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Aggregation and Chemical Reaction in Hen Lysozyme Caused by Heating at pH 6 Are Depressed by Osmolytes, Sucrose and Trehalose[1]

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We examined the effects of osmolytes, sucrose and trehalose, on the deterioration of hen lysozyme as a model protein. Sucrose and trehalose depressed the aggregation of lysozyme molecules caused by heating at 100°C at pH 6. Since lysozyme was fully denatured under these conditions, the effects of sucrose and trehalose on the denatured state of lysozyme were investigated using reduced *S*-alkylated lysozyme, a model of denatured hen lysozyme. From analyses of circular dichroism spectra and fluorescence spectra, sucrose and trehalose were found to induce α-helical conformations and some tertiary structures around tryptophan residues in the reduced *S*-alkylated lysozyme. Moreover, these compounds also depressed chemical reactions such as deamidation and racemization, which often cause the deterioration of proteins, on the reduced *S*-alkylated lysozyme. Therefore, the data suggest that sucrose and trehalose have a propensity to depress such deterioration as the aggregation of protein molecules or chemical reactions in proteins by inducing some tertiary structures (including α-helical structures) in the polypeptide chain.

Key words: aggregation, deamidation, hen lysozyme, racemization, sucrose, trehalose.

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A number of biologically important and functional proteins have marginal stability in solution, and they are easily denatured in high stress situations (*e.g.*, extreme salt concentration, high temperature). Under such circumstances, osmolytes, small organic solutes produced by the cells of all organisms (except halobacteria), maintain the structure of proteins in the cells exposed to various types of denaturing environmental stress (*1*). Osmolytes protect the macromolecular components in cells from such denaturing stresses as high temperature by increasing the thermal transition temperatures of proteins for denaturation (*2*). So far, several investigations (*3–7*) have shown how osmolytes are able to stabilize proteins against environmental stress. Namely, osmolytes destabilize the free energy level in the denatured state of a protein much more than they destabilize the free energy level in the native state, thus making denaturation more unfavorable in the presence of the osmolyte than in its absence, not by interacting directly with proteins but by the preferential exclusion of water from the vicinity of the protein (*3, 5*). On the other hand, there are few reports of the effects of osmolytes on irreversible processes, such as the deterioration of proteins through aggregation or non-enzymatic chemical reactions on their polypeptide chains, although analyses of the mechanisms of protein deterioration are of supreme importance for their medical or industrial uses.

Hen egg-white lysozyme is a common model protein used to investigate the irreversible inactivation of proteins at high temperature (*8–11*), and much information on deterioration through processes such as aggregation and deamidation have accumulated. In order to investigate whether osmolytes affect the deterioration of a protein, we examined the effect of sucrose and trehalose, naturally occurring osmolytes, on irreversible reactions in hen lysozyme.

MATERIALS AND METHODS

Materials—Hen lysozyme was donated by QP company (Tokyo). *Micrococcus luteus* was the product of Sigma. A Wakosil $_5C_{18}$-200 column, sucrose and trehalose were obtained from Wako Pure Chemicals (Osaka) and Nacali Tesque (Kyoto). TAP[2]-lysozyme was prepared by the method described in the literature (*12*). CM-Toyopearl 650S was purchased from Tosoh (Tokyo). All other chemicals were of analytical grade for biochemical use.

Analytical Methods—The enzymatic activity of lysozyme against *M. luteus* was turbidimetrically determined at 450 nm in 0.05 M phosphate buffer at pH 7 and 30°C. The time course of the irreversible inactivation of lysozyme was measured by incubation in 0.05 M phosphate buffer (pH 6) at various temperatures, while periodically removing samples and assaying them to determine their lytic activities. The determination of the transition temperature of lysozyme was carried out at pH 3 under conditions where the thermal transition is reversible, and using differential scanning calorimetry as described in a previous paper (*13*). Determinations of the amount of racemization at Asp in TAP[2]-lyso-

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Abbreviations; ANS, 1-anilino-8-naphthalene sulfonic acid; CD, circular dichroism; GdnHCl, guanidine hydrochloride; OPA-NAC, *o*-phtalaldehyde-*N*-acetyl cysteine; TAP[2]-lysozyme, reduced 20 *S-N,N*-dimethylammoniopropyl-*N',N',N'*-trimethylaminopropylated-lysozyme; TMAO, trimethylamine *N*-oxide; TPCK, *N*-tosylaminophenylalanine chloromethylketone; RP, reversed phase.

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zyme at pH 6 were carried out according to the method of Tomizawa *et al.* (9). Analyses of the OPA-NAC derivatives of L-Asp or D-Asp were carried out by RP-HPLC (Wakosil $_5C_{18}$-200, 4.6 × 250 mm). The column was isocratically eluted with 0.05 M acetate buffer containing 4% acetonitrile at pH 5.8 at a flow rate of 0.4 ml/min. The elution of the OPA-NAC derivatives of L-Asp or D-Asp was monitored by the absorbance at 350 nm. For ion-exchange HPLC, the column (CM-Toyopearl 650S, 4 × 250 mm) was eluted with a gradient of 30 ml of 0.05 M phosphate buffer, pH 7, and 30 ml of the same buffer containing 0.5 M NaCl at a flow rate of 1.0 ml/min. Elution was detected by absorbance at 280 nm.

Preparation and Analysis of Tryptic Peptides of TAP²-Lysozyme—TAP²-lysozyme (5 mg) was dissolved in 5 ml of 0.1 M phosphate buffer, pH 8. TPCK-trypsin (100 μg) was added and the solution was incubated for 2 h at 40°C. The digested sample was adsorbed on a column (Wakosil $_5C_{18}$-200, 4.6 × 250 mm) equilibrated with 40 ml of 1% acetonitrile containing 0.1% HCl, and eluted with 40 ml of 50% acetonitrile containing 0.1% HCl at a flow rate of 0.6 ml/min. The recovered tryptic peptides of TAP²-lysozyme were lyophilized for evaluation of the amounts of D-Asp present.

CD Spectra—TAP²-lysozyme (10 mM) was dissolved in 0.02 M phosphate buffer, pH 6, either with or without sucrose, trehalose, and GdnHCl. The spectra of these solutions were measured using a JASCO-J720 spectropolarimeter at 20 or 90°C.

Fluorescence Spectra—TAP²-lysozyme (10 mM) was dissolved in 0.1 mM ANS solution (ANS dissolved in 0.02 M phosphate buffer at pH 6) with or without various concentrations of sucrose or trehalose. On the other hand, *N*-acetyl tryptophan ethylester (10 mM) was dissolved in ANS solution with or without sucrose or trehalose. The fluorescence spectra of the ANS solution between 450 and 550 nm, excited at 365 nm, were measured at room temperature using a Hitachi F-2000 fluorescence spectrophotometer. For measurements of the fluorescence spectra of TAP²-lysozyme, TAP²-lysozyme was dissolved in 0.02 M phosphate buffer, pH 6, with or without sucrose or trehalose so that the concentration was 2 mM; fluorescence spectra of the solutions between 400 and 500 nm were measured upon excitation at 280 nm using the same fluorescence spectrometer.

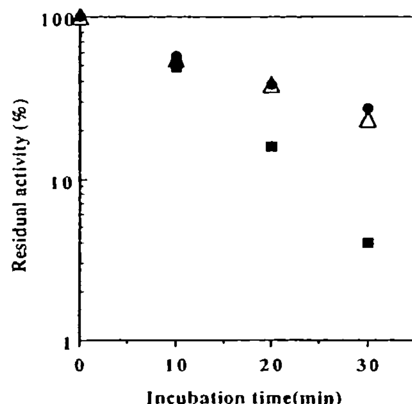

Fig. 1. **Time course of the irreversible inactivation of lysozyme solution (1 mg/ml) at 100°C, pH 6 in the absence (closed squares) or the presence of 1.5 M sucrose (open triangles) or 1.5 M trehalose (closed circles).**

RESULTS AND DISCUSSION

Sucrose and Trehalose Depress Intermolecular Interactions between Lysozyme Molecules Caused by Heating at 100°C—The transition temperatures of lysozyme in the presence of 1.7 M sucrose and 1.7 M trehalose, which are nearly saturating concentrations, were determined to be 83 and 85°C, respectively. These temperatures are about 10°C higher than that of lysozyme in the absence of additives. However, at 100°C, the population of lysozyme in the denatured state should be high even in the presence of sucrose or trehalose. In Fig. 1, the time courses of the inactivation of lysozyme (1 mg/ml) in the presence or absence of 1.5 M sucrose or 1.5 M trehalose are shown. In the absence of an additive, the residual lysozyme activity decreased steeply whereas the activity in the presence of 1.5 M sucrose or 1.5 M trehalose decreased gradually, indicating that both sucrose and trehalose depress the inactivation of lysozyme caused by heating at 100°C. In order to examine the cause of the inactivation of lysozyme, ion-exchange HPLC analysis of the lysozyme solution (1 mg/ml) after incubation at 100°C at pH 6 for 20 min in the presence or absence of an additive was carried out (Fig. 2). In the presence of 1.5 M sucrose or 1.5 M trehalose the derivatives in the peak eluted earlier than native lysozyme and deamidated lysozymes, which were identified in the previous paper (*10*), were observed, proving that the chemical reaction of lyso-

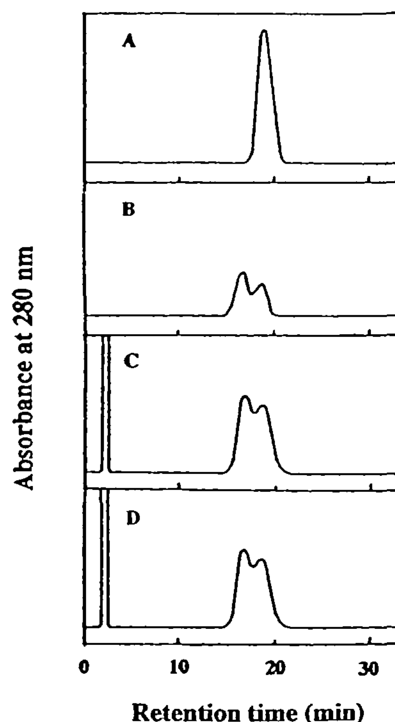

Fig. 2. **Ion-exchange HPLC of lysozyme.** The column (CM-Toyopearl 650S, 4 × 150 mm) was eluted with a gradient of 30 ml of 0.05 M phosphate buffer at pH 7 and 30 ml of the same buffer containing 0.5 M NaCl at a flow rate of 1.0 ml/min. A, intact lysozyme; B, lysozyme after incubation at 100°C, pH 6 for 20 min in the absence of an additive; C, lysozyme after incubation at 100°C, pH 6 for 20 min in the presence of 1.5 M sucrose; D, lysozyme after incubation at 100°C, pH 6 for 20 min in the presence of 1.5 M trehalose.

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zyme occurs even in the presence of 1.5 M sucrose or 1.5 M trehalose at 100°C (Fig. 2, C and D). In the absence of an additive, considerable aggregation as well as deamidation were observed upon incubating the lysozyme solution at 100°C at pH 6 for 20 min. As a result, the amount of lysozyme on ion-exchange HPLC was less than that in the presence of 1.5 M sucrose or 1.5 M trehalose (Fig. 2B).

The dependence of lysozyme concentration on inactivation after incubation at 100°C, pH 6 for 20 min was examined (Fig. 3). In the absence of an additive, the inactivation of lysozyme was clearly observed. On the other hand, in the presence of 1.5 M sucrose or 1.5 M trehalose, little inactivation was observed. Tomizawa *et al.* (*10*) demonstrated that the inactivation of lysozyme at pH 6 depends mainly on intermolecular interactions between lysozyme molecules due to sulfhydryl-disulfide interchange reactions. Moreover, they also showed that such inactivation is depressed in the presence of excess $CuCl_2$ because a small amount of copper ion suppresses intra- and intermolecular disulfide exchange by catalyzing the air oxidation of heat-induced trace amounts of free thiols. The dependence of the concentration of lysozyme *vs.* its inactivation after the addition of $CuCl_2$ (2.5 mol excess of lysozyme concentration) was thus examined (Fig. 3). As indicated in a previous paper (*10*), we confirmed that the inactivation of lysozyme depends only slightly on the lysozyme concentration in the presence of $CuCl_2$. Moreover, in the presence of both $CuCl_2$ and 1.5 M sucrose or 1.5 M trehalose at lysozyme concentrations of 0.2–4 mg/ml, the residual lysozyme activities after incubation at 100°C for 20 min were found to remain higher (above 80%) than expected for the residual activities in the presence of the respective solutes at the same lysozyme concentration. These findings indicate that the effects of $CuCl_2$ and sucrose or trehalose on the depression of lysozyme inactivation are not completely the same. Therefore, the cause of the depression of the intermolecular interactions between lysozyme molecules at 100°C at pH 6 by the addition of sucrose or trehalose was thus found not to be due solely to sulfhydryl–disulfide interchange reactions.

Sucrose and Trehalose Induce Some Tertiary Structures and α-Helical Conformations in Denatured Lysozyme—A protein in the denatured state is believed to adopt a mainly random structure. However, there is a possibility that sucrose and trehalose affect the conformation in the denatured state because we observed that the intermolecular interactions between lysozyme molecules were depressed in the presence of 1.5 M sucrose or 1.5 M trehalose at 100°C where the denatured state of lysozyme should predominate. In order to examine the effect of sucrose and trehalose on the conformation of lysozyme in the denatured state, we employed TAP^2-lysozyme, a soluble denatured lysozyme (*12*) in which all amino acid residues are fully accessible to the solvent. To date, there have been few reports on the effect of sucrose or trehalose on the conformation of proteins in denatured states, however, a few studies have reported that sucrose induces α-helical conformations in proteins in the native state (*14, 15*). First, we examined whether TAP^2-lysozyme adopts an α-helical conformation in the presence of sucrose or trehalose at 20°C (Fig. 4). The CD spectrum of TAP^2-lysozyme in aqueous solution shows a slight concave-shape around 222 nm. Moreover, there is a difference in the CD spectra between in the presence and absence of 6 M GdnHCl. These results indicate that TAP^2-lysozyme slightly adopts an α-helical conformation in aqueous solution. The presence of the α-helical conformation in TAP^2-lysozyme is supported by the finding that there is a difference in the CD spectra of TAP^2-lysozyme and its tryptic peptides, which retain no residual structure even in the presence of additives (*16*). On the other hand, in the presence of 1.5 M sucrose or 1.5 M trehalose (Fig. 4), the CD spectrum of TAP^2-lysozyme shows a concave shape at 222 nm. In addition, the molar ellipticity at 222 nm in the spectra of TAP^2-lysozyme decreases as the concentration of sucrose or trehalose increases (Fig. 5). These findings clearly indicate that an α-helical conformation of TAP^2-lysozyme is induced upon the addition of sucrose or treha-

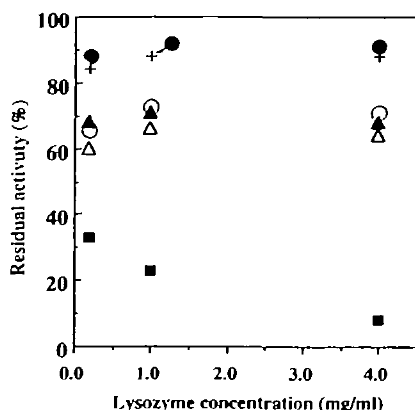

Fig. 3. **Effect of the lysozyme concentration on its irreversible inactivation by incubation at 100°C, pH 6 for 20 min in the absence (closed squares) or presence of either 1.5 M sucrose (open triangles), 1.5 M trehalose (open circles), $CuCl_2$ (closed triangles) or both 1.5 M sucrose and $CuCl_2$ (crosses), or both 1.5 M trehalose and $CuCl_2$ (closed circles).**

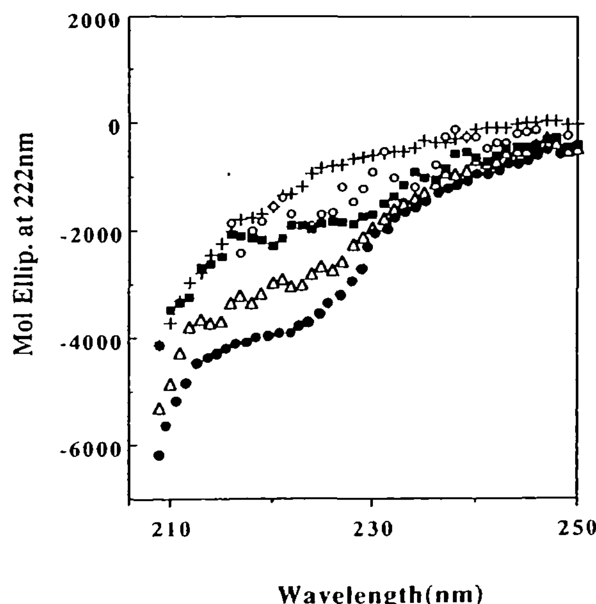

Fig. 4. **Far UV CD spectra of TAP^2-lysozyme in 0.02 M phosphate buffer (pH 6) at 20°C in the absence (closed squares) or presence of 1.5 M sucrose (open triangles), or the presence of 1.5 M trehalose (closed circles) or 6 M GdnHCl (open circles) and tryptic peptides of TAP^2-lysozyme (crosses).**

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lose. However, the extent of α-helical conformation of TAP²-lysozyme which is induced by the addition of sucrose or trehalose is less than that of the addition of trifluoroethanol, a typical inducer of α-helical protein conformations (data not shown). In any event, the present results indicate that an α-helical conformation of lysozyme in the denatured state is induced by the addition of sucrose or trehalose, indicating that the denatured structure of lysozyme is more compact than the randomly coiled state.

Next we examined the conformation of TAP²-lysozyme in the presence of sucrose or trehalose at higher temperatures. Since we could not measure the CD spectrum at 100°C where the irreversible inactivation of lysozyme was investigated, the CD spectrum of TAP²-lysozyme was obtained at 90°C (Fig. 6). Because the CD spectrum of TAP²-lysozyme in the absence of an additive was identical to that in the presence of 6 M GdnHCl, TAP²-lysozyme in the denatured state at 90°C was found not to have any ordered structure. TAP²-lysozyme in the presence of 1.5 M sucrose or 1.5 M trehalose, however, showed a different CD spectrum from that in the absence of an additive and a clear concave shape around 222 nm, indicating that TAP²-lysozyme retains an α-helical conformation in the presence of 1.5 M sucrose or 1.5 M trehalose even at 90°C. We therefore found that the structure of lysozyme in a denatured state at higher temperatures is also more compact than its randomly coiled state. It is thus suggested that the compact structure of lysozyme in the denatured state caused by the addition of sucrose or trehalose depresses the unfavorable intermolecular interactions between lysozyme molecules, resulting in a depression of aggregation.

It is known that ANS does not bind to the unfolded state or folded state of a protein, but rather to a folding intermediate (*17*). The spectra of ANS in the presence of TAP²-lysozyme on excitation at 365 nm are shown in Fig. 7. In the absence of an additive, the spectrum shows a broad peak around 490–500 nm. With an increase in the concentration of sucrose and trehalose, the spectrum shows a clear optimum at 480 nm, indicating that ANS interacts with some tertiary structure in TAP²-lysozyme and that the fluorescence intensity at 480 nm increases. In orderto deny the direct interaction of tryptophan residues with sucrose or trehalose, the fluorescence spectra of ANS in the presence of *N*-acetyltryptophan ethylester and 1 M sucroseor or 1 M trehalose were measured. The spectrum shows a small, broad optimum near 510 nm (data not shown). From the fluorescence spectrum of ANS with a maximum wavelength at 480 nm in the presence of TAP²-lysozyme, we considered that TAP²-lysozyme has a more compact structure than a randomly coil. This is supported by the result in which the maximum wavelength of the fluorescence spectra of TAP²-lysozyme shifted toward 340 nm, at which the tryptophan residue is protected from the solvent (*18*), with the

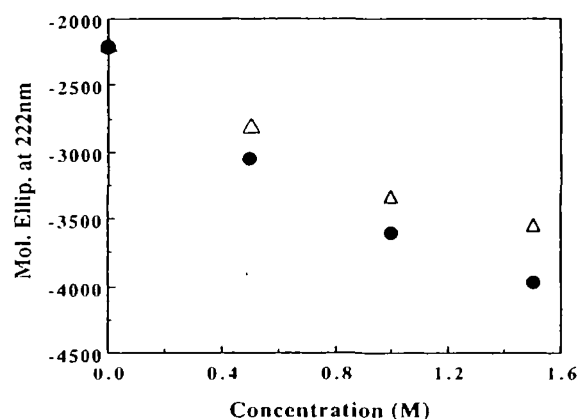

Fig. 5. **Plots of molar ellipticity at 222 nm in the spectra of TAP²-lysozyme against various concentrations of sucrose (open triangles) or trehalose (closed circles).**

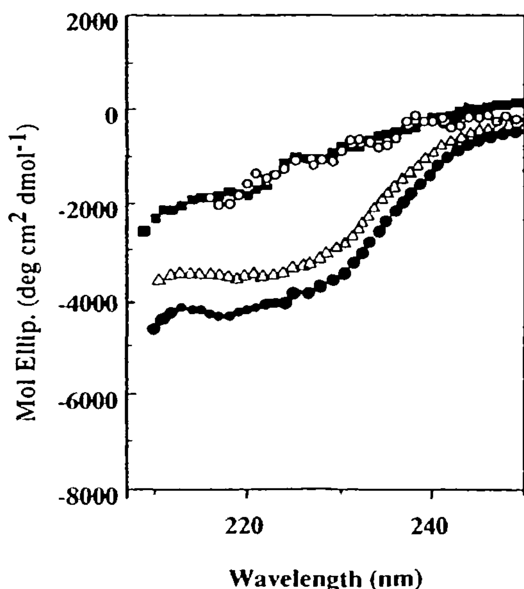

Fig. 6. **Far UV CD spectra of TAP²-lysozyme in 0.02 M phosphate buffer (pH 6) at 90°C in the absence (closed squares) or presence of 1.5 M sucrose (open triangles), 1.5 M trehalose (closed circles) or 6 M GdnHCl (open circles).**

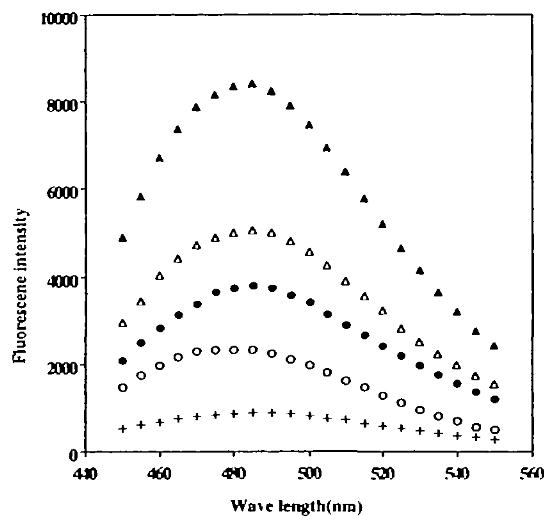

Fig. 7. **Fluorescence spectra of ANS in 0.02 M phosphate buffer (pH 6) at 20°C in the presence of only TAP²-lysozyme (crosses), in the presence of both TAP²-lysozyme and 0.5 M trehalose (closed circles), both TAP²-lysozyme and 1.0 M trehalose (closed triangles), both TAP²-lysozyme and 0.5 M sucrose (open circles), or both TAP²-lysozyme and 1.0 M sucrose (open triangles).**

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addition of 1.5 M sucrose or 1.5 M trehalose, whereas in the absence of an additive, the maximum was near 350 nm (Table I). From the above results, we confirmed that denatured lysozyme adopts a more compact structure than randomly coiled lysozyme upon the additionof 1.5 M sucrose or 1.5 M trehalose. Thus, it was concluded that the aggregation and chemical reactions in hen lysozyme caused by heating at 100°C at pH 6 are depressed due to the induction of some tertiary and α-helical structures by the addition of an additive.

Sucrose and Trehalose Depress Chemical Reactions in the Polypeptide Chain of Hen Lysozyme—It has been reported that heating hen lysozyme at 100°C at pH 6 induces chemical reactions such as deamidation and racemization (*8, 10*). Therefore, in order to examine the effect of sucrose and trehalose on the chemical reactions in the polypeptide chain of hen lysozyme, TAP[2]-lysozyme was incubated at 100°C at pH 6 in the presence or absence of 1.5 M sucrose or 1.5 M trehalose, and the extent of deamidation was analyzed by peptide analysis on RP-HPLC. Figure 8 shows the RP-HPLC patterns of a tryptic peptide (Ile98-Val99-Ser100-Asp101-Gly102-Asp103-Gly104-Met105-Ser106-Ala107-Trp108-Val109-Ala110-Trp111-Arg112) containing the most labile asparagine residue (Asn103) in hen lysozyme after incubation at pH 6 and 100°C for 1 h in the absence or presence of additives. Peak a indicates the tryptic peptide in which the peptide bond Asp101-Gly102 is changed α to β. Peak b indicates the intact tryptic peptide overlapping the tryptic peptide where Asn103 has been deamidated and also the peptide bond at Asp101-Gly102 has been changed α to β. Peak c indicates the tryptic peptide in which Asn103 is deamidated (Ile98-Val99-Ser100-Asp101-Gly102-Asp103-Gly104-Met105-Ser106-Ala107-Trp108-Val109-Ala110-Trp111-Arg112). The amount of deamidated peptide in the absence of an additive (Fig. 8A) was clearly larger than that in the presence of 1.5 M sucrose (Fig. 8B) or 1.5 M trehalose (Fig. 8C).

On the other hand, the racemization of the L-aspartyl residue to D-aspartyl in lysozyme upon incubation at 100°C has been reported to occur (*8, 10*). In order to investigate the effect of sucrose and trehalose on the racemization of aspartyl residues in lysozyme, we measured the formation of D-Asp in lysozyme after incubation of the TAP[2]-lysozyme solutions at 100°C in the presence or absence of 1.5 M sucrose or 1.5 M trehalose. As shown in Fig. 9, the formation of D-Asp was confirmed after the incubation of TAP[2]-lysozyme at 100°C, pH 6 for 1 h (Table II) whereas only a small amount of D-Asp was formed before the incubation of TAP[2]-lysozyme (Fig. 9). In the presence of each additive, only a small amount of D-Asp was observed (Table II) in samples after incubation. That is, the formation of D-aspartyl residues inTAP[2]-lysozyme due to heating was depressed by the addition of 1.5 M sucrose or 1.5 M trehalose. On the other hand, we measured the amount of D-Asp formed in the tryptic peptides of TAP[2]-lysozyme in the presence or absence of an additive after incubation at 100°C, pH 6 for 1 h (Table II). Almost the same amount of D-Asp was formed and there was no difference in the formation of D-Asp between the absence and presence of any additive (Table II). Therefore, these findings suggest that sucrose and trehalose do not depress the racemization of L-aspartyl residues in TAP[2]-lysozyme by interacting directly with the susceptible residue. In conclusion, the addition of sucrose or treha-

TABLE I. **Maximum wavelengths in the fluorescence spectra of TAP[2]-lysozyme in the absence or presence of additves.**

Additive	Maximum wavelength (nm)
None	350
1.5 M sucrose	342
1.5 M trehalose	342

TABLE II. **Amounts of D-Asp formed in TAP[2]-lysozyme or its tryptic peptides after incubation at pH 6 and 100°C for 1h in the absence or the presence of additives.**

	D-Asp (%)[a]		
	None	1.5 M sucrose	1.5 M trehalose
Whole	16	<2	<2
Trypsin digest	18	18	19

[a]Deviation was less than 5% of each detected value.

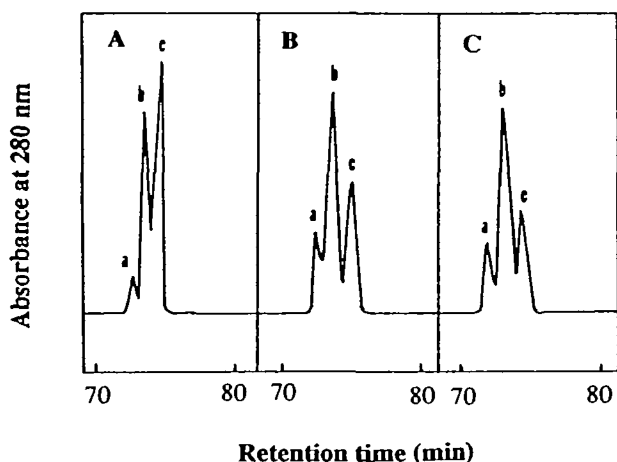

Fig. 8. **RP-HPLC of the tryptic peptides derived from TAP[2]-lysozyme after incubation at 100°C, pH 6 for 20 min in the absence (A) or presence of 1.5 M sucrose (B) or 1.5 M trehalose (C).** The column (Wakosil 5C18, 4.6 × 250 mm) was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 50% acetonitrile, both containing 0.1% HCl at a flow rate of 0.6 ml/min.

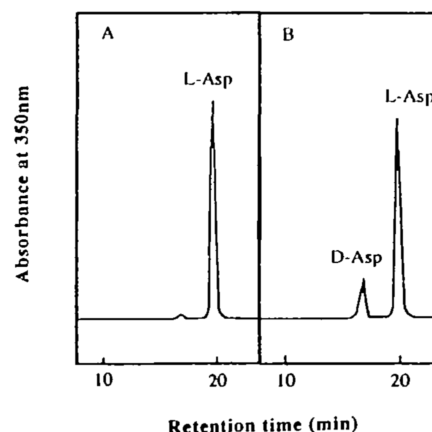

Fig. 9. **RP-HPLC of OPA-NAC derivatives of L- and D-aspartate.** Before (A) and after (B) incubation of TAP[2]-lysozyme at 100°C, pH 6 for 1 h. The column (Wakosil $_5C_{18}$-200, 4.6 × 250 mm) was isocratically eluted with 0.05 M acetate buffer containing 4% acetonitrile, pH 5.8, at a flow rate of 0.4 ml/min.

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lose appears to depress chemical reactions such as deamidation and racemization in TAP[2]-lysozyme due to the induction of some tertiary and α-helical structures in the polypeptide chain.

Significance of Sucrose and Trehalose as Osmolytes In Vivo—In this paper, we have described that the aggregation of lysozyme molecules that occurs at pH 6 at 100°C is depressed in the presence of sucrose or trehalose. A similar depression of aggregation by the addition of sucrose has been reported for TEM β-lactamase, but the mechanism was not explained (*19*). Goldberg *et al.* (*20*) have demonstrated that aggregation can occur in the early stage of folding where the population of the unfolded state of lysozyme is rich. Therefore,it may be considered that the compact structure caused by the addition of sucrose or trehalose to the unfolded state of lysozyme depresses the unfavorable intermolecular interactions and increases the solubility of the unfolded lysozyme, the dominant form at 100°C, resulting in the depression of aggregation.

Up to now the function of osmolytes in biological systems was recognized to be that a stabilizer to increase the thermal stability of a protein (*4–7*). In this paper, we found common properties of sucrose and trehalose, which induce α-helical conformations and some tertiary structures in the polypeptide chain of hen lysozyme, thus depressing the formation of aggregates and chemical reactions. This is a significant finding regarding the function of sucrose and trehalose. On the other hand, Yang *et al.* (*21*) showed recently that TMAO, a naturally occurring osmolyte, and glycerol, which mimics the effects of naturally occurring molecules, accelerate the formation of β-amyloid fibrils in Aβ1-40, which is an irreversible reaction, whereas these osmolytes act as stabilizers to increase the thermal stability proteins (*22, 23*). Therefore, further investigations of the effect of osmolytes on irreversible processes may be called for. *In vivo*, the molecular chaperone is knownto be induced by thermal stress (*24*). It plays a role in facilitating protein folding by preventing protein aggregation. As for low molecular weight compounds, sucrose and trehalose would be reasonable solutes for studying biological responses to heat stress, because both have functions that increase the thermal stability of proteins and thus reduce protein deterioration. Finally, the function of sucrose and trehalose as osmolytes may be of interest to biochemists, and our findings provide a better understand of the biological response to various types of environmental stresses.

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