Video Article

Identification of Plant Ice-binding Proteins Through Assessment of Ice-recrystallization Inhibition and Isolation Using Ice-affinity Purification

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Abstract

Ice-binding proteins (IBPs) belong to a family of stress-induced proteins that are synthesized by certain organisms exposed to subzero temperatures. In plants, freeze damage occurs when extracellular ice crystals grow, resulting in the rupture of plasma membranes and possible cell death. Adsorption of IBPs to ice crystals restricts further growth by a process known as ice-recrystallization inhibition (IRI), thereby reducing cellular damage. IBPs also demonstrate the ability to depress the freezing point of a solution below the equilibrium melting point, a property known as thermal hysteresis (TH) activity. These protective properties have raised interest in the identification of novel IBPs due to their potential use in industrial, medical and agricultural applications. This paper describes the identification of plant IBPs through 1) the induction and extraction of IBPs in plant tissue, 2) the screening of extracts for IRI activity, and 3) the isolation and purification of IBPs. Following the induction of IBPs by low temperature exposure, extracts are tested for IRI activity using a 'splat assay', which allows the observation of ice crystal growth using a standard light microscope. This assay requires a low protein concentration and generates results that are quickly obtained and easily interpreted, providing an initial screen for ice binding activity. IBPs can then be isolated from contaminating proteins by utilizing the property of IBPs to adsorb to ice, through a technique called 'ice-affinity purification'. Using cell lysates collected from plant extracts, an ice hemisphere can be slowly grown on a brass probe. This incorporates IBPs into the crystalline structure of the polycrystalline ice. Requiring no *a priori* biochemical or structural knowledge of the IBP, this method allows for recovery of active protein. Ice-purified protein fractions can be used for downstream applications including the identification of peptide sequences by mass spectrometry and the biochemical analysis of native proteins.

Video Link

The video component of this article can be found at https://www.jove.com/video/55302/

Introduction

Ice-binding proteins (IBPs) are a diverse family of protective proteins that have been discovered in a number of organisms including plants[1], insects[2], fish[3], and microbes[4]. The key feature of these proteins is their unique ability to specifically and efficiently adsorb to ice crystals, modifying their growth. IBPs have several documented properties, with the two most well characterized being thermal hysteresis (TH) and ice-recrystallization inhibition (IRI). TH activity is more readily observed in IBPs produced in freeze-intolerant animals. This activity results in lowering of the freezing point of organisms' circulatory or interstitial fluids to prevent freezing. In contrast, in freeze-tolerant organisms, which will inevitably freeze at subzero temperatures, IBPs appear to have low TH activity. Despite the low TH activity, a high IRI activity to restrict ice crystal growth is often observed with these proteins. For the freeze-tolerant organism, this IRI activity presumably helps protect cells from the uncontrolled growth of ice in extracellular compartments.

The "mattress button" model can be used to describe the mechanism by which IBPs prevent the growth of ice crystals[5]. Under this model, IBPs specifically adsorb to the ice crystal surface at intervals, such that water molecules can only incorporate with the growing ice crystal lattice in the space between bound IBPs. This creates a curvature that makes the incorporation of additional water molecules unfavorable, an event that can be described by the Gibbs-Thomson effect[6]. The anchored clathrate waters hypothesis provides a mechanism for the specific binding of IBPs to the ice crystal surface whereby the presence of charged residues, specifically positioned on the protein ice binding site, results in the reorganization of water molecules so they match one or more planes of the ice crystal lattice[7].

TH activity can be quantified by measuring the difference between the melting and freezing temperatures of a single ice crystal in the presence of an IBP. While TH activity is a widely accepted method of evaluating the activity of IBPs, the low TH gap produced by plant IBPs (typically only a fraction of a degree) normally requires a high protein concentration, specialized equipment and operator experience. Although non-IBPs may restrict ice crystal growth, it is a property shared by all IBPs and thus testing for IRI activity is an effective initial screen for the presence of IBPs, especially for those with low TH activity. The methodology used to test this activity is known as a 'splat assay', whereby a protein sample is flash frozen to produce a monolayer of small ice crystals, which are observed over a period of time to determine if ice crystal growth is restricted.

Unlike other methods used to screen a source tissue sample for the presence of IBPs, this technique is applicable to low protein concentrations in the range of 10-100 ng, utilizes easily-fabricated equipment, and generates data that is quickly and easily interpreted. However, it is important to stress that this assay provides an initial screen for IBPs that should be followed by the determination of TH and ice crystal shaping.

The isolation of native proteins is challenging, often requiring information regarding the structural and biochemical properties of a protein of interest. The affinity of IBPs for ice allows for the isolation of these proteins using ice as a substrate for purification purposes. Since the majority of molecules are pushed ahead of the ice-water boundary during ice crystal growth, slow growth of an ice-hemisphere in the presence of an IBP sample results in a highly-purified sample, devoid of large quantities of contaminating proteins and solutes. This method has been used to identify IBPs from insects[8,9,10], bacteria[11], fish[12] and plants[13,14]. Additionally, the IBP-enriched fractions achieved through this method can also be used for downstream biochemical analysis. This paper outlines the identification of IBPs in plants through the induction and extraction of IBPs, analysis of IRI activity to confirm the presence of proteins, and the isolation of proteins using ice affinity purification.

Protocol

1. Splat Apparatus Setup

1. Fill a temperature-programmable circulating water bath with ethylene glycol (50% v/v in water).
   NOTE: Green automotive ethylene glycol can be used.
2. To assemble the external chamber, use insulated plastic tubing to attach the water bath to a double-walled glass bowl. Insulate the glass bowl with polystyrene foam and cover with a plastic petri dish lid. Cut a 3-4 inch hole in the bottom of the polystyrene chamber so that the light source can be seen through the glass chamber.
3. Mount the external chamber on a dissection microscope, fitted with a camera port and a 4X polarized objective lens with a 10x ocular lens. Place an additional piece of polarized film in the bottom of the external chamber.
4. Clamp a 1-1.5 m tube (~5 cm diameter) onto a retort stand and place inside a large polystyrene container, along with a cooling block positioned underneath the tube at the base of the retort stand.

2. Preparation of Native Protein Extracts for IRI Analysis

1. To induce the expression of IBPs in freeze-tolerant grasses or other freeze-tolerant plant species, cold acclimate plants for up to 1 week at 4 °C, under low light conditions (6 h light/ 18 h dark). This does not apply to samples collected from the field that have already been exposed to low temperature conditions and acclimation periods.
2. Obtain approximately 0.1 g of leaf tissue by cutting at the base of the stem and then place in a 1.5 mL tube. Immediately flash freeze the sample in liquid nitrogen and store at -80 °C until use.
3. To lyse cells, grind tissue sample into a fine powder using a mortar and pestle under liquid nitrogen. Ensure that the liquid nitrogen does not evaporate during the homogenization process.
4. Immediately place ground samples on ice and allow the liquid nitrogen to completely evaporate. Add 1 mL of a native protein extraction buffer containing 10 mM Tris-HCl, 25 mM NaCl (pH 7.5), with two EDTA-free protease inhibitor tablets added immediately before use. Pipette to mix; do not vortex.
   NOTE: Additional additives may need to be used if the lysate contains secondary metabolites (see Discussion).
5. Gently shake samples overnight (18-24 h) at 4 °C. Conduct the remaining steps at 4 °C.
6. Remove cellular debris by centrifuging samples at ~16,000 x g for 5 min. Retain the soluble fraction and centrifuge for an additional 5 min if cellular debris persists. Clarified samples can be stored at -20 °C until use.

3. Splat Assay Pre-experiment Set up

1. Pour 100 mL of hexane into external chamber set up with the polarized film and add 5-6 desiccation beads to prevent moisture build up on the slides. Add a small amount of vacuum grease to the plate covering the bath and twist to ensure a tight seal and prevent evaporation of the hexane.
2. Cool the hexane bath between -4 °C and -6 °C using the programmable water bath approximately 2-3 h before starting the experiment. Make sure to check the temperature of the hexane with a thermometer prior to beginning the experiment.
3. Place the cooling block in a polystyrene box directly below the plastic tube and cover completely with dry ice, 40 min prior to starting the experiment.
4. Before beginning the assay, ensure that the tube is level. To determine where on the cooling block the sample will drop, first test the procedure with water and indicate where the sample drops with a marker (as described below).
5. Remove dry ice from the top of the cold block and place a glass microscope cover slide on the drop mark that has been determined with water.
6. Immediately before starting assay, turn on the light source and scrape any ice that has formed on the bottom of the glass chamber. Avoid keeping the light source on unless ice crystals are being visualized to prevent heating the hexane.

4. Conducting the Splat Assay

1. Dip the disposable tip affixed to an automatic pipette (1-20 µL) in immersion oil, using a paper towel remove any excess oil. This step will allow the sample to fall rather than adhere to the outside of the pipette.
   NOTE: Removing excess oil from the pipette is critical to ensure that oil droplets do not form on the ice crystals, making visualization difficult.
2. Pipette 10 µL of sample and place pipette directly over plastic tubing before releasing the sample. A distinct 'splat' sound should be heard when the sample hits the glass cover slide, creating a monolayer of ice crystals.

3. Quickly and carefully remove the microscope slide from the cold block using tweezers and place in the hexane bath on top of the polarizing film.
4. Looking under the microscope, put the microscope slide into the field of view and adjust the objective lens so that the cross-polarization allows for the visualization of high contrast ice crystals. Adjust magnification to 40x.
5. Attach the camera to the microscope and adjust the settings to "macro" to allow clear visualization of ice crystals and capture the image.
   NOTE: Waiting up to 1 h to allow ice crystals to grow can give a clearer picture.
6. Repeat steps 4.1-4.5 for all samples. Typically, place up to 6 slides with a different sample in the hexane chamber.
7. Turn off the light source and place a plastic plate over the hexane bath, ensuring a tight seal. Allow ice crystals to anneal overnight (12-24 h, keeping a consistent annealing period within a particular assay). Following incubation, capture images of the ice films as described above.
   NOTE: Some samples may recrystallize more quickly than others. If no recrystallization is apparent after 12 h, allow crystals to anneal for up to 24 h to ensure that no recrystallization will occur.

5. Splat Assay Data Analysis

1. Upload the photos to a computer and directly compare the size of ice crystals before and after annealing. Samples with ice recrystallization activity will not grow, whereas samples without no IRI activity will recrystallize forming noticeably larger ice crystals. Light interference will make large ice crystals appear in slightly different hues and thus are easy to see.

6. Ice-affinity Purification Equipment Setup

1. Fill a temperature-programmable circulating water bath with ethylene glycol. Green automotive ethylene glycol can be used.
2. Connect the bath to a hollow, brass probe using insulated plastic tubing[15].
3. Construct a polystyrene container into which a 150 mL beaker can fit snuggly. Create a lid for the container with a hole in the center for the brass cold finger.

7. Preparation of Samples for Ice-affinity Purification

1. Cold acclimate plant tissue as described in section 2.1.
2. Collect ~100-150 g of the ground biomass (step 2.3) in 20 mL tubes and immediately flash freeze. Samples can be kept at -80 °C before use.
3. Prepare sample as described in sections 2.3-2.5. Use a 1:1 ratio (mg of tissue: mL of native protein extraction buffer) for resuspension of leaf tissue. Use additional protease inhibitor tablets to avoid the degradation of IBPs by endogenous proteases.
   NOTE: If the tissue sample is too concentrated, adjust the ratio to 1:2 or 1:3 (mg of tissue: mL of native protein extraction buffer).
4. To remove cellular debris, sieve lysate through 2 layers of cheesecloth, 3 times. Pellet the debris by centrifugation at 30,000 x g for 40 min at 4 °C.
5. Increase the volume of sample to 120 mL using native protein extraction buffer. Use the lysate immediately for ice-affinity purification to avoid any loss of ice-binding activity.

8. Conducting Ice-affinity Purification

1. Prior to purification, set the programmable water bath to cool from -0.5 °C to -3 °C at a rate of -0.04 °C/h.
   NOTE: The cooling rate can be adjusted depending on the initial volume of sample.
2. Cool the ice finger to -0.5 °C and subsequently submerse it into a 50 mL tube containing cold distilled water. Add a few ice chips to facilitate ice nucleation and allow a thin layer of ice to form on the finger. This process typically takes between 20 and 30 min.
   NOTE: Any lumps on the ice-coated finger should be smoothed with a gloved hand prior to placing in sample. If an ice layer does not form at -0.5 °C, lower the temperature to -0.75 °C.
3. Place sample in a 150 mL glass beaker containing a small magnetic stir bar into a polystyrene container on top of the magnetic stirrer. Make sure that the ice finger is centered and lowered at least half way into the liquid sample. Seal the container.
4. Allow the program to run for approximately two days, checking every 24 h. Once 50% of the sample is frozen, stop the program. Incorporation of IBPs into the ice hemisphere will result in "ice-etching".
   NOTE: Purification experiments can be done using larger sample volumes with the length of time at which the program runs adjusted accordingly.
5. In order to remove the ice hemisphere, warm the probe to 4 °C, rinse the hemisphere with distilled water to remove unbound protein, and then thaw at 4 °C.
   NOTE: Most IBPs are not stable in water and an appropriate buffer (*i.e.* 50 mM Tris-HCl, pH 8 or 50 mM ammonium bicarbonate, pH 8) will need to be added periodically during the thawing process (approximately 1 mL/2 h). Ice hemispheres can be wrapped in aluminum foil and stored at -20 °C prior to thawing.
6. If the ice-hemisphere still contains pigment, or to increase the sample purification, repeat steps 8.1-8.5 2-3 times.
   NOTE: Although the concentration of IBP may be lower following multiple rounds of purification, purity is more valuable than yield if the purified samples will be analyzed using MS. If sufficient material is available, three rounds of ice-affinity purification is recommended.

9. Identification of IBPs

1. Since IBPs are contained in a large volume following thawing of the ice-hemisphere, concentrate the samples. Concentrate samples containing ~100 g of tissue to ~1-3 mL using centrifugal concentration tubes with a 3,000 Da molecular weight cut-off to avoid the loss of small proteins.

2. Prior to sending samples for MS, determine the protein concentration using a protein quantification assay such as a BCA or Bradford assay. Estimate the purity of samples by gel electrophoresis such as SDS-PAGE. Test the purified proteins for IRI activity prior to sending for MS to ensure that active protein has been retained through the purification and concentration steps.
3. Use concentrated samples directly for mass spectrometry analysis[10].

Representative Results

For ease of set-up, **Figure 1** and **Figure 2** are included as visual representations of the equipment used for IRI analysis and ice-affinity purification, respectively. Results of IRI analysis using extracts collected from mustard weed with and without IBPs are depicted in **Figure 3**. These results show that extracts collected from mustard weed, which is not freeze-tolerant, were unable to restrict ice crystal growth due to the lack of IBPs present. In contrast, the small ice crystals seen with transgenic plants containing IBPs from perennial ryegrass restrict ice crystal growth substantially. **Figure 4** shows ice hemispheres grown using perennial ryegrass extracts expressing IBPs after two rounds of purification. After the first round of purification the high degree of ice-etching on the hemisphere surface indicates that IBPs have adsorbed to the ice and modified growth of the ice crystals **(Figure 4A**). The second round of purification shown in **Figure 4B** has clearer ice, indicating that there was some exclusion of contaminating molecules. Ice-etching on the hemisphere surface indicates that IBPs are still present. **Figure 5** shows an SDS-PAGE following ice-affinity purification, of proteins collected from perennial ryegrass, and subsequently stained with a silver stain. A number of proteins were identified with seven distinct bands, corresponding to the most abundant proteins that adsorb to ice.

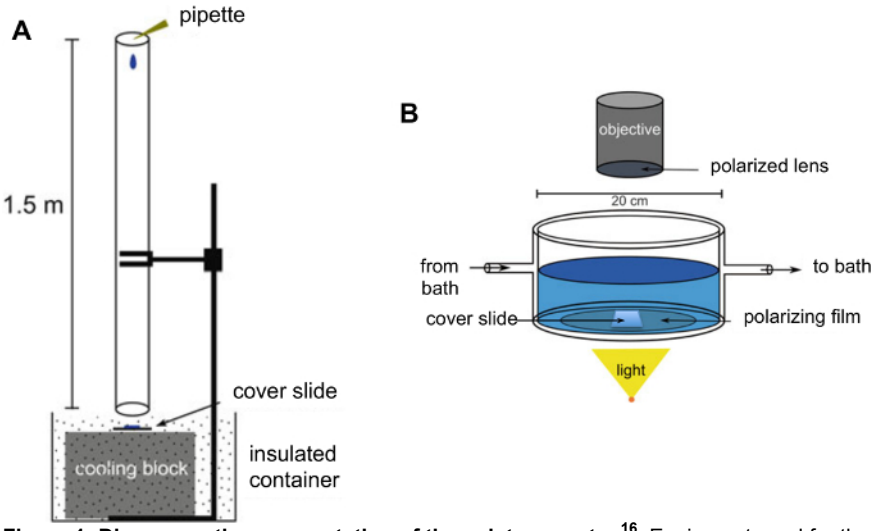

Figure 1: Diagrammatic representation of the splat apparatus[16]. Equipment used for the generation of a monolayer of ice crystals (**A**) and for the visualization of ice crystal growth (**B**). Please click here to view a larger version of this figure.

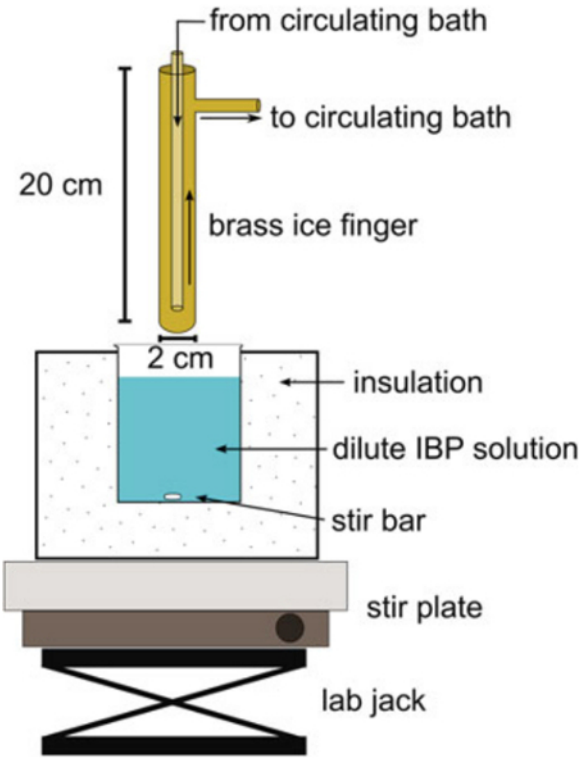

Figure 2: Apparatus used for ice-affinity purification of IBPs[16]. A brass "ice finger" attached to a programmable ethylene glycol bath is used to slowly grow an ice-hemisphere. Please click here to view a larger version of this figure.

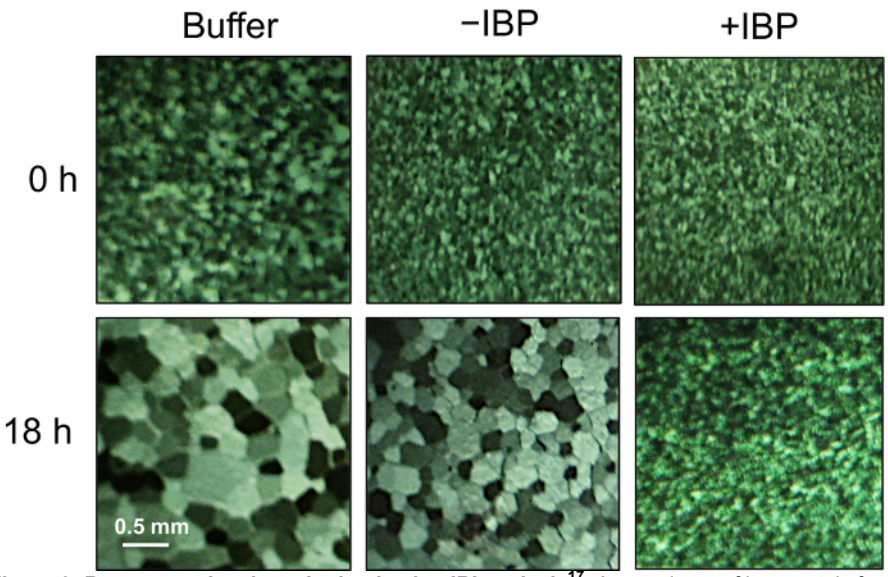

Figure 3: Representative data obtained using IRI analysis[17]. A monolayer of ice crystals formed using plant extracts is visualized at 40x magnification. Extracts from wild-type *Arabidopsis thaliana* that do not contain IBPs (−IBPs) were compared to extracts of transgenic *A. thaliana* expressing IBPs from *Lolium perenne* (+IBPs). Following an 18 h incubation period at -4 °C, ice crystals had recrystallized in the buffer and wild-type *A. thaliana* samples. In contrast, ice crystal growth was restricted in the *A. thaliana* extracts expressing an IBP. Please click here to view a larger version of this figure.

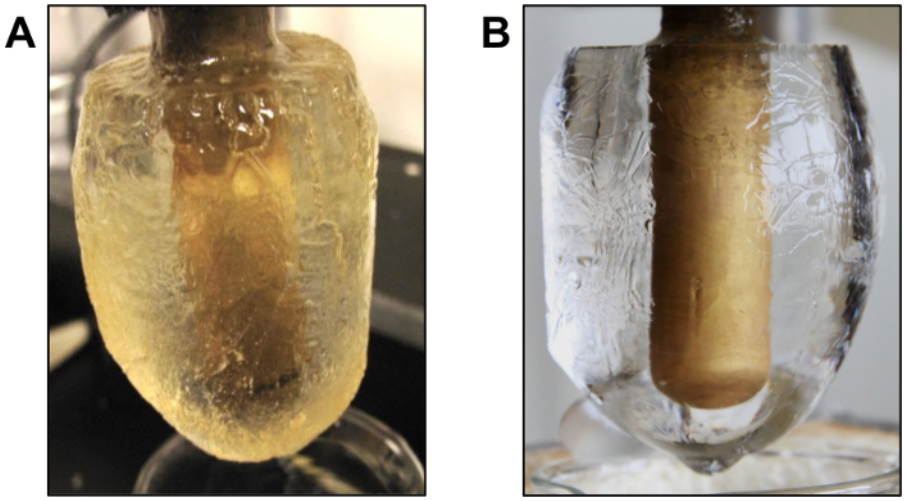

Figure 4: Ice-hemispheres grown using IBP extracts collected from the freeze-tolerant grass, *L. perenne*. After grass extracts were subjected to one round of ice-affinity purification, ice-etching of the surface of the ice hemisphere indicates the successful incorporation of IBPs (**A**). To remove additional solutes, pigments, and contaminating proteins, a second round of ice-affinity was used, resulting in a clearer ice-hemisphere (**B**). Please click here to view a larger version of this figure.

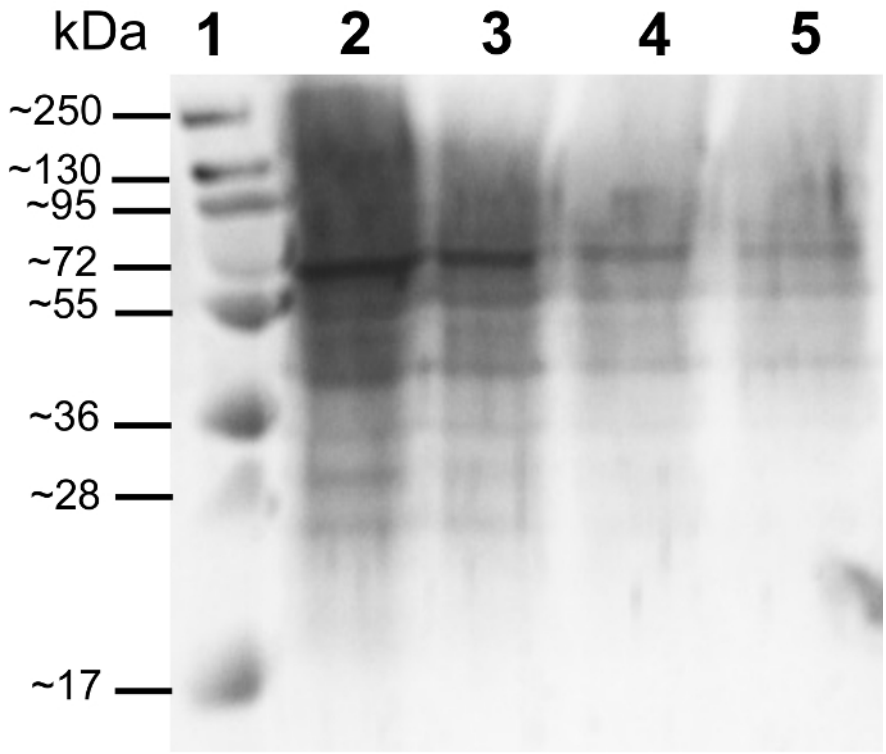

Figure 5: Monitoring the purification of native IBPs from perennial ryegrass. Following two rounds of ice-affinity purification and centrifugal concentration, native grass extracts were electrophoresed on a denaturing polyacrylamide gel (SDS-PAGE) and then visualized using silver stain. Lane 1: molecular weight protein ladder; Lane 2: undiluted extract; Lane 3-5: samples diluted in water prior to gel loading with a 1:2, 1:5, and 1:10 (sample:water) ratio. Since this particular plant species contains a number of IBPs, multiple bands are observed which may correspond to different IBP isoforms. Please click here to view a larger version of this figure.

Discussion

For the successful analysis and purification of IBPs, it is important to understand the temperature-sensitive nature of some of these proteins. Certain plant IBPs become unstable at temperatures above 0 °C, resulting in unfolding, precipitation and inactivity. In order to obtain active IBPs, it is often critical that plants are processed in a cold room (~4 °C) and samples are kept on ice during experimentation. Another factor to consider when using whole-cell crude lysates is the degradation of proteins by endogenous proteases. In plants, the expression of IBPs is generally low, and thus any loss in protein yield could result in insufficient material for future experimentation or analysis. Working quickly during

protein extraction, with the addition of a reliable protease inhibitor cocktail can reduce such protein loss. Additionally, it is important that the cold-acclimation period be optimized for the greatest accumulation of IBPs in laboratory-grown plants.

Another common problem when working with IBPs is that many of the assays are sensitive to temperature and humidity. In regards to IRI analysis, moisture buildup on the microscope cover slide can result in a layer of ice that makes visualization of ice crystals difficult. Using a dehumidifier and extra desiccation beads can resolve this difficulty; however, it is recommended that this assay is not conducted when laboratory relative humidity levels are above 70%.

The splat assay, as presented, is qualitative. By performing a dilution series prior to analysis, the IRI endpoint can be established to determine the concentration range in which the IBPs can no longer restrict ice crystal growth. Various labs have also used computer software to measure the average size of ice crystal grains[18]. As previously indicated, while testing for IRI activity is an efficient initial screen, confirmation of ice-binding activity should be established by determining the level of TH or by directly visualizing the adsorption of IBPs to ice crystals[16]. A notable limitation of IRI analysis is that several non-IBP molecules have been identified that mimic IRI activity. These molecules can include bulky proteins, phenolic glycosides, and synthetic polymers such as polyvinyl alcohol[19,20,21]. As a result, a buffer control should always be run with samples to ensure that any contaminants or additives do not result in the observed IRI activity.

While ice-affinity purification is easily performed, when not done correctly, other proteins, solutes and metabolites can predominant. An important consideration is the rate of ice crystal growth; by lowering the cooling rate (*i.e.* to -0.02 °C/h), the ice hemisphere grows more slowly and any non-ice-binding molecules are less likely to become incorporated in the ice crystal lattice. The presence of contaminating molecules can also be avoided by ensuring that only 50% of the lysate is incorporated into the hemisphere (*i.e.* 50:50, liquid to ice ratio). As previously stated, multiple rounds of ice affinity can also clarify the ice-fraction and result in a sample of higher purity. Notably, secondary metabolites are often overproduced under biotic and abiotic stress conditions in plants. During the purification of phenol-rich plant tissues, if dark brown pigments persist, 1.5% (v/v) polyvinylpyrrolidone (PVP) and 1.5% polyvinylpolypyrrolidone (w/v) (PVPP) can be added to the extraction buffer, to neutralize secondary metabolites. Antioxidant additives such as thiourea (5-10 mM) can also be used[19,21].

If information about the IBP of interest is known, additional purification steps, such as high-pressure liquid chromatography (HPLC) can also be used. Modifications to ice-affinity purification can also be considered and have been reported to optimize protein yield and gain additional insight into IBP characteristics including the faster ice shell purification[23] and fluorescence-based ice plane affinity (FIPA)[24].

When optimized, the techniques described above can yield a purified protein pool for the identification of IBPs; however, unspecific incorporation of other highly abundant proteins may be inevitable. While IBPs from the Pooideae family share sequence homology[25], due to the high diversity between IBPs from different organisms, it is typically not possible to identify an IBP by sequence homology alone. A common feature of IBPs is the presence of amino acids known to bind to ice, including threonine, serine and valine[26]; identifying peptides sequences rich in these residues may be useful when mining sequence data.

Disclosures

The authors have nothing to disclose.

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References

1. Sidebottom, C., *et al.* Heat-stable antifreeze protein from grass. *Nature.* **406** (6793), 256 (2000).
2. Duman, J.G. Antifreeze and ice nucleator proteins from terrestrial anthropods. *Annu Rev Physiol.* **63**, 327-357 (2001).
3. Davies, P.L., & Hew, C.L. Biochemistry of fish antifreeze proteins. *FASEB J.* **4** (8), 2460-2468 (1990).
4. Gilbert, J.A., Hill, P.J., Dodd, C.E., & Laybourn-Parry, J. Demonstration of antifreeze protein activity in Antarctic lake bacteria. *Microbiology.* **150** (Pt 1), 171-180 (2004).
5. Knight, C.A., Cheng, C.C., & DeVries, A.L. Adsorption of alpha-helical antifreeze peptides on specific ice crystal surface planes. *Biophys. L.* **59** (20), 409-418 (1991).
6. Yeh, Y., & Feeney, R.E. Antifreeze proteins: structures and mechanisms of function. *Chem. Rev.* **96**, 601-618 (1996).
7. Smolin, N., & Daggett, V. Formation of ice-like water structure on the surface of an antifreeze protein. *J. Phys. Chem. B.* **112**, 6193-6202 (2008).
8. Graham, L.A., & Davies. P.L. Glycine-rich antifreeze proteins from snow fleas. *Science.* **310** (5747), 461 (2005).
9. Hawes, T.C., Marshall, C.J., & Wharton, D.A. Antifreeze proteins in the Antarctic springtail, Gressittacantha terranova. *J. Comp. Physiol. B.* **181** (6), 713-719 (2011).
10. Basu, K., Graham, L.A., Campbell, R.L., & Davies, P.L. Flies expand the repertoire of protein structures that bind ice. *Proc. Natl. Acad. Sci. U.S.A.* **112** (3), 737-742 (2015).
11. Guo, S., Garnham, C.P., Whitney, J.C., Graham, L.A., & Davies, P.L. Re-evaluation of a Bacterial Antifreeze Protein as an Adhesin with Ice-Binding Activity. *PLoS One.* **7** (11), e48805 (2012).
12. Marshall, C.B., Fletcher, G.L., & Davies, P.L. Hyperactive antifreeze protein in a fish. *Nature.* **429** (6988), 153 (2004).
13. Zhang, D.Q., Liu, B., Feng, D.R, He, Y.M., & Wang, J.F. Expression, purification and antifreeze activity of carrot antifreeze protein and its mutants. *Protein Expr. Purif.* **35** (2), 257-263 (2007).
14. Gupta, R., & Dreswal, R. Low temperature stress modulated secretome analysis and purification of antifreeze protein from Hippophae rhamnoides, a Himalayan wonder plant. *Proteome Res.* **11** (5), 2684-2696 (2012).

15. Kuiper, M.J., Lankin, C., Gauthier, S.Y., Walker, V.K., & Davies, P.L. Purification of antifreeze proteins by adsorption to ice. *Biochem. Biphys. Res. Commun.* **300** (3), 645-648 (2003).
16. Middleton, A.J, *et al.* Isolation and characterization of ice-binding proteins from higher plants. *Methods Mol. Biol.* **1166**, 255-277 (2014).
17. Bredow, M., Vanderbeld, B., & Walker, V.K. Ice-binding proteins confer freezing tolerance in transgenic Arabidopsis thaliana. *Plant Biotechnol. J.* (2016).
18. Olijve, L.L, *et al.* Blocking rapid ice crystal growth through nonbasal plane adsorption of antifreeze proteins. *Proc. Natl. Acad. Sci. U.S.A.* **113** (14), 3740-3745 (2016).
19. Zakharov, B., *et al.* Ice Recrystallization in a Solution of a Cryoprotector and Its inhibition by a Protein: Synchrotron X-Ray Diffraction Study. *J. Pharm. Sci.* **105** (7), 2129-2138 (2016).
20. Capicciotti, C.J, *et al.* Small molecule ice recrystallization inhibitors enable freezing of human red blood cells with reduced glycerol concentrations. *Sci. Rep.* **5**, 9692 (2015).
21. Knight, C.A., Cheng, C.C., & DeVries, A.L. Adsorption of alpha-helical antifreeze peptides on specific ice crystal surface planes. *Biophys. J.* **59** (2), 409-418 (1991).
22. Van Driessche, E., Beeckmans, S., Dejaegere, R., & Kanarek, L. Thiourea: the antioxidant of choice for the purification of proteins from phenol rich plant tissues. *Anal. Biochem.* **141** (1), 184-188 (1984).
23. Marshall, C.J., Basu, K., & Davies, P.L. Ice-shell purification of ice-binding proteins. *Cryobiology.* **72** (3), 258-263 (2016).
24. Basu, K., *et al.* Determining the ice-binding planes of antifreeze proteins by fluorescence-based ice plane affinity. *J. Vis. Exp.* **83**, e51185 (2014).
25. Sandve, S.R., Rudi, H., Asp, T., & Rognli, O.A. Tracking the evolution of a cold stress associated gene family in cold tolerant grasses. *BMC Evol. Biol.* **8** (245) (2008).
26. Middleton, A.J., Brown, A.M., Davies, P.L., and Walker, V.K. Identification of the ice-binding face of a plant antifreeze protein. *FEBS Lett.* **583** (4), 815-819 (2009).

