Conformational Changes of α_s -Casein by Heating

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Conformational changes of α_s -casein by heating were investigated by measuring ultraviolet difference spectra. The ultraviolet difference spectra at elevated temperature against 5.5°C were measured in various ionic strengths and pHs. Thermal effects of the difference spectra were cancelled by comparing with the spectra of model compounds such as lysozyme and ribonuclease, and the blue shift of α_s -casein spectra was observed at above 30°C in these all experimental conditions. This shift was considered to mean unfolding of the α_s -casein molecule. The aggregation of α_s -casein was observed above ionic strength of 0.4 by heating. These heat-induced changes were reversible until the aggregation was observed.

Pasteurizing, sterilizing and evaporating processes are essential for milk processing and storage. These heat processes are considered to cause soft curd and cooked flavor problems, forewarming effect and *etc.*¹⁾ There are a number of investigations^{2,3)} on the factors influencing heat stability of milk. But, the study on conformational changes of α_s -casein by heating has not been carried out enough. It is owing to the following reasons. K-Casein and β -lactoglobulin contain the residues involving -SH and S-S, but α_s -casein does not contain these residues and not aggregate even if it is heated at 100°C. Since α_s -casein contained little α -helix and β -structure but contained a random structure,⁴⁾ it is difficult to detect a conformational change of α_s -casein. However, Kresheck *et al.*⁵⁾ reported that marked aggregation of α_s -casein was observed by heating at 70°C. Leslie *et al.*⁶⁾ recently showed that the increase of temperature or the addition of urea lead to expose the non-polar residues of α_s -casein by means of the NMR measurement.

In this paper, the conformational changes of α_s -casein by heating are reported as having been investigated by using ultraviolet difference spectra measurements which can detect a small conformational change.

MATERIALS AND METHODS

1. *α_s -Casein.* α_s -Casein was prepared from Holstein cows' milk by the method of Zittle and Custer,⁷⁾ and the removal of impurities by ethanol treatment was repeated twice. The solution of α_s -casein was filtered through⁸⁾ 0.45 μ Milipore filter, dialyzed against 30 mM EDTA solution (pH 7.0) and then against deionized water at 4°C, and followed lyophilization. α_s -Casein prepared was homogeneous in the disc gel electrophoresis. α_s -Casein solution used for measurements was prepared by mixing about 1% α_s -casein stock solution, 0.2 M phosphate buffer of pH 6.9 and 1 M NaCl solution: being adjusted to 0.13 or 0.4% α_s -casein in 0.02 M phosphate buffer of pH 6.9 and ionic strength 0.094 (standard buffer). These solutions were used for measurements after being allowed to stand for 1 hr, and the concentration of α_s -casein was determined spectrophotometrically: α_s -casein $E_{1\%}^{1\text{cm}} = 10.0$ at 280 nm.⁸⁾

2. *Ribonuclease, lysozyme and other chemicals.* Six times crystallized lysozyme from hen egg white (Seikagaku Kogyo Co.) and five times crystallized ribonuclease from bovine pancreas (Sigma Chemical Co.) were used without further purification. The concentration of these protein samples was determined spectrophotometrically: lysozyme $E_{1\%}^{1\text{cm}} = 26.9$ at 280 nm⁹⁾ and ribonuclease $\epsilon = 9800$ at 277.5 nm¹⁰⁾ (13,683).¹¹⁾

Guanidine hydrochloride was recrystallized from methanol, and urea from 70% aqueous ethanol. Other chemicals were of guaranteed reagent grade and used without further purification.

3. *Ultraviolet difference spectra.* Ultraviolet (UV) difference spectra were measured with a Jasco spectropolarimeter model J-10 which was improved to meas-

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ure at full scale 0.1 in optical density, and the full scale of the instrument was set to 0.2 optical density. A reference cell and a sample cell were maintained at different temperature by employing separate cell holders. α_s -casein solution was prepared to 0.13% in concentration and the solution of lysozyme or ribonuclease was prepared to $E_{1\text{cm}} = 1.5 \pm 0.2$ at 280 nm in 0.1 M NaCl. ϵ of α_s -casein was calculated from MW 27,000.¹²⁾

4. *Optical rotation and circular dichroism measurements.* Optical rotation (OR) measurements were made at 365 nm by using a Perkin Elmer-141-polarimeter with a 10 cm standard cell. The concentration of α_s -casein was 0.4% and the reduced mean residue rotations, $[\text{m}]'$, were calculated from the mean residue molecular weight 122.¹³⁾

Circular dichroism (CD) measurements were carried out by using a Jasco spectropolarimeter model J-10 with a cell holder which was maintained at definite temperature.

5. *Maintenance of temperature.* The temperature was held within $\pm 0.1^\circ\text{C}$ by connecting cell holders with a Haake KT62 (below 10°C) and a Colora ultrathermostat NB -32,741 (above 10°C). The temperature in each cell was measured by a Takara thermistor.

RESULTS

1. Measurements of UV difference spectra

UV difference spectra of α_s -casein. UV difference spectra as shown in Fig. 1 indicate the effects of temperature on α_s -casein in the standard buffer. The temperature of the reference cell was maintained at 5.5°C , and

FIG. 1. Ultraviolet Difference Spectra of α_s -Casein as a Function of Temperature.

The reference cell was at 5.5°C and the Sample's temperature gradually raised. Conditions: protein concentration, 0.12% in the standard buffer (0.02 M phosphate buffer pH 7.0, ionic strength 0.094).

that of the sample cell was gradually raised. The difference spectra of α_s -casein did not change with time at each temperature measured. Therefore, difference spectra were measured within 5 to 30 min after the cell had reached a definite temperature. These spectra had an isobestic point at 263 nm, and as the temperature of the sample cell was raised, the $\Delta\epsilon$ values became progressively greater in all wave lengths measured except for the isobestic point and 290 nm region. The $\Delta\epsilon$ values at 290 nm became smaller at the higher temperature than 30°C .

Figure 2 shows the effects of temperature on the completely unfolded α_s -casein in the standard buffer containing 5 M guanidine hydrochloride. These difference spectra have two isobestic points at 255 and 284 nm, and all $\Delta\epsilon$ values become greater in proportion to temperature. The effects of temperature on the spectra of acetyl tyrosine ethyl-ester (Fig. 3) and acetyl tryptophan ethylester are similar to these on the spectra of the completely unfolded α_s -casein, and both spectra have 2 or 3 isobestic points. It is considered that these phenomena are caused by the so-called thermal effects, not being accompanied by the conformational changes of solute. The effects can be shown by that the $\Delta\epsilon$ values become

FIG. 2. Temperature Difference Spectra of Ultraviolet Absorption of α_s -Casein in the Standard Buffer Containing 5 M Guanidine Hydrochloride.

The temperature of the reference cell was maintained at 5.5°C and the concentration of protein was 0.136%.

FIG. 3. Temperature Difference Spectra of Acetyl Tyrosin Ethylester in the Standard Buffer.

The temperature of the reference cell was maintained at 5.5°C and the concentration of acetyl tyrosin ethylester was 0.025%.

greater in proportion to temperature and that the spectra have isobestic points. Considering this fact, it seemed that the result of Fig. 2 shows also thermal effects and that Fig. 1 shows the spectra which were caused by both conformational change and the thermal effects. As to α_s -casein and the completely unfolded α_s -casein, the observed effects of temperature were perfectly reversible.

A comparison of α_s -casein with lysozyme and ribonuclease. The ribonuclease was used as a model compound which undergoes conformational change by heating, and the lysozyme as one which does not undergo conformational change by heating.¹⁶⁾ The difference spectra of these model compounds were measured with the same procedure as α_s -casein (Figs. 1 and 2). The temperature of the sample cell was gradually raised. Figure 4 shows the effect of temperature on lysozyme, and Fig. 5 shows that on ribonuclease. The spectra of lysozyme have two isobestic points and the $\Delta\epsilon$ values become greater in proportion to temperature: the effects may be caused by the thermal effect only. On the spectra of ribonuclease the $\Delta\epsilon$ values become greater in

FIG. 4. Temperature Difference Spectra of Lysozyme in 0.1 M NaCl Solution of pH 6.0.

The temperature of the reference cell was maintained at 5.5°C and the concentration of the protein was 0.043%.

FIG. 5. Temperature Difference Spectra of Ribonuclease in 0.1 M NaCl Solution of pH 4.6.

The temperature of the reference cell was maintained at 5.5°C and the concentration of the protein was 0.19%.

FIG. 6. Temperature Difference Spectra of Lysozyme in 0.1 M NaCl Solution of pH 5.2 Containing 5 M Guanidine Hydrochloride.

The temperature of the reference cell was maintained at 5.5°C and the concentration of the protein was 0.043%.

FIG. 7. Temperature Difference Spectra of Ribonuclease in 0.1 M NaCl Solution of pH 4.6 Containing 5 M Guanidine Hydrochloride.

The temperature of the reference cell was maintained at 5.5°C and the concentration of the protein was 0.19%.

FIG. 8. Change of $\Delta\epsilon$ at 290 nm of α_s -Casein, Ribonuclease, and Lysozyme as a Function of Temperature.

The temperature of the reference cells was maintained at 5.5°C. ○: α_s -casein, protein concentration of 0.126%, standard buffer. ●: ribonuclease, protein concentration of 0.19%, 0.1 M NaCl solution of pH 4.6. □: lysozyme, protein concentration of 0.04%, 0.1 M NaCl solution of pH 6.0.

proportion to temperature up to 40°C, and then decrease rapidly above 50°C. This shows a blue shift of absorption spectra, to be caused by the heat-induced change of conformation above 50°C. The blue shift of absorption spectra is usually caused by

exposing chromophor residues out of molecule. Difference spectra of lysozyme and ribonuclease in 0.1 M NaCl solution containing 5 M guanidine hydrochloride are shown in Fig. 6 and Fig. 7. These spectra have isobestic points and the $\Delta\epsilon$ values become greater in proportion to temperature, showing thermal effects only. Figure 8 shows $\Delta\epsilon$ values at 290 nm of lysozyme, ribonuclease, and α_s -casein as a function of temperature. These results show clearly that lysozyme undergoes the thermal effects only, and that ribonuclease gives rise rapidly to the blue shift by the conformational change above 50°C. Therefore, it is considered that comparing α_s -casein with lysozyme and ribonuclease, the blue shift of α_s -casein spectra occurred by heating above 30°C. And the blue shift may have been caused by the dissociation of α_s -casein and/or the unfolding of a molecule of α_s -casein, for the exposing of chromophor residues out of molecule can be caused by these two reasons.

The influences of ionic strength. The temperature difference spectra of α_s -casein were measured at various ionic strengths. Figure 9 shows the temperature difference spectra of α_s -casein in the case of ionic strength of 0.84. These spectra were similar to those shown in Fig. 1. The rise of 320 nm regions, however, occurred at 50°C and turbidity was observed. The turbidity was increased with time at the temperature where the turbidity arised. The

FIG. 9. Temperature Difference Spectra of α_s -Casein at Ionic Strength of 0.84 in 0.02 M Phosphate Buffer of pH 7.0.

The temperature of the reference cell was maintained at 5.5°C and the concentration of the protein was 0.136%.

FIG. 10. Change of $\Delta\epsilon$ at 290 nm of α_s -Casein in Various Ionic Strengths as a Function of Temperature. The temperature of the reference cells was maintained at 5.5°C and the concentration of the protein was 0.136%. Signs of $\circ$, $\triangle$, $\square$ and $\bullet$ show ionic strength of 0.094, 0.244, 0.444 and 0.844 respectively, in 0.02 M phosphate buffer of pH 7.0. Dotted lines show the region at which the turbidity was observed.

rate of formation of aggregate was increased with an increase in temperature. The turbidity formed once, was not extinguished by decrease in temperature. The extent of the increase of $\Delta\epsilon$ values at 290 nm reduced at 40°C in spite of forming the aggregate. Then $\Delta\epsilon$ of UV difference spectra at 290 nm in the buffer of various concentrations of NaCl solution were plotted against temperature in Fig. 10. The decrease of $\Delta\epsilon$ values occurred between 30°C and 40°C in all ionic strengths prepared. This shows that the blue shift occurred in all ionic strengths. The higher in the ionic strength, the greater in the $\Delta\epsilon$ values against temperature. The increase of $\Delta\epsilon$ values probably means the red shift of absorption spectra, which is caused by folding of chromophor residues inside molecule.

Then the UV difference spectra of α_s -casein in buffers of various ionic strengths against α_s -casein in the standard buffer were measured, and the temperature of both cells was raised simultaneously. The UV difference spectra in the case of ionic strength of 0.84 are shown in Fig. 11. The ionic strength higher than the one in the standard buffer caused the red shift of α_s -casein spectra. And the red shift was greater with increase in temperature, and the 320 nm region of the spectra came to rise

FIG. 11. Effect of Temperature on the Difference Spectra of α_s -Casein at Ionic Strength of 0.84 against Ionic Strength of 0.094.

The temperature of the both cells was raised simultaneously. The concentration of the protein was 0.136%. The curve ② at 60°C was obtained 10 min after the curve ①.

FIG. 12. Effect of Temperature on $\Delta\epsilon$ at 285.5 nm of α_s -Casein in Various Ionic Strengths against the Standard Buffer.

The concentration of the protein was 0.136%. The ionic strength of the standard buffer was 0.094, and those of sign $\bullet$, $\circ$, $\blacksquare$ and $\square$ were 0.24, 0.44, 0.84 and 1.24, respectively.

from 50°C; turbidity was formed. The $\Delta\epsilon$ values at 285.5 nm of α_s -casein in buffers of various ionic strengths against those in the standard buffer were plotted against temperature as shown in Fig. 12. As the ionic strength became higher, the $\Delta\epsilon$ values were greater; the red shift occurred, and the red shift was greater with increase in temperature except below ionic strength 0.24. The dotted lines show the formation of aggregate. As the red shift became greater by increasing ionic

strength, the aggregate began to form at lower temperature. Since the red shift of absorption spectra is usually caused by the association of molecules, these results would mean that α_s -casein associates one another with increase in ionic strength and the association is accelerated with temperature, and finally an aggregate is formed.

The influences of pH and α_s -casein concentration. The following experiments were carried out to make clear the influences of pH and α_s -casein concentration. The difference spectra were measured of α_s -casein solution adjusted to pH 6.2, 6.9 and 7.8. The reference cell was maintained at 5.5°C, and the temperature of sample cell was raised. The pH change from 3°C to 70°C in each solution

FIG. 13. Effect of pH on $\Delta\epsilon$ at 290 nm of α_s -Casein as a Function of Temperature.

The reference cell was maintained at 5.5°C and the concentration of the protein was 0.136%. The solution were at pH 6.2 (●), pH 6.9 (○), and pH 7.8 (□).

FIG. 14. Effect of the Concentration of α_s -Casein on $\Delta\epsilon$ at 290 nm as a Function of Temperature.

The reference cell was maintained at 5.5°C and the concentrations of the protein were 0.12% (○) and 0.27% (□).

was only 0.1 pH. Results are shown in Fig. 13. The $\Delta\epsilon$ values at pH 6.2 were similar to those at pH 6.9, but the heat-induced change was shifted to higher temperature at pH 7.8.

The temperature difference spectra were measured in the concentrations of 0.12 and 0.27%. The values at 290 nm are shown in Fig. 14, indicating similar $\Delta\epsilon$ values in both concentrations.

2. Measurements of circular dichroism and optical rotation

In the standard buffer, the CD spectra of α_s -casein at 5, 30 and 70°C showed similar

FIG. 15. Effect of Temperature on the Circular Dichroism of α_s -Casein in the Standard Buffer.

The temperatures of the solution were 5°C (○), 25°C (△), and 67°C (□); and the concentration of the protein was 0.297%.

FIG. 16. Effect of Temperature on the Optical Rotation of α_s -Casein.

○: 0.56% in the standard buffer, ●: 0.33% in the standard buffer containing 5 M guanidine hydrochloride.

patterns in the 210~250 nm region as shown in Fig. 15. Then, temperature changes of the reduced mean residue rotation of α_s -casein at 365 nm were measured and shown in Fig. 16. In the standard buffer the monotonous decrease in levorotation was observed from 5°C to 45°C, and depressed at 45°C. In the presence of 5 M guanidine hydrochloride the monotonous decrease in levorotation was observed from 5°C to 80°C. As to the decrease in levorotation accompanying the increase of temperature, Aune *et al.*¹⁷⁾ explained that these properties depend on environmental factors according to the rule of Kauzman and Eyring,¹⁸⁾ and levorotation is increased when the structure is disrupted. In Fig. 16, therefore, it is considered that the prevention of decrease in the levorotation of α_s -casein resulted from the compensation of thermal effects and the disruption of structure, and that the heat-induced change of α_s -casein occurred at 45°C.

DISCUSSION

1. On thermal effects

When one attempts to investigate heat-induced change in the conformation of protein, the following effects will contribute to an absorption spectral change with temperature:¹⁵⁾ (1) change in the real optical path length due to thermal expansion of the cells, (2) change of solute concentration due to thermal expansion of the solvent, (3) change of the degree of ionization of certain groups, (4) change of the intrinsic solvent effects, and (5) change of energy levels of electron of solute. Effect (1) will probably be negligible over usual temperature ranges. In effect (2) the concentration of α_s -casein is decreased by about 2% from 5°C to 70°C, and this amount affects little changes of $\Delta\epsilon_{290}$ because ϵ_{290} of α_s -casein is 2,070. Effect (3) was hardly recognized in the spectra of lysozyme, because the $\Delta\epsilon$ values of the spectra became greater in proportion to temperature as well as those of acetyl tyrosine ethylester and acetyl tryptophan ethylester which have the ionizing groups masked by

acetyl and ethyl residues. Effect (3) was not recognized in α_s -casein either, for the similar shifts of spectra were obtained in various ionic strengths. Effect (4) is appreciable. Increasing the temperature of water decreases its refractive index slightly ($n_D^{20}=1.333$; $n_D^{100}=1.318$) and its dielectric constant substantially ($D^{20}=80$; $D^{100}=48$). The shift of spectra by the solvent effects follows the equation of McRae,¹⁹⁾ and therefore, a change of refractive index and dielectric constant leads to the shift of spectra. By using model compounds in the same solvent as a sample solution, the conformational changes of α_s -casein can be known by deducting this effect. Effect (5) is as follows: the temperature tends to lower the peak of spectra and to shallow the trough of spectra.²⁰⁾ Comparing with the spectra of model compounds, this effect can be deducted from those of sample. Thus, the heat-induced net change of conformation can be measured.

There are a few reports^{21,22)} about the heat-induced changes of conformation of casein by UV measurement, but they have no regard for thermal effects. Yoshida²²⁾ reported that the UV difference spectra of whole-casein showed red shift by heating. It is assumed that the blue shift of spectra might have been overlooked since thermal effects were not considered in his work, and that he would take thermal effects for the red shift.

2. Effects of ionic strength

The UV difference spectra at elevated temperature against 5.5°C were measured in various ionic strengths, and the blue shift of α_s -casein spectra was observed in all ionic strengths prepared. The blue shift is caused by exposing of chromophor residues out of molecule. Though the exposing of chromophor residues is caused by either the dissociation or the unfolding of a molecule, it can not be known by which reason the blue shift of α_s -casein will be caused. Since the extent of association of α_s -casein alters by ionic strength,²³⁾ the measurements of UV difference spectra in various ionic strengths would mean the measurements on the various

extents of associated α_s -casein. Furthermore, at ionic strength above 0.4 α_s -casein exhibited a red shift and the aggregate was formed by heating. Thus, in spite of the occurrence of association and finally aggregation of α_s -casein by heating, the blue shift was observed above 30°C. Therefore, the blue shift of α_s -casein spectra was considered to be caused by the unfolding of α_s -casein.

It is known that α_s -casein associates one another with increasing in ionic strength and form the turbidity finally.²⁴⁾ It was found, however, that α_s -casein became to form aggregate with increasing in temperature at the ionic strength of above 0.4, and that the turbidity was not observed at ionic strength of zero to 0.2 even by the increasing in temperature. These phenomena are considered to be caused by that the conformation of α_s -casein is unfolded by heating and the hydrophobic regions are exposed. And the reason why the aggregate is not formed at low ionic strength (0~0.2) may be the strong repulsion among charges of molecules. The formation of aggregate at ionic strength above 0.4 will occur by weakening the repulsion by enough counter ions in solvent. It can be also understood from above explanation that the higher in ionic strength, the lower in the temperature where the aggregation is formed.

3. The aggregation process of α_s -casein by heating

From these results, it is speculated that α_s -casein is subjected to the following conformational changes by heating. The chromophore residues of α_s -casein molecules which have been buried in the internal hold are exposed to the solvent above 30°C, and by further heating α_s -casein alters to the random structure. Accompanying with the alteration of the structure, the hydrophobic residues are exposed to the solvent and α_s -casein associates and then aggregates by heating at the ionic strength where the aggregate is not formed at room temperature. These conformational changes and association of α_s -casein are reversible until the aggregate is formed.

These process are shown by a diagram as follows:

(1) This process is reversible. α_s -Casein is unfolded by heating. (2) This process is irreversible. Unfolded α_s -casein is mutually aggregated.

The degrees of the association or aggregation of α_s -casein under these conditions are not clear, and this is a problem to be investigated further.

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