Maltodextrins as Lyoprotectants in the Lyophilization of a Model Protein, LDH

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Purpose. Maltodextrins, partially hydrolysed starches, were evaluated as potential lyoprotectants and the effect of combinations of maltodextrins and PEG 8000 on the protection of lactate dehydrogenase (LDH) was examined.

Methods. LDH activity assays were performed immediately before freezing and after reconstitution. The activity recovery was used as the parameter to evaluate the lyoprotectants. Differential Scanning Calorimetry (DSC) was used to measure the glass transition temperature (Tg') of the solutions. DSC and X ray diffraction were used to characterise the freeze-dried products.

Results. Maltodextrins were found to protect LDH againt inactivation during freeze-drying. The lyoprotection obtained by these maltodextrins is dependent on their D.E. value and the concentration used. The maltodextrin formulations performed as good or better than those containing sucrose and maltose, depending on the concentration used. Freeze dried cakes of maltodextrin formulations were amorphous. In the case of low D.E. maltodextrins, lyoprotection was improved by the addition of PEG 8000 as a cryoprotectant.

Conclusions. Maltodextrins could be considered as potential lyoprotectants in lyophilization of proteins.

KEY WORDS: freeze-drying; protein; maltodextrins; PEG.

INTRODUCTION

With the recent advances in recombinant DNA technology, a great number of new therapeutic proteins have been investigated in recent years. Most of the therapeutic proteins require parenteral administration and because of limited stability in aqueous solution many protein formulations are freeze-dried to achieve long term stability (1,2,3). Although shelf lives can be improved by lyophilization, some proteins are inactivated during this process. Lyophilization involves two processes, freezing and drying; both might affect the protein structure by causing polypeptide chain unfolding and aggregation. Prevention and reduction of these in process degradations may be obtained by the use of cryo- and lyoprotectants. The cryoprotective action of different compounds was tested using lactate dehydrogenase (LDH) as a model protein (4). Polyethyleneglycol was reported to be a good cryoprotective agent, although it crystallizes during lyophilization, so it failed to protect dried proteins (5). Additives, including sugars, polyols, amino acids and surfactants have been investigated as potential lyoprotectants and mechanisms of action were discussed (6). Certain carbohydrates (e.g. dissaccharides) were effective in the protection of phosphofructokinase (PFK) during freeze-drying (7). Carpenter et al. suggested that these carbohydrates might protect dried proteins because these solutes bind to dried proteins, serving as a "water substitute" when the hydration shell of the protein is removed. Hydrogen bonding of the carbohydrate to the protein was found to be requisite for stabilisation of proteins during freeze drying (8).

Polymers as PVP and Ficoll 70 have been used as lyoprotectants in the lyophilization of RNAse (9). Polymers such as dextran, Ficoll and carboxymethylcellulose have been reported to preserve β-galactosidase activity during freeze-drying in the presence of inositol (10).

The objectives of this study were to investigate maltodextrins, partially hydrolysed starches, as potential lyoprotectants and to examine the effect of combinations of maltodextrins and PEG 8000 as stabilizers of parenteral freeze-dried formulations. LDH was selected as a model protein, since it loses 80% of its activity when freeze-dried in the absence of stabilizers (6). Ongoing research on the immunogenecity of maltodextrins should provide evidence of their possible use as excipients in parenteral formulations.

MATERIALS AND METHODS

Materials

L-lactate dehydrogenase(LDH) Type II from rabbit muscle was purchased as an ammoniumsulfate suspension from Sigma Chemical Company (St.Louis, USA). The spray dried maltodextrins (Eridania—Beghin Say—Cerestar, Vilvoorde, Belgium) were obtained by enzymatic hydrolysis of corn starch, and had different dextrose equivalents (D.E.): C★PUR01910 (D.E. = 14), C★PUR01921 (D.E. = 22) and C★PUR01934 (D.E. = 38). The carbohydrate composition and molecular weights of the different maltodextrins used in this study are summarized in Table I. Polyethyleneglycol, average MW 8000, was obtained from Union Carbide (Danbury, Connecticut US). The other chemicals used in this study were sucrose (Alpha Pharma, Zwevegem, Belgium) and maltose (Eridania—Beghin Say—Cerestar, Vilvoorde, Belgium).

Reagents used in the activity assay were obtained from Rôche Diagnostics (Brussels, Belgium).

Methods

LDH Activity Assay

LDH activity was measured spectrofotometrically with a Cobas Mira analyser (Rôche Diagnostics, Brussels, Belgium) using the Unimate LDH reagent kit. The 250 μl reaction mixture contained 80 mM tris/HCl buffer (pH 7.2) ; 1.6 mM pyruvate, 200 mM NaCl and 0.2 mM reduced nicotinamide adenine dinucleotide. The reaction was initiated by the addition of 4μl of the LDH solution and monitored by measuring the decrease in absorbance at 340 nm. Assays were performed immediately before freezing and after reconstitution.

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Table I. Composition of the Maltodextrins

Sugar	D.E.	Carbohydrate composition (%)		MWn[a]	MWw[b]
C★PUR01910	14	Dextrose	1	1733	34190
		Maltose	3		
		Maltotriose	6		
		Higher saccharides	90		
C★PUR01921	22	Dextrose	2	804	12330
		Maltose	7		
		Maltotriose	10		
		Higher saccharides	81		
C★PUR01934	38	Dextrose	2	552	7846
		Maltose	33		
		Maltotriose	20		
		Higher saccharides	4		

[a] Number average molecular weight.
[b] Weight average molecular weight.

Freeze-Drying

Protein solutions containing 5 and 25 μg/ml LDH respectively, were lyophilized. The enzyme solutions were mixed with the solutions of the various additives and 2ml of the final solution was filled into 8 ml Type I glass vials (Gaasch Packaging, Mollem, Belgium). Bromobutyl stoppers 12 mm (Helvoet Pharma, Alken, Belgium) were partially inserted into the vials and the solutions were freeze-dried in a Amsco-Finn Aqua GT4 freeze-dryer. The samples were frozen on the lyophilizer shelves to −40°C in 25 min. and were kept at this temperature for 1 hr. Primary drying was performed by keeping the vials for 12 hrs at a pressure of 0.2 mbar, a shelf temperature of −10 °C and a condensor temperature of −60°C. Secundary drying was carried out by increasing the shelf temperature to 25°C and reducing the pressure to 0.1 mbar. Secondary drying time was 10 hrs.

Lyophilization was terminated by venting the drying chamber with air and the vials were sealed by automatic stoppering in the freeze-dryer. All samples freeze dried with full retention of structure. The freeze-dried cakes were reconstituted with 2 ml distilled water. The mean activity was calculated as a percentage of the activity before lyophilization. For each cycle 10 samples were analysed and the average value was calculated (±S.D.).

DSC Analysis

The differential scanning calorimeter used was a DSC 2920 (TA Instruments,Gent, Belgium). The instrument was calibrated using the melting transition of indium. To analyse the glass transitions and crystallisation behaviour of the additives in frozen solutions, aliquots (25 μl) of each sample solution were placed in aluminium cells with a capacity of 50μl. An aluminium top was placed on the sample and the sample pan was non hermitically sealed. An empty sample container was used as the reference. The samples were cooled to −60°C at 10°C/min with liquid nitrogen and then heated at 10°C/min to 30°C. The thermogram was recorded during heating of the sample.

Samples of the freeze dried powders (ca 5–10 mg) were placed in aluminium pans, non hermetically sealed and scanned at 10°C/min from 20 to 210°C. An empty sample container was used as the reference pan.

X-ray Diffraction

The freeze-dried samples were evaluated on crystallinity using X-ray diffraction (Diffractometer D5000, Cu, Kα (Siemens, Germany)).

RESULTS AND DISCUSSION

Characterization of Solutions

Glass transition temperatures (Tg′) of 10% (w/v) maltodextrin solutions are presented in Table II. There is a linear relationship between D.E. and the glass transition temperature. The practical importance of Tg′ in the lyophilization of a non crystallizing solute is that during primary drying, product temperatures above Tg′ result in a loss of the microstructure formed during the freezing process. With the low D.E. maltodextrins (eg. D.E.14,D.E.22) in freeze drying formulations, higher product temperatures can be used during primary drying, resulting in shorter cycle times, because of an increase in the sublimation rate (11).

Maltodextrins as Lyoprotectants

Figure 1(A) shows the effect of 3 different maltodextrins on the activity recovery of LDH (25 μg/ml) after lyophilization. Maltodextrins protected the protein during freeze-drying and the activity recovery was dependent on the dextrose equivalent. An activity recovery of over 80% was observed with maltodextrin D.E. 38 concentrations above 1% (w/v). There was little influence of the maltodextrin concentration on the protein protection in the concentration range between 1%–10% (w/v). Using maltodextrins D.E. 22, however, lyoprotection increased non linearly from 22%(±5.5) to 91%(±1.4) as the maltodextrin concentration was increased from 0% to 10% (w/v). A similar relationship between maltodextrin concentration and protein protection was observed for the D.E.14 maltodextrin formulations, where the recovered activity was over 90% when concentrations above 5% (w/v) were used. A concentration dependent lyoprotection was seen with sucrose, with an optimal protein protection of

Table II. Glass Transition Temperatures

Carbohydrate[a]	Glass transition Tg′ (°C ± SD)[b]
Maltodextrin D.E. 38	−21.97 (±0.21)
Maltodextrin D.E. 22	−15.36 (±0.16)
Maltodextrin D.E. 14	−12.09 (±0.19)
Maltose	−29.30 (±0.22)
Sucrose	−34.10 (±0.26)
Trehalose	−29.61 (±0.23)

[a] 10% w/v solutions.
[b] Glass transition temperatures are the mean of 3 analysis, calculated as the midpoint of the transition.

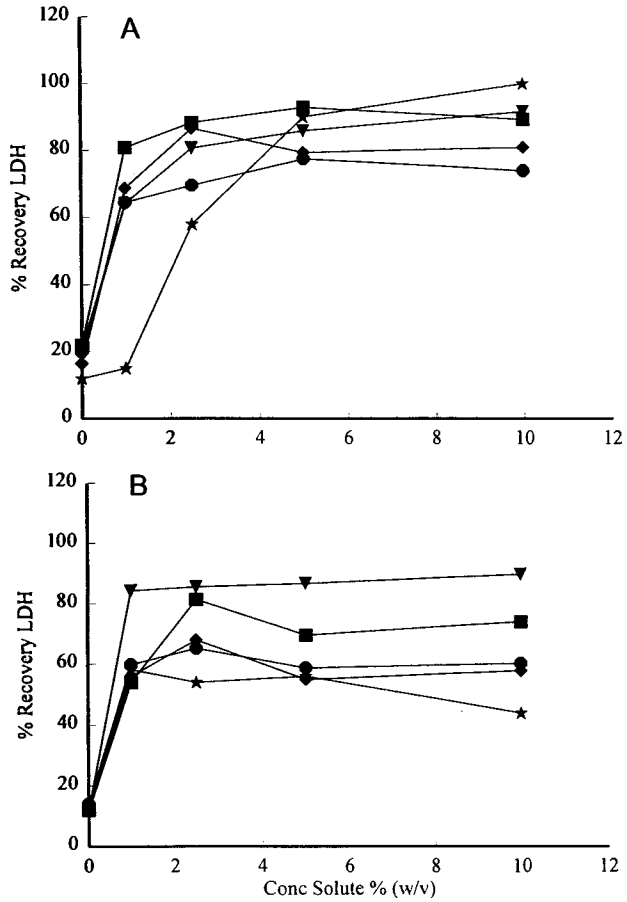

Fig. 1. Recovery of LDH activity at a concentration of 25 μg/ml (A) and 5 μg/ml (B) after lyophilization in the presence of sucrose ●,maltose ♦ and maltodextrins with different D.E. value:★ D.E. 14; ▼ D.E. 22; ■ D.E. 38.

77.3% (±3.3) using a sucrose concentration of 5% (w/v). Compared to the three maltodextrins, the capacity of sucrose to protect LDH (25 μg/ml) during freeze-drying was lower in the concentration range between 5–10% (w/v).

The maltodextrins used are obtained by enzymatic hydrolysis of corn starch. The carbohydrate composition of these maltodextrins is variable, depending on the D.E. The dissacharide maltose , a component of maltodextrins, was also evaluated as a potential lyoprotectant. Using a maltose concentration of 2.5% (w/v), a protein protection of 86.5% (±1.34) was obtained (Figure 1A). This result is comparable with the activity recovery for the D.E. 38 formulation at the same concentration. To achieve a similar protection with the D.E.14 and D.E.22, a higher concentration was required. This can partially be explained by the carbohydrate composition of these low D.E. maltodextrins. The maltose concentration of D.E.14 is only 3%(w/w), whereas the maltose concentration of D.E.38 is 33%(w/w) (Table I). The higher the concentration of low molecular wheight saccharides (e.g. maltose, maltotriose) in the maltodextrins, the lower the solute concentration needed to obtain maximal protein protection.

When a lower concentration of 5 μg/ml was used (Figure 1B) a lyoprotection of over 85% recovery was observed with the maltodextrin having a D.E. of 22. The lyoprotection was independent of the maltodextrin concentration in a concentration range between 1–10% (w/v). A similar relationship between maltodextrin concentration and lyoprotection was observed for the D.E.14 formulations, where the recovered activity was approximately 55%, when concentrations above 1% (w/v) were used. Comparing the three maltodextrin formulations, an optimal lyoprotection was achieved with the maltodextrin having a D.E. of 22.

The sucrose formulations resulted in a recovery of 60% independent of the concentration. The capacity of maltodextrins with a high D.E. of 38 and 22 to protect LDH (5μg/ml) during freeze drying was higher in comparison with the sucrose formulations.

Combinations Maltodextrins / PEG 8000

Combinations of the above mentioned maltodextrins with 1% (w/v) PEG 8000 were also freeze-dried (Figure 2). PEG / glucose and PEG / trehalose mixtures are reported to be effective at protecting PFK and LDH during freeze-drying (5). The activity of LDH freeze-dried without maltodextrins or sucrose, containing 1% PEG 8000 (w/v) was about 16% of the original solution. The addition of 1% PEG 8000 to the maltodextrin D.E. 38 formulations had no influence on lyoprotection: a recovery of LDH (25 μg/ml) activity of 80.3% (±5.1) was achieved, independent of the maltodextrin concentration. But combining PEG 8000 with the D.E. 22 and D.E. 14 maltodextrins or sucrose resulted in an improved lyoprotection. An activity recovery of 91.6% (±3.1) was achieved with the maltodextrin D.E.22 / PEG 8000 combination, even at a maltodextrin concentration of 1% (w/v). When the maltodextrin with D.E. 14 was combined with 1% PEG 8000 (w/v), it provides a high degree of protection during freeze-drying: a maltodextrin concentration of 1% (w/v) resulted in a 101.1% (±3.4) recovery. Using sucrose / PEG 8000 combinations a protein protection of 105.7% (±7.6) was achieved also at a solute concentration of 1% (w/v). The addition of PEG 8000 to maltose also resulted in an increased protection.

PEG 8000 is known to be a good cryoprotectant (5),

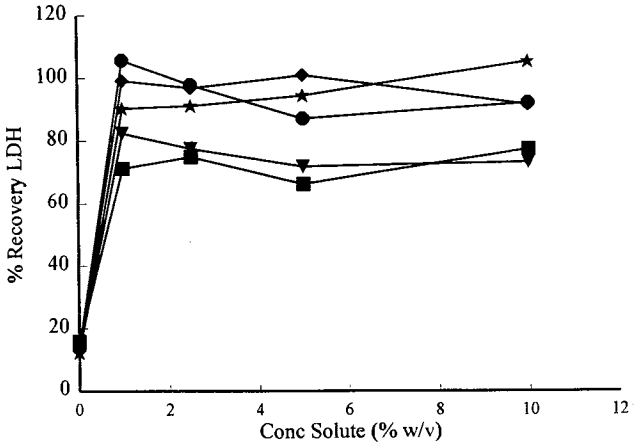

Fig. 2. Recovery of LDH activity at a concentration of 25 μg/ml after lyophilization in the presence of PEG8000 1% (w/v) and sucrose ● ,maltose ♦ and maltodextrins with different D.E. value:★ D.E. 14;▼ D.E. 22; ■ D.E. 38.

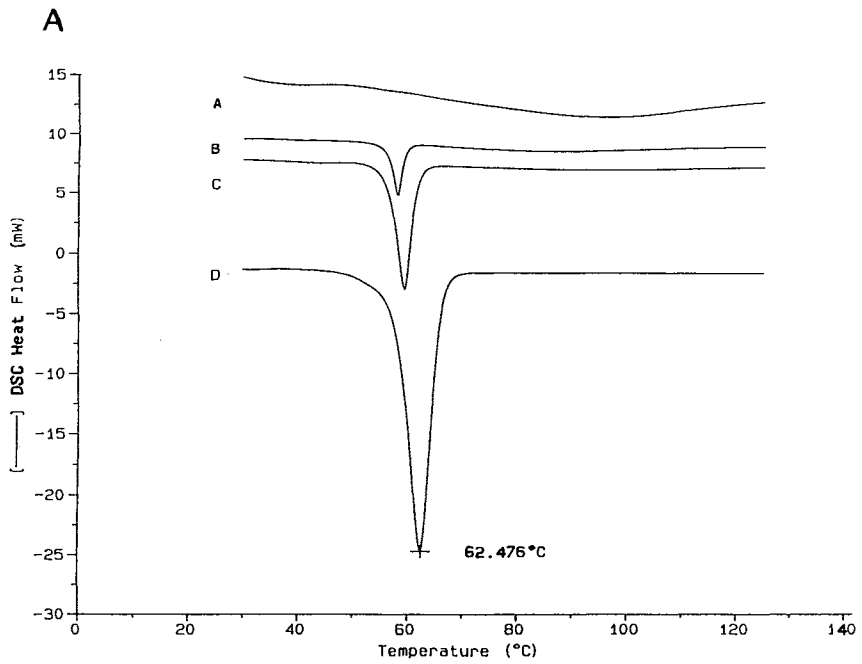

Fig. 3A. DSC thermogram of freeze dried formulations: maltodextrin D.E.22 5% (w/v) (A); D.E.22 5% (w/v) and PEG 8000 1% (w/v) (B); D.E.22 2.5% (w/v) and PEG 8000 1% (w/v) (C); PEG 8000 1% (w/v) (D).

providing protein protection during freezing, but PEG failed to protect freeze dried proteins. The ineffective protein stabilization by PEG 8000 can be explained by its crystallisation during freeze-drying. Carpenter et al. reported that, using LDH and PFK, relatively low concentrations of sugars (e.g. glucose, trehalose), which alone did not protect lyophilized proteins, were excellent stabilizers when used in combination with 1% PEG (w/v) (5).

In the study, relatively low concentrations (e.g. 1–2% (w/v)) of the maltodextrins with D.E. 14 and 22, which allone provided little protein protection, were effective protein stabilizers when used in combination with 1% (w/v) PEG 8000.

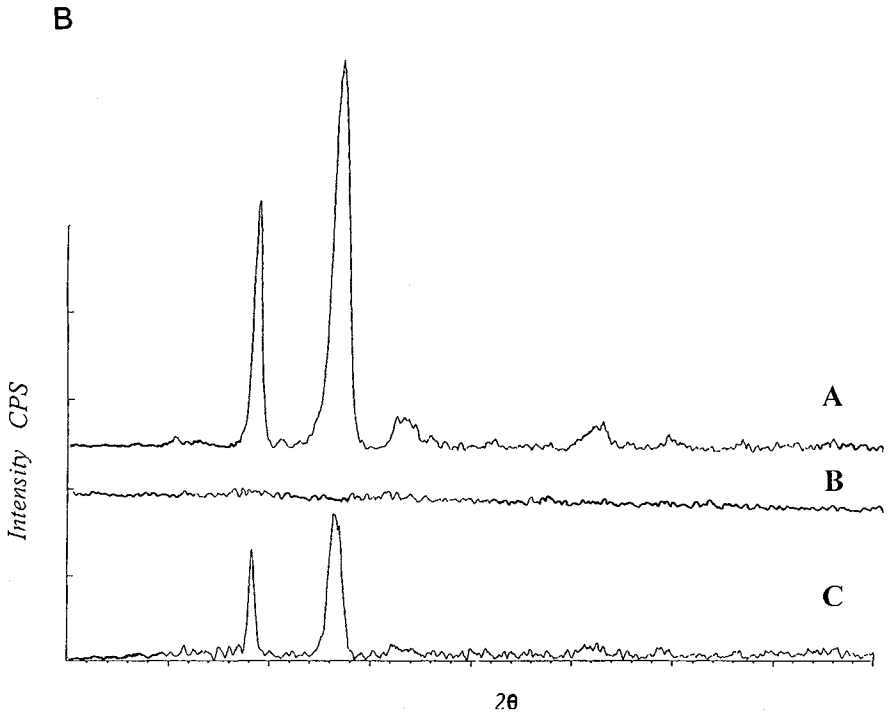

Fig. 3B. X-ray diffraction pattern of freeze dried formulations: PEG 8000 1% (w/v) (A); maltodextrin D.E.22 5% (w/v) (B) ;D.E.22 5% (w/v) and PEG 8000 1% (w/v) (C).

PEG stabilizes the proteins during freezing, due to the preferential exclusion of PEG from the protein surface. The sugars afforded protein protection during drying by interaction with the dried protein, probably serving as a water-substitute.

Characterisation of Freeze-Dried Products

DSC and X-ray diffraction patterns (Figure 3A and 3B) revealed the freeze-dried maltodextrin formulations to be amorphous. Amorphism is known to be an essential condition in the stabilisation of freeze-dried proteins. Amorphous mannitol and inositol stabilised β-galactosidase against inactivation during freeze drying, but the stabilising effects were lost when the solutes crystallised (10).

Molecular interaction between the protein and the solutes are reported to be necessary for stabilising proteins during freeze drying (6,8). Maintenance of the amorphous state results in molecular interaction with proteins, whereas crystallisation is believed to remove the additives from the protein phase (2). PEG 8000 allone failed to protect LDH during freeze-drying. The melting point of PEG 8000 is 67.4°C. DSC analysis of the freeze-dried cakes of PEG 8000 showed a melting endotherm at 62.5°C, while X-ray diffraction of the freeze-dried cakes also revealed a crystalline product.

With the addition of maltodextrin, the intensity of the X-ray diffraction peaks from PEG8000 decreased. The enthalpy of the PEG 8000 melting endotherm in the DSC thermograms also decreased in the PEG 8000/maltodextrin formulations. This was due to the diluting effect of the amorphous maltodextrins. The combinations of maltodextrins with PEG 8000 were partially amorphous.

Research on the influence of molecular weight fractionation of the maltodextrins on the protein stability is ongoing.

CONCLUSIONS

Maltodextrins were found to protect LDH against inactivation during freeze-drying. The lyoprotection obtained was dependent on the D.E. and the concentration of the maltodextrins used. The maltodextrin formulations performed as good or better than those containing sucrose and maltose, depending on the concentration used. Freeze dried cakes of maltodextrin formulations were amorphous. In the case of low D.E. maltodextrins, lyoprotection was improved by the addition of PEG 8000 as a cryoprotectant.

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