Identification of Peptides in Aggregates Formed during Hydrolysis of *β*-Lactoglobulin B with a Glu and Asp Specific Microbial Protease

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The purpose of the present study was to identify the peptides responsible for aggregate formation during hydrolysis of *β*-lactoglobulin by BLP at neutral pH. Hydrolysates taken at various stages of aggregate formation were separated into a precipitate and a soluble phase and each was analyzed by CE and mass spectrometry. The aggregates consisted of six to seven major peptides of which four were tentatively identified. The peptides were positively charged at neutral pH and had a high charge-to-mass ratio at low pH. The fragment f135−158 seemed to be the initiator of aggregation, since it was present at high concentration in the aggregates at all stages, and the concentration of this peptide remained low in the supernatant. F135−158 contains several basic and acid amino acids alternating with hydrophobic amino acids, which is in accordance with formation of noncovalently linked aggregates, as previously shown.

Keywords: *β-lactoglobulin B; enzymatic hydrolysis; aggregation; peptides; identification*

INTRODUCTION

The ability to form gels and provide texture to food products is an important functional property of whey proteins. Gelation of whey proteins can be affected by enzymatic treatment, e.g., cross-linking or hydrolysis (Chen et al., 1994; Færgemand et al., 1997). Recently, it has been shown (Ju et al., 1995) that the gelation properties of a whey protein isolate were significantly improved at neutral pH by prior limited hydrolysis with a protease from *Bacillus licheniformis* (BLP). Since this result was most unexpected, several studies have been performed to elucidate the mechanism behind this gelation process (Otte et al., 1996a,b; Ju et al., 1997). Gels formed from hydrolyzed whey proteins have been shown to consist of small aggregates, and it was speculated that the aggregates were formed during hydrolysis prior to the thermal treatment (Otte et al., 1996a). In fact, the sole process of hydrolysis of whey protein isolate by BLP leads to formation of a soft gel, with a similar particulate microstructure (Otte et al., 1996b). The formation of aggregates thus seems to be related to the increasing gelation properties of whey proteins after hydrolysis. We have shown that aggregates are also formed upon hydrolysis of a *β*-lactoglobulin isolate by BLP (Otte et al., 1997a), indicating that the aggregates take their origin in the *β*-lactoglobulin molecule. The aggregates derived from *β*-lactoglobulin were shown to consist of a range of peptides of intermediate size (2−6 kDa) held together by noncovalent bonds (Otte et al., 1997a).

The present study was undertaken in order to further detail the mechanism of aggregate formation during hydrolysis of *β*-lactoglobulin by BLP and increase our understanding of the structural features in proteins important for gelation. To increase the possibilities of structural assignment, a pure preparation of one genetic variant of *β*-lactoglobulin was used. The peptides with the strong ability to aggregate were characterized and tentatively identified.

MATERIALS AND METHODS

Materials. The *β*-lactoglobulin B (Dec.) substrate as described by Kristiansen et al. (1998) was used. The enzyme, BLP, kindly provided by T. Mathiassen, Novo Nordisk A/S, is a serine protease specific for Glu−X and Asp−X bonds, with a strong preference for Glu−X bonds (Breddam and Meldal, 1992). The enzyme powder used (SP 446, batch PPA 5542) had an activity of 20.5 Anson units/g. Chemicals used were from Merck (Darmstadt, Germany).

Hydrolysis of *β*-Lactoglobulin. *β*-Lactoglobulin was dissolved at 10 mg of protein/mL in 0.075 M Tris-HCl, pH 7.5. After preincubation at 40 °C for 15 min, the enzyme (dissolved in Tris buffer) was added at an E/S ratio of 1/100, giving a final *β*-lactoglobulin concentration of 9.8 mg/mL.

Measurement of Aggregation. Aggregate formation during hydrolysis at 40 °C was monitored by turbidity and dynamic light scattering. Turbidity was measured as the apparent absorbance at 500 nm using a Biochrom 4060 spectrophotometer (Pharmacia LKB, Cambridge, UK). Samples for dynamic light scattering were filtered (0.22 *μ*m) and equilibrated for 1 h at 40 °C before addition of enzyme. Measurements were taken with a Malvern Autosizer 2C instrument as described by Otte et al. (1997a).

Isolation of Aggregates. From a hydrolysate made as described above, 200-*μ*L aliquot samples were taken at selected times and immediately centrifuged (11.200*g*, 2 min, room temperature). The supernatant was decanted and 100 *μ*L of supernatant were transferred to an equal volume of 0.35% TFA to lower the pH to 2.3 and stop the enzyme reaction. The precipitate was washed twice with 200 *μ*L of Tris buffer and dissolved in 200 *μ*L of 0.1% TFA. Aliquots (25 *μ*L) of these samples were directly analyzed by capillary electrophoresis (CE); other aliquots (100 *μ*L) were vacuum-dried and subjected to mass spectrometry.

Capillary Electrophoresis. Capillary electrophoresis (CE) was performed as described by Otte et al. (1994), except for the use of 0.15 M phosphate buffer, pH 2.5, as run buffer and a capillary with an inner diameter of 50 *μ*m. Samples were injected hydrodynamically for 20 s and separated at 15 kV (≈49 *μ*A).

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10.1021/jf990947o CCC: $19.00
Published on Web 05/05/2000

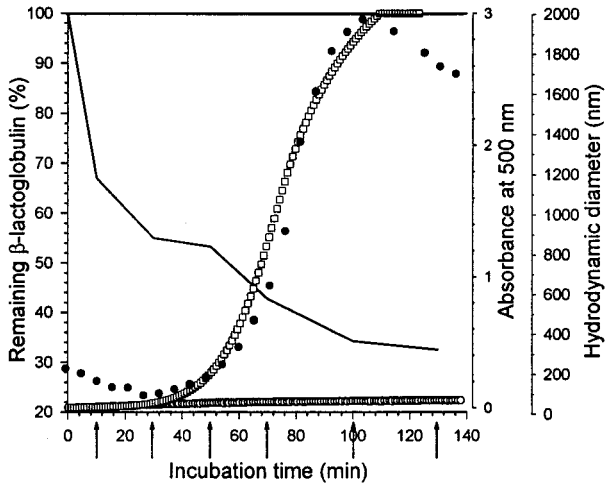

Figure 1. Hydrolysis of β-lactoglobulin during incubation with *Bacillus licheniformis* protease and development in turbidity and particle size. (—) Remaining β-lactoglobulin as determined by capillary electrophoresis; (□) turbidity in the presence of enzyme; (○) turbidity without enzyme; (●) hydrodynamic diameter as determined by dynamic light scattering. Arrows indicate the times when aggregates were harvested.

Mass Spectrometry. The vacuum-dried samples were analyzed by matrix-assisted laser-desorption ionization−time-of-flight (MALDI-TOF) mass spectrometry as described by Madsen et al. (1997). Theoretical masses and charges were calculated by using the GPMAW software version 3.04 (Lighthouse data, Engvej 35, 5230 Odense M, Denmark).

RESULTS

Hydrolysis and Aggregate Formation. During incubation at 40 °C, BLP gradually degraded the intact β-lactoglobulin (Figure 1, solid line). After approximately 30 min of hydrolysis, aggregates started to form as shown by an increasing turbidity as well as by an increasing *z*-average hydrodynamic particle diameter (Figure 1, symbols). Aggregation of peptides from pure β-lactoglobulin started at a time when approximately 45% of the protein had been degraded (Figure 1), which is a little earlier than the onset of aggregation of peptides formed by BLP treatment of a β-lactoglobulin isolate (Otte et al., 1997a).

Characterization of Aggregates. From the aggregation curves, shown in Figure 1, six times for collection of aggregates were chosen, one before the onset of aggregation (10 min), one at the very beginning of aggregate formation (30 min), and three times with increasing amounts of aggregates present (indicated by arrows in Figure 1).

Capillary Electrophoresis (CE). As shown in Figure 2A, approximately 14 peptides were formed in the supernatant after only 10 min of hydrolysis. This number is in reasonably good agreement with the number of peptides expected if complete cleavage of β-lactoglobulin occurs. The 16 Glu residues in β-lactoglobulin should be readily accessible since they are all, except one, situated on the surface of the β-lactoglobulin monomer (Brownlow et al., 1997). Of the 17 fragments formed upon complete cleavage, two are free Glu and one a dipeptide, Pro113-Glu114, which probably would not give a good detector response in CE. Accordingly, the concentration of all detected peptides increased upon hydrolysis in excess of 10 min, as seen by their increasing peak heights (Figure 2A).

In the initial phase of hydrolysis, the amount of precipitate (aggregates) was very low and no peptides were detectable by CE until 70 min of hydrolysis. Interestingly, the same peptides seemed to occur both in the supernatant and the precipitate (compare parts A and B of Figure 2), suggesting that a certain concentration of a given peptide must be present before precipitation occurs.

The time course of differences in the relative abundance of the various peptides in the supernatant and precipitate gives an idea of which peptides have the strongest precipitation potential. The peptide (or peptides) migrating next to β-lactoglobulin as a broad peak (peak no. 1) were at a maximal concentration in the supernatant after 50 min of hydrolysis, whereafter it was primarily recovered in the precipitate (Figure 2B), indicating that this peptide is involved in aggregation. Although the migration time of peak no. 1 is similar to the migration time of intact β-lactoglobulin, this peak is not expected to include intact β-lactoglobulin, since (i) native β-lactoglobulin gives a narrow peak in CE and (ii) intact β-lactoglobulin was not present in aggregates from a β-lactoglobulin isolate (Otte et al., 1997a). The peptides numbered 3−7 and 11 also seemed to preferentially precipitate since a constant low concentration remained in the supernatant, possibly the maximum concentration soluble, and the concentration of these peptides increased in the precipitate.

Opposed to the previous peptides, the concentration of peptides no. 8, 8a, 9, and 12 constantly increased in the supernatant. The peptides no. 8, 8a, and 12 were practically absent from the precipitate, indicating that they were soluble at a high concentration. The presence of peptide no. 9 in the precipitate may result from coprecipitation with other peptides.

Mass Spectrometry. After 10 min of hydrolysis, the supernatant contained around 11 components as detected by MALDI-TOF mass spectrometry. The dominating responses were at *m*/*z* values of 2825 and 4892. According to their mass and the specificity of the enzyme toward the carboxylic site of glutamic acid residues, these components were tentatively identified as the fragments f135−158 and f1−45 of β-lactoglobulin, respectively (Table 1). Clear signals were also obtained at *m*/*z* values of 2333 and 2695.2, tentatively identified as f90−108 and f135−157, and only minor signals were obtained at the *m*/*z* values 3304, 3809, 4000, and 5259.

In all supernatants obtained during 130 min of hydrolysis the signal at 4892 (f1−45) dominated, whereas the intensity of the signal at 2824 (f135−158) varied much. The other signals either remained low or decreased with increasing hydrolysis time as indicated in Table 1.

The presence of f90−108 not bound via Cys106 to a fragment containing Cys119 indicates that thiol/disulfide interchange reactions may have occurred during proteolysis.

New signals appeared during hydrolysis as indicated in the lower part of Table 1. Interestingly, also signals with higher *m*/*z* values (5630−7703) than those previously mentioned appeared after 70 min of hydrolysis (Table 1), suggesting that these fragments were released only slowly from the β-lactoglobulin molecule or that they were formed from existing peptides by noncovalent or covalent binding.

No signals were detected in the precipitate taken after 10 min of hydrolysis. After 30 min of hydrolysis, seven

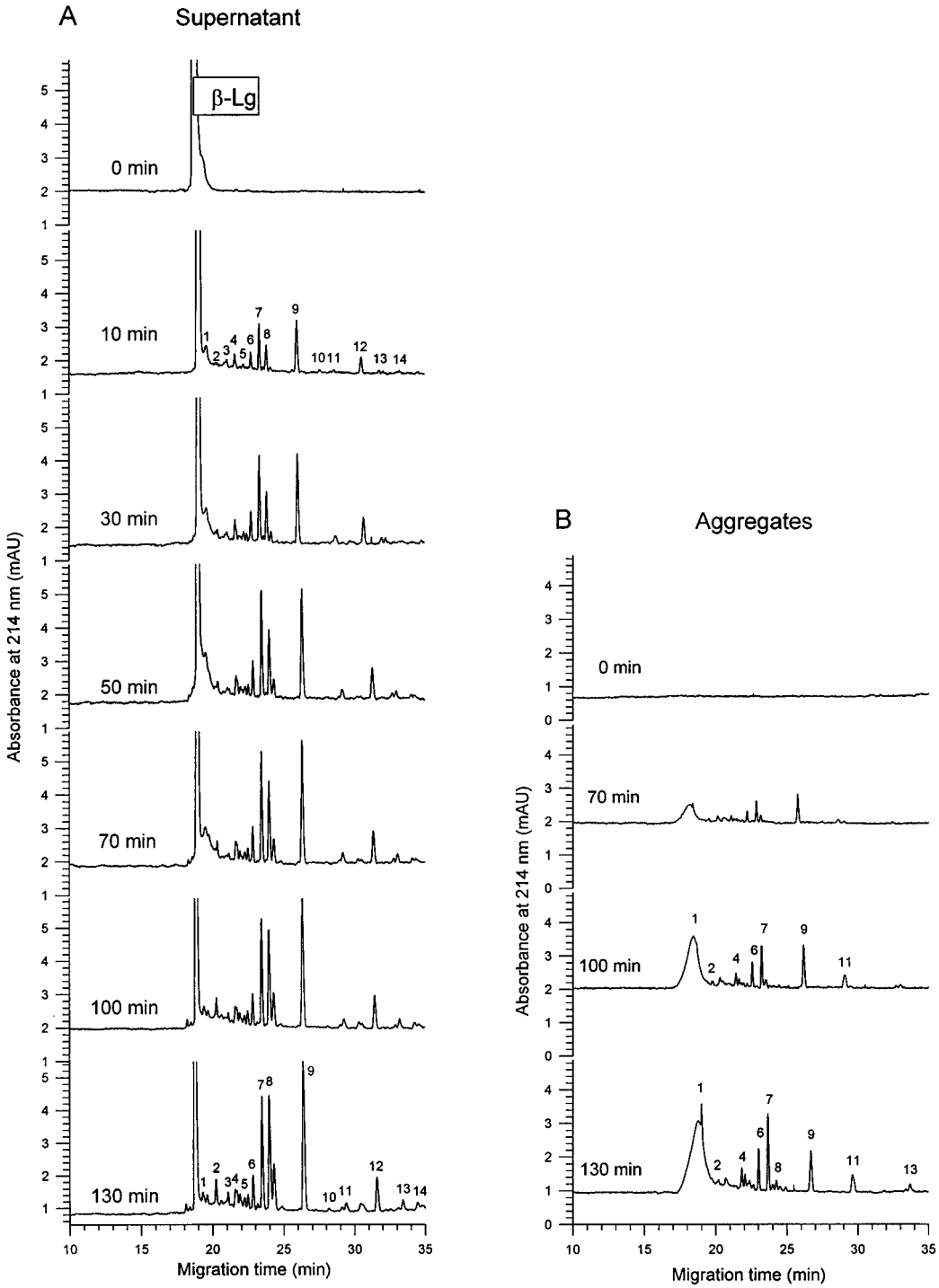

Figure 2. Capillary electrophoresis of β-lactoglobulin hydrolysate samples taken from supernatant (A) and precipitate (B) at various times after addition of *Bacillus licheniformis* protease. Major peaks are arbitrarily numbered.

major and three minor signals were detected, the major signals with *m*/*z* values as listed in Table 1 (upper and middle part). Four of the peptides were tentatively identified as f135−158, f135−157, f75−108, and f75−89 of β-lactoglobulin. The fragment f135−158 remained dominating in the precipitate throughout. The fragment with one glutamic acid residue less, f135−157, was also quite dominating in all precipitates. The fates of the other components are given in Table 1. The fragment f1−45, which was dominating in the supernatant, was also visible in the precipitate after 50 min of hydrolysis.

Like for the supernatant, components with higher molecular weights also appeared in the precipitate after extended hydrolysis (Table 1).

It thus seems that the primary fragments forming the aggregates were f135−158 and f135−157. The results suggest that after the concentration of these peptides had reached a certain low level in the supernatant, they precipitated. Upon extended hydrolysis, other peptides, like the more soluble f1−45 and later f90−108, coprecipitated with these peptides.

DISCUSSION

The increased gelation properties of whey protein hydrolysates over intact whey proteins seems to be related to the formation of noncovalently bound aggregates from peptides released during hydrolysis of β-lactoglobulin by BLP (Ju et al., 1995; Otte et al.,

Table 1. Characteristics of Components Formed during Hydrolysis of β-Lactoglobulin B with a *B. licheniformis* Protease and Detected by Mass Spectrometry[a]

Measured $[M+H]^+$	Supposed Fragment	Theoretical Mass	Charge at pH 2.5	Charge at pH 7.5	Occurrence of peptides in Supernatant	Occurrence of peptides in Precipitate	Charge/mass	$q/MW^{2/3}$
Present in supernatant								
4892	1-45	4897.7	+3.2	- 2.0	+++++ →	↗ after 50 min +++	0.65×10^{-3}	11.1×10^{-3}
2825	135-158	2827.3	+5.2	+0.9	+ ↘	+++++ →	1.84	26.0
2695	135-157	2698.2	+5.2	+1.9	++ →	+++ →	1.93	26.8
2333	90-108	2334.8	+3.1	- 0.1	++ after 70 min ↘	After 70 min ↗ +	1.33	17.6
3304	135-162	3307.9			+ ↘			
3809					+			
4000	75-108	4003.8	+6.1	+0.8	+ ↘	+ →	1.52	24.2
5256					+ →			
Present in precipitate only								
2932						++ ↘		
1689	75-89	1687.0	+3.2	+0.9		++ After 50 min ↘	1.90	22.6
1790						+ →		
4667						+ →		
Formed after prolonged hydrolysis								
2376					↗ after 70 min +			
2890						↗ ++		
3336					↗			
3607	128-158	3599.1	+5.0	-2.9		↗ +++	1.39	21.3
3693					↗			
3779						↗ +		
5506						↗ +		
5632					↗ +	↗ +		
6110					↗ after 70 min +	↗ +		
6732						↗ after 100 min +		
7703					+ at 70 and 100 min	↗ after 100 min +		

Relative concentrations are indicated by '+', where '+' is low and '+++++' very high; → present in all hydrolysates; ↘ decreases during hydrolysis; ↗ increases during hydrolysis

[a] The components are listed after initial presence in the supernatant (top) and precipitate (top and middle) and their formation only during further hydrolysis (bottom).

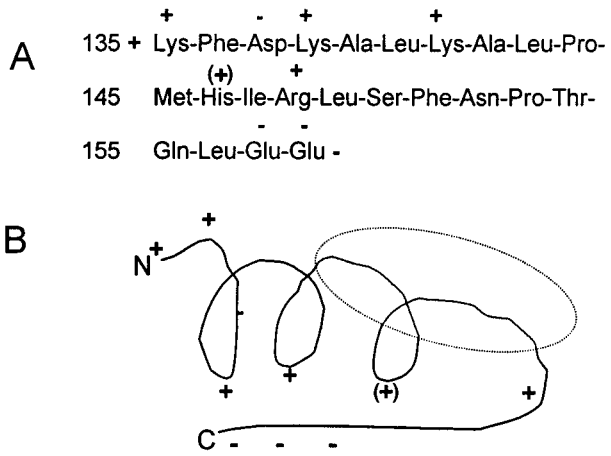

Figure 3. (A) Primary sequence of the peptide fragment (f135−158) initially present and quantitatively dominating in the aggregates formed during hydrolysis of β-lactoglobulin by *Bacillus licheniformis* protease. (B) Possible folding and charge distribution of f135−158 in solution. +, one positive charge; (+), one small positive charge; −, one negative charge at pH 7.5. Hydrophobic area is indicated as a shadowed ellipse.

1996a; Otte et al., 1997a). In the present study, the initial formation of aggregates from β-lactoglobulin was studied, and from the MS measurements the primary peptide fragment initiating aggregation seemed to be f135−158. The primary sequence of this 24 amino acid residue peptide is given in Figure 3A, showing that the peptide is characterized by a high content of basic amino acid residues and thus carries a positive net charge even at pH 7.5, the pH of hydrolysis (Table 1). It contains two positive charges at the N-terminal, another three positive charges in the first half of the molecule, and three negative charges at the C-terminal (Figure 3A). Furthermore, the peptide contains many hydrophobic residues, Phe, Leu, and Ile, alternating with the charged residues, which may also be important in aggregation.

In the native β-lactoglobulin molecule the residues 135−158 form part of an α-helix (135−140), a loop (141−146), a β-strand (147−150), and another α-helix (Brownlow et al., 1997). The Glu158 residue is situated in a very mobile exposed loop, which must be easily accessible to the enzyme. The other cleavage necessary to form f135−158 must have occurred in the middle of an α-helix structure.

It would be interesting to determine the actual conformation of this peptide in solution. The distribution of positively charged amino acid residues at every fourth position in the first part of f135−158 could favor an amphipathic α-helix conformation, like in the native state (Figure 3B; α-helix makes a turn for every 3.6 amino acids). Since peptides in solution often adopt an α-helix structure (Florance et al., 1991; Kuroda et al., 1996), it is conceivable that this would be the preferred secondary structure, at least until the Pro144 residue.

The postive charge at the N-terminal or at one side of the supposed α-helix (depending on the position of the C-terminal tail) together with the small positive charge at His146 and the hydrophobic area of the α-helix would give at least three interaction sites for other peptides (Figure 3B), which is sufficient for random aggregation. This conformation and aggregation mechanism is in agreement with previous results showing that hydrophobic and electrostatic interactions were involved in aggregation (Otte et al., 1997a).

In both mass spectrometry and CE, separation of analytes is based on their charge-to-mass ratio (q/MW). However, in CE, the frictional force exerted during migration is of major importance, implying that the surface area or size of the molecule is critical. Florance et al. (1991) have shown that for fragments of a peptide hormone with 5−22 amino acids there was a good

correlation between the migration time in CE at low pH and the logarithm of $q/MW^{2/3}$. The $q/MW^{2/3}$ ratio also correlated very well with the electrophoretic mobility of 14 peptides derived from a growth hormone at both high and low pH (Rickard et al., 1991).

The q/MW ratios of the fragments of β-lactoglobulin identified in the present study (calculated for pH 2.5) are given in Table 1. Based on the charge-to-mass ratios and the fate of the peptides as determined by mass spectrometry and CE, it is probable that the two primarily precipitating peptides, f135−157 and f135−158, which have approximately the same mass/charge ratio, were migrating as components in peak no. 1 in CE (Figure 2B). Since peak no. 7 is increasing in the precipitate (Figure 2B), this peak could represent f90−108, and the fragment dominating in the supernatant (peak no. 9, Figure 2A) could be f1−45. Peak no. 6 and 11, however, also gave a quite good response in CE of the aggregates (Figure 2B). Since these could not be attributed to a particular fragment of β-lactoglobulin, it cannot be excluded that other fragments of the β-lactoglobulin molecule, e.g., from the parts not positively recovered (f46−74 and f109−134), might participate in aggregation. The latter fragment of β-lactoglobulin seems not to be easily recovered from hydrolysates (Otte et al., 1997b). Perhaps this fragment, which contains two Cys residues, one originally free and one originally engaged in a disulfide bond with Cys106, had formed intermolecular disulfide bonds with similar or other fragments and was not easily ionized from the peptide mixture for mass spectrometry.

CONCLUSIONS

The present results based on CE and MALDI-TOF mass spectrometry measurements show that the aggregates formed during hydrolysis of β-lactoglobulin by BLP consists of six to seven main peptides with molecular weights ranging from 1700 to 4000 Da. The peptides have a relatively high charge-to-mass ratio at pH 2.5 (the pH of the CE analysis) and were mainly positively charged at pH 7.5, the pH of hydrolysis.

Four of the peptides in aggregates were tentatively identified as f75−89, f75−108, f135−157, and f135−158 of β-lactoglobulin, with f135−158 as the most significant. The clusters of positive and negative charges of the latter fragment alternating with hydrophobic areas confirms that aggregation occurs through electrostatic and hydrophobic interactions and suggests that this fragment is the initiator of aggregation.

Since some parts of the β-lactoglobulin molecule were not recovered, it cannot be excluded that other parts than those identified in the present study may have a role in aggregation.

ABBREVIATIONS USED

BLP, *Bacillus licheniformis* protease; CE, capillary electrophoresis; MALDI-TOF, matrix-assisted laser-desorption time-of-flight; q/MW, charge-to-mass ratio; $q/MW^{2/3}$, charge divided by the molecular weight to the $^2/_3$ power; TFA, trifluoroacetic acid; Tris, Tris(hydroxymethyl)-aminomethane.

ACKNOWLEDGMENT

We thank Mila Zakora, Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Denmark, and Jan de Jong, Novo Nordisk A/S, Bagsvaerd, Denmark, for their invaluable technical assistance.

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Received for review August 23, 1999. Revised manuscript received February 25, 2000. Accepted March 13, 2000. This research is part of the FØTEK Program sponsored by the Danish Ministry of Research through LMC Center for Advanced Food Studies.

JF990947O