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β-Cyclodextrin modifications as related to enzyme stability in dehydrated systems: Supramolecular transitions and molecular interactions

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ABSTRACT

The effect of β-cyclodextrin modifications (polymerization (PCD) and later carboxymethylation (CMPCD)) on their action as enzyme stabilizers was analyzed during freeze-drying and thermal treatment. Combined polymer-trehalose matrices were also employed. Due to their higher T_g values, PCD and CMPCD provided better structural stability to the freeze-dried formulations than β-CD. However, only PCD was a good excipient to protect invertase both in amorphous and supercooled systems. FT-IR revealed increased protein denaturation in the presence of CMPCD, but not in the presence of PCD. Even though all polymers inhibited/delayed trehalose crystallization, only trehalose (T) combined with PCD (PCD + T) and with β-cyclodextrin (β-CD + T) offered the best stability to the enzyme. In β-CD + T system, trehalose was the main responsible for the protection. In PCD + T system, both additives contributed to improve the enzyme stability. FT-IR and DSC were useful to analyze the combined role of molecular and supramolecular interactions on enzyme stability in dehydrated model systems.

1. Introduction

Highly thermostable enzymes are required for several industrial and biotechnological applications. Although a number of techniques have been examined for increasing protein stability, the use of water soluble polyols as thermoprotectant additives appears as the most economical approach taking into account the simplicity and low cost of this method (Fernández, Villalonga, Fragoso, & Villalonga, 2004b). The disaccharide trehalose is a good additive to protect enzymes after freeze-drying or thermal treatment (Lee, Hafeman, Debenedetti, Pethica, & Moore, 2006; Mazzobre, Buera, & Chirife, 1997; Sun & Davidson, 1998; Suzuki, Imamura, Yamamoto, Satoh, & Okazaki, 1997). However, undesired trehalose crystallization significantly affects the mechanisms by which the protective effects on biomolecules are developed and, consequently, the shelf life of a product (Mazzobre et al., 1997; Schebor, Mazzobre, & Buera, 2010; Sun & Davidson, 1998; Suzuki et al., 1997). When trehalose crystallizes, enzymes are rapidly inactivated and alternative media have to be formulated in order to expand the range of applicability towards higher relative humidities and/or temperatures. Several authors have demonstrated that the presence of polymers delays sugar crystallization processes (Iglesias & Chirife, 1978; Karmas, Buera, & Karel, 1992; Mazzobre et al., 1997). In a previous work we studied the structure/function relationship of different biopolymers in relation to their efficiency as enzyme stabilizers in freeze-dried formulations, in comparison to trehalose (Santagapita et al., 2008). Biopolymers such as alginate and dextran, and particularly a synthesized β-CD-branched alginate were employed. The modified β-CD-branched alginate combined the physical stability provided by alginate with the protective effect on hydrophobic regions of the enzyme given by cyclodextrin. β-Cyclodextrin (β-CD) is a cyclic non-reducing oligosaccharide composed of 7 glucopyranose units bonded together via α(1–4) glycoside linkages, with a hydrophobic central cavity and a hydrophilic outer surface (Szejtli, 1998). CDs are capable of including a variety of hydrophobic guest compounds, such as aromatic amino acids located at the proteins surface (Fernández, Fragoso, Cao, Baños, & Villalonga, 2002; Villalonga et al., 2003). Thermal stability of enzymes could thus be improved by chemical conjugation of the enzyme with several CD derivatives, inducing multipoint supramolecular advan-

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tageous interactions at the surface of the enzyme (Fernández et al., 2002; Villalonga et al., 2003). O-carboxymethyl-poly-β-cyclodextrin (CMPCD) has been previously employed for modifying enzymes in very diluted aqueous media by chemical conjugation (Fernández, Villalonga, Fragoso, Cao, et al., 2004a; Villalonga, Reyes, Fragoso, Cao, & Villalonga, 2005; Villalonga, Tachibana, Cao, Matos, & Asano, 2007). These neoglycoenzymes retained high activity, increasing optimum temperature, thermostability and enhancing their stability to pH in the analyzed cases. On the other hand, the assembly of PCD with dextran was recently studied as a potential component of controlled delivery systems with applications of interest in pharmacy and industry (Gref et al., 2006). However, neither PCD nor CMPCD were previously employed in freeze-dried protein formulations.

Invertase from *Saccharomyces cerevisiae* was chosen as a model enzyme to analyze its stability at considerably high temperatures, since it is a quite thermostable enzyme, widely used in the pharmaceutical and food industries (Tomotani & Vitolo, 2007), and also for the development of analytical devices (Rodríguez, Aguilar, & Pérez Padilla, 2000). For these important applications, a highly stable and reusable invertase form is desired, in order to reduce costs and increase the productivity of the overall process.

The purpose of this work was to analyze the effect of β-CD modifications – through its polymerization (PCD) and/or later carboxymethylation (CMPCD) – as invertase stabilizers in freeze-dried formulations. Combined polymer-trehalose matrices were also analyzed in order to achieve protection in supercooled systems where trehalose normally crystallizes. The results may also provide general basis for stabilizing other kind of enzymes. As both molecular and supramolecular protein/excipient interactions are involved in enzyme stabilization, the combined analysis through differential scanning calorimetry (DSC) and spectroscopic techniques such as Fourier transform-infrared (FT-IR) spectroscopy provide a global analysis of the involved phenomena.

2. Experimental

2.1. Synthesis of poly-β-cyclodextrin polymer and its later carboxymethylation

The poly-β-cyclodextrin polymer (PCD) and the O-carboxymethyl-poly-β-cyclodextrin (CMPCD) were synthesized as previously described (Fernández et al., 2004a; Renard, Deratani, Volet, & Sebille, 1997; Villalonga et al., 2005, 2007). Briefly, 5 g of β-CD (Amaizo, Hammond, IN, USA) was dissolved in 50 mL of 10% (w/v) NaOH and treated with 10 mL of epichlorohydrin. The solution was continuously stirred for 8 h, then another 5 mL of epichlorohydrin was added and stirring was kept overnight at room temperature. The solution was concentrated to about 15 mL and poured over cold ethanol in order to precipitate the polymer. The precipitate was crushed several times with ethanol in a mortar until a fine powder was obtained. The polymerized CD was then washed again with ethanol and acetone and dried under high vacuum overnight. For the introduction of the carboxymethyl groups into the polymer, 2 g of the polysaccharide was dissolved in 50 mL of 5% (w/v) NaOH and 2 g of $ClCH_2COOH$ was added. The system was stirred for 24 h, neutralized with 2 M HCl, concentrated to about 15 mL, cooled to 4 °C and filtered. The supernatant was poured over cold ethanol in order to precipitate the modified polymer. The precipitate was crushed several times with ethanol in a mortar until a fine powder was obtained. The powder was then washed two more times with ethanol and acetone and dried under high vacuum overnight.

The molecular weight of CMPCD and PCD was determined by gel permeation chromatography on Fractogel EMD BioSEC (S) (1.6 cm × 100 cm) calibrated with dextran standards as previously described (Fernández et al., 2004a; Villalonga et al., 2005, 2007). PCD and CMPCD have an average molecular weight of 13 kDa and 15 kDa, respectively. The degree of carboxymethylation of CMPCD determined by potentiometric titration was estimated as 40% (D-glucose, mol/mol) as previously described (Fernández et al., 2004a; Villalonga et al., 2005, 2007).

2.2. Preparation of the samples and thermal treatment

Solid amorphous systems were obtained by freeze-drying aqueous solutions containing 10% (w/v) α-α-trehalose dihydrate crystals (T) (Hayashibara Co, Ltd., Shimoishii, Okayama, Japan), a polymer (β-CD, PCD or CMPCD) or a blend trehalose-polymer (1:1). A suspension of powdered invertase from *S. cerevisiae* (β-fructofuranosidase, E.C. 3.2.1.26, Fluka, Buchs, Switzerland) was added to each solution. Each one of the final systems contained 117 enzymatic unit/mL. One enzyme activity unit was defined as the amount of enzyme required to hydrolyze 1.0 μmol of sucrose per minute at pH 4.6, at 37 °C. Invertase concentration was estimated spectrophotometrically by absorbance measurement at 280 nm, considering the relationship 1 mg/mL = 2.25 (absorbance unit) (Trimble & Maley, 1977).

Aliquots of 0.075 mL of each system were placed in Eppendorf tubes, frozen by immersion in liquid nitrogen (−196 °C) and 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, a chamber pressure of 30 Pa, and shelf temperature of 25 °C. The secondary drying was also performed at 25 °C. After freeze-drying the samples were transferred to vacuum desiccators and exposed to relative vapor pressure (RVP) of 22% (saturated solution of CH_3COOK) and 43% (saturated solution of K_2CO_3) at 25 ± 1 °C for one week (Greenspan, 1977). After rehumidification, the samples were hermetically sealed and stored at 87 ± 3 °C in a forced air convection oven. Being the studied invertase a quite thermostable enzyme, this temperature results adequate to perform accelerated tests to analyze the enzyme stability during practical experimental times. At suitable intervals, two samples were removed from the oven and the remaining activity of the enzyme was determined as described below; the average value was reported.

2.3. Thermal transitions

Glass transition temperature (T_g) and endothermal peak were determined by 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). For each system the endothermal baseline shift represents the glass transition and the endothermic peak (around 97 °C) correspond to the melting of crystalline trehalose dihydrate (Sussich & Cesàro, 2000). 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 hermetically sealed aluminum pans of 0.040 mL inner volume (Mettler), heated from −20 °C to 200 °C at 10 °C/min (dynamic experiment); an empty pan was used as a reference. The confidence interval estimated for temperature values was 2 °C.

In trehalose-containing systems the degree of trehalose crystallization (ϕ) was calculated from the ratio of the area of the endothermic melting peak in the sample thermogram (ΔH_m) and the melting enthalpy of pure trehalose dihydrate (ΔH_{mT}, 139 J g^{-1})

measured by dynamic DSC in the same conditions:

$$\phi = \frac{\Delta H_m}{\Delta H_{mT}} \quad (1)$$

The confidence interval estimated for enthalpy values was 10 mJ.

2.4. 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 ± 2 °C. These drying conditions were selected in previous studies (Santagapita et al., 2008) and they were adequate to determine water content in the studied systems with a confidence interval of 6% for a 95% certainty.

2.5. Fourier transform-infrared spectroscopy

The pellets for Fourier transform-infrared (FT-IR) spectroscopy analysis were prepared by mixing the freeze-dried systems with KBr. FT-IR absorption measurements were performed with a Nicolet 8700 FT-IR spectrometer (Thermo Electron Corporation Gmbh, Karlsruhe, Germany), with DTGS TEC detector. A Happ-Genzel apodization was used, with a Mertz phase correction. The optical bench was purged with dry air free of CO_2 during analysis. Spectra were recorded between 400 and 4000 cm^{-1}, and an average of 64 scans was recorded at a resolution of 1 cm^{-1} in the transmission mode. Spectral analysis was performed using the Omnic software version 7.3 (Thermo Electron Corporation Gmbh). The spectral region between 3000 and 3600 cm^{-1}, corresponding to the –OH stretching vibration (Barth, 2007; Vanderkooi, Dashnau, & Zelent, 2005), and the amide I vibration, absorbing near 1650 cm^{-1}, which arises from the C=O stretching vibration (Barth, 2007), were selected for analysis. The amide I vibration is hardly affected by the nature of the side chain. It depends, however on the secondary structure of the backbone and is therefore the amide vibration that is most commonly used for secondary structure analysis (Barth, 2007; Carpenter, Izutsu, & Randolph, 1999). In order to analyze the amide I band a narrowing technique procedure must be applied, which allows to resolve a convoluted spectrum into its component bands (Barth, 2007). The spectra were thus processed with the calculation of the second derivative, which is one of the useful recommended procedures (Carpenter et al., 1999). Also, a Norris filter was used (segment value was 9 and gap value 3), in order to decrease the noise:signal ratio and gain on signal pattern (Norris & Williams, 1984). The protein contribution to the amide I band was obtained by subtracting the polymer spectrum from the protein–polymer spectrum.

2.6. pH determination

Before freeze-drying, the pH values of the samples (0.075 mL) were measured with strips (Merk, Darmstadt, Germany) in the range of 0–6 and 5–10 pH units, with a scale graduated in 0.5 pH units.

2.7. Invertase activity

The freeze-dried/humidified samples were resuspended in 0.75 mL of 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 50 mM, 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 inactivate the enzyme. The reducing sugars were determined spectrophotometrically at 546 nm using the 3,5-dinitrosalicylic acid method (Bernfeld, 1955). For each system, the amount of hydrolyzed sucrose after a given time of thermal treatment (S) was related to the amount of sucrose hydrolyzed before the thermal treatment (S_0) and the remaining activity (R.A.) was expressed in percentage as: R.A. = 100 S/S_0. Two samples were taken for each condition and duplicate measurements were performed. The confidence interval was 7%, calculated by measuring 4 samples of the same run.

3. Results and discussion

3.1. Glass transition and hydrogen bonding capacity in β-CD polymers

In order to evaluate the effect of β-CD polymerization and carboxymethylation on the thermal properties, DSC thermograms obtained for the different samples were analyzed. Table 1 shows the glass transition temperatures, Δc_p values (difference in specific heat capacity, related to the baseline shift) and water content values obtained for the freeze-dried samples exposed at relative vapor pressure (RVP) of 22%. Data of trehalose samples were also included for comparative purposes. Both β-CD polymers presented higher T_g values than trehalose and β-CD (the values decreased in the order: CMPCD > PCD > β-CD > trehalose), as seen in Table 1. The increased T_g values, which render more stable glasses, indicated an increased thermal and physical stability of the derivatives (Ozmen & Yilmaz, 2007). It is also to be noted that the Δc_p values of the CMPCD and PCD polymers were lower than those of trehalose and of β-CD, as expected due to their higher molecular weight (Roos & Karel, 1991).

The differences between β-CD and the synthesized polymers were also reflected in the FT-IR spectra: the –OH stretching region was analyzed to study the hydrogen bonding capacity of each polymer. The absorbance corresponding to the –OH stretching region is between 3000 and 3600 cm^{-1}, and it is shown for the freeze-dried samples of CMPCD, PCD and β-CD in Fig. 1. The position of the maximum absorbance in the wave number scale varied in the following decreasing order: CMPCD > PCD > β-CD. It is interesting to note that wave number for the maximum absorbance correlates with the T_g values for β-CD polymers (shown in Table 1). This is in agreement with previous results showing that the position of the –OH band depends on the molecular weight of the polymer (Wolkers, Oliver, Tablin, & Crowe, 2004). The observed positive linear correlation between the band position of the –OH stretching mode and the T_g of the polymers has also been related to the average length of the hydrogen bonds, and hence to the molecular packing of the amorphous sugars (Wolkers et al., 2004). As T_g increases, sugars exhibit a decreased degree of molecular packing which allows to rearrange hydrogen bonds during a temperature change in the glassy state (Wolkers et al., 2004).

Table 1
Glass transition temperature (T_g), change in heat capacity at T_g (Δc_p), and water content (as percent of dry basis, % d.b.) obtained for samples exposed at 22% RVP during one week. β-CD: β-cyclodextrin; PCD: poly-β-cyclodextrin; CMPCD: carboxymethyl-poly-β-cyclodextrin.

Systems 22% RVP	Water content (% d.b.) ± 0.4	T_g (°C), onset ± 2	Δc_p (J g^{-1} K^{-1}) ± 0.07
β-CD	2.5	55	0.25
PCD	1.9	78	0.14
CMPCD	3.4	97	0.20
Trehalose	6.2	50	0.61

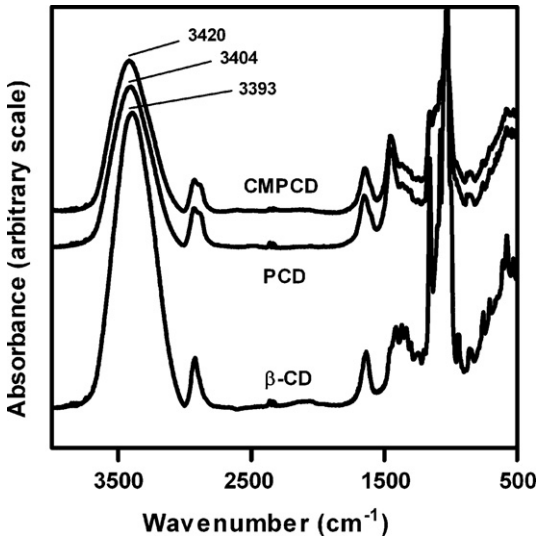

Fig. 1. FT-IR spectra of freeze-dried CMPCD, PCD and β-CD systems. IR region corresponding to –OH stretching band was located between 3000 and 3600 cm^{-1} and peak values (cm^{-1}) are indicated. CMPCD: carboxymethyl-poly-β-cyclodextrin; β-CD: β-cyclodextrin; PCD: poly-β-cyclodextrin.

3.2. β-CD polymers as excipients for invertase stabilization

Fig. 2 shows the remaining activity of invertase in the modified polymers and in their combined matrices with trehalose after freeze-drying. During the drying process the enzyme was best protected by the trehalose (T) matrix, while the polymers or their mixtures with trehalose were not as good as the disaccharide to protect the enzyme. Between 95 and 85% enzymatic activity recovery was achieved with PCD (in matrices with or without trehalose, respectively). As previously reported, the formulation containing β-CD + T showed more than 85% remaining enzymatic activity (Santagapita et al., 2008). On the other hand, CMPCD (with or without trehalose) and β-CD conferred less than 50% of enzymatic activity compared to trehalose as only excipient.

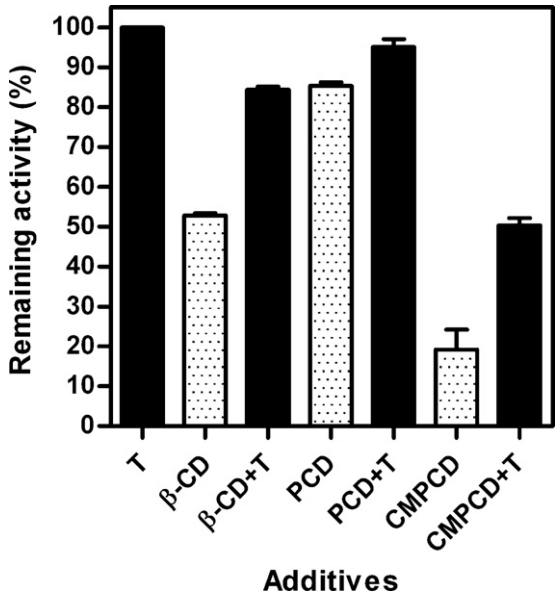

Fig. 2. Remaining activity of invertase in β-cyclodextrin additives and in their combined matrices with trehalose after freeze-drying in relation to the activity of the enzyme without any additive. The bars represent the standard deviation. T: trehalose; β-CD: β-cyclodextrin; PCD: poly-β-cyclodextrin; CMPCD: carboxymethyl-poly-β-cyclodextrin.

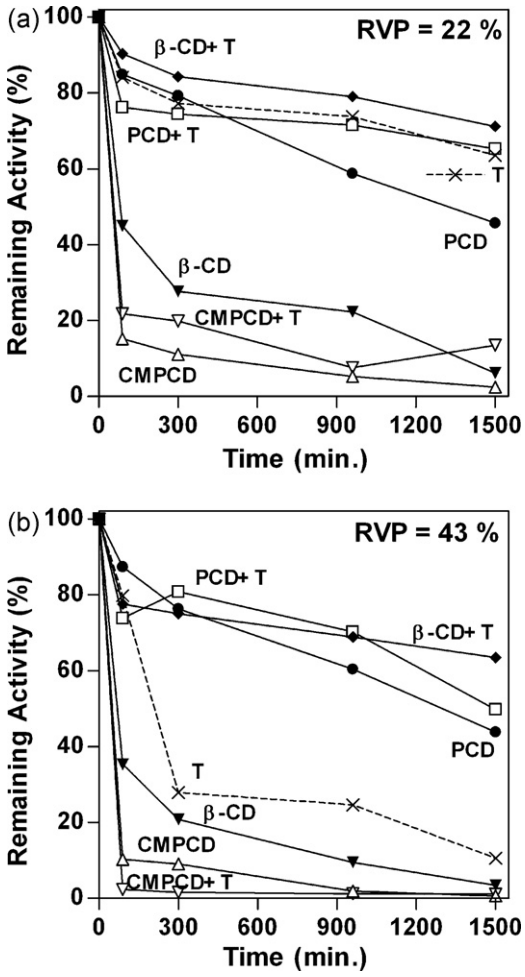

Fig. 3. Remaining activity of invertase in freeze-dried systems exposed at 22% RVP (a) and 43% RVP (b) as a function of heating time at 87 °C. The confidence interval for remaining enzyme activity was 7%. T: trehalose (×); β-CD: β-cyclodextrin (▼); PCD: poly-β-cyclodextrin (●); CMPCD: carboxymethyl-poly-β-cyclodextrin (Δ); PCD + T (□); β-CD + T (♦); CMPCD + T (▽).

Fig. 3 shows the remaining activity of invertase as a function of time of incubation at 87 °C at RVP 22 and 43%. Although trehalose was an effective protectant of the enzyme in systems exposed to RVP 22% and heated at 87 °C (Fig. 3a), the effect was lost at RVP 43% (Fig. 3b). This behavior was related to trehalose crystallization (Mazzobre & Buera, 1999; Mazzobre et al., 1997; Santagapita & Buera, 2006; Suzuki et al., 1997). As at RVP 43% the systems were above T_g value, both the molecular mobility and the amount of water were enough to allow the crystallization of the stable crystalline form of trehalose (trehalose dihydrate). Consequently, the enzyme was rapidly inactivated. On the contrary, although at RVP 22% the system was also in the supercooled state, the low water content prevented the trehalose dihydrate to crystallize and the non crystalline trehalose exhibits a better protection on the enzyme. The best protective systems during thermal treatment at both RVP were PCD, PCD + T, and β-CD + T (Fig. 3a and b). Enzyme remaining activity in the PCD system as a single additive was higher than 45% after 25 h of thermal treatment at 87 °C, at both RVP values analyzed, while it was almost negligible in CMPCD and β-CD systems. Although β-CD could interact with hydrophobic groups of the enzyme, preventing denaturation-derived changes, it was not efficient as protectant (Fig. 3a and b). The driving force of the guest-β-CD complex formation is the substitution of the high-enthalpy water molecules inside the β-CD cavity by appropriate guest molecules, less polar than water, to occupy the relatively

hydrophobic β-cyclodextrin cavity (Szejtli, 1998). Usually, the guest molecule and the β-cyclodextrin are dispersed in water and stirred to equilibrium for several hours to favor inclusion complex formation (Bilensoy, Doğan, Şen, & Hıncal, 2007), but this procedure was not considered adequate for the studied enzyme. In the conditions employed in present study for inclusion complex formation (short time, low temperature, without stirring), protein interactions with the barely soluble β-CD (1.8 g 100 mL^{-1}) were probably inadequate to assess enzyme stability. Through its polymerization or by the addition of trehalose (systems PCD, PCD + T, and β-CD + T), β-CD becomes a suitable matrix to stabilize invertase. Both the addition of trehalose and/or its polymerization seem to favor the interaction of β-CD with the enzyme probably by increasing its water solubility, which was observed through the limpid aspect of the β-CD + T, PCD and PCD + T samples. However CMPCD, being also very soluble, was a poor protectant of invertase, even in its mixture with trehalose (CMPCD + T), as shown in Fig. 3a and b. Since the measured pH values of the resuspended samples were higher than 6 in all cases, and taking into account that the isoelectric point of invertase is lower than 4.5 (Kizilyar, Akbulut, Toppare, Özden, & Yağci, 1999), the enzyme was negatively charged in the studied conditions. It is to be noted that electrostatic forces determine protein interactions with biopolymers (Basso et al., 2005; Santagapita et al., 2008), which may positively or negatively influence protein stability. CMPCD is a polyanionic polymer, and its tridimensional structure exposes negative residues to the surface of the enzyme in the dehydrated system. As previously observed with alginate (Santagapita et al., 2008), this polymer probably destabilized the native protein structure at the interface with the polymer. PCD is a neutral polymer, and the enzyme stability must be less affected by electrostatic interactions as observed previously with dextran (Santagapita et al., 2008). Comparing these neutral polymers, PCD offers a slightly better protection to the enzyme than dextran during both freeze-drying and thermal treatment, although they act by different stabilization mechanism (Basso et al., 2005). β-CD is also neutral, and can interact with hydrophobic groups of the enzyme, but was not efficient as protectant, as previously discussed. Supramolecular interactions (multiple hydrogen bonds and others less strong intermolecular interactions) generated between PCD and the enzyme are responsible of enzyme conservation. In this sense, the search of new additives or of combined stabilizing mechanism is of high impact for industry.

At RVP 22%, in trehalose systems the absence of the melting endothermal peaks in the DSC thermograms confirmed that all systems were essentially amorphous. At RVP 43% the amount of adsorbed water was sufficient for complete trehalose crystallization (which is 10.5% in dry basis). While in the trehalose/enzyme system 60 and 80% of the sugar had crystallized during humidification at RVP 43% and later thermal treatment, respectively, sugar crystallization was delayed/inhibited in the blends containing any of the studied β-CD polymers (Fig. 4). Thus, the synergistic stabilizing effect of the combined systems PCD or β-CD + T, could be, at least in part, related to the delay/inhibition of trehalose crystallization, although molecular interactions played a significant role. PCD has shown to be an appropriate additive for dehydrated formulations of invertase, allowing to protect the enzyme in conditions at which trehalose crystallizes (Fig. 3b).

3.3. Protein structure by FT-IR

Fourier transform-infrared (FT-IR) spectroscopy was employed in order to study the protein secondary structure in the presence of each polymer (Barth, 2007; Carpenter et al., 1999; Vanderkooi et al., 2005). The IR spectrum for the enzyme without additives was similar to that reported by David, Wang, Yang, and Yang (2006). The sensitivity of the amide I vibration (around 1650 cm^{-1}) to secondary protein structure makes it possible to study protein folding, unfolding and aggregation in the presence of each polymer. Fig. 5 shows the spectra in the region between 1600 and 1700 cm^{-1}, after applying band narrowing technique (the second derivative spectra plus Norris filter were applied). It is known that invertase contains two domains, mostly constituted by β-strands (one with five blades forming a propeller domain and the other with two six-stranded β-sheets), with one small α-helix on each domain (Alberto, Bignon, Sulzenbacher, Henrissat, & Czjzek, 2004). According to this, the bands corresponding to β-sheet are the most representative to analyze. After applying narrowing techniques, folded proteins depicted a structured amide I spectrum, in contrast with unfolded proteins which show a broad band around 1650 cm^{-1} (Barth, 2007). As shown in Fig. 5, in the PCD matrix the enzyme shows the peaks at 1631 and 1680 cm^{-1}, both associated to β-sheet secondary protein structure. When β-CD is present, the same peaks are observed,

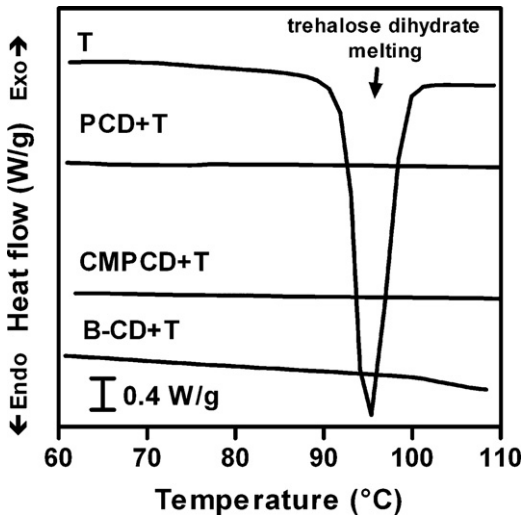

Fig. 4. Differential scanning calorimetry thermograms for selected freeze-dried samples containing trehalose exposed to 43% RVP during one week before thermal treatment. The endotherm around 97 °C corresponds to trehalose dihydrate crystals melting. T: trehalose; β-CD: β-cyclodextrin; PCD: poly-β-cyclodextrin; CMPCD: carboxymethyl-poly-β-cyclodextrin.

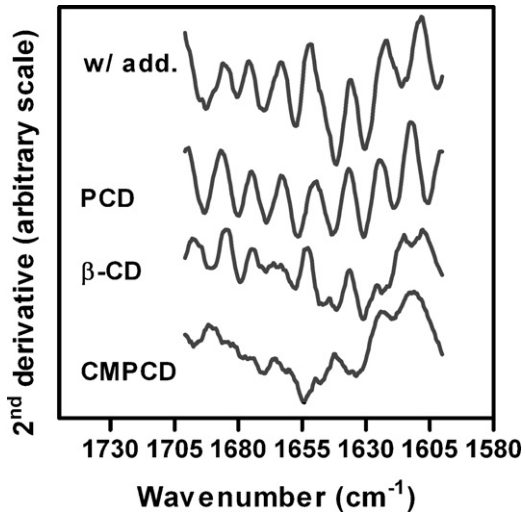

Fig. 5. Second-derivative amide I spectra of invertase with and without selected β-cyclodextrin additives. Folded proteins depict a structured amide I spectrum, in contrast with unfolded proteins, which show a broad band in the proximities of 1650 cm^{-1} (Barth, 2007). Peaks direction is downward. w/add: without additive; PCD: poly-β-cyclodextrin; β-CD: β-cyclodextrin; CMPCD: carboxymethyl-poly-β-cyclodextrin.

but with less intensity and definition. A similar analysis could be performed with the corresponding band to α-helix secondary structure (close to 1656 cm^{-1}). In the CMPCD matrix, instead, the enzyme shows a very broad band centered at 1654 cm^{-1} which is characteristic of unfolded protein. Even though the characteristic bands corresponding to β-sheet secondary structure appeared in the spectrum, their intensity is diminished. Then, the decreasing enzyme activity found after heat treatment (in the order PCD > β-CD > CMPCD) is in accord with the degree in which the amide I regions of the IR spectra are modified by each polymer.

4. Conclusion

β-CD, PCD and CMPCD were good physical stabilizers of freeze-dried formulations due to their high T_g values (compared to trehalose), but a high glass transition for the excipients was not directly related to enzyme stabilization (Pikal, 1999). Their action as single additive enzyme protectants showed a wide variety of behaviors: while PCD was one of the best excipients acting alone, enzyme remaining activity was almost negligible in CMPCD and β-CD systems. Furthermore, PCD allowed to protect the enzyme in supercooled conditions, at which trehalose crystallizes.

Among the systems composed by two additives, trehalose combined with PCD (PCD + T) and with β-cyclodextrin (β-CD + T) offered the best stability to the enzyme during both freeze-drying and thermal treatment. All polymers inhibited trehalose crystallization in supercooled media. In β-CD + T system, trehalose was the main responsible for the protection observed at both RVP, although the increased β-CD solubility observed in presence of trehalose must be also involved. In PCD + T system, both additives contributed to the protection.

It is to be noted that electrostatic forces determine protein interactions with biopolymers (Basso et al., 2005), which may positively or negatively influence protein stability. Since the enzyme was negatively charged at the studied conditions, its native structure was seriously affected by the polyanionic CMPCD. Being PCD and β-CD both neutral, PCD could interact with the enzyme probably by supramolecular interactions, enhancing protein stability. At the studied conditions, β-CD could not develop adequate interactions to protect the enzyme.

By examining the IR spectra of freeze-dried enzyme formulations both matrix and enzyme structure could be studied: supramolecular properties of the matrix were related to the position of OH stretching band and changes in the structured spectrum in the amide I regions reflected the degree in which enzyme conformation was modified by each polymer. The multilevel approach, which includes molecular and supramolecular properties of the systems, allows studying protein/excipient interactions to improve the long-term stability of biomolecules in dried formulations.

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