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Purification and Characterization of a Cryoprotective Protein (Cryoprotectin) from the Leaves of Cold-Acclimated Cabbage[1]

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We have purified a protein (cryoprotectin) from the leaves of cold-acclimated cabbage (*Brassica oleracea* L.) that protects thylakoids from nonacclimated spinach (*Spinacia oleracea* L.) against freeze-thaw damage. The procedure involves precipitations by heat, ammonium sulfate, and the glycosaminoglycan heparin and column chromatography on Polyamide 6 and a C_{18} reverse-phase matrix. After reverse-phase chromatography we obtained a single band of an apparent molecular mass of 7 kD when fractions that showed cryoprotective activity were analyzed by sodium dodecyl sulfate gel electrophoresis and silver staining. Gel-filtration experiments confirmed that the active protein is a monomer of 7 kD native molecular mass. This 7-kD protein could be purified only from cold-acclimated cabbage, but not from plants grown under nonacclimating conditions. Using peroxidase-labeled lectins, we show that cryoprotectin is a glycoprotein and that the saccharide moiety contains α1–3-linked fucose.

Most plant species from temperate climates follow an annual cycle of frost hardening and dehardening, with their maximum freezing tolerance occurring during the winter and their minimum tolerance occurring during the summer. In herbaceous plants, frost hardening can be experimentally induced by exposing the plants to a period of low, nonfreezing temperatures. The physiological and biochemical changes taking place during this cold-acclimation period have been the subject of research aimed at understanding the molecular basis of frost hardiness and ultimately at improving the frost hardiness of crop plants.

It is well established that cellular membranes, such as the plasma membrane (Steponkus, 1984), chloroplast envelope (Krause et al., 1988), and thylakoids (Hincha and Schmitt, 1992b; Hincha et al., 1996), are the primary targets of freezing injury in plant cells. During cold acclimation, changes in the lipid composition of membranes (Lynch and Steponkus, 1987; Steponkus et al., 1993) and increases in cellular sugar content (Levitt, 1980) have been observed and these have been linked to increased frost hardiness. It has also been reported that the expression levels of several genes change during cold acclimation (Guy, 1990; Thomashow, 1993). But, although a number of these genes have been cloned and sequenced, no evidence for a direct role of any of the induced gene products in cellular frost hardiness has been reported to date.

The first reports of plant proteins that directly protect isolated thylakoids against freeze-thaw damage were published more than 20 years ago (Heber and Kempfle, 1970; Volger and Heber, 1975). We have shown previously that partially purified protein fractions from cold-acclimated spinach (*Spinacia oleracea* L.) and cabbage (*Brassica oleracea* L.) prevented the freeze-thaw-induced rupture of thylakoids isolated from leaves of nonacclimated spinach plants (Hincha et al., 1989, 1990). Only very low levels of activity were found in protein fractions prepared from the leaves of nonacclimated plants, suggesting that the cryoprotective activity is cold induced (Hincha et al., 1990).

From these reports it remained unclear whether the observed cryoprotective activity was the property of one or several proteins, and no information about the nature of the involved protein(s) was available. It was also unknown whether the cold induction of cryoprotective activity was due to an activation of preexisting proteins or to the appearance of novel proteins.

We report here what to our knowledge is the first purification of a cryoprotective plant protein (cryoprotectin) and show that the cryoprotective activity is associated with a cold-induced 7-kD glycoprotein.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Spinach (*Spinacia oleracea* L. cv Monnopa) was grown under nonhardening conditions in a growth chamber with 12 h of light at 150 μmol quanta m^{-2} s^{-1} at 25°C and 12 h of dark at 15°C at 50% RH. Cabbage (*Brassica oleracea* L. cv Grüfiwi) was grown in the garden for several months and then transferred to pots and cold acclimated at a constant temperature of 4°C with a 10-h light/14-h dark cycle for 2 weeks. Plants were harvested, and leaves were either used

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Abbreviation: TFA, trifluoroacetic acid.

directly for protein extraction or were stored frozen at −20°C.

Thylakoid Isolation and Cryoprotection Assay

Thylakoids were isolated from spinach leaves as described recently (Hincha and Schmitt, 1992a). The membranes were washed three times in 5 mM NaCl. Cryoprotectin was activated either by pretreatment with 5% (v/v) ethylene glycol (Hincha et al., 1996), which was subsequently removed by gel filtration through Sephadex G-25, or by addition of $MnCl_2$ and $CaCl_2$ to a final concentration of 1 mM each. Samples (0.4 mL) containing approximately 0.5 mg chlorophyll mL^{-1}, 2.5 mM NaCl, 5 mM Suc, and additional protein as indicated in the figures were placed in a freezer at −20°C for 3 h and were rapidly (within 2–3 min) thawed in a water bath at room temperature. After thawing, samples were diluted with an equal volume of 10 mM $MgCl_2$. Aliquots (75 μL) of the thylakoid suspension were filled into glass capillaries and the packed volume was determined by hematocrit centrifugation (Hincha and Schmitt, 1992a, 1995). Two measurements were taken from each sample and averaged. Control samples without added protein were held at 0°C or at −20°C for the same time. The cryoprotective activity in the samples is expressed as percent cryoprotection, with the packed volume obtained from 0°C controls set as 100% protection and the packed volume obtained from the −20°C controls set as 0% protection.

Gel Electrophoresis

Electrophoresis of proteins was performed in polyacrylamide gels under reducing conditions in the presence of SDS as described by Schägger and von Jagow (1987). The upper part of the separating gels contained 10% and the lower part 16% acrylamide. Polypeptide bands were stained with silver nitrate (Blum et al., 1987) or Coomassie brilliant blue in the presence of copper sulfate (Görg et al., 1978). The molecular weights of the bands were estimated from the position of the bee venom peptide melittin (Sigma) and a protein standards mix from Bio-Rad (low-molecular-weight standard) on parallel lanes.

Detection of Protein Glycosylation

Proteins were fractionated by SDS-PAGE and then electroblotted on PVDF membranes (Towbin et al., 1979). Unoccupied binding sites on the membranes were blocked by incubation in 3% (w/v) milk powder in 25 mM Tris/150 mM NaCl (pH 7.5) (Johnson et al., 1984). The membranes were subsequently incubated with 5 μg mL^{-1} of the L-Fuc-specific lectins from *Lotus tetragonolobulus* or *Ulex europaeus* coupled to horseradish peroxidase (both from Sigma). The bound lectin was visualized by the insoluble dye produced by the peroxidase reaction with 0.015% (v/v) H_2O_2 and 0.43 mg mL^{-1} 4-chloro-1-naphthol. The reaction was stopped after approximately 10 min by washing the blots in distilled water. Molecular weights were estimated from a mixture of prestained protein standards (Bio-Rad).

Determination of Protein and Chlorophyll

Protein concentrations were determined by a dye-binding assay in microtiter plates (Redinbaugh and Wilbur, 1985) using BSA as a standard. Chlorophyll was determined according to Arnon (1949).

Gel-Filtration Chromatography

Gel-filtration chromatography under nondenaturing conditions was performed on a Superose 12 column (1 × 30 cm; Pharmacia) using a HPLC system (LKB, Bromma, Sweden). The running buffer consisted of 200 mM NaCl and 20 mM sodium phosphate (pH 6.8). Sample size was 200 μL containing approximately 12 μg of protein, and the flow rate was 0.5 mL min^{-1}. The absorbance of the eluate was monitored continuously at 226 nm. Details of the column calibration are given in the legend to Figure 5.

Purification of Cabbage Cryoprotectin

Cabbage leaves (1 kg) were deveined and homogenized in a blender (Ika, Staufen, Germany) in 600 mL of an ice-cold solution comprising 50 mM Tris, 2% (w/v) Polyclar AT (insoluble PVP), and 300 μM mercaptobenzothiazole (pH adjusted to 7.8 with acetic acid). The homogenate was filtered through 50-μm nylon mesh and then centrifuged for 30 min at 23,000*g*. The supernatant solution was incubated in a boiling-water bath for 8 min and immediately transferred to an ice-water bath. From the cooled solution the precipitated proteins were removed by a 15-min centrifugation as above and discarded.

The supernatant was brought to pH 4 by addition of concentrated acetic acid and was applied to a column (3 × 60 cm) of Polyamide 6 (Serva, Heidelberg, Germany), a matrix consisting of small nylon beads. The column was developed in water at 4°C. Cryoprotectin was not bound and the proteins recovered after passage through the column were precipitated by the addition of solid ammonium sulfate to 60% saturation. The solution was stirred for 1 h at 4°C and, after centrifugation as above, the pellets were resuspended in 20 mM sodium phosphate (pH 7.3), 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The solution was diluted with this buffer to a conductivity of 6 to 7 millisiemens.

Cryoprotectin was precipitated from this solution by heparin (a sulfated glycosaminoglycan consisting mainly of disaccharide units of GlcUA and GlcNAc). Heparin (100 mg; Sigma) was added, and the mixture was stirred overnight at 4°C. The heparin was removed from the solution by centrifugation (23,000*g* for 15 min), and unbound proteins were removed by washing the pellets twice in 20 mM sodium phosphate (pH 7.3). The first elution buffer consisted of 150 mM NaCl, 5 mM β-mercaptoethanol, and 50 mM Tris (pH 7.1). The second elution buffer contained an additional 300 mM β-mercaptoethanol. In each step the heparin-cryoprotectin complex was incubated for 30 min at 4°C, followed by centrifugation as above. To solubilize more strongly bound proteins, heparinase I (5 units; Sigma) was used to digest the heparin during incubation overnight at room temperature (Kohnke-Godt and Gabius, 1991).

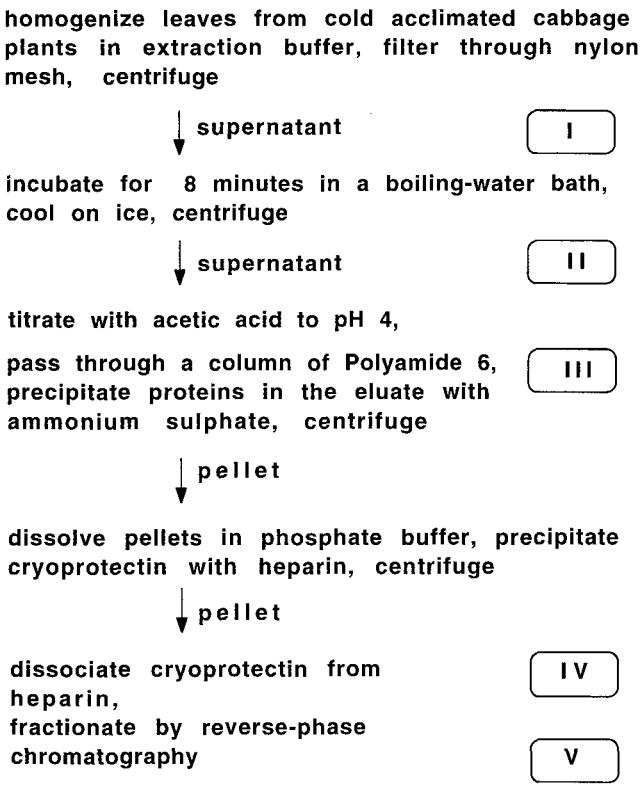

Figure 1. Flow chart for the purification of cryoprotectin from the leaves of cold-acclimated cabbage. Experimental details for the different steps are given in "Materials and Methods." Roman numerals refer to the purification steps listed in Table I.

TFA was added to the eluted proteins for a final concentration of 0.1% (v/v) at pH 2.0. The proteins were fractionated over a C_{18} Vydac (Macherey & Nagel, Düren, Germany) column (4.6 × 150 mm) using a Shimadzu (Kyoto, Japan) HPLC system with the temperature of the column oven set to 37°C. The proteins were eluted with an acetonitrile gradient from 1 to 60% in 0.1% TFA at a flow rate of 0.5 mL min^{-1}. The absorbance of the eluate was monitored at 220 nm. Fractions of 0.5 mL were collected.

RESULTS

Purification of Cryoprotectin

Methods for a partial purification of a protein fraction from cold-acclimated cabbage with cryoprotective activity for thylakoid membranes have been published from our laboratory previously (Hincha et al., 1989, 1990; Hincha and Schmitt, 1992a). These preparations, however, contained several polypeptides when analyzed by SDS-PAGE (Hincha et al., 1989). Figure 1 shows a flow chart of the newly developed method that allows the purification of a single cryoprotective protein. The experimental details for each step are given in "Materials and Methods."

The extraction process has been simplified compared to the previously published methods. The sonication of the crude extract was omitted because it was found that it did not increase the cryoprotective activity of the supernatant solution after centrifugation (data not shown). The heat stability of cryoprotectin has been described in detail before (Hincha and Schmitt, 1992a). Since most other proteins in a leaf extract are denatured by boiling, this feature was again an important part of the purification scheme (Fig. 1; Table I).

After the heat-denatured proteins were removed by centrifugation, the supernatant solution was acidified and then passed through a column of Polyamide 6. This matrix, consisting of nylon beads, very effectively binds the phenolics still present in the solution as well as some proteins (Table I). Cryoprotectin was not bound by Polyamide 6 and was recovered in the void volume of the column (data not shown). This step can be further simplified by adding the Polyamide 6 directly to the protein solution and removing it by centrifugation after several minutes of incubation. This is particularly convenient for the purification of larger amounts of cryoprotectin.

To reduce the solution volume for the next steps, cryoprotectin was precipitated with ammonium sulfate after the Polyamide 6 treatment. The precipitated protein was collected by centrifugation and the pellets were dissolved in phosphate buffer. Heparin was added to this solution in the presence of Mn^{2+} and Ca^{2+}. In the absence of the cations, no cryoprotectin-heparin complexes could be precipitated (data not shown). After an overnight incubation at 4°C, the protein-heparin complexes were collected by centrifugation. The pellets were washed twice in phosphate buffer to remove unbound proteins. After incubation with 5 mM (elution 1) and 300 mM (elution 2) β-mercaptoethanol and subsequent centrifugation, the supernatants contained cryoprotective activity (Table I). Additional activity could be released by digesting the final pellet with heparinase I. An analysis of the proteins precipitated by heparin by SDS-PAGE and silver staining of the bands revealed the presence of several polypetides in these samples (Fig. 2). The presence of prominent bands with apparent molecular masses of approximately 7 and 9 kD was noticeable. The presence of 4 M urea in the electrophoresis sample buffer led to the disappearance of the 9-kD polypeptide and a concomitant increase in the intensity of the 7-kD band (data not shown). We attribute this to an incomplete denaturation of this extremely stable protein (see below). In all subsequent experiments, cryoprotectin was completely denatured before electrophoretic analysis, resulting in a single 7-kD polypeptide.

Table I. *Purification of cryoprotectin from 1 kg of fresh cabbage leaves*

Purification Step[a]	Protein	Protein	Activity	Purification Factor	Yield
	mg mL^{-1}	*mg*	*% mL^{-1}*		*%*
I	3.8	3,420	60	1	100
II	0.4	350	60	9.5	97
III	0.3	244	50	11	80
IV	0.13	0.78	1,110[b]	534	13
V	0.005	0.01	1,860[b]	23,500	7

[a] Compare Figure 1. [b] Determined from the cryoprotective activity of a serial dilution of the samples in 10 mM Suc.

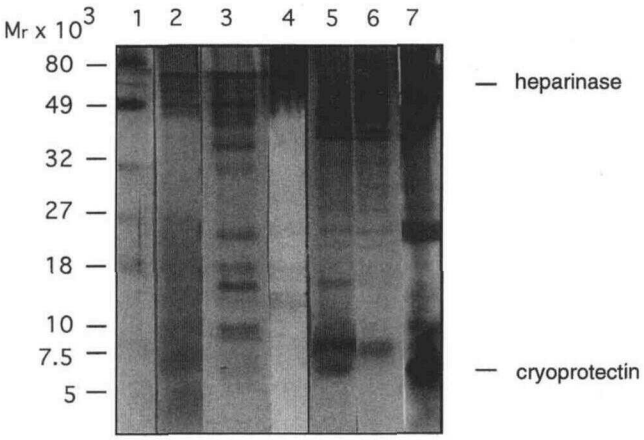

Figure 2. Precipitation of cryoprotectin with heparin. See Figure 1 and "Materials and Methods" for experimental details. The numbers on the left indicate the positions and molecular masses of the standard proteins in lanes 1 (low-molecular-mass standards from Bio-Rad) and 2 (monomer and multimers of melittin). Lane 3 shows the proteins present in the sample before heparin precipitation, and lane 4 shows the proteins not bound by heparin after the second washing step. Bound proteins were dissociated from the heparin by incubation in 5 mM β-mercaptoethanol (elution 1, lane 5) followed by an incubation in 300 mM β-mercaptoethanol (elution 2, lane 6). Lane 7 shows the pattern of proteins released from the heparin after the two mercaptoethanol elutions by enzymatic digestion of the heparin with heparinase I. The positions of the heparinase and the 7-kD cryoprotectin are indicated on the right. Aliquots of equal volume from the different fractions were analyzed by SDS-PAGE, followed by silver staining of the proteins.

The proteins recovered from the heparin precipitation after elution with 5 or 300 mM β-mercaptoethanol were further fractionated by reverse-phase chromatography on a C_{18} silica matrix. The proteins were applied to the column in the presence of 0.1% TFA (pH 2.0) and the bound proteins were eluted with a linear acetonitrile gradient. Figure 3 shows typical elution profiles of cryoprotectin from the 5 mM (Fig. 3A) and the 300 mM (Fig. 3B) mercaptoethanol fractions. The absorbance of the eluate was monitored at 220 nm and showed several peaks. After the pH in the eluted fractions had been readjusted to 7 and the organic solvent was removed by gel filtration, it was possible to measure cryoprotective activity in the samples. Activity was detected in several fractions (Fig. 3), with the highest activity in Figure 3A at 30 and 33% acetonitrile and the highest activity in Figure 3B at 36 and 38%. This elution pattern was reproducible through many preparations and under different elution conditions (changes in TFA concentration and pH). The possibility that the activity recovered from the reverse-phase column was due to the elution of heparin could be excluded. We found that heparin, even at concentrations as low as 10 μM, severely damaged thylakoids, both at 0°C and during a freeze-thaw cycle (data not shown). The possible involvement of protein glycosylation in the heterogeneity revealed by the elution profiles will be discussed below.

When the fractions constituting the major peaks after reverse-phase chromatography were analyzed by SDS-PAGE, silver staining revealed a prominent band at an apparent molecular mass of 7 kD (Fig. 4). It can be clearly seen that cryoprotectin from the 5 mM mercaptoethanol fraction was purified to homogeneity by this procedure, whereas the 300 mM mercaptoethanol fraction still contained several polypetides of a higher molecular mass after reverse-phase chromatography. We conclude from these data that the cryoprotective activity in these samples is exclusively associated with the 7-kD cryoprotectin.

Native Molecular Mass

To determine whether cryoprotective activity was associated with the 7-kD cryoprotectin monomer or whether oligomeric structures were necessary for activity, we used gel-filtration chromatography under nondenaturing conditions. A Superose 12 column was calibrated with a range of proteins of known molecular mass (Fig. 5, inset), and a cryoprotectin sample after heparin precipitation was analyzed under the same experimental conditions. The cryoprotective activity eluted from the column as a single peak associated with a single symmetrical absorbance peak (Fig.

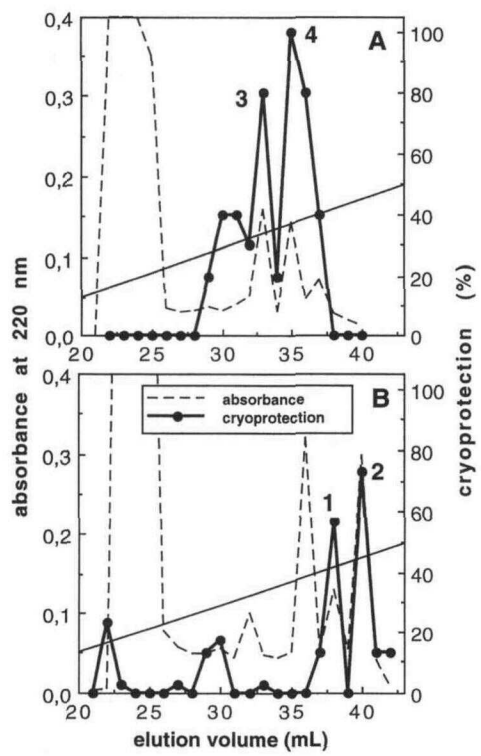

Figure 3. Purification of cryoprotectin on a C_{18} reverse-phase column. Samples after heparin precipitation (cf. Fig. 2, elution 1, with A, and Fig. 2, elution 2, with B) were brought to 0.1% TFA (pH 2.0) and applied to a C_{18} Vydac column (4.6 × 150 mm). Proteins were eluted with a linear gradient of acetonitrile (1–60%) in 0.1% TFA (pH 2.0). The absorbance of the eluate was monitored at 220 nm. The pH in the fractions was readjusted to 7 by the addition of 20 mM Tris (pH 8.6). The fractions (0.5 mL) were then transferred into 10 mM Suc, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ by passage through Sephadex G-25 (NAP-5 columns), and cryoprotective activity was measured as described in "Materials and Methods." The major activity peaks are labeled from 1 to 4.

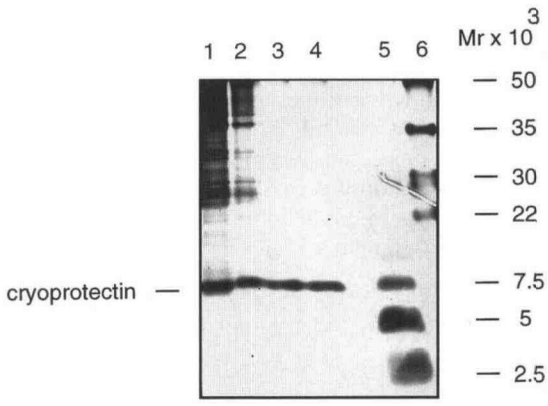

Figure 4. SDS-PAGE analysis of the purification of cryoprotectin by reverse-phase chromatography (cf. Fig. 3). In lanes 1 through 4, aliquots of equal volume from the numbered peak fractions in Figure 3 have been applied to the gel. Polypeptides were visualized by silver staining. The molecular masses of the standard proteins shown in lanes 5 and 6 are shown on the right of the figure.

5). From the regression line of the calibration curve, we calculated an apparent molecular mass of 7 kD for the active cryoprotectin (Fig. 5, inset). There was no evidence for active oligomers.

Cabbage Cryoprotectin Is Cold Induced

It has been shown previously that a high cryoprotective activity was found only in extracts from cold-acclimated plants, but not in extracts from nonacclimated plants (Heber and Kempfle, 1970; Hincha et al., 1990). Figure 6 shows that the 7-kD cryoprotectin was detectable after SDS-PAGE only when leaves from cold-acclimated cabbage were used for the purification. When proteins from the leaves of nonacclimated plants were fractionated by the same method, neither cryoprotective activity nor the 7-kD protein could be precipitated by heparin.

Glycosylation

It had been shown that some cryoprotective activity was bound to an affinity column of the immobilized lectin concanavalin A (Hincha and Schmitt, 1992b). Although binding was never complete, it suggested that cryoprotectin was glycosylated. When blots of cryoprotectin were probed with lectins, a strong signal was obtained with the peroxidase-labeled, L-Fuc-specific lectin from *Lotus tetragonolobulus* (Fig. 7). When the L-Fuc-specific lectin from *Ulex europaeus* was used to probe such blots, only a very weak signal was obtained (Fig. 7). The lectin from *L. tetragonolobulus* is specific for α1–2- and α1–3-linked Fuc and the *U. europaeus* lectin is specific for α1–2- and α1–4-linked Fuc (Debray et al., 1981). Therefore, we conclude from these experiments that cryoprotectin is glycosylated with a saccharide structure containing α1–3-linked Fuc. Binding of the lectins to cryoprotectin was completely suppressed in the presence of free L-Fuc, indicating that binding occurred via an interaction between the sugar-binding site of the lectin and a carbohydrate structure of cryoprotectin, and not by an unspecific interaction. When a protein preparation from nonacclimated cabbage was analyzed (cf. Figure 6), no binding of the labeled lectin to the membrane could be detected (Fig. 7).

DISCUSSION

In this paper we describe the purification to homogeneity of a cryoprotective plant protein. The relevance of this work to the problem of plant frost hardiness obviously rests on the assumption that our in vitro assay for cryoprotective activity is pertinent to the in vivo situation. Several lines of published evidence support this assumption (reviewed by Hincha and Schmitt, 1992b; Hincha et al., 1996). To summarize this evidence briefly, we have shown that freeze-thaw damage to leaves from nonacclimated spinach results in thylakoid membrane rupture and the release of the electron transport protein plastocyanin. Loss of plastocyanin is also observed when isolated thylakoids are frozen and thawed under the experimental conditions employed in this study. The loss of plastocyanin could be completely prevented by the presence of a partially purified cryopro-

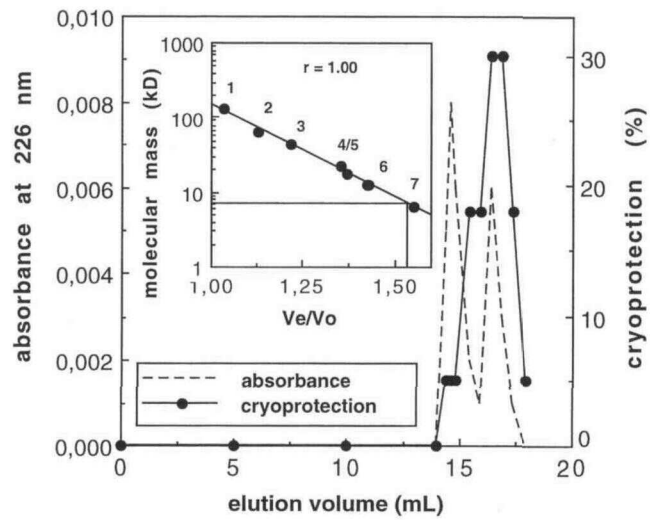

Figure 5. Determination of the molecular mass of cryoprotectin by gel-filtration chromatography under nondenaturing conditions. A cryoprotectin fraction (200 μL containing approximately 12 μg of protein) after heparin precipitation (cf. Fig. 2) was analyzed on a Superose 12 column (1 × 30 cm) in 20 mM sodium phosphate, 200 mM NaCl (pH 6.8) at a flow rate of 0.5 mL min^{-1}. Absorbance of the eluate was monitored at 226 nm. Individual fractions (0.5 mL) were transferred into 10 mM Suc, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ by passage through NAP-5 columns and were assayed for cryoprotective activity. The inset shows a calibration curve for the column, where the molecular mass of standard proteins on a logarithmic scale is plotted as a function of their relative elution volume (Ve/Vo, elution volume/column void volume). The standard proteins were: 1, BSA dimer (134 kD); 2, BSA monomer (67 kD); 3, ovalbumin (45 kD); 4, carbonic anhydrase (29 kD); 5, trypsin inhibitor (29 kD); 6, myosin (17.8 kD); and 7, aprotinin (6.5 kD). The line was fitted to the data by linear regression analysis. The relative elution volume of cryoprotectin and the resultant molecular mass deduced from the calibration curve are also indicated.

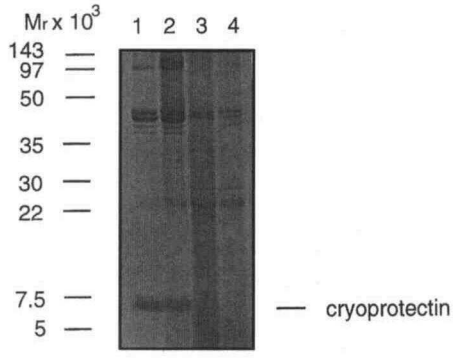

Figure 6. The 7-kD cryoprotectin is a cold-induced protein. Equal amounts of leaves from cabbage plants grown either under nonacclimating conditions or for 2 weeks under cold-acclimating conditions (see "Materials and Methods" for details) were harvested, and proteins were isolated under identical conditions following the procedure outlined in Figure 1. After heparin precipitation, the proteins from the first and second elutions (cf. Fig. 2) were freeze-dried and then dissolved in electrophoresis sample buffer. Approximately 4 μg of protein were loaded in each lane and analyzed by SDS-PAGE and staining with Coomassie blue. Lanes 1 and 2, First and second elution after heparin precipitation of a protein preparation from cold-acclimated cabbage. Lanes 3 and 4, Corresponding fractions from nonacclimated cabbage leaves. The positions and molecular masses of standard proteins are indicated on the left. The position of the 7-kD cryoprotectin is indicated on the right.

tectin fraction. Measurements of packed thylakoid volume from the same samples showed that collapse of the membrane vesicles was also prevented in the presence of sufficient amounts of cryoprotective protein. Since the cryoprotection measured by the two methods was linearly correlated, volume measurements can be used for purification and characterization purposes without compromising the validity of the results for the problem of plant frost hardiness.

Table I gives a quantitative summary of a typical cryoprotectin purification from 1 kg of leaves from cold-acclimated cabbage plants. The newly devised procedure is relatively simple, since it involves mainly precipitation steps (heat, ammonium sulfate, and heparin) and only two chromatographic steps. Since one of these, the chromatography on Polyamide 6, can be replaced by a batch procedure without any loss in protein purity, highly enriched cryoprotectin for functional analyses can be prepared without the use of sophisticated chromatographic equipment. With a single additional step of reverse-phase chromatography, cryoprotectin can be purified to apparent homogeneity (Fig. 4).

One of the crucial steps in this purification protocol is the precipitation of cryoprotectin by heparin. From the first elution with β-mercaptoethanol the protein can be purified to homogeneity by reverse-phase chromatography. The additional cryoprotectin released by treatment with heparinase I is too contaminated with other proteins and therefore not suitable for this purification. Heparin is a highly charged, sulfated glycosaminoglycan of heterogeneous structure and chain length. It has been used before for the precipitation and characterization of animal and bacterial lectins (Kohnke-Godt and Gabius, 1991; Calvete et al., 1994; Menozzi et al., 1994). Although for the binding of these lectins no divalent cations were reported to be necessary, binding of cryoprotectin to heparin occurred only in the presence of manganese and calcium ions. This makes a specific interaction through a carbohydrate recognition domain a likely possibility. A more detailed experimental analysis of the binding properties of cryoprotectin is currently in progress in our laboratory.

The final fractionation of cryoprotectin from both heparin elutions on a C_{18} reverse-phase column yielded four major peaks (Fig. 3). This apparent heterogeneity may be related to the glycosylation of the protein (Fig. 7). Most glycoproteins that have been investigated in sufficient detail show structural heterogeneity in their sugar moiety (Dwek et al., 1993). Also, the molecular mass determination for cryoprotectin, both by SDS-PAGE and gel-filtration chromatography, has to be viewed with caution, since the glycosylation of proteins can influence their electrophoretic and chromatographic behavior. The molecular mass of 7 kD derived from SDS-PAGE has been calculated using standard proteins between 2.5 and 143 kD. When the melittin bands (2.5, 5, 7.5, and 10 kD) were not included in the calculations, higher apparent molecular masses were obtained.

The data shown in Figure 3 emphasize the remarkable stability of cryoprotectin. In addition to its heat stability (Hincha and Schmitt, 1992a), it could also be recovered in an active form after exposure to TFA and acetonitrile at pH

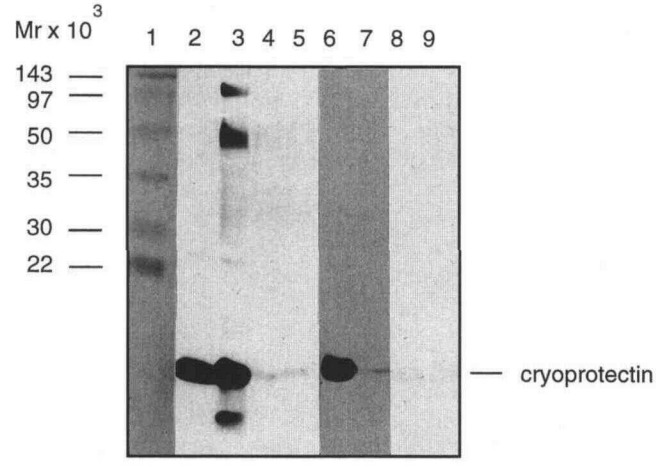

Figure 7. Detection of the glycosylation of cabbage cryoprotectin after SDS-PAGE and blotting onto a PVDF membrane. All lanes were loaded with approximately 4 μg of protein obtained by heparin precipitation (cf. Figs. 1 and 2). The peroxidase-labeled lectin from *L. tetragonolobulus* recognized a protein of approximately 7 kD from cold-acclimated cabbage obtained after the first and second elutions (lanes 2 and 3). A protein fraction from nonacclimated cabbage (cf. Fig. 6) did not contain a comparable glycoprotein (lanes 4 and 5). The lectin is specific for L-Fuc and its specificity was checked by incubating parallel blots with the lectin in the absence (lane 6) or presence (lane 7) of 50 mM L-Fuc, which inhibited binding of the lectin to the 7-kD protein. With the lectin from *U. europaeus* only a very weak signal was obtained with cryoprotectin from the first and second heparin elutions (lanes 8 and 9). The molecular masses of the standard proteins shown in lane 1 are indicated on the left and the position of cryoprotectin is shown on the right.

2.0. This is at least in part explained by the fact that no oligomeric structures are required for cryoprotective activity (Fig. 5). In an earlier publication we have reported a molecular mass of 28 kD for the cryoprotective activity in a much less purified preparation (Hincha et al., 1989). Recently, we have shown that cryoprotectin is reversibly inactivated by several organic acids and that this acid treatment also leads to the disappearance of a high-molecular-weight peak in gel filtration experiments (Hincha et al., 1996). Whether this can completely explain the different molecular weights obtained in different studies remains to be experimentally resolved. It is clear, however, that the monomeric form of cryoprotectin has strong cryoprotective activity. Of course, it is possible that in the cellular environment cryoprotectin forms oligomeric structures. Whether this would have any influence on the cryoprotective activity remains to be investigated.

The data in Figures 6 and 7 indicate that cryoprotectin may be a cold-induced protein. After precipitation of proteins from nonacclimated plants by heparin there was no evidence from activity measurements (data not shown), Coomassie brilliant blue-stained gels (Fig. 6), or lectin blots (Fig. 7) of the 7-kD cryoprotectin. Since we have no antibodies available yet for the detection of cryoprotectin in total leaf homogenates, we cannot exclude the possibility of a nonglycosylated precursor of a different molecular mass or of a precursor that would not bind to heparin. Studies with radioactively labeled amino acids will be necessary to determine whether cryoprotectin is indeed synthesized de novo during cold acclimation.

The lectin-binding studies (Fig. 7) show that cryoprotectin is a glycoprotein. From the published binding specificities of the two lectins (Debray et al., 1981), it can be inferred that the sugar structure contains α1–3-linked Fuc. A detailed analysis of the functional and structural properties of the carbohydrate associated with cryoprotectin is currently in progress.

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LITERATURE CITED

Arnon DJ (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24:** 1–15

Blum H, Beier H, Gross HJ (1987) Improved silverstaining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Calvete JJ, Solis D, Sanz L, Diaz-Maurino T, Töpfer-Petersen E (1994) Glycosylated boar spermadhesin AWN-1 isoforms. Biological origin, structural characterization by lectin mapping, localization of O-glycosylation sites, and effect of glycosylation on ligand binding. Biol Chem Hoppe-Seyler **375:** 667–673

Debray H, Decout D, Strecker G, Spik G, Montreuil J (1981) Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. Eur J Biochem **177:** 41–55

Dwek RA, Edge CJ, Harvey DJ, Wormald MR (1993) Analysis of glycoprotein-associated oligosaccharides. Annu Rev Biochem **62:** 65–100

Görg A, Postel W, Westermaier R (1978) Ultrathin-layer isoelectric focusing in polyacrylamide gels on cellophane. Anal Biochem **89:** 60–70

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

Heber U, Kempfle M (1970) Proteine als Schutzstoffe gegenüber dem Gefriertod der Zelle. Z Naturforsch **25b:** 834–842

Hincha DK, Heber U, Schmitt JM (1989) Freezing ruptures thylakoid membranes in leaves, and rupture can be prevented in vitro by cryoprotective proteins. Plant Physiol Biochem **27:** 795–801

Hincha DK, Heber U, Schmitt JM (1990) Proteins from frost-hardy leaves protect thylakoids against mechanical freeze-thaw damage in vitro. Planta **180:** 416–419

Hincha DK, Schmitt JM (1992a) Cryoprotective leaf proteins: assay methods and heat stability. J Plant Physiol **140:** 236–240

Hincha DK, Schmitt JM (1992b) Freeze-thaw injury and cryoprotection of thylakoid membranes. *In* GN Somero, CB Osmond, CL Bolis, eds, Water and Life. Springer, Berlin, pp 316–337

Hincha DK, Schmitt JM (1995) Long-term cryopreservation of thylakoid membranes by sugars. *In* JG Day, MR McLellan, eds, Methods in Molecular Biology, Vol 38: Cryopreservation and Freeze-Drying Protocols. Humana Press, Totowa, NJ, pp 71–80

Hincha DK, Sieg F, Bakaltcheva I, Köth H, Schmitt JM (1996) Freeze-thaw damage to thylakoid membranes: specific protection by sugars and proteins. *In* PL Steponkus, ed, Advances in Low-Temperature Biology, Vol 3. JAI Press, London (in press)

Johnson DA, Gautsch JW, Sportsman JR, Elder JH (1984) Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech **1:** 3–8

Kohnke-Godt B, Gabius H-J (1991) Heparin-binding lectin from human placenta: further characterization of ligand binding and structural properties and its relationship to histones and heparin-binding growth factors. Biochemistry **30:** 55–65

Krause GH, Grafflage S, Rumich-Bayer S, Somersalo S (1988) Effects of freezing on plant mesophyll cells. Symp Soc Exp Biol **42:** 311–327

Levitt J (1980) Responses of Plants to Environmental Stresses, Vol 1. Academic Press, Orlando, FL

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **83:** 761–767

Menozzi FD, Mutombo R, Renauld G, Gantiez C, Hannah JH, Leininger E, Brennan MJ, Locht C (1994) Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. Infect Immun **62:** 769–778

Redinbaugh MG, Wilbur HC (1985) Adaptation of the dye-binding protein assay to microtiter plates. Anal Biochem **147:** 144–147

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368–379

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Steponkus PL, Uemura M, Webb MS (1993) A contrast of the cryostability of the plasma membrane of winter rye and spring oat: two species that widely differ in their freezing tolerance and plasma membrane lipid composition. *In* PL Steponkus, ed, Advances in Low-Temperature Biology, Vol 2. JAI Press, London, pp 211–312

Thomashow MF (1993) Genes induced during cold acclimation in higher plants. *In* PL Steponkus, ed, Advances in Low-Temperature Biology, Vol 2. JAI Press, London, pp 183–210

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Volger HG, Heber U (1975) Cryoprotective leaf proteins. Biochim Biophys Acta **412:** 335–349