Relationship between the Glass Transition of Soy Protein and Molecular Structure

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The change in molecular structure of the soy protein samples as a result of the microbial transglutaminase treatment was studied using solid-state ^{13}C NMR spectroscopy and circular dichroism (CD), and the relation to the glass transition temperature (T_g) was examined. From NMR measurements, the structure of the local region of the C_α methine was observed to change, and the region had relatively high mobility. From CD measurements, the structural change seemed to be caused by the change in the secondary structure (disintegration of the β-structure). By comparison with the T_g of another protein, the state of the secondary structure of a protein was suggested to be a key in determining its T_g.

Keywords: *Glass transition; solid-state NMR; secondary structure; circular dichroism*

INTRODUCTION

In our previous study (Mizuno et al., 2000), we found that the glass transition temperature (T_g) of two fractions isolated from soy protein products was lowered by the microbial transglutaminase (MTG) treatment, which generates cross-links. And, we found that this phenomenon might be related to the state of water in the protein samples. These findings are contrary to the expectations, because the T_g of polymers is generally considered to be elevated by the generation of cross-links. In the MTG-treated sample, there was relatively more immobilized water, which acted as a strong plasticizer, than in the MTG-nontreated sample. The change in the state of water might be caused by the structural change in the protein as a consequence of the generation of covalent cross-links. On the other hand, the T_g of α-casein was found to be elevated by the MTG treatment (Mizuno et al., 1999). This opposite result seems to be attributable to the difference in the molecular structure of the protein samples caused by the MTG treatment and proposes the importance of a study of the relationship between glass transition and molecular structure.

There are various methods for the study of the molecular structure of a protein, which are performed in appropriate solvents. However, it is possible that these experimental conditions are inadequate for the study of the relationship between glass transition and molecular structure, because the glass transition under consideration occurs in low-moisture systems (30% wet basis, at most). To obtain more useful information concerning the molecular structure for this kind of study, measurement in a low-moisture system is preferable. From this standpoint, solid-state ^{13}C NMR spectroscopy was used for the study of the molecular structure of soy protein samples. It is a very powerful tool for conformational characterization of polypeptides and proteins in the solid state, because the ^{13}C NMR chemical shifts depend on their main-chain conformations such as the α-helix and β-sheet (Yoshimizu et al., 1991). So far, many studies concerning the molecular structure of polypeptides and proteins have been done using ^{13}C NMR in the solid state (Saito et al., 1983; Tuzi et al., 1990; Yoshimizu and Ando, 1990). Moreover, from the measurement of the relaxation behavior of ^{13}C NMR signals, the information concerning the molecular mobility of proteins can be directly obtained at the same time.

Many studies on the glass transition of food proteins, e.g., gluten (Hoseney et al., 1986; Fujio and Lim, 1989) and soy protein (Morales and Kokini, 1997; Johari and Sartor, 1998), have been done. In these studies, the T_g was determined using thermal or mechanical analysis, such as differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA), which can provide information about the apparent (macroscopic) state of the samples. In fact, the glass transition of polymers is considered to be strongly related to the molecular (microscopic) state. However, there are few studies on the relationship between the macroscopic information (T_g) and the microscopic information (molecular structure). These kinds of studies are expected to promote understanding of the mechanism of the glass transition of food proteins and to control it for practical use.

Therefore, in this study, the change in the molecular state of soy proteins with the MTG treatment was examined by solid-state ^{13}C NMR, and the relation to the change in T_g, which was reported in the preceding paper in this issue (Mizuno et al., 2000), was discussed. Moreover, we also compared the change in molecular state of casein due to the MTG treatment and discussed an idea about the mechanism of the glass transition of proteins.

MATERIALS AND METHODS

Sample Preparation. Following the method described in the preceding paper in this issue (Mizuno et al., 2000), the 7S

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10.1021/jf991151s CCC: $19.00
Published on Web 06/29/2000

fraction and 11S fraction from the soy protein were obtained, and the MTG-treated samples were prepared. In addition, the MTG-treated α-casein samples were also prepared from bovine milk α-casein (Sigma Chemical Co., St. Louis, MO). The MTG treatment of α-casein was carried out in a manner basically similar to that in the case of soy protein (Mizuno et al., 1999). The added amount of the MTG (80 units/g of substrate) and the reaction time (8 h) were different from those used for soy protein. The water contents of the dry samples prepared after lyophilization were adjusted before the NMR measurement by the same method described in the previous paper.

NMR Measurement. ^{13}C cross-polarization (CP)/magic-angle-spinning (MAS) NMR spectra were recorded on a DSX400WB NMR spectrometer (Bruker, Tsukuba, Japan) operating at 100.6 MHz. Samples (ca. 200 mg) were contained in a cylindrical rotor and spun at a speed of 6 kHz. The contact time was 1.0 ms, and the repetition time was 5 s. The spectral width and number of data points were 35 211 Hz and 4K, respectively. Spectra were usually accumulated more than 10 000 times to achieve a reasonable signal-to-noise ratio. The ^{13}C NMR chemical shifts were calibrated using the carboxyl peak of glycine (176.03 ppm) and were converted to the value from tetramethylsilane.

To examine the influence of MTG treatment on the molecular mobility in the soy protein, the measurements of ^{13}C spin−lattice relaxation time (T_1) were carried out at room temperature using a standard cross-polarization inverse-recovery pulse sequence (180°−τ−90°). The τ values of 2, 20, 40, 80, 120, 250, and 500 ms were used in each measurement.

Circular Dichroism (CD) Measurement. CD spectra were measured in the 200−250 nm range by a J-600 (JEOL) with constant nitrogen flushing at ambient temperature. The sample preparation for the CD measurement was performed following the method of Kamata et al. (1991). First, sample proteins were suspended in 0.01 M phosphate buffer (pH 7.2). The concentration of protein in the suspension was about 0.1 mM. The suspensions were filtered through 0.22 mm pore size filters before measurement.

RESULTS AND DISCUSSION

^{13}C CP/MAS NMR Measurements. The NMR spectra of the 7S fractions isolated from the soy protein isolate are shown in Figure 1. The spectra of low- and high-moisture samples are shown in parts a and b, respectively, of Figure 1. The ^{13}C NMR signals observed at ca. 172−175, 115−158, 50−60, and 10−40 ppm are assigned to the carbonyl carbons, the aromatics, the C_α methines, and side-chain aliphatics, respectively (Yoshimizu et al., 1991). In Figure 1a, there is no remarkable difference between the NMR spectra of the MTG-treated sample and the nontreated sample, except for the signal shape of the C_α methine (50−60 ppm); that is, a shoulder appeared at around 60 ppm in the C_α signal region of the MTG-treated sample. In the high-moisture samples, the signal shape difference in the C_α methine was also observed as shown in Figure 1b; that is, a local signal (indicated by the arrow) was shifted to the lower magnetic field by the MTG treatment. The signal change in the C_α region was observed in both low- and high-moisture samples, but the change seemed to be more apparent in the high-moisture samples. However, this change in the C_α signal did not range over the whole C_α region but only in the local part of the C_α signal. This suggests that the MTG treatment caused a change in the local structure of C_α in the soy protein.

In Figure 2a, the NMR spectra of the 11S fractions isolated from the soy protein isolate are shown. Comparing both samples, the slight signal shape difference in the C_α methine (50−60 ppm) was observed similarly to the case of the 7S fractions. There was no significant

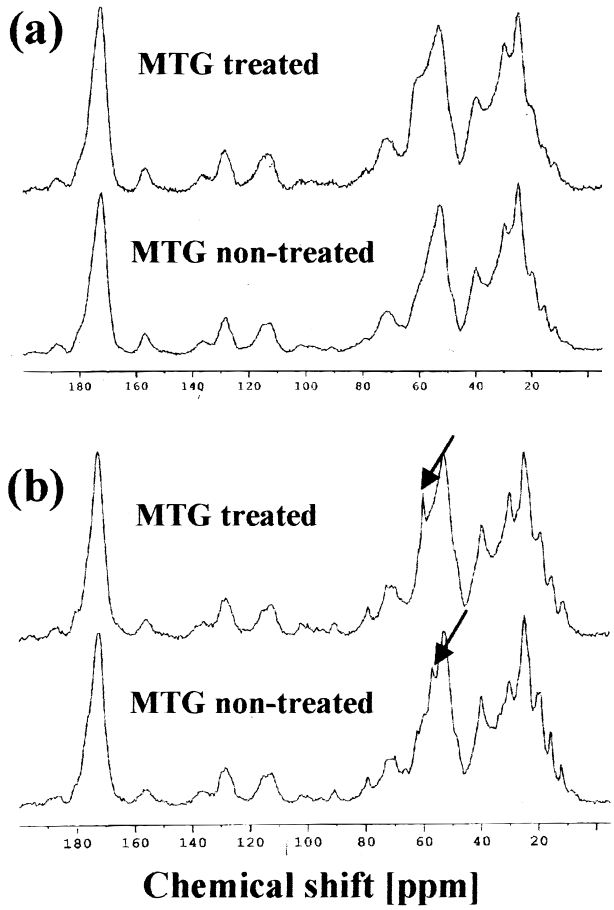

Figure 1. ^{13}C CP/MAS spectra of 7S fractions: (a) low water content samples (MTG-treated 2.5%, MTG-nontreated 3.5%); (b) high water content samples (MTG-treated 19.5%, MTG-nontreated 21.3%).

change in the other signals in the spectra. The signal change in the C_α region caused by the MTG treatment did not seem to be as clear as in the case of the 7S fractions. In the previous paper (Mizuno et al., 2000), we showed the results of the SDS−polyacrylamide gel electrophoresis in which the 7S fractions were isolated better than the 11S fractions from the soy protein isolate, and the 11S fractions seemed to have a wider distribution of molecular weight than the 7S fraction. The lack of clarity of the change in the signal by the MTG treatment in the 11S fractions may be attributable to the distribution of molecular weight.

The NMR spectra of the 7S fractions isolated from the defatted soybean flake (prepared from soybeans without heat treatment) are shown (Figure 2b). The change in the local C_α signal (indicated by the arrow) was observed in the same manner as in the other samples. In the preceding paper in this issue (Mizuno et al., 2000), we showed results in which the T_g of the fractions isolated from soy protein products was lowered by the MTG treatment and indicated that this phenomenon might be related to the state of water in the protein samples. The change in T_g related to the state of water may be caused by the local structural change of the C_α methine. The structure of the C_α methine is considered to be related to the conformation of the protein. Saito et al. (1984) studied the relationship between the conformation of polypeptides, especially the secondary structure, and ^{13}C chemical shifts in the solid state. The local structural change in the C_α methine observed in this study may also be the change in the secondary

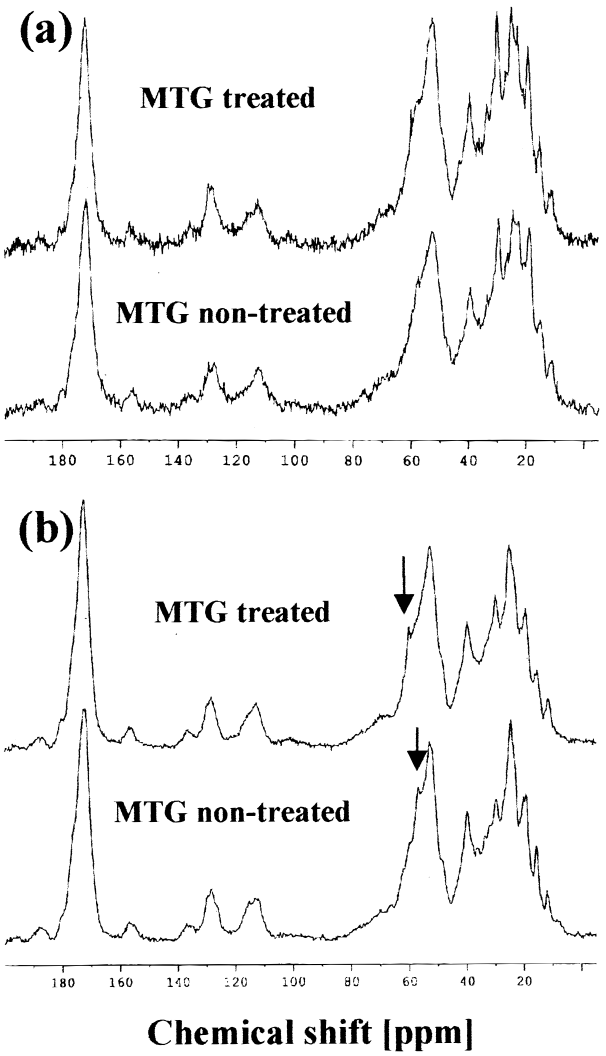

Figure 2. ^{13}C CP/MAS spectra of 11S fractions isolated from the soy protein isolate (MTG-treated 17.2% w/w water, MTG-nontreated 22.2% w/w water) (a) and 7S fractions isolated from defatted soybean flake (MTG-treated 20.3% w/w water, MTG-nontreated 21.7% w/w water) (b).

structure of the soy protein samples. The glass transition of a polymer is closely related to the molecular mobility, as well as the molecular structure, and it is possible that the change in molecular mobility of the soy protein occurred with the local structural change in the C_α methine. Therefore, we measured the relaxation behavior of ^{13}C nuclei of soy protein samples to acquire information concerning the molecular mobility.

The relaxation behavior of the ^{13}C signal of the 7S fractions isolated from soy protein isolate is shown (Figure 3). The signals of the side-chain aliphatic carbons at around 10−40 ppm were observed to decay with τ, and the side-chain aliphatic carbons seemed to have higher mobility than the other carbons. The signals of the side-chain aliphatic carbons of the MTG-treated sample appeared to decay more slowly as shown in Figure 3b, compared with those of the nontreated sample shown in Figure 3a. This shows that the side chain of the MTG-treated sample had lower mobility than in the nontreated sample. The restraint of the side chain of the MTG-treated sample was considered to be attributable to the ϵ-(γ-Glu)Lys cross-links generated by the MTG treatment (Sakamoto et al., 1994). In the spectra of the MTG-treated sample (Figure 3b), the specific local signal (indicated by the arrow) for the C_α methine (50−60 ppm) was also observed. However, it decayed in about 80 ms, and the relaxation of the specific C_α region signal seemed to occur faster than that of the other C_α methine. This implies that the local region of the C_α methine changed by the MTG treatment had relatively high mobility, compared with the other region of the C_α methine. Because the molecular mobility of an amorphous material is closely related to the glass transition (Elliot, 1990), the increase in mobility in the local C_α caused by the MTG treatment may be related to the decrease in the T_g of the soy protein. In addition, a study on the structural change which caused the change in molecular mobility may serve to control the T_g of proteins, as well as soy protein. As described above, the MTG treatment was suggested to induce a secondary structure change in the soy protein. A detailed study on the change in molecular structure due to the MTG treatment was done using CD measurements.

Relationship between T_g and Secondary Structure. The CD spectra of soy protein samples (7S fractions) are shown in Figure 4. The spectrum for the MTG-nontreated sample (Figure 4a) was similar to the results for the 7S and 11S soybean globulins (Koshiyama and Fukushima, 1973; Kamata et al., 1991) considered to be β-structure-type proteins. Thus, the secondary structure of the MTG-nontreated sample seemed to be rich in β-structure. On the other hand, in the spectrum for the MTG-treated sample (Figure 4b), the shape dramatically changed; that is, the peak at around 215 nm, which is characteristic for the MTG-nontreated sample, disappeared, and the peak top shifted to 210 nm. Generally, the secondary structure of proteins is determined by comparion with the CD data for poly-L-lysine whose secondary structure is adjustable, and the peak in the CD spectra of poly-L-lysine at around 215 nm is considered to be attributable to the α-helix or β-structure (Greenfield and Fasman, 1969). In this study, the disappearance of the peak at around 215 nm due to the MTG treatment seemed to be caused by the relative decrease in the β-structure, which was the dominant secondary structure in the sample. In other words, the MTG treatment caused the disintegration of the secondary structure in the soy protein sample. In the previous NMR study, the local structural change in the C_α methine with the change in the secondary structure was suggested to occur due to the MTG treatment. These results of CD measurement could be direct experimental evidence that the secondary structure of soy protein changed dramatically and is related to the lowering of the T_g of the soy protein due to the MTG treatment. The disintegration of secondary structure means the decrease of intramolecular interaction by hydrogen bonding which existed in the disintegrated β-structure. Since the intramolecular interaction can prevent the local molecular motion, it is possible that the mobility of a MTG-treated soy protein molecule might increase, which lowers the T_g. Moreover, it is possible that the accessibility of water to the protein might change. Urry et al. (1974) studied the effect of solvent on the functional groups in polypeptides by ^{13}C NMR and reported that carbonyl groups which participated in intramolecular hydrogen bonding in the β-structure were shielded from the solvent. This suggests that the disintegration of the β-structure of soy protein by the MTG treatment allowed the solvent

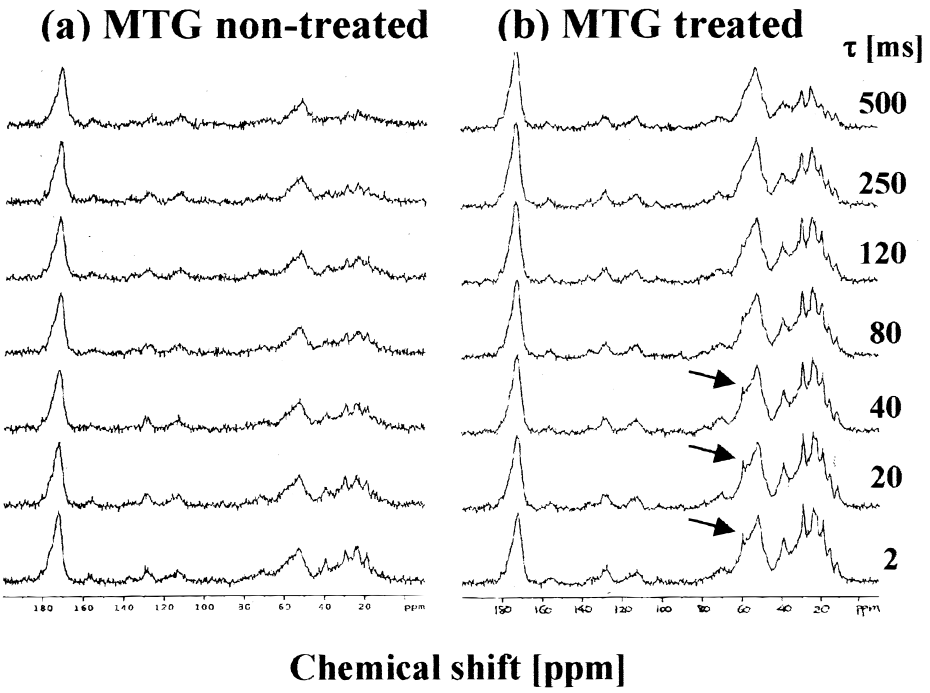

Figure 3. Relaxation behavior of ^{13}C nuclei of 7S fractions isolated from soy protein isolate: (a) MTG-nontreated sample; (b) MTG-treated sample.

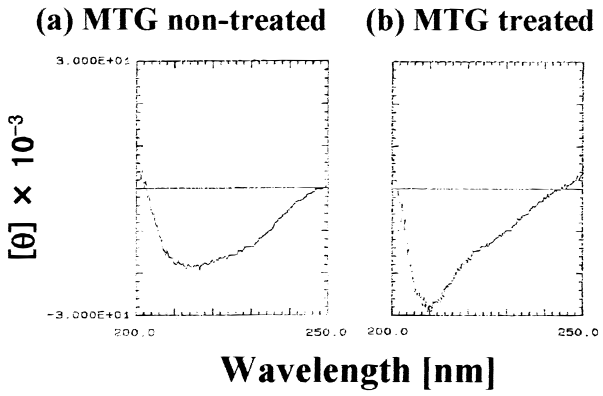

Figure 4. CD spectra of 7S fractions: (a) MTG-nontreated sample; (b) MTG-treated sample.

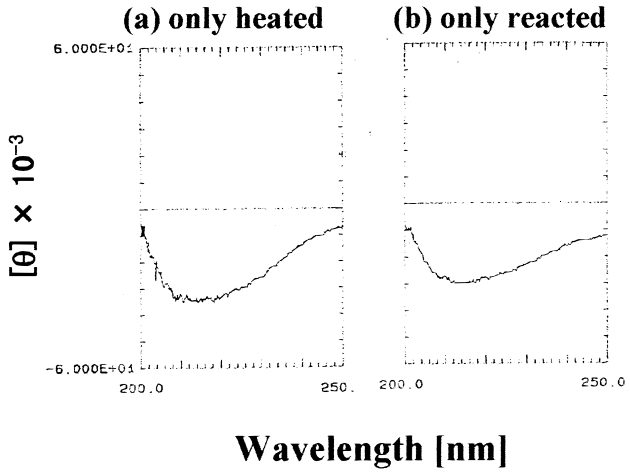

Figure 5. CD spectra of 7S fractions: (a) heated without MTG; (b) MTG reacted without heating the sample.

(water) to approach the protein sample easily. This means an increase in plasticizer around the protein molecule, and it is possible that the lowering of the T_g by the MTG treatment is closely related to the disintegration of the secondary structure. Actually, in the MTG-treated soy protein, the increase of immobilized water was observed by ^1H NMR measurement (Mizuno et al., 2000), which suggested the increase of plasticizer around the protein molecule.

The MTG treatment in this study includes two major processes, the reaction catalyzed by MTG and the inactivation of the enzyme by heating. The structural change due to the MTG treatment observed by the NMR and CD measurements was induced in a series of these processes. Next, the structural change when these processes were independently done was observed in the CD measurement. In Figure 5a, the CD spectrum of the 7S fraction heated without the reaction-catalyzed MTG is shown. No significant difference in the shape of the CD spectra between the MTG-nontreated sample (Figure 4a) and the heated sample was perceived. In addition, there was also no significant difference between the MTG-nontreated sample and the MTG reacted without heating (Figure 5b). From these results, we can see that the structural change due to the MTG treatment was not caused by the individual processes, the reaction catalyzed by MTG and the inactivation of the enzyme by heating, but by a series of these processes, the reaction catalyzed by MTG and the inactivation of the enzyme by heating after the reaction. In the case of soy protein, the structural change in the secondary structure accompanying the MTG treatment appeared to dominate the T_g. To generalize the relation between T_g and the secondary structure of proteins, a comparison with the case of another protein was done next.

Comparison with the Case of the T_g of Casein. In a previous paper (Mizuno et al., 1999), we reported that the T_g of casein which included more than 85% α_s-casein was elevated by the MTG treatment (Figure 6). In that paper, we concluded that the T_g of casein was elevated by the immobilization of the protein by inter- and intramolecular cross-linking. This result is in

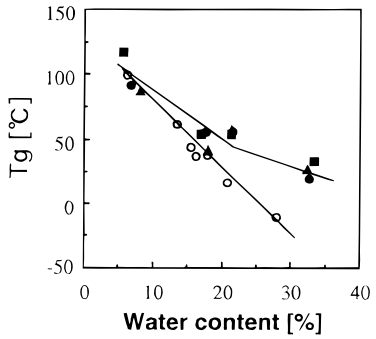

Figure 6. Dependency of the T_g of α-casein on the water content: (●) MTG-treated (high MW); (■) MTG-treated (intermediate MW); (▲) MTG-treated (low MW); (○) MTG-nontreated.

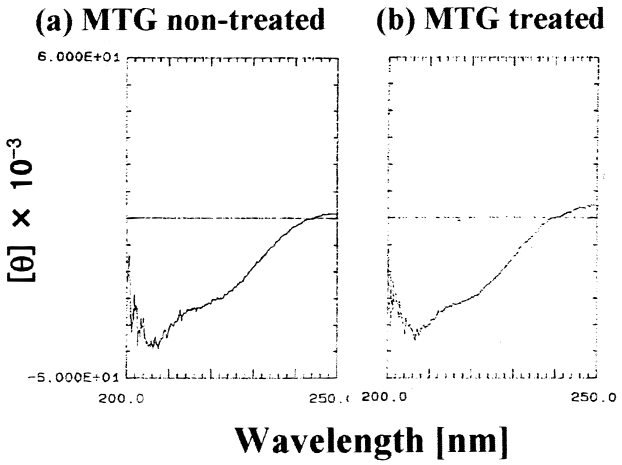

Figure 7. CD spectra of α-casein samples: (a) MTG-nontreated sample; (b) MTG-treated sample.

conflict with the result in this study, in which the T_g of soy protein was lowered by the MTG treatment. A consideration of the origin of the conflict could be useful for the understanding of the T_g of proteins in general. Thus, we studied the change in the secondary structure of casein by the MTG treatment and considered the effect of the MTG treatment on the T_g of both proteins.

The CD spectra of casein samples, MTG-nontreated (Figure 7a) and MTG-treated (Figure 7b), are shown. No difference in the shape of the spectra was observed, and the shape was similar to the case of the MTG-treated soy protein sample (Figure 4b), which was considered to be rich in random structure because of the disintegration of the β-structure. This means that casein was originally rich in random structure and that the change in secondary structure caused by the MTG treatment was slight, compared with soy protein. It is recognized that α_s-casein contains many proline residues which serve to restrict the formation of a secondary structure (Brunner, 1977). Our CD results agreed with the information on the secondary structure of casein. In the case of soy protein, we suggested that water molecules could approach the protein sample as a result of the disintegration of the β-structure accompanying the MTG treatment. On the other hand, in the case of casein, which changed little in secondary structure due to the MTG treatment, this kind of accessibility of water molecules as a result of the change in secondary structure did not seem to occur. Therefore, the immobilization of the protein by inter- and intramolecular cross-linking by the MTG treatment might be dominant in elevating the T_g of casein. From these results, it was suggested that the original secondary structure was important as a dominant factor in controlling the T_g of casein and soy protein. It is important for an understanding of glass transition of general proteins to carry out a study on the T_g of other proteins focusing on a change in the secondary structure.

Generally, the T_g of polymers is said to be elevated by cross-linking, and for example, Brinke et al. (1983) reported that the T_g of styrene was elevated due to cross-linking of covalent bonds. This is believed to be due to immobilization of the polymer network by the generation of cross-linking. However, in some cases, it is considered that generating cross-linking of covalent bonds is not necessarily effective for elevating the T_g of polymers, when the cross-linking is carried out in large excess (Furukawa, 1994). Moreover, Elliot (1990) pointed out that the mobility of some glass materials was influenced by the weaker secondary "back-bonds" binding structural units together, as well as the primary covalent bonds. In the case of biopolymers, noncovalent bonds, such as hydrogen bonding and hydrophobic interaction, which play an important role in stabilizing the structure, may be the back-bonds which dominate their T_g. Actually, Mizuno et al. (1998) suggested that the T_g of starch was closely related to hydrogen bonding, showing that the T_g of starch was elevated with increasing crystallinity. In this study, the T_g of soy protein was shown to be related to the state of the secondary structure, especially the β-structure, which was stabilized by hydrogen bondings. These results show the strong possibility that the T_g of biopolymers in general can be determined by the state of the noncovalent bonds, and in future work, the relationship between the T_g and noncovalent bonds should be focused on for controlling the glass transition of biopolymers. In addition, cross-linking of soy protein is also generated by other enzymatic methods than usage of transglutaminase, e.g., peroxidase (Stuchell and Krochta, 1994), or chemical methods (Park and Hettiarachchy, 1999; Hwang and Damodaran, 1996). In future work, it is necessary for a better understanding of the relationship between cross-linking and the T_g of soy protein to study the effect of those types of cross-linking on the T_g.

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Received for review October 20, 1999. Revised manuscript received April 20, 2000. Accepted May 4, 2000.

JF991151S