## 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 recrystallization inhibition activity. Thermal hysteresis activity of the FfIBP was approximately 2.5°C at 50uM that is 10 times higher than moderately active LeIBP. Furthermore, the Ice Recrystallization inhibition activity represent the FfIBP has ability to inhibit ice growing at low

## Results

3. preliminary X-ray crystallographic analysis



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.

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	n
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X-ray source	KBSI, Rigaku micromax-007 HF
Space group	P4 <sub>1</sub> 22
Unit-cell parameters(Å)	a = 69.4, b = 69.4, c = 178.2,
Wavelength(Å)	1.5418
Resolution Range(Å) <sup>a</sup>	69.36-2.90 (3.06-2.90)
No. of observed reflections <sup>a</sup>	74340 (9447)
No. of unique reflections <sup>a</sup>	10314 (1458)
Completeness (%) <sup>a</sup>	99.9 (100)
Redundancy <sup>a</sup>	7.2 (6.5)
$R_{\rm sym}^{\rm a,b}$	0.147 (0.405)
$I/\sigma^{a}$	9.0 (3.0)

<sup>a</sup>Numbers in parentheses indicate the statistics for the last resolution shell. <sup>b</sup> $R_{sym} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle | / \sum_h \sum_i I(h)_i$ , where *I* is the intensity of reflection *h*,  $\sum_h$  is the sum over all reflections, and  $\sum_i$  is the sum over *i* measurements of reflection *h*.



### Results

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**1.** Thermal hysteresis and Re-crystallization inhibition activity





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  $2.5^{\circ}$ 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

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  $-6^{\circ}$ C for 1hour.

2. Multiple sequence alignment and antifreeze activity of recombinant FfIBP

FfIBP	62:	LDLWNLEVAANFAILSKTGINDVYKSAITGDVGASFITGAANLLKCDBVNGTIDS	VEAAGE-ACKITDASREATAV	: 136
CoIIBP	29:	-GPYAMELCEAGTETILSKSGIADVYPSTVTGNVCTSEITGAATLLNCDEWTCAMT	VESAGELPCSINSEYLLELAV	: 105
LeIBP	21:	QRDLSWELCVASNEAILAKAGISSVPDSAILGDIGVSEAAATYITGEGLTQDSSTTMATSPOWTGLINA	AFYSTE	: 104
FpoIBP	21:	-GPAPWILLCKNEN PAILSETGVENVLDSSVNCDICVSEIGASGVTGBSLTGDSGGSBSTEKOWTCRWYA	STYGDEALASLATAV	: 103
LedIBP	67 :	-GPAAWNLCTAGNYAILAKSGISTVPESIISGNIGVSEISTTAFTGESETLDSTGKEATSCOVVGELEA	ASFAAB	: 149
TisIBP	22:	-GPTAWFLCTACNYAILASAGVSTVPOSVITCAVGLSFAAATFLTGFSLTMSSTGTFSTSTCVTGCLTA	ATYGTEALSILATAL	: 104
ChuIBP	150 :	LAVWNIRTAVNYVLIAKTAINNNPTSAVTCAIGISEAATSYITCESLTNAGGWATSSCVICHIBE	APMVSSASSNIATAL	: 229
NeIIBP	23:	CSAVELETAGEFAVISKAGVSTTGPTEVTGDIGTSPIASTALTGPALIKDSSNTASTSSLVTCKIVA	A YTAS ALSEMATAL	: 104
ShiBP	23:	PSRWNILKAGKERLUTKTGWATTGINKWKGDMCTSBIARAALTGATLVADSTNERSESPEWTCNWA	SNDAVE	: 104
FARR	107			
FILBP	137:	GDVCIAYDNAAGSENEDEENNICACTIGCKTI TEGEYKWOSTENIPTE-IAISCSSTEVWIFOVAGNEN	SSAVENTHAGGECARNIEVOT	:225
CollBP	106 :	SDNGIAYNDAAGEVPALHTEDETGEIGELTDEPGVYRWSSDVNISTD-VAFNGTMDDVWIMCISGNUNG	ANAKENTITIER/LAKNIE/Q	: 194
LeIBP	105 :	ANAETAYNCAACFVDEDELEI CACEURDCTIVEGLYKWHSSWSVPTD-IIIIFEGNGDATWWFOIAGGI SI	ADGVAFTH AGEPINSTNHABO	: 193
FpoIBP	104 :	FDVENAMKDMCDRIDEDETNEHTCALGCAINVEGLYKEREGVSITAD-HVLTCGPTDTYLFOHAGTUS	AAGVEHINIVGELLPANVVOAM	: 192
LedIBP	150 :	SD&QTAENDATEEVTEDETN JEGEEIGELVITEGLYKWAGAWSVNSTGVAIAGTPLEHEIFOIPATAGE	AAASEMTIVEEIPASNIVAA	:239
TisIBP	105 :	GDAGTAYVNAPTESGENELEEYTGAUGEKIN PEGLYKANS PAGASAD-FEIIGTSTETWIFQIAGTEGE	AAGKKHINACCOQAKNIVOV	: 193
ChuIBP	230:	NEWQTAYTDAAGEKTEDYVELETENIGEKTIQEGLYKWHSSWSVPSD-VHISGGANDVWIFQISGNISH	SAGARUTISCOLQARNIF	: 318
NgIIBP	105:	SDNSTAFTDAAGESDPDELEUCAGSTECETTVAGLYKWGADWSFTSS-UVFDGSATDVWILQVAKDFIV	GNGAQUYITCTENAENIFIQ	: 193
Shibb	105:	LEVCAAYTEAKE PDEEHINFEACSTEETILEGLYKWDAGVSFTDG-VEFECSSTEIWILQIGAGEN	GSGA KIAGO KVKNIF QU	: 193
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FfIBP	226 :	AGAMTICSTSHFECNITSCHOUNKTAASINGRMNAQTAWTICMNTTIPC	:276	
CoIIBP	195 :	AGYTALCTYASFECIVESKALISUNTGTTVNGRUDACTAWTICKN/INAPTECYEEAPL	:253	
LeIBP	194 :	GDDVTVCKGAHFEGVILLAKREVTICTCSSINGRVLSCTEVALCKATVNSPFVPAPEVVCKRSNARCWL	:261	
FDOIBP	193 :	ADSVTVAATSSECCUTUGKICVVVNTNASVEGRTLACTAVVICKATVIVPGVCGA	:247	
LedIRP	240 :	TSVWTAGAGSHIEGWUTAKUAWTHETGATWNGRITACTEWALQSATWIG	:288	
TEIRP	194 :	AGAUSTEAGAKEECVITAKTANTI KUCSSINGETTSCHAMATOKATWVCK	:243	
ChuIBP	319 :	AGTWTACTTSHIECKAUSKAGUTENWCASUKCEAUACREUTUDGNOWTOP	:368	
NellBP	194 -	SAAMNIGTTAHVECNUTSANAHANCOGSSUNGRAUSON HTUDSVILLYS-	: 242	
SLIPP	104 -	VCDAVI CTCSHUCCUTUCCUTUSSI KCAUTACTUTUSSE UTKESECOTTUCCETS	- 255	
SDIDL	134.	ALL AND A DE ALL AND A	- 200	

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* (NgIIBP) 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.

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 icebinding mechanism of FfIBP, we have carried out X-ray crystallographic experiments. Purified FfIBP was concentrated to 16.4mg ml<sup>-1</sup> and quadrangular pyramid-shaped crystals suitable for diffraction (maximum dimension of 500  $\mu$ m; 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 ( $V_{\rm M}$ ) of 4.21 Å <sup>3</sup> Da<sup>-1</sup> (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

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