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Heat-Induced Gelation of Charcoal-Treated Serum Protein

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The gel strength of charcoal-treated bovine serum and porcine serum proteins at a concentration of 8% was determined with different concentrations of NaCl after heating at 80°C for 15 min at pH 7.0. The charcoal treatment considerably enhanced the gel strength of porcine serum protein, and also improved gel strength of bovine serum protein at NaCl concentrations between 75 mM and 150 mM. The gel strength of commercial PSA and BSA and that of fractionated bovine and porcine proteins by ion-exchange chromatography were also determined at a constant NaCl concentration. With the charcoal treatment, the gel strength of fractions containing albumin increased, while that containing globulin decreased. The gel strength of charcoal-treated bovine serum and porcine serum remarkably increased by the addition of the egg white, ovalbumin and glutathione. The promotion of gel formation with glutathione and ovalbumin seemed to result from the interaction between sulfhydryl groups in glutathione and ovalbumin and intramolecular disulfide bonds in the serum protein, thereby resulting in the highly organized matrix.

Keywords: heat-induced gelation, charcoal-treatment, gel strength, serum protein

Interest in the use of animal blood and its protein components as constituents in food products has grown steadily in recent years. The utilization of blood in food processing was studied because of its good nutritional properties and functional properties such as gelation, water holding capacity and emulsifying property (Wismer-Pedersen, 1979; Nakajima & Umeda, 1984). Serum or plasma fraction can be used in foodstuffs as a functional ingredient because of its gelling properties induced by heat. The gel structure contributes to food texture in a way that it can act as a matrix retaining water, fat and other components.

Gelation involves the formation of a three-dimensional matrix through intermolecular hydrogen bonding which allows the immobilization of water within the gel structure (Gosset *et al*., 1984). In addition, electrostatic interactions, hydrophobic interactions and disulfide bond formation are also considered responsible for the gelation of proteins (Shimada & Matsushita, 1980; Ma & Holme, 1982; Gosset *et al*., 1984; Hayakawa & Nakai, 1985a, b). Factors affecting the protein gelation include the concentration of protein, temperature, pH, ionic strength, solvent composition, and reducing agents (Schmidt *et al*., 1979; Mulvihill & Kinsella, 1987; Wang & Damodaran, 1990).

The protein content of the separated serum from blood is low as substitutes of egg in food processing. The concentration or dehydration is desired, while off-flavor occurs in freeze-dried or spray-dried serum protein during storage. The components responsible for the off-flavor development were hemoglobin, phospholipids and poly-unsaturated fatty acids, which can be effectively removed by charcoal treatment at low pH (Hayakawa *et al*., 1989).

There are many reports on heat-induced gel formation in animal blood plasma or serum protein (Hermansson, 1982; Howell & Lawrie, 1984; Yasuda *et al*., 1986; Hirose *et al*., 1990; Matsudomi *et al*., 1993; Neiser *et al*. 1998), but none on the study of the effect of charcoal treatment on the gelation of serum protein. The purpose of this study was to evaluate the gel strength of heat-induced gelation of charcoal-treated bovine and porcine serum proteins as ingredients assisting gel formation. The effects of egg white, ovalbumin, glutathion and trehalose on the gel strength of charcoal-treated serum protein were investigated as well.

Materials and Methods

Chemical reagents Bovine serum albumin (BSA, A-4503), porcine serum albumin (PSA, A-2764) and glutathion-reduced form (GSH, G-4251) were purchased from Sigma Chemical Co. (St Louis, MO) and used without further purification. Charcoal (400 mesh, 076-09) and trehalose (344-13) were purchased from Nacalai Tesque Inc. (Kyoto). All other chemicals used were of reagent grade.

Serum samples Porcine and bovine blood were obtained from the local slaughterhouses (Takamatsu, Kagawa) and allowed to stand for 2 h without the anticoagulant at ambient temperature for serum separation. The serum was centrifuged at 8000 rpm for 15 min at 4°C to remove the blood cells and then frozen for later use. The serum albumin and globulin were fractionated by ion-exchange chromatography on DEAE-cellulofine A-500 (Seikagaku Co.) using continuous ionic gradient elution.

Preparation of egg white and ovalbumin Homogenized chicken egg white was diluted with an equal volume of Milli Q pure water and the pH adjusted to 6.0 with 0.1 N HCl. After dialyzing against Milli Q pure water, the solution was freeze-dried. The egg white protein solution was made up to 8% (W/V) by dissolving it in 5 mM phosphate buffered saline (PBS). The preparation of ovalbumin was based on the method developed by Sorensen and Hoyrup (Johnson & Zabik, 1981).

Charcoal treatment Bovine and porcine sera or serum protein fractions adjusted to pH 3.0 were mixed with charcoal

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(0.5 g/100 ml) and stirred for 1 h at 4°C followed by centrifugation at 10,000×*g* for 15 min (Chen, 1967). The supernatant was adjusted to pH 7.0 with 0.1 N NaOH. Concentrated phosphate buffer and solid NaCl were added to charcoal-treated or non-treated serum to make final concentrations of 5 mM phosphate buffer and 75–150 mM NaCl at pH 7.0. The prepared sera or serum protein fractions were dialyzed against 5 mM phosphate buffer containing 75–150 mM NaCl or a fixed NaCl concentration of 100 mM for 3 days at 4°C (changed twice).

Gel preparation Egg white or ovalbumin were mixed with charcoal-treated serum containing 100 mM NaCl at ratios of 4:4, 5:3, 6:2, 7:1 and 8:0, respectively. GSH concentrations were adjusted to 0, 1, 2, 4, 8 and 16 mM, and trehalose at 0, 1, 2, 3, 4 and 5% in charcoal-treated serum containing 100 mM NaCl, respectively. The final protein concentration was made up to 8% based on the method of Lowry *et al*. (1951). An aliquot of 5 ml of mixed protein solution was put into a small petri dish (2.2 cm in diameter) covered with a silicone plate and a glass plate, heated at 80°C for 15 min in a water-bath, followed by cooling in tap water for 10 min and standing at room temperature for 50 min.

Gel strength determination Gel strength was determined using a Rheometer (Yamaden RE2-3305 Rheoner II) with a 50 g load cell. A gel sample prepared in a small petri dish was placed on a stage moving upward at a speed of 0.5 mm/s. The sample was tested with a probe specially designed to fit the sample dish. This probe was made of a razor measuring 0.36×0.03 cm at the edge (Hayakawa & Nakamura, 1986). Gel strength was expressed as the force in kPa applied to the probe edge when the surface yield point was reached. Determination of gel strength was repeated at least six times for each sample.

Polyacrylamide gel electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out based on the method of Laemmli *et al*. (1970).

Determination of sulfhydryl groups Protein sulfhydryl groups were determined using DTNB according to Ellman (1959) modified by Shimada and Cheftel (1988). An aliquot of 0.1 ml protein solution was mixed with 2.9 ml of 0.086 M Tris-0.09 M glycine-4 mM EDTA (pH 8.0) buffer containing 6 M urea and 2% SDS (Hayakawa & Nakai, 1985a), 0.03 ml of DTNB solution (4 mg/ml) was added and the absorbance was read at 412 nm after standing at room temperature for 10 min. The amounts of SH groups were calculated using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. Mean values of triplicate results (with standard deviations<10%) were reported.

Results and Discussion

Effect of charcoal treatment The gel strength of charcoal treated (CT-) and non-treated (NT-) bovine serum and porcine serum with various concentrations of NaCl are shown in Fig. 1. It was observed that the gel strength of NT-porcine serum was lower than that of NT-bovine serum. The gelation of albumin and other globular proteins resulted from conformational changes induced by the thermal denaturation. Upon heating, serum protein underwent denaturation and aggregation.

The gel strength of bovine serum treated with charcoal slightly increased at various concentrations of NaCl except for 125 mM. The gel strength of non-treated bovine serum decreased at 100 mM NaCl, as reported by Kitabatake *et al*. (1987) on egg white ovalbumin. This might be due to the loss of the critical balance between attractive and repulsive forces of proteins associated with heat-induced gel formation in bovine serum, and the excessive aggregation and insolubilization which occurred at 100 mM NaCl. There was an apparent improvement in gel strength of bovine serum at 100 mM NaCl because charcoal treatment suppressed the decrease in gel strength. The gel strength of charcoal-treated porcine serum greatly increased at all levels of NaCl. Significant differences in gel strength between charcoal treated and non-treated bovine serum as well as porcine serum were found at 100 mM NaCl, so that further experiments were carried out at 100 mM NaCl.

The gel strength of CT-porcine serum was significantly higher than that of NT-porcine serum ($p<0.05$). With charcoal treatment, bovine serum did not deteriorate in gel-forming property and porcine serum improved in gel-forming property. Therefore, charcoal treatment of serum under acid condition was useful in

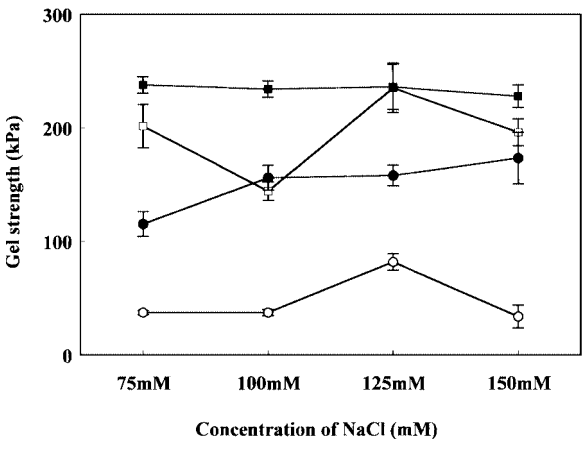

Fig. 1. Effect of charcoal treatment on gel formation of bovine and porcine sera. □: non-charcoal treated bovine serum, ■: charcoal treated bovine serum, ○: non-charcoal treated porcine serum, ●: charcoal treated porcine serum. Gelling conditions: heating at 80°C, 15 min; pH 7.0; NaCl 75–150 mM; protein 8% (w/v). Each datum represents mean±SD of 6 replicates.

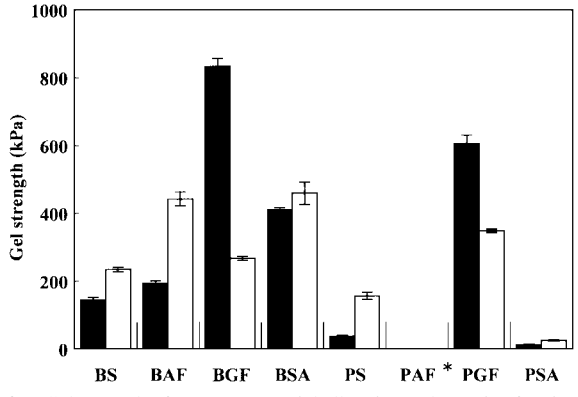

Fig. 2. Gel strength of sera, commercial albumins and proteins fractionated from bovine and porcine sera. Filled bar: non-charcoal treated, open bar: charcoal treated, BS: bovine serum, BAF: bovine albumin fraction, BGF: bovine globulin fraction, BSA: commercial bovine serum albumin, PS: porcine serum, PAF: porcine albumin fraction, PGF: porcine globulin fraction, PSA: commercial porcine serum albumin. *: not determined because of low gel strength. Gelling conditions are the same as used in Fig. 1, with NaCl 100 mM. Bars are means of 6 replicates±standard deviation.

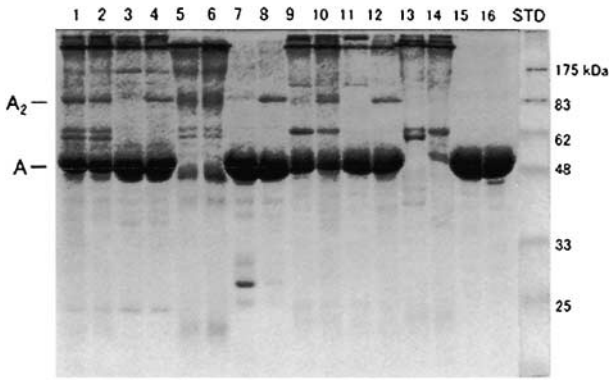

Fig. 3. SDS-PAGE patterns of sera, commercial albumins and proteins fractionated from bovine and porcine sera. Odd number lanes correspond to the non-charcoal treated BS, BAF, BGF, BSA, PS, PAF, PGF and PSA from left to right. Even number lanes correspond to the charcoal treated BS, BAF, BGF, BSA, PS, PAF, PGF and PSA from left to right. The abbreviations are identical to those described in Fig. 2. A: serum albumin; A_2: dimeric serum albumin.

improving the quality of dried serum. The gel strength of sera and commercial albumins and proteins fractionated from bovine and porcine are shown in Fig. 2. The gel strength of bovine blood serum is higher than that of porcine blood serum. With charcoal treatment, the gel strength of commercial bovine and porcine serum albumins (BSA and PSA), bovine and porcine sera (BS and PS) and albumin fractionated from bovine (BAF) increased except for albumins fractionated from porcine (PAF), and that of globulins fractionated from bovine and porcine (BGF and PGF) decreased. Conformational changes in BGF and PGF proteins by acidic charcoal treatment and excessive aggregation by heating were believed to have led to the decrease in gel strength.

The gel strength of CT-BSA and CT-BAF is much higher than that of CT-BS, and it has been suggested that bovine serum albumin plays a more important role in the gel formation of heat-induced bovine serum protein (Hermansson, 1982). Unlike bovine serum proteins, the gel strength of CT-PSA and CT-PAF was lower than CT-PS. The porcine and bovine serum albumins are composed of 583 amino acids (Weinstock & Baldwin, 1988) and there are some differences in the amino acid sequences of N and C terminals (Howell & Lawrie, 1984). It was reported that modification of the free sulfhydryl group by *N*-ethylmaleimide depressed the gel formation of serum albumin heated at neutral pH (Yasuda *et al*., 1986; Matsudomi *et al*., 1993). Therefore, the free sulfhydryl group (Cys 34) plays an important role in the gel formation. In addition, the difference of amino acid sequence around *N* terminal influences the gel formation of both bovine and porcine serum albumins. About 60% of the free sulfhydryl group in bovine serum albumin was detected whereas no sulfhydryl group was detected in porcine serum albumin by the DTNB method, which suggested that this group in porcine serum albumin was masked. Therefore, the heat-induced gel formation of porcine serum albumin was less feasible than that of bovine serum albumin. The conformational changes around Cys 34 in porcine serum albumin and the interaction of serum albumin with conformationally modified globulins upon heating are responsible for the increase in gel strength of porcine serum treated with charcoal.

Additionally, the porcine red cells can be hemolyzed more easily than bovine and a part of the hemoglobin was transferred to serum during blood collection. Porcine hemoglobin contains 6 SH groups in tetramer (1 SH/α-chain, 2 SH/β-chain) whereas bovine hemoglobin contains 2 SH groups in tetramer (no SH/α-chain, 1 SH/β-chain) (Schroeder *et al*., 1967a, b; Braunitzer *et al*., 1978). Therefore, free SH groups in residual porcine globin can interact with SS bonds in serum albumin and globulins. Furthermore, the resulting globin by charcoal treatment of hemoglobin exhibited a high ability of gel formation (Hayakawa *et al*., 1982; 1983). Therefore, the residual globin in the porcine serum could be partially responsible for the increase of gel strength by charcoal treatment of porcine serum.

Comparison of the electrophoretic patterns under reducing and non-reducing conditions is widely used to identify the presence of disulfide bond holding together aggregates of proteins. To estimate intermolecular covalent cross-linking, charcoal treated serum proteins were analyzed by SDS-PAGE in both the absence and presence of 2-mercaptoethanol (ME). The electrophoretic patterns under the non-reducing condition showed that the band of dimeric serum albumin appeared in CT-PS but not in CT-BS. A faint band of dimeric serum albumin only appeared in CT-BAF, CT-BSA, CT-PS and CT-PAF (Fig. 3). The patterns of serum proteins treated with charcoal under the reducing condition showed no band of dimeric serum albumin (not shown). This indicated that intermolecular covalent cross-linking of serum proteins occurred only slightly.

Effect of chicken egg white protein and ovalbumin The effects of mixing of egg white protein or ovalbumin with CT-bovine serum and CT-porcine serum on their gel strength are shown in Fig. 4. It was observed that the addition of a small amount of chicken egg white or ovalbumin remarkably improved the gelling properties of CT-bovine serum and those of CT-porcine serum. The highest gel strength of the mixture of CT-bovine serum and egg white protein was at a ratio of 6:2, and of the mixture of CT-bovine serum and ovalbumin was at a ratio of 5:3.

The gel strength of the mixture of CT-porcine serum and egg white protein at a ratio of 7:1 was the highest while the gel formation of the mixture of CT-porcine serum and ovalbumin at a

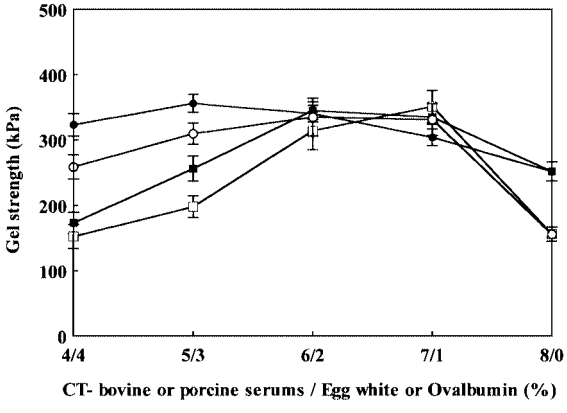

Fig. 4. Effects of mixing ratio of CT-bovine serum or CT-porcine serum with egg white or ovalbumin on gel formation. ■: CT-bovine serum/egg white, ●: CT-bovine serum/ovalbumin, □: CT-porcine serum/egg white, ○: CT-porcine serum/ovalbumin. The mixing ratio of sera with egg white proteins is (4–8)/(4–0)%(w/v). The total protein concentration is 8%(w/v). Gelling conditions same as Fig. 1, NaCl 100 mM. Data are expressed as mean ± standard deviation (n=6).

ratio of 6:2 reached maximum. The increase in gel strength of CT-porcine serum with egg white and ovalbumin was higher than that of CT-bovine serum. The highest gel strength of mixtures of bovine and porcine sera with egg white were measured at a 3 : 3 (% w/w) and 2 : 4 (% w/w) mixing ratio by Howell and Lawrie (1984). This suggested that sera and egg white easily interact following charcoal treatment.

Egg white is well known to coagulate or form gel readily when heated above 60°C. Ovalbumin is the predominant protein in egg white and responsible for the heat-induced gelation of egg white. Chicken ovalbumin contains four free sulfhydryl groups in the interior of the molecule involved in gelation (Ma&Holme, 1982). The proteins of whole blood plasma, serum and plasma fractions interacted with egg white proteins producing stronger gels (Howell & Lawrie, 1984). The aggregations of serum protein with egg white can occur during heating through the exchange reaction of sulfhydryl groups and disulfide bonds (Howell & Lawrie, 1984; Xu *et al*., 1997). The exchange reaction of free sulfhydryl groups of ovalbumin with disulfide bonds of serum proteins, especially serum albumin, occurred when a small quantity of egg white or ovalbumin was added to the serum and enhanced the gel strength. The levels of cleavage of the disulfide bonds in serum albumin can affect the strength of gel formed by heating (Hayakawa & Nakamura, 1986). These may be important in the gelling, probably by promoting more extensive intermolecular interactions (Ma & Holme, 1982; Kato *et al*., 1990a, b). Ovalbumin is a major protein for the heat-induced gelation of egg white, and gel strength of ovalbumin is much higher than that of egg white at the same protein concentration. Therefore, the gel strength of serum added with a large amount of ovalbumin was higher than that added with the same amount of egg white.

Effect of GSH The addition of a small amount of GSH increased gel strength of CT-bovine serum and CT-porcine serum (Fig. 5). It was observed that the gel strength of these sera was increased two fold by the addition of 1–2 mM GSH. The gel strength pronouncedly increased by the addition of more than 4 mM GSH for CT-bovine serum and more than 8 mM for CT-porcine. The results may demonstrate that the increase of gel strength of CT-bovine serum and CT-porcine serum added with GSH is caused by disulfide cross-links. BSA and PSA have 1 sulfhydryl group and 17 disulfide bonds per monomer (Spencer & King, 1971). GSH can react with sulfhydryl group to form disulfide bonds and reduce intramolecular disulfide bonds to sulfhydryl groups. Reduction of disulfide bonds in lysozyme and α-lactalbumin is involved in conformational exchanges and subsequent exposure of hydrophobic regions, which promotes the interaction of proteins and ultimately leads to gel formation (Hayakawa & Nakamura, 1986; Legowo *et al*., 1993). The results obtained in the present study agreed well with the suggestion that the partial cleavage of the disulfide bonds in lysozyme, bovine serum albumin and α-lactalbumin was presumably better than full cleavage for the formation of a hard gel (Hayakawa & Nakamura, 1986; Hirose *et al*., 1990; Legowo *et al*., 1993). A small amount of GSH (1–2 mM) may improve reactivity of the free sulfhydryl group in serum albumin (Cys 34) resulting in considerable improvement of gel formation. Furthermore, a moderate amount of GSH (more than 4 mM or 8 mM) reacted with several disulfide bonds of protein resulting in remarkable enhancement of gel strength of bovine and porcine sera. It seems that the requirement of high GSH concentration which can induce remarkable increase in gel strength of porcine serum and low gel strength of porcine serum at more than 8 mM GSH are due to less sensitivity compared to those of bovine serum for conformational changes upon heating.

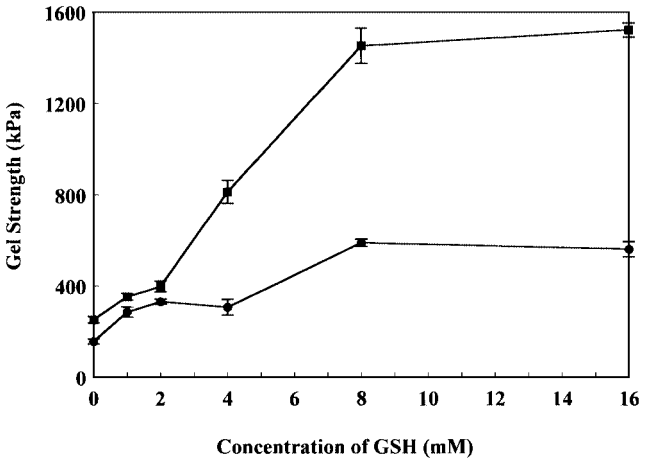

Fig. 5. Effects of glutathione on gel formation of CT-bovine serum and CT-porcine serum. ■: CT-bovine serum, ●: CT-porcine serum. Gelling conditions same as Fig. 1, NaCl 100 mM. Data are expressed as mean ± standard deviation (n=6).

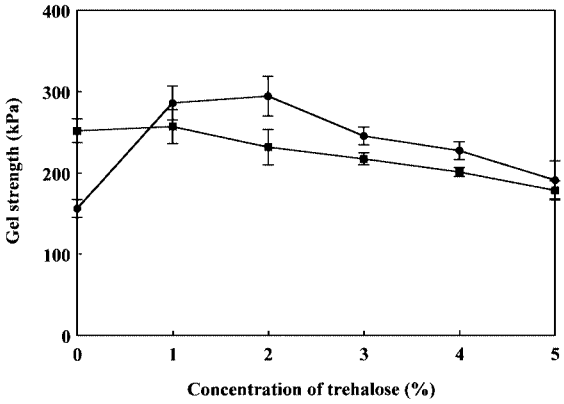

Fig. 6. Effects of trehalose on gel formation of CT-bovine serum and CT-porcine serum. ■: CT-bovine serum, ●: CT-porcine serum. Gelling conditions same as Fig. 1, NaCl 100 mM. Data are expressed as mean±standard deviation (n=6).

Effect of trehalose The addition of sugar to the protein solution is useful for protein drying because sugar can enhance the stability of protein. However, it is not advisable to add reducing sugar because of the browning reaction which occurs with this sugar in the dry-preservation. Therefore, trehalose was added, a naturally occurring non-reducing disaccharide possessing water holding and protecting properties (Miyake, 1998; Takeuchi, 1999), and the effect on heat-induced gelation of serum was determined.

The addition of trehalose increased gel strength of CT-bovine serum and CT-porcine serum (Fig. 6). The gel strength of former then gradually decreased when trehalose was added. On the other hand, the gel strength of CT-porcine serum was remarkably in-

creased by the addition of 1–2% trehalose. The rigid gel formed at a low concentration of trehalose was due to the depression of excess hydrophobic interaction and the formation of hydrogen bonds between protein and sugar molecules. It seemed that the decrease in gel strength at a high concentration of trehalose was caused by excessive weakening of the hydrophobic interactions between protein molecules.

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