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# COLD TOLERANCE OF PLANT ANTIFREEZE PROTEINS: A REVIEW

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## ABSTRACT

The life style and survival of organisms is highly affected by environmental conditions or changes in temperature, humidity and availability of resources to live in the stress environmental conditions. There are various abiotic and biotic environmental stresses, including cold, heat, drought, salinity, alkalinity and diseases caused by micro-pathogens. Every organism has his own body adjustments to resist or tolerate these environmental stress conditions. Among these, cold stress which included low temperature or freezing at sub zero temperatures caused adverse effects on the growth and development of organisms. Cold stress caused inhibition of most of the physiological functions in body which may lead towards the death of organisms. Various antifreeze proteins have been discovered in most of the micro to macro organisms to combat with freezing temperature conditions. The prescribed review article provides information about the discovery and role of antifreeze proteins in various organisms.

**Keywords: cold tolerance, antifreeze proteins, glycoproteins, ice crystallization, chilling temperature**

## INTRODUCTION

Cold stress, which includes chilling at low temperature and or freezing at sub zero temperatures, adversely affects the growth and development of plants and significantly constraints the spatial distribution of plants and agricultural productivity. Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic, chilling-induced inhibition of water uptake, freezing-induced cellular dehydration, oxidative and other stresses. Cold acclimation is a process by which plants acquire freezing tolerance upon prior exposure to low non-freezing temperatures. Most temperate plants can cold-acclimate and acquire tolerance to extracellular ice formation in their vegetative tissues. Living organisms have developed diverse strategies to enable them to survive freezing conditions. One strategy that has evolved repeatedly is the expression of antifreeze proteins. Antifreeze proteins belonged to a class of protein which is produced by different group of organism like Bacteria, Fungi, higher plants and vertebrates. Antifreeze proteins help the organism to survive in the subzero environment. Antifreeze proteins bind to the small ice crystal and inhibit in the growth of crystal and re-crystallization process which is lethal to living organism [1]. Antifreeze proteins also protect the cell membrane from cold damage [2]. Antifreeze proteins have non-colligative properties unlike the widely used automotive antifreeze, ethylene glycol. Antifreeze protein does not lower the freezing point, and it works like a non-colligative manner. It binds at the surface of ice crystal [3]. Living organism species containing Antifreeze proteins are classified as may be Freeze avoidant or freeze tolerant in both cases it is thought that Antifreeze proteins may be inhabit the process of re-crystallization and prevent the cell membrane from cold damage [4].

## ANTIFREEZE PROTEINS (AFP) AND ANTIFREEZE GLYCOPROTEINS (AFGP)

Antifreeze proteins played an important role for cold tolerance through inhibiting ice growth within and among the cells of living organisms. These proteins belong with a diverse group of proteins that have ability to inhibit the ice crystal growth though binding with ice [5-7]. So antifreeze proteins are important group of protein that played significant function in tolerance against environmental low temperature stress. Antifreeze proteins operated through intact with the ice surface which caused to decrease freezing point without equilibrium between melting and freezing points. It has been found that the thermal hysteresis (solidification of water to Ice) in degree centigrade (°C). Antifreeze proteins usually integrated within the ice crystals due to the freezing of and their attraction for surface of ice and caused to reduce the growth of large ice crystals more as compared with small ice crystals [8]. In other words antifreeze proteins are either sop the development or growth of ice crystallization process or reduce the process for crystallization of ice. The crystallization of ice is achieved as the melting of ice is started [9]. Under the temperate type of environmental conditions, antifreeze proteins played an important role for survival of cell. Antifreeze glycoproteins and antifreeze proteins have usually been used due to their anti-freezing activities in various organisms like insects, marine fish, bacteria and plants [10].

## DISCOVERY AND HISTORY OF ANTIFREEZE PROTEINS

Discovery of antifreeze proteins is quite old nearly half century agoduring 1950s, a Canadian scientist; Scholander found that the survival mechanism of Arctic fish under highly cold water of the ocean was due to antifreeze proteins. The Arctic fish is the class of animal which can survive easily below zero degree temperature. Actually, it is the fish that could survive in colder water more than freezing point of fish blood. The antifreeze protein expressed in organism and remains in the blood stream and cytoplasm. Scholander found that there should be something like "antifreeze" in fish blood [1] so it means that expressed antifreeze protein remains soluble in the blood and remain in circulation in the blood. In late 1960s, a breakthrough was came on the isolation and purification of first most antifreeze protein from antarctic fish that were belonged to the class of antifreeze proteins which were in glycoproteins in nature. He named these proteins as "glycoproteins as biological antifreeze agents" [11], in other words Biological active proteins of antifreeze are glycoproteins in nature. They were later on recognized as antifreeze glycoproteins. De Vreies also demonstrated the physical and chemical characteristics of antifreeze proteins [12].

Later on, Griffith and his colleague discovered antifreeze proteins in the leaves of winter rye [13]. Winter rye was the first plant which was used as experimental organism in plants. Later on plant biologist started extensive research on the plant Antifreeze proteins. The thermal hysteresis of antifreeze proteins was also studied in angiosperm plants [14]. Later on, antifreeze proteins were discovered from more than 23 angiosperm species. Antifreeze proteins have also been reported form bacteria and fungi. In this way antifreeze proteins were cauterized from mammals to higher plants then even in the Prokaryotes like Bacteria in recent years [15].

## CLASSIFICATION AND DIVERSITY OF AFP

Classification of Antifreeze Proteins mostly based on its different discovered antifreeze proteins from different organisms. A large number of non-homologous antifreeze proteins have been reported e.g. Type-V. Some antifreeze proteins have simple polypeptide that contains the properties or activity of antifreeze simply some are in glycoproteins in nature which have been found in various organisms.

## ANTI-FREEZE PROTEINS OF FISH

The Antifreeze glycoproteins of fish first studied in northern cod and antarctic notothenioids (a class of fish which can happily survive in sub-zero temperature or frozen environment). The protein size is ranged from 2.6-3.3 kDa [16].

## ANTIFREEZE PROTEINS TYPE-1

Type I Antifreeze proteins were discovered in well known shorthorn sculpin, winter flounder and Longhorn sculpin. These antifreeze proteins have been highly characterized antifreeze proteins with 3D protein structure. Type-I AFPs are the three faces of 3D protein structure; hydrophobic, hydrophilic and Thr-Asx face [17]. The Type I antifreeze proteins have a ling, single, and amphipathic alpha helix structures. Its size is ranged from 3.3-4.5 kDa. The Type I-hyp, is actually Type-I AFP which is present in a large number of Right-eye flounders, with molecular size of 32 kDa having two 17 kDa di-meric molecules, so they are not mono-meric in nature (Figure 1). Type-1 AFP was purified and isolated from winter flounder fish blood plasma. Type I has been reported to be better under decreased freezing point in various fish Antifreeze proteins [18].

## TYPE-II AFPS

Type II antifreeze proteins discovered in smelt, herring and sea raven (Figure 2). These are the globular proteins with five disulfide bond and cysteine-rich in nature [19].

## TYPE-III AFPS

The type-III antifreeze proteins have been found in antarctic eelpout (Figure 3). These proteins are somehow similar as that of Type-I antifreeze proteins which contained overall similar hydrophobic region at surface of ice binding. Their molecular size is relative small with only ~6kD [5,16].

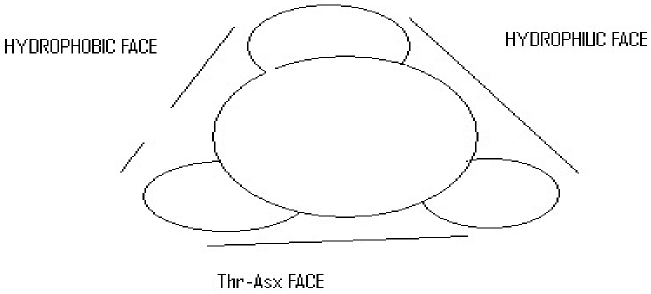


**Fig 1: Three faces of the Type-I AFPs**

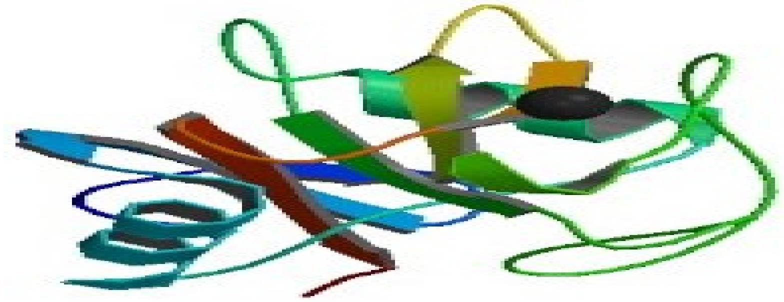


**Fig 2: Cartoonic Structure of Type II Antifreeze Protein**

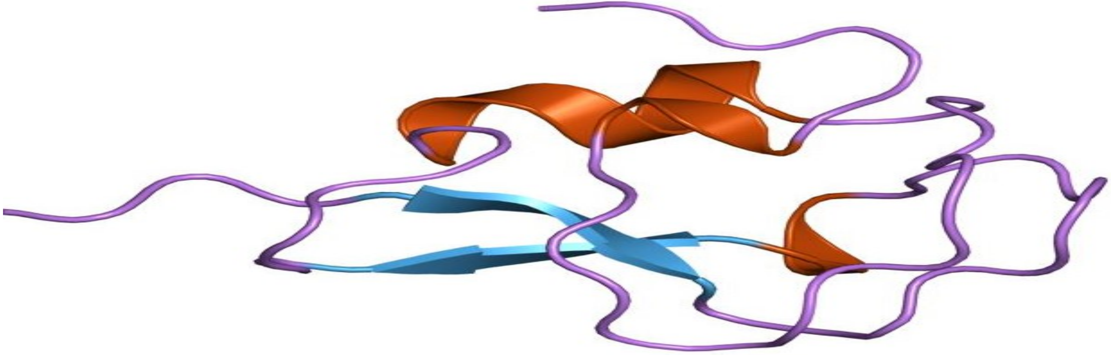


**Fig 3: Cartoon representation of the molecular structure of Type III Antifreeze Proteins**

## TYPE-IV AFPS

Type-IV antifreeze proteins have been reported form longhorn sculpin. These are alpha the helical proteins which are rich in glutamine and glutamate. The protein has nearly ~12kDa molecular size containing four helix bundles of molecules. It's thought that it is the modification of post-translation of pyroglutamate amino acid residue with a cyclic glutamine amino acid residue attached with N-terminal region [20]. Scientists from University of Guelph, Canada have recently reported role of pyroglutame amino acid residue in type-IV antifreeze protein for its activities from the longhorn sculpin species.

## ANTI-FREEZE PROTEINS OF PLANTS

Before the discovery of plant Antifreeze Protein gene the classification system was mostly based on Fish antifreeze proteins. Antifreeze proteins classification has become the more complicated one due to Griffith discovery for plant antifreeze proteins in higher plants [13]. The plant antifreeze proteins have been found different as compared with fish antifreeze proteins [21,22].

The characteristics of plant antifreeze proteins were described by them under these main points.

1.Plant Antifreeze proteins showed lower thermal hysteresis as compared with fish and insect Antifreeze proteins.

2.The physiological function of antifreeze proteins is to inhibit the ice re-crystallization rather than prevention of the formation process.

3.They have explained on the basis of DNA and Protein Homology that most of plant antifreeze proteins are evolved pathogenesis-related proteins, most of them lose the anti-pathogenic activities but some of them are still antifreeze and anti-pathogen and antifungal properties.

## AFP STRUCTURE AND FUNCTION

Peter's information on 3D structure is mostly done on the basis of mutagenesis studies of antifreeze proteins. 3D structures have been studied now for several non-homologous forms and insect antifreeze proteins. The structures of each type of antifreeze protein were analyzed, specially the binding site for ice in antifreeze proteins have been determined by technique of site-directed mutagenesis which indicated that the surface of ice is bound by the antifreeze protein. Mutagenesis studies show that the following main points are critical for biological activity of Antifreeze proteins:

1. The antifreeze proteins showed flat and substantial proportion for ice bindings
2. The antifreeze proteins showed hydrophobic edge which exposed to the solvent
3. The surface - surface complementary effects are appeared as a key for tight binding with contribution of van der Waals week hydrogen bonding [23].

## EVOLUTION

Great evolutionary distribution and diversity has been found throughout the world for antiifreeze proteins. It has been thought that various types of antifreeze proteins evolved in recent times in reaction of sea level glaciations. The glaciation process carried out about 1-2 million years ago in Northern Hemisphere of earth while 10-30 million years ago at Antarctican part of earth [2]. Regardless of evolutionary diversity, wide distribution and a large number of antifreeze proteins showed similar function. These are the sum of the reasons which indicated that why a lot of antifreeze proteins showed similar function for cold tolerance.

1. Ice is chemically made up of hydrogen and oxygen with large number of binding structures and surfaces for interaction of antifreeze proteins.
2. The 3D or tertiary structure of antifreeze proteins are usually varies due to difference in amino acid sequence, which showed interaction with ice binding sites [2].

## ANTIFREEZE PROTEIN MODE OF ACTION

The antifreeze proteins have been found that they lower process of ice formation/crystallization through adsorption–inhibition mechanism [24]. The Antifreeze proteins molecules are adsorbed to inhibit thermodynamically favored ice formation on non-basal ice planes [24]. According to physical activities of antifreeze proteins in presence of a rigid and flat surface in antifreeze proteins seemed to facilitate their interactions with ice crystals though *Van der Waals* physical applied force [25].

## BINDING TO ICE

Jorov et al studied the geometrical structure of they explained that normally the crystals of ice only exhibited the prism faces (1010) and basal (0001) which are looking round shape and flat discs shape [3]. However, it is supposed that the geometrical surface 2021 normally preferred binding ice surface for antifreeze proteins type I [8]. It is thought that due to hydrophobic interaction plays a significant role in the binding mechanism [26]. Binding mechanism antifreeze proteins with ice crystals has been studied through the use of molecular modelling programs [1,3].

The *Lolium perenne* is a perennial ryegrass, it is freeze tolerant plant .It maintain the cellular structure during the subzero temperature successfully. It maintains its because of its high activity of antifreeze proteins expression. In contrast, freeze-intolerant plants suffer lethal [27]. In *Lolium perenne* (ryegrass) a 118-Amino acids residue Antifreeze proteins discovered with superior ice recrystallization inhibition activity and has reported that consensus sequence X X N X V X G of these 7-residue repeating motif play an important role in antifreeze proteins biological activity [28]. Michael and his coworkers constructed the 3-D (Three Dimentional ) model of Ryegrass antifreeze protein. This model is indicating that it has similarities with insect antifreeze protein structure. He predicted that ryegrass antifreeze protein has two ice binding sites. Due to this it has superior ice binding recrystallization inhibition activity of ryegrass antifreeze protein (LpAFP) [29].

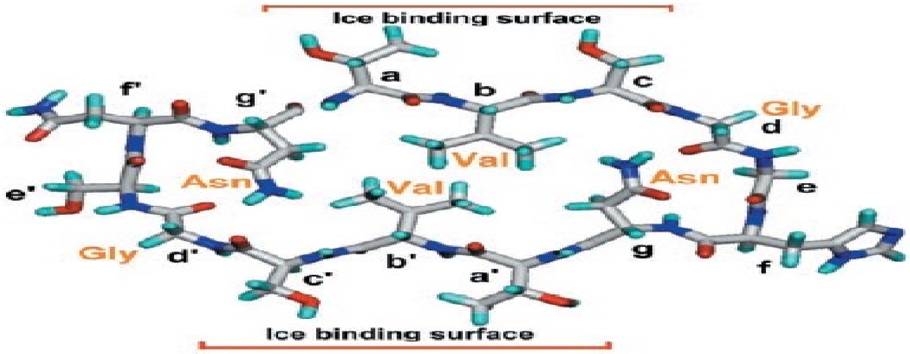


**Fig. 4: Cross-section of LpAFP *(Lolium perenne* L. (perennial ryegrass) model showing positions of conserved residues and the putative ice binding surfaces. Two repeat motifs, (X X N X V X G), are needed for each loop, each corresponding to repeat labeled *a-g* and *a_-g_*.**

## DISCOVERY OF PLANT AFP

In 1992 Griffith discovered the AFP (Antifreeze Proteins) in plant kingdom. *Secale cereale* L. (winter rye) was plant, the apoplastic extract of winter rye grass was contain the protein that was produced after the process of cold acclimation. Further studies indicate that the winter rye AFP was as non-colligative behavior that modified the ice crystal. Majors properties of *Secale cereale* was: it's a weaker thermal hysteresis activity, if it was compared with animal AFPs like fish and insect AFP, its physiological behavior was quite interesting that it inhibit the process of ice crystallization, the homological studies of winter rye AFP indicated that it was evolved from PR-proteins (Pathogenesis related Proteins) and winter rye AFP is still antifungal and anti-pathogen also [13].

## CARROT ANTI-FREEZE PROTEINS

The carrots (*Daucus carota* L.) is a biennial plants used as vegetable. The survival of carrot root at low temperatures is significant for completing life cycle of carrot plant for flowering and production of seeds for second year crop. The carrots enhanced their tolerance against freezing point with response in exposure period of low temperature, without the non-freezing temperature limits, is a process which is known as cold acclimatization [30]. The process of cold acclimatization is a complex process which involved histological adjustments [31], anatomical [32] and several plant physiological alterations regulated by gene(s) [33-35]. The plant physiological changes are involved the increase in concentrations of sugars, soluble proteins, amino- acids, organic acids and the alteration in the lipid composition of cell membranes [36,37]. The gene that encoded an antifreeze protein has been isolated from the carrot plant through the use of sequencing information obtained from protein purification. Antifreeze proteins of carrot showed higher similarity with one of the apoplastic plant leucine-rich repeated proteins, named as polygalacturonase inhibitor protein. The carrot antifreeze proteins gene is highly regulated under low temperature conditions. The expression study of antifreeze proteins gene has been carried out in transgenic *Arabidopsis thaliana* plants which leads to an increased anti-freeze activity of plant. Antifreeze protein of carrot antifreeze protein (DcAFP) showed a strong anti-freezing activity which belonged to the polygalacturonase inhibitor protein family which showed similarities in their sequence with antifreeze protein. Antifreeze protein of carrot is also included a leucine-rich repeated (LRR) motif. The electrostatic potential of carrot antifreeze protein and polygalacturonase inhibitor proteins revealed that there are many non-conservative residues within β-helix of the carrot antifreeze protein leucine-rich repeat motif which had been substituted with basic amino acids and changing the surface as positive from negative. The electrostatically prevention of binding sites of carrot antifreeze protein with the positively charged activity surface shows similar behavior of polygalacturonase.

## Theoretical structure and ice-binding sites of DcAFP

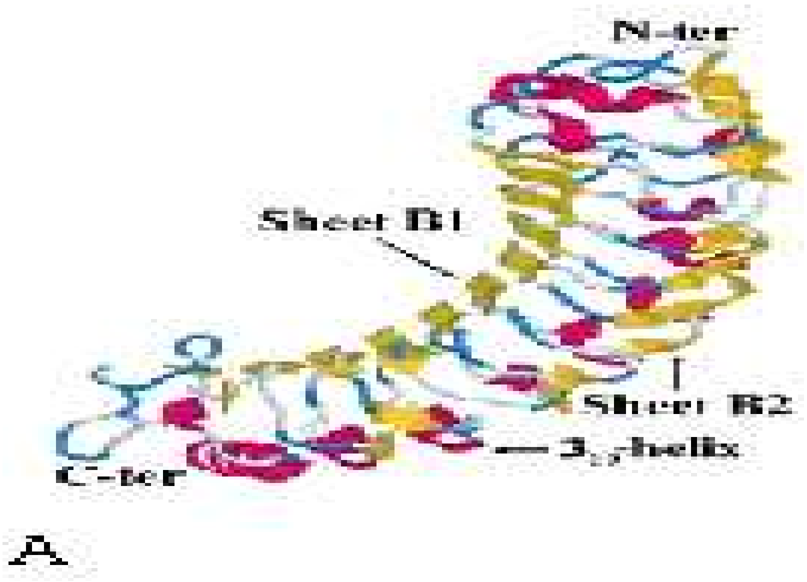
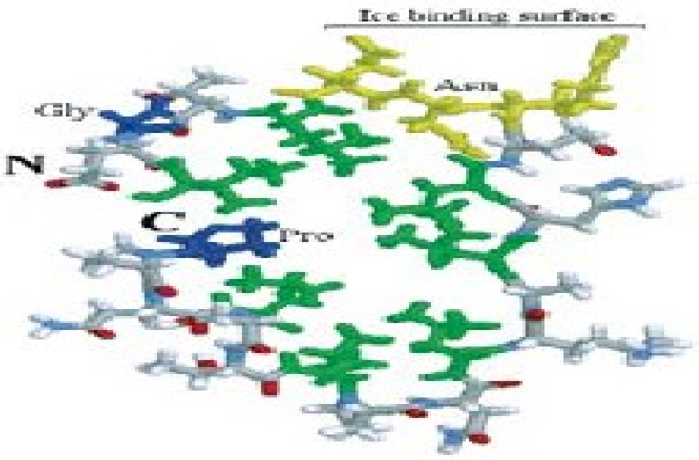
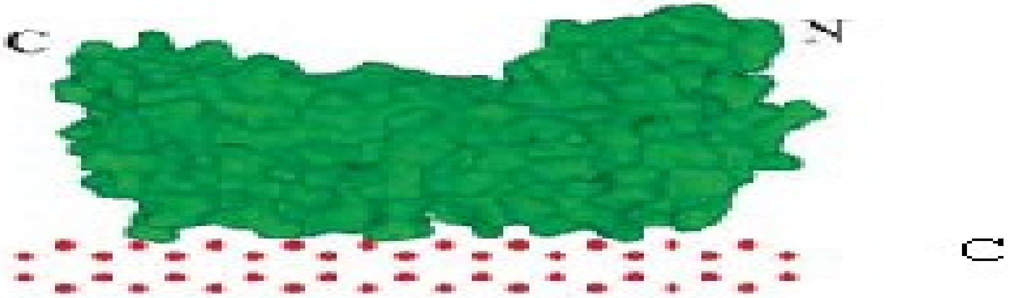


**Fig. 6 A: DcAFP 3D structure, indicating the beta sheets B1 and B2 sheets. In-between two β-sheets, the ice binding sites are there. While the ice interacting sites are not seen in the case of ryegrass and insects AFPs**



B

**Fig. 6. B: DcAFP cross-section indicating that, LRR motif and of course, ice-interacting surface. Lucine and Isoleucine amino acid are green in color, LRR is strongly hydrophobic in nature, and these Lucine repeats also play a role in DcAFP stabilization.**



**Fig/\. 6. C: Ice and DcAFP complementary surface to surface, the green color of DcAFP surface are docked to 1010 planes of prism of ice. Oxygen atoms in ice by red color dots represented. Successive loops (each) matches to the repeat of spacing of 4.5A$^{o}$ of ice and prism face**

## EXPRESSION OF AFP GENE IN *E.COLI*

Fan et al amplified the antifreeze protein gene from carrot seedling genomic DNA .They amplified ~1100bp gene of carrot antifreeze protein gene and cloned in E.coli expression vector fused with GST tag (Glutathione S Transferase). The 1100bp gene from carrot antifreeze protein (AFP) was expressed in *Escherichia coli*, after induction with IPTG (isopropyl thiogalactoside) the 60kDa fusion protein complex was seen on SDS-PAGE and Western blotting [38]. Zhang and his coworkers cloned the mature protein coading antifreeze protein gene in E.coli expression vector in pET-11. Antifreeze protein gene with Hitadine as fusion complex 36 kDa (*His-afp*).They studied the mutated Carrot antifreeze gene protein and established the new reviable assay on *E. coli*. Cold resistance *E.coli* was used in their further studies like by measurement of thermal hysteresis (TH) activity [39].

## COMMERCIAL APPLICATION OF ANTIFREEZE PROTEIN

Antifreeze proteins have vast commercial applications which help in a lot of life science fields that may be benefited due to the protection from freezing damage of tissue and cells. Some of the applications are enlisted below:

1. In temperate climates the harvesting season of crop plants has become feasible due to anti-freezing activity of crop plants
2. Under extremely cold/low temperature conditions the production of fish has been improved
3. The shelf life of frozen/preserved at freezing point has also been improved
4. Cryo-surgery has also been improved due to antifreeze proteins
5. The preservation period for tissues to be transplanted and the transfusion of medicines have also been improved
6. Antifreeze proteins have also use in therapy of hypothermia

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