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Preservation Effect of Vitreous non Reducing Carbohydrates on the Enzymatic Activity, Denaturation Temperature and Retention of Native Structure of Lysozyme

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Abstract. In order to develop more efficient methodologies to preserve protein based products at room temperature, a study on the preservation of the enzyme lysozyme in glassy matrices made from three different carbohydrates: trehalose, sucrose and raffinose was carried out. The lysozyme-carbohydrate systems were evaluated structurally by Fourier Transform Infrared (FTIR) spectroscopy, to analyze the intermolecular interactions between carbohydrate and protein. Their thermal stability was characterized using differential thermal analysis (DTA), which allowed the measurement of glass transition temperatures (T_g) of the vitreous sugars and differential scanning calorimetry (DSC), which was used to measured the T_g of sugars in the sugar enzyme systems as well as the thermal denaturation temperature of lysozyme (T_d). The structural studies revealed that sucrose is the most effective sugar for the preservation of the native conformation of lysozyme during lyophilization. Nevertheless, analysis of enzymatic activity showed, after storing the enzyme at room temperature for more than five weeks, that the highest activity retention was achieved when preserved in the presence of trehalose and raffinose. Freeze-dried lysozyme in the absence of sugars partially lost its native conformation during the lyophilization and lost 20% of its biological activity when stored at room temperature.
Keywords: Sugar glass, freeze-drying, preservation of proteins, enzymes.

Resumen. Con el propósito de encontrar métodos más eficientes para preservar productos con base a proteínas a temperatura ambiente se estabilizó la enzima lisozima en presencia de tres diferentes carbohidratos: trehalosa, sacarosa y rafinosa. Los sistemas lisozima-carbohidratos fueron evaluados estructuralmente por espectroscopía de infrarrojo por transformada de Fourier (FTIR) para analizar las interacciones intermoleculares carbohidrato-proteína. También se hizo un estudio de estabilidad térmica usando técnicas de calorimetría diferencial de barrido (DSC) y análisis térmico diferencial (DTA) con las que se determinó la temperatura de transición vítrea (T_g) de los azúcares amorfos puros y de los sistemas azúcar enzima, así como la temperatura de desnaturalización térmica de la lisozima (T_d). Los estudios estructurales demostraron que la sacarosa es capaz de preservar más efectivamente la conformación nativa de la lisozima durante el secado por liofilización; Sin embargo, el análisis de actividad biológica mostró que la enzima, después de haber sido almacenada a temperatura ambiente por más de 5 semanas, retuvo mayor actividad cuando fue preservada en presencia de trehalose y rafinosa. La lisozima liofilizada en ausencia de sacáridos perdió parcialmente su conformación nativa durante el secado y perdió un 20% de actividad biológica al ser almacenada a temperatura ambiente.
Palabras clave: Azúcares vítreos, liofilización, preservación de proteínas, enzimas.

Introduction

Some biological products, like vaccines, antibodies or enzymes, have increasing applications in the food, pharmaceutical, and biotechnological industry. Given the natural origin of these products they are most of the times labile to ambient conditions during storage. The stress conditions that these molecules are susceptible to during storage are changes in temperature, *p*H, and humidity. To overcome these problems, drying processes like freeze drying (lyophilization) or spray drying are extensively used to stabilize and to commercialize these protein based products.[1] The main difficulty faced during storage of these dried products is that even when they overcome the stress associated with drying (pressure and temperature changes) there is no guarantee that they will retain their biological activity at normal conditions; therefore, they need to be stored and transported at temperatures that range between 8°C to −20 °C; this controlled condition of temperature during supply and storage is known as cold chain.

With the purpose of providing an improvement to the current preservation process of thermolabile molecules and to eliminate the cold chain, different research groups have studied sugar glasses as stabilizers of biomolecules during drying and storage [2-9]. The idea of using sugars for preservation comes from a natural phenomenon called anhydrobiosis. The latter is a process in which some biological organisms like insects, bacteria, and fungi survive long periods of dryness and exposition to low temperatures by synthesizing non-reducing disaccharides on amorphous (or vitreous) state [10]. Although there is no an accepted mechanism that fully explains the preservation phenomenon of biomolecules in vitreous sugar matrices, it is clear that glass forming capacity and high glass transition temperature (higher than 60°C when they are anhydrous) of these carbohydrates are essential characteristics that make them suitable for their application in preservation [11-13]. There is also evidence that other parameters like specific sugar-biomolecule intermolecular interactions [14] need to be studied to find more efficient methodologies of stabilization of biomolecules like proteins.

Considering the high potential that vitreous sugars have in the preservation of protein based products and the increasing economic impact of biotechnology, several vitreous non reducing carbohydrates matrices using trehalose, sucrose and raffinose, were comparatively studied in order to evaluate their

effect on the preservation of native structure, thermal denaturation and retention of enzymatic activity. For the present study lysozyme was selected as a thermolabile protein based model system; the recommended storage temperature for this commercial freeze dried enzyme is –20 °C which makes it suitable for preservation efficiency testing of stabilizing agents during and after drying and storage at room temperature.

Fourier transform infrared spectroscopy (FTIR) was used to determine the changes in the enzyme's native structure when dried in presence and absence of sugars; differential thermal analysis (DTA) and differential scanning calorimetry (DSC) were used to measure glass transition temperature (T_g) of the carbohydrate+enzyme systems and the denaturation temperature of the enzyme (T_d) in order to correlate the thermal stability of the enzyme and thermal stability of the vitreous matrices with the fraction of enzyme activity retained as a function of time under storage at room temperature.

Results and discussion

Analysis of the thermal stability of the amorphous carbohydrates and enzyme+carbohydrate systems:

Thermal analysis provides information about the phase transitions that a material will exhibit during heating or cooling. Among these transitions, glass transition temperature (T_g) gives important information on the thermomechanical stability of amorphous solid phases since above T_g the material loses its rigidity and becomes a viscous liquid. Crystallization needs to be avoided for preservation purposes since the stabilizing agent must remain as an amorphous material in the range of storage temperature. Figure 1 shows a thermogram for amorphous sucrose in which the onset of the glass transition is observed at 67 ºC followed by an exothermic process corresponding to the crystallization of the material at 114 ºC, which is well above the practical storage temperature. In order to characterize the thermomechanical properties of the amorphous sugars used as stabilizing agents on the present study, T_g of pure trehalose, raffinose and sucrose were measured. Figure 2 shows thermograms for the three amorphous carbohydrates; prepared by microwave intermittent heating cycles of aqueous solutions. It has been previously reported that the higher the glass transition temperature of the carbohydrate, the greater the thermal stability of a protein+carbohydrate system will be [15]. To test the thermal stability of the pure amorphous carbohydrates, their glass transition temperatures were compared with the T_g's of lysozyme+carbohydrate systems prepared by freeze drying. Figure 3 presents an example of a thermogram of lysozyme+carbohydrate system (sucrose+enzyme) in which the small endothermic step at 44 °C corresponds to the glass transition temperature of the amorphous matrix. Similar thermograms were obtained for trehalose and raffinose systems. The results of the thermal analysis for all the sugars are summarized in Table 1.

Also on Figure 3 the thermogram presents an endothermic peak at 138 °C which corresponds to the denaturation temperature of the enzyme (*Td*) which is the maximum temperature at which a protein is stable. Denaturation is a process in which the secondary and tertiary structures of a protein are disrupted. This process in which interactions such as hydrogen bonding, salt

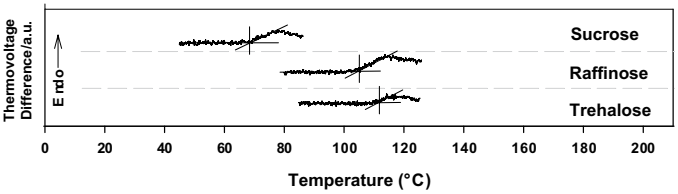

Figure 2. Experimental thermograms of amorphous sucrose, raffinose and trehalose obtained with a differential thermal analysis instrument (DTA) at a heating rate of 10ºC/min.

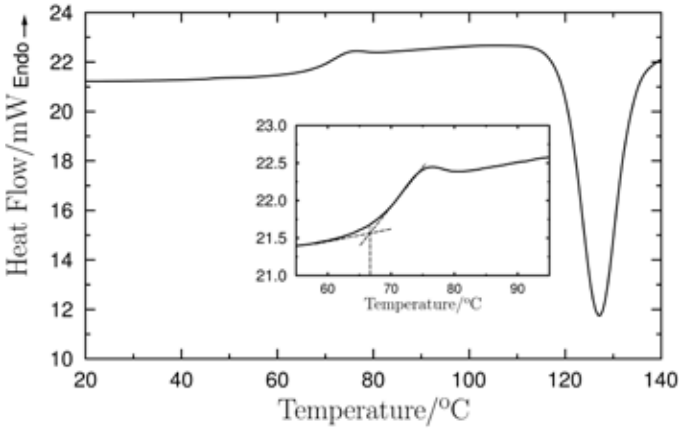

Figure 1. Experimental thermogram of amorphous sucrose obtained with a differential scanning calorimeter (DSC) at a heating rate of 10 ºC/min. Glass transition is observed at 67 ºC (see inset) and the crystallization of the glass occurs after reaching a temperature of 114 ºC.

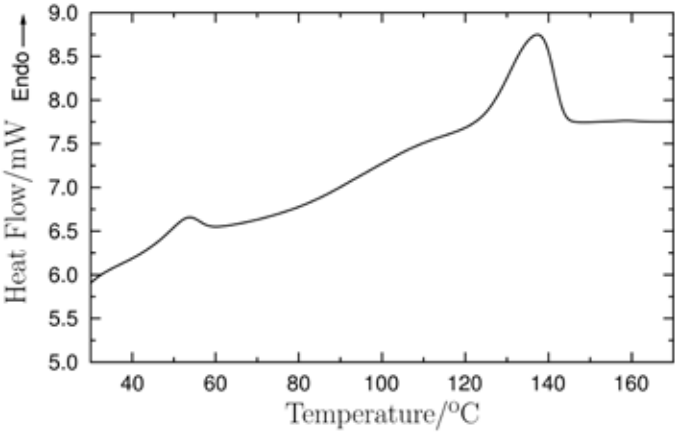

Figure 3. Thermogram of the system sucrose lysozyme, prepared by freeze-drying an aqueous solution of lysosyme in presence of sucrose. The thermogram shows glass transition temperature at 44 °C and denaturation temperature of the lisozyme at 138 °C.

Table 1. Experimental values of glass transition temperatures (T_g) of pure amorphous sugars and of sugar in the lysozyme-carbohydrate systems. Denaturation temperature (*T*d) of lysozyme in the enzyme-carbohydrate systems are presented in the last column.

Sugar	*T*g pure sugar	*T*g lysozyme-sugar system	*T*d lysozyme
Trehalose	115 °C	62 °C	157 °C
Rafinose	112 °C	62 °C	156 °C
Sucrose	67 °C	44 °C	138 °C

bridges, van der Waals interactions and sulfur-sulfur covalent bonds are broken can be described, according to the Lumry and Eyring model [16] to occur in two stages: the first is the breaking of these interactions of the protein and may be reversible, and the second involves an irreversible loss of tertiary structure such as α-helices and β-sheets to finally adopt a random coil configuration. Either of these steps involves a certain amount of energy released or absorbed, so it is possible to follow the denaturation process using a thermal analysis. Table 1 presents denaturation temperatures (T_d) of lysozyme in the three enzyme-carbohydrate systems studied. As it can be seen, trehalose and raffinose confer greater thermal stability to the enzyme than sucrose. As a means of comparison, when the lysozyme is dissolved in water without any preservative or stabilizing agent its denaturation temperature occurs around 75 °C. [17]

FTIR structural analysis of the enzyme-carbohydrate systems

In order to study the stability of the tertiary structure of the enzyme after the freeze drying and its encapsulation in the vitreous matrices, infrared spectroscopic analysis was performed to compare the enzyme's structure before and after drying process. Figure 4 shows the second derivative spectra for the region of amide I and II which allow the comparative analysis of lysozyme's structural changes as shown in Figure 4: a) in its native state in aqueous solution, (see inset); b) freeze dried in the absence of any sugar, c) freeze dried in presence of a stabilizing sugar, in this case sucrose glass; and d) denatured by thermal effect. It may be noted how the native state presents a sharp absorption band around 1653 cm^{-1} which indicates a primarily α-helical conformation for lysozyme in that state. When performing the freeze drying in the absence of carbohydrate a clear reduction of this peak is observed together with an increase of a peak around 1680 cm^{-1}, which corresponds to the formation of antiparallel β-laminar zones, and the emergence of a new peak at 1615 cm^{-1} due to the formation of a parallel β-laminar zone. This pattern of differential signals indicated a radical change in the native structure of lysozyme during the process. However, in the presence of sucrose, lysozyme seems to hold primarily an α-helical conformation similar to its native structure.

By repeating this analysis with two other stabilizing agents studied (raffinose and trehalose) it was found that although sucrose has a lower glass transition temperature (T_g) it was able to preserve the native structure of lysozyme more efficiently. This is attributed to the ability of these sugars to form hydrogen bonds of greater magnitude, according to the theory advocated by Carpenter and Crowe which suggests that the sugar can replace the stabilizing water-protein interactions [18]. The theory stipulates that the formation of hydrogen bonds between the hydroxyl groups of carbohydrate and lysozyme simulate the interactions present between the polypeptide and water, thereby enhancing the preservation of the native conformation to the extent of the magnitude of these interactions.

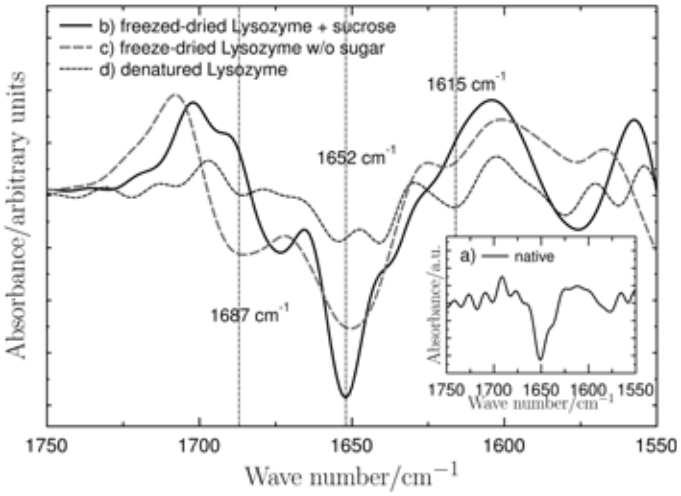

Figure 4. Second derivative FTIR spectra for lysozyme in the Amide region I and II. a) In the inset the second derivative spectrum for the native lysozyme in solution is shown for comparison purposes. The spectra **b** and **c** correspond to solid freeze-dried lysozyme in presence and absence of sucrose and spectrum d correspond to a fully denatured lysozyme.

Analysis of the retention of the biological activity of lysozyme

The lysozyme activity was monitored for more than 5 weeks of storage at 25 °C. The enzyme did not present significant loss of activity when introduced in the raffinose or trehalose glasses (see Figure 5). For the same period of time and in the absence of a stabilizing agent (without sugar), a 20% loss of activity was observed. Considering that that trehalose and raffinose presented the highest thermal stability and sucrose was the most effective sugar for the preservation of the native conformation of lysozyme during lyophilization, formulations with mixtures of sugars in which both parameters (structural and thermal stabilization) are optimized could represent an area of opportunity to find a universal stabilizing excipient with thermo-mechanical properties that allow preservation of thermolabile molecules during drying and room temperature storage. Our group is currently working on formulations with mixtures of sugars and secondary components. Results will be published on a separate publication.

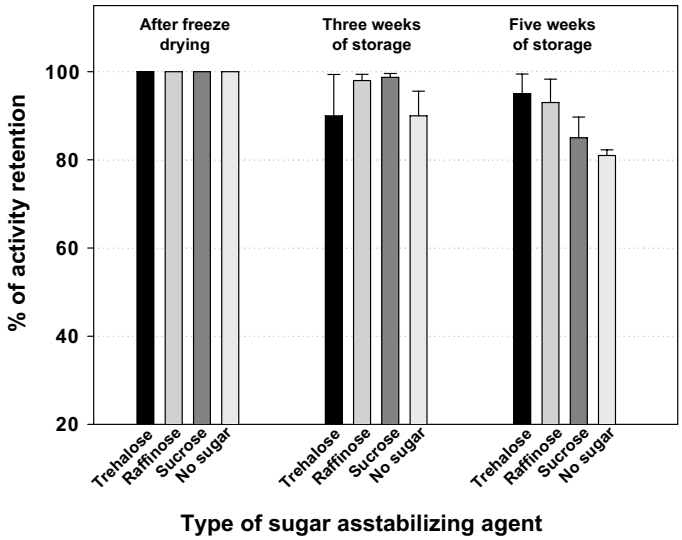

Figure 5. Retention of enzymatic activity of lysozyme freeze dried in presence and absence of sugars and stored at 25 °C.

Conclusions

Among the three studied sugars, sucrose, with the lowest glass transition temperatures was able to most effectively preserve the native conformation of lysozyme during lyophilization. However, in cases where the capacity of thermal stabilization of the sugar plays a role (as is the case of storage at room temperature), raffinose and trehalose were more effective. This leads to the need of a multi-component glassy matrix study with the purpose to achieve a formulation with high glass transition temperature but at the same time with the ability to preserve the native structure of the protein during stabilization and long term storage at room temperature.

Development of protein based products that do not need a cold chain is one of the highest priorities for technology development on biotechnology and pharmaceutical areas; sugar glasses have shown to be a great promise. Although much effort has been devoted to the understanding of the carbohydrate glass preservation, the mechanism still remains unclear. While the full understanding of the preservation phenomenon remains to be established, there are many areas of opportunity for research in this field; our group is currently working to find what are the key parameters on the characterization of amorphous carbohydrates to predict which matrices could be efficient stabilizing agents to preserve thermolabile molecules without the need of cold chain.

Experimental section

D-(+)-Trehalose dihidrated (Sigma T5251), Sucrose 99%+ (Sigma S5016) and D-(+)-Rafinose pentahidrated 98% (Sigma R0250) were used for the preparation of the vitreous matrices. Lysozyme was selected as a model biomolecule and the cell membrane of *Micrococcus lysodeikticus* was used as the substrate to measure the enzyme's activity. Both were obtained from Sigma Aldrich. All materials were used as received without further purification.

Preparation and characterization of amorphous sugars

The sugars selected as stabilizing agents for this study, sucrose, trehalose and raffinose, were used to prepare anhydrous amorphous matrices starting from their aqueous solutions which were then subjected to intermittent heating process of drying in microwave oven and vacuum according to the methodology reported by Seo *et al.* [19] and optimized by Mederos *et al.* [20]. Glass transition temperatures of the amorphous carbohydrates were determined by DTA at heating rate of 10 °C/min [21]. The vitreous samples obtained had a content of water close to 2%. Water content was determined by weight loss and confirmed by Karl Fisher titration.

Preparation and characterization of carbohydrates+enzyme systems

For the preparation of carbohydrate+enzyme systems, aqueous solutions containing amorphous carbohydrate and the enzyme were prepared in deionized water. The solutions were then frozen at −80 °C and freeze dried in a Virtis freeze-dryer and subsequently stored at room temperature (25 °C). After the freeze drying process the glass transition of the carbohydrate+enzyme systems and the denaturation temperature of lysozyme (T_d) were determined by DSC. The measurements were made using a Perkin Elmer DSC Pyris I. For these measurements the samples were sealed in aluminum pans and were heated at a rate of 10 °C/min.

Structural characterization of the ternary structure of lysozyme

The IR spectra for the freeze dried enzyme+carbohydrate systems were obtained preparing pellets of the samples with potassium bromide in a 1:100 carbohydrate: KBr ratio. The spectra were acquired at 22 °C with a resolution of 4 cm^{-1} using a Perkin-Elmer 1750-FTIR. Amide I and amide II bands were inspected analyzing second derivative FTIR spectra according to the methodology described by Dong *et al.* [22]. D_2O was used to prepare the protein solutions because it allows a better separation of the amide I and II bands. For comparison purposes native and denatured lysozyme samples were also inspected. The native enzyme was measured in solution using a calcium fluoride cell. Denatured lysozyme was produce after a heating treatment.

Measurement of the enzymatic activity

Lysozyme's activity was determined through the turbidimetric method described by Shugar *et al*. [23] This method is based on the monitoring of the reaction of degradation of the cell wall

of the bacteria *Micrococcus lysodeikticus* which is catalyzed by the lysozyme. The evaluation of biological activity was monitored measuring the absorbance of the sample at 450 nm in a UV/VIS Perkin Elmer Lambda 18 spectrophotometer.

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