Suppression of protein inactivation during freezing by minimizing pH changes using ionic cryoprotectants

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Freezing and lyophilization are often used for stabilization of biomolecules; however, this sometimes results in partial degradations and loss of biological function in these molecules. In this study we examined the effect of freezing-induced acidity changes on denaturation of the model enzyme haloalkane dehalogenase under various experimental conditions. The effective local pH of frozen solutions is shown to be the key causal factor in protein stability. To preserve the activity of frozen-thawed enzymes, acidity changes were prevented by the addition of an ionic cryoprotectant, a compound which counteracts pH changes during freezing due to selective incorporation of its ions into the ice. This approach resulted in complete recovery of enzyme activity after multiple freeze-thaw cycles. We propose the utilization of ionic cryoprotectants as a new and effective cryopreservation method in research laboratories as well as in industrial processes.

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1. Introduction

Although lyophilization is a key fundamental process in protein production, it still faces serious challenges in the freezing and drying process (Cicerone et al., 2015; Kasper et al., 2013; Kolhe et al., 2010b); some of the stresses that proteins undergo during lyophilization are unavoidable (low temperature, crystal formation, increased concentration, water removal), but changes in pH during freezing can be minimized to mitigate unnecessary degradation (Bhatnagar et al., 2007; Cicerone et al., 2015; Kasper and Friess, 2011). Up to now, no general approach has existed for regulating the pH of frozen solutions. If uncontrolled, the pH in the freeze-concentrated solution (FCS) in the veins surrounding ice crystals can be greatly altered compared to that of the original aqueous solution and this can detrimentally influence the other solutes present (Bronstheyn and Chernov, 1991; Heger et al., 2006; Reinsch et al., 2015; Takenaka et al., 2006; Vajda, 1999; Williams-Smith et al., 1977). Such pH changes can be of the utmost importance, not only in protein storage and production, but also in the fields of food conservation (Soltanizadeh et al., 2014), transfusion medicine (Asghar et al., 2014), and studies on the origin of self-replicating life (Attwater et al., 2013; Mutschler et al., 2015) as well as for bromine activation and ozone depletion events in natural ice (Abbatt et al., 2012; Bartels-Rausch et al., 2014; Pratt et al., 2013).

A constant pH is essential for the stability of many biomolecules. Buffers are typically used to maintain pH values in solutions; however, many buffers cease working in frozen solutions (Murase and Franks, 1989; Sundaramurthy et al., 2010b). The reason is that during freezing, ice forms essentially pure crystals, resulting in a concentration increased by several orders of magnitude in the FCS (Bartels-Rausch et al., 2014; Bogdan et al., 2014; Heger et al., 2005; Kania et al., 2014; Kasper and Friess, 2011; Krausko et al., 2015a,b, 2014). This FCS will eventually also solidify, depending on the temperature. Often, the solubility limits of the buffer are reached, causing crystallization of the less soluble component and thus pronounced acidity changes.

Another mechanism leading to changes in acidity during freezing of salt solutions is the difference in the distribution of ions between the FCS and the ice (Bronstheyn and Chernov, 1991).
One type of ion (either the cation or the anion) is preferentially incorporated into the ice lattice, whereas the excess of counterions accumulates in the FCS. The process is known as selective ion incorporation. As a result, an electrical potential called freezing potential (FP) develops between the FCS and the ice when a solution of an ionic compound is frozen (Bronshhteyn and Chernov, 1991; Lefebre, 1967; Lodge et al., 1956; Murphy, 1970; Wilson and Haymet, 2008; Workman and Reynolds, 1950). This charge imbalance is then neutralized by the diffusion of H⁺ or OH⁻ from the liquid into the ice. If the anions of the salt are incorporated into the ice more than the cations, the excess negative charge in the ice is neutralized by a flow of protons from the solution into the ice and the FCS becomes more basic. An example of such salt is NaCl (Bronshhteyn and Chernov, 1991; Heger et al., 2006; Sola and Corti, 1993; Takenaka et al., 2006; Workman and Reynolds, 1950). In contrast, greater incorporation of the cations into the ice leads to positively charged ice, and the consequent flow of OH⁻ from the solution to the ice results in acidification of the FCS if salts like NH₄Cl (Heger et al., 2006), Na₂SO₄ (Robinson et al., 2006), or (NH₄)₂SO₄ (Robinson et al., 2006) are used.

The desire to protect the compounds being lyophilized from degradation is achieved by combination of solution composition and freeze-drying procedure. Numerous factors contribute to the success of the lyophilization, e.g., choice of the freezing agents, buffers and stabilizers. Previously, the change of pH during freezing and lyophilization was monitored and then minimized by the proper choice of buffer, or its dilution by other compounds e.g. sugars. Despite invested effort, the problem of pH shift during the freezing has not been solved systematically till now and remains an empirical endeavor (Badawy and Hussain, 2007; Reinsch et al., 2015).

For the formulation processes the strongest most recommended method to avoid the pH jump is the right choice of buffer. It was well documented that partial crystallization of Na₃H₅PO₄·12H₂O results in sharp decrease by 3 units of pH during freezing of sodium phosphate (Na-P) buffer from initially neutral solution, whereas potassium phosphate (K-P) buffer showed much smaller pH shift (Murase and Franks, 1989; Sundaramurthi et al., 2010a; Van den Berg, 1959). TRIS buffer (2-Amino-2-hydroxyethyl-propane-1,3-diol) was observed to increase the pH by as much as 2 units at freezing (Kolhe et al., 2010a; Williams-Smith et al., 1977). Succinate buffer of initial pH 4 exhibited consequent pH increase to 8 followed be decrease to 2 at freezing (Sundaramurthi et al., 2010b). Sodium citrate and acetate, histidine hydrochloride and acetate accounts among the buffers not altering the pH considerably at freezing and are therefore most recommendable for these purposes (Izutsu et al., 2009; Jameel, 2010; Kolhe et al., 2010a). The temperature independent pH buffer was formed as a mixture of HEPES and K-P (Sieracki et al., 2008). This blend retains the pH close to 7 also after the freezing and down to 77 K as measured by absorption spectra changes of two pH indicator dyes. The protection ability of the buffer was shown on oxacillin and methemoglobin. Both of these degrade at 253 K in individual HEPES and K-P but nearly do not degrade in the blended buffer. For most of these buffers, the pH shift was observed also to depend on the initial solution pH and concentration of the buffer (Gomez et al., 2001).

The methods determining the apparent change of pH during freezing and lyophilization were reviewed (Badawy and Hussain, 2007); these are the slurry low-temperature electrode method, and the techniques employing molecular probes. The strengths and weaknesses of the methods were compared and possible problems of reciprocal validation were discussed. Less commonly applied approaches to assess the microenvironmental pH utilizes bare eye perception (Orii and Morita, 1977) or measurement of fluorescence (Fu et al., 2000; Wren and Donaldson, 2012), nuclear magnetic resonance (Robinson et al., 2006) or electron paramagnetic resonance (Williams-Smith et al., 1977) characteristics in frozen or lyophilized state.

The pH shift caused by buffer partial crystallization was often observed to be decreased by the presence of sugars added as cryoprotectant or bulking agents (Lu et al., 2009; Orii and Morita, 1977). The sugars are often added as bulking agents into the formulation anyhow, so they may have served as freezing pH shift eliminators tacitly. In this respect the role of bovine serum albumin, polyvinylpyrrolidone (Anchordoy and Carpenter, 1996) trehalose and inulin were also studied (Eriksson et al., 2003). The role of glycerol as a dilution medium of the salts was recognized already by Lovelock who proposed the use of it as a “salt buffer” to protect the red blood cells at freezing (Lovelock, 1953). The addition of polymers was also shown to influence the pH jump substantially; in one study of citrate buffer the polyvinylpyrrolidone was found to cause pH increase whereas the dextran pH decrease (Lu et al., 2009). The successful use (glycol) protein was employed on red blood cells cryopreservation (Deller et al., 2013).

Here we present a practical empirical approach to protein cryopreservation based on experimental estimation of the effective pH in the frozen state and consequent minimization of freezing-induced pH changes by addition of an appropriately chosen ionic compound to play the role of ionic cryoprotectant that minimizes the change in effective pH via the selective ion incorporation mechanism in such a way that it opposes the acidity change connected to the proteins and the excipients. This original approach leads to complete recovery of the activity of the model enzyme, haloalkane dehalogenase DbjA, after multiple freeze-thaw cycles. The close relationship observed between the freezing-induced pH change and the activity of the frozen-thawed enzyme provides a persuasive picture of the power and usefulness of this newly proposed cryopreservation strategy.

2. Methods

2.1. Model enzyme

Hydrolitic haloalkane dehalogenase DbjA (EC 3.8.1.5) from Bradyrhizobium japonicum USDA110 was used as the model enzyme in our experiments. It catalyzes hydrolytic cleavage of carbon-halogen bond in halogenated aliphatic hydrocarbons. DbjA has a broad pH optimum with maximum activity at pH 9.7, and retains more than 90% of the maximum activity in the pH range from 7.7 to 10.4. DbjA preserves both secondary and tertiary structure in the pH range 6.2–10.1. In acidic conditions, DbjA loses its tertiary structure at pH below 6.2 and it aggregates when pH is decreased below 5.3. Under alkaline conditions, the enzyme loses most of its tertiary structure at pH above 10.7. At the pH 10.3–11.5 DbjA occurs in disordered conformation and remains soluble (Chaloupkova et al., 2011).

2.2. Enzyme preparation

His-tagged DbjA was overexpressed in Escherichia coli BL21 using a previously described method (Sato et al., 2005) and purified using the Ni-NTA Superflow Cartridge (Qagen, Hilden, Germany). The enzyme was bound to the resin in equilibrating buffer (20 mM potassium phosphate buffer, pH 7.5, containing 0.5 M sodium chloride, and 10 mM imidazole). Unbound and weakly bound proteins were washed out with the buffer containing 10 mM imidazole. The target enzyme was eluted by a buffer containing 500 mM imidazole. The active fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.5). The enzyme was kept at 277 K during the purification procedure. Enzyme concentration was determined by the Bradford
assay (Sigma-Aldrich, St. Louis, USA) \cite{Bradford,1976}. Purity was checked by SDS-PAGE. The enzyme in 50 mM sodium phosphate buffer (pH 7.5) was prepared by dialysis from the purified enzyme solution. The enzyme was stored in 50 mM potassium or sodium phosphate buffer at 277 K until use.

2.3. Activity assay

Dehalogenation activity of DbjA was assayed as a rate of dehalogenation of 1,2-dibromoethane to 1-bromoethanol either by Iwasaki method \cite{Iwasaki,1952} or by GC analysis. In the Iwasaki method, bromides released from the substrate during enzymatic reaction were analyzed spectrophotometrically at 460 nm after the reaction with mercuric thiocyanate and ferric ammonium sulfate. Sunrise microplate reader (Tecan, Grödig/Salzburg, Austria) was used for the measurements. In case of addition of tetramethylammonium chloride (TMACl) to the solution of enzyme, Iwasaki method could not be used due to the interference of chlorides. Then a loss of the substrate in time was quantified by GC analysis (HP 6890, Hewlett-Packard). To perform a reaction, 10 µL of substrate 1,2-dibromoethane was injected into 10 ml of glycine buffer (100 mM, pH = 8.6) in 25 ml Reacti flask closed by Mininert valve. The reaction mixture was incubated in a water bath at 310 K. The dehalogenation reaction was initiated by addition of 0.2 ml of enzyme solution in the concentration of 0.1–0.2 mg/ml. The reaction was monitored by withdrawing 1 ml samples at 4, 8, 12, 16, and 20 min from the reaction mixture. The aliquots were immediately mixed with 35% nitric acid (for Iwasaki analysis) or with methanol (for GC analysis) to terminate the reaction. Each activity was measured in at least three independent replicates for both non-frozen and frozen-thawed samples. The ratio of specific activity of frozen-thawed sample to specific activity of non-frozen sample is denoted as recovery activity.

2.4. Freeze-thaw cycle

1 ml of DbjA enzyme solution in a concentration of 0.1–0.2 mg/ ml and initial pH of 7.5 was frozen in a 15 ml polypropylene tube immersed for 10 min into liquid nitrogen (77 K) or ethanol cooling bath with a desired temperature (typically 233 K). To achieve very slow cooling rates, 1 ml of the enzyme solution in a vial was frozen in a cryostat (Ostistat DN2, Oxford Instruments). The sample was precooled to 273 K and then the temperature of the sample was lowered by 0.5 K/min to 223 K. The temperature was monitored by a sensor located above the sample. In this case, ice nucleation temperature was also recorded; it ranged between 258 and 263 K. Afterwards, the frozen solutions were thawed in the water bath (295 K) for 15 min. The freeze-thaw cycles were repeated if a more pronounce effect of enzyme degradation was desired. If not stated otherwise, data from 3 freeze-thaw cycles are presented.

2.5. UV–vis measurements

The acidities of frozen solutions were estimated by measuring UV–vis spectra of the molecular probe cresol red (CR). CR belongs to the group of sulfonephthalein indicators that are known to work at frozen conditions and in a dry state \cite{Govindarajan,2006;Heger,2006}. In solution it exists in three different forms depending on pH: diprotonated (A, λmax = 516 nm), monoprotonated (B, λmax = 434 nm), and deprotonated (C, λmax = 573 nm). pkA and pkB of CR were reported to be 1.10 \cite{Dean,1992} and 8.15 \cite{Perrin,1983}, respectively. The three forms of CR are distinguishable also in frozen samples. At 77 K, the absorption maxima of all three forms are red-shifted to some extent and the absorption bands belonging to species A and C are split in two, probably due to restricted rotation along the partial double bond between the central carbon and the benzene rings \cite{Heger,2006}. CR in a final concentration of 2.5 × 10⁻⁶ M was added to the solutions and was used as an internal probe \cite{Heger,2006}. The samples were frozen directly in Plastibrand cuvettes with a path length of 1 cm. The UV–vis spectra were recorded immediately after removing the cuvettes from a cooling bath using Cary 5000 UV–vis-NIR spectrophotometer with integrating sphere DRA 2500 (both Agilent, Santa Clara, USA). Although the sample temperature was not controlled during the recording of absorption spectra, no changes in the spectra were observed within the time period which was necessary for the measurement. Each sample was measured in at least three independent replicates. Pure ice was used as a reference.

2.6. Hammet acidity functions H₀

Since the pH scale is used as a measure of acidity only for dilute aqueous solutions, Hammet acidity functions, H₀, were used for quantification of the acidity of frozen solutions. The extent of indicator protonation in a frozen solution with a given H₀ value is the same as that in a dilute liquid solution of the same pH. The UV–vis spectra of frozen CR solutions with initial pH of 11.6 and 12.1, respectively, were measured to get the spectra of pure forms A, B, and C in ice. Those were fitted into the spectra of frozen buffer solutions and the relative abundances (cA, cB, and cC) of forms A, B, and C were obtained. From these relative abundances the acidity functions H₀ were calculated using equations

\[H_0 = pK_A + \log \frac{c_B}{c_A},\]

for the dissociation A ⇌ B

\[\text{or}\]

\[H_0 = pK_C + \log \frac{c_B}{c_C},\]

for the dissociation B ⇌ C

\[H_0 = pK_A + \log \frac{c_C}{c_B},\]

for the dissociation C ⇌ B

Since the pKₐ values of CR frozen in ice are not known (and may differ from the values in liquid solution), pKₐ values of CR in solution were used for calculation of H₀ of frozen samples. Therefore, absolute H₀ values may differ from the pH values of partially frozen solutions measured with a low temperature electrode. However, H₀ values can be used for comparing the acidity of frozen solutions in relative terms \cite{Williams-Smith,1977}. Similar approach was used to evaluate H₀ of lyophilizes \cite{Govindarajan,2006}.

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded at room temperature (293 K) using a Chirascan spectropolarimeter (Applied Photophysics, Leatherhead, United Kingdom) equipped with a Peltier thermostat. Data were collected from 185 to 260 nm (in pure 50 mM phosphate buffer, pH 7.5) at a scan rate of 100 nm/min, 1 s response time and 2 nm bandwidth using a 0.1 cm quartz cuvette containing the enzyme. Each spectrum shown is the average of five individual scans and was corrected for absorbance caused by the buffer. CD data were expressed in terms of the mean residue ellipticity (θMRE) using the Eq. (1):

\[\theta_{\text{MRE}} = \frac{\theta_{\text{obs}} - M_w 100}{c \cdot l \cdot T},\]

(1)

where θobs is the observed ellipticity in degrees, M_w is the polypeptide molecular weight in g/mol, n is number of residues, c is the polypeptide concentration, l is the cell path length and the
factor 100 originates from the conversion of the molecular weight to mg/dmol. Secondary structure content was calculated from the spectra by using of CDSSTR (Compton and Johnson, 1986; Manavalan and Johnson, 1987; Sreerama et al., 2000), Selcon3 (Sreerama et al., 1999, 2000), K2D (Andrade et al., 1993) and CONTIN (Provencher and Glockner, 1981; Vanstokkum et al., 1990) methods implemented in DichroWeb server (Lobley et al., 2002; Whitmore and Wallace, 2004, 2008).

2.8. Freezing potential measurements

Freezing potential was measured with an apparatus described in a published paper (Roubal et al., 2011). A sample was put into a glass tube (height 20 cm, internal diameter 3 mm) with two platinum wire electrodes placed inside the tube. One electrode was sealed through the bottom of the tube, the other was placed in an upper part of the tube. The tube was cooled from the bottom and the voltage between the upper electrode (in contact with a liquid sample) and bottom electrode (in contact with ice) was measured. Solutions of NaCl, NH₄Cl, MgCl₂, TMACI in a concentration range from 10⁻³ M to 10⁻⁷ M were used as the samples.

3. Results

3.1. Effect of freezing rate and buffer type on DbjA activity

Samples of DbjA were exposed to freeze-thaw stress under various conditions to study the impact of freezing-induced acidity changes on denaturation of this model enzyme. Three freezing methods were used: fast freezing in liquid nitrogen (77 K) and slower freezing in an ethanol cooling bath (233 K) and very slow controlled freezing in cryostat (0.5 K/min). Under all conditions examined, the loss of activity from DbjA fast frozen at 77 K was significantly higher than that from samples frozen at 233 K or higher temperatures (Fig. 1a and S1, S4). Additional cooling of the samples frozen at 233–77 K and subsequent holding in the frozen state had no effect on the recovery of DbjA activity (Figs. S1 and S2). There is thus compelling evidence that the enzyme degradation occurs during the freezing process. DbjA denaturation was irreversible — the enzyme did not regain its activity within 24 h after thawing when incubated at 277 K (Fig. S3). Throughout the experiments, several batches of DbjA enzyme were used. Although the specific and recovered activities of DbjA were consistent within

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**Fig. 1.** Effect of freezing rate and buffer type on activity of DbjA. a. Recovered activities and preserved α-helical contents of DbjA frozen in 50 mM Na-P or K-P at 77 K and at 233 K. Preserved α-helical content is the ratio of α-helical content in frozen-thawed sample to that in non-frozen sample, calculated from the CD spectra presented in panels c and d. Error bars represent standard deviations of recovered activities from 6, 6, 9, and 6 replicates, respectively. Two-way ANOVA revealed the effects of temperature (p < 0.001) and buffer (p = 0.001) on the recovered activity. b, UV–vis spectra of cresol red (CR) in 50 mM Na-P and K-P, with an initial pH of 7.5. The grey spectrum represents liquid solutions of buffers before freezing; red and orange spectra represent frozen solutions of Na-P and K-P, respectively, at 77 K. Peaks belonging to forms A, B, and C of CR are indicated. c and d. Comparison of CD spectra of DbjA in 50 mM K-P (c) or 50 mM Na-P (d) before freezing and after 5 freeze-thaw cycles at 77 K and at 233 K. A decrease in the magnitude of negative peaks at 208 and 222 nm indicates loss of α-helical conformation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
a batch, they differed in absolute values among various batches of the enzyme. The reproducibility of the results reported throughout the paper is expressed by the standard deviations being indicated in the Figures by error bars.

In the experiments, two types of phosphate buffer were used – sodium phosphate (Na-P) and potassium phosphate (K-P). They are similar in composition in the liquid state, but differ substantially when frozen. Na-P acidifies extensively during freezing due to precipitation of Na₂HPO₄, while there is only a minor change in acidity during freezing of K-P (Murase and Franks, 1989). Thus this pair of buffers makes it possible to evaluate only the effect of pH change, with other factors being very similar. We observed a clear relationship between acidity function $H_0$ of the frozen samples and recovery of DbjA activity (Fig. 2). DbjA frozen in 50 mM K-P buffer ($H_0 = 6.6$; recovery $(66 ± 6\%)$) exhibits greater recovery of activity than DbjA frozen in 50 mM Na-P ($H_0 = 1.4$; recovery $(16 ± 9\%)$). As DbjA in solutions loses its tertiary structure at pH values below 6.2 and aggregates when the pH is decreased to below 5.3 (Chaloupkova et al., 2011), an increase in the acidity of the FCS is therefore expected to denature the enzyme. Lowering the Na-P concentration to 1 mM resulted in both smaller acidity increases during freezing and much better recovery of activity after thawing ($H_0 = 6.1$; recovery $(36 ± 5\%)$). This effect was even more pronounced when the enzyme was dialyzed against pure water with an initial pH of 7.5 adjusted using NaOH ($H_0 = 6.8$; recovery $(61 ± 8\%)$) as shown in Fig. 2. All the data obtained in all our experiments show a relationship between the loss in enzymatic activity after freezing and thawing and the change in acidity during freezing.

To investigate the relationship between inactivation and structural changes in DbjA upon freezing, CD spectra of the enzyme in 50 mM Na-P and K-P buffers were measured before and after multiple freeze-thaw cycles at 233 and 77 K (Fig. 2). Far-UV CD spectra of the native enzyme, measured in both buffers before freezing, showed one positive peak at 195 nm and two negative features at 208 and 222 nm, characteristic of an $\alpha$-helix (Fasman, 1989). The $\alpha$-helical content of the native enzyme estimated from measured spectra was about 25%. Inspection of CD spectra recorded after subjecting DbjA in both buffers to multiple freeze-thaw cycles revealed changes in intensity in comparison with the CD spectra recorded under native conditions. Structural changes in DbjA caused by multiple freeze-thaw cycles at 77 K were significantly more pronounced than those at 233 K. The estimated content of $\alpha$-helical structure preserved in DbjA after 5 freeze-thaw cycles is displayed in Fig. 1a. The observed conformational changes in the enzyme are in good agreement with the inactivation of DbjA upon multiple freeze-thaw cycles at 233 and 77 K (Fig. 1a).

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**Fig. 2.** Effect of acidity of FCS on recovery of DbjA activity. a. Recovery of DbjA activities in various buffer solutions with an initial pH of 7.5 after 3 freeze-thawing cycles at 77 K, and corresponding $H_0$ values for frozen buffer solutions at 77 K. Tukey test revealed significant differences ($p < 0.001$) between all the means except 50 mM K-P and water. Error bars represent standard deviations of recovery activities from 9, 12, 6, and 6 replicates, respectively. b. UV–vis spectra of CR in frozen solutions corresponding to those in panel a. The grey spectrum represents liquid solutions of buffers before freezing. Peaks belonging to forms A, B, and C of CR are indicated. c. Effect of adding TMACl on recovery of DbjA activity after 3 freeze-thaw cycles at 77 K. Recovered activities of DbjA in 50 mM phosphate buffers are significantly lower than recovered activities of DbjA in 50 mM phosphate buffers with the addition of 0.1 M TMACl, frozen–thawed under otherwise identical conditions ($p < 0.001$). d. UV–vis spectra of CR in frozen solutions (77 K) of 0.1 M TMACl, 50 mM Na-P with 0.1 M TMACl, and 50 mM K-P with 0.1 M TMACl. The initial pH of all the solutions was 7.5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.2. Acidity changes caused by freezing of salts

A series of common salts were examined for freezing-induced acidity changes associated with selective ion incorporation (Figs. 3b). These experiments were undertaken to probe the effect of the salt alone and therefore the solutions used in this experiments contained only the studied salts and CR as the pH indicator. Initial pH of the solutions was 5.0 or 6.0 as indicated in Fig. 3b. Since the pKₐ values of CR in ice are not known, calculated H₀ values may be offset from absolute pH values measured with low temperature electrode. Therefore the resulting UV–vis spectra and H₀ values of the frozen samples are compared to the spectra and H₀ of the frozen CR solution at the same conditions. In general, salts with bulky anions, e.g. sulfates, increased the acidity of the frozen solution, while halides increased the basicity of the frozen solution (Fig. 3b). This correlates with the results of FP measurements, where relatively small anions (e.g. halides) were usually incorporated better into the ice lattice than almost any cation, while relatively large anions (nitrate, acetate) were not usually incorporated into the ice (Workman and Reynolds, 1950). Ammonium salts do not follow this trend, since NH₄⁺ is incorporated into the ice to a large extent, more than, for example, Cl⁻, thus making the FCS acidic (Workman and Reynolds, 1950). A very high level of basification was observed when TMACI was frozen. This is not surprising, since the bulky tetramethylammonium cation would be expelled from the ice more than the chloride anion. The extent of this basification was dependent on the concentration of TMACI, with a higher concentration of TMACI resulting in a more basic frozen solution: solutions of 0.001 M, 0.01 M and 0.1 M TMACI, all with an initial pH of 5.0, resulted in H₀ values of 7.5, 7.9 and 8.1, respectively, when the solution was frozen (Fig. S5).

3.3. Neutralization of freezing-induced acidity changes by addition of ionic cryoprotectant

We have proposed and investigated a new method of neutralizing buffer-induced acidity changes by the addition of a carefully chosen salt in the role of ionic cryoprotectant; this does not change pH of a non-frozen solution, but during freezing it causes an acidity change opposite to that resulting from the buffer crystallization, so that the overall change in acidity is negligible. To counterbalance buffer-induced acidification by the mechanism of crystallization of individual buffer component, an ionic cryoprotectant that causes the basification of the FCS must be applied. For this purpose, TMACI at various concentrations was added to the 50 mM Na-P solution and the acidity of the frozen mixed solution was monitored. The addition of TMACI even at low concentrations resulted in reduced acidification during freezing in comparison with that undergone by the buffer solution alone. Almost complete inhibition of freezing-induced acidification was observed at a TMACI concentration of 0.1 M (Fig. S6). This concentration of TMACI had no negative effect on the specific activity of non-frozen enzyme (Fig. S7).

The effect of adding TMACI on the recovery of DbjA activity after freezing and thawing was positive over the whole range of tested concentrations (from 0.01 M to 1 M). Recovered activities of DbjA after freezing and thawing in the presence of TMACI were higher than those in the buffer alone. When slow freezing (0.5 K/min) was applied, the recovered activity of DbjA enzyme in 50 mM Na-P

Fig. 3. Selective ion incorporation. a. Scheme showing selective ion incorporation into ice and consequent charge neutralization leading to acidity change. Top—non-frozen solution, middle—onset of freezing, bottom—neutralization of charge imbalance. Left-hand column: During freezing of NH₄Cl solution, ammonium cations are preferentially incorporated into the ice leaving an excess of negatively charged chloride anions in the freeze-concentrated solution (FCS). A negative freezing potential (FP) can be measured. Subsequent neutralization of the charge imbalance results in acidification of the FCS. Right-hand column: The opposite situation applies during freezing of TMACI solution, where chloride anions are incorporated into the ice to a greater extent than bulky tetramethylammonium cations. b. Effects of various salts on the FP and acidities (H₀) of frozen solutions. Signs (+ or −) of the FP were taken from the literature (Workman and Reynolds, 1950) or measured. FPs are expressed as potentials of the FCS with respect to ice. A negative FP is associated with acidification of the FCS, while a positive FP is associated with basification of the FCS. H₀ values were calculated from UV–vis spectra of CR in frozen solutions (77 K) of 0.01 M salts with an initial pH of 5.0 or 6.0.
buffer without addition of TMACl was (43 ± 16)%. If 0.1 M TMACl was added to the solution to counterbalance the pH shift on freezing, the recovered activity of DbjA was increased to (112 ± 27)%. To demonstrate the protective effect of the ionic cryoprotectant more clearly, fast freezing at 77 K was used, as this method of freezing caused more damage to the unprotected enzyme than slower rates of freezing. The effect of adding 0.1 M TMACl to 50 mM phosphate buffers on the recovered activity of DbjA after 3 freeze-thaw cycles is shown in Fig. 2c. The recovered activity of DbjA in 50 mM Na-P buffer alone was (16 ± 9)%. The addition of 0.1 M TMACl prevented the loss of activity of DbjA in phosphate buffers during freezing and thawing; DbjA activity was completely recovered to (105 ± 24)% Similarly, the recovered activity of DbjA in 50 mM K-P increased from (66 ± 6)% to (106 ± 14)% when 0.1 M TMACl was added to the solution prior to freezing.

4. Discussion

We demonstrate a strong link between losses in the activity of the model enzyme DbjA and an increase in the acidity of FCS under various experimental conditions. The enzymatic activity loss, which was irreversible, was accompanied by changes in the structure of the enzyme, as confirmed by CD spectroscopy. When the enzyme was frozen in Na-P buffer, which acidifies more than K-P during freezing, there was a greater loss of the activity in comparison with the enzyme frozen in K-P buffer under otherwise identical conditions. Lowering of the Na-P concentration resulted in less change in acidity during freezing and also led to greater recovery of enzyme activity. These findings support those of previous research in which minimization of protein losses during freezing was found to be linked to minimizing acidity changes in the FCS by making an optimal choice of buffer salts and using them at a low concentration (Bhatnagar et al., 2007; Jiang and NaiL, 1998; Kasper and Friess, 2011; Sieracki et al., 2008). Lower freezing rates resulted in lower losses of DbjA activity in comparison with higher freezing rates when the enzyme was frozen in K-P, in Na-P and also in water without any buffer. This is in agreement with previous studies in which lower losses of enzyme activities were observed during slow freezing (Bhatnagar et al., 2008; Cao et al., 2003; Chang et al., 1996; Chen and Cui, 2006; Jiang and NaiL, 1998; Strambini and Gabellieri, 1996). However, there are other studies that show the opposite trend (Pikal-Cleland et al., 2000; Wei et al., 2012). We believe that this contradiction is caused by two mutually counteracting phenomena that are expected to occur during slow freezing: a) formation of larger ice crystals, hence a smaller ice surface area, and b) more extensive precipitation of the less soluble buffer component, hence a greater acidity change (Williams-Smith et al., 1977). Smaller ice surface area results in an increase enzyme recovery after freezing and thawing since the contact of protein with the ice surface is thought to be responsible for surface-induced denaturation (Jameel, 2010), while acidity changes cause protein degradation.

Here we validated a new approach to minimize the acidity change in the FCS by addition of the ionic cryoprotectant that affects the acidity of the frozen sample in a manner opposite to that of the protein and excipients. The increase in acidity related to sequential crystallization of Na-P was canceled out by the addition of TMACl, which turns the FCS more basic during freezing due to selective ion incorporation. Addition of 0.1 M TMACl to 50 mM Na-P led to complete recovery of DbjA activity after 3 freeze-thaw cycles when both very slow freezing (0.5 K/min) and very fast freezing (77 K) were applied. Since all the other conditions (the rate of freezing, concentration of the enzyme and the buffer etc.) are preserved for freezing of the samples with and without addition of TMACl to the solutions, the excessive degradation of the enzyme frozen in the buffer alone can be assigned mostly to the pH shift. All the other effects (ice crystal formation, self-buffering capacity of protein etc.) are not supposed to be greatly affected by the presence of TMACl and therefore their impact on the extent of degradation of the enzyme during freezing should be approximately the same in the samples with and without the addition of TMACl.

TMACl was proved to be a useful ionic cryoprotectant for DbjA enzyme during freezing in phosphate buffers due to increase of $H_0$ of the frozen solution and eliminator of buffer-induced acidification. Similar effect can be expected when other salts will be used. To prevent unwanted acidification, salts with the anion incorporating more into the ice than the cation (generating positive PF) should be used as the ionic cryoprotectants. On the other hand, salts with the cation incorporating more into the ice than the anion (generating negative PF) should be used as the ionic cryoprotectants to prevent unwanted basification. Examples of such salts are given in Fig. 3. It should be emphasized, that any salts present in the solution to be frozen, for example those used to adjust ionic strength, may substantially change the resulting acidity of frozen samples. Since pH change related to buffer crystallization, and also to selective ion incorporation, is dependent on the rate of freezing, the initial pH, type and concentration of the buffer/salt used, the amount of the ionic cryoprotectant which is necessary for neutralization of the buffer-induced pH change is dependent on the experimental conditions during the process of freezing. The resulting acidity of the frozen solution should be monitored and compared to the pH optimum of the protein, because the addition of the ionic cryoprotectant to the buffer solution may affect the solubility of the individual buffer substances during freezing (Larsen, 1973) and cause undesirable effects.

Previously, the freezing-induced pH change, if recognized as a problematic factor, was empirically minimized either by the choice of different buffer or by its dilution by the bulking agent (Badawy and Hussain, 2007; Lu et al., 2009). On the other hand, our newly proposed method of applying ionic cryoprotectant is based on the understanding of physicochemical phenomenon of selective ion incorporation. Many studies of microenvironmental pH conclude, that the freezing or lyophilization optimization deserve a priori pH test since there are little rules available to predict the pH behavior in the microenvironment of frozen active pharmaceutical compound (Badawy and Hussain, 2007). The method of applying ionic cryoprotectants may give to the researches one of the tools that can help in the design of successful freezing protocols.

Conjunction of already established methods and our newly proposed ionic cryoprotective method may bring some practical recommendations and new possibilities for the formulation protocols. Among the common features of all cryoprotection methods is the choice of the buffers known to exhibit a small pH shift upon freezing and applying the buffer in the smallest possible concentration that suffice the solution buffering capacity since high buffer concentrations at freezing can become problematic. The distinct element of proposed ionic cryoprotection method from previous methods is the possibility to neutralize the remaining pH shifts by justified choice of ionic compounds. The choice can be made in a way that the FCS would have properties desirable for future workup of the frozen or lyophilized formulation (e.g., keeping high glass transition temperature). The use of ionic cryoprotectants seems to be practical for most of the freezing cycles as it proved operational for extremely fast (immersion into the liquid nitrogen) as well as very slow (0.5 K/min) freezing methods. It should be stressed out, that our proposed approach needs to be tested and verified for each particular case of cryoprotectant and its possible industrial application. The cooperative effect of the ionic cryoprotectant and the bulking agent (e.g., saccharides) is a matter of our further research; we expect further
protein stabilization in comparison to saccharides alone (Lu et al., 2009). Also the effect of added salts to glass transition temperature should be considered.

In this work, we intentionally applied the harshest freezing conditions (fast freezing and extensively crystallizing buffer) to demonstrate that even these conditions can be rendered benign by an appropriate choice of the ionic cryoprotectant. Thus the availability of fast freezing methods, which previously caused extensive protein degradation, may now become advantageous, because under these conditions protein aggregation can be minimized and dissolution of a protein in its active form facilitated. We believe that a properly designed approach to freezing using ionic cryoprotectants will pave the way to more efficient storage of proteins both in research laboratories and in industry.

Conflict of interest

Authors declare they have no conflict of interest.

Author's contributions

D.H. devised the idea for the project and directed the research. J. D. and R.C. provided the enzyme, methodology and facilities for activity measurements. I.K. conducted and coordinated the experimental work, analyzed the data and wrote the initial draft of the manuscript. J.P., L. F. and M.K. conducted parts of the experimental work and analyzed the data. R.C. and M.K. measured and R.C. analyzed the CD spectra. S.M. evaluated the data statistically. All authors contributed to discussions and writing of the final paper.

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Appendix A. Supplementary data

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References


