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Denaturation temperature of dried lens tissue rises with trehalose pretreatment

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Abstract Trehalose, a non-reducing disaccharide, is known as an exceptional protector against desiccation and heat stress. Differential scanning calorimetry is a simple tool to determine thermodynamic parameters concerning the structural state of proteins. In this study, we measured denaturation temperature (or melting temperature) of dried lenses to know the effect of trehalose pretreatment. Isolated porcine lenses were incubated in wells without any solution or with saline, 100 mM trehalose, or 100 mM cyclic tetrasaccharide in saline at room temperature for 150 min. The solutions were removed, and all lens samples were dried at room temperature and in room humidity until the weight showed no change. The nucleus of each sample was taken out and placed on a measuring platform for differential scanning calorimetry. The denaturation temperature of the dried lenses significantly rose by about 10 °C with 100 mM trehalose pretreatment, compared to no pretreatment, pretreatment with saline or 100 mM cyclic tetrasaccharide ($P < 0.05$, analysis of variance, $P < 0.05$, Student–Newman–Keuls tests, $n = 7$). The denaturation temperature showed no difference among the dried lenses with no pretreatment or pretreatment by saline or cyclic tetrasaccharide. In conclusion, pretreatment with trehalose raises denaturation temperature of the dried porcine lens. Trehalose might stabilize the dried tissue structure to get a higher denaturation temperature.

Keywords Trehalose · Dehydration · Denaturation temperature · Lens · Differential scanning calorimetry

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Introduction

Cataract formation is a major problem in health systems, and still today, cataract is the most common cause of blindness [1]. Although the surgical lens replacement represents the only available cure, it is of great pharmacological interest to identify new molecules that are able to counteract the lens opacity which might be attributed to conformational changes of the tissue structure [2]. Crystallins, which are the major proteins of the lens, are known to constitute about 90% of water-soluble proteins of the lens and contribute to the transparency and refractive properties by forming a uniform concentration gradient in the lens [3].

Trehalose, a disaccharide formed by alpha-1,1 linkage of two D-glucose molecules, has been known to protect proteins against chemical and thermal denaturation, and hence, against loss of the function [4]. For instance, trehalose stabilizes native structure of alpha-crystallin and also inhibits alpha-crystallin aggregation to maintain its chaperone activity under stress conditions [2]. In our previous studies, trehalose was shown to keep the corneal epithelial surface smooth in drying condition [5]. However, our studies also showed that trehalose could not delay the development of lens opacity in vitro, but that cyclic

tetrasaccharide could [6]. Cyclic tetrasaccharide, present naturally at least in yeast, has a ring structure, consisting of four glucose residues joined by alternate alpha-1,3 and alpha-1,6 linkages, which is rigid and resistant to enzymatic hydrolysis by glycosidases such as amylases and alpha-glucosidases [6]. Under the circumstances, it is interesting to know which one, either trehalose or cyclic tetrasaccharide, could better stabilize lens tissues in drying condition.

Differential scanning calorimetry is a simple and reliable technique to study conformational transitions of biological macromolecules since it can determine, in a single measurement, the temperature, the enthalpy and the heat capacity changes in the transition process [7]. These changes are believed to originate from the disruption of forces stabilizing native protein structures such as hydrogen bonds. Thus, the thermodynamic parameters, such as denaturation temperature (melting temperature), obtained from differential scanning calorimetric experiments, are sensitive to the structural state of proteins. In this study, we determined by differential scanning calorimetry the denaturation temperature of air-dried porcine lens tissues with different pretreatments.

Materials and methods

Enucleated porcine eyes were obtained from a local slaughterhouse, stored at 4 °C, and used within 6 h. A 10-mm wide incision at the midperiphery of the eyeball was made to extract the lens. The vitreous gel and the lens were squeezed out of the eyeball by being pushed with fingers gently. The released lenses were placed in saline without direct touching and washed for 10 min. The lenses were transferred with a spoon to wells of a 24-well multidish containing either saline, trehalose at 100 mM concentration dissolved in saline, or cyclic tetrasaccharide at 100 mM concentration dissolved in saline, or without solution. The lenses were incubated for 150 min at room temperature, and then, all solutions were moved out of the wells by aspiration. The lenses were dried at room temperature and in room humidity until the weight showed no change (Fig. 1). It took about eight days to reach this state of dryness. Trehalose and cyclic tetrasaccharide were provided by Hayashibara Biochemical Laboratories Inc. (Okayama City, Japan).

Before proceeding to differential scanning calorimetric measurements, the dried lenses were cut into halves with a razor blade, and the nuclei were removed with a forceps. In order to make samples have close contact with the bottom of an aluminum platform pan, we ground the nucleus into fine powder on a workbench with a metal bar. Each sample with the weight ranging from 10.0 to 11.2 mg was

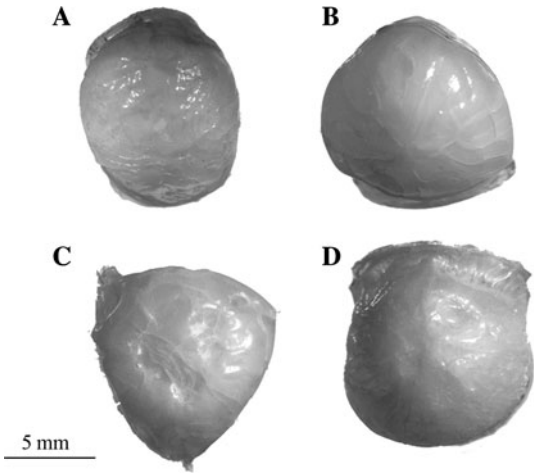

Fig. 1 Dried porcine lenses at room temperature and in room humidity used for differential scanning calorimetric measurement. The lens with no pretreatment (**A**), pretreated for 150 min with saline (**B**), 100 mM trehalose in saline (**C**), and 100 mM cyclic tetrasaccharide in saline (**D**)

transferred onto an aluminum platform pan by a medicine spoon, and then the pan was sealed by a sample press kit. We used an empty aluminum pan as a reference for differential scanning calorimetric measurements. The measurements were carried out on a Seiko DSC-220 assembled with an SSC-5300 thermal controller (Seiko Instruments Inc., Chiba, Japan). The samples were cooled at a scan rate of 10 K/min from 30 to 0 °C, held for 1 minute at 0 °C, and then heated at a scan rate of 10 K/min from 0 to 200 °C, under the atmosphere of nitrogen at a flow rate of 50 mL/min to stabilize the temperature in measurements. The denaturation temperature was identified as an endothermic peak on the thermogram, represented by the temperature of the peak maximum, which disappeared upon rescanning of the sample. For statistical analysis, the significance was calculated with one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test as a post-hoc test for multiple comparisons with a statistical software (SPSS 15.0 for windows, SPSS Inc., Chicago, USA).

Results

Thermograms for no pretreatment and trehalose-pretreated dried lenses are showed in Fig. 2. The denaturation temperature of lenses pretreated either with 100 mM trehalose, 100 mM cyclic tetrasaccharide or saline, and with no pretreatment were 90.2 ± 3.4, 78.5 ± 2.7, 78.0 ± 2.2, and 77.1 ± 2.5 °C, respectively, as a mean and standard deviation (Fig. 3). The denaturation temperature of dried lens tissues pretreated with 100 mM trehalose was significantly higher than that of dried lens tissues pretreated with

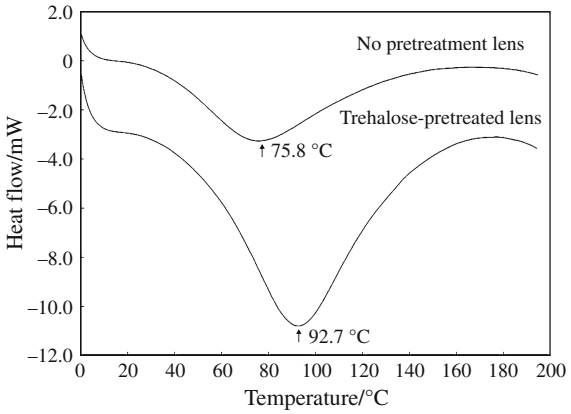

Fig. 2 Differential scanning calorimetric curves (thermograms). *Upper* and *lower curves* correspond to no pretreatment and trehalose-pretreated lens tissues, respectively. The denaturation temperature is identified as an endothermic peak and presented by the temperature of the peak maximum (*trough*, *arrows*)

100 mM cyclic tetrasaccharide or saline, or with no pretreatment. ($P < 0.05$, ANOVA, $P < 0.05$, Student–Newman–Keuls test, $n = 7$, Fig. 3). No significant difference was found in denaturation temperatures among dried lens tissues pretreated by saline or cyclic tetrasaccharide at 100 mM, and with no pretreatment. (Student–Newman–Keuls tests, $n = 7$).

Discussion

Denaturation temperatures and glass transition temperatures, which provide us with helpful information of the

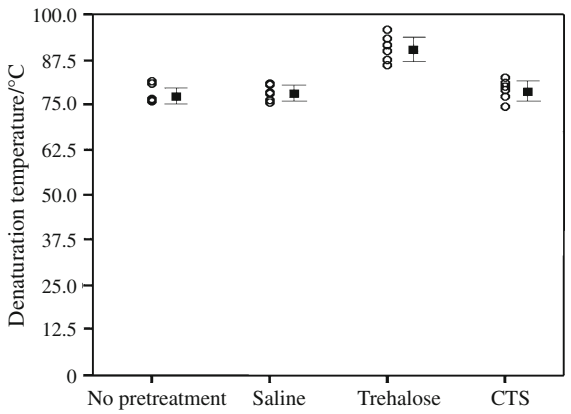

Fig. 3 Actual measurements (*open circles*), mean (*closed squares*) and standard deviation (T bars) of denaturation temperatures of porcine lens tissues pretreated with saline, trehalose or cyclic tetrasaccharide (CTS), or with no pretreatment. Trehalose pretreatment results in a significantly higher denaturation temperature (by about 10 °C), compared with saline or cyclic tetrasaccharide pretreatment or no pretreatment ($P < 0.05$, ANOVA, $P < 0.05$, Student–Newman–Keuls test, $n = 7$)

polymers, involve energy changes or heat capacity changes that can be detected by the differential scanning calorimetry with great sensitivity. Therefore, it has been widely used in many fields, such as industry and pharmaceutical science, for decades. Recently, the differential scanning calorimetry has become a useful method for the study of protein structures and nucleic acids since it can detect small differences in thermal signatures of small samples in simple and rapid measurements [7, 8]. Detailed studies with differential scanning calorimetry have dealt with the thermodynamics of ligand-binding interactions, particularly, intramolecular interactions in large proteins and the effect of single mutations on the thermal stability of proteins [9]. The denaturation temperature, at which 50% of the protein molecules are in the denatured state [7], measured by the differential scanning calorimetry, is a key indicator of protein stability [10]. The denaturation temperature of the biological tissue would be defined as the sum of the denaturation temperature of proteins and the other macromolecules which constitute the tissue.

The porcine lens has been shown to serve as a suitable model for the human lens [11, 12]. The total protein content of the lens is about 33.7–35.8% of the wet weight. Crystallin proteins, designated as α, β-, and γ-crystallins, represent about 35%, 40–45%, and 12%, respectively, of the total soluble protein content in the porcine lens. In addition, the proportion of insoluble protein increases from around 8% in the outer layers to about 25% in the inner layers [11]. In this study, the denaturation temperature of the lens tissue would be thus determined mainly by the denaturation of crystallins which are the primary components of the lens. Increased conformational rigidity of proteins would increase their thermal stability, and hence, would raise the denaturation temperature [13].

The original aims of this study were to determine denaturation temperatures of the intact lens tissues pretreated with different solutions and to understand the trehalose effects on stabilizing the lens tissue as a whole. Nonetheless, we had to dry the lens tissue to measure the denaturation temperature, since the wet tissues were vulnerable to temperature changes caused by evaporation during the measurement. The state of dehydration would be also ideal to measure the denaturation of the biological tissues which consist of different kinds of macromolecules. In addition, because the aluminum pan was too small to hold a whole lens, we had no choice but to crash the dried lens into small pieces. The reasons why we chose 100 mM concentrations of trehalose and cyclic tetrasaccharide were that 100 mM cyclic tetrasaccharide could delay the lens opacification in vitro [6], and that 100 mM trehalose was the best concentration of protecting corneal epithelial cells from death by drying as shown in our previous studies including clinical trials [14–16].

In one preceding study, denaturation temperatures of the disrupted human lens tissues obtained by phacoemulsification at cataract surgeries were measured by differential scanning calorimetry [1]. The measurements were done at a heating rate of 0.3 K/min in the range of temperature from 0 to 100 °C, and the samples were cataractous lens tissue suspensions in the fluid, with the irrigation fluid used as the reference. In that study, denaturation temperatures of the cataractous lens suspensions fell into two major peaks around 67 and 85 °C, which are comparable to the denaturation temperatures of the dried porcine lens tissues obtained without trehalose pretreatment in the present study. The slower heating rate, used in that study, was more suitable to determine the denaturation temperature of biological materials. Such a faster heating rate of 10 K/min as used in this study was, indeed, adopted to determine the denaturation temperature of dehydrated globular proteins [13]. A concern in the preceding study [1] would regard the supposition that heating the samples in the fluid state would cause evaporation, which would, in turn, influence the accuracy of the measurement of denaturation temperature. Since we measured the denaturation temperature of dehydrated samples, it was not required to use reference pans filled with corresponding solutions.

A large number of studies have used differential scanning calorimetry to determine denaturation temperature of purified proteins [4, 7, 8, 13]. One study showed that 2 M trehalose could raise the denaturation temperature of a protein, ribonuclease A, by as much as 18 °C, at pH 2.5 [4]. In this study, the trehalose-treated dried lens tissues had a denaturation temperature of about 10 °C higher, indicating that macromolecules in these lens tissues, including crystallins, are kept in a more stable condition. Furthermore, the pretreatment with trehalose gave a thermogram with the more apparent peak maximum, suggesting that macromolecules in the lens would be arranged more regularly in the presence of trehalose.

The mechanisms by which trehalose protects biological molecules can be divided into three categories, water replacement, glass transition, and chemical stability. These three mechanisms are not mutually exclusive and all may contribute to the stabilizing effects of trehalose [17]. In this study, the presence of trehalose raised the denaturation temperature of the dried lens. If trehalose would replace water in the dried tissue, the content of water would become lower, and hence, the denaturation temperature would rise. Based on the current results, trehalose might raise the denaturation temperature by replacing water molecules which surround proteins such as crystallins.

Another mechanism underlying the rise of the denaturation temperature of the lens tissue in the dehydration would be the stable state of proteins in the presence of trehalose, as discussed below. Hydrogen bonding among proteins as well as among the other macromolecules and the formation of a glass state in the presence of trehalose are mandatory for protection from damage during dehydration [17]. Hydrogen bonding between proteins and disaccharides was indeed shown to be responsible for the inhibition of dehydration-induced protein unfolding [18]. Fourier transform infrared (FT-IR) spectroscopic studies, carried out with trehalose and proteins, have confirmed that hydrogen bonding did occur between the sugar and the protein and that this interaction is the requirement for the preservation of labile proteins during desiccation [19]. Moreover, protein stabilization by trehalose has been explained in term of free energy changes [2]. In future studies, by measuring not only the denaturation temperature, but also the glass transition temperature of viable tissues pretreated by trehalose, we might have a hint to understand the reason why trehalose protects biologic structures from damage in freezing or dehydration.

The major limitations in this study are that the measurement of denaturation temperature could be affected not only by the scanning speed of heating temperature but also by the protein water content [10, 20]. In this study, we did not measure the water content of the samples in more accurate ways, which might influence the results. The presence of residual water would decrease the denaturation temperature of proteins or protein-containing tissues. Another influential factor which we have to consider is the fact that every sample was not necessarily derived from the exactly same part of the lens tissues. In addition, we had to grind each sample into fine powder to make sure to have close contact with the bottom of the measuring pan. By doing this, we might damage the macromolecules consisting of the lens. At present, it remains unknown whether trehalose or cyclic tetrasaccharide could penetrate into the lens tissues uniformly in the incubation period of 150 min. In order to prove that trehalose might increase the denaturation temperature of the lens tissue by interacting with crystallins located inside the lens fiber cells, we have to know whether the lens fiber cell membranes would be permeable to trehalose or not. Until now, no study has addressed the route for transport of trehalose into the lens. In future studies, it is necessary to know what are obstacles for the transport of trehalose in the lens.

In conclusion, this study is the first to measure the denaturation temperature of the biological tissue by differential scanning calorimetry. Trehalose at 100 mM could increase the denaturation temperature of the dried lens tissues, which would give a key to understanding the mechanism of trehalose effects on stabilizing the tissue under extreme conditions such as complete drying. The trehalose-induced rise in the denaturation temperature of the lens tissue would also give a hint to consider cataract formation from the viewpoint of macromolecular stability.

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