  Bull. Chem. Soc. Jpn., 53, 2451—2455 (1980)

The Effect of Sugars on the Thermal Denaturation of Lysozyme

Hatsuho UEDAIRA* and Hisashi UEDAIRA

Research Institute for Polymers and Textiles, 1-1-4, Yatabe-Higashi, Tsukuba, Ibaraki 305

(Received January 5, 1980)

Thermal denaturation of lysozyme (hen egg-white) in aqueous sugar solution was studied by DSC measurements at pH 5 and by CD measurements at pH 2.12. All of the sugars investigated show a stabilizing influence on the native conformation. The effect of sugars on the transition temperature of lysozyme almost linearly increased with the increase of the numbers of equatorial hydroxyl groups per molecule of sugars. The ellipticity of the solutions at 294 nm of lysozyme and glucose or arabinose was additive in the value of each component at pH 5. The effect of sugars in stabilizing the protein structure may be due to the intensification of the hydrophobic interactions inside the protein molecule.

There have been many investigations on the effects of alcohols and sugars on the stability of globular proteins to elucidate the nature of the interactions that stabilize the native conformation of proteins.

The effects of mono- and polyhydric alcohols on the reversible thermal denaturation of enzymes were investigated by Gelsma and Stuur,[1] Velicelebi and Sturtevant,[2] and Gekko, Morikawa and Noguchi.[3] The thermal stability of tropocollagen in these alcohols has also been measured by Harrap.[4]

A retarding effect of sucrose and glycerol on the rate of change of the optical rotation of ovalbumin in urea solution was found by Simpson and Kauzmann.[5] The effect of glucose on the denaturation of haemoglobin by urea was also tested and characterized by Simons and Naftalin.[6] The influence of polyhydric alcohols and sugars on the rate of subunit dissociation of tetrameric L-asparaginase in the presence of urea was investigated by Shifrin and Parrott.[7] In general, polyhydric alcohols have stabilizing influences on the native conformation of proteins.

No systematic investigations are available concerning the relation between the molecular conformation of sugars and their effect on the stability of globular proteins. In this work an attempt was made to explain the relation by studying the effect of various sugars on the thermal denaturation of lysozyme by differential scanning calorimetry. The interactions between some sugars and lysozyme were also studied by circular dichroism spectrometry.

Experimental

Materials. The hen egg-white lysozyme used in the present experiments was a six times recrystallized preparation obtained from Seikagaku Kogyo Co. The molecular weight of lysozyme was assumed to be 14307 and its average residue weight to be 110.9. Saccharides (G. R.) were recrystallized once or twice from water–ethanol solutions. Solutions were prepared with deionized and distilled water. The concentration of lysozyme was determined spectrophotometrically using an extinction of $E^{1\%}_{1cm}=26.9$ at 280 nm.[8] A Hitachi automatic recording spectrophotometer, model ESP-3T, or a Shimadzu double beam spectrophotometer UV 220 was used.

DSC Measurements. A Perkin-Elmer DSC Model II Calorimeter was used. 20 μl samples were packed in a stainless steel cell for high pressure use. Concentration of lysozyme was 6.188—7.986% at pH 5.0 (adjusted by adding HCl) and ionic strength was adjusted to be 0.1 by adding NaCl. It is known that aggregation of native lysozyme is favored at high pH values, above about 5.[9] On the other hand the stainless steel pan reacts with the acid solution at high temperature. Consequently, the thermal denaturation of lysozyme was measured at pH 5 with DSC.

CD Measurements. CD spectra were measured using a JASCO J-40A spectropolarimeter with a J-DPZ data processor. The recording of each spectrum was repeated four times, and the averaged spectra were obtained on the data processor. The temperature of the sample was controlled by using a Thermocircular TC-100 (Tokyo Riko Co.). The spectra were measured at protein concentrations of 0.1—0.5% in the spectral region above 250 nm and of 0.013% in the region between 200—250 nm.

Results and Discussion

Thermal Denaturation of Lysozyme without Sugars. The heating rate dependence of the denaturation temperature was observed by DSC. In Fig. 1, the foot and peak temperatures in the DSC curves for the denaturation of lysozyme at pH 5 are plotted against heating rate. The denaturation temperature increased linearly with heating rate; the extrapolated foot and peak temperatures were 64.5 and 75.4 °C, respectively. According to Velicelebi and Sturtevant,[2] T_d, the temperature at which the transition is half completed, and T_p, the temperature of maximal excess heat capacity, do not coincide, even for a two-

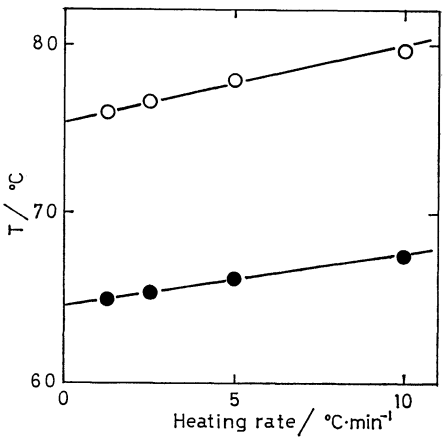

Fig. 1. Heating rate dependency of the foot (—●—) and peak (—○—) temperatures in the DSC curves for the thermal denaturation of lysozyme at pH 5 and $I=0.1$.

state process with no permanent change in heat capacity. In their work, T_d differs from T_p by 0.75—1.0 °C, but our results show the difference is within 0.1 °C. The calibration was made using the melting of In (156.7 °C) and water (0 °C). The foot and peak temperatures extrapolated to zero heating rate show 0.5 and 0.8 °C higher values for In (0.269 mg) and 1.2 and 2.3 °C higher values for water (0.7 mg), respectively. Consequently, the value of 75.4 °C of the extrapolated T_p for the denaturation of lysozyme may be a higher value still. However, due to the large difference in sample weight of lysozyme solution and of calibration substances, we use the T_p values extrapolated to zero heating rate as the denaturation temperature of lysozyme.

In Table 1, the denaturation temperature, T_d, obtained by various methods is shown. Our results are in good agreement with those in the literature.

Table 1. Comparison of the denaturation temperatures of lysozyme at pH 2.12 and 5.0 obtained by various methods

pH	2.12	5.0
T_d/°C	59.3[a]	78.5[a]
	46.8[b]	75.0[b]
	52.0[c]	
	55.8[d]	
	53.8[e]	75.4[f]

a) Calorimetry: Ref. 10. b) ORD: Ref. 11. c) UV: Ref. 1. d) Viscometry: Ref. 15. e) CD: Results of this study. f) DSC: Results of this study.

Considering the S/N ratio, we chose the heating rate of 5 degrees per minute under these conditions: The DSC sensitivity of 1 mcal/s, 20 μl of sample volume, at lysozyme concentration of 8% and ionic strength of 0.1. At this heating rate, as shown in Fig. 1, the meausured value for denaturation temperature is higher than the extrapolated value by 2.2 °C. The base line was not perfectly straight, so the evaluation of the peak area contains some uncertainty. The calorimetric enthalpy of denaturation, ΔH_{cal}, obtained from the area of DSC curve was not changed for heating rates from 1.25 to 20 degrees per minute, within experimental errors; the average value was 477±8 kJ/mol. The denaturation temperature was not changed at repeated heating about 24 h after the first run.

As most investigations are made at a more acidic condition, the denaturation was also measured by CD spectrum at pH 2.12 and ionic strength at 0.1. The value of molar ellipticity of the native lysozyme was in good agreement with the data of Shimaki *et al.*[12] and of Chen *et al.*[13]

Changes in the values of molar ellipticity, $[\theta]$, with temperature for lysozyme solution at pH 2.12 are shown in Fig. 2. The values of $[\theta]$ at 294 nm decrease with temperature and the transition temperature is 54 °C. The value of $[\theta]$ at 206 nm increases with temperature and the transition temperature is 54 °C too. The values of $[\theta]$ at 294 and 206 nm are caused by trp 62 and 108, and by the helix portion of lysozyme, respectively.[13,14] Figure 2 shows the cooperative nature of the thermal denaturation. The transition temperature T_d is taken as the temperature at which the transition is half complete. The obtained T_d values are compared with the literature values in Table 1. Table 1 shows that our results are within the values in the literature. The value of $[\theta]$ at 294 nm for the sample which was kept at 25 °C for more than 15 h after heating became the same value as that of the initial native one.

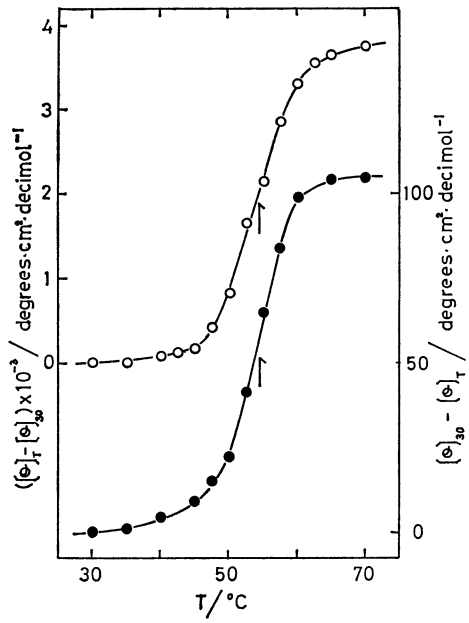

Fig. 2. Changes in the values of molar ellipticity for the lysozyme with temperature at pH 2.12 and I=0.1. —○—: At 206 nm, —●—: at 294 nm.

Plots of ln K against $1/T$, where K is the equilibrium constant for the denaturation obtained from the CD measurements, were almost linear. The values of van't Hoff enthalpy of denaturation, ΔH_{vh}, obtained from the plots were 280±8 kJ/mol from the absorption at 206 nm and 267±16 kJ/mol from the absorption at 294 nm, respectively. These values and the value of ΔH_{cal} are less than that which are obtained by Privalov[10] and by Velicelebi and Sturtevant,[2] but close to the values observed by O' Reilly and Karasz[15] and others.[16–19]

Thermal Denaturation of Lysozyme in the Presence of Sugars. DSC curves for the thermal denaturation of lysozyme (8.04%) in 1 mol kg^{-1} sugar solutions at pH 5 and I=0.1 at the heating rate of 5 degrees per minute are shown in Fig. 3. In every case studied, the thermal denaturation temperature is elevated by the addition of sugars. The values of the increasing temperature of the denaturation of lysozyme by 1 mol kg^{-1} sugar solution, ΔT_d (°C/mol), are plotted against the numbers of equatorial OH groups per molecule of sugars in Fig. 4. The numbers of equatorial OH groups are calculated from the NMR data of Angyal *et al.*[20] The values of ΔT_d are found to be closely related to the molecular conformation of sugars. From the slope in Fig. 4, T_d of lysozyme is raised about 0.8 °C per equatorial OH group. The values of ΔT_d of lysozyme by 1 mol dm^{-3} polyhydric alcohol

solutions are measured by Gelsma and Stuur,[1] and their results show that the T_d is raised by 0.8 °C per OH group, regardless of which polyhydric alcohol is added.

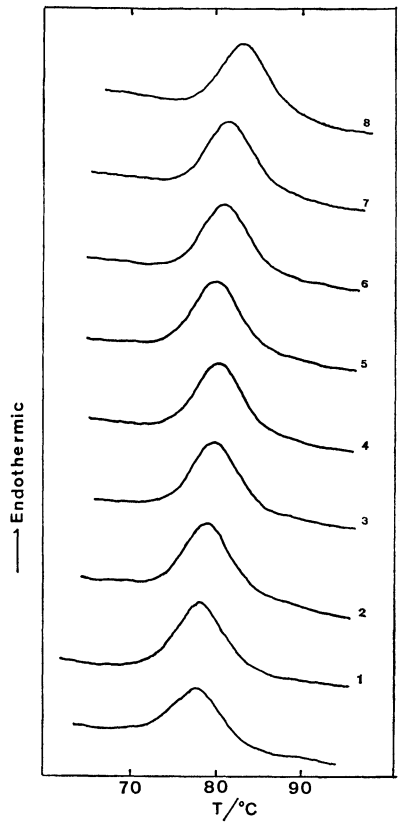

Fig. 3. DSC curves for the thermal denaturation of lysozyme in 1 mol kg^{-1} sugar solutions at pH 5 and I=0.1.
The lowest curve is for a solution without a sugar. Curves 1: with deoxyribose, 2: ribose, 3: lyxose, 4: arabinose, 5: xylose, 6: fructose, 7: glucose, and 8: sucrose.

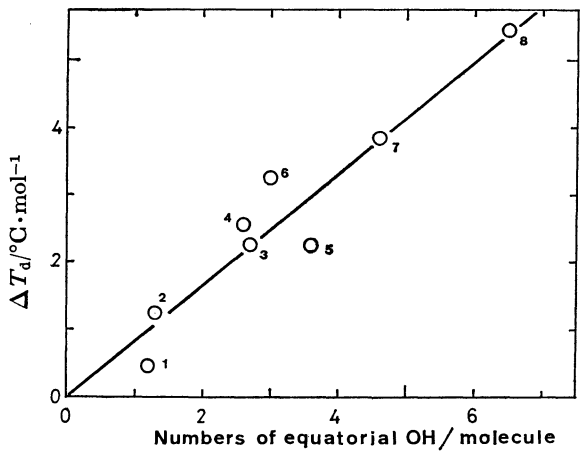

Fig. 4. The relation between the changes in the denaturation temperature of lysozyme in the presence of 1 mol kg^{-1} sugar solutions and the numbers of equatorial OH groups in a sugar molecule.
The numbers in the figure denote the same sugar as in Fig. 3.

The values of ΔH_{cal} of lysozyme in sugar solutions did not show a clear relation to the number of equatorial OH groups per added sugar molecule. In every case, sugars increased the value of ΔH_{cal} by about 10%.

Thermal denaturation for the lysozyme solution at pH 2.12 in 1 mol dm^{-1} glucose solution was also measured by the change in the values of molar ellipticity of lysozyme at 294 nm. Denaturation temperature and ΔH_{vh} were 58.5 °C and 310±16 kJ/mol, respectively. In this case, the molar ellipticity for renatured lysozyme solution was slightly lower than that for the native one. Comparing the data obtained for lysozyme with and without glucose, T_d is increased by about 5 degrees, and ΔH_{vh} increased by about 10%. The effect of the increase of T_d and ΔH_{vh} at pH 2.12 is nearly equal to the effect obtained at pH 5 by DSC measurements. The effect of polyhydric alcohols on the thermal denaturation of lysozyme is also found to be independent of pH.[1]

The Interaction between Lysozyme and Sugars. To elucidate the cause of the stabilizing effect of sugars we measured CD spectra at various concentrations of glucose and arabinose at pH 5, ionic strength 0.1, and 25 °C. CD spectra for lysozyme and arabinose solutions at various concentrations of arabinose are shown in Fig. 5. Arabinose shows a fairly negative broad band in these region, as curve h shows. Curve a is the CD spectrum for lysozyme solution and curves from b to g are the CD spectra for the mixed solutions of lysozyme and arabinose with increasing concentration of arabinose. Similarly, CD spectra for lysozyme in glucose solutions showed slightly less positive

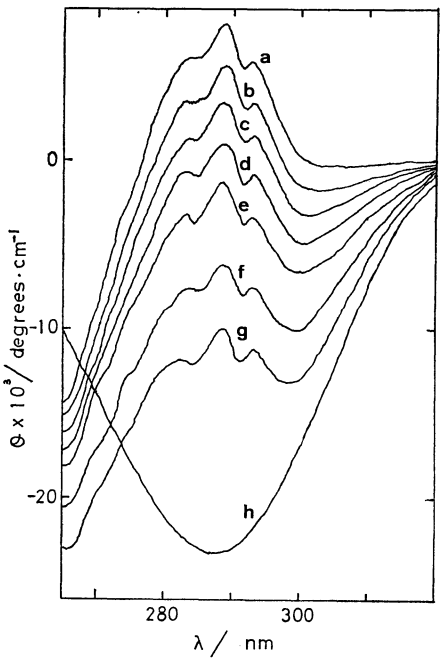

Fig. 5. The ellipticity, θ, for lysozyme and arabinose solutions at pH 5, I=0.1 and 25 °C.
Concentration of lysozyme is 8.782×10^{-3} residue mol dm^{-3}. Concentrations of arabinose are, a: 0 M (1 M=1 mol dm^{-3}), b: 0.1613 M, c: 0.3226 M, d: 0.4839 M, e: 0.6452 M, f: 0.9678 M, g: 1.290 M, and h: 1.613 M (h: without lysozyme).

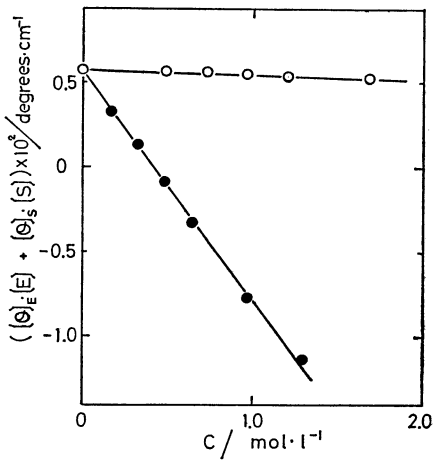

Fig. 6. The changes in the value of $[\theta]_E[E]+[\theta]_S[S]$ with sugar concentration.
Where $[\theta]_E$ and $[\theta]_S$ are molar ellipticity of lysozyme and a sugar, respectively, and [E] and [S] are molar concentrations. —○—: With glucose, —●—: with arabinose.

absorption by glucose in these wave lengths. The values of the ellipticity for the mixed solutions at 294 nm obtained by these experiments are plotted against sugar concentration in Fig. 6. Solid lines are values calculated on the assumption that the mixed solution shows the ellipticity which is simply the sum of the values of each component. The agreement between experimental and calculated values is good. These data suggest that sugars may not be bound to lysozyme at pH 5.

There have been many investigations which show the complex formation of lysozyme with sugars. Ikeda and Hamaguchi[21] found that sucrose enhanced the CD band of lyxozyme at 294 nm at pH 7.4, and that 1 mol of sucrose interacts with 1 mol of lysozyme, the association constant for the interaction was 0.4 (M^{-1}) at 25 °C. Kuramitsu *et al.*[22] showed that the binding constant for glucose and α-methyl xyloside to lysozyme was 0.5 and 1.6, respectively, from the absorption in CD spectra at 295 or 305 nm at pH 7.3 and 25 °C. Visible excited Raman spectra of lysozyme showed considerable differences in the relative intensities between that for lysozyme–glucose system and pure lysozyme, suggesting glucose–lysozyme complex formation.[23]

On the other hand, Rupley *et al.*[24] found that monosaccharide binding requires an acetamide group, and that glucose and maltose did not bind measurably. The binding constant for glucose to lysozyme is tabulated to be less than 0.1 (M^{-1}) by Chipman and Sharon.[25]

At pH 7.0, the values of ellipticity of the mixed solution of glucose and lysozyme at 304 and 294 nm are higher (less negative) that the values calculated on the assumption of additivity of $[\theta]$ of each component, in agreement with the results of Kuramitsu *et al.*[22] Also according to them, the molar ellipticity of lysozyme–α-methyl xyloside complex at 295 nm is nearly equal to that of lysozyme. The possibility remains that at pH 5, a complex is also formed but the molar ellipticity of the complex is the same as the sum of the values of each component.

As the reference values of the association constant for the complex formation of lysozyme and the saccharides studied here are very small, even if the complexes are formed, we would like to point out that there exist other interactions which stabilize the native conformation of protein in the system protein–sugar–water. The results of isopiestic measurements for the model systems[26,27] indicate that the sucrose solution environment is a more unfavorable solvent than water for amino acid with aliphatic and aromatic side chains. Consequently, it would require much more work for nonpolar groups in the interior of the protein to be exposed in the sucrose solution than in water; they would thus be caused by the sucrose solution to enter into the interior of the protein. Bull and Breese[28] suggest that the substances which stabilize proteins, such as sucrose, enhance the water structure in the immediate neighborhood of the protein and consequently contribute to protein stability. According to Tait *et al.*,[29] the distance between O–O atoms in equatorial hydroxyl groups of β-D-glucose coincides with the distance between O–O atoms in the tridimite structure of water. The structure making effect of sugars in aqueous solutions is more marked for the one which has more equatorial OH groups in the molecule.[30] Figure 4 shows that the sugars which strongly enhance the water structure raise the denaturation temperature of lysozyme more markedly. Thus in the presence of sugars the environmental effect or intensification of hydrophobic interaction inside a protein may promote a resistance against thermal denaturation of a protein.

References

1) S. Y. Gelsma and E. R. Stuur, *Int. J. Pept. Protein Res.*, **4**, 377 (1972).
2) G. Velicelebi and J. M. Sturtevant, *Biochemistry*, **18**, 1180 (1979).
3) K. Gekko, T. Morikawa, and H. Noguchi, 17th Annual Meeting of the Biophys. Soc. Japan, Nagoya, Oct. 1979, Abstr. p. 305.
4) B. S. Harrap, *Int. J. Pept. Protein Res.*, **1**, 253 (1969).
5) R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.*, **75**, 5139 (1953).
6) N. L. Simmons and R. J. Naftalin, *Biochim. Biophys. Acta*, **419**, 493 (1976).
7) S. Shifrin and C. L. Parrott, *Arch. Biochem. Biophys.*, **166**, 426 (1975).
8) K. Imai, T. Takagi, and T. Isemura, *J. Biochem.*, **53**, 1 (1963).
9) A. J. Sophianopoulos and D. E. Van Holde, *J. Biol. Chem.*, **239**, 2516 (1964).
10) P. L. Privalov, *J. Mol. Biol.*, **86**, 665 (1974).
11) K. Hamaguchi and H. Sakai, *J. Biochem.*, **57**, 721 (1965).
12) N. Shimaki, K. Ikeda, and K. Hamaguchi, *J. Biochem.*, **71**, 265 (1972).
13) Y. H. Chen, J. T. Yang, and K. H. Chen, *Biochemstry*, **13**, 3350 (1974).
14) For example, K. Hayashi and T. Imoto, "Lysozyme," Nankodo, Tokyo (1974).

15) J. M. O'Reilly and F. E. Karasz, *Biopolymers*, **9**, 1429 (1970).
16) A. J. Sophyanopoulos and B. J. Weiss, *Biochemistry*, **3**, 1920 (1964).
17) Y. Fujita and Y. Noda, *Bull. Chem. Soc. Jpn.*, **52**, 2349 (1979).
18) C. C. McDonald, W. D. Philips, and J. D. Glickson, *J. Am. Chem. Soc.*, **93**, 235 (1971).
19) C. Tanford and K. C. Aune, *Biochemistry*, **9**, 206 (1970).
20) S. J. Angyal, *Angew. Chem. Int. Ed. Engl.*, **8**, 157 (1969); S. J. Angyal and V. A. Pickles, *Aust. J. Chem.*, **25**, 1171, 1695 (1972).
21) K. Ikeda and K. Hamaguchi, *J. Biochem.*, **68**, 785 (1970).
22) S. Kuramitsu, K. Ikeda, and K. Hamaguchi, *J. Biochem.*, **74**, 143 (1973).
23) K. G. Brown, E. B. Brown, and W. B. Person, *J. Am. Chem. Soc.*, **99**, 3128 (1977).
24) J. A. Rupley, L. Butter, M. Gerring, F. J. Hartdegen, and R. Pecoraro, *Proc. Natl. Acad. Sci. U. S. A.*, **57**, 1088 (1967).
25) D. M. Chipman and N. Sharon, *Science*, **165**, 454 (1969).
26) H. Uedaira, *Bull. Chem. Soc. Jpn.*, **50**, 1298 (1977).
27) T. S. Lakshimi and P. K. Nandi, *J. Phys. Chem.*, **80**, 249 (1975).
28) H. B. Bull and K. Breese, *Biopolymers*, **17**, 2121 (1978).
29) M. J. Tait, A. Sugget, F. Franks, S. Ablett, and P. A. Quichenden, *J. Solution Chem.*, **1**, 131 (1972).
30) H. Uedaira, Kagaku Sosetsu, No. 11, 191 (1976).