Purification of goat β-lactoglobulin from whey by an ultrafiltration membrane enzymic reactor

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Summary. This paper presents a novel contribution to the purification of goat β-lactoglobulin by using an ultrafiltration membrane enzymic reactor. The basis of the purification process was the enzymic hydrolysis of contaminating proteins, α-lactalbumin and traces of serum albumin, by pepsin at 40 °C and pH 2, conditions under which β-lactoglobulin is resistant to peptic digestion. Simultaneously, β-lactoglobulin and peptides were separated by ultrafiltration. β-Lactoglobulin was retained in the reactor while peptides generated by hydrolysis from α-lactalbumin and serum albumin permeated through the membrane. The process was made continuous by the addition of fresh whey to replace the lost permeate. Three mineral membranes with 10, 30 and 50 kDa molecular mass cut-off were tested and the 30 kDa membrane was selected for the continuous process. The simultaneous purification and concentration of β-lactoglobulin from clarified goats' whey was achieved in a single step. The ultrafiltration membrane enzymic reactor could treat eight reactor volumes of clarified whey. The recovery of β-lactoglobulin was 74 %, its purity was 84 % and its concentration 6·6-fold that in the initial clarified whey.

β-Lactoglobulin (β-lg) is a well known protein, usually isolated from bovine whey, whose biological and functional properties have been much studied (Creamer & MacGibbon, 1996; Guimont *et al.* 1997). Bovine β-lg has excellent heat set gelation properties that can be immediately applied in formulated foods. Its high solubility at low pH makes it useful as an active agent in protein-fortified acidic beverages (Smithers *et al.* 1996). Composed of nine β-strands and one α-helix, which forms a hydrophobic pocket, β-lg is probably involved in the binding of many hydrophobic compounds such as retinol and fatty acids (Pérez & Calvo, 1995).

Numerous techniques can be used to separate bovine β-lg from α-lactalbumin (α-la), serum albumin (SA) and other minor components. Some of these methods are more suitable for the laboratory preparation of β-lg: chromatographic fractionation of whey proteins with anion-exchange resins (Outinen *et al.* 1996*a*) and centrifugation (Tupasela *et al.* 1997) have been used successfully for this purpose.

On a larger scale, thermal precipitation of α-la near its isoelectric point gives a supernatant fraction enriched in β-lg (Pearce, 1983; Bramaud *et al.* 1995, 1997). Preferential enrichment of β-lg is also obtained when whey is partly hydrolysed by trypsin before heat treatment (Sato *et al.* 1996; Caessens *et al.* 1997). Two

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chromatographic methods were tested and considered to be readily adaptable to commercial scale production of α-la; however, β-lg is denatured (Outinen *et al.* 1996*b*). Sequential precipitation of whey proteins by the addition of $FeCl_3$ or NaCl was used with success to prepare pure bovine β-lg from whey (Kuwata *et al.* 1985; Mailliart & Ribadeau-Dumas, 1988).

Another method for purifying β-lg takes advantage of its resistance to digestion by pepsin and some bacterial enzymes (Mohedano *et al.* 1996; Santoro & Faccia, 1996) that readily hydrolyse other milk or whey components. This resistance is explained by the particularly stable conformation of the core of β-lg (Reddy *et al.* 1988; Schmidt & Poll, 1991). Kinekawa & Kitabakate (1996) developed a purification method for β-lg based on its resistance to peptic digestion. The whey was first incubated with pepsin, which hydrolysed α-la and SA. The remaining protein was then precipitated by salting-out and dialysed or filtered through an ultrafiltration membrane. We have applied the principle of this discontinuous purification process for bovine β-lg to develop a continuous process that makes possible the simultaneous purification and concentration of goat β-lg. The purification of β-lg was based on the selective hydrolysis by pepsin at 40 °C and pH 2 of the contaminating proteins, α-la and SA, together with the simultaneous removal of their products by ultrafiltration. β-Lactoglobulin should remain unaffected and be retained by the ultrafiltration membrane. The behaviour of goats' whey proteins towards hydrolysis by pepsin was studied first and then the separation of β-lg and peptides was tested with mineral membranes of 10, 30 and 50 kDa molecular mass cut-off (MMCO).

materials and methods

All reagents were of analytical grade. Fresh raw milk was collected from a herd in west central France composed of 200 Saanen and Alpine–Saanen goats. The milk was stored at 3 °C for 1 d before being treated. Goats' milk (11 l) was first skimmed by heating at 40 °C and centrifuging at 6250 ***g*** for 15 min (KR 22i, Jouan, F-44805 Saint-Herblain, France). Caseins were precipitated by acidification of whey at pH 4·2 (the pH_i of goat caseins; unpublished results of Institut Technique des Produits Laitiers Caprins, F-17700 Surgères, France) with 5 M-HCl and removed by centrifugation (6250 ***g*** for 10 min). The remaining lipids and caseins were removed by thermocalcic destabilization (Fauquant *et al.* 1985). The pH was adjusted to 7·3 with 5 M-NaOH and the resulting whey was cooled to 10 °C; the lipoprotein precipitate was removed by centrifugation (6250 ***g*** for 10 min) and the whey clarified by filtration on a Zeta-plus filter (Cuno Europe, F-62730 Les Attaques, France). The turbidity was determined as A_{600}, a value of $< 0{\cdot}02$ being considered acceptable for further studies. From 11 l milk, 6 l clarified whey were obtained and stored at −18 °C.

Whey analysis

Proteins and peptides from goats' whey hydrolysate were separated and quantitated by reversed-phase HPLC. The system (Waters, Milford, MA 01757, USA) consisted of a Waters 600 automated gradient controller-pump module, a Waters Wisp 717 automatic sampling device, and a Waters 996 photodiode array detector. The mobile phase for the reversed-phase Delta-Pak C4 column (3·9 × 150 mm, 5 μm, 100 Å; Waters) consisted of a binary gradient of trifluoroacetic acid in water (1 ml/l) for solvent A and trifluoroacetic acid in acetonitrile (1 ml/l) for solvent B. The gradients applied were 0–300 ml B/l in 5 min, 300–350 ml B/l in

5 min, 350–470 ml B/l in 35 min, 470–1000 ml B/l in 5 min and finally pure B for 10 min. The flow rate was 0·8 ml/min. Between injections, the column was re-equilibrated with pure A for 10 min. Before injection, each sample was filtered through a 0·22 μm filter (Millex GV; Millipore, Bedford, MA 01730, USA) and 50 μl was injected. Detection was by measuring absorbance at 215 nm.

The amounts of β-lg and α-la in the whey, the reactor retentate and the permeate were calculated from their chromatographic peak areas relative to standard curves of the pure protein using Millenium software (Waters). The amounts of peptides generated by α-la hydrolysis were calculated by taking into account the ratio between the peak area of α-la and the sum of the peak areas of α-la peptides after hydrolysis. Chromatographs were carried out three times.

The photodiode array detector and the Millenium software were used to compare untreated β-lg and the β-lg resulting from the purification process. The comparison was performed by u.v. spectral analysis between 250 and 300 nm (Zhao *et al.* 1995).

SDS gel electrophoresis

SDS gel electrophoresis was performed in a Mini-Protean II cell (Bio-Rad, F-94203 Ivry-sur-Seine, France) according to the standard procedure (Laemmli, 1970). Gels contained 140 g acrylamide/l and were stained with Coomassie blue. Low molecular mass markers (6·5–66 kDa) were from Sigma (St Louis, MO 63178, USA). Chromatographic fractions were assigned by SDS-PAGE according to their molecular masses.

Batch hydrolysis of whey

Clarified whey (100 ml) was adjusted to pH 2 with 1 M-HCl in a 150 ml thermostatted and stirred reactor. When the temperature reached 40 °C, enzyme (porcine pepsin A, EC 3.4.23.1; Sigma) was added at an enzyme:substrate ratio of 1:50 (w/w, relative to total protein content). The pH was kept at 2 by a Titrino 716 pH-stat (Metrohm, CH-9100 Herisau, Switzerland) delivering 0·197 M-HCl and the temperature at 40 °C with a cryostat (Julabo, D-77960 Seelbach, Germany). Samples (0·5 ml) were collected regularly and added to 1 ml 0·5 M-Tris-HCl buffer, pH 7·0 to inactivate pepsin before analysis of protein content by HPLC.

Ultrafiltration operations

The 150 ml reactor used for batch reactions was coupled to a Carbosep module composed of a Tuthill D7428 (Tuthill, Concord, CA 94520, USA) magnetically coupled gear pump and a Carbosep mineral membrane (a single tube 0·4 m long, i.d. 6 mm, surface area 8000 mm^2; Carbosep, F-01703 St-Maurice de Beynost, France). Three different MMCO membranes were used: M5, M7 and M8 (10, 30 and 50 kDa respectively). In these experiments permeate was recycled to the tank reactor. Ultrafiltration experiments were performed both with and without pepsin; those without pepsin were termed recycled ultrafiltration (recycled UF) and those with pepsin recycled hydrolysis.

The whey circulated in the reactor and the transmembrane pressure was set to 0·1 MPa by adjusting the pump speed. The resulting tangential velocity was 4 m/s. When the temperature was stabilized at 40 °C (after 5 min), recycled UF began. The same conditions were used for recycled hydrolysis as for batch hydrolysis. Samples of permeate and retentate (0·5 ml) were taken at regular intervals and the protein composition of each sample was analysed by reversed-phase HPLC.

For both types of experiments, the transmission (*Tr*) was calculated as

$100 \times A_p/A_r$, where A represents the chromatographic peak area for protein or peptides in the retentate (r) and permeate (p) for the 50 μl injected.

Continuous hydrolysis

The method described above was used except that the permeate was not recycled to the reactor but collected in a refrigerated container. A level controller maintained the reactor volume (100 ml) by adding fresh clarified whey. To compensate for the loss of enzymic activity by pepsin inactivation or leakage through the membrane (Sannier *et al.* 1994), and to ensure the complete hydrolysis of α-la during the process, a second addition of pepsin (the same amount as that added initially) was made after 1 h of reaction. No α-la was detected in the retentate or the filtrate during the 4 h of the reaction.

The normalized volume was defined as V/V_r, where V is the volume that passed through the membrane and V_r the reactor volume. The amounts of β-lg, α-la and peptides deposited on or within the pores of the membrane were calculated from the differences between the amounts recovered in the reactor retentate and permeate and the amounts introduced into the reactor. The concentration factor was defined as the ratio of chromatographic β-lg area in the final retentate to that in the clarified whey. The purity of β-lg was defined by the concentration ratio $(\beta\text{-lg}/(\beta\text{-lg} + \text{peptides})) \times 100$.

After each experiment, the ultrafiltration unit was washed first with water until a clear permeate was obtained and then with 1 M-NaOH solution at 40 °C for 30 min. The membrane was then rinsed with water until the original water flux was restored.

Validity of results

All experiments were performed in duplicate. The experimental errors on surface determination were $< 3\%$. Consequently, the experimental errors for the concentrations of α-la, β-lg and peptides and the recovery of β-lg were $< 3\%$. Precision was better than 6% for transmission, concentration factor and purity.

RESULTS

Separation of goat whey protein

The goats' whey was resolved by reversed-phase HPLC. Five distinct fractions were separated, collected and freeze dried (Fig. 1) and then analysed by SDS-PAGE and compared with the electrophoretic pattern of raw milk and goats' whey. The whey fraction was free from casein. No bands appeared for fractions 1 and 2. Fraction 1 was probably an injection peak and fraction 2 contained little protein or peptides too small (< 6 kDa) to be detected under our electrophoresis conditions. Fraction 3 contained a single band corresponding to α-la. Fraction 4 was identified as SA and fraction 5 as β-lg. Using the standard peak area curves for each protein, the contents of the main whey proteins in the clarified whey were found to be 3·2 g β-lg/l and 1·9 g α-la/l. SA was not quantified.

Peptic hydrolysis of goat whey proteins in a batch reactor

The changes in the α-la and β-lg chromatographic peak areas during peptic hydrolysis of clarified whey were followed as a function of time. β-Lactoglobulin remained constant for > 3 h at 40 °C and pH 2 whereas α-la was completely hydrolysed within 60 min. The traces of SA disappeared during the first few minutes (results not shown).

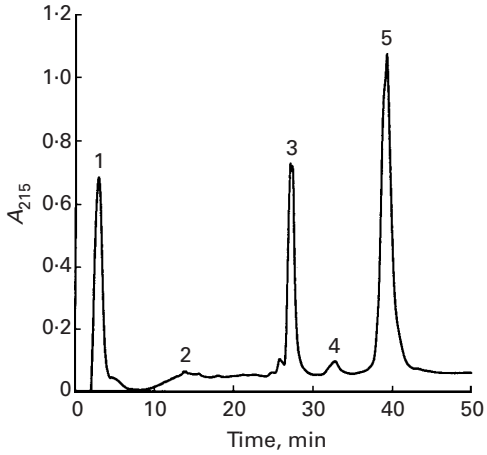

Fig. 1. HPLC chromatogram of clarified goats' whey. For separation conditions, see Materials and Methods. 1, Injection peak; 2, not determined; 3, α-lactalbumin; 4, serum albumin; 5, β-lactoglobulin.

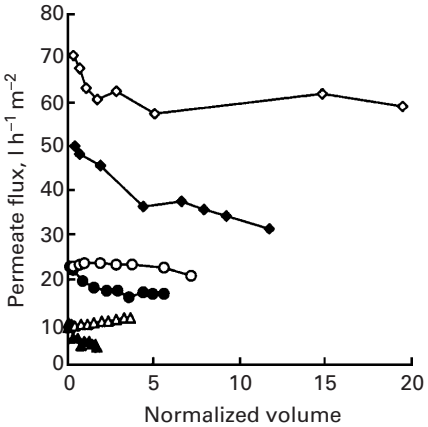

Fig. 2. Changes in permeate flux during ultrafiltration of ▲, ●, ◆, untreated and △, ○, ◇, hydrolysed goats' whey using membranes with molecular mass cut-off of ▲, △, 10; ●, ○, 30 and ◆, ◇, 50 kDa. Normalized volume is defined as V/V_r, where V is the volume passed through the membrane and V_r is the reactor volume.

Recycled ultrafiltration and recycled hydrolysis

Effect of molecular mass cut-off on recycled ultrafiltration. For the three membranes, there was a rapid decrease in protein concentration in the reactor during the first few minutes (~7%) followed by a steady state (results not shown). The supposition is that this protein was deposited at the membrane surface to form the polarization layer. The initial permeate fluxes were related to the MMCO of the membrane, the highest MMCO giving the highest permeate flux (Fig. 2). The *Tr* of α-la and β-lg were also related to the MMCO, the highest MMCO giving the highest *Tr* (Fig. 3). It should therefore be noted that for the M7 (Fig. 3*b*, *e*) and M8 (Fig. 3*c*, *f*) membranes, the *Tr* steadily decreased whereas it had a tendency to increase for the M5 (Fig. 3*a*, *d*) membrane. For the M5 membrane, the increase in *Tr* for both β-lg and α-la was related to a steady increase in their concentrations in the permeate (results not shown).

Effect of molecular mass cut-off on recycled hydrolysis. For all three membranes, the

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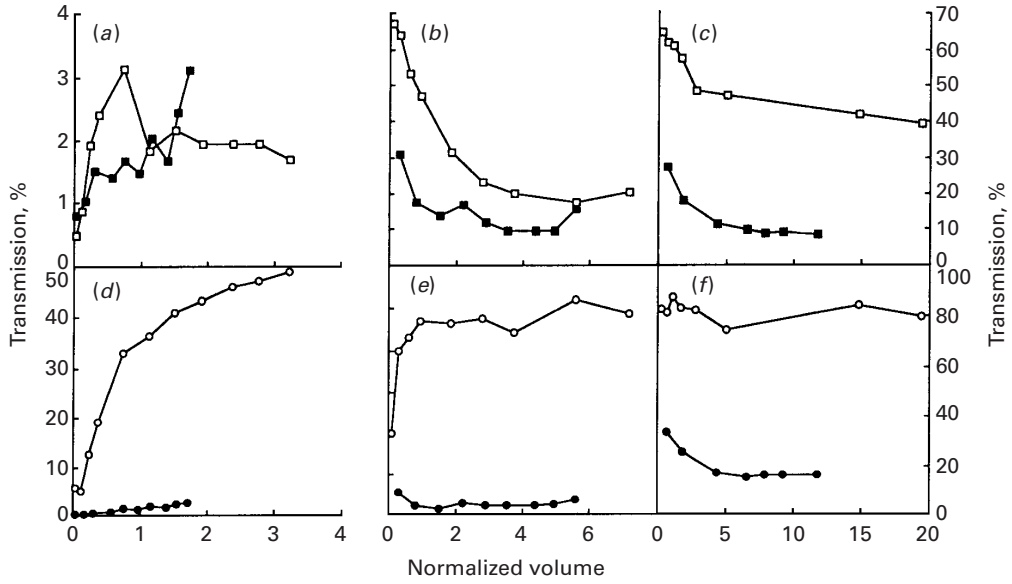

Fig. 3. Changes in the transmission during ultrafiltration of goats' whey using membranes with molecular mass cut-off of (*a*, *d*) 10, (*b*, *e*) 30 and (*c*, *f*) 50 kDa of ■, β-lactoglobulin, recycled ultrafiltration; □, β-lactoglobulin, recycled hydrolysis, ●, α-lactalbumin, recycled ultrafiltration; ○, α-lactalbumin peptides. Recycled hydrolysis and recycled ultrafiltration refer to trials with and without pepsin respectively. Normalized volume is defined as V/V_r, where V is the volume passed through the membrane and V_r is the reactor volume.

final permeate fluxes in the presence of pepsin were significantly higher than for recycled UF (Fig. 2). The initial *Tr* for β-lg was a function of the MMCO: similar to that for recycled UF for the M5 membrane (Fig. 3*a*), but higher for the M7 (Fig. 3*b*) and far higher for the M8 (Fig. 3*c*). Peptides *Tr* for the M5 and M7 membranes increased gradually to 50% (Fig. 3*d*, *e*) but the plateau was reached more rapidly with the M7 membrane. This *Tr* increase was accompanied by an increase in permeate flux. The highest peptide *Tr* was obtained with the M8 membrane (Fig. 3*f*).

Continuous hydrolysis in an ultrafiltration reactor

The choice of the membrane MMCO for the continuous process was made according to the constraints of the process: the membrane should retain β-lg while allowing peptides generated by α-la hydrolysis to permeate through it. Although the M5 membrane efficiently retained β-lg, it also retained peptides, especially at the beginning of hydrolysis (normalized volume < 1). Permeate fluxes were also too low with the membrane area used for this study. The M8 membrane gave a high permeate flux and high peptide permeation but also high leakage of β-lg. The best compromise was found with the M7 membrane with *Tr* at the end of the reaction of 4% for β-lg and 48% for α-la peptides. Hence the M7 membrane was selected for continuous hydrolysis.

For continuous hydrolysis, 800 ml clarified whey was hydrolysed in the reactor, which was equivalent to seven normalized volumes passing through the membrane in 270 min. The permeate flux decreased from 23 to 15 l h^{-1} m^{-2}. The *Tr* of β-lg and peptides decreased from 7 to 4% and from 56 to 48% respectively. The distribution of β-lg and peptides in the three compartments of the reactor were as follows: 74% of the β-lg was recovered in the retentate, 18·6% in the permeate and 7·4% on the

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membrane (0·024 mg/mm²). For the peptides, 22·7 % was recovered in the retentate, 61·4 % in the permeate and 15·9 % on the membrane (0·03 mg/mm²). The purity of the β-lg was 84 % and the concentration factor was 6·6 (19·2 g/l in the retentate). The β-lg obtained by this process was identical to untreated β-lg according to u.v. spectral analysis using the photodiode array detector.

DISCUSSION

The effect of pepsin on goats' whey proteins was in agreement with previous studies that reported the resistance of bovine β-lg towards peptic hydrolysis (Schmidt & Poll, 1991; Guo *et al.* 1995) whereas α-la was hydrolysed (Reddy *et al.* 1988; Miranda *et al.* 1989; Schmidt & van Markwijk, 1993). The goat β-lg peptide chain differs from the bovine β-lg B, C and D variants by 6, 7 and 8 changes respectively. None of these substitutions occurs in the sequence regions that are thought in the bovine proteins to be involved in α-helices or the β-structure that contains the thiol group. The conformations of goat and bovine β-lg must be very similar (Préaux *et al.* 1979). Goat α-la is more similar to bovine α-la than any other known variant, differing from it by only 12 amino acid residues out of 123 (MacGillivray *et al.* 1979).

During the recycled UF experiments the decreases in permeate flux were ascribed to membrane fouling. This causes reductions in the thermodynamic driving force across the membrane by protein osmotic pressure, pore constriction or blockage by protein deposition or gel layer formation (Marshall *et al.* 1993). This flux reduction was not correlated with protein transmission since *Tr* increased with M5 membranes (Fig. 3*a*) but decreased with M7 (Fig. 3*b*) and M8 (Fig. 3*c*) membranes. The low *Tr* for protein for M5 membranes ($< 5\%$) resulted in concentration polarization at the membrane surface as the cumulative volumes passed through the membrane. The protein concentration at the membrane surface increased and the mass flux to the membrane (defined as protein concentration at the membrane surface × permeate flux) increased in the same manner. This resulted in an increase in the *Tr* that was only apparent, since it did not take into account the actual concentration at the membrane surface. It is likely that the same protein deposition occurred on M7 and M8 membrane surfaces, since the composition of the mineral filtering layer, the whey composition and the transmembrane pressure that could have compressed the deposits were all the same. Nevertheless protein *Tr* decreased with M7 and M8 membranes. We suggest that the decrease in *Tr* for M7 and M8 membranes was the result of protein deposition within the membrane (irreversible fouling). This phenomenon was much more important than the apparent mass flux increase due to protein deposition at the membrane surface (reversible fouling). It is probable that the higher pore sizes of M7 and M8 membranes were responsible for the overall decrease in protein transmission, since it has been shown that membranes are more subject to irreversible fouling by protein deposition within the pores when they are larger (Marshall *et al.* 1993). The difference between the M5 membrane and the other two was probably the result of competition between reversible and irreversible fouling.

In recycled hydrolysis the final permeate flux and *Tr* were greater than for recycled UF. As the only difference between the two was the conversion of α-la into peptides by pepsin, it seems that α-la peptides were less fouling than intact α-la. Although considerable work has been done on α-la and β-lg ultrafiltration, there is no further information available for experiments performed at pH 2.

For the continuous hydrolysis procedure, the recovery yield of β-lg from the reactor was comparable to that for the precipitation methods using trichloroacetic acid (Caessens *et al.* 1997), $FeCl_3$ (Kuwata *et al.* 1985), NaCl (Mailliart & Ribadeau-Dumas, 1988) or differential hydrolysis followed by ammonium sulphate precipitation (Kinekawa & Kitabatake, 1996). These methods give an overall β-lg recovery of 60–84 %, but need a purification step of ultrafiltration or diafiltration to remove the excess trichloroacetic acid, iron or salt. With the ultrafiltration membrane enzymic reactor, no further treatment was needed, since the contaminants were peptides that would not prevent the direct use of the β-lg-enriched fraction as a food ingredient. Adjusting the pH to neutrality could inactivate any residual pepsin. The purity of the β-lg could be increased by removing the remaining peptides by diafiltration with the same reactor after the simultaneous purification and concentration of β-lg. Bearing in mind the efficiency of this process with a clarified acid goats' whey, it would be interesting to apply it to bovine whey or wheys produced during the manufacture of speciality goat cheeses.

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