β-Lactoglobulin Hydrolysis. 1. Peptide Composition and Functional Properties of Hydrolysates Obtained by the Action of Plasmin, Trypsin, and *Staphylococcus aureus* V8 Protease

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β-Lactoglobulin (*β*Lg) was subjected to limited hydrolysis by trypsin, plasmin, and endoproteinase from *Staphylococcus aureus* V8 (*S.aur*.V8) to degrees of hydrolysis (DH) of 1, 2, and 4%. The several hydrolysates had different peptide compositions (determined by reversed-phase HPLC and gel-permeation chromatography [GPC]). GPC under nondenaturing, denaturing, and denaturing plus reducing conditions showed that the peptides formed were linked by hydrophobic interactions or by disulfide bonds or were not linked at all. At very low protein concentration, some differences in emulsion-forming properties were observed: only the plasmin hydrolysates could form emulsions with a uniform particle-size distribution. The emulsions formed with *S.aur*.V8 hydrolysates had poor emulsion-stabilizing properties. Some hydrolysates showed increased foam-forming properties in comparison with the intact protein. All foams formed were stable. Overall, the plasmin hydrolysate (DH4) contained relatively much larger molecules and/or hydrophobic molecules. Many molecules were disulfide-linked peptides. This hydrolysate also had the best functional properties.

Keywords: *β-Lactoglobulin; hydrolysis; plasmin; trypsin, Staphylococcus aureus V8 protease; foam; emulsions*

INTRODUCTION

In the food industry, milk proteins are applied for their functional properties, such as foam- and emulsion-forming and -stabilizing properties. Enzymatic hydrolysis can be used to alter the functional properties of proteins. It generally results in a decrease of molecular weight, an increase of ionizable groups, and an increased exposure of hydrophobic groups (Panyam and Kilara, 1996). The choice of the enzyme used will determine which peptides will be formed because of differences in enzyme specificities. As a result, hydrolysates that have been formed by various enzymes may have different functionalities.

In 1974, it was reported that enzymatic hydrolysis of whey protein concentrate (WPC) with prolase, pronase, or pepsin resulted in improved foaming properties but caused decreased emulsifying properties compared to the nonhydrolyzed WPC. In addition, there seemed to exist an optimal degree of hydrolysis (DH) for improving functional properties (Kuehler and Stine, 1974). The influence of hydrolysis by several enzymes (Jost and Monti, 1982) to different DHs (Chobert et al., 1988) and the influence of hydrolysis and subsequent thermal treatment and/or fractionation of the WPC hydrolysates (Turgeon et al., 1991; Althouse et al., 1995; Lieske and Konrad, 1996) on functional properties of whey protein mixtures have been investigated ever since. However, the results from these studies are difficult to compare because of the complexity of the protein mixture used and because different methods to determine the functional properties were used.

Approximately 50% of the whey proteins is composed of *β*-lactoglobulin (*β*Lg). In its native form, *β*Lg is a globular protein with a monomer molecular weight of approximately 18.3 kDa. *β*Lg has two disulfide bonds and a free thiol group; above pH 7.5, this free SH group has increased reactivity. At neutral pH, native *β*Lg occurs as a dimer in solution, and its associated form changes at different pHs (Swaisgood, 1982; Hambling et al., 1992). Native *β*Lg is resistant to peptic and chymotryptic hydrolysis, but the susceptibility of *β*Lg to enzymatic hydrolysis often increases after protein denaturation by temperature, high pressure, or different solvent conditions (Reddy et al., 1988; Schmidt and van Markwijk, 1993; Chobert et al., 1995; Dalgalarrondo et al., 1995; Dib et al., 1996; Stapelfeldt et al., 1996). Research concerning the influence of enzymatic hydrolysis of purified *β*Lg on the functional properties has been limited to tryptic hydrolysis. It was reported that *β*Lg hydrolysates formed by the action of trypsin have altered interfacial properties (Turgeon et al., 1992), improved gelation properties (Chen et al., 1994), and decreased emulsion-stabilizing properties (Agboola and Dalgleish, 1996) when compared to nonhydrolyzed *β*Lg.

The objective of the present study was to investigate the influence of enzymatic hydrolysis of purified *β*Lg by different enzymes on the foam- and emulsion-forming and -stabilizing properties (further denoted functional properties) in a systematic way. Since it is generally accepted that the DH should be low for the hydrolysates to retain functionality (Panyam and Kilara, 1996), *β*Lg was hydrolyzed to DH 1, 2, and 4%. The enzymes used

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10.1021/jf981229p CCC: $18.00
Published on Web 07/02/1999

to hydrolyze βLg were plasmin, trypsin, and *Staphylococcus aureus* V8 protease (*S.aur*.V8). Plasmin and trypsin both hydrolyze Lys−X and Arg−X bonds (of which 15 and 3 are present in βLg, respectively), but plasmin is more selective and preferentially attacks Lys−X bonds (Bastian and Brown, 1996). *S.aur*.V8 cleaves Glu−X and Asp−X bonds (of which 9 and 11-(10) are present in βLgA(B), respectively). At pH 4, only Glu residues are hydrolyzed, whereas at around pH 8 both Glu and Asp residues will be attacked (Drapeau, 1977). The peptide composition of the hydrolysates formed by the different enzymes was analyzed with several chromatographic methods, and the functional properties of the hydrolysates were determined and discussed in view of the peptide composition.

MATERIALS AND METHODS

Materials. Bovine βLg (95% based on dry weight, w/w) was purified from a whey protein fraction by precipitating all whey proteins except βLg and the caseinomacropeptide (CMP) and subsequent ultrafiltration to remove the CMP (Caessens et al., 1997a). The protein contained the genetic variants A and B in approximately equal amounts. Bovine plasmin (EC 3.4.21.7), trypsin (EC 3.4.21.4), aprotinin, and soybean trypsin inhibitor were obtained from Sigma (No. P-7911, T-8642, A-6012, and T-9777, respectively). Endoproteinase Glu-C from *S.aur*.V8 (EC 3.4.21.19) and *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Boehringer Mannheim (Nos. 791 156 and 874 485, respectively). Unless stated otherwise, all other chemicals were of analytical grade and were purchased from Merck, Aldrich, or BDH.

β-Lactoglobulin Hydrolysis. A βLg solution (1% w/v; filtered through 0.22 μm filters (Schleicher and Schuell GmbH, Germany)) was hydrolyzed by plasmin, trypsin, and *S.aur*.V8 at 40 °C and pH 8 to a DH of 1, 2, and 4% using a pH-stat method (Adler-Nissen, 1986). The plasmin hydrolysates are called PlDH1, PlDH2, and PlDH4, the trypsin hydrolysates TrDH1, TrDH2, and TrDH4, and the *S.aur*.V8 hydrolysates V8DH1, V8DH2, and V8DH4. The DH was calculated from the amount of base (0.1 M NaOH) used in the pH-stat technique (Adler-Nissen, 1986). Incubation times were between 10 min and 4 h for the hydrolysates of the different DHs. The enzyme/substrate ratios (E/S, on crude weight basis) used for the plasmin hydrolysis were 1/200 (w/w), 1/100 (w/w), and 1/50 (w/w) for PlDH1, PlDH2, and PlDH4, respectively. Aprotinin was added to the incubates (ratio 1/200 (v/v) of a 10 trypsin inhibitor unit/mL solution) to stop the hydrolyses. For trypsin hydrolysis, the E/S ratios were 1/400 (w/w) for TrDH1 and TrDH2 and 1/200 (w/w) for TrDH4. Soy bean trypsin inhibitor (ratio 1/170 (v/v) of a 12 mg/mL solution) was added to stop these hydrolyses. For the *S.aur*.V8 hydrolysis, the E/S ratios were 1/450 (w/w) for V8DH1 and V8DH2 and 1/250 (w/w) for V8DH4. The pH of the incubates was adjusted to pH 6 (0.5 M HCl), and TLCK was added (ratio 1/200 (v/v) of a 50 mg/mL solution) to stop these hydrolyses. The hydrolysates were then lyophilized and stored at 4 °C before further analysis.

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, USA) was used. 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) formed the eluent in the following linear gradient steps: after 3 min isocratic elution at 100% A from 0% to 50% B over 50 min, then to 70% B over 5 min, after 5 min isocratic elution at 70% B to 100% A over 5 min, and finishing with 17 min equilibration at 100% A before the next run was started. The column temperature was 30 °C, and a flow rate of 0.8 mL/min was applied. Peak detection and quantitation was at 220 nm using Turbochrom data-acquisition and processing software (Perkin-Elmer, Germany). Generally, 50 μL of a 1 mg/mL sample solution was injected onto the column.

Gel-Permeation Chromatography (GPC). The hydrolysates were analyzed by GPC under nondenaturing conditions (0.125 M potassium−phosphate buffer containing 0.125 M sodium sulfate; pH 6.65) using a Superdex 75 column (Pharmacia, Sweden) on a SMART-system (Pharmacia). A flow rate of 40 μL/min was applied, 50 μL of an 1 mg/mL sample solution was injected, and detection was at 220 and/or 280 nm. Using a Superdex 75 column on an FPLC system (Pharmacia), GPC was also performed under denaturing conditions (0.125 M potassium phosphate buffer containing 0.125 M sodium sulfate and 6 M urea; pH 6.65), and at denaturing plus reducing conditions (0.125 M potassium phosphate buffer containing 0.125 M sodium sulfate, 6 M urea and 15 mM dithiothreitol [DTT]; pH 6.65). The flow rate was 0.44 mL/min, 200 μL of an 1 mg/mL sample solution was injected, and detection was at 280 nm. Prior to analysis under denaturing and denaturing plus reducing conditions, the samples were incubated overnight at ambient temperature in the denaturing GPC buffer (the buffer for the denaturing plus reducing conditions contained 0.13 M DTT). Fresh buffers were prepared each day. The performance of the columns was tested using a mixture of markers containing the proteins BSA (molecular weight (MW) = 67 kDa), chymotrypsin A (MW = 25 kDa), ribonuclease A (MW = 13.7 kDa), aprotinin (MW = 6.5 kDa), horse myoglobulin peptide (MW = 2.5 kDa), and vitamin C (MW = 176 Da). All GPC analyses were performed at 20 °C.

Functional Properties. Foam and emulsion properties of the hydrolysates were tested in screening tests, which have been described previously (Caessens et al., 1997b). Conditions used were pH 6.7 and 4.0, ionic strength (*I*) of 75 mM, and 20 °C. Briefly, the screening tests were performed as follows. Emulsions (tricaprylin oil/water = 1/9; 0.2% and 0.05% w/v protein) were made using a laboratory high-pressure homogenizer and applying two passages at 60 bar. The emulsion-forming ability was determined immediately after homogenization by measuring the particle-size distribution (Malvern MasterSizerX, Malvern Instruments Limited, U.K.). The emulsion stability against coalescence was determined immediately after homogenization and again after 1 and 24 h by measuring the turbidity at 500 nm. The emulsions were also examined for the presence of aggregates and/or flocs using a light microscope. Foam-forming and -stabilizing ability of a 0.01% (w/v) protein solution was tested using a whipping method as described previously (Caessens et al., 1997b), and the foam height was monitored for 1 h.

RESULTS

Peptide Composition of the βLg Hydrolysates. RP-HPLC was performed in order to characterize the peptide composition of the hydrolysates. Figure 1 shows the RP-HPLC chromatograms of the plasmin (a), trypsin (b), and *S.aur*.V8 (c) hydrolysates. Intact βLg B and A have retention times of 58 and 60 min, respectively. From Figure 1, it follows that the peptide composition of the hydrolysates produced by the three enzymes was different. Besides the peaks having the same retention time as intact βLg (which is present in all hydrolysates), the peaks in the chromatogram of V8DH4 are distributed over the whole chromatogram, while the chromatogram of TrDH4 shows mainly some peaks around 30 min and around 50 min retention time. The chromatogram of PlDH4 has mostly peaks with higher retention times (55−63 min). The peptide composition of the DH1, DH2, and DH4 hydrolysates produced by each enzyme did not change dramatically with increasing DH value; only the ratio of the peptides present differed. In each case, the peak of βLgA decreased more rapidly than that of βLgB, indicating that βLgA was degraded faster by enzymatic hydrolysis than βLgB. In all hydrolysates, peaks with the same retention time as intact βLg remained, making up approximately 33%, 36%, and 18%

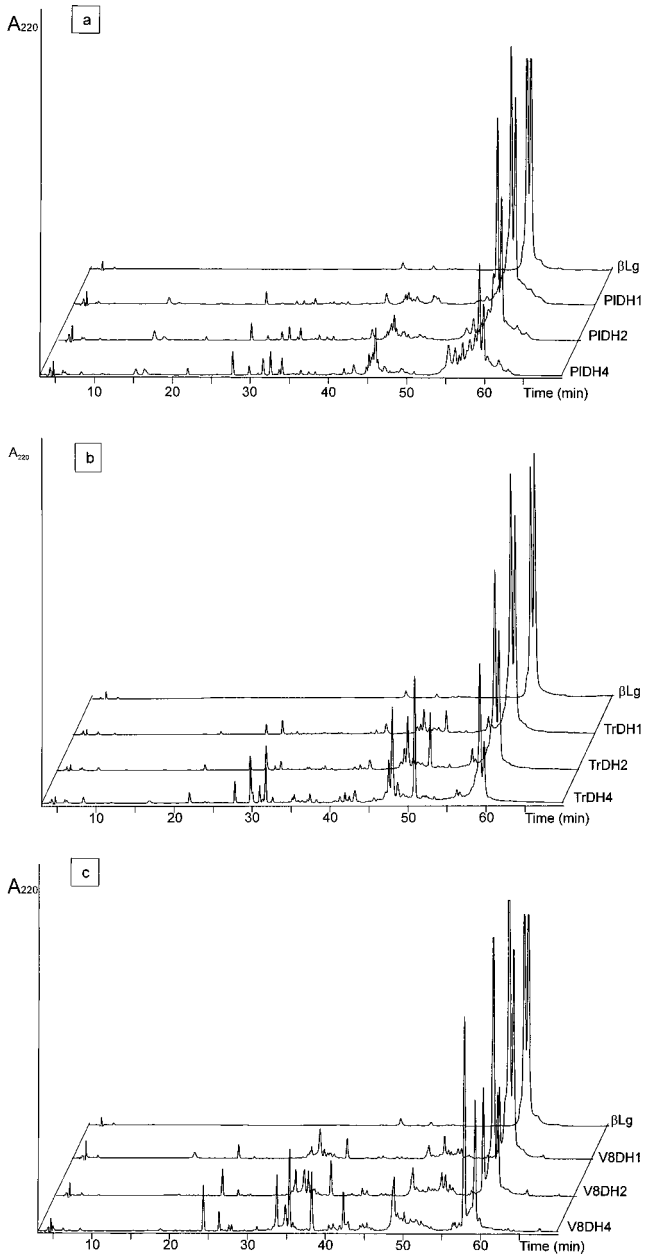

Figure 1. RP-HPLC chromatograms of the βLg hydrolysate fractions obtained by plasmin (a), trypsin (b) and *S.aur.*V8 hydrolysis (c); for abbreviations see text; for conditions used see Materials and Methods.

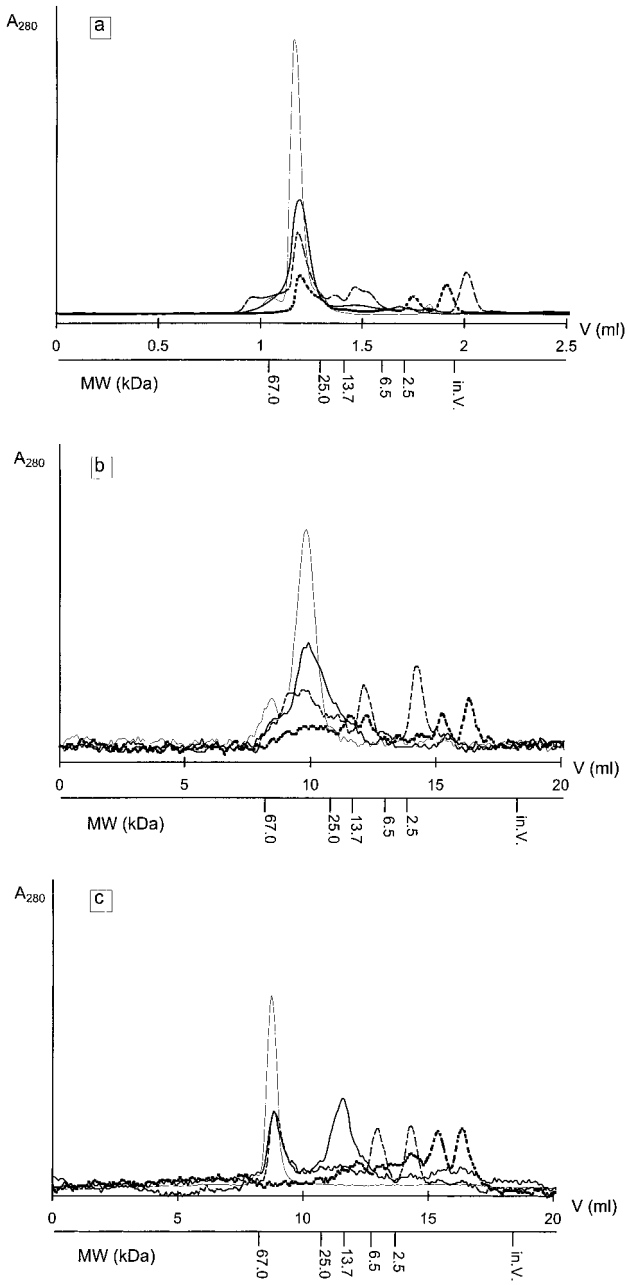

Figure 2. GPC chromatograms of PlDH4 (—), TrDH4 (– – –) and V8DH4 (- - -) hydrolysate fractions and intact βLg (–) obtained under nondenaturing (a), denaturing (b), and denaturing + reducing (c) conditions; in.V. means included volume of the column, for further abbreviations see text; for conditions used see Materials and Methods.

of the total peak area in the RP-HPLC chromatograms of the PlDH4, TrDH4, and V8DH4, respectively.

Figure 2a shows the GPC chromatograms of the PlDH4, TrDH4, and V8DH4 hydrolysates obtained under nondenaturing conditions. From these results, it follows that PlDH4 contained the lowest amount of small peptides (i.e., elution volumes of >1.7 mL). The main peak in the PlDH4 chromatogram had a retention time similar to that of the intact protein, suggesting that PlDH4 contained either a high amount of nondegraded βLg or degradation products having a retention time similar to that of βLg. V8DH4 contained the highest amount of smaller peptides and the smallest peak at a retention time similar to that of βLg.

The chromatograms in Figure 2a show peaks after the included volume, indicating nonspecific interaction of peptides with the column. Nonspecific interactions could be avoided by analyzing under denaturing conditions (with 6 M urea added). Furthermore, by analyzing in the presence of urea, hydrophobic interactions between peptides were prevented. Figure 2b shows the results obtained under denaturing conditions. The chromatogram of PlDH4 still had a main peak at the same elution volume as intact βLg (approximately 10 mL), whereas the chromatogram of TrDH4 showed two distinct peaks at larger elution volumes (approximately 12 and 14 mL). The V8DH4 hydrolysate contained a peptide composition that under denaturing conditions showed the overall smallest apparent MW and the lowest extinction at the elution volume of βLg.

Figure 2c shows the GPC results obtained under denaturing plus reducing conditions (after breaking both the intermolecular and intramolecular disulfide bonds in the hydrolysates). The chromatogram of PlDH4

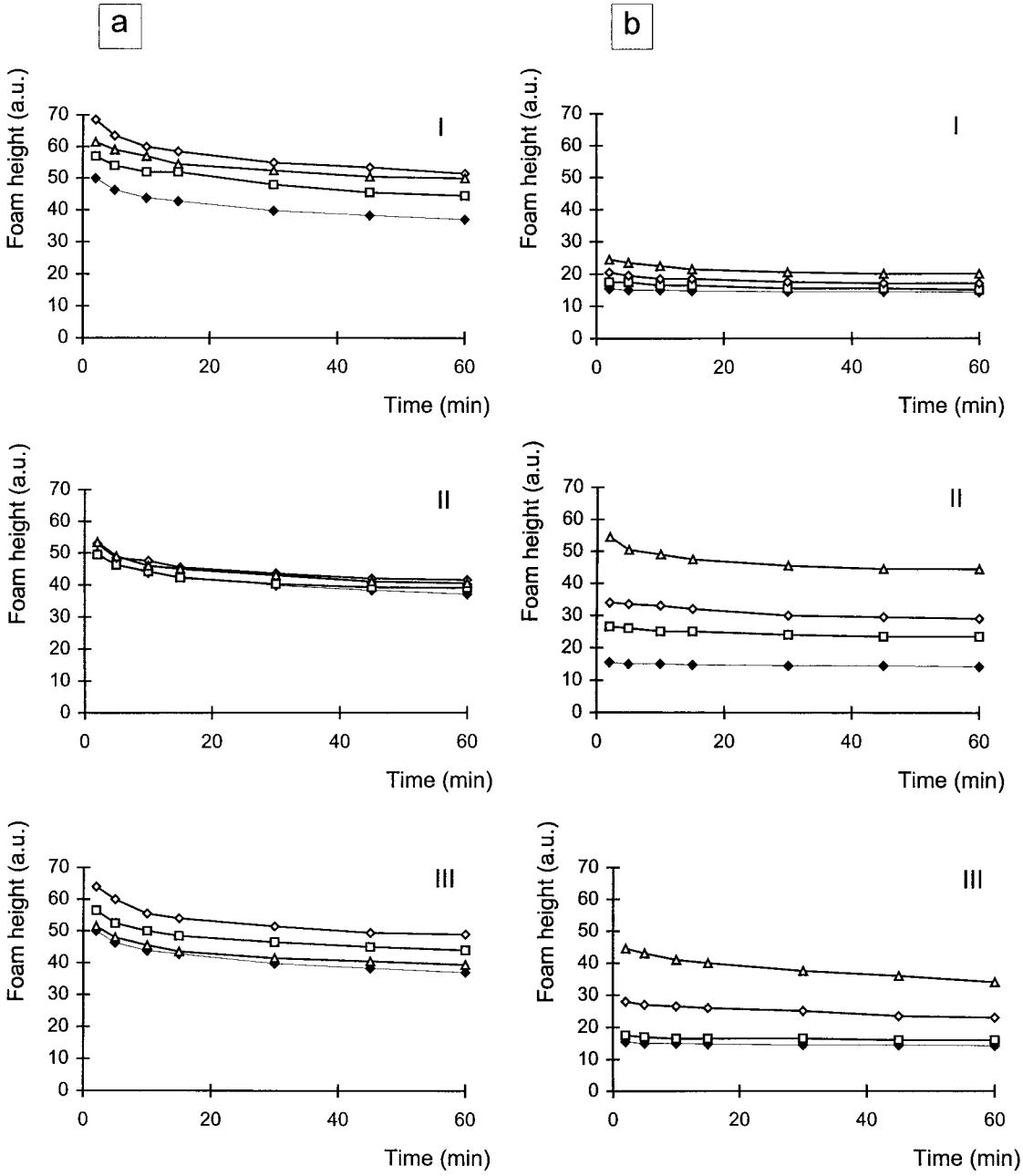

Figure 3. Foam height at pH 6.7 (a) and pH 4.0 (b) as produced with the βLg/plasmin (I), βLg/trypsin (II), and βLg/*S.aur.*V8 (III) hydrolysate fractions, as a function of time after whipping (means of duplicate measurements), intact βLg (◆), DH1 (□), DH2 (◇), DH4 (△): for abbreviations see text; for conditions used see Materials and Methods.

showed a distribution of the peptides into two main peaks, while TrDH4 and especially V8DH4 had a broader distribution of the peptides. The latter hydrolysate contained no peak with a retention time similar to that of βLg, indicating that βLg was degraded completely. In the PlDH4 and TrDH4 chromatograms, peaks with the same retention time as βLg were present, making up approximately 29% and 28% of the total peak area.

The GPC chromatograms under nondenaturing, denaturing, and denaturing plus reducing conditions of the DH1, DH2, and DH4 hydrolysates of each enzyme showed a similar peptide composition; only the peak ratios differed (no further results shown).

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

Figure 3 shows the results of the foam screening test at pH 6.7 (a) and pH 4.0 (b). At pH 6.7, the plasmin (Figure 3a-I) and *S.aur.*V8 (Figure 3a-III) hydrolysates formed more foam than intact βLg, whereas the amount of foam formed by the trypsin hydrolysates (Figure 3a-II) was about the same as that formed by βLg. In most cases, less foam was formed at pH 4 than at pH 6.7 (compare Figure 3, parts a and b). At the acidic pH, the trypsin (Figure 3b-II) and *S.aur.*V8 (Figure 3b-III) hydrolysates formed more foam than intact βLg, whereas

Table 1. Screening Test Results of Emulsions Made at pH 6.7 with βLg Hydrolysates[a,b]

	concentration					
	0.02%			0.05%		
sample	d_{32}[c] (μm)	span[c]	stability[d]	d_{32}[c] (μm)	first/second peak[e]	stability[d]
βLg	1.7	2.6	++	2.7[e]	2.5/10	++
PlDH1	1.9	1.5	++	2.5[e]	tails to 10	++
PlDH2	1.8	1.5	++	2.3		+
PlDH4	1.7	1.6	++	2.3		+
TrDH1	1.9	1.9	++	2.7[e]	2.5/10	++
TrDH2	1.8	1.5	++	2.6[e]	tails to 10	+ cr
TrDH4	1.9	1.6	++	2.3		± cr
V8DH1	1.7	1.4	++	2.2		+
V8DH2	1.7	1.4	± cr	2.6[e]	1.8/3.3	± cr
V8DH4	1.8	1.2	− cr	3.2[e]	1.7/6.2	− cr

[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; the span indicates the distribution width. [d] +, ±, and − indicate the extent of stability; cr means creaming. [e] The measurement of the average particle-size showed a double peak (values of the separate peaks are shown in parentheses).

the plasmin hydrolysate (Figure 3b-I) had foam-forming properties similar to those of βLg. At both pHs tested, the foam-stabilizing properties of all hydrolysates were good: the foams were stable during 1 h, and no coalescence was observed during the measurement.

Table 1 shows the results of the emulsion screening test at pH 6.7. At the highest concentration tested (0.2% w/v), the average particle size of the emulsion droplets formed by the hydrolysates and by βLg was similar; only the particle-size distribution (span) produced by the hydrolysates was smaller when compared to that produced by βLg. The stability (determined by measuring the turbidity at 500 nm) of these emulsions did not change during 24 h. Visually, the emulsions formed with V8DH4 and V8DH2 showed creaming after 1 and 24 h, respectively. Since no clear differences were observed at 0.2% w/v, emulsions were also made at a lower concentration (0.05% w/v). At this very low concentration, the emulsion-forming properties of the hydrolysates differed (Table 1). Only the plasmin hydrolysates (especially PlDH2 and PlDH4) formed emulsions with a uniform particle-size distribution, whereas the other hydrolysates formed emulsions containing a double peak in the particle-size distribution. Smaller and larger droplets were detected by microscopic analysis in the samples containing the double peak in the particle-size distribution (βLg, TrDH1, TrDH2, V8DH2, and V8DH4), explaining the bimodal particle-size distribution. The turbidity (500 nm) of the emulsions formed by V8DH4, V8DH2, and TrDH4 decreased during 24 h. Visually, the emulsion formed by V8DH4 already showed creaming after 1 h. Besides V8DH4, the hydrolysates V8DH2, TrDH4, and TrDH2 showed considerable creaming after 24 h, while βLg and the other hydrolysates formed emulsions with a minor amount of creaming after 24 h.

At pH 4 and 0.2% w/v, all hydrolysates and the intact βLg formed aggregated emulsions, and the particle-size distribution could, therefore, not be measured with the Malvern MasterSizer X (no further results shown).

DISCUSSION

Peptide Composition. The RP-HPLC chromatograms (Figure 1) showed that the peptide composition of the hydrolysates formed by the three different enzymes differed, which was to be expected, since the enzymes used had different specificities (see the Introduction). The PlDH4 fraction has many peaks with high retention times (55−60 min), indicating that the peaks contain large peptides and/or peptides with many hydrophobic groups. The TrDH4 fraction and particularly the V8DH4 fraction contain more peaks between 20 and 50 min and probably contain smaller, less hydrophobic peptides than the PlDH4 fraction. Other authors also analyzed βLg hydrolysates by RP-HPLC, e.g., tryptic hydrolysate fractions (Dalgalarrondo et al., 1990; Turgeon et al., 1992). The former group hydrolyzed βLg extensively, resulting in no residual βLg in the final hydrolysate. The latter group also found peaks at the end of the chromatogram of the total hydrolysate (similar to our PlDH4 hydrolysate). It was suggested that these peaks might contain peptide aggregates, possibly induced by SH/SS exchange (Turgeon et al., 1992).

The RP-HPLC results (Figure 1) further show that βLgA is degraded faster by enzymatic hydrolysis than βLgB. This was already described in the literature (Schmidt and van Markwijk, 1993), and the more flexible or less stable tertiary structure of βLgA was mentioned as a reason for its higher susceptibility to enzymatic degradation (Huang et al., 1994).

The amounts of βLg remaining in the DH4 hydrolysates determined by RP-HPLC were higher than those determined by GPC under denaturing plus reducing conditions. This could indicate that peaks having the same RP-HPLC retention time as βLg contain not only intact βLg but also large, hydrophobic peptides, which have different GPC retention times. At the same DH, the βLg/*S.aur*.V8 hydrolysate overall has the lowest molecular sizes of the peptides compared to the βLg/plasmin and βLg/trypsin hydrolysates and contains the lowest amounts of intact βLg. As discussed above, peaks having the same retention time as βLg can also be large, hydrophobic peptides. Apparently, *S.aur*.V8 hydrolyzes βLg in such a way that smaller peptides are released and the larger peptides have a different chromatographic behavior than intact βLg.

The GPC results of the hydrolysates obtained under nondenaturing conditions show an apparent molecular size similar to that of the intact βLg. However, GPC of the hydrolysates obtained under denaturing and denaturing plus reducing conditions showed that the peptides can be linked by hydrophobic interactions (in the case of the trypsin and *S.aur*.V8 hydrolysates, Figure 2b) as well as by disulfide bonds (most evident in the case of plasmin hydrolysates, Figure 2c). Turgeon et al. (1992) identified peptides present in a tryptic βLg hydrolysate but could not assign the tryptic fragment βLg[f 102−124] to any of the peaks identified in the chromatogram. Since this part of the βLg sequence contains a disulfide group and a free thiol group, the possibility of SH/SS interactions between βLg peptides was mentioned (Turgeon et al., 1992). It is likely that similar SH/SS interactions occurred in the hydrolysates described in this study and that they were most evident in the plasmin hydrolysate.

Functional Properties. Our results showed that the βLg hydrolysates had similar or increased functional properties compared to those of βLg. Increased functional properties of βLg after tryptic hydrolysis and subsequent fractionation of the hydrolysate have been observed previously (Turgeon et al., 1992; Chen et al., 1994; Huang et al., 1996). In this study, the plasmin

hydrolysates had the best foam- and emulsion-forming properties at pH 6.7. The functional properties of the plasmin hydrolysates decreased with increasing DH (no further results shown). Our results suggest an optimal DH for the functional properties, which has been suggested before (Kuehler and Stine, 1974; Arai and Fujimaki, 1991). However, it is generally accepted that besides the DH other factors, such as hydrophobicity, molecular size, and amphipathicity, might determine the functional properties (Panyam and Kilara, 1996).

It should be noted that residual βLg, if present in the hydrolysates (Figures 1 and 2b), might account for part of the functional properties determined. However, the improvement of the functionality of the hydrolysates compared to the intact protein had to be caused by the peptides. The reason for the improved emulsion-forming properties compared to those of βLg could be the higher concentration of molecules present in the hydrolysates. However, this cannot be the only reason, since the *S.aur*.V8 and trypsin hydrolysates did not have improved emulsion-forming properties. Other reasons (such as the influence of the hydrolysates on the surface tension at the air−water and oil−water interface or the prevention of coalescence during the formation of the air bubbles and oil droplets) may be important for the formation as well (Walstra and Smulders, 1998) but have not been investigated for the hydrolysates in this study. The emulsion stability of the *S.aur*.V8 hydrolysates is poor. An explanation could be the low apparent MW of the peptides present (as analyzed by GPC, Figure 2), since it is generally accepted that peptides must have a certain minimum MW to retain some functionality (Turgeon et al., 1992).

The main results found for the βLg hydrolysates formed by the action of the different enzymes can be summarized as follows:

residual amount of intact βlg:
S.aur.V8 < trypsin ≈ plasmin

presence of hydrophobic groups:
plasmin > trypsin ≈ *S.aur*.V8

presence of disulfide-linked peptides:
plasmin > trypsin ≈ *S.aur*.V8

functional properties:
plasmin > trypsin > *S.aur*.V8

Limited hydrolysis by trypsin, plasmin, and *S.aur*.V8 may improve the functional properties of βLg (the summarized functional properties mentioned above are mainly based on the increased emulsion-forming properties of the plasmin hydrolysates and the decreased emulsion-stabilizing properties of the *S.aur*.V8 hydrolysates).

In conclusion, βLg hydrolysis by the action of plasmin resulted in a hydrolysate containing relatively large peptides with many hydrophobic groups, of which many were disulfide-linked peptides. This plasmin hydrolysate also had the best functional properties. To establish a structure−function relationship, further research has been focused on the fractionation of the PlDH4 hydrolysate, the functionality of the fractions, the identification of the peptides in the fractions, and the importance of the presence of the disulfide bonds (Caessens et al., 1999).

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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.

JF981229P