Non-equivalence of D- and L-trehalose in stabilising alkaline phosphatase against freeze-drying and thermal stress. Is chiral recognition involved?

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Comparison of the ability of the enantiomeric forms of trehalose to stabilise alkaline phosphatase towards dehydration and heat showed that natural D-trehalose is superior to L-trehalose, although both disaccharides provide some protection for the enzyme. The result of this novel experiment suggests a chiral differentiation between carbohydrate and protein and thus lends support for the water replacement hypothesis of solute-based stabilisation of biomolecules, but the non-crystallinity and the physical form of the L-isomer may also be a key factor.

Introduction

The mechanism by which organisms can survive virtually complete dehydration, a physical state termed anhydrobiosis, has been the subject of intensive study for over twenty years yielding a voluminous literature on the subject which fortunately has been well reviewed,[1–8] but it is noteworthy that the first description of the overall phenomenon was made over three hundred years ago by the Dutch naturalist Antoni van Leeuwenhoek. Clearly, effective stabilisation of key biomaterials such as proteins, membranes, and their constituent lipids must be achieved in a manner such that they can return to their native state upon re-hydration, a valuable property which has considerable potential for exploitation, especially in the field of medicine and pharmacy. This stability is also attained when certain organisms are subjected to related heat stress under hostile conditions, for example the resurrection plants *Craterostigma plantagineum* and *Selaginella lepidophylla* found in the desert, cysts of the brine shrimp *Artemia*, dry baker's yeast, and some species of nematodes found in soil. A common feature of such organisms is the accumulation of disaccharides, most commonly trehalose and, in higher plants, sucrose, as a result of such stresses, although most recent research has shown[9] that some organisms, for example bdelloid rotifers, undergo anhydrobiosis without production of trehalose or related saccharides and the special properties often ascribed to trehalose and sucrose have been questioned.[8,10,11]

Cumulative research through *in vitro* experiments into the stability conferred on biomolecules by natural trehalose (D-trehalose) and sucrose led firstly to the widely held opinions that one of two major factors is involved (i) water replacement or (ii) vitrification, but a more recent view[7] is that these two factors are not mutually exclusive, both being required. The water replacement hypothesis involves stabilisation of proteins and membranes in the dry state by the direct interaction (*e.g.* through hydrogen bonding) of the disaccharides to polar residues in the biomolecules, thus maintaining their physical state similar to that under aqueous conditions *in vivo*. Since proteins and lipids and the relevant disaccharides are chiral entities it seems possible that evidence for such direct interaction might be obtained by comparing the ability of enantiomeric forms of the disaccharides to stabilise proteins when the latter are subjected to dehydration and accelerated degradation by storage at temperatures above ambient, a method of simulating over a relatively short time period the effect of long term storage at lower temperatures. A close involvement of saccharide and biomolecules by non-covalent bonding in the anhydrous state would be reflected in differing interactions of the enantiomeric forms of a given saccharide with the chiral biomolecule, leading to a non-equivalence of the enantiomers in their stabilising influence. However, should vitrification be the sole cause of bio-stabilisation, it would seem reasonable to expect that a chiral influence would not be effectively transmitted by the amorphous glassy medium and that such media formed from each of the enantiomeric saccharides would be equally effective in protecting biomolecules to dehydration and heat. To our knowledge, a search for such a chirality dependence has not been undertaken previously, presumably because of difficulty in synthesising the L-forms of trehalose and sucrose, enantiomeric forms of the natural sugars, but the availability of L-trehalose from our own work[12] prompted this present investigation, utilising the enzyme, alkaline phosphatase. This enzyme has been used previously to assess the effect of dehydration by freeze-drying followed by storage of the dried preparations at elevated temperatures in the absence and presence of added carbohydrates, including D-trehalose, a saccharide which was shown to possess notable preservative properties towards activity of the enzyme.[13] Use of the phosphatase provides a reliable and simple method of assaying residual enzyme activity by measurement of the rate of liberation of 4-nitrophenol from 4-nitrophenyl phosphate.

Results and discussion

In view of potential variability in the state of hydration of D-trehalose[14–26] —it crystallises as the dihydrate or in an anhydrous form but may be obtained containing varying proportions of water, or anhydrous, by drying *in vacuo* at an elevated temperature— a study was undertaken to make a direct comparison between D- and L-trehalose, with respect to their efficiency in stabilising

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alkaline phosphatase, by treating separate test samples of the enzyme containing the enantiomeric sugars in an identical manner. Thus, to separate equal amounts of the enantiomeric disaccharides was added the same volume of freshly prepared aqueous, buffered enzyme solution and the resultant solutions were freeze-dried for the same length of time to give solids which were then stored in stoppered flasks under identical conditions for equal times at a given constant temperature before assay of enzyme activity. After dissolution of the stored preparations in an appropriate buffer solution and addition of 4-nitrophenyl phosphate solution, comparison of enzyme activities was made by measuring the initial rates of liberation of 4-nitrophenol measured as its anion at 405 nm. A comparison was also made with the activity of the enzyme stored at 4 °C for the same time period to provide a control.

In agreement with the earlier related study,[13] freeze-drying and storage of the enzyme at above ambient temperature in the presence of D-trehalose led to a lower loss of enzyme activity than when the disaccharide was omitted, although phosphatase activity was still reduced compared to that of the initial solution. Thus, after storage of alkaline phosphatase at 37 °C for 10.75 days in the presence and absence of D-trehalose, residual activity was 82% and 35%, respectively, of that of the enzyme solution stored at 4 °C for the same period of time (Fig. 1). In a similar experiment (37 °C for 14 days) conducted with D- and L-trehalose, the retained enzyme activities were 62% and 52%, respectively, of the control maintained at 4 °C, with the activity of the enzyme stored in the absence of either disaccharide being only 33% of the control (Fig. 2). Repetition of the latter experiment but at a higher temperature (45 °C) and for a shorter time (7 days) with D- and L-trehalose gave enzyme activities of 67% and 32%, respectively, of that of the low temperature control, with the enzyme stored at 45 °C without additive having an activity of 24% that of the low temperature control (Fig. 3). In contrast, the activities of solutions containing the enzyme alone, the enzyme plus D-trehalose, and the enzyme plus L-trehalose, measured immediately after preparation and at ambient temperature (~27 °C), were virtually identical.

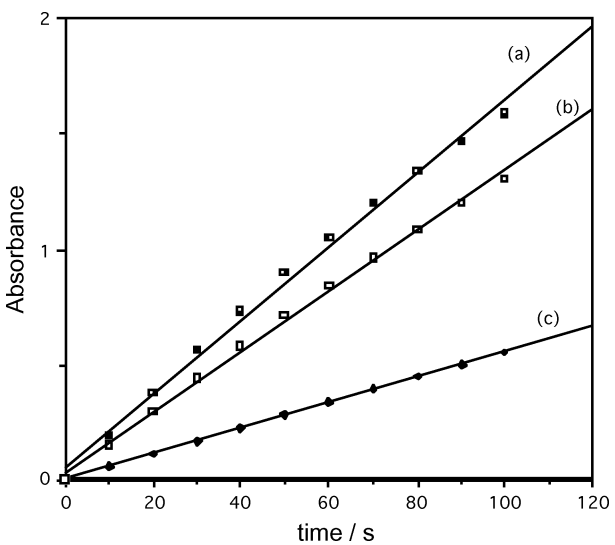

Fig. 1 Rate of liberation of 4-nitrophenol from 4-nitrophenyl phosphate by alkaline phosphatase after storage for 10.75 days: (a) at 4 °C, (b) at 37 °C in the presence of D-trehalose, (c) at 37 °C in the absence of D-trehalose.

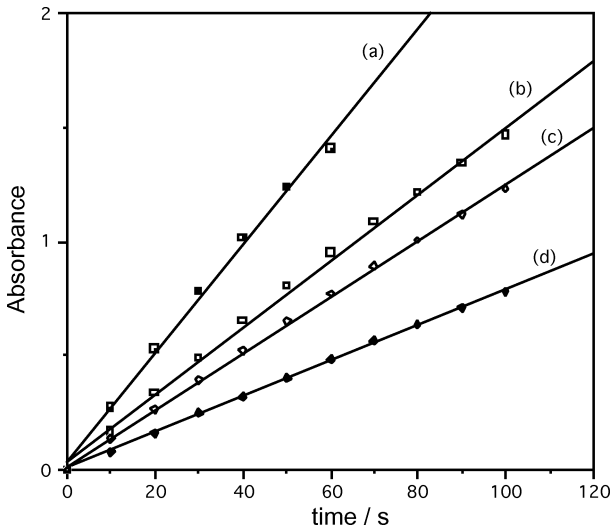

Fig. 2 Rate of liberation of 4-nitrophenol from 4-nitrophenyl phosphate by alkaline phosphatase after storage for 14 days: (a) at 4 °C, (b) at 37 °C in the presence of D-trehalose, (c) at 37 °C in the presence of L-trehalose, (d) at 37 °C in the absence of D- and L-trehalose.

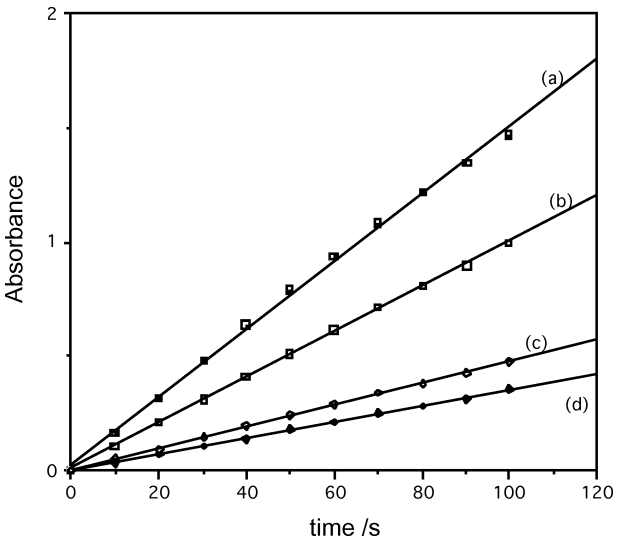

Fig. 3 Rate of liberation of 4-nitrophenol from 4-nitrophenyl phosphate by alkaline phosphatase after storage for 7 days: (a) at 4 °C, (b) at 45 °C in the presence of D-trehalose, (c) at 45 °C in the presence of L-trehalose, (d) at 45 °C in the absence of D- and L-trehalose.

These experiments indicate that the enantiomeric forms of trehalose are not equivalent in aiding the preservation of the enzymic activity of alkaline phosphatase under the conditions of dehydration by freeze-drying and thermal stress, and provide evidence for, at least in part, a direct interaction, *i.e.* of the water replacement type, between the disaccharide and the protein structures. Significantly, evidence from other research with lysozyme suggests[27] that direct carbohydrate–protein hydrogen bonding is responsible for the inhibition of dehydration-induced protein damage. The water replacement hypothesis envisages that key water molecules in the hydration shell of a biomolecule might be replaced to an extent by hydroxy groups, suitably oriented in space,

of a saccharide or other substance aiding preservation of biological activity, thereby replacing hydrogen-bond type interactions to water molecules which are crucial to the integrity of the molecular architecture of a biomolecule in its native state. Since biomolecules (*e.g.* proteins, lipids, *etc.*) are chiral, it is reasonable to expect that an associated hydration shell would be influenced in its spatial orientation by the chiral nature of the biomolecule to which the water molecules are bonded, and therefore that there would be chiral demands on molecules which might act as replacements for some or all of the water molecules. In contrast, if the only rôle of the saccharide is to form a glass (typically spatially homogeneous but without any long range lattice order) which then entraps the key water molecules of the hydration shell, it would appear that the chirality of the molecules forming the glass would have no or minor influence on the effectiveness of the additive regarding biostabilisation.

Although it appears that glass formation (vitrification) is required but is not in itself sufficient to stabilise dry biomaterials,[7] the often noted remarkable effectiveness of D-trehalose has led to speculation that the high glass transition temperature (T_g) of the anhydrous material[7,28] (*ca.* 115 °C compared to sucrose at 65 °C) might be an important factor, coupled with the fact that the presence of a similar water content in the two disaccharides would cause T_g of sucrose to fall below a given storage temperature before that of D-trehalose. Further, the latter appears anomalous in that on addition of small quantities of water to the glassy sugar, T_g remains unchanged, and it is possible that a crystalline dihydrate forms which shields the remaining glassy D-trehalose from contact with additional water.[28] It would seem reasonable to assume that a similar situation should be obtained with the L-enantiomer, and therefore it might not be expected that the observed differences in ability of the enantiomers to stabilise the enzyme can be rationalised on this basis.

Nevertheless, despite the attraction of an interpretation of our results based on chiral recognition between protein and protectant, an alternative explanation must be considered based on the possibility that non-identical physical forms of the disaccharide medium are produced on freeze-drying of the separate solutions of the two enantiomers and that the differing forms are not equal in their ability to stabilise a co-evaporated protein. The literature on the polymorphic forms of D-trehalose obtained under different conditions (*e.g.* freeze-drying, spray-drying, dehydration at different temperatures, pressures and relative humidities, and melt quenching) is extensive[14–26] and the subject is of crucial importance to the pharmaceutical industry for which an amorphous form of carbohydrates is usually required for incorporation into pharmaceutical products as excipients in freeze-drying to stabilise labile bioactive material.[29] Two crystalline forms of D-trehalose, the dihydrate rhombic form[30,31] (T_h) and the anhydrous monoclinic form[32] (T_β, mp ~ 213 °C) are well characterised through X-ray diffraction studies and it is clear that another metastable anhydrous form (T_α, mp ~ 125 °C) may be obtained by careful drying of the dihydrate under specific conditions.[14,16–18,21–24,26] Melting of this form, as yet not characterised crystallographically, leads to an amorphous form which undergoes 'cold crystallisation' from 150 °C to give trehalose in the anhydrous T_β state.[26] The latter modification is produced directly from the dihydrate (T_h) at 90 °C—strangely under highly humid atmospheres—and it has been shown that rehydration of T_α occurs much more readily than that of T_β, and that it is very hygroscopic.[26] There is even a dependence on the size and concentration of solutions of D-trehalose subject to air-drying as regards whether a dihydrate or glass is formed,[28] a curious variation that has been attributed to a differing ability of such samples to nucleate and crystallise into the dihydrate. Thus, there is much opportunity for interconversion amongst the various crystalline and amorphous phases, depending amongst other things on the temperature, the rate and length of heating, the relative humidity of the surrounding atmosphere, and the length of storage.†

In the present research, an attempt has been made to obtain comparative data on the physical state of freeze-dried D- and L-trehalose samples by visual comparison of the solid forms, and through DSC and IR measurements. A point which may be of some importance is that despite a full chemical and spectroscopic identification of L-trehalose,[12] we were unable to induce its crystallisation under conditions which allowed recrystallisation of D-trehalose to give its dihydrate (T_h). However, this failure may be understood in view of the lack of seed crystals (there was no previous synthesis of the L-enantiomer) and the known difficulty in crystallising carbohydrates. It is possible that the absence of seed crystals could have an important influence on the solid state of the L-isomer obtained on freeze-drying. An analysis by DSC of the freeze-dried sample (a light, expanded amorphous solid which, in contrast to the D-isomer, was hygroscopic) indicated it to be anhydrous with a major endotherm around 200 °C but with some variability from sample to sample. Analysis of the freeze-dried D-isomer (a solid appearing to be a mixture of crystalline and some amorphous material) indicated only a minor water content with a major endotherm around 200 °C, but measurement on different samples from the same batch showed distinct differences, indicating that it was not homogeneous. It is noteworthy that heterogeneity has been observed in solid D-trehalose samples obtained by air-drying.[28]

As expected, the FT-IR spectra of freeze-dried samples of the enantiomeric forms were virtually identical in their overall features, but the less well defined spectrum of the L-isomer contained the characteristic peaks within broader envelopes as compared to those of the D-isomer, pointing to its amorphous rather than crystalline nature.

One of two important deductions can be made from results of the present study: either (i) there is a chiral recognition phenomenon between enzyme and its protectant medium, or (ii) that the enantiomers afford different solid forms on freeze-drying with the enzyme, and that the effectiveness regarding biostabilisation is crucially dependent on the physical form of trehalose, in particular under conditions where anhydrous material is obtained, on whether T_α, T_β, or an amorphous form makes up the medium surrounding the protein. An argument against (ii)—and thus support for (i)—arises from the observations[7,22,23,28] that effective protection of proteins during the terminal stages of dehydration by compounds such as trehalose depends on the initial formation of an amorphous, glassy or metastable anhydrous (but hygrospcopic) crystal form (T_α) rather than a well recognised crystalline phase (T_h and T_β); our preparation of L-trehalose has

† An unidentified state T_ε obtained by dehydration of T_h under intermediate humid atmospheres has also been reported.[26]

not yielded crystals in contrast to the D-isomer, appears to be amorphous and hygroscopic, and yet it is *less* efficient in stabilising the enzyme.‡ Further effort is being made in order to obtain L-trehalose in crystalline form to gain supporting evidence on this matter.

Although the utility of D-trehalose for biostabilisation has been much explored, the influence of its physical form on effectiveness in this regard would merit further systematic investigation. Clearly, experiments on the stabilisation of alkaline phosphatase by D-trehalose under conditions which lead exclusively to T_α, T_β, or an amorphous form of the disaccharide will provide valuable data to aid selection of the most favourable conditions for the long-term storage of biomaterials which are sensitive to environmental stress. Further, it would be instructive to compare the properties of the enantiomeric trehaloses as regards protein protection in the presence of bulk water. The thermodynamic mechanism of protein stabilisation at above ambient temperatures by trehalose in aqueous solution, examined in the case of ribonuclease A,[33] was attributed to the smaller preferential binding to the unfolded protein than the native one; the exact nature of this binding, as yet not clearly defined, could be probed with the use of the enantiomeric substrates. In addition, the direct correlation observed[34] between the surface tension of D-trehalose solutions and the thermal stability of various proteins, including ribonuclease A, should not be chirality dependent, a prediction which could be tested with the enantiomeric disaccharides. Further, D-trehalose is known to have superior structure-breaking effects on the tetrahedral hydrogen bonding network of water, effects which lead to inhibition of ice formation and which could explain its properties as a cryoprotectant for biomolecules.[35,36] Since both enantiomers would be expected to behave similarly in this regard, a comparison of their efficacy would be informative regarding the importance of this mechanism in cryoprotection.

Conclusion

Evidence has been obtained which suggests that enantiomeric forms of trehalose are not equally effective in stabilising the enzyme alkaline phosphatase to stress by dehydration during freeze-drying and storage of the resultant solid at above ambient temperatures. The observation could be interpreted as support for the involvement of a direct carbohydrate–protein interaction in the dehydrated state, with chiral discrimination as an explanation for the difference in effectiveness of the enantiomers. This research utilising enantiomeric bio-protectants highlights a novel approach for detecting important interactions with chiral pharmaceutical and biotechnological products and may provide useful evidence regarding the mechanism of the survival of biological functions resulting from dehydration and heat stress.

‡ However, this argument against explanation (ii) may be invalid *if* T_α is the important physical form inducing stabilisation rather than a glassy or amorphous form *and* if this is derived during the freeze-drying process by dehydration of the dihydrate T_h. Preparations of T_α reported to date have required dehydration of T_h at temperatures above ambient, typically 50–105 °C, although IR evidence suggests that it might be present in freeze-dried samples.[23]

Experimental

Alkaline phosphatase (EC 3.1.3.1, Type 1S from bovine intestinal mucosa-10–30 DEA units per mg solid; catalogue number P 7640) and the phosphatase substrate *p*-nitrophenyl phosphate disodium hexahydrate (catalogue number P 4744) were obtained from Sigma. D-Trehalose dihydrate was purchased from Aldrich. UV spectra were recorded at 25 ± 1 °C temperature on a Hitachi U-2000 spectrophotometer, and FTIR spectra in the frequency range 4000 to 400 cm^{-1} (2 cm^{-1} resolution) on a Biorad FTS-165C spectrometer equipped with an attenuated total reflectance (ATR) ZeSe crystal. DSC data for sealed samples were obtained on a Thermal Analysis 2920 instrument, calibrated with indium and octadecane.

Preparation of freeze-dried samples of alkaline phosphatase for storage at above ambient temperatures

In a 25 ml round-bottom flask was weighed D- or L-trehalose (11 mg) from samples of the disaccharide which had previously been freeze-dried at 13.3–1.33 Pa from aqueous solution for 4 h, then stored over P_2O_5 under vacuum. To the flask was then added 2 ml of a freshly prepared solution of alkaline phosphatase, made by dissolving the enzyme (5 mg) in 0.1 M TRIS buffer (10 ml) which had previously been brought to pH 7 with 2 M hydrochloric acid. A control, lacking the disaccharide, was prepared by placing 2 ml of the buffered enzyme solution in a similar flask. Solutions were then freeze-dried for 4 h at 13.3–1.33 Pa and the flasks containing the solids were then placed in a constant temperature environment for a measured time. A sample of the freshly prepared phosphatase solution was stored at 4 °C for a comparative measurement.

Measurement of residual enzyme activity

After storage of the enzyme containing solid at the elevated temperature, 2 ml of a buffered aqueous solution (pH 9.5, adjusted with 2 M hydrochloric acid) of 0.1 M diethanolamine and 0.25 mM $MgCl_2$ was added to yield the reconstituted enzyme solution.

A buffer solution (buffer A) at pH 10.25 was prepared by dissolving diethanolamine (9.66 ml) in water (60 ml), adding 0.25 ml of 0.1 M aqueous $MgCl_2$ solution, adjusting the pH to 10.1 with 2 M hydrochloric acid, and then diluting to 100 ml with water.

A 3.3 mM solution of the substrate *p*-nitrophenyl phosphate was freshly prepared in buffer A. To a UV cuvette (1 cm path length, 3 ml capacity) was added 2.6 ml of buffer A, then 0.3 ml of the substrate solution, and the cuvette was placed in the cell holder of the UV spectrophotometer to achieve temperature equilibration. After 5 min, 0.1 ml of the enzyme solution was added to the cuvette, which was then stoppered and inverted three times to ensure efficient mixing before returning to the cell holder, when monitoring of the absorption at 405 nm was commenced immediately and followed for 120 s, during which time the plot was linear and the substrate conversion was less than 25%. At least two runs were made for each determination of enzyme activity and the readings averaged. Linear regression analyses of the plots of absorbance *versus* time yielded correlation coefficients in the range 0.995–1.000, and measurement of the slope of a line afforded a measure of the enzyme activity.

A similar measurement of enzyme activity was made utilising the enzyme solution that had been stored at 4 °C for the duration of the elevated temperature experiment, and enzyme activities relative to this sample were obtained by comparison of the slopes obtained from the linear regression analyses.

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