β-Lactoglobulin Hydrolysis. 2. Peptide Identification, SH/SS Exchange, and Functional Properties of Hydrolysate Fractions Formed by the Action of Plasmin

Petra W. J. R. Caessens,[†,‡] Willeke F. Daamen,[†] Harry Gruppen,[†] Servaas Visser,[‡] and Alphons G. J. Voragen*[,†]

Department of Food Technology and Nutritional Sciences, Division of Food Science, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, and Department of Product Technology, NIZO Food Research, P.O. Box 20, 6710 BA Ede, The Netherlands

β-Lactoglobulin (βLg) was hydrolyzed by plasmin to a degree of hydrolysis of 4%. The hydrolysate was fractionated by ion-exchange chromatography and subsequent hydrophobic-interaction chromatography. The βLg peptide fraction consisting of smaller peptides (mostly <2 kDa) had poor foam- and emulsion-forming and -stabilizing properties. Most of the βLg peptides were identified (in either the nonreduced or reduced form) by mass spectrometry on the basis of the known primary structure of the intact protein and the specificity of the enzyme. The peptides formed during βLg/plasmin-hydrolysis were (1) peptides lacking a cysteyl residue, (2) peptides composed of a single amino acid chain containing intramolecular disulfide bonds, and (3) peptides composed of two amino acid chains linked by an intermolecular disulfide bond. It appeared that significant SH/SS-exchange had taken place during hydrolysis. Many of the peptides present in the peptide fraction that exhibited good functional properties were disulfide-linked fragments.

Keywords: *β-Lactoglobulin; plasmin; peptide identification; SH/SS exchange; mass spectrometry; foam; emulsions*

INTRODUCTION

The formation and stabilization of foam and emulsions by β-lactoglobulin (βLg)/plasmin, βLg/trypsin, and βLg/*Staphylococcus aureus* V8 protease hydrolysates were described in the first part of this study (Caessens et al., 1999a). The functional properties at pH 6.7 of the βLg/plasmin hydrolysate with a degree of hydrolysis of 4% (DH4) were improved or similar compared to those of the intact molecule. Reversed-phase HPLC showed that this βLg/plasmin DH4 hydrolysate contained peptides eluting in the second part of the chromatogram, indicating that the peptide material present was hydrophobic and/or had a rather high molecular weight. Furthermore, this hydrolysate contained many peptides that were linked by disulfide bonds as determined by comparative gel-permeation chromatography under nondenaturing, denaturing, and denaturing plus reducing conditions (Caessens et al., 1999a).

βLg has a monomer molecular mass of about 18.3 kDa (βLgA = 18362 Da and βLgB = 18277 Da). The protein has two disulfide bonds, between residues 106 and 119, and between residues 66 and 160, and a free thiol group at residue 121 (Swaisgood, 1982; Hambling et al., 1992). The free thiol group may contribute to the maintenance of βLg's tertiary structure via water-mediated H-bonding (Burova et al., 1998). In the native protein, the free thiol group is buried in the interior of the molecule, but at higher pH (i.e., above pH 7.5; Tanford et al., 1959) and/or elevated temperatures (Iametti et al., 1996), the protein undergoes conformational changes resulting in an increased reactivity of the thiol group. Thiol/disulfide exchange plays a role in the heat-induced aggregation of βLg (Roefs and De Kruif, 1994; Sawyer et al., 1994). Chen et al. (1994) found that tryptic βLg hydrolysates had a lower gel point and gelled more rapidly than native βLg. One could hypothesize that after βLg hydrolysis the free thiol group is more exposed, initiating SH/SS reshuffling, which would subsequently induce the aggregation and gelling behavior described above.

Several authors have identified peptides produced by βLg/trypsin hydrolysis. Some of these peptides were disulfide linked, such as βLg[f 41−69] −S−S− [f 149−162] (Dalgalarrondo et al., 1990), βLg[f 61−69/70] −S−S− [f 149−162] (Turgeon et al., 1992), βLg[f 41−100] −S−S− [f 149−162] (Chen et al., 1993), and βLg[41−70] −S−S− [f 149−162] (Otte et al., 1997a). The disulfide bond present in these fragments was originally present in the parent protein (i.e., between residues 66 and 160). No disulfide-linked peptides have been identified in βLg hydrolysates formed by other enzymes, such as bromelain, papain, pepsin, or endoproteinase Arg-C (Otte et al., 1997a).

Turgeon et al. (1992) suggested the formation of new disulfide bonds during hydrolysis of βLg at pH 8 due to the increased reactivity of the free thiol group. Recently, strong indications of the occurrence of a newly formed disulfide bond in a βLg peptide have been published (Maynard et al., 1998). The first part of our study (Caessens et al., 1999a) showed that the βLg/plasmin DH4 hydrolysate had good functional properties. In this part of the study, the βLg/plasmin DH4 hydrolysate was fractionated, the functional properties of the fractions were determined, and the peptides present therein were

* To whom correspondence should be addressed. Fax: +31(0)317 484893. E-mail: Fons.Voragen@chem.fdsci.wau.nl.
† Wageningen Agricultural University.
‡ NIZO Food Research.

10.1021/jf981230o CCC: $18.00
Published on Web 07/02/1999

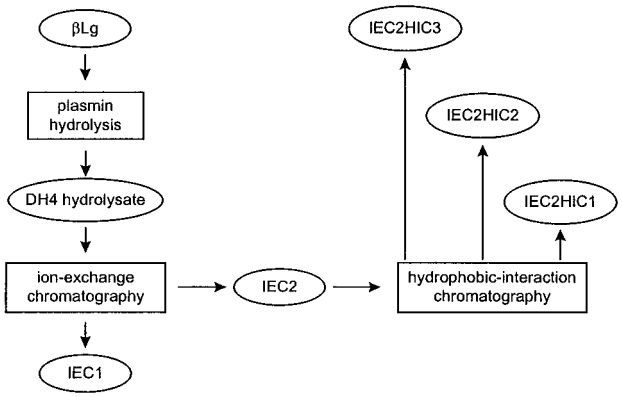

Figure 1. Outline of the βLg hydrolysate fractionation; for abbreviations used see text.

identified to investigate the occurrence and importance of the SH/SS exchange between the βLg peptides. This allows a discussion of the functional properties in relation to the peptide composition, aiming at the establishment of a structure−function relationship.

MATERIALS AND METHODS

Materials. Bovine βLg (95% based on dry weight, w/w) was purified as described previously (Caessens et al., 1997a) and was a mixture of the genetic variants A and B. Bovine plasmin (EC 3.4.21.7) and aprotinin were obtained from Sigma (No. P-7911 and A-6012, respectively). Unless stated otherwise, all other chemicals were of analytical grade and were purchased from Sigma, Merck, Aldrich, or BDH.

***β*-Lactoglobulin Hydrolysis and Fractionation of the Hydrolysate.** βLg was hydrolyzed by plasmin at pH 8.0 and 40 °C to a DH of 4% as described previously (Caessens et al., 1999a). After incubation, the reaction was inhibited by aprotinin (ratio 1/200 v/v of a 10 trypsin inhibitor unit/mL solution); the hydrolysate was lyophilized and stored at 4 °C prior to analysis or fractionation. The latter was performed by ion-exchange chromatography (IEC) and subsequent hydrophobic interaction chromatography (HIC). Figure 1 shows a brief outline of this fractionation.

Preparative IEC was performed on an ÄKTA-explorer, controlled by a UNICORN-control system (Pharmacia, Sweden), using a SourceQ column (280 mL bed volume; Pharmacia) at 20 °C. Solvent A (20 mM bis-Tris/HCl buffer, pH 6.0) and solvent B (20 mM bis-Tris/HCl buffer containing 0.5 M NaCl, pH 6.0) formed the eluent in the following linear gradient steps: 2 min sample injection (25 mL/min); 5 min isocratic elution at 100% A, over 2.5 min to 100% B followed by 2 min isocratic elution at 100% B, over 1 min to 100% A, and finishing with 7.5 min equilibration of the column at 100% A before the next run was started. The column was loaded with maximally 4 mg per mL bed volume. Except as stated otherwise, a flow rate of 44 mL/min was applied, and detection was at 220 and 280 nm. Appropriate IEC fractions were pooled. For analysis and testing, the fractions were desalted by ultrafiltration (OMEGA membrane MWCO of 1 kDa for the IEC1 fraction (Figure 1) and 3 kDa for the IEC2 fraction (Figure 2), Pall Filtron Corp., MA), lyophilized, and stored at 4 °C. Part of the pooled IEC2 fraction was not desalted, but further used for HIC purification.

Preparative HIC was performed on the ÄKTA-explorer described, using a Phenyl Sepharose high-performance column (150 mL bed volume, Pharmacia) at 20 °C. The sample to be purified, i.e., fraction IEC2 (Figure 1), was collected from the preparative IEC runs; ammonium sulfate was added to 1 M, and the pH was adjusted to pH 7.0. The protein load of the HIC column was approximately 1 mg per milliliter of bed volume. Solvent A (50 mM sodium phosphate buffer containing 1.7 M ammonium sulfate, pH 7.0) and solvent B (50 mM sodium phosphate buffer, pH 7.0) formed the eluent in the following linear gradient steps: 20 min sample injection (10 mL/min), 5 min isocratic elution at 35% B, over 1 min to 50% B, after 10 min isocratic elution over 10 min to 100% B, after 15 min isocratic elution over 2 min to 100% A, after 8 min isocratic elution over 2 min to 35% B, and 12 min equilibration of the column before the next run was started. Except when stated otherwise, a flow rate of 21 mL/min was applied, and detection was at 220 and 280 nm. Appropriate HIC fractions were pooled, desalted by ultrafiltration (OMEGA membrane 5 kDa MWCO), lyophilized, and stored at 4 °C prior to analysis.

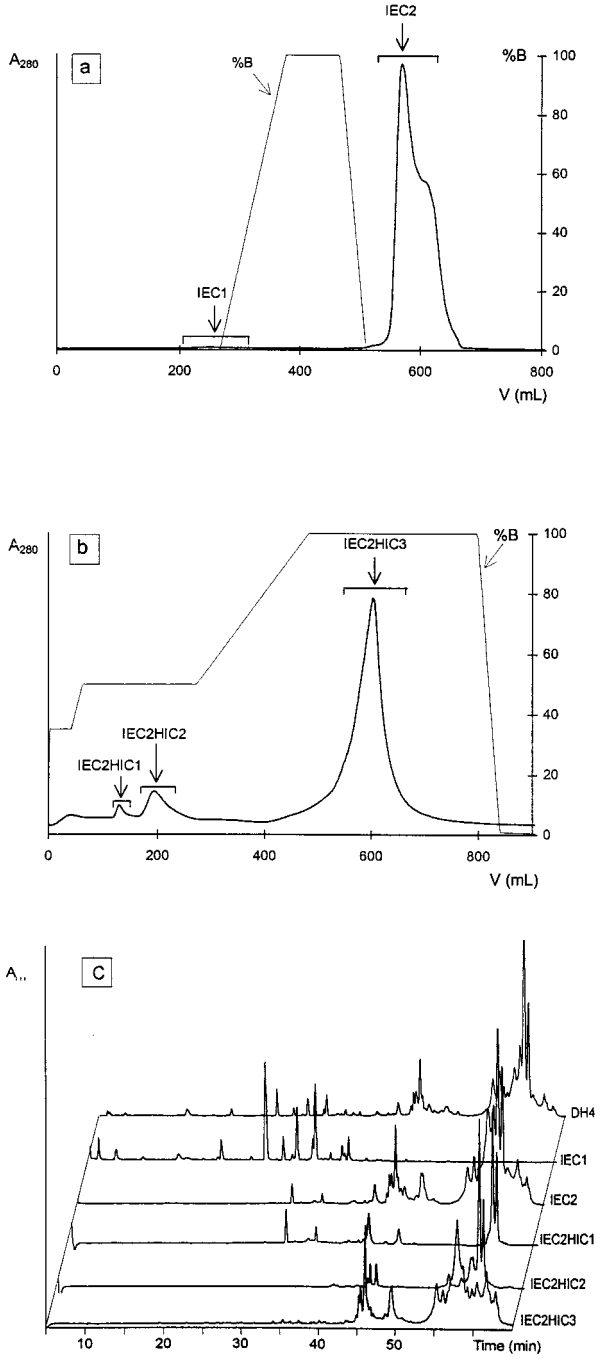

Figure 2. IEC chromatogram of the βLg/plasmin DH4 hydrolysate (a); HIC chromatogram of IEC2 (b); RP-HPLC chromatograms of the peptide fractions obtained by IEC and HIC (c); parts a and b are not adjusted for the void volumes of the columns; for abbreviations see text; for conditions used see Materials and Methods; RP-HPLC gradient RP1.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The RP-HPLC equipment used was described by Visser et al. (1991). A 250 × 4.6 mm i.d. HiPore RP-318 column (Bio-Rad) was used. To analyze the peptide composition of the different βLg peptide fractions (Figure 1),

the same gradient as described before (Caessens et al., 1999a) was used (denoted RP1), and generally 50 μL of a 1 mg/mL peptide solution was injected onto the column for these analytical runs. Gradient RP1 was also used for the semipreparative RP-HPLC (on the same column) of smaller, more hydrophilic peptides present in IEC1 (Figure 1), and 150 μL of a 1 mg/mL peptide solution was injected onto the column. Gradient RP-2, used for the semipreparative RP-HPLC of larger peptides containing many hydrophobic groups, present in IEC2HIC3 (Figure 1) was formed by solvent A [0.1% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile, v/v] and solvent B [0.08% TFA in 90% aqueous acetonitrile, v/v] in the following linear gradient steps: from 18% B to 20% B over 2 min, to 54% B over 104 min, to 70% B over 4 min; after 5 min to 18% B over 5 min and finishing with 17 min isocratic elution at 18% B before the next run was started. A flow rate of 0.8 mL/min was applied. The column temperature was 30 °C, and 150 μL of a 5 mg/mL peptide solution was injected onto the column. Peak detection and quantitation was at 220 nm using Turbochrom data acquisition and processing software (Perkin-Elmer, Germany).

Peptide Identification. The peptides collected by semipreparative RP-HPLC using gradient RP1 were analyzed by electrospray-ionization mass spectroscopy (ESI-MS) on a Quattro II triple quadrupole instrument (Micromass, U.K.) as described before (Caessens et al., 1999b). Peptide identification was obtained from the molecular mass determined combined with the sequence data of intact βLg (Hambling et al., 1992) and the known specificity of plasmin (Arg-X and Lys-X; Bastian and Brown, 1996). The peptides collected by semipreparative RP-HPLC using gradient RP2 were analyzed under nonreducing conditions by ESI-MS as described before (Caessens et al., 1999b) and under both nonreducing and reducing conditions by matrix-assisted laser-desorption/ionization time-of-flight MS (MALDI-TOF MS). The latter was performed on a Voyager-DERP (PerSeptive Biosystems) in the linear mode, controlled by Voyager RP software. The samples were dissolved in a 20 mM tris/HCl buffer (pH 7) with and without 20 mM dithiothreitol for the reducing and nonreducing conditions (reduction for approximately 30 min at ambient temperature), respectively; 1 μL of this peptide solution was mixed with 9 μL of matrix solution. The matrix solution consisted of sinapinic acid (10−15 mg/mL 3,5 dimethoxy-4-hydroxycinnamic acid in acetonitrile/3% w/v TFA/water = 3:1:6). The final mixture (2 μL) was loaded on a welled plate and was allowed to dry in air. All samples were applied in duplicate. At least two different spots were analyzed from each well, and the masses obtained (representing protonated molecules, i.e., actual mass + 1 H^+) were averaged. External calibration was performed, under the same conditions as used for the analysis, using bovine insulin (5734.6 and 2867.8 Da for single- and double-protonated molecules, respectively), thioredoxin from *E. coli* (11674.5 and 5837.7 Da for single- and double-protonated molecules, respectively), and horse apomyoglobin (16952.6 and 8476.8 Da for single- and double-protonated molecules, respectively). Horse heart cytochrome *c* (single protonated mass 12361.5 Da) was used as an external reference protein.

Functional Properties. Foam and emulsion properties of the hydrolysates were tested (at pH 6.7, ionic strength of 75 mM and 20 °C) in screening tests, which have been described previously (Caessens et al., 1997b; 1999a). The protein/peptide concentration used for the emulsion screening test was 0.05% (w/v) and for the foam screening test was 0.005% (w/v). The foam screening test was also performed under reducing conditions. To that end, the foam test was performed under nitrogen in the presence of 30 mM DTT with preceding overnight incubation of the sample at ambient temperature.

RESULTS

Fractionation of the βLg/Plasmin DH4 Hydrolysate. The DH4 hydrolysate could be separated by IEC into an unbound and a bound fraction, IEC1 and IEC2, respectively (Figure 2a). Fractions IEC1 and IEC2 made up about 1% and 99% of the total peak area in the IEC chromatogram, respectively. Fraction IEC2 was further separated by HIC (Figure 2b), resulting in three peaks denoted IEC2HIC1, IEC2HIC2, and the major peak IEC2HIC3, making up approximately 2%, 10%, and 88% of the total peak area in the HIC chromatogram, respectively. Figure 2c shows the RP-HPLC chromatograms of the several fractions obtained by IEC and HIC. Fraction IEC1 contained material eluting in the first part of the chromatogram, representing smaller and/or more hydrophilic peptides. The DH4 hydrolysate and IEC2 still contained some intact βLg, which was removed from IEC2 with the fractions IEC2HIC1 and IEC2HIC2. Fraction IEC2HIC3 did not contain detectable amounts of βLg (as analyzed by RP-HPLC using an adjusted gradient, results not shown) and was also free of smaller, more hydrophilic peptides (based on RP-HPLC retention time).

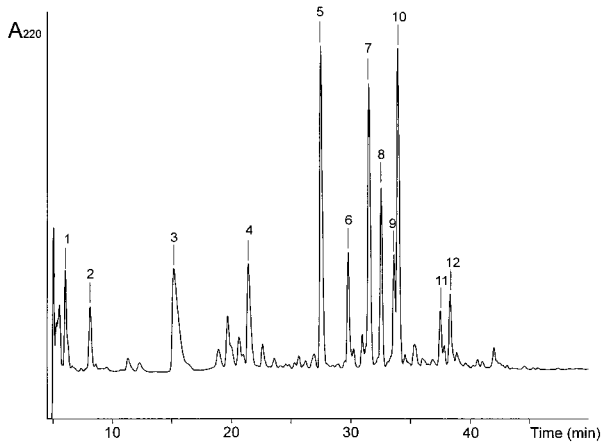

Figure 3. Semipreparative RP-HPLC chromatogram of IEC1; for conditions used see Materials and Methods; RP-HPLC gradient RP1. Code of the peaks refers to Table 1.

Several other scaleable fractionation methods (precipitation, ultrafiltration, and chromatographic techniques) were tried to achieve a further fractionation of IEC2HIC3, but none of them resulted in an efficient separation of the peptides (results not shown).

Identification of the βLg Peptides. Figure 3 shows the semipreparative RP-HPLC chromatogram of IEC1. The codes of the peaks refer to Table 1, which shows the ESI-MS results of the peptides present in this fraction. The identification was based on the masses determined in combination with the primary structure of βLg, and the specificity of plasmin. All peaks present in IEC1 could be identified, except for peak 6 (Figure 3). The latter peptide may result from nonspecific hydrolysis (Table 1), but no identification purely based on the mass determined can be given. It appeared that the peptides present in IEC1 were all small peptides (<2400 Da), and none of the identified peptides contained a cysteyl residue.

Figure 4 shows the semipreparative RP-HPLC chromatogram of IEC2HIC3. Fraction IEC2HIC3 contains many different peaks, of which 50 have been collected by semipreparative RP-HPLC, and the masses of the peptide components present have been determined. The masses (ESI-MS) of the samples allowed the identification of only a few peptides (i.e., some of the peptide material present in peaks 18−22/26/29/36, see below). To identify the other peptides, the disulfide bonds present had to be split using reduction with DTT. As

Table 1. ESI-MS Results for the Peptide Components of Fraction IEC1, As Separated by RP-HPLC (Figure 3)

RP-HPLC peak	measured value (Da)	peptide sequence (from both genetic variant A and B)	calcd value (Da)
1	330.3	[f 139−141]	330.2
2	572.3	[f 71−75]	572.3
3	916.0	[f 84−91]	915.4
4	672.3	[f 9−14]	672.3
5	932.6	[f 1−8]	932.5
6	652.5	*a*	
7	903.1	[f 76−83]	902.5
8	1065.2	[f 92−100]	1064.5
9	1585.5	[f 70−83]	1585.0
10	1457.3	[f 71−83]	1456.9
11	1800.7	[f 76−91]	1801.1
12	2355.4	[f 71−91]	2355.8

[a] When specificity of plasmin was taken into account, no fragment was found for this measured mass. When the specificity of the enzyme was ignored, several peptides could be assigned (with an accuracy of at least 2 Da): AB[f 144−148], AB[f 42−46], AB[f 97−101], AB[f 106−111], and AB[f 133−137], with calculated masses of 652.8, 651.7, 653.7, 653.7, and 650.7 Da, respectively.

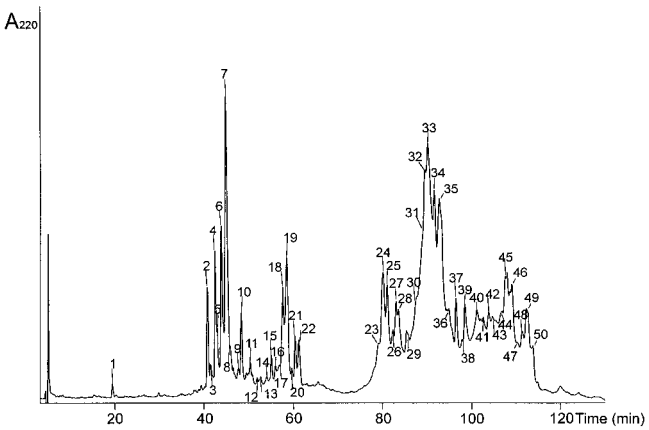

Figure 4. Semipreparative RP-HPLC chromatogram of IEC2HIC3; for conditions used see Materials and Methods; RP-HPLC gradient RP2. Code of the peaks refers to Appendix 1 and Table 2.

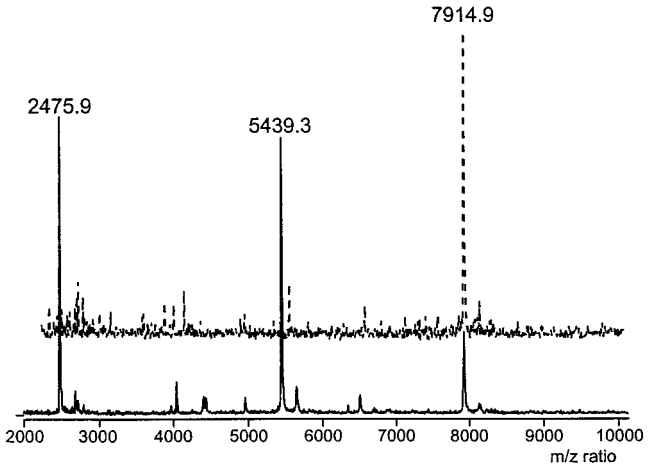

Figure 5. MALDI-TOF MS spectra of the peptide component present in peak 4 of the RP-HPLC chromatogram shown in Figure 4; dashed line for nonreduced sample and solid line for reduced sample; for conditions used see Materials and Methods.

an example, Figure 5 shows the MALDI-TOF MS spectra obtained for the material from peak 4 of Figure 4 before and after reduction. The spectrum of the nonreduced sample contained one main peak (protonated molecule 7914.9 Da). The spectrum of the reduced sample contained two main peaks (protonated molecules 2475.9 and 5439.3 Da) besides the small peak with the same mass as the nonreduced peptide. These two peaks could be identified by using the masses determined, the primary structure of βLg, and the specificity of plasmin (i.e., βLg[f 142−162], calculated mass 2478.9 Da, and βLgB[f 92−138], calculated mass 5442.2 Da). The nonreduced peptide appeared to be these two fragments linked together by an intermolecular disulfide bond (with fragment βLgB[f 92−138] also having an intramolecular disulfide bond), resulting in a calculated mass of 7916.1 Da (protonated form). All other peaks collected from IEC2HIC3 (Figure 4) were analyzed in the same way, and most of the peaks could be identified. The MS results and the identification of the peptide components present in IEC2HIC3 (Figure 4) are shown in Appendix 1 and in Table 2, the latter being a summary of the results presented in Appendix 1. The results show that many peptides are fragments linked by newly formed disulfide bonds; only a few of the peptides are linked by the original disulfide bonds present in βLg between residues 66 and 160 (i.e., βLg[f 9/15−69/70] −S−S− βLg[f 142/149−162].

Functional Properties. The screening tests used were reproducible enough to detect differences in functionality between the several peptide fractions (reproducibility tested with the intact protein). The standard deviation of the particle-size distribution (d_{32}) in the emulsion test was approximately 0.1 μm, and the maximum difference in foam height was about 5 au (obtained for the initial amount of foam produced).

Figure 6 shows the results of the foam screening test (performed at 0.005% w/v). The DH4 hydrolysate formed more foam than βLg did (as shown before at 0.01% w/v, Caessens et al., 1999a). Fraction IEC2HIC3, containing the larger, hydrophobic peptides (based on RP-HPLC retention time, see above), had the best foam-forming properties. The fractions IEC2HIC1 and IEC2HIC2, containing mainly intact βLg and some smaller, more hydrophilic peptides (Figure 2c), had even worse foam-forming properties than βLg. The foam formed by IEC1 was extremely unstable. The foam-stabilizing properties of all other fractions was good; no coalescence was observed during the measurements (60 min). Under reducing conditions, the foam-forming properties of βLg increased (approximately 18%), whereas those of IEC2HIC3 decreased (approximately 13%); reduction had no measurable influence on the foam-stabilizing properties of these samples (no further results shown).

Table 3 shows the results of the emulsion screening test. At the low protein concentration used (0.05% w/v), only the emulsions formed by the DH4 hydrolysate and the fractions IEC2, IEC2HIC2, and IEC2HIC3 had a uniform particle-size distribution (of which the DH4 hydrolysate, IEC2, and IEC2HIC3 formed the smallest droplets). The other fractions (βLg, IEC1, and IEC2HIC1) formed emulsions containing a double peak in the particle-size distribution (indicating the presence of smaller and larger droplets at the same time; Caessens et al., 1999a). At this low concentration, the emulsions formed by the latter fractions were rather unstable and showed creaming after 1 h. The other fractions formed stable emulsions (according to the turbidity measurements), showing only a minor amount of creaming after 24 h (IEC2HIC3 was most stable against creaming). At higher peptide concentration (0.2%), the emulsions formed with the DH4 hydrolysate and with the fractions

Table 2. Summary of MS Results for the Peptide Components of Fraction IEC2HIC3, As Separated by RP-HPLC (Figure 4) (Complete Results Are Shown in Appendix 1)

HPLC peaks	HPLC retention time (min)	sequences identified (mostly from both genetic variant A and B)
2−7	40.4−46.1	[f 139/142−162] −S−S− [f 84/92/101−135/138, SS][a]
10−12/15	48.2−52.1/54.9−55.5	[f 142−162] −S−S− [f 71/76/92/101/102−138/141, SS][a]
18−22	57.2−61.5	[f 70/71/76/84/92−162, 2SS][b]
24−25	79.5−81.8	[f 15−70/83] −S−S− [f 92/101−138, SS]
26	82.0−82.6	[f 15−60][c]
27−29	82.8−85.7	[f 142/149−162] −S−S− [f 15−69/70]
		[f 101−141, SS] −S−S− [f 101−141, SS]
		[f 101−141, SS] −S−S− [f 92−138, SS]
		[f 71−162, 2SS][b]
31−34	87.9−92.3	[f 84/92/101−138/141, SS] −S−S− [f 9−69/70/83/91][d]
		[f 76−135, SS] −S−S− [f 84−138, SS]
35−36	92.3−95.4	[f 9−70] −S−S− [f 142−162]
		[f 9−60][c]
41	101.9−102.9	[f 15−70] −S−S− [f 15−70]
44−46	106.3−109.6	[f 9/15−69/70] −S−S− [f 9−69/70/77]
49	111.9−113.1	[f 9−70] −S−S− [f 9−70]

[a] A variant elutes later than B variant. [b] Sequence contains only intramolecular disulfide bond. [c] Sequence without Cys-residue. [d] Sequences including [f 9−69/70] elute later than those including [f 9−83/91].

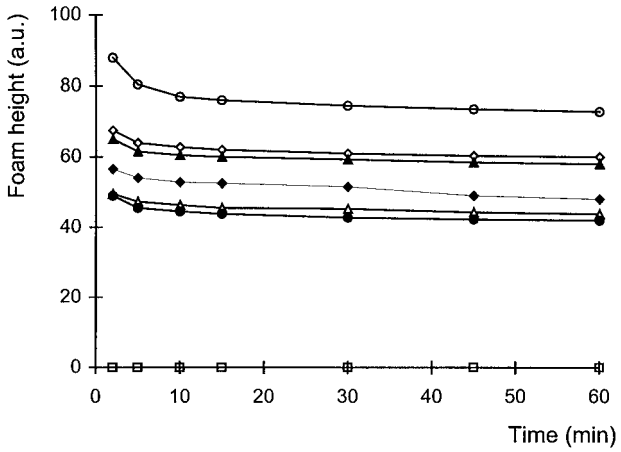

Figure 6. Foam height as produced with the various βLg fractions (Figures 1 and 2) at pH 6.7, as a function of time after whipping (means of duplicate measurements), intact βLg (◆), DH4 hydrolysate (◇), IEC1 (□), IEC2 (▲), IEC2HIC1 (△), IEC2HIC2 (●), IEC2HIC3 (○); for conditions used see Materials and Methods.

Table 3. Screening Test Results of Emulsions Made at pH 6.7 with βLg Hydrolysate Fractions[a,b]

	concentration = 0.05%		
sample	d_{32}[c] (μm)	(first/second peak)[d]	stability[e]
βLg	2.7	(2/6)	++
DH4 hydrolysate	2.3		+
IEC1	6.7	(3/14)	− −
IEC2	2.1		+
IEC2HIC1	2.9	(2/10)	−
IEC2HIC2	2.4		±
IEC2HIC3	1.9		+

[a] For conditions used, see Materials and Methods. [b] For abbreviations used, see text. [c] d_{32} is the average particle size of the emulsion droplets. [d] The measurement of the average particle size had a double peak (values of the separate peaks). [e] +, ±, and − indicate the extent of stability.

IEC2 and IEC2HIC3 possessed stability similar to that of intact βLg (no further results shown).

DISCUSSION

SH/SS Exchange. Although a large number of peptides were present in IEC1 and IEC2HIC3, RP-HPLC in combination with MS enabled almost complete identification of the peptides present. From the results in Appendix 1, it appeared that approximately 10% of the peptides present in IEC2HIC3 (proportion estimated from RP-HPLC peak area) could be identified without the presence of reduction agent, because they either did not contain a cysteyl residue, i.e., βLg[f 9/15−60], or had just intramolecular disulfide bonds, e.g., βLgA[f 70/71/76/84/90−162]. Other peptides identified were combinations of two separate fragments of the βLg molecule linked by an intermolecular disulfide bond. One of the masses determined in peak 39 (Figure 4) indicated the presence of traces of intact βLgB; no traces of βLgA were found.

The enormous number of peptides formed during hydrolysis of βLg as shown in Table 1 and Appendix 1 can tentatively be classified as follows: (1) peptides composed of a single amino acid chain without cysteyl residues (e.g., βLg[f 9/15−60]); (2) peptides composed of a single amino acid chain containing two or four cysteyl residues and having intramolecular disulfide bonds (e.g., βLgA[f 70/71/76/84/90−162]); and (3) peptides composed of two amino acid chains, each containing one to three cysteyl residues, linked by an intermolecular disulfide bond; these intermolecular disulfide bonds can either be originally present or newly formed.

Figure 7 shows a schematic picture of the peptides, mostly disulfide linked, originating from βLgA and present in IEC2HIC3 following the above-mentioned classification. Peptides comprised of two fragments linked by an original βLg disulfide bond (between 66 and 160) were identified previously in βLg/trypsin hydrolysates (Dalgalarrondo et al., 1990; Turgeon et al., 1992; Chen et al., 1993; Otte et al., 1997a). Recently, a βLg peptide having a mass of 4302 Da was assigned to the sequence βLg[f 102−124] −S−S− [f 149−162] having a newly formed disulfide bond (probably between the free thiol group, residue 121, and the cysteyl residue 160 present in the [f 149−162] subsequence; Maynard et al., 1998). Part of the peptides identified in the present study might contain original βLg disulfide bonds (between 66 and 160 and 106−119). However, many peptides also contain newly formed disulfide bonds, since the peptides having one to three cysteyl residues appear to be extremely reactive in forming new disulfide linkages (Appendix 1). Peptides from the N-terminal half of the molecule, βLg[f 10/15−69/70], are linked to peptides of the middle part of the molecule, βLg[f 71/77/91/101/102−138/141], in many different combinations

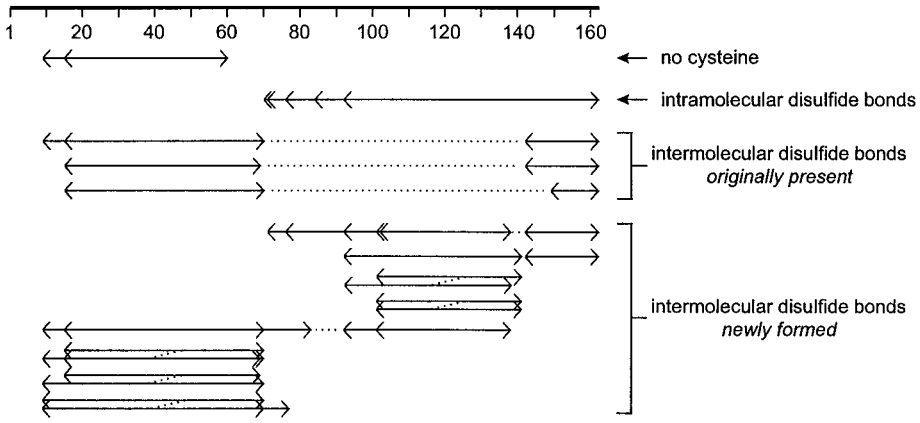

Figure 7. Schematic representation of the βLgA peptides present in IEC2HIC3; dashed line indicates intermolecular disulfide bond.

with a newly formed intermolecular disulfide bond between residues 66 and 106, 119, or 121. Apparently, a lot of reshuffling of disulfide bonds has taken place during the plasmin hydrolysis of βLg. Since the pK_a of the thiol group is rather high (approximately 9.0−9.5, depending on its surroundings; Creighton, 1993), the reactivity of the thiol group is negligible at neutral pH. Therefore, it is concluded that the reshuffling took place during hydrolysis (pH 8) rather than during fractionation (pH 6 and 7). It is remarkable that fragment βLg[f 142−162] is linked to almost all other fragments formed. Apparently, this fragment is quite reactive in forming new disulfide bonds. In the native βLg, the original disulfide bond between residues 66 and 160 is on the outer surface, whereas the other disulfide bond (106−119) is buried in the interior of the native molecule (Papiz et al., 1986). The exposure of the disulfide bond might be a reason for its reactivity (Hoffmann and Van Mil, 1997). This may also hold for the reactivity of βLg[f 142−162].

The peptides identified in this study never consisted of more than two intermolecular disulfide-linked βLg fragments. So, although SH/SS exchange does take place during βLg hydrolysis, this seems not to result in large peptide aggregates, which is in agreement with the literature (Otte et al., 1997b). Although disulfide exchange reactions are thought to play an initiating role in the thermally induced aggregation of βLg, their role is considered to be minor compared with other factors involved such as hydrophobic and electrostatic interactions (Roefs and De Kruif; 1994; Sawyer et al., 1994). Also, Otte et al. (1997b) showed that mainly the noncovalent interactions were the interacting forces in the aggregates formed during βLg hydrolysis with protease from *Bacillus licheniformis*.

Some peptides present in IEC2HIC3 eluted in two different regions of the RP-HPLC chromatogram (Appendix 1). For instance, peptide βLg[f 142−162] −S−S− βLgB[f 101−138, SS] appeared in peak 2/3 and in peak 10 of Figure 4 (retention times 40.4−41.9 min and 48.2−48.8 min, respectively). A similar phenomenon could be observed for the peptide βLg[f 142−162] −S−S− βLgB[f 92−138, SS] (appearing in the peaks 4/5 and 15 of Figure 4) and for the peptide βLgA[f 71−162, 2SS] (eluting in the peaks 22 and 29 of Figure 4). A possible explanation might be that different disulfide bonds are present within the peptide. For instance, in the case of the latter example different combinations between the cysteyl residues present at positions 106, 119, 121, and 160 are possible, which will result in an altered surface hydrophobicity of the total peptide and, consequently, in a different RP-HPLC retention behavior. Since no Arg and/or Lys residues are present between the residues 106 and 121, the results in this study on the βLg/plasmin hydrolysate give no information whether the originally present disulfide bond between 106 and 119 remains intact or that it also takes part in the reshuffling.

Fractionation. Fractionation of βLg DH4 hydrolysate resulted in several peptide fractions. Fraction IEC1 contained a mixture of smaller and/or hydrophilic peptides (Table 1), fraction IEC2HIC3 contained a complex mixture of larger, hydrophobic peptides (based on RP-HPLC retention time), and many of the peptides contained disulfide bonds (Appendix 1). Further fractionation of IEC2HIC3 by scalable methods was not successful (results not shown). Other papers (Turgeon et al., 1992; Chen et al., 1993) described the fractionation (by ultrafiltration and by IEC) of tryptic βLg hydrolysates, in which no SH/SS reshuffling was observed, although the hydrolysis had also been performed at pH 8. The peptides identified by Turgeon et al. (1992) were smaller than the peptides present in IEC2HIC3, which resulted in more peptides lacking a cysteyl residue. Probably, both the presence of smaller peptides and the absence of SH/SS exchange made it possible to fractionate that tryptic hydrolysate. Chen et al. (1993) hydrolyzed βLg at low temperature (5−10 °C), which might have prevented reshuffling in the hydrolysate, despite the larger size of the peptides (6.7, 9.6, and 13.8 kDa) and the presence of cysteyl residues in these peptides. In our previous study (Caessens et al., 1999a), it was shown that the βLg hydrolysates formed by the action of trypsin and *Staphylococcus aureus* V8 protease contained less disulfide-linked peptides than the hydrolysates formed by the action of plasmin (as determined by gel-permeation chromatography under denaturing plus reducing conditions). Probably, the use of a less selective enzyme than plasmin results in a βLg hydrolysate containing smaller peptides of which many lack a cysteyl residue. Therefore, a large number of peptides is present that cannot take place in the SH/SS exchange. Hence, more distinct differences between several peptides will exist, which might enable the fractionation of such a hydrolysate.

Functional Properties. The DH4 hydrolysate already had improved foam-and emulsion-forming properties, when compared to βLg. Fraction IEC1, containing smaller peptides (mostly <2 kDa), had poor foam- and emulsion-forming and -stabilizing properties. These

results are in agreement with the concept that a minimum molecular weight of 2000 Da is essential for good interfacial and emulsifying properties (Turgeon et al., 1992). Fraction IEC2HIC3 was formed after removing mainly intact βLg with IEC2HIC1 and IEC2HIC2 from IEC2 and contained larger peptides (mostly between 7 and 14 kDa). Fraction IEC2HIC3 possessed improved foam- and emulsion-forming properties and similar foam- and emulsion-stabilizing properties when compared to βLg, DH4 hydrolysate, and IEC2.

Reduction of the disulfide bonds in βLg seemed to improve its foam-forming properties, but in IEC2HIC3 to decrease foam-forming properties (results not shown). By reducing the disulfide bonds in βLg, the molecule becomes a more flexible structure, which apparently favors the foam-forming properties. The molecules present in IEC2HIC3 already have a more flexible structure (due to the hydrolysis), which could explain the improved functionality. The reduction of the disulfide bonds in IEC2HIC3 resulted in smaller peptides (many between 2 and 7 kDa), possessing decreased functional properties. Earlier, it was suggested that the poor interfacial properties of a βLg peptide fraction that mainly contained disulfide-linked peptides was caused by the rigid structure due to the disulfide bond (Turgeon et al., 1992). However, in our study it was found that the presence of many disulfide bonds in IEC2HIC3 was not detrimental for the functional properties of this peptide fraction.

In conclusion, it is shown that most of the peptides present in a βLg/plasmin hydrolysate are disulfide-linked βLg fragments and considerable SH/SS exchange takes place during plasmin hydrolysis of βLg. In contrast to the earlier reports on the negative effect of the presence of disulfide bonds on the interfacial properties (Turgeon et al., 1992), the results presented in this paper showed good functional properties of a peptide fraction containing many disulfide bonds.

ACKNOWLEDGMENT

Peter Wierenga is thanked for performing the foam experiments under reducing conditions.

APPENDIX 1. ESI-MS and MALDI-TOF MS Results for the Peptide Components of Fraction IEC2HIC3, As Separated by RP-HPLC (Figure 4)

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
1	x			x		
2	2477.8	AB[f 142-162]	2477.9	6867.1	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	4392.9	B[f 101-138]	4394.0			
3	2478.4	AB[f 142-162]	2477.9	6867.3[b]	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	2789.9	AB[f 139-162]	2791.3	7179.3	AB[f 139-162] -S-S- B[f 101-138, SS]	7180.3
	4395.5	B[f 101-138]	4394.0	7524.0	A[f (142-162] -S-S- B[f 92-135, SS]	7524.7
	5050.6	B[f 92-135]	5050.8	7957.7[c]		
4	2477.8	AB[f 142-162]	2477.9	7914.2	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	5442.6	B[f 92-138]	5441.2			
5	2478.2	AB[f 142-162]	2477.9	6899.3	AB[f 142-162] -S-S- A[f 101-138, SS]	6895.8
	2789.9	AB[f 139-162]	2790.3	7914.1	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	4423.9	A[f 101-138]	4422.1	8226.0	AB[f 139-162] -S-S- B[f 92-138, SS]	8227.5
	5443.4	B[f 92-138]	5441.2	8811.9	AB[f 142-162] -S-S- B[f 84-138, SS]	8813.1
	6342.7	B[f 84-138]	6339.2			
6	2481.7	AB[f 142-162]	2477.9	6895.1	AB[f 142-162] -S-S- A[f 101-138, SS]	6895.8
	4419.6	A[f 101-138]	4422.1			
	6891.4[d]	A[f 9-70]	6890.9		A[f 9-70]SH[d,e]	6891.9[e]
7	2475.6	AB[f 142-162]	2477.9	7942.3	AB[f 142-162] -S-S- A[f 92-138, SS]	7943.2
	5465.8	A[f 92-138]	5469.3			
	7940.4[d]					
8	2478.3[b]	AB[f 142-162]	2477.9	7942.8[b]		
	2703.1[c]			8254.2[c]		
	5442.2	B[f 92-138][f]	5441.2			
9	2476.8[b]	AB[f 142-162]	2477.9	7207.0[c]		
				8578.1[c]		
				10379.9[c]		
10	2477.7	AB[f 142-162]	2477.9	6767.6	AB[f 142-162] -S-S- A[f 102-138, SS]	6767.8
	4295.6	A[f 102-138]	4293.9	8254.6	AB[f 142-162] -S-S- A[f 92-141, SS]	8255.6
	5784.4	A[f 92-141]	5781.7	9151.8[c]		
	6771.3[d]			10251.8[c]		
11	2479.6	AB[f 142-162]	2477.9	6865.5	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	4392.7	B[f 101-138]	4394.0	9726.0	AB[f 142-162] -S-S- A[f 76-138, SS]	9725.3
	4421.7	A[f 101-138][f]	4422.1	10281.1	AB[f 142-162] -S-S- A[f 71-138, SS][h]	10280.0
	5444.8	B[f 92-138][f]	5441.2	15529.8[c]		
	5466.3	A[f 92-138][f]	5469.3			
	7249.5	A[f 76-138]	7252.4			
	7307.3	B[f 78-141][f]	7307.5			
	7804.4[g]	A[f 1-70][g]	7806.0			
		A[f 71-138][g]	7807.1			
	9726.1[d]					
	10278.3[d]	A[f 9-100]	10275.9		A[f 9-100]SH[d,e]	10275.9[e]

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
12	x			7895.8[c]		
				10280.6[b]	AB[f 142-162] -S-S- A[f 71-138, SS][h]	10280.0
				10720.9[c]		
13	x			10037.8[c]		
				10592.3[c]		
14	x			6021.1[c]		
				8829.7[c]		
15	2478.0	AB[f 142-162]	2477.9	7923.3	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	4394.3	B[f 101-138][f]	4394.0	8129.7	B[f 78-148, SS]SH[e]	8124.5[e]
	4421.3	A[f 101-138][f]	4422.1	8856.1[c]		
	5447.2	B[f 92-138]	5441.2			
	5468.9	A[f 92-138][f]	5469.3			
	7925.4[d]					
16	2478.6	AB[f 142-162][b,f]	2477.9	8827.6[c]		
	4395.5	B[f 101-138][f]	4394.0	9381.9[b,c]		
	4426.2	A[f 101-138][f]	4422.1			
	8834.7[d]					
	9396.5[d]					
17	x			7773.0	B[f 71-138, SS]SH[e]	7777.0[e]
				8560.7	B[f 61-135, SS]SH[e]	8560.9
				9168.1	B[f 9-91]SH[e]	9170.6[e]
				9382.2[b,c]		
18	8209.3	B[f 92-162]	8213.5	8208.6	B[f 92-162, 2SS]	8209.5
	9108.4	B[f 84-162]	9111.5	9106.2	B[f 84-162, 2SS]	9107.5
19	8239.2	A[f 92-162]	8241.57	8236.2	A[f 92-162, 2SS]	8237.6
	9136.9	A[f 84-162]	9139.6	9134.5	A[f 84-162, 2SS]	9135.6
20	7731.3			7736.1[c]		
	8236.1[b]			8237.2[b]		
	10673.7	B[f 70-162]	10679.5	10674.2	B[f 70-162, 2SS]	10675.5
21	9996.0	B[f 76-162]	9996.6	9990.4	B[f 76-162, 2SS]	9992.6
	10551.2	B[f 71-162]	10551.3	10545.3	B[f 71-162, 2SS]	10547.3
	10711.7	A[f 70-162]	10707.5	10702.3	A[f 70-162, 2SS]	10703.5
22	10019.3	A[f 76-162]	10024.7	10019.3	A[f 76-162, 2SS]	10020.7
	10574.5	A[f 71-162]	10579.4	10573.9	A[f 71-162, 2SS]	10575.4
23	x			x		
24	4394.3	B[f 101-138]	4394.0	7616.9[i]		
	4423.6	A[f 101-138]	4422.1	10570.3	B[f 15-70] -S-S- B[f 101-138, SS]	10568.1
	5441.4	B[f 92-138]	5441.2	10594.6	B[f 15-70] -S-S- A[f 101-138, SS]	10596.3
	5467.7	A[f 92-138]	5469.3	11615.6	B[f 15-70] -S-S- B[f 92-138, SS]	11615.3
	6178.1	B[f 15-70]	6178.1	11642.7	B[f 15-70] -S-S- A[f 92-138, SS]	11643.3
	6232.2	A[f 15-70][b,f]	6236.1	12009.1	B[f 15-83][h] -S-S- B[f 101-138, SS]	12006.8
	7619.5[g]	B[f 1-69][g]	7619.8	12035.8	B[f 15-83][h] -S-S- A[f 101-138, SS]	12034.9
		B[f 9-77][g]	7616.8	13056.6	B[f 15-83][h] -S-S- B[f 92-138, SS]	13054.0
		B[f 15-83][g]	7617.8	13084.8	B[f 15-83][h] -S-S- A[f 92-138, SS]	13082.1
25	2477.2	AB[f 142-162][g]	2477.9	10653.0	A[f 15-70] -S-S- A[f 101-138, SS]	10654.2
	3205.4[c]			11700.7	A[f 15-70] -S-S- A[f 92-138, SS]	11701.4
	4419.4	A[f 101-138]	4422.1	12095.8	A[f 15-83][h] -S-S- A[f 101-138, SS]	12092.0
	5466.8	A[f 92-138]	5469.3	13140.6	A[f 15-83][h] -S-S- A[f 92-138, SS]	13141.2
	6240.8	A[f 15-70]	6236.1			
	7675.4[g]	A[f 1-69][g]	7677.9			
		A[f 9-77][g]	7674.8			
		A[f 15-83][g]	7675.9			
26	2477.8	AB[f 142-162][b,f]	2477.9	5002.7	AB[f 15-60]	5003.8
	4395.7	B[f 101-138][f]	4394.0			
	4426.5	A[f 101-138][b,f]	4422.1			
	5007.3	AB[f 15-60]	5003.8			
	5447.1	B[f 92-138][f]	5441.2			
	5471.2	A[f 92-138][b,f]	5469.3			
	6053.6	B[f 15-69][f]	6049.9			
	6113.8	A[f 15-69][f]	6107.9			
	6184.0	B[f 15-70][b,f]	6189.3			
	6239.8	A[f 15-70][b,f]	6236.1			
27	2475.4	AB[f 142-162]	2477.9	4634.5[i]		
	4632.5	A[f 101-141]	4635.4	8652.7	AB[f 142-162] -S-S- B[f 15-70]	8654.0
	5469.2	A[f 92-138]	5469.3	8710.9[b]	AB[f 142-162] -S-S- A[f 15-70]	8712.0
	6177.1	B[f 15-70]	6178.1	9266.8	A[f 101-141, SS]-S-S-A[f 101-141, SS]	9264.7
	6236.4	A[f 15-70]	6236.1	10092.3	A[f 101-141, SS] -S-S- A[f 92-138, SS]	10098.7

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
28	1661.3	AB[f 149-162]	1658.9	7896.2	AB[f 149-162] -S-S- A[f 15-70]	7893.0
	2478.4	AB[f 142-162]	2477.9	8711.4[b]	AB[f 142-162] -S-S- A[f 15-70]	8712.0
	4394.7	B[f 101-138][b,f]	4394.0			
	6239.8	A[f 15-70]	6236.1			
29	2478.0	AB[f 142-162]	2477.9	8586.5	AB[f 142-162] -S-S- A[f 15-69]	8583.8
	4394.6	B[f 101-138][b,f]	4394.0	10578.4	A[f 71-162, 2SS]	10575.4
	4421.2	A[f 101-138][b,f]	4422.1	10735.8[c]		
	5442.0	B[f 92-138][b,f]	5441.2			
	5470.1	A[f 92-138][b,f]	5469.3			
	6110.4	A[f 15-69]	6107.9			
30	2477.3	AB[f 142-162][b]	2477.9	10054.3[c]		
	4394.0	B[f 101-138][b,f]	4394.0	10604.7[c]		
	4424.7	A[f 101-138][b,f]	4422.1			
	5441.3	B[f 92-138][b,f]	5441.2			
	5469.4	A[f 92-138][b,f]	5469.3			
31	2478.4	AB[f 142-162][f]	2477.9	8271.0[i]		
	4394.6	B[f 101-138]	4394.0	13168.4	B[f 84-138, SS] -S-S- B[f 76-135,, SS]	13167.1
	4422.4	A[f 101-138]	4422.1	13196.9	A[f 84-138, SS] -S-S- B[f 76-135, SS]	13195.2
	5441.7	B[f 92-138]	5441.2	13560.7	B[f 9-91] -S-S- B[f 101-138, SS]	13560.6
	5470.2	A[f 92-138]	5469.3	13589.2	B[f 9-91] -S-S- A[f 101-138, SS]	13588.7
	5757.9	B[f 92-141]	5757.9	14054.3	B[f 9-83] -S-S- A[f 92-141, SS]	14050.3
	5772.7	A[f 92-141]	5781.7	14606.7	B[f 9-83] -S-S- B[f 84-138, SS]	14607.9
	6340.4	B[f 84-138]	6339.2		B[f 9-91] -S-S- B[f 92-138, SS]	14607.8
	6367.5	A[f 84-138]	6367.3	14636.8[g]	B[f 9-83] -S-S- A[f 84-138, SS]	14634.9
	6655.3	B[f 84-141]	6651.6		B[f 9-91] -S-S- A[f 92-138, SS]	14635.9
	6834.4	B[f 76-135]	6833.9	14921.9[g]	B[f 9-83] -S-S- B[f 84-141, SS]	14920.3
	8275.3	B[f 9-83]	8272.6		B[f 9-91] -S-S- B[f 92-141, SS]	14920.3
	9172.0	B[f 9-91]	9170.6			
32	2477.7	AB[f 142-162][f]	2477.9	8270.9[i]		
	4396.4	B[f 101-138]	4394.0	12667.3	B[f 9-83] -S-S- B[f 101-138, SS]	12662.6
	4422.4	A[f 101-138]	4422.1	12690.6	B[f 9-83] -S-S- A[f 101-138, SS]	12690.7
	5444.5	B[f 92-138]	5441.2	13711.1	B[f 9-83] -S-S- B[f 92-138, SS]	13709.9
	5470.7	A[f 92-138]	5469.3	13736.0	B[f 9-83] -S-S- A[f 92-138, SS]	13737.9
	8274.1	B[f 9-83]	8272.6			
33	2478.6	AB[f 142-162][b,f]	2477.9	11227.3	B[f 9-70][h] -S-S- B[f 101-138, SS]	11222.8
	2791.5	AB[f 139-162][b,f]	2790.3	11253.9	B[f 9-70][h] -S-S- A[f 101-138, SS]	11250.9
	4394.5	B[f 101-138]	4394.0	11282.7	A[f 9-70] -S-S- B[f 101-138, SS]	11280.9
	4422.1	A[f 101-138]	4422.1	11312.3	A[f 9-70] -S-S- A[f 101-138, SS]	11308.9
	5441.9	B[f 92-138]	5441.2	12272.7	B[f 9-70][h] -S-S- B[f 92-138, SS]	12270.1
	5468.3	B[f 92-138]	5469.3	12299.9	B[f 9-70][h] -S-S- A[f 92-138, SS]	12298.1
	6832.4[f]	B[f 9-70][g]	6832.8	12326.6	A[f 9-70] -S-S- B[f 92-138, SS]	12328.1
		B[f 76-135][g]	6833.9	12359.9	A[f 9-70] -S-S- A[f 92-138, SS]	12356.1
	6892.6	A[f 9-70]	6890.9	12670.0	B[f 9-83] -S-S- B[f 101-138, SS]	12662.6
	8272.8	B[f 9-83]	8272.6	12724.5	A[f 9-83] -S-S- B[f 101-138, SS]	12720.7
	8328.5	A[f 9-83]	8330.7	12749.1	A[f 9-83] -S-S- A[f 101-138, SS]	12748.8
				13797.9	A[f 9-83] -S-S- A[f 92-138, SS]	13796.0
34	2477.7	AB[f 142-162][b,f]	2477.9	11125.9	B[f 9-69] -S-S- A[f 101-138, SS]	11122.8
	2789.5	AB[f 139-162][b,f]	2790.3	11313.5[b]	A[f 9-70] -S-S- A[f 101-138, SS]	11308.9
	4394.5	B[f 101-138][b,f]	4394.0	12171.2	B[f 9-69] -S-S- A[f 92-138, SS]	12170.0
	4420.6	A[f 101-138]	4422.1	14591.0[c]		
	5442.9	B[f 92-138][b,f]	5441.2	14819.7[c]		
	5469.7	A[f 92-138]	5469.3	14903.2[c]		
	6706.5	B[f 9-69]	6704.7	19840.4[c]		
	6834.0[g]	B[f 9-70][b,f,g]	6832.8			
		B[f 76-135][g]	6833.9			
	6893.3	A[f 9-70][b,f]	6890.9			
	7294.2[c]					
	8274.5	B[f 9-83][b,f]	8272.6			
	9171.2	B[f 9-91][b,f]	9170.6			
35	2476.7	AB[f 142-162]	2477.9	9310.6	AB[f 142-162] -S-S- B[f 9-70]	9309.8
	6834.1[g]	B[f 9-70][g]	6832.8	9368.4	AB[f 142-162] -S-S- A[f 9-70]	9366.8
		B[f 76-135][g]	6833.9	10747.6[c]		
	6889.8	A[f 9-70]	6890.9	20751.5[c]		
36	2476.3	AB[f 142-162][b]	2477.9	5657.4	AB[f 9-60]	5658.5
	2489.3	AB[f 70-91][f]	2484.0	9182.9	B[f 1-83]SH[e]	9187.8[e]
	4393.9	B[f 101-138][f]	4394.0	9237.5[c]		
	5656.7	AB[f 9-60]	5658.5	10281.1[c]		

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
37	2477.7	AB[f 142-162][b,f]	2477.9	5288.2[c]		
	5290.9[c]			5497.7[c]		
	5497.1[c]					
38	x			6203.2[c]		
				18343.0[c]		
39	2479.3	AB[f 142-162][f]	2477.9	5529.5[i]		
	5531.4	B[f 101-148][f]	5525.5	17358.5[c]		
	6179.2	B[f 15-70][f]	6178.1	18276.9	B[f 1-162, 2SS]SH[e]	18277.3[e]
	6237.5	A[f 15-70][f]	6236.1			
40	2480.5	AB[f 142-162][f]	2477.9	8722.8[c]		
	6180.1	B[f 15-70][b,f]	6178.1			
	6238.1	A[f 15-70][b,f]	6236.1			
	6893.4	A[f 9-70][f]	6890.9			
41	2479.3	AB[f 142-162][f]	2477.9	12410.2	A[f 15-70] -S-S- B[f 15-70]	12412.2
	6180.4	B[f 15-70]	6178.1	12468.7	A[f 15-70] -S-S- A[f 15-70]	12470.2
	6238.5	A[f 15-70]	6239.1			
42	4397.8	B[f 101-138][f]	4394.0	12243.7[c]		
	4421.4	A[f 101-138][f]	4422.1	12382.1[c]		
	5444.1	B[f 92-138][f]	5441.2			
	5470.9	A[f 92-138][f]	5469.3			
	6107.6	A[f 15-69][f]	6107.9			
	6178.7	B[f 15-70][f]	6178.1			
	6235.1	A[f 15-70][f]	6236.1			
	6836.5[g]	B[f 9-70][f,g]	6832.8			
		B[f 76-135][f,g]	6833.9			
	6889.1	A[f 9-70][f]	6890.9			
43	x			x		
44	6183.0	B[f 15-70][b,f]	6178.1	14448.2	B[f 9-70] -S-S- B[f 9-77][h]	14447.6
	6234.1	A[f 15-70][b,f]	6236.1	14505.0[h]	B[f 9-70] -S-S- A[f 9-77][h]	14505.7
	6834.3[g]	B[f 9-70][g]	6832.8		A[f 9-70] -S-S- B[f 9-77][h]	14505.7
		B[f 76-135][g]	6833.9	14526.8[c]		
	6890.5	A[f 9-70]	6890.9	14562.9	A[f 9-70] -S-S- A[f 9-77][h]	14563.7
	7619.7[g]	B[f 1-69][g]	7619.8			
		B[f 9-77][g]	7616.8			
		B[f 15-83][g]	7617.9			
	7675.0[g]	A[f 1-69][g]	7677.9			
		A[f 9-77][g]	7674.8			
		A[f 15-83][g]	7675.9			
45	6177.4	B[f 15-70]	6178.1	13065.4[g]	B[f 15-70] -S-S- A[f 9-70]	13066.9
	6236.1	A[f 15-70]	6236.1		A[f 15-70] -S-S- B[f 9-70]	13066.9
	6832.6[g]	B[f 9-70][g]	6832.8	13123.2	A[f 15-70] -S-S- A[f 9-70]	13125.0
		B[76-135][g]	6833.9			
	6891.2	A[f 9-70]	6890.9			
46	6110.9	A[f 15-69]	6107.9	12938.0	A[f 15-69] -S-S- B[f 9-70][h]	12938.7
	6237.7	A[f 15-70]	6236.1	12996.1[g]	A[f 15-69] -S-S- A[f 9-70]	12996.8
	6766.5	A[f 9-69]	6762.7		A[f 15-70] -S-S- A[f 9-69]	12996.8
	6838.9[g]	B[f 9-70][g]	6832.8			
		B[f 76-135][g]	6833.9			
	6893.4	A[f 9-70]	6891.9			
47	x			9286.3[c]		
				11687.2[c]		
				12640.4[c]		
				12778.2[c]		
48	x			x		
49	6834.4[g]	B[f 9-70][g]	6832.8	6831.7[i]		
		B[f 76-135][g]	6833.9	13720.3	A[f 9-70] -S-S- B[f 9-70]	13721.7
	6890.3	A[f 9-70]	6891.9	13778.5	A[f 9-70] -S-S- A[f 9-70]	13779.7
50	x			13650.2[c]		

x bad spectrum, no masses found

a reduced samples determined by MALDI-TOF MS; non-reduced samples determined by MALDI-TOF MS and/or ESI MS; both ESI-MS and MALDI-TOF MS measured masses are of the non-protonized form

b traces from former or following peak

c could not be identified

d probably mass of non-reduced peptide

e sequence contains free SH

f peptide not used to recombine non-reduced peptide

g similar mass found for more peptides

h one sequence is given; other theoretical possibilities[g]

i probably mass of reduced peptide

SS sequence contains intramolecular disulfide bond

SH sequence contains free thiol group

-S-S- two sequences linked together by intermolecular disulfide bond

LITERATURE CITED

Bastian, E. D.; Brown, R. J. Plasmin in milk and dairy products: an update. *Int. Dairy J.* **1996**, *6*, 435−457.

Burova, T. V.; Choiset, Y.; Tran, V.; Haertlé, T. Role of free Cys121 in stabilization of bovine β-lactoglobulin B. *Protein Eng.* **1998**, *11*, 1065−1073.

Caessens, P. W. J. R.; Visser, S.; Gruppen, H. Method for the isolation of bovine β-lactoglobulin from a cheese whey protein fraction and physicochemical characterization of the purified product. *Int. Dairy J.* **1997a**, *7*, 229−235.

Caessens, P. W. J. R.; Gruppen, H.; Visser, S.; van Aken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997b**, *45*, 2935−2941.

Caessens, P. W. J. R.; Visser, S.; Gruppen, H.; Voragen, A. G. J. β-Lactoglobulin Hydrolysis. 1. Peptide Composition and Functional Properties of Hydrolysates Obtained by the Action of Plasmin, Trypsin, and *Staphylococcus aureus* V8 Protease. *J. Agric. Food Chem.* **1999a**, *47*, 2973−2979.

Caessens, P. W. J. R.; Gruppen, H.; Slangen, Ch. J.; Visser, S.; Voragen, A. G. J. Functionality of β-casein peptides: importance of amphipathicity for emulsion-stabilizing properties. *J. Agric. Food Chem.* **1999b**, *47*, 1856−1862.

Chen, S. X.; Hardin, C. C.; Swaisgood, H. E. Purification and characterization of β-structural domains of β-lactoglobulin liberated by limited proteolysis. *J. Protein Chem.* **1993**, *12*, 613−625.

Chen, S. X.; Swaisgood, H. E.; Foegeding, E. A. Gelation of β-lactoglobulin treated with limited proteolysis by immobilized trypsin. *J. Agric. Food Chem.* **1994**, *42*, 234−239.

Creighton, T. E. *Proteins: Structures and Molecular Properties*, 2nd ed.; Freeman and Company: New York, 1993; Chapter 1, pp 1−47.

Dalgalarrondo, M.; Chobert, J.-M.; Dufour, E., Bertrand-Harb, C.; Dumont, J.-P.; Haertlé, T. Characterization of bovine β-lactoglobulin B tryptic peptides by reversed-phase high performance liquid chromatography. *Milchwissenschaft* **1990**, *45*, 212−216.

Hambling, S. G.; McAlphine, A. S.; Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry, Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, 1992; Vol. 1, pp 141−190.

Hoffmann, M. A. M.; Van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942−2948.

Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat-denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.

Maynard, F.; Weingard, A.; Hau, J.; Jost, R. Effect of high-pressure treatment on the tryptic hydrolysis of bovine β-lactoglobulin AB. *Int. Dairy J.* **1998**, *8*, 125−133.

Otte, J.; Zakora, M.; Qvist, K. B.; Olsen, C. E.; Barkholt, V. Hydrolysis of bovine β-lactoglobulin by various proteases and identification of selected peptides. *Int. Dairy J.* **1997a**, *7*, 835−848.

Otte, J.; Lomholt, S. T.; Ipsen, R.; Stapelfeldt, H.; Bukrinsky, J. T.; Qvist, K. B. Aggregate formation during hydrolysis of β-lactoglobulin with a Glu and Asp specific protease from *Bacillus licheniformis*. *J. Agric. Food Chem.* **1997b**, *45*, 4889−4896.

Papiz, M. Z.; Sawyer, L.; Eliopoulos E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

Roefs, S. F. M.; De Kruif, C. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.

Sawyer, L.; Morais Cabral, J. H.; Batt, C. A. Protein engineering studies of β-lactoglobulin. In *Biochemistry of milk products*; Andrews, A. T., Varley, J., Eds.; The Royal Society of Chemistry: Cambridge, 1994; pp 114−126.

Swaisgood, H. E. Chemistry of milk protein. In *Developments in Dairy Chemistry, Part I, Proteins*; Fox, P. F., Ed.; Applied Science Publishers: London, 1982; Chapter 1.

Tanford, C.; Bunville, L. G.; Nozaki, Y. The reversible transformation of β-lactoglobulin at pH 7.5. *J. Am. Chem. Soc.* **1959**, *81*, 4032−4036.

Turgeon, S. L.; Gauthier, S. F.; Mollé, D.; Léonil, J. Interfacial properties of tryptic peptides of β-lactoglobulin. *J. Agric. Food Chem.* **1992**, *40*, 669−675.

Visser, S.; Slangen, K. J.; Rollema, H. S. Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1991**, *548*, 361−370.

Received for review November 16, 1998. Revised manuscript received May 7, 1999. Accepted May 10, 1999. This research was supported by the Dutch Ministry of Economic Affairs, through the program IOP−Industrial Proteins, and by DMV−International. The mass spectrometry facilities and the ÄKTA-explorer were both funded by PPS MIBITON.

JF981230O