Impact of supramolecular interactions of dextran-β-cyclodextrin polymers on invertase activity in freeze-dried systems

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Abstract

β-cyclodextrin-grafted dextrans with spacer arms of different length were employed to evaluate the impact of supramolecular interactions on invertase activity. The modified dextrans were used as single additives or combined with trehalose in freeze-dried formulations containing invertase. Enzyme activity conservation was analyzed after freeze-drying and thermal treatment. The change of glass transition temperatures (T_g) was also evaluated and related to effective interactions. Outstanding differences on enzyme stability were mainly related to the effect of the spacer arm length on polymer-enzyme interactions, since both the degree of substitution and the molecular weight were similar for the two polymers. This change of effective interactions was also manifested in the pronounced reduction of T_g values, and were related to the chemical modification of the backbone during oxidation, and to the attachment of the β-cyclodextrin units with spacer arms of different length on dextran.

Keywords: supramolecular interactions; dextran; β-cyclodextrin; glass transition temperature (T_g); enzyme stability.

1. Introduction

Protection of labile compounds during dehydration is based on the formation of an amorphous glassy matrix and its maintenance over storage, and by the presence of adequate molecular and supramolecular interactions between the excipients and the labile compound.[1-4] Previous reports showed that the formation of an amorphous matrix does not guarantee the stabilization of immobilized labile materials, which requires the development of adequate protein-excipient interactions.[5,6] In this way, biomolecules are kinetically stabilized even though they are not under a thermodynamically stable condition. However, once the glassy systems are above their glass transition temperature, they become supercooled and protein denaturation may be accelerated.[7]

Dehydration and freezing of proteins in the presence of polyols is frequently used for increasing protein stability, where the disaccharide trehalose is one of the most used excipients.[2,4] Besides trehalose, previous works[2,8] showed that dextran (of M_w 70,000), a D-glucose linear polymer composed of 95% $\alpha(1 \rightarrow 6)$ linkage, is a very good enzyme stabilizer in freeze-dried formulations. After freeze-drying, more than 80% of invertase,[2] 75% of α-chymotrypsin,[8] and 50% of catalase activities[8] were recovered.

A less frequent approach for increasing protein stability in dehydrated formulations is the use of cyclodextrins (CDs). CDs have a hydrophobic central cavity and a hydrophilic outer surface,[9] and are capable to include a variety of hydrophobic guest compounds such as aromatic amino acids located at the protein's surface.[10] The effect of dextran modification with several mono-6-alkylenediamino- 6-deoxy-β-cyclodextrin on trypsin thermo-resistance in diluted aqueous media was related to changes on supramolecular interactions.[11,12] Then, the combination of the physical stability provided by dextran with the protective effect on hydrophobic regions of the enzyme provided by cyclodextrin (i.e., the combination of both molecular and supramolecular interactions) could offer improved protection to enzymes subjected to dehydration.

Invertase from *Saccharomyces cerevisiae* was selected as a model enzyme to analyze its stability at considerably high temperatures, it is a well-known enzyme, and was previously used as a model in similar works.[2,3] Besides, invertase has extensive industrial applications in food industry (such as in confectionary) and biotecnology (fermentation of cane molasses for ethanol production).[13] Several of its hydrobobic aminoacids (for instance, those having a key role in membrane translocation)[14] could be a target for CDs complexation.

In present work two types of supramolecular interactions were considered in relation to invertase stability: on one side, the induction of multipoint interactions at the surface of enzymes by adding CD-modified polysaccharides to protein solutions has been considered as an interesting supramolecular alternative for stabilizing enzymes.[10,12] On the other side, the glass transition and crystallization of the matrices in which the invertase is immersed are cooperative phenomena involving the concerted movements of several molecules.[15]

The purpose of this work was to evaluate the impact of enzyme-polymer supramolecular interactions by analyzing enzyme activity conservation and glass transitions of β-CD-modified dextrans with alkyl spacer arms of different lengths. The results may contribute to foster the knowledge on the structure/function relationships involved in the dehydro-protective effect of polymers on the activity of freeze-dried enzymes.

2. Materials and Methods

2.1. Materials

Dextran 70, a D-glucose linear polymer composed of 95% α(1→ 6) linkage, of M_w = 70,000 g mol^{-1}, (Sigma-Aldrich, St. Louis, M.O., USA) was employed. β-cyclodextrin (β-CD), a cyclic non-reducing oligosaccharide composed of seven glucopyranose units bonded together via α(1→4) glycoside linkages, was from Amaizo, Hammond, IN, USA and α-α-trehalose dihydrate (Tr) from Hayashibara Co, Ltd., Shimoishii, Okayama, Japan/Cargill Inc., Minneapolis, Minnesota, USA. The enzyme invertase from *Saccharomyces cerevisiae* (β-fructofuranosidase, E.C. 3.2.1.26, 1840 U/mg, molecular mass 270 kDa) was from Fluka, Buchs, Switzerland. One unit of activity was defined as the amount of enzyme required to hydrolyze 1.0 μmol of sucrose per minute at pH 4.6, at 37 °C.

2.2. Synthesis of dextran-ethane-1,2-diamine-β-cyclodextrin and dextran-hexane-1,6-diamine-β-cyclodextrin polymers

A two-step synthesis procedure was employed by following Ramírez et al.,[16] as showed in Scheme 1: i) dextran was oxidized; ii) β-CD derivatives (previously prepared) were introduced into the polysaccharide chains after reduction.

Briefly, the polysaccharide was oxidized by dissolving 500 mg of polymers in 15 ml H_2O and treated with 500 mg m-NaIO$_4$ under continuous stirring at 0°C in the dark

for 1 h. The oxidation reactions were stopped by adding 0.5 mL of ethylene glycol and stirred for another 1 h, and further dialyzed in dark and cold conditions against distilled H_2O in a semi-permeable membrane of 10 kDa. The degree of dextran degradation by oxidation was negligible, since the oxidation conditions were very soft (1 hour, in darkness).

β-CD was purified by cation exchange chromatography on CM-Shephadex C-25 (NH_4^+ form) and characterized by conventional NMR techniques. Mono-6-(ethane-1,2-diamine)-6-deoxy-β-CD and mono-6-(hexane-1,6-diamine)-6-deoxy-β-CD were previously obtained by treating mono-6-O-tosyl-β-CD[17] with ethane-1,2-diamine and hexane-1,6-diamine, respectively, in dimethylformamide,[18-20] and the mono-substitution was confirmed by NMR (Figures S1- S2, and Tables S1- S2). For introducing the β-CD derivatives into the polysaccharide chains, 350 mg of mono-6-(ethane-1,2-diamine)-6-deoxy-β-CD or mono-6-(hexane-1,6-diamine)-6-deoxy-β-CD was added to 5 mL of the activated polymer solutions and then treated with 20 mg $NaBH_4$ for 4 h under continuous stirring (Scheme 1). The modified polymers solutions were further dialyzed against distilled H_2O and finally freeze-dried (frozen at -26 °C for 24 h and finally freeze-dried in a Heto Holten A/S, cooling trap model CT 110 freeze-dryer (Heto Lab Equipment, Denmark) operating at a condenser plate temperature of -111 °C and a chamber pressure of $4\cdot10^{-4}$ mbar); the secondary drying was performed at 25 °C).

The final products, dextran-ethane-1,2-diamine-β-cyclodextrin (DECD) and dextran-hexane-1,6-diamine-β-cyclodextrin (DHCD) were characterized by ^{1}H-NMR and its average molecular weight was obtained by viscosimetry.
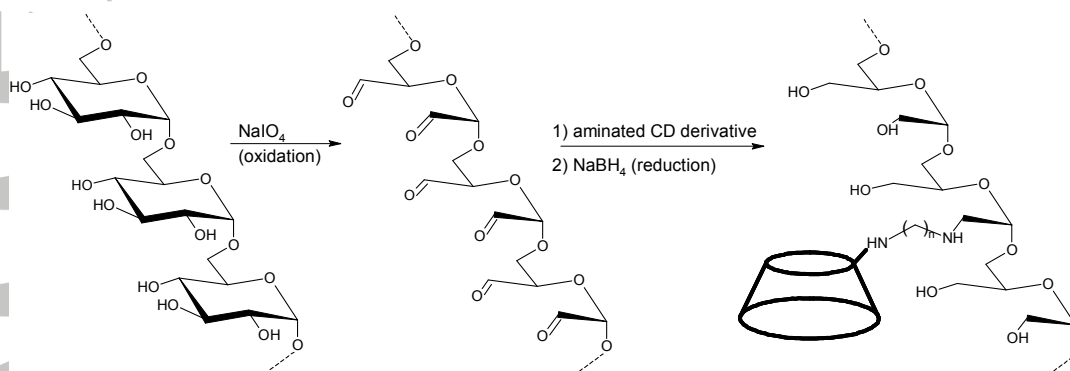
Scheme 1. Representation of the reaction steps for the dextran-β-CD polymers. The spacer arms used were ethane-1,2-diamine (n = 2) for DECD and hexane-1,6-

diamine (n = 6) for DHCD. In the scheme the size of the truncated cone corresponding to the β-CD is not in scale with respect to the dextran chain.

2.3. Preparation of the samples and thermal treatment

Solid systems consisted of freeze-dried solutions containing 10 % (w/v) of Tr, the polymer (dextran 70, DECD and DHCD) or the blend trehalose-polymer (1:1). A suspension of powdered invertase was added to each solution. Each one of the final systems contained 117 enzymatic units $\cdot mL^{-1}$. Aliquots of 75 μL of each model were placed in micro-centrifuge tubes, frozen by immersion in liquid nitrogen and freeze-dried. After freeze-drying the samples were transferred to vacuum dryers containing dried silica, and some of them were exposed to relative humidities (RH) 22 and 43 %, corresponding to saturated solutions of CH_3COOK and of K_2CO_3, respectively, at 25 ± 1 ºC for one week.[21] After rehumidification, the samples were tightly sealed and stored at 87 ± 3 ºC in a forced air convection oven for 16 h. The remaining activity of the enzyme and the thermal transitions were determined as describe below.

2.4. Invertase activity

Invertase activity was determined spectrophotometrically at 546 nm using 3,5-dinitrosalicylic acid method for reducing sugars,[22] as previously reported.[2,3] Briefly, the freeze-dried/humidified samples were reconstituted to their original volume with 50 mM sodium acetate buffer pH 4.6 and were kept at 5 °C until complete dissolution was achieved. The enzymatic activity of invertase was determined by adding 0.40 mL of sucrose solution (200 mM in sodium acetate buffer 50mM, pH 4.6) to 0.10 mL of the dissolution sample. After incubation (10 min at 37 °C), the samples were exposed at 100 °C during 10 min to denature the enzyme. For each system, the amount of hydrolyzed sucrose after any treatment (S) was related to the amount of sucrose before the treatment (S_0) and the remaining activity (R.A.) was expressed in percentage as: R.A. = 100 S/S_0. Duplicate measurements were performed for each analysis. The confidence intervals were calculated by measuring 4 samples of the same run and were 7 %. Average values were reported.

2.5. Thermal transitions

Glass transition temperature (T_g) and trehalose dihydrate melting were determined by dynamic differential scanning calorimetry (DSC) by means of a Mettler Toledo 822 equipment (Mettler Toledo AG, Switzerland) and STARe Thermal Analysis System version 3.1 software (Mettler Toledo AG). Freeze-dried samples as obtained and freeze-dried samples exposed to 22 and 43 % RH were analyzed. Glass transitions were recorded as the onset temperatures of the discontinuities (baseline shift) in the curves of heat-flow versus temperature. The endothermal peak around 97 °C corresponds to the melting of crystalline trehalose dihydrate.[23] The instrument was calibrated using standard compounds (indium and zinc) of defined melting point and heat of melting. All measurements were made in duplicate with 5-10 mg sample mass, using tightly sealed aluminum pans of 40 μL inner volume (Mettler), heated from -50 to 120 °C at 10 °C/min under nitrogen atmosphere; an empty pan was used as a reference. The confidence intervals estimated for temperature values and for enthalpy values were 1 °C and 10 mJ, respectively.

The degree of trehalose crystallization (ϕ) was calculated from the ratio of the area of the endothermal melting peak in the sample thermogram (ΔH_m) and the specific melting enthalpy of pure trehalose dihydrate (ΔH_{mTr} = 139 J/g) measured by dynamic DSC in the same condition. As shown in equation 1:

$$\phi = \Delta H_m / \Delta H_{mTr} \qquad \text{(eq. 1)}$$

2.6. Determination of water content

The total water content of the samples was determined gravimetrically by difference in weight before and after drying in vacuum oven for 48 h at 96 °C $\pm$ 2 °C. These drying conditions were selected in previous studies[2-4] and they were adequate to determine water content in the studied systems with a confidence interval of 6 % for a 95 % certainty.

2.7. Polymer characterization

2.7.1. Nuclear magnetic resonance

The DECD, DHCD, CD and dextran were characterized by ^{1}H-NMR (500 MHz Bruker equipment from Bruker Optics, Rheinstetten, Germany) in solutions of 20 mg/mL in D_2O at 25 °C. The experiments were conducted by using a Bruker's defined pulse

program (zg30), the acquisition mode was DQD (digital quadrature detection), the number of scans and dummy scans were 16 and 2, respectively, the size of the free induction decay was 65536, the spectral width was 15 ppm, and the acquisition time was around 4.4 s. The spectra were analyzed by TopSpin 2.0.b.5 software (Bruker Biospin, Germany) with exponential apodization (0.1 Hz line broadening was used). A standard Fourier transform was performed (32768 points) and the phase and baseline were corrected as required in each case. The water signal was removed for clarity.

2.7.2. Molecular weight determination by viscosimetry

The intrinsic viscosity [η] of a very diluted solution is one of the key parameters from which it is possible to obtain the average molecular weight (M_w) of a polymer. [η] is calculated by using equation 2.[24]

$$[\eta] = \lim_{c \to 0} \frac{\eta - \eta_0}{\eta_0 c} \qquad \text{(eq. 2)}$$

Where η_0 is the viscosity of the pure solvent and c is the concentration of the polymer (expressed in g/mL).

For flexible polymers with expanded chains, the [η] is related to the average M_w through the empiric Mark-Houwink-Staundinger-Sakurada relationship[24] defined in equation 3.

$$\eta = K \times M_w{}^a \qquad \text{(eq. 3)}$$

Where *K* and *a* are particular constants for each polymer-solvent pair and temperature.[25]

The *K* and *a* values chosen for the two synthesized polymers (DECD and DHCD) were a = 0.45 and K = $1.36 \cdot 10^{-3}$ mL/g.[26]

Viscosity was measured in triplicate in a Brookfield viscometer model DV-II+ (Brookfield Engineering Laboratories, Stoughton, MA, USA) in 0.5 mL of 1-0.01 % w/v solutions using a CPE 40 plate.

2.8. Statistical analysis

Significance of the effect of matrix composition on invertase activity after freeze-drying were evaluated by t test (significance level $\alpha = 0.05$ %) using Prism 5 (GrapPad Software Inc., San Diego, CA).

3. Results and Discussion

3.1. Polymers characterization

Dextran derivatives containing β-CD were synthesized. The synthesis involved the oxidation and subsequent binding of dextran with β-CD units previously aminated using two different spacer arms of 2 and 6 carbons, giving DECD and DHCD, respectively. The structure of both compounds was characterized by ^{1}H- NMR, as shown in Figure 1. The spectra of DECD and DHCD were very similar to each other, especially in the area between 3.4 to 4 ppm, where the observed peaks correspond to both dextran and β-CD. The main differences between DECD and DHCD were manifested in the signals corresponding to the different spacer arms. The spectrum of dextran (Fig. 1) shows five main peaks, which is in agreement with those reported in literature.[27] The degree of substitution of the two synthesized biopolymers was obtained by calculating the ratio between the integration signal corresponding to the anomeric protons of the CD units (5.01 ppm, signal F of Figure 1, which integrates for 7 protons) and the signal of the anomeric proton of dextran (4.92 ppm, signal A of Figure 1, which integrates for 4 protons).[16,28] This implies that there is one unit of β-CD every four glucose units of dextran. The obtained substitution degree was similar for both derivatives, with approx. 100 units of β-CD per mole of polymer, which corresponds to a 164% mass increment of the biopolymer.
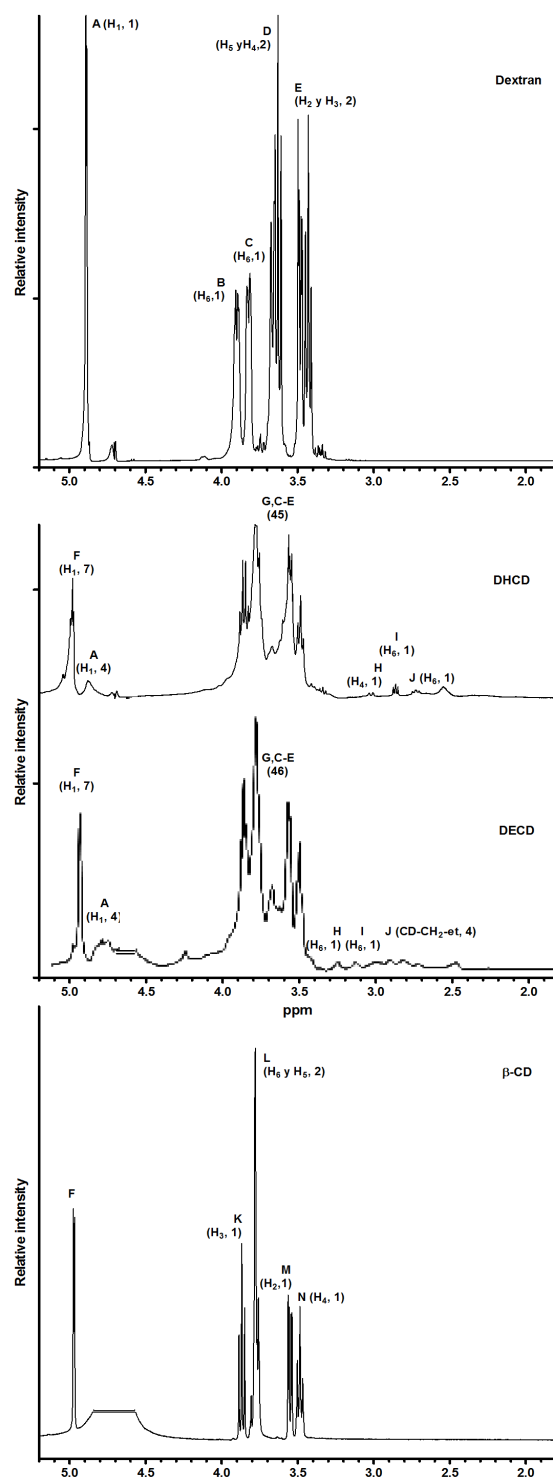
Figure 1. ^{1}H-NMR spectra of dextran 70, β-CD and the two synthesized dextran derivatives (DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin). Letters were included in relevant peaks, with the

assignment and the number of integrated protons. The water peak at 4.7 ppm was removed for clarity.

The viscosity average molecular weights (M_v) of the synthesized polymers were calculated using the empirical Mark-Houwink-Sakurada-Staundinger (MHSS) equation based on viscosity measurement. The obtained values were in the same confidence interval of $11.2 \pm 0.7 \cdot 10^4$ g/mol, and could be considered coincident for both derivatives. These values were lower than those expected on the basis of the substitution degree obtained by ^{1}H-NMR were $18.8 \cdot 10^4$ and $19.5 \cdot 10^4$ g/mol for DECD and DHCD, respectively. However, it is important to keep in mind that the constants (*K* and *a*) used in the MHSS equation were those corresponding to pure dextran. The opening of the ring by oxidation and the attachment of a voluminous group such as the CD provokes changes in the structural conformation of the dextran backbone in the new polymers, altering their flexibility.

3.2. Influence of the spacer arm length on T_g values and on enzyme-polymer interactions

T_g is related to the capacity of a compound to perform hydrogen bonding interactions with itself (establishing an extensive hydrogen bonding network in the amorphous state)[29], and also to interact with a labile compound (i.e., proteins). As expected, all systems were essentially amorphous after freeze-drying, which was confirmed by the absence of the melting endothermal peaks, and the presence of a clear glass transition in the DSC thermograms. Figure 2 shows the T_g values obtained for D, DECD and DHCD freeze-dried samples exposed at 22 and 43% RH as a function of water content. The data for dextran 70 previously reported from literature,[30] which were in agreement with those of D systems, were also included. The chemical modification of DECD promoted a higher water adsorption and lower T_g values in comparison to dextran, in samples exposed to the same RH. The effect of increasing the spacer arm length was very pronounced in the low T_g values obtained for DHCD. This T_g reduction could be related to the chemical modification of the backbone during oxidation, increasing backbone flexibility (see Scheme 1), and to a steric factor introduced by the attachment of the CD-spacer arm units on dextran, reducing the packing in comparison with dextran.[15]
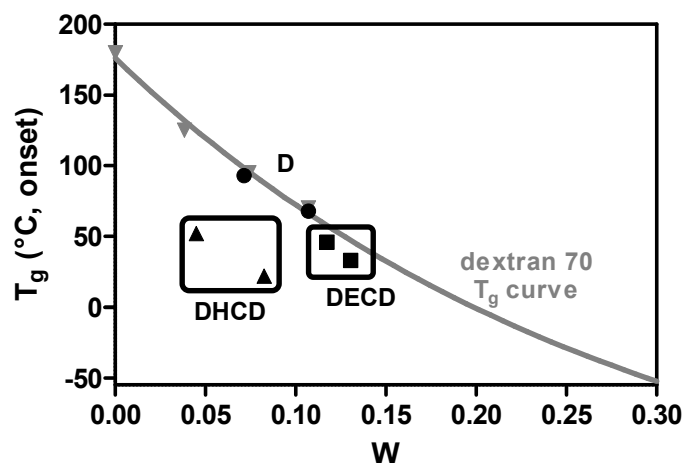
Figure 2. Glass transition temperature (T_g) and water content values (W) obtained for freeze-dried systems of dextran 70 (D) and dextran-CD modified polymers humidified at 22 and 43% RH. Literature T_g values for dextran 70 were included in grey (Icoz et al., 2005). D (black circles): dextran 70; DECD (black squares): dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD (black triangles): dextran-hexane-1,6-diamine-β-cyclodextrin.

The spacer arm length influenced the T_g values of each synthesized polymer and their capacity to interact adequately with invertase. The T_g value of polymers and sugars has been related to the average length of the hydrogen bonds, and hence to the molecular packing of the amorphous sugars.[31] How amorphous matrices are packed determines the presence of voids or defects, influencing their protective effect. Matrices formed by smaller molecules (e.g., disaccharides) are more densely packed than those corresponding to higher molecular weight saccharides.[32] Therefore, the former matrices are more effective in restricting mobility of immobilized molecular compounds with respect to matrices consisting of compounds that provide a lesser degree of molecular packing (such as β-CD, high molecular weight polymers or starches, for example). Then, a compromise is established between the benefit of having a high T_g value and a well-packaged molecular structure. Also, it is important to consider that the enzyme stability in dehydrated matrices is not only related to supramolecular aspects or to the molecular mobility of the matrix, but also depends on other factors such as chemical reactivity, molar mass and molecular structure of the matrix.[33]

Since the spacer arm length influenced T_g values and, as stated before, T_g is connected to the extent of hydrogen bonding, we studied the effect of the additives as stabilizers on enzyme activity after freeze-drying and after thermal treatment. Invertase, an extracellular and highly glycosylated enzyme, was chosen as a target model since its properties are well known, and it is easy to handle and to analyze.

The invertase activity recovered after freeze-drying in the biopolymers and in their combined matrices with trehalose is shown in Figure 3. Trehalose, one of the best biomolecule dehydro-protective agent, was chosen as a reference in order to evaluate the efficiency of the synthesized polymers. Combinations of trehalose and polymers were also studied, since it is a practical use to combine sugars and biopolymers in order to provide both physical and chemical stability to biomolecules.[34]
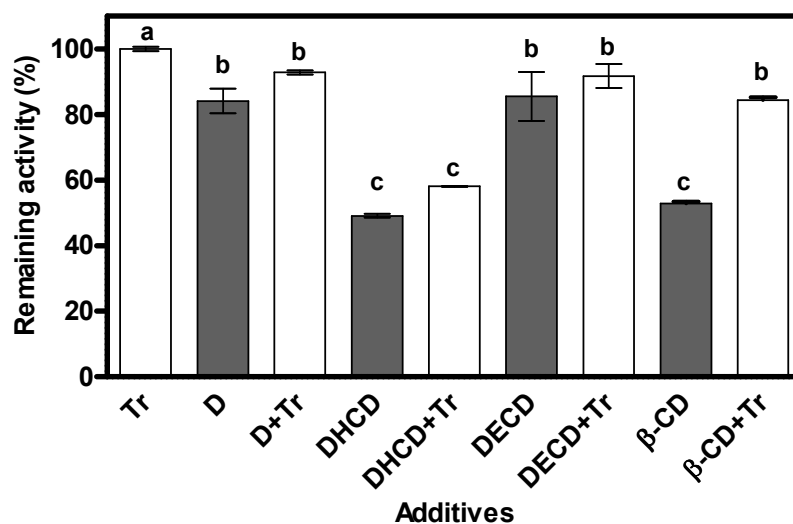

Figure 3. Remaining activity of invertase in the biopolymers and in their combined matrices with trehalose after freeze-drying. The bars represent the standard deviation. Significant differences between remaining activity values are indicated with different letters (a-c; $P < 0.05$). Tr: trehalose, D: dextran 70; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin.

As expected,[2,3] the enzyme was completely preserved in the trehalose matrix during freeze-drying. The polymers or their mixtures with trehalose were not as good as trehalose (Tr) to protect the enzyme. An activity recovery over 80% of the initial activity was obtained in D and D + Tr systems, showing the suitability of dextran as a dehydro-protectant. The synthesized polymer with the short spacer arm (DECD) gave

good enzyme recoveries after freeze-drying (either with or without trehalose). However, DHCD had a negative effect on the enzyme preservation. The activity recovered in the DHCD systems was low, in agreement to that previously observed in β-CD.[2-4] The improved recovery observed by the addition of trehalose to the β-CD formulation was not obtained by adding trehalose to DHCD systems. It was hypothesized that the non polar groups of invertase could be anchored in the β-CD cavities, avoiding enzyme inactivation.[10] Instead, the attachment of CD to dextran by using the long spacer arm in DHCD systems precluded the favorable interactions developed by dextran, as shown in Figure 3. In the combined DHCD +Tr system, it is possible that the protein could be anchored in an unfavorable conformation through supramolecular interactions between β-CD and protein aromatic groups, avoiding trehalose stabilizing effect. In the case of DECD, the presence of β-CD did not affect the dextran-protein adequate interactions during freeze-drying.

The remaining activity of invertase in the freeze-dried systems (humidified at 22 and 43% RH) treated at 87 °C (T) for 16 h is shown in Figure 4, as a function of $T-T_g$. The remaining activity of systems D and DECD (with or without trehalose) decreased as a function of $T-T_g$. However, two clear trends are observed in this figure: the systems D and DECD containing trehalose showed higher remaining activity values than the same systems without this sugar. On the other hand, DHCD systems showed a smaller variation as a function of $T-T_g$ than DECD, with lower values than the corresponding trend (with or w/o trehalose) at low $T-T_g$ values. Trehalose systems humidified at 43% RH (lower closed diamond in Fig. 4) showed a much smaller remaining activity value in comparison with the rest of the samples containing trehalose. This behavior was related to trehalose crystallization, which significantly affects the mechanisms by which the protective effects on biomolecules are developed and, consequently, enzymes are rapidly inactivated in crystallized matrices.[2-4,6,35-37] It has to be noted that in all trehalose systems the amount of adsorbed water at RH 43% was sufficient for complete trehalose dihydrate crystallization (the amount of water to form the dihydrate crystal is 10.5% in dry basis). However, only the pure trehalose system at 43% RH had crystallized to a high extent (up to 80%), as shown in Figure 5. As previously observed with other β-CD modifications on polymers,[2-4] sugar crystallization was delayed/inhibited in the blends containing any of the studied polymers at 43% RH (Figure 5). It has been reported that

the presence of certain polymers, salts and sugars could delay/inhibit crystallization of amorphous sugars in freeze-dried formulations by affecting the extent and kinetic of sugar crystallization.[34] Then, one of the advantages of adding polymers to trehalose in the combined matrices is to extend the RH range at which this sugar could be exposed, extending the stability range of the matrix for protein stabilization.
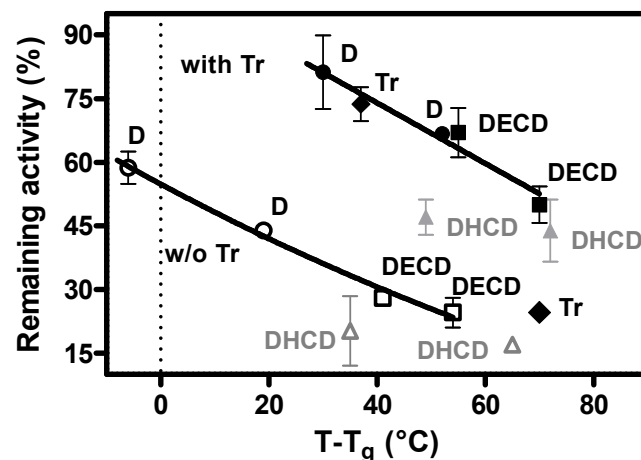

Figure 4. Remaining activity of invertase in freeze-dried systems exposed at 22 and 43 % RH and treated for 16 h at T = 87°C, as a function of $T-T_g$ (treatment temperature minus glass transition temperature). The bars represent the standard deviation. Lines are included only to show general trends. Closed symbols: systems with trehalose (Tr); open symbols: systems without trehalose; D: dextran 70; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin.

DHCD was a good stabilizer from a physical point of view, showing similar T_g values than one of the most used excipients as trehalose. However, Pikal[1] showed that a high glass transition for an excipient was not directly related to enzyme stabilization. The destabilization of invertase produced by the DHCD is related to both the modification of hydrogen bond interactions and/or molecular packing, and to the type of supramolecular interactions generated between the additive and the protein (mainly undesirable hydrophobic interactions). The presence of trehalose should improve the molecular packing, but not affect the already established undesirable interactions produced by DHCD. This work supports previous reports[5,6] which showed that the formation of an amorphous matrix does not guarantee the stabilization of immobilized

labile materials, which requires the development of adequate protein-excipient interactions.
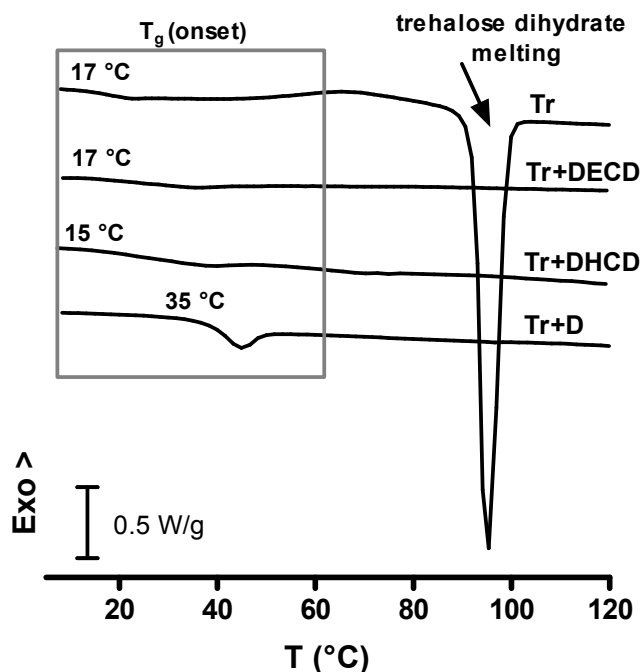

Figure 5. Differential scanning calorimetry (DSC) thermograms for selected freeze-dried samples exposed to RH 43% during one week. Glass transition temperature (T_g) and trehalose melting are indicated. Tr: trehalose; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin; D: dextran.

4. Conclusions

The different physical properties of the two modified dextran-CD polymers were also manifested on their efficiency as enzyme stabilizers. These differences were mainly related to the effect of the spacer arm length on polymer-enzyme interactions, since both the degree of substitution and the molecular weight were similar for the two polymers. The short spacer arm (2C) increased water adsorption capacity and decreased T_g values, without affecting the stabilizing properties of the polymer in freeze-dried formulations containing invertase. The long spacer arm (6C) drastically reduced the polymer efficiency as enzyme stabilizer. This change of effective interactions was also manifested in the pronounced reduction of T_g values, related to a less extend of hydrogen bond interactions between the dextran chains, and therefore, with the enzyme.

The presence of trehalose should improve the molecular packing and the overall protection of the enzyme during freeze-drying. However, its presence did not preclude the already established undesirable interactions produced by DHCD with the enzyme. The CD attachment (especially with the 6C spacer) developed supramolecular specific interactions between CD and the protein surface which limited the possibility of the protein to interact with the dextran backbone. By reducing the length of the spacer arm, the interactions between protein and dextran backbone were possible, stabilizing the protein during freeze-drying. This works shows how a small modification in the spacer arm length between a biopolymer and an attached moiety strongly influenced the capacity to interact adequately with an enzyme.

Acknowledgment

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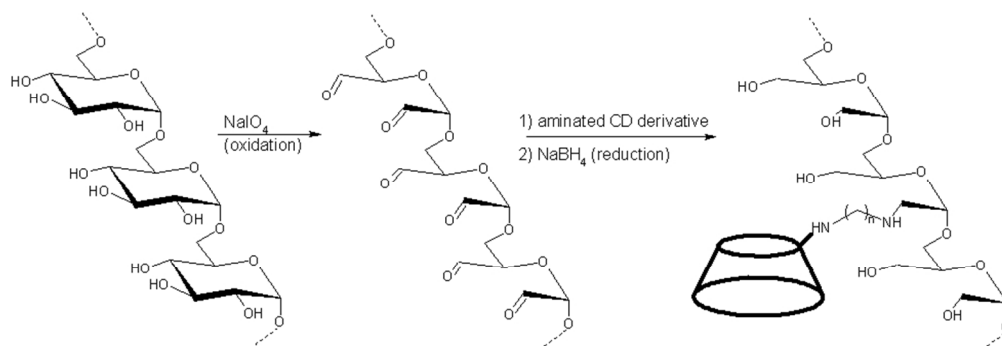
Scheme 1. Representation of the reaction steps for the dextran-β-CD polymers. The spacer arms used were ethane-1,2-diamine (n = 2) for DECD and hexane-1,6-diamine (n = 6) for DHCD. In the scheme the size of the truncated cone corresponding to the β-CD is not in scale with respect to the dextran chain.
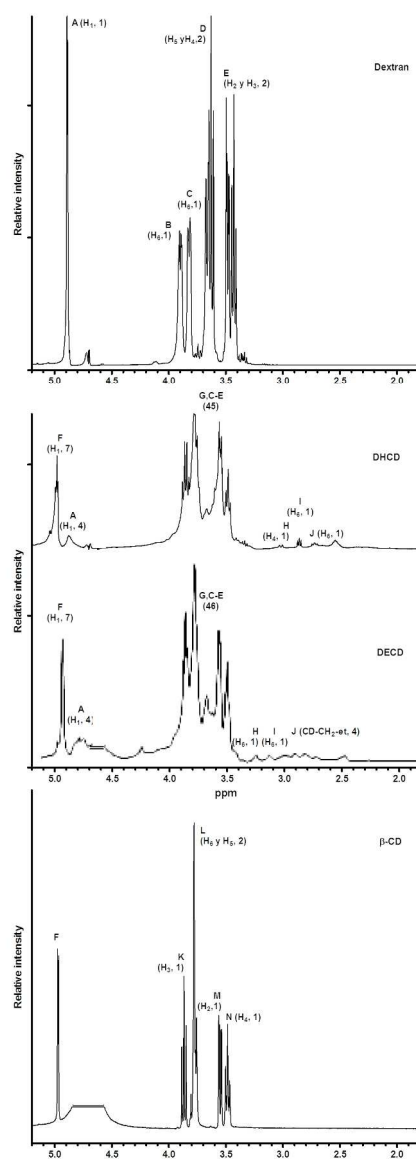82x28mm (300 x 300 DPI)

Figure 1. 1H-NMR spectra of dextran 70, β-CD and the two synthesized dextran derivatives (DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin). Letters were included in relevant peaks, with the assignment and the number of integrated protons. The water peak at 4.7 ppm was removed for clarity.
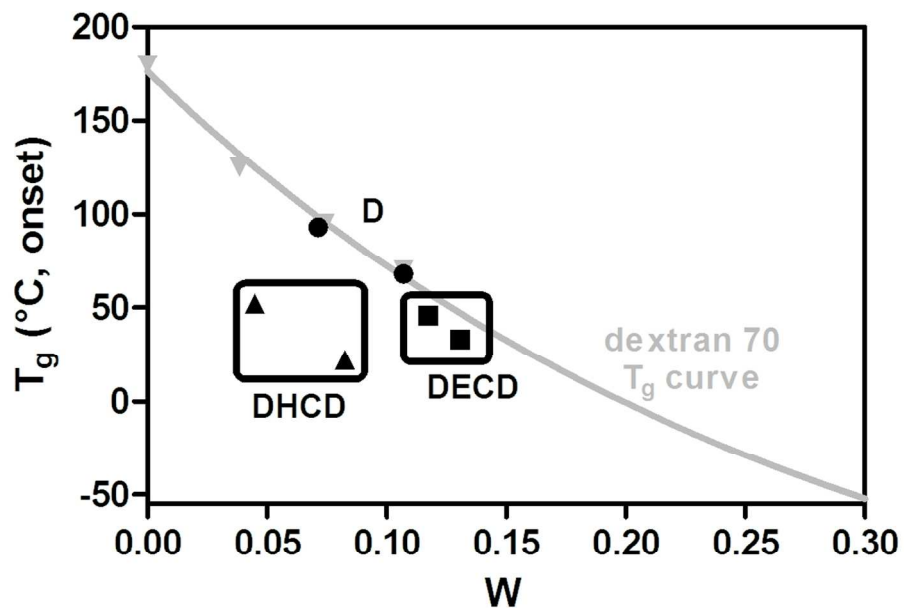105x264mm (300 x 300 DPI)

Figure 2. Glass transition temperature (Tg) and water content values (W) obtained for freeze-dried systems of dextran 70 (D) and dextran-CD modified polymers humidified at 22 and 43% RH. Literature Tg values for dextran 70 were included in grey (Icoz et al., 2005). D (black circles): dextran 70; DECD (black squares): dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD (black triangles): dextran-hexane-1,6-diamine-β-cyclodextrin.
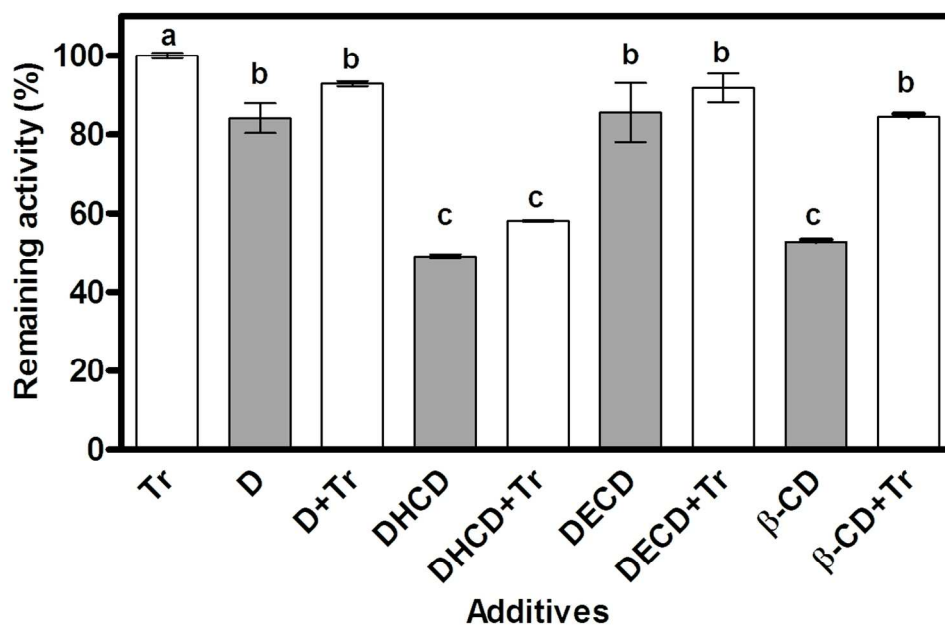106x73mm (300 x 300 DPI)

Figure 3. Remaining activity of invertase in the biopolymers and in their combined matrices with trehalose after freeze-drying. The bars represent the standard deviation. Significant differences between remaining activity values are indicated with different letters (a-c; $P < 0.05$). Tr: trehalose, D: dextran 70; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin. 117x83mm (300 x 300 DPI)
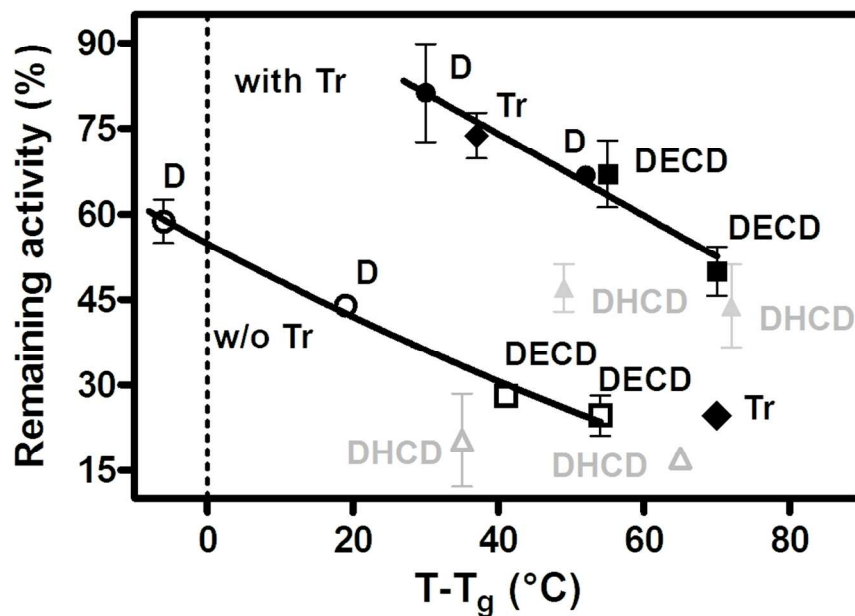
Figure 4. Remaining activity of invertase in freeze-dried systems exposed at 22 and 43 % RH and treated for 16 h at T = 87°C, as a function of T-Tg (treatment temperature minus glass transition temperature). The bars represent the standard deviation. Lines are included only to show general trends. Closed symbols: systems with trehalose (Tr); open symbols: systems without trehalose; D: dextran 70; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin.
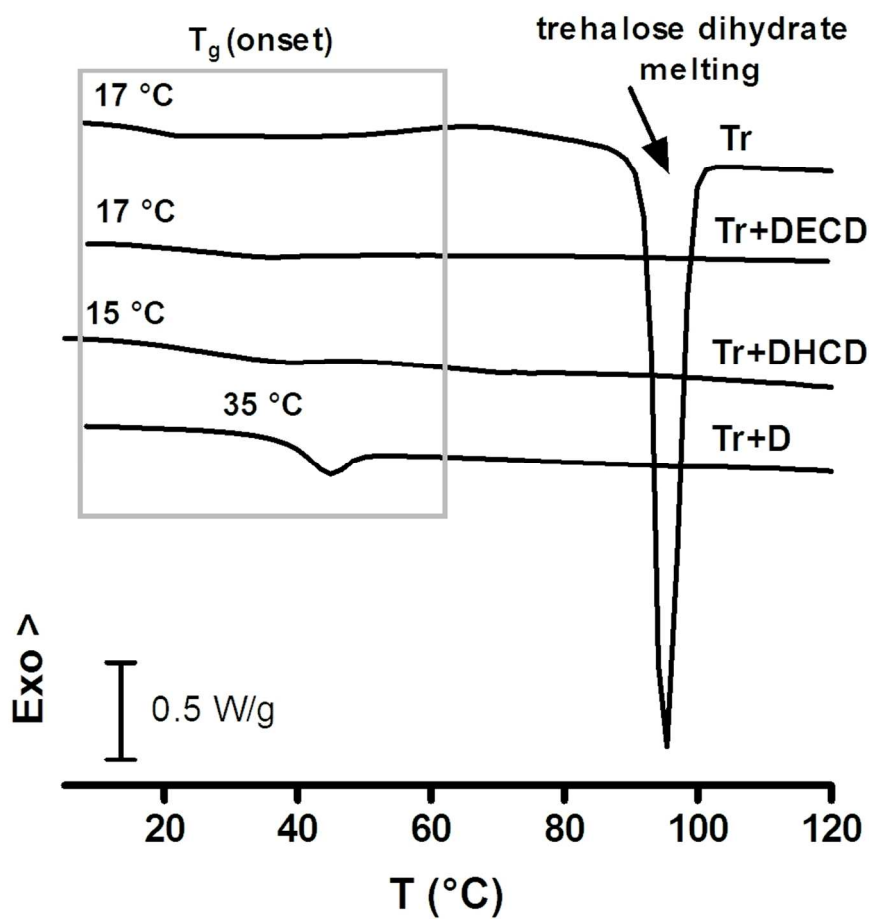105x74mm (300 x 300 DPI)

Figure 5. Differential scanning calorimetry (DSC) thermograms for selected freeze-dried samples exposed to RH 43% during one week. Glass transition temperature (Tg) and trehalose melting are indicated. Tr: trehalose; DECD: dextran-ethane-1,2-diamine-β-cyclodextrin; DHCD: dextran-hexane-1,6-diamine-β-cyclodextrin; D: dextran.
99x98mm (300 x 300 DPI)