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Aqueous solutions of proline and NaCl studied by differential scanning calorimetry at subzero temperatures

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Abstract

The hydration properties of proline are studied by differential scanning calorimetry (DSC) in aqueous solutions during freezing to −60°C and subsequent heating to +20°C. The concentration of proline in the freeze concentrated solution was estimated to approximately 50 wt% (w/w) indicating a high water solubility of proline at subzero temperatures. No glass transition was observed within the concentration range 0.9–40.1 wt% (w/w), neither at a low scanning rate of 2.5°C/min nor at a higher scanning rate of 10°C/min. Eutectic crystallization of proline was not observed during freezing or melting which shows that proline has the ability to stay in solution at subzero temperatures. Samples containing proline–NaCl–water were also investigated by DSC and it was shown that the solubility of proline is maintained in aqueous salt solutions at temperatures as low as −60°C. From DSC measurements it was found that the eutectic crystallization of NaCl is prevented by the presence of proline, even when NaCl (initially) is present in molar excess ([NaCl]/[proline] = 2.6). The possible association of these findings with the occurrence of proline accumulation in some plants and insects living under water stress conditions is discussed. © 1997 Elsevier Science B.V.

Keywords: Differential scanning calorimetry (DSC); Freezing; Proline; Sodium chloride

1. Introduction

The imino acid proline plays a crucial role in the protection against cell injuries caused by freezing and dehydration in various plants [1,2]. This also applies to overwintering poikilotherms, especially insects, which make up the most thoroughly investigated group [3,4]. It has been suggested that proline accumulation could provide a quick mechanism for maintaining osmoticum of cells and tissues in response to stress [5]. In addition, proline has been shown to stabilize several proteins during exposure to subzero temperatures as for example calcium ATPase [6] and lactate dehydrogenase [7]. The mechanism by which proline and other cryoprotectives prevent damage to proteins and different cellular structures during freezing or dehydration is not well understood [8,9]. However, the hydration of the solutes is believed to be of importance for the cryoprotective properties [10,11]. Many of the known cryoprotectives are polyhydroxy compounds which do not crystallize from aqueous solutions during freezing. Instead they are characterized by the development of a glass state with extre-

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mely high viscosity which strongly suppress the rate at which chemical reactions as well as ice re-crystallization can occur [12,13]. It has been shown that some glycerol–water [14] and trehalose–water glasses [15] formed at temperatures below 0°C have the ability to prevent the eutectic crystallization of NaCl. This behaviour has been proposed to be associated with the cryoprotective of these compounds in biological systems [15]. The available literature is almost exclusive about the hydration and glass forming properties of carbohydrate/water mixtures, and little attention has been given to the crystallization properties of the imino and amino acids. In the present study, the hydration properties of proline and the effects of proline on aqueous solutions of NaCl are elucidated at subzero temperatures by the use of differential scanning calorimetry.

2. Materials and methods

2.1. Reagents

L-Proline and NaCl were of analytical grade and were purchased from Sigma and Merck, respectively. Gallium (99.9999%, Mp. 29.78°C, melting enthalpy: 80.1 J/g) and decane (99.9%, Mp. −29.66°C) were purchased from Aldrich and Fluka Chemie, respectively. Double distilled water for preparation of solutions was passed through a millipore filtration system equipped with a 0.2 μm nylon filter (Belford, MA, USA) to remove any residual particles. The filtered water was then used immediately for preparation of the solutions.

2.2. Differential scanning calorimetry (DSC)

Calorimetric measurements were made with a Perkin–Elmer DSC-7 differential scanning calorimeter (DSC), equipped with intercooler II and Perkin–Elmer 3600 Data Station. Sealed aluminum pans were used in all experiments according to the procedures recommended by the manufacturer. An empty aluminum pan was used as reference. Gallium was used for enthalpy calibration and temperature was performed with decane. The calibrations were performed by using a scanning rate of 5°C/min. The dry box of the DSC was flushed with dry nitrogen and the sample head was purged with a constant dry nitrogen flow (20 ml/min) to avoid condensation of moisture from the air.

2.3. Sample preparation and running conditions

The sample (5–20 mg) was placed in the sample holder of the calorimeter and held at 10°C for 4 min in order to stabilize the initial heat flow. Scans involved cooling from the isothermal hold temperature to −60°C, followed by an isothermal hold at −60°C for 10 min to allow sample state equilibration. The samples were then heated to 10°C using a linear scanning rate of 2.5°C/min.

2.4. Transition temperature and enthalpy

The water crystallization temperature (T_f) was determined as the onset temperature of the first freezing exotherm observed during cooling. The ice melting temperature (T_m) was obtained as the onset temperature of the first warming endotherm observed during heating of the sample. The enthalpy of a transition was estimated from the integrated heat flow over the temperature range of the transition. The DSC-7 standard program is used for the calculation. The freezing enthalpy (ΔH_{freeze}) of water was obtained by integration from the onset temperature (T_f) to the end temperature of the exothermic peak from the crystallization. The melting enthalpy (ΔH_{melt}) of ice was determined by integration from the onset temperature (T_m) to the end temperature of the endothermic peak from ice melting according to the method described by O'Neil [16]. The enthalpy changes for ΔH_{freeze} and ΔH_{melt} are expressed as J per gram sample (J/g). Precautions to measure rapid exothermic process, such as the freezing of supercooled water, were not taken, since these would also decrease sensitivity and peak resolution [16]. All measurements were performed in duplicate.

2.5. Freezable water

The amount of freezable water in the sample was determined either from the heat liberated during freezing or from the heat absorbed during melting. The temperature dependence of the enthalpy of water crystallization was taken into consideration when determining the ice content. The following approx-

imation was used to calculate the freezing enthalpy of pure water at T_f [17]:

$$\Delta H(T_f) = \left[\Delta H_0 - \int_{T_f}^{273} \Delta C_p dT\right] \sim [-334 - 2.05(T_f - 273)]\frac{J}{g}$$

where T_f is the freezing temperature in K, ΔH_0 the freezing enthalpy at 273 K corresponding to −334 J/g, and ΔC_p the difference in heat capacity between liquid water and ice at T_f. The ratio between the freezing enthalpy measured by DSC (ΔH_{freeze}) and the freezing enthalpy of pure water is a measure of the amount of ice separated per gram sample at T_f. For the determination of ice content from ΔH_{melt} the melting enthalpy of pure water at 273 K was used ($\Delta H_{273} = 334$ J/g). The amount of ice melted per gram sample was calculated as: $\Delta H_{melt}/\Delta H_{273}$. The coefficient of variation for determination of ice content from the freezing and melting enthalpies estimated by repeated analyses of an aqueous solution containing 10.4 wt% (w/w) proline on different days was approximately 10% ($n = 5$).

The amount of solute in the freeze concentrated solution was calculated by an extrapolation method described by Simatros et al. [18]. In brief, series of solutions covering a range of solute concentrations (0.9–40.1 wt% (w/w) proline) was analyzed, and the areas of the freezing exotherm and the ice melting peak were determined at each concentration. The individual areas which decreased (numerically) with increasing solute concentration were extrapolated to the solute concentration corresponding to zero peak area.

2.6. Glass transition

Solutions containing different amounts of proline varying between 0.9 and 40.1 wt% were cooled (5°C/min), and exposed to −60°C in the DSC chamber for 20 min at −60°C. The glass transition properties were studied by analyzing the heat capacity changes during heating of the frozen aqueous solutions according to the PE-Delta software, version 6.10 (Perkin–Elmer, Norwalk, USA). Scanning rates of 2.5 and 10°C/min were used. The calorimeter sensitivity for measurement of heat capacity changes during the glass transition has been determined to approximately 0.05 J/g°C when referred to a glass forming aqueous solution of protein hydrolysate with a glass transition temperature at −40°C [7].

3. Results

3.1. DSC measurements on proline solutions

The thermal behaviour of proline was studied in aqueous solutions by DSC. Fig. 1 shows the thermoanalytical curves (heat flow vs. temperature) measured during cooling and subsequent heating of an aqueous solution containing 0.104 g proline per gram solution. The exotherm observed during cooling (I) is associated with the freezing of solvent water. The crystallization temperature (T_f) for transition I was determined to −22.5°C. The low value obtained for T_f is due to freezing of supercooled bulk water. As shown in Fig. 1, no further exothermic events occurred during cooling. Transition II in Fig. 1 was the melting endotherm associated with the melting of ice. This transition was broad. The melting process began at approximately −14°C which is the onset

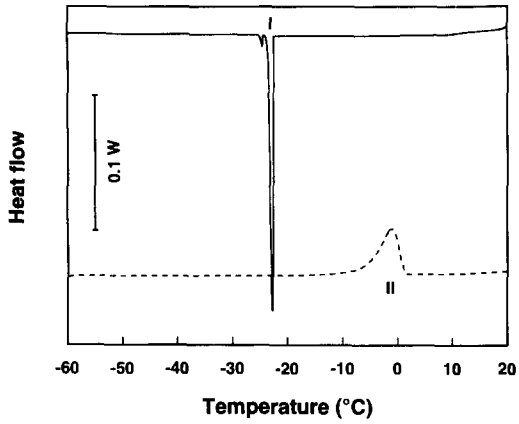

Fig. 1. Analytical DSC curves (*heat flow vs. temperature*) obtained by cooling (upper curve) from 20 to −60°C and subsequent rewarming (lower curve) from −60 to 20°C of an aqueous solution containing 0.104 g proline per gram solution. Scanning rate: 2.5°C/min. I: exotherm obtained during cooling; II: endotherm obtained during heating. The area of the freezing exotherm is −196 J/g which corresponds to the freezing of 0.67 g water per gram sample. The area of the melting endotherm indicates that 0.64 g water melts at approximately 0°C per gram of sample.

temperature for ice melting. The value for ΔH_{melt} reaches a maximum at approximately 0°C which is the equilibrium melting point for ice under normal standard conditions. The melting of pure ice dilutes the freeze concentrated solution surrounding it and produces dilution heat, which also contributes to the experimentally determined value for ΔH_{melt}. As shown in Fig. 1, practically no change in enthalpy was observed before and after transition I. During the melting process, a small increase in the enthalpy before and after the transition was found indicating that the heat capacity of the sample is slightly temperature dependent. The areas of the freezing and melting enthalpies were −196 and 213 J/g corresponding to the crystallization of 0.67 g water per gram sample and melting of 0.64 g ice. For DSC measurements of the ice content, these values are not considered as being significantly different.

It was noted that none of DSC curves within the investigated concentration range from 0.9 to 40.1 wt% (w/w) proline showed evidence of a glass transition below T_m, neither at a low scanning rate of 2.5°C/min, nor at higher scanning rate of 10°C/min. Due to the high sensitivity of the calorimeter used for measuring heat capacity changes during glass transitions, it is unlikely that the absence of a glass transition in aqueous solutions of proline could be caused by a detection problem.

3.2. Hydration properties

The values of T_f, ΔH_{freeze}, and ΔH_{melt} were estimated from thermoanalytical curves. Fig. 2 shows the experimentally determined values of ΔH_{freeze} and ΔH_{melt} measured as functions of the weight fraction of proline. The intercepts with the abscissa corresponded to approximately 52% and 48 wt% (w/w) proline when referred to the values obtained from ΔH_{freeze} and ΔH_{melt}, respectively. These compositions give the apparent concentration of proline that would not allow ice formation on cooling, because all the water present in the solution would be unfrozen. The freezing and melting enthalpies for pure water are also shown, and it appears that low concentrations of proline had a strong effect on the measured enthalpies. In the presence of 0.9 wt% (w/w) proline the measured values for $-\Delta H_{freeze}$ and ΔH_{melt} decreased from approximately 294 to 236 J/g and from 331 to 284 J/g, respectively.

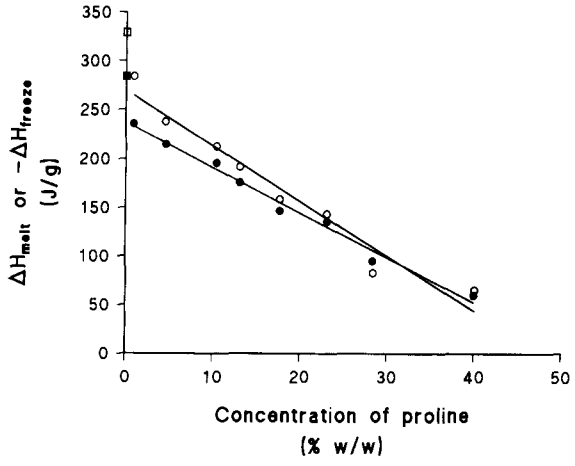

Fig. 2. ΔH_{melt} (0-0) and $-\Delta H_{freeze}$ (•-•) plotted as functions of the wt% (w/w) of proline dissolved in water. □ and ■ denote the melting and freezing enthalpies measured in pure water. Scanning rate: ±2.5°C/min.

Table 1
Percentage (%) of freezable water determined either during freezing exotherm or during melting endotherm in aqueous solutions of proline

Concentration (wt%)	T_f (°C)	% of water frozen during freezing exotherm	% of water melted during melting endotherm
0.9	−21.6	82.7 (0.82)	85.8 (0.85)
4.6	−22.7	78.6 (0.75)	74.4 (0.71)
10.4	−22.5	74.8 (0.67)	71.4 (0.64)
13.1	−21.4	70.2 (0.61)	65.6 (0.57)
17.7	−24.4	63.2 (0.52)	58.3 (0.48)
23.1	−25.5	62.5 (0.48)	56.0 (0.43)
28.4	−25.0	47.5 (0.34)	33.5 (0.24)
40.1	−31.7	36.7 (0.22)	33.4 (0.20)

The concentration of proline is expressed as the amount of dry matter per 100 g solution (wt%). The amount of freezable water is expressed as the weight percentage of the total water content in the sample determined from ΔH_{freeze} or ΔH_{melt}. The values in brackets are gram freezable water per gram sample.

The freezable water fraction as determined from the freezing and melting enthalpies is shown in Table 1 for various concentrations of proline. By increasing the concentration of proline from 0.9 to 40.1 wt% (w/w) the percentage of water that freeze or melt decreased from 84.3% (average value of 85.8 and 82.7%) to approximately 35.1% (average value of

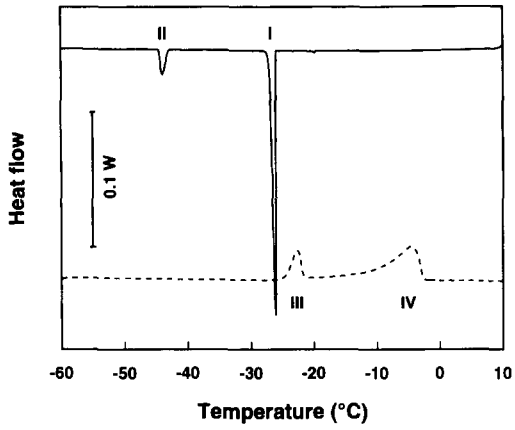

Fig. 3. Analytical curves of 5 wt% NaCl in water measured during cooling to −60°C (upper curve) followed by subsequent rewarming to +10°C (lower curve). The numbers I–IV are explained in the text. The scanning rate was 2.5°C/min.

33.4 and 36.7%) of the total water content in the samples. The reduction in ice content with increasing concentration of proline is due to the accumulation of proline in the unfrozen water phase which will decrease the rate of ice crystal growth and reduce the fraction of water crystallized. As shown in Table 1, the values for the amount of freezable water determined either from ΔH_{freeze} or ΔH_{melt} are similar although these appear to be slightly lower when the melting enthalpies are used for the calculations.

3.3. DSC measurements on NaCl solutions

Fig. 3 shows typical thermoanalytical curves measured during cooling and subsequent warming of an aqueous solution of NaCl containing 5 wt% (w/w) NaCl. In the following, the peaks and the corresponding thermal transitions are identified by a number, I–IV according to their successive appearance in a cooling–heating cycle. The numbering is illustrated in Fig. 3. The transition temperature T(I) at approximately −25°C is the temperature at which ice crystals appear in the NaCl solution. The enthalpy of the transition was −205 J/g. During cooling no transition was observed at the eutectic temperature of NaCl–H_2O which is −21°C [19]. As shown in Fig. 3, an exotherm was observed at T(II), close to −42°C with an enthalpy of −26.6 J/g. This peak is considered to represent the eutectic crystallization of NaCl. The low value of T(II) is in accordance with previous investigations of the thermal behaviour of NaCl in aqueous solutions [20,21]. The low crystallization temperature of NaCl is due to supersaturation which is often observed at cooling with precipitation of NaCl occurring well below the eutectic temperature [20]. This means that during freeze concentration a liquid phase is established which is supersaturated with NaCl. The weight fraction of NaCl in the remaining liquid phase can be estimated from $0.05/(1 - \Delta H(\text{I}))/\Delta H_{\text{freeze}}(T_f)$ which corresponds to 0.14 g NaCl per gram aqueous supersaturated solution. During heating two melting endotherms were observed at T(III) and T(IV), respectively. The endotherm at approximately −22°C is close to the equilibrium eutectic melting temperature of NaCl and this peak represents the eutectic melting temperature of NaCl. The enthalpy associated with the transition was 49 J/g. Transition IV has the same cause as previously described for proline and ΔH(IV) primarily comes from the melting of ice that is in equilibrium with the NaCl solution.

3.4. The proline–NaCl–water system

The interaction between proline and NaCl in aqueous solutions was also studied. As already mentioned the thermal events that occurred at T(II) and T(III) are suggested to be due to NaCl. By using the same notation for the peaks I–IV as in Fig. 3, the effect of increasing the concentration of proline from 0.99 to 9.08 wt% (w/w) in the presence of 5.01 wt% (w/w) NaCl in water is shown in Table 2. The crystallization and melting temperature for NaCl occurring at T(II) and T(III) decreased with increasing concentration of proline up to 2.14 and 3.83 wt% (w/w), respectively. At the same time the transition enthalpy of ΔH(II) and ΔH(III) decreased (numerically) with increasing concentration of proline. In the presence of 3.83 wt% (w/w) proline, no transitions were observed at T(II) or T(III). An initial composition of 5.01 wt% (w/w) NaCl and 3.83 wt% (w/w) proline corresponded to a molar concentration of 0.86 and 0.33 M for NaCl and proline, respectively. This shows that proline had the ability to prevent crystallization of NaCl during freeze concentration even when NaCl was present in molar excess compared with proline. Moreover, the results show that the solubility of proline was maintained in aqueous salt solutions since no crystalliza-

Table 2
Data of the thermal transitions in aqueous solutions of NaCl and proline as determined from analytical DSC curves

NaCl (wt%)	Pro (wt%)	ΔH(I) (J/g)	ΔH(II) (J/g)	ΔH(III) (J/g)	ΔH(IV) (J/g)	T(I) (°C)	T(II) (°C)	T(III) (°C)
0	0	−294	—	—	331	−11.4	—	—
5.01	—	−213	−29	49	198	−19.8	−42.5	−22.9
5.01	0.99	−214	−25	35	206	−18.0	−45.7	−24.4
5.01	2.14	−205	−16	26	206	−20.5	−50.0	−25.9
5.01	3.83	−199	—	—	193	−17.0	—	—
5.01	7.40	−190	—	—	194	−19.2	—	—
5.01	9.08	−176	—	—	175	−18.2	—	—

The specifications of the transitions I–IV are explained in the text. Cases where no transitions were observed are denoted by the symbol −. The concentrations are expressed as g dry matter per 100 g of solution (wt%).

tion peaks due to the presence of proline were observed during freezing or thawing.

4. Discussion

Calorimetric investigations of proline have been described by Rudolph and Crowe [22]. They found that aqueous solutions containing high concentrations of proline (> 0.5 M) did form an endotherm transition during cooling at approximately −53°C which was proposed to be a glass transition. But in their thermograms the characteristic change in heat capacity occurring at the glass transition point for glass forming compounds [12] was not seen during the heating scans, neither at low nor at high concentrations of proline. The same is true for our calorimetric results on aqueous solutions of proline where it is revealed that none of the DSC thermograms showed evidence of a glass transition at least not for temperatures higher than −60°C which is the lowest working temperature of the calorimeter used. This strongly suggests that aqueous solutions of proline did not form a glass at temperatures higher than −60°C.

The concentration of proline in freeze-concentrated aqueous solutions is approximately 50 wt% (w/w) when determined by the extrapolation method (Fig. 2). No exothermic or endothermic transitions due to crystallization of proline were observed within the investigated concentration range neither during cooling to −60°C nor during subsequent heating. In one experiment (results not shown) an aqueous solution containing 40.1 wt% (w/w) of proline was cooled, and exposed to −60°C in the DSC chamber for 3 h. During subsequent rewarming to room temperature no eutectic melting endotherm was observed. This shows that rather concentrated aqueous solutions of proline are stabilized at low subzero temperatures which will allow proline to stay in solution even when most of the solvent water is converted to ice. These findings are in accordance with the high solubility of proline in water, 86% by weight at 25°C [23]. The concentration determined by the use of extrapolation method represents the composition of the freeze concentrate but not necessarily the composition of the maximally freeze concentrate solution because no glass transition was observed for aqueous solutions of proline. The concentration of proline in the maximally freeze concentrated solution may therefore be even higher than the value measured above. A reduction of the temperature below −60°C could probably improve the thermal characterization of the freeze concentrated solution.

The unfrozen water (UFW) content can be calculated by the difference between the total water content of the sample and the amount of freezable water as determined by DSC. The weight percentage of proline in the freeze concentrated solution thus corresponds to approximately 0.8 and 1.0 g UFW per gram solid when referred to the values obtained by extrapolating the measured freezing and melting enthalpies to zero peak area. These values are considerably higher than the corresponding values for many other naturally occurring cryoprotectives, e.g. sucrose (0.205 g UFW/g [24]), trehalose (0.33 g UFW/g [15]) and glucose (0.41 g UFW/g, [13]) which indicates a stronger interaction of solvent water with proline than with the different polyhydroxy compounds. Moreover, low amounts of proline has a strong effect on the measured freezing and melting enthalpies (Fig. 2),

which further suggest that the water structure is highly influenced in the presence of proline. Further investigations on the solvent interactions with different cryoprotectives studied under the same experimental conditions would be useful.

As shown in Table 1, the presence of proline strongly influenced the ice formation process by altering the freezable water function in such a way that ice formation was minimized at elevated concentrations of proline. A similar relationship between accumulation of low molecular weight polyhydroxy compounds (e.g., glycerol and trehalose) and resistance to frost injury have been described for several freezing tolerant insects [4]. As already mentioned, the cryoprotective mechanism for the polyhydroxy compounds is believed to involve the development of a glassy state during freezing concentration. The present study shows that other reaction mechanisms are involved for proline regarding the hydration properties at temperatures higher than −60°C. Schobert and Tschesche [25] found that the viscosity in aqueous solutions of proline increased steeply with increasing concentrations up to 6 M at 25°C which was proposed to be due to strong hydrogen bonding interactions between solvent water and proline. From this point of view it can be suggested that the presence of high concentration of proline in the unfrozen fraction will impede the mobility of solvent water and hence the rate of ice crystal growth. This may thus indicate some similarities with the glass forming compounds.

It is proposed that freezing injuries of biological materials (e.g., membranes) is associated with eutectic crystallization of NaCl [15,26]. As shown in Table 2 eutectic crystallization of NaCl did not occur in the presence of sufficient amounts of proline even when NaCl was present in molar excess. A similar behaviour has been described for aqueous solutions of NaCl containing glycerol [14] and other polyhydroxy compounds such as sucrose and trehalose [15]. The mechanism by which the polyhydroxy compounds exert their cryoprotective effect is still under investigation. But it possibly involves the ability of such agents to reduce or to prevent the eutectic freezing of NaCl by trapping the salt within a highly viscous, or even glass-like phase [12,15]. Although the ability of proline to prevent eutectic crystallization of NaCl cannot be explained by the development of a glass it can be concluded that the presence of proline alters the interaction between solvent water and NaCl in such a way that the solubility of NaCl is increased.

Acknowledgements

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References

[1] T.M.M. Chu, D. Jusaitis, D. Aspinall and L.G. Paleg, Accumulation of free proline at low temperatures, Physiol. Plant., 43 (1978) 254–260.
[2] D. Levy, Water deficit enhancement of proline and amino nitrogen accumulation in potato plants and its association with susceptibility to drought, Physiol. Plant., 57 (1983) 169–173.
[3] K.B. Storey and J.M. Storey, Freeze tolerance in animals, Physiol. Rev., 68 (1988) 27–84.
[4] R.E. Lee, Principles of insect. Low temperature tolerance, in D.L. Denlinger, R.E. Lee (Eds.) Insects at Low Temperature, Chapmann and Hall, New York, 1991, p. 17.
[5] V. Rajagopal, V. Balasubramanian and K. Sinha, Diurnal fluctuations in relative water content, nitrate reductase and proline content in water-stressed and non-stressed wheat, Physiol. Plant., 40 (1977) 69–71.
[6] R.J. Lalonde, J.R. Lepock and J. Kruuv, Site of freeze-thaw damage and cryoprotection by amino acids of the calcium ATPase of sacroplasmatic reticulum, Biochim. Biophys. Acta, 1079 (1991) 128–138.
[7] P.H. Rasmussen, Characterization and stabilization of fish protein during frozen storage, Ph.D. Thesis, The Technical University of Denmark, Denmark, 1995.
[8] T.E. Creighton, Stability of folded conformations, Current Opinion in Structural Biology, 1 (1991) 5–16.
[9] A. Hvidt and P. Westh, Stabilization and destabilization of protein conformations, in Protein Interactions, H. Visser (Ed.), Protein Interactions, VCH Weinheim, 1992, p. 327.
[10] F. Franks, The properties of aqueous solutions at subzero temperatures, in F. Franks (Ed.), Water: A Comprehensive Treatise, Plenum Vol. 7, New York, 1982, p. 215.
[11] F. Franks, Solute-water interactions. Do polyhydroxy compounds alter the properties of water, Cryobiology, 20 (1983) 298–309.
[12] B.J. Luyet, Physical changes occurring in frozen solutions during rewarming and melting, in G.E.W. Wolstenholme, M. O'Connor (Eds.), The Frozen Cell, Churchill J&A, London, 1970, p. 27.
[13] L. Slade and H. Levine, Beyond water activity: Recent advances based on an alternative approach to the assessment of food quality and safety, Crit. Rev. Food Sci. Nutr., 30 (1991) 115–360.

[14] F.H. Cocks, W.H. Hildebrandt and L. Shepard, Comparison of the low-temperature crystallization of glasses in the ternary systems H_2O-NaCl-dimethyl sulfoxide and H_2O-NaCl-glycerol, J. App. Phys., 46 (1975) 3444–3449.

[15] H. Nicolajsen and A. Hvidt, Phase behaviour of the system trehalose–NaCl–water, Cryobiology, 31 (1994) 199–205.

[16] M.J. O'Neil, Differential scanning calorimetry for rapid exothermic transition, Anal. Chem., 57 (1985) 2005–2007.

[17] D.H. Rasmussen and A.P. MacKenzie, Clustering in supercooled water, J. Chem. Phys., 59 (1973) 5003–5013.

[18] D. Simatros, M. Faure, E. Bonjour and M. Couach, The physical state of water at low temperatures in plasma with different water contents as studied by differential thermal analysis and differential scanning calorimetry, Cryobiology, 12 (1975) 202–208.

[19] International Critical Tables 1928, Vol. IV, 1. edn., 1928, p. 235.

[20] A. Hvidt and K. Borch, NaCl-H_2O systems at temperatures below 273 K, studied by differential scanning calorimetry, Thermochim. Acta, 175 (1991) 53–58.

[21] J. Kristiansen and P. Westh, Freezing behaviour of multilamellar vesisles on 0.9% sodium chloride, Cryo-Letters, 12 (1991) 167–176.

[22] A.S. Rudolph and J.H. Crowe, A calorimetric and infrared spectroscopic study of the stabilizing solute proline, Biophys. J., 50 (1986) 423–430.

[23] J.P. Greenstein and M. Winitz, Chemistry of amino acids, John Wiley & Sons, New York, 1961, p. 523.

[24] R.H.M. Hatley, C.V.N. Berg and F. Franks, The unfrozen water content of maximally freeze concentrated carbohydrate solutions: validity of the methods used for its determination, Cryo-Letters, 12 (1991) 113–124.

[25] B. Schobert and H. Tschesche, Unusual solution properties of proline and its interaction with proteins, Biochim. Biophys. Acta, 541 (1978) 270–277.

[26] J. Kristiansen, Leakage of a trapped fluorescent marker from liposomes: Effects of eutectic crystallization of NaCl and internal freezing, Cryobiology, 29 (1992) 575–584.