Review Article

Application of Antifreeze Proteins in Foods of Animal Origin: A Review

Annada Das[1*], Geeta Chauhan[2], Kaushik Satyaprakash[3] and Serlene Tomar[4]

[1]Department of Livestock Products Technology, WBUAFS, Kolkata-700037, West Bengal, INDIA
[2]Division of LPT, IVRI, Izatnagar, Bareilly-243122, Uttar Pradesh, INDIA
[3]Division of Veterinary Public Health, IVRI, Izatnagar, Bareilly-243122, Uttar Pradesh, INDIA
[4]Division of Livestock Products Technology, IVRI, Izatnagar, Bareilly-243122, Uttar Pradesh, INDIA

***Corresponding author:** dasannada.555@gmail.com

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Abstract

This paper reviews the origin, functionality and use of antifreeze proteins (AFPs) in frozen foods of animal origin. AFPs inhibit recrystallization and reduce cellular damage, thus safeguarding the quality of frozen foods without structural and organoleptic alterations. The conventional food cryoprotectants are of synthetic/chemical origin and may be hazardous to health. In spite of the limitations like cost and time involvement for small scale purification, the AFPs of natural origin are attracting the interest of researchers for their mass commercial production and application in frozen food. A small amount of purified AFPs are sufficient to exhibit activity without much taste alterations, owing to its commercial benefit. Therefore, naturally occurring AFPs can be used as potent cryopreservatives in frozen meat, fish and dairy products to minimize the structural and functional damages during freezing and thawing.

Key words: Antifreeze Proteins, Cryoprotectants, Freezing, Frozen Foods, Recrystallization

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Introduction

Antifreeze proteins (AFPs) are the proteins with the ability to retard ice crystal growth, thereby stabilizing ice crystals and inhibiting recrystallization under freezing conditions. These are also called as Thermal hysteresis proteins (THPs), Non-freeze proteins (NFPs) or Ice binding proteins (IBPs). The term "ice-structuring proteins" (ISPs) has been also proposed as a suitable name based on the fact that all AFPs bind to and influence the growth of ice crystals and change the ice structure and hence protecting the texture of frozen food (Crevel *et al.*, 2007). AFPs are present in a wide range of cold adapted organisms in nature. There are many types of AFPs synthesized within each organism. The quality of frozen food can be improved by using AFPs which work by inhibiting recrystallization and maintaining the organoleptic

properties intact like smooth texture, desirable taste etc. AFPs reduce cellular damage and minimize the loss of nutrients through drip. AFPs may be introduced into other food products either by physical processes or by gene transfer. However, the high cost of production and purification of these proteins limit their potential commercial application in frozen food systems. But mass production of AFPs may solve this problem and make their food usage cost effective. Apart from few limitations, AFPs possess many commercial benefits, which suggest the use of AFPs as an emerging ingredient for frozen food preservation in the future. Increasingly though, it is recognized that these proteins could offer many technological advances with significant economic potential.

Historical Background of AFPs

In the 1950s, Norwegian scientist Scholander explained about antifreeze proteins in the blood of Arctic fish. In late 1960s, Arthur DeVries, isolated AFPs from the Antarctic notothenioids (DeVries and Wohlschlag, 1969). In 1969, he identified that in the blood of fish living in frozen sea, these proteins apparently lowered the freezing point of the fish blood to below the freezing point of seawater, without significantly increasing the osmotic pressure of the plasma, hence termed as AFPs.

Evolution of AFPs

The diversity and distribution of AFPs suggests that they might have evolved in response to sea level glaciation in the Northern hemisphere and Antarctica. This development of adaptations is referred to as convergent evolution. Some scientists claim that the origin of the AFPs may be due to Molecular Parallelism. Chen *et al.* (1997) reported that the notothenioid AFP gene may have had its first function of preventing the freezing of intestinal fluid. Chen *et al.* (1997) demonstrated that an antifreeze glycoprotein (AFGP) gene from the Antarctic notothenioid *Dissostichus mawsoni* was derived from a gene encoding a pancreatic trypsinogen. It is believed that the portion of the AFGP gene (encoding the ice-binding function) was derived from small region between the first intron and second exon of the trypsinogen gene. This newborn segment was expanded and duplicated to produce 41 tandem repeated segments. The contemporary AFGP gene retains 5′ end of the trypsinogen gene as its birthmark sequences at both ends which are identical to trypsinogen (Logsdon and Doolittle, 1997).

Sources of AFPs

AFPs have been found in a wide variety of organisms in nature which need to protect themselves against freeze damage. Teleost fishes were among the first organisms in which AFPs were identified which include a range of species such as cod, herring and Ocean pout (Crevel *et al.*, 2007). Since the discovery of the first AFP, these proteins have been identified not only in fishes but also in numerous plants, bacteria, fungi, diatoms and insects of colder areas (Jin-Yao *et al.*, 2006; Hassas-Roudsari and Goff, 2012).

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AFP content of different organisms varies widely. Only limited data are available on AFP content. Crevel *et al.* (2007) reported up to 0.307 mg/g AFPs of fresh weight in winter rye leaves. In both *T. borgrevinki* and *D. mawsoni,* the total AFP content is about 25 mg/ml of which approximately 25% is due to AFP 1–5 with the remaining 75% containing the smaller AFPs (Harding *et al.,* 2003). The type III AFP content in the blood of Ocean Pout is about 30 mg/ml, while that of Atlantic Cod ranges from approximately 7 mg/ml in adult fishes to 14 mg/ml in juveniles (Crevel *et al.*, 2007).

Unfortunately humans and other homeotherms of the colder areas do not contain these natural AFPs in their body.

Classification of AFPs

Many types of AFPs are synthesized within each organism. Therefore, AFP with the appropriate characteristics and a suitable level of activity should be selected for a particular food product. Five different types of fish AFPs have been isolated and characterized, termed AFP type I–IV and antifreeze glycoproteins (AFGPs) (Hassas-Roudsari and Goff, 2012). There are also two types of insect-derived AFPs (Jin-Yao *et al.*, 2005). These macromolecular AFPs vary greatly in terms of their protein structures and composition of the amino acids (Crevel *et al.,* 2007; Jin-Yao *et al.,* 2006).

Recombinant Antifreeze Proteins (R-AFPs)

As sourcing the AFPs from nature was not sustainable or economically feasible, the yeast extraction method of producing R-AFPs was developed as an alternative. The yeast is removed during processing hence the resulting protein does not contain any residual genetically modified (GM) yeast. GS-5 is an R-AFP obtained by expressing the type 1 AFP gene of polar fish Grubby Sculpin (*Myoxocephalus aenaeus*) in industrial baker's yeast (*Saccharomyces cerevisiae*). Scientists have tried to develop a method for using R-AFPs in frozen dough products by using an expression vector containing an AFP coding sequence. The main strategy of the method was to overlap two 10 base pair oligonucleotides which contain the GS–5 sequence. After introducing the GS–5 sequence into yeast, there was extention of the fragments followed by amplification of the desired fragment by PCR (Panadero *et al.*, 2005). Genes were transferred from Ocean Pout (*Macrozoarces americanus*) to industrial baker's yeast (*Saccharomyces cerevisiae*) in laboratory for production of R-AFPs to be used in ice cream. Loewen *et al.* (1997) described the shake-flask expression of the cystine-rich Sea Raven serum antifreeze proteins (SRAFPs) in Pichia resulted in secretion of R-AFPs up to 5 mg/L which had similar activity to SRAFPs. It may be more acceptable to consumers to transfer an AFP gene from *Pseudomonas putida* to *Lactobacillus spp.* for using in frozen yogurt.

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Properties Unique to AFPs

In contrast to many traditional cryoprotectant solutes, AFPs kinetically depress the temperature at which ice crystal grows in a non-colligative manner and, thereby, exhibiting thermal hysteresis (TH), i.e., a positive difference between the equilibrium melting point and ice growth temperature. By virtue of these properties the polar fish survives in subzero water which is colder than the equilibrium freezing point of their blood and other body fluids by modifying or suppressing ice crystal growth and protecting cells from cold-induced damage (Harding *et al.*, 2003).

Non-Colligative Properties

AFPs ability to lower freezing point does not depend on concentration, unlike the conventional antifreeze, ethylene glycol, rather they work in a non-colligative manner at very low concentrations (at concentrations $1/300^{th}$ to $1/500^{th}$ of those of other dissolved solutes) and such low concentrations minimize their effect on osmotic pressure (Fletcher *et al.*, 2000).

Thermal Hysteresis

Creation of a difference between the melting point and freezing point is called as thermal hysteresis. The unique properties of AFPs are attributed to their affinity for specific ice crystal surfaces. The ice crystal growth is inhibited as the water-accessible surfaces of ice are covered by AFPs (Jorov *et al.*, 2004). TH is easily measured in the laboratory with an instrument called Nanolitre Osmometer. The fish AFPs have highest level of TH that decreased approximately -1.5 °C (29.3 °F). However, insect AFPs are 10–30 times more active than fish proteins, the reason being the much lower temperatures encountered by insects than fishes (e.g. during the extreme winter, the spruce budworm resists freezing at temperatures about -30 °C) (Fletcher *et al.*, 2000).

Mechanism of Action of AFPs

AFPs are found in organisms such as Antarctic fishes, where they inhibit ice crystal growth below a solution's freezing point. Ice inhibition occurs when multiple AFPs bind to the same ice crystal, and small curvatures are created along the ice surface blocking all faces except c- axis. Then it becomes energetically unfavorable for more water molecules to bind, thereby inhibiting the growth of the ice crystal. AFPs further inhibit recrystallization (the smaller ice crystals to join and form a larger ice crystal) which causes structural damage causing drip loss in frozen food. Because AFPs lower the freezing point, a gap between the melting and freezing points is created, which is known as the thermal hysteresis (TH) gap. A TH gap can thus be used to identify novel anti-freeze proteins, and the magnitude of the TH gap can provide idea for the quantitative assessment of the AFP activity. Like their structures, the TH activity or functionality of AFPs also varies greatly.

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Application in Animal Origin Foods

AFPs have several advantages that include inhibition of ice crystal growth and recrystallization, protection from freeze damage, preservative action, maintaining organoleptic properties like texture and taste intact, effective at lower concentrations, no taste alteration by pure AFPs and healthier alternative to salt and sugar which encourage their use in frozen foods and as cryoprotectants in super chilling of food. There is a limited published literature on AFPs in animal food application and most of the food uses are found in patents (Table 1). Most of the previous studies included use of AFPs in frozen dairy or ice cream, meat and fish products.

Table 1: Potential use of AFPs in animal origin foods (in research findings)

S. No.	Sources	Product	References
1	Antarctic cod and flounder	Frozen and chilled meat	Payne *et al.* (1994)
2	Antarctic cod	Frozen meat	Payne and Young (1995)
3	Fish	Ice cream	Feeney and Yeh (1998)
4	Antarctic cod & flounder	Lamb meat	Feeney and Yeh (1998)
5	Fish (type III AFP)	Ice cream	Clarke *et al.* (2003)
6	Tilapia hybrids (type III AFP)	Frozen & chilled fish	Boonsupthip and Lee (2003)
7	Winter wheat grass	Ice cream	Regand and Goff (2006)
8	Japanese radish sprout	Chicken leg meat, Pork boston butt	Fukuoka *et al.* (2013)

Ice-Cream

AFPs are tried in various milk containing frozen confections like ice cream, frozen yoghurt, sherbet, ice milk, frozen custard, frozen fruit purees etc., with the maximum application in ice creams.

In a study conducted by Regand and Goff (2006), the semi-frozen ice cream mix containing AFPs were packed and quickly frozen to temperatures between -35 to -50 ˚C. This is done to avoid the formation of large ice crystals (Huang *et al.*, 1992). The ISP activity was not destroyed by its inclusion in ice cream mix prior to pasteurization. A synergistic effect between ISP and stabilizer was observed and the ISP activity was reduced in the absence of stabilizer in ice cream formulations. A significant smoother texture for ice creams containing ISP after heat-shock storage was evident by sensory evaluation. Another study by Feeney and Yeh (1998) showed that vanilla ice creams with AFPs showed a very little ice crystal growth whereas the control sample without AFPs showed a marked increase in ice crystal size. Clarke *et al.* (2003) examined the scanning electron microscope images of the ice cream structure containing type III AFP stored between -10 and -20˚C for 3 weeks. As a result, it was evident that prior to storage, there was very little difference between frozen samples in terms of ice crystal size. At the end of the storage, the samples with AFPs showed very little ice crystal growth, but the control samples had relatively larger ice crystals.

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In the study conducted by Regand and Goff (2006), there was a noticeable difference between the solutions containing Cold Acclimated Winter Wheatgrass Extract (AWWE) and the solutions without AWWE, indicating that AFPs inhibit the ice growth, and improve the texture of the mixtures. Significant ISP activity in retarding ice crystal growth was observed in all solutions containing 0.25% total protein from AWWE. In heat-shocked ice cream mix, ice recrystallization rates were significantly reduced by 40–46% with the addition of AFPs from AWWE.

Unilever is the multinational British-Dutch company behind ice cream brands like Breyer's Ice cream, Ben and Jerry's, Good Humor, Klondike, Popsicle, Streets etc. Recently, AFPs were incorporated into some of the Unilever's ice cream and ice lolly products, including Popsicles and a new line of Breyer's Light Double Churned ice cream bars that allows the production of very creamy, dense, smooth textured ice creams. They control ice crystal growth during thawing on the loading dock or kitchen table which drastically reduces its quality (Regand and Goff, 2006).

Meat and Fish

Ice recrystallization inhibition property of AFPs can be greatly utilized in frozen meat and fish. In a study by Payne *et al.* (1994), small pieces of bovine meat were soaked in a concentrated AFP solution and drained to dry. AFP helped to maintain the ice crystal size in the frozen meat but the soaking time of the meat in the AFP solution was too long (2 weeks), causing the sample to deteriorate after 2 weeks. To overcome this problem, Feeney and Yeh (1998) tried injecting AFP solutions to lambs before slaughter. In another study, the effects of pre-slaughter administration of AFGPs to lambs on lamb meat quality were evaluated after thawing. AFGPs from Antarctic Cod were injected intravenously into lambs pre-slaughter and upon thawing the meat samples were assessed for the effects of AFGPs on meat quality during freezing and thawing. Samples were vacuum packaged and frozen at −20 ˚C for 2–16 weeks. It was observed that injection of AFGP at either 1 or 24 hours before slaughter reduced drip loss and ice crystal size in the meat samples. Smallest ice crystals were found in the lambs injected with 0.01 mg/kg AFGP 24 hours before slaughter. These results suggested that a cost-effective and consumer-acceptable method of addition of AFPs in meat could minimize damage to meat quality on freezing (Payne and Young, 1995).

In a work done by Fukuoka *et al.* (2013), chilled chicken leg was cut into 2 cm lengths, and chilled pork boston butt was diced into 1.5cm pieces. The Japanese radish sprout extract solution (with AFPs) containing 1% salt (diluted so as to be 100 mg/l per kg of meat) was added in an amount of 20% mass of the raw material in both the meat samples, then the mixtures were tumbled, frozen by individually quick-frozen (IQF) method and then allowed to stand still at 4˚ C for 24 hours to be thawed and the amount of drip after thawing were measured in both meat samples. As a control, a solution containing only 1% salt was used (Table 2).

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Table 2: Amount of drip loss in chicken meat and pork with and without the addition of AFPs from Japanese radish sprout extract (Fukuoka *et al.*, 2013)

Solution to be added	Amount of drip in chicken	Amount of drip in pork
1% salt + Japanese radish sprout extract (with AFPs) (100 mg/l per kg of meat)	2.90%	5.60%
1% salt : control (without AFPs)	5.00%	5.80%

As shown in Table 2, it is clear that when Japanese radish sprout extract solution was added, the amount of drip decreased in case of chicken meat, although much difference was not seen in the pork samples. However, in both cases the sensory quality after thawing was improved. Each meat sample after thawing was cooked and served to five subjects, and the texture was evaluated by the following 5-levels. Evaluation Standard - 1: hard, 2: somewhat hard, 3: no difference, 4: somewhat soft, 5: soft. As a result, all subjects evaluated the texture of both the meat samples as "soft" or "somewhat soft". It was obvious from the result obtained that the AFPs inhibited the ice crystal growth and protected cell damage and thereby improving eating quality of frozen chicken meat and pork (Fukuoka *et al.*, 2013).

The study conducted by Boonsupthip and Lee (2003) first demonstrated the potential usefulness of a type III AFP in preservation of the gel-forming properties of fish muscle under frozen and chilled conditions. The results showed that AFP provided better protection than conventional cryoprotectants even after frozen temperature abuse at a relatively smaller concentration, without imparting sweetness, which is a commercial benefit.

Level of Incorporation

Relatively small amounts of pure AFPs are sufficient to exhibit activity without much taste alterations, which is a commercial benefit and suggests AFPs as possible novel preservatives and additives for frozen food in the future. Different levels of incorporation of antifreeze proteins in livestock products tried by researchers to get desired quality of frozen foods is given as follows:

In heat-shocked ice cream, ice recrystallization rates were significantly reduced by 40–46% with the addition of 0.25–0.37% total protein from AWWE. Significant ISP activity in retarding ice crystal growth was observed in all solutions containing 0.25% total protein from AWWE (Regand and Goff, 2006). Ice crystals were smallest in the meat of lambs injected with 0.01 microgram/kg AFGP, particularly when AFGP injected 24 h before slaughter (Payne and Young, 1995). As given in Table 2, the Japanese radish sprout extract solution (with AFPs) containing 1% salt diluted so as to be 100 mg/1 per kg of meat was added in an amount of 20% mass of chicken and pork samples, which were individually quick-frozen and thawed resulting in lower drip losses (Fukuoka *et al.*, 2013).

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Antifreeze Biomolecules, but not AFPs

In November 2009, the proceedings of the National Academy of Sciences published the discovery of a molecule in an Alaskan beetle (survive at -60˚C) that behaves like AFPs, but is composed of saccharides and fatty acids. The new antifreeze is based on Xylomannan - a mixture of saccharides and a fatty acid. The extracted antifreeze had a molecular weight of 240,000–310,000 and a xylose-to-mannose ratio of 0.5. Researchers have found that the saccharide-based antifreeze is more stable than the protein-based antifreeze at high processing temperatures and over a wide pH range, thus making it suitable for a wider range of applications. It was also confirmed that frying at more than 160°C did not influence its antifreeze quality in frozen fried food (Walters *et al.*, 2009).

Limitations and Future Prospects

During the past decade, AFPs have demonstrated tremendous potential for many industrial, commercial and medical applications. However, there are several factors like sensitivity to temperature and pH limits the AFPs in mass commercial application. They show weak functions after cooking for 20 minutes at 100˚C. Their activity is weaker in acidic pH (<5) and show better function above pH 5 and hence these can't be used in acidic food, but these can effectively be used in low acid and alkaline food systems requiring low processing temperatures. Extraction and purification efficiency of AFPs with 99 % purity is 3g/5 days/person which are both time and labour consuming. Pure AFPs are expensive (99 % purity costing US $50.00/g) (Fukuoka *et al.*, 2013). The problem of time, labour and cost can be solved by going for mass commercial production and development of newer cost effective methods. On application by physical means to food there is less penetration of AFPs to deeper parts. Therefore, improved methods like gene transfer should be used for better efficacy. Some people are allergic to AFPs (Crevel *et al.*, 2007) but allergenicity in the studies conducted may be due to small sample size of test population and is purely individual specific. On the other hand, AFPs are present in many food consumed in colder areas and people are consuming as part of their natural diet since time immemorial without showing any allergic reactions.

Because of ISP labeling, the AFP containing products fetch less consumer acceptance due to the GM issue. Consumer awareness is needed regarding the fact that the frozen food products with ISPs don't contain any residual GM yeast. Use of rare Antarctic fishes for AFP production is environmentally prohibitive. So, more emphasis should be given for production of R-AFPs from yeasts and bacteria (microbial origin). The use of AFPs is currently effective in selected frozen food systems. So, efforts should be made for exploration of their use in most frozen and chilled food systems. Lastly, their limited production in cold adapted species limits their mass commercial production for industrial and food usage. The need of the hour is to search for other sources and AFP analogues and go for mass commercial production for their large scale applications in frozen food industries (Harding *et al.*, 2003).

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Conclusion

Antifreeze proteins (AFPs) are promising ingredients for frozen food systems. AFPs increase the storage time and thawing quality of frozen food and desserts. These are highly active at low concentrations, which would make them cost effective. The inhabitants of colder regions have consumed it for many years as part of their natural diet. Presently, the only major route of obtaining AFPs involves the time-consuming and expensive process of isolation and purification from deep sea polar fishes and some cold adapted plants. Unfortunately, this is not amenable to mass production and commercial applications. Due to the minimal requirements, AFPs and AFP analogues can be considered as future additives for frozen foods by scientific interventions. In spite of a few limitations, AFPs are emerging as possible novel preservatives for frozen foods in future.

Conflict of Interest

There is no conflict of interest to disclose.

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