The stabilisation of purified, reconstituted P-glycoprotein by freeze drying with disaccharides ☆

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Abstract

The drug efflux pump P-glycoprotein (P-gp) (ABCB1) confers multidrug resistance, a major cause of failure in the chemotherapy of tumours, exacerbated by a shortage of potent and selective inhibitors. A high throughput assay using purified P-gp to screen and characterise potential inhibitors would greatly accelerate their development. However, long-term stability of purified reconstituted ABCB1 can only be reliably achieved with storage at −80 °C. For example, at 20 °C, the activity of ABCB1 was abrogated with a half-life of <1 day. The aim of this investigation was to stabilise purified, reconstituted ABCB1 to enable storage at higher temperatures and thereby enable design of a high throughput assay system. The ABCB1 purification procedure was optimised to allow successful freeze drying by substitution of glycerol with the disaccharides trehalose or maltose. Addition of disaccharides resulted in ATPase activity being retained immediately following lyophilisation with no significant difference between the two disaccharides. However, during storage trehalose preserved ATPase activity for several months regardless of the temperature (e.g. 60% retention at 150 days), whereas ATPase activity in maltose purified P-gp was affected by both storage time and temperature. The data provide an effective mechanism for the production of resilient purified, reconstituted ABCB1.

Keywords

ABCB1; Multidrug resistance; Lyophilisation; Trehalose; High throughput; Liposome; ATPase; Proteoliposome; Chemotherapy; Purification

Malignant cells are seen to employ a number of strategies to evade the cytotoxic effects of drugs used in chemotherapy. The generic term for these strategies is multidrug resistance (MDR) and is a significant clinical barrier to effective chemotherapy. Multidrug efflux pumps belonging to the ATP binding cassette (ABC) family of proteins such as P-glycoprotein (P-gp), multidrug resistance proteins 1–5 and breast cancer resistance protein confer the MDR phenotype to some cancers [19,42]. P-gp has been the focus of study following the discovery over 30 years ago that it mediated the efflux of chemotherapy drugs

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[21] as it has also been shown to affect the absorption, distribution and elimination of a wide range of pharmaceutical agents [22,45].

During these 30 years there has been a major effort to produce clinical inhibition of ABCB1 as a mechanism to restore sensitivity to chemotherapy. A large number of inhibitors have been developed from (i) existing drugs, (ii) chemical modifications of existing drugs, (iii) combinatorial chemistry and (iv) natural sources [26,28]. Whilst a large number of drugs are successful in vitro, the translation to clinical success has been poor. Reasons include, lack of potency, low selectivity, unwanted drug interactions and poor pharmacokinetic properties [19]. Combinatorial chemistry has been used to create one of the most promising inhibitors, tariquidar (XR9576). Tariquidar shows great selectivity, duration of inhibition and potency of interaction with P-gp and restored the efficacy of anticancer drugs in solid tumour models [49], yet even this drug has failed to progress through clinical trials [34].

There is, therefore, a real need to screen greater numbers of potential inhibitors against P-gp, whether generated by combinatorial chemistry or rational, structure based drug design. A genuine specific, high throughput screen would be hugely advantageous in this search. Current P-gp screens include the use of P-gp expressing cell lines (Caco-2, Madine-Derby canine kidney) which mimic the gastrointestinal tract and are used for bi-directional flux assays [17]. The information is not specific to P-gp-drug interactions as these cell layers contain many drug transporters, including several related ABC proteins [46]. The fact that P-gp is an ATPase has been exploited to measure drug efflux in both cell membrane fragments and pure P-gp containing proteoliposomes [2,3,40,43]. However, the membrane fragments contain many ATP dependent drug efflux pumps and P-gp must be inhibited in a separate experiment to yield specific data. Proteoliposomal systems utilising pure P-gp do not suffer from a lack of specificity and as an ATPase assay have a microtitre plate layout. These would seem to be a good candidate for the development of a high throughput screen. However, the instability of purified membrane proteins is a well established phenomenon and samples must be used immediately after purification or stored at −80 °C. This requirement precludes the development of a high throughput system that may have commercial application.

Stabilisation of labile proteins during freeze drying has been well documented, commonly involving the use of excipients such as mono and disaccharides [1,6,8,7,23,24]. The interaction of excipients with membranes and specifically liposomes during freeze drying has also been studied in some detail [11,14,13,20,30,48]. However, there has to date been very little investigation into the stabilisation of membrane bound proteins and no reported success in freeze drying a proteoliposomal system. The present investigation provides a method to generate a freeze drying protocol for the generation of stable ABCB1 preparations.

Materials and methods

Materials

Cholesterol, disodium adenosine triphosphate (Na_2ATP), nicardipine and buffer salts were obtained from Sigma (Poole, UK). DC detergent compatible protein assay kit and SM-2 Biobeads from BioRad (Hemel Hemstead, UK). Octyl-β-d-glucoside (OG), leupeptin hemisulfate, pepstatin, benzamide HCl and nickel-nitrilotriacetic acid (Ni-NTA) resin from Calbiochem (Nottingham, UK). *Escherichia coli* total lipid extract was purchased from Avanti Polar Lipids (Alabama, USA). [^{3}H]-phosphatidylcholine (84 Ci/mmol) was from Amersham Biosciences (UK) the silver staining kits for SDS PAGE were from ICN (Thame, UK) and PageBlue™ gel staining system was from Fermentas Life Sciences (Maryland, USA).

Purification and reconstitution of P-gp from insect cell membranes

Trichoplusia ni (High Five) cells were infected with recombinant baculovirus and used for expression of hexahistidine tagged (His$_6$) P-gp [47]. Cell membranes were isolated by centrifugation and ABCB1 was purified by immobilised metal affinity chromatography (IMAC) as previously described [47,38,44]. Briefly 50 mg total membrane proteins were solubilised in buffer 1 (20 mM Tris pH 6.8, 150 mM NaCl, 1.5 mM MgCl$_2$ and 2% (w/v) octyl-β-d-glucoside) supplemented with 20% (v/v) glycerol and 0.4% (w/v) of a 4:1 mixture of *E. coli* lipid and cholesterol. Solubilised proteins were added to 0.5 ml Ni-NTA resin and washed with buffer containing increasing concentrations of imidazole before elution at 120 mM imidazole. P-gp containing fractions were then reconstituted into proteoliposomes using detergent adsorption to SM-2 Bio-Beads and the efficiency was assessed by sucrose density gradient centrifugation as described [47,38,44]. Protein concentration was determined by densitometric analysis of SDS PAGE stained with Coomassie blue. Buffer 1, used in purification, was modified to examine the properties of disaccharides in freeze-drying experiments by substituting glycerol for trehalose or maltose, as required.

Measurement of P-gp ATPase activity

Hydrolysis of ATP was assessed by colorimetric assay based on the measurement of inorganic phosphate (Pi) [9]. ATPase activity was measured over varying concentrations of ATP (0–2 mM) in the absence (basal activity) and presence (drug stimulated activity) of 30 μM nicardipine. The effect of different nicardipine concentrations (0–100 μM) was also measured in the presence of 2 mM ATP. P-gp containing proteoliposomes were incubated for 20 min at 37 °C before the reaction was quenched, color developed and measured. To assess their effect on function, trehalose or maltose were added to ATPase buffer (50 mM Tris pH 7.4, 150 mM NH$_4$Cl, 5 mM MgSO$_4$, 0.02% (w/v) NaN$_3$) in the range 0–300 mM. Following freeze drying experiments samples were rehydrated with room temperature deionised water before ATPase activity was measured.

Freeze drying

Freeze drying was carried out using a CHRIST Alpha 2–4 (MartinChrist, Germany) with the condenser temperature set to −80 °C. Product was frozen and dried in 2 ml freeze-drying vials with 13 mm freeze drying stoppers (Fisher Scientific, UK). Following purification and reconstitution 400 μl aliquots were frozen in the vials with stoppers on in a −80 °C freezer. After initial optimisation, the drying cycle used was 0.011 mBar for 72 h with vials sealed under vacuum. Shelf temperature during secondary drying stabilised at 8.6 ± 0.17 °C though this was outside of direct control due to limitations with the freeze drying apparatus.

Determination of residual water

Residual water content of freeze dried products was determined by coulometric Karl Fischer titration using an AF7 Coulometric Karl Fischer (QCL Ltd., UK). Residual water was calculated as a percentage of the total weight of the dry product.

Stability testing

Following freeze drying, samples were sealed under vacuum and stored at 4 °C, 20 °C or 37 °C for up to 240 days. At various time intervals the ATPase activity was assessed and the thermal properties investigated by DSC.

Differential scanning calorimetry (DSC)

The thermal properties of the freeze dried product were investigated at various time points and storage temperature by DSC performed on a Polymer Laboratories Thermal Science Division DSC Gold (UK). Samples (3 mg protein/lipid/disaccharide mixture) were prepared

in aluminum pans and heated at 10 °C/min from −100 °C to 200 °C with 15–20 ml/min nitrogen purge gas. The glass transition temperature (T_g) was taken to be the mid point between the onset and end of the glass transition as this is in agreement with the assertion that T_g should be considered as the temperature of half vitrification on cooling [50].

Analysis of data

Analysis of ATPase data was by nonlinear regression performed by GraphPad Prism 4.0 (GraphPad Software Inc., USA). ATPase activity with varying ATP concentration was fitted with the Michaelis–Menten equation whilst activity with varying nicardipine concentration was fitted with the general dose–response equation [16]. Statistical analysis was done via by two way analysis of variance (ANOVA) and paired t-test using GraphPad Prism 4.0.

Results

Purification and reconstitution of P-gp proteoliposomes

A starting amount of 50 mg total membrane protein from High-5 insect cells generated a yield of 115 ± 41 µg ABCB1 purified to greater than 85%. Following reconstitution, ABCB1 displayed a basal ATPase activity of $V_{max} = 0.5 \pm 0.1$ µmol Pi/min/mg with an affinity constant for ATP of $K_m = 0.4 \pm 0.1$ mM. In the presence of the modulator nicardipine (30 µM), the V_{max} for ATP was stimulated approximately fivefold to a $V_{max} = 2.6 \pm 0.2$ µmol Pi/min/mg and a K_m (ATP) of 0.6 ± 0.1 mM ATP. These values are similar to several previous reports using a number of ABCB1 isoforms [47,44].

(Fig. 1) shows the ATPase activity of reconstituted ABCB1 stored at a number of temperatures between −80 and 20 °C for up to 250 days. All values of ATP hydrolysis were obtained in the presence of nicardipine and normalised against the activity prior to storage (100%). The ATPase activity decreased in a time-dependent fashion at storage temperatures other than −80 °C and measurements were ceased once activity was less than 10% of the initial value. Activity at +20 °C declined most rapidly for a half-life of inactivation of $t_{1/2} = 0.88$ days, whereas the rate of decay was considerably lower at 4 °C ($t_{1/2} = 11.6$ days) and −20 °C ($t_{1/2} = 22.7$ days). At a storage temperature of −80 °C, there was a 12–15% reduction upon thawing of the samples, but this did not change over 250 days and indicated a high level of stability for the protein at this storage temperature.

Activity of ABCB1 purified in the presence of glycerol following freeze drying

ABCB1 purified in buffer supplemented with glycerol was subjected to freeze drying and subsequent resuspension in water. The Michealis–Menten characteristics of drug stimulated ATPase activity were measured as shown in Fig. 2 panel A. V_{max} obtained for control samples not subjected to freeze drying was assigned a value of 100% and other values normalised accordingly. Following freeze drying in the presence of glycerol, the ATPase activity of resuspended ABCB1 was less than 5% of the control values, thereby indicating dramatic functional perturbation by the treatment.

Effects of trehalose and maltose on ABCB1 ATPase activity

Alternatively, the disaccharides trehalose and maltose were explored given the inability to recover ATPase activity following freeze drying in glycerol. Prior to embarking on purification of ABCB1 in the presence of trehalose and maltose required the demonstration that there were no major deleterious effects on function. Consequently, the ATPase activity of ABCB1 was determined in the presence of a range of trehalose/maltose concentrations. (Fig. 3) demonstrates the effects of these two disaccharides on the V_{max} of nicardipine stimulated ABCB1 activity, with the values normalised to that in the absence of added disaccharide. In both cases, addition of the disaccharides in the concentration range 10–30

mM produced 30–40% increases in the maximal ATPase activity. Higher doses were associated with a drop in this activity, which was more pronounced at lower concentrations of maltose. However, the maximal reduction in ATPase activity was in the range 20–25%, even at concentrations as high as 300 mM disaccharide. The data warrant further analysis of maltose and trehalose as lyoprotectants for ABCB1.

Purification of ABCB1 using trehalose or maltose

ABCB1 was purified in identical buffer systems to those described above with the exception of replacement of 20% (v/v) with an equivalent concentration of either trehalose or maltose at the point of elution from the column. (Fig. 4) shows the protein profile during a typical IMAC purification scheme for trehalose (A) and maltose (C) containing buffers. ABCB1 eluted at identical imidazole levels, regardless of the disaccharide in the buffers and the degree of purity was also unaffected. The gels in panels (B) and (D) represent quantitative analyses of the purified samples, using BSA as a densitometric standard. Using 50 mg of High-5 insect cell crude membranes gave ABCB1 yields of 115 ± 41 μg, 108 ± 29 μg or 116 ± 10 μg for buffers containing glycerol, trehalose or maltose, respectively. The basal ATPase activity of purified trehalose ($V_{max} = 0.52 \pm 0.14$ μmolPi/min/mg) or maltose (0.50 ± 0.14 μmol - Pi/min/mg) containing buffers was not significantly altered from the values obtained using a standard glycerol based procedure. Similarly, the extent of nicardipine stimulation of ATPase activity was unaffected by the presence of alternative disaccharides in the buffers (data not shown).

Freeze drying of ABCB1 proteoliposomes in trehalose or maltose buffers

ABCB1 containing proteoliposomes were freeze dried in trehalose or maltose based elution buffers at a chamber pressure of 0.011 mBar over a period of 72 h to a residual moisture content of $4.2 \pm 0.4\%$ ($n = 4$) and $4.8 \pm 0.3\%$ ($n = 4$) respectively. Longer drying times produced only a very small further decrease in residual moisture and the shorter drying time was deemed preferable for the efficiency of the cycle.

The freeze dried proteoliposomes were immediately rehydrated and ATPase activity determined to assess any functional perturbation. (Fig. 2) demonstrates that unlike glycerol, both maltose (panel B) and trehalose (panel C) afforded some degree of protection to the system as ATPase activity was retained on rehydration. Whilst less than 5% of the original activity was retained in glycerol, the recovery from freeze drying in a maltose based buffer system was 69%. However, the greatest degree of recovery was associated with trehalose, where V_{max} for ATP hydrolysis reached 83% of that before freeze drying. The data indicate that purification of ABCB1 in the presence maltose or trehalose renders it amenable to freeze drying, with significant recovery of activity upon rehydration.

Stability of ABCB1 following freeze drying in trehalose and maltose buffers

Freeze dried ABCB1 was stored at 37 °C, 20 °C and 4 °C for up to 150 days to ascertain long-term stability of the preparations. (Fig. 5) shows the ATPase activity of rehydrated ABCB1 following various storage times with either a maltose (panel A) or trehalose (panel B) based buffer systems. Maltose samples lost activity rapidly at all three temperatures with no activity remaining after 60 days. The decay in activity was temperature dependent as evidenced by the retention of 62% activity following 24 h incubation at 4 °C, whereas incubation at 37 °C was associated with only 24% recovery.

Samples freeze dried in trehalose also showed an initial loss of activity at all three temperatures tested. Samples tested after 60 days storage at 4 °C displayed $73 \pm 12\%$ ($n = 4$) of their pre-freeze drying ATPase activity whilst those at +20 °C displayed $64 \pm 14\%$ ($n = 4$) and those at 37 °C only $37 \pm 4\%$ ($n = 4$). Prolongation of storage to 150 days was associated

with further loss in ATPase activity, although the magnitude of reduction was considerably less. Significantly, the freeze dried ABCB1 retained considerable activity despite the incubation time. For example, samples stored at 4 °C retained $52 \pm 9\%$ ($n = 4$) activity and storage at a relatively high temperature of 37 °C, was associated with retention of $33 \pm 16\%$ ($n = 4$) ATPase activity.

Comparison of the storage data between trehalose and maltose reveals that maltose is considerably less effective at preserving ATPase activity in the dried state. ABCB1 purified in maltose not only retains less ATPase activity following freeze drying initially but activity also declines more rapidly than the trehalose equivalent at all temperatures tested. Whilst the V_{max} dropped to some extent over the time periods measured in both trehalose and maltose eluted samples the K_m values did not change. This suggests that whilst the amount of ATP hydrolysed over time decreased, the affinity of ABCB1 for ATP was not altered further reflecting the protein's stability.

DSC of stored freeze dried ABCB1 proteoliposomes

DSC carried out on samples immediately post-freeze drying revealed that both trehalose and maltose eluted P-gp proteoliposomes had very similar glass transition temperatures (T_gs) of ~28 °C for trehalose and ~29 °C for maltose. Whilst at first glance these are lower than many reported values it should be remembered that the system is not a simple one with additional salts and buffering molecules present. (Fig. 6) shows DSC performed on aqueous 20% (w/v) trehalose or maltose solutions following freeze drying gave T_gs of ~58 and ~48 °C, respectively. On addition of buffering molecules and salts, without P-gp proteoliposomes to the system the T_gs fell significantly so it appears the relatively low T_gs of the overall system can be attributed to the complex mixture of molecules. As described previously following the end of freeze drying the samples were sealed under vacuum and stored at 4 °C, 20 °C and 37 °C. DSC was performed periodically on the samples in order to investigate the effects of time and storage temperature on the T_gs. The data are summarised in Table 1. The T_gs of samples stored for 4 days were 26.3 ± 0.9 °C for trehalose samples and 27.0 ± 2.7 °C for maltose samples. These results suggest that the storage temperature had no consistent effect of the T_g of the system after 4 days storage. However, samples that were tested over extended periods of time displayed a further drop in the T_g, particularly in the trehalose material.

Further Karl Fischer analysis on long-term storage samples revealed that the residual moisture content had indeed risen to ~6% for both sugars, presumably plasticising the system with a consequent lowering of T_g. In addition to this macroscopic changes were visible and most marked in samples stored at 37 °C. The solid white cake structure observed immediately following freeze drying and in samples stored at the lower temperature disappeared and instead samples collapsed and became glassy and highly viscous with bubbles. This was true of both sugars at 37 °C but additionally in maltose there was discolouration with samples becoming yellow.

Discussion

The purified, reconstituted multidrug efflux pump ABCB1 displayed a temperature and time-dependent inactivation at storage temperatures between –20 and 20 °C. This inactivation could not be overcome with freeze drying despite the presence of the cryoprotectant additive glycerol. This lack of lyoprotection of glycerol was completely expected based on a review of previous studies in the literature and was merely carried out as a control to confirm the necessity to investigate other protective strategies. Therefore, the purification scheme for ABCB1 was altered to incorporate lyoprotectants; namely trehalose and maltose. Both of these disaccharides were associated with considerable recovery of

ABCB1 activity following freeze drying. However, long-term storage of freeze dried ABCB1 that had been obtained in maltose containing buffers was associated with progressive inactivation. In contrast, storage of ABCB1 isolated from trehalose containing buffers was considerably more successful. The recovery of activity displayed a dependence on storage temperature and considerable recovery of activity was possible even following storage at 37 °C for 150 days. Our investigation has therefore detailed a procedure for the production and long-term storage of purified, reconstituted ABCB1.

Purified proteins are often stored in the frozen state with the assumption that this environment provides structural stability and reduced activity of harmful proteases. The advent of therapeutic strategies that are protein based has seen lyophilisation (freeze drying) being employed as a means towards maintaining protein stability. A great deal of effort has been devoted to understanding the process of freezing and lyophilisation and moreover, their effects on protein stability [4,29,32,39]. The freezing process actually causes a number of stresses on proteins due to (i) increased solute concentration, (ii) increased solute viscosity, (iii) phase separation and (iv) large changes in local pH due to the changing solute environment. Any or all of these stresses may impact on protein structure and stability to produce marked perturbation of activity. Consequently, a number of additives have been used to maintain the stability of proteins during freezing; typically these include glycerol, ethylene glycols and dimethyl sulfoxide to name a few. There is still some confusion regarding the mechanisms underlying lyoprotection by these compounds, that is protection from both freezing and drying stresses. Whilst there are several hypotheses surrounding the sugar-mediated protection of biological molecules there is now by and large a consensus that no one mechanism acts independently of another and that the hypotheses are not mutually exclusive. These hypotheses are reviewed by Crowe [10]. One theory [5,33] suggests that they compensate for the loss of the hydration shell by forming hydrogen bonds to the exposed polar amino acid residues. The water entrapment theory proposes that rather than sugars replacing water molecules they in fact concentrate them near surfaces thus preserving the hydration of biomolecules [35]. Another theory suggests that the lyoprotectants form a vitrified environment around the protein to reduce molecular motion [18]. All these theories purport to stabilise protein activity by impairing the thermodynamics and/or kinetics associated with unfolding of protein structure.

Comparatively little information on freezing and lyophilisation of membrane proteins is available in the literature though work in the 1980s, discussed below, stimulated the interest in trehalose which led to it being believed to be superior to other disaccharides in protecting systems in freeze drying [12]. Isolated integral membrane proteins are likely to be embedded within a detergent micelle or reconstituted into artificial lipid membranes, thereby adding a further complexity to the protein–local environment interactions. We have previously demonstrated in the case of human ABCB1 that reconstitution into a lipid bilayer is associated with a considerable increase in ATPase activity of the protein [47]. Furthermore, there is a great deal of evidence indicating that the activity of ABCB1 shares a complex interaction with the lipid environment [25,27,37] The presence of a lipid environment will impact on conformational flexibility of the protein and thereby impact on the propensity to assume inactive configurations. Our data have demonstrated that the mere presence of a lipid environment is not sufficient to render the protein resistant to the harmful effects of freezing and lyophilisation. However, the inclusion of lyoprotectants, in particular trehalose, can greatly increase the protein viability.

Clearly, there is a need for future detailed studies on the lyopreservation of medically important proteins such as ABCB1. DSC was employed in the present investigation to determine the nature of the two disaccharide systems following freeze drying. Neither disaccharide displayed crystalline melting peaks indicating that they had formed amorphous

glasses. Samples of proteoliposomes containing ABCB1 obtained in either trehalose or maltose buffers were stored above and below their T_gs. Over time the T_gs of samples in both sugars fell due to the increase in moisture content. The vials however, remained sealed throughout the storage period so some explanation of the increased moisture is necessary. The similarity of the final moisture contents of both sugars gives some clue as to the origin of the moisture. It is likely that the increase in product moisture content can be attributed to the transfer of water from the stoppers used to seal the vials to the product [31]. The vitrification hypothesis alone [18] would suggest that those samples stored below their T_g retained more activity than those stored above. However, trehalose samples retained ATPase activity independent of whether the T_g was below or above the storage temperature. Maltose samples lost ATPase activity more rapidly than trehalose regardless of whether their storage temperature was above or below the measured T_g. Initially both sugars demonstrated the ability to preserve ATPase activity with trehalose only proving superior in the long term when conditions were far from optimal. It is tempting therefore to arrive at the conclusion that trehalose is a far superior lyoprotectant. Indeed early work with lobster sarcoplasmic reticulum membranes suggested that trehalose was uniquely efficacious in preserving membranes [12]. However, further work revealed that trehalose alone was being transported across the bilayer giving it access to both sides of the membrane. Having reviewed the early work the authors suggest that it is possible if other sugars had access to both sides of the membrane they would have been equally as effective as trehalose [15]. In the proteoliposomal system presented above both disaccharides had equal access to both sides of the bilayer and this may why both sugars met with initial success in preserving protein activity.

The 1–1 glycosidic bond of trehalose means it has no reactive hemi-acetal group and is a non-reducing sugar, whereas maltose is a reducing sugar. As such it seems possible that despite the reduced molecular mobility experienced in a sugar glass maltose was capable of reacting with the primary amino groups of exposed lysine or arginine residues on P-gp via the Maillard reaction, otherwise known as non-enzymatic browning. In a study investigating the protection of restriction enzyme EcoR1 during drying, maltose was found to be less effective than the non-reducing sucrose and there was no relationship between enzyme stability and T_g [36]. In addition to this the authors go on to say that samples stored above their T_g were either a dense paste or a very viscous syrup. During this investigation the samples stored at 37 °C, for both sugar systems, also exhibited the same macroscopic changes but maltose samples became took on a yellow/brown colouration. The accumulation of this color is positively correlated to the loss of protein activity [41] and the data showing rapid loss of activity at 37 °C in maltose in combination with the colouration of maltose sample supports the theory that the Maillard reaction was responsible.

It is likely then that under optimal conditions maltose may well have performed as effectively as trehalose in preserving ATPase activity. However, as demonstrated, under sub-optimal conditions trehalose has demonstrated again its ability to function as a more effective lyoprotectant. Over the last 25 years or so a large body of evidence has built surrounding this unusual property of trehalose and by and large investigators are agreed that no single mechanism is entirely responsible for lyoprotection yet trehalose is still preferred due its ability to function in less than ideal circumstances [10].

In conclusion, this investigation has provided a means to generate purified, reconstituted ABCB1 that retains activity following freeze drying. Moreover, the activity is retained following long-term storage at temperatures between 4 and 20 °C. This paves the way to generating a high throughput assay system for the investigation of ABCB1 pharmacology in order to develop a greater number of selective and potent inhibitors that will restore the efficacy of chemotherapy in cancer.

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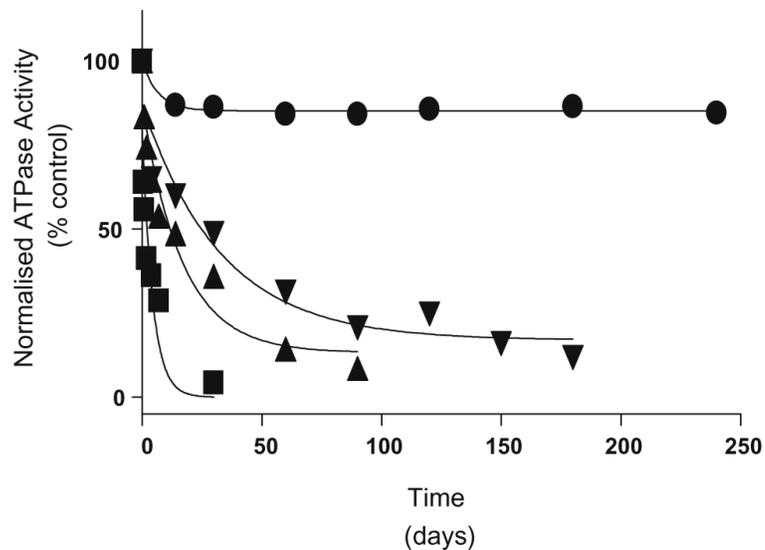
Fig. 1.
ATPase activity of ABCB1: effects of long-term storage. Purified and reconstituted ABCB1 was stored from 1 to 250 days at 20 °C (■), 4 °C (▲), −20 °C (▼) or −80 °C (●). Following incubation at the specific temperature, the ATPase activity was measured as a function of ATP concentration in the presence of nicardipine (30 μM). The maximal activity (V_{max}) was normalised to the value obtained prior to incubation (i.e. 100%) and plotted as a function of incubation time. Values represent the mean of triplicate measurements.
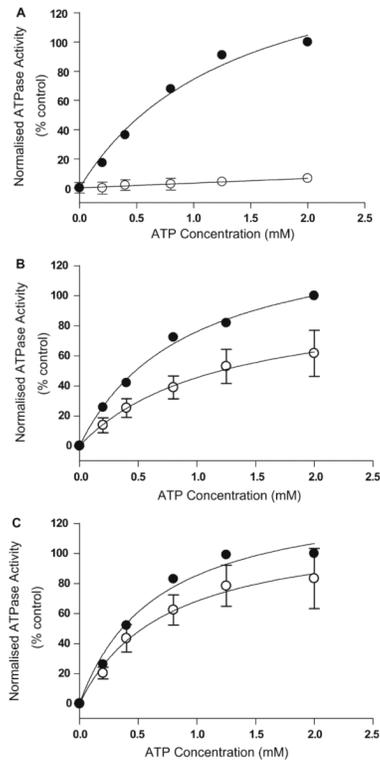
Fig. 2.
Effects of disaccharides on recovery of ABCB1 activity following freeze drying. ABCB1 was purified in buffer containing glycerol (A), maltose (B) or trehalose (C), and subjected to freeze drying. Lyophilised samples were rehydrated and the nicardipine stimulated ATPase activity measured as a function of nucleotide concentration. Closed circlesrefer to activity prior to freeze drying and open circles correspond to post-freeze drying. Nonlinear least-squares regression of the Michealis–Menten equation was fitted to each curve and the values represent the mean ± SE of four independent protein preparations.
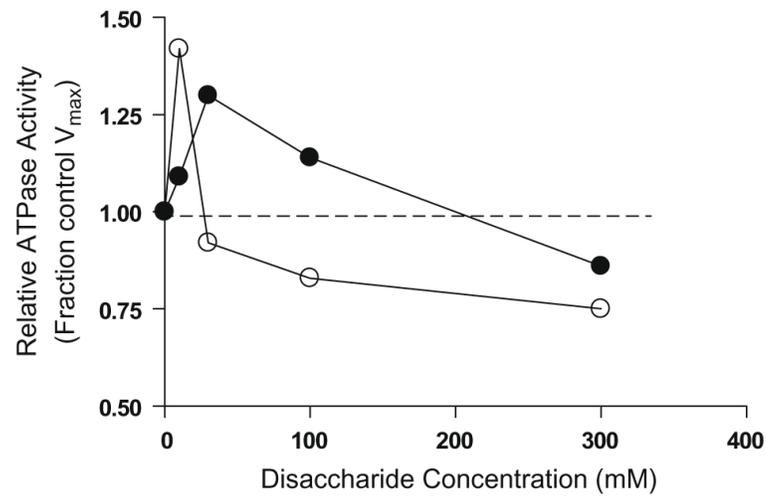
Fig. 3.
Effects of trehalose or maltose addition on ABCB1 ATPase activity. The ATPase activity of purified, reconstituted ABCB1 was measured in the presence of 2 mM ATP, 20 μM nicardipine and a range of maltose (○) or trehalose (●) concentrations. The maximal activity (V_{max}) was determined at each disaccharide concentration by nonlinear regression of the Michaelis–Menten equation. The V_{max} values were normalised to the activity obtained in the untreated control and plotted as a function of disaccharide concentration. The dotted line signifies untreated control activity.
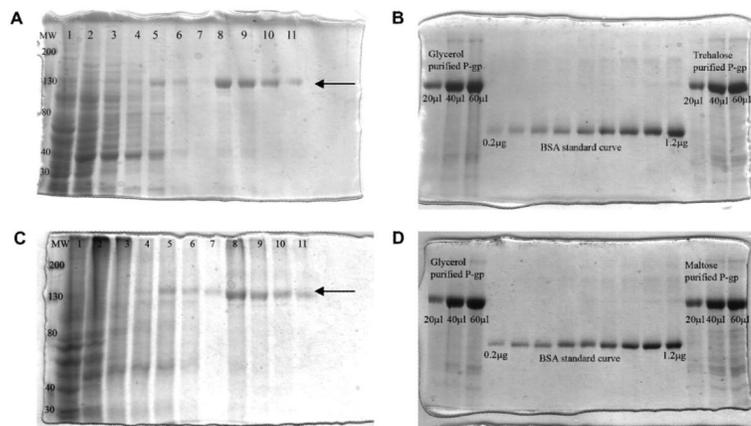
Fig. 4.
Purity and yield of ABCB1 obtained using alternative disaccharide buffers. The purification of His$_6$-tagged ABCB1 by IMAC was monitored using SDS-PAGE with Coomassie-Blue staining. Panel A contains ABCB1 eluted in buffer containing 20% (w/v) trehalose, whilst panel C shows ABCB1 eluted in 20% (w/v) maltose. Lane markings from left to right: MW - molecular weight markers, lane 1: unbound protein pH 8, lane 2: 10 mM imidazole pH 8, lane 3: 10 mM imidazole pH 8, lane 4: 20 mM imidazole pH 8, lane 5: 30 mM imidazole pH 8, lane 6: 2 mM imidazole pH 6.8, lane 7: 120 mM imidazole flow through pH 6.8, lanes 8–11: 120 mM imidazole pH 6.8. Arrow indicates position of ABCB1. SDS-PAGE analysis was used to compare the yield for ABCB1 purified in either trehalose (B) or maltose (D) with standard conditions using glycerol buffer. The central lanes contain a series of BSA containing samples (0.2–1.2 μg) used to generate a standard curve for densitometric analysis of protein content in the purified samples (20–60 μL).
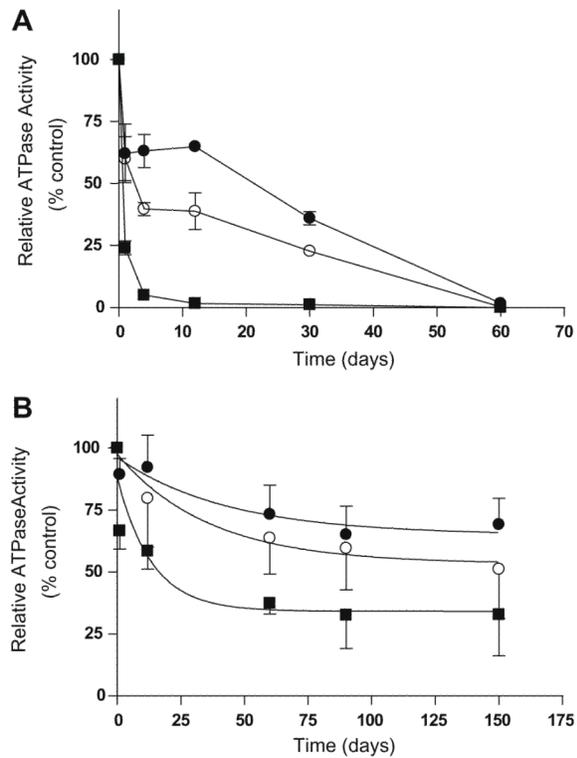
Fig. 5.
Stability of purified, reconstituted ABCB1 following freeze drying; effects of maltose and trehalose. ABCB1 was purified in buffers containing maltose (panel A) or trehalose (panel B) and following freeze drying the samples were stored at 4 °C (●), 20 °C (○) or 37 °C (■) for various times up to 150 days, after which the samples were rehydrated and the nicardipine stimulated ATPase activity. The graphs show the V_{max} values (mean ± SE) corresponding to each incubation period.
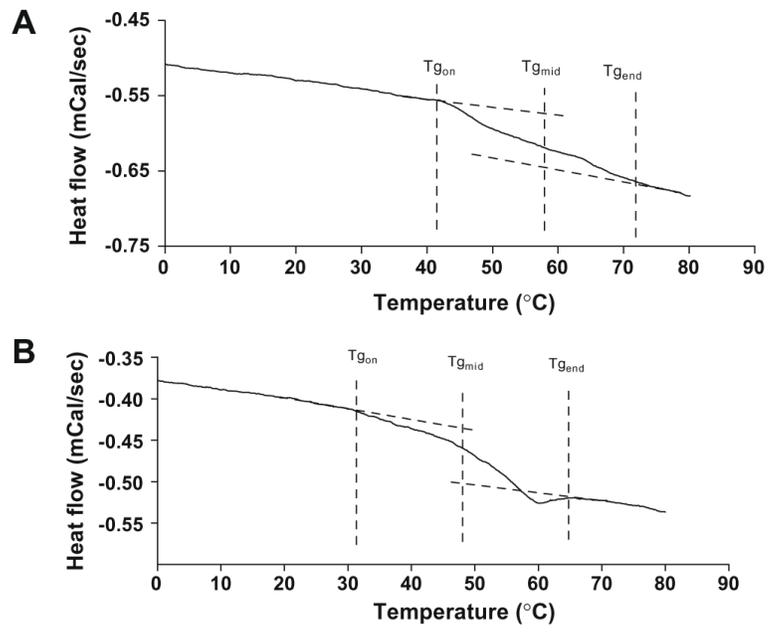
Fig. 6.
DSC of freeze dried 20% (w/v) trehalose in water (A) and 20% (w/v) maltose in water (B) displaying T_gs of ~58 and ~48 °C, respectively.

Table 1

Effects of storage conditions on the glass transition temperature. Comparison of mean T_g for freeze dried ABCB1 containing proteoliposomes obtained in buffers containing either trehalose or maltose and stored at different temperatures over time. Day 0 measurements were taken immediately following the end of freeze drying; as such it was considered all samples were at 20 °C. All values are the average obtained from two independent protein preparations

Incubation time (days)	Glass transition temperature (°C)						
	Trehalose			Maltose			
	4	20	37	4	20	37	
0	-	28	-	-	29	-	
4	26	28	25	31	28	22	
60	-	-	-	25	25	24	
90	13	14	11	-	-	-	
240	15	12	9	-	-	-	