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Structure and Applications of Antifreeze Proteins from Fishes: A Mini Review

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Antifreeze proteins (AFPs) are a unique class of proteins that enables certain fish species to survive in extremely cold environments. These proteins can bind to ice crystals and inhibit their growth, thereby preventing freezing. Fish produce a range of AFPs with different structures and mechanisms of action, making them a fascinating research subject. The study of AFPs in fish has provided valuable insights into the molecular mechanisms of protein-ice interactions, which have potential applications in the fields of biotechnology and cryopreservation. This study aimed to review the current understanding of AFPs in fish, including their structure, function, and evolutionary history. This paper also discusses the potential applications of AFPs in biotechnology, such as in the development of antifreeze materials and cryopreservation of biological samples.

Keywords
Antifreeze proteins, applications, biotechnological role, fish species

Introduction

Numerous creatures use unique adaptive methods to survive in extremely cold conditions. Water covers over two-thirds of the earth's surface, with typical surface temperatures ranging from -2 to 30 degrees Celsius depending on latitude (Bhat et al., 2016; Eskandari *et al.,* 2020; Sundaray *et al.,* 2022; Rather *et al.,* 2023). The antifreeze proteins (AFPs) are naturally occurring cryoprotectants formed by many organisms, such as fish, plants, and insects, in order to survive subzero temperatures (Kontogiorgos *et al.,* 2007; Petzold and Aguilera, 2009). In 1969, DeVries discovered them in the blood of fish living in areas where the sea froze. They were given the name AFPs after it was discovered that they reduced the freezing point of fish blood below that of sea water without significantly raising the osmotic pressure of the plasma. Since their discovery in fish (Fletcher *et al.,* 2001), AFPs have been found in fungi (Hoshino *et al.,* 2003), plants (Gilbert *et al.,* 2004), and bacteria (Kawahara *et al.,* 2004). Because of the many potential commercial uses, such as preservation, transgenic production, and cryosurgery (Wang *et al.,* 1995), AFPs' antifreeze activity has received a lot of attention. The food industry is one of the most promising areas for AFP application. AFPs can be used as cryoprotectants to prevent ice crystal formation during the freezing and thawing of food products. By inhibiting the formation of large ice crystals (Crevel *et al.,* 2002: Harding *et al.,* 2003), AFPs can improve the texture and flavour of frozen foods and increase their shelf life by preventing freezer burn, which occurs when ice crystals sublimate from the surface of the food (Ding *et al.,* 2015). AFP also has potential applications in medicine, where they can be used to preserve the viability of cells, tissues, and organs during cryopreservation. AFPs can prevent the formation of ice crystals and increase the viability of biological material after thawing, which can have significant implications for organ transplantationand tissue engineering.

AFPs are typically small, globular proteins that are highly flexible and hydrophobic, allowing them to bind to ice crystals of various sizes and shapes. In addition to their varied sources, various AFPs with distinct structural traits have independently evolved. Five structurally unique AFPs have been discovered in fish to date, and these five AFPs have been categorized as AFP types I, II, III, IV, and AFGPs based on their unique structural features and physicochemical properties (Fletcher et al. 2001).

Type I AFP

In Type I, which is alanine-rich, flounders and sculpins are prevalent (Petzold and Aguilera 2009; Crevel *et al.,* 2002). This is the most basic AFP that is known. There are a total of 37 residues, 23 of which are alanines. There are three Thr-Ala-Ala-X- Ala-X-X-Ala-X-X-Ala-X-X-Ala-X-X- repeats, with alanine predominating in the Ala position. This protein has a 58 A° straight -helix, according to Ramlov and Johnsen (2014). Several authors reported that molecular masses range from 3.3 to 4.5 kDa (Davies and Hew, 1990; Hassas-Roudsari and Goff, 2012; Crevel *et al.,* 2002). Earlier studies carried out by Tyshenko *et al.,* (1997), indicated that the main distinction between the flounder and sculpin sub-types is that the former is composed of a repeat of 11 amino acids with a distinct structure (TxxNxxxxxxx), where x is primarily alanine and N is occasionally aspartate or threonine, whereas the latter is non-repetitive and more amphipathic with multiple lysine and arginine sidechains extending from the same face of the helix. It is unclear whether the two subtypes are homologous if their high amino acid identity is explained by the need for alanine for helix stabilization because the two subtypes also bind to different planes of ice (Duman, 1994).

Type II AFPs

The main sources of type II AFPs are long-nosed poachers, smelt, herring, and sea ravens. Type II fish AFPs are globular, high in cysteine, and have a molecular weight range of 11 to 24 kDa. There are many structural similarities between type II AFPs and C-type lectin-like domains (CTLDs). In the two helices and nine strands of type II AFPs, particular cysteines form disulfide bonds. Those disulfide bonds are known for their capacity to enhance the structural stability of type II AFPs (Liu *et al.,* 2007; Nishimiya *et al.,* 2008). While there is little homology in the amino acid sequences of different types II AFP groups, structural comparison studies between these groups have shown that these AFPs share similar structures and function.

According to Ewart *et al.,* (1999), type II AFPs originated from CTLDs' structural core. Due to their dependence on calcium ions for antifreeze activity, type II AFPs are distinguished from other types.

Type III AFPs

In contrast to other AFPs, type III AFPs are small, globular proteins with an average molecular weight of 6.5 kDa that are found in wolf fish, Antarctic eelpouts (*Macrozoarces americanus*), and ocean pouts (Antson *et al.,* 2001). They also contain no cysteine residues and have fewer alanine residues than the other AFPs. There were no carbohydrates and no distinct primary structures in the AFP III molecules. According to Baardsnes and Davies (2001), type III AFPs most likely descended from sialic acid synthase (SAS) through gene duplication and divergence (Deng *et al.,* 2010). Type III AFPs are homologous to SAS C-terminal domain of SAS. There is evidence of gene duplication in the fish genome, and SAS from an Antarctic eelpout that produces type III AFP has been found to have ice-binding activity. Fish AFP III are globular proteins with helical turns and several short -strands. Quaternary-amino-ethyl (QAE) and sulfopropyl (SP) sephadex-binding isoforms of type III AFPs have been separated into two subsets based on their isoelectric point differences (Hew *et al.,* 1988), and they may be further divided into QAE1 and QAE2 subgroups (Nishimiya et al. 2005). The ice-binding surface of a type III AFP is composed of solvent-exposed residues from two sections of the amino acid sequence (residues 9-21 and 41-44) (Baardsnes *et al.,* 2002; Garnham *et al.,* 2010).

The physiochemical properties and secondary structure of AFP in zebrafish is presented in Table 1 and Table 2 (Figure 1) respectively. Moreover, 3D Structure of antifreeze protein and protein-protein interaction in Zebrafish is presented in Figure 2 and 3 respectively.

Table 1: Physicochemical properties of AFP in Zebrafish

AFP	Properties
Number of amino acids	130
Molecular weight	14194.45
Theoretical pI	5.15
Total number of negatively charged residues (Asp + Glu)	16
Total number of positively charged residues (Arg + Lys)	13
The estimated half-life is	30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (*Escherichia coli*, in vivo).
Instability index (II)	47.99
Aliphatic index	101.38
Grand average of hydropathicity (GRAVY)	-0.032

Type IV AFPs

The AFPs in long-horned sculpins are type IV. They are alpha helical proteins that are high in glutamate and glutamine. Four helix bundles make up the proteins 12KDa molecular weight (Ng and Hew, 1992). Bloodstream export was connected to a 20 amino acid N-terminal signal sequence. Additionally, according to Deng *et al.,* (1997), the sequence resembled other four-helix bundles of serum/hemolymph apolipoproteins like guinea pig apolipoprotein E and African locust apolipophorin III.

AFGPs

AFGPs are AFPs that evolved in various

polar and subpolar marine teleost lineages to enable survival in frozen, cold saltwater (Fletcher *et al.*, 2001). AFGPs have been found in numerous populations of northern and Arctic Atlantic cod for a long time (Goddard *et al.*, 1999; Hew *et al.*, 1981). According to DeVries *et al.*, (1970), the AFGPs are divided into eight subcategories, with AFGP1 having the highest molecular mass (33.8 kDa) and AFPG8 having the lowest (3 kDa). In these proteins, the Ala-Ala-Tr triad sequence is repeated 4 to 50 times, with each hydroxyl group on the side chains of the threonine atoms accompanied by a sugar branch (galactose N-acetylgalactosamine).

Table 2: Secondary Structure of AFP in Zebrafish

Alpha helix	**94.62% (123)**
Extended Strand	**3.08% (4)**
Random Coil	**2.31% (3)**

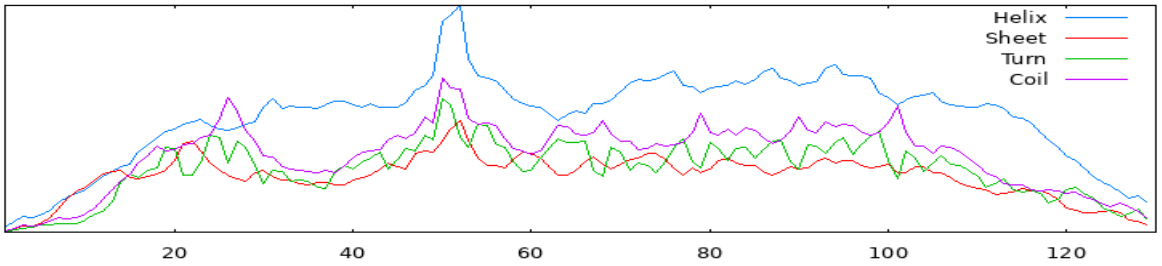

Figure 1: Secondary Structure of AFP in Zebrafish

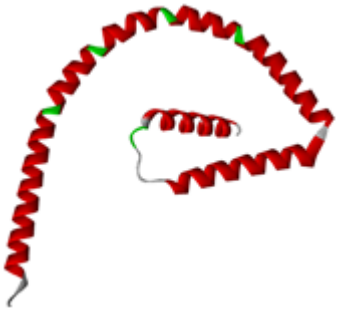

Figure 1: 3D Structure of Antifreeze protein in Zebrafish

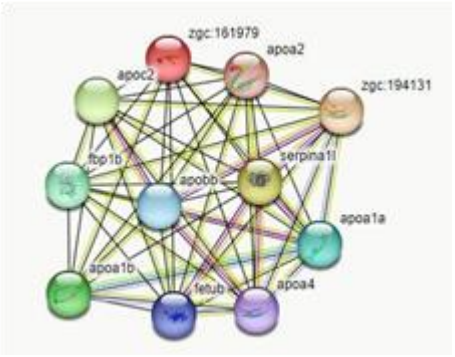

Figure 3: Protein-Protein Interaction of antifreeze protein in Zebrafish

Mechanism of Action

The two main stages of crystallization are the development of a stable crystal nucleus (embryo) and the growth of ice crystals by the nucleus. In atomically pure water, nucleation typically occurs around an outside molecule (heterogeneous nucleation) or as a result of a process in which molecules spontaneously form nuclei during intrinsic fluctuations (homogeneous nucleation). When the temperature varies within the subzero range, ice recrystallizes via a variety of mechanisms, including the growth of larger ice crystals and the disappearance of smaller ice crystals. According to Hassas- Roudsari and Goff (2012), these compounds are more potent than smaller ones in physically harming tissues and cells. Seawater in cold climate freezes at -1.9^{0}C rather than at 0^{0}C because of the dissolved sodium chloride and other substances. Arctic polar cod, Antarctic cod, Antarctic and Arctic eel pout, and winter flounder are a few examples of fish that require a blood freezing point below this level to survive in harsh environments. However, a decrease in freezing point greater than 1.4^{0}C cannot be attributed to low molecular weight dissolved solutes in fish blood. The freezing point drops by 0.5^{0}C or more as a result of the AFPs. In addition to lowering the freezing point of body fluids and preventing damage from ice recrystallization, AFPs are believed to protect membranes from cold-induced damage by inhibiting thermotropic phase transition and preventing leakage by blocking ion channels.

Owing to the peculiar structure of AFPs, they can bind to ice crystals in a precise and reversible manner. A long, flexible chain of amino acid residues that forms a flat, hydrophobic surface makes up the protein, which is also composed of a specific arrangement of amino acid residues. This surface adheres to the ice crystal surface, forming a layer of water molecules that serves as a barrier and stops the crystal from growing further.

In addition, AFPs cause fish body fluids to freeze at lower temperatures. The difference between the freezing and melting points of water, known as "thermal hysteresis," is what makes this possible. AFPs allow fish to survive at temperatures well below the freezing point of freshwater by lowering the freezing point of water by up to several degrees Celsius (Crevel *et al.,* 2002). The ability of AFPs to bind to small ice crystals, stop further ice crystal growth and aggregation, and lower the freezing point of body fluids in fish allows them to survive at subzero temperatures.

Applications of Antifreeze proteins

Antifreeze proteins (AFPs) are widely used in numerous industries because of their exceptional capacity to prevent the growth and development of ice crystals or to enhance supercooling. Some potential applications ofAFPs are discussed below.

Cryopreservation: Cryopreservation is the process of freezing and storing biological material for future use. AFPs have potential applications in cryopreservation because they can prevent ice crystal formation and damage to biological tissues during freezing and thawing. AFPs have been successfully used to improve cryopreservation of sperm, eggs, and embryos in various animal species (Chao *et al.*, 1996; Venketesh and Dayananda, 2008; Budke *et al.*, 2009).

Food industry: AFPs have potential applications in the food industry, particularly for frozen food products. By inhibiting ice crystal formation and growth, AFPs can improve the texture, flavor, and shelf life of frozen food. Because smaller ice crystals generally cause less cell damage than larger ones, adding a higher concentration of AFPs to foods that should not be frozen at all can also be beneficial. Meats have been reported to contain smaller ice crystals after treatment with either fish AFPs or AFGPs (Ghalamara *et al.,* 2022). The increased AFP concentration will improve the inhibition of ice crystal growth, allowing foods, such as strawberries, to be stored at low

temperatures with a lower risk of quality loss.

Cryosurgery: It is also referred to as cryotherapy or cryoablation, is a technique for removing unwanted or nonviable tissues by freezing them. Owing to insufficient or improper freezing, freezing by itself may only completely eradicate the targeted tumor in certain clinical situations. Because of the intense cold emitted by the freezing probe, nearby healthy tissues may also sustain freezing injury (Yiu *et al.*, 2007). Several adjunctive therapies have been proposed to increase the effectiveness of cryosurgery. These include chemical adjuvants such as cancer chemotherapeutic agents, AFP I, and amino acid adjuvants such as glycine and tumor necrosis factor alpha (TNF-alpha).

Materials science: AFPs have potential applications in materials science, particularly in the development of antifreeze coatings and deicing agents. By inhibiting ice crystal formation and growth, AFPs can prevent ice accumulation on surfaces and improve the safety and efficiency of industrial processes.

Biotechnological role: AFPs have potential applications in biotechnology, particularly in the development of cold-adapted enzymes and biocatalysts. By promoting supercooling or inhibiting ice crystal formation and growth, AFPs allow enzymes and biocatalysts to remain active at low temperatures, which is crucial for various biotechnological applications.

Conclusion

A particular kind of protein is produced in the bodies of many creatures that live in cold climates during the winter. These substances are classified as either antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs). These proteins have so far been identified in a wide range of fish, insect, bacterial, and plant species (AFPs and AFGPs will be referred to collectively as AFPs). AFPs act as ice growth inhibitors. As a result, liquid water that has dissolved AFPs does not completely freeze, even at temperatures below the freezing point of ice. When it comes to food processing, cryopreservation, ice slurries, and organisms' ability to tolerate and avoid freezing, AFPs have drawn a lot of attention. Such AFPs might have a few commercial uses. At the same concentrations, these compounds are approximately 300 times more effective at preventing freezing than traditional chemical anti- freezing agents. The effectiveness of AFPs in preventing the growth of ice suggests that they may find use in a variety of situations to stop food from freezing and injuries caused by freezing. In addition to enabling cryopreservation of tissues and organs, they could be used to increase the cold resistance of living plants or cryopreserve foods that would otherwise become inedible due to ice crystal damage. Dessert storage and quality during thawing could both be enhanced by AFPs used in food preservation. Additionally, their high activity at low concentrations may help the products' overall cost-effectiveness.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of interest

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