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Research paper

Trehalose and hyaluronic acid coordinately stabilized freeze-dried pancreatic kininogenase

Yuhua Zhang [a,b], Baoping Ji [a,*], Peixue Ling [c], Tianmin Zhang [c]

[a] College of Food Science & Nutritional Engineering, China Agricultural University, Beijing, China
[b] Shandong Institute of Commerce and Technology, Jinan, China
[c] Working Station for Postdoctoral Scientific Research, Institute of Biopharmaceuticals of Shandong Province, Jinan, China

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Abstract

The ability and mechanisms of stabilization of freeze-dried formulations of pancreatic kininogenase (PKase) by carbohydrates were evaluated. Activity and structure of PKase were examined after freeze-drying and rehydration in presence with or without a carbohydrate. Addition of trehalose, lactose, sucrose, hyaluronic acid (HA) or a combination of trehalose and HA to PKase formulations prior to freeze-drying step increases the stability of PKase during freeze-drying, storage and rehydration as measured by activity preservation. The combination of trehalose and HA is the most effective for the stabilization of PKase. Addition of HA alone to a formulation does not affect protein structure, but it increases glass-transition temperature (T_g) and stability of lyophilized PKase in presence of trehalose during dehydration, storage and rehydration processes. Therefore, trehalose and HA offer complementary properties that improve the stability of PKase during dehydration, storage and rehydration.

Keywords: Trehalose; Hyaluronic acid; Kininogenase; Freeze-drying; Stabilization

1. Introduction

The application of proteins derived from recombinant and natural sources in food industry and pharmaceutics has been increased. However, the inherent unstability of these proteins limits their usage [1]. Thus there is a need to develop stabilized protein formulations. One of the most common procedures to stabilize protein is consisting of preparing protein in a solid state by freeze-drying [2]. This method, although rendering reasonably stable products, has deleterious effects on the protein structure and loss of protein activity upon rehydration [3,4]. However, it has been found that addition of excipients to protein solutions prior to lyophilization can increase the stability of dried protein due to preventing freezing- and drying-induced damage and preserve the native structure and biological activity of these proteins during the dehydration, storage and rehydration processes [2,5]. A number of different molecules, usually low molecular weight carbohydrates, and disaccharides in particular forms, have been used as the excipients [4]. Two hypotheses have been used to explain the roles of these additives. One is ''the water substitution hypothesis'' proposed by Carpenter and Crowe [6,7], which suggests that carbohydrate molecules interact with the dried protein by hydrogen bonding in the place of lost water molecules and, therefore, inhibit unfolding of the proteins during drying. Another one is ''the glassy state hypothesis'', which contends that carbohydrate molecules form highly viscous glassy state around protein molecules and then prevent protein unfolding and aggregation [8–10].

It is proposed that both the hydrogen bonding between carbohydrate and protein and the glass formation are

* Corresponding author. College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China. Tel.: +86 10 62737129; fax: +86 10 62347334.
E-mail address: Baopingj@163.com (B. Ji).

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required for protein stabilization during dehydration and storage [11]. Therefore, carbohydrate is required to minimize lyophilization-induced structural perturbations and maximize glass transition temperature (T_g) of the formulation. In practice, it is difficult to meet the both conditions using a single carbohydrate as a stabilizer since as T_g increases along with molecular weight, the capacity of carbohydrates to inhibit lyophilization-induced protein unfolding decreases [12]. Combination of a disaccharide with a high T_g component (e.g. polysaccharide) might be necessary to stabilize protein adequately [4]. However, the potential effects of a polysaccharide on the stabilizing capacity of disaccharide and their combined effects on the stability of protein need to be further tested.

The combined effects and the mechanisms of trehalose and hyaluronic acid (HA) on the stabilization of biologic activity and native structure of a model protein, pancreatic kininogenase (PKase), were investigated.

2. Materials and methods

2.1. Materials

PKase (EC 3.4.21.8) was obtained from Jinan Welcome Biochem Pharmaceutical Co., Ltd., China. HA was obtained from Freda Biochemical Engineering Co., Ltd., China. Trehalose was obtained from Hayashibara Co., Ltd., Japan. Lactose and sucrose of analytical grade were obtained from Beijing Chemical Co.

2.2. Sample preparation

PKase was dialyzed against 10 mM, pH 7.0 phosphate buffer for several hours at 4 °C, and then a stock enzyme solution was made with buffer in presence or absence of carbohydrates. The final enzyme concentration was 5.5 mg/ml. The additive concentrations used in the formulations were 3.0 mg disaccharides (trehalose, lactose or sucrose)/mg PKase or 0.2 mg HA/mg PKase. In the formulation containing a mixture of trehalose and HA, the concentrations used were 3.0 mg trehalose/mg PKase and 0.1 mg HA/mg PKase, respectively.

2.3. Lyophilization

Sample solutions were loaded in glass vials, frozen and then lyophilized using a Labconco FreeZone 6 L lyophilizer (Labconco, USA) at a condenser temperature of −50 °C and a pressure of 6.9 Pa for at least 48 h.

2.4. Storage study

Lyophilized samples in glass vials were sealed immediately and loaded into ovens at 45 °C. Triplicate samples of each formulation were removed at the indicated time points and stored in P_2O_5-dessicated containers at −18 °C until analyzed.

2.5. PKase activity assay

PKase activity was assayed based on the hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) in 10 mM phosphate buffer, pH 7.0 at 25 °C [13]. The reaction was initiated by injecting a given volume of enzyme into the BAEE solution preheated at the reaction temperature. An increment in absorbance at the first and third minute was measured at 253 nm using an Agilent 8453 spectrophotometer (Agilent Technologies, Germany). The results were expressed as the percentage of the pre-freeze activity recovered after freeze-drying and rehydration. The lyophilized samples were rehydrated to their original volumes with deionized, distilled water prior to enzyme activity measurement. Each sample solution was tested three times and a mean value was calculated.

2.6. Fourier transformation infra-red spectroscopy (FT-IR)

Spectra were obtained with a Nicolet Magna-IR 750 Fourier transformation infra-red spectrometer (Nicolet, Madison, WI, USA) at a resolution of 4 cm^{-1} at room temperature. The dried samples about 0.5–1.0 mg were mixed and ground with 300 mg KBr. The spectrum for native, aqueous PKase was obtained by injecting 20 mg/ml solution into a CaF_2 cell with a 6-μm spacer. Second derivative spectra [14] were calculated with the derivative function of Nicolet Omnic software [15]. The final protein spectra were smoothed with a 6-point function to remove white noise.

2.7. Differential scanning calorimetry (DSC)

The T_g of the freeze-dried formulations containing carbohydrates and protein was measured using a DSC 822^e differential scanning calorimeter (Mettler Toledo AG, Greifensee, Switzerland). Prior to measurement, the dried samples were transferred into vacuum desiccators and equilibrated for 1 week over P_2O_5 for 'zero' moisture content. Dried protein sample (2–10 mg) was hermetically sealed in an aluminum sample pan, and an empty pan was used as a reference. Samples were scanned at 4 °C every min under N_2 stream from 0 to 200 °C. The T_g was calculated at the midpoint of the endothermic shift based on the reported method [16].

2.8. Circular dichroism (CD) spectroscopy

The secondary structure of PKase before drying and after rehydration was monitored using a JASCO J-810 CD spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Initial aqueous and rehydrated samples were diluted to 0.2 mg/ml of PKase. Samples were loaded into 1-mm-pathlength quartz cells and scanned from 260 to 190 nm at 0.2 nm intervals with a 3-s averaging time at room temperature. Spectra were background corrected and converted to residue ellipticity (deg cm^2/dmol). The α-helix content was

computed from mean residue ellipticity at 222 nm based on linear relationship between signal intensity and α-helix content described before [17,18].

2.9. Residual moisture content analysis

Residual water content of the lyophilized protein was measured by the weight loss of the samples when the temperature was raised to 105 °C in vacuum.

3. Results and discussion

3.1. Effect of trehalose and HA on PKase activity recovery during lyophilization and storage

The capacity of various carbohydrates to preserve the activity of lyophilized PKase was examined (Fig. 1). Lyophilization of PKase in absence of carbohydrate resulted in about 30% loss of enzymatic activity upon rehydration. Addition of trehalose, lactose, sucrose or HA to PKase prior to freeze-drying prevented the loss of PKase activity. 13%, 20%, 15%, and 23% of activity decrease was observed when addition of trehalose, lactose, sucrose or HA to PKase, respectively. Trehalose is the most active, and HA is the less active. However, a stronger protection of activity in freeze-dried sample was obtained when trehalose and HA used together than each agent used alone.

To test whether these carbohydrates can protect the activity at higher temperature and long-term storage, these formulations were stored at 45 °C for 7 weeks. The PKase activity decreased in all formulations as storage time increasing. However, addition of trehalose, lactose, sucrose, HA or a combination of trehalose and HA prevented the activity loss, and the combination of trehalose and HA had the best protection ability. These results suggest that a combination of trehalose and HA may be used to prevent the activity loss during the freeze-drying and high-temperature storage processes.

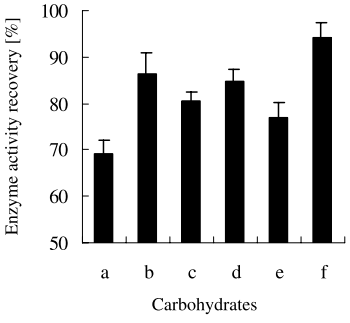

Fig. 1. Activity recovery of PKase lyophilized with various carbohydrates. (a) No carbohydrate; (b) trehalose; (c) lactose; (d) sucrose; (e) HA; (f) the combination of trehalose and HA. The PKase formulations as indicated were taken immediately after freeze-drying and rehydration. The activity was measured as described in Section 2. The data shown are mean values plus SD.

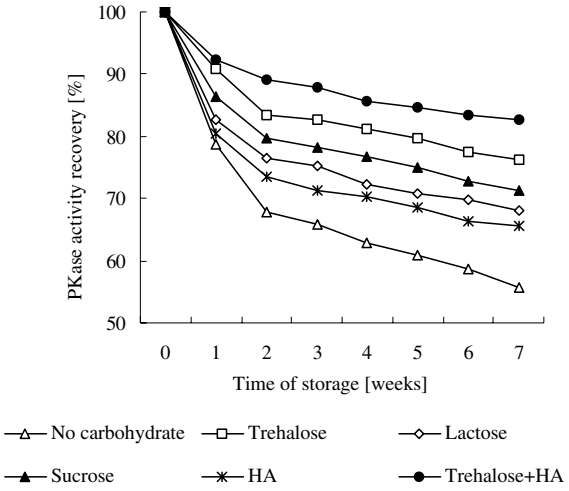

Fig. 2. Activity recovery of freeze-dried PKase during storage at 45 °C. The PKase formulations as indicated were taken during storage at 45 °C for 7 weeks and rehydration. The activity was measured as described in Section 2. The data shown are mean values plus SD.

3.2. Structure stability of PKase during freeze-drying and storage

To ask whether the protection of the PKase activity loss is due to structure stabilization of PKase during lyophilization and storage by these carbohydrates, the second derivative infra-red spectra of PKase, in the amide I region, in aqueous states, in lyophilized states, in presence or absence of these carbohydrates were measured using a Fourier transformation infra-red spectrometer (Fig. 3).

There were several alterations in the FT-IR spectra of lyophilized PKase relative to that of the native, aqueous enzyme. The bands at 1637 and 1671 cm^{-1} were shifted to 1639 and 1673 cm^{-1}, respectively, and the absorbance decreased. Two new bands appeared at 1698 and 1693 cm^{-1}. The intensities in the region from 1625 to 1600 cm^{-1}, assigned to intermolecular β-sheet, increased and indicated the presence of aggregated protein in the dried solid [12]. In presence of trehalose, these transitions were partially inhibited that reflected by an increase in the intensity at 1639 cm^{-1} and the disappearance of the bands in 1698 and 1693 cm^{-1}. However, the shift of the dominant band from 1637 to 1639 cm^{-1}, the disappearance of bands in 1671 and 1644 cm^{-1}, and the increase of intensity in 1687 cm^{-1} suggested that the structure of PKase was still changed comparing with the native one. Similar results were noted with addition of lactose or sucrose. The spectrum of PKase in presence of HA, comparing with aqueous PKase, revealed a decrease in intensities and shifts from 1671, 1668 and 1656 cm^{-1} to 1675, 1670 and 1660 cm^{-1}, respectively, and a compensatory increase in intensities in the regions around 1625–1600 cm^{-1} and 1653–1648 cm^{-1}, and the appearance of a new band centered at 1652 cm^{-1}. However, the spectrum of PKase in presence of trehalose and HA was less changed comparing with the native, aqueous PKase structure. These results

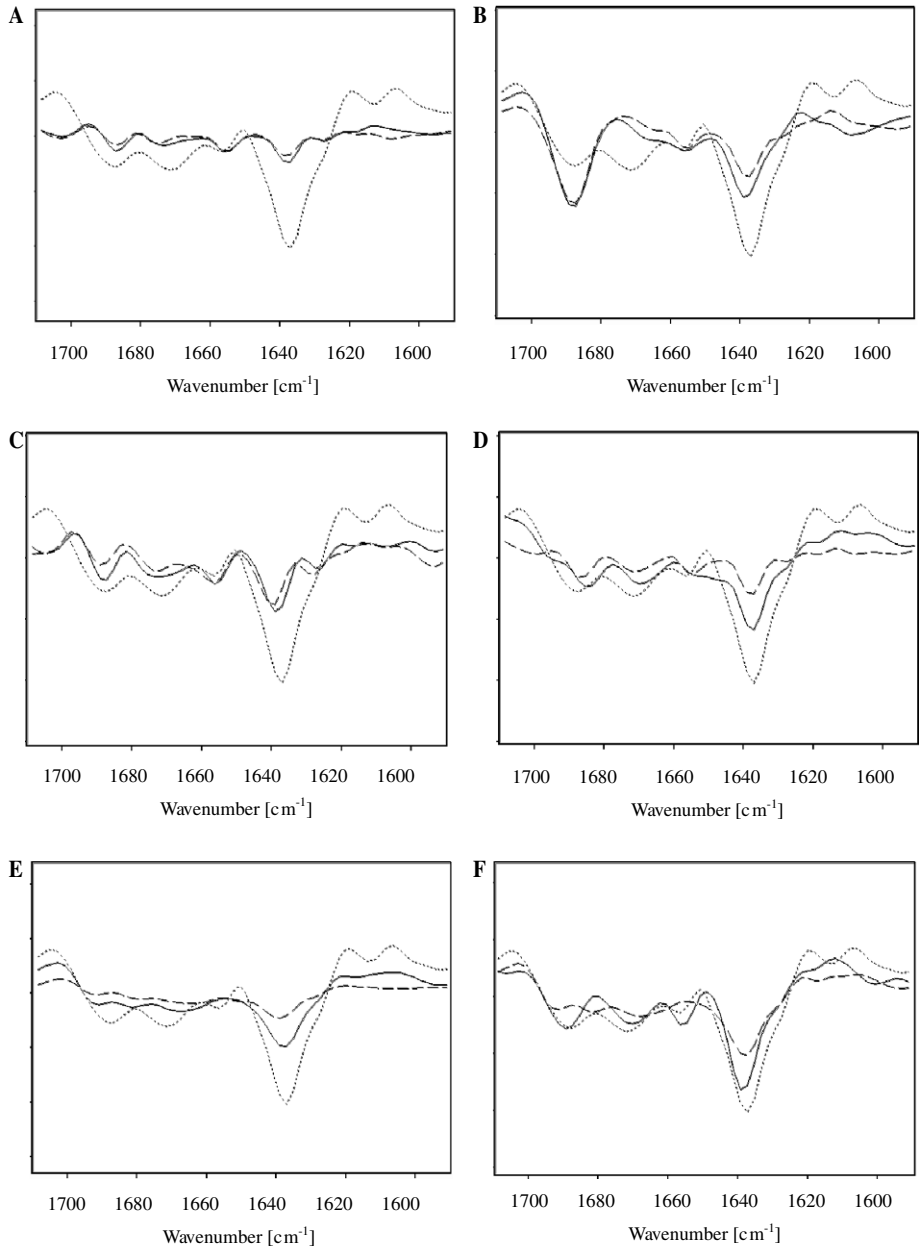

Fig. 3. Second derivative amide I spectra of dried PKase: (dotted lines) native, aqueous PKase; (solid lines) spectra taken immediately after freeze-drying; (dashed lines) spectra taken after 7 weeks at 45 °C. (A) No carbohydrate; (B) trehalose; (C) lactose; (D) sucrose; (E) HA; (F) the combination of trehalose and HA.

suggested that the PKase in presence of trehalose, sucrose, lactose or the mixtures of trehalose and HA maintained more native-like secondary structure (though there were changes compared with native structure) than that of PKase dried alone or in presence of HA. It is possible that these carbohydrates protected the enzyme during drying via hydrogen bonding as described by Carpenter and Crowe [6,7]. This can be further demonstrated from the effect of protein on IR spectra of carbohydrates (Fig. 4). The spectra of all the samples after 7 weeks of storage at 45 °C were less native-like comparing with the corresponding samples taken immediately after freeze-drying (data not shown), which were consistent with the results of activity recovery (Fig. 2). Based on these data, it seems that the enzymatic activity recovery ability by these carbohydrates is due to maintenance of the native structure of PKase protein.

3.3. The interaction of PKase with carbohydrates

To test whether there is hydrogen bonding between the carbohydrate and PKase that maintains the naive structure of protein during dehydration [4,6,7], the –OH plane-bending deformations of the infra-red spectra for carbohydrates lyophilized alone was compared with that of carbohydrates lyophilized in presence of PKase and

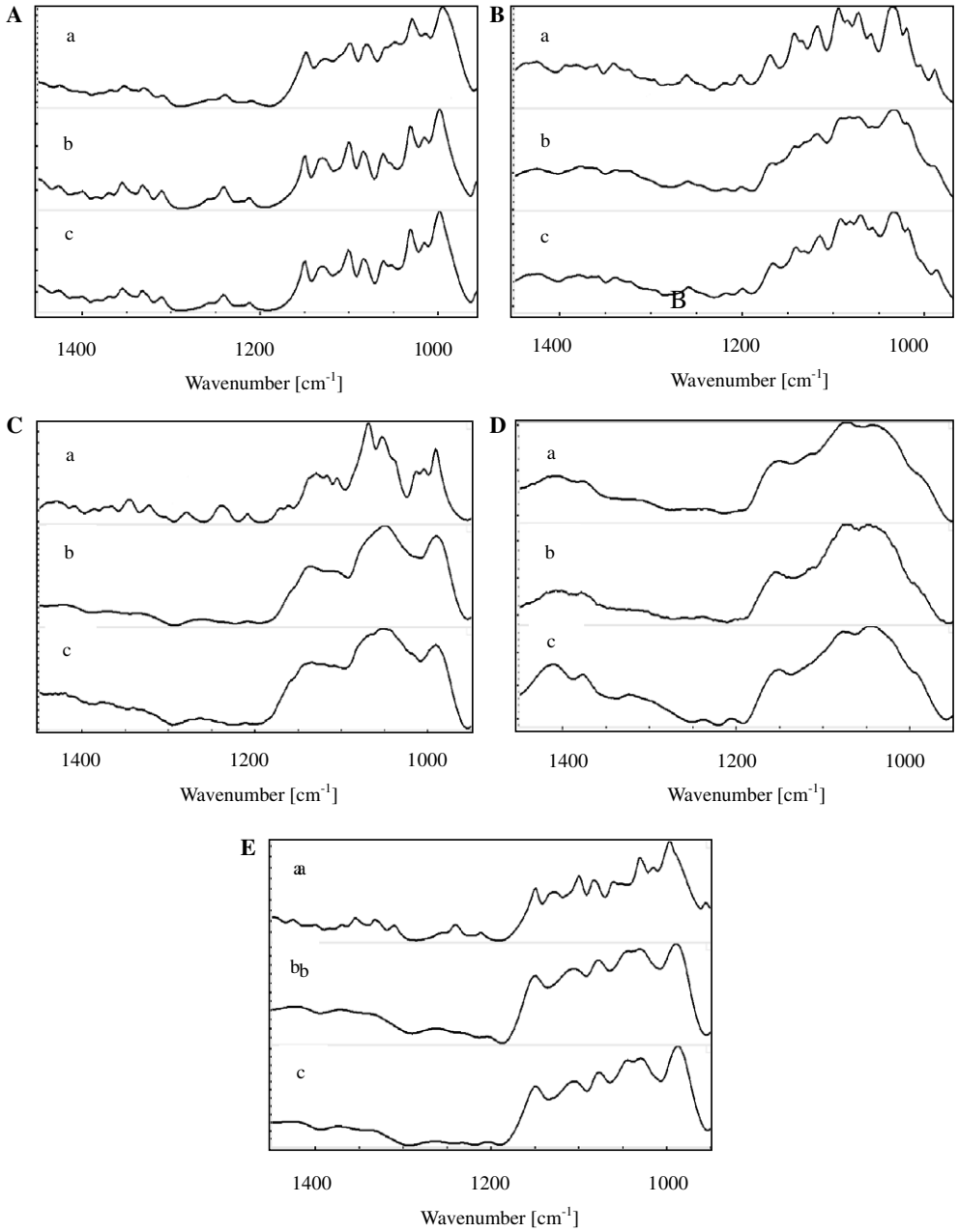

Fig. 4. Infra-red spectra of -OH plane-bending deformations for carbohydrates. (a) Freeze-dried carbohydrate alone; (b) freeze-dried carbohydrate in presence of PKase; (c) hydrated carbohydrate. (A) Trehalose; (B) lactose; (C) sucrose; (D) HA; (E) the combination of trehalose and HA.

hydrated carbohydrates (Fig. 4). The presence of PKase leaded to a decrease in absorbance in the entire region and a loss of band splitting. The spectrum of trehalose lyophilized in presence of PKase was remarkably similar to that of trehalose in water. Similar results were observed with lactose, sucrose and the mixtures of trehalose and HA, but not HA. Thus, protein plays the same role for the dried disaccharides (trehalose, lactose and sucrose) as dose water for the hydrated disaccharides. Presumably, there is interaction between protein and disaccharides [6,7]. The interaction might be formed between -OH groups in the disaccharides and polar groups in the PKase by hydrogen bonding [19]. The spectrum of HA lyophilized in presence of PKase was similar to that of HA lyophilized alone but not to that of hydrated HA. This suggests that there is no direct interaction between HA and PKase lyophilized together. Moreover, HA did not inhibit the formation of hydrogen bonding between trehalose and PKase in the dried sample containing all of them. It has been found that the capacity of carbohydrate protecting lyophilized catalase activity decreased as carbohydrate molecular weight increased [20] since larger carbohydrate molecules have steric hindrance, which will prevent effective hydrogen bonding of the carbohydrate to a dried protein. Consistent with this observation, we have found that HA with the largest molecular was least effective of all the carbohydrates tested.

Table 1
The T_g values of lyophilized PKase in presence of various carbohydrates

Formulation	T_g (°C)
Trehalose	70.78
Lactose	59.49
Sucrose	63.59
HA	106.18
Trehalose + HA	85.42

3.4. T_g of lyophilized PKase and storage stability

One of the physical factors that have been found to be important in determining the stability of lyophilized protein formulations during long-term storage is the T_g of the amorphous phase containing protein [21,22]. Protein is simply mechanically immobilized in the glassy, solid matrix in the dried state. This restriction of translational

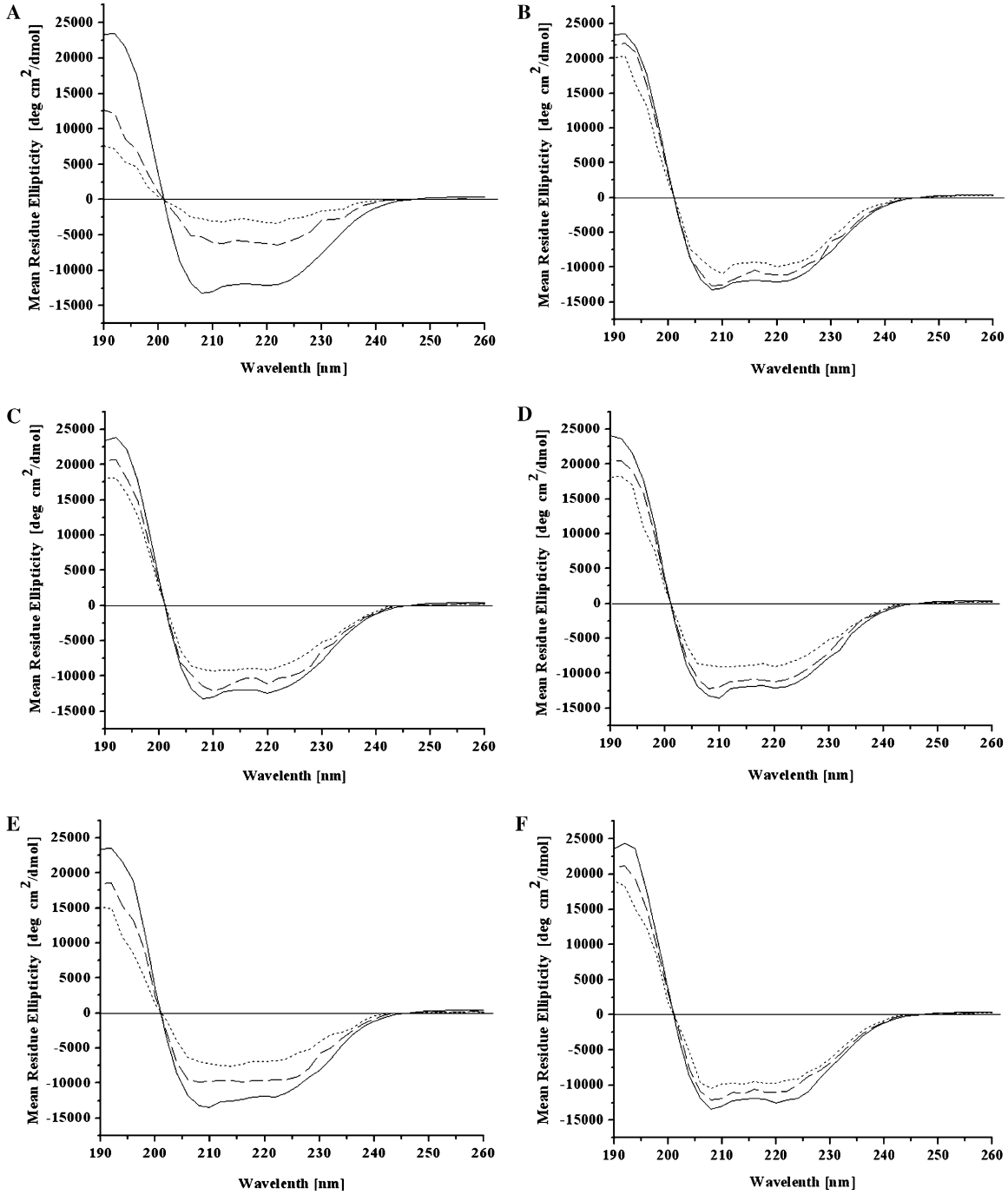

Fig. 5. CD spectra of rehydrated PKase after freeze-drying with: (A) No carbohydrate; (B) trehalose; (C) lactose; (D) sucrose; (E) HA; (F) the combination of trehalose and HA. (Solid lines) native, aqueous PKase; (dashed lines) PKase rehydrated immediately after freeze-drying; (dotted lines) PKase rehydrated after 7 weeks of storage at 45 °C.

and relaxation processes is thought to prevent protein unfolding [10]. In addition, spatial separation by glassy carbohydrates between protein molecules is proposed to prevent aggregation [21] and denaturation should only take place at a comparative slow rate with the mixtures locked in the glassy form [10,11]. Thus storage in the glassy state below the sample T_g is necessary for maintaining stability of the dry solid proteins. The values of T_g determined by DSC for these formulations, with the residual moisture content between 0.02 and 0.05 g H_2O/g dry mass, are shown in Table 1. The measured T_g values of these formulations exceed the storage temperature of 45 °C adopted in the current study.

At a given storage temperature, a protein in a higher T_g formulation should be more stable [23,24]. PKase freeze-dried with HA had a higher T_g, but protein unfolding was unable to be prevented by HA during dehydration and storage (Fig. 3). It has been found that glassy state alone was insufficient to stabilize PKase during dehydration and storage [10]. Enthalpic relaxation measurements of glasses have shown that the molecular mobility still exists even below the T_g 50 °C [25]. Therefore, it was not sufficient to maintain the stability by simply storing the protein formulation well below its T_g, especially if the protein is unfolded during initial lyophilization process [26].

3.5. CD spectra of PKase after rehydration

To test the effect of rehydration on the secondary structure of freeze-dried PKase, CD spectra of rehydrated samples and native, aqueous ones were compared. Upon rehydration immediately after freeze-drying, the CD spectra of these formulations dried in presence of a carbohydrate were similar as native PKase (Fig. 5, Table 2), indicating that the majority of non-native molecules refolded into their native conformation in these formulations. In contrast, the spectrum of the PKase lyophilized in absence of a carbohydrate was relatively less native-like. It has been recognized that a protein, which remains as native form in the dried solid still remains the form after rehydration, but if a protein form is perturbed in the dried solid it will denature irreversibly or readopt the native conformation [27]. In our results, it seems that a carbohydrate is presumably in favor of the retention of the native structure of PKase during rehydration. However, the CD spectra of these formulations rehydrated after 7 weeks of storage at 45 °C indicate that non-native PKase content increases in all the samples. These data suggest that the reversibility of secondary structure changes induced by dehydration decreases as increased storage time. Addition of HA to a trehalose-containing formulation slightly improves the recovery of native secondary structure of PKase after 7 weeks of storage at 45 °C.

Table 2
The α-helix content of rehydrated PKase formulations

Formulation	Rehydrated immediately after freeze-drying	Rehydrated after 7 weeks at 45 °C
No carbohydrate	0.75	0.62
Trehalose	1.00	0.88
Lactose	0.95	0.82
Sucrose	0.98	0.85
HA	0.90	0.76
Trehalose + HA	0.99	0.91

The numbers are values relative to native, aqueous PKase.

The α-helix content of the samples freeze-dried without carbohydrates and stored after 7 weeks at 45 °C in the dry state measured by infrared spectroscopy decreased evidently. However the decrease of α-helix content in these formulations was prevented by addition of a carbohydrate (Table 2).

4. Conclusions

1. HA provides only minimal protective effect on PKase activity due to its unable to form adequate hydrogen bonding with the dried protein.
2. Trehalose inhibits lyophilization-induced protein unfolding and effectively protects the activity of PKase.
3. Addition of HA alone to a formulation does not affect protein structure, but it increases T_g and stability of lyophilized PKase in presence of trehalose during dehydration, storage and rehydration processes. Therefore, trehalose and HA offer complementary properties that improve the stability of PKase during dehydration, storage and rehydration when the two types of additives are used together.

Acknowledgements

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