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Microparticulation of whey protein: related factors affecting the solubility

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Mikropartikulierung von Molkenprotein: Faktoren, die die Löslichkeit beeinflussen

Zusammenfassung. Simplesse 100, Fettsubstitut auf Molkenbasis, zeigt ein gutes Lösungsverhalten. Da die Entstehung von Mikropartikeln mit thermischen Verfahrensschritten verbunden ist, ist ein solches Verhalten ungewöhnlich. Zur Klärung dieses Phänomens wurden gelchromatographische Trennungen an Sephadex G-100 bzw. an Sephacryl S-1000 sowie die SDS-PAGE herangezogen, unterstützt durch UV-Studien, analytische Techniken und Zentrifugation bei 23.000 *g*. Die Ergebnisse lassen den Schluß zu, daß das Lösungsverhalten mikropartikulierten Molkenproteins im wesentlichen von zwei Effekten bestimmt wird, (1) der optimalen Entfaltung der Proteinmoleküle und (2) der Stabilisierung der entfalteten Proteinmoleküle durch Kohlenhydrate. Beide Effekte favorisieren nicht-kovalente Bindungsmechanismen im mikropartikulierten Protein und sind verantwortlich für physiko-funktionelle und nutritive Eigenschaften.

Abstract. Solubility of Simplesse 100, the only whey-based fat substitute, was found to be good, considering the fact that technology for preparation of Simplesse 100 is a sequence of thermal steps. To characterize this phenomen, gel chromatography on Sephadex G-100, Sephacryl S-1000 and SDS-PAGE were used, supported by high-speed separation, UV studies and analytical procedures. Results show that the unusual solubility characteristic of microparticulated whey protein is related to two molecular effects: (1) optimal defolding of protein molecules and (2) stabilization of the defolded status by carbohydrate. Both effects were considered to favour non-covalent bonds, which contribute to the outstanding physico-functional and nutritive properties of microparticles.

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Introduction

Simplesse 100 is the only protein-based fat substitute obtained from whey protein as a result of denaturing treatments, like thermodenaturation under moderate acidic conditions, followed by thermocoagulation and microparticulation [1, 2]. The sizes in whey protein particles produced in this way are comparable with those of homogenized milk fat globules. In the narrow range between 0.5 μm and 3.0 μm particle size, Simplesse 100 is perceived orally to be like high-quality fats, as well as exhibiting their structural characteristics. Thus, this feature represents a new kind of function of whey protein.

Considering that Simplesse 100 is made from relatively crude whey protein concentrate of about 50% protein in the dry matter and that this protein has gone through several denaturing thermal steps, it still retains rather good solubility and digestibility [3]. This can be put down to the fact that: (1) whey protein remains soluble after thermal denaturation under acidic conditions [4–6] because any disulphide interaction is excluded, and (2) lactose seems to be important for the thermal stability of microparticulated whey protein, as described previously [7] for various sugars in connection with thermal denaturation and coagulation.

In this work both factors were elucidated to find out inter-relations between solubility characteristics and corresponding structural orientation as prerequisites for microparticulation.

Materials and methods

Whey products. Simplesse 100 dry, a microparticulated whey protein powder containing 50.4% protein and 27.1% lactose, was obtained from Nutrasweet, Germany. Lacprodan 80, containing 78.0% protein and 4.0% lactose, is an ultrafiltered whey protein powder produced by Danmark Protein. Raw milk whey was obtained by isoelectric precipitation of casein at pH 4.6 using 1 N HCl. The coagulate was separated at 6000 *g* for 10 min. The acidic modification of raw milk whey was carried out at 90°C for 10 min at pH 3.8. After thermal treatment the modified whey was rapidly cooled down to room temperature and neutralized to pH 6.4 with 1 N NaOH.

Table 1. The nitrogen solubility index (NSI) of different preparations of whey protein at various pH (data in %)

pH	NSI of whey protein preparation:		
	Lacprodan 80	Lacprodan 80 pH 6.5/90°C/5 min	Simplesse 100 dry
2.0	97.6	47.8	76.6
3.0	89.9	43.1	55.5
4.0	81.3	31.5	53.5
5.0	80.5	32.1	51.8
6.0	92.1	55.2	57.5
7.0	94.5	53.4	58.3
8.0	94.8	55.4	66.2

In the *third column* Lacprodan 80 has been previously heat treated at pH 6.5 at 90°C for 5 min

Determination of solubility. The nitrogen solubility index (NSI) was examined by the centrifugation method using 2.5% protein solutions and determination of the nitrogen content in the supernatant [8]. Turbidimetric measurements of the absorbance index (AI) were carried out at 900 nm [9] using the Novaspec photometer (Pharmacia).

Chromatographic fractionations. Gel permeation chromatography was performed using Sephadex G-100 in a glass column (100 × 2.5 cm) and 0.02 mol/l sodium citrate at pH 6.0 containing 0.02% sodium azide as a preservative. The effluents were continuously monitored by measuring the absorbance at 280 nm and collected in 3.0-ml fractions at a flow rate of 35 ml/h. Gel permeation chromatography on Sephacryl S-1000 was performed in a glass column (50 × 2.5 cm). The elution buffer contained 3 mol/l urea, 20 mmol/l imidazole and 50 mmol/l NaCl and was adjusted to pH 6.9 with 1 N HCl. The effluents were collected in 3.0-ml fractions at a flow rate of 60 ml/h. The absorbances of all fractions were measured at 280 nm. Fractions were further investigated by chemical methods and SDS-PAGE.

Chemical methods. Protein was determined by the Bio-Rad protein assay, whereby calibrations were made using solutions of 0.05%, 0.1% and 0.2% Simplesse 100 or Lacprodan 80 prepared in elution buffer for chromatography. As a microassay for lactose, a modified procedure of the phenol-sulphuric acid method was used [10].

Polyacrylamide gel electrophoresis. SDS-PAGE using tricin, according to Schägger and Jagow [11], was done in the Multiphor II Electrophoresis System (Pharmacia). The resolving and stacking gels were composed of 10% respo 4% acrylamide made up in TRIS/HCl buffer, pH 8.45. The 1.5-mm-thick gels were run at a constant voltage of 200 V for 40 min, stained by Coomassie Blue R 200 and destained for 12 h and documented using the Gelimage Scanning System (Pharmacia).

Results and discussion

Solubility and structural features

One of the most intrinsic physical properties of proteins is their solubility. For the majority of proteins it is found best in the native status, whereas upon thermal processing most proteins become more or less denatured. This is accompanied by loss of solubility. In Table 1 the NSI values were compared for undenatured and denatured Lacprodan 80 as well as for Simplesse 100 dry.

Results in Table 1 show that microparticulated protein is less soluble than Lacprodan 80 but more soluble than whey protein that has been thermally denatured at pH 6.5.

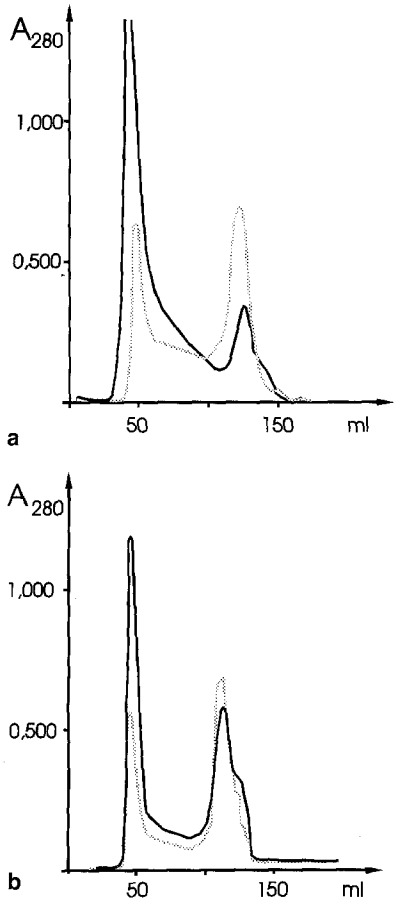

Fig. 1. a Elution profiles of Simplesse 100 (*solid line*) and Lacprodan 80 (*dotted line*) on a Sephacryl S-1000 column (0.2 mol/l NaCl, pH 6.9). **b** Elution profiles of Simplesse 100 and Lacprodan 80 on a Sephacryl S-1000 column (3 mol/l urea, 20 mmol/l imidazole, 50 mmol/l NaCl, pH 6.9). The absorbance of all fractions were measured at 280 nm (A_{280})

Nevertheless, microparticulated whey protein still has enough molecular flexibility to exhibit quite reasonable physico-functionality and digestibility [3]. To elucidate the effects involved in aggregation of whey proteins to form microparticles, more advanced methods are required, as will be shown with chromatographic and electrophoretic techniques. In Fig. 1 a typical elution profiles were depicted comparing the patterns between Simplesse 100 and Lacprodan 80 under physiological conditions (0.2 mol/l NaCl, pH 6.9) on Sephacryl S-1000.

Both Simplesse 100 and Lacprodan 80 were resolved with two peaks having maxima close to 50 ml (> 1 million Da) and 125 ml (< 100 kDa). Microparticles were eluted within the first peak as a turbid fraction. The second peak was found to be β-lactoglobulin and α-lactalbumin in associated forms but also as monomers. Further extended studies with different rates of protein denaturation revealed a remarkable similarity between Simplesse 100 and heat-denatured whey protein (pH 6.5, 90°C for 10 min) (not shown). From these chromatographic findings it might be concluded that both microparticulation and the generally practiced whey protein denaturation would cause comparable irreversible denaturations. However, it looked different in the presence of a disintegrating buffer like 3 mol/l

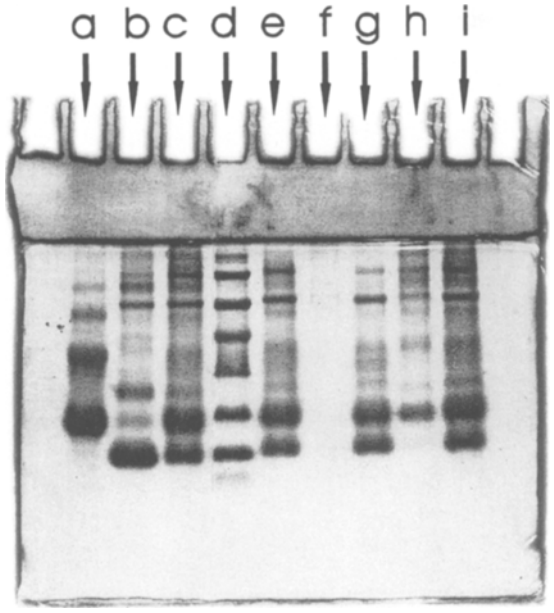

Fig. 2. SDS-PAGE of Simplesse 100 and Lacprodan 80 and their chromatographic fractions obtained by gel permeation chromatography (data from Fig. 1b). *a*, enriched β-lactoglobulin; *b*, enriched α-lactalbumin; *c*, Simplesse 100 1.5%; *d*, low molecular weight (LMW) marker (Pharmacia); *e*, Lacprodan 80 fraction 2; *f*, Lacprodan 80 fraction 1; *g*, Simplesse 100 fraction 2; *h*, Simplesse 100 fraction 1; *i*, Simplesse 100 1.5%

urea, which was effective in dissolving Simplesse 100 (Fig. 1b), but not heat-denatured Lacprodan 80. This may be causally related to non-covalent bonds in Simplesse 100 favoured by the acidic denaturation step. Urea reduces the first peak in such a manner that the profile becomes more similar to that of Lacprodan 80 (Fig. 1b). However, SDS has been found to be effective also in reducing the first peak, emphasizing that with microparticulation the disulphide mechanism does not play a role.

Further characterization of the urea Sephacryl S-1000 fractions was made by SDS-PAGE (Fig. 2). In the first Simplesse 100 peak (50 ml) only β-lactoglobulin was revealed, whereas the second peak (125 ml) contained both α-lactalbumin and β-lactoglobulin. The subtly differentiated presence of α-lactalbumin and β-lactoglobulin in the Sephacryl S-1000 fractions might be of significance in the arrangement between non-covalent bonds. Assuming that the molecular contribution shown in Fig. 1b concerns only the effects of split H bonds, then hydrophobic forces must be the other non-covalent forces responsible for the molecular arrangement of β-lactoglobulin in the first peak. The assumption is supported by the electrophoretic findings obtained with SDS-PAGE (Fig. 2, lanes c, e, i), showing similar profiles for Lacprodan 80 and Simplesse 100.

Extensive studies in solubility were made by separation of diluted protein (1.5%) at 23.000 *g* for 30 min. Both the cleared supernatant as well as the sedimented protein were investigated by gel permeation chromatography on Sephacryl S-1000 in the presence of 3 mol/l urea. Results are shown in Fig. 3a, b. Protein which remained soluble after the separation step is marked by dotted lines. Full lines indicate the sedimented protein. Considering the Simplesse

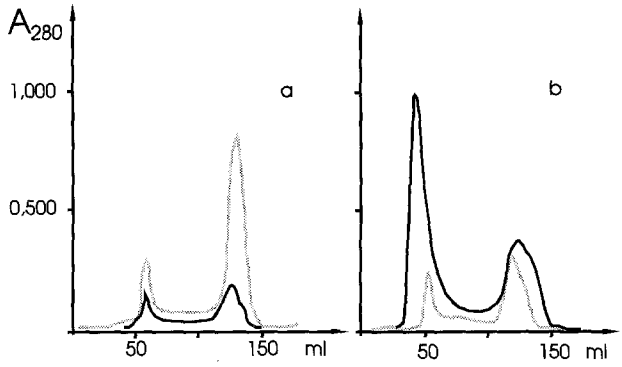

Fig. 3. Elution profiles of supernatants (23,000 *g*/30 min) (*dotted line*) and sediments (*solid line*) of Lacprodan 80 (**a**) and Simplesse 100 (**b**) on a Sephacryl S-1000 column (3 mol/l urea, 20 mmol/l imidazole, 50 mmol/l NaCl, pH 6.9). The absorbances of all fractions were measured at 280 nm (A_{280})

100 profiles, apparently most of the whey protein was associated in microparticles, whereas the amount of soluble protein remained low.

The role of disaccharides

The ability of lactose to protect milk protein against thermocoagulation has been demonstrated by several workers [7, 12, 13]. In practice it is comprehensible by the fact that high-purity whey protein very often contains higher proportions of denatured protein compared with others having higher lactose contents. Moreover, lactose may affect the mode and characteristics of whey protein aggregates due to its ability to replace expelled water molecules in exposed hydrophobic pockets [13]. It is still an open question as to whether and how lactose interacts with denatured protein under the conditions of microparticulation. However, the good solubility of lactose proved to be a hindrance in detecting or quantifying interactions between protein and lactose.

First attempts with Simplesse 100 using native PAGE combined with fuchsin staining failed to detect any protein-associated lactose with microparticles. So chromatographic procedures were the methods of choice again with Sephadex G-100 or Sephacryl S-1000 followed by a colorimetric determination of lactose in the eluents. Results obtained with Sephadex G-100 are shown in Fig. 4a, b. These results show that lactose remains associated with the protein to a certain degree under physiological conditions. Both whey protein preparations contain lactose maxima within the first protein peak associated with aggregated whey protein. Lactose is also associated with free whey protein (165 ml β-lactoglobulin, 175 ml α-lactalbumin). It seems that β-lactoglobulin does have an increased affinity for lactose.

In Fig. 5 the elution pattern of Simplesse 100 obtained on Sephacryl S-1000 using 3 mol/l urea buffer is shown. All the lactose is embedded in the last part of the second peak, associated with α-lactalbumin and β-lactoglobulin as shown above. It seemed that the embedded lactose might signify a specific affinity with whey proteins eluting as the second peak. Extended chromatographic studies with whey

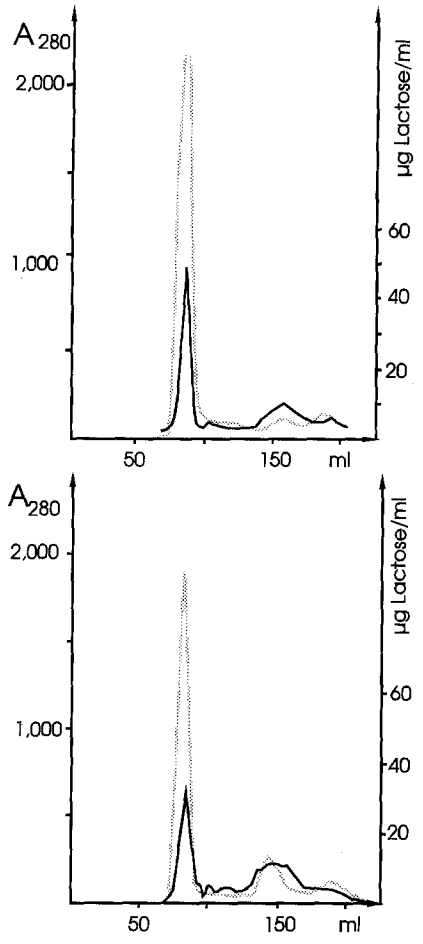

Fig. 4. Elution profiles of Simplesse 100 (**a**) and Lacprodan 80 (**b**) on a Sephadex G-100 column (0.2 mol/l NaCl, pH 6.9) visualized by absorbance at 280 nm (A_{280}, *dotted line*) and lactose concentration (µg lactose/ml, *solid line*)

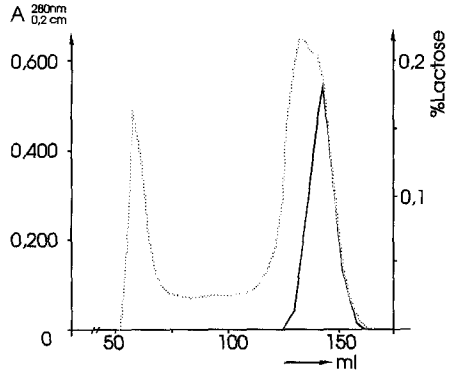

Fig. 5. Elution profile of Simplesse 100 on a Sephacryl S-1000 column (3 mol/l urea, 20 mmol/l imidazole, 50 mmol/l NaCl, pH 6.9) visualized by absorbance at 280 nm (A_{280}, *dotted line*) and lactose concentration (% lactose, *solid line*)

proteins in the presence of increasing amounts of lactose revealed that lactose eluted at the same position as shown with Simplesse 100 (Fig. 5), but describing a much wider amplitude. These results supported the assumption that lactose stabilizes whey protein via non-specific preferential hydration. Related effects were observed with sour

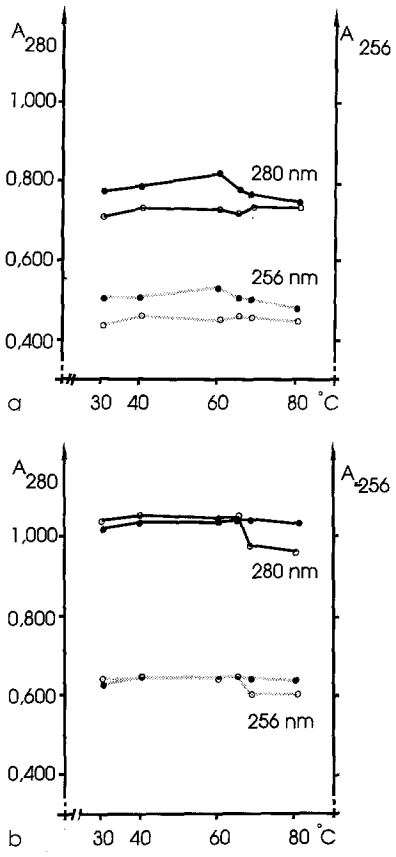

Fig. 6. Effect of heat treatment on the absorbance at 280 nm (A_{280}) or 256 nm (A_{256}) of raw milk whey (**a**) and the same whey after a preliminary thermal modification (**b**) in the presence or absence of 0.1% sucrose.

whey obtained by isoelectric precipitation of raw milk and with the same whey after heating under acidic conditions.

UV studies

Whey was heated to different temperatures (30–80° C) for 1 min, cooled with ice-water and centrifuged at 10,000 *g* for 20 min to remove any precipitate formed. The protein concentration was estimated by the Bio-Rad procedure and diluted to 0.1% protein in 0.05% phosphate buffer. The same experiment was repeated but in the presence of 0.1% sucrose. The UV absorbance of the diluted whey samples was read at 280 nm and 256 nm and plotted against temperature. The results are shown in Fig. 6a, b.

Heat-induced aggregations are mostly preceded by conformational changes in proteins and are detectable by increases in UV absorbance, like the exposure of tryptophan residues visible at 280 nm, or the contribution of SH groups at 256 nm [14, 15]. The results in Fig. 6 show that carbohydrates promote conformational changes in whey protein. At 60° C the structural defolding reached a maximum with minimal denaturation (Fig. 6a). Increasing temperature further to 70° C breaks the promotional effect of carbohydrates and whey protein becomes more and more irreversibly denatured.

These effects are not relevant to microparticulation because the process is preceded by a thermal treatment under acidic conditions. Related conformational changes are best characterized by UV readings at 30° C (Fig. 6b). Heating under acidic conditions caused a high degree of molecular defolding which is stabilized by the hydroxyl groups of lactose as reported with ovalbumin [16]. At temperatures higher than 65° C a surplus of carbohydrate affects the thermocoagulation by smoothing out the sharp transition between 65° C and 70° C. It is conceivable that an increased thermoflexibility might be advantageous to a more controlled aggregation of whey protein.

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