Multistep Purification of an Antifreeze Protein from *Ammopiptanthus mongolicus* by Chromatographic and Electrophoretic Methods

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Abstract

Antifreeze proteins (AFPs) are known as thermal hysteresis proteins, which can depress the freezing points of the solution by noncolligative effects, but do not affect the melting points. Although some AFPs have been found in some plants, the identity of most proteins remains unclear, owing to insufficient quantity and quality to characterize them. In this report, we describe the purification of an AFP from the winter leaves of *Ammopiptanthus mongolicus* using a combination of column chromatography and gel electrophoresis. After homogenization in ascorbate-acid-containing Tris buffers (pH 7.4) the soluble proteins are captured by (diethylamino)ethyl-cellulose 52 material. An elution with 0.1–0.3M KCl leads to a crude active fraction. The crude fraction is further purified on a Superdex 75 prep-grade column and finally a Poros 20HP2 column. A complex, consisting of two proteins with relative molecular masses of 34,700 and 37,100, respectively, in sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis, is obtained by this protein purification protocol. The recovery of two proteins from the gel is carried out by electrophoresis. The purified protein, with a molecular mass of 37,100, shows thermal hysteresis activity (THA) and can modify the normal growth of ice crystals. The THA of this purified antifreeze protein is 0.24°C at the concentration of 5 mg/mL.

Introduction

Antifreeze proteins (AFPs) are known as thermal hysteresis proteins (THPs), which can depress the freezing points of the solution by noncolligative effects, but do not affect the melting points. The difference between freezing points and melting points is known as thermal hysteresis (TH) and is used as a measure of AFP activity (1). AFPs can influence ice crystal growth effectively. In the absence of AFP activity, ice crystals grow as spherical discs. At low AFP concentrations, ice crystals grow along both the *a*- and *c*-axes to produce a flat hexagonal shape. At higher AFP concentrations, ice crystals grow along only the *c*-axis to generate hexagonal bipyramidal or multifacetted and spear-like shapes (2). AFPs play an essential role in preventing organisms from sub-zero-temperature damage, and they are diverse in their structures, functions, and activities found in fish, plants, insects, spiders, fungi, and centipedes (3). There is substantial information about structure, mechanism of action, biological function, and regulation of gene expression of several AFPs in fish (4). In contrast, little is known about AFPs in plants.

Several reports demonstrated the occurrence of AFPs in plant tissues (5,6). Some proteins with antifreeze activity were also purified and partially characterized (7,8). In addition, complementary DNAs (cDNAs) of AFPs were isolated from cDNA libraries of carrot (9,10) and ryegrass (11).

During the past years, THPs were noted in *A. mongolicus* (12,13). Although an AFP with molecular mass of 50,000 has been purified from *A. mongolicus* by (diethylamino)ethyl (DEAE)-cellulose 52 column, Sephacryl S-300 column, and preparative isotachophoresis (13), purification of high quality and quantity AFP from *A. mongolicus*, sufficient to allow a detailed analysis by biophysical methods, remained a challenging problem. Published purification protocols for AFPs from plants usually involved preparative isoelectric focusing (IEF)–sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and chromatography including ion-exchange chromatography (IEC), gel permeation chromatography, and reverse-phase high-performance liquid chromatography (HPLC) (7–9). AFPs are strongly hydrophilic. The advantages of porous material were already demonstrated for the purification of soluble proteins (14). The application of hydrophobic interaction chromatography (HIC) with porous support material for purification of AFPs has not been described so far. In this contribution, we described the purification of an AFP using a protocol with three chromatographic steps, followed by an electrophoretic method.

Experimental

Materials and equipment

The leaves of *A. mongolicus* were collected in January, 2000, in

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the suburb of Wuhai, Inner Mongolia, China and stored at –80ºC.

The materials used were: *N,N,N',N'*-tetramethylethylenediamine (TEMED); trihydroxymethyl aminomethane (Tris); glycine (Gly); sodium dodecyl sulphate (SDS); acrylamide (Acr); *N,N,N',N'*-methylenebisacrylamide (Bis); bovine serum albumin (BSA); formaldehyde solution (37%, w/w); glutaraldehyde; and Coomassie Brilliant Blue R-250 (Sigma Chemical Corp., St. Louis, MO). We also used ethylenediamine tetraacetic acid (EDTA), ammonium persulfate (APS), and ascorbate acid (AA) (Beijing Chemical Reagents Factory, Beijing, China). DEAE-cellulose 52 (Whatman International, Kent, U.K.), Superdex 75 prep grade (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Poros 20HP2 (Boehringer Mannheim, Mannhein, Germany), low-molecular-mass marker (Dongfeng Corp., Shanghai, China), Milli-Q water (Millipore, Freehold, NJ), and a kit of Roche protein assay (Roche Biochemicals, Mannheim, Germany) were used, and double-deionized water (ddH_2O) was obtained from our laboratory distillation facility. If no company is specified, the chemical was purchased from Sino-American Biotech Corp. (Beijing, China).

Manual columns (45 × 2.5 cm, 70- × 1.6-cm i.d.) and a Peristaltic pump P-3 were from Amersham Pharmacia Biotech AB. A Bio-Rad 2000/200 electrophoresis unit (Bio-Rad Laboratories, Hercules, CA) was used. A UV-1601 photospectrometer was obtained from Shimadzu (Tokyo, Japan). Freeze-drying equipment was purchased from Northfield Precision Instrument (Island Park, NY). Gel-Pro Imager kits were from Media Cybernetics (Silver Spring, MD). The dialysis tubing of regenerated cellulose with a molecular cutoff of relative molecular mass (M_r) 10,000 was obtained from the Institute of Beijing Chemical Agents (Beijing, China). A nanoliter osmometer (Clifton Technical Physics, Hartford, NY) was used. A phase-contrast photomicroscope (Olympus BHT Carsen Medical and Scientific Corp., Ontario, Canada) was also used. The ultracentrifuge was purchased from Beckman Instruments (Fullerton, CA). A blender (Shengqi Corp., Taipei, Taiwan), ultrafiltration cell containing an M_r 7000 cutoff hollow cellulose membrane (Institute of Beijing Medicine, Beijing, China), and 0.22-µm syringe filter unit (Institute of Shanghai Medicine, Shanghai, China) were used. An autofraction collector from Huxi Instruments (Shanghai, China) was also used.

HPLC was carried out using a Delta Prep 4000 HPLC system (Waters, Milford, MA). The system was equipped with two pumps (constant flow rate, 0.5–150 mL/min), 2487 dual channel detector, fraction collector, and Rheodyne 7725 injector (Rohnert Park, CA). Run programming and real-time and post-run analyses were carried out through BioCAD Workstation for Windows 98 on the Great Wall computer (Great Wall Computer Co., Shenzhen, China). Self-packed Poros 20HP2 column (100- × 4.6-mm i.d.) for HIC was from Boehringer Mannheim.

Purification of antifreeze protein

The primary leaves were ground for 10 min at 4ºC with a blender in a 2 volume (w/v) ice-cold medium consisting of 50mM Tris-HCl (pH 7.4), 10mM EDTA, and 20mM AA. For disruption, the leaves were homogenized. The resulting homogenate was filtered through four layers of muslin and then centrifuged at 12,000 rpm at 4°C for 30 min. The pellets were ground again for 5 min in 1 volume of the same buffer and centrifuged as described previously. The combined supernatant was a crude extract and loaded onto a column.

All chromatographic steps were performed at room temperature. Three subsequent chromatographic separations were established. Initially, the crude extract was adjusted to anion-exchange loading buffer consisting of 5mM Tris-HCl (pH 7.4), 100mM EDTA, and 0.1M KCl and subjected to a manual DEAE-cellulose 52 column (45 × 2.5 cm) previously equilibrated with the same buffer. Elution was achieved by a gradient of KCl from 0.1M up to 0.3M (500 mL) and 0.3M (until the absorbance of 280 nm near to zero) with elution buffer consisting of 5mM Tris-HCl (pH 7.4) and 100mM EDTA. The flow-rate was 0.4 mL/min, and 8-mL fractions were collected continuously. After screening the fractions for antifreeze activity by observing ice crystal morphology, the contents of positive tubes corresponding to a single peak were pooled, ultrafiltrated, lyophilized, and exchanged into gel permeation buffer consisting of 150mM NaCl and 50mM Tris-HCl (pH 7.4).

In the next step, gel permeation chromatography was performed using a manual Superdex 75 prep-grade column (70 × 1.6 cm) equilibrated with the same buffer. The flow rate was 0.4 mL/min, and 4-mL fractions were collected and analyzed for activities. Fractions showing antifreeze activity were pooled and concentrated. During this procedure, a buffer exchange was performed to the loading buffer of the following chromatography step.

The concentrated protein solution was further purified by HIC using a Poros 20HP2 HPLC column (100 mm × 4.6 mm). For HIC, all buffers were made in Milli-Q water, filtered through a 0.22-µm syringe filter unit, and degassed under sparging with helium. The concentrated active fractions were applied to the column that was pre-equilibrated with the loading buffer consisting of 3.0M ammonium sulfate and 20mM sodium phosphate (pH 7.0). A linearly decreasing salt gradient (3–0M ammonium sulfate in 20mM sodium phosphate, pH 7.0) was used to elute the AFP from the Poros 20HP2 column at a flow rate of 1 mL/min. Fractions were collected every 1 min. Fractions corresponding to antifreeze activity were pooled, lyophilized, dialyzed against ddH_2O, and analyzed by SDS–PAGE.

Electrophoresis and determination of protein content

Continuous SDS–PAGE, according to LaemmLi, was used to determine the grade of purification (15). The gel stock solution, which contained 29.1% Acr and 0.9% Bis, was sterilized through membrane filter and stored at 4ºC. A gel containing 3% stacking (500mM Tris, adjusted to pH 6.8 with HCl, 0.1% SDS, 0.05% APS, and 0.05% TEMED) and 13.5% separation gels [1.5M Tris-HCl (pH 8.8), 0.15% SDS, 0.075% APS, and 0.075% TEMED] was set up in a Bio-Rad 2000/200 electrophoresis unit and run at 20 m*A* for 4 h. The following buffers were used: 100mM Tris-HCl, (pH 6.8), 4% SDS, 2% 2-mercaptoethanol (2-ME), 0.1 mg/L bromophenol blue, 20% glycerol (2× sample buffer), 1% SDS, 3.22% Tris, and 14.1% Gly (pH 8.3) (running buffer). Denaturation of proteins was performed by diluting the samples with sample buffer (1:2) and heating the mixtures at 100°C for 5 min. After electrophoresis, gels were stained in silver staining solution (16). Briefly, the gels were fixed with 50% methanol–5% acetic acid for 30 min and sensitized by incubating in 30% ethanol, 6.8%

sodium acetic acid, 0.125% glutaraldehyde, and 0.2% sodium thiosulfate for 30 min, followed by several washes with ddH_2O for 15 min. The gels were incubated in 0.1% silver nitrate and 0.002% formaldehyde for 20 min, followed by thorough rinsing with ddH_2O. The gels were developed in 2.5% sodium carbonate and 0.001% formaldehyde, and the reaction was terminated with 1.46% EDTA for 10 min, followed by thorough washes with ddH_2O. All gels were stored with 10% glycerol at 4°C. Standard proteins served as markers for molecular mass. Gel images were taken with a charge-coupled device camera on Gel-Pro Imager kits. Protein concentrations for samples were determined using a kit of the Roche protein assay according to the manufacturer's instructions with a standard curve of BSA.

Recovery of proteins from SDS–PAGE

After electrophoresis, the gel was stained in staining solution consisting of 0.1 g/L Commassie Brilliant Blue R-250, 50% ethanol, and 10% acetic acid for 20 min and stained again in 250mM KCl until protein bands became milk white. The protein bands were then rinsed a few times with water and cut into pieces. Next, the protein bands were put into dialysis tubes in solution consisting of 25mM Tris, 192mM Gly, and 0.5% SDS (pH 8.3) and run at 80 m*A* in a Bio-Rad 2000/200 electrophoresis unit until the protein band color disappeared. Subsequently, the gel pieces were centrifuged at 5000 rpm for 5 min, and supernatants were precipitated in 5-volume cold acetone at –20ºC overnight and centrifuged again at 16,000 rpm at 4ºC for 20 min. The antifreeze activity of the pellets was determined.

Assay of antifreeze activity

THA and the morphology of ice crystals were measured using a Clifton nanoliter osmometer and a phase-contrast photomicroscope following procedure described by Hon et al. (7).

Results and Discussion

Morphology of ice crystal of the crude extraction from *A. mongolicus*

Besides producing TH activity, AFP can modify the morphology of ice crystal growth as described previously. Ice crystals formed in distilled water were round discs (Figure 1A) because ice grows only along the *a*-axis, with little growth along the *c*-axis, whereas ice crystals grown in soluble proteins from winter *A. mongolicus* were hexagonal discs (Figure 1B). They showed that soluble proteins from winter *A. mongolicus* functioned to change the morphology of ice crystals in the solution and had antifreeze activity.

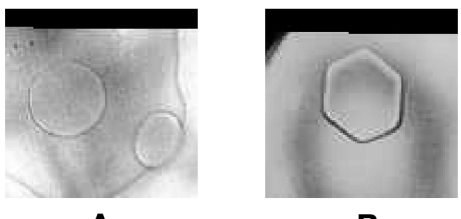

Figure 1. Effect of antifreeze proteins on the growth of an ice crystal in solution: (A) an ice crystal grown in distilled water and (B) an ice crystal grown in a solution containing antifreeze proteins obtained from winter *A. mongolicus*.

Purification of the antifreeze protein

For the preparation of soluble proteins, cells were disrupted in medium consisting of 50mM Tris-HCl (pH 7.4), 10mM EDTA, and 20mM AA. AA was antioxidant and maybe attributed to the cell lysis. The reducing agents, like 2-ME, in the extraction step were not appropriate because antifreeze activities of several AFPs from fish and insects disappeared.

After the crude extract was adjusted to an anion-exchange loading buffer, the soluble proteins were separated by anion-exchange chromatography on a DEAE-cellulose 52 column (Figure 2). Proteins bound to the DEAE-cellulose 52 column were eluted with a KCl gradient in 5mM Tris-HCl (pH 7.4) and 100mM EDTA buffers. Under these conditions, several peaks appeared and desorption of active fractions occurred at 0.1–0.3M KCl as a narrow peak, indicated by the arrow. The other peaks representing many different proteins were not further characterized. In contrast, DEAE-cellulose 52 chromatography in 50mM Tris-HCl buffers (pH 7.4) was not successful. One big peak was obtained; the activities were distributed over a wide range of different fractions. The AFP (molecular mass of 50,000) from *A. mongolicus* was eluted on the DEAE-cellulose 52 column at 6.0M KCl, which bound to the column (13).

Afterwards, the fractions containing the desired antifreeze activities were concentrated. After a buffer exchange, they were separated by gel permeation chromatography in a Superdex 75-pg column, which has a separation range of M_r 3000–70,000. The running buffer contained 150mM sodium chloride to improve the separation of soluble proteins. The chromatography in Figure 3 shows the absorbance at 280 nm, and the activity, indicated by the arrow, is found in the third peak.

After gel permeation chromatography, TH-active fractions still contained contaminants (see below), which make an additional purification step necessary. HIC was not applied to the purification of AFP before. HIC was chosen as the purification step because little protein activity was lost and the yield was higher. To

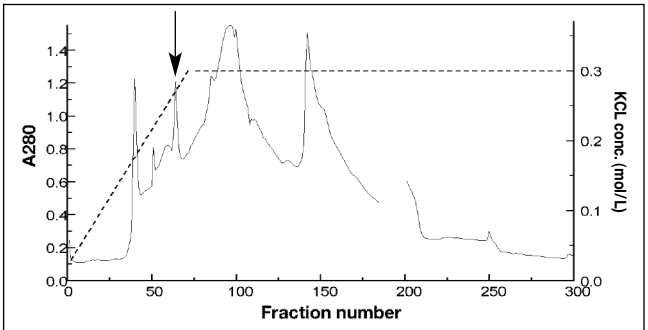

Figure 2. Anion-exchange chromatography of *A. mongolicus* proteins on a DEAE-cellulose 52 column (45- × 2.5-cm i.d.) equilibrated with running buffer [5mM Tris-HCl, 100mM EDTA, and 0.1M KCl (pH 7.4)] at 0.4 mL/min. The crude extract proteins were applied to the column after adjusting the salt concentration to running buffer. Elution of bound proteins was achieved by a linear KCl gradient (0.1–0.3M, dashed line, right axis) in the same buffer. Proteins were detected by absorbance at 280 nm (straight line, left axis). The arrow marks the active peak.

completely purify protein, the pooled fractions from Superdex 75 pg were fractionated on a Poros 20HP2 column for HIC. Porous materials contain large pores (> 1000 Å), which make mass transport far more important than diffusion of conventional materials, in which pores are less than 1000 Å. This leads to an independent process on the flow rate and speeds up the chromatographic separations efficiently. Poros 20HP2 was a very strong hydrophobic material. In order to separate the active protein from other proteins well, we used a linear 3.0M $(NH_4)_2SO_4$ gradient. Active fraction was eluted between 9 and 10 min and separated from other proteins (Figure 4). The hydrophilic proteins do not interact with the stationary phase. In contrast, less hydrophilic peptides interact with the support. It showed that active proteins were strongly hydrophilic.

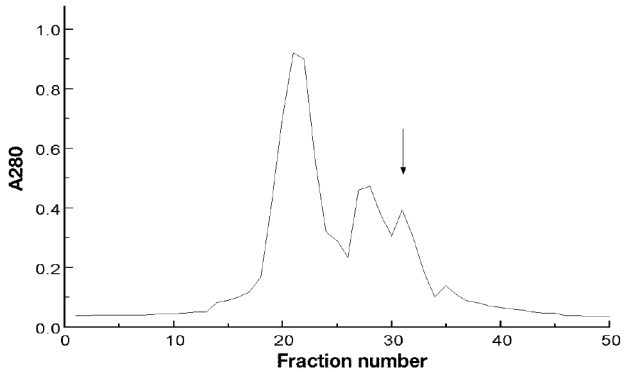

Figure 3. Intermediate purification of 1 mL of a pool of TH-active DEAE-cellulose 52 fractions on a Superdex 75 prep-grade column (70- × 1.6-cm i.d.) equilibrated and run with the running buffer [150mM NaCl and 50mM Tris-HCl (pH 7.4)] at 0.4 mL/min. Proteins were detected by absorbance at 280 nm. The arrow indicates the active peak.

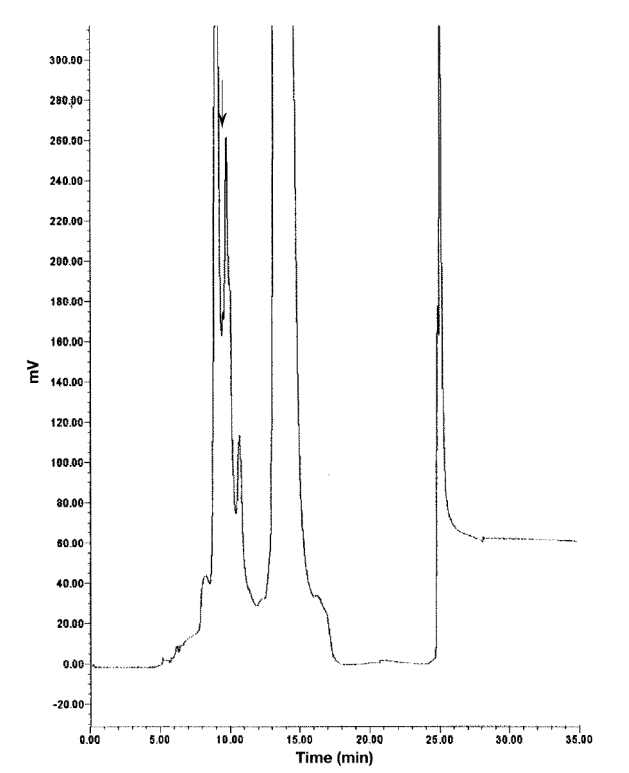

Figure 4. Polishing step of 1 mL of a pool of TH-active Superdex 75-pg fractions on a preparative Poros 20HP2 hydrophobic interaction HPLC column (100- × 4.6-mm i.d.). Elution was achieved by a linear $(NH_4)2SO_4$ gradient (3–0M, within 25 min) at a flow rate of 1 mL/min. Detection was at 280 nm, with simultaneous measurement of the conductivity. The arrowed peak with higher absorbance shows antifreeze activity.

To check the purification, samples were taken at all steps and analyzed by SDS–PAGE (Figure 5). The silver-stained gel showed the efficiency of the separation strategy. The crude extract and IEC purification showed many proteins (Figure 5A and 5B). After gel permeation chromatography, TH-active fractions still contained other proteins as contaminants (Figure 5C). Therefore, a second purification step was necessary. After HIC, two proteins close to each other were visible at approximately molecular masses of 34,700 and 37,100 (Figure 5D). To identify which protein had antifreeze activity, the recovery of proteins from SDS–PAGE gel was conducted by electrophoresis. The protein with 37,100 showed TH activity and could modify the normal growth of ice crystals. The TH activity of this purified antifreeze protein was 0.24ºC at the concentration of 5 mg/mL. As compared with whole cell protein of the plant, the crude extract lacked some high-molecular-mass proteins (Figure 5A).

Duman (16) once found several AFPs from bittersweet nightshade and purified an AFP with molecular mass of 67,000 using DEAE-Sepharose CL-6B and preparative IEF, however, other active fractions from DEAE-Sepharose CL-6B were purified, with difficulty, to a single band by IEF. Hon et al. (7) also found multiple antifreeze polypeptides with similar amino acid compositions in rye leaves. These multiple antifreeze polypeptides formed oligomeric complexes to interact with ice to inhibit ice growth and recrystallization effectively. Many such active proteins were also observed in fish (17) and insect species (18). The presence of multiple AFPs in an individual may be the rule in AFPs in some animals and plants, with slight variation in sequences and sometimes major variations in molecular masses. Several AFPs with different activities and structures in the same organism may function cooperatively to protect organisms from freeze damage in different compartments and at different times.

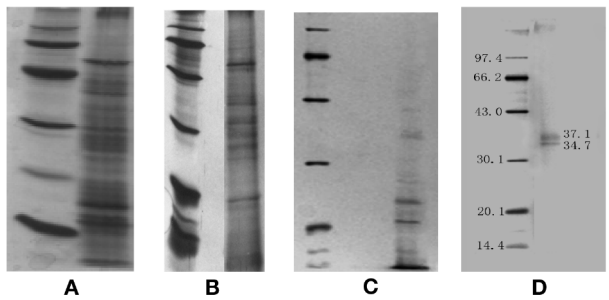

Figure 5. SDS–PAGE gel analysis of proteins from different chromatographic steps of the purification. The proteins were analyzed on 13.5% gels and visualized by staining with silver. (A) The supernatant of crude extraction, (B) pooled active fractions after DEAE-cellulose 52 column, (C) pooled active fractions after Superdex 75 prep-grade column, and (D) purified AFP in pooled active fractions after Poros 20HP2 column. For the left lane, protein molecular mass markers (from top to bottom) were 97.4, 66.2, 43.0, 30.1, 20.1, and 14.4 kDa. The right lane identifies the pooled active fractions from different chromatographic steps.

The antifreeze activity of AFP is critical for the survival of organisms under subzero temperatures. However, the TH activity of AFPs from plants (generally 0.2–0.5°C) is lower than that of AFPs from fish (generally 0.7–1.5°C) or insects (generally 3–6°C) (6), and AFP from rye grass shows higher activity of ice recrystallization inhibition, which is at least 200 times that of the type III AFP from ocean pout (11). Low THA suggests that the major function of plants may not be to suppress the apoplastic freezing, but to inhibit ice recrystallization, which is the growth of larger ice crystals at the expense of smaller ones. Ice recrystallization is considered to be a factor in damaging tissues at high subzero temperatures and during the thawing process. The ice recrystallization inhibition of AFPs occurs at very low concentrations (1).

Conclusion

In summary, we developed a protocol to purify AFP using widely known standard chromatographic techniques such as anion exchange, gel permeation, and hydrophobic interaction, followed by electrophoresis with the modification of ice crystal morphology.

Acknowledgments

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