Antifreeze Proteins: Characteristics and Potential Applications

Bimo Ario Tejo  
*Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia,*  
bimo@ucsiuniversity.edu.my

Azren Aida Asmawi  
*Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

Mohd Basyaruddin Abdul Rahman  
*Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

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Antifreeze Proteins: Characteristics and Potential Applications

Bimo Ario Tejo¹,²*, Azren Aida Asmawi², and Mohd Basyaruddin Abdul Rahman²

¹. Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia
². Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

*E-mail: bimo@ucsiuniversity.edu.my

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Abstract

The freezing of water is usually fatal to most organisms because it causes extensive damage to cell membranes due to the formation of ice crystals. However, several structurally different classes of antifreeze proteins (AFPs) found in fish, insects, plants, and microorganisms, including bacteria, yeast, and fungi, have been found to be capable of modifying the growth of ice crystals by thermal hysteresis and ice recrystallization inhibition. This unique property could potentially be applied to medicine and the industry as it is useful when low-temperature storage is required and ice crystallization must be avoided. However, the application of AFPs today is not economically viable due to the complexity of the large proteins, the laborious procedures required, and the low yields obtained. A wide range of peptides mimicking their parent proteins were recently successfully designed and chemically synthesized. The developed approaches present new opportunities to understand the structure–function relationship of small-structured peptides with antifreeze properties. This mini-review highlights the diversity, classification, and properties of AFPs. The emerging applications of short mimetic peptides of AFPs and their potential application are also described.

Keywords: antifreeze peptide; antifreeze protein; ice recrystallization inhibition; thermal hysteresis

Introduction

Subzero temperatures cause extensive damage to living organisms. At such temperatures, extracellular ice crystals form on tissues and could result in cell membrane injury. If freezing persists, intracellular water passes into extracellular spaces, leading to cell dehydration and increased osmolarity due to a higher concentration of solutes [1]. However, organisms found in both polar and subpolar seawaters, where temperatures are consistently lower than the freezing point of physiological solutions of most organisms, are able to survive because of a unique adaptation. Over 30 years ago, an antifreeze protein (AFP) discovered in Antarctic notothenioids by biologists Eastment and DeVries first established the essential role of such proteins in the survival of marine teleosts in icy seawater [2]. This discovery initiated a field of challenging and exciting research that examines these proteins and the mechanisms by which they prevent or reduce damage to organisms living in subzero temperatures.

The effects of AFPs were initially thought to include reduction of the extracellular fluid temperature and prevention of fish from being affected by icy conditions. However, scientists were led to re-evaluate this theory when two antifreeze gene families were identified in winter flounder. The first family imparts antifreeze properties to blood and is expressed in the liver (HPLC6 and AFP9), while the second protects cells and tissues directly in contact with ice (SAFP2) and is mainly expressed by the gills and skin epithelia [3]. Research has demonstrated that, apart from AFPs providing defense against the effect of freezing to the whole organism and its external epithelia, mammalian cell membranes are also protected from damage by cold [4]. These membranes are believed to assist in the physiological adjustment of cells to lower temperatures. Nevertheless, the exact mechanisms by which AFPs offer protection at low temperatures remain incompletely understood.

Several researchers have attempted to solve this enigma by modeling AFP effects, investigating new AFP structures, and identifying factors affecting antifreeze activity to understand the exact mechanisms behind these effects. The outstanding biological activity found in some polypeptide α-helices has generated significant interest in the design and synthesis of short α-helical peptide mimetics [5-7]. A major benefit of using
Antifreeze peptides is that these smaller molecules can act as molecular tools to potentially obtain a new understanding of important sequences in AFPs and their synthesis. Hence, this mini-review is intended to describe the current understanding of AFPs, the challenges associated with designing short peptides capable of mimicking AFPs, and their potential applications to the medical and industrial fields.

**Classification of Fish Antifreeze Proteins**

Hew and Yang [8] classified AFPs into two main groups: non-glycosylated AFPs and antifreeze glycoproteins (AFGPs). Fractionation of proteins in fish blood serum revealed that the molecular masses of AFGPs range from 2.6 kDa to 33.7 kDa [9]. These proteins are composed of a repeating three-residue peptide containing a disaccharide unit attached to each third residue. Non-glycosylated AFPs can either be thermal hysteresis proteins (THPs) or ice structuring proteins (ISPs), and these terms are sometimes used interchangeably in the literature to describe AFPs. Prevention of ice crystal formation by depressing the point at which water freezes is attributed to the action of THPs [10], and the control of ice crystal size, shape, and aggregation is an inherent ISP property [11].

Fish AFPs are classified into four main types (Figure 1) according to the sequence in which they were discovered. Type I proteins, with molecular masses ranging from 3.3 kDa to 4.5 kDa, are found in abundance in several fish species. They are typically folded in a slightly curved amphipathic α helix and have a high alanine residue content [9, 12]. Type II proteins have molecular masses ranging from 11 kDa to 24 kDa and exist in a number of fish species, including Atlantic herring, smelt, and sea raven. Their three-dimensional structure consists of helices and β strands organized in a single globular domain stabilized by a disulfide bridge [13]. Type III proteins contain no particularly dominant amino acids, and a large number of their isoforms are found in Antarctic and Arctic zoarid fish (ocean pout or eel pout). The proteins form a compact β-sandwich fold [14]. Type IV protein is a newly discovered AFP with a molecular mass of 2.3 kDa. It is found in the longhorn sculpin and forms a helical bundle structure [12].

**Properties of Antifreeze Proteins**

AFPs have similar physical properties, but their structures and sources differ. The main activities of AFPs are thermal hysteresis (TH) and ice recrystallization inhibition (IRI). TH activity is typically used to determine and quantify antifreeze activity; however, a higher concentration of AFPs is needed to demonstrate this activity compared with that required to demonstrate IRI. TH refers to a thermodynamic interruption at the point where a solution melts or freezes, whereas IRI takes place through changes in the shape or structure of ice crystals and affects the growth rate of ice crystals [16, 17]. The binding of proteins to certain planes of an ice crystal’s surface is responsible for such inhibition. Thus, TH and IRI activity in cold-adapted organisms that produce AFPs is believed to reduce tissue damage due to the formation of ice crystals.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Type IV</th>
<th>AFGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (kDa)</td>
<td>3.3 – 4.5</td>
<td>11 – 24</td>
<td>6.5</td>
<td>12</td>
<td>2.6 – 33</td>
</tr>
<tr>
<td>Key properties</td>
<td>Alanine-rich α-helix</td>
<td>Disulfide-bonded Cysteine rich</td>
<td>β-sandwich Globular</td>
<td>Alanine-rich Helical bundle</td>
<td>AAT repeat Disaccharide</td>
</tr>
<tr>
<td>Representative structure</td>
<td><img src="image1" alt="Type I" /></td>
<td><img src="image2" alt="Type II" /></td>
<td><img src="image3" alt="Type III" /></td>
<td><img src="image4" alt="Type IV" /></td>
<td><img src="image5" alt="AFGP" /></td>
</tr>
<tr>
<td>Natural source</td>
<td>Right-eyed flounders, sculpins</td>
<td>Sea raven, smelt, herring</td>
<td>Ocean pout, wolfish, eel pout</td>
<td>Longhorn sculpin</td>
<td>Antarctic Notothenioids, northern cods</td>
</tr>
</tbody>
</table>

Figure 1. Diversity of Antifreeze Proteins (AFPs). Summary of Characteristic Differences between AFPs and Antifreeze Glycoproteins (Modified from [15])
Eastman and DeVries [2] were the first to demonstrate TH using the concept of nanoliter osmometry in their studies of ice crystal morphology; indeed, this concept is the accepted method for AFP research. The TH activity of AFPs varies in different organisms and the environmental conditions in which they survive; fish AFPs have higher TH capacity (between 0.6 and 1.5 °C) than plant AFPs (where TH only occurs between 0.2 and 0.4 °C) [18, 19]. Recent research reveals that insect AFPs are significantly more active than fish AFPs. At low concentrations, for example, the TH activity of beetle AFPs may be up to 100 times greater than the TH activity of fish AFPs at the same concentration; thus, the AFPs of beetle are called hyperactive [20, 21]. As temperatures fluctuate within the subzero range, ice recrystallization by various mechanisms takes place through the formation of large crystals with the concurrent disappearance of small crystals (Figure 2). Physical damage to tissues and cells is more likely to occur from large crystals generated during recrystallization than small ones [22]. AFPs at low concentrations react with and inhibit the recrystallization process by binding with the boundaries of ice crystals [11].

Ice crystal growth takes place in slightly supercooled water, and flat circular plates are formed along the ice crystal’s a-axis when AFPs are not present. When AFPs are present in the solution, ice crystal growth perpendicular to the basal plane along the c-axis occurs in the form of prism faces or in other directions not along the basal faces [23]. The morphology of the resulting crystals is hexagonal or needle-like when AFP is present. Ice-etching experiments have been carried out to explain this process and better understand the ice crystal faces targeted by AFPs [24]. This type of experiment involves slowly growing an ice crystal in a low-concentration AFP solution. Over time, certain ice crystal planes become visible and reflect where the protein has particular binding affinity. The results of such experiments indicate that the binding process across the crystal surface is not random.

### Proposed Antifreeze Protein Mechanism

The generally accepted mechanism by which AFPs interfere with ice crystal growth involves binding of the proteins to the ice surface and restriction of ice crystal growth in covered areas on the ice surface [25]. Areas of ice not covered with AFPs continue to grow, while other areas show interrupted growth. This mechanism leads to the curvature of the ice crystal surface by increasing the surface area-to-volume ratio until it can no longer thermodynamically support spontaneous ice growth. The potential of an ice crystal to grow is, therefore, arrested by the adsorption–inhibition mechanism [26] illustrated in Figure 3.

Sönnichsen et al. [14] initially suggested that the major force driving AFP ice-binding is related to the hydrophobic effect. The ice-binding site of Type III AFPs has been revealed to be more hydrophobic compared with the rest of the molecule. The hydrophobic model proposes that the interaction of all AFPs with ice takes place on large proportions of the total surface area of the protein molecule [28]. Furthermore, the sites where ice-binding could be observed are noticeably planar, do not have protrusions or other interruptions, and tend to be the most hydrophobic AFP surfaces [5, 28]. The two central interactions in this model are Van der Waals forces and the adsorption capability of AFPs, both of which facilitate close contact [14]. Hydrogen bonds may also

![Figure 2. Ice Recrystallization Inhibition Assay. Ice Crystal Formation (A) in a Buffer Solution without Antifreeze Protein (AFP), (B) in the Presence of 0.1 mM Glaciozyma antarctica AFP and (C, D) in the Presence of Different Antifreeze Peptides (10 mM) (Adapted from [5])](image)

![Figure 3. Schematic Demonstrating the Adsorption–inhibition Effect of Antifreeze Proteins (AFPs) on Ice Crystal Growth. The Arrows Indicate the front of Ice Growth. (A) Ice Growing as a Disc and (B–D) Retardation of Ice Growth at the Crystal Face to which AFPs have been Adsorbed. This Mechanism Produces the Characteristic Facets Observed when AFPs Shape Ice Crystals (modified from [27]))](image)
Antifreeze Peptide

The outstanding biological activity found in some polypeptide α-helices has generated significant interest in the design and synthesis of short α-helical peptide mimetics. Garner and Harding [6] were the first to design small-structured peptides with antifreeze properties. Harding et al. [30] suggested that the helical structures of AFPs are important for ice crystal growth inhibition. A key element for growth inhibitor proteins is the hydrophobic effect, as demonstrated in the results of recent structure–activity studies. The binding interaction of a helical AFP is assumed to require the high conservation of the ice-binding face. The structures of all Type I AFPs, similar to winter flounder HPLC6 proteins, have regularly spaced threonine residues, but the hydrophilic residues vary [30]. All of these proteins exhibit a hydrophobic face located in individual α-helical regions where an alanine-rich surface can be found. Therefore, the ice-binding face of the helix could be hypothesized to be found where this type of surface is present.

As a result of the advancing understanding of the characteristics of stabilized α-helix mimetics, various applications may potentially be developed by using short antifreeze peptide properties through routine design and synthesis for cost-effective commercial production. Harrison et al. showed that short peptides can be modified and fixed in highly α-helical structures in water using (i, i+4) or (i, i+7) lactam bridges. The biological activity of native proteins is stabilized and sustained by the lactam bridges, thereby enabling the design of small-structured polypeptides with antifreeze properties. Up to 60% of the natural antifreeze activity was reported to have been achieved from a short segment of Type I AFP isolated from HPLC6 protein [32].

The efficacy of peptides corresponds to their length and helicity, and at least 25 residues are needed to demonstrate antifreeze activity [6, 30]. In addition, a relatively flat binding site is important to increase the surface area of AFPs because such a site produces a good fit for binding to ice planes [12, 33]. Although the designed peptides are not especially effective, they can still select and bind to ice surfaces, similar to their antifreeze parent proteins. However, weak affinity is observed, likely because the ice-binding surface was not developed under optimal conditions. Modification of peptides and improved binding results should be possible once greater knowledge of the interaction between ice and AFPs is achieved.

Our group previously reported the relationship between peptide structure and activity via NMR spectroscopic exploration and molecular dynamics simulation [5]. NMR results showed that the antifreeze activity of the peptides is strongly associated with their geometrical linearity and helicity. Molecular dynamics simulation results suggested that the activity of the designed peptides could be described in terms of their structural rigidity/flexibility, i.e., the most active peptides exhibit higher structural stability and flexibility compared with other peptides with lower activity and greater rigidity.

Potential Applications of Antifreeze Proteins

The potential applications of AFPs to the medical and industrial fields have stimulated much interest on account of the unique and useful properties these proteins, which may be taken advantage of when low-temperature storage is required and ice crystallization must be avoided. AFP-related discoveries indicating that the proteins may contribute to the survival of hypothermia at temperatures below freezing point are often applied to cryopreservation [34-36]. However, the effects observed are contingent on the type and concentration of the AFP, and the proteins present some risk of cytotoxicity to cells. Successful subzero cryopreservation of rat liver has been reported [37]. A controlled test revealed that tumor cells exposed to AFP are destroyed regardless of the freezing treatment applied whereas control samples exhibit a high rate of cell survival [38, 39]. AFPs have shown to reduce complications due to unexpected cell death caused by intracellular freezing in both organ preservation and research-based cell and tissue culture [40]. Although the precise cause of this effect is unclear, the results have been replicated in follow-up research [41]. Besides their successful implementation in cryosurgery, AFPs could also protect not only organs but also blood platelets against intracellular freezing [42, 43], thus ensuring that the number of blood platelets remains within the optimal range. Unfortunately, the applications of AFPs to human organ transplantation, especially kidney
transplantation, requires further extensive research as the structure of human organs is much more complicated than that of rodents [9].

The food industry benefits from the suppression of ice crystal growth during freezing, storage, transport, and thawing. The presence of AFPs, for example, can prevent deterioration of food products, such as undesirable changes in food quality caused by the loss of nutrients or cellular damage due to dripping [44, 45]. This approach has been used to produce ice cream, where ice crystal growth is minimal compared with the control product [11, 46]. Commercially produced ice creams containing AFPs are now available to consumers [19]. While frozen storage can potentially increase the shelf-life of bakery products, such as bread dough, some problems that must still be considered include the deterioration of bread quality due to reduced volume, weakening of the dough structure, poor texture, and decreased gas content. According to Panadero et al. [47], the use of AFPs could improve the quality of frozen sweet dough by enhancing its total gas content and gassing rate. High concentrations of carrot AFPs have been shown to enhance the quality of dough by improving its softness and maintaining loaf volume during freezing [48].

AFPs genes harvested from many organisms, including cold-adapted insects, plants, and fish, can be used by other organisms that do not have the AFP genome to survive in low or subzero temperatures. Research has been undertaken to apply AFPs to aquaculture and agriculture, but the developed methods are currently not being used for production. The fishing industry in eastern Canada is affected by icy seawater, particularly in areas where Atlantic salmon is farmed. This species does not produce AFPs and its cold tolerance limit is ~0.9°C; beyond this temperature, the fish die. A Type I AFP from the winter flounder gene was previously inserted into the salmon genome to improve the latter’s resistance to cold temperatures; as a result, fewer fish died from hypothermia and the economics of the participating farms improved [49, 50]. Similarly, some transgenic plants express AFPs from other organisms, which could potentially increase geographic growing regions by extending crop growing seasons and horticultural production. Corn was the first plant to be developed to successfully express animal AFPs in plant material [51]; however, the reported recrystallization inhibition rate indicated that the AFP activity of the crop is limited. Thus, further research in this area is needed.

Conclusion

Overall, the survival of many organisms in freezing temperatures is solely dependent on the interaction between proteins and ice. However, what is responsible for this activity remains unclear. The design of short small-structured AFPs will shed light on the peptide structure–antifreeze activity relationship and promote the development of novel short mimetic peptides of AFPs that can be easily synthesized at low cost for commercialization.

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Makara J. Sci.


