



Review Article

Source, mechanisms, preparation and potential applications of the antifreeze peptide: a review

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Abstract: Antifreeze peptides (AFP) constitute a unique category of small-molecule proteins or protein hydrolysates that shield organisms from harm induced by freezing or sub-freezing conditions. The heightened effectiveness of AFP compared to traditional commercial antifreeze agents and natural antifreeze proteins has sparked a surge in research interest in recent years. This burgeoning enthusiasm has driven significant advancements in AFP research, paving the way for their potential industrial utilization within the food sector. This review provides a comprehensive exploration of the source of AFP, assesses their antifreeze capabilities, elucidates their mechanisms of action, outlines various preparation methods, and scrutinizes their diverse applications in the food industry. Furthermore, it delves into the prospects for the development and application of AFP, shedding light on the trajectory for ongoing and future research endeavors.

Keywords: antifreeze peptide; mechanism of action; preparation method; potential applications

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1 Introduction

As the global economy continues to develop, and living standards improve, there is a concomitant rise in demand for higher-quality food. However, maintaining the original food quality over extended periods presents a significant challenge due to its seasonal, regional, and perishable nature^[1,2]. The food cold chain, a systematic innovation that emerged with the advancement of refrigeration technology in the 20th century, addresses this challenge. This modern and comprehensive low-temperature system, underpinned by food low-temperature technology and realized through sophisticated refrigeration technology and equipment, ensures that food quality is preserved across the entire production and distribution spectrum^[3,4]. From raw materials through processing, storage, transportation, and sales, the food cold chain maintains consistently high quality, meeting the burgeoning demand for superior food products^[5]. The application of frozen processing in the food industry holds considerable significance. AFP effectively inhibits food spoilage caused by microbial proliferation, extends shelf life, and largely preserves the original nutritional content of food. Furthermore, it enhances the stability and safety of food products, ensuring they meet the rigorous standards expected by consumers and regulatory bodies alike^[6,7].

However, the freezing of food leads to the formation of large ice crystals within its tissues, causing internal cells to become deformed, ruptured, and extruded, ultimately compromising the structural integrity. During thawing, this damage results in

significant juice loss, which negatively impacts the food's appearance and freshness. As a result, the quality of food after thawing is generally inferior to its pre-frozen state^[8,9]. Therefore, the growing research interest in minimizing damage caused by large ice crystals during food refrigeration and transportation has led to the development of several effective techniques. On the one hand, the commonly employed freezing technologies in the food industry include ultrasound-assisted freezing, high-pressure freezing, and electrostatic field-assisted freezing, among others^[9,10]. Zhu *et al.*^[11] found that ultrasound and adenosine monophosphate (AMP) treatment of frozen surimi maintained a stable microstructure, improved water retention, and slowed myofibrillar protein denaturation during 60 days of storage. Similarly, Dadashi *et al.*^[12] reported that high-pressure freezing (200 MPa at -20 °C) of egg whites and yolks reduced proteolysis, shortened phase transition time, increased viscoelasticity, and enhanced protein aggregation compared to conventional freezing. However, in industrial production, the implementation of these technologies will significantly increase the cost of product ingredients. On the other hand, traditional freezing approaches often involve using antifreeze agents such as complex phosphates, sugars, and phenols^[13]. However, polyphosphates are heavily regulated due to health risks, including impaired calcium absorption, bitter taste, high blood pressure, and worsened chronic kidney disease^[14]. Meanwhile, sucrose-based agents are less used due to their high calorie and sweetness, which conflict with low-sugar trends and pose risks for diabetes, hypertension, and obesity^[15]. Therefore, it is important to explore more economical, healthy, and practical antifreeze methods.

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Antifreeze peptides (AFP) are a unique group of small-molecule proteins or protein hydrolysates that protect organisms from damage caused by freezing and sub-zero temperatures. Compared with ice nucleation bacteria technology, which has safety risks, ultrasonic freezing technology, which has complex solution mechanisms and equipment that needs to be developed, and high-pressure freezing technology, which requires large capital investment and unstable ice crystals, AFP extracted from food-derived proteins have the advantages of being safe and non-toxic, easy to prepare, stable in terms of nutrition, ice crystals and physicochemical properties, and easy to industrialize and apply on a large scale^[16]. Their remarkable effectiveness compared to traditional antifreeze agents and natural antifreeze proteins has spurred significant research interest^[17]. AFP can adhere to ice crystal surfaces, inhibiting recrystallization and resulting in the formation of consistently small, uniform ice crystals. This prevents ice crystals from penetrating the internal structure of food, thereby significantly reducing the adverse effects of freezing on food quality^[18,19]. In 2006, the Chinese Ministry of Health listed antifreeze as a new type of food additive that can be used in frozen foods. Qu *et al.*^[20] added 0.5% oat antifreeze protein to wheat gluten protein. The results showed that the antifreeze protein group had a reduced degree of water retention, emulsification, and foaming, and a significant decrease in free sulfhydryl groups, which can effectively reduce the loss of wheat gluten protein during the freeze-thaw cycle. Incorporating AFP into food products has been demonstrated to effectively reduce freeze-thaw losses, highlighting their significant potential for widespread application of freezing technology within the food industry^[21-23]. The antifreeze activity of AFP largely depends on their source and preparation method, but current experimental methods make it difficult to describe in detail the mechanism and process of the interaction between AFP and ice crystals. Therefore, molecular dynamics simulation technology using computers has become the main trend in the study of the antifreeze mechanism of AFP. For example, Li *et al.*^[24,25] performed molecular dynamics simulation found that the Pv-AFP 1 (KAADSFNHNKAFKVG) and Pv-AFP 2 (KAADSFNHNKAF) sequences are similar, but their mechanisms of action are not the same. Their activity may be related to the

α -helix structure, their amphiphilicity and their affinity for interacting with water molecules and ice crystals, binding sites and patterns. However, despite growing research in this field, comprehensive data remains limited, and several challenges persist. This paper provides an in-depth overview of the sources, evaluation methods, preparation techniques, mechanisms of action, and applications of AFP in the food industry. In addition, this article also discusses the future prospects and applications of AFP in the field of food development.

2 Source of AFP and antifreeze properties assessment

2.1 Source of AFP

Since the late 1960s, scholars have discerned that organism inhabiting cold environments—ranging from fish and insects to bacteria and plants—undergo protracted periods of natural selection. This process leads to the synthesis of a unique class of active proteins within their biological frameworks, which act as protective shields against stressors, commonly referred to as antifreeze proteins. These AFP can be delineated into four principal categories based on their origins: those sourced from fish, insects, plants, and bacteria. As shown in Fig. 1, Scholander *et al.*^[26] pioneered the identification of a specialized protein facilitating Arctic fish in their adaptation to sub-zero temperatures; Ramsay^[27] uncovered the existence of antifreeze proteins within yellow mealworms; Fei *et al.*^[28] validated the effectiveness of antifreeze proteins sourced from sardonyx in plant organisms; Naomi *et al.*^[29] isolated antifreeze proteins with a molecular weight of 164 kDa originating from *Pseudomonas putida* GR12-2.

Based on their activity, antifreeze proteins can be categorized into AFPI, AFPII, AFPIII, and AFPIV^[31-35]. Please refer to Table 1 for additional insights. Extracting natural antifreeze proteins from living organisms presents challenges, as they are not only limited in abundance but also labor-intensive and expensive to procure, rendering large-scale production arduous. As a result, the quest for easily accessible and cost-effective means of obtaining

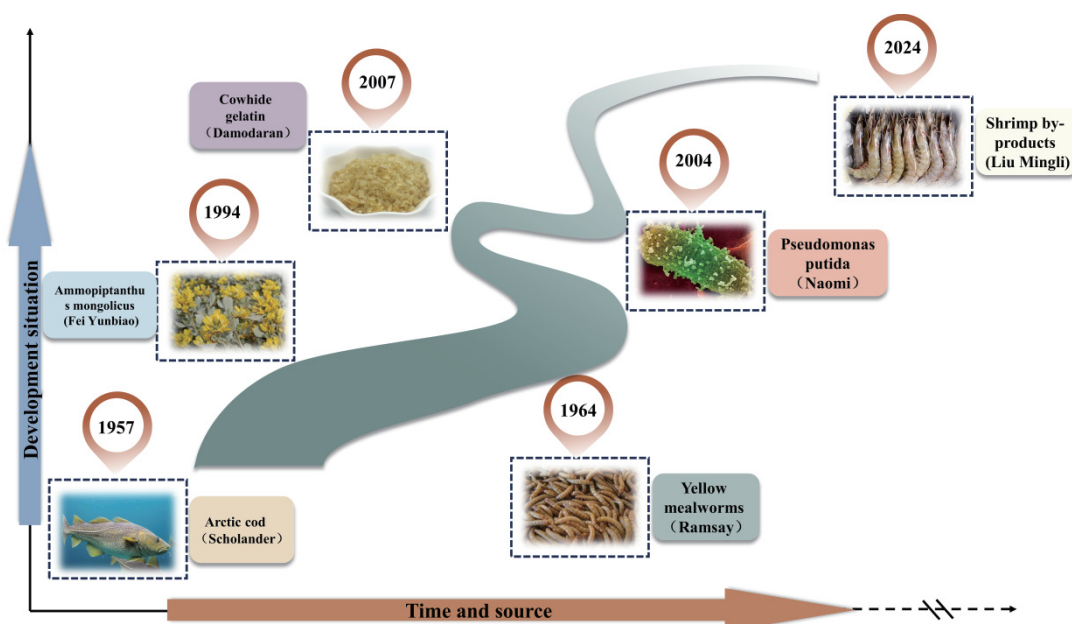
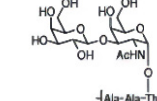


Figure 1 Development of antifreeze proteins and AFP.

Table 1 Characteristics of different types of antifreeze proteins^[30]

Characteristic	AFPI	AFPII	AFPIII	AFPIV	AFGP
Mass (Da)	3,300–4,500	11,000–24,000	6,500	12,000	2,600–33,000
Key properties	Alanine rich; α -helix	Disulfide bonded	β -Sandwich	Alanine rich; helical bundle	AAT repeat; disaccharide
Representative structure					
Natural source	Right-eyed flounders	Sea raven; smelt	Ocean pout; wolfish	Longhorn sculpin	Antarctic notothenioid

antifreeze proteins has emerged as a formidable hurdle for researchers.

AFP predominantly originate from food-based proteins, which undergo enzymatic hydrolysis to generate protein hydrolysates. Subsequent screening of these hydrolysates for peptide fragments showcasing antifreeze activity leads to the isolation of AFP. AFP exhibit a classification based on their structural and compositional attributes, delineated into four distinct types: 1) characterized by alanine-rich sequences and an α -helical configuration; 2) featuring cystine-rich compositions with a β -folded structure; 3) displaying no discernible structural pattern; 4) encompassing antifreeze glycoproteins (AFGP). Duman and DeVries^[36] successfully isolated alanine-rich AFP, with molecular weights of 12, 8, and 6 kDa, respectively, from the serum of American flounder. Slaughter *et al.*^[37] extracted AFP from sea crows, revealing molecular weights spanning from 14 kDa to 16 kDa. These peptides boasted a notable abundance of cysteine and hydrophilic amino acids. Hew *et al.*^[38] meticulously isolated eight antifreeze polypeptides within the American giant shearwater. Interestingly, these polypeptides showcased a balanced amino acid composition devoid of notable biases and lacked the presence of alanine, cystine, or discernible secondary structures. The Antarctic cod stands as a reservoir of antifreeze glycoprotein peptides, from which Komatsu *et al.*^[39] expertly extracted AFP showcasing molecular weights spanning from 2.7 kDa to 34 kDa. Intriguingly, each of these peptides features the recurring structural motif (Ala-Thr-Ala).

The primary sources utilized for the extraction of AFP include collagen^[40], fish skin^[41], shrimp by-products^[21], and similar substrates. Damodaran and Srinivasan^[42] successfully obtained the desired AFP through the hydrolysis of bovine protoplasts using papain as the enzymatic catalyst. These peptides were subsequently utilized in the storage of ice cream. A comprehensive review of the literature elucidates that AFP structures commonly feature Gly-Pro-x tripeptide repeats, accompanied by specific amino acid lengths and relative molecular masses typically falling below 2,000 kDa^[43].

2.2 Assessment of the antifreeze efficacy based on thermal hysteresis activity

AFP are well-known for their ability to lower the freezing point of a solution without affecting the melting point. This phenomenon creates a notable difference between the two points, known as thermal hysteresis activity. The magnitude of this variance hinges upon the interplay between AFP and ice crystals see Fig. 2 for details^[44].

2.2.1 Ice crystal morphology image method

The ice crystal morphology imaging technique involves microscopic observation of ice crystal nuclei during the melting phase, alongside the determination of freezing and melting points of antifreeze proteins/peptides. This meticulous process allows for the quantitative assessment of their thermal hysteresis activity. DeVries^[45] corroborated the presence of antifreeze activity in the

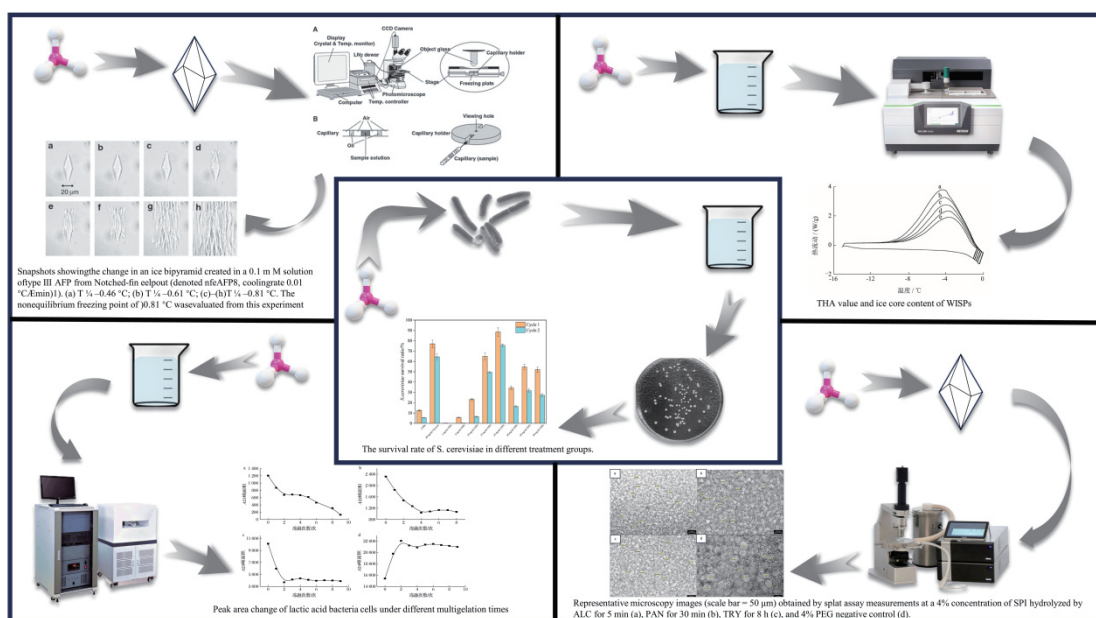


Figure 2 Schematic diagram of the evaluation method of frost resistance^[21,54,58]

plasma of Antarctic fish utilizing the microscopic capillary single crystal observation, commonly referred to as the deVries method. While the method is straightforward in its application, it is susceptible to a considerable degree of human error. This is attributed in part to the significant fluctuations in the rate of heating/cooling of the sample within the capillary, which are challenging to regulate. Additionally, the frozen sample tends to adhere to the capillary, thereby impeding the detection of changes in morphology.

Tomalty and Walker^[46] assessed the thermal hysteresis activity of ryegrass antifreeze proteins employing the Clifton nanolitre osmometer method. This involved the utilization of a microscope to observe the growth of ice crystals at a protein concentration of 1 mg/mL.

2.2.2 Differential scanning calorimetry (DSC)

The underlying principle of DSC for evaluating thermal hysteresis activity entails recording the heat absorption or exothermic changes within the solution system during the crystallization process. This involves delineating the freezing and melting points of the sample, identifying the onset temperature of crystallization, and subsequently deriving the thermal hysteresis activity of the sample^[47]. Tan *et al.*^[48] synthesized yak bone collagen peptides sourced from yak bone, employing DSC to ascertain the thermal hysteresis activity. Their findings revealed a heat hysteresis activity value of 5.76 °C, alongside a protein yield of 48.1% achieved under optimal conditions. Qu *et al.*^[20] utilized antifreeze proteins derived from winter wheat, exhibiting a thermal hysteresis activity of 0.46 °C as determined by DSC, in the treatment of frozen tofu. Remarkably, the results showcased a substantial enhancement in various attributes of frozen tofu compared to the control group. Specifically, water retention, rehydration capacity, steam resistance, elasticity, and tensile properties exhibited notable increases of 114.17%, 57.42%, 56.25%, 21.52%, and 23.71%, respectively. Meanwhile, hardness and shear strength experienced reductions of 24.83% and 23.75%, respectively. These findings collectively indicate a significant improvement in the quality and structural characteristics of frozen tofu.

2.2.3 Low-field magnetic resonance imaging (LFMRI)

The LFMRI technology serves to discern the spatial distribution of mobile water by detecting positive proton signals. During the melting process, a heightened signal intensity in the frozen AFP solution correlates with a greater concentration of mobile water. In contrast, solid ice exhibits no discernible signal. This enables the precise elucidation of the impact of AFP on the melting dynamics of the frozen solution^[49]. Masselot *et al.*^[50] have demonstrated a correlation between the rate of ice recrystallization and the molecular mobility of sugar solutions. LFMRI allows for the quick determination of both the size of initial ice crystals and the recrystallization process within a sample. This method offers advantages over microscopic observation, requiring less time and being associated with lower error rates^[51, 52].

2.3 Assessment of antifreeze effect based on the principle of ice crystal recrystallization inhibition

During the freeze-thaw cycle, temperature fluctuations induce the melting of small ice crystals, increasing supercooled water. Supercooled molecules then aggregate on the surface of existing ice crystals, causing the original small ice crystals to dissolve and coalesce into larger ice crystals. This process represents ice crystal

recrystallization^[53]. Ice recrystallization is a thermodynamically driven process that causes the total free energy of the entire system to decrease, in line with the law that all spontaneous processes proceed in the direction of free energy decrease. Therefore, ice recrystallization is an important indicator for evaluating the antifreeze activity of AFP. Ice crystal nucleation depends on nuclei, while ice crystal recrystallization is the disappearance of small ice crystals to form large ice crystals, which is unrelated to the nucleation process. This is the main difference between the two, and ice crystal recrystallization is the most sensitive method for evaluating the antifreeze activity of AFP.

The recrystallization inhibition activity of antifreeze proteins/peptides can be discerned through splat cooling at low temperatures, employing cold-stage polarized light microscopy to observe ice crystal growth. Fomich *et al.*^[54] conducted enzymatic hydrolysis of soy isolate proteins and examined their ice crystal growth utilizing the Splat method. The researchers discovered that all soy isolate protein hydrolysates showed ice recrystallization inhibition (IRI) activity. This activity resulted in a decrease in ice crystal diameters by a significant percentage when compared to natural soy isolate protein. Specifically, the reduction in ice crystal diameters ranged from 57% to 29% across the different soy isolate protein hydrolysates studied. These results provide compelling evidence for the ability of soy isolate protein hydrolysates to effectively inhibit ice crystal growth. Yuan *et al.*^[55] used trypsin and alkaline protease to hydrolyze wheat gluten and measured the inhibition of ice crystal recrystallization activity by the splash method. The results showed that regardless of the dispersing medium used, the IRI activity of the trypsin hydrolysate was always better than that of the alkaline protease product. The increase in IRI activity was related to changes in the secondary structure, which was manifested as an increase in α -helix content and a decrease in β -turn content. This indicates that there is a close relationship between the structural rigidity of the molecule, the antifreeze activity of the peptide, and the IRI activity.

2.4 Cryoprotection method for organisms

The bacterial cryoactivity test entails employing the colony counting method to quantify the survival rate of bacteria before and after undergoing AFP cryogenic treatment. A higher survival rate post-treatment serves as compelling evidence of the antifreeze activity exhibited by AFP^[56]. Bacterial systems are conventionally identified by assessing the optical density (OD) value at 600 nm, offering insight into the viability of the colony from a lateral perspective. Cao *et al.*^[57] employed squid skin collagen as the primary raw material, subjecting it to enzymatic treatment to obtain the hydrolyzed product. Subsequently, this hydrolyzed product was introduced into *Escherichia coli*. The assessment of antifreeze activity was accomplished by quantifying the survival rate of *E. coli*.

3 Preparation of AFP

Antifreeze proteins were initially extracted directly from organisms capable of surviving in cold environments. However, the limited availability, small quantities, and high production costs have hindered the development and widespread application of these proteins. Presently, AFP are garnering significant attention from researchers due to their similar efficacy to antifreeze proteins. Additionally, AFP are characterized by their abundant sources, high safety, biological activity, and other advantageous properties. The potential applications of AFP in medicine and the food

industry are particularly promising. Currently, there are three primary methods for preparing AFP (Fig. 3): 1) Direct extraction of natural biopeptides and other active ingredients from the organisms themselves; 2) Obtaining biopeptides with various physiological functions through the degradation of proteins; 3) Synthesizing biologically active peptides using chemical methods and recombinant DNA technology.

3.1 Biological extraction method

Bio-extraction involves directly isolating natural AFP from plants, animals, bacteria, fungi, and other organisms using solvents alongside various separation and purification techniques. The process typically begins with introducing the biological material into a suitable solvent, followed by the removal of impurities such as proteins and salts through dissolution. This is achieved through repeated centrifugation, pH adjustments, and other preparatory steps, culminating in the acquisition of a crude extract. This extract then undergoes further separation and purification to isolate the target active peptide. Common extraction solvents include water, perchloric acid, acetic acid, ammonium acetate and other buffer solutions.

Ramsay^[27] utilized the bio-extraction method to isolate AFP from the yellow mealworm. Their research demonstrated that these AFP exhibited antifreeze activity 10–100 times greater than that of typical fish, as determined by thermal hysteresis activity. Guo^[59] used water as a solvent to extract eucommia leaf protein, and the optimal conditions were obtained: a material-liquid ratio of 1:20, 10 flash extractions, an extraction time of 60 min, and extraction at 4 °C. The extraction rate can reach 0.72%, so that the antioxidant capacity, antibacterial activity, and tumor cell inhibition effect of eucommia leaf protein can be further determined. Ding^[60] used a $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ solution as a buffer to extract AFP from barley grains. After ultrafiltration and a series of column chromatography purifications, the relative molecular mass of the barley grain AFPs was 13,370 Da, and the THA was 1.04 °C. The sequence was compared and showed high homology with proteins from 12 genera in the grass family Triticeae. The fundamental principle of this method is

straightforward and easily comprehensible. Most biological extraction methods are used to extract endogenous AFP, because endogenous AFP come from the organism itself and are present in very small amounts in the body. Although the process is relatively simple, a large amount of organic solvent is required for separation and purification. This makes the process costly and poses a significant risk to the environment. It also causes problems such as the actual residue of the extracted AFP causing toxicity, which in turn limits the feasibility of large-scale industrial production.

3.2 Proteolytic method

3.2.1 Biological enzymatic method

Biological enzymatic digestion involves the use of specific or non-specific proteases to break down proteins into small molecule peptides. This method employs a variety of proteases, including trypsin, pepsin, neutral protease, alkaline protease, papain, and complex protease mixtures, to synthesize AFP. Cai *et al.*^[61] conducted an enzymatic hydrolysis of squid skin collagen, initially using alkaline protease followed by papain treatment. The conditions of the enzymatic process were optimized using the cryoprotective activity of peroxidase as an indicator of antifreeze efficacy. The amino acid sequences of the primary components were identified as either GPYPAAANSPE or EPSNAAPYYPG, which are characteristic of antifreeze proteins due to their richness in hydrophilic amino acids. Liu *et al.*^[62] treated crayfish shells with alkaline protease and trypsin, demonstrating that the resulting crayfish peptides exhibited significant antifreeze activity, as evidenced by the survival rate of yeast used as an indicator. Two peptide sequences, YWDHPIRDGAFPH and GPPGKPGIPDIVDW, were identified and subsequently confirmed to possess antifreeze activity. The use of enzymes as extraction reagents renders this method relatively expensive. Nevertheless, it remains the most prevalent technique for the preparation of AFP due to its inherent safety and eco-friendliness, aligning well with the anticipated advancements in the food industry.

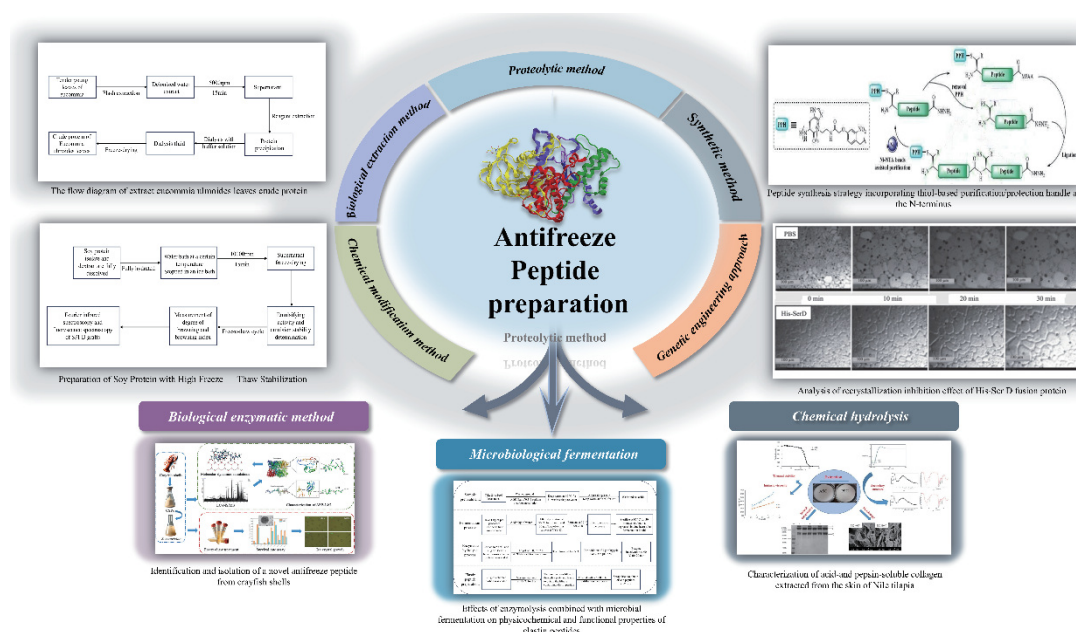


Figure 3 Preparation method of AFP^[15, 59, 62-64, 67, 73].

3.2.2 Microbiological fermentation

The microbial fermentation method entails the biochemical metabolic reaction of microorganisms to convert large molecule proteins into small molecule protein active peptides. During this process, AFP are generated with different amino acid sequences and molecular weights, influenced by the microbial species, metabolism, and fermentation conditions employed. Lu *et al.*^[63] integrated enzymatic and microbial fermentation methods, comparing them with a control group utilizing a single protease. Their results showcased the advantages of the microbial fermentation approach in enhancing the physicochemical and functional properties of elastin. Moreover, the flexibility to select different strains enables the preparation of a diverse array of AFP. This method efficiently harnesses and further refines production waste in the food industry. However, owing to the relatively limited yield of AFP produced by microorganisms and concerns regarding the uncertainty and safety of fermentation processes, the microbial fermentation method still possesses considerable scope for development.

3.2.3 Chemical hydrolysis

Chemical hydrolysis involves subjecting proteins to specific temperature conditions using acid or alkali solutions of appropriate concentrations to obtain small molecule peptide fragments. This process entails breaking protein peptide bonds and disrupting the spatial structure of proteins. Commonly used acid and alkali solutions include hydrochloric acid, phosphoric acid, sodium hydroxide, and others. Sun *et al.*^[64] showcased the potential of chemically hydrolyzing structural protein-rich raw materials like collagen and keratin to broaden the applications of collagen. While this method offers cost-effectiveness and simplicity, the presence of acid and alkali reagents can compromise amino acids and diminish the nutritional value of the resulting product. Moreover, the need to remove these reagents post-hydrolysis represents a drawback. Consequently, this method finds limited application in AFP preparation.

3.3 Synthetic method

Synthetic methods are pivotal in laboratory settings, streamlining the preparation of peptides with precise amino acid sequences. These methods typically fall into three categories: liquid-phase synthesis, solid-phase synthesis, and genetic recombination techniques.

Polypeptide synthesis is often a repetitive procedure involving the sequential addition of amino acids, commonly from the C-terminus to the N-terminus in solid-phase syntheses. Solid phase synthesis stands out as highly efficient for small-scale peptide synthesis, accommodating peptides ranging from 10 residues to 100 residues. Notably, all reactions can be conducted within a single vessel, streamlining the process. Following coupling, any unreacted solvents and by-products can be readily flushed out, significantly simplifying the purification process.

Liquid phase synthesis, an early and widely utilized method, involves peptide synthesis in a homogeneous solution. Liu *et al.*^[65] introduced a dual-function handle with both protection/purification at the N-terminus. This processing strategy successfully constructed glycoproteins with tandem repeat sequences through several chemical linkages and affinity purification cycles. Contains 4 mucins 1 with 20 amino acids as repeating units and antifreeze glycoprotein AFGP with 80 repeating tripeptide sequences and a total of 240 amino acids. Each of the threonine residues of the

latter contains an α -linked O-GalNAc, and the antifreeze ability of the synthesized antifreeze protein has been verified^[15]. RGD is a sequence peptide composed of three amino acids: arginine (Arg), glycine (Gly), and aspartic acid (Asp). It has high biological activity and can competitively inhibit the binding of various adhesion proteins, including Fib, to platelets. Song Mingli and others obtained a crystalline dry product of the tripeptide (Arg-Gly-Asp) with a total yield of 57.2% by optimizing the reaction conditions^[66]. The synthetic method has the advantages of high flexibility, high yield and high purity. However, after peptide attachment, it's crucial to separate, purify, or crystallize the product to eliminate any unreacted raw materials and by-products. This approach proves to be expensive, time-consuming, and operationally challenging, rendering it less suitable for widespread use in the preparation of AFP.

3.4 Genetic engineering approach

With the continuous advancement and evolution of biotechnology and its broadening applications, a novel method for producing bioactive peptides has emerged: introducing exogenous bioactive peptides into other organisms *via* genetic engineering techniques. This induces the expression of such peptides, enabling the synthesis and secretion of large quantities of antifreeze-active peptides. Jin *et al.*^[67] ingeniously introduced the silk gum AFP gene into the plasmid, followed by expression through *E. coli*. The resultant target protein was meticulously purified and subjected to rigorous antifreeze analysis. Their groundbreaking work showcased that the newly obtained fusion protein remarkably diminished the size of ice crystal particles in solution, demonstrating exceptional recrystallization inhibition properties. Zhang^[68] ingeniously utilized *Lactococcus lactis* as an expression vector to construct AFP sequences aligned with *Lactococcus lactis* codons, and subsequently expressed antifreeze proteins induced by *Streptococcus lactis*. The outcomes of this groundbreaking study showcased that the recombinant bacterium displayed augmented cell survival and remarkable heat-lagging activity. Notably, it was observed that the bacterium proficiently shielded the intracellular vitality of crucial enzymes and preserved the integrity of cell membranes, thereby manifesting a significant protective effect against freezing stress. Through the strategic implementation of transgenic technology, organisms are genetically modified to enable the efficient expression of the AFP within the vector. This innovative approach unlocks the potential for vast increases in the yield of AFP extraction, while concurrently driving down production costs. Such advancements are pivotal in meeting the burgeoning demands for industrial-scale applications.

3.5 Chemical modification method

Chemical modification, a technique harnessed in protein science, entails the precise introduction of distinct functional groups into proteins *via* chemical methodologies. This process can also involve the disruption and polymerization of amino acid residue side chain groups. Such modifications serve to intricately alter the physical, chemical, and functional attributes of proteins, thereby expanding their potential applications in various domains^[69]. Commonly employed chemical modification techniques include alkali treatment, acid treatment, acylation, deacylation, phosphorylation, glycosylation, thiolation, and various others. These methods facilitate precise alterations to protein structures, enhancing their versatility and functionality for diverse applications^[70-72].

Glycosylation within AFGP stands as a pivotal phenomenon,

constituting the primary determinant of the antifreeze activity inherent in these proteins. Despite its significance, the preparation and purification of AFGP pose notable challenges. Presently, chemical modification emerges as a prevalent method utilized to procure AFGP and unravel the intricate relationship between its structural attributes and antifreeze efficacy. Sun *et al.*^[73] conducted a study wherein they subjected soy protein to glycosylation through a wet melted reaction. Their research revealed that glycosylated soy protein significantly bolstered the freeze-thaw stability of grafted products.

4 Mechanism of action of AFP

The efficacy of AFP in preserving the quality of frozen goods predominantly stems from their thermal hysteresis activity. This phenomenon involves the remarkable capability of antifreeze proteins to decrease the freezing point of a substance while keeping the melting point unchanged, thereby establishing a noticeable difference between the two temperatures. Antifreeze proteins or peptides can bind to the surface of ice crystals, thereby augmenting their curvature and surface tension. This augmentation subsequently elevates the vapor pressure, consequently resulting in a reduction of the freezing point—an effect commonly attributed to the Kelvin effect^[74]. Moreover, below the melting point, small ice crystals tend to coalesce, forming larger crystals through a process termed recrystallization. This phenomenon has the potential to compromise the original structure and quality of the product. Nevertheless, the inclusion of AFP or proteins serves to impede the recrystallization effect, effectively curbing the growth of ice crystals and preserving their size and uniformity^[75]. Research has elucidated that the antifreeze activity of AFP is not contingent upon the entirety of the antifreeze protein, but rather on specific peptide domain sites^[76].

AFPPs can adhere to the surface of ice crystals through a diverse array of molecular forces, encompassing hydrogen bonding and van der Waals interactions. This impedes the mobility of ice crystals at the solid-liquid interface and the fusion of water molecules with ice crystals, thereby effectively thwarting ice recrystallization^[77-79]. Furthermore, AFPP binds to phosphate

groups present in the cell membrane, consequently elevating the phase transition temperature of the cell membrane. This action serves to mitigate the mechanical damage inflicted by ice crystals upon the cell. As shown in Fig. 4, this mechanism prevents the leakage of cellular contents outward, thereby preserving the ionic balance within and outside the cell membrane. Such preservation enhances the cell's resilience to cold temperatures, enabling it to survive below the freezing point^[80].

4.1 Adsorption inhibition model

The adsorption inhibition model was initially formulated by Raymond and deVries in 1977^[74]. Their theory posited that the attachment of antifreeze proteins to the surface of ice crystals, facilitated by the Kelvin effect, plays a pivotal role in safeguarding the frozen product. The mechanism operates on the premise that ice crystals typically grow perpendicular to the surface of the ice crystal. Yet, when impurity molecules adhere to the ice crystal's surface, a reduction in the freezing point becomes imperative to enable ice crystal growth amidst the impurities. This phenomenon can be ascribed to the simultaneous augmentation in the curvature of the ice crystals, leading to an expansion of the surface area. The influence of surface tension disrupts the equilibrium of the surface area, consequently reducing the freezing point. Antifreeze proteins or peptides can intercalate between the interfaces of ice and water, thereby modulating the growth of ice crystals and influencing their formation and expansion^[81]. Guo^[82] employed *Tenebrio molitor* antifreeze protein (TmAFP) and AFPIII in their study. They found that AFP have a unique ability to selectively adhere to the surface of ice crystals. They further elucidated an irreversible adsorption model, elucidating the process by which antifreeze proteins accumulate on the prismatic surface of ice crystals under the influence of water molecules.

4.2 Surface complementarity model

The surface complementarity model, also recognized as the “receptor-ligand model,” was introduced by Leinla in 2002^[83]. In this conceptual framework, antifreeze proteins assume the role of receptors, whereas ice crystals function as ligands. The theory

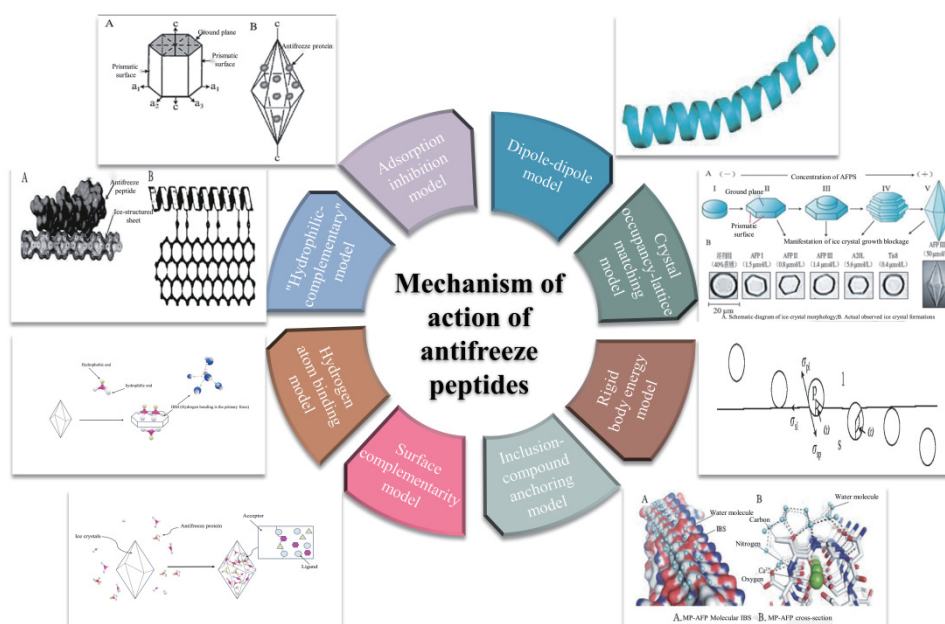


Figure 4 Schematic diagram of AFP action mechanism^[75,90].

postulates that the surface generated by the ice crystal binding sites of antifreeze proteins exhibits a complementary structure to that of ice crystals. This complementarity is governed by a myriad of forces, primarily hydrophobic and van der Waals forces, with hydrogen bonding playing a secondary role. These forces act upon the surface of the ice crystals, resulting in the irreversible binding of antifreeze proteins to the ice crystal surface. Consequently, this prevents the growth of ice crystals. This model effectively elucidates the capacity of various types of AFP to adhere to the surface of ice crystals, despite their distinct structures. It suggests the presence of diverse topologies on the surface of ice crystals, which selectively and complementarily bind to AFP possessing specific structural and functional attributes. Thus, the model of surface complementarity applies to all antifreeze proteins^[84].

4.3 Hydrogen atom binding model

The model posits that the antifreeze protein molecule exhibits distinct hydrophobic and hydrophilic sides. The hydrophobic end engages with the aqueous phase, whereas the hydrophilic end forms hydrogen bonds with the ice phase. Antifreeze proteins and ice crystals conjoin to create a composite structural entity, consisting of four analogous ice-binding modules (ice-binding motifs, IBM). These modules are constructed from Thr/Asp and Thr/Asn/Leu repeats, which confine the IBM residues, thereby giving rise to a flat ice-binding surface. The flat and rigid structure of this binding surface plays a crucial role in facilitating the mechanism of binding between antifreeze proteins and ice crystals. The former facilitates the augmentation of antifreeze binding to ice crystals, while the latter guarantees the uniformity and steadfastness of antifreeze binding^[85]. The structural attributes of antifreeze proteins, pivotal in enabling their attachment to ice crystals and bolstering the stability of the helix, constitute the fundamental rationale behind the inhibition of ice crystal growth by antifreeze proteins.

4.4 Dipole-dipole model

This model was first introduced by Yang *et al.*^[86]. According to their proposition, antifreeze proteins exhibit dipoles oriented parallel to the hydrophilic and hydrophobic groups of the helix axis. Their investigation unveiled that AFP sourced from winter halibut are abundant in Ala. Within Ala, the secondary structure of AFP is predominantly confined to a solitary α -helix, facilitating their interaction with water molecules through dipole interactions near the interface between free water and the ice phase. The dipolar interaction between α -helices and water molecules propels AFP towards the interface between free water and the ice phase. Subsequently, AFP establish hydrogen bonds with both free water and ice crystals. This dual bonding mechanism effectively curtails the recrystallization effect of ice crystals and decelerates their growth *via* the Kelvin effect. Graham *et al.*^[87] examined multiple AFP from various species and convincingly demonstrated their common ability to inhibit ice crystal growth by forming hydrogen bonds between α -helices and ice surfaces. The dipole-dipole model presents a compelling depiction of the interaction between antifreeze proteins and ice crystals, offering a fresh perspective for the exploration of AFP mechanisms.

4.5 Crystal occupancy-lattice matching model

DeVries^[88] initially introduced the lattice-matching model, which was further elaborated upon by Knight^[89] in the form of the crystal occupancy model, drawing inspiration from its predecessor. The lattice-matching model proposes that during the

growth of ice crystals, new surfaces of ice are continually formed. These surface progressively irreversibly bind AFP molecules until the entire ice crystal surface is fully enveloped by AFP, thereby halting further growth. The crystal occupancy theory model can be viewed as a complementary and expansive counterpart to the dipole-dipole model. Whereas the dipole-dipole model suggests that the hydrophilic residues of AFP form hydrogen bonds with ice crystals, the crystal occupancy theory posits that these residues occupy positions within the crystal lattice structure previously held by oxygen atoms on the crystal surface. This dual perspective provides a more comprehensive understanding of the intricate interactions between AFP and ice crystals. This impedes the growth of ice crystals that have been adsorbed by AFP along the growth axis, leading to a modification in cross-section owing to variations in the growth rate of each prismatic plane. Over time, this process gradually converges to a hexagonal shape^[90].

4.6 Rigid body energy model

The rigid-body energy model posits that AFP exhibit minimal potential energy between ice-water interfaces within an ice-water system. It contends that entropy energy serves as the impetus propelling the movement of AFP. Furthermore, it emphasizes the necessity for AFP to align precisely with ice crystals; otherwise, water molecules would diffuse to the interface. Moreover, it is proposed that as ice crystals grow, AFP advance alongside them, while water molecules diffuse behind the particles. This collective movement pushes the particles forward, ultimately facilitating the formation of ice crystals within the solution^[90]. While the rigid-body energy model satisfactorily elucidates the driving force behind AFP movement toward the ice-water interface, it falls short of comprehensively elucidating the entire mechanism by which AFP interact with ice crystals.

4.7 Inclusion-compound anchoring model

Li and Ma^[91] discovered that the inclusion-anchoring model predominantly revolves around AFP inducing water molecules to trail the lattice via hydrophobic interaction. Subsequently, these AFP anchor the water molecule lattice through hydrogen bonding. This anchored lattice then aligns with the surface of the specific ice crystal, facilitating its binding to the AFP. Garnham *et al.*^[92] introduced and supported the inclusion-anchoring model by studying the mechanism of action of AFP derived from Antarctic bacteria introduced. Their research uncovered that the structural segment of AFP linked with antifreeze activity features an ice-binding site (IBS) constituted by the Thr-Gly-Asx motif within the Ca^{2+} binding sequence. This IBS is characterized by its hydrophobic nature, with hydrophobic interactions acting as the driving force behind the organization of water molecules into a lattice. Subsequently, this lattice is anchored to the AFP *via* hydrogen bonding. Overall, this description aligns well with the earlier theory.

4.8 “Hydrophilic-complementary” model

Wang *et al.*^[93] developed a theoretical framework detailing the interaction between AFP and the surface of ice crystals employing molecular dynamics simulation technology. Their investigations reveal that AFP engage with ice crystals through the “hydrophilic-complementary” model, delineating the mechanism of action. The model posits that AFP, characterized by specific amino acid lengths and structures, engage with water molecules situated within the prismatic surfaces of ice crystals, forming hydrogen bonds. Through these bonds, peptide molecules attach to the

surfaces of ice crystals. Peptide chains with a molecular mass of less than 2,000 Da possess ample hydrophilicity and flexibility to counteract the competitive hydrogen bonding interactions between ice and water levels. This ability is attributed to the stabilizing hydrogen bonding facilitated by the proline- and alanine-rich residues of the side chains. Consequently, these AFP demonstrate pronounced ice crystal inhibitory activity.

5 Potential applications of AFP

Antifreeze peptides are widely used in food applications, mainly in frozen ice cream, frozen dough, frozen meat products and so on (Fig. 5).

5.1 Frozen yogurt

The challenge of ice crystal growth during the freezing of ice cream has long plagued researchers seeking solutions. With prolonged freezing, ice crystals tend to undergo recrystallization, resulting in larger and more abundant crystals. These enlarged crystals pose a threat to the texture and taste of the ice cream, compromising its overall quality. The size and morphology of ice crystals play a crucial role in determining the final structure of the ice cream. Hence, it is paramount to minimize ice crystal size and the occurrence of recrystallization at every stage of the ice cream manufacturing process^[94]. Seo and Oh^[95] showed that stabilizers interacting with milk proteins in ice cream can form a strong gel network structure together with polysaccharides. This interaction effectively inhibits ice crystal recrystallization and promotes the formation of fine ice crystals with regular morphology during both freezing and storage of the ice cream. Consequently, this process ensures the desired texture of the ice cream. Paglia *et al.*^[96] conducted an experiment where milk protein concentrates were applied to ice cream during frozen storage. The results showed that ice cream treated with milk protein concentrates exhibited a remarkable anti-collapse effect.

5.2 Frozen dough

Presently, freezing technology stands as a pivotal solution for combating the rapid degradation and restricted shelf life often

encountered in staple foods. However, prolonged freezing can lead to the recrystallization of small ice crystals, resulting in the formation of larger ice crystals that disrupt the original gluten network-like structure of the frozen dough. Consequently, yeast cells may sustain damage or even perish as a result. This phenomenon ultimately impedes the rising of the dough. In recent years, researchers have delved into various antifreeze agents bolstering the storage stability and quality of frozen dough^[97].

Liu *et al.*^[98] conducted a pivotal experiment wherein fish collagen was integrated into yeast culture and subsequently introduced into the frozen dough. The outcomes unveiled a significant improvement: after enduring six freeze-thaw cycles, the survival rate of yeast surged from a mere 9% to an impressive 47%, alongside a notable enhancement in relative fermentation potency. Moreover, the dough infused with fish collagen peptides showed a 12% reduction in gluten content, accompanied by an 8% increase in free sulfhydryl content. Remarkably, these findings mirrored those obtained from the control alginate group, underscoring the promising potential of fish collagen peptides as expansive-scale antifreeze agents for the storage of frozen dough. Zhang *et al.*^[99] introduced gluten antifreeze polypeptide into the frozen dough, yielding noteworthy results. Their study showcased the efficacy of gluten antifreeze polypeptide in preserving the secondary structure of gluten proteins and the orderly molecular arrangement. Notably, compared to the control group, the presence of the antifreeze polypeptide led to a 3% augmentation in the mass fraction of the α -helical structure. Moreover, the addition of 1% and 2.5% of the polypeptide corresponded to substantial increases of 8.8% and 9.8%, respectively, in the mass fraction of the disulfide bond G-G configuration. This preservation mechanism fortified the structural integrity of the gluten network, thereby upholding the pivotal role of gluten proteins within the dough matrix.

5.3 Frozen meat products

In today's global market, where meat exports surpass \$13 billion, refrigeration technology plays a crucial role in ensuring the safety of meat products during distribution

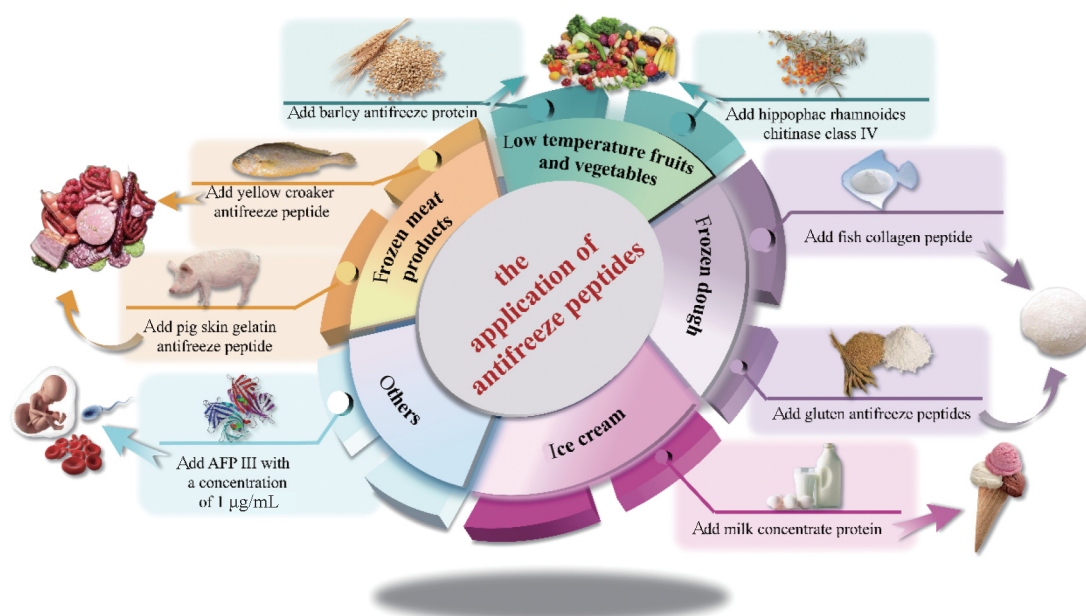


Figure 5 Schematic application of AFP.

worldwide. However, the detrimental effects of freeze-thaw cycles on meat quality and the resulting loss of meat remain major concerns^[100]. The cyclical process of freezing and thawing predominantly impacts the moisture content of meat, which resides within muscle fibers and interstitial spaces. As moisture freezes, the concentration of remaining solutes—comprising proteins, lipids, carbohydrates, minerals, and vitamins—elevates. Concurrently, ice crystals undergo growth and recrystallization throughout the freezing process and during transportation in the cold chain. This disturbs the intricate equilibrium of the meat system, leading to a deterioration in its original organization and ultimately compromising the quality of the meat^[101,102]. Myofibrillar proteins represent a class of structural proteins intrinsic to animal tissues, endowed with distinctive biological functionalities. These proteins not only furnish vital amino acids and calories but also wield a direct influence over the quality and sensory attributes of meat products^[103].

Xu *et al.*^[104] employed trypsin-treated yellow croaker protein hydrolysate alongside myofibrillar fibrillar proteins in their investigation. Their results unveiled the hydrolysate's efficacy in retarding the oxidation and aggregation processes of myofibrillar fibrillar proteins. Furthermore, it exhibited the capacity to stabilize the secondary structure and tertiary conformation of these proteins while maintaining the color, water-holding capacity, and texture properties of the meat product. Lu^[105] incorporated pigskin gelatin hydrolysate (PGH) into pork as a raw material in their study. Significantly, the results highlighted the protective effects of injecting 4% PGH into pork, resulting in a 5.32% reduction in thawing loss, a 15.54% decrease in ice crystal area, and preservation of the meat's secondary structure integrity. Consequently, myofibrillar protein loss in pork was effectively minimized. This application demonstrated its efficacy in mitigating freezing-induced damage while ensuring the stability and integrity of myofibrillar proteins.

5.4 Low-temperature fruits and vegetables

Given the persistent emphasis on agricultural productivity, there has been a marked surge in domestic fruit and vegetable cultivation. However, ensuring the freshness of these produce items from harvesting to transportation, storage, and marketing is essential for consumers seeking to enjoy them in their freshest state^[106]. Despite advancements in various sectors, China's fruit and vegetable preservation technology has been slow to mature, and its optimization remains incomplete. Consequently, substantial losses occur, with approximately 30% of annual fruit and vegetable storage ending in damage, potentially resulting in losses worth hundreds of billions of dollars^[107]. Presently, the predominant method for transporting and storing fruits and vegetables involves low temperatures. However, prolonged exposure to these low temperatures can lead to the formation of ice crystals, which in turn can harm the original tissue structure of these perishable items.

Kashyap *et al.*^[108] investigated the antifreeze activity of hippophae rhamnoides chitinase class IV (Hr CHI4) by subjecting freeze-thawed green peas to its addition. The researchers scrutinized the tissue structure of green peas *via* scanning electron microscopy and gas chromatography-mass spectrometry. Their findings unveiled a reduction in drip loss and electrolyte leakage post-pretreatment with Hr CHI4, alongside the preservation of cell membrane integrity in green peas. Kashyap and Kumar^[109] showcased that pretreating peas with barley AFP led to substantially reduced water and vitamin loss compared to the

control group.

5.5 Others

Given its remarkable antifreeze characteristics, AFP has garnered attention as a potential safeguarding agent for cryopreserving biological samples, offering promising avenues across a spectrum of disciplines including agriculture and biomedicine^[110].

In a study conducted by Mehdipour *et al.*^[111] the incorporation of AFP III at a concentration of 1 µg/mL into chicken semen dilutions yielded significant enhancements in the viability, fertilization rate, and hatchability of chicken semen. Attention to dosage and conditions is paramount when utilizing AFP. Generally, the optimal temperature range for achieving the highest quality of pig semen and fertilization capacity falls between 0–0.8 °C^[112]. Chao *et al.*^[113] introduced 1.54 mg/mL of AFP into human erythrocytes during preservation. The outcomes revealed AFP's efficacy in impeding ice crystal recrystallization. However, this intervention led to an elevated hemolysis rate of erythrocytes, consequently diminishing their survival rate. Nguyen *et al.*^[114] supplemented porcine mulberry embryos with 1 µg/mL of AFP. This addition did not compromise the DNA integrity of the mulberry embryo cells. However, it did lead to a reduction in developmental capacity compared to the control group. However, the bulk of experiments suggest that AFP can indeed offer advantages in the cryopreservation of animal embryos. For instance, Liang *et al.*^[115] discovered that the addition of 1 mmol/L AFGP notably boosted the survival rate of bovine embryos.

6 Summary and outlook

The incorporation of AFP has proven effective in mitigating losses incurred by frozen products due to repetitive freeze-thaw cycles. Contrasted with conventional food antifreeze methods like high-sugar and high-salt solutions, AFP present several advantages, notably in terms of safety, minimal impact on human health, and other desirable attributes. With an increasing focus on the nutritional quality of food items, the continued advancement of AFP within the realms of the food industry and research and development emerges as an inevitable trajectory.

While AFP hold immense promise for development, the predominant method for their production—bio-enzymatic digestion—remains comparatively costly compared to other commercial food antifreeze options. Additionally, their procurement and preparation pose significant challenges, given the limited of resources available. These hurdles impede large-scale preparation and widespread promotion of AFP in the current landscape. A paramount challenge confronting researchers is devising a streamlined approach for preparing AFP that not only attains high concentrations but also ensures robust cryoprotective effectiveness. The cell-free translation system (CFPS) is used to prepare AFPs. CFPS uses exogenous DNA or mRNA as a template, supplemented with substrates and energy substances in a cell-free protein expression system. The system uses organelles provided by cell extracts in combination with T7/SP6 RNA polymerase to express the target protein *in vitro*^[94]. In CFPS, the absence of cell membranes helps protein folding, which in turn leads to better synthesis of AFPs for high-throughput production. CFPS also produces fewer impurities, which is beneficial for purifying proteins. This avoids a decrease in the target protein during purification and allows for higher protein yields^[116]. Therefore, the use of CFPS has good prospects and

potential for the large-scale preparation and promotion of AFP. In addition, integrating AFP with innovative food antifreeze technologies holds promise for augmenting cryoprotection capabilities. This paper provides a comprehensive overview of the origins of antifreeze proteins and peptides, assesses their efficacy in cryoprotection, and elucidates their mechanisms of action. Its objective is to furnish valuable insights for the advancement and broad implementation of AFP within the realm of food science.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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