma contains factors supporting sperm functions such as motility and fertility, but that it also contains factors detrimental to sperm storage (Shannon 1965; Dott 1974; Way et al. 2000). The major proteins of bull seminal plasma (BSP proteins: BSP-A1/A2, BSP-30-kDa) bind to the spermatozoal surface at ejaculation and stimulate cholesterol and phospholipid elimination from the spermatozoal membrane. Because cholesterol efflux is time and concentration dependent; continuous exposure to seminal plasma that contains BSP may be detrimental to spermatozoal membranes. Manjunath et al. (2002) and Bergeron et al (2004) proposed that LDLs prevent the detrimental effect of BSP protein by binding them. They also suggest that the lipid from LDL or whole LDL could associate with the spermatozoal membrane and preserve the integrity of the plasma membrane during sperm preservation. Many workers have corroborated this hypothesis (Watson 1976; Foulkes 1977; Watson 1981, MacDonald and Foulkes 1981; Cookson et al. 1984). Another hypothesis is that LDL prevents the loss of membrane phospholipids, which increases the resistance of spermatozoa to cold shock (Parks and Graham 1992).

Amirat et al. (2005) have compared the morphological modifications induced spermatozoa by egg yolk and LDL extenders after cooling to 4 °C and after freezing at –196 °C. Alterations were visualized by electron microscopy. The ultrastructure of frozen spermatozoa were thus observed in cryofixed,

cryosubstituted samples. Yolk extender was more deleterious for bull spermatozoa than LDL. Granular components of the egg yolk were seen to be rapidly adsorbed by the plasma membranes, first on the sperm heads, then on the flagellae. The acrosomes were modified or destroyed, which could result from intrusion of the calcium present in high concentration in egg yolk and which rapidly enters the cells when the temperature is below 30 °C (Courtens et al. 1989). Contrastingly, LDLs were less aggressive to cells, there were no alterations of the plasma membranes and very little acrosome disruption.

The effect of LDL on semen cryopreservation has been evaluated for bull semen (Moussa et al. 2002; Amirat et al. 2004). A patent concerning the extraction of LDL and the constitution of a new extender for bovine spermatozoa has been recently granted (Anton et al. 2001). The use of LDL for other species has by now been intensively evaluated, particularly for horses (Khlifaoui 2004) and dogs (Bencharif et al. 2006), in view of proposing new commercial extenders that are not presently available commercially for species that have presented some difficulties in sperm cryoprotection. Hen egg yolk LDL could easily replace egg yolk per se in semen extenders with regard to all the advantages described in this review. Moreover, it can be envisaged that the use of LDL can be extended to other cells, such as those of embryonal, tissues, to mention other possibilities.

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Chapter 31 Egg-Protein-Based Films and Coatings

Catherine Guérin-Dubiard* and Jean-Luc Audic

1 Introduction

Increased use of synthetic polymers in various applications like packaging films and coatings has led to serious ecological problems due to their non-biodegradability (Mathlouthi 1986). In consequence, natural biopolymers are attractive for innovative uses as "green" materials, considering their renewable and biodegradable nature (De Graaf and Kolster 1998). Thus, research aimed at the use of biodegradable polymers in technical applications has expanded considerably in the recent years (Cuq et al. 1998; Krochta and Mulder-Johnston 1997), not only to solve environmental problems associated with the use of synthetic polymers but also to find new markets for Western agriculture (over-) production.

Naturally occurring biopolymers available for forming films and coatings fall generally into the categories of animal or vegetal proteins, polysaccharides (sometimes from microbial sources), lipids, and fats. These biopolymeric materials used in films and coating formulations are shown in Fig. 1. Until now, usual synthetic petrochemical polymers like polyolefins, polyesters, polyamides, or polypropylene are those mainly used in packaging applications because they are available in great quantities, at low cost, and present some favorable functionality characteristics such as good mechanical and physical properties. Nevertheless, compared to these non-biodegradable polymers, biopolymers present the principal advantage of preserving the environment and of being obtained from renewable resources. Of late, films and coatings obtained from biopolymers also present specific properties relevant for technical applications. In this section, only films and coatings using proteins and in particular egg proteins will be presented.

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2 Protein-Based Films and Coatings

Among the film-forming agents presented in Fig. 1, proteins combine the most important and commercially exploitable properties. In fact, proteins present good film-forming ability and protein films generally exhibit meaningful mechanical properties, sometimes comparable to those of petroleum polymer films (Audic and Chaufer 2005). Protein-based films and coatings also present high barrier properties for polar gases such as O_2 or CO_2, surface active properties, and qualities of adhesion to various materials and substances (Chen 1995; Park 1999). Further, the properties of proteins can be adjusted for the specific requirements of various applications. For such purposes, because proteins are composed of approximately 20 amino acid monomers that have different side groups attached to the central carbon, they can be submitted to chemical, enzymatic, and/or mechanical modifications to allow film formation or to improve film properties (Kolster et al. 1998).

Table 1 presents the main technical applications of proteins in accord with their specific properties. Many uses are historical, such as in paint, ink, paper, and glue (De Graaf and Kolster 1998; Audic et al. 2003). Others are of more recent vintage, such as in edible films and coatings used in packaging. This is the case for protective albumen coatings on raisins (Bolin 1976), on egg shell (Wongs et al. 1996), on meat (Reutimann 1996), on potato slices to reduce oil uptake and water retention (Aminlari et al. 2005), and in orange coating (Sothornvit 2005). Protein-based films always exhibit low oxygen permeability compared to conventional synthetic polymers like polyethylene but a bit

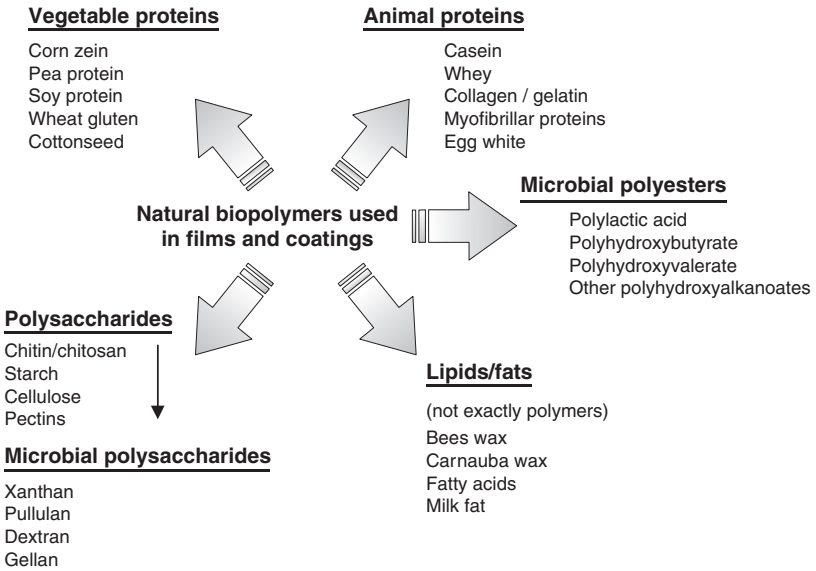

Fig. 1. Biopolymeric materials used in films and coating formulations

Table 1. Principal technical applications of proteins films and coatings. From Audic and De Graaf (Audic et al. 2003; De Graaf and Kolster 1998)

Product	Property	Applications	Remarks/importance
Coating	Film forming ability Adhesion Technical properties	Paint Ink	Still used in some paints [a] Still used
	Pigments/ink binding ability	Paper	Still used [a]
		Packaging	**In use / To be developed**
		Leather finishing	Historical
		Textile coating	Historical
Adhesive	Good processability Bond strength Water resistance obtained by crosslinking	Water-based glue	Historical [a] Still used in some few applications
Films	Strength	Rigid applications	Historical
	Good mechanical properties	Edible films Coating	Historical. In development [a] To be developed [a]
	Water resistance obtained by crosslinking	Film/foil in packaging application	Mainly at laboratory scale
	Gas barrier properties (O2, CO2)	Water soluble pouches	In use

[a] Egg protein applications

higher than ethylene vinyl alcohol (EVOH), which is used in the manufacture of gas barrier films. Table 2 compares the properties of some protein-based films, including those of egg white proteins, to those of conventional synthetic polymer films. However, compared to synthetic polymers, proteins present two major drawbacks:

1. –they are generally stiff and brittle
2. –they are sensible to water and moisture

Thus, in order to find technical applications for protein films, protein properties sometimes need to be adjusted by incorporation of additives into the film-forming formulation (e.g., plasticizers) and/or by chemical modification of the protein backbone (e.g., by crosslinking).

3 Formulation of Films and Coatings

Egg products, in particular albumen, are very significant sources of proteins for the formation of films and coatings. Actually, egg white is an aqueous solution of 10.5% protein. Ovalbumin, which constitutes more than half of

Table 2. Comparison of the oxygen permeability and elongation at break of protein and synthetic polymer films

Film	Eb[a](%)	OP[b] (cm^3, $\mu m/m^2$, d.kPa)	Reference
SC/Gly (2:1)[c]	30–70	<10	Chen 1995; Chick and Ustunol 1998
SC/TEA (2:1)	175		
SC/TEA (2:1)+HCHO[d]	250	–	Audic and Chaufer 2005; Audic et al. 2003
WG/Gly (2.5:1)	25	3.82	Chick and Ustunol 1998
WPI/Gly (2.3:1)	31	76.1	Chen 1995; Chick and Ustunol 1998; McHugh and Krochta 1994; McHugh et al. 1994
Egg A/Gly (1:0.3)	12	–	Gennadios et al. 1996a
Egg A/Gly (1:0.5)	32	–	Gennadios et al. 1996a
LDPE	200–500	1870	Miller and Krochta 1997
HDPE	200–500	427	Miller and Krochta 1997
EVOH (56% VOH)	200–350	0.066	Chick and Ustunol 1998

[a] Eb = elongation at break; [b] OP = oxygen permeability; [c] x:y = protein to plasticizer ratio; [d] HCHO used as crosslinker. SC = sodium caseinate; Gly = glycerol; Egg A = egg albumen; WG = Wheat gluten; TEA = triethanolamine; WPI = Whey protein isolate; LDPE = low density polyethylene; HDPE = high density polyethylene; EVOH = ethylene vinyl alcohol

egg white proteins, contains four free sulfhydryl (SH) groups. Other major proteins such as ovotransferrin, ovomucoid, and lysozyme contain many disulfide (S-S) bonds (15, 9, and 4, respectively). The mechanism of film formation is hypothesized to involve inter- and intramolecular S-S bonds and SH groups. Moreover, due to low frequency of secondary structure (α helix and β-sheets), ovalbumin is mostly random coil polypeptide conferring egg white good film-forming ability.

Most methods for preparing egg white films involve, as a first step, denaturation of egg white proteins by adjusting solution to pH 10.5 to 11.5, and heating at 40 °C for 30 minutes (Handa et al. 1999a). The increase surface SH concentration with thermal and alkaline denaturation allows formation of S-S bonding by oxidation and sulfhydryl-disulfide interchange reactions (Mine 1992; Gennadios et al. 1996a) and makes film more stretchable. Then, film formation can be obtained by solvent casting or by extrusion. At laboratory scale, protein films are generally obtained by the solvent casting method. The protein and additives are dissolved in a solvent (mainly water for water-soluble proteins) and the film or the coating is recovered after evaporation of the solvent. Drying rates can be improved by low-relative-humidity (RH) heated air. The conventional extrusion techniques used for transformation of

synthetic polymers should be of practical and economical value for protein-based materials. In any case, even if some protein behave like thermoplastics, little has been published until now on this approach.

4 Optimization of Egg Protein Films by Incorporation of Additives

4.1 Plasticizers

The plasticizer, generally a polyol type (Sanchez et al. 1998), reduces interactions (such as hydrogen bonding or electrostatic interactions) between protein chains causing an increase in the polymer-free volume and a decrease in the protein glass transition temperature (Tg; Di Gioia and Guilbert 1999). Plasticizers are added to protein-based biomaterials to overcome weakness and brittleness, and to enhance workability, elasticity, and flexibility. Most common plasticizers are di- and tri-ethanolamine, water, sugars, starches, and fatty acids, such as octanoic or palmitic acids owing to their miscibility with protein and their ability to increase elongation, elasticity, and flexibility (Gennadios 1996b; Handa et al. 1999b). On the other hand, by decreasing intermolecular forces (Irissin-Mangata et al. 2001), plasticizers cause a decrease in barrier properties due to increasing free volume. Plasticizers also induce a decrease in protein films' tensile strength, which may cause some problems for materials already exhibiting low tensile properties. As seen in Fig. 2, showing typical stress-strain curves obtained from tensile tests realized on plasticized caseinate films (Audic and Chaufer 2005), the incorporation of triethanolamine (TEA) or glycerol (Gly) as plasticizer in casein causes a

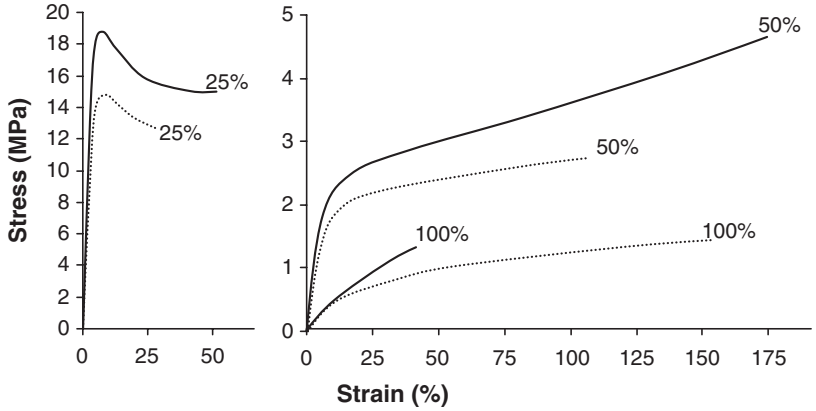

Fig. 2. Stress-strain curves of TEA (*solid line*) and Glycerol (*dotted line*) plasticized samples at 53% relative humidity. The plasticizer content is indicated beside each curve

decrease in tensile strength and generally an increase in ultimate elongation. Water is also a plasticizer for protein (Gontard and Ring 1996). Like polyol plasticizers, water molecules contain hydroxyl groups that interact with protein through hydrogen bonding. Equilibrium moisture content also affects the mechanical strength of protein films through water plasticization: water molecules soften the structure of films, affording materials a high molecular lubricacy. This film moisture content is related to the surrounding relative humidity (RH) and tensile tests must be recorded at a fixed RH.

4.2 Protein Chemical Modification: Crosslinking

As mentioned above, plasticized protein films present two important drawbacks: first, extensible protein films break at rather low stresses, lower than those disrupting synthetic polymers. Secondly, such plasticized protein films are water-soluble which greatly limit their application. In order to improve tensile properties as well as reduce water sensibility, protein-based films can be crosslinked. Crosslinking agents are bifunctional compounds able to create linkages between protein chains; network strength improves as a result of crosslinking between polypeptide chains. Most cited crosslinkers include formaldehyde (Audic and Chaufer 2005); dialdehydes (Rhim et al. 1998) or diacidchlorides (Lens et al. 1999), which are specific for amine groups; carbodiimides, which are specific for carboxylic groups; and bismaleimides, which are specific for sulfhydryl groups (Kolster et al. 1998). Gennadios et al. (1998) suggest occurrence of crosslinking between dialdehyde starch and egg white to increase film tensile strength and yellowness. Protein can also be γ-irradiated or UV irradiated to create crosslinks between proteins chains (Ressouany et al. 1998). According to Rhim et al. (1999), UV treatments increase tensile strength and reduce water vapor permeability of albumin films. In some cases, protein chains are covalently linked together by the action of enzymes. For example, transglutaminase is able to link polypeptides through the formation of ε-(γ-glutamyl) lysyl crosslinks (Ikura et al. 1980). Globular proteins, e.g., β-lactoglobulin, α-lactalbumin, and also egg white proteins, have compact structures that limit the accessibility of transglutaminase to the target glutamine and lysine residues (Matsumora et al. 1996). So, in the case of egg white, Lim et al. (1998) proposed to improve tensile and oxygen barrier properties, with a methodology based on partial denaturation of proteins by a preheating treatment at pH 10.5, followed by enzymatic polymerization of the proteins at 50 °C using a Ca^{2+}-independent microbial transglutaminase. Crosslinking of egg white proteins were also to be possible by using divalent ions such as calcium (Ghorpade et al. 1995) or copper (Kumar et al. 2002). Sulfur compounds such as CS_2 ethylene di- or tri-thiocarbonate and thiourea have also been used as ycrosslinkers (Kumar et al. 2002).

In order to control water sensibility of protein-based films, proteins can be hydrophobized by the attachment of long apolar (aliphatic) chains (Ghorpade 1995; De Graaf et al. 1998). The functionality of the protein is then modified by chemical reaction; the amino group of lysine residues especially

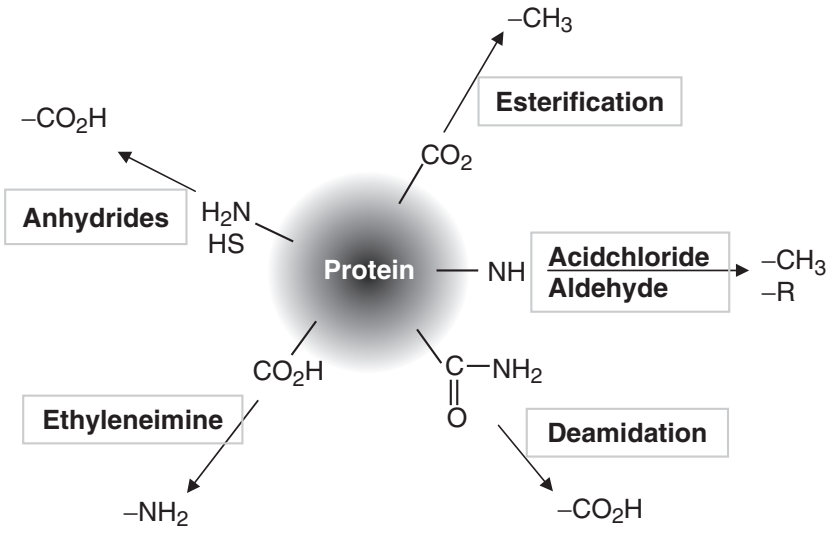

Fig. 3. Chemical modification of proteins

have a great potential for modification. For example, protein can be acylated with anhydrides of dicarboxylic acids (Ghorpade et al. 1995). Protein can also be silanated to improve its adhesion to hydrophobic substrates (Liang and Wang 1999). In fact, proteins contain a large number of reactive groups on which many chemical modification can be performed (Kolster et al. 1998), as indicated in Fig. 3.

4.3 Other Additives

Mixing proteins with oils, waxes, or acetylated monoglycerides is an easy route to drastically reduce water sensitivity of protein films and coatings (Kamper et al. 1984; Krochta et al. 1990; De Graaf et al. 1998). In film formulations for active or "intelligent" packaging, proteins can also be mixed with antioxidants, antimicrobials (Nakamura and Kato 2000; Mecitoglu et al. 2006), nutrients, flavors, time-temperature indicators, and colors to enhance food safety, nutrition, and quality (Vermeiren et al. 1999).

5 Conclusion

Egg white proteins have a real potential in commercial film and coating applications. Films are easily formed by alkaline and thermal treatment of egg white protein solutions. Their properties can be improved by addition of

plasticizers or by chemical modifications such as crosslinking reactions catalyzed with transglutaminase. Such films can be used as active packaging when natural antimicrobial molecules are incorporated.

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Chapter 32 Magnetic Particles for Egg Research

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1 Introduction

The progress in new technologies and new materials has influenced the "classical" food technology processes and related science. Egg-related research and technology is no exception, as can be seen from current scientific papers.

Different types of new materials (both in the nanometer and micrometer range) have been developed and used in various areas. Magnetic nano- and microparticles represent extremely interesting and useful materials for bioapplications due to their specific properties (e.g., possible manipulation in an external magnetic field, "visibility" in magnetic resonance imaging, heat production in an alternating magnetic field, biocompatibility, etc.). Up to now, not many magnetic-particle applications have been introduced into egg-related research and technology; however, this field of activity can substantially benefit from the use of magnetic nano- and microparticles in the future. In this chapter we have tried to summarize the current state of art.

Magnetic nano- and microparticles used in different areas of biosciences and biotechnology usually use magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), or various type of ferrites ($MeO.Fe_2O_3$, where Me = Ni, Co, Mg, Zn, or Mn) as magnetic moieties. Many different types of magnetic particles and devices for their manipulation are produced commercially; for introductory information see recent papers (Safarik and Safarikova 1999, 2004). The areas of egg-related research that follow have already benefited from the application of magnetic particles.

2 Immobilization of Egg-Related Biologically Active Compounds

Immobilization of enzymes, antibodies, and other biologically active compounds is a very important technique used in various areas of life sciences and biotechnology. Biologically active compounds immobilized on magnetic

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

carriers can be removed from the system by using an external magnetic field or can be directed to the desired place. The immobilized compounds can be used to express their activities in a desired process (e.g., immobilized enzymes), or can be used as affinity ligands enabling one to capture or modify the target molecules or cells (Safarik and Safarikova 2002).

Several egg proteins (e.g., avidin, lysozyme, ovalbumin, IgY) have been immobilized on magnetic carriers and used for selected applications (for typical examples see Table 1). Among them, magnetic avidin derivative is especially important because of its ability to tightly and specifically bind biotin and biotinylated compounds. Avidin is a tetrameric protein (molecular weight 68 kDa), composed of subunits of identical amino acid composition and sequence. Each subunit of avidin binds to a molecule of biotin. Due to its high isoelectric point (10.5) and glycosylation avidin exhibits some nonspecific interactions (Andreoni and Delaage 1992), and that's why its microbial analogue streptavidin is used predominantly. Commercially produced magnetic particles with immobilized avidin are available (see Table 2).

Lysozyme and ovalbumin immobilized on magnetic particles have mainly been used to separate compounds or complexes with specificity for the immobilized protein, such as anti-lysozyme aptamers or ribosome complexes, or to study antigen presentation by macrophages.

Chicken egg yolk antibodies (IgYs) represent a promising alternative to animal antibodies because eggs are obtained noninvasively , and the phylogenetic distance between chickens and mammals results in differences in antibody specificities. Currently the IgY antibodies are used successfully in immunochemistry for the detection of antigens of viral, bacterial, plant, and animal origin, of intestinal parasites in domestic animals, and of contamination of foods with toxins or drugs (Schade et al. 2005). Surprisingly, there are only a few reports describing immobilization of IgYs on magnetic particles and the subsequent application of these complexes, mainly focused on their use in immunomagnetic separation of microbial pathogens.

Not only individual proteins, but also the whole egg white complex can be transferred into magnetic hen egg white beads by a simple procedure. During the bead formation other components can also be immobilized (Kubal et al. 1986).

3 Isolation of Egg-Related Biologically Active Compounds

Considerable attention is being paid to the isolation, separation, and purification of various types of biologically active compounds, such as proteins, peptides, nucleic acids, nucleotides, and polysaccharides. New separation techniques, capable of treating difficult-to-handle materials, even in the presence of particulate matter, are necessary. Magnetic separation processes and magnetic adsorbents can fulfil this requirement.

Table 1. Examples of egg proteins immobilized on magnetic nano- and microparticles

Immobilized protein	Magnetic carrier	Application	Reference
Avidin	Magnetite nanoparticles activated with carbodiimide	Binding of biotinylated DNA fragments	(Wang and Lee 2003)
Avidin	Iron-dextran microspheres	Removal of target compounds or cells	(Lawless and Williams 1993)
Avidin	Carboxylated superparamagnetic microspheres	Aequorin-linked assay for biotin	(Feltus et al. 1997)
Avidin	Magnetic alginate beads activated with chloroacetic anhydride	Binding of biotinylated goat anti-mouse (B-GAM) antibody	(Pope et al. 1994)
Avidin	Magnetic nanoparticles containing quantum dots	Binding of biotinylated compounds	(Wang et al. 2005)
Avidin	Polyethylene magnetic nanoparticles	Detailed particles' characterization	(Chatterjee et al. 2002)
IgY (*S. enteritidis* specific)	Dynabeads M-280 tosylactivated	Immunomagnetic separation of *Salmonella enteritidis* from eggs	(Mine 1997)
IgY (*Campylobacter jejuni* specific)	Magnetic poly (glycidyl methacrylate) microspheres	*Campylobacter jejuni* detection in food	(Horak and Hochel 2005)
Lysozyme (mononitrated at Tyr-23)	Tosylactivated Dynabeads	Development of new immobilization procedure	(Walton et al. 1998)
Lysozyme	Tosylactivated Dynabeads	Recovery of ribosome complexes with specificity to lysozyme	(Coia et al. 2001)
Lysozyme (biotinylated)	Dynabeads-streptavidin	Selection of anti-lysozyme aptamers	(Cox and Ellington 2001)
Ovalbumin	Magnetic beads	Study of antigen presentation by macrophages	(Lord et al. 1998)

Table 2. Examples of commercially available magnetic particles with immobilized avidin

Name	Diameter (μm)	Particle composition	Manufacturer/supplier
MPG Avidin	5	Magnetic porous glass	PureBiotech LLC, Middlesex, NJ, USA
Avidin coated magnetic beads	0.91	Estapor polysterene magnetic beads	Biocode Hycel, Liege, Belgium
Sphero–avidin coated magnetic particles	Many types	Magnetic polystyrene particles	Spherotech, Inc., Libertyville, IL, USA
Avidin magnetic nanocrystals	0.025	Magnetic nanoparticles coated with biocompatible polymer	Nanocs Inc., New York
Avidin coated magnetic particles	Many types	Magnetic polystyrene particles	G. Kisker GbR, Steinfurt, Germany

Batch separation techniques are preferentially used for magnetic separation of proteins and peptides; alternatively magnetically stabilized fluidized beds can be used. Magnetic adsorbents of different types (affinity, ion exchange, hydrophobic, metal chelate, dye-binding, etc.) have been successfully used (Safarik and Safarikova 2004).

Lysozyme has been very often used as a model enzyme in the course of magnetic separation process development. In most cases partially purified lysozyme has been a starting material; only exceptionally diluted egg white was used. Table 3 shows typical examples of magnetic separation of egg proteins.

Also nonmagnetic chromatographic adsorbents can be used in magnetically stabilized fluidized beds, if a sufficient amount of magnetically susceptible particles is also present, as shown on the example of separation of lysozyme from a model mixture using nonmagnetic affinity resin (Chetty and Burns 1991).

4 Magnetic Bioassays of Egg Related Biologically Active Compounds

Egg-related proteins are sometimes used as model analytes and food allergens in the course of development of new analytical procedures. Recently, using antibody-conjugated bacterial magnetic particles, a high-performance and fully automated system has been developed for rapid chemiluminescence immunoassay for lysozyme. This system contains a reaction station, tip rack, and an eight-tip pipettor able to attach a strong magnet to the tip surface. The

Table 3. Examples of egg white proteins isolated by magnetic separation techniques. Both diluted egg white and commercially available preparations were used as starting materials

Purified protein	Magnetic carrier	Affinity ligand	Further details	Reference
Avidin	Maghemite nanoparticles	Biotin	Particles diameter 13 nm	(Fan et al. 2003)
Lysozyme	Magnetic chitin, magnetic acetylated chitosan	–	Elution with 0.01 M HCl	(Safarik 1991; Safarik and Safarikova 1993)
Lysozyme	Magnetic poly (2-hydroxyethyl methacrylate)	Cibacron Blue F3GA[a]	Elution with 1 M KSCN	(Odabasi and Denizli 2004)
Lysozyme	Magnetic chitosan beads	–	Magnetically stabilized fluidized bed	(Goto et al. 1995)
Lysozyme	Magnetic cross-linked polyvinylalcohol	Cibacron Blue 3GA[a]	Elution with high salt buffer	(Tong et al. 2001)
Lysozyme	Magnetite – polyacrylic acid nanoparticles	–	Ion-exchange separation	(Liao and Chen 2002)
Lysozyme	Magnetic cross-linked polyvinylalcohol beads	–	Adsorption study	(Xue and Sun 2001)
Lysozyme	Magnetic agarose beads	Cibacron Blue 3GA[a]	Magnetically stabilized fluidized bed	(Tong and Sun 2003)
Lysozyme	Magnetic chitosan	Cibacron Blue 3GA[a]	Study of adsorption properties	(Yu et al. 2000)
Lysozyme	Ferrofluid modified sawdust	–	Elution with 0.5 M NaCl	(Safarik et al. 2005)
Lysozyme	Nano-sized magnetic particles	–	Elution with NaH2PO4 and NaSCN	(Peng et al. 2004)

[a] Polysciences Inc., Warrington, PA, USA

immunoreaction time is 5 min, and the assay is completed within 20 min. The detection limit for lysozyme is 10 ng/mL (Matsunaga et al. 2001).

In other experiments change of the Brownian relaxation (which shows up as a peak when magnetic susceptibility is plotted as a function of frequency) of magnetic nanoparticles with immobilized protein (avidin used as a model compound) was studied. After specific binding of appropriate reagent (biotin

in this case), the diameter of the magnetic nanoparticles increased, which resulted in a shift of the peak to lower frequencies. The method could thus be used to detect different target molecules because each leads to a characteristic decrease in the frequency of the peak (Chung et al. 2004).

Highly specific and sensitive label-free detection of the target protein was achieved by combining aptamer-coated magnetic beads and chronopotentiometric stripping measurements of the captured protein. Model protein lysozyme has been detected selectively and with high sensitivity in a mixture containing a large excess of proteins and amino acids (Kawde et al. 2005).

5 Immunomagnetic Separation of Egg-Related Microbial Pathogens

As with any food of animal origin, eggs and egg products may be contaminated with pathogenic microorganisms. Standard microbiology procedures for their detection usually require four stages and at least four different growth media; hence the total time from sampling the food to obtaining a result can be measured in days. One of the possibilities for shortening the isolation and detection period is to replace the selective enrichment stage with a non-growth-related procedure. This can be achieved by specific magnetic separation of the target organism directly from the sample or the pre-enrichment medium. Isolated cells can than be identified by standard microbiological procedures.

Immunomagnetic separation (IMS) has already found many applications, especially in food, clinical, veterinary, and environmental microbiology (Safarik and Safarikova 1999). Table 4 shows examples of successful applications of IMS in egg technology. IMS is not only faster but also usually gives higher number of positive samples. Also, sublethally injured microbial cells can be isolated using IMS. Commercially available immunomagnetic particles for *Salmonella* detection are produced, e.g., by Dynal (Oslo, Norway; Dynabeads anti-*Salmonella*) and Vicam (Watertown, MA, USA; *Salmonella* Immunobeads).

Magnetically separated cells can be inoculated on selective agars or liquid nutrient media and tested in the standard way. Magnetically captured cells can also be detected using an impedance technique, enzyme-linked immunosorbent assay (ELISA), etc. IMS can be effectively combined with the polymerase chain reaction (PCR). The process combining these two procedures is sometimes abbreviated as MIPA (magnetic immuno PCR assay). The main purpose of IMS is to remove the PCR-inhibitory compounds from a sample without loss of sensitivity through dilution. The oligonucleotide primers should be specific either for the target genus (e.g., detection of different strains of *Salmonella*) or for the individual strain of interest. Various modifications of PCR for MIPA technique, e.g., nested PCR with two nested pairs of primers in a two-step PCR, are described (Safarik and Safarikova 1999).

Table 4. Examples of immunomagnetic separations of pathogenic bacteria from egg products

Microorganism	Analysed sample	Magnetic particles	References
Salmonella	Powdered egg products	Dynabeads anti-Salmonella	(Cudjoe et al. 1995)
Salmonella enteritidis	Raw blanded eggs	Dynabeads anti-Salmonella	(Cudjoe et al. 1994)
Salmonella	Egg samples	Dynabeads anti-Salmonella	(del Cerro et al. 2002)
Salmonella enteritidis	Pooled liquid egg samples	Dynabeads anti-Salmonella	(Holt et al. 1995)
Salmonella	Dried and shell eggs	Dynabeads anti-Salmonella	(Karpiskova and Holasova 1999)
Salmonella	Egg products	Dynabeads anti-Salmonella	(Mercanoglu and Aytac 2002)
Salmonella enteritidis	Contaminated liquid eggs	Tosylactivated Dynabeads with bound *S. enteritidis* specific IgY	(Mine 1997)
Salmonella	Raw egg products	Dynabeads anti-Salmonella	(Molla et al. 1994)
Salmonella	Pasteurised egg yolk, egg yolk powder, whole eggs, egg white	Dynabeads anti-Salmonella	(Rijpens et al. 1999)
Salmonella	Egg products	Dynabeads anti-Salmonella	(Shaw et al. 1998)
Salmonella	Dried eggs	Dynabeads anti-Salmonella	(Spanova et al. 2001)
Salmonella enteritidis, S. Typhimurium, S. Newport	Liquid eggs, shell eggs, dry eggs	Vicam anti-Salmonella beads	(Tan and Shelef 1999)

6 Magnetoliposomes

Magnetoliposomes are magnetic derivatives of liposomes and can be prepared by entrapment of magnetic fluids within the core of liposomes. Alternatively, magnetoliposomes are prepared using the phospholipids vesicles as nanoreactors for the in situ precipitation of magnetic nanoparticles. Affinity magnetoliposomes can be produced by covalent attachment of ligands to the surface of vesicles or by incorporation of target lipids in the matrix of structural phospholipids. Magnetoliposomes have been used for the immobilization of membrane-bound enzymes or antibodies, as well as for entrapment of various drugs (Safarik and Safarikova 2002). Also activated particles, containing decanal

incorporated among the lipid molecules in the bilayer with their aldehyde groups exposed to the aqueous phase, allowing straight attachment of protein molecules, have been prepared (Elmi and Sarbolouki 2001).

In many cases the origin of phospholipids used for the magnetoliposomes' preparation is not precisely specified. Nevertheless, egg yolk phosphatidylcholine or phosphatidylethanolamine can be used efficiently, as documented in several papers (Domingo et al. 2001; Elmi and Sarbolouki 2001; Lesieur et al. 2003; Matsuo et al. 2003). Alternatively, egg-yolk sphingomyelin (primarily N-hexadecanoyl-o-sphingosine-1-phosphocholine) was used for the same purpose (Bogdanov et al. 1994). These experiments have shown that liposomes made of egg phosphatidylcholine have a strong resistance against aggregation, in comparison with liposomes composed of other phospholipids (Matsumura and Furusawa 1995).

7 Other Applications of Magnetic Particles

Protein crystallization is important in several applications: as a key step in determining tertiary structure by diffraction techniques, as a way of generating stable protein for storage, and as a method of protein purification in downstream processing. The effects of exogenous mineral substrates (including magnetite) on the induction time for nucleation, and on the number, morphology, and purity of protein crystals were investigated in a series of experiments using chicken egg-white lysozyme as a model protein. Presence of magnetite causes earlier lysozyme nucleation in comparison with controls (Kimble et al. 1995; Kimble et al. 1998).

Ovalbumin was used as a model antigen to study the process of antibody production in mammals using different types of inorganic adjuvants, including magnetite. Experiments showed that magnetite exhibited adjuvancy similar to routinely used inorganic adjuvants and simultaneously elicited a slightly smaller granulomatous reaction than Al_2O_3. This diminished tissue reaction coupled with a fairly robust humoral adjuvancy response makes magnetite a clinical adjuvant candidate that warrants further study (Naim et al. 1997).

8 Future Trends

As can be seen, not many applications of magnetic particles in egg research and technology are described in the literature. Currently the greatest potential has been observed in the area of immunomagnetic separation of microbial pathogens (especially *Salmonella*) in raw eggs and egg products. Also yolk phospholipids have been used for the construction of different types of magnetoliposomes. The situation can, however, change dramatically in the future.

The most promising interactions between magnetic end egg related research and technologies can be expected to appear in the following several areas. First of all, egg-yolk-derived immunoglobulins (IgY) can become extremely important part of new immunomagnetic particles and in their related procedures. Currently (in December 2006) there are 2,565 papers in PubMed and 2,230 papers in Web of Science to be found upon searching for the term "immunomagnetic." These data clearly show that many important applications of magnetic particles with bound IgY can be expected in human and veterinary medicine, cell biology, microbiology, virology, parasitology, analytical, bioanalytical and environmental chemistry, biotechnology, etc. Secondly, analytical techniques employing magnetic beads could be used more often for the detection and quantification of important egg-related compounds, especially if present in low concentrations (e.g., the existence of ovalbumin and lysozyme as food allergens in special types of food). Thirdly, specific magnetic particles could be used for the isolation of minor protein components from egg white and specific IgYs from egg yolk.

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Chapter 33 Avidin–Biotin Technology

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1 The Avidin–Biotin System

As early as 1941, when the extraordinary affinity of avidin for biotin was recognized (see Chap. 11, "Avidin"), the idea of using avidin–biotin technology as a research tool emerged. Indeed, because of the absence of interaction between avidin and the carboxy-containing side chain of biotin, biotin can be linked to a wide spectrum of biologically active molecules, without modifying biotin-avidin interaction. On the other hand, avidin can be also attached to a solid phase or to many molecules, such as probes. This is the basis of avidin–biotin technology: a target molecule in an experimental system is labeled with a biotinylated binder molecule, which is in turn labeled, either with an avidin-conjugated probe, or with free avidin followed by a biotinylated probe.

2 Biochemical Applications

In the early 1970s the first applications of this technology were published. Biotin, covalently attached to bacteriophages, was shown to be available for subsequent interaction with avidin, resulting in phage inactivation (Becker and Wilchek 1972). Heitzmann and Richards (1974) used the avidin–biotin complex for a new, rapid, stable, and specific staining of biological membranes intended for electron microscopy observation. This technique was applied to visualize ghosts of *Acholeplasma laidlawii* thanks to labeled protein amino groups, and erythrocytes thanks to aldehyde groups resulting from the oxidation of membrane oligosaccharides. In 1976, Bayer et al. localized lectin and antibody receptors on erythrocytes via the avidin–biotin complex and introduced the term "affinity cytochemistry". But it was only in the mid 1980s that avidin–biotin technology really entered the laboratory for routine analyses, especially for clinical diagnostics. For that purpose, different test formats

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Bioactive Egg Compounds (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade)

have been developed for serum/plasma and tissue/blood cell analysis. For body fluid analyses, avidin–biotin technology has been mainly used as a matrix, which is widely spread and well accepted in clinical laboratories. Test sensitivity can be up to ten times higher than with classical tests in which antibodies and antigens are directly bound to solid phases, and much shorter incubation times are needed (a few minutes versus a few hours). Applications for tissue and cell analysis are the concern of pathology and hematology laboratories, where avidin–biotin technology is mainly used for signal amplification (Schetters 1999).

More recently, avidin–biotin interaction has become increasingly popular for a variety of specific applications and techniques in the life-science and the nanoscience fields. Included are biochemical applications such as immunoassay, blotting, signal amplification, affinity cytochemistry, imaging, and bioseparation (Bayer and Wilchek 1994). Among the most recent developments, one can point out the sensitive and specific method for quantifying DNA and proteins in biological samples such as foods and tissue extracts, including genetically modified crops and other plants in neighboring fields (Masarik et al. 2003). A combination of the DNA non-crosslinking aggregation and biotin–avidin technology was also efficient enough for the detection of single nucleotide polymorphism (Sato et al. 2004). Hytonen et al. (2005) constructed novel avidins: dual-chain avidins were obtained by joining two avidin monomers into a single polypeptide chain (pseudodimer), in which one of the two binding sites was genetically modified to have lower binding affinity for biotin. The resulting pseudotetramer (dimer of dual-chain avidins) then had two high and two lower affinity binding sites. This molecular scaffold was demonstrated to be useful for novel bioseparation tools by immobilizing dual-affinity avidin with its high affinity sites, thus leaving the sites with lower affinity available for lower interaction with biotinylated molecules.

3 Biomedical Applications

Many of the recent and most promising developments for avidin–biotin applications concern biomedicine. While immunolocalization, imaging, nucleic acid blotting, and protein labeling are by now familiar applications (Wilchek and Bayer 1989), avidin-biotin technology has recently been adapted for use in gene therapy vector applications to add proteins or cell-targeting ligands to non-viral and viral vectors (Barry et al. 2003). Similarly, Han et al. (2005) developed a new but slightly invasive cell manipulation and gene or molecule transfer system in a single living cell by using a nanoneedle on which DNA is immobilized by covalent bonding and avidin–biotin-affinity binding. Cell surface functionality using avidin–biotin technology, in combination with biotinylated neurotransmitter analogs has been proposed for the controlled activation of neuronal post-synaptic receptors (Vu et al. 2005). Avidin–biotin technology also participated in the construction of a neurotrophin

peptide–antibody conjugate used as a delivery vector of a neurotrophin designed to overcome the poor blood–brain barrier permeability and diffuse into the brain tissue (Wu 2005). In a like manner, Aktas et al. (2005) have designed functionalized chitosan nanospheres for the transport of a specific caspase inhibitor into the brain. This peptide significantly reduces vulnerability to neuronal cell death.

Many recent developments also deal with cancer treatment. Because of its glycosylation and thus its affinity for some lectins, the ability of avidin to specifically target intraperitoneal tumors has been tested (Yao et al. 1998). The promising results let the authors to suggest avidin as a vehicle for the delivery of radioisotopes, drugs, toxins, or therapeutic genes to tumors. Tumor pre-targeting with avidin was also proposed to increase the uptake of tumor necrosis factor (TNF) conjugated to biotin, and to improve the activity of systemically administered biotin-TNF conjugates in their use as local or locoregional anticancer drugs. Such a pretargeting process was found to increase the binding and persistence of biotin-TNF on the surface of tumor cells in vivo (Gasparri et al. 1999). The binding of biotinylated anti-CD3 antibody was achieved by avidin–biotin complex formation on the surface of biodegradable gelatin nanoparticles, which were efficiently internalized into CD3-expressing cells, i.e. human T-cell leukemia cells and primary T-lymphocytes. A receptor-mediated endocytotic process was demonstrated, indicating that such functionalized nanoparticles could be a promising selective drug delivery system for specific cell types (Dinauer et al. 2005).

Likewise, Nobs et al. (2006) proposed a selective cancer cell targeting of biodegradable poly(lactic acid) nanoparticles, the surfaces of which were attached to monoclonal antibodies, either directly or with the use of the avidin–biotin technology. However, pharmacokinetics and immunological properties of chicken egg white avidin, as well as of streptavidin, do not always seem optimal for pretargeting cancer treatments. It was to get around this problem that Hytonen et al. (2003) screened egg white avidins from duck, goose, ostrich, and turkey. Whereas all these avidins exhibit biotin-binding properties similar to those of chicken avidin, duck, goose, and ostrich avidins showed lower immunological cross-reactivities, suggesting that the use of these proteins could offer advantages over chicken avidin and streptavidin in pretargeting applications.

4 How to Get Around the Nonspecific Binding of Avidin

For some applications, avidin creates the problem of biotin-independent binding of various extraneous macromolecules in some target systems. For instance, because of its glycosylated moiety, avidin interacts with lectin-like molecules derived from cellular systems, and because of its very high pI (10.5) the strong positively charged avidin interacts with negatively charged molecules such as acidic proteins or nucleic acids. This is the reason why

many efforts have been necessary seeking to eliminate such nonspecific bindings. Kaplan et al. (1983) proposed to use acetylated avidin to decrease the nonspecific binding of avidin to negative cell surfaces. Bussolati and Gugliotta (1983) prevented spurious staining by using the avidin–biotin interaction at pH 9.4, without affecting the previous binding of primary antibodies nor the affinity of avidin to biotin. A bit later Clark et al. (1986) suggested addition of extra salt to buffers to decrease the affinity of avidin to proteins electroblotted to nitrocellulose. Selective detection of sialoglycoproteins by avidin–biotin technology became possible by first using a nonglycosylated basic protein (lysozyme) for quenching acidic groups on erythrocyte membranes (Bayer et al. 1987). Another method explored circumvention of interference due to nonspecific binding activity by substituting streptavidin for avidin. Indeed, this protein isolated from culture broth of *Streptomyces avidinii* (Chaiet and Wolf 1964) offers quite the same affinity for biotin as does avidin, but does not possess any carbohydrate moiety and has a pI of approximately 5–6. Thus, in the early 1980s, despite the higher cost of streptavidin, its use became popular. But this protein exhibits other drawbacks: the biotin binding site in streptavidin is deeper than in avidin, involving some steric hindrances (Gitlin et al. 1989), and some strong biotin-independent interactions can be observed between streptavidin and cell surfaces. These interactions have been attributed to the Arg-Tyr-Asp sequence contained in streptavidin, which mimics the universal recognition domain (Arg-Gly-Asp) present in fibronectin and other adhesion-related molecules (Alon et al. 1990). These streptavidin disadvantages finally led to new developments for egg white avidin in avidin–biotin technology. Hence, blocking of free amino groups was proposed to reduce the charge of avidin. This counteraction preferentially concerned arginine residues, which are generally not involved in the crosslinking to the functional reagents or probes. Such derivatives of avidin are commercially available (Bayer and Wilchek 1994). A simple procedure for the preparation of deglycosylated avidin was also described, based on the enzymatic activity of a mixed microbial culture, containing especially a strain of *Flavobacterium meningosepticum* (Bayer et al. 1995). The combination of deglycosylation and chemical arginine modification led to the creation of NeutraLite Avidin, a product of Belovo Chemicals (Bastogne, Belgium), which is a modified avidin without sugar moiety, with a neutral pI, while still bearing free lysine groups for further derivatization (Bayer and Wilchek 1994).

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