

Effects of freezing and the cryoprotectant lactobionic acid in the structure of GlnK protein evaluated by circular dichroism (CD) and isothermal titration calorimetry (ITC)

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Abstract Freezing is a widely applied method in food preservation. The technique has negative effects on sensory and textural properties of some foods. In this study the effects of the freeze–thaw process and lactobionic acid (LBA) as a cryoprotectant on GlnK protein solution were evaluated by circular dichroism (CD) analysis and isothermal titration calorimetry (ITC). The freeze–thaw cycles caused changes in GlnK conformation and interactions with small ligands (adenosine triphosphate, ATP). CD assay demonstrated changes in the molar ellipticity values of the samples subjected to freezing, indicating conformational changes to the GlnK protein. Additionally, ITC analysis indicated that the freeze–thaw process caused changes in the interaction properties of GlnK with its ligand ATP. LBA cryoprotectant activity was also evaluated and with both of the techniques it was demonstrated that the compound prevented the damage caused by the freeze–thaw process, thereby maintaining the characteristics of the samples.

Keywords Protein solutions · Freezing cycles · Molecular interaction · Thermodynamics

Introduction

Freezing is one of the most frequently used techniques for preserving the chemical and microbiological stability of food products. Although the process is widely employed, it still causes some negative effects in some foods and results in a loss of quality in these products. These negative effects include the degradation of proteins and changes in the texture and taste in some foods (Rawson et al. 2012; Kaale and Eikevik 2013).

In order to understand these changes and search for solutions to minimize the damages, many studies have been carried out to evaluate the effects of freezing on proteins. García-Arias et al. (2003) showed that the freeze–thaw process results in a decrease in the protein content of sardine fillets. Compared with fresh samples, the thawed ones presented a decrease in the number of SH groups, characteristic of cysteine protein. Herrera et al. (2001) showed by using DSC that the actin and myosin proteins from “blue whiting” fish exhibited thermal denaturation in conditions of freezing and storage. Wang et al. (2014a, b) reported a decrease in the water sorption capacity in gluten samples as a result of the freezing process. The results suggested an increase in the hydrophobic site exposure in proteins caused by the freezing process. With regard to the physicochemical properties of these proteins, a depolymerization of glutenin caused by the deterioration of gluten and glutenin in the freezing and storage processes was observed (Wang et al. 2014a, b).

To improve the freezing technique, some cryoprotectant substances can be used to prevent the protein denaturation of biological tissue from freezing damage (Hubálek 2003; Ariyaprakai and Tananuwong 2015). This effect can be achieved in different ways, depending on the mechanism of action of the cryoprotectant. These mechanisms include

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freezing rate reduction (Alvarez et al. 2010), modification in the ice crystal structure (Furukawa et al. 2005) and a decrease in the solution's freezing point (Iijima 1998).

There are several studies at low temperatures using cryoprotectants to prevent freezing damage. Maltodextrins were added to different types of frozen fish, and the compound promoted less denaturation in the samples through the inhibition of formaldehyde formation (Herrera et al. 2001). Glycerol is a compound with cryoprotectant properties that is widely employed in microbiology. It is one of the most widely used substances in the field for reducing freezing damage (Hubálek 2003). Iijima (1998) presented the effect of glycerol as a cryoprotectant in the preservation of frozen blood cells. Samples with the glycerol cryoprotectant in concentrations above 55% were completely free of ice crystals. Recently, antioxidant components of grape were used to increase the shelf life of Indian mackerel in iced conditions and the results presented these components as a promising natural preservative (Sofi et al. 2016).

One food additive that has attracted attention in the freezing area is lactobionic acid (LBA). There are reports of its beneficial effect when employed in the preservation of transplanted organs at low temperatures (Isaacson et al. 1989; Charloux et al. 1995), in the reduction of hypothermically induced cell swelling (Shepherd et al. 1993) and as an additive to meat products (Nielsen 2009). LBA is a compound obtained from chemical or microbial oxidation of lactose. It has created great interest among the food, pharmaceutical, chemical and cosmetics industries due to its functional properties (Gutiérrez et al. 2012). The additive is approved by the US FDA (Food and Drug Administration) (FDA 2014) and has chelating, moisturizing and antioxidant properties (Isaacson et al. 1989; Green et al. 2009; Tasic-Kostov et al. 2012).

GlnK from *Herbaspirillum seropedicae* is a protein belonging to the PII family. This family has an important biological role in the control of the nitrogen metabolism and is widely distributed in nature (Arcondéguy et al. 2001). In this sense, studies about the structure of GlnK and its interactions are important in understanding the protein's behavior and its reaction mechanism (Xu et al. 1998; Bonatto et al. 2012). Moreover, GlnK has been studied in this research group; it can be overexpressed, easily purified and is relatively stable, so it is suitable for use as a protein model in this study.

Isothermal titration calorimetry (ITC) is a technique that measures the thermodynamic parameters involved in the interactions between molecules (Perozzo et al. 2004). The method is versatile and can be applied to many systems and conditions. Examples include GlnK and ATP binding in the presence of 2-oxoglutarate (2-OG) (Radchenko et al. 2010), the effect of pH and the conformation in the complexation of bovine α -lactalbumin and oleic acid (Park et al. 2015), and the

toxicity of the sarafloxacin compound to the catalase enzyme (Cao et al. 2013). In light of its potential, the technique deserves to be explored in the field of the freezing process.

Circular dichroism (CD) is a spectroscopic technique that evaluates the conformation and stability of proteins in different temperatures, with different ionic strength and in the presence of small molecules (Corrêa and Ramos 2009). The method can be used to evaluate the frozen storage effect on proteins in accordance with Wang et al. (2014a); however, up to now, no studies have been found regarding GlnK protein evaluated by this technique.

The aim of this study was to evaluate the effects of the freeze–thaw process and the LBA cryoprotectant effect on the structure of the GlnK protein using the combined techniques of ITC and CD.

Materials and methods

Materials

Lactobionic acid 97% (LBA) (PubChem CID: 7314), adenosine triphosphate 99% (ATP) (PubChem CID: 5957) and tris(hydroxymethyl)aminomethane (tris) >99.8% (PubChem CID: 6503) were purchased from Sigma-Aldrich and used without further purification. Ultrapure water, produced by Merck Millipore ultrapure water systems (Millipore Corp., Redford, MA, USA), was used throughout the entire experiment and all other chemicals and reagents were analytical grade unless stated otherwise.

Purification of GlnK from *Herbaspirillum seropedicae*

Herbaspirillum seropedicae GlnK was expressed in the native form of the plasmid pEMB200 containing the GlnK gene into pET29a + vector. Protein expression was induced in the empty cell of *Escherichia coli* BL21. After induction, the proteins were purified on a fast protein liquid chromatography (FPLC) AKTA system (GE Healthcare) using a Hi-Trap Heparin column (GE Healthcare) (Bonatto et al. 2007). Twenty protein fractions were obtained and eluted with an increasing NaCl gradient (50–1000 mmol L⁻¹). After purification, the proteins were dialyzed against buffer (50 mmol L⁻¹ Tris pH 8.0; 100 mmol L⁻¹ NaCl; 5 mmol L⁻¹ MgCl₂) and stored at 5 °C.

GlnK protein characterization

Electrophoresis analysis

The protein fractions' purity was evaluated by electrophoresis technique. The procedure was performed in a

denaturing polyacrylamide gel according to the protocol described by Laemmli (1970). For all the protein electrophoresis essays the gel separator concentration was 12% (w/v) and the stacking gel concentration was 4%. Electrophoresis assays were performed in a vertical system according to the manufacturer's instructions (BioRad). Samples were mixed with the sample buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 0.0625 mol L⁻¹ Tris-HCl pH 6.8, 5% β-mercaptoethanol) and boiled before application. The buffer used was Laemmli (3 g L⁻¹ Tris base, 14 g L⁻¹ of glycine and 1 g L⁻¹ SDS) and the device voltage was 200 V. After electrophoresis, the proteins from the gel were stained with Coomassie blue stain R-250 and destained with a 50% (v/v) methanol and 10% (v/v) acetic acid solution.

Determination of the protein concentration (spectrophotometrically)

The protein quantifications were performed by spectrophotometry (UV) at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The buffer solution in which the protein was dialyzed was used as the reference for the spectrophotometer. For the protein concentration determination the molar weight and the molar extinction coefficient parameters employed were estimated by the online software ProtParam (Gasteiger et al. 2005).

Freezing assays

Sample preparation

GlnK samples at 0.1 mmol L⁻¹ were dialyzed overnight at 5 °C against a buffer solution containing Tris-Cl 50 mmol L⁻¹ pH 8.0, NaCl 100 mmol L⁻¹ and MgCl₂ 5 mmol L⁻¹. GlnK samples with LBA cryoprotector were made with GlnK samples at 0.1 mmol L⁻¹ that were dialyzed overnight at 5 °C against a buffer solution containing Tris-Cl 50 mmol L⁻¹ pH 8.0, NaCl 100 mmol L⁻¹, MgCl₂ 5 mmol L⁻¹ and LBA 10 mmol L⁻¹.

Freeze-thaw cycles procedure

For the freezing essays, GlnK aliquots of the dialyzed protein were used with and without cryoprotectants. The samples were removed from the refrigerator, quickly prepared in 1.5 mL polypropylene tubes and subjected to one or two freezing cycles. The freezer temperature during the freezing essays was set at -27 °C and the freezing time was 1 h. The thawing procedure was performed at 25 °C

for 10 min. Right after the samples had been thawed, they were evaluated using the ITC and CD techniques.

Evaluation of the secondary structure of GlnK samples by CD after freeze-thaw cycles

A CD spectropolarimeter (J-815, Jasco International Co., Tokyo, Japan) was used to evaluate the GlnK conformation and stability before and after one and two freezing cycles in the presence or not of the LBA additive.

Spectra were obtained using quartz cells with an optical path of 0.01 and 1 cm wide. Nitrogen was used as purge gas at a flow rate of 10 L min⁻¹. For the assays, the protein solution concentrations were adjusted to GlnK 0.5 mg mL⁻¹ with ultrapure water.

The mean residue ellipticity (MRE) was calculated in accordance with Eq. (1). The molecular weight values (12 kDa) and residue numbers (112 per monomer) for the GlnK protein were estimated using the online software ProtParam (Gasteiger et al. 2005).

$$MRE = \frac{\theta \times 100 \times MM}{C \times l \times n}, \quad (1)$$

where θ is the ellipticity degree (deg), l is the optical length (cm), C is the concentration (mg mL⁻¹), MM is the molar mass (kDa) and n is the number of protein residues. The average molar residual ellipticity (MRE) is given in deg cm² dmol⁻¹ (Corrêa and Ramos 2009).

GlnK protein and ATP interaction evaluated by ITC

The experiments were conducted using an ITC₂₀₀ (MicrocalTM) from GE Healthcare. The temperature was set at 20 °C and stirring was maintained constantly at 300 rpm. The reference cell was filled with 200 μL of ultrapure water and the sample cell was filled with 200 μL of dialyzed 0.1 mmol L⁻¹ GlnK solution. The 3 mmol L⁻¹ ATP solution was injected into the sample cell from a 40 μL capacity syringe. The titration was performed in the cell containing the GlnK protein with a first injection of ATP solution at a volume of 0.4 μL followed by 24 successive injections of ATP at a volume of 1.5 μL. The duration of each injection was 3 s and the intervals between the injections were 150 s. The experiments were performed in duplicate.

Statistical analyses

All measurements from each sample in the CD and ITC equipment were conducted in duplicate. The Action fuction of Excel 2010 was used to perform ANOVA using Tukey's *multiple comparison test*. Significance was noted at $p < 0.05$.

Results and discussion

Purification and characterization of GlnK protein from *Herbaspirillum seropedicae*

Electrophoresis of the protein fractions obtained from the column is presented in Fig. 1. Lane 1 contains the molecular weight markers (GE Healthcare) with their respective masses in kDa. Lane 2—crude extract; lane 3—soluble protein; lane 4—insoluble protein; lane 5—proteins not bound to the column; lane 6—proteins not bound after rinsing with buffer; lanes 7–21—fractions of the elution with NaCl. The fractions corresponding to lanes 14–18 of the gel have been chosen for use in the subsequent analyses. In these fractions, GlnK proteins were found in greater quantity and with few impurities. The presence of the GlnK protein was detected from its monomer molecular weight of approximately 12 kDa. These fractions were pooled and dialyzed overnight against a buffer of Tris–Cl 50 mmol L⁻¹ pH 8.0, NaCl 100 mmol L⁻¹ and MgCl₂ 5 mmol L⁻¹ for further analysis.

The molar absorption coefficient parameters (2.98 M⁻¹ cm⁻¹) and the GlnK trimer molar mass (36 kDa) were estimated using the online software ProtParam (Gasteiger et al. 2005). These parameters were used for the protein concentration measurement and the obtained value was 0.3 mmol L⁻¹ of protein. The quantity, concentration and purity of the GlnK protein obtained were suitable for the CD and ITC analysis.

Freeze–thaw effect on the secondary structure of GlnK protein evaluated by CD

CD spectra of GlnK 0.5 mg mL⁻¹ control subjected to one and two freezing cycles are presented in Fig. 2. The CD

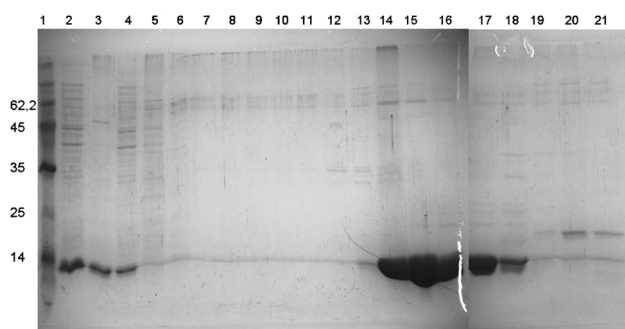


Fig. 1 Electrophoresis of the GlnK protein from *H. seropedicae* fractions obtained from the purification in the heparin column (Hitrap GE Healthcare), using the SDS-PAGE 12% gel. Lane 1—molecular weight marker (GE Healthcare) with the respective molar masses in kDa. Lane 2—crude extract; lane 3—soluble proteins; lane 4—insoluble proteins; lane 5—proteins not bound to the column; lane 6—proteins not bounded after the rinsing with buffer; lanes 7–21—elution fractions with NaCl (GlnK solution)

curves of the GlnK secondary structure did not exhibit typical α -helix or β -sheet conformation characteristics, but a combination of them. The two most common secondary structures of the polypeptide chain were α -helix and β -sheet. The α -helix structures were characterized by a double minimum at 208 and 222 nm and a maximum absorption at 191–193 nm, and β -sheet structures present a minimum at 215 nm and a positive maximum at 195 nm (Corrêa and Ramos 2009). Different protein sources have specific percentages and ratios of α -helix and β -sheet in their protein secondary structures. Some studies also indicated that these structures can be modified by the protein environment as a solvent, temperature and binder (Whamond and Thornton 2006; Güler et al. 2016). The GlnK structure curves obtained in this study present a positive maximum at 195 nm, a negative minimum at 206 nm and shoulder at 217 nm (Fig. 2).

The K2D algorithm was employed on the CD data (Whitmore and Wallace 2008) to quantify the different contents of GlnK secondary structures. The percentage compositions of α -helix, β -sheet and random coils obtained for the GlnK control sample were 50 \pm 1% coil random, 37 \pm 1% β -sheet and 12 \pm 1% α -helix.

The freeze–thaw cycles caused changes in the protein secondary structure, which can be observed in the molar ellipticity values of CD spectra (Fig. 2). The samples that were subjected to freezing had reductions in the intensity of the peaks at 195 nm (positive), and an increase at 206 nm (negative) and at the shoulder at 217 nm (negative). These

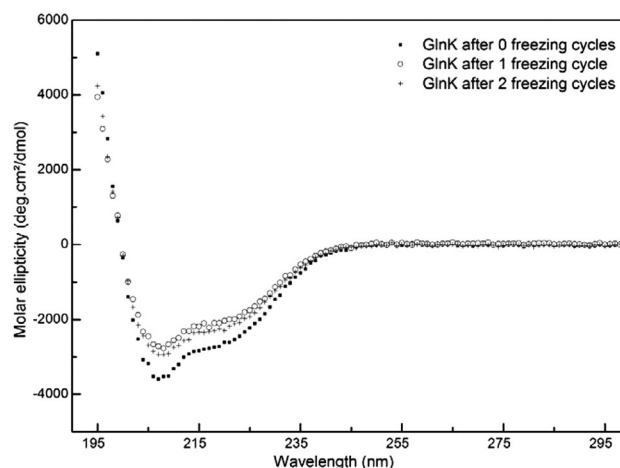


Fig. 2 UV CD spectrum evaluating the effect of freezing cycles on the secondary structure of GlnK 0.5 mg mL⁻¹ solutions. Black squares GlnK not subjected to freeze–thaw cycles (native), open circles GlnK subjected to one freeze–thaw cycle, plus symbols GlnK subjected to two freeze–thaw cycles. Through Tukey statistical test it was observed significant differences between the samples at 0.05 level. Black squares samples were different from open circles and plus symbols samples and open circles and plus symbols samples presented no difference at 0.05 level

changes at the intensity of the peaks in the CD spectra can be due to conformational changes in the protein structure. Similar results were obtained by Zhao et al. (2015) for soy protein isolate that was subjected to the freezing process. It was suggested that the freeze–thaw cycle processes modify the structural characteristics of the soy protein isolate. Samples subjected to 0–2 freeze–thaw cycles showed a decrease in the ellipticity values in the positive peak, resulting in a decrease in the β -sheet structure. Nevertheless, these authors suggest that the freeze–thaw cycles caused a change in the molecule structure and a bigger exposure of the hydrophobic side-chain groups. Changes in the protein structures were also studied by Güler et al. (2016) in terms of enzymatic breakdown of the bovine serum albumin. Although proteolysis reaction causes more damage to protein structures than freeze–thaw cycles, both processes result in similar changes to CD spectra. Güler et al. (2016) demonstrated that the decrease of the ellipticity to around 190–195 nm indicated a loss of α -helix structure resulting from the breakdown of the enzyme into smaller fragments. At the same time, increase of the ellipticity values to 222 nm indicates a breakdown of secondary structure elements and therefore the degradation of BSA.

In this experiment, by using the CD technique, the effects of the freeze–thaw procedure on the GlnK conformation could be evaluated.

Freezing effect on the GlnK and ATP binding evaluated by ITC

The binding between a 3 mmol L⁻¹ ATP solution with a 0.1 mmol L⁻¹ GlnK solution subjected to one and two freezing cycles and not (nature) is shown in Fig. 3. Two main differences were found between the samples. The first regards the difference in the interaction enthalpy values between the samples subjected to freezing and the control. In Fig. 3, the enthalpy values, represented by the indicators, for all samples were negative, which indicates favorable interactions for all systems. Since the enthalpy values of the control samples were smaller than the samples subjected to the freeze–thaw process, this indicated that control samples were more stable than frozen samples. This suggested that the bindings in these samples were more likely to occurred than samples those subjected to freezing. This change in the enthalpy values was possibly caused by a change in the binding mechanism or modification to the interaction between the sites (Brown et al. 2009). Changes in the protein mechanism interactions can be caused by heat treatment and evaluated by ITC as presented by Rispen et al. (2008). In that study molecular energy changes were observed in the Immunoglobulin G protein as a result of thermal treatment. It was verified by

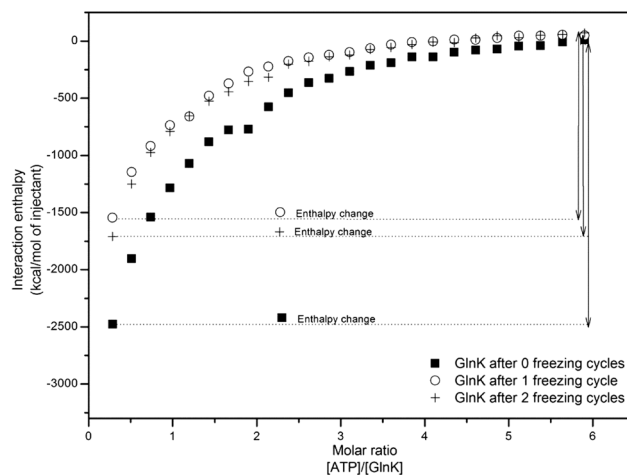


Fig. 3 ITC curves evaluating the freeze–thaw cycles effect in the interactions between solutions of 0.1 mmol L⁻¹ GlnK protein and 3 mmol L⁻¹ ATP. *Black squares* GlnK not subjected to freeze–thaw cycles (nature), *open circles* GlnK subjected to one freeze–thaw cycle, *plus symbols* GlnK subjected to two freeze–thaw cycles. *Arrows* represent the respective enthalpy values of the samples. Through Tukey statistical test it were observed significant differences between the samples at 0.05 level. *Black squares* samples were different from *open circles* and *plus symbols* samples and *open circles* and *plus symbols* samples presented no difference at 0.05 level

ITC analysis that samples subjected to heat treatment exhibited greater heat interaction values than the control samples. The authors suggest that the heat treatment caused some conformational changes in the protein and resulted in an increase in the binding affinity. Comparing Rispen's and this work, in both cases the heat value changes were due to heat treatment. The difference was that samples that had an increase in the temperature also had an increase in the binding affinity and samples that were frozen had a decrease in this parameter.

Another aspect observed in ITC curves that differentiates the GlnK and ATP binding subjected to freezing treatment is the system saturation. The amount of ATP solution required to saturate the system was lower in protein samples subjected to freezing (1 and 2 cycles of freezing) than in control. The saturation of the system is analyzed in Fig. 3 in a similar way to that used by Dimitrova et al. (2002). The system saturation was identified in the ITC graphics as the region where the interaction enthalpy values of the samples remained constant. Analyzing the control sample in Fig. 3, the system saturation could not be identified. However, for samples that were subjected to freezing, there was a tendency of saturation in the molar ATP/GlnK ratio at around 4.5. This change in the system saturation may be the result of a conformational change in the protein structure. The CD technique supports this result, since it changes in the molar ellipticity values for the samples subjected to the freeze–thaw procedure were observed.

Through the ITC and CD techniques it was possible to assess the effects on the GlnK protein caused by freeze–thaw cycles. Likewise, in what follows in the sequence the effects of the LBA cryoprotectant on the system will be presented.

LBA cryoprotectant effect on GlnK secondary structure subjected to freeze–thaw process evaluated by CD

The LBA cryoprotectant effect on the secondary structure of the GlnK protein before and after the freeze–thaw procedure was evaluated by CD. The CD spectra of 0.5 mg mL^{-1} GlnK solutions with 10 mmol L^{-1} LBA additive in nature form and subjected to one and two freezing cycles are shown in Fig. 4. In the same way as occurs for the GlnK spectra without the additive, the spectra of GlnK samples with LBA also have characteristic conformations of α -helix and β -sheet. The CD curves for samples in nature and subjected to freezing cycles do not significantly differ, practically overlapping each other. The results obtained in this study show that the freeze–thaw cycles did not influence the GlnK secondary structure in the presence of the LBA cryoprotectant.

Cryoprotectant effect of LBA in GlnK and ATP binding evaluated by ITC

The LBA cryoprotectant effects were also studied using the ITC technique. The bindings between GlnK added to LBA

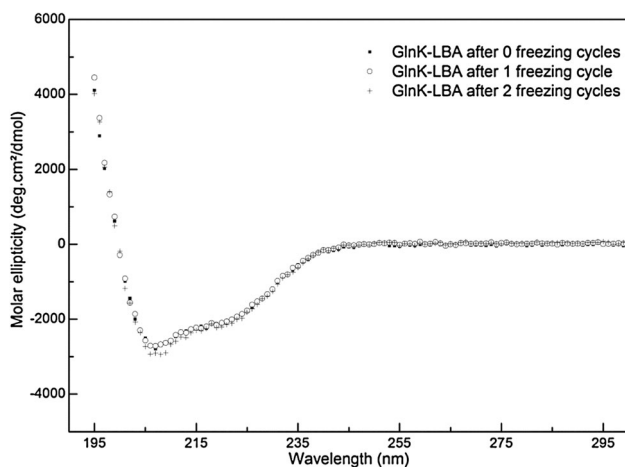


Fig. 4 UV CD spectrum of the cryoprotectant effect of the 10 mmol L^{-1} lactobionic acid on the secondary structure of 0.5 mg mL^{-1} GlnK solutions. *Black squares* GlnK with additive not subjected to freeze–thaw cycles *open circles* GlnK with additive subjected to one freeze–thaw cycle, *plus symbols* GlnK with additive subjected to two freeze–thaw cycles. Through Tukey statistical test it were not observed significant differences between the samples at 0.05 level

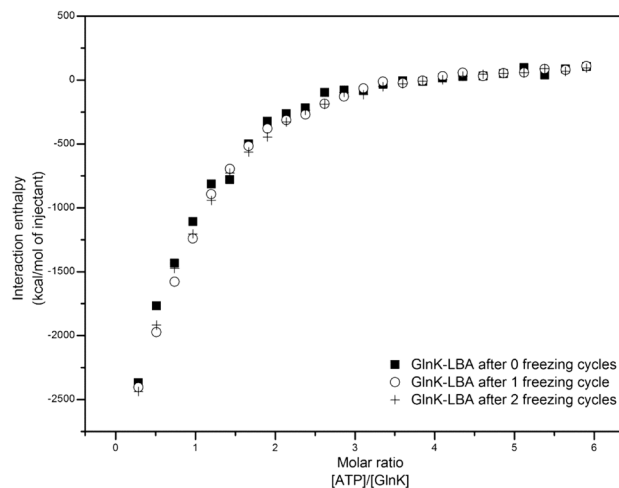


Fig. 5 ITC curves of the cryoprotectant effect of 10 mmol L^{-1} lactobionic acid in 0.1 mmol L^{-1} GlnK solutions subjected to freeze–thaw cycles by the interactions between the protein and the 3 mmol L^{-1} ATP. *Black squares* GlnK not subjected to freeze–thaw cycles, *open circles* GlnK subjected to one freeze–thaw cycle, *plus symbols* GlnK subjected to two freeze–thaw cycles. Through Tukey statistical test it were not observed significant differences between the samples at 0.05 level

samples with ATP samples in different freezing conditions are presented in Fig. 5.

LBA presented a cryoprotectant effect on GlnK-ATP binding after the freeze–thaw procedure. In contrast to what occurred with no additive samples, protein samples with LBA subjected to freezing cycles maintained their initial characteristics. The binding heat values did not change with the freezing exposure. This indicates that the interaction mechanism of GlnK-ATP remained unchanged. In addition, the freezing cycles did not cause negative effects that affected the protein binding with ATP, as there were no observed changes in the system saturation. These events support the CD analysis of the LBA cryoprotection of the GlnK protein.

Conclusion

The effects of the freeze–thaw process and LBA cryoprotectant activity on the GlnK protein structure were evaluated by the ITC and CD techniques.

The changes in the molar ellipticity values of the protein samples subjected to freeze–thaw cycles were observed. These changes were related to conformational changes in the GlnK protein caused by the freezing process. Additionally, the ITC technique showed changes in the GlnK and ATP bindings of samples exposed to freezing. Freezing cycles modified the ITC curves for GlnK-ATP interaction, particularly for the enthalpy interaction values and the saturation point. Therefore, it can be assumed that the

freeze–thaw process causes changes in GlnK interactions and conformation.

Having confirmed the changes in the GlnK protein caused by the freezing process, the LBA cryoprotectant activity was also evaluated. No significant differences between GlnK samples added from LBA subjected or not to freezing cycles were found. These results show the cryoprotectant effect of the additive in the conditions studied using the CD technique. Similar results were obtained by ITC analysis. ITC curves for GlnK with LBA interacting with ATP maintained the same characteristics before and after the freeze–thaw procedure, reinforcing the cryoprotective effect of the additive. It can be concluded that the CD and ITC techniques are appropriate for evaluating the freezing effects and the presence of cryoprotectants in the studied system. The freezing cycles changed the secondary structure of the GlnK protein and its binding with ATP, and the cryoprotectant effect of LBA was observed using both techniques in the studied conditions.

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References

- Alvarez MD, Fernández C, Canet W (2010) Oscillatory rheological properties of fresh and frozen/thawed mashed potatoes as modified by different cryoprotectants. *Food Bioprocess Technol* 3:55–70. doi:10.1007/s11947-007-0051-9
- Arcondéguy T, Jack R, Merrick M, Arconde T (2001) P II signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiol Mol Biol Rev* 65:80–105. doi:10.1128/MMBR.65.1.80
- Ariyaprakai S, Tananuwong K (2015) Freeze–thaw stability of edible oil-in-water emulsions stabilized by sucrose esters and Tweens. *J Food Eng* 152:57–64. doi:10.1016/j.jfoodeng.2014.11.023
- Bonatto AC, Couto GH, Souza EM, Araújo LM, Pedrosa FO, Noindorf L, Benelli EM (2007) Purification and characterization of the bifunctional uridylyltransferase and the signal transducing proteins GlnB and GlnK from *Herbaspirillum seropedicae*. *Protein Expr Purif* 55:293–299. doi:10.1016/j.pep.2007.04.012
- Bonatto AC, Souza EM, Oliveira MAS, Monteiro RA, Chubatsu LS, Huergo LF, Pedrosa FO (2012) Uridylylation of *Herbaspirillum seropedicae* GlnB and GlnK proteins is differentially affected by ATP, ADP and 2-oxoglutarate in vitro. *Arch Microbiol* 194:643–652. doi:10.1007/s00203-012-0799-9
- Brown RK, Brandts JM, O'Brien R, Peters WB (2009) ITC-derived binding constants: using microgram quantities of protein, label-free biosensors techniques and applications. *Label-Free Biosens*. doi:10.1017/CBO9780511626531.012
- Cao Z, Liu R, Yang B (2013) Potential toxicity of sarafloxacin to catalase: spectroscopic, ITC and molecular docking descriptions. *Spectrochim Acta Part A Mol Biomol Spectrosc* 115:457–463. doi:10.1016/j.saa.2013.06.093
- Charloux C, Paul M, Loisanec D, Astier A (1995) Inhibition of hydroxyl radical production. *Free Radic Biol Med* 19:699–704
- Corrêa DHA, Ramos CHI (2009) The use of circular dichroism spectroscopy to study protein folding, form and function. *Afr J Biochem Res* 3:164–173
- Dimitrova MN, Matsumura H, Terezova N, Neytchev V (2002) Binding of globular proteins to lipid membranes studied by isothermal titration calorimetry and fluorescence. *Colloids Surf B Biointerfaces* 24:53–61. doi:10.1016/S0927-7765(01)00248-X
- Furukawa Y, Inohara N, Yokoyama E (2005) Growth patterns and interfacial kinetic supercooling at ice/water interfaces at which anti-freeze glycoprotein molecules are adsorbed. *J Cryst Growth* 275:167–174. doi:10.1016/j.jcrysgro.2004.10.085
- García-Arias MT, Pontes EA, García-Linares MC, García-Fernández MC, Sánchez-Muniz FJ (2003) Grilling of sardine filets. Effects of frozen and thawed modality on their protein quality. *LWT Food Sci Technol* 36:763–769. doi:10.1016/S0023-6438(03)00097-5
- Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. *Proteomics Protoc Handb*. doi:10.1385/1-59259-890-0:571
- Green BA, Ruey JY, Scott EJV (2009) Clinical and cosmeceutical uses of hydroxyacids. *Clin Dermatol* 27:495–501. doi:10.1016/j.clindermatol.2009.06.023
- Güler G, Vorob MM, Vogel V, Mäntele W (2016) Proteolytically-induced changes of secondary structural protein conformation of bovine serum albumin monitored by Fourier transform infrared (FT-IR) and UV-circular dichroism spectroscopy. *Spectrochim Acta Part A Mol Biomol Spectrosc* 161:8–18. doi:10.1016/j.saa.2016.02.013
- Gutiérrez LF, Hamoudi S, Belkacemi K (2012) Lactobionic acid: a high value-added lactose derivative for food and pharmaceutical applications. *Int Dairy J* 26:103–111. doi:10.1016/j.idairyj.2012.05.003
- Herrera JJ, Pastoriza L, Sampedro G (2001) A DSC study on the effects of various maltodextrins and sucrose on protein changes in frozen-stored minced blue whiting muscle. *J Sci Food Agric* 81:377–384. doi:10.1002/1097-0010(200103)81:4<377:AID-JSFA820>3.0.CO;2-0
- Hubálek Z (2003) Protectants used in the cryopreservation of microorganisms. *Cryobiology* 46:205–229. doi:10.1016/S0011-2240(03)00046-4
- Iijima T (1998) Thermal analysis of cryoprotective solutions for red blood cells. *Cryobiology* 36:165–173. doi:10.1006/cryo.1998.2075
- Isaacson Y, Salem O, Shepherd RE, Thiel DHV (1989) Lactobionic acid as an iron chelator: a rationale for its effectiveness as an organ preservative. *Life Sci* 45:2373–2380
- Kaale LD, Eikevik TM (2013) A study of the ice crystal sizes of red muscle of pre-rigor Atlantic salmon (*Salmo salar*) filets during superchilled storage. *J Food Eng* 119:544–551. doi:10.1016/j.jfoodeng.2013.06.002
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685
- Nielsen PM (2009) Meat based food product comprising lactobionic acid. United States patent application pub. no.: US 2009/0214752 A1. 371: 4–7. <http://www.google.ch/patents/US20090214752>. Accessed 26 June 2015
- Park YJ, Kim KH, Lim DW, Lee EK (2015) Effects of pH and protein conformation on in-solution complexation between bovine α -lactalbumin and oleic acid: binding trend analysis by using SPR and ITC. *Process Biochem* 50:1379–1387. doi:10.1016/j.procbio.2015.05.018
- Perozzo R, Folkers G, Scapozza L (2004) Thermodynamics of protein–ligand interactions: history, presence, and future aspects.

- J Recept Signal Transduct Res 24:1–52. doi:[10.1081/RRS-120037896](https://doi.org/10.1081/RRS-120037896)
- Radchenko MV, Thornton J, Merrick M (2010) Control of AmtB–GlnK complex formation by intracellular levels of ATP, ADP, and 2-oxoglutarate. *J Biol Chem* 285:31037–31045. doi:[10.1074/jbc.M110.153908](https://doi.org/10.1074/jbc.M110.153908)
- Rawson A, Tiwari BK, Tuohy M, Brunton N (2012) Impact of frozen storage on polyacetylene content, texture and colour in carrots disks. *J Food Eng* 108:563–569. doi:[10.1016/j.jfoodeng.2011.09.003](https://doi.org/10.1016/j.jfoodeng.2011.09.003)
- Rispens T, Lakemond CMM, Derksen NIL, Aalberse RC (2008) Detection of conformational changes in immunoglobulin G using isothermal titration calorimetry with low-molecular-weight probes. *Anal Biochem* 380:303–309. doi:[10.1016/j.ab.2008.06.001](https://doi.org/10.1016/j.ab.2008.06.001)
- Shepherd RE, Isaacson Y, Chensny L, Zhang S, Kortés R, John K (1993) Lactobionic and gluconic acid complexes of FeII and FeIII; control of oxidation pathways by an organ transplantation preservative. *J Inorg Biochem* 49:23–48. doi:[10.1016/0162-0134\(93\)80046-C](https://doi.org/10.1016/0162-0134(93)80046-C)
- Sofi FR, Raju CV, Lakshmisha IP, Singh RR (2016) Antioxidant and antimicrobial properties of grape and papaya seed extracts and their application on the preservation of Indian mackerel (*Rastrelliger kanagurta*) during ice storage. *J Food Sci Technol* 53:104–117. doi:[10.1007/s13197-015-1983-0](https://doi.org/10.1007/s13197-015-1983-0)
- Tasic-Kostov M, Pavlovic D, Lukic M, Jaksic I, Arsic I, Savic S (2012) Lactobionic acid as antioxidant and moisturizing active in alkyl polyglucoside-based topical emulsions: the colloidal structure, stability and efficacy evaluation. *Int J Cosmet Sci* 34:424–434. doi:[10.1111/j.1468-2494.2012.00732.x](https://doi.org/10.1111/j.1468-2494.2012.00732.x)
- Wang P, Chen H, Mohanad B, Xu L, Ning Y, Xu J, Wu F, Yang N, Jin Z, Xu X (2014a) Effect of frozen storage on physico-chemistry of wheat gluten proteins: studies on gluten-, glutenin- and gliadin-rich fractions. *Food Hydrocolloids* 39:187–194. doi:[10.1016/j.foodhyd.2014.01.009](https://doi.org/10.1016/j.foodhyd.2014.01.009)
- Wang P, Xu L, Nikoo M, Ocen D, Wu F, Yang N, Jin Z, Xu X (2014b) Effect of frozen storage on the conformational, thermal and microscopic properties of gluten: Comparative studies on gluten-, glutenin- and gliadin-rich fractions. *Food Hydrocolloids* 35:238–246. doi:[10.1016/j.foodhyd.2013.05.015](https://doi.org/10.1016/j.foodhyd.2013.05.015)
- Whamond GS, Thornton JM (2006) An analysis of intron positions in relation to nucleotides, amino acids, and protein secondary structure. *J Mol Biol*. doi:[10.1016/j.jmb.2006.03.029](https://doi.org/10.1016/j.jmb.2006.03.029)
- Whitmore L, Wallace B (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers* 89:392–400. doi:[10.1002/bip.20853](https://doi.org/10.1002/bip.20853)
- Xu Y, Cheah E, Carr PD, Heeswijk WCV, Westerhoff HV, Vasudevan SG, Ollis DL (1998) GlnK, a PII-homologue: structure reveals ATP binding site and indicates how the T-loops may be involved in molecular recognition. *J Mol Biol* 282:149–165. doi:[10.1006/jmbi.1998.1979](https://doi.org/10.1006/jmbi.1998.1979)
- Zhao J, Dong F, Li Y, Kong B, Liu Q (2015) Effect of freeze–thaw cycles on the emulsion activity and structural characteristics of soy protein isolate. *Process Biochem* 50:1607–1613. doi:[10.1016/j.procbio.2015.06.021](https://doi.org/10.1016/j.procbio.2015.06.021)