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Author(s)	MAHATABUDDIN, SK.
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Abstract of Doctoral Dissertation

博士の専攻分野の名称　博士（**生命科学**）　氏 名　シエイク マハタウッディン

Degree requested　Doctor of Life Science　Applicant name　SK. MAHATABUDDIN

学 位 論 文 題 名

Title of Doctoral Dissertation

Functional Analysis of a Supersoluble Fish Antifreeze Protein

(高溶解性魚類不凍タンパク質の機能解析)

Modification of ice crystal morphology and controlling their growth-kinetics have been accepted as major scientific challenges. The biological ice crystal modifiers, antifreeze proteins (AFPs), can bind onto the surface of ice crystals to alter their morphology that facilitates organisms to survive in ice-laden environments. This binding of AFPs impeded the growth-kinetics of the ice crystals and finally terminated their growth through non-colligative freezing point depression according to Gibbs-Thomson effect. The mechanism of such binding of these peptides and their evolution have been studied by scientists from multiple disciplines for past few decades. Their tremendous effort led us to identify AFPs in various organisms ranging from bacteria to fish, since the discovery of AFP in 1969. Discovery of new species and their characterization advanced our knowledge regarding their mechanism of action, structural diversity and evolutionary precursors. A new species of biological ice crystal modifier has been identified and characterized in this dissertation.

The activities of ordinary AFPs have been evaluated using two concentration-dependent parameters, thermal hysteresis (TH) and ice recrystallization inhibition (IRI). The former parameter (TH) represents the hysteresis gap between the melting (T_m) and freezing temperature (T_f) (in the absence of AFPs $T_m \approx T_f$) of an AFP bound ice crystal (TH = T_m - T_f) generated by the adsorbed AFPs. The AFPs can exhibit 0.5-1.0^{o}C of TH at millimolar concentrations (moderately active AFPs) to ~6.0^{o}C of TH at sub-millimolar concentrations (hyperactive AFPs). The adsorption followed by inhibition is widely accepted mechanism for the TH caused by AFPs. However, the mechanism of this adsorption of AFPs onto the ice surfaces are still under investigation. To date, at least three different hypothesis have been proposed to explain the adsorption mechanism of AFPs, namely hydrogen bonding hypothesis, hydrophobic effect and anchored clathrate hypothesis. The latter one is one of the major hypothesis employed to explain the mechanism of AFP adsorption in recent years. However, adsorption of AFPs not only suppress the ice crystal growth but also impeded the grain boundary migration to stop Ostwald ripening, which is denoted as ice recrystallization. This function is known as IRI activity of AFPs. It protects the organisms and water containing materials (foods, paints) from the detrimental effect of formation of large ice crystals.

Fishes living in the Polar Regions and its proximal temperature area are the potential source of the AFPs. To date, five class of fish AFPs have been reported: type I, α-helical alanine rich (Mw: 3.3-4.5-kDa) proteins; type II, ~14-kDa proteins; type III, ~7-kDa globular proteins; type IV, ~12-kDa helix bundle protein; type Ih, (~17-kDa) four helix bundle protein (such as Maxi). A new fish AFP (bpAFP) has been identified from a right eye flounder, barfin plaice, *Liposetta pinnifasciata*. The large-scale purification methodology of this AFP from the fish muscle homogenate and preparation of its recombinant protein (rbpAFP) are described in ***Chapter two***. The sequence of bpAFP showed that, it composed of 40 amino acid residues that contains three tandem repeats of the 11 consensus sequence TX_{10} (where X is mostly alanine) which presumably construct the monomeric α-helical structure (details description is in the dissertation). It consists just five polar residues (D, S, D, K and E) excepting the four regularly spaced threonines and the C-terminal arginine. Three out of these five (D, S and D) are located in the N-terminus and the rest two K (i) and E (i+4) will presumably form a salt bridge. That is, the residual alanines along with threonines are principal constituent of bpAFP, implying that assembly of only methyl groups with four equally spaced hydroxyl groups construct an extremely regular surface of this molecule. These sequential features categorized this peptide as type I AFP with lowest polar residues as other type I AFPs have more polar residues to acquire amphipathic nature of their α-helices. By contrast, this AFP showed ultra-high solubility in pure water, to date it is the highest dissolution limit of any AFP. The solubilized native or recombinant bpAFP altered the ice crystal morphology to hexagonal trapezohedron.

The embryonic ice crystals, the target ligand of AFPs, formed at atmospheric pressure are mainly hexagonal. These hexagonal ice crystals contains numerous planes defined by Miller-Bravais indices in which different patterns of oxygen atoms are presented on the surface of distinct planes. The ice-binding specificities of AFPs on those planes

are the major factor that governs their activities. Therefore, the fluorescence-based ice plane affinity (FIPA) analysis of bpAFP, rbpAFP and a recombinant type III (nfeAFP6) AFP have been performed for understanding the crystallography of adsorption of these AFPs in ***Chapter three***. The FIPA images visualized that nfeAFP6 was unable to show any concentration-dependent ice plane specificity within the concentration range 0.02-0.2 mg·mL^{-1}. While, bpAFP and rbpAFP displayed concentration-dependent interminable ice-binding specificity within concentration range 0.01-0.1 mg·mL^{-1}. At lower concentrations ([bpAFP] ≤ 0.02), it showed similar but not identical FIPA pattern obtained for moderately active type I AFPs whereas entirely fluorescent ice hemispheres were obtained at the latter concentration (0.10 mg·mL^{-1}) of bpAFP. Such entirely fluorescent ice hemispheres implies the multiple ice-plane (including the basal plane) binding of bpAFP similarly to hyperactive species. This interminable ice-binding further affects the ice crystal size and shapes obtained in the presence of bpAFP. The basal plane binding of this rod like α-helical protein presumably be originated from the self-assembly or oligomerization of this peptide similarly to the Maxi, a 33-kDa four helix bundle protein.

In ***Chapter four***, the TH activities and dependence of the α-helical structure on pH and temperature of bpAFP and rbpAFP has been investigated. The concentration dependence of the TH activities of bpAFP and rbpAFP displayed ordinary hyperbolic profile similarly to other fish AFPs. While, the bpAFP and rbpAFP showed a very high TH activity (2.5-3°C) compared to the known moderately active fish AFPs at higher protein concentrations. It also visualize both along (observed for moderately active AFPs) and normal (observed for hyperactive AFPs) to the *c*-axis ice crystal busting, rapid ice growth observed at the temperatures ≤T_f, pattern at the lower and higher bpAFP concentrations, respectively (details description is in the dissertation). The bpAFP showed significant TH activity even after heating at 100°C. In addition, their α-helices displayed reversible thermal denaturation within temperature range 0-90°C. The α-helicity and TH activities were remain similar within a long range of pH.

The role of different amino acid residues of AFP sequence in ice-binding have been investigated through mutational analysis and advanced our knowledge about mechanism of action of AFPs. On this perspective, mutated bpAFP has been prepared and characterized which described in ***Chapter five***. The point mutation did not significantly affect the α-helical secondary structure and its reversible thermal denaturation and pH stability of bpAFP. By contrast, this mutation reduced 30% of the TH activity and eradicate the basal plane binding ability of bpAFP. These results point out the important role of threonine^2 for the functional activities of bpAFP.

Then again, it has not been subjected a parameter to correlate TH and IRI, which helps understanding of the ice-binding ability of AFPs including defective species that cannot perfectly arrest the ice crystal growth. In the ***Chapter six***, the author introduced a new parameter named "critical ice shaping concentration (CISC)", a minimum concentration of an AFP species necessary to alter the shape of the ice crystals. It could be useful for the comparative studies among the active and defective AFP species. The CISC and effective IRI concentrations (C_{eff}) of natural and recombinant type I, II and III fish AFPs were also examined in this chapter. The new parameter CISC is interrelated with the IRI and TH activity. Type I, II, III fish AFPs and their recombinant proteins can exhibit effective IRI at two fold concentrations of their CISC.

In brief, in this dissertation a new supersoluble type I AFP has been identified from barfin plaice (*Liposetta pinnifasciata*) and characterized. Unlike all other antifreeze proteins characterized to date, the bpAFP transitions from moderately-active to hyperactive with increasing the concentration. Its hyperactivity is documented here by high freezing point depression, changes in ice morphology, and the ability to bind the basal plane of ice. It can be reliably predicted the α-helical structure of this supersoluble peptide in which the peptide bonds along the helix backbone are slightly bifurcated and able to hydrogen bond to solvent water. Hydration of this helix is helped by its extreme alanine-rich property (75%), since the small side chain of alanine (methyl group) is insufficient to block access to solvent. It is proposed that the gain in hyperactivity, which includes binding to additional planes of ice, is the result of bpAFP oligomerization. This research work also showed that threonine^2 plays an important role for interminable ice-binding of bpAFP. Its C_{eff} is double of CISC concentration, this relationship was true for both the type II and III AFPs, for which the author made the assumptions that 1) CISC of AFP is a measure of primary adsorption ability onto the ice crystal surfaces to cause ice-shaping, while 2) IRI and TH are related with more AFP concentrations needed for both primary and secondary adsorptions to halt the ice crystal growth.