Extraction and Isolation of Antifreeze Proteins from Winter Rye (Secale cereale L.) Leaves

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Apoplastic extracts of cold-acclimated winter rye (Secale cereale L. cv Musketeer) leaves were previously shown to exhibit antifreeze activity. The objectives of the present study were to identify and characterize individual antifreeze proteins present in the apoplastic extracts. The highest protein concentrations and antifreeze activity were obtained when the leaf apoplast was extracted with ascorbic acid and either CaCl₂ or MgSO₄. Seven major polypeptides were purified from these extracts by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under nonreducing conditions. The five larger polypeptides, of 19, 26, 32, 34, and 36 kD, exhibited significant levels of antifreeze activity, whereas the 11- and 13-kDa polypeptides showed only weak activity. Five of these polypeptides migrated with higher apparent molecular masses on SDS gels after treatment with 0.1 M dithiothreitol, which indicated the presence of intramolecular disulfide bonds. The apoplastic extracts contained antifreeze proteins from fish and insects, and no common epitopes were detected. We conclude that cold-acclimated winter rye leaves produce multiple polypeptides with antifreeze activity that appear to be distinct from antifreezes produced by fish and insects.

AFPs have been discovered in a wide range of overwintering organisms, including marine teleost fishes (reviewed by Davies and Hew, 1990; DeVries and Cheng, 1992), terrestrial arthropods (reviewed by Duman et al., 1991), vascular plants (Griffith et al., 1992; Urrutia et al., 1992; Duman and Olsen, 1993), and nonvascular plants, fungi, and bacteria (Duman and Olsen, 1993). AFPs accumulate in most of these organisms during cold acclimation; therefore, it has been hypothesized that these proteins play an integral role in the mechanism of frost resistance (Duman et al., 1991; Duman and Olsen, 1993). Their biological role in fish that survive in icy polar oceans is to prevent freezing of the body fluids by lowering the freezing temperature noncolligatively (DeVries, 1986). On the other hand, ice actually forms in the intercellular spaces of freezing-tolerant organisms exposed to sub-zero temperatures (Pearce, 1988; Duman et al., 1991). In this case, the proteins are thought to modify rather than prevent the growth of ice. This may promote winter survival by decreasing the rate at which ice forms during the initial freezing event or by preventing recrystallization (the condensation of many small crystals into a few large crystals) during prolonged freezing events (Knight and Duman, 1986; Urrutia et al., 1992).

AFPs exhibit two unique characteristics in the way they interact with ice (reviewed by Yang, 1993). First, they adsorb onto the nonbasal planes of ice at the ice-water interface (Raymond et al., 1989) and exert a concentration-dependent effect on ice crystal growth morphology (DeVries, 1986). When ice crystals are grown in the presence of relatively low concentrations of AFPs, the pyramidal faces of the crystal are expressed, and the crystal appears hexagonal in shape. At sufficiently high concentrations of AFPs, ice grows parallel to the c axis of the crystal lattice with limited growth along the a axes to form long narrow spicules (DeVries, 1986). Second, AFPs depress the freezing temperature of a solution noncolligatively in that they lower the freezing temperature more than they lower the melting temperature. The difference between freezing and melting temperatures is termed thermal hysteresis and is measured by observing the growth of a seed crystal microscopically (DeVries, 1986). AFPs are often referred to as thermal hysteresis proteins in frost-tolerant organisms to differentiate proteins with similar in vitro properties but apparently different biological functions (Duman et al., 1991).

Overwintering organisms produce AFPs with different characteristic compositions and structures (reviewed by Davies and Hew, 1990). AFPs from fish have been classified as AFGPs and as type I, type II, and type III AFPs. AFGPs obtained from Antarctic fish range from 2.5 to 33 kD and are characterized by the presence of a repeated tripeptide, (Ala-Ala-Thr), in which the Thr is linked to a disaccharide. Type I AFPs from flounders and scallops are Ala-rich polypeptides of 3 to 5 kD, whereas type II AFPs are characterized as being

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Abbreviations: AA, ascorbic acid; AFGP, antifreeze glycoprotein; AFP, antifreeze protein; CA, citric acid.
rich in half-cystines, and their activities are decreased by sulfhydryl reagents. These AFPs are found in sea raven and Atlantic herring, both with a molecular mass of about 14 kD, and in smelt, with a molecular mass of 24 kD (Ewart and Fletcher, 1990). Type III AFPs have been found in ocean out, are 6 to 7 kD in size, and have no distinctive composition. Fewer AFPs have been purified from insects; however, these AFPs have been categorized into two groups that are based on the presence or absence of Cys (Duman et al., 1991). Structural studies of AFPs have served to emphasize the differences rather than the similarities among AFPs from different organisms. The x-ray crystal structure of type I AFP from flounder revealed that this protein is a single \(\alpha\) helix (Yang et al., 1988), whereas the NMR structure of type III AFP from ocean out showed that this protein contains \(\beta\) sheets with no \(\alpha\) helices (Sonnichsen et al., 1993). How AFPs with such different compositions and structures and found in such a diverse spectrum of ectothermic organisms can have similar properties in their interactions with ice is a fascinating aspect of this area of research, and various models of mechanisms by which AFPs bind to ice have been proposed (reviewed by Yang, 1993).

We have previously shown that winter rye (Secale cereale L.) leaves accumulate proteins in the apoplast during cold acclimation and that apoplastic extracts containing these proteins have the ability to modify ice (Griffith et al., 1992; Marentes et al., 1993). Partially purified apoplastic proteins obtained from winter rye leaves form ice crystals in solution that are hexagonal bipyramids and exhibit a thermal hysteresis of 0.3°C at a concentration of 60 mg of protein mL\(^{-1}\). This antifreeze activity was eliminated by treating the extracts with protease, a result that suggests that AFPs are produced by winter rye (Griffith et al., 1992). In the work presented here, we demonstrate that a number of individual polypeptides isolated from apoplastic extracts obtained from winter rye leaves have antifreeze activity. We also present a comparison of these polypeptides with AFPs previously isolated from fish and insects.

**MATERIALS AND METHODS**

**Plant Material**

Winter rye (Secale cereale L. cv Musketeer) plants were grown and cold acclimated as described before (Griffith et al., 1992; Griffith and McIntyre, 1993). About 20 seeds were sown in coarse vermiculite in 15-cm pots and placed in a 20/16°C (day/night) growth chamber with a 16-h daytime for 1 week to initiate germination. The plants were then transferred to 5/2°C with a 16-h daytime for 7 to 8 weeks before the leaves were harvested for extraction. The plants were watered weekly with modified Hoagland solution.

**Extraction of Apoplastic Proteins**

Noncovalently bound proteins were extracted from the apoplast of rye leaves using a modification of the method of Rohringer et al. (1983). Rye leaves were cut into 3-cm sections, placed in a Miracloth (Calbiochem, San Diego, CA) bag, and rinsed well with several changes of deionized water (type I water; Barnstead/Termolyne Corp., Dubuque, IA) to wash cellular proteins from the cut ends. The leaves were then vacuum infiltrated using a DuoSeal vacuum pump (model 1402; Welch Scientific Company, Skokie, IL) for 30 min in a buffer that contained 20 mm AA (pH 3) alone or with 20 mm CaCl\(_2\), EDTA, or MgSO\(_4\); 20 mm CA (pH 3); or 50 mm Tris-HCl (pH 8). The leaves were blotted dry between two layers of paper towels and inserted in bundles into plastic cones (Centricon; Amicon Canada Ltd., Oakville, Ontario, Canada). The cones were placed in centrifuge tubes, and the apoplastic extract was collected from the bottom of the tubes after the leaves were centrifuged at 2000g for 10 min.

**Protein Electrophoresis**

Extracted proteins were added to sample buffer (Laemmli, 1970) with or without 0.1 M DTT and then heated at 95°C for 5 min before loading. Polypeptides were separated on 12 or 15% polyacrylamide gels (7 \(\times\) 7 \(\times\) 0.15 cm) according to the method described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250.

**Isolation of Apoplastic Polypeptides**

Apoplastic polypeptides were isolated by elution from polyacrylamide gels (Hager and Burgess, 1980). The polypeptides were separated by preparative SDS-PAGE (16-18-18 0.15-cm gels) using 12 and 15% polyacrylamide gels to resolve all of the major polypeptides under reducing and nonreducing conditions, respectively. After electrophoresis, the gels were briefly rinsed in ice-cold deionized water, stained with 0.25 m ice-cold KCl for 10 min, and destained with ice-cold deionized water. Bands that could be observed by staining with KCl were excised, rinsed twice with deionized water, and crushed. The polypeptides were eluted overnight by diffusion with Tris-HCl (50 mm, pH 8) and 0.1% SDS. The crushed gel pieces were separated from the elution buffer by centrifugation, and the polypeptides were concentrated by precipitation with 4 volumes of acetone. The pellets were redissolved in 10 \(\mu\)L of 0.1 m NH\(_4\)HCO\(_3\) (pH 8). Two sets of elution experiments were carried out to investigate the effect of disulfide bond reduction on antifreeze activity. Approximately 1 mg of protein was separated under nonreducing conditions using four preparative gels and 0.6 mg under reducing conditions using two preparative gels.

**Antifreeze Activity Assay**

We routinely assayed antifreeze activity in extracts and during the course of purification by monitoring the ability of the polypeptides to modify the growth habit of ice crystals in a manner similar to that observed in fish antifreezes (DeVries, 1986). Each sample (10 mL) was loaded into the wells of a silver sample holder that had been placed on a freezing stage mounted on the stage of a phase-contrast photomicroscope (Olympus BHT; Carsen Medical and Scientific Co., Markham, Ontario, Canada). The temperature was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY). The samples were flash frozen to \(-40°C\) to form small ice crystals and then thawed until only one ice crystal remained in the well. The temperature was
then slowly decreased, and the morphology of the ice crystals was photographed. To avoid contamination from previous samples, the sample holder was cleaned by sonication after each assay. The sample holder was then checked for residual antifreeze activity with deionized water, which should form a round ice crystal, before a fresh sample was assayed.

To demonstrate the characteristic habit of ice crystal growth in the presence of winter rye AFPs, the growth sequence of an ice crystal formed in an apoplastic extract obtained from cold-acclimated winter rye leaves is shown in Figure 1. The ice crystal is viewed normal to the a axis, which are parallel to the basal plane of the ice crystal, and therefore, the crystal shown in Figure 1 is hexagonal in cross-section. As the temperature is lowered, the ice crystal grows from a truncated to a full hexagonal bipyramid along the c axis, which is normal to the basal plane (Fig. 1, top, left to right). The ice crystal does not increase noticeably in size upon further cooling until a threshold temperature is reached, at which point the crystal grows explosively along the a axes (Fig. 1, bottom).

The level of antifreeze activity depends on both the type and the concentration of antifreezes present in a sample. In this assay, lower activity is detected as a decrease in the extent of ice crystal growth along the c axis. For example, partial bipyramidal ice crystals may expand along the a axes without attaining a complete bipyramidal shape. Even lower activity is shown by ice crystals that are shaped like a hexagonal plate with well-defined faces and little c axis growth. An ice crystal that is characteristic of very low antifreeze activity forms a hexagonal plate, but the corners of the ice crystal become round as the crystal expands along the a axes. Examples of changes in ice crystal morphology that occur in a dilution series are shown in Figure 3B.

**Immunoblotting**

Immunoblotting was performed according to the method of Ghosh et al. (1989) with modifications. Apoplastic extracts were separated by SDS-PAGE and electroblotted onto 0.45-µm nitrocellulose membranes (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) using the Mini Trans-Blot cell (Bio-Rad) with a transfer buffer of 16 mM Gly, 25 mM Tris-HCl (pH 8.7), 0.02% (w/v) SDS, and 20% (v/v) methanol. The transfer was performed at 300 mA of constant current for 2 h. The blots were blocked in a buffer of 25 mM Tris-HCl (pH 7.6), 140 mM NaCl, and 1% (w/v) milk powder. Completeness of transfer was monitored by the appearance of prestained mol wt markers (Bio-Rad) on the blot. Anti serum against *Euglena Rubisco was used in a 1:5000 dilution. Rabbit antisera against the type II fish AFPs, namely AFPs from sea raven (SR2) and Atlantic herring (H2), were used in a 1:2000 dilution. Sea raven and Atlantic herring AFPs were included in these immunoblots as positive controls. The sea raven AFP (14 kD) was partially purified from serum by HPLC using a C18 column and a 0.05% TFA:acetonitrile gradient (Ng et al., 1986). Atlantic herring AFP (14.6 kD) was partially purified from serum by size exclusion chromatography using a Sephadex G-150 column with 0.1 M NH4HCO3 buffer (Ewart and Fletcher, 1990). The immunoreactions were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG with 5-bromo-4-chloro-3-indolyphosphate-toluidine salt and tetrazolium blue chloride. Rabbit antisera against insect AFPs, including anti- *Tenebrio molitor* AFP and anti- *Dendroides canadensis* AFP, were used at a dilution of 1:50 to increase the sensitivity of the immunodetection (J.G. Duman, personal communication).

**Amino Acid Composition**

Apoplastic extracts were electrophoresed on 12% polyacrylamide gels without reducing agent and electroblotted onto a membrane (Bio-Rad) using 3-[cyclohexylamin]-2-hydroxy-1-propanesulfonic acid transfer buffer (Matsudaera, 1987). The membrane was briefly stained with 0.1% Coomassie blue R-250 in 1% (v/v) acetic acid and 40% (v/v) methanol, destained with 50% (v/v) methanol, and rinsed thoroughly with deionized water. The bands corresponding to the eluted polypeptides were excised, hydrolyzed with 5.7 N HCl and 1% phenol at 100°C for 24 h, derivatized with phenyl isothiocyanate, and analyzed for amino acid compositions using the Waters Pico-Tag system (Waters Canada, Mississauga, Ontario, Canada). Cys was determined in a separate experiment using a polyvinylidenefluoride blot of the membrane. Rabbit antisera against Rubisco was used at a dilution of 1:5000. Bands containing individual polypeptides were excised from the blot, wetted with methanol followed by fresh performic acid.
acid at 5°C to oxidize Cys to cysteic acid, dried under vacuum, and then hydrolyzed and analyzed by the Waters Pico-Tag system as described above.

Assay of Protein Concentration

Protein concentrations of apoplastic extracts were determined by the Bradford (1976) method as modified by Bio-Rad. Concentrations of the eluted polypeptides were estimated using the method of Lowry et al. (1951) as modified by the Bio-Rad Detergent-Compatible Protein Assay. BSA was used as the standard protein in both cases.

RESULTS

Apoplastic Extraction of AFPs

The solution formerly used by our group to obtain apoplastic extracts was based on the procedure of Mauch and Staehelin (1989) and contained AA, EDTA, β-mercaptoethanol, and protease inhibitors (Griffith et al., 1992). In an optimal extraction of apoplastic protein, the extraction solution should yield high levels of extracted protein and low levels of intracellular contamination, as determined by the presence of Rubisco (Holden and Rohringer, 1985). An extraction buffer containing 50 mM Tris-HCl (pH 8), which was similar to that used by other researchers using the method (Rohringer et al., 1983), yielded an extract that appeared similar to that used by other researchers using the method (Staehelin, 1989) and contained AA, EDTA, β-mercaptoethanol, and proteinase inhibitors (Rohringer et al., 1983). Adding salts to the extraction buffer resulted in a qualitative and quantitative increase in the proteins eluted from cold-acclimated plants (Fig. 3A). When either 20 mM CaCl$_2$ or MgSO$_4$ was included in the extraction buffer, the protein concentrations of the apoplastic extracts were approximately twice as high (0.63 and 0.60 mg mL$^{-1}$, respectively) as that obtained with AA alone (0.32 mg mL$^{-1}$). The same set of polypeptides that migrated between 10 and 40 kD in SDS-PAGE was present in every extract, with the exception that the smallest polypeptide (10 kD) was observed only when the leaves were extracted with CaCl$_2$ or MgSO$_4$ and the polypeptides were separated under reducing conditions (Fig. 3A).

Antifreeze activity was compared semiquantitatively in the apoplastic extracts by serially diluting the extracts with the corresponding extraction buffer to determine at what dilution the activity would disappear (Fig. 3B). The endpoint of dilution was chosen such that, upon further dilution, hexagonal ice crystals did not form when the concentration was decreased. The extracts were adjusted to the same protein concentration (0.32 mg mL$^{-1}$) before activity was assayed. Extracts obtained with AA and CaCl$_2$ had higher ice modification activity than extracts obtained with AA alone, judging by the fact that a higher degree of dilution was required before antifreeze activity disappeared (Fig. 3B). Using the same technique, we observed that extracts containing EDTA and AA had activities similar to those of the ascobate extracts. Extracts containing MgSO$_4$ and AA and extracts containing CaCl$_2$ and AA exhibited similar activities. The addition of CaCl$_2$ or MgSO$_4$ to the AA or EDTA extracts did not enhance ice crystal modification (results not shown).

Isolation of Individual Antifreeze Polypeptides

Size exclusion chromatography (Sephacryl S-200; Pharmacia LKB Biotechnology, Uppsala, Sweden) was initially used to fractionate proteins exhibiting antifreeze activity (Griffith et al., 1992). However, all of the major apoplastic polypeptides co-eluted in two overlapping peaks, and all of the fractions within the peaks contained active antifreezes. Subsequent efforts to isolate individual proteins with antifreeze activity from the apoplastic extracts by conventional column chromatography were unsuccessful because of the similar elution properties of the polypeptides. We then discovered that antifreeze activity could be readily recovered from apoplastic extracts treated with 2% SDS and heat, followed by acetone precipitation and resolubilization in NH$_4$HCO$_3$. As a result, we decided to separate and elute the individual major polypeptides in their denatured forms from SDS-polyacrylamide gels. After apoplastic polypeptides were separated by SDS-PAGE under nonreducing conditions, a total of seven bands could be observed clearly with the KCl stain, and these were excised. Five of the eluted polypeptides,
Isolation of Antifreeze Proteins from Winter Rye Leaves

Figure 3. Comparison of apoplastic extracts obtained with different extraction buffers from cold-acclimated rye leaves. A, SDS-PAGE of apoplastic proteins denatured in the presence or absence of DTT. Protein concentrations for the extracts obtained with 20 mM AA (AA) alone, with 20 mM AA and 20 mM EDTA, CaCl₂, or MgSO₄, and with 50 mM Tris-HCl were 0.32, 0.51, 0.63, 0.60, and 0.66 mg mL⁻¹, respectively. Each lane was loaded with an equal volume (5 µL) of extract, and the gel was stained with Coomassie brilliant blue. Molecular mass markers (M) were separated in the middle lane. B, Antifreeze activity of apoplastic extracts obtained with or without salt present in the extraction medium. The extract obtained with AA plus CaCl₂ was diluted to the same protein concentration as the extract obtained with AA alone. Antifreeze activity was quantitated by serial dilution of the extracts, and, therefore, the ratios denote dilution factors. In all cases, the morphology of the ice crystal was photographed before explosive growth along the a axis occurred. Magnification bars = 10 µm.

with molecular masses of 19, 26, 32, 34, and 36 kD, showed a high degree of antifreeze activity as they modified the normal growth habit of ice crystals so that partial or full hexagonal bipyramids formed in solution (Fig. 4). The 13- and 11-kD polypeptides exhibited very little antifreeze activity (Fig. 4). The protein concentrations of the five more active polypeptides in solution ranged from 0.41 to 0.53 mg mL⁻¹, whereas the less active polypeptides were about 2 to 3 times more concentrated (Fig. 4). Thus, the difference in antifreeze activity between the 19-, 26-, 32-, 34-, and 36-kD polypeptides and the 11- and 13-kD polypeptides is not due to differences in protein concentration. However, one interesting feature is that the bipyramidal ice crystals that formed in solutions of individual polypeptides have more clearly defined pyramidal faces (Fig. 4) than the rounder ice crystals that formed in crude apoplastic extracts (Fig. 1).

Two negative controls were performed to ascertain that the ability to modify ice crystal morphology was not conferred upon the polypeptides by the isolation process. First, BSA was separated by SDS-PAGE, eluted from the gel, precipitated with acetonitrile, and resolubilized in the same manner as the AFPs. Second, a piece of gel from the side that was not used to separate proteins was excised and treated similarly. Neither BSA nor the polycrylamide gel itself modified the normal growth habit of ice crystals (Fig. 5), which indicated that antifreeze activities observed in the eluted polypeptides were not conferred by the experimental procedures.

Effect of Disulfide Bond Reduction on Antifreeze Activity

Disulfide-reducing agents have been reported to decrease the activity of AFPs isolated from certain insects, plants (Duman et al., 1991), and fish (Slaughter et al., 1981; Ewart and Fletcher, 1989); therefore, it was necessary to determine the effect of reducing agents on AFPs from rye leaves. As shown in Figure 6, the electrophoretic patterns of the major apoplastic polypeptides were different in extracts treated with...
Eluted BSA Empty gel

Figure 5. Negative control experiments used to test the purification procedures. No antifreeze activity was observed in 1 mg of BSA, which had been denatured, electrophoresed, stained with KCl, eluted from the polyacrylamide gel, precipitated with acetone, and redissolved in 0.1 M NH₄HCO₃. No antifreeze activity was observed in eluates of the SDS-polyacrylamide gels themselves.

the disulfide-reducing agent 0.1 M DTT. In these experiments, apoplastic extracts were denatured in sample buffers with and without DTT and then electrophoresed in adjacent lanes and in widely separated lanes (Fig. 6). We observed that polypeptides electrophoresed in the absence of DTT underwent disulfide bond reduction as DTT diffused in from an adjacent lane, resulting in a lane that was half-reduced and half-nonreduced (Fig. 6, right lane). This lane showed that five polypeptides, corresponding to the 32-, 26-, 19-, 13-, and 11-kD polypeptides in Figure 4, migrated as polypeptides with higher molecular masses after disulfide reduction. These results indicate that these five polypeptides possess intramolecular disulfide bonds because further unfolding of the polypeptides upon cleavage of disulfide bonds allows binding of more SDS, resulting in higher negative charge/mass ratios. To investigate whether reduction of disulfide bonds affected antifreeze activity, seven major polypeptides were excised from SDS-polyacrylamide gels run under reducing conditions (Fig. 7). The polypeptides of 38, 36, 29, and 26 kD, which corresponded to the nonreduced 36-, 34- and 32-, 26-, and 19-kD bands, respectively, modified the normal growth pattern of ice crystals in that partial or complete hexagonal bipyramids were formed. The lower molecular mass polypeptides, which migrated at 15 and 13 kD when they were reduced and at 13 and 11 kD when they were not reduced, exhibited little activity (Fig. 7). The 10-kD polypeptide was clearly resolved only when it was reduced, and it exhibited little activity as well (Fig. 7).

Comparison of AFPs from Winter Rye, Fish, and Insects

As shown in Table I, the AFPs from winter rye have similar amino acid compositions. They are all relatively enriched in the amino acid residues of Asp/Asn, Glu/Gln, Ser, Gly, Thr, and Ala. In addition, the rye AFPs all lack His, except for the presence of 0.63 mol% His in the 26-kD nonreduced polypeptide. When the amino acid analysis was repeated after separating the polypeptides in their reduced form and oxidizing Cys with performic acid, the 38-, 36-, 29-, 26-, and 15-kD polypeptides shown in Figure 7 were determined to contain between 2 and 5 mol% Cys.

Figure 6. Effect of DTT on polypeptides present in an apoplastic extract obtained from cold-acclimated winter rye leaves. Apoplastic polypeptides denatured in the presence or absence of 0.1 M DTT were loaded onto polyacrylamide gels (6 μg per lane) either one lane apart (left side of gel) or side by side (right side of gel) to determine the effect of DTT on the apparent molecular mass of the polypeptides. Molecular mass markers (M) were separated in the middle lane. Apoplastic polypeptides electrophoresed in the far right lane were affected by DTT that diffused from the next lane; therefore, that half of the lane under reducing conditions and the other half was run under nonreducing conditions. Arrows indicate polypeptides that migrated to different positions after cleavage of disulfide bonds. The gel was stained with Coomassie brilliant blue.

Figure 7. Antifreeze activity of reduced apoplastic polypeptides. An apoplastic extract containing approximately 0.6 mg of protein was denatured in sample buffer containing 0.1 M DTT at 95°C for 5 min and was then electrophoresed on two 15% preparative polyacrylamide gels. All of the major polypeptides were isolated in the same manner as those shown in Figure 4. Molecular masses were determined for the reduced forms of the polypeptides by comparison with known standards (Bio-Rad). Magnification bar =
We examined immunoreactivity between AFPs from winter rye and antibodies to the cystine-rich AFPs from fish. None of the rye polypeptides that exhibited antifreeze activity when eluted from polyacrylamide gels reacted with antibodies raised against either the sea raven AFP or the Atlantic herring AFP (Fig. 8). However, a group of polypeptides that migrated at approximately 90 kD reacted positively when probed with antisera to the AFPs from both fish (Fig. 8). Immunoblots of rye polypeptides probed with antisera to

### Table I. Amino acid compositions (mo%) of apoplastic polypeptides obtained from cold-acclimated winter rye leaves

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<th>Amino Acid Residue</th>
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</table>

Representative amino acid compositions of AFPs from fish and insects are shown in Table II. The chemical compositions of the winter rye AFPs are obviously distinct from those of the fish AFGPs and type I AFPs, which are highly enriched in Ala, and type III AFPs, which, like the former two groups, possess no Cys residues. The cystine-containing AFPs, either from fish or insects, typically have higher contents of Cys residues, ranging from 8 to 28 mol%, than observed in the AFPs from winter rye.

### Table II. Representative amino acid compositions (mo%) of AFPs from fish and insects: AFGP 8 from polar cod (Osuga and Feeney, 1978); type I AFP from winter flounder (Hew et al., 1986); type II AFP (SR2) from sea raven (Ng et al., 1986); type II AFP (H2) from Atlantic herring (Ewart and Fletcher, 1990); type III AFP (SP-1-A) from ocean pout (Li et al., 1985); TC-2 and T4 from the beetle Tenebrio molitor (Patterson and Duman, 1982; Tomchaney et al., 1982); H1 from the beetle Dendroides canadensis (Wu et al., 1991)

<table>
<thead>
<tr>
<th>Amino Acid Residue</th>
<th>Fish</th>
<th>Insects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFGP</td>
<td>Type I</td>
</tr>
<tr>
<td>Asp/Asn</td>
<td>0</td>
<td>11.5</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>Ser</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Gly</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arg</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>Thr</td>
<td>33.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Ala</td>
<td>50</td>
<td>62.8</td>
</tr>
<tr>
<td>Pro</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td>Tyr</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Val</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Met</td>
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<td>0</td>
</tr>
<tr>
<td>Cys</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leu</td>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>Phe</td>
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<tr>
<td>Lys</td>
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<td>3.3</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>33.3</td>
<td></td>
</tr>
</tbody>
</table>

* Values are equal to Thr (assuming one disaccharide per Thr).
The method used to extract the leaf apoplast has been well established and is widely used by researchers studying proteins secreted by plants in response to pathogen invasion (reviewed by Carr and Klessig, 1989; Bol et al., 1990). The concern of intracellular contamination from the cut edges of the leaves was addressed by Holden and Rohringer (1985), who showed that washing the cut leaves is essential to reducing contamination and that Rubisco is an appropriate marker to use to assay contamination of the apoplastic extracts. In our experiments, the leaves are well washed, and it appears that intracellular contamination is dependent on the buffer used to extract the apoplast. The presence of 20 mM AA in the extraction buffer yields a clear, colorless extract uncontaminated by Rubisco (Figs. 2 and 3A). The role that a low concentration of AA plays in preventing cell lysis during the extraction process is unclear.

It is apparent that the majority of apoplastic polypeptides extracted from cold-acclimated winter rye leaves exhibit ice-binding activity. Five major polypeptides were recovered from the extraction process is unclear.

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reducing contamination and that Rubisco is an appropriate

Apoplastic polypeptides (7 μg of protein), Atlantic herring AFP (4 μg of protein), and sea raven AFP (4 μg of protein) were electrophoresed on a 15% polyacrylamide gel and then immunoblotted with polyclonal antisera against sea raven and Atlantic herring AFPs. Both antisera were used in a 1:2000 dilution. The positive controls, Atlantic herring AFP and sea raven AFP, have molecular masses of 14.6 and 14.0 kD, respectively (indicated by arrows).

Figure 8. Western blot analysis of winter rye apoplastic extracts probed with antisera against cystine-containing AFPs from fish. Apoplastic polypeptides (7 μg of protein), Atlantic herring AFP (4 μg of protein), and sea raven AFP (4 μg of protein) were electrophoresed on a 15% polyacrylamide gel and then immunoblotted with polyclonal antisera against sea raven and Atlantic herring AFPs. Both antisera were used in a 1:2000 dilution. The positive controls, Atlantic herring AFP and sea raven AFP, have molecular masses of 14.6 and 14.0 kD, respectively (indicated by arrows).

AFPs from Tenebrio molitor and Dendroides canadensis were entirely negative, which suggests that there are no common epitopes between the AFPs from winter rye and the AFPs of these two insects (J.G. Duman, personal communication).

**DISCUSSION**

We have identified a number of antifreeze polypeptides in apoplastic extracts from cold-acclimated winter rye leaves by improving the extraction buffer and using an unconventional isolation method. We have also characterized these polypeptides by observing the effect of disulfide bond reduction on antifreeze activity (Figs. 4, 6, and 7), by determining the amino acid compositions (Table I), and by analyzing immunoblots obtained using antibodies against cystine-containing AFPs from other organisms (Fig. 8).

The method used to extract the leaf apoplast has been well established and is widely used by researchers studying proteins secreted by plants in response to pathogen invasion (reviewed by Carr and Klessig, 1989; Bol et al., 1990). The concern of intracellular contamination from the cut edges of the leaves was addressed by Holden and Rohringer (1985), who showed that washing the cut leaves is essential to reducing contamination and that Rubisco is an appropriate marker to use to assay contamination of the apoplastic extracts. In our experiments, the leaves are well washed, and it appears that intracellular contamination is dependent on the buffer used to extract the apoplast.

SDS-polyacrylamide gels (19, 26, 32, 34, and 36 kD, nonreduced) showed high levels of antifreeze activity (Fig. 4). These polypeptides also have similar amino acid compositions (Table I). The presence of multiple antifreeze polypeptides with closely related amino acid compositions is not uncommon in the other overwintering organisms that also produce AFPs. Microheterogeneity has been observed for all type I, II, and III AFPs in fish because slight variations in amino acid sequences occur in AFPs of similar molecular mass in the same family (Fourney et al., 1983; Ng et al., 1986; Hew et al., 1988). The heterogeneity in AFPs has been attributed to the expression of multigene families and to posttranslational modification of the proteins. Another example occurs in the AFPs purified from Antarctic fish, in which a family of AFPs of various sizes has been characterized as having different numbers of a repeated glycosylated tripeptide unit (DeVries, 1983). However, in the beetle Tenebrio molitor, AFPs that vary significantly in their amino acid compositions have been identified (Tomchaney et al., 1982).

Four of the five aforementioned active antifreeze polypeptides have half-cystines, and their slower migration rates on SDS-polyacrylamide gels upon treatment with DTT suggest that they possess intramolecular disulfide bonds (Fig. 6). The most spectacular change in apparent molecular mass occurs in the 19-kD polypeptide, which appears to be 7 kD larger on SDS-polyacrylamide gels after treatment with DTT (Fig. 6). These results indicate that substantial additional unfolding of the polypeptides occurs when the disulfide bonds are reduced. Nevertheless, the corresponding reduced polypeptides eluted from SDS-polyacrylamide gels also exhibit high levels of antifreeze activity. The recovery of activity was not due to the reoxidation of disulfide bonds, as shown by the fact that the molecular masses of eluted polypeptides corresponded to the molecular masses of fully reduced polypeptides when the eluted polypeptides were separated again by SDS-PAGE under nonreducing conditions (data not shown). This observation was unexpected because sulfhydryl agents have been reported to decrease antifreeze activity of the type II fish AFPs (Slaughter et al., 1981; Ewart and Fletcher, 1990) and to completely eliminate thermal hysteresis activity of AFPs from certain insects and plants (Wu et al., 1991; Urrutia et al., 1992). At this time, we are not certain if the ability of winter rye AFPs to modify the growth habit of ice in their reduced forms is a unique property among the AFPs because the reported DTT-induced reduction of antifreeze activity has been based on changes in thermal hysteresis rather than on changes in ice crystal morphology (Ewart and Fletcher, 1990; Urrutia et al., 1992). However, this property is worth further investigation during studies of the structures of the cystine-containing AFPs and the mechanisms by which they bind ice.

The presence of cystines seems to be a common structural theme among AFPs identified in diverse families of organisms in both the animal and the plant kingdoms. Immuno-cross-reactivities have been reported to occur among the three groups of fish type II AFPs that share a high degree of sequence homology among themselves (Ewart and Fletcher, 1990; K.V. Ewart, personal communication). In addition, the cystine-containing AFPs from the insect spruce budworm Choristoneura fumiferana were found to immunoprecipitate AFPs from other organisms (Fig. 8).
with the anti-sea raven AFP antibodies (Hew et al., 1983). Positive reactions were also observed when an immunoblot of the stem exudate of Solanum dulcamara was probed with antiserum to the AFPs from the beetle Tenebrio molitor (Urutia et al., 1992). However, none of the major rye apoplastic polypeptides that have antifreeze activity cross-reacted with the antiserum to the fish type II AFPs (Fig. 8) or with those to AFPs from the insects Tenebrio molitor and Dendroides canaden sis (J.G. Duman, personal communication). Although the rye polypeptides of approximately 90 kD did cross-react with antibodies to type I1 AFPs, we do not know whether these polypeptides are active antifreezes because they did not stain with KCl; therefore, it was not possible to excise them from the gel and assay the eluate for activity. At this time, it would be premature to infer that cystine-containing AFPs all have common conformations that bind to ice. Disulfide bonds might play a more probable role in stabilizing the folding of the proteins, thus rendering them less susceptible to denaturation.

The heterogeneity of AFPs within the same organism is also an interesting finding and may be related to the capacity of the AFPs to function cooperatively. Such cooperativity has been observed among the fish AFGPs in which the antifreeze activity of the highly active AFGPs 1 to 5 greatly increased when they were mixed with the smaller AFGP 8, which has only weak activity when assayed alone (Osuga et al., 1978; Mulvihill et al., 1979). In our experiments, we found that antifreeze activity is higher in apoplastic extracts obtained with salts (CaCl2 or MgSO4) that contain an extra 10-kD polypeptide, which has shown little antifreeze activity on its own (Figs. 3 and 7). At this point, we do not have enough purified protein to test whether this small polypeptide produces a potentiating effect when mixed with the larger, more active AFPs because of the low recovery rate of our purification methods. However, some cooperativity in ice binding can be inferred from the morphologies of ice crystals grown in whole extracts and in solutions of isolated AFPs, where better defined pyramidal faces could be observed in the latter than in the former (Figs. 1, 4, and 7). We hypothesize that individual AFPs have certain preferred planes on the ice lattice onto which they adsorb, so that more pyramidal planes are expressed when different AFPs are combined. This results in a rounder appearance of the bipyramidal ice crystals grown in unpurified apoplastic extracts. This phenomenon of cooperativity in the interactions between AFPs and ice may have biological significance in the apoplast of the plant, where the control of ice growth is a critical factor in the mechanism of frost tolerance.

The winter rye AFPs are extracted from the apoplast and may be associated with the cell wall, which is largely composed of carbohydrates. Thus, the rye AFPs might hydrogen bond to hydroxyl groups present in the cell wall in much the same way that these proteins form hydrogen bonds with the ice crystal lattice. If there are common mechanisms in the interactions between AFPs and either ice or the cell wall, then this raises the possibility that the ability to bind to ice may be a common feature of proteins secreted into the apoplast.

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