Protein Preferential Solvation in (Sucralose + Water) Mixtures

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Protein preferential solvation in (sucralose + water) mixtures

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ABSTRACT: Addition of sugars such as sucrose to aqueous protein solutions generally stabilizes proteins against thermal denaturation, by preferential exclusion of sugars from proteins (preferential hydration of proteins). In this study, we investigated the effect of sucralose, a chlorinated sucrose derivative, on protein stability and preferential solvation. Circular dichroism and small-angle X-ray scattering measurements showed that sucrose increased the denaturation temperature of myoglobin and was preferentially excluded from the protein, whereas sucralose decreased the denaturation temperature of myoglobin and was preferentially adsorbed to the protein. No clear evidence was obtained for indirect effects of sucralose on protein destabilization via the structure and properties of solvent water from the physicochemical properties (mass density, sound velocity, viscosity, osmolality) of aqueous sucralose solutions; therefore, we concluded that a direct protein-sucralose interaction induced protein destabilization.

1. INTRODUCTION

The stability of protein structure in aqueous solution is affected by coexisting small molecules (cosolvents). For example, the addition of sugars such as sucrose protects proteins against denaturation.[1-3] This enhancement of protein stability by the addition of sugars has been applied in both the food and pharmaceutical industries.[4,5] The stabilizing effect of sugars on proteins has also attracted attention in the field of biology, with some organisms shown to accumulate sugars in cells to help them tolerate extreme environments.[6]

Sucralose (1,6-dichloro-1,6-dideoxyfructose-4-chloro-4-deoxygalactose) is a chlorinated derivative of

sucrose, with three hydroxyl groups substituted by chlorine atoms. It is approximately 600 times sweeter than sucrose and has been widely used as a non-nutritive sweetener.[7] In spite of structural similarity between the two sugars, several recent studies have reported that, in contrast with its precursor sucrose, sucralose reduces protein stability.[8-10]

It has been known for some time that the effect of cosolvents on protein stability is correlated with preferential solvation: stabilizers (e.g., sugars, polyols) are preferentially excluded from proteins (preferential hydration of proteins) and so stabilize the native state that exposes less surface area to solvent contact with respect to the denatured state, whereas destabilizers (e.g., ureas, guanidine hydrochloride) are preferentially adsorbed to proteins and so stabilize the denatured state that exposes more surface area to solvent contact with respect to the native state.[1,3,11-13] The preferential exclusion/adsorption of cosolvents by proteins has been observed by densitometry,[1,11,12] osmometry,[14] calorimetry,[15] and scattering techniques.[16-19] However, the preferential solvation of protein by sucralose has not been clarified experimentally. The modulation of protein stability by cosolvents is not only caused by direct interactions between the protein and the cosolvents but also by indirect effects via the change in the structure and properties of the solvent water.[2,3,20,21] There have been far fewer studies investigating the properties of aqueous solutions of sucralose compared with the number of studies that have investigated aqueous solutions of sucrose.[22]

In the present study, we investigated the thermal stability and the preferential solvation behavior of myoglobin in mixtures of sucralose and water. Physical properties of aqueous sucralose solutions (mass density, sound velocity, viscosity, and osmolality) were measured to investigate the mechanism underlying the effect of sucralose on protein stability. Circular dichroism (CD) measurements showed that the thermal stability of myoglobin was decreased by sucralose. Small-angle X-ray scattering (SAXS) measurements indicated that sucralose had a greater tendency than sucrose to be adsorbed to myoglobin. The physical properties of aqueous solutions of sucralose were not significantly different from those of sucrose, suggesting that sucralose destabilized protein structure through direct interaction with proteins rather than indirect effects on the structure and properties of the solvent water.

2. EXPERIMENTAL METHODS

Myoglobin from horse skeletal muscle (Sigma-Aldrich), sucralose (Sigma-Aldrich), and sucrose (FUJIFILM Wako Pure Chemical Co.) were used without further purification. The buffer solvents used were 20 mM Na_2HPO_4/NaH_2PO_4 at pH 7.0 for CD measurements and 10 mM HEPES (*N*-(2-hydroxymethyl)-piperazine-*N*′-(2-ethane-sulfonic acid)) and 50 mM NaCl at pH 7.0 for SAXS measurements. The stock solution of myoglobin (25 mg/ml for CD and 100 mg/ml for SAXS) and sugar

solutions of various concentrations were mixed in a 1:4 ratio to prepare protein solutions of 5 and 20 mg/ml for CD and SAXS, respectively. The protein solutions were clarified by centrifugation at 16,000 × g for 15 min, before the measurements were made. The sugar concentration ranged from 0 to 20 w/v% for sucralose and 0 to 40 w/v% for sucrose.

The CD spectra were measured from 260 to 180 nm under an N_2 gas atmosphere using a vacuum-ultraviolet circular dichroism spectrophotometer (BL12) at the Hiroshima Synchrotron Radiation Center (HiSOR, Higashi-Hiroshima, Japan).[23] An optical cell with CaF_2 windows was used, and the path length of the cell was adjusted with a Teflon spacer to 50 μm. The temperature of the cell was controlled in the range from 20℃ to 90℃ using a Peltier temperature control unit.

The SAXS measurements were performed using the BL-6A[24] and BL-10C[25] spectrometers at the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan). The X-ray wavelength was 1.5 Å at BL-6A and 1.0 Å at BL-10C. The sample-to-detector distance was 50 cm. The X-ray scattering intensity was recorded with a PILATUS 1M detector (Dectris) at BL-6A and a PILATUS 2M detector (Dectris) at BL-10C. The exposure time was 20 to 30 s. The temperature of the solutions in the sample cells was maintained at 25℃ using a temperature controller (mK2000, Instec). The two-dimensional SAXS data were circularly averaged, converted into one-dimensional scattering intensity data, and background subtracted to obtain the scattered intensity profile $I(q)$ as a function of the scattering vector length q, defined as $q = (4\pi/\lambda)\sin(\theta/2)$, where θ and λ are the scattering angle and the wavelength of the X-rays, respectively. The zero-angle scattered intensity $I(0)$ and the radius of gyration R_{g} were calculated by applying the Guinier approximation formula to the scattering data in the small-angle region.[26,27]

$$\ln I(q) = \ln I(0) - \frac{1}{3}R_g^2 q^2. \tag{1}$$

The mass density of the sugar solutions and the speed of sound in the sugar solutions were measured using a density and sound velocity meter (DSA 5000, Anton-Paar) at 20℃. The average electron-density of the sugar solutions was calculated from the mass-density data, as shown in the Results and Discussion section. The hydration number of sugars was also estimated from the mass density and sound velocity data, as follows. The adiabatic compressibility coefficient κ can be calculated from Laplace's equation:

$$\kappa = \frac{1}{\rho u^2} \tag{2}$$

where ρ and u are the mass density and the speed of sound, respectively.[28] According to the Passynsky method,[28,29] the hydration number n_{H} of a sugar molecule in a sugar solution consisting of n_{water} moles of water and n_{sugar} moles of sugar was calculated from the following equation:

$$n_{\mathrm{H}} = \frac{n_{\mathrm{water}}}{n_{\mathrm{sugar}}}\left(1 - \frac{\kappa}{\kappa_{\mathrm{water}}}\right) \quad (3)$$

where κ_{water} is the compressibility of water. The hydration number defined by eq. 3 represents the average number of water molecules that are affected by interaction between the sugar and water and show strongly reduced compressibility compared with pure bulk water.[28]

The osmolality of sugar solutions was measured using a vapor pressure osmometer (Vapro 5600, Wescor). The water activity a_{w} in the sugar solutions was calculated from the following equation:

$$a_{\mathrm{w}} = \exp(-V_{\mathrm{water}} \cdot \mathrm{Osm}) \quad (4)$$

where V_{water} and Osm represent the molar volume of water and the osmolality, respectively.[30] The viscosity measurement of sucralose solutions was performed with an Ubbelohde-type capillary viscometer at 20°C.

3. RESULTS AND DISCUSSION

3.1 Effect of Sucralose on the Thermal Stability of Myoglobin

Figure 1 shows the CD spectra of myoglobin in the buffer solvents with and without sugars. The spectra showed negative peaks at 208 and 222 nm, stemming from the α-helix structure in myoglobin, independent of the addition of sugars. The shape of the spectra below 200 nm for the myoglobin solutions containing sucralose or sucrose was different from that without sugars, which was due to the CD signals of sucralose and sucrose (Fig. S1). On the other hand, the spectra at 20°C with and without sugars coincide with each other at wavelengths above 200 nm, indicating that both sucralose and sucrose did not affect the secondary structure of myoglobin. The temperature dependences of the ellipticity at 222 nm are shown in Fig. 2. The abrupt change in the ellipticity with increasing temperature was attributed to the denaturation of myoglobin. The denaturation temperature of myoglobin was increased by the addition of sucrose but decreased by the addition of sucralose. This result shows that sucralose destabilized the structure of myoglobin, whereas sucrose stabilized it.

3.2 Excluded Molecular Volume and Electron Density of Sugars

In the X-ray scattering of solutions, the scattered intensity depends on the difference between the average electron densities of the solute and the solvent (referred to as contrast). The electron density of myoglobin was calculated to be 0.424 e/Å^3 on the basis of the crystal structure of myoglobin (code 1WLA, registered in the Protein Data Bank (PDB)). The electron density of aqueous sugar solutions at the concentration C_{sugar} (w/w%) can be calculated using the following equation:[19]

$$D = \frac{N_A \rho}{100}\left[\frac{C_{sugar} v_{sugar} D_{sugar}}{M_{sugar}} + \frac{(100 - C_{sugar}) v_{water} D_{water}}{M_{water}}\right], \quad (5)$$

where v_{sugar}, v_{water}, D_{sugar}, D_{water}, M_{sugar}, and M_{water} are the excluded molecular volume, the electron density, and the molar masses of sugar and water molecules, respectively; N_A is Avogadro's number.

Figure 3 shows the concentration dependences of the mass density for aqueous solutions of sucralose and sucrose. The mass density of a mixture of sugar and water is given by the following equation:[19]

$$\rho = \frac{100}{N_A\left[\left(\frac{C_{sugar} v_{sugar}}{M_{sugar}} + \frac{(100 - C_{sugar}) v_{water}}{M_{water}}\right)\right]}. \quad (6)$$

The value of v_{sugar} was determined by fitting eq. 6 to the data shown in Fig. 3. The values of v_{sugar} and D_{sugar} obtained for sucralose and sucrose are shown in Table 1. The electron density of the aqueous solution of sucralose calculated using eq. 5 and the values in Table 1 coincided with that of sucrose (Fig. S2). The calculated values for the electron density were used in the SAXS simulation, as described later.

3.3 Effect of Sugars on SAXS of Myoglobin Solutions

Figure 4 shows the Guinier plots of the SAXS curves of myoglobin in the aqueous solutions of sucralose and sucrose. As the sugar concentration increased, the scattered intensities of X-rays slightly decreased for sucralose (Fig. 4a) but significantly decreased for sucrose (Fig. 4b). As shown in eq. 1, the slope and the intercept of the Guinier plot give $I(0)$ and R_g, respectively. Figure 5 shows the plots of $I(0)$ and R_g as a function of the sugar concentration. The addition of sucrose clearly decreased $I(0)^{1/2}$ but did not change R_g significantly, which is consistent with previous reports.[19] On the other hand, the addition of sucralose decreased $I(0)^{1/2}$ less sharply and clearly increased R_g.

Hirai et al. investigated the effect of sugars and polyols on the SAXS of protein solutions.[18,19] They showed that the change in the observed scattering function (and therefore the values of $I(0)$ and R_g) could be attributed to the change in the contrast between protein and solvent, when the solvation shell surrounding the protein surface was taken into consideration. They simulated X-ray scattering curves from the atomic structure of protein registered in PDB, using the CRYSOL program developed by Svergun et al.,[32] and assuming that the electron densities of solvent and the solvation shell are variable. They compared the results of experiments and simulations and concluded that sugars and polyols were preferentially excluded from protein (preferential hydration). In the present study, we adopted a similar approach to investigate the preferential solvation of myoglobin in a mixture of sucralose and water. We used the CRYSOL program to calculate theoretical SAXS curves based on the structure of myoglobin

(1WLA in PDB) and the electron densities obtained in Section 3.2. The electron density D_{shell} of the solvation shell was given by:

$$D_{\mathrm{shell}} = \beta\phi D_{\mathrm{sugar}} + (1 - \beta\phi) D_{\mathrm{hydration}}, \quad (7)$$

where ϕ is the volume fraction of sugar, β is a parameter, and $D_{\mathrm{hydration}}$ is the electron density of the hydration shell around the protein. The value of $D_{\mathrm{hydration}}$ was set to be 1.1-times higher than that of the bulk water, based on the value found in the literature.[33] The parameter β in eq. 7 represents the preferential solvation behavior: the preferential exclusion of sugar from protein occurs when $0 \leq \beta < 1$, and the preferential adsorption of sugar to protein occurs when $\beta > 1$. The values of $I(0)$ and R_{g} for the theoretical scattering curves were obtained from the Guinier plots. Figure 6 compares $I(0)^{1/2}$ and R_{g} calculated for various values of β with those obtained experimentally. The experimental data of $I(0)^{1/2}$ fall on theoretical curves of $\beta > 1$ for sucralose and those of $\beta < 1$ for sucrose. which concludes that sucralose was preferentially adsorbed to the protein and sucrose was preferentially excluded from the protein (Fig. 6a). Although the experimental data for R_{g} are scattered, the larger R_{g} values for sucralose than for sucrose also indicate that sucralose had a tendency toward preferential adsorption in comparison with sucrose (Fig. 6b). In this analysis, the experimental values of R_{g} were slightly larger than the theoretical ones calculated for the value of β that gave a theoretical curve of $I(0)^{1/2}$ fit to the experimental data. This discrepancy could be attributed to the fact that the width of the solvation shell is fixed at 3 Å in the CRYSOL program.[32] The present study showed that protein structure was destabilized by sucralose exhibiting preferential adsorption and stabilized by sucrose exhibiting preferential exclusion. This result is consistent with theoretical considerations based on thermodynamics[1,11,12] and statistical thermodynamics.[13] The observed adsorption of sucralose to protein is also in agreement with the results of a recent molecular dynamics simulation.[34]

3.4 Physical Properties of Aqueous Sucralose Solution and a Mechanism for the Destabilization of Protein by Sucralose

Recent studies have reported that the addition of sucralose lowered the denaturation temperature of proteins, including bovine serum albumin,[8] staphylococcal nuclease,[8] lysozyme[9], and whey protein isolate.[10] However, the mechanism via which sucralose destabilizes protein has not yet been clarified. In general, the effects of cosolvents on proteins are twofold: direct binding to proteins and indirect effects through changes to the structure and properties of water. In terms of direct binding, the binding of sucralose to proteins via electrostatic interactions,[8,9,34] hydrophobic interactions,[9] hydrogen bonds[10,34], and van der Waals interactions[10] has been suggested based on the results of docking simulations,[9,10] molecular dynamics simulations,[34] time-resolved fluorescence anisotropy of tryptophan,[8] and isothermal

titration calorimetry.[9,34] The SAXS measurements in the present study showed that sucralose was preferentially adsorbed to myoglobin, which supports the notion of direct binding of sucralose to proteins. For indirect effects, although they have been mentioned in the literature,[9] they have not been thoroughly investigated, probably because of a lack of data about the physical properties of aqueous sucralose solutions.

One of the indirect effects of cosolvents on the stability of proteins comes from the entrapment of water molecules by the cosolvents. Sugars and polyols are strongly hydrated, therefore they reduce the water molecules available to hydrate proteins. This leads proteins to assume their folded (native) state, which suffers less from water loss. Figure 7 shows the hydration number, n_{H}, of sucralose and sucrose as a function of the sugar concentration obtained from the sound velocity data. The values of n_{H} of sucralose and sucrose were 14–16 and 12–14, respectively, depending on the sugar concentration. These values are similar to those reported in the literature.[28] The larger hydration number for sucralose corresponds to the results of neutron diffraction experiments[35] that showed sucralose had a greater number of water molecules in its hydration shell compared with sucrose. As this large hydration number for sucralose compared with sucrose predicts enhancement of protein stability, the water-entrapment hypothesis does not seem to explain the protein destabilization caused by sucralose.

The hydration of sugars in aqueous solution lowers water activity.[36] Miyawaki proposed a thermodynamic model to analyze protein stability with explicit consideration of the change in the hydration state of proteins following the unfolding process; this showed that protein stability was enhanced by a decrease in water activity.[37] Figure 8 shows the water activity determined from the osmolality of the sucralose and sucrose solutions. The water activity monotonically decreased with the sugar concentration for both sucralose and sucrose, with the decrease in the water activity of sucralose being slightly less than that of sucrose. This result indicates that the water-activity effect on the enhancement of protein stability was slightly less for sucralose than for sucrose but did not lead to a decrease in protein stability following the addition of sucralose.

Another indirect effect of cosolvents is the enhancement/disruption of water structure that they induce. Sugars and polyols enhance the hydrogen-bonded structure of water. This strengthens hydrophobic interactions within proteins, resulting in the stabilization of protein structure.[2,3] In contrast, cosolvents that disrupt the water structure destabilize proteins.[21] The degree of water structuration is given by the parameter $B/(M_2V_2)$, in which M_2, V_2, and B are, respectively, the molecular weight, the partial specific volume of the solute, and the viscosity coefficient B, defined as

$$\mu/\mu_0 = 1 + Bm + Cm^2, \tag{8}$$

where μ and μ_0 are the viscosity of the solution and water, respectively, m is the molality of the

solute, and C is a coefficient.[38] In Fig. 9, the viscosity of aqueous sucralose solutions measured in the present study is compared with viscosity values for sucrose obtained from the literature.[31] The viscosity data for the solutions of sucralose and sucrose coincided with each other. The values of $B/(M_2V_2)$ calculated from the data in Fig. 9 were 4.0×10^{-3} g/cm^3 for sucralose and 4.1×10^{-3} g/cm^3 for sucrose, which are almost the same. The viscosity data appeared to show no differences in the degree of water structuration between the sucralose and sucrose solutions. This suggests that, even though sucralose reduces protein stability, sucralose is, like sucrose, a water-structure maker. The enhancement of water structure by sucralose has previously been suggested by Shukla et al., on the basis of results they obtained showing the bulk hydration dynamics of tryptophan in a sucralose solution, measured using time-resolved fluorescence spectroscopy.[9]

Oshima and Kinoshita conducted a theoretical investigation into the stability of proteins in sugar solutions. They showed that the higher denaturation temperature of proteins in sugar solutions was due to the increase in the solvent-entropy gain upon protein folding, which was induced by the enhancement of the entropic correlations among solvent molecules (solvent crowding) in the presence of sugar molecules.[39] A similar theoretical explanation for the stabilizing effect of sugars based on the solvent-entropy change induced by the solvent-excluded volume effect has been proposed by Graziano.[40] Oshima et al. also indicated that the effect of cosolvents on the thermal stability of proteins was determined by a complex function of the diameter of cosolvent molecules and the total packing fraction of the solution. According to their method,[39] the diameter of sugar molecules d_{sugar} and the total packing fraction of the solutions η for both sucralose and sucrose were calculated from the data of the crystal density[31,41] and the mass density of the solutions (Fig. 3). The calculated value of d_{sugar} for sucralose was 0.83 nm, which was almost the same as that of sucrose (0.80 nm). The value of η for the 20 w/w% aqueous solution was 0.43 for both sucralose and sucrose. These results show that the addition of sucralose or sucrose contributes equally to the solvent-entropy gain upon protein folding. Murakami and Kinoshita[42] showed that the addition of polyols and monohydric alcohols stabilized and destabilized proteins, respectively, due to the enhancement and the reduction of the solvent-entropy gain upon protein folding, respectively, using the same theoretical procedure as Oshima et al.[39] Murakami et al.[42] also found that, similar to sugars and polyols, the addition of urea increases the solvent-entropy gain upon protein folding, although urea destabilizes the native structure of proteins. They proposed two essential factors that affect protein stability: the solvent-entropy gain and the loss of protein-solvent interaction energy upon protein folding and concluded that the latter factor was dominant for urea, i.e., the effect of attractive interaction energy between protein and urea surpassed that of the solvent-entropy gain. The case of sucralose in the present study is analogous to that of urea. The physical properties of the sucralose solutions were similar

to those of the sucrose solutions, and there was no evidence that sucralose destabilized proteins via indirect effects through changes in the structure or properties of water. The preferential adsorption revealed by our SAXS measurements indicates that the attractive interaction between protein and sucralose is stronger than that between protein and water. Therefore, the destabilization of protein by sucralose is mainly driven by the direct binding of sucralose to protein rather than indirect effects on the structure and properties of water.

Types of interactions and sites in the binding of sucralose to proteins, though they could not be deduced from the results of the present study, have been a subject of controversy. Shukla et al. demonstrated by using the docking simulation that sucralose bound at the hydrophobic pocket on the surface of lysozyme, at which sucrose did not bind.[9] Simončič and Lukšič carried out the molecular dynamics simulation and showed that sucralose formed more hydrogen bonds with acidic/basic and polar surface amino acid residues of lysozyme.[34] They inferred from the results that electrostatic forces (dipole-dipole, ion-dipole, hydrogen bonds) were predominantly responsible for the binding between sucralose and lysozyme. Zhang et al. concluded that both hydrophobic and hydrophilic amino acid residues took part in the binding interaction between sucralose and whey protein isolate, based on their docking simulation results.[10] Most recently, it emerged that urea, a common chemical denaturant of proteins, interacted favorably with both polar and nonpolar residues of proteins.[43] As sucralose has a dipole moment which is larger than that of sucrose and close to that of urea[8,34] and, at the same time, appears more hydrophobic than sucrose,[9] sucralose would be able to interact with various protein amino acid residues, independently of their physico-chemical properties. The attractive interaction energy destabilizes the native structure of proteins because a greater number of binding sites is available on the surface of unfolded proteins.

4. CONCLUSIONS

Although sugars such as sucrose are commonly used as stabilizer molecules for proteins, we found that sucralose decreased the thermal stability of myoglobin. Protein destabilization by sucralose was mainly due to the preferential adsorption of sucralose to proteins, which we observed using the SAXS technique.

Supporting Information. CD spectra of sucrose and sucralose, electron density of aqueous solutions containing sucrose and sucralose.

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Table 1. Excluded volumes and electron densities of sucralose and sucrose

	Sucralose	Sucrose
Excluded volume ($Å^3$)	397.5	353.7
Electron density ($e/Å^3$)	0.518	0.515

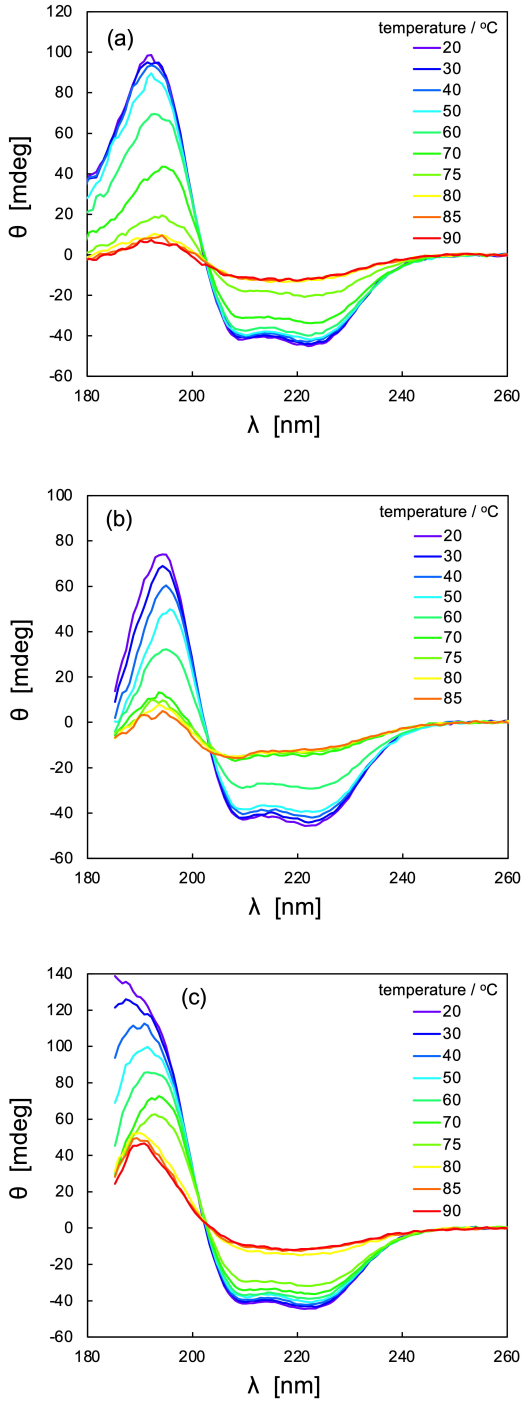


Figure 1. CD spectra of myoglobin in phosphate buffer solvents with and without sugars and measured at various temperatures: (a) without sugars, (b) with 20 w/v% sucralose, and (c) with 20 w/v% sucrose.

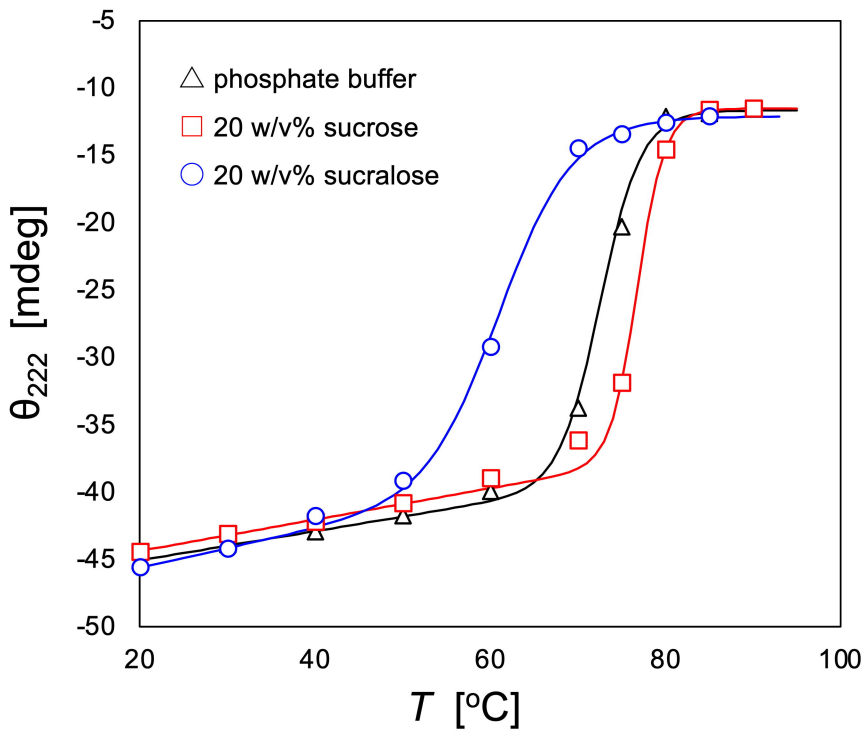


Figure 2. Temperature dependences of ellipticity at 222 nm for myoglobin in phosphate buffer solution without sugars (triangles), with 20 w/v% sucrose (squares), and with 20 w/v% sucralose (circles).

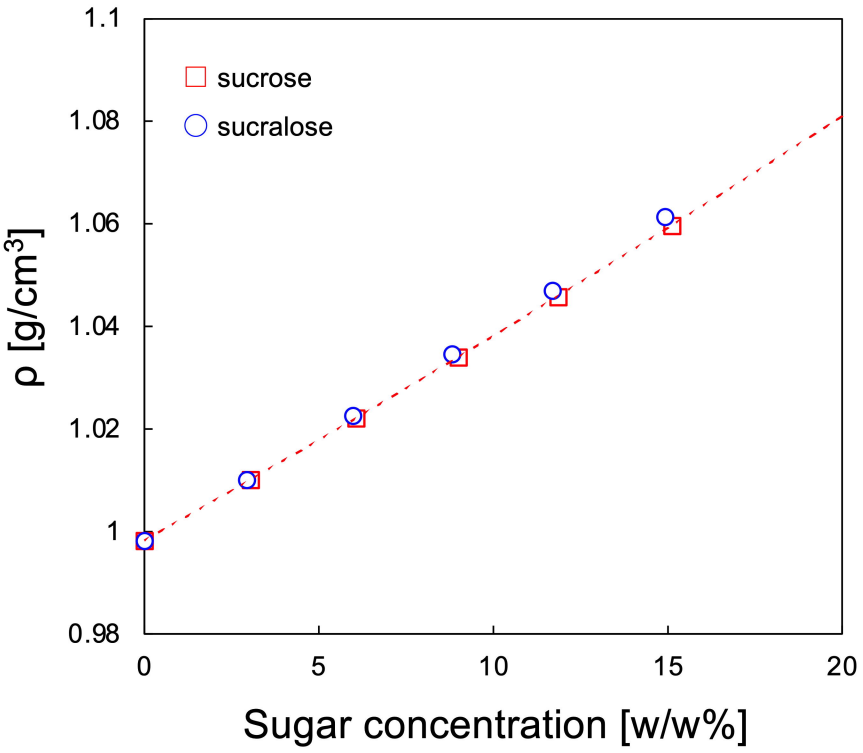


Figure 3. Mass density of aqueous solutions containing sucrose (squares) and sucralose (circles) at various concentrations. The dashed line shows data for sucrose obtained from the literature.[31]

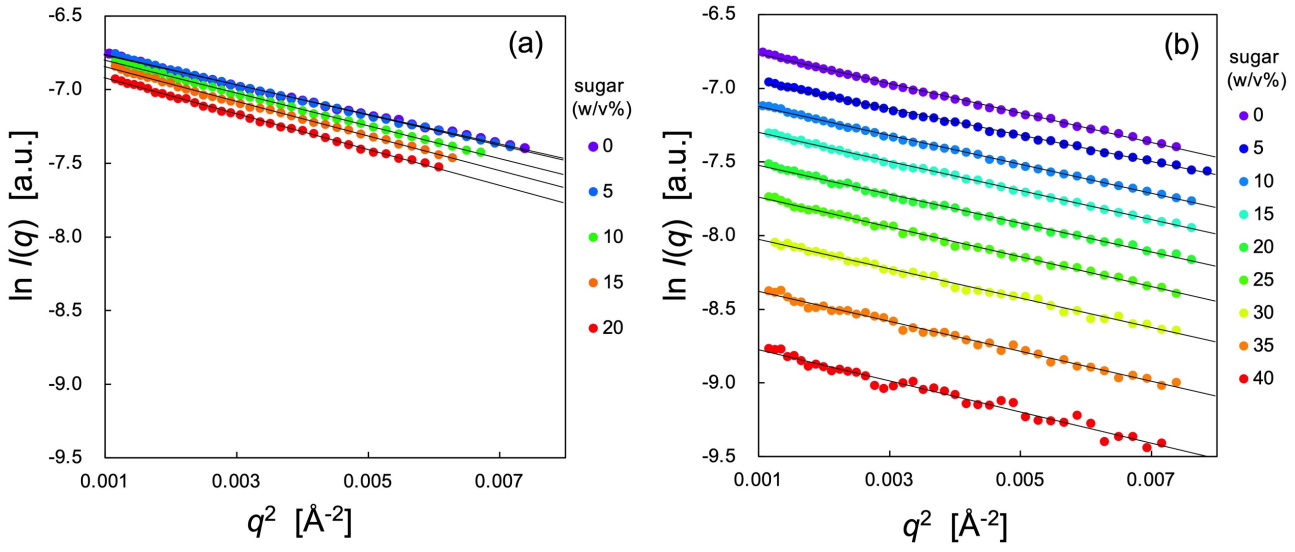


Figure 4. Guinier plots of SAXS curves of myoglobin in HEPES buffer solution containing sucralose (a) and sucrose (b) at various concentrations.

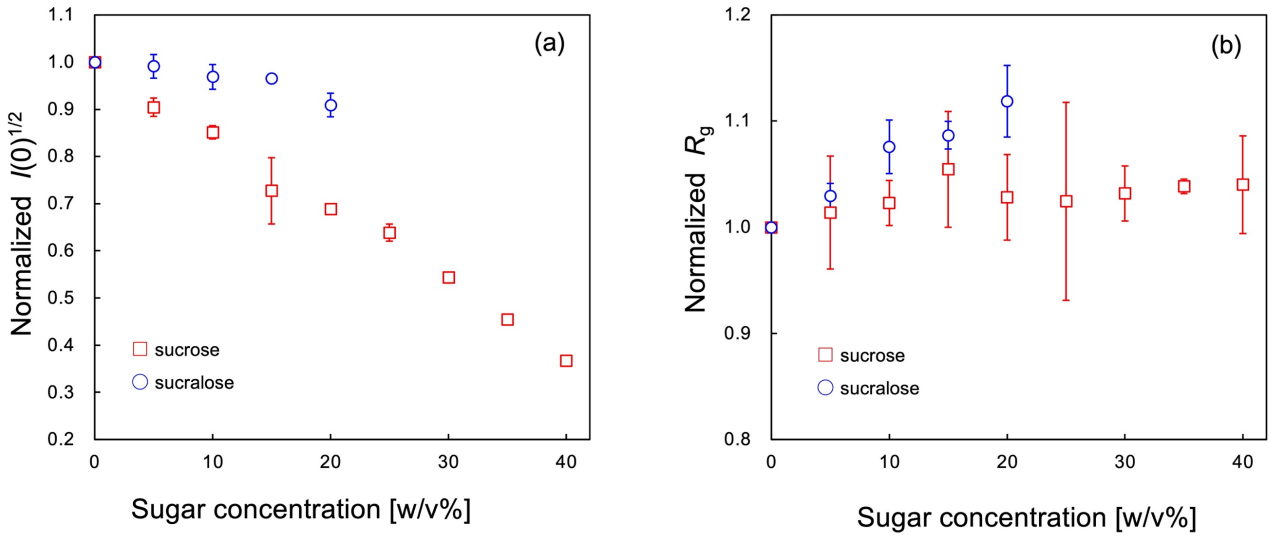


Figure 5. Normalized values of squared zero-scattering intensity (a) and radius of gyration (b) for myoglobin in HEPES buffer solution containing sucrose (squares) and sucralose (circles) at various concentrations.

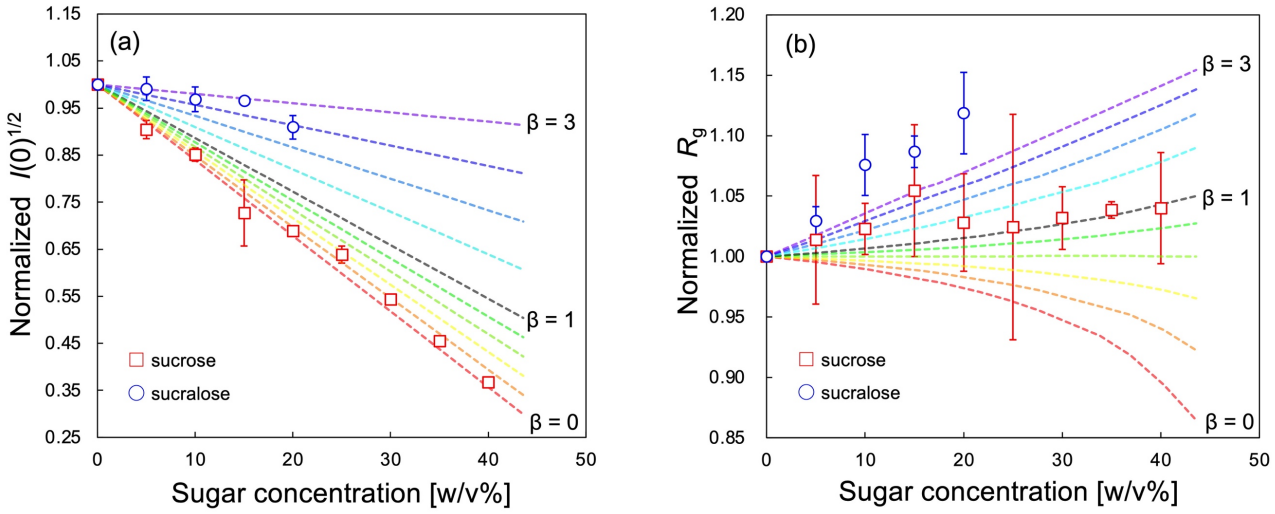


Figure 6. Comparison between the experimental (symbols) and theoretical (dashed lines) values of the normalized squared zero-scattering intensity (a) and the normalized radius of gyration (b). The symbols are the same as in Fig. 5. The theoretical curves obtained are for β = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, and 3.0, from the bottom to the top.

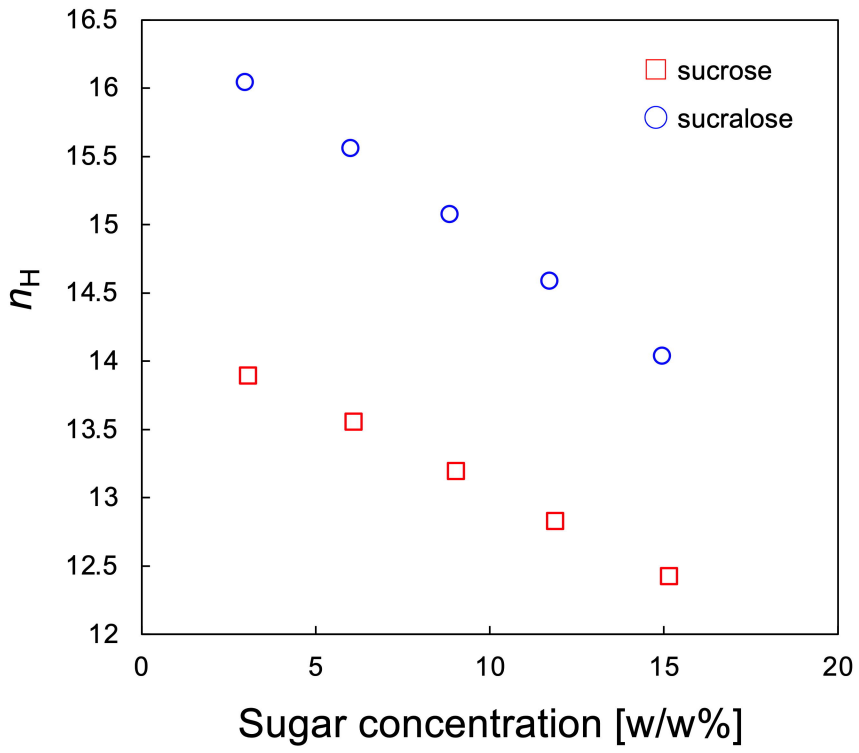


Figure 7. Hydration number of aqueous solutions containing sucrose (squares) and sucralose (circles) at various concentrations.

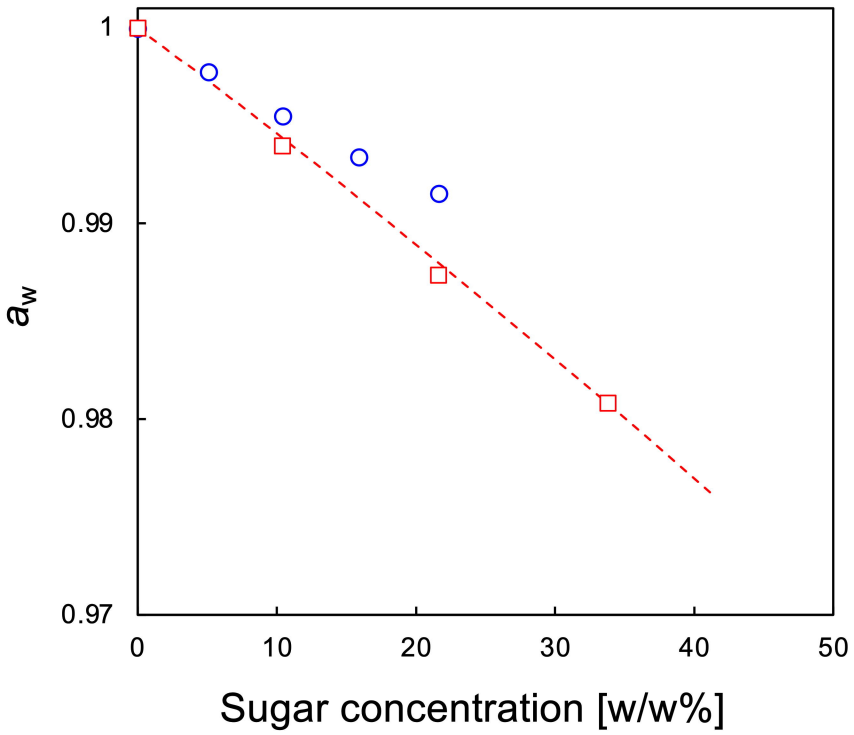


Fig. 8. Water activity of aqueous solutions containing sucrose (squares) and sucralose (circles) at various concentrations. The dashed line shows data for sucrose obtained from the literature.[31]

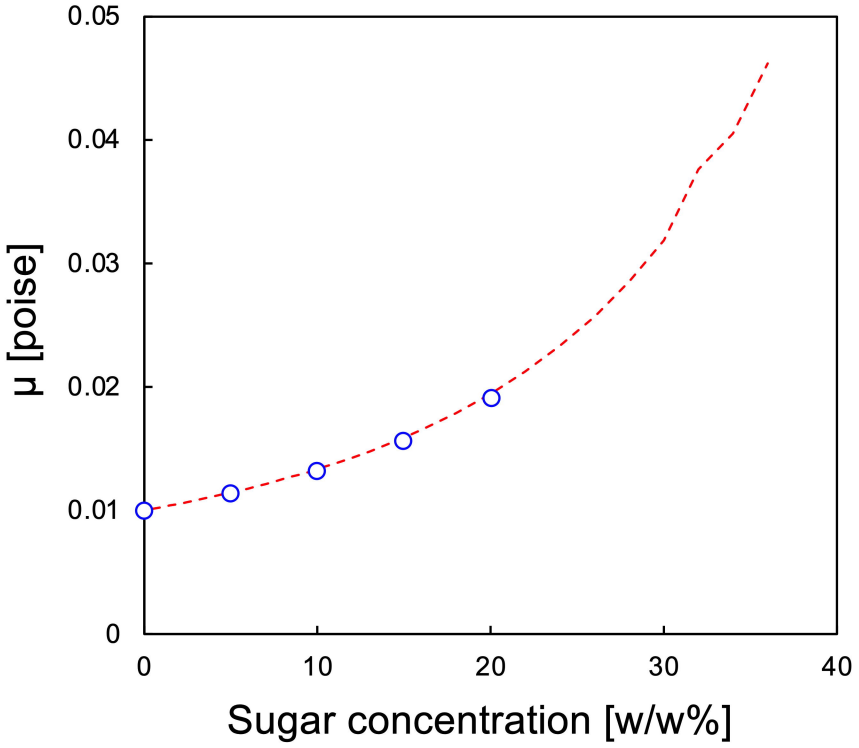
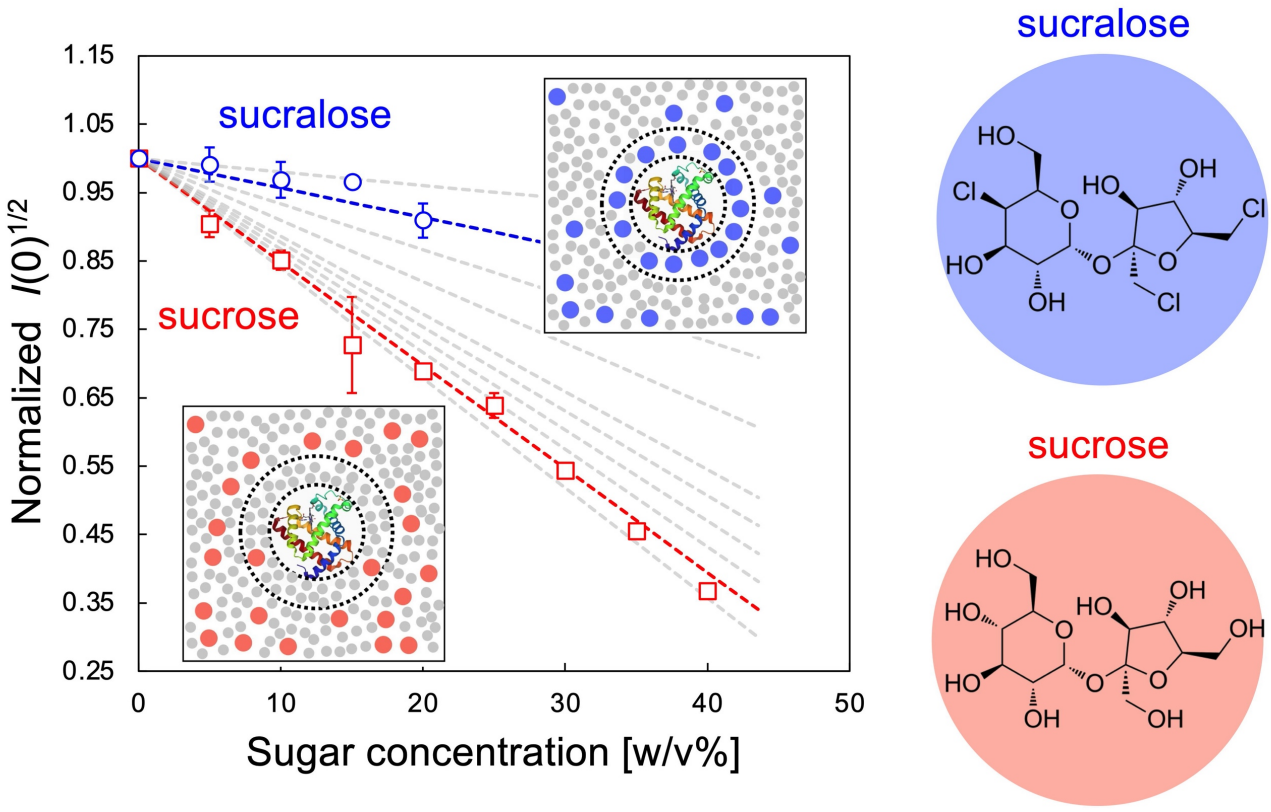


Fig. 9. Viscosity of aqueous sucralose solutions at various concentrations. The dashed line shows data for sucrose obtained from the literature.[31]


sucralose
sucrose
Normalized I(0)^1/2
Sugar concentration [w/v%]
1.15
1.05
0.95
0.85
0.75
0.65
0.55
0.45
0.35
0.25
0
10
20
30
40
50
HO
Cl
HO
OH
HO
OH
Cl
O
O
O
Cl
HO
HO
HO
OH
HO
OH
OH
O
O
O
OH
OH

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