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Effect of Sugars on Rabbit Serum Albumin Stability and Induction of Secondary Structure

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Abstract—The effect of sugars (sucrose, maltose, and glucose) on the thermal and chemical denaturation of rabbit serum albumin (RSA) has been examined by viscosity and far UV circular dichroism measurements. The viscosity measurements indicate a change in the reduced viscosity from 4.18 to 16.23 ml/g in the temperature range from 20 to 90°C. The T_m value for RSA obtained by viscosity measurements in the absence of sugar was found to be 63.2°C, but this value increased to 68.4, 70.3, and 73.2°C in the presence of 0.5 M sucrose, 0.5 M glucose, and 0.5 M maltose, respectively. Further, the stability of RSA in the presence of 0.5 M sugars was also investigated by measuring the mean residue ellipticity at 222 nm (MRE_{222}) using chemical (0-6 M guanidine hydrochloride) and thermal (20-90°C) transition processes. At the midpoint of the chemical denaturation, the increase in the MRE values at 222 nm in the presence of 0.5 M sugars were of the same order as the increase in the T_m values, i.e., maltose > glucose > sucrose. Interestingly, a mixture of 0.25 M glucose and 0.25 M fructose showed a cumulative effect on the thermal as well as chemical stability as compared to 0.5 M sucrose alone. In the case of both thermal and chemical denaturation, there was an increase in the MRE_{222} values upon addition of various sugars, this indicating induction of secondary structure in the protein.

Key words: osmolytes, protein stabilization, rabbit serum albumin, circular dichroism, viscosity

In recent years, proteins and other biological systems are finding increasing applications in food industry and as therapeutic agents, clinical diagnostic materials, analytical tools, and biocatalysts. For understanding of many biological phenomena, stability of native structure and conformation of proteins is of fundamental importance [1-3]. Proteins that find their end use as biocatalysts need to be able to function over a considerable period of time. Therefore, they must be rendered stable against deteriorating conditions and, hence, study of thermal as well as chemical inactivation of proteins has gained impetus [4-6]. The stability of proteins depends on the particular environment and the exposure to conditions that can promote chemical deterioration or conformational changes [1, 7]. Conformational destabilization is produced by varying different external conditions, such as extremes of pH, temperature, pressure, and solvent conditions [8, 9]. The ability of naturally occurring osmolytes to protect proteins without disturbing their biological activity would appear to provide an important and general selective advantage for organisms to adapt to environmental stresses. Sugars (polyhydroxy compounds) are among the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure and freezing [10, 11]. The same sugars have been found to be effective stabilizers of native conformation when added at >1 M concentration [12-14]. Hence, studies on the effect of osmolytes on the stability of proteins are important to understand their mechanism of action. In this paper, we present the studies on the effect of various sugars on the stability of RSA.

MATERIALS AND METHODS

Materials. Rabbit serum albumin (RSA) and bovine serum albumin (BSA) used in this study were obtained from Sigma Chemical Co. (USA). After gel chromatography on a Sephadex G-200 column (90 × 1.8 cm), RSA was defatted by the procedure of Chen [15]. All other reagents used were of analytical grade.

Protein estimation. Protein concentration was determined by the method of Lowry et al. [16] and by the gravimetric method.

Viscosity measurements. Viscosity measurements of the protein, in 20 mM sodium phosphate buffer, pH 7.0, were made in a KIMAX viscometer (size 25) with a flow time of 511 seconds for 5 ml distilled water (25°C) assem-

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bled in a Neslab RTE 110 water bath, which was attached to a CD spectrophotometer. The temperature was controlled within ±0.2°C. The times of fall of the protein solutions (t) and the times of free fall for the corresponding blanks (t_0) were determined at least 5 times, and the average values were taken for calculation. The reduced viscosity (η_r) for each sample was calculated using the following equation:

$$\eta_r = \frac{t - t_0}{t_0 c},$$

where c is protein concentration in g/ml. Further, the fraction of denatured protein f_D was calculated using the following equation:

$$f_D = \frac{Y_N - Y}{Y_N - Y_D},$$

where Y_N, Y_D, and Y are the observed parameters for the protein in native, denatured, and transition states, respectively. The equilibrium constant (K_d) was calculated from the following equation:

$$K_d = f_D/(1 - f_D).$$

The K_d values thus obtained were used for the calculation of free energy change (ΔG) by the equation:

$$\Delta G = -RT \ln K_d,$$

where R is the gas constant (0.002 kcal/mol) and T is the absolute temperature. The free energy of stabilization (ΔG_D^0) was obtained by the linear extrapolation of the plot of ΔG versus denaturant to its zero value [17].

CD measurements in the far UV region were made on a Jasco J-720 spectropolarimeter using a quartz cell of 0.1 cm pathlength. A thermostatically controlled Neslab RTE-110 circulating water bath maintained the temperature of the cell holder as desired. CD results were expressed in terms of mean residue ellipticity (MRE) in deg·cm^2·dmol^{-1} according to the equation:

$$MRE = \frac{\text{CD (mdeg)}}{10\, nC_p l},$$

where C_p is the molar concentration of the protein, n is the number of amino acid residues (576 for RSA), and l is the pathlength (0.1 cm for far UV). The percent α-helix was calculated by the method of Chen et al. [18] according to the equation:

$$\alpha = \frac{MRE_{222} - 2340}{30300} \cdot 100.$$

RESULTS AND DISCUSSION

Thermal transition of RSA under native conditions measured by reduced viscosity. The thermal transition of RSA was studied by viscosity measurements in the temperature range 20 to 90°C. As seen from Fig. 1, the transition started at above 40°C with a sharp increase in the reduced viscosity up to 75°C and then remained constant up to 90°C. The reduced viscosity of RSA above 90°C was found to be 16.23 ml/g, which indicates that the protein loses its native conformation. In this case the T_m value was found to be 63.2°C.

Effect of sugars on the thermal stability of RSA. The effect of fixed concentration (0.5 M) of sugars on the T_m values of RSA under native conditions was investigated in 20 mM sodium phosphate buffer, pH 7.0, by reduced viscosity measurements. Figure 2 shows the effect of sucrose, maltose, and glucose on the temperature-induced (20-90°C) denaturation of RSA. For the sake of comparison, the data have been processed in terms of the fraction of denatured protein (f_D), and the curve of the f_D versus temperature suggests that the transition occurs in one step (N↔D). The thermal denaturation of RSA in the above temperature range was found to be reversible as same value of time of fall was observed at 40°C after the temperature of the sample was cooled from 80°C. Maltose was found to be most effective in stabilizing the protein against temperature-induced denaturation, this being evident from Fig. 2, which shows a shift in the transition curve towards higher temperature. Sucrose was found to be least effective in stabilizing the protein. The midpoint of temperature transition for RSA in the absence of sugar changes from 63.2 to 68.4, 70.3, and 73.2°C in the presence of sucrose, glucose, and maltose, respectively.

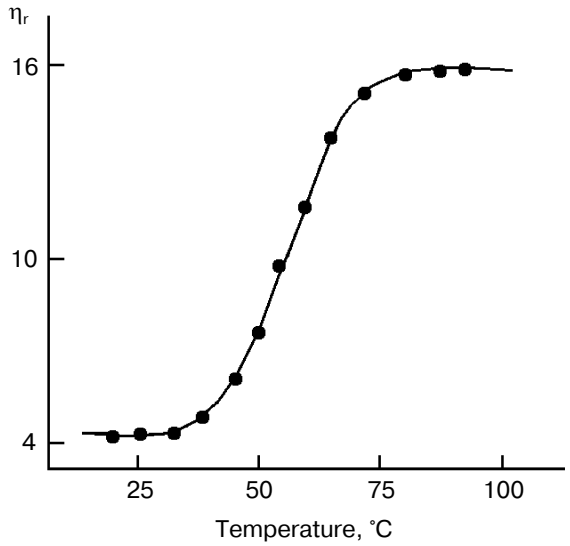

Fig. 1. Effect of temperature on the reduced viscosity (η_r) of RSA in 20 mM sodium phosphate buffer, pH 7.0.

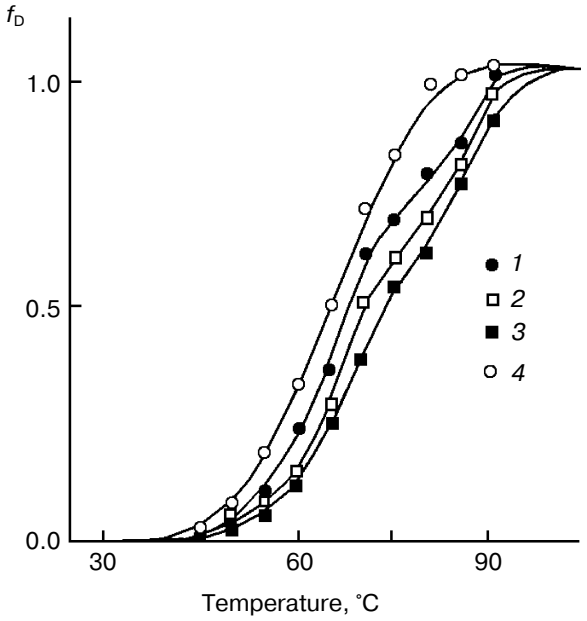

Fig. 2. Effect of different sugars (0.5 M) on the thermal stability of RSA: *1*) sucrose; *2*) glucose; *3*) maltose. The results are expressed in terms of the fraction unfolded as measured by reduced viscosity measurements at various temperatures and compared with those obtained in the absence of sugars (*4*).

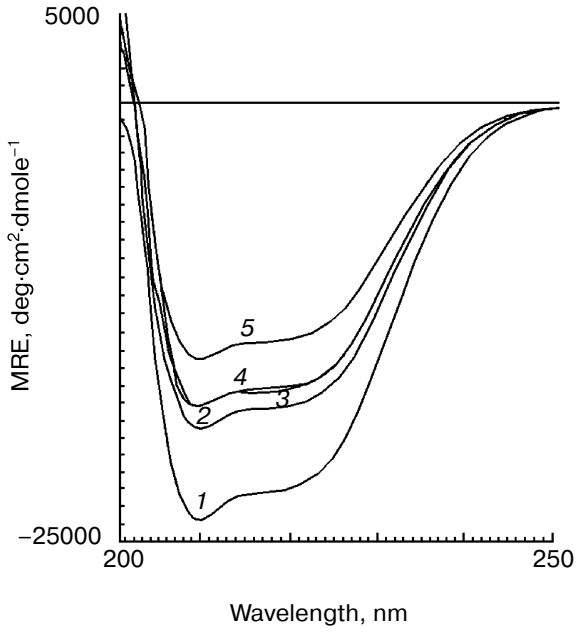

Fig. 3. Far UV CD spectra: *1*) native RSA; RSA at 65°C (*5*) and RSA at 65°C in the presence of 0.5 M sucrose (*4*), glucose (*3*), and maltose (*2*) in 20 mM sodium phosphate buffer, pH 7.0.

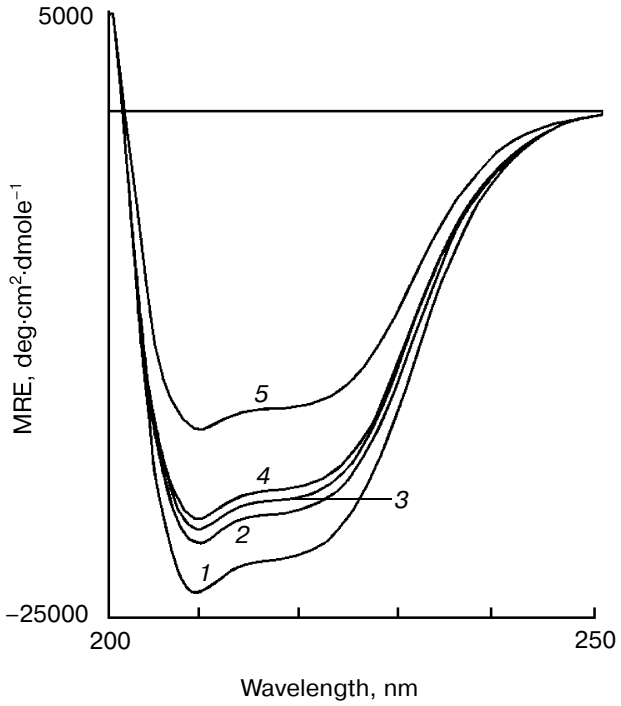

Fig. 4. Far UV CD spectra: *1*) native RSA; RSA in the presence of 1.9 M GnHCl alone (*5*) and in the presence of 0.5 M sucrose (*4*), glucose (*3*), or maltose (*2*) at 25°C.

Effect of sugars on the T_m value as monitored by far UV CD measurements. The midpoint of thermal transition evaluated by MRE_{222} measurement of RSA occurs at around 65°C (data not shown). This is 2°C higher than measured by viscosity measurement, indicating that the tertiary structure is lost much before the secondary structure. We also studied the effect of different sugars on the T_m of RSA by monitoring ellipticity at 222 nm. The MRE value at the melting temperature of RSA was found to be 13,000 deg·cm^2·dmole^{-1}. In the presence of 0.5 M sucrose, 0.5 M glucose, or 0.5 M maltose, the ellipticity at 222 nm at the melting temperature increased to 15,819, 15,912, and 16,849, respectively (Fig. 3 and Table 1). The order of effectiveness in enhancing the thermal stability at the melting temperature is thus as follows: maltose > glucose > sucrose.

The stabilizing effect of the following sugars seems to occur by the preferential exclusion of the respective sugar from the surface of the protein, which increases the chemical potential of the protein. Thus, in the presence of the sugars increase in protein surface area is thermodynamically more unfavorable than in water, and the equilibrium between states is shifted towards that with the smallest surface area [1]. Timasheff et al. have reported that protein structure stabilizing agents are preferentially excluded from the domain of the protein [19]. The preferential hydration of protein in aqueous sucrose medium and their stabilization appear to be related to the increase

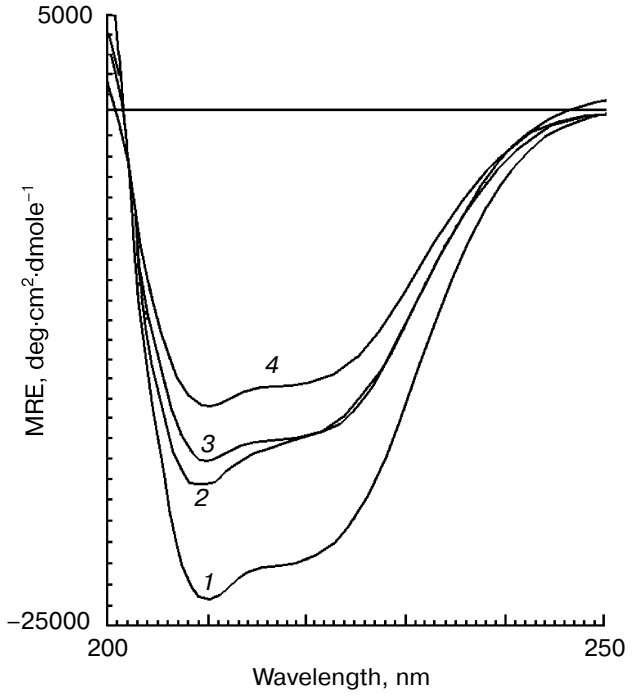

Fig. 5. Far UV CD spectra: *1*) native RSA; *2*) RSA in the presence of 1.9 M GnHCl and 0.25 M glucose + 0.25 M fructose; *3*) RSA in the presence of 1.9 M GnHCl + 0.5 M sucrose; *4*) RSA in presence of 1.9 M GnHCl.

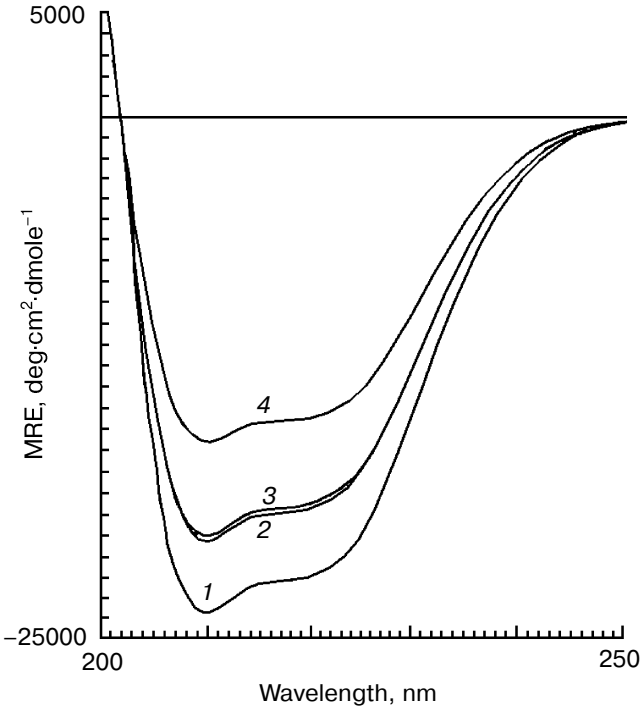

Fig. 6. Far-UV CD spectra of RSA under similar conditions as mentioned in the legend of Fig. 5, except that GnHCl was not added and the spectra were recorded at 65°C.

in the surface tension of water induced by the addition of sucrose. Alternatively, it has been proposed that the stabilizing effect of sugars is the result of decreased hydrogen bond rupturing capacity of the medium [20-23]. The dominant factor in protein stabilization is the enhancement of the structure of the medium or of the solvation layer of the protein.

Chemical denaturation of RSA. The chemical denaturation of RSA was monitored by far UV CD measurements at 222 nm. Effect of the sugars was also investigated by the chemical denaturation method in the presence of guanidine hydrochloride (GnHCl). The midpoint of transition (1.9 M) was taken for further experiments. The increase in MRE value was found to be in the following order: maltose > glucose > sucrose as shown in Fig. 4. Interestingly, these results indicate resistance to the loss of secondary structure in the presence of different sugars during GnHCl-induced denaturation, which means that

Table 1. Effect of different sugars on the stability of RSA at 65°C as measured by MRE at 222 nm

Conditions	MRE_{222}, deg·cm²·dmol⁻¹	α-Helix content, %
RSA	−21 500	61.0
RSA at 65°C	−13 000	33.9
RSA at 65°C + 0.5 M sucrose	−15 800	42.9
RSA at 65°C + 0.5 M glucose	−15 900	43.2
RSA at 65°C + 0.5 M maltose	−16 800	46.2

Table 2. Effect of different sugars on the stability of RSA in 1.9 M GnHCl as measured by MRE_{222}

Conditions	MRE_{222}, deg·cm²·dmol⁻¹	α-Helix content, %
RSA	−21 500	61.0
RSA + 1.9 M GnHCl	−14 100	37.2
RSA + 1.9 M GnHCl + 0.5 M sucrose	−18 400	51.1
RSA + 1.9 M GnHCl + 0.5 M glucose	−18 700	52.0
RSA + 1.9 M GnHCl + 0.5 M maltose	−19 400	54.3

the sugars provided marked stability to RSA against chemical denaturation. The values of MRE at 222 nm and the α-helical contents in the presence of different sugars are listed in Table 2.

Cumulative effect of sugars on protein stability. The far UV CD spectra of RSA at the midpoint for GnHCl (1.9 M) and at the midpoint of thermal denaturation show the same amount of increase in the MRE value at 222 nm in the presence of 0.5 M sucrose as in the presence of a mixture of 0.25 M glucose and 0.25 M fructose, as shown in Figs. 5 and 6. These results indicate that the sugars brought about induction of secondary structure and stabilization of the protein. GnHCl denaturation was also performed in the presence of a mixture of 0.25 M glucose + 0.25 M fructose and 0.5 M sucrose alone (data not shown). The ΔG_D^0 for (0.25 M glucose + 0.25 M fructose) was 5.31 kcal/mol as compared to 4.92 kcal/mol for 0.5 M sucrose alone.

In conclusion, we can say that three different sugars taken in this study show thermal stabilization of RSA and induction of secondary structure to some extent. Interestingly, 0.25 M glucose and 0.25 M fructose show the same stabilizing effect as 0.5 M sucrose.

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