Characterization and preliminary X-ray crystallographic analysis of an Ice-binding protein (FfIBP) from phychrophilic bacteria, *Flavobacterium frigoris*

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Abstract

The Ice binding protein (IBP) is a prerequisite material for organism to be allowed to live in a subzero environment. Ice growth in a cold environment is fatal for organism which is not just physically destruction of inner cell organelle but also chemical damage such an osmotic shock. IBP has been characterized property of which ability inhibit the ice growth by binding to specific ice plane.

Flavobacterium frigoris isolated from Antarctic area produce an ice-binding protein (FfIBP) to survive and reduce damage from ice growth. The FfIBP has been cloned and over-expressed in *Escherichia coli*. To diagnose ice-binding mechanism, we measured thermal hysteresis (TH) activity which is numerical value of a gap between freezing and melting point as well as ice re-crystallization inhibition activity. Thermal hysteresis activity of the FfIBP was approximately 2.5℃ at 50uM that is 10 times higher than moderately active LeIBP. Furthermore, the Ice Re-crystallization inhibition activity represent the FfIBP has ability to inhibit ice growing at low concentration 2.5uM limit. Consequently the FfIBP was classified as a hyper-active Ice binding protein. Also, preliminary X-ray crystallography was performed to reveal the ice-binding site of FfIBP at the molecular level.

Introduction

Many plants, insects, animals and other organisms have evolved with unique adaptive mechanisms that allow them to survive in harsh environments at the extremes of temperature (Rothschild & Mancinelli, 2001). In order to survive under extremely cold environments, many organisms produce antifreeze proteins (AFPs) and ice binding proteins (IBPs). These proteins can lower the freezing point of a solution noncolligatively without affecting the melting point (thermal hysteresis), thus freezing of body fluids can be prevented. Also, these proteins can inhibit ice re-crystallization, of which the large ice crystals grow with the expense of smaller ones, thus prevent cell damages during freeze-thaw cycles (Knight *et al.*, 1984; Knight *et al.*, 1991). It is generally accepted that these proteins function through adsorption of their flat ice-binding surfaces onto particular planes of ice crystals and prevent or inhibit further ice growth(Yeh & Feeney, 1996). Notably, the antifreeze activity of AFPs and IBPs is various and dependent on its protein structure. Therefore, structural studies are essential to understand their antifreeze and ice-binding mechanism.

AFPs were characterized according to their structure and thermal hysteresis (TH) values, and have been categorized into several types like Type I-IV from fish, insect AFP, bacterial AFP and plant AFP (Griffith *et al.*, 1997; Davies & Hew, 1990; Graether *et al.*, 2000). Recently identified IBPs from bacteria, diatoms, and fungi formed a distinct cluster in a phylogenetic analysis (Raymond & Janech, 2009). The first ice-active fungal protein (~25 kDa) was found in a snow mold, *Typhula ishikariensis* (Hoshino *et al.*, 2003) and similar ice-binding proteins were subsequently found in sea ice diatoms (Janech *et al.*, 2006), a sea ice bacterium (Raymond *et al.*, 2007), a bacterium from a deep ice-core in the Antarctic ice sheet (Raymond *et al.*, 2008) and an Arctic yeast, Leucosporidium sp. AY30 (Lee *et al.*, 2010). Recently, the crystal structure of IBP (LeIBP) from Leucosporidium sp. AY30 was determined in our lab. The LeIBP structure revealed that IBPs have unique structural features compared to previously available AFPs and bind to ice lattice in a different manner (Lee *et al.*, 2012).

In this study, we succeeded in the expression and purification of another IBP (FfIBP) from Antarctic bacteria, *Flavobacterium frigoris*. Ice binding property of FfIBP was already confirmed by ice pitting experiment (Raymond *et al.*, 1989). The FfIBP shows a 36% sequence similarity to its family member LeIBP. However, FfIBP has up to 10-fold higher antifreeze activity as compared with the LeIBP. To investigate why FfIBP has stronger activity, we have carried out structural and functional studies. As the first step toward its structural elucidation, we report here the results of preliminary X-ray crystallographic experiments of the FfIBP.

Results

1. Thermal hysteresis and Re-crystallization inhibition activity

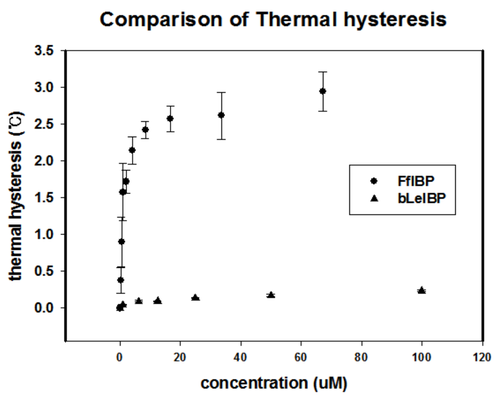

Fig 1. Thermal hysteresis activity of FfIBP.. Activity was ten times higher than moderately active LeIBP.

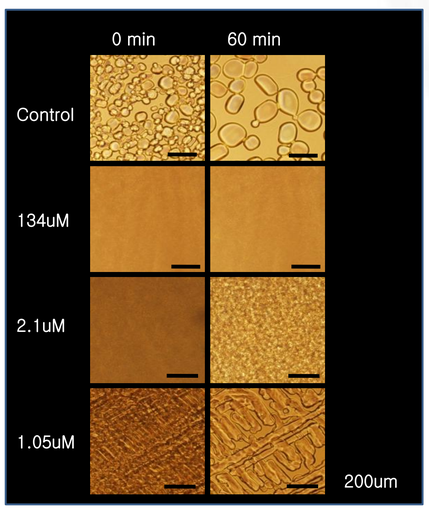

Fig 2. Ice re-crystallization inhibition assay of FfIBP. was mixed same volume of sucrose (60%). Ice granules are sustained at low concentration of FfIBP (2.1uM) at -6℃ for 1hour.

2. Multiple sequence alignment and antifreeze activity of recombinant FfIBP

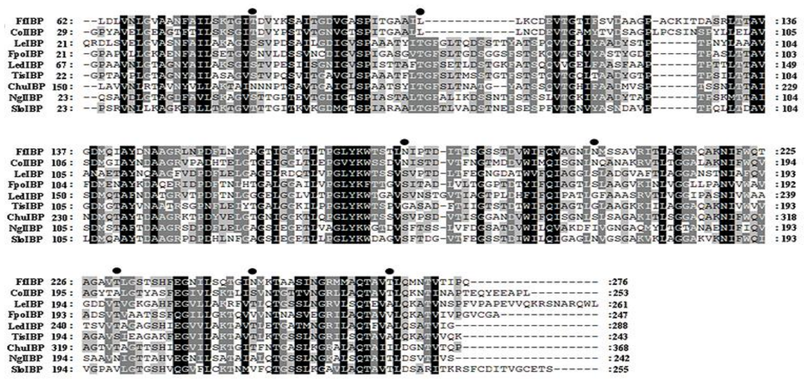

Fig 3. Sequence alignment of FfIBP and other IBPs from *Colwellia psychrerythraea* (ColIBP), *Leucosporidium* sp. AY30 (LeIBP), *Flammulina populicola* (FpoIBP), *Lentinula edodes* (LedIBP), *Typhula ishikariensis* (TisIBP), *Cytophaga hutchinsonii* ATCC 33406 (ChuIBP), *Navicula glaciei* (NglIBP) and *Stephos longipes* (SloIBP) using ClustalX program. Residues constituting the ice-binding sites in LeIBP structure are indicated by filled circles. Highly conserved residues are shaded grey and black.

Results

3. preliminary X-ray crystallographic analysis

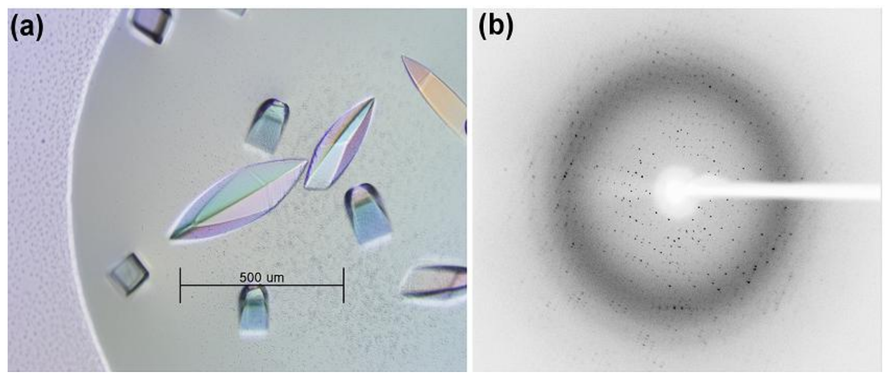

Figure 3. Crystal morphology and diffraction pattern of recombinant FfIBP. (A) FfIBP crystals grown with 0.1M sodium acetate pH4.4 and 3M sodium chloride. (B) X-ray diffracted image (2.9 Å resolution) obtained during data collection from the FfIBP crystal.

Table.1 Data collection statistics

Data collection	
X-ray source	KBSI, Rigaku micromax-007 HF
Space group	$P4_122$
Unit-cell parameters(Å)	a = 69.4, b = 69.4, c = 178.2,
Wavelength(Å)	1.5418
Resolution Range(Å) [a]	69.36-2.90 (3.06-2.90)
No. of observed reflections [a]	74340 (9447)
No. of unique reflections [a]	10314 (1458)
Completeness (%)[a]	99.9 (100)
Redundancy [a]	7.2 (6.5)
R_{sym} [a,b]	0.147 (0.405)
I/σ [a]	9.0 (3.0)

[a]Numbers in parentheses indicate the statistics for the last resolution shell.
[b]$R_{sym}=\Sigma_h\Sigma_i \left| I(h)_i-<I(h)> \right| / \Sigma_h\Sigma_i I(h)_i$, where I is the intensity of reflection h, Σ_h is the sum over all reflections, and Σ_i is the sum over i measurements of reflection h.

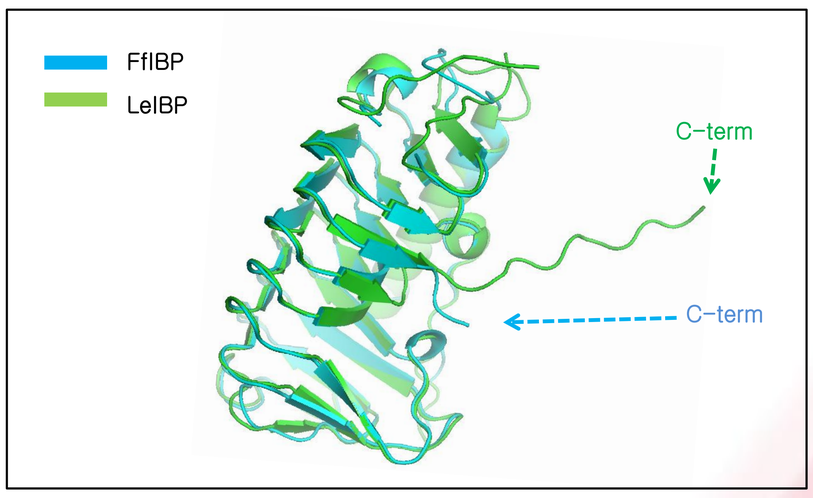

Figure 4. Structural comparison of FfIBP and LeIBP. Photograph shows that two IBPs are very similar except C-terminus region.

Results

An ice-binding protein (FfIBP) from *Flavobacterium frigoris* PS1 was cloned, over-expressed, and purified in *E. coli* BL21 (DE3). The purified FfIBP appears to be a monomer as suggested by the elution profile in the size-exclusion chromatography and the analytic ultracentrifugation results (data not shown here). In LeIBP structure, a C-terminal hydrophobic loop region adopts an extended conformation and plays in dimer formation (Lee *et al.*, 2012). However, the C-terminal region is not present in FfIBP that may render the FfIBP behave as monomer in solution. Notably, the C-terminally truncated LeIBP was more active to the effects of TH than the wild type. We had predicted that its removal break the dimer interaction and does seem to be freely accessible for ice binding in the monomeric state, which may confer higher TH activity to the C-terminal truncated LeIBP mutant (Fig. 4). Most strikingly, the recombinant FfIBP displayed a TH activity of 2.5°C at 50u*M* that is 10 times higher than moderately active LeIBP which is about 0.17 at 50uM (Fig. 1) (Lee *et al.*, 2012). Although the FfIBP shares protein sequence similarity with the LeIBP and is expected to have a similar overall three-dimensional structure, it exhibits distinct differences from LeIBP, including its oligomerization and TH activity.

To obtain structural information about the reasons of hyper-antifreeze activity and different ice-binding mechanism of FfIBP, we have carried out X-ray crystallographic experiments. Purified FfIBP was concentrated to 16.4mg ml^{-1} and quadrangular pyramid-shaped crystals suitable for diffraction (maximum dimension of 500 μm; Fig. 3A) were grown in 0.1M sodium acetate pH4.4 and 3M sodium chloride. Complete diffraction data was collected to 2.9 Å resolution (Fig. 3B) in space group $P4_122$, with unit-cell parameters $a = b$ = 69.4 Å, c = 178.2 Å (Table 1). Calculation of the Matthews coefficient suggests the presence of one molecules in the asymmetric unit with a solvent content of 70.8% and a Matthews coefficient (V_M) of 4.21 $Å^3$ Da^{-1} (Matthews, 1968). A good molecular replacement solution (translation-function contrast value of 18.8 and R-factor of 48%) was obtained using the monomeric model of LeIBP structure (PDB code 3UYU) in the MOLREP software. The resulting PDB file was refined against the original data set in REFMAC (Murshudov *et al.*, 2011) and showed reasonable R_{work} and R_{free} values of 25.5% and 32.3%, respectively. Currently, further structure refinement and model building of FfIBP is in progress and the structural details will be reported elsewhere.

Reference

Lee, J. H., Park, A. K., Do, H., Park, K. S., Moh, S. H., Chi, Y. M. & Kim, H. J. (2012). *Journal of Biological Chemistry* In press.
Raymond, J. A., Christner, B. C. & Schuster, S. C. (2008). *Extremophiles* 12, 713-717.