

Thermal Denaturation and Coagulation of Whey Proteins: Effect of Sugars¹

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ABSTRACT

The thermal coagulation of unfractionated whey proteins was inhibited by various sugars. The disaccharides, sucrose and lactose, were most effective, and the amino sugar, glucosamine, least effective in this respect. Ultraviolet absorption and light-scattering measurements on the thermal denaturation and coagulation of both unfractionated and individual whey proteins (α -lactalbumin, β -lactoglobulin, and bovine serum albumin) showed that sucrose promotes the denaturation of these proteins but inhibits their subsequent coagulation. These results are interpreted in terms of the effect of sucrose on the hydrophobic interactions between solvent and protein.

INTRODUCTION

Although sugars are extremely common biochemicals, comparatively little interest has been shown in their effect on protein structure and denaturation. Beilinson (5) noticed the inhibition of thermal denaturation of ovalbumin by sucrose. Ball et al. (4) found that many sugars inhibit the appearance of sulfhydryl groups on heating ovalbumin. In an extensive study on the effect of many organic molecules on the rate of ovalbumin denaturation by urea, Simpson and Kauzmann (19) noticed that molecules with a large number of hydroxyl groups (e.g., sucrose, glycerol) inhibited protein denaturation by urea. In contrast, molecules with more nonpolar character (e.g., dioxane, ethanol) accelerated the rate of ovalbumin denaturation in the same conditions.

The involvement of monosaccharides and disaccharides in protein chemistry is evident. Sucrose stabilizes tubulin and other proteins through nonspecific preferential hydration (21). α -Lactalbumin (α -LA) binds to regions on lactose synthase with an increased affinity for glucose (13). Up to 8 mol of glycosyl residues can be covalently attached to bovine β -lactoglobulin (β -LG) per mole via gluconic or melibiononic acids, resulting in high solubility even at low ionic strength or at the isoelectric pH, and improved heat stability (11).

Sucrose inhibits sulfhydryl-disulfide exchange of α -LA and β -LG during heat treatment (22). Hillier (8) showed that lactose inhibits whey protein aggregation on heat treatment. As heat treatment is used widely as a means of improving the storage qualities of milk products, the effect of sugars on the thermal stability of whey proteins is of great interest. In this work the effect of various sugars on whey protein coagulation has been studied and a more detailed analysis of the effect of sucrose on whey protein thermal denaturation is given.

MATERIALS

Lyophilized bovine serum albumin (BSA), α -LA, and β -LG were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were analytical grade.

Acid whey was prepared by the method of McKenzie (17) from partly skimmed milk (2% milk fat) (Silverwoods Dairy, Peterborough, Ont., Can.). The whey solution (adjusted to pH 6 with .1 M NaOH) was dialyzed against 4 L of .05 M sodium phosphate buffer pH 7, for 24 h. The protein concentration was diluted to 3.5 mg/ml in all preparations.

METHODS

Determination of Protein Coagulation.

Ten-milliliter samples of whey protein solution were heated in test tubes in a water bath to 80°C and maintained at this temperature for 5 min. The solutions were cooled on ice and centrifuged at 10,000 $\times$ g in a

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TABLE 1. Effect of sucrose on thermal coagulation of whey proteins.

Sucrose concentration	Protein concentration unprecipitated ¹	Precipitated	Summary ²
(mg/ml)	(mg/ml)		
0	1.0	1.9	2.9
25	1.5	1.7	3.2
40	1.8	1.6	3.4
60	2.1	1.6	3.7
80	2.4	1.6	4.0
100	2.6	1.5	4.1

¹ Lowry assay as bovine serum albumin.² Initial concentration of protein was 3.5 mg/ml.

Sorvall superspeed bench centrifuge for 20 min. The supernatant was collected and the protein estimated by the Lowry method. The precipitate was resuspended in 10 ml of .05 M phosphate buffer, pH 7, and recentrifuged under the same conditions. The supernatant was discarded and the tubes dried and weighed. Sugar concentrations were from 20 to 200 mg/ml. Typical results obtained for sucrose are listed (Table 1) in detail to show the progressive effect of increased sucrose content and to show the mass balance. Results obtained for all the sugars are compared in Table 2. Recoveries exceed 100% in the presence of high sucrose concentration. Some sugar appears to be bound.

Ultraviolet Spectroscopy

Experiment 1. 100 μ g/ml solutions of α -LA, β -LG, and BSA in .05 M phosphate buffer, pH 7, were heated to various temperatures in the range 25 to 100°C in test tubes in a water bath and maintained at constant temperature for 1 min. Solutions were then cooled on ice. Absorption spectra of the heat-treated samples were measured on a Pye Unicam SP1800 spectrophotometer. The experiment was repeated with sucrose (100 μ g/ml) added to the protein preparations. Maximum absorbance ($\lambda_{\max}$), with and without added sucrose, was plotted against temperature in Figure 1.

Experiment 2. Samples of whey (10 ml) were heated to a given temperature, maintained at that temperature for 1 min, cooled on ice,

and any precipitate centrifuged at 10,000 $\times$ g for 20 min. Supernatants were collected and the protein concentration in each tube estimated by a Lowry assay. The supernatants were diluted to 400 μ g/ml in .05 M phosphate buffer. The experiment was repeated with 100 μ g/ml sucrose added to the whey samples. The UV absorbance spectra of the diluted, heat-treated samples were taken. The absorbance at $\lambda_{\max}$ (278 nm) was plotted against temperature in Figure 1.

Experiment 3. Samples of whey preparations (1 mg protein/ml) were placed in the sample cell of the spectrophotometer and the temperature raised stepwise at a rate of 1°C/min using an electrically heated cuvette holder controlled by a rheostat. The temperature of the sample and reference cell was measured using a YSI telethermometer (model 42SC) with a YSI 400 probe (Yellow Springs Instruments, OH). The absorbance spectrum was measured at 10°C intervals until the temperature reached 60°C and at 4°C intervals thereafter. The method of Leach and Scheraga (14) was used to calculate the contribution of light scattering to the absorbance between 250 and 300 nm. The absorbance (corrected for light scattering) and the contribution of light scattering to the spectrum at 280 nm are plotted against temperature in Figure 2. The experiment was repeated with 100 μ g/ml of sucrose added to the whey samples.

TABLE 2. Effect of sugars on the thermal coagulation of whey proteins.¹

Sugar	mg Protein precipitate	mg Protein precipitate
	(50 mg sugar/ml)	(100 mg sugar/ml)
Sucrose	16.2	14.7
Fructose	16.5	15.7
Glucose	16.2	14.9
Maltose	16.4	15.3
Lactose	16.3	14.9
Glucosamine	17.1	16.5
None	19.0	19.0

¹ Ten milliliters of whey solution (3.5 mg protein/ml) were heated to and maintained at 80°C for 5 min in the presence of various sugars. The precipitate was collected by centrifugation, dried, and weighed.

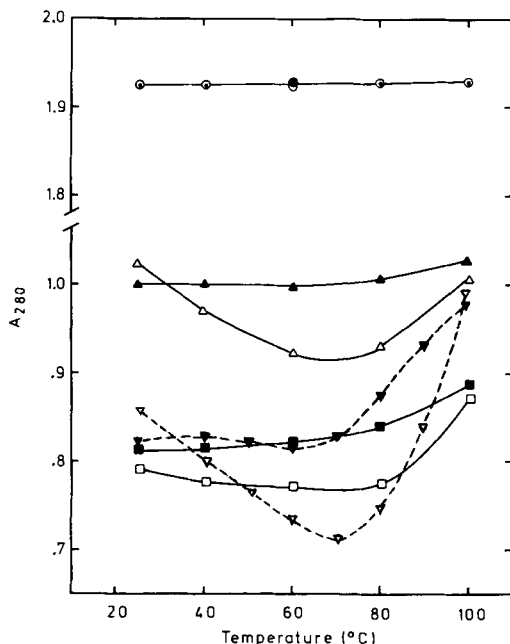


Figure 1. Effect of heat treatment on the absorbance of commercial solutions of individual whey proteins (100 $\mu\text{g}/\text{ml}$) and of whey protein solution (400 $\mu\text{g}/\text{ml}$) at maximum absorbance (277 to 281 nm) with and without 100 $\mu\text{g}/\text{ml}$ added sucrose: α -lactalbumin (α -LA) ($\circ$ - $\circ$); α -lactalbumin plus sucrose ($\bullet$ - $\bullet$); β -lactoglobulin (β -LG) (Δ - Δ); β -LG plus sucrose ($\blacktriangle$ - $\blacktriangle$); bovine serum albumin (BSA) ($\square$ - $\square$); BSA plus sucrose ($\blacksquare$ - $\blacksquare$); whey solution (∇ - ∇); whey solution plus sucrose ($\blacktriangledown$ - $\blacktriangledown$).

RESULTS

The initial experiment showed that sugars inhibited the thermal coagulation of whey proteins when the whey solution was heated to 80°C for 5 min. The effect of increasing sucrose concentrations on the amount of protein coagulation is given in Table 1. There was monotonic decrease in the amount of precipitate and an increased amount of protein remaining in solution as sucrose concentration increased from 20 to 200 mg/ml. This experiment was repeated with several sugars, both monosaccharides and disaccharides, and the results are given in Table 2. Sucrose, lactose, and glucose were the most effective at inhibiting whey protein coagulation in these conditions. The amino-sugar glucosamine was far less effective in this respect than the non-substituted sugars. Sucrose was chosen for further experiments.

Ultraviolet analysis of standard whey proteins heated to 25 to 100°C in the presence and absence of sucrose (Figure 1) showed changes in the absorbance spectrum of β -LG and BSA when sucrose was present. Absorbance of β -LG at λ_{max} (277 nm) increased when the protein was heated to 60 to 90°C in the presence of sucrose than when the protein alone was heated. The effect of sucrose on the spectrum of BSA was not as marked. The difference in absorbance of both β -LG and BSA samples disappeared when the samples were heated to 100°C. No change was noticed in the spectrum of α -LA on heating with or without sucrose present.

A parallel experiment was attempted using the whey preparation, but on heating to 70°C, the protein started to precipitate out of solution, which made the solution turbid and hard to analyse. One method of overcoming this difficulty was by centrifuging off any precipitate formed. The solutions were then diluted, after Lowry protein determination, to 400 $\mu\text{g}/\text{ml}$ so that any change in absorbance would be a function of the absorbance characteristics of the protein and not due to concentration differences. As seen in Figure 1, absorbance of the whey solution, without added sugar, decreased gradually at λ_{max} (other wavelengths, not shown, gave similar curves) when heated to temperatures up to 80°C. Between 80 and 100°C absorbance increased quite sharply, indicating a change in the environment of one or more of the aromatic residues. The addition of sucrose to the preparation changed the spectra quite significantly. In this case, absorbance at λ_{max} did not decrease, but gradually increased from 20 to 80°C, then increased more rapidly from 80 to 100°C.

Figure 2 shows the effect of sucrose on both the absorbance and light scattering of whey protein solutions heated within the spectrophotometer. In the absence of sugar, the absorbance at 270 and 260 nm gave similar curves). There was a large light scattering contribution to all spectra at all temperatures, but this increased sharply when the temperature of the solution rose above 72°C, which shows that protein aggregation increases above this temperature.

The addition of sucrose to the whey solution inhibited protein aggregation as measured by

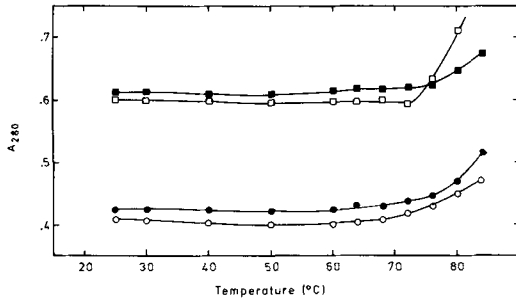


Figure 2. The effect of temperature on the absorbance and light scattering of whey protein solution. One milligram per milliliter whey solution at absorbance at 280 nm (A_{280}) (○-○); turbidity at 280 nm (T_{280}) (□-□); 1 mg/ml whey solution + 100 µg/ml sucrose A_{280} (●-●); T_{280} (■-■). See text for details.

the increase in light scattering. The observed increase in the absorbance at 280 nm became evident between 50 and 60°C in contrast with 64°C in the absence of sucrose.

DISCUSSION

Figure 1 shows that the diluted whey mixture had absorption characteristics similar to those of β -LG on heating with and without sucrose present. Addition of sucrose to the whey sample gave increased absorbance at λ_{max} (278 nm) after heat treatment up to 90°C. The difference disappeared after heating to 100°C. Heat-induced aggregation is usually preceded by a conformational change in the protein (1), shown in this case by the increase in absorbance at 278 nm characteristic of the exposure of a tryptophan residue. This occurs in the whey mixture on heating the protein molecules to temperatures in excess of 60°C. However, in the presence of sucrose these changes are found at lower temperatures.

Heat treatment to 100°C is sufficiently severe to eliminate the effect of sugars in promoting the conformational change. Hillier (8) showed that although the kinetic constants of whey denaturation at 80°C were affected by increased lactose concentrations, no change was observed during denaturation at 133°C. Holt et al. (9) found that the addition of aldohexoses and ketohexoses did not increase the stability of skim milk at 120°C.

The results in Figure 2 support the conclusions of the earlier experiments. Addition of sucrose inhibits whey coagulation but also

favors a conformational change in at least one of the protein molecules; β -LG probably provides the most significant contribution to this effect.

Figure 3 illustrates a postulated mechanism for the effect of sugars on whey denaturation and coagulation. In the absence of sugar β -LG and BSA unfold on heating to denatured monomers. This denaturation was shown by UV analysis on standard whey proteins. Alpha-LA and β -LG form a complex by sulfhydryl-disulfide exchange (10, 22) and BSA aggregates by a similar mechanism (2). Hillier (8), in a kinetic analysis of whey denaturation between 40 to 100°C, showed that although the three whey proteins denatured at different rates there were remarkable similarities in the temperature dependence curves. She concluded that the denaturation of each protein was intimately connected with the denaturation of the others. It seems likely that BSA is also involved in the overall complex formation to give an α -LA- β -LG-BSA complex. However, these complexes have not been isolated or identified.

The addition of sugars stabilizes a denatured form of β -LG (6), probably the monomers (Stainsby, 1978, personal communication), against further unfolding. The exposure of sulphhydryl groups or disulphide bridges and the subsequent aggregation of the molecules is thus prevented.

It is now accepted that hydrophobic interactions play an important role in maintaining the conformational integrity of globular proteins, and they also appear to be involved in the formation of complex intramolecular structures, e.g., α -helices (7). The main effect of sugars on the conformation of proteins involves changes in hydrophobic interactions (12). Two types of hydrophobic interaction have been recognized: "pairwise" interaction in which hydrophobic groups associate but otherwise remain in contact with water, and complete transfer of hydrophobic groups from water to a nonpolar environment (3).

The free energy of pairwise hydrophobic interaction between CH_2 groups was found by studying ion-pair formation by long-chain alkyltrimethylammonium carboxylates; (18) ion pairs were stronger in 25% (wt/wt) sucrose than in water. However, the driving force (decrease in Gibbs free energy) for transfer

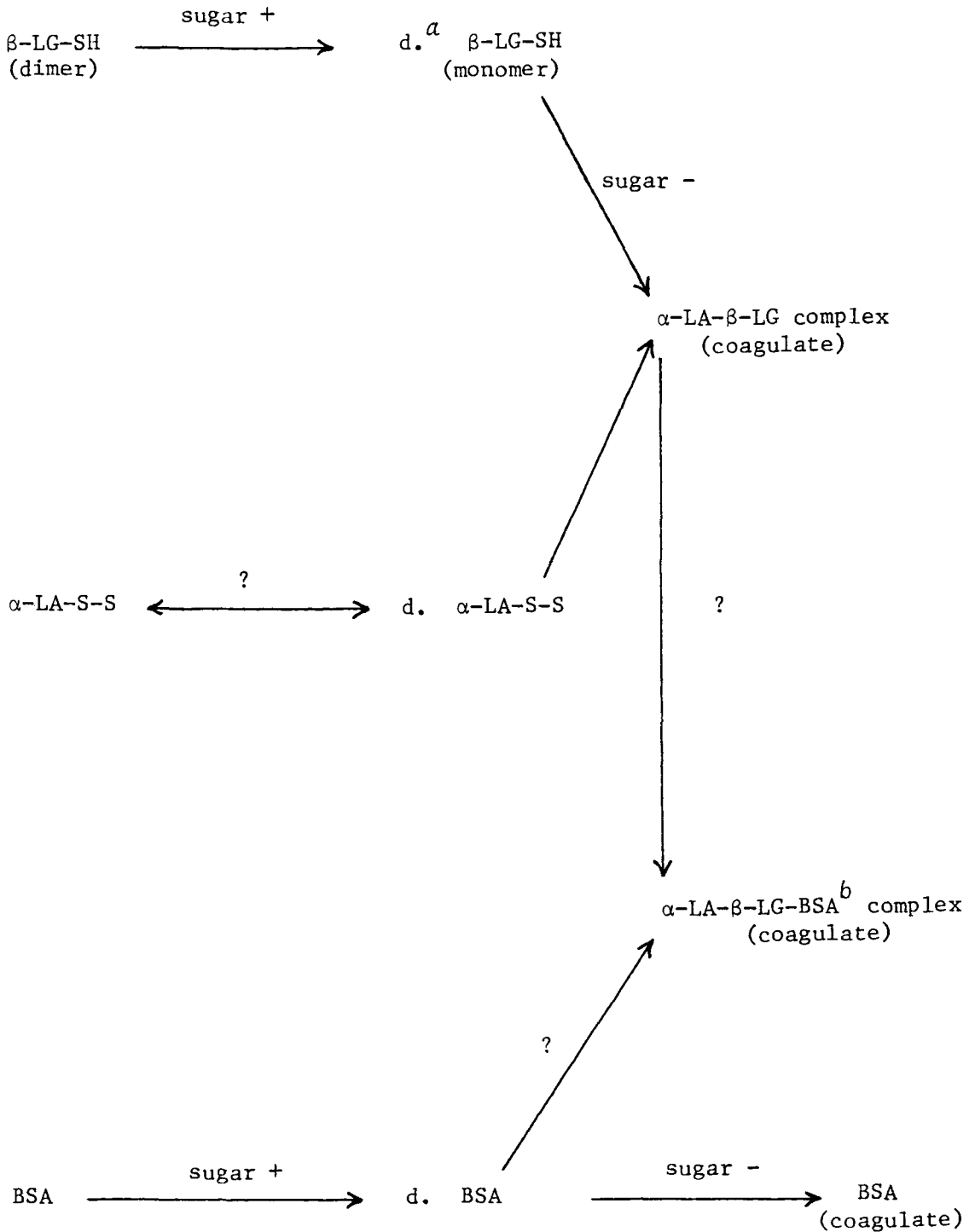


Figure 3. Inhibition of whey protein coagulation by sugars: proposed mechanism: a) d = denatured molecule; b) The order of complex formation and the composition of the complex are not known. α -Lactalbumin (α -LA)- β -lactoglobulin (β -LG)-bovine serum albumin (BSA) has been chosen to represent the final coagulated product, which contains some of each of the whey proteins, and is not meant to imply that a complex containing one molecule of α -LA, β -LG, and BSA linked in this way has been isolated.

of nonpolar molecules from water to a nonpolar environment was decreased in sucrose solutions as compared with water (20). Sucrose promotes pairwise hydrophobic interaction between nonpolar molecules or side chains but not their complete transfer to the hydrophobic core. Thus, addition of sucrose to a protein solution would relax the driving force, pushing hydrophobic residues to the nonpolar environment of the center of the molecule and stabilize pairwise interaction. In this way an unfolding of the native molecule to maximize pairwise interaction would be favorable in sugar solution.

The conclusions reached in this paper, that sugars inhibit whey protein thermal coagulation but promote a conformational change in at least one of the protein molecules, can be explained by the phenomena just described. Heat treatment of any solution increases the motion of the constituent molecules, thus increasing the strain on the weak interactions, e.g., hydrogen bonds, which maintain the tertiary structure of a protein (1). The strength of the forces maintaining tertiary structure, therefore, determine the heat treatment required to denature the molecule. Addition of sucrose to a protein solution decreases the tendency of hydrophobic residues to occupy the interior of the molecule and favors pairwise interactions between hydrophobic residues (i.e., a denatured state). If the strength of the hydrophobic interactions maintaining tertiary structure is decreased on addition of sucrose, the temperature required to denature the molecule would also be lowered.

The strength of pairwise interactions between hydrophobic residues has not yet been determined, but is stronger in sugar solution than in water. Increased stability of denatured protein molecules to further unfolding and aggregation in the presence of sucrose would be a logical outcome of this greater strength of pairwise interactions.

The major obstacle in understanding the mechanism of the effect of sugars on the thermal denaturation and coagulation of proteins is a lack of knowledge on the nature of protein-solvent, particularly protein-water, interactions (16). Until this problem is solved, the effect of other cosolvent molecules on proteins will not be fully understood.

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