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Review Article

ANTIFREEZE PROTEINS IN PLANTS: AN OVERVIEW WITH AN INSIGHT INTO THE DETECTION TECHNIQUES INCLUDING NANOBIOTECHNOLOGY

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Abstract: Antifreeze proteins (AFPs) are a class of polypeptides which enables various organisms to survive subzero temperatures and have been found in vertebrates, invertebrates, plants, fungi and lichens. AFPs possess the characteristic thermal hysteresis (TH) and ice recrystallization inhibition (IRI) properties which allow them to adsorb the surface of ice crystals and inhibit their growth and recrystallization. AFPs are also known as ice restructuring proteins due to their ability to modify ice crystal morphology which leads to formation of hexagonal shape ice crystals in the presence of AFPs and disc shape AFPs in its absence. AFPs have various applications in medical, agricultural, industrial and biotechnological field. This review provides an overview of the AFPs, their TH and IRI properties and potential biotechnological applications of AFPs. Various conventional detection methods like Capillary assay and Differential Scanning Calorimetry (DSC) with their advantages and disadvantages are discussed in detail along with the commonly used Splat assay and Nanoliter osmometer. Moreover, a novel, high-throughput and efficient nanobiotechnological method for AFP detection is also discussed. The method is based on colorimetric detection of freeze-labile gold nanoparticles and can provide an alternative to overcome the limitations of conventional methods by providing quick and easy way to screen AFPs in multiple systems simultaneously.

Keywords: Antifreeze; IRI; Splat assay; Nanoliter osmometer; Nanoparticles.

1. Introduction

Different areas on earth face extreme temperature ranging from 55 ºC in Sahara desert to –89 ºC in Arctic and Antarctic regions. Temperature stress can greatly affect the crop productivity and low temperature is one such limiting environmental factor which affects the growth and distribution of plants. Understanding the phenomenon of adaptation during temperature stress has been a topic of considerable importance (Guy, 1985; Thomashow, 1993). Various organisms have evolved different strategies to protect themselves from such environmental extremes. They either avoid or develop tolerance against the undesired stress conditions.

Plants being poikilothermic may allow their temperature to vary with the temperature of the environment to survive these temperature extremes and various kinds of biotic, abiotic stress. Overwintering plants found in Arctic, Antarctic and Alpine climates have developed a high level of freezing tolerance and can survive even when the temperature reaches –50 °C in winter (Griffith *et al.*, 1999). Plants acquire cold tolerance by cold acclimation, a phenomenon which involves various physiological and metabolic changes including alterations in lipid composition, accumulation of amino acids and soluble sugar, changes in enzyme activities,

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production of various antifreeze compounds, dehydrins, kinase regulators, carbohydrate metabolism proteins and cryoprotective proteins which protect intracellular proteins to membrane during the freeze-thaw process and maintain their functional state (Ewart *et al.*, 1999; Yu and Griffith, 2001).

Additional proteins which accumulate in extracellular spaces during cold acclimation include cell wall-modifying proteins which identify the changes occurring at membrane level, influx of Ca^{2+} and start the regulatory signal transduction pathway, pathogenesis-related (PR) proteins which protect the plants against disease causing organisms and AFPs which interact with ice and protect the cells against fatal intracellular and intercellular ice formation (Hiilovaara-Teijo *et al.*, 1999; Griffith *et al.*, 1992). Freeze-tolerant cultivar of winter wheat can survive –25 °C (Antikainen and Griffith, 1997) while some grasses can survive even at – 30 °C establishing that plants have special mechanisms to protect themselves against freezing stress. These overwintering plants survive low temperature stress by employing strategies like freeze avoidance or freeze tolerance for freezing resistance (Levitt, 1972). Freeze-avoiding plants avoid complete freezing in the tissues by supercooling (a phenomenon where aqueous solutions remain in liquid state even when cooled below their melting point) or lowering of the freezing point with the help of antifreeze substances while freezing tolerant plants secrete ice nucleating proteins in their apoplast, which allows ice formation only in the extracellular region at low level of supercooling thereby avoiding intracellular ice formation by decreasing ice nucleation temperature at high level of supercooling i.e. below the temperature at which extracellular ice formation occurs (Steponkus *et al.*, 1984; Parody-Morreale *et al.*, 1988; Zamecnik and Janacek, 1992). This review focuses on plants AFPs, their properties, biotechnological applications and various antifreeze assays to detect these potential AFPs.

2. Antifreeze proteins

AFPs were first discovered by De Vries in 1969 as macromolecules in the blood of Antarctic fishes capable of preventing ice growth and surviving subzero (–1.9 ºC) temperatures (DeVries and Wohlschlang, 1969). Since then proteins with similar properties have been discovered in a variety of biological organisms ranging from plants (Griffith, 1992) fungi, bacteria (Duman and Olsen, 1993) to arthropods (Duman 1979, Block and Duman, 1989). Four different types of AFPs and Antifreeze glycoproteins (AFGPs) are reported in fishes (Ewart *et al.*, 1999; Yeh and Feeney, 1996; Davies and Sykes, 1997 and Cheng, 1998).

AFPs include non-homologous, structurally diverse class of proteins. AFPs present in different organisms often have no structural and sequence similiarity. These proteins are classified into two major groups: AFGPs and AFPs. AFPs interact with ice and modify the ice crystal morphology owing to the presence of two unique properties, thermal hysteresis (TH) and ice recrystallisation inhibition (IRI) due to which they are also known as ice structuring proteins (ISPs), Thermal Hysteresis proteins (THPs) and Ice Recrystallisation Inhibition Proteins (IRIPs). AFPs act in a non-colligative manner and prevent ice formation by depressing the freezing point of a solution without affecting the melting point (March and Reisman, 1995; Antikainen and Griffith, 1997). The difference between the freezing and melting point is known as thermal hysteresis. By virtue of TH activity, AFPs protect the plants by depressing the freezing point and allowing supercooling of water but once ice is formed another property, IRI comes into play. Rather than preventing water from freezing, it controls the size, shape and aggregation of smaller ice crystals at high subzero temperature, by binding the surface of nascent ice crystals and inhibiting ice recrystallisation which could cause physical damage to the tissue (Knight *et al.*, 1995; Griffith and Ewart 1995; Zachariassen and Kristiansen, 2000; Clarke *et al.*, 2002). AFPs adsorb to the prism planes of ice and change the ice crystal morphology (Raymond et al, 1989). AFPs bind along the a-axes and allow formation of hexagonal shaped crystals while hyperactive AFPs allows growth of ice preferably along the c-axis rather than a-axis of the ice crystal lattice leading to the formation of narrow spicules (De Vries, 1986). The relationship between TH activity and AFPs concentration is hyperbolic suggesting

both to be related in a non-linear fashion. AFPs are effective even at a lower concentration of 100µg/lt in inhibiting ice recrystallisation and this concentration is 100 times lower than required to show TH activity (Knight *et al.*, 1988; Yeh *et al.*, 1994). Presence of putative signal peptide sequences suggest that these AFPs are secreted. However, AFPs are present in a variety of organisms but the TH and IRI property and mode of action is specific to the type of AFP present. It has been found that plant AFPs work in an all together different manner than insect and fish AFPs and possess highest IRI activities which is detailed below.

2.1. Plant AFPs

In plants AFPs were first reported by Griffith *et al.*, in 1992. These have been found on exposure to low temperature (during cold acclimation in apoplast) in various overwintering plants in different parts including seeds, stems, crowns, barks, branches, buds, petioles leaf blades, flowers, berries, roots and tubers. Atleast 23 species of angiosperms like oats (*Avena sativa*), winter rye (*Secale cereale*) (Hon *et al.*, 1994), barley (*Hordeum vulgare*) (Antikainen and Griffith, 1997), carrot (*Daucus carota*) (Smallwood *et al.*, 1999), potato (*Solanum tuberosum*), Bittersweet nightshade (*Solanum dulcamara*) (Duman, 1994; Urrutia *et al.*, 1992); Seabuckthorn (*Hippophae rhamnoides)* (Gupta and Deswal, 2012), *Picea pungens* (Jarzabeck *et al.*, 2009), *Picea abies* (Sabala *et al.*, 1996), Peach (*Prunus persica*) (Wisniewski *et al.*, 1999), *Forsythia suspensa* (Simpson *et al.*, 2005), *Ammopiptanthus mongolicus* (Fei *et al.*, 1994), *Arachis hypogaea* (Dave and Mitra, 1998), Perennial ryegrass (*Lolium perenne*) (Pudney *et al.*, 2003; Sidebottom *et al.*, 2000), Winter wheat (*Triticum aestivum*) (Chun *et al.*, 1998; Atıcı and Nalbantoglu, 1999), kale (*Brassica oleracea*) (Huang and Duman, 1995), *Rhodiola algida* (Lu *et al.*, 1998) and many more were found to possess these proteins. In freeze tolerant species, plant AFPs are induced after cold acclimation in the extracellular compartment where ice crystallization occurs and prevents intercellular ice crystallization which could be lethal to the tissues.

AFPs are structurally diverse and each type differs in their TH activity. Plant AFPs show lower TH activity (0.2 to 0.5 ºC) as compared to fish which show TH in the range of 0.6 - 1.5 ºC (Fletcher *et al.*, 1998; Doucet *et al.*, 2000; Ewart *et al.*, 1999) and insect AFPs show TH range of 3 - 6 ºC while hyperactive AFPs can show TH as high as 5 - 10 ºC, (Walker *et al.*, 200) but possess extraordinary high IRI activity (Venketesh & Dayananda, 2008). Insect AFPs possess considerably higher activity 100-1000 times than fish. Hyperactive AFPs from insect *Choristoneura fumiferana* binds to both prism and basal planes thereby helping them to survive a temperature range of even –40 to –70 °C. For example, winter rye AFPs exhibit IRI activity at concentration as low as 25 µg/l (Griffith and Ewart, 1995). Several AFPs have been isolated, characterized and cloned in higher plants like *Solanum dulcamara,* Carrot, Winter rye, *Lolium perrene.* Plant AFPs are an example of convergent and parallel evolution with nearly identical proteins evolved from two totally different genes and perform same function without structural and sequential similiarity. Several plant AFPs show homology with pathogenesis proteins like glucanases, chitinases, thaumatins and perform both enzymatic and antifreeze functions. Many such proteins found in plants exhibit dual functions to provide antifreeze activity (freeze tolerance) along with the protection against psychrophilic pathogens and disease resistance while some THPs homologs still exist which retain pathogenesis activity but show no antifreeze activity thereby suggesting that plant AFPs represent intermediate stage of evolution with original function retained by these AFP homologs. Various pathogenesis responsive AFPs have been isolated from plants. In winter rye 3 such proteins have been isolated including Class I and Class II endochitinase AFPs, thaumatin-like proteins and β-1,3-endoglucanase AFPs (Pihakaski-Maunsbach *et al.*, 2001,Griffith and Yaish, 2004). Moreover, Chitinase genes encoding AFPs of 31.7 kDa and 24.8 kDa were cloned from winter rye leaves (Yeh *et al.*, 2000). These proteins have also been reported in *Daucus carota, Solanum dulcamara* and *Arachis hypogaea*. AFP from *Daucus carota* shows sequence similarity with polygalacturonase inhibitor proteins (PGIPs) which belongs to a large family of PR-proteins (Meyer *et al.*, 1999; Worrall *et al.*, 1998). Although

not much is known about the regulation of AFPs, their dual functioning and how these AFPs bind to ice crystal to exhibit antifreeze activity have been described which is discussed briefly here.

3. Mechanism of action of AFPs

Different AFPs possess different ice-binding faces which are relatively flat, hydrophobic and bind to different planes of the ice. None of the single mechanism is found sufficient to clearly explain the interaction and ice binding affinity of AFPs. AFPs possess amphipathic ice binding faces and majorly affect the growth and morphology of ice crystals through adsorption-inhibition mechanism, surface complementarity between the ice binding site of AFPs and the specific ice planes inhibits further growth of ice at the facets adsorbed with these AFPs while the exposed ice surface between bound AFPs grow into a curvature which causes a thermodynamically unfavourable condition known as Kelvin effect (Wen and Laursen, 1993; Wilson *et al.*, 1993; Davies and Sykes, 1997). The curvature formed has a larger surface area to volume ratio which allows depression of freezing point at that surface as compared to remaining solution. AFPs exert their effect by gathering at ice-water interface, forming hydrogen bonding on the outside with ice and hydrophobic interactions (buried inside) on opposite side contribute to this binding with ice thereby preventing ice recrystallization and changing ice crystal morphology (Barrett, 2001). The forces involved in the binding are provided by vander waal's interaction, hydrogen bonding and gain in entropy due to release of water from the surface after hydrophobic ice binding site adsorb to the ice. Different AFP molecules possess independent activity and do not require protein-protein interactions for ice binding.

Kuiper *et al.*, (2001) first presented a theoretical three-dimensional model of plant AFPs from rye grass (*Lolium perenne*). The model partially explained the protein's low thermal hysteresis but high ice recrystallization inhibition activity based on a β-roll domain with 8 loops of 14 - 15 amino acids, which display opposite-facing and composite ice-binding sites complementary to ice prism face and this interaction leads to high ice recrystallisation inhibition. Ice growth may occur in two directions along the c-axis (normal to the basal plane) or along the a-axis (prism facets) and different type of AFPs bind to different axes based on which the ice crystal morphology is modified to hexagonal or needle shape. However the trend of ice binding followed by diverse AFPs is same, hyperactive AFPs remain stable at low temperature and prevent growth of ice by binding along the c-axis (Figure 1) and grow explosively when the temperature is lowered below the non equilibirium freezing point due to attachment of undercooled water in the direction where ice growth is normal to c-axis while moderately active AFPs direct ice growth along c-axis and prevents ice growth at prism plane (Scotter *et al.*, 2006).

4. Methods to Detect Antifreeze Activity

Antifreeze activity can be quantified and observed by various conventional methods like Splat assay, Nanoliter osmometer, Differential Scanning Calorimetry and Capillary assays. However, each method has its own benefits and drawbacks but splat assays and Nanoliter osmometer are commonly used methods to determine IRI and TH activity. A novel nanoparticle based colorimetric detection has been introduced recently, and could be used effectively to measure AFP activity. All these methods are discussed in detail.

4.1. Splat assay

"Splat cooling" allows a reliable assessment tool for analyzing the effect of solutes on recrystallization of ice. The dependency and control of ice grain growth rate with grain size, solute concentration and temperature was observed. RI activity of AFPs was first demonstrated by Knight *et al.* (1986) using a "splat" assay. The original technique used to study recrystallization inhibition effect involves sealing the solution between 300 μm layers of glass slides to form a film and allowing nucleation at the range of –6 to –8 °C followed by freezing by placing the sandwich on an aluminium block maintained at –12 °C and annealing at –4 °C for 24 hrs. The grain size obtained was used to measure the effect of the solute upon spontaneous recrystallization. The trouble with this method is

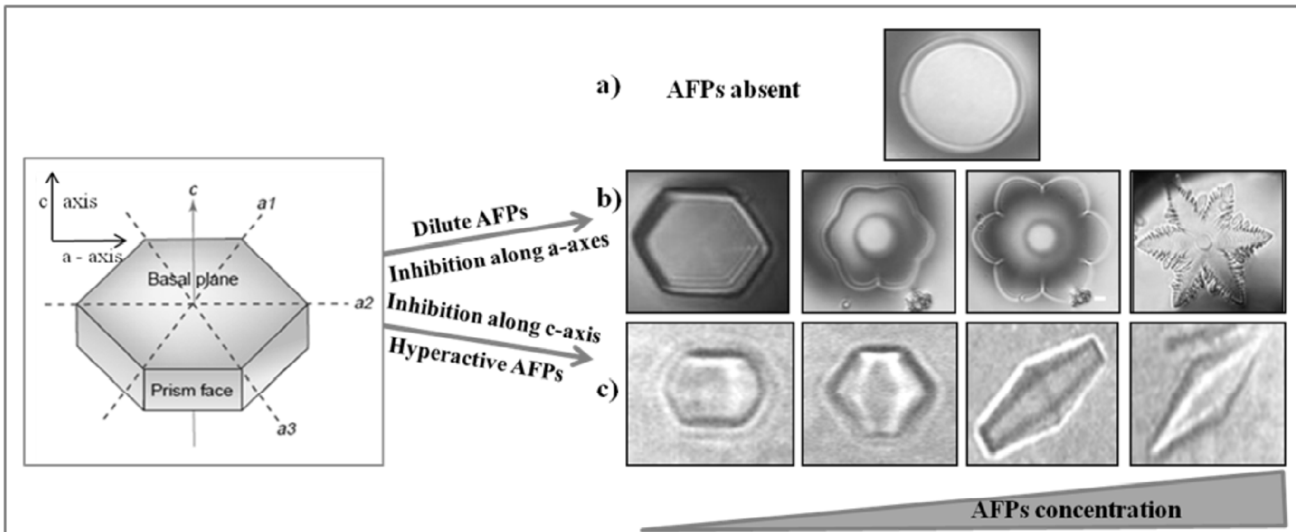

Figure 1: **Mechanism of action of AFP**

The mechanism of ice growth and changes in ice crystal morphology in the presence of AFPs. The ice crystal grow acquire a particular shape as the temperature is lowered, which occurs due to growth along the three a - axes i.e. the prism plane and the c-axis known as basal plane. (a) In the absence of AFPs, ice grow in the shape of circular disk. (b) While in the dilute solution, the AFPs preferentially bind to the prism face of ice and inhibit binding of water molecules along a-axes which allows water to bind to the basal plane and grow along the c-axis. This type of AFP binding allows formation of hexagonal to flower to star-shaped ice crystals depending on AFPs concentration. (c) At higher concentrations of AFPs, the ice crystals due to AFP binding towards the basal plane rather than prism plane and allow growth of ice along a-axes thus forming bipyramids or spicules, that are hexagonal in cross-section, a characteristic feature of Hyperactive AFPs.

the involvement of two separate processes: the nucleation of new grains and their recrystallization which depends on both annealing temperature and time and the final size of ice crystal is the result of above processes but due to the uncertainity involved in the nucleation, the results produced are unsatisfactory showing no reproducibility. At certain annealing temperature and protein concentration either no recrystallization at all was observed or the ice texture changes completely to polyhedral grains.

In order to overcome this problem a new standard procedure was introduced in the field of cryobiology to measure ice recrystallization by Knight *et al.* (1988) known as "Splat Cooling Assay" which was already being used by the metallurgists for sample preparation. It involved release of 10 µl droplet of AFP solution from 2.6 m above a polished aluminium plate cooled to –78 °C by dry ice and used immediately to minimize frost on the surface. The droplets immediately froze after hitting the metal surface and form a thin and slightly concave disc of 5 mm width and 50 µm thickness. The disc was covered with coverslip and was later transferred from the plate to a cold microscope stage with a cold knife blade at high subzero temperature. Ice recrystallization during annealing were observed over time in small, flat-bottomed glass or polystyrene dishes at –8 °C and photographed using cross-polaroids method to determine changes in the average size of the ice crystals grains with time. To improve the photograph quality the ice discs in the dishes could be immersed in hexane. The method showed reproducible grain size coarsening during annealing, and this amount of coarsening is a measure of grain boundary mobility and energy. Inhomogenity in grain size was obtained in few cases which could be due to non-uniform stress in the ice disc but this problem could be solved by picking a maximum grain diameter.

Disadvantages: This method is very time consuming as the steps involved in preparing splat and conducting annealing are very complex and the factors on which the method is dependent like sublimation of ice from splat unless kept under hexane or a coverslip, and condensation of water vapour on the splat during the transfer to the cold stage are very complex.

4.1.1. Modified splat assay: sucrose sandwich splat assay

This method was later modified by Smallwood *et al.* (1999) and was named **"sucrose-sandwich splat assay"** and is extensively used by researchers to studying plant AFPs and their IRI activity. The sample was sandwiched evenly between two round glass coverslips, snap-frozen in dry ice-cooled anhydrous 2,2,4-trimethylpentane, immersed in another jacketed beaker containing the solvent and were incubated at –6 °C. (Smallwood *et al.* 1999). Digital images of all samples were taken using immediately after they were immersed in the –6 °C bath and after their annealing (Knight *et al.*, 1995; Worrall *et al.*, 1998; Sidebottom *et al.*, 2000; Raymond and Fritsen, 2001).

Another variant after slight modifications was deviced which retain the essential features of Splat assay and is used extensively nowadays to quantitatively measure the endpoint of RI activity which is defined as the concentration below which the degree of ice recrystallization was indistinguishable from that in the control and was known as "Sucrose sandwich splat assay". In the method the protein solution in 30% sucrose was allowed to be sandwiched between round coverslips and snapfrozen in heptane kept at –80 °C. High solute concentration was used to avoid non-specific activity and ice boundary migration due to other non-AFP solutes. Sandwich was immediately transferred into a glass viewing chamber containing heptane,, maintained at –6 °C by a circulating water bath (Figure 2A). Growth of ice crystals was observed in the microscope fitted with camera after incubating the samples for an hour to allow annealing of ice crystals and then capturing the images (Smallwood and Worall, 1999; Pudney 2003). The method can be used semiquantitatively to analyze the RI endpoints by making serial dilutions of samples and determining the concentration at which RI activity was no longer detected.

Advantages: Both the splat and modified splat assays demonstrate the IRI activity of AFPs and the endpoint of IRI activity. The method is simple, easy to use and does not require a skilled person to handle the equipment. The assay could be used as a basic method to screen the presence or absence of AFPs in the samples.

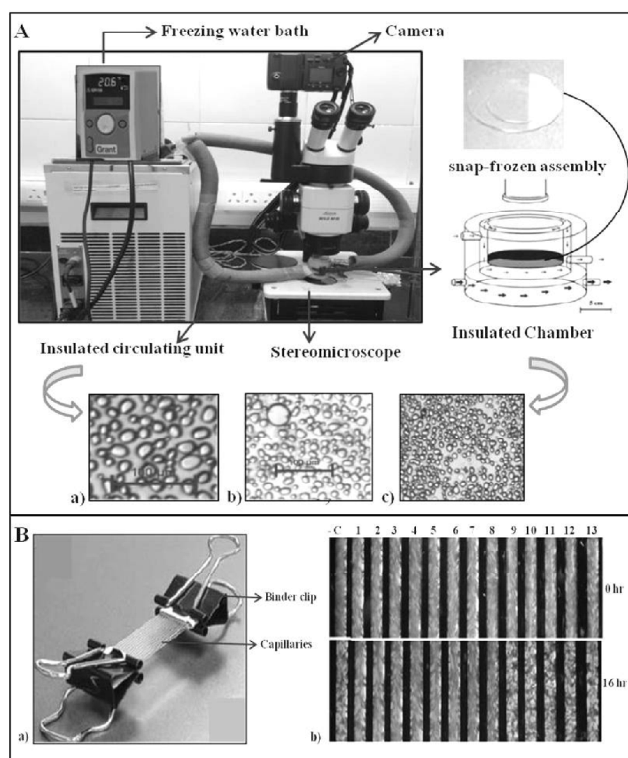

Figure 2: **IRI endpoint determination using A) Sucrose sandwich splat assay and B) Capillary assay.**

A) The instrumental setup showing stereomicroscope along with the insulated chamber connected with freezing water bath and snap-frozen sandwich for performing sucrose sandwich splat assay. RI assay using modified splat assay a) Control b) and c) Sample containing 30 % sucrose is annealed for 1 h at –6 °C to allow ice recrystallization. AFP free solution show higher ice recrystallization as compared to AFP containing solution (depending on the concentration of AFPs) which shows controlled growth of ice crystals. B) Apparatus showing a set of 10 µl capillaries aligned together and clipped by binder clips used for IRI assays. IRI assay using Capillary method. Serial dilutions (1-13) of AFP concentrations are prepared, snap frozen to form multicrsyatalline ice pattern and incubated for 16 hr at –6 °C to allow annealing of ice crystals and then compared with negative control (AFP free solution) to determine the endpoint of IRI. Pictures were taken immediately after snap freezing at 0 h and after 16 h incubation. The figure is adapted and modified from Tomczack *et al.* (2003).

Disadvantages: Only one sample at a time could be analyzed and a direct comparison of results is not possible by this method until recorded images are assembled and analyzed next to each other. Moreover the sample once used has to be discarded and could not be stored for future use or reference. It requires larger annealing times and is time consuming method.

4.2. Capillary assay

Tomczak *et al.* (2003) devised an efficient assay to determine the IRI activity endpoints of AFPs. This method is simple, allow direct visualization and comparison of multiple series of samples in the same field of view and also allow retrieval of the sample for future use. Here IRI activity of an AFP was determined using 10 µl glass capillaries (51mm long, 1mm outer diameter) into which samples were loaded by capillary action. The capillaries contain AFP dilution series in the desired buffer, buffer as control and a negative control, BSA for comparision which could be sealed and aligned next to each other by clamping them together at each end (Figure 2B) and each series were snap-frozen in anhydrous 2,2,4-trimethylpentane cooled to –50 °C by dry ice, which were later immersed in a jacketed beaker filled with the same solvent maintained at –6 °C by circulating refrigerated bath (Tomczack et al, 2003). Samples were incubated at –6 °C overnight for approximately 16 hr and their images were taken for analysis as soon as they were immersed in the –6 °C bath and again at the conclusion of the experiment at 16 hr. The method allows visualisation of 10 -15 samples simultaneously thereby increasing its applicability for rapid screening of AFPs in fractions collected from column chromatography. Moreover, serial dilutions prepared could be used to determine the RI endpoint.

Advantages : The capillary method has several advantages over the splat or sucrose sandwich splat assays.

First, a series of samples could be prepared, viewed and analysed simultaneously, thereby allowing determination of RI endpoint for multiple samples within one field of view. This method uses a simple set-up and is efficient as it doesn't require any special and complex instrument like nanoliter osmometer. Moreover, results obtained corresponded well with the TH values measured by nanoliter osmometer and similar endpoints were observed as that obtained from modified splat assay performed in TBS. The method could be used to analyze and find antifreeze protein in column fractions by making serial dilutions of column fractions for identifying the antifreeze protein peak, because even small quantities of AFP found in fractions between peaks have RI activity before dilution. Once prepared, the capillaries loaded with samples can be used reproducibly over several weeks in subsequent RI assays and can be archived in a freezer for future reference hence it is possible to prepare a standard AFP sample set that will allow the activity of AFPs to be compared directly from batch to batch.

4.3. TH measurement

Nanoliter osmometer is used to measure TH activity and to observe ice crystal morphology. TH activity of AFPs could be used for quantitative analysis (DeVries, 1986) while the ice crystal morphology provides a qualitative analysis of AFP activity (Atici and Nalbantoğlu, 2003). The device consists of a controller box, a cooling stage coupled with a temperature controller unit and a sample holder. Temperature of the stage can be adjusted to as low as –20 °C –40 °C with a precision of ± 0.01 °C. Syringes capable of loading nanoliter volumes are used to apply mineral oil at the base of the well of disc to prevent the sample from leaking and dehydration. Later nanoliter volume of the protein sample is loaded onto the oil droplet held by surface tension in the wells of a sample holder disc which is then mounted on a cooling stage of a phase-contrast photomicroscope carefully where the temperature of the sample is controlled using Peltier device. The sample is rapidly frozen at –20 °C to form multicrystalline ice crystals and the temperature is gradually increased (0.1 °C/min) to allow thawing of the frozen sample till only a single ice crystal is left in the well. The temperature is further decreased slowly to allow this single ice crystal to grow and the change in its morphology was observed during TH measurement and recorded using video photography though the microscope (Fletcher et al., 2001, Gwak *et al.*, 2010; Lee *et al.*, 2012). After each assay, the sample holder disc is rinsed with organic solvent to avoid contamination from previous samples and is later checked for residual antifreeze activity with deionized water, which should form a round ice crystal, before assaying any fresh sample. In the presence of AFPs the ice crystal are hexagonal whereas they are disc shaped in the absence of AFPs (Figure 3).

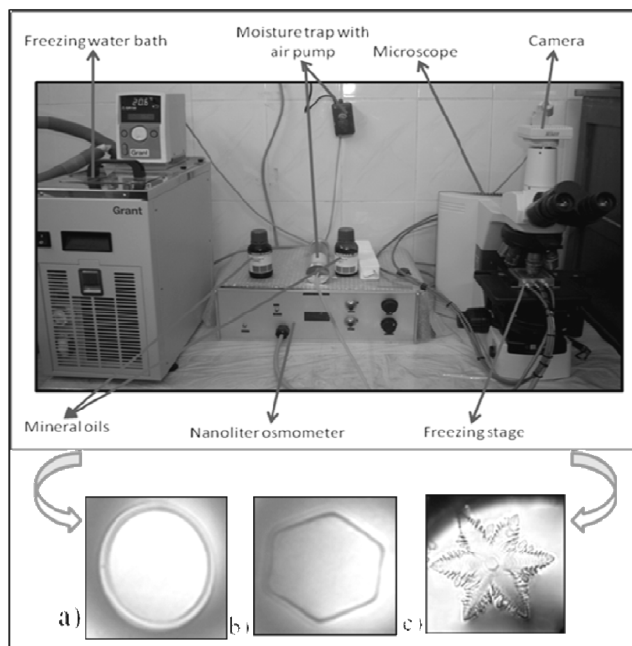

Figure 3: **TH activity and ice crystal morphology detection using Nanoliter osmometer.**

The instrumental setup showing Nanoliter osmometer and microscope with the freezing stage connected to the temperature controlled freezing water bath used to measure TH activity and antifreeze activity of the sample. Ice crystal morphology of a) Control, b) and c) Sample where disk shape ice crystal suggests the absence while hexagonal and star shaped ice crystal suggests presence of antifreeze activity in the sample.

Advantages: Along with the THPs, non–THPs can also show IRI activity by inhibiting water mobility at the ice grain boundaries thereby creating an artefact for AFPs. So IRI is not a diagnostic feature of THPs rather, TH activity and ICM is a better tool for AFPs detection.

Disadvantage: It may lead to observer dependent errors and require long observations of the crystals using naked eyes over a temperature gradient. Moreover, it needs specialised equipment and trained person to handle the nanoliter carefully. ICM and TH measurement obtained may vary depending on the ice content thereby producing non-reproducible results.

4.4. Differential Scanning Calorimetry (DSC)

Calorimetry is a primary technique to measure thermal properties of materials to determine the enthalpy associated with the desired substance. Different calorimeters have been used frequently to measure thermodynamic properties of the biomolecules in various fields of Science. DSC is a thermodynamical tool to determine enthalpy change associated with change in properties of reference and sample material on variation in temperature and time. The instrument has multiple applications in bioscience and nanoscience like thermodynamical analysis of stability of biomolecules under different environmental conditions like pH, ionic strength etc., determination of structural-phase transitions, thermal transition temperatures, melting point heat of fusion, factors involved in folding and stability of biomolecules and oxidative stability, to study conformational changes of molecules in biochemical reactions. Moreover, a recent application of DSC includes analysis of interactions between biomolecules and nanoparticles. The methodology used in a basic DSC experiment involves introduction of energy into a sample cell and a reference cell simultaneously and their temperatures are raised equally for a particular period. Since more energy is required to bring the sample to the same temperature as that of reference, this energy required is equivalent to the amount of energy change during the process. DSC increases or decreases the temperature of the system at a given rate automatically and monitoring the temperature difference which arises between the two cells. Minute differences between the amount of heat absorbed or released by the sample cell with respect to reference cell can be measured and related to sample.

DSC Instrumentation : A general diagram of the basic components of a high sensitivity DSC instrument includes capillary shaped sample and reference cells which could contain sample or reference volumes from 0.15 to 0.8 ml of solution surrounded by a shield to maintain the temperature of scanning to run in an adiabatic mode with no heat transfer to the surroundings and scan rate using (Privalov *et al.*, 1995; Plotnikov *et al.*, 1997) and software controls during the run. It determines a differential heat absorbed or released (enthalpic changes) by the sample and the reference cell using heat monitoring system which also has a temperature sensor on varying the pressure periodically using a controller (Figure 4). Enthalpic changes in the sample with

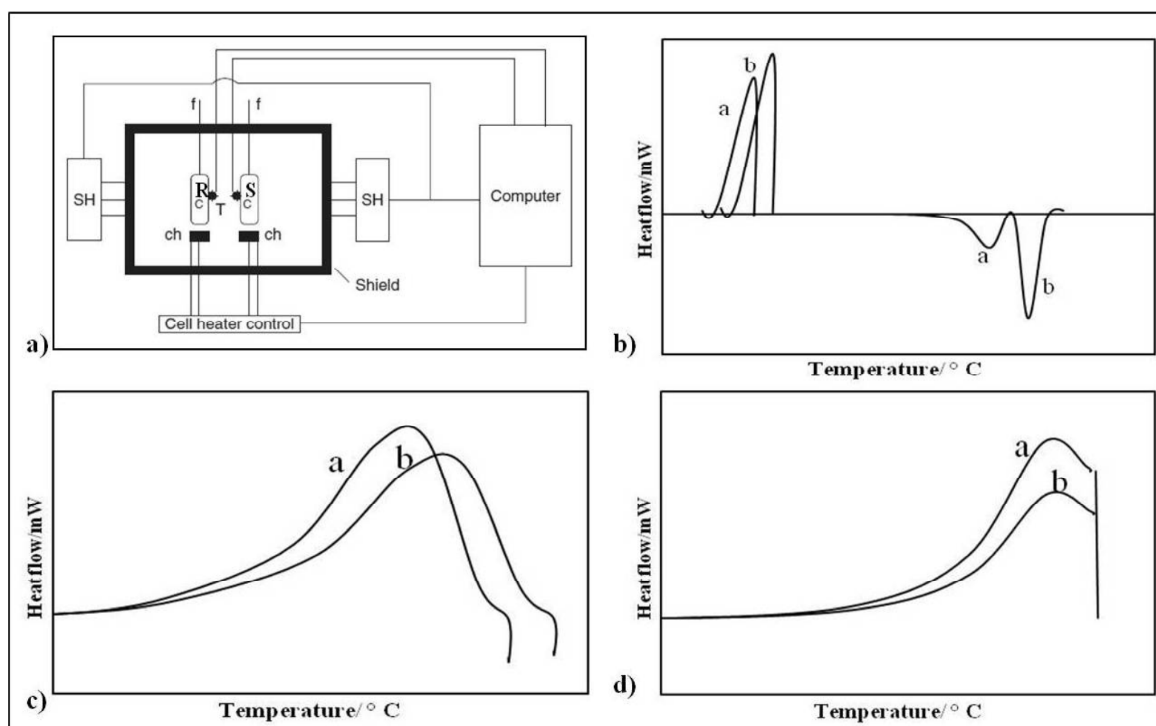

Figure 4 : **Differential Scanning calorimetry to determine TH activity.**

a) Schematic representation of a Differential scanning calorimeter. Reference (R) and sample (S) cells are insulated by shield connected with shield heaters (SH). The temperature difference between the cells is determined by temperature sensors (T) which is then controlled through individual cell heaters (ch) located at the base of cells, regulated via computer softwares to provide compensating energy and maintain zero temperature difference between the cells. Calorimetric signal is obtained as compensating energy per unit time. b) Exothermic freezing and endothermic melting DSC curves for a) AFP containing and b) AFP free BSA solution. Refreezing curves of partially melted c) AFP and d) AFP free BSA solution obtained at different hold temperature (Th). For AFP containing sample curves show TH activity while TH activity was absent for the curves of BSA.

respect to reference over temperature and/or time can be monitored using temperature sensors and the signal is recorded as the calorimetric output. Nowadays, the equipments have become more automated and have autosampler and can perform unattended operation (Plotnikov *et al.*, 2002) for 24 h/day. This high throughput technology enables DSC to be used for drug-biopolymer screening studies as well as in basic research.

In 1988, Hansen and Baust proposed Differential scanning calorimetry to determine the TH activity of AFPs by calorimetric analysis of the solutions in a liquid nitrogen-cooled calorimeter. Samples (1-5 µl) containing AFP solutions were placed in 20 µl immersion oil-filled aluminium pans for volatile samples to prevent their dehydration during the isothermal annealing experiments and BSA was used as a standard AFP-free solution for comparison. An empty aluminium pan was placed in the reference cell.

The sample was first allowed to cool from 28 to –15 °C at the rate of 1 K/min to allow freezing at and then warmed slowly at 0.5 K/min to allow complete melting of the sample. The enthalpy of melting (ΔHm), their melting and freezing points were calculated from the DSC curve. The sample was again frozen at –15 °C, followed by slow warming upto a temperature which allows partial melting of ice crystals and hold (T_h) to allow annealing for 15 mins for interaction of these crystals with AFPs and then slowly recooled to determine the onset temperature (To) at which crystallization exotherm appeared and the enthalpy released on refreezing (ΔHr) and TH activity of AFP were calculated as the difference between the hold or annealing temperature and the onset temperature of recrystallisation. The experimental parameters used in the experiments

could be varied to get more reliable, reproducible results for analysing plant AFPs system. Oil baselines were run before and after sample analysis which showed no TH activity. The freeze exotherm and the melt endothermic area were compared to calculate the ice percentage of the sample (Hansen and Baust, 1989). Thermograms of the samples were analysed and it was observed that higher hold temperatures lead to delay in the onset of the freeze exotherm and increase in area under exothermic curves whereas high temperature allow an increase in the area under the curves suggesting decrease in content of ice present in the equilibrium sample. Solutions without AFP show narrow melting peak and no delay in the onset of freeze exotherm when ice (< 5%) was present in the sample with occurrence of immediate recrystallization of the melted part after temperature drop suggesting absence of TH activity (DeVries and Price, 1984; Hirsh *et al.*, 1985). However on testing AFP efficacy in presence of larger content of ice (>5%) in the system, the AFPs were unable to suppress the ice growth on lowering of temperature and explosive crystallization was observed while AFP containing samples in the presence of < 5% ice fraction which resembles the conditions plants face during the freezing, showed smooth and spreaded exothermic melting peak and a delay in the freeze onset suggesting strong interaction of AFPs with ice water interface and decrease in ice fraction with increase in TH by reducing the size of ice-crystals due to binding of AFPs to the prism face thereby inhibiting ice crystal growth effectively because of high TH activity (Zachariassen and Husby, 1982). Recently, a quick, qualitative and simple method which does not require intense observations and uses low amount of sample (1 µl) has been proposed by Hassas-Roudsari and Goff (2012) which uses DSC based on RI assay. In this method the rate of activity is measured for an hour in isothermal conditions and the samples with AFPs show exothermic thermograms while the ones without activity do not show exothermic thermograms and could be used to measure TH. However, the samples once used cannot be recycled and no data is provided for ice crystal sizes.

Advantages: This method can help in revealing much higher activity up to 10 °C by using higher cooling rates, with minimum variation within a sample whereas other methods report high level of variation, due to ice-crystal size variability. It is a greater quantitative analysis method to provide accurate estimate of ice crystal volume present in the solution and could analyse higher ice fractions (10 - 30%).

Microscopic observations are unable to quantify ice content and variations in activity due to resolution limitations, observer-dependent problem, surface area/volume relationships, non-functional quantifiable assessment of ice fraction due to the discrepancy between real and ideal crystal.

Disadvantage: DSC analysis requires more time for analysis and involves complex procedure and equipments while other methods could help in rapid screening of antifreeze activity.

4.5. Nanobiotechnology

Nanoparticles differ from the bulk materials from which they are derived as these physical, chemical, optical, electrical, mechanical and magnetic properties are size dependent. Colloidal gold nanoparticle is a suspension of nano sized gold particles in water. The liquid vary in color depending on its size due to Surface Plasmon Resonance. In this method gold salt solutions are citrate reduced from Au^{+3} ions to nanoparticles of gold metals whereas excess citrate anions in solution adhere the surface of gold metal to provide negative charge thereby creating electrostatic repulsion amongst them to keep them separate from each other. In this state solution absorbs green light at 520 nm and exhibits red color while on removal of this repulsive force leads to aggregation of the nanoparticles which now absorbs longer wavelength at 650 nm and appear blue (Figure 5). Nanoparticles have various applications in different fields like immunocytochemistry, detection of AFPs, drugs and pharmaceuticals etc.

A novel, highly sensitive and rapid method based on colorimetric assay using freeze-labile gold nanoparticles was used to detect concentration dependent activity and stability of the AFPs. The assay is based on a change in color of AuNPs solution due to the frozen self assembly and light scattering-assisted surface plasmon at

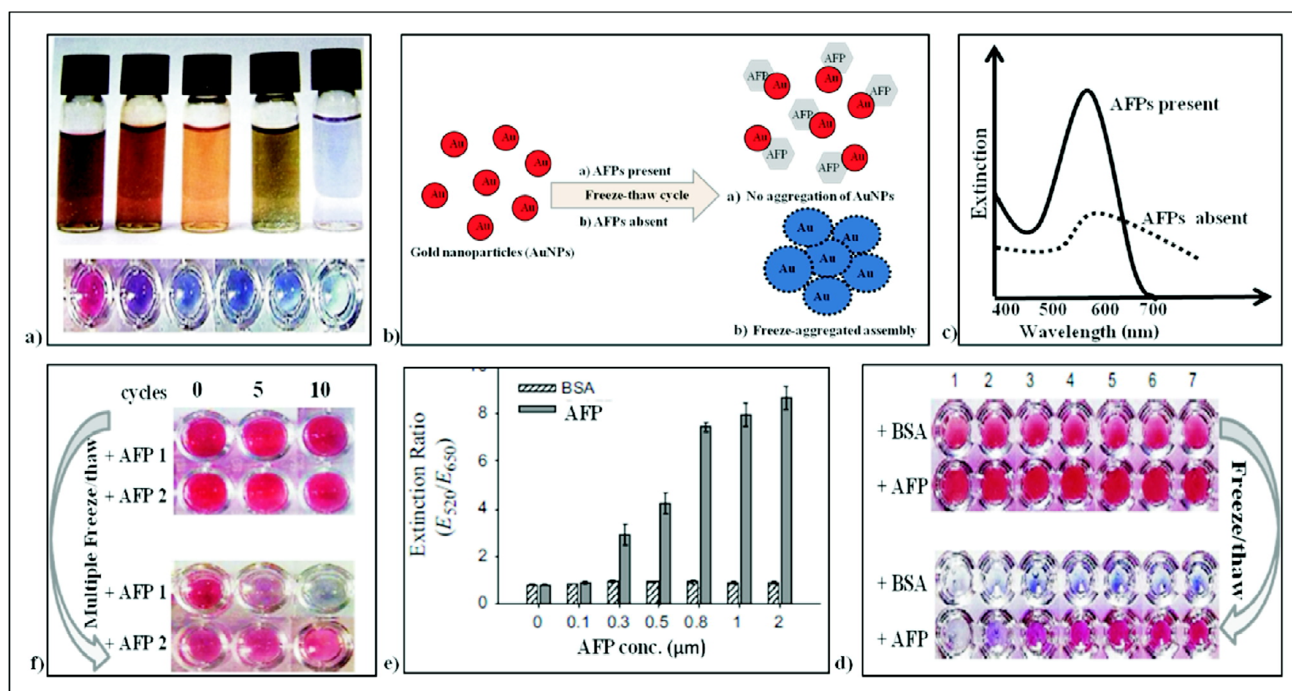

Figure 5: **Colorimetric assay of AFP activity using gold nanoparticles.**

(a) Gold nanoparticles exhibiting different colors at different wavelengths due to surface plasmon resonance (b) Schematic representation showing principle of gold nanoparticles based colorimetric assay to determine antifreeze activity. Freeze induced aggregation (blue) of AuNPs (red) is inhibited after freeze/thaw cycle due to the presence of AFPs (c) extinction spectrum showing the quantification of freeze-induced assembly in the presence and absence of AFPs (d) In the assay MSA- AuNP solution was mixed with either non AFP (BSA) or AFP solution in the microwell plate and freeze/thaw of the mixture was allowed for an hour at 20 °C and10 min at 37 °C and pictures were taken before and after thawing. It was observed that AuNP aggregation was inhibited in presence of AFPs while aggregation occurred in non AFP containing solutions (e) Quantification of aggregation measured by the extinction ratio (E520/E650) (f) The method could also allow comparative stability analysis between two AFPs after multiple freeze/thaw cycles. The figure is modified and adapted from Park *et al.* (2013).

subzero temperature due to surface disruption by ice crystals and can be observed directly with naked eyes and could be quantified through the extinction spectrum. Herein, addition of AFPs which acts as cryoprotectants, to mercaptosuccinic acid-capped (MSA) gold nanoparticles AuNPs prevent their self assembly, aggregation and damage even after a freezing/thawingcycle, therefore allowing no color change in the AuNP solution and in retaining the original color of the solution. The method is simple and cost effective and has received particular attention in the field of clinical and environmental diagnosis.

4.5.1 Methodology to form AuNPs: MSA-capped citrate-AuNPs were synthesised by citrate reduction and stabilization of gold chloride in water and further modification with 2-mercaptosuccinic acid to produce nanogold particles with a narrow size distribution (Grabar *et al.*, 1995; Zhu *et al.*, 2003). A brief methodology involves addition of 1mM $HAuCl_4.3H_2O$ stock solution (20 ml) to distilled water (50 ml) to make a final concentration of 300 nM after boiling with vigorous stirring. Citrate-AuNPs were formed by rapid addition of (2 mL) 30 mM sodium citrate dehydrate to above solution, followed by boiling for 20 mins and cooling with continuous stirring which changes the color of the solution from pale yellow to red brown. Cit-AuNPs are further modified by adding equal amount of 2-mercaptosuccinic acid as that of sodium citrate to the Cit-AuNP solution and stirring the solution at RT for 1h. The molar ratio of final solution contains tetrachloroaurate, sodium citrate and MSA in a ratio of 1: 200:200 to form the AuNPs of average size size 15.8±1.3 nm estimated using a field emission transmission electron microscope. The final concentration of the AuNPs in solution was calculated using the molar extinction coefficient ($1.7 \times 10^8 M^{-1}cm^{-1}$) at 520 nm using UV-Visible spectrophotometer.

4.5.2. Colorimetric assay of AFP activity

The assay could be performed in a microwell plate containing MSA-AuNP solution (100 µl) and AFP containing solution according to its effective concentration could be added to the wells and the final volume in each well was made upto 200 µl while BSA was used as AFP free solution to be taken as a negative control. The sample was allowed to freeze (–20 °C) for 1 hour followed by a thaw cycle at 37 °C for 10 min and the extinction spectrum or aggregation parameter (E_{520}/E_{650}) of AuNP solution, before and after the freeze thaw cycle was quantified by taking absorbance through UV-Vis spectrophotometer (Figure 5). A linear relation is present between the extinction ratio and AFP concentration. The method could also be used to determine the AFP activity and compare the thermal stability of different AFPs by either subjecting them to undergo rapid repetitive (5-10) cycles of freeze/thaw cycles or without freeze-thaw of AFPs to be taken as control which were added to MSA-AuNP solution followed by a cycle of freezing at –20 °C for an hour and thawing at 37 °C for 10 min leading to even and complete aggregation of AuNPs changing its color to blue. It was observed that AFPs containing solution inhibit aggregation of AuNPs at subzero temperature by covering the surface of ice crystals which showed a strong scattering peak near 520 nm in the extinction spectrum and no color change of AuNP solution from red to blue was observed while AuNPs without AFPs self assembled leading to color change hence concentration dependent color change could be observed using the colorimetric assay (Park *et al.*, 2013).

MSA capped Cit-AuNPs has several advantages over traditional citrate reduced gold nanoparticles as being a thiol derivative chemically adsorb via Au-S bonding, its structural similiarity with citric acid and dissociable carboxylic groups allow electrostatic repulsion to maintain the colloid stability after modification in wide pH range and show homogenous growth of particles. These were used to provide a significant difference in the frozen assembly and aggregation of AuNPs as compared to other AuNPs like Cit-AuNPs which are unable to show a marked difference in the frozen assembly between AFP containing and AFP free BSA solution used as negative control as BSA strongly binds with AuNPs surface via cysteine residues thereby making AuNPs stable at subzero temperatures. Moreover, PEG modified AuNPs were highly stable and does not allow self assembly even after freeze/thaw cycles therefore MSA capped Cit-AuNPs due to their loose binding to AuNPs were the best choice to detect AFP activity and stability. To further confirm whether this protection of AuNPs was due to binding of AFPs or some other factors are responsible for it, AFP mutants were formed and the assay was performed which show no TH and AFP activity thus confirming AFPs to act as cryoprotectants for AuNPs at freezing temperature.

Advantages: This novel technique has multiple advantages over conventional TH measuring methods. The method is simple, cost-effective, less complex to operate, sensitive, not labor intensive, high throughput and 4 fold faster screening method for analyzing AFP activity.

TH measurements using other detection methods require high protein concentration in the micromolar range and more time is invested for the observations which take into account only one AFP per observation. However, use of this novel method requires 100-fold lower detection range than other methods and not only leads to faster screening but could also allow real time analysis of multiple AFPs simultaneously.

Disadvantages: At times self assembly may also form due to surface unfunctionality of the AuNPs or freezing conditions rather than ice crystallization. Moreover, the inhibition of self assembly may also occur due to other factors like strong bonding of modified AuNPs with the ligands thereby not allowing significant difference in AFP activity.

4.6. Biotechnological Applications of AFPs

The ability of AFPs to influence ice crystal growth and recrystallization has opened doors for their usage in a wide variety of applications like cold storage in food technology by improving the texture and properties of frozen food and dairy products and reducing leakage of nutrients from

them, improving cold tolerance in plants by making transgenics or directly by vaccum infilteration, cryopreservation of cells, tissues, cell lines and transplant organs, cryosurgery for cancer cells destruction, de-icing agents and to induce cold tolerance in freeze susceptible organisms, aquaculture of cold tolerant fishes (Grandum and Nakagomi 1999; Koushafar and Pham, 1997; Fletcher, 1999; Cheng and Chen, 1999). However, many results have shown that AFPs depending on the type and concentration used may either protect or harm cells.

4.6.1. Transgenic plants: Transgenic expression of Type I AFPs have been done in potatoes, tomatoes and tobacco plants and vaccum infilteration of potato leaves has led to development of freezing tolerance in potatoes (Kenward, 1993; Hightower, 1991; Wallis, 1997). Directly injecting AFP type I in rainbow trout increased their freeze tolerance (Fletcher *et al.*, 1997; Wu *et al.*, 1998). However, in some cases AFPs transferred failed to increase cold tolerance and provide protection against freeze damage which may be either due to absence of suitable enzyme to convert the proteins in their active form or due to lower concentration of the desired protein produced in the transgenics (Larese *et al.*, 1996). Expression of the carrot AFP gene has been done to produce transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants which showed accumulation of antifreeeze activity and freeze tolerance (Worrall *et al.*, 1998). Moreover, an insect AFP in *A. thaliana* (Huang *et al.*, 2002) results in an accumulation of antifreeeze activity species (Meyer *et al.*, 1999; Ewart *et al.*, 1999). Although the plant AFPs possess less effective TH activity than found in fishes and insects it possess high IRI activity which has profound effect on agriculture, which adds to the relevance of wide analysis of plant AFPs. These AFPs could be used to engineer cold susceptible plant species to make them cold tolerant by expression of AFPs. Transgenic tomato expressing genes encoding AFPs show improved freezing tolerance and ice recrystallization inhibition was also observed in the transgenic tissue (Hightower, 1991).

4.6.2. Aquaculture: It refers to the cultivation of fishes in controlled environmental conditions for their commercial exploitation. AFPs exist in many fishes naturally and these AFPs has been consumed as part of the human diet (Atící & Nalbantoğlu, 2003; Crevel *et al.*, 2002; Griffith & Yaish, 2004). In colder regions, aquaculture of some of the commercial fishes during winter is very difficult as they are not able to survive the extreme cold and hence migrate. Problem can be rectified by generating cold tolerant fishes thereby making aquaculture running throughout the year. Rainbow trout, a cold susceptible fish, was transformed into cold tolerant by transferring an AFP gene from winter flounder and could allow its survival even at a freezing temperature of –1.4 °C.

4.6.3. Frozen food products : Since frozen storage is a widely used approach to enhance the shelf life of many frozen items and multiple freezing/thawing leading to formation of large ice crystals could have detrimental effects on the quality, volume, structure, a texture, gas content and nutrient content. AFPs (ISPs) could provide a wide potential and opportunity in food technology and their addition in frozen foods physically, could control structure and size of ice crystal during freezing, storage, transport and thawing which prevent quality loss such as deterioration in food texture and quality by reducing cellular damage and loss of nutrients by reducing drip (Griffith & Ewart, 1995; Feeney & Yeh, 1998; Wathen & Jia, 2005). Moreover, several ISPs from edible parts of plant or fishes have been consumed by humans and are found safe. Nowadays, many such commercial products containing AFPs/ISPs are permitting very little ice crystal growth as compared to control sample which show increase in ice crystal size (Warren *et al.*, 1992). Soaking of bovine meat in type I AFPs prior to freezing reduced the growth of intracellular ice crystals thereby preventing their cell damage and loss of nutrition during thawing (Payne *et al.*, 1994). Similiarly, injecting AFGPs intravenously in lambs before either 1 or 24 h of slaughtering and then vacuum packing and freezing the meat after slaughtering reduced drip loss, protein loss and ice crystal size and could help in storing the meat for 2 to 16 weeks (Payne & Young, 1995). Expression of ISPs in lactic acid bacteria during fermentation helps in direct usage of this organism for production of yogurt, sauerkraut, pickles and fermented sausages

without any need to add purified AFPs in the food itself (Fletcher *et al.*, 1997; Venketesh & Dayananda, 2008). ISPs has been also used to improve the quality of frozen dough used in baker's industries by increase the gassing rate and total gas production in frozen sweet dough (Panadero *et al.*, 2005). Concentrated carrot AFPs have shown some beneficial effects on the quality of the dough by holding the loaf volume and improving the softness of the dough while frozen storage due to lower freezable water content. Moreover, these proteins did not show any negative effect on volatile compounds also (Zhang *et al.*, 2007) while ISPs from winter wheat reduce structural deterioration in the gluten-ice structure and improve mechanical properties of frozen hydrated gluten network by recrystallization inhibition, inducing freezing point depression and decreasing the amount of freezable water (Kontogiorgos, Goff, & Kasapis, 2007; Xu *et al.*, 2009). Recently, the addition of ISP (0.5%; w/w) to corn and wheat starch gels has shown better structural composition. Despite the advantages of using AFPs for cryopreservation of food items, the major drawback of this method is the cost-effectiveness and the acceptance by vegetarian section of consumers due to ethical issues related with using animal AFPs. Introduction of recombinant AFP from the polar fish *Myoxocephalus aenaeus* in industrial baker's yeast strains of *Saccharomyces cerevisiae* increased freezing tolerance. Moreover, the gassing rate and total gas production in frozen doughs was also increased by this yeast by reducing the osmotic damage during freezing/ thawing.

4.6.4. Cryopreservation of tissues and organs

***Oocytes and embryos*:** Many studies have shown the cryopreservative effects of AFPs on various tissues and organs. Role of AFPs in cryopreservation of mammalian cells was first observed by Arav *et al.*, in 1993. Treatment of Porcine oocytes during vitrification with each of the four fish AFPs, exhibited increased cell survival (75 %) even after their thawing while the untreated oocytes were unable to survive freezing (Arav *et al.*, 1993). Recently, type III AFPs at concentration as low as 500 ng/ml were used to supplement vitrification of immature germinal vesicle-stage mouse oocyte and have shown to promote freeze injury resistance in the oocytes even after thawing thereby increasing survival, in vitro maturation, fertilization, embryonic development up to the blastocyst stage, better membrane integrity and higher proportion of oocytes with normal spindle and chromosome morphology as compared to untreated oocytes (Jo *et al.*, 2012). Injecting AFPs into mammalian oocytes and embryos could improve their cryopreservation by inhibiting ice crystals growth and recrystallization during thawing and stabilization of the cellular membranes during chilling. Moreover such cryopreserved embryos or oocytes have been transferred in at least 25 species to produce healthy offspring (Rubinsky *et al.*, 1991; Knight *et al.*, 1984; Robles *et al.*, 2006; Gajdal and Smorąg, 2009). Paramo *et al.* (2008) reported use of AFPs for cryopreservation of fish embryos which are difficult to freeze due to their sensitivity to freezing and complex structure, with multiple compartments and permeability barriers. Type I and III FITC labelled AFPs were introduced in zebrafish embryos at early developmental stages by incubation in media containing proteins and evaluated by fluorescence and confocal microscopy. The method followed was simple, harmless, easy, non-invasive and effective than conventional microinjection method which makes it possible to treat several embryos at the same time and improved the cryoprotection of the cellular compartment.

Sperm: Various reports have been presented about the use of AFPs and AFGPs for sperm cryopreservation in ram, mouse, chimpanzee and bull. Payne *et al.* (1994) assessed that addition of type I AFP and AFGPs at lower concentration of 0.1 to 10 μg/ml to the freezing medium reduced the loss of motility in Ram spermatozoa that occurs during freeze thaw process but prior chilling before addition of AFPs leads to cytotoxicity and AFPs only at a concentration of 10 μg/ml were able to increase the percentage of motile spermatozoa significantly after freezing and thawing. Addition of fish AFPs to bull sperm at different concentrations 0.1, 1, 10 and 100 mg/ ml was able to maintain the viability of sperm, its osmotic resistance and integrity of acrosome after freeze thaw cycle. It was observed that

addition of type I AFP helped in increasing the osmotic resistance and reduced the mechanical stress to the cell membrane at as low as 0.1–10 mg/ml when compared to their control (Prathalingam, 2006). In another report it was suggested that addition of type III AFP from fish increased the velocity, linearity of movement, and percentage of viable cells in sperm of a teleost, *Sparus aurata* during freezing/thawing without changing the lipid composition of plasma membrane during cryopreservation thereby increasing the sperm quality after thawing (Beirao *et al.*, 2012).

Platelets: Blood platelets loose activities in hypothermic conditions. Currently platelets are stored at 22°C, which limits their shelf-life to 5 days. Incubating platelets with AFGPs prevented granule secretion in a concentration-dependent manner and platelets showed that these can be stored at 4°C without losing their activity and their shelf life can be increased up to 21 days (Tablin *et al.*, 1996). Knight and Duman, (1986) have characterized the influence of AFP on cryopreservation of red blood cells, which can survive cooling but on thawing damage to RBCs occur by ice crystal growth and recrystallisation. It was observed that even low concentrations of AFPs (μg) from winter flounder were able to allow increase in survival rate of RBCs cryopreserved in hydroxyethyl starch solutions even after thawing by inhibiting ice recrystallization in the extracellular regions during warming cycle.

4.6.5. Cryosurgery: Cryosurgery is a minimally invasive surgical technique that allow freezing for obliteration of the undesirable tissue to protect the healthy tissue. Pham *et al.* (1999) observed that injecting type I AFPs at 10 mg/ml along with (1 ml) PBS solution into the frozen subcutaneous metastatic prostate adenocarcinoma tumors of nude mice enhances severe destruction of cellular and connective structures, including the nuclei membrane after thawing by formation of needle shaped ice crystals leading to survival of healthy tissue. The study suggests that adjunctive use of AFPs in cryosurgery may reduce problems which may occur due to undesirable tissues that survive freezing.

5. Conclusion

Plant AFPs are proteins which features low TH and considerably higher IRI activity. These proteins interact with ice water interface and inhibit their growth by forming facets on ice crystals due to a thermodynamically unfavourable Kelvin effect. The higher IRI activity allows usage of this protein in the various areas like frozen food storage, cryopreservation of cellular entities and genetic engineering to produce cold tolerant crops and organisms where ice recrystallization has serious detrimental effect and which can be controlled and inhibited by AFPs. These proteins also show other beneficial effects like providing disease resistance due to their homology with pathogenesis-related proteins. However, many techniques have been proposed for detection of AFPs like splat assay, nanoliter osmometer, capillary assay, DSC and very recent Nanobiotechnology, which involves calorimetric assay using gold nanoparticles but each of these identification method is not perfect enough and comes with its own set of advantages and disadvantages associated with them and to choose any particular technique is practical but require various trials and experiments before incorporating best of all these techniques in one. Right now, splat assay which calculate the endpoint of IRI and nanoliter osmometer which show TH and ice crystal morphology characteristic of AFPs are frequently used methods for determining antifreeze properties of AFPs despite being time consuming. However, use of novel method nanotechnology needs more exploration and optimisation to be used frequently for AFP detection. Since AFPs show no consensus ice binding motif, their PCR studies are not possible and the classical assays used for detection and purification of AFPs are very slow and tedious to perform. No common mechanism has been formulated to show the ice binding and inhibition mechanism of structurally diverse AFPs and how it leads to production of diverse ice crystal shape (Needle spicules or hexagonal disc). Innovative and novel techniques like fluorescent labelling of AFPs could possibly give an idea about the real time binding and inhibition of these proteins.

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Abbreviations

AFPs, Antifreeze proteins; AFGPs, Antifreeze glycoproteins; TH, Thermal Hysteresis; THPs, Thermal hysteresis proteins; IRI, Ice recrystallisaton inhibition; ISPs, Ice structuring proteins; Cit-AuNPs, Citrate gold nanoparticles; PBS, Phosphate buffer saline; RBCs, Red blood corpuscles; ICM, Ice Crystal Morphology.

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