Freezing and Ice Recrystallization Properties of Sucrose Solutions Containing Ice Structuring Proteins from Cold-Acclimated Winter Wheat Grass Extract

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ABSTRACT: The freezing properties of sucrose solutions containing ice structuring proteins (ISPs) from cold-acclimated winter wheat grass extract (AWWE) were evaluated. Neither significant ice nucleation nor thermal hysteresis activity in unpurified AWWE were detected (P > 0.05). Ice recrystallization in sucrose solutions was assessed by bright field microscopy. Ice crystal growth was significantly reduced with the addition of more than 0.05% total protein from AWWE in 23% sucrose solutions frozen under static conditions and temperature cycled under long periods of time (60 min). Ice recrystallization inhibition was not evident when the samples were temperature cycled for shorter periods of time (10 min), indicating that an adsorption time may be required for the ISPs to be significantly active. The ice recrystallization was reduced with the increased on ISP concentration until reaching a plateau after adding 0.13% total protein from AWWE, representing a surface coverage of 9 mg protein/m² ice (assuming 100% adsorption). The reduction of ice crystal growth was as high as 74% compared with the control. This ice recrystallization inhibition offers significant opportunity for the use of ISPs from AWWE in frozen foods.

Keywords: freezing, ice recrystallization, ice structuring proteins, ice crystal growth

Introduction

All plants that tolerate exposure to freezing temperatures have evolved mechanisms to allow them to avoid intracellular ice formation because of the loss of compartmentalization that occurs when growing ice crystals rupture cellular membranes. The process of freezing tolerance can be considered to be a 2-stage mechanism (Griffith and Antikainen 1996). In the 1st stage, ice nucleating proteins are produced extracellularly by the plant (Pearce 2001). These proteins exert a protective role by inducing ice nucleation at a temperature that gives the optimal cooling rate, and also by promoting ice nucleation in the extracellular space to ensure that ice formation takes place in compartments where little damage is caused. Although heterogeneity in the distribution of ice in plant tissues has been observed using various techniques (Wcisniewski and others 1997), intrinsic ice nucleators have been characterized in only a few freezing-tolerant plants (Ashworth and others 1985; Brush and others 1994; Lundheim and Wahlberg 1998). In the 2nd stage of the freezing-tolerance process in plants, ice crystal growth is mainly controlled by another category of plant ice structuring proteins (ISPs) (Clarke and others 2002): the recrystallization inhibition proteins. The presence of recrystallization inhibition proteins has been reported in more than 27 species of higher plants (Griffith and others 1992, 1997; Urrutia and others 1992; Duman and Olsen 1993; Duman 1994). They are extracellular, apoplastic proteins found in the xylem, cell walls, and intercellular spaces of various plants (Marentes and others 1993). Their activity is observed only when the plants are acclimated at low temperatures (Urrutia and others 1992).

The abilities of plant ISPs to promote ice nucleation and to inhibit recrystallization during freezing and thawing suggest possibilities for their use as natural ice modulators in the cold storage of food (Griffith and Ewart 1995; Goff and others 2002). The texture of frozen foods could be improved by the inhibition of ice recrystallization, especially foods such as ice cream and popsicles that are eaten while frozen. The inhibition of ice crystal growth may preserve the smooth, creamy texture of a high-quality product. The reduction of ice recrystallization may also be important in foods that are eaten after they have thawed. The quality of baked products from frozen dough often suffers as a result of ice crystal damage. Large intracellular ice crystals in tissues such as meat and fish may damage cell membranes and cause increased drip during thawing. This could result in a lower-quality frozen product due to reduced water-holding capacity and loss of nutrients from the tissue (Griffith and Ewart 1995). Many of these applications may be improved by the use of ISPs. Using a plant extract as the source of ISPs brings considerable advantage in availability and consumer acceptance versus the use of similar active compounds derived from other sources such as gene transfer technology (Feeney and Yeh 1998).

Our objectives in this study were therefore to characterize the freezing properties (ice nucleation, freezing point, and enthalpy of crystallization) of cold-acclimated winter wheat grass extract (AWWE) in sucrose solutions, to analyze the concentration effect of AWWE on the ice crystal growth in quiescently frozen sucrose solutions and to determine the effect of heated acclimated (HAWWE) and non-cold acclimated (NAWWE) winter wheat grass extract on the ice recrystallization of sucrose solutions.
Materials and Methods

Freezing properties of AWWE by differential scanning calorimetry

The freezing properties (onset of freezing, freezing point depression, and enthalpy of freezing) of (1) AWWE (Ice Biotech, Flamborough, Canada), (2) heated AWWE (85 °C, 10 min), (3) AWWE containing 23% w/w sucrose (Fisher, Toronto, Canada), and (4) 25% sucrose solution in water (control with the same freezing point depression as the sucrose solution + AWWE) were determined by differential scanning calorimetry (Q1000 DSC, TA Instruments, New Castle, Del., U.S.A.). The AWWE contained 4.33% total solids, of which 12.05% were proteins (or 0.52% wet basis). Nucleation was evaluated by annealing the sample (between 6 and 8 mg) at −3 °C for 1 h and then cooling to −30 °C at 0.3 °C/min. All samples were made in triplicate and the determination of statistically significant differences for each sample was carried out using the analysis of variance (ANOVA) single factor and the least significant difference (LSD) test.

Ice recrystallization in sucrose solutions containing AWWE

Solutions of varying concentrations (Table 1) were prepared with AWWE for ice recrystallization assays. The solutions were pre-cooled and quenched to −50 °C. The solutions were then warmed to −4 °C, and recrystallization was stimulated by cooling the samples to −8 °C at −0.2 °C/min. The freezing properties (onset of freezing, freezing point depression, and enthalpy of freezing) of 23% sucrose solutions and AWWE were prepared for ice recrystallization assays. The freezing point depression and the enthalpy of freezing were determined using the Image Processing Tool Kit. The % water frozen in the solution was calculated from the total initial ice mass, the solutions at time zero were used to calculate the total initial ice volume and, having the density of ice, the total initial ice mass that was observed in the image at time zero was also estimated. The surface area coverage of the protein was calculated by dividing the mg of protein present in the image acquired between the mean total ice surface area estimated from the image.

Results and Discussion

Freezing properties of AWWE by DSC

The freezing properties (onset of freezing, freezing point depression, and enthalphy of freezing) of 23% sucrose solutions and AWWE in 23% sucrose solutions, heated or not heated, were not significantly different (P > 0.05, Table 1). Therefore, it can be assumed that ice nucleators were not present in the extract or at least not in a considerable amount. This freezing curve of this solution was created and from this, the % water frozen (in mass) at the experimental temperature (−4 °C) was extrapolated. The mean total ice area and the mean ice crystal equivalent diameter (X50) of the solutions at time zero were used to calculate the total initial ice volume and, having the density of ice, the total initial ice mass that was observed in the image at time zero was also estimated. Considering the % water frozen in the solution and the initial total ice mass on the image at time zero, the total amount of solution and the mg of protein present in the acquired image were determined. The surface area coverage of the protein was calculated by dividing the mg of protein present in the image acquired between the mean total ice surface area estimated from the image.

Ice recrystallization in sucrose solutions containing AWWE

The results from ice recrystallization tests in sucrose solutions

Table 1—Freezing properties of cold-acclimated winter wheat grass extract (AWWE) containing 23% sucrose, its control (25% sucrose in water), and AWWE before and after heating (mean ± SD values)*

<table>
<thead>
<tr>
<th>Freezing properties</th>
<th>AWWE + 23% sucrose</th>
<th>25% Sucrose</th>
<th>AWWE</th>
<th>HAWWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset freezing point (°C)</td>
<td>−11 ± 2.65a</td>
<td>−12.07 ± 0.45a</td>
<td>−10.44 ± 0.5b</td>
<td>−10.08 ± 0.69b</td>
</tr>
<tr>
<td>Peak freezing point (°C)</td>
<td>−7.83 ± 2.03a</td>
<td>−8.4 ± 0.4a</td>
<td>−4.74 ± 0.27b</td>
<td>−4.79 ± 0.49b</td>
</tr>
<tr>
<td>Enthalpy of freezing (kJ/kg)</td>
<td>125 ± 20a</td>
<td>152 ± 3a</td>
<td>242 ± 11b</td>
<td>227 ± 7b</td>
</tr>
</tbody>
</table>

*Same letter in the same row on each effect indicate no significant difference with P ≥ 0.05.

**HAWWE = heated acclimated winter wheat grass extract.
containing AWWE demonstrated that the short temperature cycles at high frequency cycling resulted in no evident reduction of ice crystal growth with the addition of AWWE ($P > 0.05$) (Figure 1b).

However, after the long cycles and low frequency temperature treatment (Figure 1c), a significant inhibition of recrystallization was detected when more than 0.05% of total protein from AWWE was added to the sucrose solution ($P \leq 0.05$), after which, at approximately 0.13%, a plateau was reached (Figure 2 and 3). Assuming a 100% adsorption of the protein from AWWE to the ice crystal, the ISP coverage on the ice surface area at 0.13% would be about 9 mg/m². The values used to calculate this number are shown in Table 2. In this case, as much as 74% reduction of ice crystal growth in sucrose solutions containing AWWE compared with the sucrose solutions without AWWE was observed (Figure 3). The effect on the reduction of ice crystal growth was mainly in the 1st cycle.

Ice crystal growth curves for 23% sucrose solutions with and without AWWE, heated AWWE, and NAWWE from the ice recrystallization assay are shown in Figure 4. The activity of the AWWE in inhibiting recrystallization was reduced significantly after heating the extract ($P \leq 0.05$). The final ice crystal size of NAWWE was significantly smaller than the 1 for the control; however, the % ice crystal growth was not significantly reduced ($P > 0.05$).

To understand better the mechanism of action of ISPs in our model systems, an analysis of the expected physicochemical changes occurring in the system through the recrystallization assay will be attempted. The 1st step in the assay involves a quench cooling step to avoid crystallization in a dendritic shape and promote the formation of spherical crystals. When freezing occurs rapidly, the system can follow any arbitrary path on the phase diagram. Very rapid freezing causes the system to cool well below the freezing point depression curve. If cooling is to an extremely low temperature (as in our case, –50 °C), the system will remain as small ice crystals that may be imbedded in a glassy matrix and will remain as such until temperature is increased (Hartel 2001). Until this point, and assuming that the presence of the AWWE in the sucrose solution does not promote considerable ice nucleation, both of the systems.

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)

![Table 2](table2.csv)

**Table 2—Calculations for protein coverage of ice surface area.** The values were calculated from the total image area ($6.1 \times 10^5 \mu m^2$) considering a crystal monolayer and assuming circular geometry*  

| Initial total ice area | $2.9 \times 10^5 \mu m^2$ | Initial X50 value | 18.2 | Total ice surface area | $1.2 \times 10^6 \mu m^2$ | Total ice volume | $3.5 \times 10^6 \mu m^3$ | Density of ice | 0.917 g/cm³ | Total ice mass | 3.23 μg | Total liquid water mass | 3.11 μg | Total initial water in solution | 6.34 μg | Total mass of solution | 8.34 μg | Total protein from AWWE | 0.01 μg | Surface coverage | $9.3 \times 10^{-9} \mu g/\mu m^2$ | 9.33 mg/m² |
|----------------------|--------------------------|------------------|-----------|------------------------|--------------------------|---------------------|----------------------|---------------|-----------------|-----------------|------------------|-----------------|--------------------------------|----------------|---------------------------|-----------------|------------------------|-----------------|------------------------|

*AWWE = cold-acclimated winter wheat grass extract.
with and without AWWE, could be considered very similar (apart from the fact that some of the ISP proteins could get trapped in the ice crystals because of the fast crystallization of the sample).

As the temperature is raised to –10 °C, irruptive recrystallization is promoted with the subsequent increase in the mean ice crystal size. Irruptive recrystallization occurs when the full amount of crystalline material has not been formed due to some type of constraint (molecular mobility) during processing, but then subsequent rewarming causes additional crystallization (Hartel 2001). During the holding time of 10 min at –10 °C, the sample will try to reach a phase equilibrium dictated by the freezing curve. Accretion and Ostwald ripening will be the favored ice recrystallization mechanisms under constant temperature conditions. Due to the small difference in thermodynamic equilibrium between large and small crystals defined by the Kelvin or the Gibbs-Thomson equation (Yeh and Feeney 1996), the small crystals will disappear and the large ones will grow (Fennema 1973). The inhibitory influence of ISPs on ice crystal growth has been proposed to derive from local ice surface curvature effects induced by the adsorption of these proteins at the ice-solution interface. This is referred as the adsorption-inhibition hypothesis (Brown and others 1985; DeVries 1986) and it is explained by the same thermodynamic principle: the Kelvin effect. During this holding period at –10 °C, the effect in the retardation of ice recrystallization from the ISPs adsorption could start to be evident.

During temperature cycling, the ice volume fraction in the sample will vary according to the temperature, following the freezing curve. In the pre-cycling treatment, warming up will cause ice to melt and ISPs to be desorbed from the ice interface. No published data about ISP desorption have been found. However, it has been suggested that ISPs are released from the ice crystal surface with the same ease as water molecules (equally thermodynamically favorable), supported by the fact that the melting points of solutions with or without ISPs have been shown to be the same (Knight and Wierzbicki 2001), although, the diffusion kinetics of ISPs and water may be still different. Upon cooling, the rate will be a major factor influencing the ability of ISPs to retard ice crystal growth. If the cooling rate is high, the ice growing front may engulf some ISPs before they are adsorbed into the ice surface and, as a result, the ice crystal growth will not be inhibited. Conversely, if the cooling rate is low enough to allow the ISP molecules to migrate from the bulk solution to the crystal interface, orient into the proper conformation and then diffuse around the ice crystal surface to find an appropriate kink site for incorporation into the ice lattice, then the ISPs adsorption will be favored. Simultaneously, other solute molecules must diffuse away to allow ISP adsorption (or growth of the ice crystal). With increasing the number of cycles, the differences in diffusion rates of water and ISPs could cause the formation of a local concentration gradient at the ice-interface surroundings, promoting the ISP adsorption (or trapping).

It is important to keep in mind that the driving force for ISP adsorption is governed by the difference in the interaction energy (interfacial energy) of the portion of the ISP molecule that contacts the ice against its interfacial energy with water (Knight and Wierzbicki 2001). Even if the conditions permit the diffusion and proper orientation of ISPs molecules in solution, the irreversible interaction of an ISP molecule with ice would be favored only when the total free energy in the system is reduced. If the adsorption has occurred into a non-thermodynamically favorable binding site, the ISP will desorb and water molecules will be attached to the ice crystal instead. If the binding site is the most favorable, an irreversible ISP adsorption instead of a dynamic equilibrium has been proposed to take place because if this did not occur, in freezing hysteresis experiments, every time an ISP detaches from the surface, the interface where it had been attached would advance irreversibly because the water is supercooled (Knight and others 1991).

In summary, in the recrystallization assay applied in this research, during any cycling temperature treatment, the ISP adsorption may retard ice crystal growth by either (1) inhibiting the formation of more ice in the system when cooling down: an analogous behavior to the non-colligative freezing point depression, mainly explained by the Kelvin or the Gibbs-Thomson equation (Yeh and Feeney 1996), or (2) reducing the ice recrystallization mechanisms occurring during temperature cycling: shrink-melt-grow, shrink-melt-regrow, and accretion. Because recrystallization is mainly caused by the size-dependence of the melting point through the Kelvin effect, its inhibition is also through freezing hysteresis. However, only a very small amount of freezing hysteresis is needed because the amounts of supercooling involved in the process are of the order of 0.01 °C (Knight and Wierzbicki 2001). This thought explains why an ISP can be effective in retarding recrystallization at very low concentrations and show no thermal hysteresis, as is the case in most of plant ISPs.

In our experiments, the effect of ISPs in the inhibition of ice crystal growth was not significant (P > 0.05) at the end of the pre-cycling period (time zero). The purpose of the pre-cycling period in the recrystallization assay was to obtain a measurable ice crystal distribution as a starting point for the cycling temperature treatment that followed (long cycles at low frequency or short cycles at high frequency). The fact that the ice crystal growth observed in samples containing similar concentrations of ISPs subjected to the long cycling treatment (192 min; comprising 2 cycles of 60-min intervals) is less than after the short cycle treatment (138 min; comprising 5 cycles of 10-min intervals), suggests that the number of cycles could be determinant in the effectiveness of the ISPs and/or an adsorption time may be required for the ISPs to be considerably active (longer periods of time at constant temperature). This implies that the ISPs offer low resistance against the ice recrystallization mechanisms occurring during temperature cycling and their most important effect occurs at constant temperature.

ISP showed activity retarding recrystallization only in the 1st cycle of the recrystallization assay, and this is explained by the as-

Figure 4—Ice crystal growth curves for 23% sucrose solutions containing 0.4% total protein from (D) cold-acclimated winter wheat grass extract (AWWE); (A) heated acclimated winter wheat grass extract (HAWWE); (D) non-cold-acclimated winter wheat grass extract (NAWWE) and (x) their control (0% total protein) when solutions were subjected to 2 temperature cycles of 60 min from –4 °C to –10 °C.
ymptotic behavior of the ice crystal growth at constant temperature. At longer times the ice crystal size reaches a plateau because the thermodynamic driving force for Ostwald ripening is lower at larger ice crystal sizes than at smaller sizes.

The increase of inhibition of ice recrystallization with the increase of ISP concentration agrees with the adsorption-inhibition hypothesis, ice crystal growth ceases when the surface concentration of adsorbed ISP molecules exceeds a critical concentration, the value of which is a decreasing function of temperature. In our study, we calculated the maximum % of ice coverage that ISP could achieve assuming 100% adsorption of the protein in their critical concentration. In fact, the ISP activity at retarding ice recrystallization could also be increased in the later periods at constant temperature because the reduction in the total ice surface area (due to the melting of the small crystals and growth of the larger ones) will promote an increase in the local ISP concentration on the ice-water interface of the remaining larger crystals.

The results shown in Figure 4 suggest that the cold-acclimation in winter wheat grass promotes ISP synthesis, as has been previously reported (Griffith and others 1992; Urrutia and others 1992; Marentes and others 1993). In the same figure, the elimination of ISP activity with the heat treatment implies ISP heat denaturation. However, because the AWWE has not been purified, this loss of activity could also be due to the formation of complexes between the other solids present in the raw AWWE and the ISPs.

Conclusions

The freezing profiles of sucrose solutions with or without winter wheat grass extract were evaluated before and after a heat treatment. The onset of freezing, freezing point, or enthalpy of crystallization were not significantly modified with the addition of the AWWE or the thermal treatment of the extract. Similar onsets of freezing suggest that there is not a strong ice nucleation activity in the plant and/or that the efficacy in the extraction of ice nucleators from the leaf is low.

Ice crystal growth was significantly reduced with the addition of more than 0.05% total protein from AWWE in 23% sucrose solutions frozen under static conditions and cycled with long periods at low frequency. At more than 0.13% of total protein from AWWE a plateau in the ability to retard recrystallization with the increasing of the AWWE concentration was reached, suggesting the formation of a saturated monolayer by the adsorption of the ISPs to the ice-water interface. The protein coverage of the ice surface was estimated to be 9 mg/m². Higher efficiency of the ISPs with longer periods at constant temperature indicates that the kinetics of diffusion and adsorption of the ISP to the ice crystal interface may play an important role in its activity at retarding ice recrystallization.

As proposed by Griffith and Ewart (1995) it appears that ISPs from AWWE offer enormous potential for their use in frozen foods as ice recrystallization inhibitors.

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References


