Comparison of the Size and Rate of Formation of Peptides Released by Limited Proteolysis of β-Lactoglobulins A and B with Immobilized Trypsin†

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Proteolytic susceptibility of β-lactoglobulin (β-Lg) genetic variants A and B was determined by subjection to immobilized trypsin. Products of limited proteolysis, performed in 50 mM Tris-HCl buffer (pH 8.0) at 4 °C, were characterized by fractionation with anion-exchange chromatography and by denaturing gel electrophoresis of the isolated fractions. Both the rate of appearance of products and the peptides formed were different for the two variants. Two major peptides, with estimated molecular weights of 7500 and 8200 were obtained in homogeneous form by ion-exchange fractionation of β-Lg A hydrolysates. Similar fractionation of β-Lg B hydrolysates yielded fractions that were heterogeneous, containing larger peptides that were not present in β-Lg A hydrolysates. The increased rate of appearance of products with β-Lg A and the release of larger peptides from β-Lg B suggest that variant A has a lower structural stability, perhaps in localized areas of the molecule, causing a change in the relative susceptibilities of the 17 tryptic hydrolysis sites.

INTRODUCTION

Genetic variants of β-lactoglobulin (β-Lg A and B) in bovine whey are composed of 162 amino acid residues but differ at residue 64 (A/B, Asp/Gly) and at residue 118 (A/B, Val/Ala) (Swaisgood, 1982). Recently (Huang et al., 1994), the relative structural stabilities of genetic variants A and B were investigated using immobilized trypsin as a probe and differential scanning calorimetry (DSC). It was found that β-Lg A has a greater susceptibility to tryptic hydrolysis and lower thermal stability than β-Lg B, suggesting that, thermodynamically, the β-Lg A variant has a more flexible or less stable tertiary structure than β-Lg B. This result reflects the overall rate of hydrolysis since the disappearance of substrate was quantitatively measured in that study. In the present study the hydrolysis products were examined. Since both variants contain the same 17 potential tryptic hydrolysis sites (Swaisgood, 1982), we asked whether the difference in susceptibility resulted from a similar change in susceptibilities at all tryptic sites or from a change in the relative susceptibilities at various cleavage sites. In the former case, trypsin would release the same initial products with both variants, whereas, in the latter case, different initial products would appear in the reaction.

Thus, the present study was conducted to determine the initial products of tryptic hydrolysis of β-Lg A and B by comparison of the fractionation profiles resulting from ion-exchange FPLC. The isolated fractions were further characterized by denaturing gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade, and distilled-deionized water was used throughout. Genetic variants β-Lg A and B were purified from homozygous cow's milk as previously described (Fox et al., 1967). The purity of both variants was greater than 95% as determined by peak areas obtained with FPLC. Trypsin (type XIII, TPCK treated from bovine pancreas) was obtained from Sigma Chemical Co. (St. Louis, MO).

Immobilization of Trypsin. Trypsin was covalently immobilized on succinamidopropyl controlled-pore glass beads as previously described (Janolino and Swaisgood, 1982; Huang et al., 1994). The tryptic activity of the biocatalyst was determined according to the method of Taylor and Swaisgood (1980) using *p*-tosyl-L-arginine methyl ester (TAME, Sigma) as the substrate. The measured activity was 37 μmol min^{-1} mL^{-1} of the biocatalyst.

Tryptic Hydrolysis of β-Lg A and B. Both genetic variants were hydrolyzed in 50 mM Tris-HCl buffer, pH 8.0, at a concentration of 5 mg/mL. Lyophilized protein (40 mg) was dissolved in 8.0 mL of 50 mM Tris-HCl, pH 8.0, containing 0.02% NaN_3. After filtration with a 0.45-μm filter, 6 mL of the β-Lg solution was recirculated through a fluidized-bed bioreactor containing 2 mL of immobilized trypsin beads at 4 °C. Samples were withdrawn at various time intervals from the reaction mixture and centrifuged (13600*g* for 5 min) prior to analysis by ion-exchange FPLC.

FPLC Chromatography. Tryptic hydrolysate solutions were analyzed by FPLC with a Mono Q HR 5/5 anion-exchange column (Pharmacia LKB, Piscataway, NJ) that had been equilibrated with 20 mM piperazine, pH 6.0 (buffer A). The FPLC system was a Waters Model 510 pump, a Model UK6 injector, an automated gradient controller system, and a Model 990 photodiode array detector (Waters, Milford, MA). The detector was equipped with an APC IV series computer (NEC Information System, Inc., Foxborough, MA) for data acquisition and spectral analysis. Buffers were filtered through 0.2-μm filters and degassed with an ultrasonic cleaner under vacuum. After centrifugation at 13600*g* for 5 min, 150-μL samples were injected and eluted at room temperature using a flow rate of 1.0 mL/min with a salt gradient formed with 0.3 M NaCl in 20 mM piperazine, pH 6.0 (buffer B). The gradient was formed using 0–60% buffer B for 30 min and 60–100% buffer B for 10 min, followed by 100% for 5 min. The column was returned to the original condition by 100–0% buffer B for 5 min. The absorbance of the eluate was monitored at 280 nm. Each peak was collected and lyophilized for further analysis by SDS-PAGE.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the peptides isolated by FPLC fractionation was performed on an LKB 2050 Midget electrophoresis unit (LKB Produkter AB, Bromma, Sweden) with a gel gradient of 16–27% acrylamide. Each lyophilized fraction from

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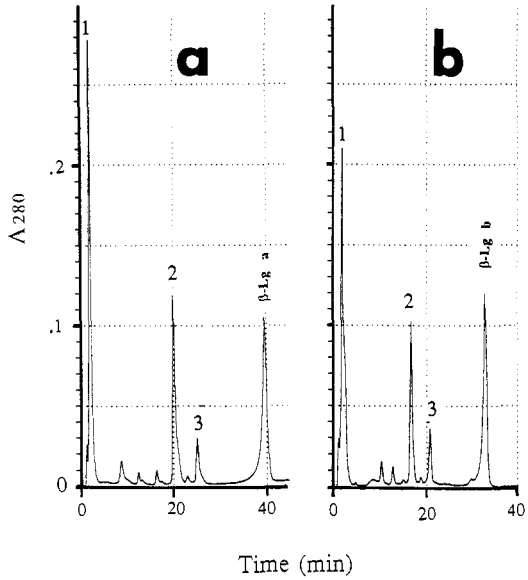

Figure 1. Typical fractionation profiles of tryptic hydrolysates (180-min hydrolysis time) of β-Lg A (a) and B (b) obtained by chromatography on a Mono Q HR 5/5 anion-exchange column. Samples (150 μL) were loaded onto the column in 20 mM piperazine, pH 6.0, and eluted with a salt gradient formed with 0.3 M NaCl in the buffer. Three major fractions were designated 1, 2, and 3 according to the order of increasing retention times for both β-Lg A and B. Fractions 2 and 3 for further analyses were obtained by fractionation of 1-mL samples.

FPLC was dissolved in SDS–PAGE buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol with or without 2-mercaptoethanol). Approximately 2–10 μg was loaded into the gel. The peptide or protein bands were visualized by staining with Coomassie Brilliant Blue R250. Molecular weight standards were from Sigma. Relative migration (R_f) values were measured with respect to a dye front. A calibration curve was prepared from triplicate measurements of the position of the molecular weight standards by fitting to the relationship $\log M_r = a + bR_f$, where M_r is the subunit molecular weight and a and b are constants defining the linear fit. This calibration was then used to estimate the molecular weight of the peptides.

RESULTS

FPLC Fractionation of Tryptic Digests. Under the same reaction conditions with immobilized trypsin, the FPLC elution profiles for hydrolysates of β-Lg A and B are different, indicating that different peptide products are released (Figure 1). Intact β-Lg exhibited a retention time of 39.5 min for β-Lg A, while that of β-Lg B was 32.9 min, which is consistent with the two additional negative charges per dimer of variant A (Swaisgood, 1982). Both hydrolysate profiles displayed three major fractions in addition to the intact protein. However, the magnitudes and elution times for peaks 2 and 3 were quite different, suggesting that the peptides released initially were different. The retention times for the three major fractions, designated 1, 2, and 3 in order of increasing times, were 1.7, 20.0, and 25.1 min, respectively, for β-Lg A (Figure 1a), while those for the B variant were 1.8, 16.6, and 20.8 min (Figure 1b).

Kinetics of Tryptic Hydrolysis. Both the disappearance of β-Lg and the appearance of peptide products as identified by FPLC were followed as a function of time. Changes in the relative concentrations of these components with time are shown for both variants A and B in Figure 2. It is apparent from these data that β-Lg A is degraded more rapidly than β-Lg B under these conditions. For example, the times for 50% conversion of substrate were

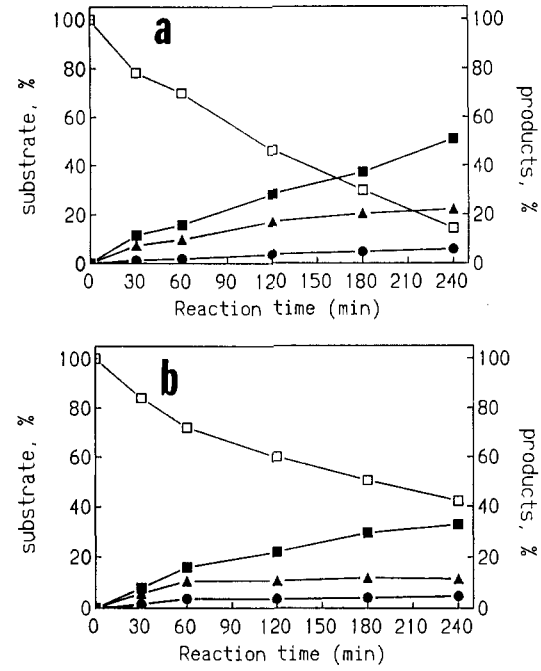

Figure 2. Progress curves for hydrolysis of β-Lg A (a) and B (b) obtained by limited proteolysis with immobilized trypsin. Proteolysis was performed in 50 mM Tris-HCl buffer (containing 0.02% NaN_3), pH 8.0, at 4 °C using a substrate concentration of 5 mg/mL and 2 mL of immobilized trypsin beads. (□) Fraction of the total area for intact substrate; (■) fraction 1; (▲) fraction 2; (●) fraction 3.

110 min for β-Lg A compared to 160 min for β-Lg B. At a hydrolysis time of 240 min the amounts of variants A and B degraded were 85% and 60%, respectively.

Likewise, the rate of appearance of products is faster for β-Lg A than for B (Figure 2). Furthermore, the relative concentrations of peptides in fractions 2 and 3 for β-Lg A appear to steadily increase, whereas the peptide concentrations in fractions 2 and 3 of β-Lg B seem to plateau after 60 min.

Analysis of Hydrolysates by SDS–PAGE. Typical results of SDS–PAGE analyses of proteolysis mixtures and FPLC fractions 2 and 3 obtained for both β-Lg A and B in the presence and absence of 2-mercaptoethanol are shown in Figure 3. Examination of FPLC fraction 1 indicated that it contained small peptides with molecular weights of less than 3000 (data not shown). As shown in Figure 3a, unfractionated hydrolysates of β-Lg A exhibited two major peptides in the presence of 2-mercaptoethanol. Unfractionated hydrolysates of β-Lg B contained several major peptides with larger molecular weights than those in β-Lg A hydrolysates in the presence of 2-mercaptoethanol. Fractions 2 and 3 obtained by FPLC of β-Lg A hydrolysates appeared homogeneous by SDS–PAGE with bands corresponding to the 6800- and 5900-Da peptides, respectively (Table 1). On the other hand, fractions 2 and 3 from FPLC of β-Lg B hydrolysates were heterogeneous. Fraction 2 appeared to contain three peptides with the sizes given in Table 1, while fraction 3 exhibited one large and one smaller peptide.

Since β-Lg contains disulfide bonds linking cysteinyl residues 66 to 160 and 106 to 119 (Hambling et al., 1992), it is possible that, following proteolysis, two peptides could remain linked through a disulfide. Therefore, the hydrolysates were also analyzed by SDS–PAGE in the absence of 2-mercaptoethanol. Patterns shown in Figure 3b are similar to those with 2-mercaptoethanol, but the estimated sizes of some peptides are slightly larger (Table

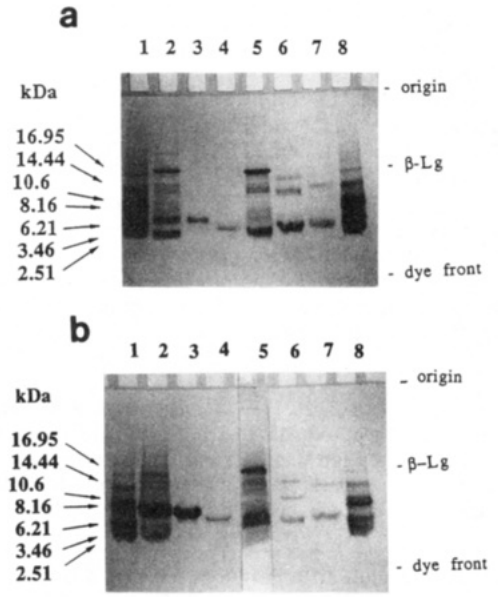

Figure 3. SDS–polyacrylamide gel electrophoretograms of β-Lg hydrolysates and peptide fractions isolated by anion-exchange chromatography on Mono Q. A hydrolysis time of 240 min was chosen to give optimum amounts of the peptides released in the early phase of proteolysis. Lanes 1 and 8 are protein markers; lanes 2 and 5 are total tryptic limited hydrolysates of β-Lg A and B, respectively; lanes 3 and 4 are chromatographic fractions 2 and 3 from β-Lg A (see Figure 1a); lanes 6 and 7 are chromatographic fractions 2 and 3 from β-Lg B (see Figure 1b). (a) Sample buffer contained 2-mercaptoethanol. (b) Sample buffer did not contain disulfide reductant.

Table 1. Molecular Weights Estimated from SDS–PAGE for the Initial Peptides Released from β-Lg A and B by Immobilized Trypsin

chromatographic fraction	estimated molecular weight[a] (×10⁻³) with 2-ME, M_r ± SD	no 2-ME, M_r ± SD	increase
β-Lg A			
fraction 2	6.8 ± 0.7	8.2 ± 0.2	1.4
fraction 3	5.9 ± 0.3	7.5 ± 0.3	1.6
β-Lg B			
fraction 2	16.4 ± 0.7	15.6 ± 1.3	−0.8
	12.4 ± 0.7	11.9 ± 1.3	−0.5
	5.8 ± 0.6	7.4 ± 0.5	1.6
fraction 3	13.3 ± 0.8	14.6 ± 1.1	1.3
	5.6 ± 0.4	7.8 ± 0.6	2.2

[a] Mean value and standard deviations calculated from triplicate gels. The middle of the stained zone was used to estimate its position.

1). Thus, the estimated sizes of the two peptides obtained from β-Lg A are 8200 and 7500 Da as isolated in fractions 2 and 3, respectively, representing increases of 1400 and 1600 Da. With β-Lg B, the two larger peptides in fraction 2 did not appear to increase in size, while the smallest peptide increased by 1600 Da. Both peptides in Fraction 3 from β-Lg B were larger when 2-mercaptoethanol was omitted.

DISCUSSION

Analysis of the tryptic hydrolysates of β-Lg A and B by ion-exchange FPLC and by SDS–PAGE indicate that the initial peptides released are different for the two variants. Electrophoretic analyses show that chromatographic fractions 2 and 3 from variants A and B are quite different even though the retention times of fraction 2 from variant A and fraction 3 from variant B are similar. Thus, fraction 2 from variant A is homogeneous, while fraction 3 from variant B contains two peptides. Also, although the size of one of the peptides in these two fractions is similar, the decrease in size upon disulfide reduction is substantially greater for the peptide in fraction 3 of β-Lg B.

Finally, several of the peptides present in a limited hydrolysate of β-Lg B are larger than those in variant A hydrolysates. This observation is consistent with the lesser susceptibility of β-Lg B to trypsinolysis as noted here and in the previous study (Huang et al., 1994). It appears that the larger peptides formed initially with trypinolysis of variant A are very susceptible to further degradation and hence not observed in the hydrolysates analyzed. These results lead to the conclusion that the relative susceptibilities of the 17 tryptic sites in β-Lg must be different in variants A and B. Peptide products appear more rapidly with variant A, and also the relative amounts of the various peptides produced are different. Furthermore, larger peptides were detected in early hydrolysates from β-Lg B that could not be detected from β-Lg A.

Recently, the major peptide fraction produced by limited trypsinolysis of a mixture of β-Lg A and B was isolated and characterized (Chen et al., 1993). Analysis of the reduced peptides by mass spectrometry indicated that β-Lg A(f48-101) and β-Lg A(f41-100) with masses of 6198 and 6931 Da, respectively, were the major peptides present. None of the major peptides detected were derived from β-Lg B. These observations support the conclusions of the present study suggesting that β-Lg A is more susceptible to proteolysis. Moreover, the estimated sizes of the peptides in fractions 2 and 3 isolated from β-Lg A hydrolysates are consistent with the reported masses.

The increased estimated sizes obtained in the absence of 2-mercaptoethanol suggest that a small peptide is attached to the larger peptide by a disulfide bond. Alternatively, the apparent increase in molecular weight estimated by SDS–PAGE could result from molecular expansion upon disulfide reduction. However, the linkage of a small peptide via a disulfide to the larger peptide was confirmed by mass spectrometry in the previously reported study of peptides isolated from a limited trypsinolysis of a mixture of variants (Chen et al., 1993). Moreover, the sizes estimated by SDS–PAGE are consistent with the reported masses of 7900 and 8600 Da for the peptides with disulfides intact.

Correlation of susceptibility of a peptide bond to proteolysis with the flexibility of residues surrounding that bond has been well documented, and hence susceptibility to proteolysis has been used as a probe of protein structure (Matthyssens et al., 1972; Mihalyi, 1978; Burgess et al., 1975; Church et al., 1982; Swaisgood and Catignani, 1987). The present study further illustrates the sensitivity of this method to protein structure or structural stability. Since no differences in secondary or tertiary structure could be discerned between β-Lg variants A and B (Monaco et al. 1987), we suggest that the subtle difference in primary structure results in a small difference in structural stability. Furthermore, the changes in stability may be localized, resulting in changes of the relative susceptibilities of the tryptic sites to proteolysis.

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