Gelation of the Complex between κ -Casein and β -Lactoglobulin

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Summary The gelation of the complex between κ -casein and β -lactoglobulin was examined. The apparent viscosity of the mixture of κ -casein and β -lactoglobulin increased by heat treatment. The mixture formed a gel at the concentration of 8% (w/w). The gel formation was accelerated by the chymosin treatment. The mixture of κ -casein and β -lactoglobulin in each concentration of 1.5% gelled by the addition of chymosin after heat treatment. Forming the complex between κ -casein and β -lactoglobulin was necessary to the gel formation. Therefore, heat treatment of above 70°C was demanded. Turbidity increased and the relative content of soluble protein decreased accompanied with gel formation. The addition of calcium ion to the heated solution produced an increase in turbidity. The larger the protein concentration was, the larger the breaking stress of the formed gel was. The addition of calcium ion weakened the breaking stress of the gel.

Key Words gelation, κ -casein, β -lactoglobulin, κ -casein- β -lactoglobulin complex

κ -Casein is an important casein component that stabilizes casein micelles in the presence of calcium ion. β -Lactoglobulin comprises about 50% of the whey protein and does not have any biological role other than a nutritional function. Tobias *et al.* (1) and Slatter and van Winkle (2) have described the possibility of the interaction between κ -casein and β -lactoglobulin induced by heat treatment. This interaction results in a complex formation between κ -casein and β -lactoglobulin (3). We reported that the hydrophobic bond took part in the complex formation as well as the interchange of an -SH/-S-S- (4). A long term preservation of ultra-high temperature sterilized milk leads to the production of precipitates and the gelation.

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These phenomena are considered to be due to the interaction among milk constituents. Especially, the interaction between κ -casein and β -lactoglobulin is well known. It is considered that a κ -casein- β -lactoglobulin complex determines some of the characteristics of milk.

The use of whey is restricted in the present day. It is significant to make valuable products from whey. Now many gels are used as food, such as gelatin gel, pudding and soy curd. Gel formation has always been important in the production of cheese, yogurt, *etc.* (5). Casein is a heat stable protein and does not normally gel on heating. On the contrary, β -lactoglobulin precipitates by heat treatment. It is considered that many varieties of processing and application of β -lactoglobulin will be expected when it is endowed with gelation function. The gelation of β -lactoglobulin with κ -casein by heating is examined in this paper. The gel formed from κ -casein and β -lactoglobulin is expected to be available as a valuable food protein from the standpoint of utilization of whey.

MATERIALS AND METHODS

Materials. κ -Casein was prepared by the method described in our previous paper (6). Crystals of β -lactoglobulin was obtained from bovine milk by the method of Aschaffenburg *et al.* (7). Chymosin [EC 3.4.23.4] was purchased from Nakarai Chemical Co.

Viscosity measurements. The mixture of κ -casein and β -lactoglobulin or the κ -casein solution was heated at 90°C for 20 min. The viscosity of native and heated protein solutions was measured with Tokyo Keiki Visconic, model ELD, at 20°C. The shear rate of the viscometer was between 1.92 and 383 s⁻¹. Viscosity is indicated as an apparent viscosity, as the heat denatured protein solutions often show thixotropic properties (8).

Gelation of protein solution. Proteins were solubilized in 10 mM imidazole-HCl buffer, pH 7.1, containing 70 mM KCl. The weight ratio of κ -casein to β -lactoglobulin was 1. The mixture of κ -casein and β -lactoglobulin was heated at various temperatures for 20 min. Heat treatment was carried out with a Scinics dry block bath, model AL-250, in the test tube or a plastic cubic cell. After heat treatment, chymosin powder was added to the protein solution in the weight ratio of 1 : 50. The protein solution containing chymosin was incubated at 37°C.

Determination of protein. Protein was determined by the method of Lowry *et al.* (9) after destruction of the gel using a glass stick. The mixture of κ -casein and β -lactoglobulin (1 : 1, w/w) was used as a standard protein.

Measurement of turbidity. Absorbance at 610 nm of the gel was measured in a plastic cubic cell as the parameter of turbidity.

Measurement of breaking stress of gel. The breaking stress of the gel was measured with an Iio Electric Curd meter, model M3014A, equipped with a weight of 50 or 100 g after the storage of 2 days at 4°C of gel formed. The container used for measurement was a plastic cubic cell.

RESULTS

Apparent viscosities of native and heated protein solutions

β -Lactoglobulin is heat labile protein and precipitates by heat treatment, while κ -casein does not. The precipitation is not observed by heating of the mixture of κ -casein and β -lactoglobulin, as a κ -casein- β -lactoglobulin complex is induced by heat treatment. In the gelation, a prior aggregation appears to be necessary (10). Viscosity of the protein solution is affected by many factors. One of the factors is the molecular weight. The results are shown in Fig. 1. Viscosity of β -lactoglobulin solution was not measured because the protein precipitated under given conditions of heating. Gelation of κ -casein solution was enhanced rapidly in proportion to the concentration in comparison to that of the unheated one. The value of 8% κ -casein solution heated was 87.7 centi Poises, while that of 4% solution heated was 11.2 centi Poises. The apparent viscosity of native κ -casein solution increased from 2.57 centi Poises at 4% solution to 7.70 centi Poises at 8% solution. On the other hand, the apparent viscosity of the mixture of both proteins increased more remarkably by heat treatment. Though the value of the unheated mixture was 2.17 centi Poises, that of the heated mixture was 897.6 centi Poises at the concentration of 4%. The fact that the viscosity of the mixture is enhanced by heating at a low concentration suggests that the mixture forms a gel at a high concentration by heating.

Gelation of the mixture of κ -casein and β -lactoglobulin

Heat treatment of the mixture was carried out at various protein concentrations (1–10%) in test tubes. Figure 2 shows the photographs of test tubes fallen sideways after heat treatment. It is known that a high concentration of protein is demanded for gel formation. At the concentration of 8%, a κ -casein- β -lactoglobulin

Fig. 1. Effects of protein concentration and heat treatment on apparent viscosity. A: κ -casein solution, B: mixture of κ -casein and β -lactoglobulin. ●, ▲, heated at 90°C for 20 min; ○, △, unheated.

Fig. 2. Photograph of test tubes fallen sideways after heat treatment, which contain the mixture of κ -casein and β -lactoglobulin. Protein mixture was heated at 90°C for 20 min.

Fig. 3. Photograph of test tubes fallen sideways, which contain the mixture of κ -casein and β -lactoglobulin treated with chymosin after heating at 90°C for 20 min. Chymosin treatment was performed at 37°C for 3 h.

gel was formed, but the gel was very soft. It is necessary for protein molecules to aggregate in the form entrapping water in order to form gel. It is considered that the aggregation of κ -casein- β -lactoglobulin complex enough to make the gel may not take place at a low concentration. Heating the mixture in 10% concentration, the solution did not flow even if the test tube was inclined. This fact indicates that a κ -casein- β -lactoglobulin gel was perfectly induced at the concentration of 10% by heat treatment.

It is well known that gel is formed by chymosin treatment of concentrated casein solution (11). Chymosin powder was added to the mixture heated at 90°C for 20 min. As shown in Fig. 3, the gelation was observed at the mixture in a 3%

Fig. 4. Effect of heating temperature on gel formation of complex between κ -casein and β -lactoglobulin. The mixtures of κ -casein and β -lactoglobulin was heated at indicated temperatures for 20 min. The protein concentration was 3%. Chymosin powder was then added and the reaction mixture was incubated at 37°C for 3 h. nt*, non treatment.

Fig. 5. Effect of heating temperature on soluble protein content. The protein solution was heated and then treated with chymosin as described in Fig. 4, and then centrifuged at 3,000 rpm for 20 min. The gel formed was destroyed physically using a glass stick and subjected to the centrifugation. Soluble protein was determined by measuring the amount of protein in the supernatant.

concentration. The protein concentration demanded for gelation to occur is thus lowered by chymosin treatment. In gelation the coagulation occurs not in the form of relatively compact aggregates but by binding at selective sites along the chain of the linear macromolecules or chains forming a network which is capable of entrapping the dispersing medium. The result indicates that it may be favorable for forming a network structure to be constructed by chymosin acting on a κ -casein- β -lactoglobulin complex.

Effect of heating temperature on gel formation

The effect of the heating temperature on gelation of the mixture in a 3% concentration was examined. As shown in Fig. 4, gel was formed by heating at above

70°C, while the mixture heated below 65°C did not form a gel even if chymosin was added to the mixture. The complex formation between κ -casein and β -lactoglobulin does not occur at the temperature below 65°C (12). Long *et al.* suggested that the complex formation occurs on heating above 68°C (13). In fact, the complex is formed by heating at 70°C for 20 min (3). When κ -casein is attacked by chymosin, caseinoglycopeptide and para- κ -casein are formed, and then the formation of precipitates is observed. The precipitate observed in Fig. 4 is para- κ -casein. The fact obtained in this experiment indicates that it is necessary to form the complex between κ -casein and β -lactoglobulin for the gelation by heat treatment and chymosin treatment.

The effect of heating temperature on soluble protein content was also examined. As shown in Fig. 5, soluble protein content decreased as the gel was formed. The complex between κ -casein and β -lactoglobulin is soluble. About 60% of the protein became insoluble. This indicates the formation of very large aggregates containing water.

Turbidity changes with gelation

The effect of protein concentration on turbidity was examined after a 2-day incubation at 4°C with chymosin. The result is shown in Fig. 6. Turbidity increased with concentration until the gel was formed. On the contrary, turbidity decreased with concentration after gelation. It is considered that the κ -casein- β -lactoglobulin complex was induced by heating and then the complex aggregated. The increase of the turbidity until gelation is considered to be due to the progress of aggregation of the complex, and the decrease after gelation is due to the conformation change with the formation of a network entrapping water.

Figure 7 shows the change of turbidity in the process of gel formation. In this experiment, about 4 min was necessary for the gelation. A rapid change in turbidity was observed between before and after gelation. The turbidity hardly changed after

Fig. 6. Effect of protein concentration on turbidity of gel formed by heating at 90°C for 20 min followed by chymosin treatment.

Fig. 7. Changes of turbidity in process of gel formation. The mixture of κ -casein and β -lactoglobulin was heated at 90°C for 20 min, in the plastic cubic cell. Protein concentration was 3% (w/w). Absorbance at 610 nm was measured after the addition of chymosin.

Fig. 8. Changes of turbidity of protein mixture containing calcium ion after chymosin treatment. The mixture of κ -casein and β -lactoglobulin was heated at 90°C for 20 min in the plastic cubic cell, and then calcium ion as calcium chloride was added. Protein concentration was 3% (w/w). Absorbance at 610 nm was measured after the addition of chymosin.

a perfect gelation of about 10 min (Fig. 7). It was considered from the result that a partial destruction of κ -casein- β -lactoglobulin complex by chymosin resulted in the rearrangement of the protein molecules accompanying the increase of turbidity.

Effect calcium ion on gelation

Calcium ion is an important constituent of milk in nutrition and interacts with casein components. Further, calcium ion inhibits the complex formation between κ -

Fig. 9. Effect of protein concentration on the breaking stress of κ -casein- β -lactoglobulin gel formed by chymosin treatment after heating at 90°C for 20 min.

casein and β -lactoglobulin (14). Therefore, the effect of calcium ion on gelation was examined by the addition after heating of the mixture. A gel was formed by the chymosin treatment of the heated mixture containing calcium ion. Figure 8 shows the change of turbidity of the protein solution in the 3% concentration containing calcium chloride as calcium ion after the chymosin treatment. In the cases containing calcium ion, the rate of turbidity change increased with calcium ion concentration. This fact implies that calcium ion promotes the aggregation of protein molecules and peptides. It was suggested that gel formation was accelerated by calcium ion.

Breaking stress of gel

The breaking stress of κ -casein- β -lactoglobulin gel induced by heating and chymosin treatment was measured as the parameter of gel strength. The results are shown in Fig. 9. Gel was formed by the incubation with chymosin for 3 h after heating at 90°C for 20 min. The measurement was carried out after further storage for 2 days at 4°C. In the case of the 3% gel, the breaking stress was 2.65×10^4 dyne/cm². The values of 4% and 7% gels were 4.38 and 14.56×10^4 dyne/cm², respectively. Strength of gel formed from a κ -casein- β -lactoglobulin complex increased with protein concentration.

The effect of calcium ion on gel strength was also examined. The addition of calcium ion was carried out as described above. The breaking stress of the gel containing calcium ion was smaller than that of the gel having no calcium ion (Fig. 10). It is considered that a rough network might be formed, because calcium ion promoted the aggregation.

Fig. 10. Effect of calcium ion on the breaking stress of κ -casein- β -lactoglobulin gel formed by chymosin treatment. The mixture was heated at 90°C for 20 min, and then calcium ion was added. Protein concentration was 3% (w/w). The mixture containing calcium ion was incubated with chymosin at 37°C for 3 h.

DISCUSSION

In the case of soybean proteins, the precipitate is formed by heat treatment at a low concentration and the gel is formed at a high concentration (15). On the other hand, a κ -casein- β -lactoglobulin complex is formed at a low concentration as described above, and the gelation of these protein mixtures has not been studied. In this paper, the gelation of the mixture of κ -casein and β -lactoglobulin at a high concentration and the acceleration of gelation by chymosin treatment were examined.

Apparent viscosity increased with the increase of protein concentration not only in the case of κ -casein solution but also in the case of the mixture of κ -casein and β -lactoglobulin. When the protein solutions were heated, the increase of apparent viscosity with protein concentration was very large compared with that of unheated protein solution. Especially, this increase of apparent viscosity was remarkable in the case of the heated protein mixture (Fig. 1). In general, many proteins are denatured by heat treatment and then aggregate. The aggregation of κ -casein would also be induced by heating. In the case of the mixture of κ -casein and β -lactoglobulin, first, the complex consisting of both proteins was formed by heat treatment and second, the aggregation of the complex by heating resulted in the gelation.

Gel was formed from the mixture at a high concentration by heat treatment, while the gelation was not observed in the case of the κ -casein solution. It is said that gel is formed by entrapping water among a network structure constructed after the aggregation of protein molecules. It may be considered that the extent of aggregation of κ -casein was smaller compared with the mixture of κ -casein and β -lactoglobulin from the apparent viscosity. Self-association of κ -casein is due to a hydrophobic bond, while an -SH/-S-S- interchange as well as the hydrophobic bond participates in the formation of a κ -casein- β -lactoglobulin complex. Therefore, the

molecular weight of the aggregates of the complex may be large enough to form a network structure compared with that of the κ -casein aggregates.

Chymosin treatment lowered the protein concentration demanded for gelation. A gel was formed from only the 3% protein solution by heating following the addition of chymosin, while gel formation was not observed until an 8% concentration without chymosin treatment. This fact indicated that a network structure was formed easily by the destruction of complex using chymosin. The phenomenon of gelation usually requires that the macromolecules involved are highly asymmetric either in the form of random chain or rods (10). It may be considered that a globular complex changes to a rod or a linear macromolecule by partial hydrolysis. It is said that the hydrophobic bond is easily formed with the increase of temperature. In our experiments, about 70% of the protein used was converted into the complex by heating at 70°C for 20 min (3). On the other hand, most of the protein was converted into the complex by heating at 80°C for 20 min. It is considered that the complex formed at 80°C was larger than that formed at 70°C, because of the fact that more protein was incorporated into the gel formed by heating at 80°C compared with that formed by heating at 70°C (Fig. 5).

Gel strength was inversely proportional to the turbidity of the gel in both cases of the addition and lack of addition of calcium ion (Figs. 6, 8, 9, and 10). The gel consisting of rods with a small molecular weight is stronger than that composed of large globular molecules. Also, the former is considered to have a smaller turbidity than the latter. Therefore, the strong gel may be formed from a protein solution in high concentration by the reconstruction of the molecule chain because of the hydrolysis by chymosin. Further, calcium ion promotes the aggregation of molecules resulting in forming a large network consisting of large aggregates. Therefore, the addition of calcium ion weakened the gel strength.

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