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EFFECT OF TETRASODIUM TRIPOLYPHOSPHATE ON THE FREEZE-CONCENTRATED GLASS-LIKE TRANSITION TEMPERATURE OF SUGAR AQUEOUS SOLUTIONS

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Abstract

The freeze-concentrated glass-like transition temperatures (T_g'$_2$s), so-called "ante-melting temperature" or "ice-melting temperature", of tripolyphosphate-sugar aqueous solutions prepared with various sugars (ribose, sorbitol, glucose, maltose, sucrose, and trehalose) were investigated by using differential scanning calorimetry to evaluate the effect of tetrasodium tripolyphosphate on the T_g'$_2$ of sugar aqueous solutions. The T_g'$_2$s of tripolyphosphate-sugar aqueous solutions were higher than those of tripolyphosphate or sugar aqueous solutions and converged in a narrow temperature range of 238 to 243 K. Furthermore, a study of the T_g'$_2$s of tripolyphosphate-glucose aqueous solutions adjusted to various ratios indicated that the T_g'$_2$ increment depended on the ratio and that another glass-like transition appeared at a temperature below the T_g'$_2$ by increasing the ratio of tripolyphosphate. The drastic increase in the T_g'$_2$s of sugars with the addition of tripolyphosphate will be useful for improving the cryostabilization of biomaterials.

Keywords: tripolyphosphate, sugar aqueous solution, freeze-concentrated glass-like transition, cryostabilization, DSC

INTRODUCTION

When an aqueous solution freezes, the crystallization of water causes freeze-concentration, and eutectic separates out from the freeze-concentrated solution phase at the eutectic temperature (T_e) under the equilibrium condition by further cooling. Some kinds of aqueous solution (*e.g.*, sugar or polymer), however, cannot form eutectic at the T_e in the usual cooling process because of high viscosity caused by the freeze-concentration; it turns into a glassy state at the maximally freeze-concentrated glass transition temperature (T_g'), instead (1-3). In this glassy state, molecular mobility is extremely limited by the high viscosity of 10^{12} Pa s or more. Thus, an unstable material or a reactive molecule trapped in the maximally freeze-concentrated glass is very stable for a long term. This "glass transition concept" has been widely recognized as a cryostabilization mechanism of frozen biochemical, pharmaceutical, and food materials. To accumulate basic knowledge on the glass transition behavior of related materials, the T_g's of many kinds of aqueous solutions have been examined extensively (4-20).

The glass transition of amorphous materials has been often investigated by using differential scanning calorimetry (DSC). In the case of freeze-concentrated systems, however, DSC heating thermograms usually exhibit two glass-like transitions that are independent of the initial solute content of the aqueous solutions; first a small endothermic shift and subsequent large one are observed. In this paper these glass-like transition temperatures are denoted as $T_g'_1$ and $T_g'_2$, respectively. The origin of two glass-like transitions has been discussed extensively, and four major interpretations are proposed as following: 1, $T_g'_1$ corresponds to a maximally freeze-concentrated glass transition and $T_g'_2$ corresponds to an ante-melting temperature where the viscous flow initiates in the amorphous region occurring a few Kelvin below the beginning of ice-melting (4-6); 2, $T_g'_1$ corresponds to a maximally freeze-concentrated glass transition and $T_g'_2$ is the temperature of the beginning of ice-melting (7-10); 3, $T_g'_1$ and $T_g'_2$ correspond to glass transitions of two phases of different solute concentrations, and the former is due to the transition in a freeze-concentrated region containing a large amount of unfrozen water and the latter is due to the transition in a maximally freeze-concentrated region (11,12); 4, $T_g'_1$ and $T_g'_2$ corresponds to a single maximally freeze-concentrated glass transition involving an enthalpy relaxation process (13,14). It seems that the origin of two glass-like transitions is not completely understood. However, the facts that the collapse and recrystallization of freeze-concentrated solutes usually occur at a temperature above the $T_g'_2$ mean that $T_g'_2$ is a more important parameter than $T_g'_1$ in the industrial application to the cryopreservation and lyophilizing of biomaterials (17,18).

It was recently reported that oxyanions (*e.g.*, borate and phosphate) mixed with sugars exhibit an increase in viscosity of the solution, $T_g'_2$ of the frozen system, and glass transition temperature (T_g) in the hydrated state, compared with those of single sugar systems (21-28). Miller *et al*. demonstrated that the viscosity or the T_g of a sodium tetraborate-trehalose mixed aqueous system increases more than that of trehalose alone (21,22), and that the storage stability of enzyme (21) or bacteria (23) trapped in the glassy state improved with decreased molecular mobility. Furthermore, Izutsu *et al*. investigated the effect of sodium tetraborate on the $T_g'_2$s of sugar and polyol aqueous solutions and reported a drastic increase in the $T_g'_2$s with the addition of borate (24,25). It was considered that the borate accomplishes this by forming a reversible cross-linked network between hydroxyl compounds (21-25,29-31). On the other hand, Ohtake *et al*. pointed out a toxicity problem of borate in pharmaceutical and/or food applications; they focused on monophosphate (*i.e.*, KH_2PO_4 and K_2HPO_4), which was expected to have an effect similar to that of borate from the viewpoint of molecular structure (26,27). They demonstrated that the T_g of monophosphate-trehalose or sucrose mixtures is higher than that of trehalose or sucrose alone; increasing the T_g improved the thermal stability of phospholipids (26). In addition, Ekdawi-Sever *et al*. found that the viscosity of monophosphate-sugar aqueous solution increases exponentially with the monophosphate content (28). Although little is known about the effect of monophosphate on the $T_g'_2$ of sugar aqueous solution, it was expected that the addition of a monophosphate would drastically raise the $T_g'_2$ of the sugar aqueous solution as well as those of the borate-sugar systems. However, since monophosphates easily form eutectic (17,32,33), we have misgivings about the possibility of the devitrification during freeze-thawing or freeze-drying. Then, the use of a tripolyphosphate as a substitute for a monophosphate is proposed because the tripolyphosphate aqueous solution easily turns into a glassy state by freeze-concentration (34) and is expected to raise the $T_g'_2$ of sugar systems as well as that of borates or monophosphates.

The purpose of this study is to evaluate the effect of tetrasodium tripolyphosphate on the $T_g'_2$ of sugar aqueous solutions. For this purpose, first the $T_g'_2$s of tripolyphosphate-sugar aqueous solutions prepared with six kinds of sugar widely used in various fields (ribose,

sorbitol, glucose, maltose, sucrose, and trehalose) were examined by using a DSC. Second, the $T_g'_2$s of tripolyphosphate-glucose aqueous solutions of various ratio of tripolyphosphate were examined in order to investigate the effect of tripolyphosphate on the $T_g'_2$ of the aqueous sugar solution, using glucose as a typical sugar.

MATERIALS AND METHODS

Analytical grade glucose, sorbitol, and trehalose dihydrate were purchased from Sigma Chem. Co., and the other reagents were purchased from Wako Pure Chem. Ind. Ltd.

First, to investigate the effect of tripolyphosphate on the $T_g'_2$s of various sugar aqueous solutions, 5.0% (w/w) aqueous solutions containing a 1.0:1.0 weight ratio of tripolyphosphate:one of the sugars abovementioned were prepared as a typical system. The $T_g'_2$ was examined by using a Shimadzu heat-flux DSC-50. The temperature and heat capacity for the DSC measurement were calibrated with indium and distilled water. As a reference material, α-alumina powder was used. Approximately 10 mg of sample was weighed and sealed in an aluminum DSC pan. The sample was cooled to 210 K at 6 K/min and then heat-scanned up to 300 K at 2 K/min. As a control, the $T_g'_2$s of 5.0% (w/w) tripolyphosphate aqueous solution and 5.0% (w/w) of each sugar aqueous solution were also examined in this study.

Second, to examine the effect of the ratio of tripolyphosphate and glucose on the $T_g'_2$ of the mixture system, 17.5 to 25.0% (w/w) aqueous solutions containing 6.0:1.0, 2.0:1.0, 1.0:1.5, 1.0:1.0, 1.0:4.0, 1.0:6.0, and 1.0:8.0 weight ratio of tripolyphosphate:glucose were prepared. The effect of the initial total solute content of the aqueous solution on the $T_g'_2$ was not taken into consideration since $T_g'_2$ is a thermal property of the maximally freeze-concentrated system. The $T_g'_2$ was examined by the DSC procedure, similar to the above-mentioned method.

RESULTS

DSC heating curves for 5.0% (w/w) tripolyphosphate and sugar aqueous solutions are presented in Fig. 1. Each aqueous solution indicated an endothermic shift in the range of the scanned temperature. Since onset points of the endothermic shifts agreed with the $T_g'_2$s reported by previous studies (1,10,34), we judged the shift to be the freeze-concentrated glass-like transition due to the $T_g'_2$. The other freeze-concentrated glass-like transition that should have been observed at the lower temperature (*i.e.*, $T_g'_1$) was not apparent in this study probably because the initial solute contents of the aqueous solutions were too low to detect the small endothermic shift. The observed $T_g'_2$ values are summarized in Table 1 with the $T_g'_1$ and $T_g'_2$ values in references. DSC heating curves of 5.0% (w/w) tripolyphosphate-sugar aqueous solution containing a 1.0:1.0 weight ratio of tripolyphosphate:sugar are shown in Fig. 2. Although the solution indicated an apparent freeze-concentrated glass-like transition characterized as the $T_g'_2$ as well as the tripolyphosphate or sugar aqueous solution, it should be noted that the $T_g'_2$s of the mixed aqueous solutions were higher than those of tripolyphosphate or sugar aqueous solutions and converged in a narrow temperature range of 238 to 243 K. The $T_g'_2$ increments for the aqueous sugar system with the addition of tripolyphosphate ($\Delta T_g'_2$) are summarized in Table 1. On the other hand, a small endothermic shift was also observed at a temperature below the $T_g'_2$. This observation is discussed afterward.

The effect of the ratio of tripolyphosphate to glucose on the $T_g'_2$ of the aqueous solutions was next examined. DSC heating curves for tripolyphosphate-glucose aqueous solutions of various weight ratios are shown in Fig. 3. It was assumed that the difference in total solute

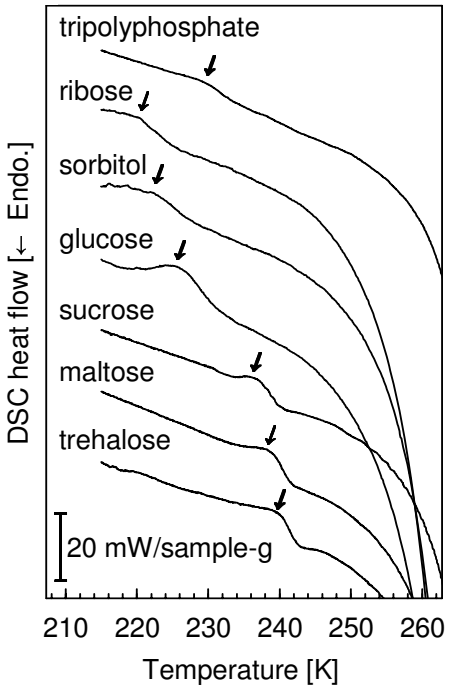

Fig. 1. DSC heating curves for 5.0% (w/w) various sugar and tripolyphosphate aqueous solutions. Arrows indicate glass-like transition.

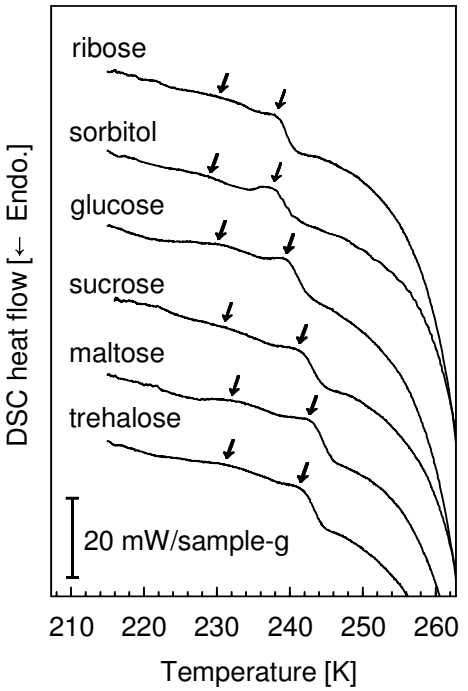

Fig. 2. DSC heating curves for 5.0% (w/w) tripolyphosphate-various sugar aqueous solutions with the weight ratio of 1:1. Arrows indicate glass-like transitions.

Table 1 $T_g'_2$s and $T_g'_1$s of tripolyphosphate, sugar, and the mixed aqueous solutions

compound	$T_g'_2$ (this work) [K]	$T_g'_2$ (references) [K]	$T_g'_1$ (reference) [K]	$T_g'_2$ of the mixture (weight ratio of 1:1) [K]	$\Delta T_g'_2$ [K]
tripolyphsphate	229.5	230.0 (34)	-	-	-
ribose	220.2	226.2 (1), 220.2 (10)	206.2 (10)	238.6	18.4
sorbitol	223.1	229.7 (1), 224.2 (10)	210.2 (10)	238.0	14.9
glucose	226.5	230.2 (1), 227.2 (10)	216.2 (10)	239.8	13.3
sucrose	237.4	241.2 (1), 239.2 (10)	227.2 (10)	241.8	4.4
maltose	239.1	243.7 (1), 241.2 (10)	231.2 (10)	243.3	4.2
trehalose	240.0	243.7 (1), 243.2 (10)	233.2 (10)	242.1	2.1

content of the mixture solutions does not affect the $T_g'_2$ values because $T_g'_2$ is independent of the initial solute content of aqueous solution. Actually, the $T_g'_2$ of 5.0% aqueous solution of 1.0:1.0 weight ratio of tripolyphosphate:glucose, as shown in Fig. 2, agreed with that of the 20.0% solution of the same weight ratio, as presented in Fig. 3. On the other hand, a glass-like transition scarcely observed at a temperature below the $T_g'_2$ of the 20.0% solution became much clearer than that of the 5.0 % solution, because the endothermic effect induced by glass transition becomes larger with increasing of the initial solute content of aqueous solutions (9). For the mixed aqueous solution containing tripolyphosphate more than the ratio of tripolyphosphate:glucose=1.0:1.5, the $T_g'_2$ became higher as the ratio of tripolyphosphate decreased to the ratio of 1.0:1.0. At the weight ratio of 1.0:1.0, $T_g'_2$ was the highest among the

examined systems. The other glass-like transition temperature observed at a temperature below the $T_g'_2$ also changed depending on the glucose content within only a few Kelvin. In the contrast, the $T_g'_2$ of the aqueous system containing tripolyphosphate less than the weight ratio of tripolyphosphate:glucose=1.0:1.5 became lower as the ratio of tripolyphosphate decreased to the ratio of 1.0:8.0, and the other glass-like transition, which should have been observed at a temperature below the $T_g'_2$, disappeared.

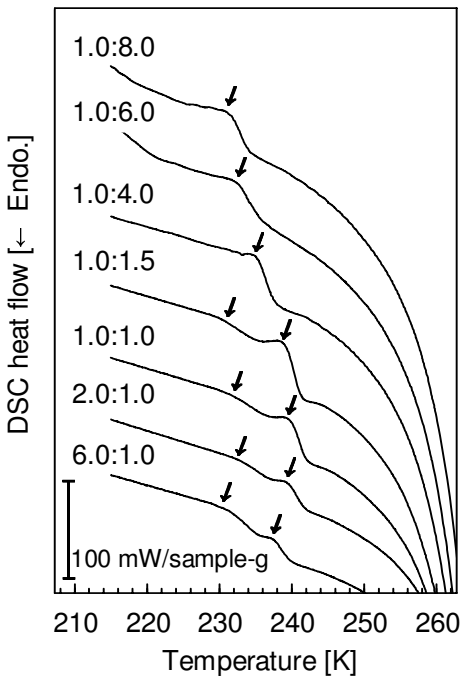

Fig. 3. DSC heating curves of tripolyphosphate-glucose aqueous solutions of various tripolyphosphate:glucose ratios. Arrows indicate glass-like transitions.

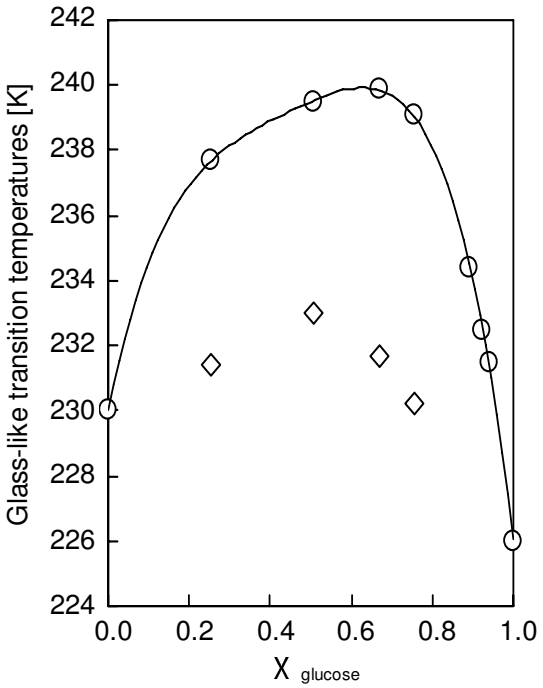

Fig. 4. $T_g'_2$ of tripolyphosphate-glucose aqueous solution as a function of glucose mole fraction. Circle and diamond indicate $T_g'_2$ of the tripolyphosphate-glucose aqueous system and the other glass-like transition temperature observed at a temperature below the $T_g'_2$, respectively.

Table 2 Glass-like transition temperatures of tripolyphosphate-glucose aqueous solutions

total solute content [% (w/w)]	weight ratio of tripolyohosphate:glucose	$X_{glucose}$	$T_g'_2$ [K]	the other T_g' [K]
22.5	1.0:8.0	0.94	231.5	-
17.5	1.0:6.0	0.92	232.5	-
25.0	1.0:4.0	0.89	234.4	-
25.0	1.0:1.5	0.75	239.1	230.2
20.0	1.0:1.0	0.67	239.9	231.7
15.0	2.0:1.0	0.51	239.5	233.0
17.5	6.0:1.0	0.25	237.7	231.4

These glass-like transition temperatures are presented as a function of glucose mole fraction ($\chi_{glucose}$) in Fig. 4 for a more detailed analysis of the glucose ratio dependency of the glass-like transition temperatures of the mixture solution. It was confirmed from the figure that the $T_g'_2$ increment with the addition of tripolyphosphate indicated a peak at approximately $\chi_{glucose} = 0.70$, and the other glass-like transition appears below $\chi_{glucose} = 0.75$. Table 2 summarizes these results.

DISCUSSION

There are studies for $T_g'_2$ of aqueous solutions containing two kinds of solute. Chang and Randall (17) investigated the $T_g'_2$ of aqueous solutions containing bovine serum albumin (BSA) and various kinds of solute by using DSC and thermal mechanical analysis. They demonstrated that $T_g'_2$ of BSA-solute aqueous solutions ranged between $T_g'_2$ of BSA aqueous solutions and $T_g'_2$ of the solute aqueous solutions. Similar results were reported for $T_g'_2$ of sucrose-dextran aqueous solutions (14), for $T_g'_2$ and $T_g'_1$ of sucrose-gelatin and sucrose-peptone aqueous solutions (20), and for $T_g'_2$ and $T_g'_1$ of sucrose-glycine aqueous solutions (6,19). $T_g'_2$s of tripolyphosphate-sugar aqueous solutions, however, were higher than those of tripolyphosphate or sugar aqueous solutions. This result suggests that such interaction as was indicated between borate and sugar or phosphate and sugar also works between the tripolyphosphate and sugar. $T_g'_2$s of aqueous sugar systems examined in this study were raised by adding the tripolyphosphate, as indicated in Fig. 2. The $T_g'_2$ increment for ribose, sorbitol, and glucose was especially remarkable in comparison with those of disaccharides. Since aqueous sugar systems originally have a low $T_g'_2$, the drastic increase in $T_g'_2$ will be useful in preventing collapse or devitrification during freeze-thawing or freeze-drying. It was found that the $T_g'_2$ increment of tripolyphosphate-glucose systems depends on the ratio, as indicated in Figs. 3 and 4. Although it was not investigated in this study, the $T_g'_2$s of the mixture systems of tripolyphosphate and other sugars are also expected to exhibit a change of $T_g'_2$ depending on the ratio. A tripolyphosphate salt is actually used with sugars as a cryoprotectant for frozen foods (35,36), however, the mechanism of the cryoprotective effect is not clear (37). The drastic increase in the $T_g'_2$s of aqueous sugar systems with the addition of tripolyphosphate observed in this study suggests a possible answer to the problem.

The formation of a complex between borates and hydroxyl compounds has been well-established (29-31), and it has been recognized that the formation of a complex causes a greater increase in viscosity of the aqueous solution, $T_g'_2$ of the frozen system, and T_g in the dehydrated state of the mixture system (21-25). Although a salt that cannot form a complex with hydroxyl compounds (*e.g.*, NaCl) also raises the viscosity of the solution and the T_g in the hydrated state of the hydroxyl compound only by a few Kelvin due to the effect of ionic charges (16,22), T_g' of the frozen system was reduced by increasing the amount of unfrozen water of the freeze-concentrated solute phase (38,39). For a monophosphate-sugar mixture, it was found that adding a monophosphate also could drastically raise the viscosities of the solution and the T_gs in the dehydrated state of disaccharides, depending on the ratio and the pH (26,27). Previous studies have indicated that a complex between monophosphate and sugar, as well as a borate-hydroxyl compound system, is formed from the consideration of the similarity of molecular structure between monophosphate and borate. Although these interpretations can be reasonably applied to tripolyphosphate-sugar systems, the effect of water on the mixture system should also be considered in the case of $T_g'_2$. Since water plays a role of superior plasticizer, the amount of unfrozen water in the freeze-concentrated system significantly affects the $T_g'_2$ value (1,14,38,39). According to these considerations, we concluded that the tripolyphosphate-sugar mixture system exhibited an increase in $T_g'_2$

because water was excluded from the freeze-concentrated system by the complex formation between tripolyphosphate and sugar.

The other glass-like transition was observed at a temperature below the $T_g'_2$ as shown in Figures 2 and 3. The following two interpretations are suggested as the reason. One interpretation is that the glass-like transition observed at a temperature below the $T_g'_2$ corresponds to $T_g'_1$ of the mixed aqueous systems. Although $T_g'_1$ of glucose (10) and tripolyphosphate (34) was lower than the scanned temperature, $T_g'_1$ of the mixed aqueous systems may have been raised up to 230 K to 233 K by the complex formation between tripolyphosphate and sugar. In fact, Izutsu *et al*. reported that addition of sodium tertaborate to glycerol aqueous solution raised $T_g'_1$ and $T_g'_2$ of the glycerol aqueous solution by the complex formation between borate and glycerol (24). The other interpretation is that increasing the ratio of tripolyphosphate in tripolyphosphate-glucose aqueous system causes a phase separation of the freeze-concentrated region between a tripolyphosphate-glucose complex region and that of tripolyphosphate-rich, and that the glass-like transition observed at a temperature below the $T_g'_2$ is originated from a freeze-concentrated tripolyphosphate-rich phase. There is a report that dehydrated monophosphate-disaccharide systems exhibit two glass transitions depending on the ratio of mixtures; Ohtake *et al*. stated that the mixtures containing monophosphate more than the molar ratio of monophosphate:disaccharide=1.0:1.0 exhibited two glass transitions induced by a phase separation (27). This result is roughly in agreement with those found in tripolyphosphate-glucose aqueous system; the mixed aqueous systems containing tripolyphosphate more than $\chi_{glucose}$ = 0.75 exhibited two glass-like transitions. Furthermore, the onset points of the glass-like transition observed at a lower temperature than the $T_g'_2$ for tripolyphosphate-glucose aqueous solutions were in a narrow temperature range of 230 K to 233 K; the temperatures are in agreement with the $T_g'_2$ for the tripolyphosphate aqueous solution. In this case, it can be thought that the $T_g'_1$ of the tripolyphosphate-glucose aqueous systems was lower than the scanned temperature. In order to understand the origin of the two glass-like transitions, further studies are required.

In conclusion, we emphasize the importance of adding tripolyphosphate to the sugar solution. Sugar has been widely used as an effective cryo- or lyo-protectant for biochemical, pharmaceutical, and food materials. Since increasing $T_g'_2$ of the aqueous sugar system by adding tripolyphosphate is related to a decrease in molecular mobility and an increase in the collapse temperature or devitrification temperature, the tripolyphosphate addition is expected to improve storage stability during cryopreservation and processing stability during freeze-thawing or freeze-drying of unstable biomaterials. The effects of pH and cation on the $T_g'_2$ should also be considered in future. A foreseeable extension of this research would involve clarification of the remaining problems and investigating the effect of tripolyphosphate on the cryoprotection of sugars.

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