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A New Multistep Ca^{2+}-Induced Cold Gelation Process for β-Lactoglobulin

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The objective of this study was to obtain β-lactoglobulin (β-lg) gels at very low protein concentrations using a new multistep Ca^{2+}-induced cold gelation process. In the conventional cold gelation process, salt free β-lg solutions were heated at neutral pH, cooled, and cross-linked by adding salts. In our new process, first, long linear β-lg fibrils were formed at pH 2. Solutions of these fibrils were cooled, and subsequently, the pH was adjusted to 7 or 8. Transmission electron microscopy studies showed that the long linear fibrils formed at pH 2 were stable when the pH was adjusted to 7 or 8. In the final step, the fibrils were cross-linked using $CaCl_2$. Using rheological measurements, the critical percolation concentration was determined. In the new multistep cold gelation process, the critical percolation concentration was an order of magnitude lower than in the conventional cold gelation method.

KEYWORDS: **β-lactoglobulin; cold gelation; fibrils; critical percolation concentration**

1. INTRODUCTION

Many globular food proteins have the ability to form a gel (*1*). The gel properties depend on protein concentration, ionic strength, pH, and heating procedure (*2*, *3*). One distinguishes heat-induced gelation and cold set gelation. The heat-induced gelation consists of heating the protein solution and subsequent cooling, during which gelation takes place (*4*−*10*). The cold set gelation consists of heating the protein solution and subsequent cooling, resulting in a solution from which a gel can be obtained after addition of salt (*11*−*14*). Cold set gelation can lead to new uses for whey proteins in a variety of foods (*15*).

To obtain cold set gels, it is necessary to prepare a heat-denatured solution with a protein concentration below the critical gelation concentration at a temperature above the denaturation temperature. The gelation is induced at low temperatures by the addition of mono- or polyvalent cations (*1*, *3*, *13*, *16*, *17*).

In the cold set gelation method, as is applied in many papers to salt free whey protein isolate or β-lactoglobulin (β-lg), the protein solution was heated at pH 7, cooled, and cross-linked by the addition of NaCl or $CaCl_2$ (*1*, *3*, *13*, *16*, *17*). In the rest of the paper, we will refer to this type of cold set gelation as "conventional cold gelation". The cold set gels formed using this conventional cold gelation method showed a fine-stranded structure, better water-holding capacity, and higher gel strength than gels formed by the heat-induced gelation (*15*, *17*, *18*).

Cold set gels with different properties, like transparency, shear stress, and strain at fracture, and water-holding capacities were formed by varying the preheating time and/or temperature and the amount and type of salt (*3*, *12*, *15*, *17*−*21*). Monovalent and divalent salt ions both screen electrostatic interactions between charged protein molecules. Divalent cations, such as Ca^{2+}, can in addition form intermolecular ion bridges between negatively charged or carboxylic groups of β-lg (*3*, *12*, *22*). This ability, combined with a larger screening effect, enables divalent cations to induce gelation at much lower salt concentrations than monovalent cations (*12*, *13*).

Using $CaCl_2$ as a gelling agent, there is a difference in the extent of aggregation, at varying $CaCl_2$ levels, which can be due to differences in the rate of aggregation (*13*). For concentrations <0.03 M $CaCl_2$, the screening effect dominates (*14*). Screening will lead to an increase in aggregation rate. At higher $CaCl_2$ concentrations, the formation of salt bridges takes place. To form a salt bridge, molecules must be properly aligned. This effect can lead to a decrease in the aggregation rate (*13*). The difference in the gelation mechanism, caused by different $CaCl_2$ concentrations, could possibly be responsible for the difference in the network building process at the microscopic level (*14*).

The objective of this study was to obtain β-lg gels at a very low protein concentration using a new multistep cold gelation procedure. In the new procedure, long linear fibrils were formed at pH 2 and low ionic strength, after heating at 80 °C for 10 h (*23*, *25*). Solutions of these fibrils were cooled, and subsequently, the pH was adjusted to 7 or 8. Transmission electron microscopy (TEM) micrographs showed that these long linear fibrils were stable against changes of pH. In the final step, the fibrils were cross-linked using $CaCl_2$. Using rheological measurements, the critical percolation concentration was determined using the method described by van der Linden and Sagis (*26*).

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The new multistep cold gelation procedure is compared with conventional cold gelation and heat-induced gelation, as defined above.

2. MATERIALS AND METHODS

2.1. Sample Preparation. β-lg was obtained from Sigma (L-0130) and was a mixture of the genetic variants A and B. The protein was dissolved in bidistilled water, which was adjusted to pH 2 using a 6 M HCl solution. The protein solution was subsequently adjusted to pH 2 by the addition of a 6 M HCl solution. To remove traces of calcium ions from the β-lg and to obtain a protein solution with the same pH and ionic strength as the solvent, the protein was diluted repeatedly with HCl solvent and filtered through a 3K filter in an Omegacell membrane cell (Filtron; membrane consisting of polyethersulfone) at 4 °C and a maximum pressure of 3 bar. The procedure was stopped when the pH and conductivity of the eluated solution and the solvent were the same. The β-lg solution was centrifuged at 22 600*g* for 30 min. To remove any traces of undissolved protein, the β-lg supernatant was filtered through a protein filter (FP 030/2, 0.45 μm, Schleicher & Schuell). A UV spectrophotometer was used to determine the β-lg concentration at a wavelength of 278 nm. A molar extinction coefficient of 16.8 mM^{-1} cm^{-1} was used. The obtained stock protein solution ($10 \pm 2\%$ w/w) at pH 2 was used for all measurements. We checked the pH during the conduction of our experiments.

2.2. TEM. Five different β-lg samples were prepared for TEM experiments. A 2% (w/w) β-lg sample at pH 2 was heated at 80 °C for 10 h in a water bath and cooled on ice, i.e., to 0 °C (I). After the sample was cooled, the pH was adjusted to 7 (II) or 8 (III) with 0.1 and 1 M NaOH. To compare the new multistep cold gelation method with the conventional cold gelation method, 3% β-lg samples at pH 7 (IV) or pH 8 (V) were heated at 80 °C for 30 min in a water bath and cooled on ice, i.e., to 0 °C.

All samples were diluted to 0.04% β-lg. The TEM samples were prepared by negative staining. A drop of the diluted solution was deposited onto a carbon support film on a copper grid. The excess was removed after 30 s using a piece of filter paper. A droplet of 2% uranyl acetate, pH 3.8, was added for 15 s; any excess was removed as before. Electron micrographs were made using a Philips CM 12 Transmission Electron Microscope operating at 80 kV.

2.3. Conversion Experiments. A series of test tubes with 3% β-lg at pH 7 or 8, without added salt, were heated at 80 °C for 30 min. The pH was adjusted from pH 2 (stock protein solution) to pH 7 or 8 by the addition of 0.1 and 1 M NaOH solution before heating. After various time periods between 0 and 30 min, tubes were taken out of the water bath and immediately cooled in ice water.

To determine the concentration of nonaggregated β-lg, the heated samples were diluted in a 0.1 M citric acid/phosphate buffer at pH 4.8. After the aggregates precipitated overnight, the supernatant was centrifuged for 10 min at 20 000*g*. The nonaggregated β-lg concentration present in the supernatant was determined by a UV spectrophotometer at a wavelength of 278 nm. c_t/c_0 was determined by dividing the concentration of nonaggregated β-lg at a certain heating time (c_t) to the concentration of β-lg before heating (c_0).

2.4. Rheological Measurements. A strain-controlled rheometer (VOR, Bohlin) with a concentric cylinder geometry (C14: cup diameter, 15.4 mm; bob diameter, 14 mm; bob height, 21 mm; depth, 6.9 mm; cone angle, 15°) was used to determine the storage modulus (G') as a function of strain (frequency, 1 Hz; temperature, 25 °C; strain, 0.000206−0.206). A 2% β-lg sample at pH 2 was heated at 80 °C for 10 h in a water bath. After the sample was cooled on ice, i.e., to 0 °C, the pH was adjusted to 7 or 8 with 0.1 and 1 M NaOH. Various $CaCl_2$ concentrations (0.005−0.1 M) were added very carefully on ice, and the solution was mixed well. To some samples, NaCl was added instead of $CaCl_2$ (0.03 or 0.15 M). After this procedure, the solution was poured into the rheometer. The rheometer was heated from 3 to 25 °C. After 3 h in rest, a strain sweep was performed.

To compare this new multistep cold gelation method with the conventional cold gelation method, 3% β-lg samples at pH 7 or 8 were heated at 80 °C for 30 min (this heating condition was chosen because it was often used in papers on cold gelation) (*1*, *13*, *17*, *19*, *21*, *27*−*29*). After the samples were cooled on ice, i.e., to 0 °C, various $CaCl_2$ concentrations (0.01 or 0.05 M) were added very carefully on ice, and the solution was mixed well. After this procedure, the solution was poured in the rheometer. The rheometer was heated from 3 to 25 °C. After 3 h in rest, a strain sweep was performed.

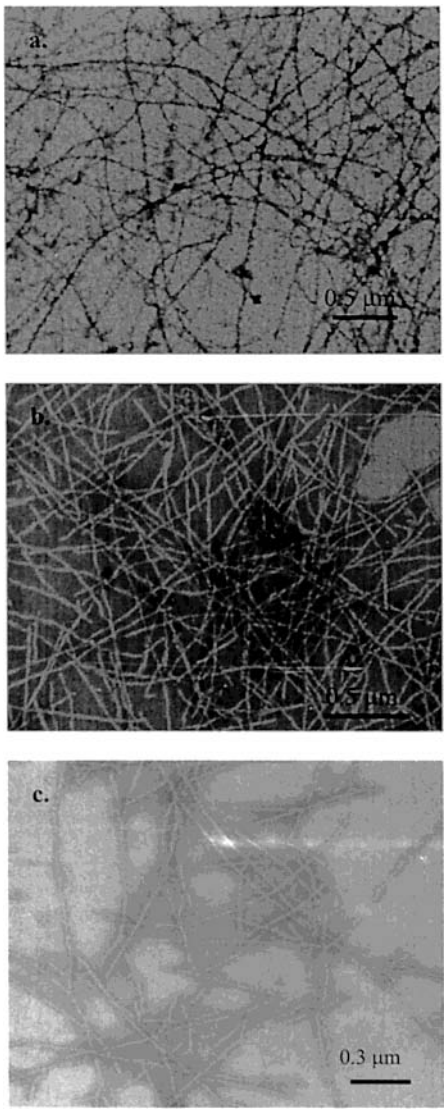

Figure 1. TEM micrographs of 2% β-lg at pH 2 heated at 80 °C for 10 h and subsequently cooled on ice to 0 °C (**a**); afterward, the pH was adjusted to pH 7 (**b**) or pH 8 (**c**).

3. RESULTS AND DISCUSSION

3.1. TEM. TEM micrographs were made to obtain insight in the β-lg structures formed in the different steps of the cold gelation process. **Figure 1a** shows structures formed after heating 2% β-lg at pH 2, at 80 °C for 10 h, and subsequently cooled on ice to 0 °C. Long linear fibrils were formed with a contour length of about 2−7 μm. When the pH of this heated β-lg solution was adjusted to 7 (**Figure 1b**) or 8 (**Figure 1c**), long linear fibrils were still observed. The long linear fibrils formed at pH 2 were stable against changes of pH for at least a week.

To compare the β-lg structure formed, after it was heated, using the new multistep cold gelation method with the conventional cold gelation method, TEM micrographs were made of 3% β-lg, heated at pH 7 or 8 at 80 °C for 30 min, and subsequently cooled on ice to 0 °C (**Figure 2a**,**b**). The structures

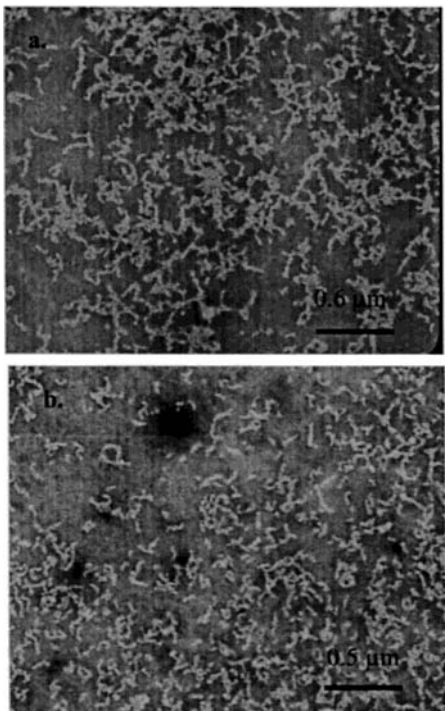

Figure 2. TEM micrographs of 2% β-lg at pH 7 (**a**) or pH 8 (**b**) heated at 80 °C for 30 min and subsequently cooled on ice to 0 °C.

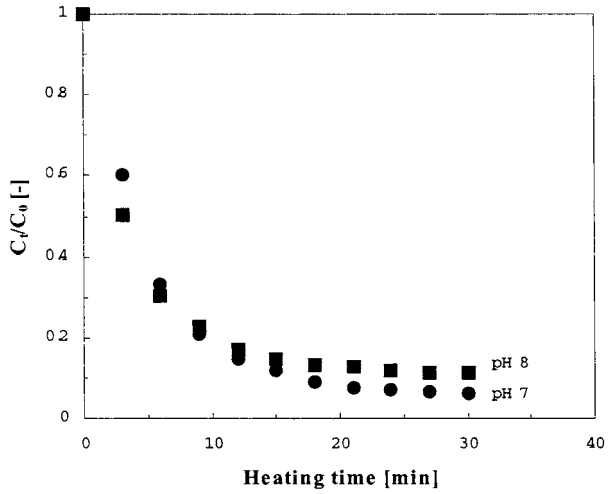

Figure 3. c_t/c_0 vs heating time for 3% β-lg at pH 7 or pH 8, heated at 80 °C for 30 min, and subsequently cooled on ice to 0 °C.

formed at pH 7 or 8 were more than a factor of 10 shorter as compared to the fibrils formed after heating at pH 2. For pH 8, short fibrillar-like structures were observed, whereas for pH 7 a sort of intermediate structure between fibrils and random aggregates was formed. Thus, the structures obtained using the new multistep cold gelation procedure were longer and stiffer than the conventional cold gelation procedure.

3.2. Conversion Experiments. The effect of conversion of the monomers into aggregates on the critical percolation concentration (determined in section 3.3) was investigated, using conversion experiments. The conversion for 3%, salt free β-lg, at pH 7 or 8 heated at 80 °C during 30 min, was determined (**Figure 3**). For pH 7, 94% of the monomers was converted after 30 min, while for pH 8, 89% was converted. Conversion experiments performed for β-lg at pH 2 showed that only 60% of the monomers was converted into fibrils, after heating at 80 °C for 10 h (*25*).

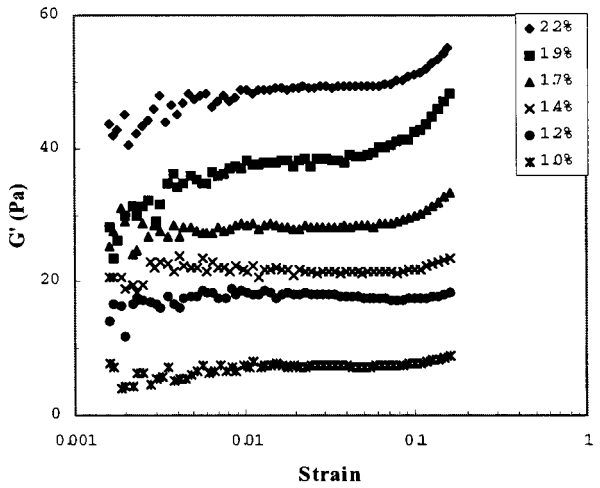

Figure 4. G' vs strain for various β-lg concentrations at pH 2, heated at 80 °C for 10 h and subsequently cooled on ice to 0 °C, after which the pH was adjusted to pH 7 and 0.01 M $CaCl_2$ was added.

An explanation for the difference in conversion between pH 2 and pH 7 or pH 8 can be the difference in denaturation temperature. At neutral pH, the denaturation temperature for β-lg is 65−75 °C (*30*−*32*), whereas the denaturation temperature for β-lg at pH 2 is about 80 °C (determined with differential scanning calorimetry, unpublished results). For the conversion experiments, all samples were heated at 80 °C. This temperature was above the denaturation temperature for β-lg at pH 7 and 8, resulting in a high conversion of the monomers into aggregates. For β-lg at pH 2, the heating temperature of 80 °C was around the denaturation temperature, resulting in a lower conversion of monomers, as compared to the conversion at pH 7 or 8.

3.3. Rheological Measurements. Rheological measurements were performed for β-lg samples prepared according to the new multistep cold gelation process and for samples prepared according to the conventional cold gelation method. **Figure 4** shows the storage modulus, G', vs strain for 2% β-lg heated at pH 2, where after it was cooled, the pH was adjusted to pH 7 and 0.01 M $CaCl_2$ was added. We waited 3 h before measurements were performed. We note that G' as a function of time, at low strain after addition of $CaCl_2$, did not show a significant change after 2 h. The heated β-lg solution was diluted to various concentrations before $CaCl_2$ was added, resulting in the various concentration curves in **Figure 4**. From the linear regime of each curve (i.e., the regime where G' is independent of the strain), G' can be determined. Using G', obtained from the various concentration curves, the critical percolation concentration, c_p, and a scaling exponent, t, can be calculated. We used a scaling relation $G' \sim (c - c_p)^t$, where c is the concentration of monomers (*26*). We calculated c_p and t using the method described in van der Linden and Sagis (*26*). This method is a graphical method that uses plots of $(G')^{1/t}$ vs c_p and extrapolates these plots to $(G')^{1/t} = 0$. This procedure makes use of the fact that independent of the value of t the curves must all intersect the concentration axis at the same value. When the assumed value for t is close to the actual value, the plot will be linear. If t is too small or too large, the lines are curved. From the plots of $(G')^{1/t}$ vs c_p for various t, we selected those t values that give an approximately straight line. From these plots, an average value of c_p was determined. We plotted log G' vs log$(c - c_p)$, using the different values for c_p obtained from the estimated t values. For each of the values for c_p, we determined the t value and averaged these values. **Table 1** shows the

Table 1. Calculated Values for c_p, t, and c_p Corrected for Conversion

heating condition	final pH	$CaCl_2$ (M)	c_p (% (w/w))	conversion (%)[a]	c_p corrected for conversion	t (–)
pH 2, 10 h, 80 °C	7	0.005	>2	60	>1.2	
pH 2, 10 h, 80 °C	7	0.0075	>2	60	>1.2	
pH 2, 10 h, 80 °C	7	0.01	0.12 ± 0.07	60	0.07 ± 0.04	2.0 ± 0.1
pH 2, 10 h, 80 °C	7	0.05	0.62 ± 0.06	60	0.37 ± 0.04	1.9 ± 0.2
pH 2, 10 h, 80 °C	7	0.1	0.67 ± 0.06	60	0.40 ± 0.04	1.9 ± 0.2
pH 2, 10 h, 80 °C	8	0.005	>2	60	>1.20	
pH 2, 10 h, 80 °C	8	0.0075	>2	60	>1.20	
pH 2, 10 h, 80 °C	8	0.01	0.44 ± 0.08	60	0.27 ± 0.05	1.8 ± 0.2
pH 2, 10 h, 80 °C	8	0.05	0.61 ± 0.06	60	0.37 ± 0.04	1.8 ± 0.2
pH 2, 10 h, 80 °C	8	0.1	0.89 ± 0.05	60	0.53 ± 0.03	1.9 ± 0.2
pH 7, 0.5 h, 80 °C	7	0.01	>3	94	>2.82	
pH 7, 0.5 h, 80 °C	7	0.05	0.51 ± 0.07	94	0.48 ± 0.07	1.9 ± 0.2
pH 8, 0.5 h, 80 °C	8	0.01	>3	89	>2.67	
pH 8, 0.5 h, 80 °C	8	0.05	0.60 ± 0.06	89	0.53 ± 0.05	2.0 ± 0.2

[a] The conversion of β-lg monomers into fibrils at pH 2 was determined in ref *25*. Conversion experiments were performed at pH 7 and pH 8; see section 3.2.

calculated values for c_p, t, and the value for c_p corrected for conversion. The calculated values for t were all about 2, indicating isotropic force percolation and a homogeneous network (*26*).

Table 1 shows that the lowest value for c_p was found for a 2% β-lg solution heated at pH 2, where after it was cooled, the pH was adjusted to 7 and 0.01 M $CaCl_2$ was added. For this condition, there is an optimal interplay between the screening of electrostatic interactions between the fibrils and the formation of intermolecular ion bridges between charged or carboxylic groups of β-lg by Ca^{2+} ions. Using the new multistep cold gelation method, no gel was formed when $CaCl_2$ concentrations lower than 0.01 M were added. For $CaCl_2$ concentrations higher than 0.01 M, c_p increases with increasing ionic strength. This may be a result of clustering of the fibrils (bundle formation), caused by increased screening of the repulsive forces and ion bridge formation.

We investigated the relative importance of the screening of electrostatic interactions between the fibrils and the formation of ion bridges. Samples of 2% β-lg were heated at pH 2, and after they were cooled, the pH was adjusted to 7. NaCl was added to these samples instead of $CaCl_2$. We added 0.03 M NaCl or 0.15 M NaCl, which has an ionic strength equivalent to the ionic strength of 0.01 M $CaCl_2$ or 0.05 M $CaCl_2$, respectively. The ionic strength of the samples was equal to 0.03 and 0.15 M, which is equal to the ionic strength of a 0.01 and 0.05 M $CaCl_2$ sample. No gel was observed after the addition of 0.03 or 0.15 M NaCl, which indicates that for these conditions screening of electrostatic interactions between the fibrils is not the driving force to form a gel. The presence of polyvalent cations, which can form salt bridges, is necessary for the formation of a gel network.

For all conditions in **Table 1** where a "larger than" sign is noted, no gel was formed after $CaCl_2$ was added to the heated solutions. For these conditions, it is impossible to obtain a gel using the cold gelation method. To use this method, it is necessary to obtain a heat-denatured solution. When we increased the β-lg concentration, a heat-denatured gel was formed immediately after heating.

The calculated values for c_p for β-lg samples heated at pH 2, where the pH afterward was adjusted to 7 or 8, all were within the same range (**Table 1**). The net negative charge of a β-lg monomer at pH 8 is slightly higher than the charge at pH 7 (−9 for pH 8; −7.8 for pH 7), resulting in a higher electrostatic repulsion between the structures formed. This effect did not result in a much different value for c_p at 0.05 and 0.1 M added $CaCl_2$. For 0.01 M added $CaCl_2$, the c_p at pH 8 was about three times higher.

We can compare the new multistep cold gelation method with the conventional cold gelation method. The c_p corrected for conversion, which indicates the effective amount of structures necessary to form a network, was a factor of 7 lower for β-lg samples prepared according to the new multistep cold gelation method (0.01 M $CaCl_2$ was added) than for β-lg samples prepared according to the conventional cold gelation method (0.05 M $CaCl_2$ was added) (**Table 1**). The new multistep cold gelation method results in a value for c_p (corrected for conversion), which is an order of magnitude lower than the c_p (corrected for conversion) found using the conventional cold gelation method.

We compared samples prepared according to both cold gelation methods for the condition where 0.01 M $CaCl_2$ was added. A gel with the lowest measured c_p was formed using the new multistep cold gelation method, whereas no gel was formed using the conventional cold gelation method. This may be explained in terms of an adjusted random contact model for semiflexible fibrils (*25*). This model yields $c_p \sim 1/P$, where P denotes the persistence length of the fibrils (*25*). The persistence length consists of two contributions. One contribution is the bare persistence length while the other is an electrostatic persistence length. For fibrils made at pH 2 and at ionic strength between 0.01−0.08 M, the bare persistence length is much larger than the electrostatic persistence length (*25*). Addition of 0.01 M $CaCl_2$ to fibrils made according to the new multistep cold gelation method will therefore not significantly influence P. Thus, the persistence length of the fibrils in **Figure 1** (made at pH 2) will be roughly equal to the persistence length of the fibrils made according to the new multistep cold gelation method and to which 0.01 M $CaCl_2$ has been added. From the above and the TEM micrographs, it follows that the persistence length P of fibrils formed according to the new multistep cold gelation method (heated at pH 2, **Figure 1**) is much larger than that of fibrils formed according to the conventional cold gelation method (heated at pH 7 or pH 8, **Figure 2**). Thus, using $c_p \sim 1/P$, one expects that using the new multistep cold gelation method would result in a much lower c_p than using the conventional cold gelation method.

Comparing the results obtained using the conventional cold gelation method with previous papers on cold gelation, previous papers showed the formation of a gel after the addition of 0.01 M $CaCl_2$, whereas no gel was formed when the conventional cold gelation method was used in this paper (*1*, *17*, *19*, *27*, *28*).

This difference may be the result of a different way of adding $CaCl_2$ to the heated solution. In this paper, $CaCl_2$ was added very carefully on ice and mixed well. Previous papers reported that $CaCl_2$ was added by dialysis for 24 h (*1*, *17*, *19*, *27*, *28*). The combination of slowly adding $CaCl_2$ with a longer time to form a gel will result in gel formation at lower $CaCl_2$ concentrations. For the experiments performed in this paper, it was not possible to add $CaCl_2$ via dialysis, because the samples needed to be poured into the VOR before a gel was formed.

When the results of the new multistep cold gelation method are compared with heat-induced gelation, a much lower c_p was found using this new multistep cold gelation method. For β-lg at pH 7 and low ionic strength, a critical gel concentration of 8% was found after heating at 100 °C for 1 h (*33*) and a critical gel concentration of 10% was observed after heating at 95 °C for 1 h (*9*). Also, heat-induced gelation of β-lg at pH 2 resulted in a higher c_p (1.4%, at 0.01 M ionic strength) as compared with using the multistep cold gelation process (*25*).

4. CONCLUSION

TEM micrographs showed that the long linear fibrils formed for β-lg at pH 2 (without added salt) after heating at 80 °C for 10 h were stable when the pH was adjusted to 7 or 8. Short fibrils were observed when β-lg was heated at 80 °C for 30 min at pH 8 (without added salt), and a sort of intermediate structure between fibrillar and random aggregates was found for β-lg heated at 80 °C for 30 min at pH 7 (without added salt). Conversion experiments showed a conversion of 94% for β-lg heated at pH 7 and 89% for β-lg heated at pH 8, whereas the conversion was 60% when β-lg was heated at pH 2 (*25*).

Using rheological measurements, the c_p was calculated. The lowest value for c_p was found when a β-lg sample was heated at pH 2, and afterward, the pH was adjusted to 7, and 0.01 M $CaCl_2$ was added. The lowest value for c_p obtained for β-lg samples prepared according to the new multistep cold gelation method was an order of magnitude lower than the lowest value for c_p obtained by using the conventional cold gelation method.

Using this new multistep cold gelation process, a gel network can be formed at much lower concentrations as compared to the conventional cold gelation method or heat-induced gelation. This novel route opens possibilities for efficient use of food ingredients in general.

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