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Protection of the enzyme L-asparaginase during lyophilisation—a molecular modelling approach to predict required level of lyoprotectant

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Abstract

Many novel therapeutic agents are proteins and peptides which need stabilisation due to their inherent instability in aqueous solution. Freeze-drying is an established method for protein stabilisation, although the use of additives is often necessary in order to preserve protein structure and activity during lyophilisation itself. The molecular interactions between protein and protective additive are as yet unclear. In this study, we examined the use of a range of saccharide additives to stabilise the model multi-subunit enzyme L-asparaginase during lyophilisation, assessed post-drying enzyme activity and quaternary structure, and related the extrapolated levels of additive necessary to provide full stabilisation to the theoretical levels predicted from an existing hypothesis using molecular modelling. It was found that each of the saccharides tested here displayed similar levels of protection towards L-asparaginase under the conditions used. Amounts of additive required to give full stabilisation to the enzyme were extrapolated from the activity data and were found to be in good agreement with theoretical amounts calculated from molecular modelling studies. Our data suggest that the existing hypothesis may be relevant to the prediction of optimum levels of lyoprotectant for the freeze-drying of proteins. However, further studies would be necessary in order to obtain a full picture of protein-additive interactions at the molecular level.

Keywords: Enzyme activity; Lyoprotectant; Multi-subunit protein; Molecular modelling

1. Introduction

Many novel therapeutic agents emerging from molecular drug design and recombinant DNA technology are proteins and peptides (Pikal, 1990a,b; Adams, 1991; Arakawa et al., 1993; Nail and Gatlin, 1993). The process of freeze-drying is often used to stabilise such agents which prove to be unstable for long periods when stored as aqueous solutions. However, the process itself places stresses on proteins (and other biologically sensitive materials) which are often not considered during the formulation design process. Freeze-

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drying may cause stability problems by inducing conformational instability in many proteins subjected to freezing and subsequent dehydration stresses (Crowe et al., 1990). In the case of multisubunit proteins, freeze-drying can often cause a breakdown in the quaternary structure, leading to the production of inactive subunits. Numerous studies have demonstrated that additives may be used which preserve the structure and biological activity of such molecules, as evidenced in reviews by Arakawa et al. (1993), Skrabanja et al. (1994), Izutsu and Yoshioka (1995) and Carpenter et al. (1997). However, the precise protective mechanism by which these additives function has not been fully elucidated. Indeed, it is possible that there is not one universal mechanism which applies to all biological entities; the sheer number of studies carried out into the stabilisation of a diverse range of proteins and other biomolecules does not provide the reader with a clear pattern from which a mechanism may be elucidated. Some of the entities studied to date include: alkaline phosphatase (Ford and Allahiary, 1993; Ford and Dawson, 1994), L-asparaginase (Hellman et al., 1983; Adams and Irons, 1993), β-galactosidase (Izutsu et al., 1991, 1993a,b, 1994a,b), catalase (Tanaka et al., 1991), lactate dehydrogenase (Izutsu et al., 1994c, 1995), PFK (Carpenter and Crowe, 1989) and numerous other enzymes and biologicals (Pikal et al., 1991; Prestrelski et al., 1993a,b; Carpenter et al., 1993; Chang et al., 1993; Vemuri et al., 1994; Constantino et al., 1995; Draber et al., 1995; Suzuki et al., 1997).

In this study, we examine the freeze-drying characteristics of a model polymeric protein, L-asparaginase, a well-characterised enzyme exhibiting a pattern of freeze-drying instability which is typical of many such entities. When lyophilised in the absence of stabilising additives, the biologically active tetramer (134 kDa) of L-asparaginase is broken down to give the inactive monomer (34 kDa) (Hellman et al., 1983). The aim of this study was to evaluate the lyoprotective (or damaging) effects of a number of pharmaceutically relevant excipients (Table 1) on the structure and biological activity of L-asparaginase. Here, we discuss our findings in the light of a molecular modelling approach used to predict the levels of protective additive needed to provide full protection of our model protein.

2. Experimental

2.1. Materials

L-asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 24.3 mg/ml and activity 15 360 iu/ml, by CAMR, Porton Down, Wiltshire, UK. All other excipients were purchased from Sigma, Poole, Dorset, UK.

2.2. Freeze-drying

Aliquots (1 ml) of diluted aqueous solutions of L-asparaginase (1.45 mg/ml) containing excipients at a range of concentrations (0.05–1.00 mg/ml) were dispensed into glass lyophilisation vials and frozen at −70°C for 1 h. The frozen samples were transferred to an Edwards Modulyo freeze-dryer and lyophilised at a chamber pressure of ~0.2 millibar for 4 h at a shelf temperature of 30°C, followed by a further 12 h at the same chamber pressure with no shelf-heating applied. Dried products were sealed under vacuum and stored at 4°C. For the enzyme activity assay and for FPLC analysis, dried samples were reconstituted to original volume with Sørensen's Glycine II buffer (0.1 M, pH 10.0), a buffer shown to stabilise any inactive monomer present (Marlborough et al., 1975).

Table 1
Classification and typical freezing behaviour of additives used in this study

Additive	Description	Typical Frozen Matrix
Trehalose	Disaccharide	Amorphous
Lactose	Disaccharide*	Amorphous
Maltose	Disaccharide*	Amorphous
Sucrose	Disaccharide	Amorphous
Glucose	Monosaccharide*	Amorphous
Mannitol	Monosaccharide Alcohol	Crystalline

* Denotes reducing sugar.

2.3. Moisture content determination

Moisture contents of a representative batch of lyophilised cakes were determined by thermogravimetric analysis (TGA). Briefly, samples of freeze-dried product (2–3 mg) were heated to 130°C at a constant rate of 10°C/min, in a Perkin-Elmer TGS-2 thermogravimetric analyser, linked to a Perkin-Elmer TADS 3600 thermal analysis data station with related software. Sample weight was plotted as a function of temperature, and moisture content determined using system software.

2.4. FPLC of reconstituted products

Reconstituted solutions were introduced into two Sepharose HR 10/30 columns (Pharmacia, Sweden) connected in series. Sørensen's Glycine II buffer (0.1 M, pH 10.0) was used as eluent (flow rate 0.40 ml/min). Column eluate was monitored by a UV/visible flow-through detector at wavelength 280 nm, and absorbance plotted against time.

2.5. Enzyme activity determination

The biological activity of L-asparaginase was determined by the rate of turnover of the substrate L-aspartyl-β-hydroxamic acid (AHA), using a method based on that first reported by Frohwein et al. (1971). Briefly, aliquots of analyte solutions (100 μl) diluted 1 in 1000 with Sørensen's glycine II buffer (0.1 M glycine + 0.1 M NaCl, adjusted to pH 10.0 with NaOH) were pipetted into the wells of a 96-well microtitre plate, to which a solution of AHA (10 μmol/ml, 25 μl/well) was added. After an incubation period of 10 min at 37°C, a solution of ferric chloride reagent (25g $FeCl_3.6H_2O$ + 4.50 ml hydrochloric acid (sg 1.16) to 500 ml with d.H_2O) was added to each well (125 μl) to quench the reaction by forming a complex with excess AHA present. The absorbance of the resulting complex was analysed at a wavelength of 550 nm using a microtitre plate reader (Anthos Instruments, Austria). Calibration standards were prepared by dilution of original L-asparaginase/additive solutions (pre-freeze-drying) and the retained activity of L-asparaginase in reconstituted samples expressed as a percentage of original activity. A negative control was prepared by replacing AHA with filtered distilled water (thus giving no complexes) and a positive control prepared by performing the addition of AHA to wells containing L-asparaginase solution after the ferric chloride reagent had been added (thus giving full complexation). Good linearity was demonstrated between optical density readings and complexed AHA remaining in calibration standards.

2.6. FT-IR analysis of dried products

FT-IR spectrophotometry was carried out on KBr discs of freeze-dried samples, using a Mattson Fourier-Transform Infrared spectrophotometer (Mattson Instruments, UK) and related software.

2.7. Molecular modelling

Molecular modelling of *Erwinia* L-asparaginase was carried out by Dr Melanie Duffield at CAMR, Porton Down, following the publication of the crystal structure of the protein by Miller et al. (1993).

3. Results and discussion

Following drying, sample moisture contents were found to be typically between 3 and 5% (w/w), as determined by TGA. FPLC analysis of quaternary protein structure gave retention ratios (V_e/V_0) for the monomer and tetramer of 1.94 ($\pm$ 0.02) and 1.76 ($\pm$ 0.02), respectively. The raw peak heights for monomer and tetramer components in analyte solutions suggested that the tetramer:monomer ratio had increased with increasing concentration of additive used (Fig. 1(A)), but that each additive gave similar results. A plot of tetramer peak height against additive concentration (Fig. 1(B)) demonstrates the apparently similar protective abilities of the chosen excipients at each concentration.

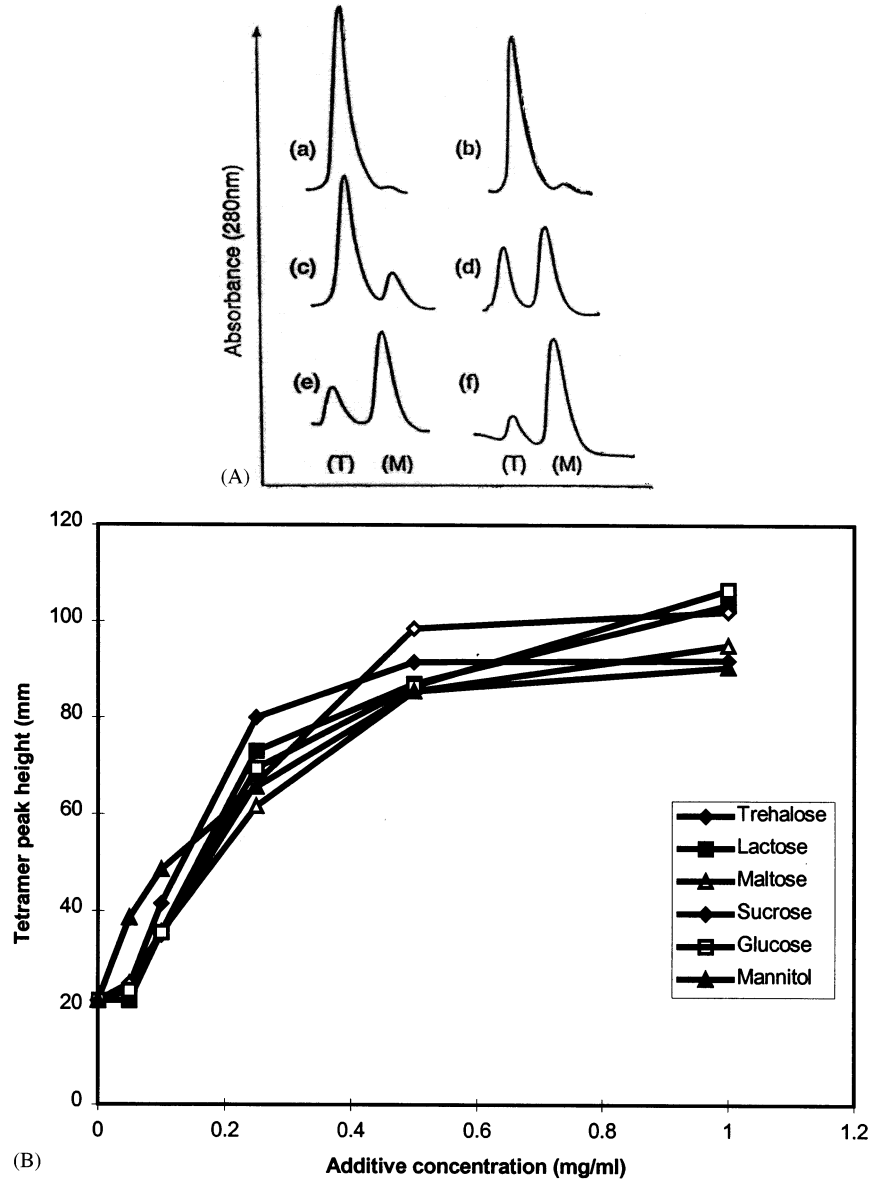

Fig. 1. FPLC profiles (A) for reconstituted solutions of L-asparaginase (1.45 mg/ml) which had been lyophilised in the presence of lactose at concentrations of: (a), 1.0 (b), 0.50 (c), 0.25 (d), 0.10 and (e) 0.05 mg/ml and in the absence of additive (f). Profiles provided a semi-quantitative assessment of the relative proportions of (active) tetramer (T) and (inactive) monomer (M) present in reconstituted solutions. A plot of tetramer peak height against additive concentration (B) demonstrates the similarity of the protective abilities of the saccharides used in this study.

In concordance with the quaternary structural data from the FPLC assay, the measurement of residual enzyme activity showed that each disaccharide appeared to confer similar levels of freeze-dry stability on L-asparaginase, and that the level of protection was dependent on the concentration of additive used (Fig. 2). Glucose and mannitol appeared to be equally as effective as the disaccharides at all concentrations used, in contrast to stability data reported for PFK (Carpenter and

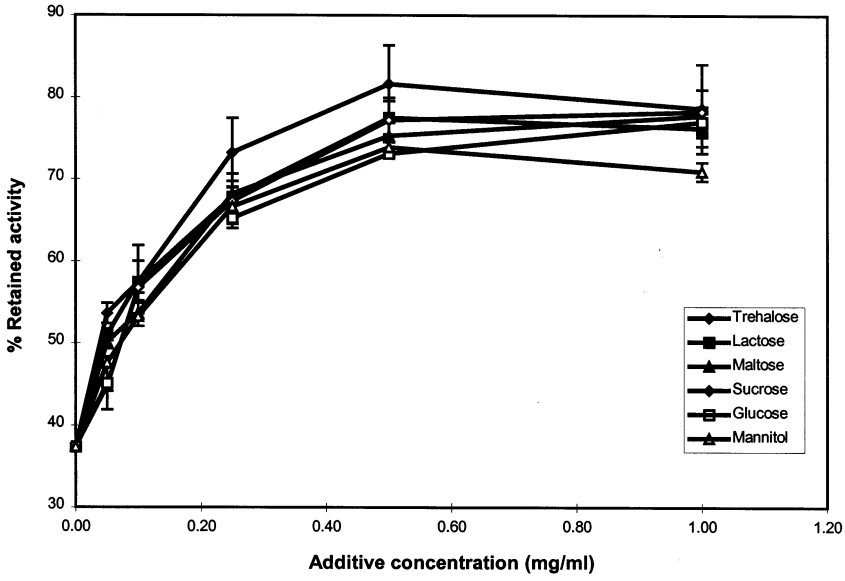

Fig. 2. Effect of saccharides on the retention of enzyme activity of L-asparaginase immediately following freeze-drying, as determined using the AHA assay developed from Frohwein et al. (1971) ($n = 3$, mean $\pm$ S.D.).

Crowe, 1989), which was stabilised only by disaccharides. The apparent success of mannitol as a protectant in the present study also contrasts with the findings of Hellman et al. (1983).

FT-IR spectra provided evidence of possible hydrogen bonding between L-asparaginase and trehalose (Fig. 3), an observation which has been previously reported for trehalose with other proteins (Carpenter and Crowe, 1989). For the enzyme freeze-dried alone (a), where little intermolecular hydrogen bonding would be expected, the amide (II) band showed a maximum at 1538 cm^{-1}. The shifting of this band in spectra (b) and (c) to around 1548 cm^{-1} suggests that levels of hydrogen bonding between L-asparaginase and trehalose when freeze-dried together (b) were similar to those present in the hydrated L-asparaginase itself (c). While these spectral features are similar to those previously reported for lysozyme and trehalose (Carpenter and Crowe, 1988), we remain unconvinced that it is possible to determine from these spectra alone whether any protective effects are due to the presence of residual water in the dried product, or to a direct interaction between protein and additive.

Molecular modelling of L-asparaginase was carried out in order to allow an estimation to be made of the number of highly polar residues

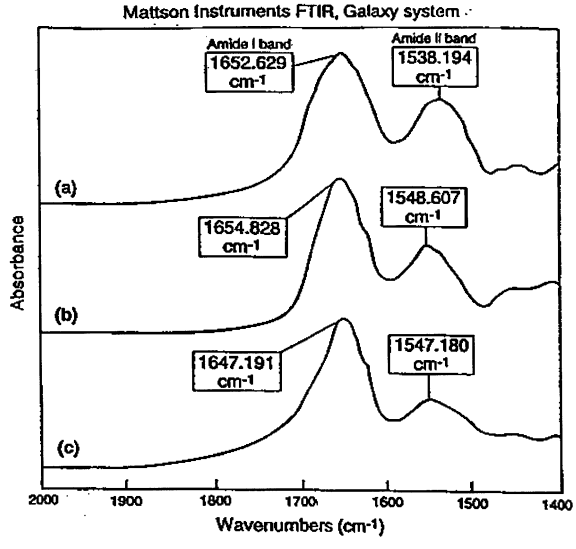

Fig. 3. Amide band region for dried samples, as determined by FT-IR spectroscopy. Spectra shown are of L-asparaginase freeze-dried alone (a), freeze-dried in the presence of trehalose (1.0 mg/ml) (b) and hydrated L-asparaginase (c). All spectra have been normalised with respect to the amide I bands.

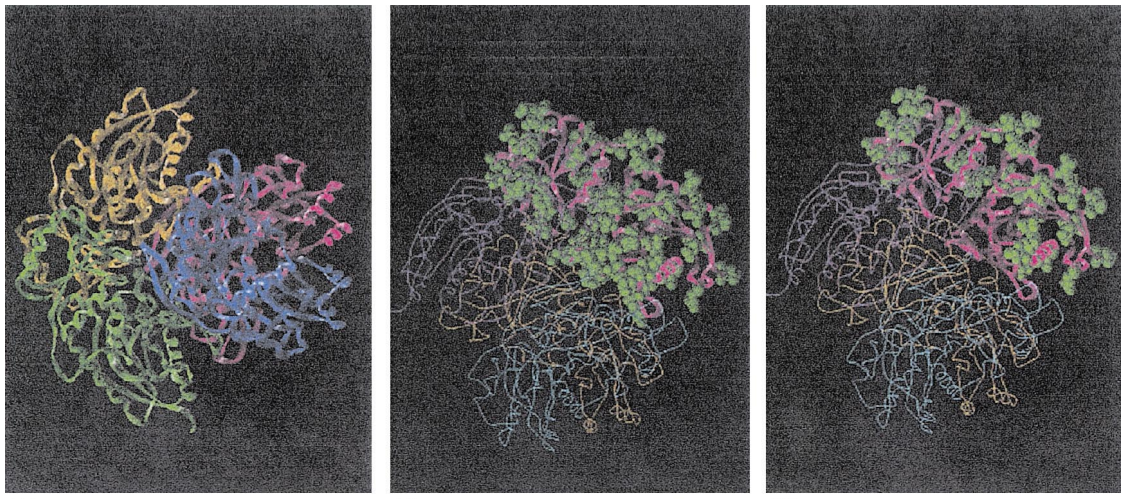

Fig. 4. (A) Structure of L-asparaginase tetramer depicted in ribbon form, with each monomer sub-unit highlighted in a different colour; (B) L-asparaginase tetramer, with one monomer sub-unit highlighted in pink, the HPR exposed in unassociated form depicted in green, remaining three sub-units depicted as ribbons; (C) as for (B) except the HPR exposed in associated form are depicted in green.

(HPR) exposed on the protein surface in both monomeric and tetrameric conformations. Following his studies on the dehydration of calcein, Pauling (1945) suggested that the protein should not be dried exhaustively, and that certain HPR on the protein surface should be maintained in the hydrated state in order that in should not become denatured during drying. The theory that HPR should be maintained in the hydrated state was more recently taken up by Hsu et al. (1991), who investigated the optimum residual moisture levels in lyophilised protein pharmaceuticals.

The structures of the L-asparaginase monomer and tetramer, together with a prediction of exposed HPR, are shown in Fig. 4. Fig. 4(A) shows the L-asparaginase tetramer as a ribbon, with each monomer subunit highlighted in a different colour. Fig. 4(B) shows the L-asparaginase tetramer, with one monomer sub-unit highlighted in pink and the HPR exposed in unassociated form depicted in green. The remaining three sub-units are depicted as single strands. Fig. 4(C) shows the molecule in a similar manner to Fig. 4(B), but highlights the HPR exposed in the associated (tetramer) form, as opposed to the unassociated form. The calculated distribution of HPR in the monomeric and tetrameric forms are given in Table 2.

On the arbitrary basis that one molecule of saccharide additive is required for each HPR exposed on the surface of the L-asparaginase monomer, it was calculated that for the concentration of protein employed in the present study (1.45 mg/ml), the concentrations of additive required to interact with all exposed HPR were 1.096 mg/ml disaccharide (MW 342.3) and 0.577 mg/ml monosaccharide (MW 180.2). Similarly, on the assumption that only HPR exposed on the surface of the tetramer would protectively interact with additive, it was calculated that under the conditions of this study, 0.622 mg/ml disaccharide and 0.328 mg/ml monosaccharide should be required to achieve full interaction. In either case, the range of concentrations of additive found to be required experimentally in the present study for the lyoprotection of L-asparaginase suggested that the amounts of additive required to provide full protection to the protein during drying would be in reasonable agreement with these theoretical concentrations. Any discrepancy between theoretical and experimental values may be attributed to a number of factors. Firstly, the HPR defined

above may not be the only residues which require the presence of water during drying, since the basis on which residues were defined as 'highly polar' was somewhat arbitrary. However, it is also possible that not all of the HPR defined above may require the presence of water during drying. This might in turn be dependent upon further factors such as neighbouring residues to the HPR, or additives present in a protein solution, particularly where the protein has been isolated from an organism and trace levels of compounds such as glucose may be present. Secondly, it is possible that some of the residues at the monomer-monomer interface (which may or may not fall into the category of HPR as defined above) may have also required the presence of water in order to maintain the quaternary structure of the protein. The elucidation of the precise interactions at the molecular level might perhaps be facilitated by the use of more sensitive analytical techniques, such as microcalorimetry.

Further studies carried out in our laboratory using PEG (mw 10 kDa) as additive indicate that in the case of PEG, the adoption of the 1:1 assumption used above for the saccharides leads to our experimental observations being far removed from the calculated theoretical values (data not shown). Without further experimental evidence of PEG as a lyoprotectant, we are unable to explain why this is the case; however, we propose that PEG protectively interacts with L-asparaginase via a different mechanism to the saccharides tested here. Results from preliminary thermal studies using differential scanning calorimetry (DSC) in our laboratories (data not shown) suggest that one of the distinct crystal forms of PEG may be responsible for the protection of L-asparaginase, although this conflicts with the prevailing theory that only amorphous excipients may provide lyoprotection to proteins.

The water replacement hypothesis suggests that a lyoprotectant mimics the water in the hydration shell of the protein molecule (Arakawa et al., 1993). Since water molecules form hydrogen bonds, it is possible that the interactions between proteins and co-solute molecules occur via hydrogen bonding, and that successful lyoprotectants should be chosen on this basis. If this were the sole criterion which needed to be fulfilled, then it is difficult to understand why other compounds capable of forming hydrogen bonds with amino residues of proteins (including monosaccharides) were not equally effective lyoprotectants in the case of PFK. It seems possible that the water-replacement hypothesis may help partially explain lyoprotectant activity, but that simple mimicking of water in the hydration shell may be insufficient for successful stabilisation of a protein during the removal of the hydration shell itself. Even when a compound is able to form hydrogen bonds with

Table 2
Pattern of HPR distribution in *Erwinia* L-asparaginase

Residue	Total number of HPR exposed in unassociated form (per monomer)	Number of HPR remaining exposed in associated form (per monomer)	Number of HPR becoming hidden upon association (per monomer)
Aspartic acid (D)	18	11	7
Glutamic acid (E)	14	8	6
Serine (S)	13	9	4
Threonine (T)	20	11	9
Tyrosine (Y)	9	3	6
∴Total per monomer	74	42	32
∴Total per tetramer	296	168	128

other molecules, it may also have to fulfil further criteria in order to be able to confer stability on a protein during lyophilisation. Such additional criteria could include steric factors and surface charge profiles, and thereby may differ for each protein studied. It is also possible that cryoprotectant and lyoprotectant additives do not function by mimicking water molecules and their interactions with proteins, but operate by a simpler 'water-sharing' mechanism. In such cases, the protectant molecule might act by holding water molecules (possibly those initially present in the hydration shell) close to the protein in such a way as to render the water molecules difficult to remove under normal freeze-drying conditions. Steric criteria and other factors particular to each protein may also have to be satisfied.

Since quaternary, tertiary, secondary and primary structure varies markedly from protein to protein, it is possible that different protective criteria need to be satisfied when stabilising different proteins. Consequently, when attempting to develop general guidelines for screening potential protective compounds, it is possible that there are more examples which are exceptions to, than those conforming to, a general pattern. Nevertheless, there are some factors which are applicable to protein stability during freeze-drying. In particular, the role of residual water, its interaction with the protein molecule and retention in the dry matrix are all crucial to the success of the freeze-drying process (Franks, 1982). It appears from the present data that such interactions in the presence of additives investigated were stabilising, when compared to those in the absence of additive.

4. Conclusions

We conclude that each of the excipients investigated here appeared to provide similar levels of freeze-drying protection to L-asparaginase at all (excipient) concentrations tested. This similarity also holds true when these additives are assessed on the basis of the number of free hydroxyl groups per mol, suggesting that protective interaction between L-asparaginase and additives in this instance might imply a direct involvement of these hydroxyl groups. However, the relative availabilities of the various hydroxyl groups on each molecule for such interaction have not been quantified, and so further studies would be necessary to test this hypothesis. It was interesting to note that the amounts of additive required to provide the maximum levels of protein preservation observed in the current study ($\approx 75\%$) compared well with the theoretical amounts of additive calculated to be required using the molecular modelling approach together with the HPR theory. We therefore believe that the HPR hypothesis may be significant in explaining protein-co-solute interactions, and may also be relevant for both protein-stabilising interactions (such as lyoprotection) and protein-destabilising processes (such as the Maillard reaction). It is anticipated that the use of currently-available sensitive techniques (for example, micro-thermal analysis) should provide a more vigorous test of the HPR hypothesis, by providing quantitative real-time data for thermal events occurring in protein-excipient solutions; such investigations may allow a fuller elucidation of protein-co-solute interactions at the molecular level.

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