Isolation and purification of serum and interfacial peptides of a trypsinolyzed β-lactoglobulin oil-in-water emulsion

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Abstract

Information on the conformation of proteins adsorbed to an oil–water interface is usually determined by following the time course of enzymatic hydrolysis of the protein in an oil-in-water emulsion. Unlike previous works reported in the literature, the research presented in this paper provides information on which peptides are actually in contact with the lipid bilayer (interfacial peptides) and those segments that project into the aqueous phase (serum peptides). In order to achieve this classification of peptides, we present a method to separate serum peptides from interfacial peptides by initial centrifugation steps followed by reversed-phase high-performance liquid chromatography. The effectiveness of the method was ascertained by performing proteolysis on β-lactoglobulin adsorbed to an oil-water interface in a soybean oil-water emulsion. It was found that more peptides are qualitatively and quantitatively found adsorbed to the oil–water interface as compared to peptides released into the serum. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Oil-in-water; Peptides; β-Lactoglobulin

1. Introduction

Protein adsorption is a very important area of research, as many proteins readily adsorb to solid/liquid interfaces and at liquid/liquid interfaces (e.g. in the preparation of oil-in-water emulsions). Oil-in-water (o/w) emulsions are oil droplets dispersed in water and are stabilized by surfactants (e.g. proteins adsorbed to the oil-water interface). An understanding of how such proteins contribute to the stability of emulsions requires knowledge of the molecular structure of the adsorbed protein. For emulsions, proteolysis of the adsorbed protein is the only suitable method for the determination of information on its conformation [1–6]. The initial work by Shimizu et al. [1] outlined the use of the proteolytic enzyme, trypsin, in performing protein structure studies of α_{s1}-casein adsorbed to a soybean o/w interface (a single pure-protein model system). Later work by Leaver and Dalgleish [4,5] concentrated on determining the conformation of β-casein, also on a soybean o/w interface. The results of these previous studies do not provide specific information on secondary, tertiary or quaternary structures, but instead

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give information on the change in conformation of protein molecules when they are adsorbed to an interface, by identifying the portions of the molecule which were particularly susceptible to enzymatic attack. Although, the time courses of formation and degradation of various peptides were followed during the enzymatic hydrolysis of the emulsion, no distinction was made as to which peptides were experimentally derived from the interface to those that were in the serum fraction of the hydrolysed emulsion. The separation of serum peptides from peptides attached at the interface (interfacial peptides) is important, as it will provide information on segments of the protein that are actually in contact at the o/w interface and those that are projecting into the serum, at specific times of hydrolysis. In addition, knowledge of serum and interfacial peptides can also facilitate the designing of better protein surfactants for food and drug products. Therefore, we present a technique to isolate peptides that remained adsorbed to the o/w interface from peptides liberated into the serum by performing proteolysis on β-lactoglobulin (a globular protein; unlike α_{s1}- and β-casein which possess minimum secondary structural elements) adsorbed to an o/w interface.

2. Experimental

Unless specified elsewhere, water was of Milli-Q quality and GS filters were obtained from Millipore Corporation, Mississauga ON, Canada. All chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA. Chromatography columns were purchased from Pharmacia Biotech, Baié d'Urfé, Quebec, Canada. Centrifugation was carried out using an Eppendorf Centrifuge 5415C (Brinkmann Instruments Inc., N.Y., USA).

2.1. Purification of β-lactoglobulin

Crude β-lactoglobulin was obtained from Protose Separation Inc., Teeswater, Ontario at 80% purity and was purified by anion-exchange chromatography using Q Sepharose® Fast Flow gel [7,8]. The purified protein was dialysed against water using a Spectra/Por porous membrane (molecular weight cutoff: 6–8

kDa, Fisher Scientific Corporation, Mississauga, ON).

2.2. Emulsion preparation

Oil-in-water emulsions (20% v/v; oil:protein solution) were made fresh each day with a Microfluidizer (Model M 110S, Microfluidics Corporation, Newton, MA). The effective homogenization pressure was 42 Mpa. The first release of emulsion was collected and then quickly poured back into the inlet chamber of the Microfluidizer and re-circulated through the homogenizing chamber for ten strokes of the pump. Unadsorbed protein was removed by ultracentrifugation at 20 600 *g* for 1 h at 4°C using a Beckman L8-M Ultracentrifuge (Beckman ON, Canada). Upon centrifugation the emulsion separated into two phases, a white creamy upper layer and a clear lower liquid layer. The subnatant (serum) was removed with the use of a 18 mm gauge syringe and discarded. The remaining cream fraction was then resuspended in 20 m*M* potassium phosphate buffer (0.1% sodium azide, pH 7.0) and this resuspended emulsion was used in all later experiments.

2.3. Separation of interfacial peptides from serum peptides

Initial experiments on the trypsinolysis of adsorbed β-lactoglobulin produced many peptides, some of which were highly hydrophobic and tended to remain at the emulsion interface. To obtain serum and interfacial peptides, an Eppendorf tube containing 1.5 ml of hydrolysed emulsion was centrifuged at 19 500 *g* for 10 min at room temperature. The cream settled at the top and the serum formed the lower aqueous layer. With a syringe the serum fraction was removed and placed into a fresh Eppendorf tube. This serum fraction was treated with a chloroform/methanol (vol. ratio 2:1) to remove lipids and centrifuged for 10 min at 19 500 *g*. The aqueous phase containing serum peptides was removed and lyophilized. The cream fraction was resuspended in 1.0 ml of phosphate buffer and Tween 20 was added to a final concentration 6.0%. The sample was stirred for 1 h followed by addition of 0.5 ml delipidating mixture and centrifuged for 10 min at 19 500 *g*. The upper aqueous-methanol phase

containing peptides from the interface was extracted and lyophilized.

2.4. Qualitative assessment of peptide removal from an oil–water interface by reversed-phase high-performance liquid chromatography (RP-HPLC)

To determine the efficiency of recovery of interfacial peptides an aliquot of resuspended emulsion was incubated at 37°C with TPCK type XIII trypsin from bovine pancreas (EC 3.4.21.4) at an enzyme:substrate ratio of 1:40. The reaction was inhibited with 1 mM PMSF after 24 h of enzyme hydrolysis. A 1.0 ml aliquot was treated to isolate interfacial peptides and serum peptides using Tween 20. Another 1.0 ml aliquot was immediately used to remove peptides from the whole emulsion by adding 0.5 ml chloroform/methanol (2:1) mixture and then centrifuging at 19 500 g for 10 min. The upper methanol fraction was removed and lyophilized. Peptides from the serum, interface and the whole emulsion were analyzed by RP-HPLC.

2.5. Trypsinolysis of a 1.0% aqueous solution of β-lactoglobulin

A 1.0% (w/v) solution of β-lactoglobulin was prepared by dissolving the protein in phosphate buffer. This stock solution was stored at 4°C and was used for a maximum of 1 week. Protein hydrolysis was carried out at 37°C by adding trypsin at an enzyme:substrate ratio of 1:1000. After 5 s, 60 s and 60 min intervals, 1.5 ml aliquots were removed from the hydrolysed mixture and the reaction stopped by addition of 1 mM PMSF. The hydrolysates were then lyophilized and later prepared for RP-HPLC.

2.6. Trypsinolysis of 1.0% β-lactoglobulin oil–water emulsion

Emulsions were prepared, trypsinolysed, and the serum and interfacial peptides were separated. Excess Tween 20 from interfacial peptides were removed with the use of C$_{18}$ Sep-Pak cartridges No. 51910. (Waters Associates, Millipore Corporation, Milford, MA). The serum and interfacial peptides were prepared for RP-HPLC in the same way as was done for peptides from the hydrolysis of β-lactoglobulin in solution.

2.7. Amino acid analysis

Individual peptides obtained from HPLC analysis were repurified on an Ultropack Lichrosorb RP18 column (4×250 mm) kept at a temperature of 40°C. The flow-rate was set at 0.01 ml/min and absorbance readings taken at 220 nm. Amino acid analysis of individual peptides was carried out on a PicoTag Work Station according to the Manufacturer's protocol (Millipore Corporation, Waters Chromatography Division, Milford, MA).

3. Results and discussion

3.1. Qualitative analysis of peptides extracted from the oil–water interface using Tween 20

The peptide profile of the serum phase of the emulsion (Fig. 1a) was similar qualitatively to the profile of the peptides extracted by just using the chloroform/methanol mixture to the whole hydrolysed emulsion (Fig. 1c). However, there were larger quantities of peptides still adsorbed to the interface (Fig. 1b) which were not recovered by using the chloroform/methanol mixture on the whole hydrolysed emulsion. The method involving the use of Tween 20 showed that a large amount and number of different peptide products of trypsinolysis remained associated with the emulsion interface. Previous methods [1–5] did not consider the production of peptides that remained attached to the interface and hence product peptides analyzed in these methods were mainly those of the serum fraction. It is possible to suggest that the delipidating procedure does not totally break up the cream structure and release peptides that are attached to the interface. Instead, the delipidating mixture causes the formation of an intermediate precipitate layer (containing peptides and protein) that separates the aqueous–methanol fraction from the chloroform–lipid fraction. The presence of large quantities of peptides at the interface implies that β-lactoglobulin is a good source of surface-active peptides as stated by Turgeon et al. [9].
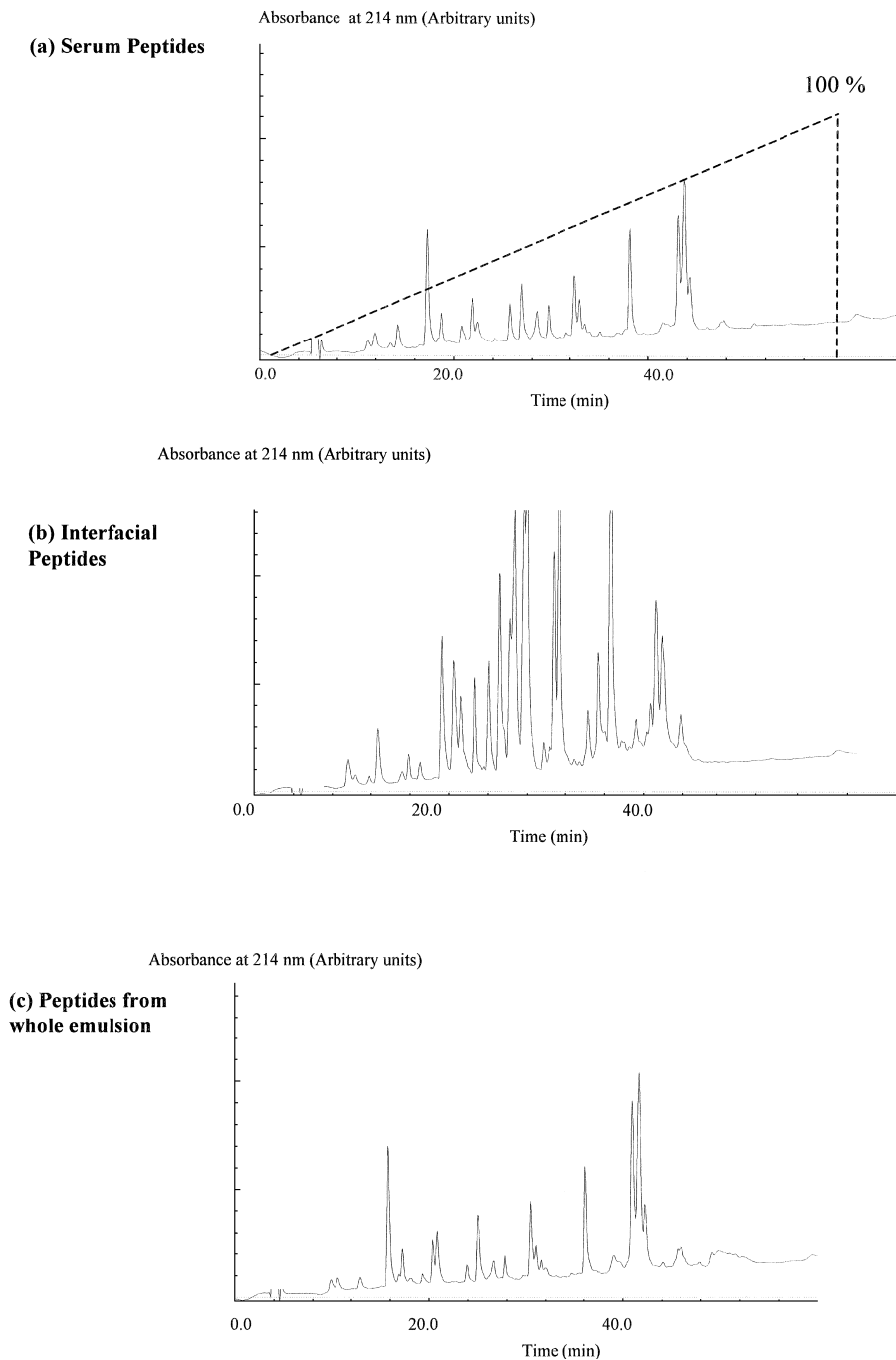
Absorbance at 214 nm (Arbitrary units)

(a) Serum Peptides

100 %

0.0   20.0   40.0

Time (min)

Absorbance at 214 nm (Arbitrary units)

**(b) Interfacial
Peptides**

0.0   20.0   40.0

Time (min)

Absorbance at 214 nm (Arbitrary units)

**(c) Peptides from
whole emulsion**

0.0   20.0   40.0

Time (min)

Fig. 1. The RP-HPLC peptide profile of the (a) serum phase and (b) interfacial peptides from an hydrolyzed emulsion. (c) Shows the peptide profile obtained from adding chloroform/methanol to the whole hydrolyzed emulsion without separation of serum and interfacial peptides. Peptides were eluted on a reverse phase PepRPC HR 5/5 column (Pharmacia FPLC System, Pharmacia LKB Biotechnology) using an acetonitrile gradient. Absorbance was measured at 214 nm with a sensitivity setting of 0.2.
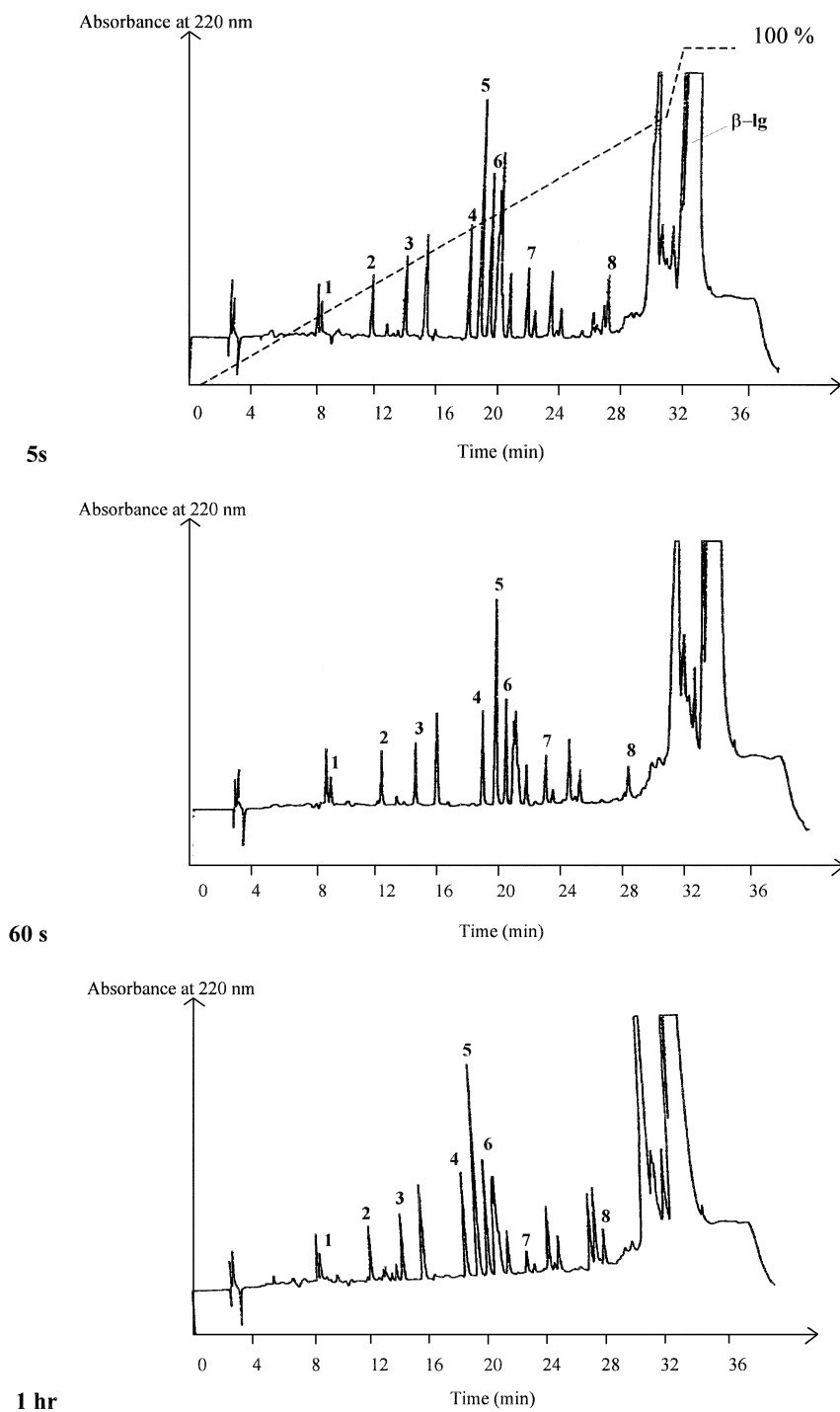
Fig. 2. RP-HPLC chromatograms for trypsinolysis of β-lactoglobulin (in solution) at different times (enzyme:substrate ratio 1:1000). The hydrolysates were loaded onto a Ultropack Lichrosorb RP 18 column (4×250 mm) at a flow-rate of 1 ml/min. Elution was carried out using an acetonitrile gradient.

3.2. Hydrolysis of β-lactoglobulin in solution and in an emulsion

At very low enzyme concentration the hydrolysis occurred only for a short time (Fig. 2). There was an initial rapid enzymatic attack within the first 5 s, after which the enzyme was either inhibited or inactivated by peptide products. Alternatively, only a small fraction of the substrate may be in a physical state, which is susceptible to trypsinolysis. The slow hydrolysis in solution of β-lactoglobulin has also been observed in other works [10,11]. Some of the peaks collected and later identified by amino acid analysis are numbered from 1 to 8 (Table 1).

3.3. Hydrolysis of β-lactoglobulin adsorbed to an oil–water interface in an emulsion

During the first 5 s of hydrolysis (Fig. 3) few peptides in small quantities, appear in the serum. However, the intensity of the identified peaks slowly increased within the first hour. There was no β-lactoglobulin in the serum phase, which means that all the β-lactoglobulin was probably adsorbed to the o/w interface of the emulsion. The pattern of peptides released into the serum (Fig. 3) was different from that found for the dissolved β-lactoglobulin (Fig. 2). In particular, the group of peptides 4, 5 and 7 (in Fig. 2) was difficult to detect in the peptides released into the serum especially in the early stages of the reaction. In contrast, peptides 2 and 8 were

always present in the serum fraction. For interfacial peptides (Fig. 4) it was found that during the first 60 s, a large number of peptides were formed, after which the pattern did not change much. This supports the observation that the enzyme becomes inhibited after the early stages of hydrolysis of the emulsion, as was found for the hydrolysis of β-lactoglobulin in solution. When the chromatographic pattern of the interfacial peptides are compared with those chromatograms of the serum peptides, it can be seen that there are qualitatively and quantitatively more peptide products found on the interface than in the serum at any particular time of enzyme hydrolysis. Specifically, it can be seen that peptides 1, 3 and 6, which are not found in the serum of the proteolyzed emulsion, are present at the interface, at an early stage. It is reasonable to infer that some of the areas of attachment of the adsorbed β-lactoglobulin to the oil–water interface occurs at segments 139–141 (peptide 1) and 84–101 (peptides 3 and 6). Overall, analysis of both serum and emulsion interface is required to understand the behavior of the adsorbed protein. We have outlined in this research an improved method for the determination of structural information of adsorbed proteins at an oil–water interface. Future work would concentrate more on the early phase of trypsinolysis (along with statistical analysis) to follow the kinetics of release of peptides during the first minute. The hydrolysis of β-lactoglobulin by trypsin only identifies regions where positively charged lysine and arginine residues are present. The location of these residues may be only a part of a region of the protein that may undergo many other conformational changes, i.e. loops and trains [12,13]. Therefore, it is necessary to use other proteases to get a complete peptide mapping of the adsorbed protein. This can be achieved with the use of proteases with different peptide bond specificities, to provide more information about specific regions and so add to the information already obtained from the work on trypsin.

Acknowledgements

Table 1
The amino acid sequence of major peptides collected and re-purified from RP-HPLC (see text for details)

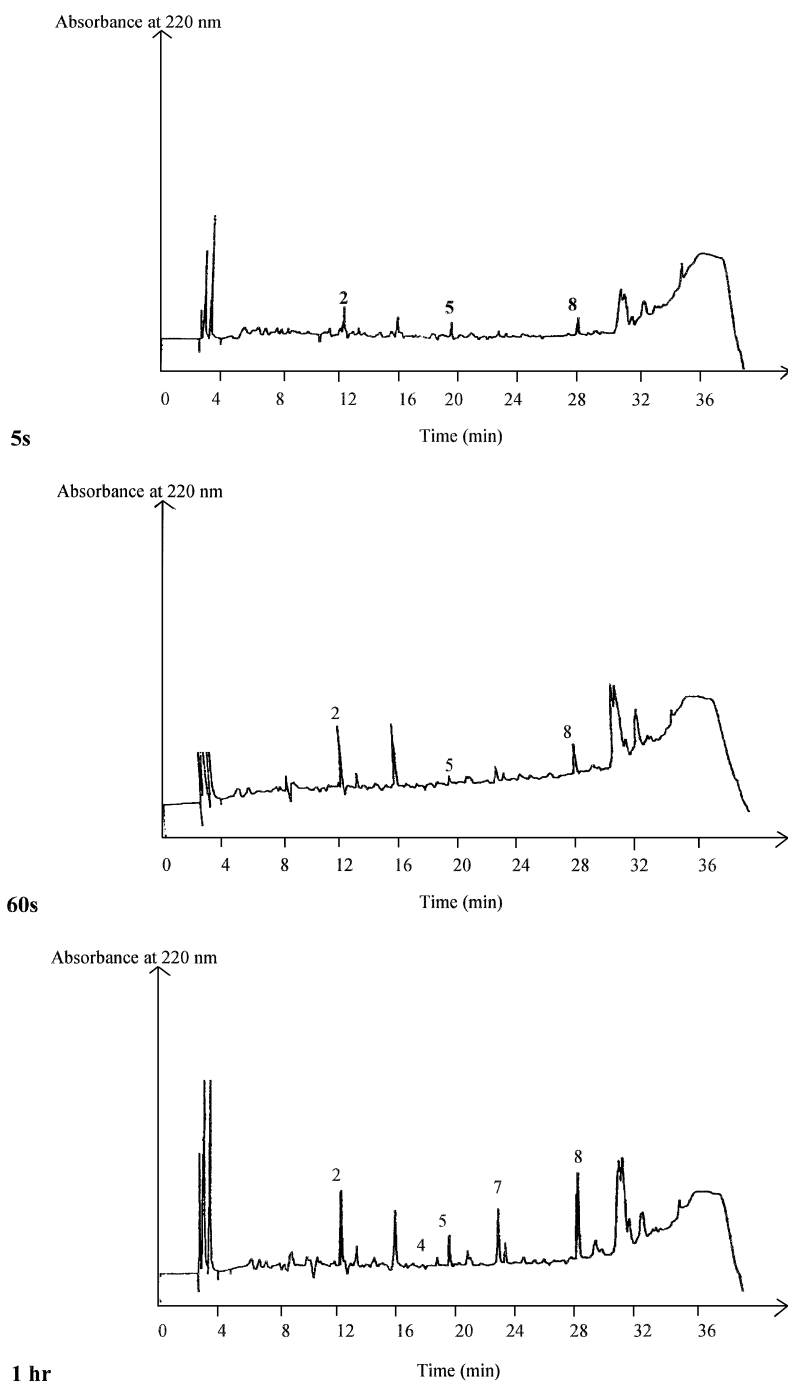| Peptides		Amino Acid Sequence
1	(139-141)	A-L-K
2	(71-75)	I-I-A-E-K
3	(84-91)	L-D-A-I-N-E-N-K
4	(1-8)	L-I-V-T-Q-T-M-K
5	(142-148)	A-L-P-M-H-I-R
6	(92-101)	V-L-V-L-D-T-D-Y-K-K
7	(61-69 + 149-162)	W-E-N-D-E-C-A-Q-K ⌐ L-S-F-N-P-T-L-Q-E-E-Q-C-H-I
8	(41-60)	V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K

Fig. 3. RP-HPLC chromatograms for serum peptides obtained from the trypsinolysis of β-lactoglobulin in an emulsion (enzyme:substrate ratio 1:1000). The serum peptides were obtained by first centrifugating the hydrolysed emulsion and removing the subnatant with a 18 mm needle syringe The hydrolysates were loaded onto a Ultropack Lichrosorb RP 18 column (4×250 mm) at a flow-rate of 1 ml/min. Absorbance was measured at 220 nm.
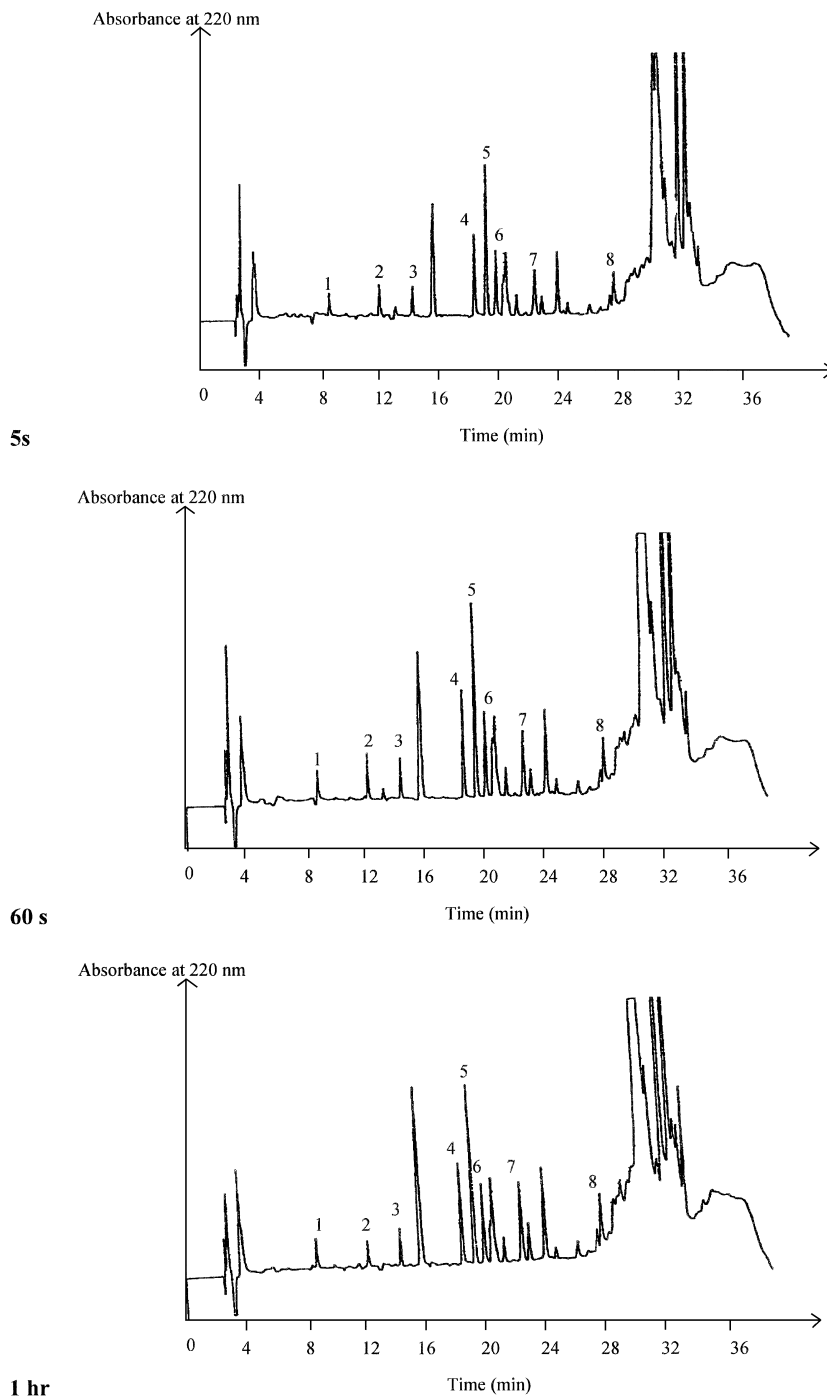
Fig. 4. RP-HPLC chromatograms for interfacial peptides obtained from the trypsinolysis of β-lactoglobulin in an emulsion (enzyme:substrate ratio 1:1000). The interfacial peptides were obtained by adding Tween 20 to the cream phase after first centrifugating the hydrolysed emulsion. The Tween 20 dissolved hydrolysates were loaded onto a Ultropack Lichrosorb RP 18 column (4×250 mm) at a flow-rate of 1 ml/min. Absorbance was measured at 220 nm.

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