Extraction and Purification of IgY

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Abstract

Immunoglobulin Y (IgY) is an antibody present in the yolk of the egg and to obtain it at high purity levels, while retaining its biological activity, multi-step processes are required. The initial step is removal of the lipid fraction from the yolk. The delipidation methods routinely used are water dilution or precipitation by either polyethylene glycol (PEG), anionic polysaccharides or organic solvents. The second step is the extraction of the IgY from the remaining proteins in the mixture, which can be achieved by precipitation with a variety of salts, or additional PEG steps or by aqueous biphasic systems. The final step in IgY purification involves chromatographic methods based on different separation techniques such as cation exchange, hydrophobic charge-induction and various affinity systems giving IgY with >90% purity. In this chapter, comparisons

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among different methods of IgY extraction applied to a single batch of eggs and the commercial extraction kits currently available are discussed. Finally, the techniques for the characterization of the IgY with regard to purity and activity are outlined as well as the appropriate storage conditions.

Keywords

Extraction · Purification · Chromatography · Characterization · Storage

11.1   Introduction

In this chapter, various methods for IgY extraction and purification from hen egg yolk are discussed. The knowledge of the composition of the original biological matrix and properties of the solutes are fundamental to rationalise the best extraction and purification conditions aiming at attaining antibodies of high purity and biological activity (Walsh 2013). The formation of a chicken egg is a complex process, starting from the growth of a single follicle and culminating in the passage of the egg through the oviduct and finally egg laying (Chap. 2). The egg must contain all the nutrients (proteins, lipids, minerals and vitamins) necessary for the development of the chick and antibodies, namely IgY, for its protection until it develops its own immune response. These nutrients are derived from the hen plasma and transported to the ovary and then into the egg (Schjeide et al. 1963). The yolk is surrounded by a two-layer vitelline membrane (Sunwoo and Gujral 2015). The composition of the yolk is approximately 48% water, 33% lipids and 17% proteins (Burley 1991). The yolk, after aqueous dilution, can be separated into two main fractions by low speed centrifugation (Fig. 11.1): (i) the major fraction is an aqueous
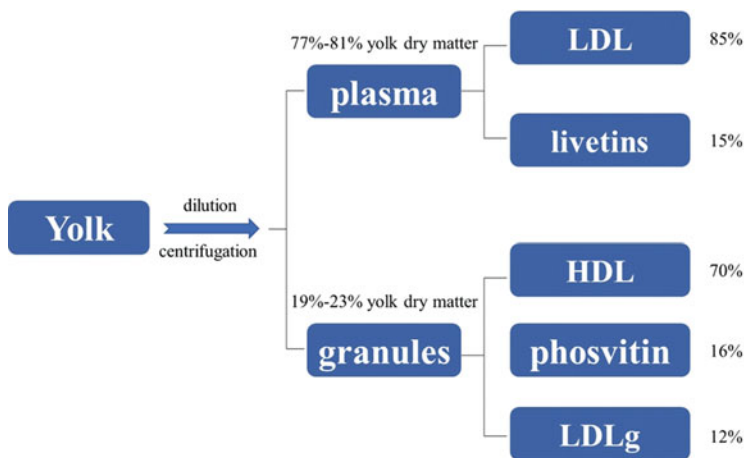

Fig. 11.1 Constituents of plasma and granules from hen egg yolk (LDL, low-density lipoprotein; HDL, high-density lipoprotein). Adapted from Anton (2013)

plasma consisting of 85% low density lipoproteins and the remainder α-livetin (serum albumin), β-livetin (α2-glycoprotein), and γ-livetin (IgY); (ii) the second fraction contains granules of varying sizes composed of 70% high-density lipoproteins (α- and β-lipovitellins), 16% phosvitin (glycophosphoprotein), and 12% low-density lipoproteins (LDL) (Anton 2013). All of the livetins are water-soluble and correspond to serum proteins (Williams 1962).

Low density lipoproteins are protein-lipid complexes which are spherical nanoparticles (17–60 nm) with a lipid core composed of 71% triacylglycerol, and 4% cholesterol esters surrounded by a monofilm of phospholipids and proteins; due to their low density (0.982) LDLs are soluble in aqueous solutions (Anton et al. 2003).

11.1.1 Properties of IgY

Numerous methods of extraction and fractionation of proteins are based on their physicochemical and structural characteristics such as solubility, hydrophobicity, molecular weight, and isoelectric point (pI). The structure and biology of IgY is reviewed in Chap. 5. However, the properties of particular interest for the extraction of IgY are summarized in Table 11.1. Generally, protein solubility is affected by the pH of the aqueous medium and is a result of the electrostatic and hydrophobic interactions between the protein molecules. Solubility increases if the electrostatic repulsion is greater than the hydrophobic interactions. At the isoelectric point proteins have a net zero charge and molecules tend to associate resulting in insolubility and precipitation out of solution (Scopes 1993). Above or below the pI, the net charge is negative or positive, respectively and solubility is enhanced. Values for the pI of IgY have been reported to be in the range of 6.7 ± 0.9 (Dávalos-Pantoja et al. 2000) and 5.5–7.0 (Chalamaiah et al. 2017) and 5.8 (Polson et al. 1980). It would be worthwhile to determine the exact value experimentally as this would provide a better theoretical basis for the extraction.

Table 11.1 Properties of IgY important for extraction

Property	Value
Molecular weight [a]	180 kD
Isoelectric point [b]	5.5–7.6
Molar extinction coefficient [c]	1.094, 1.33, 1.35
pH stability [d]	4–11
Solubility [e]	> 86% at pH 2–5 and 8–12
Thermostability [f]	4 °C > 6 months
	65 °C > 2 months
	100 °C > 6 months
Pepsin stability [g]	pH 4.0, 91% for 1 h; 63% after 10 h

Notes: Data according to [a] Amro et al. 2018; [b] Dávalos-Pantoja et al. 2000; [c] Stålberg and Larsson 2001, Pauly et al. 2011, Hodek et al. 2013 respectively; [d] Schade et al. 2005; [e] Chalamaiah et al. 2017 [f] Ren et al. 2016; Ge et al. 2020; [g] Gadde et al. 2015

11.1.2 General Considerations for the Extraction of IgY

A variety of processes may be used, alone or in combination, to extract IgY from egg yolk. The choice is influenced by a number of factors such as: the preservation of the activity of the immune specific immunoglobulins and the degree of purity; the scale of extraction either laboratory or industrial; the costs and the equipment required; and the potential use of other yolk products after the extraction of the IgY (Huang and Ahn 2019). There is a balance to be struck between the purity and the amount of IgY as every purification step results in some loss of the protein (Schade et al. 2001). Two earlier review articles about the methods for the isolation and purification of IgY are published (Meulenaer and Huyghebaert 2001; Kovacs-Nolan and Mine 2004). A comparison of the different methods of extraction and purification is made difficult as the yield and purity of IgY are not always specified as the emphasis in many of the publications is on the biological activity of the antibody against its target antigen. The most valuable information on the choice of a method for the isolation of IgY is obtained when researchers have compared the different methods on the extraction of a single batch of egg yolks. These comparative studies are presented in Sect. 11.6. In general, care must be taken in reviewing the yields as although the data are usually expressed as mg of IgY/mL of egg yolk or mg IgY/mg egg yolk, it may also be expressed as mg IgY mg/mL water in yolk (Kitaguchi et al. 2008) or mg IgY/mL of the water soluble fraction (Kassim et al. 2012); these latter two reporting methods will give yields which appear to be much higher.

The protein content of the various fractions obtained during the extraction are also estimated by a variety of methods. To determine the protein concentration based on the UV absorbance at 280 nm (Table 11.1) different percent extinction coefficients (now referred to as molar absorptivity) of 1.35 for a 1% solution of IgY (Stålberg and Larsson 2001) or an extinction coefficient of 1.33 for IgY (Pauly et al. 2011) or 1.094 (Hodek et al. 2013) were used; this probably reflects the solvent in which the molar absorptivity is determined as there is a solvent effect even in water (Bohman and Arnold 2016) so care needs to be taken to ensure the correct value is used. Protein concentrations are also determined using a number of assays; the Lowry assay using BSA as a standard protein (Deignan et al. 2000); the Biuret method (Akita and Nakai 1993) or the Bradford assay (Chang et al. 2000). Different methods of protein estimation, which interact with different specific amino acids of the protein (Bocian et al. 2020), are likely to give different results and may explain some of the variation in IgY levels recorded. The standard curves for the protein estimation should ideally be performed with a purified IgY rather than another protein such as BSA which is often used.

11.2   Egg Collection and Separation of Yolk from Egg White

Eggs are collected daily from the hens after immunization (Chap. 10). The egg shells are numbered, recorded and stored at 4 °C for two to three weeks, or at room temperature for days until used. This avoids fouling of the material (Fishman and

Fig. 11.2 An egg separator used to remove the egg white from the yolk

Berg 2018). Before removing the egg from the shell, the egg is washed with clean water. Alternatively, for aseptic collection of the egg yolk, the egg can be washed with clean water, soaked in a solution of dodecyl dimethyl benzyl ammonium bromide for 1–3 min, wiped dry with a light cotton fabric (Etamine), and finally cleaned with 75% ethanol to allow for natural air drying. For industrial scale processes, a commercial egg washing machine and sheller is used. The first step in the extraction of IgY from yolk, is the separation of the yolk from the egg white; this is an important step as egg white is mainly composed of proteins. The major proteins in egg white are ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), G2 and G3 globulin (4% each), ovomucin (3.5%) and lysozyme (3.4%) (Ji et al. 2020). The shell is carefully cracked, and the egg white is initially removed using an egg separator (Fig. 11.2).

To remove any adhering egg white, the yolk may be rinsed in flowing water or a buffer or rolled on filter paper (Schade et al. 2001). The outer layer of the vitelline membrane surrounding the egg yolk is composed of ovomucin, lysozyme and vitelline membrane outer proteins I and II while the inner layer is principally composed of glycoproteins (Sunwoo and Gujral 2015). The yolk contents are released by puncturing the vitelline membrane, which is retained on filter paper, and the volume of egg yolk is measured.

11.3 Delipidation of Egg Yolk

One of the main challenges in the extraction of IgY is the removal of the lipids present in the egg yolk. The general strategy for IgY extraction from yolk involves a preliminary step where the granules are removed by one of a number of different methods and the IgY remains in the plasma (Fig. 11.1; Table 11.2).

11.3.1 Water Dilution

Depending on the potential use of the IgY it may be preferable to extract using biocompatible methods. For example, the water dilution method is based on the

Table 11.2 Summary of methods used to remove lipids from egg yolk

Method	Variable conditions
Water dilution method	Degree of dilution
	+/− pH adjustment
	+/− freeze/thaw cycle
3.5% Polyethylene glycol precipitation (PEG)	+/− Chloroform
Anionic polysaccharide precipitation	Dextran, Na-alginate, λ-carrageenan, Na-carboxymethyl cellulose, Pectin
Organic solvent extraction	2-propanol and acetone
Specific chemicals	Phosphotungstic acid and magnesium chloride Caprylic acid

Note: +/− indicates with/without

aggregation of yolk lipoproteins at low ionic strengths resulting in their being insoluble in water (Sunwoo and Gujral 2015). The water-soluble fraction (WSF) is then separated from the lipid components by centrifugation or filtration. Two main factors have been assessed for delipidation using water dilution, the extent of dilution and the pH of the solution. The following conditions have been used: addition of deionized water to give a ten-fold dilution (Jensenius et al. 1981; Nilsson et al. 2008); addition of tap water (eight-fold dilution) followed by freezing, and filtration (Hodek et al. 2013); six-fold water dilution at pH between 5.0–5.2 for 6 hours at 4 °C (Akita and Nakai 1992). Acidic conditions have been shown to effect changes in the yolk granules which increases their ability to bind lipids and thus at the lower pH of 5, the recovery of IgY increases and the amount of LDL in the WSF decreases (Sunwoo and Gujral 2015). Freezing of the diluted yolk results in structural changes in the lipoprotein particles, due to less availability of water, and leads to the formation of lipid aggregates which are large enough to be removed by low-speed centrifugation (Zivkovic et al. 2009). Water dilution methods have yielded 9.8 mg IgY/mL of egg yolk with 93%–96% recovery (Akita and Nakai 1992) and 13.1 mg of IgY per mL of egg yolk at a purity of 71% (Deignan et al. 2000). The water dilution method has several advantages for the purification of IgY for oral administration (Nilsson et al. 2008); these include no toxic products are used in the purification; it is rapid, efficient and suitable for large-scale production. Removal of the aggregated yolk lipids is most commonly achieved by the following methods: centrifugation (Jensenius et al. 1981; Akita and Nakai 1992) or filtration (Sect. 11.4.3).

11.3.2　Polyethylene Glycol Precipitation

Polyethylene glycol (PEG), a polymer of ethylene oxide with the general formula of $H(OCH_2CH_2)_nOH$, can be synthesised to yield straight chain or branched polymers and is one of the most useful protein precipitants (Arakawa and Timasheff 1985).

PEG is non-toxic and non-immunogenic and as such has been widely used in the biotechnology industry. Polson were the first to develop this method where the addition of 3.5% PEG was used to extract IgY from egg yolk (Polson et al. 1980). At this concentration of PEG, the lipid is displaced from the solution and removed by centrifugation. This method was superseded by the addition of chloroform to a dilute suspension of egg yolk which resulted in nearly a three-fold increase in the IgY recovered (Polson 1990) without denaturing the protein. A modification of the chloroform PEG method in which PEG was replaced by the addition of 25% trichloroacetic acid was reported to yield twice as much IgY (200–250 mg IgY/egg yolk) compared to the chloroform PEG method (Asemota et al. 2013).

11.3.3 Anionic Polysaccharides

The use of the anionic polysaccharides, such as dextran sulphate, which interacts with the egg yolk lipoproteins to form a precipitate has also been used; the excess dextran sulphate is then precipitated by the addition of calcium chloride (Jensenius et al. 1981). IgY recovery was compared to the addition of the anionic polysaccharides Na-alginate, λ-carrageenan, Na-carboxymethyl cellulose, and pectin to six-fold diluted yolk at various pH levels (Chang et al. 2000). They reported that the interactions of the polysaccharides and lipoproteins were determined by ionic bonding, hydrophobic interactions and hydrogen bonding. The combination of pectin at 0.15% level at pH 5.0 gave an IgY recovery of 74%. Polysaccharides, as safe and nontoxic substance, can be applied for the isolation of the IgY in biomedical fields.

11.3.4 Organic Solvents

Organic solvents which are miscible with water, such as ethanol (C_2H_5OH), acetone ((CH_3)$_2CO$) and 2-propanol (C_3H_8O) are classical protein precipitants. However, care must be exercised to avoid denaturation of the protein by the solvent which involves working at $-20\ °C$ and finally careful removal of the solvent is necessary. The results of Deignan et al. showed that a yield of 7.11 mg (range 6.20–7.80) per mL of egg yolk with a purity of 57%IgY was obtained after precipitation using 2-propanol followed by acetone (Deignan et al. 2000). In general, the requirement to work at very low temperatures and the use of organic solvents which are deemed undesirable in the food, pharmaceutical and nutraceutical environment restricts the scale of use of this method.

11.3.5 Specific Chemicals

Phosphotungstic acid and magnesium chloride have been used to precipitate lipoproteins (Vieira et al. 1984). Peak IgY yields of 15.1 mg of IgY per mL of egg

yolk, with a purity above 60% purity, were obtained after lipid removal using phosphotungstic acid and magnesium chloride (Deignan et al. 2000). Some acids such as caprylic acid were also successfully used to eliminate lipids (Mclaren et al. 1994).

11.4 Extraction of IgY Following Delipidation

The concentrated fraction containing IgY obtained after the delipidation step, contains other water-soluble proteins and some minor lipids or lipoproteins (Fig. 11.1). An additional step to remove remaining lipoproteins is achieved by addition of 0.1% charcoal at pH 4.0 (Ko and Ahn 2007). The major protein components of the plasma are IgY, α-livetin (chicken serum albumin), and β-livetin (α-2-glycoprotein) and the relative amounts in the yolk are 3:5:2 (Chalamaiah et al. 2017). A more detailed proteomic analysis of egg yolk (Mann and Mann 2008) identified 119 proteins of which 100 were in the plasma fraction (Fig. 11.1). The plasma proteins in highest abundance were as follows: serum albumin, vitellogenin-cleavage products, apovitellenins, IgY, ovalbumin and a 12 kDa serum protein with cross-reactivity to β2-microglobulin. A number of methods are commonly used to extract IgY from the delipidated plasma such as precipitation, filtration, column chromatography and aqueous biphasic systems (Table 11.3).
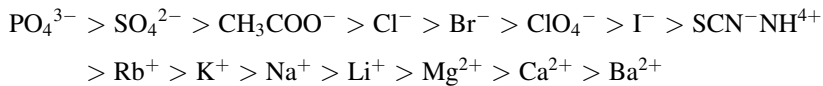
11.4.1 Salt Precipitation

The second step to extract IgY after delipidation is carried out by salt precipitation using saturated salt solutions. Selective precipitation may be achieved by adjusting the pH of the solution to the isoelectric point of IgY (pI = 5.7) where it is least soluble (Chalamaiah et al. 2017). Proteins are usually soluble in solutions with low concentrations of ions which ensures the protein remains folded and stable (Scopes

Table 11.3 Summary of IgY extraction methods

Method	Variable conditions
Salt precipitation	Ammonium sulphate
	Lithium sulphate
	Sodium sulphate
	Sodium chloride
PEG precipitation	8.5% and 12%
Filtration methods	Funnel
	Column gel filtration
	Ultrafiltration
Aqueous biphasic systems	Good's buffer and polypropylene glycol 400
	Phosphate salt and Triton X 100
	Polyethylene glycol (PEG) 1500 and potassium phosphate

1993). At the lower salt concentrations, the anions and cations neutralize the charge on the protein, and it is soluble; at higher salt concentrations the surface will become charged again and aggregate which is the salting out effect. The salting out ability of anions and cations usually follows the Hofmeister series shown below:

$$PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > ClO_4^- > I^- > SCN^-NH^{4+}$$
$$> Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$

Diverse salt solutions have been used to purify IgY such as ammonium sulphate (Ko and Ahn 2007; Xu et al. 2013), lithium sulphate (Bizhanov et al. 2004), sodium sulphate (Jensenius et al. 1981; Deignan et al. 2000) and sodium chloride (Hodek et al. 2013). Comparisons are made among these salts based on the yield and purity of the IgY obtained. Ammonium sulphate, $(NH_4)_2SO_4$, is often the first choice for salting out because it is soluble at high ionic strength and the cost is comparatively low (Duong-Ly and Gabelli 2014). Ammonium sulphate precipitation with two steps (50% and 30%) yielded 9.2 mg IgY/mL egg yolk and a purity of 98% (Xu et al. 2013); two steps (55% and 32%) yielded 4.7–9.2 mg/mL egg yolk however, the purity was not determined (Dai et al. 2013); two steps (both at 40% at pH 9.0) yielded 70–80% of IgY present in the WSF with higher purity (units not given) (Ko and Ahn 2007). Lithium sulphate precipitation in two steps (34% each step) yielded 7.7 mg IgY/mL egg yolk and a purity of 94% (Bizhanov et al. 2004). Sodium sulphate yielded 10–15 mg IgY/mL egg yolk representing a recovery of 70–80% of the total yolk IgY (Jensenius et al. 1981). Sodium chloride at a final concentration of 8.8. yielded 8.9 mg of IgY per mL of yolk and a purity of 97% purity (Hodek et al. 2013).

Sodium sulphate precipitation performed after various delipidation methods showed yields of IgY from 4.9 to 7.5 mg of IgY/mL of yolk with purities ranging from 87% to 94% (Akita and Nakai 1993). Removal of the salt at the end may be achieved by dialysis, often for prolonged periods of time, depending on the salt utilized, or by the quicker method of gel filtration chromatography.

11.4.2 PEG Precipitation

This method involves the extraction of IgY from the delipidated solution by means of two further PEG precipitation steps (Polson et al. 1980; Pauly et al. 2011). In the initial step 3.5% PEG-6000 is used to remove lipids. At higher concentrations of PEG, the protein molecules are excluded from the solvent region occupied by the PEG molecules and as result the protein becomes concentrated and precipitates out of solution when its solubility limit is exceeded (Kumar et al. 2009). In this method there are two further precipitation steps: the addition of 8.5% PEG-6000 yields a pellet following centrifugation for 20 min at 10,000 g; in the final step, 12% PEG-6000 is added to the recovered pellet, the solution is centrifuged and the final pellet is recovered and dissolved in buffer and stored at −20 °C. The IgY purity of

the extract is around 80%, which means, depending on the age of the laying hen (Chap. 2), 40–80 mg per egg. Other reports on the use of this method showed a IgY yield of 4.9 mg/mL per egg yolk, at a purity of 89% (Akita and Nakai 1993) and a range of 8.39–8.83 mg of IgY per mL of egg yolk. Moreover, SDS-PAGE analysis shows no contaminating bands (Deignan et al. 2000). IgY PEG precipitation at 3.5% and twice at 12% followed by cryo-ethanol yielded 4.9 mg/mL egg yolk with a purity of 89% (Akita and Nakai 1993). The addition of the cryo-ethanol served to remove the PEG from the pellet containing the IgY and any contaminating proteins which have a higher solubility than IgY in the alcohol (Polson et al. 1985). This is a mild method and can stabilize the three-dimensional confirmation of the protein which is beneficial in the extraction of bioactive proteins. PEG is a relatively inexpensive raw material and thus can be effectively used in scaled up processes. Resolubilization of the final pellet after centrifugation steps, rather than filtration, is sometimes more time consuming (Gagnon et al. 1996). Contamination of the pellet with PEG may alter the retention behaviour in ion exchange chromatography (Gagnon et al. 1996).

11.4.3 Filtration

Filtration is a separation process based on a barrier that retains some species based on their size and shape while allowing other species in the solution to pass through it (Saxena et al. 2009). The common filtration methods for IgY purification are funnel filtration, gel filtration on a column and ultrafiltration through membranes with different pore sizes (Meulenaer and Huyghebaert 2001). A filtration system was proposed by Kim and Nakai 1998 to separate IgY from egg yolk. A WSF was delipidated using a cellulose powder column in conjunction with an octadecyl column (Kim and Nakai 1998); IgY was precipitated with 1.5 M NaCl pH 9.0 and purified by ultrafiltration through different membranes with cut-offs of 300 kDa–30 kDa with the following results: Amicon, 74%–99% purity, 80%–85% recovery; Harp, 81%–84% purity, 72%–74% recovery; A/G, 89% purity, 75% recovery (Kim and Nakai 1998).

IgY was purified from the WSF by ultrafiltration using polyethersulfone and modified polyethersulfone membranes (Hernández-Campos et al. 2010). The authors concluded that both pH and ionic strength affect the purification and recovery of IgY. When NaCl was added (150 and 1500 mM) the purity of IgY decreased, mainly at pH values close to or higher than the isoelectric point of IgY. The best results were obtained in the absence of salt at pH values of 5.7 (purification factor > 3.5 and IgY recovery of 83%) for polyethersulfone and pH 6.7 (purification factor > 4 and IgY recovery of 94%) for and modified polyethersulfone membranes (Hernández-Campos et al. 2010). Filtration methods are considered feasible for IgY purification for large-scale and industrial applications and have the added advantage of concentrating the IgY solution (Akita and Nakai 1992; Meulenaer and Huyghebaert 2001).

11.4.4 Aqueous Biphasic Systems (ABS)

Liquid-liquid extraction applying aqueous biphasic systems (ABS) represents a viable alternative for the extraction of biomolecules (Freire et al. 2010; Pereira et al. 2015, 2016). Due to their biocompatible water-rich environment, ABS are a promising alternative to classical liquid-liquid extraction methodologies where volatile organic solvents are often used (Albertsson 1970). In addition, ABS allow the combination of extraction, purification and concentration in a single step. Typical ABS consist of two immiscible aqueous-rich phases based on polymer/polymer, polymer/salt or salt/salt combinations (Freire et al. 2012). The use of ABS for the purification of IgG antibodies was largely investigated using conventional polymer-polymer- or polymer-salt-based ABS (Azevedo et al. 2009) and as there is a restricted polarity difference between the coexisting phases this has limited their success in the purification of IgG antibodies (Ayyar et al. 2012). To overcome this limitation, the addition of specific ligands to the polymer used in the ABS creation or the use of hybrid processes have been described (Ruiz-Ruiz et al. 2012; Ferreira et al. 2016). More recently, ionic-liquid-(IL)-based ABS have been proposed as more efficient separation processes than polymer-based ABS, particularly due to the possibility of tailoring the phases polarities and affinities by a proper manipulation of the IL cation/anion combinations (Freire et al. 2012). ILs are organic salts, most of them water-soluble, with circa millions of different cation-anion combinations (Shi and Wang 2016). The effective use of ABS for antibody purification requires knowledge of the molecular-level mechanisms behind the biomolecules partitioning between the two aqueous phases (Freire et al. 2012). Usually, the biomolecule partition is relatively complex due to solute-solvent interactions, such as van der Waals, hydrogen-bonding, and electrostatic effects (Freire et al. 2010). Although less investigated, some reports can be found in the literature on the use of ABS to extract IgY. In this work, novel ABS constituted by cholinium (Ch)-based Good's buffer ILs (GB-ILs) ([Ch] [Tricine], [Ch][HEPES], [Ch][MES], and [Ch][TES]) and polypropylene glycol 400 (PPG 400) were used for the recovery of IgY from the WSF of egg yolk. In all the ABS evaluated, IgY revealed a preferential partitioning to the IL-rich phase. Computational tools were used to confirm that hydrogen bonding and van der Waals interactions between IgY and the IL were the main interactions responsible for the partition of IgY to the IL-rich phase. Extraction efficiencies of IgY in the range of 79%–94% were reported (Taha et al. 2015).

Micelle-based ABS composed of a phosphate salt, NaCl and Triton X-100 have been described for the extraction of IgY. Lipids were extracted into the surfactant-rich top phase, whereas IgY migrated to the phosphate-rich bottom phase. At optimal conditions, the yield of IgY was 97%, corresponding to 11.1–14.9 mg IgY/g egg yolk. The amount of lipids in the bottom phase was 22.9% of the total amount in the egg yolk (Stålberg and Larsson 2001). An integrated system for the recovery of IgY from egg yolk was developed by (Priyanka et al. 2014). In this work, IgY was extracted from immunized chicken egg yolks using aqueous two-phase and aqueous three-phase systems followed by a precipitation step with PEG 6000. Polyethylene glycol (PEG) 1500 and potassium phosphate were used as phase-forming
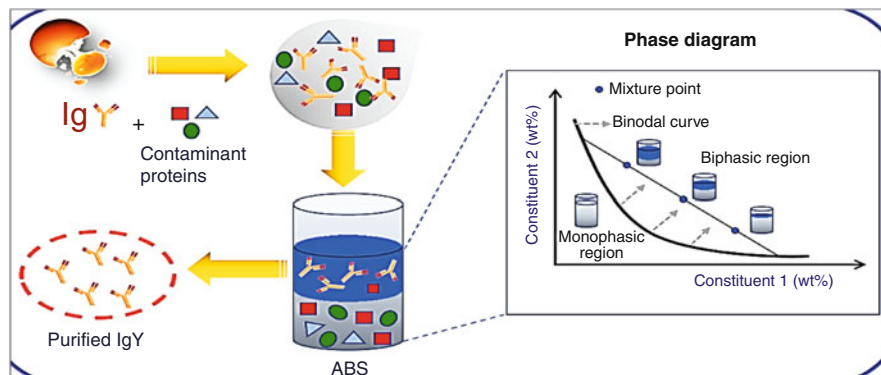
Fig. 11.3 Schematic diagram representing the extraction of IgY from egg yolk using aqueous biphasic systems. Note: The picture in the right side corresponds to an example of an ABS phase diagram shown in an orthogonal representation

constituents of ABS. Butanol and ammonium sulphate were added to the diluted yolk to obtain a three-phase system (two liquid phases and one solid intermediate phase) in two consecutive stages: (i) composed of butanol and 19% ammonium sulphate; and (ii) composed of butanol and 14% ammonium sulphate. In the two-phase system, IgY preferentially migrated to the PEG-rich top phase with a yield of 9.0 mg/mL of egg yolk, while for the three-phase system the yield was 6.0 mg/mL. The authors suggested that the lower yield obtained with the three-phase system was due to the multiple stages involved during the IgY extraction. It was demonstrated that in both approaches the IgY purity increased, as addressed by SDS-PAGE (Priyanka et al. 2014) (Fig. 11.3).

In summary, the results achieved to date with ABS reveal that these systems can be designed to act as effective extraction platforms for IgY. Improved ABS can be achieved by a tailoring of the phases' polarities and affinities by the proper selection of the ABS phase-forming constituents and mixture compositions.

11.5 Purification of IgY

There is ample background information available as the widespread manufacturing of antibodies as biopharmaceuticals has led to the use of chromatographic techniques in the downstream stage due to their high resolution and selectivity, efficiency and applicability in continuous processing (Liu et al. 2010; Cramer and Holstein 2011). To obtain a sample of IgY at the highest purity, additional polishing steps are required after its extraction. In general, chromatographic methods are usually used. The various chromatographic methods rely on the relative affinities of the charged protein for a stationary or mobile phase.

11.5.1 Cation Exchange Chromatography

Ion exchange chromatography is a separation method where the resin is either positively (anion) or negatively (cation) charged and the analyte to be separated has the opposite charge (Pratap 2017). Ko and Ahn evaluated the performance of cation exchange chromatography for IgY purification from diluted egg yolk against the ammonium sulphate precipitation method (Ko and Ahn 2007). The column was packed with pre-swollen carboxymethyl cellulose. The purity of IgY obtained from the ammonium sulphate method was 70%–80%, and much higher than the 30%–40% obtained by cation exchange chromatography. For IgY purification ammonium sulphate precipitation can be scaled up whereas cation exchange chromatography is limited by the volumes that can be separated (Ko and Ahn 2007).

11.5.2 Hydrophobic Charge-Induction Chromatography

Hydrophobic charge-induction chromatography (HCIC) is characterized by the adsorption of proteins over a hydrophobic surface in the presence of high concentrations of lyotropic salts (e.g. ammonium sulphate) (Burton and Harding 1998). In this type of chromatography, ligands may establish hydrophobic, thiophilic and electrostatic interactions with target proteins (Tong et al. 2011). HCIC chromatography was used to purify IgY antibodies from egg yolk, specifically applying macroporous cellulose-tungsten carbide composite beads activated by allyl bromide or divinyl sulfone, and then coupled with 4-mercaptoethyl-pyridine hydrochloride, 2-mercapto-l-methyl-imidazole, and 2-mercapto-benzimidazole as the HCIC ligands (Feng et al. 2008). High adsorption capacities of IgY between 100.6 and 137.6 mgIgY/mL adsorbent were reported for four adsorbents evaluated (Feng et al. 2008). Packed bed and expanded bed HCIC with a macroporous cellulose-tungsten carbide composite activated with allyl bromide and 4-mercapto-ethyl-pyridine hydrochloride were additionally investigated to improve the separation efficiency of IgY (Xia et al. 2012). For the packed bed mode, IgY was extracted with a purity of 92% and 58.4% yield, whilst for the expanded bed mode, a purity of 90% and a yield of 59.5% was obtained. The authors concluded that the expanded bed mode is a more appropriate purification platform for IgY (Xia et al. 2012).

11.5.3 Affinity Chromatography

Among the several chromatographic approaches, affinity chromatography is one of the most selective and efficient processes for purification of antibodies (Ayyar et al. 2012; Coskun 2016). This chromatographic method exploits the interactions of a specific ligand to an antibody; if a highly selective ligand is identified it will retain the antibody while contaminants flow through the column; this leads to high levels of purity in a single step (Liu et al. 2010; Fitzgerald et al. 2011; Ayyar et al. 2012). Native immunoglobulin binding proteins, such as protein A and protein G, effective

Table 11.4 Affinity chromatography methods developed for IgY purification

Ligand	pH of elution buffer	Purity (%)	Recovery (%)	Reference
Elastin-like polypeptide (ELP)-fused C2 domain of streptococcal protein G	2.5–9.5	96.3	64.0	(Xia et al. 2017)
A synthetic ligand (TG19318)	3.0	90.0	90.0–95.0	(Verdoliva et al. 2000)
A stable synthetic ligand[a]	7.0–7.2	92.1	78.2	(Dong et al. 2008)
Protein M[b]	2.8–3.5	98.7		(Jiang et al. 2016)
IgY-binding peptides [c]	3.0	93.0	70.0	(Khan et al. 2017)

[a] Detailed ligand information is not provided; [b] A transmembrane protein from human mycoplasma; [c] IgY-specific peptides identified by T7 phage display technology, with amino acid sequences: GVKCTWSSIVDWVCVDM, GTRCDWSAAYGWLCYDY, RSVCVWTAVTGWDCRND

in the purification of IgG, are not suitable for IgY purification because of the differences between the specific amino acid sequence of IgY-Fc and IgG-Fc (Jiang et al. 2016). A number of ligands have been developed specifically for the affinity purification of IgY (Table. 11.4).

The use of an elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G to purify IgY was shown to give high levels of purity (96.3%) and a recovery which was significantly higher than that by ammonium sulphate precipitation or ethanol fractionation; the purification could be completed within 3 hours which is an additional advantage (Xia et al. 2017). In another study, a synthetic ligand (TG19318) was used to purify IgY, after extraction by the water dilution method, and gave a purity of greater than 90% and a yield of IgY of 10.2 mg/mL egg yolk (Verdoliva et al. 2000). A wide screening of 700 synthetic ligands, synthesized by epichlorohydrin and cyanuric chloride methods was evaluated for IgY purification from the chloroform extract of egg yolk (Dong et al. 2008). A highly efficient ligand for IgY was identified, leading to a purity level of 92.1% with a recovery yield of 78.2%. This ligand has an adsorption capacity of 74.8 mg IgY/mL (Dong et al. 2008). A specific IgY binding peptide (without cross-reactivity to human or mouse/rat IgG) was developed (Khan et al. 2017). The peptide-conjugated column was prepared by immobilization of the biotinylated Y4–4 peptide in a HiTrap Streptavidin HP column. The column was used to purify IgY which had been extracted by the water dilution method followed by ammonium sulphate precipitation (35%) and gave a purity 93% and recovery of 70% (Table 11.4). Purified protein M coupled to NHS-activated Sepharose 4FF was shown to bind to the IgY variable region and IgY from the WSF was recovered with 98.7% purity from egg yolk (Jiang et al. 2016). An approximate 125 times increase in the effective IgY in the eluent was obtained by this method. The method is also applicable to the purification of monoclonal and engineered antibodies. The

capacity for IgY adsorption (2 mg IgY/mL agarose) was low when compared to HiTrap Protein G HP for IgG adsorption (25 mg IgG/mL agarose) and could be further optimised (Jiang et al. 2016). Barroso achieved a recovery of 96% of IgY by using an affinity chromatographic column 4B Sepharose gel activated with divinyl sulfone (DVS), with an adsorption capacity of 26 mg of IgY/mL of gel (Barroso et al. 2005). Metal affinity chromatography for IgY purification from chicken egg yolk was also used in an IDA-Cu^{2+}-cryogel system. The maximum adsorption capacity of IDA-Cu^{2+} cryogel was 27 mg of IgY/g of resin (Junior et al. 2015), similar to the values provided by Barroso (Barroso et al. 2005). Chen attempted the purification of IgY from immunized chicken egg white lysozyme (LS) by an immunoaffinity column LS-Sepharose 4. The capacity of the column for specific IgY against LS was 0.68 mg of IgY/mL of wet gel (0.54 mg of IgY/mg of LS). IgY was effectively isolated with a purification factor of 3380 (Chen et al. 2002). The advantages of affinity chromatography with regard to high specificity and purity of the IgY, often in a single step, are likely to promote continuing research in this domain. Finally, it is important to highlight the use of a different chromatographic approach, namely a three-zone simulated moving-bed (SMB) platform for the separation of IgY from egg yolk. The three-zone SMB equipment was set up by connecting three columns. The highest purity of IgY achieved was 98% with a productivity ca. 0.0067 g IgY/(g adsorbent h) (Song and Kim 2013).

11.6 Comparison of Methods Used for Delipidation and Extraction of IgY

As discussed earlier (Sect. 11.1.2) there are two criteria related to extraction of IgY, which are the variety of methods used for protein determination which are likely to yield different results and how the concentration of IgY is reported, that make comparison of results of different methods difficult. However, a number of researchers have undertaken comparative studies of extraction methods on a single batch of egg yolk and these studies will now be discussed (Akita and Nakai 1993; Deignan et al. 2000; Bizhanov and Vyshniauskis 2000; Ren et al. 2016). Comparisons are made within each study but there is also some difficulty in comparing across studies due to the various methods for presentation of results. The results of the first comparative study in 1993 showed that the water dilution method was the most efficient for the extraction of IgY in terms of yield and functionally active protein and can be readily scaled up (Table 11.5). The procedure

Table 11.5 Summary of results of methods compared

Method	Water dilution	PEG	Dextran sulphate	Xanthan gum
Yield (mg IgY/mL egg yolk)	9.8	4.9	7.5	7.3
Purity	94%	89%	87%	89%

Data according to (Akita and Nakai 1993)

Table 11.6 Summary of results of methods compared

Method	Freeze & Thaw, pH 7.0	3.5% PEG	Dextran sulphate & calcium chloride	Phosphotungstic acid & magnesium chloride	2-propanol & Acetone
Yield mg IgY/mL egg yolk	13.1	11.0	15.6	15.1	4.0
Purity	71%	57.1%	64.3%	69.8%	57%
12% PEG	+		+	+	+
Yield mg IgY/mL egg yolk	7.49		8.8	8.6	2.0

+ Indicates addition of PEG. Data according to (Deignan et al. 2000)

Table 11.7 Summary of results of methods compared

Method	PEG	Chloroform	Dextran blue & calcium chloride
Yield mg IgY/mL egg yolk	4.7	9.0	4.1
Purity (IgY/protein%)	94%	52%	49%
Major bands by SDS-PAGE (kDa)	66	46, 75	35, 41, 66
Minor bands by SDS PAGE (kDa)	6 in total. Ranges 41–57 and 75–80	46, 50, 75	45

Data according to (Bizhanov and Vyshniauskis 2000)

also allows for the use of the rest of the egg yolk as a food product or the extraction of other biologically active molecules.

A comparison of five methods for delipidation, followed by three methods of IgY precipitation was reported by Deignan (Deignan et al. 2000); Table 11.6). Based on yield and purity of IgY, the two best methods of delipidation were precipitation with dextran sulphate and calcium chloride and phosphotungstic acid and magnesium chloride. For Ig precipitation 12% PEG gave the highest yield of protein compared to precipitation with sodium sulphate or ammonium sulphate (results not shown). However, freezing and thawing at pH 7.0 followed by 12% PEG, while giving slightly lower yields of similar purity, represents a very cost-effective method.

A comparison of three IgY extraction methods by Bizhanov (Bizhanov and Vyshniauskis 2000) showed a higher IgY yield using chloroform but with a lower level of purity (Table 11.7). Similar levels of specific activity of the IgY preparations were obtained for all three methods suggesting the integrity of the antigen binding sites was maintained during the extraction. Each of the methods resulted in multiple bands by SDS-PAGE (Sect. 11.8.1) indicating the presence of protein contaminants.

Six principal extraction methods were compared and the highest yield was obtained by the water dilution or carrageenan methods by Ren (Ren et al. 2016) Table 11.8). The organic solvents (chloroform and phenol) gave the lowest lipid

Table 11.8 Summary of results of methods compared

Method	Water Dilution	PEG	Caprylic acid	Chloroform	Phenol	Carrageenan
Yield (units not given)	++					++
SDS-PAGE purity		++				++
% Lipid residue	10.8	10.0	9.1	7.3	7.8	11.2
Filtration rate	++++	+++	+++	+++++	+++	++
Protein concentration mg/mL	+++	++	+++	++++	++	+

Data according to (Ren et al. 2016)

residues but the antibody titre was particularly affected by storage at $-20\,^{\circ}C$ for one month. The water dilution method and caprylic acid yielded protein concentrations of 20 mg/mL while the carrageenan extraction gave only 10 mg/mL. The IgY preparation extracted by using the water dilution method had the highest titre compared to the other five methods.

The final choice of a method to extract IgY depends on the use of the product, and the water dilution and PEG methods are the most commonly used. For some uses, extraction may not be necessary. For example, IgY used for passive immunization of animals (Chap. 15), may be delivered either as liquid yolk or egg yolk powder (Ge et al. 2020).

11.7   Commercially Available IgY Extraction Kits

A number of companies supply kits containing all the reagents necessary to extract and purify IgY. The components of the kits are not disclosed, due to commercial sensitivity. However, a review of the protocols presented in Table 11.9 shows that two kits involve a delipidation step, followed by IgY precipitation with relatively high yields and purity. The other methods rely on thiophilic adsorption chromatography (Chen et al. 2002). The term "thiophilic" refers to an affinity for sulfone groups that lie in close proximity to thioether groups. Thiophilic adsorption chromatography is essentially a resin-based alternative to ammonium sulphate precipitation and yields a concentrated, essentially salt-free, highly purified IgY. The gentle binding and elution conditions ensure a high protein recovery with excellent preservation of antibody activity. The binding capacity is in the range of 20–30 mg IgY/mL of settled beads depending on the supplier and the columns can be regenerated and reused a number of times.

Table 11.9 Commercially available IgY extraction kits

Company	Steps	Amount of IgY	Yield	Purity
Thermo Scientific™ Pierce™ ChickenIgY Purification Kit	Delipidation Precipitation	80 to 120 mg IgY per egg yolk	80%	85% ∼ 95%
Exalpha Biologicals, Inc. IgY EggsPress Purification Kit	Delipidation Precipitation	4–7 mg IgY per mL egg yolk. 60–105 mg/ egg yolk		90%
HiTrap IgY Purification HP	Delipidation step* Column separation based on thiophilic adsorption		78%	>70%
Affiland egg yolk purification kit	Column separation of egg yolk	50–100 mg IgY/egg yolk		98%
BioVision's Hi-Bind™ T-Gel Agarose	Thiophilic Adsorption Chromatography			
G-Biosciences' Thiophilic Resin	Thiophilic Adsorption Chromatography			
Pierce™ Thiophilic Adsorption Kit	Thiophilic Adsorption Chromatography			76% by HPLC

aReagents not supplied with kit

11.8 Methods Used to Confirm Purity and Activity of IgY

Antibodies may be characterized by different methods to determine their degree of purity and their biological activity. These parameters are determined independently and confirmation of the result by a single analytical method is usually acceptable for polyclonal antibodies. For antibodies used to treat human diseases the degree of characterization required is much greater with regard to structure, charge state and microheterogeneity and each property must be determined by at least two analytical methods (Staub et al. 2011).

11.8.1 Molecular Weight and Structure

The molecular weight of IgY is accepted to be180 kDa (Table 11.1) and after extraction and purification of IgY it is usual to confirm the molecular weight experimentally. The sequence of amino acids (primary structure) in a protein determines how it folds into secondary and tertiary structures; IgY also has a quaternary structure composed of two heavy and two light chains and is glycosylated. To confirm the molecular weight of IgY, it is necessary to disrupt the secondary and tertiary structures, and this is achieved by the addition of sodium

dodecyl sulphate (SDS) and then by electrophoresis which separates charged macromolecules under an electric field. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation is then directly related to the molecular weight of the intact protein. For example, SDS-PAGE of IgY recovered by affinity purification exhibited a single band near 180 kDa (Edupuganti et al. 2013). SDS-PAGE may also be performed under reducing conditions, which disrupts the di-sulphide bonds between the heavy and light chains (Chap. 5) which are then separated by electrophoresis giving bands at 65–67 kDa (Heavy chain) and 21–23 kDa (Light chain) (Sheng et al. 2018). The appearance of a number of additional bands after SDS-PAGE indicates that other protein contaminants are present (Table 11.6). The molecular weight can also be determined using mass spectrometry and the average molecular weight of IgY was determined in an early study as 167 kDa, with heavy chains of 65 kDa and the light chains of 19 kDa (Sun et al. 2001); however, it is generally accepted that the molecular weight of IgY is approximately 180 kDa with heavy chains of 67 kDa and light chains of 25 kDa (Sheng et al. 2018). A more detailed analysis of the purity of an IgY preparation delipidated using the water dilution method was performed: the protocol involved two-dimensional gel electrophoresis and nanoflow liquid chromatography coupled offline to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry; 25 individual proteins were identified in that preparation as well as IgY (Nilsson et al. 2008). Capillary electrophoresis, operates on the same principal as gel electrophoresis, but has the advantages of a smaller sample size, improved resolution, decreased separation time and real-time detection (Staub et al. 2011). It has been used to investigate IgY separated by Protein M affinity chromatography (Jiang et al. 2016). Circular dichroism (CD) is a technique used to evaluate the secondary structure of purified IgY. The work of Liu et al. showed that, after two-stage ultrafiltration steps (Liu et al. 2010). IgY as evaluated by CD displayed a typical β-sheet curve (∼45% of the sheet content), suggesting that the IgY folded with a reasonable secondary structure (Liu et al. 2010). The effects of enzymatic deglycosylation on the conformation and stability of IgY were also evaluated by CD (Sheng et al. 2017). In this work, IgY was recovered from egg yolk by precipitation with PEG 6000, followed by further purification by gel permeation chromatography. The CD spectra of IgY and deglycosylated IgY suggested a β-sheet content for native IgY of 42%, and an α-helix structure in IgY (Sheng et al. 2017). However, conformational changes after enzymatic treatment were observed, with a decrease in the α-helix and β-sheet contents of 23.2% and 23.3%, respectively (Sheng et al. 2017). On the other hand, the random coil increased from 5% to 28.5%, indicating that the IgY structure became more flexible and disordered after the removal of N-glycan, meaning that glycosylation is fundamental to maintain the structure and stability of IgY (Sheng et al. 2017).

11.8.2 Biological Activity of IgY

After extraction and purification it is necessary to show that the IgY binds to the specific antigen used in the immunization protocol (Chap. 10). The most common

bioassay used is an *enzyme-linked immunosorbent assay* (ELISA) where binding of the IgY to the antigen of interest is determined quantitatively. It is also important to ensure that the IgY does not bind other possible contaminants present in the sample to be measured. *Western blot analysis* may also be used to qualitatively characterize the purified IgY from egg yolk. In this methodology, the protein bands from the SDS-PAGE gel are eluted into a nitrocellulose filter, allowing the identification of specific proteins in a complex matrix by antibody labelling (Yang and Mahmood 2012). For example Western blot analysis of IgY purified by the PEG 6000 precipitation method, specifically recognized the purified canine IgG used but did not recognize IgG from other animal species, such as cat IgG, guinea pig IgG, rabbit IgG, goat IgG, sheep IgG and horse IgG (Santos et al. 2014). The activity of the IgY may also be determined by *immunodiffusion assays*. These methods, for the measurement of antibody and antigen binding, are confirmed by the presence of a precipitation arc following diffusion through a gel. The two related methods used are Ouchterlony's double diffusion method (Ouchterlony 1949) or radial immunodiffusion (Hudson and Hay 1980). Both methods have been applied to evaluate IgY after extraction and purification (Chen et al. 2002). The real-time analysis of the interaction of an antibody with an antigen is possible using *surface plasmon resonance analysis* (SPR). IgY is immobilized on a prepared gold sensor surface and binding of the antigen results in a change in polarized light as the molecules bind and dissociate resulting in a real-time sensogram (Edupuganti et al. 2013; Khan et al. 2017). The specific conditions for the immobilization of IgY at a flow rate of 5 μL/min, pH 4.0 at concentration of 100 μg/mL gave a linear range over 50–500 ng/mL and a detection limit of 40 ng/mL (Chun-Yu et al. 2012).

11.9 Purification and Characterization of Monoclonal IgY

The methods used for the production of monoclonal IgY and antibody fragments are outlined in Chap. 14. The first step is to harvest the antibody from the cell culture by removal of the cells and cell debris, either by centrifugation, depth filtration and sterile filtration to yield a clarified fluid suitable for chromatography (Liu et al. 2010).

While IgY does not bind to protein A or G, protein M has been shown to very effective in the purification of scFv IgY (Jiang et al. 2016) as well as specific antigen affinity chromatography (Khan et al. 2017). In common with polyclonal IgY, monoclonal IgY antibodies are routinely assayed by ELISA, Western blotting and SPR. There are a range of other techniques which are applied to the analysis of monoclonal antibodies which due to space limitations we cannot describe in detail. Spectroscopy is commonly used to assess the secondary and tertiary structure of antibodies. Numerous techniques are available, such as X-ray, nuclear magnetic resonance, absorption, fluorescence, Circular Dichroism (CD), dynamic light scattering, and infrared spectroscopy (Baudys and Kim 2000). CD spectroscopy is the technique of choice for studying chirality, particularly for monitoring and characterizing protein interactions in solution (Bertucci et al. 2010). IgY aggregates

can also be analysed by CD (Joshi et al. 2014). Capillary isoelectric focussing may be used to determine the pI of proteins, characterize impurities, and monitor protein charge heterogeneity of monoclonal antibodies. It can distinguish between two proteins whose pI differs by as few as 0.005 pH units (Fonslow et al. 2010).

11.10 The Storage and Stability of IgY Product

It is important to maintain the stability and bioactivity of IgY in different storage conditions. IgY solutions can be stored for months to years at 4 °C, ideally with the addition of bacterial growth inhibitors such as sodium azide, thimerosal and gentamicin (Schade et al. 2005). Two important points should be considered when storing IgY. IgY preparations stored with sodium azide need to be dialyzed for use when labelling with horseradish peroxidase as it can inhibit its activity at dilutions of primary antibody of lower than 1:1000 (Schade et al. 2005). The repeated freeze-thawing of IgY antibody between −70 °C and room temperature results in a 50% loss of activity, which can be avoided by the addition of glycerol or by freezing in a small aliquots which once thawed are stored at 4 °C (Staak et al. 2000; Gadde et al. 2015). Longer term storage of large scale antibody preparations is usually by lyophilization (freeze-drying) (Fishman and Berg 2019). Consideration should be given to the concentration of the protein solution to be freeze dried and the inclusion of molecules which can help stabilize the protein, such as sucrose, trehalose or polysorbate 80 (Fishman and Berg 2019).

IgY solutions show a slight tendency toward self-aggregation, but this does not seem to influence the overall IgY antibody activity, and the activity of highly purified IgY decreases faster than that of partially purified IgY (Schade et al. 2005).

For oral delivery of IgY, it is notable that pepsin causes higher inactivation of IgY compared to trypsin (Gadde et al. 2015). Several methods have been developed to protect IgY from degradation by pH and pepsin present in the stomach (Chap. 12), allowing it to arrive unscathed at the small intestine, which is especially important for the use of IgY against enteric pathogens (Gadde et al. 2015). IgY in liquid yolk preparations retains its activity for up to 12 weeks at 4 °C but may become contaminated by bacterial growth (Ge et al. 2020). Yolk powder is prepared by dilution of the yolk with an equal volume of distilled water at 4 °C, freezing at −80 °C and lyophilization at −70 °C and can be stored free from bacterial contamination for up to 1 year (Ge et al. 2020). Further extraction of IgY from yolk, either by the water dilution or the PEG methods, reduced the antibody titre as compared to the liquid yolk or egg powder and suggests that for veterinary products IgY delivered as egg yolk powder may be optimal (Ge et al. 2020).

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