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Abstract of Doctoral Dissertation

Degree requested Doctor of (Life Science) / Pharmaceutical Science / Clinical Pharmacy **Applicant's name: Nour Md Mofiz Uddin Khan**

Title of Doctoral Dissertation

Molecular Basis of Ice-binding Mechanism of Microbial Antifreeze Proteins
(微生物由来不凍タンパク質の氷結晶結合メカニズムの分子基盤)

Antifreeze proteins (AFPs) are unique biomolecules with diverse molecular structure evolved and adopted by many organisms including fishes, insects, plants, microbes, as a guard against cold damage. AFPs bind on ice surface to stop its growth by depressing freezing point non-colligatively to create a gap between equilibrium melting (T_m) and freezing (T_f) point. The temperature difference (T_m ~ T_f) in the gap is termed as thermal hysteresis (TH) and define the AFPs as hyperactive or moderately active. Most of the hyperactive AFPs adopted β-spiral structure forming highly regular surface with definitive array of Threonine (T), Asparagine (N) as TxT or xGTGND motifs ('x' is arbitrary amino acid, G-Glycine, D-Aspartic acid). Those conserved residues can order water with hydrophobic effect exerted by methyl group of sidechains and hold on its surface via hydrogen bonds. These surface bound waters have spatial position that can allow the AFP to bind to multiple planes of ice, one of which is the basal plane that is a common target for hyperactive AFPs and refer to its high activity. The surface consists of amino acids that interact with ice is called ice-binding site (IBS). In the recent years, it is come to an inference that microbes that include bacteria, algae, diatoms, and fungi, take up AFPs with high amino acid sequence conservation. Unlike with known hyperactive AFPs, this group of AFPs have β-helical structure constituting IBS with less regularity without any repeating motif. However, they have a wide range of activity from moderate to hyperactive. In my PhD, I study these activity variances in three isoforms of AFP produced by a species instead of AFPs from different species of microorganisms.
Typhula ishikariensis, a snow mold fungus produces seven isoforms of antifreeze protein denoted by *Tis*AFP2-8. The isoform 6 (*Tis*AFP6) identified as moderately active which possesses ~ 99% sequence identity with isoforms 2-5. Another isoform *Tis*AFP8 has 83% sequence identity with *Tis*AFP6 shows hyperactivity. It is not yet clearly understood what factors make a sharp change in activity of fungal AFPs. To reveal their ice-binding mechanism *Tis*AFP7 isoform was studied, and characterized in terms of biochemical, structural, molecular dynamic simulation study and compare with other two isoforms *Tis*AFP8 and *Tis*AFP6.

To evaluate the antifreeze activity, *Tis*AFP7 gene was transformed into bacterial cell (*E. coli*) which expressed as a soluble protein. TH was measured using purified *Tis*AFP7 solution for a series of concentration (0.022 to 0.23 mM) which gives a maximum value of 0.95 °C at 0.23 mM. When TH of *Tis*AFP7 was plotted against concentration with *Tis*AFP8 and *Tis*AFP6 isoforms, the TH curve of *Tis*AFP7 lies at the intermediate position between *Tis*AFP8 and *Tis*AFP6. During TH measurement of *Tis*AFP7 at 0.23 mM concentration, the ice crystal bursting appeared as a six-direction manner. It is known that when AFP binds on and protect basal plane of ice, a hexagonal symmetry is formed after bursting of the crystal which is commonly observed for hyperactive AFPs. By contrast *Tis*AFP7 showed two-direction growing pattern at 0.01 mM which means basal plane is not protected at lower concentration. This discrepancy of ice bursting as a concentration dependent manner was not exhibited by *Tis*AFP8 and *Tis*AFP6, which were always showed six-direction and two-direction bursting pattern relating to their activity. Furthermore, *Tis*AFP7 was tagged with fluorescence dye

and used to adsorb on single ice-crystal hemisphere to determine the ice planes *Tis*AFP7 binds with. The fluorescence dye visualized on entire ice hemisphere which means *Tis*AFP7 can bind on basal plane as well as prism and pyramidal planes, in other words, entire ice planes. It is a unique binding pattern for AFP with TH below 1 °C which is assumed to appear with weak basal plane affinity for what two-direction bursting might be occurred at lower concentration of the protein solution. In addition, thermal stability was examined to find any relation with antifreeze activity of the AFP molecules. *Tis*AFP7 showed least stability (47.5 °C) whereas *Tis*AFP6 exhibited highest (53.5 °C) then *Tis*AFP8 (50.0 °C) indicating no direct correlation with their activity.

To understand the mechanism of *Tis*AFP7 at molecular level, 3D structure of *Tis*AFP7 was elucidated. Compare the structure of *Tis*AFP7 with other two isoforms flat, extensive face of the triangular β-helical molecule was assumed as IBS which consist of loop and sheet structure. Hydration waters that bind on protein surface via hydrogens bonds are observed higher number on IBS with specific orientation. On the loop IBS, a zigzag water network which shown close geometrical similarity with waters on ice basal-plane is termed as ice-like water. This network also found in *Tis*AFP8 and *Tis*AFP6, however, only *Tis*AFP8 can extend this network at the outer region of IBS. A ring-like water network of 10 water molecules also obtained surrounding the hydrophobic Phenylalanine43 residue on the top of the loop IBS of *Tis*AFP7 which was not present in *Tis*AFP8 due to crystal packing and in *Tis*AFP6 due to replacement of Phenylalanine43 by Serine. It is assumed that *Tis*AFPs approach to and bind on basal plane through this ice-like water network which may enhanced by hydrophobic hydration (ring-like network) for *Tis*AFP7 and *Tis*AFP8. To approach closer to the original mechanism an amino acid residue Threonine20 on loop IBS replaced by bulky sidechain amino acid Tyrosine which reduced TH (60%) and two-direction bursting occurred even at high concentration indicating loss of basal plane binding. Fluorescence-tagged T20Y mutant of *Tis*AFP7 showed no basal plane binding as well. It was also noticed from crystal structure of T20Y mutant that the zigzag network of hydration water is disrupted by the defective mutation which might lack the mutant to attach the basal plane.

In addition with above results, molecular dynamic simulation for residence time of hydration waters on IBS show higher staying time than rest of the waters on protein surface. When compare the residence time for IBS of three isoforms it comes as *Tis*AFP7 (79.43 ps), *Tis*AFP6 (73.16 ps), and *Tis*AFP8 (85.98 ps).

Taken together here concluded the different activities of *Tis*AFP isoforms that associated with the hydration water structure surrounding IBS and they offer the host to be a habitat in the icy environment.