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Protease-induced gelation of unheated and heated whey proteins: effects of pH, temperature, and concentrations of protein, enzyme and salts

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Abstract

The influence of various reaction conditions on the gelation of whey protein isolate (WPI) induced by a protease from *Bacillus licheniformis* (BLP) was investigated by following the gelation with a Formagraph and a controlled stress rheometer. As little as 0.5% of denatured WP, or 2% of untreated WP, was able to gel upon the enzymatic treatment (pH 7.0, 50°C, 1% BLP, no salt added). The enzyme-induced gelation process was significantly enhanced by increasing protein and enzyme concentration and by increasing temperature. The presence of salt, up to 15 mM CaCl$_2$ or 100 mM NaCl, decreased the gel time for the enzyme-induced gelation process. Higher salt concentrations, however, led to a more coagulum-like gel. The gelation properties of the enzyme-induced gels varied with pH, due to a combination of effects on the net charge and on the enzyme activity. In all conditions tested, the denatured WPI solution had a higher rate of gelation and a higher gel strength than the gels from untreated WPI solution. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Whey protein; Proteolysis; Gelation; Salts

1. Introduction

Gelation of whey proteins (WP) can be induced by various chemical, physical and enzymatic treatments (Sato, Nakamura, Kawanari & Nakajima, 1995; Van Camp & Huyghebaert, 1995; Færgemand, Otte & Qvist, 1997; Katsuta, Hatakeyama & Hiiraki, 1997). For food applications, gels induced physically, e.g. thermally or by high hydrostatic pressure, or enzymatically are preferred, due to the lower risk of toxic byproduct formation. Recently, it has been shown that gelation of a whey protein isolate (WPI) can be induced enzymatically by a protease from *Bacillus licheniformis* (BLP) upon incubation at neutral pH and 40°C (Otte, Færgemand, Lomholt & Qvist, 1996a). Industrial application of the BLP-induced gelation has been proposed, because relatively strong gels are formed at a protein concentration of 9%, at which concentration heating at 80°C for 30 min does not induce gelation of native WP at neutral pH (Ju, Otte, Zakora & Qvist, 1997).

In order to utilize the enzyme-induced gelation for food applications, information on the influence of various compositional and environmental factors relevant to food manufacture must be available. Factors known to affect the thermal gelation of whey proteins are protein concentration and composition, pre-denaturation, pH, heating rate and temperature, as well as addition of salts, such as NaCl and CaCl$_2$ (Mulvihill & Kinsella, 1987). For heat-induced gelation of WPI, a minimum of 4–12% protein is required for gelation, depending on pH, ionic strength and heating intensity (Morr & Foegeding, 1990). When the concentration of WP is increased above the minimum, or when the heating temperature is increased (up to a certain limit), the time of gelation is reduced and the gel strength is increased (McSwiney, Sing & Campanella, 1994; Tang, McCarthy & Munro, 1994; Taylor, Gladden & Fryer, 1994). Thermal pre-denaturation of the proteins may affect the heat-induced gelation (Mulvihill & Kinsella, 1987; Beuschel, Partridge & Smith, 1992), as well as the Ca$^{2+}$-induced cold gelation.
(Hongsprabhas & Barbut, 1997) of WP. Addition of 10–20 mM CaCl$_2$ increases the hardness of WPI gels set thermally at pH near 7 (Lupano, Dumay & Cheftel, 1992), whereas higher concentrations of NaCl (100–200 mM) are necessary to obtain maximum hardness of heat-set gels from a WP concentrate or $\beta$-lactoglobulin solution (Zayas, 1997).

Thermal pre-treatment has also been shown to have a major impact on the enzyme-induced gelation as well as on the microstructure and appearance of gels made from WPI by the action of BLP (Ju et al., 1997). It is not known how other factors affect the BLP-induced gelation of WPI. In the present study, the effects of pH, temperature, and concentrations of salts, protein and protease on the BLP-induced gelation of both unheated and pre-heated WPI solutions were assessed.

2. Materials and methods

2.1. Materials

A commercial whey protein isolate, BiPro (batch no. 157B-3-TP 27-341, Bio-Isolates PLC, Deeside, UK) with the specifications described by Ju, Otte, Madsen and Qvist (1995) was used. The enzyme used was a serine protease from Bacillus licheniformis (BLP), specific for Glu–X and Asp–X bonds (Breddam & Meldal, 1992). The lyophilized enzyme preparation, kindly provided by T. Mathiassen (Novo Nordisk A/S, Denmark), had an activity of 20.5 Anson units/g. Ethylenediamine tetraacetic acid tetra sodium salt (EDTA), trifluoroacetic acid (TFA) and all other chemicals used were of analytical grade from Merck (E. Merck, Darmstadt, Germany).

Highly purified water (MilliQ Plus, Millipore Corporation) was used for all solutions.

2.2. WPI solutions

Solutions of 9% WPI, pH 7.0, were made as described by Ju et al. (1997). Denatured WPI solutions were obtained by heating at 80°C for 30 min. As judged from size-exclusion HPLC peak areas 93% of the $\beta$-lactoglobulin was denatured by this heat treatment. The pH of the heated WPI solution was readjusted to 7.0, if necessary. The samples of WPI with varying pH were prepared by slow adjustment to the required pH (5.0–8.0) using 4 M HCl or 2 M NaOH, respectively, and stirring to prevent local effects. The denatured WPI solution instantly formed a gel when the pH was adjusted to below 6.2. WPI solutions with 1–8% WP were made by dilution of the 9% WPI solutions with MilliQ water. To obtain desired salt concentrations, small amounts of stock salt solutions (4 M CaCl$_2$ or NaCl) were added to the WPI solutions under vigorous stirring, and the pH was readjusted to 7.0 using a few drops of 4 M or 1 M NaOH. The enzyme solutions were prepared just before use by dissolving 100 mg BLP powder in 0.5 mL of MilliQ water. For varying the enzyme concentration, aliquots of this enzyme solution (4.5–135 μL) were added to 10 mL of WPI solution. Control samples were prepared with heat-inactivated (100°C, 20 min) enzyme.

2.3. Enzyme-induced gelation

The characteristics of the BLP-induced gelation of WPI solutions were assessed using a Formagraph (Type 11700, Foss Electric, Hillerød, Denmark) as described previously (Ju et al., 1997). The standard conditions for the enzyme-induced gelation were 9% WPI solution, pH 7.0, 50°C, and an enzyme to substrate (E/S) ratio of 1%.

Gel time was determined as the time that elapsed from enzyme addition until the amplitude of the Formagraph curve was 1 mm, and the rate of gel firming was determined as the reciprocal of $k_{10}$ (Ju et al., 1997). The mean and standard deviation from triplicate measurements were determined. The gelation of selected samples from each experiment was also assayed by dynamic oscillation measurements using a controlled stress rheometer (Bohlin CVO, Bohlin Ltd., Cirencester, UK) in the autostrain mode. The 14-C measuring system was used with a frequency of 0.5 Hz, and an autostrain set to 0.1. A sample volume of 2.8 mL was used, with a thin layer of vegetable oil added to prevent evaporation. Due to the inappropriacy of the Formagraph for determination of high gel strengths (Ipsen, Otte & Schumacher, 1997), the gel strength was calculated from the oscillation measurements as the complex modulus, $G^*$, at two times the gel time, which was defined as the time when the phase angle, $\delta$, was equal to 45°. All dynamic determinations were made in duplicate.

2.4. Hydrolysis determination

To determine the rate of hydrolysis at the varying conditions used for gelation, separate experiments were set up with selected values of the variables pH, temperature, and concentration of enzyme, NaCl and CaCl$_2$. Samples were taken prior to and at varying times after enzyme addition until the WPI solution had turned into a gel. The samples from the hydrolysates of the unheated WPI solution were immediately added to 19 volumes of 20 mM CaCl$_2$-lactose solution. Control samples were prepared with heat-inactivated (100°C, 20 min) enzyme.

Preliminary tests showed that no further hydrolysis took place during this period.
3. Results

Under the standard conditions (9% WPI, pH 7.0, 50°C), the gels formed from the untreated WPI solutions were soft, white and opaque, and the gels formed from the denatured WPI solutions were brownish, translucent and stronger.

3.1. Effect of whey protein concentration

Decreasing concentrations of both unheated and denatured WPI from 9 to 0.5% increased the time of gelation and decreased the rate of gelation and the gel strength (Fig. 1). At a concentration of 9% WP, the gel times for the denatured and unheated WPI solutions were 40 and 75 min, respectively (Fig. 1A). The 2% denatured WPI solution gelled after 125 min, whereas the 2% untreated WPI solution needed 210 min to form a gel. None of the control samples (with inactive enzyme) formed gels within 16 h of incubation, showing that the enzyme plays an essential role in gelation.

Since the product used in the present study required a minimum protein concentration of 9% for thermal gelation at pH 7.0 and 80°C/30 min (Ju et al., 1995), these results suggest that much lower protein concentrations would be needed in food systems for the enzyme-induced gelation than for the heat-induced gelation. Solutions of only 2% of unheated WPI or 1% of denatured WPI were sufficient to form an enzyme-induced gel (in about 4 h).

The relationships between gel strength and protein concentration of the BLP-induced gels are shown in Fig. 1C. Increasing protein concentration increases the number of protein-particle collisions and more cross-links are formed leading to both a higher rate of gelation and a higher final gel strength.

3.2. Effect of enzyme concentration

With both untreated and denatured WPI solutions, increasing enzyme concentration (E/S ratio) from 0.1 to 3.0% resulted in non-linearly decreasing gel time; the gel time of the native 9% WPI solution decreasing from 240 min at an E/S ratio of 0.1% to 30 min at an E/S ratio of 3% (Fig. 2A, circles). The 9% denatured WPI solution with an E/S ratio of 3% gelled after 20 min (Fig. 2A, squares). None of the control solutions containing 1% or 2% denatured enzyme formed gels within 16 h of incubation. However, the WPI solutions (both native and denatured) containing 3% inactivated enzyme formed a very soft gel after about 13 h of incubation. This may be due to a small amount of residual enzyme activity or to low molecular weight substances present in the enzyme preparation causing gelation after prolonged incubation.

The rate of gel firming for both the untreated and denatured WPI increased with the enzyme concentration —although with a much higher slope for the denatured WPI solution (Fig. 2B). This is in line with the increasing rate of hydrolysis of both native and denatured WP with increasing enzyme concentration (Fig. 3), seen most clearly by the formation of peptides (Fig. 3B and D).

Analogous to the rennet-catalysed coagulation of casein micelles, the BLP-induced gelation of WP may be divided into a primary step (hydrolysis) and a secondary step (aggregation; Carlson, Hill Jr. & Olson, 1987). Plotting the gel time versus the reciprocal to the enzyme concentration...
concentration shows an intercept \( >0 \) for gelation of the unheated WP (Fig. 2D, circles), indicating that the aggregation reaction does take a finite time (Carlson et al., 1987). The fact that the curve for gel time versus the reciprocal to the enzyme concentration for the denatured WP passes through the origin (Fig. 2D, squares) suggests that aggregation of the hydrolysis products formed from the denatured WPI occurs immediately. These results indicate that different gelation mechanisms act for the native and denatured WPI solutions.

The denatured WPI hydrolysate seemed to turn into a gel when a certain concentration of peptide material had been formed (Fig. 3D). Interestingly, the gel firmness was nearly unaffected by the enzyme concentration (Fig. 2C). This suggests that not the rate of gelation, but rather the extent of hydrolysis at the gel point, and thus the nature of the aggregates, determines the gel strength within each type of gel (untreated and denatured WPI).

3.3. Effect of temperature

Increasing the temperature had a significant positive effect on the gelation reactions of both the untreated and the denatured WPI solutions. From 30 to 60°C the time for gelation decreased from 7 h to 30 min for the untreated WPI solution and from 270 to 20 min for the denatured WPI solution (Fig. 4A). With inactivated BLP, none of the WPI solutions (untreated and denatured) formed gels within the observation periods (900 min at 30°C, 450 min at 50°C and 90 min at 60°C).

Reduced gel times with increasing temperature might stem from (i) increased enzyme activity due to both
a higher substrate accessibility from partial unfolding of the compact globular structure of the WP and to increased catalytic rate at higher temperature, and from (ii) increased aggregation rates at the higher temperature, because of increased number of collisions.

In accordance with the high-temperature optimum of BLP (70°C according to Madsen & Qvist, 1997), the unheated WP was hydrolysed faster with increasing temperature (Fig. 5A). The simultaneously higher rate of appearance of hydrolysis products (Fig. 5B) confirms that the decreased peak area of native β-lactoglobulin in Fig. 5A was due to hydrolysis and not just denaturation at elevated temperatures.

The rate of gel firming and the gel strength of denatured and unheated WPI solutions were both highly affected by increased temperature, increasing in a nearly sigmoid manner (Fig. 4B and C). The higher gel strength obtained from the native WP at higher temperature (Fig. 4C) is probably caused by more thiol/disulphide interchange reactions taking place, as the buried thiol group of β-lactoglobulin becomes more reactive (Monahan, German & Kinsella, 1995; Brownlow et al., 1997). The nature of WP gels made from combined thermal and enzymatic treatments is currently under investigation in our laboratory.

3.4. Effect of pH

Varying pH influenced the BLP-induced gelation of the unheated and the denatured WPI solutions differently. When the pH of unheated 9% WPI solution was adjusted to below 6.2, the WPI solution appeared milky and opaque, and it became more opaque towards lower pH values; but no gel formation occurred even at pH 5.0. This indicates formation of acid-induced aggregates due to lowering of electrostatic repulsive forces with decreasing pH.

When the pH of the denatured WPI solution was adjusted to 6.0 or lower, acid-induced gelation occurred almost instantly. At pH 6.2, the denatured WPI samples formed a gel after 90 min when incubated at 50°C without active enzyme (Fig. 6A, grey square), whereas the denatured WPI samples with higher pH did not form gels within 560 min of incubation at 50°C without enzyme. Acid-induced gelation of WPC or WPI pre-heated at 80°C for 30 min has been reported previously (Abd El-Salam & El-Etriby, 1996; Ju & Kilara, 1998b). As judged from the translucent nature of the gels from the denatured WPI, the denatured protein aggregates must have aggregated in an ordered manner to form a fine-stranded gel when the pH was lowered.
The gelation behaviour of the WPI solutions that formed gels in the presence of active enzyme is shown in Fig. 6 (black symbols). With increasing pH from 5.0 to 6.0, the gel time of the enzyme-induced gel from the untreated WPI solution dramatically decreased, and with further increase in pH the gel time increased, until pH 6.8 above which it slowly decreased (Fig. 6A, circles). This behaviour was observed repeatedly by both Formagraph and Bohlin measurements (Ipsen et al., 1997), and was not due to experimental error, as further indicated by
the small standard deviations from triple measurement (Fig. 6A). Due to excessive attractive protein–protein interactions, the gels formed at and below pH 6.0 were more like a coagulum, in accordance with the appearance of acid-induced aggregates at this pH value. At pH 5, protein aggregation was excessive resulting in precipitation rather than gelation, as also indicated by the high standard deviation obtained at pH 5 (Fig. 6A and B). However, none of the untreated WPI samples with inactive enzyme formed gels at 50°C during 16 h of incubation, showing that the enzyme retains a small activity at the lower pH values.

Both the rate of hydrolysis of native β-lactoglobulin in WPI and of formation of hydrolysis products increased with pH (Fig. 7A and B). In the lower pH range from 5.6 to 6.2, the enzyme activity is low (Madsen & Qvist, 1997), and gelation is determined by the reduced electrostatic repulsive forces in the acid environment. The reduced gel times of the untreated WPI solutions with increasing pH from 6.8 to 8.0 probably reflects increased rates of hydrolysis. The rate of gel firming of the native WPI solutions was proportional to pH (Fig. 6B, circles), probably due to increased enzyme activity at higher pH values (as discussed above).

Contrary to the untreated WPI solution, the gel time of the denatured WPI solution increased with increasing pH from pH 6.2 to 8.0 (Fig. 6A, squares). However, an increased rate of formation of hydrolysis products was observed at higher pH (Fig. 7D), indicating that the charge of the proteins has greater influence than the enzymatic reaction on the gel time of denatured WPI. This is also supported by the fact that only a very small concentration of peptides is released at pH 6.8 and 6.4 before gelation occurs (Fig. 7D).

The rate of gelation of the denatured WPI solutions increased only up to pH 6.8, whereafter it decreased with pH up to pH 8.0 (Fig. 6B, squares). This concurs with the charge of the protein being more important for the gelation process of the denatured WPI than the enzymatic reaction. However, as the absence of enzyme did not lead to gelation, the enzymatic reaction still plays a role.

The strength of the gels made from denatured and unheated WPI continuously increased with pH from 6.4 to 8.0. Increasing firmness with increasing pH is in contrast to the decreasing gel firmness of heat-set WPI gels (Shimada & Cheftel, 1988), suggesting that this pH effect is exerted mainly via the enzymatic reaction. In addition, increasing exposure and reactivity of the free –SH group in β-lactoglobulin with increasing pH, with the possible formation of disulphide bonds, may also partly explain the strengthening of the gel network with increased pH.

3.5. Effect of salts

Salt additon up to 500 mM NaCl and 30 mM CaCl₂ had a dramatic effect on gelation of the denatured WPI solutions, whereas the effects on unheated WPI solutions were more limited.

3.5.1. NaCl

Salt addition led to increasing milkiness of the untreated WPI solutions, but no gels were formed from the unheated WPI solutions with 400 and 500 mM NaCl added, even during 950 min of incubation at 50°C with inactive enzyme. NaCl acts by shielding the negative charges on the proteins thereby reducing the electrostatic repulsive forces, and attractive forces become dominant,
resulting in the formation of random aggregates and white, opaque solutions.

Upon addition of NaCl (≥ 200 mM) to the 9% denatured WPI solution a gel was formed at room temperature before addition of enzyme. The salt-induced gel-building process continued after readjustment of the pH to 7.0. Salt-induced gelation of predenatured WPI has also been reported by McClements and Keogh (1995), Kitabatake, Fujita and Kinekawa (1996), and Ju and Kilara (1998b). The NaCl-induced gels from the denatured WPI were all brownish and translucent, and the gel strength appeared to increase with increasing salt content, and also over time.

The gelation time for the enzyme-induced gel from the unheated WPI solution tended to decrease with increasing sodium addition, up to 100 mM (Fig. 8A). However, both the rate of gelation, which was very low, and the gel firmness decreased with increasing Na concentration (Fig. 8B and C). This indicates that at higher salt concentration more coagulate gels were formed, and at the highest NaCl concentrations the gel network collapsed because of excessive protein–protein interactions. The HPLC results show that the enzymatic reaction proceeded faster at low NaCl concentration (results not shown), which could be the reason for the decreased rates of gelation of the unheated WP with increasing NaCl concentration (Fig. 8B).

The effect of NaCl on the enzyme-induced gelation of the denatured WPI could be analysed only at NaCl concentrations below 200 mM. Addition of NaCl to the denatured WPI solution decreased the time for the enzymatically induced gelation to approximately 20 min at 100 mM. Control solutions of denatured WP with 10–50 mM NaCl and inactive enzyme did not form gels within 150 min of incubation at 50°C, showing that for gelation at low salt concentration both the enzymatic cleavage and the charge shielding effect of the sodium ions are important. Similar to the untreated WPI, the rate of gelation and gel firmness decreased with increasing sodium level due to excessive protein–protein interactions resulting in a more aggregate type of gel or coagulum (Fig. 8B and C, squares). Decreasing gel strength of the enzyme-induced gels is in contrast to the increasing gel strength of heat-set WPI gels with up to 20 mM NaCl (Fernandes, 1994; Tang et al., 1994). However, high NaCl concentrations also result in the formation of a coagulum during heating of WP (Tang, McCarthy & Munro, 1995b).
3.5.2. CaCl₂

Addition of CaCl₂, up to 30 mM, to the untreated WPI solution did not lead to salt-induced gelation at room temperature but only to slight changes in opaqueness. However, with CaCl₂ concentrations ≥ 15 mM the untreated WPI solution formed a gel in the absence of active enzyme when incubated at 50°C, but only after 3 h (30 mM) to 6 1/2 h (15 mM CaCl₂). These gels were weak with a white, opaque appearance and of similar low firmness as the enzyme-induced gels (Formagraph results not shown). It has been shown that Ca-induced aggregation of WP is temperature dependent, and takes place at ≤50°C and pH ~ 7.0 (Zhu & Damodaran, 1994; Sherwin & Foegeding, 1997; Ju & Kilara, 1998a). Sherwin and Foegeding (1997) suggest that calcium favours a structural change in β-lactoglobulin at 40-45°C which increases intermolecular interactions. Calcium is also believed to be involved in the formation of bridges between negative charges on protein aggregates resulting in network formation (Jeyarajah & Allen, 1994; Barbut, 1995; Tang et al., 1995b; Twomey, Keogh, Mehra & O'Kennedy, 1997).

The WPI gelation that took place in the presence of active enzyme only was also highly affected by the presence of calcium (Fig. 9, black symbols). The gel time of the unheated WPI decreased from 84 min (no salt added) to a minimum of 9 min with 15 mM CaCl₂ added (Fig. 9A). Higher calcium concentrations (20–30 mM) did not reduce the enzyme-induced gelation time of the untreated WPI further. The rate of gel firming of the unheated WPI solution was very low and decreased with increasing CaCl₂ concentrations (Fig. 9B). Apart from a slightly lower rate of hydrolysis at the highest CaCl₂ concentration (30 mM), the rate of the enzymatic hydrolysis of β-lactoglobulin and formation of hydrolysis products seemed unaffected by addition of Ca²⁺ (not shown), indicating that the effect of Ca²⁺ is exerted specifically on the protein rather than on the enzymatic reaction.

The gels made from native WP with ≥ 10 mM CaCl₂ added had a very low G* value (Fig. 9C), and a soft coagulum was formed. Decreasing gel strength with increasing CaCl₂ concentration up to 40 mM has also been observed for heat-induced WPI gels (Twomey et al., 1997), confirming that the effect of Ca²⁺ is exerted directly on the protein. Similar to Na⁺ ions, Ca²⁺ ions shield negative charges on the proteins, which reduces electrostatic repulsion and causes aggregation, but since CaCl₂ is effective at 10 times lower concentration than NaCl, despite the ionic strength of CaCl₂ being only three times higher than that of NaCl, Ca²⁺ also seems to have a more specific effect as discussed above.

Calcium chloride addition at ≥ 10 mM caused the heat-denatured 9% WPI solution to form a gel instantaneously at room temperature. The gel formed by addition of 10 mM CaCl₂ was translucent and elastic and with increasing CaCl₂ concentration (up to 30 mM) the gels became increasingly turbid and less elastic, which is in accordance with the observations made by Hongprasrhas and Barbut (1997) on Ca²⁺-induced whey protein gels.Probably, Ca²⁺ acted by bridging the pre-denatured aggregates.

When active enzyme was present, the gel times of the denatured WPI solution with 2.5 and 5.0 mM CaCl₂ added were 24 and 12 min, respectively (Fig. 9A, squares). The control solutions with 5 mM CaCl₂ did not form a gel within 560 min. Interestingly, addition of these low levels of CaCl₂ markedly increased the rate of the enzyme-induced gel firming of the denatured WPI (Fig. 9B). Since the enzymatic hydrolysis of soluble aggregates seemed almost unaffected by the presence of CaCl₂...
Fig. 9. Effect of CaCl$_2$ on the enzyme-induced gelation of 9% unheated (●) and denatured (■) WPI solutions. (A) Gel time, (B) rate of gelation, and (C) Gel strength, $G^*$, at two times the gel time. Bars represent standard deviations from three determinations (two for $G^*$). Results for control solutions with heat-inactivated enzyme are shown in grey. Results obtained with EDTA added are shown at 3.75 mM CaCl$_2$.

(results not shown), this may stem from the charge shielding and bridging effects of Ca$^{2+}$. As for the native WP, the gel firmness decreased with CaCl$_2$ level up to 5 mM (Fig. 9C), probably because Ca$^{2+}$ caused excessive aggregation of the pre-denatured WP.

3.5.3. EDTA

The inherent calcium concentration of the 9% WPI solutions with no salt added was about 4 mM. To assess if this calcium concentration already had an effect, the enzyme-induced gelation was performed with a WPI solution containing 5 mM EDTA to complex all Ca$^{2+}$ ions present. Removing the Ca continued the already seen phenomenon that occurred by reducing the added Ca$^{2+}$ concentrations from 10 to 2.5 mM, i.e. increasing gel time and decreasing rate of gelation (Fig. 9A and B), and increasing gel strength (Formagraph results not shown).

The appearance of the gels made from untreated WPI solution hardly changed by addition of EDTA, they were still smooth and white, whereas the gels made from denatured WPI solutions in the presence of EDTA appeared less turbid, indicating that the small amount of Ca$^{2+}$ already present had caused some aggregation of the denatured WP, in accordance with the observation of Hongsprabhas and Barbut (1997) and Ju and Kilara (1998b).

4. Discussion

The BLP-induced gelation of WPI results in very different gels depending on whether the WP were unheated or pre-denatured. According to the results on the microstructure of similar gels (obtained at 40°C by Ju et al. (1997)), gel formed from the native WP consisted of small aggregates (~0.1 μm) formed during hydrolysis, whereas the gel from the denatured WP was fine-stranded and thus similar to heat-set gels from intact WP or β-lactoglobulin (Langton & Hermansson, 1992; Otte, Ju, Skriver & Qvist, 1996b).

The mechanism of gelation of the unheated WPI by the action of BLP is being further examined in our laboratory. Our hypothesis is that peptides released during proteolysis of native WP associate into aggregates which in turn associate into a gel network (Otte, Lomholt, Ipsen, Stapelfeldt, Bukrinsky & Qvist, 1997).

Pre-denaturation of β-lactoglobulin and other globular proteins at near-neutral pH may result in the formation of linear aggregates (Doi, 1993; Griffin, Griffin, Martin & Price, 1993; Roefs & De Kruijf, 1994; Gezimati, Creamer & Singh, 1997), from which fine-stranded, transparent gels are formed under appropriate conditions. In the present study, limited hydrolysis by the BLP enzyme was sufficient to cause gelation. The role of the enzyme in the gelation of denatured WPI probably is to cleave only limited fragment(s) from the linear protein aggregates with a highly negative charge. This creates the right balance between repulsive and attractive forces for ordered association and formation of a fine-stranded, translucent gel with high strength.

Under all conditions examined, the enzyme-induced gelation properties of the denatured WPI solutions were markedly better than those of the native WPI solutions, indicated by a lower gel time, an increased rate of gelation, and a higher gel firmness.

Both gelation processes occur in two steps, i.e. hydrolysis and aggregation. The aggregation step, however,
is much faster for the denatured WP as shown by the lines in Fig. 2D. This might explain why, in all conditions examined, the solutions of denatured WPI incubated with BLP gelled faster than the solutions of unheated WPI. The significance of the two reaction steps in gelation of both substrates is further explored in a separate paper (Ipsen, Otte, Lomholt & Qvist, 2000).

The higher gel strength of the gels from the denatured WP could be due to the presence of intermolecular disulphide linkages (Roefs & de Kruijf, 1994) which are believed to confer elasticity to WP gels (Tang, McCarthy & Munro, 1995a), or it may be a physical phenomenon resulting from the increased chain length of the denatured protein aggregates, which by physical entanglement restricts the mobility and thus enhances non-covalent interactions (Wang & Damodaran, 1990).

The present study, reporting on the effects of pH, temperature, and concentrations of protein, protease, NaCl and CaCl₂, has increased our knowledge on which application of this enzyme-induced gelation procedure in food products can be based. It is suggested that the enzyme-induced gelation is advantageous in products with a low salt content and that do not tolerate extensive heating, because gels can be obtained at a lower protein concentration than that needed for thermal gelation. Since the enzyme is not active at 5°C, application in non-acid desserts, that could be stored at this temperature, is suggested.

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