JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzyme-Induced Gelation of Extensively Hydrolyzed Whey Proteins by Alcalase: Comparison with the Plastein Reaction and Characterization of Interactions

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Extensive hydrolysis of whey protein isolate by Alcalase 2.4L produces a gel. The objectives of this study were to compare enzyme-induced gelation with the plastein reaction by determining the types of interactions involved in gelation. The average chain length of the peptides did not increase during hydrolysis and reached a plateau after 30 min to be ~4 residues, suggesting that the gel was formed by small molecular weight peptides held together by non-covalent interactions. The enzyme-induced gel network was stable over a wide range of pH and ionic strength and, therefore, showed some similarities with the plastein reaction. Disulfide bonds were not involved in the gel network. The gelation seems to be caused by physical aggregation, mainly via hydrophobic interactions with hydrogen bonding and electrostatic interactions playing a minor role.

KEYWORDS: Whey proteins; enzymatic hydrolysis; Alcalase; aggregation; plastein reaction; peptides; hydrophobic interaction

INTRODUCTION

Functional ingredients and nutraceuticals are gaining in popularity (*1*). Whey protein hydrolysates (WPH) have been on the market for many years because they possess excellent nutritional value and functional properties. WPH are widely used in sports nutrition because it has been established that low molecular weight peptides are easily absorbed, and therefore muscle catabolism may be reduced or eliminated during performance (*2*). Enzymatic hydrolysis is generally used to produce WPH because it occurs under mild conditions and thus retains nutritional quality.

Previous work (*3*) has shown that gelation occurs during extensive hydrolysis of whey protein isolate (WPI) with Alcalase 2.4L at high solid content (20% w/v). This phenomenon is unexpected and creates a hurdle for the industrial production of WPH, where high protein concentration is required to reduce costs of drying. The structure of the enzyme-induced gel was stable over a wide range of temperature (10−65 °C), and the types of interactions involved in the gelation process were unclear.

Proteases catalyze the hydrolysis of peptide bonds in proteins giving rise to products of lower molecular weight than the initial substrate, which are usually soluble in water. On the other hand, when highly concentrated solutions of protein hydrolysates are incubated with proteases, water-insoluble and gel-forming products may be formed. These products, normally called plasteins, were discovered almost 100 years ago, but the mechanism of their formation has been very controversial. Some researchers have reported evidence for peptide synthesis by either condensation or transpeptidation (*4*, *5*), whereas others have showed that the mechanism was mainly driven by physical aggregation (*6*, *7*).

The plastein products form gels or thixotropic solutions, depending on concentration, which are stable over a wide pH range (*6*, *8*) and a temperature range from at least 0 to 70 °C (*9*, *10*). Some similarities between the enzyme-induced gel and the plastein reaction were observed (*3*). However, gel formation was in a one-step procedure, whereas plastein formation is usually a three-step process. For the plastein reaction, the first step involves an enzymatic hydrolysis of proteins at low substrate concentrations (3−5%) and at the optimum pH of the protease. The most frequent enzymes used are pepsin, α-chymotrypsin, trypsin, and papain. It is also possible to use partial acid hydrolysis. The second step is concentration of the hydrolysate, by either evaporation, freeze-drying, or spray-drying. Finally, the third step is an incubation of the hydrolysate with the same or other enzymes at high substrate concentrations (30−50%) (*6*, *7*). Fujimaki et al. (*11*) found that plastein reactions occur most rapidly between pH 4 and 7. Also, the rate of the reaction is influenced by the average molecular weight of the peptide in the hydrolysate.

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10.1021/jf026041r CCC: $25.00
Published on Web 08/22/2003

More recently, Lorenzen and Schlimme (*12*) determined that aggregates were formed in the course of hydrolysis of sodium caseinate with pancreas proteinases. Aggregates had properties almost identical to plasteins prepared from sodium caseinate hydrolysate (*13*, *14*). The objectives of this study were to investigate the similarities between enzyme-induced gelation and the plastein reaction and to determine the type of interactions involved.

MATERIALS AND METHODS

Substrate. WPI was obtained from Davisco Foods International (Le Sueur, MN). The protein content was 92.7% (w/w), determined by combustion method with a conversion factor of 6.38 × N = % protein (*15*). Alcalase 2.4L (a liquid preparation from *Subtilisin carlsberg*) was obtained from Novozymes (Franklinton, NC). McIlvain buffers of various pH values (2.5−8.0) were prepared by mixing 0.1 M citric acid and 0.2 M disodium phosphate to appropriate pH values (*16*). All other chemicals were of reagent grade quality.

Hydrolysis of WPI. The WPI hydrolysate was made as described by Doucet et al. (*3*). WPI solutions (20% w/v) were heated to 45 °C and adjusted to pH 8.0 before the addition of Alcalase 2.4L. The ratio of enzyme preparation to whey protein was 1:10 (v/w). The final pH of the hydrolysate was 6.0 (after 5 h of hydrolysis) because the pH was not controlled during the reaction. The enzyme-induced gel obtained after 5 h of hydrolysis was diluted with an equal volume of deionized water. Proteolysis was terminated according to the method of Adler-Nissen (*17*). The pH of the solution was lowered to 4.0, and the temperature was increased to 50 °C for 15 min to stop the enzyme reaction. The hydrolysates were then frozen, lyophilized, and stored at 4 °C before further analysis.

Degree of Hydrolysis (DH) and Average Chain Length of Peptides. The *o*-phthaldialdehyde (OPA) method described by Church et al. (*18*) was used to determine α-amino groups and to calculate the DH. The average chain length was determined before and after acid hydrolysis. For the acid hydrolysis, 100 μL of hydrolysate solution was taken at various times, mixed with 2 mL of 6 M HCl in a 5 mL glass ampule, sealed under vacuum, and incubated at 110 °C for 24 h. Hydrolyzed solutions were neutralized with 2 mL of 6 M NaOH. The average chain length was obtained from the following formula (*19*):

$$\frac{100}{\text{av chain length}} = \frac{(\%\ \alpha\text{-amino N before acid hydrolysis}) \times 100}{\%\ \alpha\text{-amino N after hydrolysis}}$$

Turbidity Measurements. Turbidity measurements were made at room temperature with a Shimadzu UV-160U spectrophotometer (Shimadzu Corp., Tokyo, Japan). The turbidity was measured as the optical density at 420 nm. For experiments with McIlvain buffers, samples from the 20% w/v WPI hydrolysate were taken at different times after protease addition and diluted 60× with buffers of desired pH (to 3 mg of protein/mL) and held for 15 min before measurement. All other turbidity measurements in different buffers and reagents were conducted on the enzyme-induced gel produced after 5 h of hydrolysis. Samples were also diluted 60× (to 3 mg of protein/mL), mixed, and allowed to stand for 15 min. The resulting suspensions were homogenized and immediately checked for turbidity at 420 nm. Buffers, alone or with enzyme, did not show any absorbance at 420 nm (results not shown). The control solution was the enzyme-induced gel obtained after 5 h of hydrolysis and dispersed in water to a final concentration of 3 mg/mL. All measurements used water as a reference. Each experiment was replicated three times. A new solution was made for each replication.

Fluorescence Spectroscopy. Fluorescence measurements were done on a System 3 scanning spectroflurometer (Optical Technology Devices Inc., Elmsford, NY). Samples of 10 μL were taken at different times of hydrolysis and immediately mixed with 2840 μL of 0.1 M Bis-Tris buffer, pH 6.0. After mixing, 150 μL of a 0.8 mM 1-anilinonaphthalene-8-sulfonate (ANS) solution prepared in the same buffer was added, and samples were equilibrated for 1 h at room temperature. The ANS was excited at 350 nm, and fluorescence spectra were recorded between 400 and 600 nm. Fluorescence intensity was reported as the intensity relative to the unhydrolyzed WPI solution.

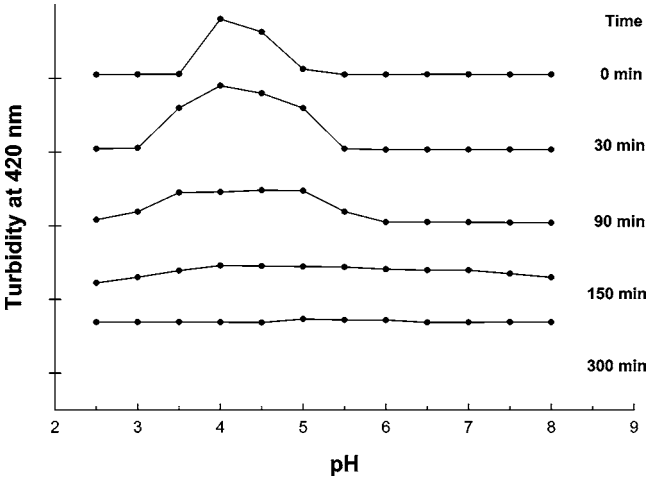

Figure 1. Turbidity (OD_{420nm}) of the whey protein isolate solution as a function of pH and time of hydrolysis with Alcalase 2.4L. Samples of whey protein solution (20% w/v) were taken at the corresponding time of hydrolysis and diluted (3 mg of protein/mL) with McIlvain buffers of respective pH. The optical density range for each time is 0–1.

Statistical Analysis. Data are presented as the mean and standard deviation. A single-factor ANOVA was performed ($\alpha = 0.05$) in comparing treatments, and values were reported as significantly different at $p < 0.001$.

RESULTS AND DISCUSSION

Aggregate Formation. The turbidity as a function of pH and time of hydrolysis was followed to determine if the hydrolysate behavior was the same as that of intact whey proteins. Samples were taken at different times during hydrolysis and diluted with McIlvain buffers of different pH values. The turbidity of the solution before the addition of the enzyme (0 min) was very low except for the pH range of 3.5−5.0, where a high turbidity was observed (**Figure 1**). This is in accordance with Stading and Hermannson (*20*) for β-lactoglobulin (β-LG) and suggests that the behavior of proteins in WPI is mainly due to β-LG at pH values near the p*I*. However, after 30 min of hydrolysis, the turbidity peak in the acid pH range (3.0−5.5) increased, whereas turbidity remained very low for pH values out of this range.

In previous work (*3*) we have shown that extensive hydrolysis of WPI with Alcalase 2.4L in the same conditions caused a dramatic increase in turbidity and viscosity after a critical point and further led to the formation of a gel. In the earlier study, the turbidity was measured at the pH of the hydrolysate and started increasing at ∼100 min. The apparent gel point was reached at 104 min, indicating a rapid reaction leading to gelation. It can be seen from **Figure 1** that the turbidity peak in the pH range of 3.0−5.5 decreased before gelation (104 min) and tended to disappear with further incubation. After 150 min of hydrolysis, the turbidity peak (pH 3.0−5.5) decreased but the overall turbidity was still high for pH values between 3 and 8 (**Figure 1**). Rheological experiments (*3*) have shown that the gel reached an apparent plateau at 300 min. Unlike the WPI solution, the turbidity of the hydrolysate at 300 min is high, independent of pH (**Figure 1**). Otte et al. (*21*) have shown that partial hydrolysis of WPI solutions with BLP, a purified form of Alcalase 2.4L specific for Glu and Asp residues, led to aggregation and caused a new secondary turbidity maximum at pH 5.6 in addition to the turbidity maximum at pH 4.2. With further hydrolysis, this secondary turbidity peak moved to higher

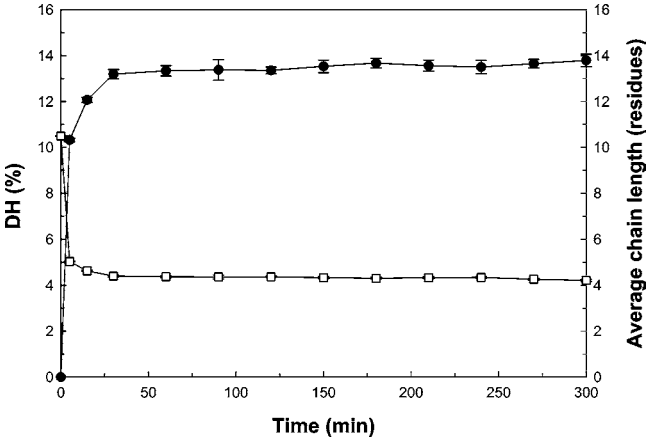

Figure 2. Degree of hydrolysis (●) and average chain length (□) measured during hydrolysis of the whey protein isolate solution with Alcalase 2.4L.

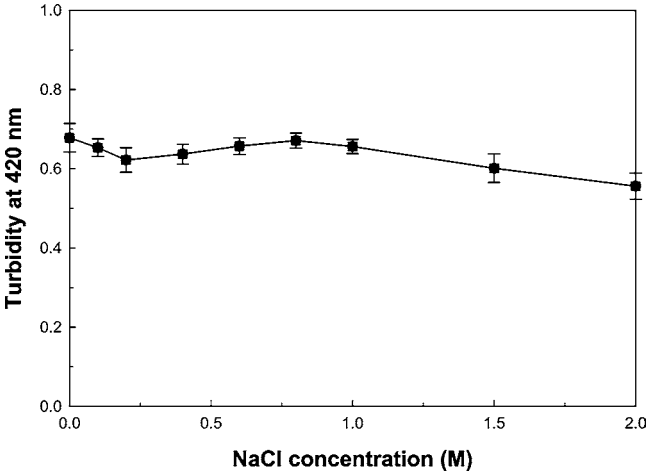

Figure 3. Turbidity (OD_{420nm}) of the enzyme-induced gel as a function of ionic strength. Samples were diluted (3 mg of protein/mL) with Bis-Tris buffer (pH 6.0) containing various amounts of NaCl.

pH, reaching pH 7.0 after 210 min of hydrolysis and indicating aggregate formation at neutral pH. These results indicate different conditions of aggregation from this study where no secondary turbidity maximum is formed and no change in turbidity is noticed when the gel is cured.

The stability over a wide pH range, that is, 2.5−8.0 (**Figure 1**), reinforces the similarity between the enzyme-induced gel and the plastein reaction. Other researchers have reported the stability of plastein toward pH. von Hofsten and Lalasidis (*22*) have shown that gels and precipitates formed during plastein reaction with whey protein and fish protein hydrolysates were almost insoluble in water or buffer solutions of various ionic strengths in the pH range of 3−9. Sukan and Andrews (*9*) have studied the solubility at various pH values for a plastein prepared with sodium caseinate and shown that the plastein was stable over the pH range of 1.5−9.0. Brownsell et al. (*23*) have also shown that plastein formed with mycoprotein peptide hydrolysates was stable over the pH range of 2−11.

Degree of Hydrolysis and Average Chain Length of Peptides. In a previous paper (*3*), the hydrolysis of the WPI solution by Alcalase 2.4L was followed by osmometry. Osmolality measurements have the advantage that they can be measured during hydrolysis, but it was impossible to follow the DH with this method when the viscosity started to increase. Thus, the DH was followed by the OPA method described by Church et al. (*18*). The presence of 1% SDS in the OPA solution served to inactivate the enzyme and ensured full exposure of amino groups. It also solubilized aggregates and gels. Therefore, it was possible to measure the DH and the average chain length for the entire reaction. As reported before (*3*), the DH increased rapidly in the first 5 min due to the high protein content and enzyme concentration (**Figure 2**). The DH reached a plateau after only 30 min and was constant for the rest of the reaction (**Figure 2**).

The average chain length derived from OPA-sensitive groups was measured to determine the size of peptides over time. The average chain length of the substrate decreased rapidly (5.0 residues after 5 min) and reached a plateau after 30 min to 4.3 residues (**Figure 2**). There was no change in the average chain length before or after the gelation (< or >104 min) (**Figure 2**). This is in agreement with other studies that found that a plastein reaction does not lead to an increase in the average chain length (*14*, *19*). Moreover, it reinforces that the enzyme-induced gel is formed by small molecular weight peptides held together by non-covalent interactions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions was performed on the enzyme-induced gel, and results are not shown because the peptides formed during hydrolysis were not retained or stained in the polyacrylamide gel, even when the most sensitive silver stain was used. However, electrophoretic gels showed a very small amount of aggregates not entering the gel and suggests that some aggregates survived heating in the presence of SDS even though it is considered as one of the most vigorous treatments to disrupt physical aggregation. The presence of a small amount of high molecular weight material is in accordance with other investigations that established physical aggregation as the main mechanism involved. Sukan and Andrews (*10*) reported protein bands remaining close to the origin when plastein products formed from a sodium caseinate hydrolysate were analyzed by SDS-PAGE. Glee and Hazen (*24*) have also reported that heating in the presence of SDS and 2-ME did not disrupt preformed aggregates from hydrophobic cell wall proteins.

Characterization of Enzyme-Induced Gel. For the remainder of this paper, the enzyme-induced gel obtained after 5 h of hydrolysis will be referred to as the "enzyme-induced gel". Properties of the enzyme-induced gel before and after lyophilization were the same. Therefore, the following experiments were conducted on the rehydrated powder for practical reasons.

Aso et al. (*25*) have reported that the plastein reaction product from a peptic hydrolysate of a soybean globulin fraction was not solubilized or salted-out by increasing ionic strength. Hence, the turbidity of the enzyme-induced gel was measured as a function of ionic strength in order to investigate effects of electrostatic interactions. Electrostatic repulsion can be affected by pH and salt concentration but, as observed in **Figure 1**, the pH did not have any major influence on turbidity in the range chosen (2.5−8.0). Therefore, the enzyme-induced gel was diluted with a Bis-Tris buffer at pH 6.0 to recreate conditions similar to the final hydrolysate pH, and various amounts of NaCl were added to obtain various ionic strengths (**Figure 3**). As the NaCl concentration was increased from 0.1 to 2.0 M, a slight decrease in turbidity was observed, but overall no major changes were noticed. Thus, NaCl, which is able to break weak ionic bonding, did not lead to the dissociation of the aggregates and did not salt-out the peptides. This suggests that electrostatic interactions are not the main forces involved in aggregation.

To establish more similarities with the plastein reaction, the ability of various reagents to disrupt the aggregates was investigated and compared to the enzyme-induced gel dispersed

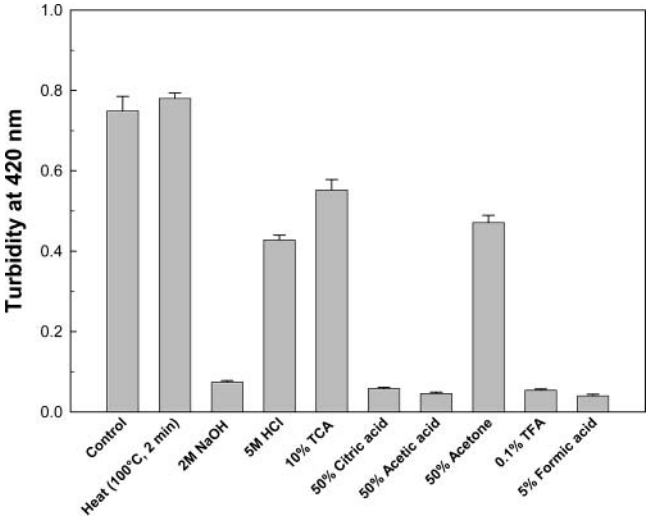

Figure 4. Ability of various reagents or treatments to disrupt the structure of the enzyme-induced gel as evaluated by turbidity measurements (OD_{420nm}).

in water (control) (**Figure 4**). When the control solution was heated for 2 min at 100 °C and measured at that temperature, the turbidity did not increase significantly. This suggests that hydrogen bonds are not the main force involved in the formation of aggregates because they would have been broken during heating. This is in agreement with previous findings when the plastein reaction was applied to sodium caseinate hydrolysates (*10*, *14*). The enzyme-induced gel was solubilized in 2 M NaOH (**Figure 4**). von Hofsten and Lalasidis (*22*) have also shown that plastein from whey protein concentrate was soluble at pH >10. Turbidity was also decreased by treatments with 5 M HCl or 10% trichloroacetic acid (TCA), but aggregates were not fully solubilized (**Figure 4**). Aggregates were disrupted at low and high pH, indicating that electrostatic interactions between amino groups and carboxylic acids could play a role in the network formation. However, at the pH at which gelation occurs (6.0), aggregates were insoluble when the ionic strength was increased (**Figure 3**), indicating that electrostatic interactions are probably not the only force involved. The insolubility in 10% TCA is considered to be one of the parameters for describing plastein reaction (*26*). TCA is known to have no affinity to low molecular weight peptides and is used for the quantitative analysis of proteins and to determine the plastein reaction yield (*11*). Sukan and Andrews (*10*) have shown that the addition of neither 5 M HCl nor 10% TCA could completely solubilize a sodium caseinate plastein. As can be seen from **Figure 4**, organic acids such as citric acid and acetic acid could completely solubilize the enzyme-induced gel. This confirmed that organic acids are more effective than inorganic acids (e.g., HCl) in dissolving aggregates. Therefore, solubilization at low pH is dependent on the type of acid used. Other workers have found similar results for plastein reaction products (*14*, *23*). The stability to acetone (50 vol %) is considered to be another criterion of the presence of plasteins (*6*). This reagent is known to break weak hydrophobic interactions. It can be seen from **Figure 4** that the turbidity was decreased but aggregates were not completely solubilized. Commonly used acids in chromatography buffers such as 0.1% TFA and 5% formic acid were able to fully dissociate the aggregates and reinforce their use as solubilizing buffers when plastein reaction products or enzyme-induced gel has to be characterized (**Figure 4**) (*27*).

Turbidity of the enzyme-induced gel was also measured after treatment with different concentrations of dissociating reagents

Table 1. Turbidity (OD_{420nm}) of the Enzyme-Induced Gel Treated with Different Concentrations of Dissociating Reagents: Urea, Guanidine Hydrochloride (Gn-HCl), Sodium Dodecyl Sulfate (SDS), and β-Mercaptoethanol (2-ME)

molar concn	turbidity (OD_{420nm}) after treatment with			
	urea[a]	Gn-HCl[a]	SDS[a]	2-ME[a]
0	0.644 ± 0.032a	0.644 ± 0.032a	0.644 ± 0.032a	0.644 ± 0.032a
0.1	0.649 ± 0.067a	0.694 ± 0.039a	0.050 ± 0.002b	0.698 ± 0.012a
0.5	0.651 ± 0.035a	0.730 ± 0.028a	0.066 ± 0.008c	0.681 ± 0.075a
1	0.696 ± 0.009a	0.639 ± 0.011b	0.079 ± 0.005c	0.684 ± 0.054a
2	0.708 ± 0.060a	0.536 ± 0.047c		0.676 ± 0.031a
4	0.657 ± 0.054a	0.185 ± 0.023d		
6	0.468 ± 0.018b	0.042 ± 0.004e		
8	0.371 ± 0.069b	0.046 ± 0.008e		

[a] Values are means of three measurements ± standard deviation. Values followed by a different letter in each column are significantly different ($p < 0.001$) from one another.

in a Bis-Tris buffer pH 6.0 (**Table 1**). Treatment with an 8 M urea solution was not enough to fully solubilize aggregates (solution $OD_{420nm} = 0.371$). Guanidine hydrochloride, which is generally considered to be a more effective denaturant than urea (*28*), was able to fully solubilize the enzyme-induced gel at a concentration of 6 M (**Table 1**). These two reagents are known to be effective in loosening protein conformations maintained by hydrogen bonding, but it has been reported that hydrophobic interactions are also weakened at the same time as a result of the breakdown of the hydrogen bonding (*29*). A much lower concentration of SDS (0.1 M) was required to increase the solubility of aggregates (**Table 1**). It is known that SDS, bearing both hydrophilic and hydrophobic groups in the same molecule, is capable of binding with water-insoluble polypeptides through the apolar interaction and solubilizing them as a result of the full hydration at the surface of the micelle (*30*). Therefore, SDS is able to reduce hydrophobic protein−protein interactions. The results suggest that hydrophobic forces could be the main factor contributing to network assembly. Aso et al. (*25*) have shown that turbidity of the plastein reaction product from a peptic hydrolysate of a soybean globulin fraction decreases with increasing concentrations of urea, guanidine hydrochloride, and SDS. However, lower concentrations of these three dissociating reagents were required to decrease turbidity compared to the enzyme-induced gel. Otte et al. (*31*) have shown that aggregates formed during the hydrolysis of β-LG with a Glu- and Asp-specific protease from *Bacillus licheniformis* could be dissolved in either 2% SDS or 8 M urea, which are expected to break down both hydrogen bonds and hydrophobic interactions, suggesting that such interactions play a significant role in aggregate formation.

The turbidity of the enzyme-induced gel was also measured after treatment with different concentrations of the reducing agent β-mercaptoethanol to determine if disulfide bonds linked molecules in aggregates (**Table 1**). Reducing agents are usually effective at low concentrations, but high concentrations were also used in this study to break all intermolecular and intramolecular disulfide bonds present in the enzyme-induced gel. No significant differences were observed up to a concentration in β-mercaptoethanol of 2 M (**Table 1**). Similar results were observed with dithiothreitol, another reducing agent (results not shown).

Many authors have shown that peptides produced from β-LG hydrolysis with trypsin are disulfide linked (*32*, *33*). However, the hydrolysis of β-LG by other enzymes, such as bromelain, papain, pepsin, or endoproteinase Arg-C, does not lead to disulfide-linked peptides (*34*). Caessens et al. (*35*) have found

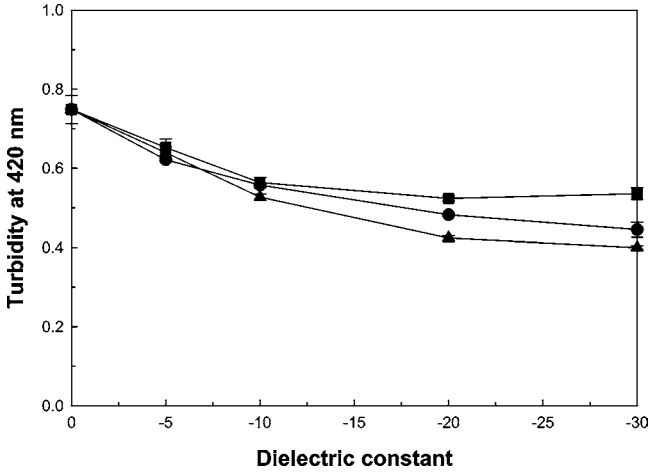

Figure 5. Turbidity (OD_{420nm}) of the enzyme-induced gel in methanol (■), ethanol (●), and propanol (▲) with decreased dielectric constants.

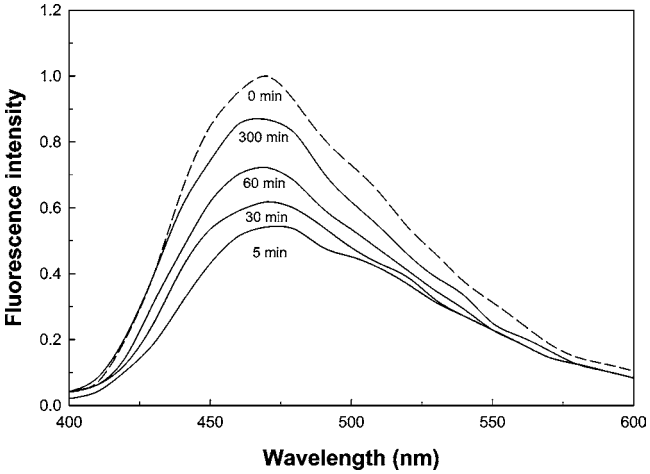

Figure 6. Fluorescence emission intensity spectra of 1-anilinonaphthalene-8-sulfonate (ANS) at different hydrolysis times. The spectrum for unhydrolyzed whey protein isolate (0 min) is indicated with a broken line.

that significant SH/SS exchange had taken place during hydrolysis of β-LG by plasmin. The peptides they identified never consisted of more than two intermolecular disulfide-linked β-LG fragments, and they showed that SH/SS does take place during β-LG hydrolysis, but this seems not to result in large peptides aggregates, which is in agreement with the literature (*34*). Caessens et al. (*36*) have also shown that β-LG hydrolysates formed by *Staphylococcus aureus* V8 protease contained fewer disulfide-linked peptides than the hydrolysates formed by the action of plasmin (as determined by gel permeation chromatography under denaturing plus reducing conditions). They suggested that the use of a less selective enzyme than plasmin results in a β-LG hydrolysate containing smaller peptides of which many lack a cysteyl residue. Therefore, a large number of peptides are present that cannot take place in the SH/SS exchange. Alcalase 2.4L is also known to have a broad specificity, and disulfide bonds do not seem to be involved in aggregate and gel network formation (**Table 1**).

A protic solvent is a solvent capable of acting as a hydrogen bond donor. Weak protic solvents such as alcohol may change protein−solvent interactions (*37*, *38*). Alcohol also contributes to the decrease of the bulk dielectric constant of the solvent. The dielectric constant of a solvent is a measure of its ability to keep opposite charges apart. The turbidity of the enzyme-induced gel was thus measured in the presence of methanol, ethanol, and 1-propanol (**Figure 5**). For each of these aqueous alcohols, the turbidity decreased with increasing concentration, that is, with decreasing dielectric constant. These results were expected because SDS solubilized the enzyme-induced gel and hydrophobic interactions were suggested as the main force leading to gelation. These results are similar to those seen with plastein reaction products from a peptic hydrolysate of a soybean globulin fraction (*25*). Addition of aqueous alcohols caused the turbidity to decrease as the dielectric constant decreased. They concluded that the formation of the water-insoluble product is due to the hydrophobic assembly of plasteins chains. In contrast, alcohols were much less effective in decreasing the turbidity of the enzyme-induced gel (**Figure 5**) than plastein reaction product (*25*), indicating other forces contributing to the network.

Hydrophobicity can be defined as the tendency of nonpolar molecules to adhere to each other in an aqueous environment (*39*). Generally, the number and relative size of hydrophobic sites on a protein surface dictate its solubility and tendency to aggregate under physiological conditions of pH, temperature, and ionic strength (*40*). ANS is a probe known to undergo an increase in fluorescence on binding to hydrophobic surfaces and has been used to detect and study conformational changes under different conditions for whey proteins and β-LG (*41*−*43*). The binding of ANS at various times of the hydrolysis reaction was measured to confirm the contribution of hydrophobic interactions in the enzyme-induced gel (**Figure 6**). A rapid decrease in the fluorescence intensity was observed after 5 min of hydrolysis (**Figure 6**). As seen previously, most of the hydrolysis occurs in the first 5 min (**Figure 2**), and the resulting mixture of peptides did not have as many effective binding sites as the unhydrolyzed WPI. However, after 30 min of hydrolysis an increase in the fluorescence intensity was detected and the intensity increased gradually for the rest of the reaction but never reached the intensity of the unhydrolyzed WPI (**Figure 6**). β-LG, the major protein in WPI, has two different biological binding sites for ANS, one external site in proximity of a hydrophobic patch on the protein surface and an internal site located in the calyx (*44*). On the other hand, binding of the ANS probe to a peptide solution can be associated with a general and nonspecific binding. The lower intensity compared to the unhydrolyzed WPI could be due to loss of biological binding sites or an inaccessibility of the ANS probe to the inside of aggregates formed during the reaction. Other researchers (*31*) have reported the same limit for the probe *cis*-parinaric acid when aggregates were formed during hydrolysis of β-LG with a protease from *B. licheniformis*.

Table 2. Summary of the Similarities between Enzyme-Induced Gel and Plastein Reaction

plastein reaction characteristic	enzyme-induced gelation similarity[a]
substrate concentration	+
pH stability	+++
average chain length	+++
electrostatic interactions	+++
hydrogen bonds	+++
hydrophobic interactions	+++
dielectric constant decrease	++

[a] An increase in + indicates an increase in similarity.

Table 2 presents a summary of the similarities between the enzyme-induced gel and the plastein reaction. Even though the substrate concentration used to obtain the enzyme-induced gel (20% w/v) is lower than the plastein reaction (30−50% w/v), many similarities were identified. Hydrophobic interactions seem to be the main force involved in the gelation with hydrogen bonding and electrostatic interactions playing a minor role.

Research is currently being conducted to identify peptides produced by hydrolysis. This will allow for a better understanding of aggregation and gelation during the extensive hydrolysis of whey proteins.

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Received for review October 16, 2002. Revised manuscript received May 19, 2003. Accepted July 29, 2003. This is paper FSR-02-40 in the Journal Series of the Department of Food Science, North Carolina State University, Raleigh NC. This work was supported by grants from the Southeast Dairy Foods and Research Center, Dairy Manangement, Inc., the Natural Sciences and Engineering Research Council of Canada (NSERC), and the Fonds Quebecois de la Recherche sur la Nature et les Technologies. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultutal Research Service of the products named or criticism of similar ones not mentioned.

JF026041R