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Aggregation Properties of Whey Protein Hydrolysates Generated with *Bacillus licheniformis* Proteinase Activities

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Hydrolysis of whey protein concentrate (WPC) with Alcalase 2.4 L, a *Bacillus licheniformis* proteinase preparation, induces gelation. The aggregation behavior of WPC hydrolysates generated with Alcalase and Prolyve 1000, a *Bacillus licheniformis* proteinase that did not induce gelation, were studied by turbidity and particle size analysis. With the use of synthetic peptide substrates, it was shown that Alcalase contains a glutamyl endopeptidase (GE) activity not present in Prolyve. Comparison of the aggregation behavior of WPC hydrolysates generated with Alcalase, Prolyve, and combinations of Prolyve with a GE activity isolated from Alcalase showed that GE was responsible for the observed enzyme-induced peptide aggregation in Alcalase hydrolysates. Hydrolysates generated with Prolyve, having a degree of hydrolysis (DH) of 11.8% and 10.4% of peptide material greater than 10 kDa, could be induced to aggregate by the addition of GE. These results emphasize the contribution of enzyme specificity to the physicochemical and functional characteristics of proteinase hydrolysates of WPC.

KEYWORDS: Hydrolysis, whey protein, aggregation, gelation

INTRODUCTION

Proteins provide many functions in food including the formation of interfacial films to stabilize foams and emulsions and the structural networks associated with gels and edible films (*1*). Whey proteins represent a rich and heterogeneous protein source with wide ranging functional attributes for nutritional, biological, and food purposes (*2*). Whey protein concentrates (WPC) and isolates (WPI) are widely used as ingredients to impart desirable functional properties to a variety of foods such as fortified beverages, desserts, meat, and bakery products (*3*).

The ability of whey proteins to form gel matrixes capable of holding large amounts of water and other ingredients, thereby imparting various textural and sensory properties to foods, is extremely important to the food industry. However gelation is an unwanted functional property in certain food products such as beverages and frozen desserts. Therefore the ability to manipulate the gelation properties of whey proteins can greatly extend the application of whey protein as ingredients in various foods (*4*).

The formation of whey protein gels can be achieved by heating (*5*−*7*), by high-pressure treatment (*8*−*10*), or by acidification (*11*−*13*) and addition of salts (*14*−*16*) to predenatured whey proteins. However whey protein gelation has also been induced by enzymatic treatments such as cross-linking with transglutaminase (*17*) and more recently by enzymatic proteolysis (*18*−*20*).

A considerable amount of research has been carried out into the modification of whey protein gelation by hydrolysis with a proteinase from *Bacillus licheniformis* (BLP) that specifically cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues. Otte et al. (*18*) showed that hydrolysis of WPI with BLP leads to the formation of peptide aggregates, which ultimately formed a particulate gel at 40 °C, pH 7.0. The hydrolysis of a purified preparation of β-lactoglobulin (β-lg) has also been shown to form aggregates on incubation with BLP, and a gel was formed at β-lg concentrations as low as 7 g (100 mL)$^{-1}$ (*21*). The gel formed appeared to consist of peptides with molecular masses ranging from 2 to 6 kDa, which were held together by noncovalent, mainly hydrophobic and electrostatic interactions (*21*). Otte et al. (*22*) identified several peptides released from β-lg on hydrolysis with BLP and suggested that β-lg f(135−158) may be the initiator of aggregation.

Extensive hydrolysis of whey protein with the commercially available proteinase preparation Alcalase has also been shown to lead to the formation of an enzyme-induced gel (*23*). Gelation of Alcalase hydrolysates of WPI occurred after a critical DH (18%) had been reached, and the mechanism of gelation required the formation of aggregates, which subsequently formed a gel (*20*). The enzyme-induced gels formed were physically strong and similar to heat-induced gels. Very little intact α-lactalbumin or β-lg remained when the hydrolysate became viscous, indicating that intact proteins were not involved in aggregate formation (*20*). Doucet et al. (*24*) reported that the Alcalase-induced WPI

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gel consisted of low molecular mass peptides held together by noncovalent (mainly hydrophobic) interactions and that the average peptide chain length at the time of gelation was 4.3 residues. They also identified 130 peptides from an Alcalase digest of WPI and calculated that most peptides had an average hydrophobicity of 1.0−1.5 kcal per residue and a net charge of zero at the pH at which gelation occurred (*25*).

Although there has been a good deal of research into the formation of an enzyme-induced gel during the hydrolysis of whey proteins with Alcalase 2.4 L, the importance of enzyme specificity in the formation of these gels has not been shown. The object of this study was to characterize the aggregation behavior of whey protein hydrolysates generated with two *Bacillus licheniformis* proteinase preparations (Prolyve 1000 and Alcalase 2.4 L) and to relate the observed differences in the specificity of the proteolytic activities to differences in the aggregation properties of hydrolysates. The overall objective was to develop an understanding of the contribution of enzyme specificity to Alcalase-induced gelation of whey proteins.

MATERIALS AND METHODS

Whey protein concentrate (WPC 80, 80.17% protein) manufactured from sweet whey was purchased from a commercial supplier. Trifluoroacetic acid (TFA), HPLC grade acetonitrile, and L-leucine were obtained from BDH (Poole, Dorset, U.K.). HPLC grade methanol, HPLC grade water, L-tyrosine hydrochloride, thyroglobulin, glycerol, calcium chloride ($CaCl_2$), ammonium sulfate, potassium chloride (KCl), and caffeine were from Sigma Chemical Co. (Poole, Dorset, U.K.). Suc-Ala-Ala-Phe.pNA (s.AAF.pNA) and acetyl-Glu.pNA (Ac.glu.pNA) were from Bachem (Bubendorf, Switzerland). Puradisc 25 AS disposable syringe filters (0.2 μm) were from Whatman (Maidestone, U.K.). Supor hydrophilic membrane filters (47 mm, 0.2 μm) were from Pall Corporation (Ann Arbor, MI).

Alcalase 2.4 L was from Novozymes A/S (Bagsvaerd, Denmark). Prolyve 1000 was kindly supplied by Lyven Enzymes Industrielles (Caen, France).

All other reagents were of analytical grade, unless otherwise stated.

Enzymatic Hydrolysis of WPC. A 20 g (100 mL)$^{-1}$ aqueous solution of WPC was allowed to hydrate for 1 h at room temperature with gentle mixing. The protein solution was equilibrated at 50 °C, and the pH was adjusted to 7.0 with 2.0 N NaOH before addition of the enzyme. Initially, 1 mL of Alcalase was added per 100 mL of WPC solution. The levels of addition of the other enzyme activities were adjusted to give addition levels equivalent to 1 mL of Alcalase per 100 mL of WPC as determined by assay with synthetic proteinase substrates (azocasein, Ac.glu.pNA, s.AAF.pNA). The pH of the reaction mixture was maintained constant throughout hydrolysis using a pH stat (718 Stat Titrino, Metrohm, Herisau, Switzerland). Degree of hydrolysis (DH) was calculated from the volume of base required to maintain the pH at 7.0, as previously described (*26*). Each WPC hydrolysis experiment was performed in duplicate. The average standard deviation between individual hydrolysis experiments carried out under the same conditions was less than 0.4% of a DH unit. Hydrolysate samples were taken at 30 min intervals, diluted to 0.8 g per 100 mL in distilled H_2O, and analyzed for turbidity and particle size distribution before heat inactivation at 90 °C for 20 min.

Determination of Enzyme Activity. The general proteinase activity in Alcalase 2.4 L and Prolyve 1000 was determined by assay with azocasein (*27*). The assay was performed by the addition of 100 μL of the enzyme activity (diluted in 50 mM phosphate buffer, pH 7.0) to 1 mL of 0.5 g (100 mL)$^{-1}$ azocasein dissolved in 50 mM phosphate buffer, pH 7.0. After 5 min incubation at 50 °C, the reaction was terminated by the addition of 400 μL of 24 g (100 mL)$^{-1}$ trichloroacetic acid followed by microfugation at 14 000 rpm for 5 min (Sigma model 1-15, SIGMA Laborzentrifugen, Osterode am Harz, Germany). A sample of supernatant (500 μL) was removed and added to a cuvette containing 500 μL of 1 M NaOH. Absorbance at 440 nm was measured using an Ultraspec 2000 (Pharmacia Biotech, Cambridge, England). Activity was expressed as the change in absorbance (ΔAbs) at 440 nm per minute per mg of enzyme protein.

Subtilisin and glutamyl endopeptidase (GE) activities were determined by assay with s.AAF.pNA and Ac.glu.pNA, respectively (*28*). Assays were performed by the addition of 50 μL of enzyme solution to 450 μL of 1.11 mM substrate in 50 mM phosphate buffer, pH 7.0. The assay mixture was incubated at 50 °C for 5 min before termination by the addition of 1 mL of 1.5 M acetic acid. Where necessary, enzyme preparations were diluted in 50 mM phosphate buffer prior to assay. The quantity of paranitroanilide (pNA) released was determined by measuring absorbance at 410 nm with an Ultraspec 2000 (Pharmacia Biotech, Cambridge, England) using an extinction coefficient of 8800 M^{-1} cm^{-1} for pNA at 410 nm (*29*). Activity was expressed on the basis of the quantity of pNA released per min per mg of enzyme protein.

Separation and Isolation of Enzyme Activities. Proteolytic activities were separated and isolated from commercially available enzyme preparations by hydrophobic interaction fast protein liquid chromatography (FPLC) using a HiLoad 16/10 Phenyl Sepharose HP column (Pharmacia Biotech, Cambridge, England) at a flow rate of 1 mL min^{-1}. The FPLC system consisted of two P-500 pumps, a LCC-501 controller and FRAC-100 fraction collector. Conductivity and absorbance at 280 nm were monitored throughout the separation procedure using a Pharmacia conductivity monitor and a UV-MII detector. The system was interfaced with FPLC Director software (Pharmacia Biotech, Cambridge, England).

The column was first equilibrated with buffer A (50 mM phosphate buffer, pH 7.0, containing 500 mM ammonium sulfate and 500 mM KCl). All buffers were filtered through 0.2 μm membrane filters prior to use. Commercially available enzyme preparations were diluted 1:4 in buffer A and filtered through 0.2 μm syringe filters prior to injection. For the initial separation of activities, 2 mL aliquots of diluted enzyme preparation were injected, but for isolation of specific activities from Alcalase 2.4 L, 5 × 2 mL injections of diluted enzyme preparation were applied for each run. Separation was by an increasing gradient of buffer B (50 mM phosphate buffer, pH 7.0). Fractions (3 mL) were collected and immediately analyzed for proteolytic activity using synthetic substrates.

For the isolation of specific activities from Alcalase 2.4 L, fractions identified as having high GE activity were pooled and rechromatographed twice to remove subtilisin activity as determined by assay with s.AAF.pNA. The purified GE fractions were applied to Sephadex PD-10 desalting columns (Pharmacia Biotech, Cambridge, England), eluted with 50 mM phosphate buffer, pH 7.0, and lyophilized using a FreeZone 4.5 freeze-dryer system (Labconco, Kansas City, MO). Lyophilized samples were resuspended in 30% glycerol (containing 5 mM $CaCl_2$) and stored at 4 °C.

Fractions identified as having high levels of subtilisin activity were free of GE activity, and no further chromatography steps were required. Because these fractions were eluted in 100% buffer B, no desalting steps were required, and the pooled fractions were immediately lyophilized and resuspended in 30% glycerol, 5 mM $CaCl_2$ and stored at 4 °C. Both purified subtilisin and GE activities maintained >95% of activity over 3 months under the storage conditions.

Determination of Protein Content. The protein content of the enzyme preparations was determined by the method of Lowry et al. (*30*) using bovine serum albumin as standard. The protein content of WPC was determined by the macro-Kjeldahl method ($N \times 6.38$) (*31*).

Determination of Hydrolysate Turbidity. Turbidity of hydrolysate solutions was determined by monitoring absorbance at 420 nm (*18*). Hydrolysate samples were diluted to 0.1 g (100 mL)$^{-1}$ protein equivalent with distilled water prior to turbidity analysis. Each sample was analyzed in triplicate.

Particle Size Analysis. Particle size analysis of hydrolysates was performed using a Malvern Mastersizer 2000 with a Hydro 2000S sample dispersion system, interfaced with Mastersizer 2000, version 5.1, software (Malvern Instruments Ltd, Malvern, U.K.). Hydrolysate samples were added to the dispersion unit, which was set at a speed of 1500 rpm, until a laser obscuration of between 5% and 10% had been achieved. Relative refractive index and absorption values of 1.52 and 0.000, respectively, were used for hydrolysate samples. Each sample

Table 1. Proteinase Activities of Various Enzyme Preparations Determined at 50 °C, pH 7.0 (Mean ± SD of Four Determinations)

	proteinase substrate		
	azocasein (ΔAbs min^{-1} mg^{-1})	s.AAF.pNA[a] (μmol min^{-1} mg^{-1})	ac.glu.pNA[b] (nmol min^{-1} mg^{-1})
Alcalase	23.04 ± 0.59	47.39 ± 0.28	14.45 ± 0.45
Prolyve	18.90 ± 0.29	52.55 ± 1.41	0.00
subtilisin[c]	15.19 ± 0.95	35.28 ± 0.38	0.00
GE[c]	*d*	0.05 ± 0.00	267.72 ± 9.65

[a] Succinyl-Ala-Ala-Phe-paranitroanilide. [b] Acetyl-Glu-paranitroanilide. [c] The subtilisin and glutamyl endopeptidase (GE) activities were isolated from Alcalase 2.4 L. [d] Not determined.

was analyzed in triplicate. The volume weighted mean ($d_{4,3}$) of the particle size distribution was taken as the mean particle size of the hydrolysate.

Gel Permeation HPLC. Molecular mass distribution profiles of hydrolysate samples were obtained by gel permeation high performance liquid chromatography (GP−HPLC) using a Waters HPLC system, comprising a model 1525 binary pump, a model 717 Plus autosampler and a model 2487 dual λ absorbance detector interfaced with a Breeze data-handling package (Milford, MA). Hydrolysate samples (0.80 g of protein equivalent (100 mL)$^{-1}$ H_2O) were filtered through 0.2 μm syringe filters and 20 μL applied to a TSK G2000 SW separating column (600 mm × 7.5 mm I.D.) connected to a TSKGEL SW guard column (75 mm × 7.5 mm I.D.) (*32*). Separation was by isocratic elution with a mobile phase of 0.1% TFA in 30% acetonitrile at a flow rate of 1.0 mL min^{-1}. Detector response was monitored at 214 nm. A calibration curve was prepared from the average retention times of standard proteins and peptides (0.25 g (100 mL)$^{-1}$) (*33*). The void volume (V_o) was estimated with thyroglobulin (600 000 Da), and the total column volume (V_t) was estimated with L-tyrosine.HCl (218 Da).

Reversed-Phase HPLC. Reversed-phase (RP) HPLC was performed using the Waters HPLC system previously described. The column used was a Phenomenex Jupiter (C18, 250 mm × 4.6 mm I.D., 5 μm particle size, 300 Å pore size) with a Security Guard system containing a C18 (ODS) wide pore cartridge (4 mm × 3 mm I.D.) (Phenomenex, Cheshire, U.K.). The column was equilibrated with solvent A (0.1% TFA) at a flow rate of 1.0 mL min^{-1}, and peptides were eluted with an increasing gradient of solvent B (0.1% TFA, 80% acetonitrile) as follows: 0−4 min, 0% B; 4−54 min, 0−60% B; 54−55 min, 60−100% B; 55−59 min, 100% B; 59−60 min, 100−0% B. Detector response was monitored at 214 nm. Hydrolysate samples (0.80 g of protein equivalent (100 mL)$^{-1}$ of H_2O) were filtered through 0.2 μm syringe filters, and 20 μL was applied to the column.

Statistical Analysis. Statistical analysis (independent-samples *t*-tests) was performed on turbidity and particle size data using SPSS, version 11.0.

RESULTS AND DISCUSSION

The azocasein assay was used to quantify the general proteinase activity in the two commercially available *Bacillus licheniformis* proteinase preparations, Alcalase 2.4 L and Prolyve 1000 (**Table 1**). It was observed that, under the conditions of assay (pH 7.0, 50 °C), Alcalase had higher levels of general proteinase activity (23.04 units per mg of enzyme) than Prolyve (18.90 units per mg of enzyme). WPC 80 hydrolysates were generated with both preparations at equivalent proteinase addition levels using the results obtained from the azocasein assay. However, even at equivalent proteinase addition levels, hydrolysates generated with Alcalase reached a higher DH (19.2% DH after 5 h) than hydrolysates generated with Prolyve 1000 (15.9% DH after 5 h) (**Figure 1**). These results suggested that a broader range of peptide bonds in the intact whey proteins were susceptible to hydrolysis by the proteinase activity in Alcalase compared to the proteinase activity in Prolyve.

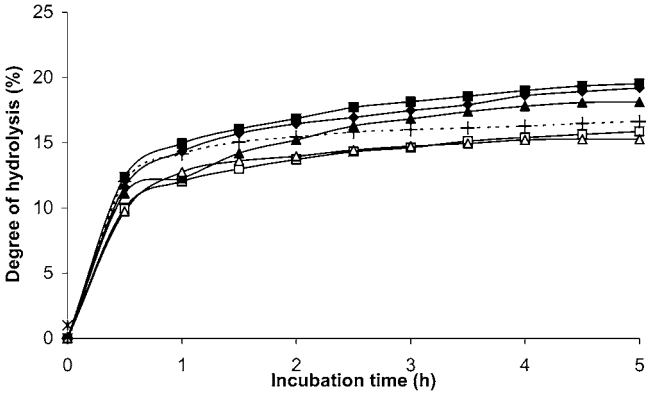

Figure 1. Degree of hydrolysis (DH, %) for whey protein hydrolysates generated with Alcalase 2.4 L (◆), Prolyve 1000 (□), subtilisin (△), Prolyve and glutamyl endopeptidase (at start of hydrolysis) (■), Prolyve and glutamyl endopeptidase (after 1 h hydrolysis) (▲), and subtilisin at twice the original addition level (+) (pH 7.0, 50 °C). Values shown are mean for duplicate hydrolysis experiments.

In the present study, Alcalase hydrolysis of WPC visibly resulted in the formation of a soft gel on reaching a DH of 16.9%. Doucet et al. (*20*) previously showed that WPI hydrolysates generated with Alcalase formed a gel on reaching a DH of 18%. However, the two DH values cannot be directly compared since in the previous study (*20*) DH was monitored osmometrically, the hydrolysis conditions (pH 8.0, 45 °C) and the substrates (WPC vs WPI) were different, and pH was not controlled during the hydrolysis reaction. The Prolyve hydrolysates, however, showed no evidence of increasing viscosity or gel formation, again indicating a difference in the specificity of the proteolytic activities in these two enzyme preparations.

According to the manufacturers, the main proteolytic component of both Alcalase and Prolyve is Subtilisin Carlsberg (EC 3.4.21.62). Subtilisin activities are relatively nonspecific proteinases but preferentially cleave peptide bonds after large non-β-branched hydrophobic residues (*34*). Previous studies on Alcalase specificity show that a significant number of peptides present in Alcalase digests of casein phosphopeptides (*35*) and whey protein isolate (*25*) had a glutamic acid residue at the C-terminus. This could be explained by the presence of a GE activity, which has previously been isolated from Alcalase (*28*, *36*) and was shown to specifically cleave peptide bonds after glutamic acid and to a lesser extent aspartic acid residues in peptides (*37*). Both Alcalase and Prolyve were incubated with the synthetic substrates Ac.glu.pNA, which is specific for GE activities, and s.AAF.pNA, which is readily hydrolyzed by subtilisin. The results showed high levels of subtilisin activity in both Alcalase and Prolyve (47.38 and 52.55 units per mg of enzyme, respectively); however, GE activity was only present in Alcalase (14.45 units per mg of enzyme) (**Table 1**).

The Alcalase and Prolyve preparations were subjected to hydrophobic interaction FPLC using Phenyl Sepharose (**Figure 2**). Three peaks of absorbance at 280 nm were observed for Alcalase, but only two peaks were evident for the Prolyve preparation. A distinct peak in absorbance at 280 nm with a retention volume of approximately 100 mL was observed in the Alcalase profile (**Figure 2a**) that was not present in the Prolyve preparation (**Figure 2b**). Fractions collected from FPLC of the two preparations were immediately analyzed with Ac.glu.pNA and s.AAF.pNA to determine the elution volume of the subtilisin and GE activities. The results indicated that

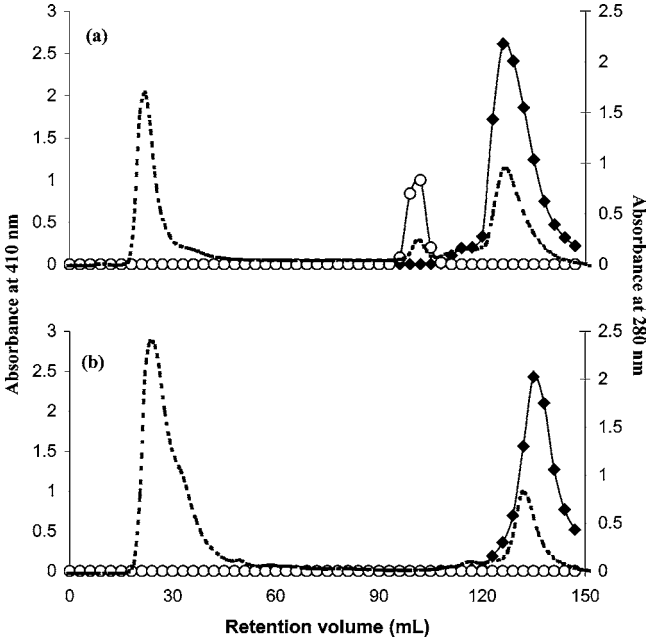

Figure 2. Absorbance values at 280 nm (– – –) and activity against succinyl-Ala-Ala-Phe.pNA (◆) and acetyl-Glu.pNA (○) for the separation of (**a**) Alcalase 2.4 L and (**b**) Prolyve 1000 by Phenyl Sepharose fast protein liquid chromatography. Activity values are expressed as absorbance at 410 nm per mL of fraction for acetyl-Glu.pNA activity and absorbance at 410 nm per μL of fraction for succinyl-Ala-Ala-Phe.pNA activity.

the peak in absorbance at 280 nm eluting at approximately 130 mL was subtilisin, while the large peak eluting at the start of both chromatograms (at approx 30 mL) did not contain any proteolytic activity. A peak of activity against Ac.glu.pNA was detected in fractions eluting at approximately 100 mL for the Alcalase sample (**Figure 2a**) that was absent in the corresponding fractions collected from Prolyve (**Figure 2b**). Fractions collected from multiple injections of Alcalase were pooled, lyophilized, and resuspended in 30% glycerol (containing 5 mM $CaCl_2$) to give purified preparations of GE and subtilisin. The isolated subtilisin preparation had a lower specific activity against s.AAF.pNA than both Alcalase and Prolyve (**Table 1**). This may be due to the loss of some activity during the purification process. The isolated GE fraction had high levels of activity against Ac.glu.pNA (267.72 units per mg of protein) and very low levels of activity against s.AAF.pNA (0.05 units per mg of protein).

The subtilisin activity isolated from Alcalase 2.4 L was used to generate WPC hydrolysates to determine whether hydrolysis with subtilisin would induce gel formation. The level of subtilisin activity added was equivalent to the units of activity added for the hydrolysis of WPC with Alcalase, as determined by assay with azocasein. It was observed that the hydrolysate generated with isolated subtilisin did not form a gel, which suggested that the GE activity present in Alcalase may be responsible for the aggregation behavior of whey protein hydrolysates generated with Alcalase. This finding is in agreement with the observation that hydrolysis with a proteinase with a similar specificity as GE (BLP) induced gelation of WPI (*18*, *19*).

WPC hydrolysates were subsequently generated using Alcalase, Prolyve, and the isolated subtilisin activity at equivalent addition levels and also with the isolated subtilisin activity at twice the addition level of Alcalase (subtilisin (×2)). Hydrolysates were also generated with Prolyve in combination with the GE activity isolated from Alcalase. The Prolyve and GE

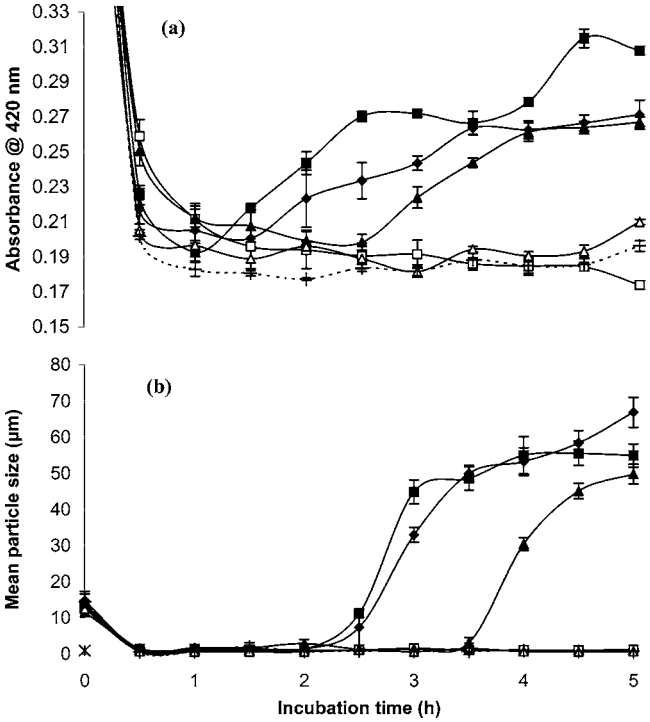

Figure 3. Turbidity (**a**) and mean particle size values (**b**) ($d_{4,3}$, μm) for whey protein hydrolysates generated with Alcalase 2.4 L (◆), Prolyve 1000 (□), subtilisin (△), Prolyve and glutamyl endopeptidase (at start of hydrolysis) (■), Prolyve and glutamyl endopeptidase (after 1 h hydrolysis) (▲), and subtilisin at twice the original addition level (+) (pH 7.0, 50 °C). Values are means of triplicate determinations; error bars show standard deviation.

activities were added at levels equivalent to the subtilisin and GE activity present in Alcalase. Two different sets of hydrolysates were generated using the Prolyve and GE combination, one by the addition of both activities at the start of hydrolysis (ProGE0h) and the other by hydrolysis with Prolyve for 1 h followed by the addition of GE for the remaining 4 h (ProGE1h).

As expected, hydrolysates generated with Alcalase and combinations of Prolyve and GE reached higher DH values than the hydrolysates generated with Prolyve and subtilisin (**Figure 1**). After 5 h of hydrolysis, DH values of 19.2%, 19.5%, and 18.1% were reached for hydrolysates generated with Alcalase, ProGE0h, and ProGE1h compared to DH values of 15.9%, 15.3%, and 16.6% for hydrolysates generated with Prolyve, subtilisin, and subtilisin (×2), respectively. A marginally higher DH value was obtained for subtilisin (×2) hydrolysates, but the DH still did not reach the DH values obtained for the Alcalase or Prolyve and GE hydrolysates.

Throughout hydrolysis, the aggregation behavior of the different hydrolysates was observed by turbidity (at 420 nm) and particle size analysis. These techniques have previously been used to monitor enzyme-induced gelation of WPI during hydrolysis with BLP (*18*, *19*). All hydrolysates showed an initial decrease in turbidity (**Figure 3a**), and hydrolysates generated with Prolyve, subtilisin, and subtilisin (×2) did not increase in turbidity during further hydrolysis. Furthermore, these hydrolysates showed no visible evidence of increasing viscosity or gelation. Alcalase hydrolysates also decreased in turbidity initially, but turbidity began to increase again after 1.5 h hydrolysis, corresponding to a DH of 15.7%. This hydrolysate eventually formed a soft gel after about 2.5 h hydrolysis (DH = 16.9%). Doucet et al. (*20*) showed that the turbidity (at 600 nm) of Alcalase hydrolysates of WPI greatly increased when a

DH of 18% was reached. ProGE0h hydrolysates showed an increase in turbidity after 1 h hydrolysis (14.9% DH) and had a higher turbidity than Alcalase hydrolysates over the entire incubation period. These hydrolysates were also observed to visibly form a soft gel indicating that it was the GE activity in Alcalase 2.4 L that was responsible for the aggregation of Alcalase hydrolysates of WPC. It was previously reported (*25*) that numerous peptides present in Alcalase hydrolysates of WPI had C-terminal glutamate residues, but no previous study has shown the importance of the GE activity in the Alcalase-induced gelation of whey protein hydrolysates. ProGE1h hydrolysates also showed an increase in turbidity, but only after 2.5 h hydrolysis (15.9% DH). This showed that even hydrolysates generated after 1 h hydrolysis with subtilisin, having a DH of 11.8%, can be induced to aggregate by the addition of GE. Turbidity values of Alcalase, ProGE0h, and ProGE1h hydrolysates were significantly higher ($P < 0.0005$) than the turbidity values of Prolyve, subtilisin, and subtilisin ($\times 2$) hydrolysates after a 5 h incubation. Hydrolysates generated with isolated GE only also displayed an increase in turbidity, and a gel was formed even though the final DH achieved was only 3.3% (data not shown). Previous studies (*18*, *19*) have indicated that hydrolysis of WPI with BLP induces gel formation at a DH of approximately 2%. However, because DH was quantified osmometrically and pH was not controlled during the hydrolysis reaction, it is difficult to directly compare DH values with those obtained in this study.

The mean particle size of all hydrolysates initially decreased, but the particle size of Alcalase and ProGE0h hydrolysates began to increase after about 2.5 h incubation at 50 °C (**Figure 3b**). The mean particle size of Alcalase hydrolysates after 5 h hydrolysis was 67.0 μm compared to a mean particle size of 55.0 μm for the ProGE0h hydrolysate. The ProGE1h hydrolysate also showed an increase in mean particle size but only after 3.5 h hydrolysis again showing that hydrolysates generated after 1 h hydrolysis with Prolyve can be induced to aggregate by the addition of GE. The addition of the GE activity after 1 h hydrolysis did however appear to delay the onset of aggregation by 1 h. A mean particle size of 49.9 μm was achieved after 5 h hydrolysis with ProGE1h. Both the Prolyve and subtilisin hydrolysates showed no increase in particle size throughout the hydrolysis period resulting in mean particle size values of 1.2 μm and 0.7 μm after 5 h hydrolysis. Furthermore, the subtilisin ($\times 2$) hydrolysate showed no increase in particle size (0.8 μm after 5 h) again showing the requirement for the specific cleavage of peptide bonds by GE for peptide aggregation to occur. The mean particle size values of Alcalase, ProGE0h, and ProGE1h hydrolysates were significantly higher ($P < 0.0005$) than the mean particle size of Prolyve, subtilisin, and subtilisin ($\times 2$) hydrolysates after 5 h incubation.

It was noted for hydrolysates in which aggregates were formed that aggregate formation was detected earlier by turbidity than by particle size analysis (**Figure 3a,b**). This may be because during particle size analysis the sample is placed in a dispersion unit to obtain representative particle size distributions. This dispersion may disrupt weaker aggregates formed at the beginning of aggregation, which may be detected by the turbidity analysis where no sample dispersion takes place.

The development of peptide aggregates in WPC hydrolysates can also be observed in **Figure 4**, which shows the particle size distribution profiles of all hydrolysates after 0.5, 2.5, and 5 h hydrolysis. After 0.5 h hydrolysis (**Figure 4a**), it can be seen that all hydrolysates contained mixtures of small particles (~1 μm). After 2.5 h hydrolysis (**Figure 4b**), the onset of peptide aggregation is clearly visible in hydrolysates generated with Alcalase and ProGE0h. These hydrolysates now appear to contain mixtures of the original small particles, but larger aggregates are also visible in both cases. The hydrolysates generated with the Prolyve and subtilisin activities only contain small particles. The ProGE1h hydrolysate did not begin to aggregate until after 3.5 h and appears similar to the subtilisin hydrolysates after 2.5 h hydrolysis. After 5 h hydrolysis (**Figure 4c**), the particle size distribution profiles had changed dramatically for hydrolysates generated with Alcalase, ProGE0h, and ProGE1h. No small particles are visible for these three hydrolysates, and the particle size distribution profiles only show the presence of large aggregates. The particle size distribution profiles for the Prolyve and subtilisin hydrolysates did not change over the 5 h incubation time.

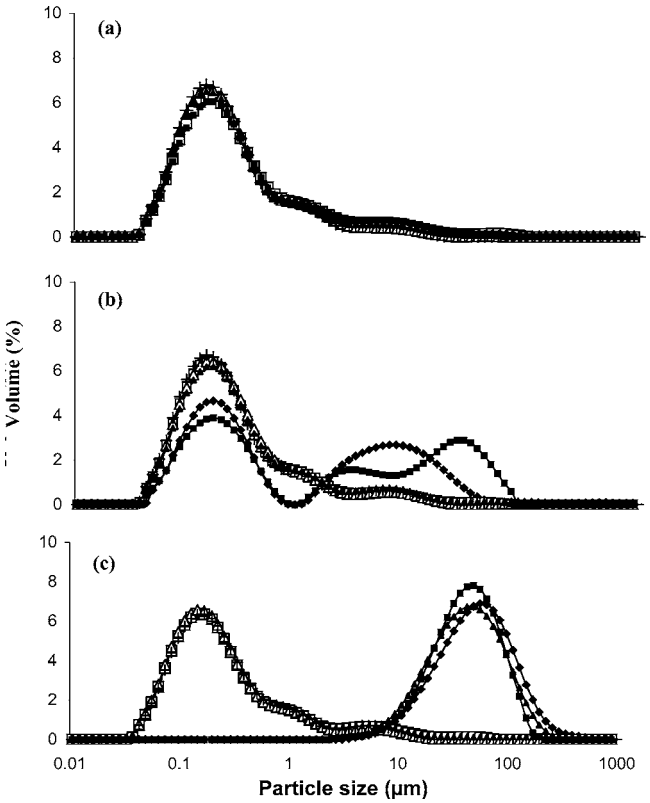

Figure 4. Particle size distribution profiles for whey protein hydrolysates after (**a**) 30 min (**b**) 2.5 h, and (**c**) 5 h hydrolysis with Alcalase 2.4 L (◆), Prolyve 1000 (□), subtilisin (△), Prolyve and glutamyl endopeptidase (at start of hydrolysis) (■), Prolyve and glutamyl endopeptidase (after 1 h hydrolysis) (▲), and subtilisin at twice the original addition level (+) (pH 7.0, 50 °C).

Gel permeation HPLC showed that after 1 h hydrolysis, Alcalase and ProGE0h hydrolysates had approximately 10% more peptide material <1 kDa than the other hydrolysates (**Table 2**). These hydrolysates also had less material in the mass ranges 1−5 kDa, 5−10 kDa, and >10 kDa than the other hydrolysates, although the subtilisin ($\times 2$) hydrolysate had less material >10 kDa than the Alcalase hydrolysate after 1 h hydrolysis. The ProGE1h hydrolysate had 10.4% of peptide material >10 kDa after 1 h hydrolysis. Therefore, at the time the GE activity was added to this hydrolysate, only 10.4% of the protein in WPC was intact as all the major proteins in whey have molecular masses >10 kDa. This would suggest that the addition of GE to a subtilisin hydrolysate may induce peptide aggregation by the further hydrolysis of peptides released from intact whey proteins by the action of subtilisin, and not by the

Table 2. Molecular Mass Distributions (Determined by Gel Permeation HPLC) of Whey Protein Hydrolysates after 1, 2.5, and 5 h Incubation with Various Enzyme Preparations (pH 7.0, 50 °C)

enzyme	hydrolysis time (h)	molecular mass distribution (kDa)[a] >10	5–10	1–5	<1[b]
Alcalase	1.0	8.2	4.5	23.6	63.7
ProGE0h		7.0	3.8	22.8	66.4
ProGE1h		10.4	7.0	27.4	55.1
Prolyve		10.4	6.7	27.5	55.4
subtilisin		9.8	6.8	28.1	55.2
subtilisin (×2)		7.4	5.3	29.7	57.7
Alcalase	2.5	4.4	2.7	18.9	74.1
ProGE0h		3.6	2.4	18.3	75.8
ProGE1h		7.0	3.8	22.6	66.7
Prolyve		9.5	5.3	25.8	59.4
subtilisin		9.2	5.0	26.6	59.2
subtilisin (×2)		7.1	4.2	27.2	61.5
Alcalase	5.0	1.5	1.7	15.9	81.0
ProGE0h		1.4	1.5	14.9	82.2
ProGE1h		2.1	2.1	18.7	77.0
Prolyve		9.1	4.7	23.6	62.6
subtilisin		8.8	4.3	23.7	63.2
subtilisin (×2)		6.9	3.6	24.4	65.1

[a] Values are areas within a defined molecular mass distribution, expressed as % of total area of a chromatogram at 214 nm. [b] Detection at 214 nm can lead to an underestimation of the percentage material <1 kDa because most free amino acids do not show significant absorbance at 214 nm.

hydrolysis of intact protein by GE. Doucet et al. (*20*) have suggested that intact proteins do not play a major role in the gelation of WPI induced by hydrolysis with Alcalase.

After 2.5 h incubation, the hydrolysates that had started to aggregate (Alcalase and ProGE0h) again had much less peptide material in the molecular mass ranges 1 −5 kDa, 5−10 kDa, and >10 kDa than those for hydrolysates that did not aggregate. These hydrolysates consequently had more peptide material <1 kDa, for example, Alcalase hydrolysates had 14.7% more material <1 kDa than Prolyve hydrolysates (**Table 2**).

After 5 h hydrolysis, a much higher percentage of the peptide material in the hydrolysates that formed aggregates was <1 kDa when compared to hydrolysates that did not form aggregates (**Table 2**). For example, Alcalase hydrolysates had 81.0% of peptide material <1 kDa after 5 h hydrolysis compared to Prolyve hydrolysates, which had 62.6% <1 kDa. This difference can also be observed at the higher molecular mass ranges, with Alcalase having a total of only 19.1% of peptides >1 kDa (3.2% of which were >5 kDa) compared to 37.4% of peptides in Prolyve hydrolysates >1kDa (13.8% of which were >5 kDa). These results would appear to rule out the participation of large peptides (>5 kDa) in the enzyme-induced aggregation of WPC by hydrolysis with Alcalase. Doucet et al. (*24*, *25*) suggested that small peptides (<2 kDa) were responsible for the formation of aggregates during the hydrolysis of WPI with Alcalase.

Reversed-phase profiles for Alcalase, ProGE0h, and ProGE1h hydrolysates (**Figure 5**, parts **a**, **b**, and **c**, respectively) show distinct differences from the Prolyve and subtilisin hydrolysate profiles (**Figure 5**, parts **d** and **e**, respectively). For example, the Prolyve hydrolysate has two peptide peaks with retention times of approximately 40 min, which are not present in the Alcalase hydrolysate. The main peak present in the Prolyve and subtilisin chromatograms elutes at approximately 31 min. A peak eluting at this time is also present in the Alcalase hydrolysate, but it is very much diminished in comparison to the Prolyve hydrolysate.

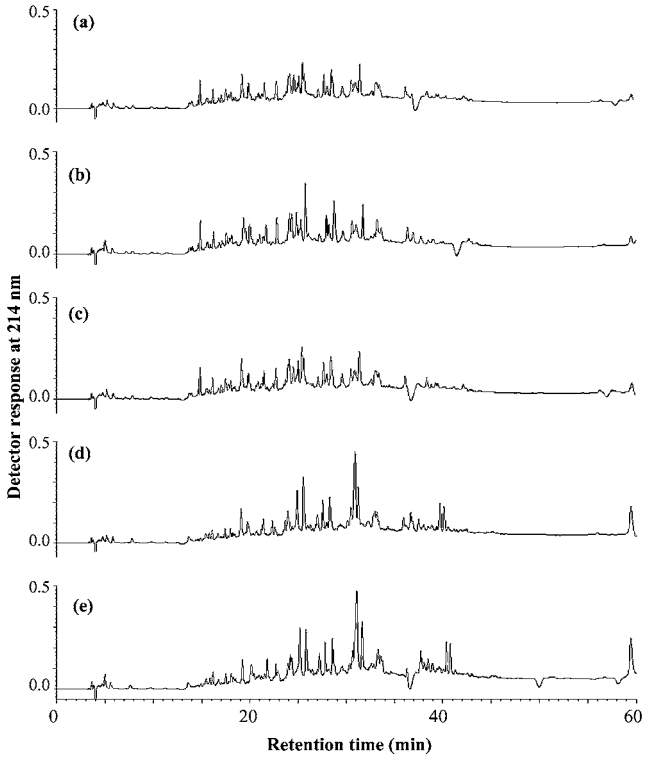

Figure 5. Reversed-phase HPLC profiles for whey protein hydrolysates generated after 5 h incubation (pH 7.0, 50 °C) with (**a**) Alcalase 2.4 L, (**b**) Prolyve 1000 and glutamyl endopeptidase (at start of hydrolysis), (**c**) Prolyve 1000 and glutamyl endopeptidase (after 1 h hydrolysis), (**d**) Prolyve 1000, and (**e**) subtilisin.

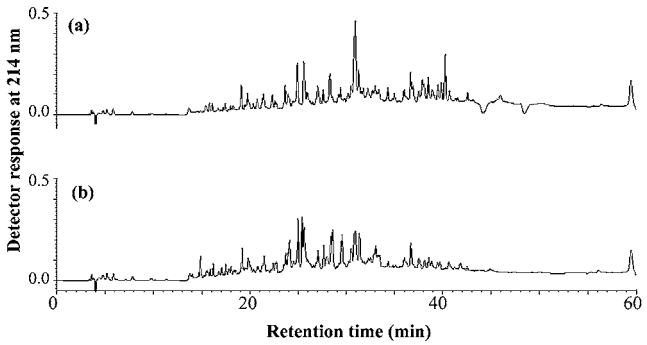

Figure 6. Reversed-phase HPLC profiles for whey protein hydrolysates after (**a**) 1 h incubation with Prolyve 1000 and (**b**) 1 h incubation with Prolyve 1000 followed by 30 min incubation with Prolyve 1000 and glutamyl endopeptidase (pH 7.0, 50 °C).

Figure 6 shows RP-HPLC profiles for ProGE1h hydrolysates generated after 1 and 1.5 h incubation (i.e., immediately before and 30 min after the addition of GE to the Prolyve hydrolysate). These chromatograms again help to identify peaks that are specifically hydrolyzed by GE and may lead to the release of peptides that induce hydrolysate aggregation. The peak eluting at 31 min after 1 h hydrolysis with Prolyve (**Figure 6a**) was significantly degraded after 30 min incubation with GE (**Figure 6b**), whereas the peptide eluting at approximately 40 min in **Figure 6a** was completely degraded after 30 min incubation with GE. The peptide eluting at 40 min in **Figure 6a** was isolated from Prolyve hydrolysates by semipreparative RP-HPLC and identified as β-lactoglobulin f(43−57) as previously described (*38*). The isolated peptide was hydrolyzed into four fragments on incubation with GE but did not appear to be further hydrolyzed on incubation with subtilisin (data not shown).

These results show the influence of the proteinase specificity on the characteristics of hydrolysates generated with commercially available proteinase preparations. It was shown that hydrolysates generated with the subtilisin activity from Alcalase did not aggregate, but hydrolysates generated with Prolyve could be induced to aggregate by the addition of a GE activity. The presence of two prominent peaks in subtilisin and Prolyve hydrolysates (eluting at 31 and 40 min), which are readily hydrolyzed by GE, indicates that GE hydrolysis of these peptides may be a key event in the initiation of peptide aggregation.

ABBREVIATIONS USED

Abs, absorbance; Ac.glu.pNA, acetyl-Glu.pNA; BLP, *Bacillus licheniformis* proteinase; β-lg, β-lactoglobulin; DH, degree of hydrolysis; FPLC, fast protein liquid chromatography; GE, glutamyl endopeptidase; HPLC, high-performance liquid chromatography; pNA, paranitroanilide; ProGE0h, WPC hydrolysates generated with a combination of Prolyve and GE with both enzymes added at the start of hydrolysis; ProGE1h, WPC hydrolysates generated with a combination of Prolyve and GE with the GE activity added after 1 h hydrolysis with Prolyve; RP, reversed-phase; s.AAF.pNA, succinyl-Ala-Ala-Phe.pNA; Subtilisin ($\times$2)WPC, hydrolysates generated with subtilisin at twice the original addition level; TFA, trifluoroacetic acid; WPC, whey protein concentrate; WPI, whey protein isolate.

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