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New small endothermic peaks with hysteresis commonly observed in the differential scanning calorimetric study of biopolymer–water systems

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Abstract

New small endothermic transitions with remarkable hysteresis were found in many biopolymer–water systems including proteins and polysaccharides by using a high sensitivity differential scanning microcalorimeter. The transition temperatures are usually around 40°C for fresh samples and they shift to the temperatures from 20 to 60°C according to the preservation at various temperatures. The transition enthalpies per gram of water (ΔH_w) in the samples lie between 2 and 20 J/g water, and are not so diverse from species to species for the fresh biopolymer–water systems. The ΔH_w decreases rapidly with the increase of water content of the samples. The remarkable narrowing of the band width of ^{1}H-NMR signals of lysozyme–water and starch–water systems were observed at the temperature ranges of the endothermic transitions. The band widths showed fine hysteresis corresponding to the hysteresis behavior observed in the DSC measurements. The endothermic transitions observed in the DSC measurements must be brought about by the release of the cooperative motion of water and the biological macromolecules.

Keywords: Biopolymer; Water; Endothermic transitions; DSC; ^{1}H-NMR

1. Introduction

More than 20 years ago, Jollès and Berthou [1] reported the crystalline polymorphism of lysozyme produced by merely changing the temperature of the super-saturated mother solution. In those days, we studied the partial specific volume and partial specific heat of lysozyme in the aqueous state, and observed small anomaly at the critical temperature corresponding to the crystalline polymorphism [2,3]. We supposed that the anomalies were ascribed to the change of the hydration of lysozyme produced by the conformation change of the surface of lysozyme molecule [4].

Recently we studied the anomalies by using a high performance differential scanning calorimeter (DSC) in the sensitivity and baseline stability, and observed small endothermic peaks with remarkable hysteresis at the temperature ranges of interest of the crystalline polymorphism [5]. The subsequent studies revealed that the endothermic anomalies were also observed even in the lyophilized powder of lysozyme.

In addition to the study of lysozyme, we have carried out DSC studies of various biological macro-

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molecules including enzymes and polysaccharides, and observed the same type of anomalies with remarkable hysteresis. In this report, we show the small endothermic peaks with remarkable hysteresis commonly observed in the DSC curves of the biopolymer–water systems.

2. Experimental

Dialyzed and lyophilized lysozyme powder after crystallization of six times was obtained from Seikagaku Kogyo. For comparison, three times recrystallized sample of lysozyme from Sigma Chemical was used. Both samples gave the same result in the DSC and NMR measurements. Globular proteins such as bovine albumin, myoglobin, α-chymotrypsin and RNAase were obtained from Sigma Chemical in forms of lyophilized powders. Starch samples from potatoes were obtained from Wako Chemicals and Sigma Chemical and also from some food industry companies. All of the samples gave the same results in DSC measurements under the same conditions of their thermal hysteresis. Hyaluronic acid from cockscomb was obtained from Sigma Chemical. Water contents of the samples were measured by drying the samples at 100°C for more than 5 h under vacuum of about 1 Pa.

We used a differential scanning microcalorimeter of heat flow type specially designed by Tokyo Riko that utilized the semiconductor thermopiles from Melcor. as the sensitive detectors for detecting the small endo- or exothermic heat flows. The sensitivity of the sensors were 13.4 mV/°C. The temperature of the furnace was controlled by Chino's temperature program controller of KP1000, which actualized a constant rate of increase of the furnace temperature with high accuracy. The sample cells were made of silver with screwed caps and the capacity were as large as 6 mm in diameter and 11 mm in height. The inside of the cells were sealed by using thin silicon rubber packing. In ordinary measurements, the sample of about 50 mg were packed in the sample cells, and the cell was sealed after pressing the sample powder. The heating rate was 1°C/min. The transition enthalpy of the sample was calibrated by the melting enthalpy of benzophenone ($T_m = 321.4$ K, $\Delta H = 17.9$ kJ/mol). The ^{1}H-NMR measurements were carried out using a JOEL-FX90Q-FTNMR spectrometer operating at 89.6 MHz with a standard technique of induction decay Fourier transform. The sample tubes were carefully sealed to prevent leakage of the water vapor from the tube.

3. Results and discussion

DSC curves of a concentrated solution of lysozyme at pH 4.65 and 0.1 M NaCl are shown in Fig. 1. These curves are the accurate traces of the original DSC curves with a reduced scale. A broad endothermic transition is observed at the temperature ranging from 30 to 50°C at the first DSC scanning, corresponding to the anomaly previously reported. The enthalpy of the transition is about 11 kJ/(mol of lysozyme). The transition becomes obscure at the second scanning as shown in the curve b.

The anomaly was more strikingly observed in the DSC curve of the paste-like sample which was prepared by dropping 0.5 M NaCl solution onto the same weight of lyophilized powder of lysozyme and mixing up (see Fig. 2). We see a small sharp endothermic peak in the temperature between 40 and 50°C in the first scanning curve. This curve clearly shows that the transition is independent of the sharp denaturational transition of lysozyme which begins at the higher temperature above 65°C. The enthalpy of the transition is about 24 kJ/(mol of lysozyme). However, no transition was observed at the second scanning as shown in the curve b in Fig. 2.

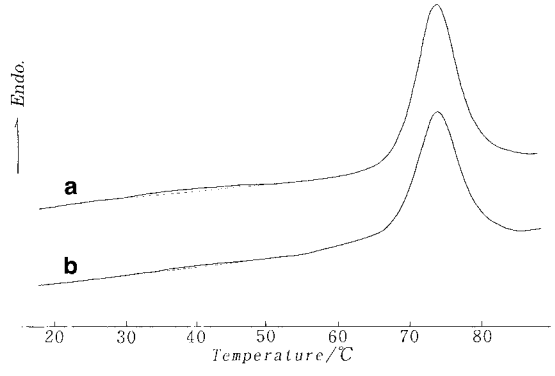

Fig. 1. DSC curves of concentrated lysozyme solution (16 wt.%, pH 4.65, 0.1 M NaCl): (a) first scanning, and (b) second scanning soon after the first scanning.

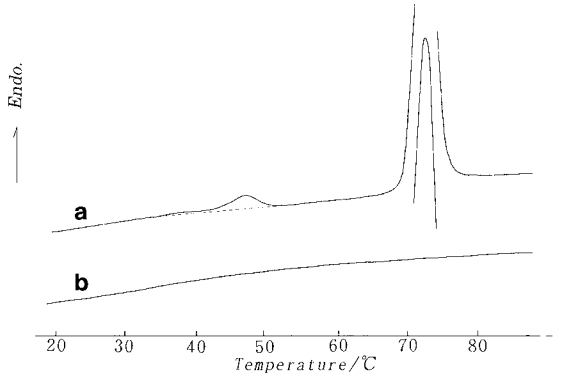

Fig. 2. DSC curves of lysozyme paste (lyophilized lysozyme/0.5 M NaCl=1/1 (w/w)): (a) first scanning, and (b) second scanning soon after the first scanning.

We observed a clear transition peak in the lyophilized lysozyme sample too, and the transition plays a remarkable hysteresis in the DSC measurements. An example of such hysteresis is shown in Fig. 3, following the sequences of DSC measurements. The measurements were carried out after packing lyophilized lysozyme sample (water content: 10.2%) in a sample cell. The result of the first run is shown in the curve a in Fig. 3, in which a relatively large and broad endothermic peak is observed. The transition enthalpy of 22 kJ/(mol of lysozyme) was estimated from the peak area. No peak appeared in the second run of DSC which starts immediately after cooling the sample rapidly from the final temperature of the first run. After the sample cell was kept at 20°C for about 20 h, however, the endothermic peak appeared again at the same temperature range, though the peak area decreased. The fourth and fifth runs were carried out after the sample cell was kept 30°C for 16 h and 5 days, respectively, and we observed the endothermic peaks at the temperatures higher than those of the first and second runs. The sample cell was kept at 20°C after the fifth run was over, then we observed that the endothermic temperature returned to the position of original sample (see the curve f). The highest peak temperature over 60°C was observed when the seventh run was carried out after the sample was kept at 40°C for 2 days. After the seventh DSC run was over, the sample cell was opened and the sample in the cell was dried at room temperature under vacuum and sealed again. DSC was carried out for the dried sample, and

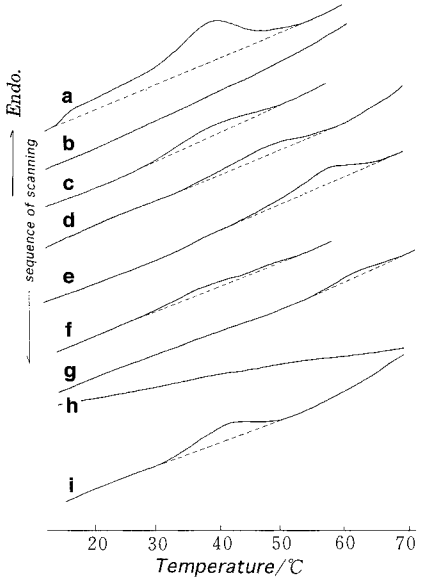

Fig. 3. DSC curves of lyophilized lysozyme (water content: 10.2 wt.%): (a) first scanning, (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 20°C for 20 h, (d) forth scanning after having preserved at 30°C for 16 h, (e) fifth scanning after having preserved at 30 h for 5 days, (f) sixth scanning after having preserved at 20°C for 18 h, (g) seventh scanning after having preserved at 40 h for 2 days, (h) eighth scanning after having dried and (i) ninth scanning after water regain (water content: 10.2 wt.%).

no endothermic peak was observed (the curve h). However, the endothermic peak appeared again when the sample regained water to the initial level of 10.2 wt.% (the curves i).

The anomalous small endothermic peaks as mentioned above were also observed in the DSC curves of many globular proteins. The DSC curves of the commercially available lyophilized powder of bovine albumin are shown in Fig. 4. The water content of the sample was 29.6 wt.%. A clear endothermic peak was observed at about 40°C at the first scanning. However, the endothermic anomaly scarcely appeared at the second scanning started immediately after the rapid cooling of the sample cell. The third scanning was taken after holding the sample cell at 20°C for a day after the second scanning finished. We see that the endothermic peak appeared again, though the peak was somewhat broad (the curve c). The third scanning was carried out up to the temperature of denaturation. After the sample cell was kept at 4°C for 3 days after

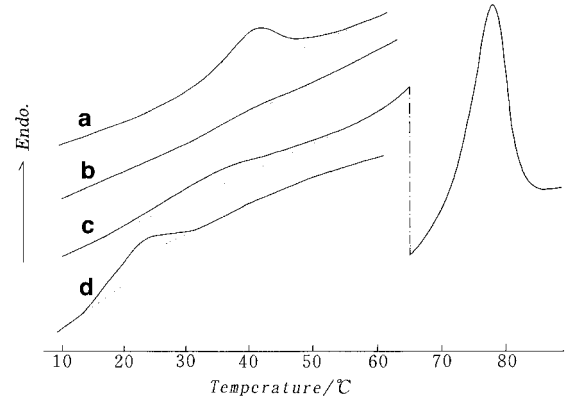

Fig. 4. DSC curves of lyophilized albumin (water content: 29.6 wt.%): (a) first scanning, and (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 20°C for a day and (d) fourth scanning after having preserved at 4°C for 3 days.

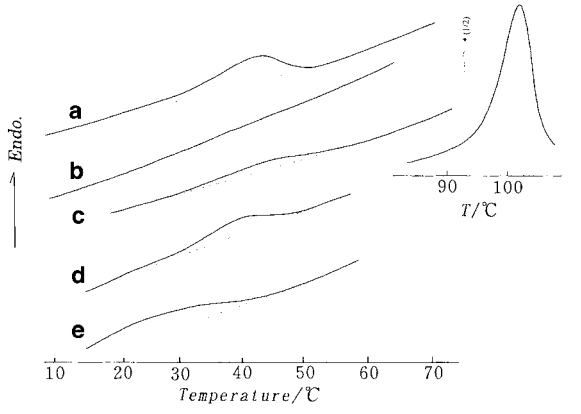

Fig. 6. DSC curves of lyophilized ribonuclease (water content: 11 wt.%): (a) first scanning, (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 20°C for a day, denaturation peak appearing at about 100°C (scale of the longitudinal axis is 1/2 of the left), (d) fourth scanning after having denatured at the preceding scanning and preserved at 20°C for a day, and (e) fifth scanning after having preserved at 4°C for 3 days.

the third scanning, the endothermic peak appeared at rather lower temperature of about 20°C.

Figs. 5 and 6 are the DSC curves of lyophilized powders of myoglobin and ribonuclease, whose water contents were 15 and 11 wt.%, respectively. The third scanning for ribonuclease was carried out over the denaturation temperature. The denaturation peak is shown at the left side of the curve c in Fig. 6. The fourth scanning was carried out after keeping the sample at 20°C for a day, and we observed an endothermic peak at about 40°C like that in the first scanning curve (see the curve d). Denaturation of the protein in the lyophilized powder is usually an irreversible process. Then, the recovery of the endothermic peak at about 40°C implies that the appearance of the anomalous peak is not an inherent characteristic to the native conformation of the protein.

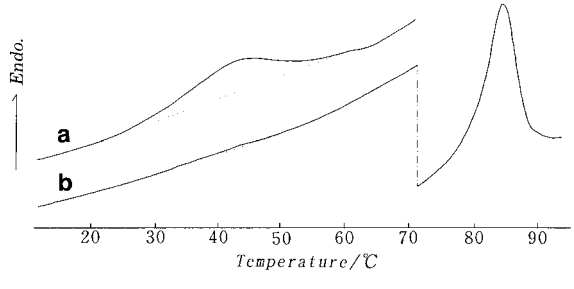

Fig. 5. DSC curves of lyophilized myoglobin (water content: 15 wt.%): (a) first scanning, and (b) second scanning soon after the first scanning, the denaturation peak appears at about 85°C.

It is noticed that the peak temperatures in the DSC curves of the original lyophilized samples appear nearly at the same temperature ranges of around 40°C, irrespective of the kind of proteins.

We estimated the enthalpies of the thermal transitions of these globular protein–water systems. The molar enthalpy per protein or per water was calculated by assuming that the same total heat was generated only by protein or by water, respectively. The results for some of the lyophilized powder samples at the first scanning are listed in Table 1, where T_w is the peak temperature of the small anomalous endothermic transition, ΔH_p the transition enthalpy per mole of proteins, and ΔH_w is the transition enthalpy per gram of water, respectively. These values of ΔH_p and ΔH_w are very small compared to the denaturation enthalpies of those proteins or melting enthalpy of ice, respectively, and they are not so diverse from species to species of the proteins.

The transition enthalpies of ΔH_p and ΔH_w change according to the water contents of the samples. Table 2 shows the water content dependence of the transition enthalpy of the lysozyme–water systems. The table shows that both of the transition enthalpies, ΔH_p and ΔH_w, take maximum values at the intermediate ranges

Table 1
Thermodynamic parameters of the small endothermic transitions of globular protein–water systems

	Water content (w/w, %)	T_s (°C)	ΔH_p (kJ/mol protein)	ΔH_w (kJ/g H_2O)
Lysozyme	10.2	40	20	12
Ribonuclease	11.1	42	11	8.9
Chymotripsin	13.5	46	22	5.5
Myoglobin	14.9	42	28	9

of water contents of 10–13 wt.%. It is noticed that ΔH_w decreases rapidly as the water content of the sample increases.

Next, we show the result of DSC studies of starches. The DSC curves of a starch sample with saturated water under various scanning conditions are shown in Fig. 7. We observed an endothermic peak at about 40°C at the first scanning. However, no peak appeared at the second scanning, same as in the case of globular proteins. After the second scanning, the sample was preserved at 4°C for 6 days. Then at the third scanning, an endothermic peak appeared at about 20°C as shown in the curve c. The fourth scanning after keeping the sample at 20°C for 20 h gave nearly the same endothermic peak as observed at the first scanning (the curve d). Preservation of the sample at −30°C for 3 days gave no endothermic peak as shown in the fifth curve (e).

The endothermic peak grows gradually by preservation of the sample. Such tendency is shown by the results of sixth and seventh scanning measurements (the curves f and g). The sample at the seventh scanning was preserved at 4°C more than six times longer than that at sixth scanning, then the seventh endothermic peak is much larger than that of sixth one. Keeping the sample at 20°C for 3 days returns DSC curve nearly to the original one, though the baseline is somewhat different (the curve h).

Table 2
Change of the enthalpies of the small endothermic transitions in lysozyme–water systems as a function of water content

Water content (w/w, %)	ΔH_p (kJ/mol protein)	ΔH_w (kJ/g H_2O)
7	9.3	8.7
10	20	12
13	25	11
20	7.3	2.0
Solution (16 wt.%)	11	–

The same tendencies were observed in the DSC curves of the starch samples with less content of water as shown in Fig. 8 (sample with 14.6 wt.% water) and Fig. 9 (7.8 wt.% water). The notable endothermic peaks appeared at the first scanning as shown at the top curve in both the figures. The recovering process becomes slower as the water contents of the sample decreases. For example, even the long preservation at 20°C over 8 days were not sufficient to recover the endothermic peak to the original one as shown in the curve c in Fig. 8.

The enthalpies of the endothermic transitions of starch–water systems are listed in Table 3. The transition enthalpies per gram of water in the starch–water systems are in the same order as those in globular protein–water systems listed in Table 1, though the

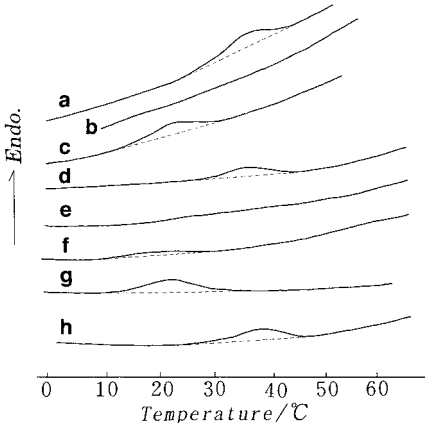

Fig. 7. DSC curves of starch (water content: 20.3 wt.%): (a) first scanning, (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 4°C for 6 days, (d) fourth scanning after having preserved at 20°C for 20 h, (e) fifth scanning after having preserved at −30°C for 3 days, (f) sixth scanning after having preserved at 4°C for 18 h, (g) seventh scanning after having preserved at 4°C for 5 days and (h) eighth scanning after having preserved at 20°C for 3 days.

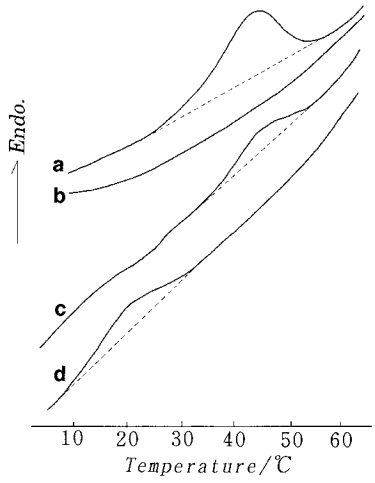

Fig. 8. DSC curves of starch (water content: 14.6 wt.%): (a) first scanning, (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 20°C for 8 days, and (d) fourth scanning after having preserved at 4°C for 3 days.

magnitudes in the starch–water system are about twice as large as those in protein–water systems for the samples with water contents of 10–15 wt.%. The transition enthalpy decreases drastically for the sample with saturated water as shown in Table 3.

We have studied various biological substances including functionally interesting polysaccharides like hyaluronic acid. This substance is widely distributed

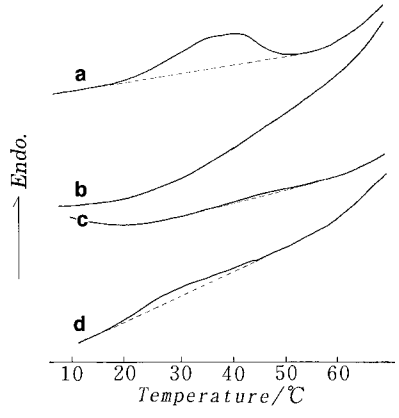

Fig. 9. DSC curves of starch (water content: 7.8 wt.%): (a) first scanning, (b) second scanning soon after the first scanning, (c) third scanning after having preserved at 20°C for 7 days, and (d) fourth scanning after having preserved at 4°C for 3 days.

Table 3
Enthalpies of the small endothermic transitions of starch–water systems

Water content (w/w, %)	ΔH_w (kJ/g H_2O)
20.3 (fully wet)	2.5
14.6 (original)	18.5
7.8 (partially dried)	20.0

among the connecting tissues of animals, forming gel-like structure or highly viscous liquid. We observed a notable endothermic peak with remarkable hysteresis as shown in Fig. 10. The characteristics of the thermal hysteresis are the same as in the globular proteins and starches. The interval over a week was not sufficient for the sample to return to the initial state at 20°C (see the curve f in Fig. 10).

Water in biological substances gives usually rather sharp proton NMR signals even at relatively low content of water, and we can detect the signals by using usual high resolution NMR spectrometer. We obtained the ^{1}H-NMR signals of starch–water and lyophilized powder lysozyme–water systems by changing temperature using JOEL 90 MHz NMR spectrometer. Fig. 11 shows the results for starch with water

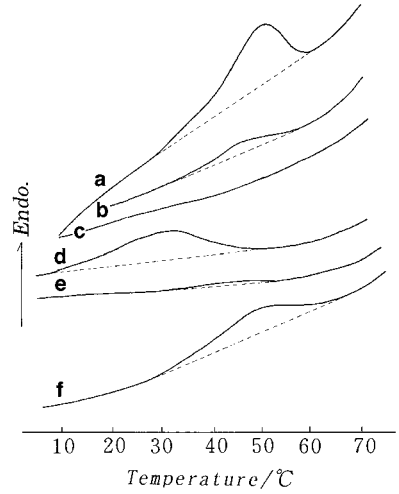

Fig. 10. DSC curves of hyaluronic acid (water content: 15.7 wt.%): (a) first scanning, (b) second scanning after preserved at 20°C for 3 days, (c) third scanning soon after the second scanning, (d) fourth scanning after preserved at 4°C for 4 days, (e) fifth scanning after preserved at 20°C for 28 h, and (f) sixth scanning after preserved at 20°C for 7 days.

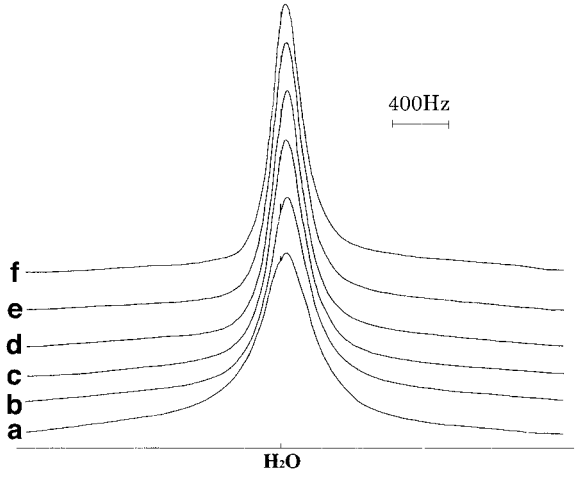

Fig. 11. Temperature dependence of the ^{1}H-NMR signals from starch (water content: 14 wt.%). 90 MHz FT-NMR spectroscopy; the spectral width is indicated in the figure ((a) 22°C, (b) 32°C, (c) 42°C, (d) 52°C, (e) 62°C, (f) 72°C).

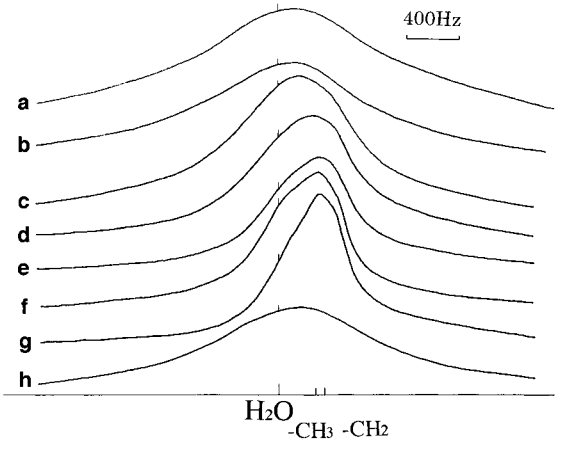

Fig. 13. Temperature dependence of the ^{1}H-NMR signals from lyophilized lysozyme (water content: 11 wt.%; main water is replaced by D_2O). 90 MHz FT-NMR spectroscopy; the spectral width is indicated in the figure ((a) 5°C, (b) 23°C, (c) 38°C, (d) 46°C, (e) 56°C, (f) 66°C, (g) 75°C, (h) 23°C).

content of 14 wt.%. We used double NMR sample tube for the NMR locking, and the positions of the curves in Fig. 11 are normalized by the residual proton in the external standard of D_2O. A remarkable narrowing of the bandwidth is observed in this figure. The band width is plotted as a function of temperature (see Fig. 12). The half width of the signal decreases rapidly as temperature rises, as shown in the left side curve of Fig. 12. The curve in the right side shows the recovering process of the band width. The time interval of 20 h is not enough for the bandwidth to return to the initial state.

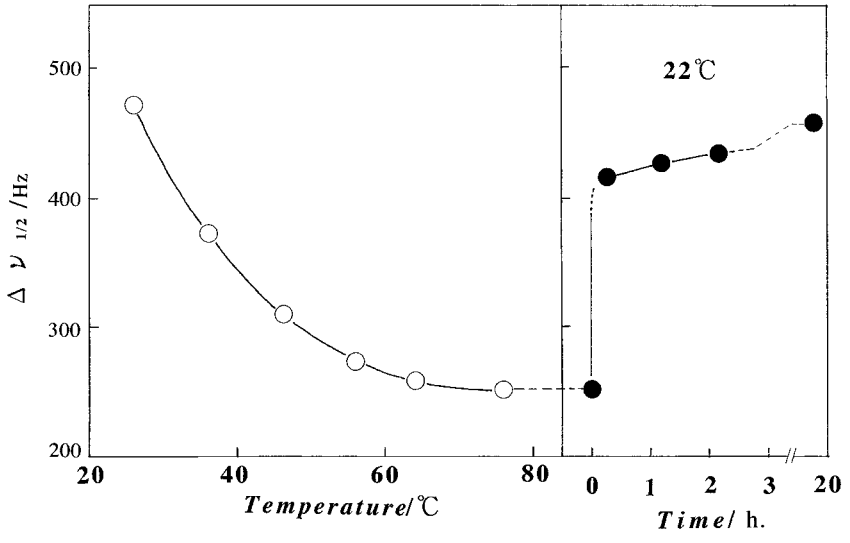

Fig. 12. Left side: band width narrowing of the ^{1}H-NMR signal from starch sample (water content: 14 wt.%) with increase of temperature. Right side: recovering process of the band width at 22°C.

Temperature dependence of the NMR signal was also taken about lyophilized lysozyme sample. In this measurement, H_2O in the original lyophilized powder had been replaced by D_2O. The results are shown in Fig. 13. In addition to narrowing of the signal originated in the residual proton of HDO or H_2O, the proton signals originated in peptide chains become separately observed with the increase of temperature. These facts implies that the endothermic transition in the DSC measurements is brought about by the release of the cooperative motion of water and the biological macromolecules.

Many important phase transitions have been reported concerning biopolymer–water systems, such as glass transitions, melting of bound water and free water, denaturation of proteins, and gelatinization of starches [6–8]. However, the small endothermic transitions with remarkable hysteresis observed in this study are not ascribed to any established transitions in the biopolymer–water systems mentioned above. Recently, Tanaka et al. carried out DSC studies on water restrained in hydroxyethylcellulose and hydrophobically modified hydroxyethylcellulose and recognized the small endothermic transitions at nearly same temperature region as mentioned in this study. They attributed the endothermic transitions to the deformation of the liquid crystalline state in those systems [9]. Although their temperature ranges and magnitudes of the endothermic peaks are similar with our systems, large differences exist between them in the hysteresis behaviors in the sequences of the DSC runs; no hysteresis behavior was observed in the sequential DSC runs reported by Tanaka et al., while remarkable hysteresis was observed in our systems.

Though the molecular basis of the transitions, especially the remarkable hysteresis phenomena of the transitions observed in this study are not yet clear, it must be a new type of phase transition in which weak interaction of biopolymers and water molecules may be essentially important. The study of the fine transition may bring a new field in understanding important functional properties of biopolymer–water systems.

4. Conclusion

The existence of the broad endothermic peak was confirmed in the lysozyme solution at 30–50°C corresponding to the occurrence of the crystalline polymorphism at the temperature range reported by Jollès and Berthou. The more distinct endothermic peaks were observed for lysozyme samples with less water content. These results imply that the polymorphism is produced by the structural change of the lysozyme–water system at the temperature range of the endothermic peaks. Subsequent study revealed that the small endothermic transitions were commonly observed at the same temperature range in many biopolymer–water systems. We suppose that the small endothermic peaks originate from the structural changes of the systems with some kinds of weak interactions (WIs) of the biopolymers and water, though the structure of the weak interaction is not yet clear.

The characteristics of the WI transitions are as follows: (1) The WI transitions usually appear at around 40°C for the fresh sample. (2) The WI transition temperature shifts according to the pre-heat treatments of the samples, lying generally between 20 and 60°C. (3) The enthalpies of the WI transitions are less than about 20 J/(g of water). High content of water in the sample lowers magnitude of the enthalpy markedly. (4) The remarkable hysteresis were observed in the WI transitions. The thermal hysteresis recovers partially with long relaxation time (several hours or days). (5) The band width of ^{1}H-NMR signal of the biopolymer–water system (10–15 wt.%) sets in narrowing remarkably at the temperature range corresponding to the WI transition temperatures in DSC measurements, which shows that the cooperative release of the molecular motion both of water and biopolymers is related to the WI transitions.

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