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Aggregate Formation during Hydrolysis of β-Lactoglobulin with a Glu and Asp Specific Protease from *Bacillus licheniformis*

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The hydrolysis of isolated β-lactoglobulin (9 and 70−200 mg/mL) by a *Bacillus licheniformis* protease was followed to assess whether aggregates and gels, respectively, were formed during hydrolysis. Changes during hydrolysis were monitored by electrophoresis, dynamic light scattering, and fluorescence and circular dichroism spectroscopy. Gelation was monitored by dynamic oscillation rheology. Upon hydrolysis of a β-lactoglobulin preparation with the *B. licheniformis* protease aggregates were formed and a soft gel resulted from only 70 mg/mL of β-lactoglobulin. The aggregates consisted of a number of peptides with molecular weight ranging from 2000 to 6000 and p*I* from 5 to 8. As the aggregates were solubilized in either SDS or urea or at extreme pH values, it is proposed that noncovalent interactions, mainly electrostatic and hydrophobic, are major interacting forces. These kinds of aggregates are thought to be important in protease-induced gelation of whey protein isolate solutions.

Keywords: *β-Lactoglobulin; proteolysis; aggregation; fluorescence; circular dichroism*

INTRODUCTION

Extensive enzymatic hydrolysis of proteins leads to fragmentation of the protein into a number of peptides, normally resulting in formation of smaller molecules. It has been shown recently that hydrolysis of a whey protein isolate solution by a Glu and Asp specific protease from *Bacillus licheniformis* (BLP) led to the formation of aggregates, ultimately forming a gel (Otte et al., 1996a). At present, it is not clear why hydrolysis with this enzyme results in formation of larger particles. It was hypothesized by Otte et al. (1996b) that one or a few cuts in the major proteins may cause them to unfold, thereby exposing hydrophobic areas, which could bind to hydrophobic areas of other molecules and in this way lead to aggregation and gelation. As a first step toward relating protein structure to gelation ability, the outcome of hydrolysis as well as the nature of the aggregates formed during hydrolysis should be investigated.

β-Lactoglobulin, the most abundant whey protein in bovine milk, is believed to have a major impact on the heat-set gelation properties of whey protein products (Mulvihill and Donovan, 1987; McSwiney et al., 1994). It is not known, however, whether β-lactoglobulin is largely responsible for the enzyme-induced gelation properties of whey protein isolate (Otte et al., 1996a). Hydrolysis of β-lactoglobulin in dilute solutions by BLP has been characterized by Madsen et al. (1997), who did not mention aggregate formation. In their work, after 4 h of hydrolysis at 40 °C, about half of the β-lactoglobulin had been degraded to ≈25 peptides, as shown by both reversed-phase HPLC (RP-HPLC) and capillary electrophoresis. Size exclusion HPLC (SE-HPLC) showed that the resulting peptides were of various sizes, most with molecular weights around 2000 or 5500.

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The purpose of the present study was to assess whether aggregates and finally gels were formed when a β-lactoglobulin preparation was hydrolyzed by BLP, as well as to characterize the aggregates formed. Initially, aggregate formation was followed during hydrolysis under conditons as used by Madsen et al. (1997). Gel formation induced by BLP was studied and compared to the gelation of whey protein isolate as described by Otte et al. (1996a). Further, the hydrolysate was characterized by RP-HPLC and aggregates were isolated and characterized by dissolution in various buffers.

MATERIALS AND METHODS

Materials. The β-lactoglobulin preparation was PSDI 24 from MD Foods Ingredients amba (Videbaek, Denmark). The preparation contained 95% dry matter of which 92% was protein. The content of lactose and fat was <1%, and the mineral content consisted of approximately 1.1% Na, 0.35% P, and 0.02% Ca and <0.1% of other minerals. Capillary electrophoresis and RP-HPLC showed that the product contained ≈70% β-lactoglobulin and contaminations of peptides, mainly caseinomacropeptide (data not shown). Electrospray ionization mass spectrometry and anion exchange chromatography showed that the genetic variants A and B of β-lactoglobulin were present in the approximate proportion 60:40 (data not shown).

The protease from *B. licheniformis* (BLP), obtained from Novo Nordisk A/S (Bagsvaerd, Denmark), was a lyophilized powder with an activity of 8.25 Anson units/g and a specificity toward Glu−X and Asp−X bonds, with a strong preference for Glu−X bonds (Svendsen and Breddam, 1992). The whey permeate powder used was Variolac-83 (MD Foods Ingredients amba). The calcium content of the permeate powder was 1 ppm as determined by atomic absorption spectrophotometry of La^{3+}-containing samples. *cis*-Parinaric acid was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade from Merck (Darmstadt, Germany), and highly purified water (MilliQ Plus; Millipore Corp., Bedford, MA) was used throughout.

Hydrolysis and Aggregate Formation. To follow the hydrolysis of β-lactoglobulin and formation of aggregates, β-lactoglobulin was dissolved at 10.5 mg/mL in 0.075 M Tris-

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HCl buffer, pH 7.5. After equilibration for 10 min at 40 °C, BLP was added at an enzyme-to-protein ratio of 2% (w/w), resulting in a substrate concentration of 8.7 mg of protein/mL, and the mixture was incubated at 40 °C. All hydrolysates were made from the same substrate and enzyme preparations, kept at −20 °C until use. Identical hydrolysates were used for dynamic light scattering, fluorescence spectroscopy, and circular dichroism measurements. Hydrolysate samples (50 μL) for analysis by capillary electrophoresis, RP-HPLC, and isoelectric focusing were added to 150 μL of aqueous 0.35% trifluoroacetic acid (TFA) to lower the pH to ≤2.5, and inactivate the enzyme, and kept at 5 °C or frozen until analysis. Another hydrolysate, initially diluted 10 times as the sample used for circular dichroism measurements, was made. Aliquots from this hydrolysate were analyzed by RP-HPLC (diluted 1:1 with 1% TFA) as well as by SE-HPLC (injected immediately without lowering of pH) using a mobile phase consisting of Tris buffers to mimic the conditions existing in the hydrolysate and thus avoid effects of extreme conditions on the association of peptides formed.

Gelation. Gelation studies were performed with 70, 120, and 200 mg/mL of protein. The β-lactoglobulin powder was dissolved in a whey permeate solution of the same conductivity (1.45 mS) as the 120 mg/mL whey protein isolate solution used by Otte et al. (1996a), the pH was adjusted to 7.0 with a few drops of 1 M NaOH, and gelation was monitored at 40 °C. In preliminary experiments aggregate formation and gelation were observed visually also at 50 and 60 °C. Dynamic oscillation studies were performed at 40 °C as described by Otte et al. (1996a) using a Bohlin VOR rheometer (Bohlin Instruments Ltd., Cirencester, U.K.) equipped with a C_{14} measuring system. An amount of enzyme (dissolved in 100 μL of permeate) equivalent to 2% (w/w) of the protein was rapidly mixed into 3 mL of sample, of which 2.8 mL was immediately transferred to the rheometer and covered with a thin layer of vegetable oil, and measurements were taken every 300 s. An additional experiment was performed using a 0.075 M Tris-HCl buffer, pH 7.5, as used for the hydrolytic experiments (see above). For all rheological measurements, identical solutions of β-lactoglobulin with heat-inactivated enzyme (boiled for 20 min) served as control. All experiments were performed at least twice. The gel time was defined as the time of the first measurement when the phase angle, δ, remained below 45°, the rate of gelation was calculated from the slope of the steepest part of the complex modulus (G^*) versus time curve, and the gel strength was defined as the highest G^* value measured.

Characterization of a Concentrated Hydrolysate. A solution of β-lactoglobulin (120 mg of protein/mL) in water adjusted to pH 7 was hydrolyzed with 2% (w/w) BLP for 4 h. After the pH was lowered to 2 by addition of 0.5% TFA, 2 mL of the hydrolysate was fractionated the next day by semi-preparative RP-HPLC. Fractions of 1.2 mL were collected, evaporated, frozen, and freeze-dried. The dried fractions were redissolved in 100 μL of water, the pH was adjusted to 7.5 with 0.1 M NaOH (0.5−100 μL), and visual turbidity was noted. Finally, the fractions were diluted 100 times with water and their absorbance at 220, 280, and 420 nm was measured.

Characterization of Aggregates. Aggregates were isolated after incubation of β-lactoglobulin with 2% (w/w) BLP under standard conditions for 18 h or at 50 °C for 3.5 h. The hydrolysate was centrifuged (1900*g*, 10 min, ambient temperature), and the supernatant was discarded. The precipitate was washed with water, the pH was lowered to 2.5 with 0.1% TFA, and the suspension was finally freeze-dried. The freeze-dried samples were redissolved at 2 mg/mL in one of the following buffers: (1) 10 mM Tris-HCl, 1 mM EDTA, pH 8; (2) buffer 1 with 2% (w/v) sodium dodecyl sulfate (SDS); (3) buffer 1 with 2% (w/v) SDS and 0.5% (w/v) dithioerythritol (DTE); (4) buffer 1 with 8 M urea; (5) buffer 1 with 8 M urea and 0.5% (w/v) DTE. For isoelectric focusing, a concentration of 100 mg/mL in buffer 4, however, was necessary to dissolve enough material for visual observation of bands in the gel. The solutions, in which pH varied from 7.1 to 7.5, were thoroughly mixed and left at 39 °C for 1 h. Any undissolved material was removed by centrifugation (10 min, 1900*g*, ambient temperature); the supernatants were analyzed by SDS−PAGE, and turbidity was measured as the apparent absorbance at 420 nm using water as reference. The amount of dissolved protein was measured in 10× diluted samples at 280 nm using the respective buffers (also diluted) as reference. In another experiment, aggregates harvested without addition of TFA were suspended at 3 mg/mL in a range of McIlvain (pH 2.2−8.0) and 0.1 M borate (pH 9.0 and 10.0) buffers with and without 1 M NaCl. After thorough mixing, the samples were allowed to equilibrate for 1 h at room temperature and overnight at 5 °C, and undissolved material was sedimented by centrifugation (11000*g*, 3 min, ambient temperature) before measurement of the absorbance at 280 and 420 nm using the respective buffers as reference.

Dynamic Light Scattering. The β-lactoglobulin sample for dynamic light scattering was filtered (0.22 μm) and equilibrated at 40 °C for 10 min before addition of enzyme at 2% (w/w). Measurements were taken in situ every 5 min using a Malvern Autosizer 2C instrument connected to a Malvern Model 7032 64-channel digital autocorrelator. The light source was a 632.8 nm He−Ne laser, and the scattering angle was 90°. Sizes were calculated from correlation functions by the method of cumulants.

Fluorescence Spectroscopy. Samples (15 μL) of hydrolysate were withdrawn at fixed times and immediately added to 2985 μL of 0.075 M Tris-HCl buffer, pH 7.5. Intrinsic fluorescence was measured using a SLM48000S spectrofluorometer (SLM Instruments Inc., Urbana, IL) as described by Stapelfeldt et al. (1996), with 290 nm excitation wavelength, and emission was recorded at 90° in the wavelength interval 300−400 nm. For measurement of hydrophobicity, hydrolysate samples of 15 μL taken at various times were immediately added to 2950 μL of the Tris-HCl buffer and 35 μL of a 1 mM solution of *cis*-parinaric acid in dimethyl sulfoxide. After mixing, samples were left for 15 min at 25 °C prior to measurement of emission spectra (excitation wavelength = 325 nm). Fluorescence intensity was measured relative to the intensity of the unhydrolyzed sample.

Circular Dichroism Spectroscopy. For circular dichroism measurements a hydrolysate prepared on ice was immediately diluted 10 times with water, flushed with N_2 for 2 min, and transferred to a Suprasil quartz cell with a path length of 100 μm (Helma, Germany). The cell was mounted in the spectrometer and equilibrated at 40 °C with N_2 purging for 20 min. Measurements were taken every 20 min using a Jasco-710 spectropolarimeter recording from 260 to 180 nm with 0.5 nm resolution and 4 s response time. The concentration of β-lactoglobulin was determined spectrophotometrically using the molar absorption coefficient at 280 nm of 17 600 M^{-1} cm^{-1} (Sitohy et al., 1995). The results are expressed as $\Delta\epsilon$ per mole of amino acids in β-lactoglobulin. The method of Hennessey and Johnson (1981) was used to estimate the content of secondary structure.

Electrophoresis. Capillary zone electrophoresis was performed on a Hewlett-Packard HP3DCE instrument (G1602A, Hewlett-Packard A/S, Waldbronn, Germany) with the HP ChemStation software revision 04.01, otherwise using the same conditions as described by Madsen et al. (1997). Gel electrophoresis was performed on a PhastSystem using materials supplied by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). SDS−PAGE was performed on Phast Gel high-density gels according to the manufacturer's instructions (Separation Technique File 112). The gels were stained with Coomassie Blue R according to the procedure given in Development Technique File 201. Molecular weights were estimated using molecular weight markers (LMW electrophoresis calibration kit). Isoelectric focusing was performed on PhastGel IEF 3−9 gels according to the manufacturer's instructions (Application Note 380). Isoelectric focusing gels with hydrolysates were silver stained according to the instruction manual (PhastGel silver kit), and the IEF gels with dissolved aggregates were stained with Coomassie Blue. The p*I* of hydrolysis products was estimated from standard p*I* markers (IEF calibration kit pH 3−9).

Chromatography. All chromatographic analyses were performed on a Waters HPLC system as described by Madsen

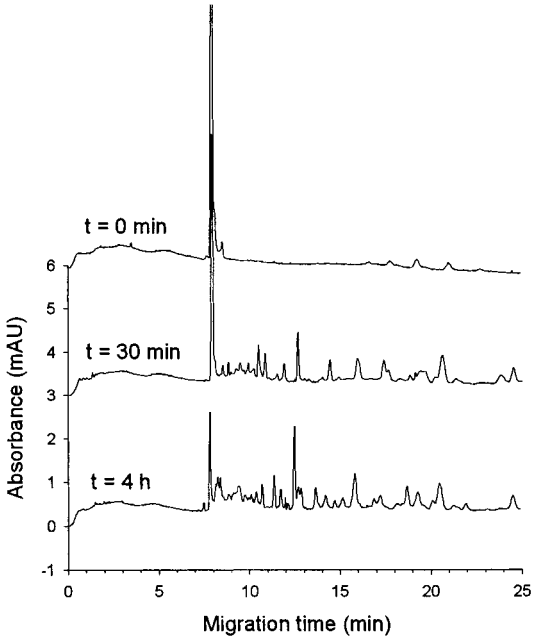

Figure 1. Capillary electrophoresis of β-lactoglobulin (9 mg of protein/mL 0.075 M Tris-HCl) hydrolyzed for various times (*t*) by the *B. licheniformis* protease. Analysis was performed in an untreated fused-silica capillary with a 0.1 M phosphate buffer, pH 2.0, at 18 kV and detection at 214 nm.

et al. (1997). Analytical RP-HPLC was performed on a Nova-Pak C_{18}, 4 μm, 3.9 × 150 mm (Waters Corp., Milford, MA) with the eluents as described by Madsen et al. (1997). Semi-preparative HPLC was performed with a μBondapak C_{18} radial compression column (8 × 100 mm, 10 μm, WAT085721, Waters) and a flow rate of 0.6 mL/min. Buffer A was 0.1% TFA in water; buffer B was 0.08% TFA in 80% 2-propanol. Elution was performed with a linear gradient from 5 to 90% B in 85 min. SE-HPLC was performed on a TSK gel G-2000SWXL column with a combination of (A) 0.0075 M Tris, pH 7.1, and (B) 0.075 M Tris containing 0.15 M NaCl, pH 7.3. Elution was with buffer A for 5 min followed by buffer B for 10 min and finally buffer A for 25 min.

Spectrophotometry. Spectrophotometric measurements were performed on a Biochrom 4060 (Pharmacia LKB Biotech) using either a semimicrocell (0.7 mL) or a flow cell, both with a path lengths of 10 mm. Using the Rayleigh scattering theory on the intensity of scattered light depending on the wavelength to the fourth power, the 280 nm absorbance values were corrected for turbidity according to the formula

$$\epsilon = \epsilon_{app} - \frac{\tau}{10}\left(\frac{\lambda_\tau}{\lambda_\epsilon}\right)^4$$

where ϵ is absorbance, ϵ_{app} is the measured absorbance including turbidity, τ is turbidity (apparent absorbance at 420 nm), λ_ϵ is the wavelength of absorbance measurement, and λ_τ is the wavelength of turbidity measurement.

RESULTS AND DISCUSSION

Hydrolysis of β-Lactoglobulin and Aggregate Formation. During incubation with BLP, β-lactoglobulin was gradually hydrolyzed (Figures 1 and 2). Capillary electrophoresis showed that after 4 h of hydrolysis, only ≈14% of the β-lactoglobulin remained intact and a large number of peptides had been formed (Figures 1 and 2A). This is in perfect accordance with the findings of Madsen et al. (1997). By isoelectric focusing also ≈10% β-lactoglobulin was shown to remain intact after 4 h of hydrolysis, and at least one peptide with a higher p*I* than β-lactoglobulin was formed (Figure 2B). Probably most of the peptides formed during hydrolysis were not retained or stained in the isoelectric focusing gel, even when using the sensitive silver stain.

Measurements of the intrinsic fluorescence showed that the emission wavelength maximum ($\lambda_{em,max}$) initially increased (red-shifted) during hydrolysis, indicating increased exposure of tryptophan to the solvent, most likely due to unfolding of the β-lactoglobulin molecule (Figure 2C, squares). The $\lambda_{em,max}$ value of 342 nm obtained after 1 h of hydrolysis corresponds to λ_{max} of β-lactoglobulin in 5−6 M urea (Stapelfeldt and Skibsted, 1997) or at 300 MPa (Stapelfeldt et al., 1996). Upon further incubation, $\lambda_{em,max}$ decreased again, indicating that the tryptophanyl residues became less exposed to the solvent. This would be the case upon association of either unfolded β-lactoglobulin with other β-lactoglobulin molecules or tryptophan-containing peptides, in effect shielding the indole moieties from the solvent. In fact, the decrease in λ_{max} occurred at a time when aggregates had just started to form, as seen by an increase in the *z*-average hydrodynamic particle size (Figure 2C, circles). Although the initial average hydrodynamic radius was much larger than expected, probably due to a small fraction (<1%) being aggregated (Otte et al., 1996a), it can easily be seen from Figure 2C that aggregation started after ∼1 h of incubation, at which time approximately 60% of the β-lactoglobulin had been degraded (Figure 2A).

The initial increase in the $\lambda_{em,max}$ shown in Figure 2C (squares) may indicate that hydrophobic areas of the β-lactoglobulin molecule were exposed, suggesting that hydrophobic interactions are involved in aggregate formation. Another indication of transitory exposure of hydrophobic sites comes from studies using *cis*-parinaric acid as a probe. The fluorescence quantum yield of *cis*-parinaric acid is known to be very sensitive to its microenvironments, thus increasing in apolar surroundings and decreasing to almost zero in water (Sklar et al., 1977). As may be seen from the emission spectra in Figure 2D, *cis*-parinaric acid binds to β-lactoglobulin (0 min), giving rise to a structureless emission spectrum with $\lambda_{em,max}$ = 415 nm. Initially, the action of the enzyme upon β-lactoglobulin decreased the polarity at the binding site of *cis*-parinaric acid, which is seen by doubling of the quantum yield after 2 min. Further action of the enzyme on β-lactoglobulin gradually impaired the binding of *cis*-parinaric acid, causing *cis*-parinaric acid at time 45 min to sense a polarity corresponding to that of the initial binding site, while prolonged hydrolysis (4 h) ultimately left *cis*-parinaric acid in a microenvironment corresponding to an aqueous peptide solution without effective binding sites for the probe. The fatty acid analogue *cis*-parinaric acid is believed to bind to β-lactoglobulin in a manner corresponding to the binding of natural fatty acids (Spector and Fletcher, 1970; Perez et al., 1989). However, it remains unclear whether the binding site is in the center of the β-barrel formed by eight of the nine β-strands occurring in the native β-lactoglobulin molecule (Papiz et al., 1986) or in an outer hydrophobic binding site (Dufour et al., 1994), maybe close to the α-helix (L. Sawyer, Department of Biochemistry, University of Edinburgh, 1997, personal communication). The accessibility of *cis*-parinaric acid for its binding site in β-lactoglobulin might be increased by the initial cleavages which could lead to partial unfolding (demasking of the binding site) or change the surroundings. The subsequently decreased quantum yield could be due

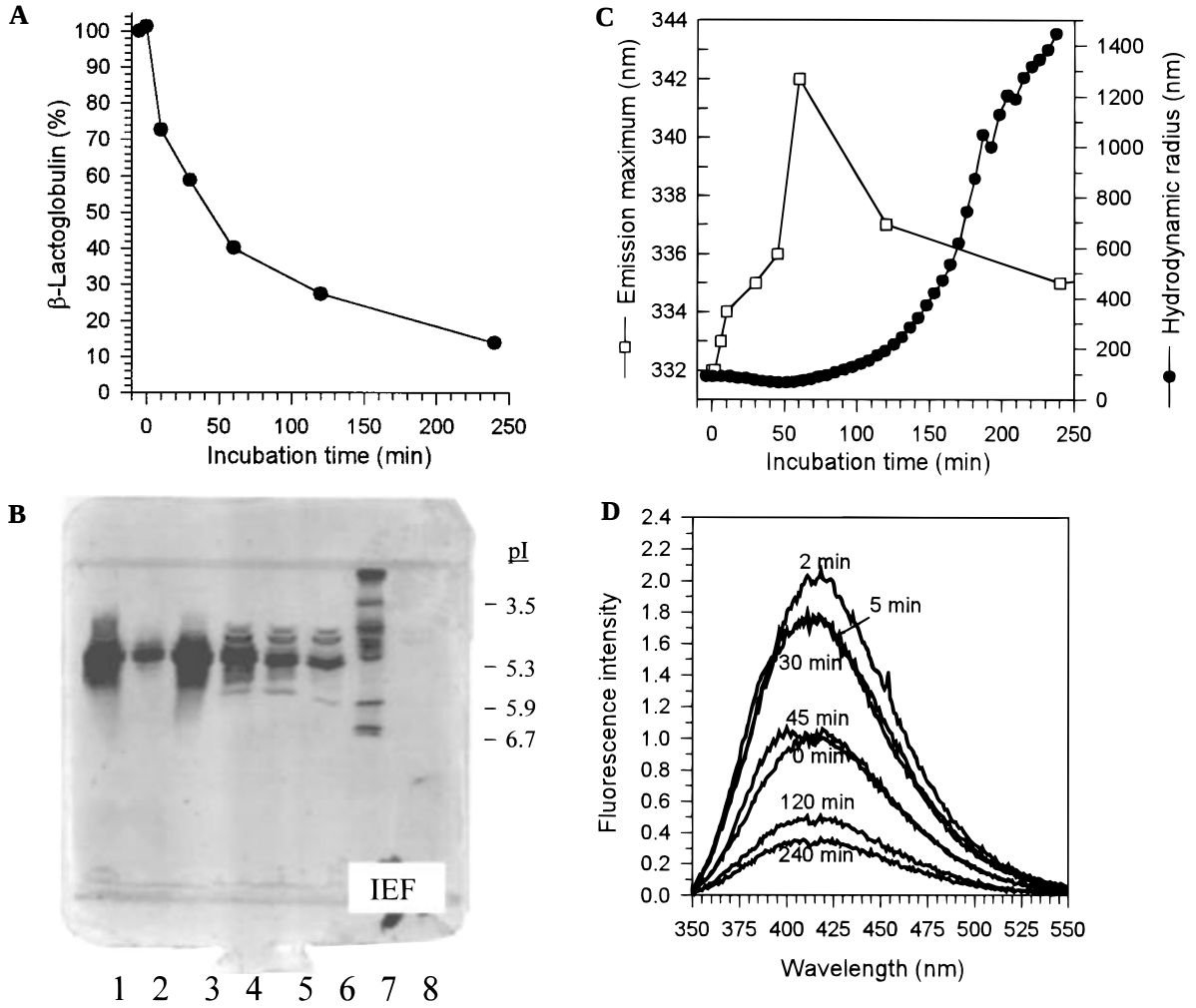

Figure 2. Characteristics of the hydrolysis of *β*-lactoglobulin in 0.075 M Tris-HCl by the protease from *B. licheniformis:* (A) remaining *β*-lactoglobulin (*β*-LG) as measured by capillary electrophoresis as described in the legend for Figure 1; (B) isoelectric focusing gel, silver stained (lanes 1 and 2, *β*-lactoglobulin, 10 and 1 mg/mL, without enzyme; lanes 3−6, hydrolysates taken after 0, 0.5, 2, and 4 h of incubation; lane 7, p*I* markers; lane 8, enzyme at the concentration used); (C) fluorescence emission maximum wavelength (□) and the weight-average hydrodynamic radius as measured by dynamic light scattering (●); (D) fluorescence intensity of added *cis*-parinaric acid at various hydrolysis times (relative to unhydrolyzed *β*-lactoglobulin with *cis*-parinaric acid).

to cleavage in the binding site and/or to inaccessibility inside the aggregates.

The fate of the secondary structure during hydrolysis was investigated by circular dichroism measurements. Selected circular dichroism spectra obtained during hydrolysis are shown in Figure 3A. Before addition of enzyme ($t = 0$) the content of *β*-sheet was found to be 54% at 40 °C (Figure 3B), which is close to the value of 51% obtained by Qi et al. (1997) at pH 6.7 using circular dichroism in the interval 170−260 nm. The α-helix content of 27% obtained in the present work probably includes a small amount of structure from the contaminating caseinomacropeptide in the *β*-lactoglobulin preparation.

During hydrolysis the content of *β*-sheet gradually decreased to 17% after 15 h, whereas the content of α-helix only slowly decreased to 15% within the same period. Compared to the chromatographically determined degradation of intact *β*-lactoglobulin, the amount of secondary structure changed much more slowly than expected if *β*-lactoglobulin was hydrolyzed to a number of peptides without secondary structure (Figure 3B). This indicates that released peptides retained secondary structure, compatible with the enzyme cleaving preferentially at the Glu residues situated in the unordered structure, or that the peptides formed were not immediately released. According to the primary structure published by Eigel et al. (1984) and the secondary structure segments assigned by Papiz et al. (1986), eight Glu residues are situated in the *β*-strands forming the *β*-barrel (three of these are located next to *β*-turns), two Glu residues are in the α-helix segment, and five (six in the B variant) in the less defined structure. The number of peptides as detected by capillary electrophoresis analysis (Figure 2A), however, suggests that more peptides were formed than expected from cleavage only at the five to nine Glu residues located in or near *β*-turns, even if a few extra peptides derived from partial cleavage at some of these sites are considered. Taking into account the low ionic strength of the sample, it is more probable that hydrolysis in the tight *β*-sheet structure at the sites mentioned above would not immediately disrupt the relatively stable structure. This is in accordance with the slower apparent degradation of *β*-lactoglobulin under the mild conditions of SE-HPLC (Figure 3B, open squares) compared to the dissociating conditions of RP-HPLC (Figure 3B, open circles).

Gelation Studies. Whether or not the aggregates formed during hydrolysis ultimately formed a gel when present at higher concentrations was investigated using protein concentrations of 70, 120, and 200 mg/mL, which are below, near, and well above, respectively, the least

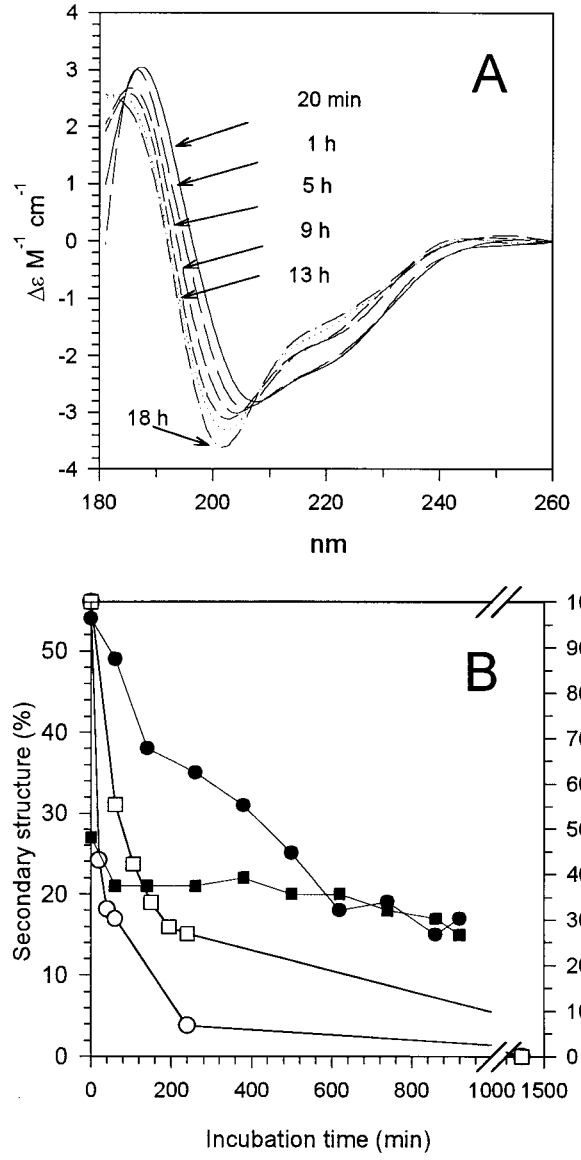

Figure 3. Changes in secondary structure during hydrolysis of 1 mg/mL β-lactoglobulin in 0.0075 M Tris-HCl by the protease from *B. licheniformis*: (A) circular dichroism spectra; (B) changes in β-sheet (●) and α-helix (■) contents and degradation of β-lactoglobulin under similar conditions measured by SE-HPLC peak area at 280 nm (□) and RP-HPLC peak area at 220 nm (○).

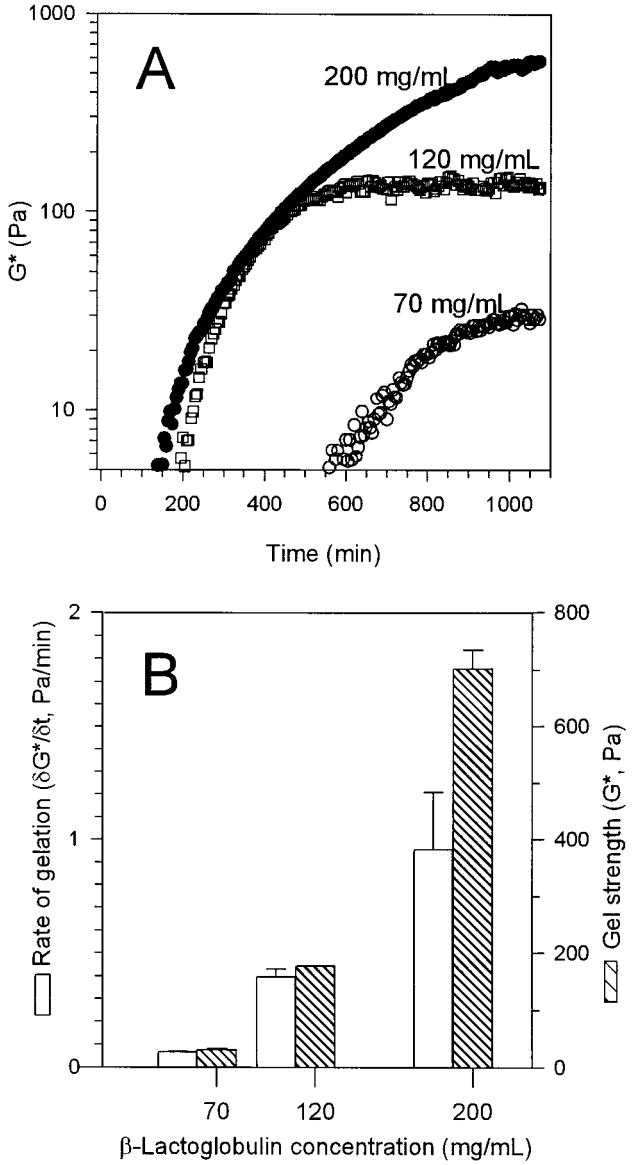

Figure 4. Gelation of β-lactoglobulin during hydrolysis with the *B. licheniformis* protease at pH 7.0 and 40 °C: (A) examples of gelation curves as a function of incubation time and protein concentration (○, 70 mg/mL; □, 120 mg/mL; and ●, 200 mg/mL); (B) The influence of β-lactoglobulin concentration on the rate of gelation and maximum gel strength. Error bars indicate the standard error of the mean from two determinations.

gelling concentration of a whey protein isolate for thermal gelation at 80 °C and pH 7 (Ju et al., 1997). The two lower concentrations correspond to the concentrations of β-lactoglobulin and whey protein, respectively, in the whey protein isolate solution used by Otte et al. (1996a). Preliminary results showed that all β-lactoglobulin hydrolysates became turbid after 50 min of incubation at 40 °C, indicating formation of a considerable number of aggregates, and after 21 h of incubation a white precipitate or soft gel was formed. At higher temperatures the visually observed gelation times were significantly reduced, being 9 min for a 200 mg/mL β-lactoglobulin solution incubated with BLP at 60 °C. To assess whether a true gel or just a precipitate was formed during incubation of β-lactoglobulin with BLP, a range of hydrolysate mixtures was monitored using dynamic oscillation rheometry.

The rheological measurements (Figure 4) showed that even 70 mg/mL of β-lactoglobulin formed a gel upon incubation with BLP at 40 °C. The times of gelation were 580, 190, and 110 min, respectively, for the 70, 120, and 200 mg/mL β-lactoglobulin/permeate solutions. The final strength of the gel formed from 70 mg/mL β-lactoglobulin was very low and the gel very soft and pourable. With increasing β-lactoglobulin concentration both the rate of gelation and the final gel strength increased (Figure 4B), resulting in stronger, but still pourable, gels. The control solutions of β-lactoglobulin in permeate with inactive enzyme were milky, but translucent, and no gel was formed after 20 h of incubation at 40 °C, except for the β-lactoglobulin concentration of 200 mg/mL that formed a very weak pourable gel after 11 h of incubation. When dissolved in Tris buffer, pH 7.5, the appearance and the rheological properties measured of the β-lactoglobulin solution with inactive enzyme did not change at all during incubation for 18 h at 40 °C.

The gel time of a whey protein isolate solution (120 mg/mL; Otte et al., 1996a) when incubated with 5% BLP

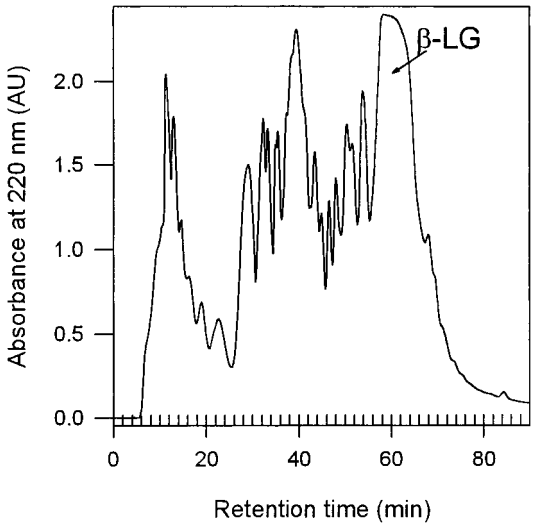

Figure 5. Semipreparative RP-HPLC of β-lactoglobulin (β-LG; 120 mg/mL) hydrolyzed with *B. licheniformis* protease for 4 h and acidified to pH 2. Fractions collected are indicated by minor ticks.

was 103 min, corresponding to 258 min with 2% BLP as used in the present study, which is intermediate between the gelation times obtained with 70 and 120 mg/mL of β-lactoglobulin (Figure 4). However, the final gel strengths as represented by the complex modulus (G^*) were much lower for the 70 and 120 mg/mL β-lactoglobulin gels than for the 120 mg/mL whey protein isolate gel. The observed difference in gelation behavior of β-lactoglobulin in the present study and whey protein isolate (Otte et al., 1996a) may be due to the presence of other proteins in the whey protein isolate, to differences in the state of protein aggregation, and/or to differences in the composition of the low molecular weight phase. Although the β-lactoglobulin preparation was dissolved in permeate to mimic the low molecular phase of the whey protein isolate, differences were found in the contents of, e.g., calcium (1.5 mM in the 120 mg/mL β-lactoglobulin solution versus 5 mM in the 120 mg/mL whey protein isolate solution), which has been shown to significantly affect the enzyme-induced gelation of whey protein isolate (unpublished results from our laboratory). The gelation curve of the 120 mg/mL β-lactoglobulin in Tris buffer, pH 7.5 (not shown), was practically identical with the gelation curve of the 200 mg/mL β-lactoglobulin in permeate (Figure 4A), showing that the gelation properties are highly affected by the low molecular phase (and pH) of the solution.

Although the gels formed from β-lactoglobulin during hydrolysis with BLP were soft in comparison to the whey protein isolate gels, it is supposed that the same mechanism is involved in aggregation and gelation, and it is probable that the effects of the enzyme on β-lactoglobulin dominate the properties of the BLP-induced gelation of the whey protein isolate. Rheological studies are in progress on the effect of increased temperatures on this BLP-induced gelation process of both β-lactoglobulin and whey protein isolate solutions.

Characteristics of a Concentrated Hydrolysate. A hydrolysate containing a considerable amount of aggregates was fractionated using RP-HPLC. As shown in Figure 5, the hydrolysate contained a large number of peptides in addition to intact β-lactoglobulin. None of the individual fractions (collected for 2 min) seemed to form aggregates upon increase of the pH to 7.5 as

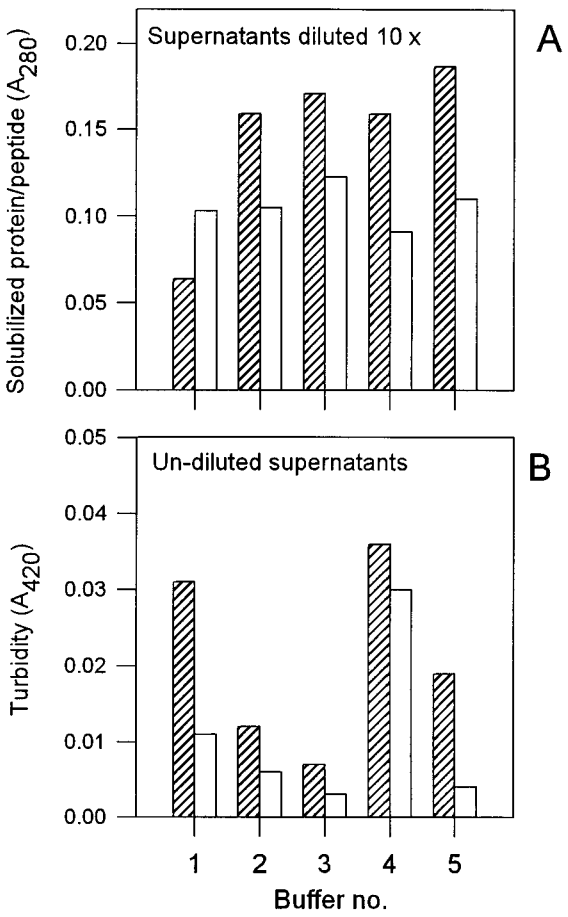

Figure 6. UV absorbance (A) and turbidity (B) of the supernatants from *B. licheniformis* protease induced aggregates from β-lactoglobulin (crosshatched bars) and untreated β-lactoglobulin (empty bars) solubilized in various buffers at 2 mg/mL. Buffer 1: 10 mM Tris-HCl, 1 mM EDTA, pH 8. Buffer 2: with 2% (w/v) sodium dodecyl sulfate (SDS). Buffer 3: with 2% (w/v) SDS and 0.5% (w/v) dithioerythritol. Buffer 4: with 8 M urea. Buffer 5: with 8 M urea and 0.5% (w/v) dithioerythritol.

judged visually and by turbidity measurements (not shown), suggesting that not just one single but several peptides are involved in formation of aggregates, perhaps in combination with intact β-lactoglobulin.

Characterization of Aggregates. Isolated aggregates were dissolved (2 mg/mL) in various dissociating buffers and the dissolved components analyzed by UV measurements and SDS−PAGE (i) to establish if β-lactoglobulin participated in aggregation, (ii) to determine the number and characteristics of the peptides forming the aggregates, and (iii) to get an idea of the kind of forces involved. The aggregates were not fully dissolved in the Tris buffer at pH 7.5 (with 1 mM EDTA, buffer 1) as indicated by the milky appearance of the solution. However, all other solutions became clear, indicating the disappearance of large particles. This was confirmed by spectrophotometry showing that only little material absorbing at 280 nm was solubilized in buffer 1, whereas more than double this amount was dissolved in the other buffers (Figure 6A, crosshatched bars). The fact that the aggregates could be dissolved in either SDS or urea, which are expected to break down both hydrogen bonds and hydrophobic interactions, suggests that such interactions play a significant role in the aggregate formation.

As both β-lactoglobulin and the aggregates were dispersed at 2 mg/mL, it is surprising that the 280-nm

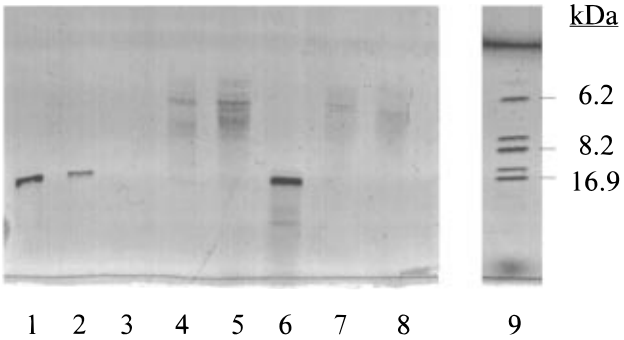

Figure 7. SDS−PAGE of β-lactoglobulin and aggregates of β-lactoglobulin formed upon hydrolysis with *B. licheniformis* protease (both at 2 mg/mL): lane 1, β-lactoglobulin in buffer 1; lane 2, β-lactoglobulin in buffer 2; lane 3, aggregates in buffer 1; lane 4, aggregates in buffer 2 (with SDS); lane 5, aggregates in buffer 3 (with SDS and DTE); lane 6, β-lactoglobulin in buffer 3; lane 7, aggregates in buffer 4 (with urea); lane 8, aggregates in buffer 5 (with urea and DTE); lane 9, molecular weight markers from a separate gel.

absorbance of the aggregates in buffers 2−5 is larger than that of β-lactoglobulin. This is probably due to contamination of the β-lactoglobulin preparation by caseinomacropeptide, which contains no aromatic amino acids (indicating that caseinomacropeptide does not take part in aggregation). The turbidimetric measurements showed that aggregation was more pronounced in the buffers without DTE than with DTE (Figure 6B, buffer 2 vs 3 and buffer 4 vs 5), suggesting that solubilization of aggregates was more profound in the presence of DTE. Similar results, however, were obtained with intact β-lactoglobulin, which should be fully soluble in all buffers (Figure 6B, empty bars). The various turbidities of the solutions were, therefore, likely due to the presence of aggregates formed through intermolecular thiol/disulfide interchange reactions favored by the partial unfolding of the proteins/peptides caused by SDS and urea. The much lower turbidity of β-lactoglobulin in the presence of the disulfide-breaking agent DTE (buffer 5) confirms the presence of disulfide linked aggregates in the buffers without DTE (buffers 2 and 4).

Further analysis of the solubilized material by SDS−PAGE (Figure 7) showed that β-lactoglobulin was not a major constituent of the aggregates, which were built from a large number of lower molecular weight peptides. The molecular weight of these peptides was found to be in the range of 2000−6000, as evidenced by analysis together with molecular weight markers. This is in aggreement with results from MALDI-TOF mass spectrometry of a 5-h hydrolysate showing a number of components with masses of 2.0−5.2 kDa (results not shown).

In accordance with the SDS−PAGE results, isoelectric focusing of aggregates dissolved in urea showed no clear band from intact β-lactoglobulin, but many components with pI > 6.7 were present (results not shown).

The aggregates thus seemed to be formed from a great number of peptides held together by noncovalent interactions. Electrostatic interactions could play a role in aggregation as attractive forces between negatively charged carboxylic groups and positively charged amino groups or by bridging of carboxylic groups through the protease, which is positively charged at pH <9, or through divalent cations. Unpublished results from our laboratory with immobilized enzyme have shown that aggregates were also formed when the enzyme was not present in the reaction mixture, excluding the enzyme as a bridging agent. Due to the specificity of the enzyme toward acidic amino acid residues, peptides containing two carboxylic acid groups only three C atoms apart are formed, and two such peptides in tail to tail orientation may complex with divalent cations such as Ca^{2+}. On complete hydrolysis at all Glu residues of β-lactoglobulin (10 mg/mL), ≈6 mM of peptides with two carboxylic acid groups would be formed. Although this is much higher than the 0.06 mM of calcium present, it can be speculated that calcium may interact with part of the peptides to initiate or contribute to formation of aggregates.

To evaluate the role of electrostatic forces in aggregate formation, the effect of pH on dissociation of the aggregates was assessed by dispersing isolated aggregates in buffers ranging from pH 2 to 10. The aggregates were insoluble in the pH interval 3−8, whereas aggregates were dissolved at pH ≤2 and pH >9 (results not shown). At pH 9 the supernatant was turbid, indicating that the aggregates were only partly solubilized.

The two histidyl residues present in β-lactoglobulin, being the only titratable groups from pH 3 to 8, apparently were not essential for aggregate formation or were buried inside the aggregates and experienced an abnormal pK_a value. As the aggregates were dissolved at low pH as well as at high pH, these results indicate that electrostatic interactions between amino groups and carboxylic acids play an essential role in aggregate formation, whereas the role of divalent cations interacting with carboxylic acids, although present, is less significant. This is further substantiated by the fact that less than half of the peptides forming the aggregates were dissolved in a buffer containing 1 mM EDTA (Figure 6A, buffer 1), which should complex all calcium present.

When the aggregates were dispersed in buffers also containing 1.0 M sodium chloride, they were insoluble at all pH values (not shown). The substantial increase in ionic strength provided by the salt was sufficient to mask exposed ionic groups and to strengthen the hydrophobic interactions between nonpolar residues. Thus, at high ionic strength the hydrophobic interactions seemed to play a major role in aggregation of the peptides formed from β-lactoglobulin, whereas at low ionic strength the electrostatic attractive forces seemed of major importance. The contribution of other noncovalent interactions in maintenance of the aggregates could not be deduced from the present experiments.

Studies with a highly purified β-lactoglobulin preparation to identify the peptides with the strong tendency to aggregate have been planned to further reveal the mechanism of aggregation.

ABBREVIATIONS USED

BLP, *Bacillus licheniformis* protease; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization−time-of-flight mass spectrometry; RP-HPLC, reversed-phase HPLC; SDS, sodium dodecyl sulfate; SE-HPLC, size exclusion HPLC; TFA, trifluoroacetic acid.

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