PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
2017, VOL. 47, NO. 3, 299–304
http://dx.doi.org/10.1080/10826068.2016.1244682

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Improving thermal hysteresis activity of antifreeze protein from recombinant *Pichia pastoris* by removal of N-glycosylation

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ABSTRACT

To survive in a subzero environment, polar organisms produce ice-binding proteins (IBPs). These IBPs prevent the formation of large intracellular ice crystals, which may be fatal to the organism. Recently, a recombinant FfIBP (an IBP from *Flavobacterium frigoris* PS1) was cloned and produced in *Pichia pastoris* using fed-batch fermentation with methanol feeding. In this study, we demonstrate that FfIBP produced by *P. pastoris* has a glycosylation site, which diminishes the thermal hysteresis activity of FfIBP. The FfIBP expressed by *P. pastoris* exhibited a doublet on SDS-PAGE. The results of a glycosidase reaction suggested that FfIBP possesses complex N-linked oligosaccharides. These results indicate that the residues of the glycosylated site could disturb the binding of FfIBP to ice molecules. The findings of this study could be utilized to produce highly active antifreeze proteins on a large scale.

KEYWORDS

Antifreeze protein; *Flavobacterium frigoris*; glycosylation; ice-binding protein; *Pichia pastoris*

Introduction

The both edges of the Earth, the Antarctic and Arctic, have harsh environments that are dark, strong winds and blizzards, and very cold. The marine and freshwater organisms living in polar regions are consistently exposed to low temperatures, ranging from −1.9 to 2.0 in seawater, and 0 to 5°C in freshwater.[1] Psychrophiles produce active enzymes under low temperatures and accumulate polyunsaturated lipids to compose high-fluidity cell membranes.[2] It was revealed that some creatures found in subfreezing environments produce antifreeze proteins (AFPs) or ice-binding proteins (IBPs) to circumvent cryodamage such as intracellular ice formation.[3] AFPs induce a gap between freezing and melting points, which is known as thermal hysteresis. AFPs inhibit the growth of the ice crystal by interfering the surface of solid ice and liquid water phases. This mechanism allows cells to protect themselves from cryodamage.[4] Typically, IBPs are superset of AFPs and other ice-nucleating proteins. Several IBPs have been found from psychrophilic organisms including Antarctic sea ice algae, yeast, fungi, insects, and bacteria.[4–6] Some of these IBPs are secreted out of the cell membrane and may reduce cell damage by inhibiting recrystallization of extracellular ice.[3] Because of the many potential benefits of protecting cells from freezing damage, there has been growing interest in these proteins as candidates for industrial applications such as frozen food storage.[7]

Obtaining IBPs and AFPs from natural resources, including fish, insects, and plants, is limited and difficult due to cost ineffectiveness. Therefore, recombinant protein production is generally performed using heterologous expression system.[8] To date, our research team has found several IBPs, including LeIBP, FfIBP, and Type-III AFP from diverse Antarctic and Arctic organisms, and we investigated their properties in previous studies.[4,5,9] One of the IBPs, the hyperactive FfIBP, was isolated from a gram-negative Antarctic bacterium *Flavobacterium frigoris* PS1, with a molecular mass of approximately 24.2 kDa. FfIBP presented the highest thermal hysteresis (TH) activity among the IBPs and AFP that we have found.[5,9]

The yeast expression system yields high protein expression levels and secretion levels of the target protein, allowing for easy protein purification.[10] Therefore, we used *Pichia pastoris* as an expression host. A plasmid harboring codon-optimized FfIBP was constructed and transformed into *P. pastoris* for laboratory-scale production to determine the optimal production conditions in a 7-L fed-batch culture.[11] Interestingly, the thermal hysteresis activity of FfIBP produced by *P. pastoris* was considerably lower than that produced by *Escherichia coli*.[5] We anticipated that the difference was due to glycosylation. Glycosylation is a complex post-translational modification found in all domains of life and usually occurs in the endoplasmic reticulum and Golgi apparatus of eukaryotic cells.[12] Glycans play various structural and functional roles in membrane and secreted proteins. Protein glycosylation is also involved in correct folding, solubility, antigenicity, stability, and biological activity.[13–17] It showed critical effects on heterologous expression of cellobiohydrolase I in *P. pastoris*.[16] The present study aims to investigate if the FfIBP produced by *P. pastoris* is glycosylated and if the antifreeze activity increases by removing the glycosylation.

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Supplemental data for this article can be accessed on the publisher's website at http://dx.doi.org/10.1080/10826068.2016.1244682.

Materials and methods

Microorganism, plasmids, and media

The pPICZαA vector (Invitrogen, Carlsbad, CA, USA) and *P. pastoris* expression system (Invitrogen, Carlsbad, CA, USA) were used for FfIBP production. Recombinant *P. pastoris* was cultured in yeast extract–peptone–dextrose (YPD) medium (yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L). Fermentation basal salt (FBS) medium (glycerol, 40 g/L; $CaSO_4$, 0.93 g/L; K_2SO_4, 18.2 g/L; $MgSO_4{\cdot}7H_2O$, 14.9 g/L; KOH, 4.13 g/L; H_3PO_4 [85%], 26.7 mL/L) containing trace metal solution ($CuSO_4{\cdot}5H_2O$, 6.0 g/L; NaI, 0.08 g/L; $MnSO_4{\cdot}H_2O$, 3.0 g/L; $NaMo_2{\cdot}H_2O$, 0.2 g/L; H_3BO_4, 0.02 g/L; $CoCl_2$, 0.5 g/L; $ZnCl_2$, 20.0 g/L; $FeSO_4{\cdot}7H_2O$, 65.0 g/L; biotin, 0.2 g/L; H_2SO_4, 5.0 ml/L) was used for 7-L fed-batch culture.

Cloning and expression of FfIBP

The coding region of *FfIBP* was optimized according to the codon usage of *Pichia*. *FfIBP* (amino acids 61–276: the first 60 residues were removed, because they were predicted to form a signal sequence and flexible loop regions) was amplified by polymerase chain reaction (PCR) using *F. frigoris* PS1 genomic DNA as a template.[5] Amplified fragments were cleaved with XhoI and NdeI and ligated into the pPICZαA vector. The resulting expression vector contained an N-terminal 6×His-tag fusion protein for purification of FfIBP. The construction and transformation of FfIBP expression vector were performed using the EasySelect Pichia Expression Kit (Invitrogen, Carlsbad, CA, USA). The pPICZαA vector expressing FfIBP was designed to secret the 216 amino acids mature FfIBP into the culture media.

Production of FfIBP in P. pastoris

The laboratory-scale production of FfIBP was performed under optimized culture conditions (pH 5, 30°C) as described previously[11] using a 7-L-jar bottom magnet-drive fermenter (KF-7L model, Kobiotech, Korea). The seed culture grown in 300 mL of YPD was transferred into a 7-L fermenter containing 3 L of FBS medium with 13.1 mL of trace metal solution. The dissolved oxygen (DO)-stat glycerol–methanol fed-batch was initiated by supplying the glycerol-feed medium [250 mL of 50% glycerol containing 3 mL of trace metal solution] for 9 hr. The DO-stat methanol fed-batch was started by adding 2 L of 100% methanol feed with 24 mL of trace metal solution after glycerol feeding was done. The methanol feed rate was set to 3.6 mL/hr/L for 120 hr. DO-stat methanol fed-batch was controlled as follows: methanol feeding was paused when the DO value dropped below 20% of air saturation, while continued when the DO recovered to above the set point by measuring the concentration of DO at each minute.[9] The cell density was expressed as dry cell weight (DCW) and optical density at 600 nm with a UV/VIS spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Sweden). The ratio of DCW (g/L) to optical density was 0.23. The expression and activity of FfIBP were confirmed by SDS-PAGE and TH activity assay.

Purification of FfIBP

The fermentation broth was harvested by centrifugation at 8,000*g* for 10 min, and the supernatant was filtered through a 3.0-μm filter (Whatman, Fisher Scientific, USA). The filtered broth was loaded onto a Ni-NTA agarose column pre-equilibrated with NPI-5 buffer (50 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 5 mM imidazole) and washed with 10-bed volumes of NPI-20 buffer (50 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole). The bound FfIBP was eluted with NPI-300 buffer (50 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 300 mM imidazole). The final elute was buffer-exchanged with 50 mM Tris-HCl (pH 8.0) and concentrated on a 10-kDa centrifugal filter (Amicon Ultra Centrifugal filter, Millipore, Bedford, MA, USA).

Analysis of glycosylated protein

The glycosylation of expressed proteins was verified by periodic acid-Schiff (PAS) staining the SDS-polyacrylamide gel using the Pierce™ Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, USA). After electrophoresis, the gel was fixed with 50% ethanol for 30 min and washed with 3% acetic acid solution for 10 min. It was agitated in oxidizing solution (Pierce Biotechnology, Rockford, USA) for 15 min and then agitated for 15 min again in glycoprotein staining reagent before being transferred into reducing solution (Pierce Biotechnology, Rockford, USA). Finally, the gel was washed with 3% acetic acid and ultrapure water. Glycoprotein was confirmed with the appearance of magenta bands. To verify the binding of N-glycans to the protein, FfIBP was incubated with 5 unit of the Peptide-N-Glycosidase F (PNGase F, Sigma-Aldrich, USA) at 37°C for 1 hr. Glycan cleaving of FfIBP was confirmed by SDS-PAGE analysis.

Expression of nonglycosylated FfIBP

Glycosylation sites were predicted using web-based NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc) for N-glycosylation. FfIBP mutagenesis was performed using mutagenic primers: 5′ - GTT GCA GGA AAC CTG GCT ATG AGT TCT GCA GTT - 3′, 5′ - AAC TGC AGA ACT CAT AGC CAG GTT TCC TGC AAC - 3′ for N203A replacement, 5′ - GTT GCA GGA AAC CTG CAA ATG AGT TCT GCA GTT - 3′, 5′ - AAC TGC AGA ACT CAT TTG CAG GTT TCC TGC AAC - 3′ for N203Q replacement. Plasmids for nonglycosylated FfIBP were used as template DNA for mutagenesis. The mutant proteins were expressed from *Pichia* and purified using the same procedure as described previously.[4]

Thermal hysteresis assay

The TH activity of FfIBP was measured using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand). The value of TH activity was defined as the maximum difference between the melting and freezing temperatures of the sample. The TH activity was measured in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl buffer and the protein concentration was ranged between 0 and 110 μM. The ice-crystal morphology was

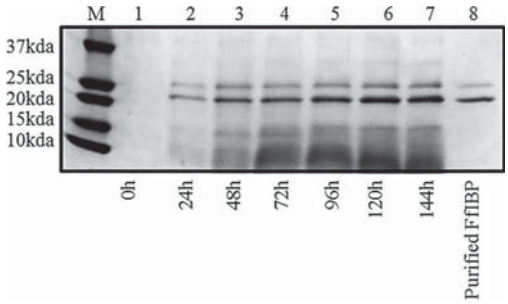

Figure 1. Expression of FfIBP by *P. pastoris* cultured in a 7-L fermentation. FfIBP expression was visualized using SDS-PAGE by running the supernatant of culture medium. Lane M, size marker; lane 1–7, 0–144 hr postinduction; lane 8, purified FfIBP.

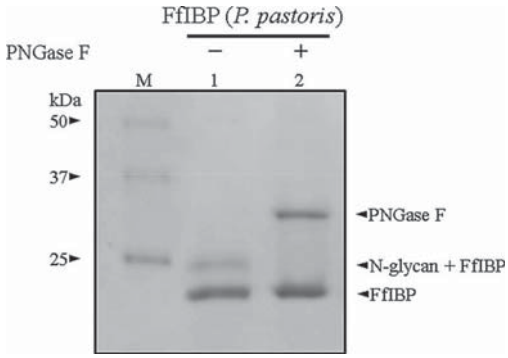

Figure 2. Enzymatic digestion of N-linked glycosylated FfIBP expressed by *P. pastoris*. Lane M, size marker; lane 1, purified FfIBP; lane 2, purified FfIBP treated with PNGase F. The doublet disappeared following enzymatic digestion, exhibiting a single protein band and enzyme band.

captured using Canon PowerShot A620 (Canon Inc., Tokyo, Japan).

Results and discussion

Construction of FfIBP-expression vector

An ice-binding protein (*FfIBP*) gene from *F. frigoris* PS1 was cloned, overexpressed, and purified in *P. pastoris* X-33 cells. To achieve high-level secretory expression, the *FfIBP* gene (residues 61–276; the first 60 residues, which constitute a signal-peptide sequence, were removed) was subcloned into the pPICZαA vector, which contains the α-factor secretion signal and Zeocin-resistance gene for selection. An N-terminal His_6-tag was added to the gene product, with the sequence MGSSHH HHHHSSGLVPRGSHMASMTGGQQMGRGS (Supplementary Fig. 1a). The expression of FfIBP, which was regulated by AOX1 promoter, was induced with methanol (Supplementary Fig. 1b).

Laboratory-scale production of FfIBP and purification

The FfIBP was produced by fed-batch culture of *P. pastoris* in a 7-L jar fermenter at pH 5 and 30°C with methanol feeding for 144 hr. The cell growth increased steadily and total protein concentration reached 495 ± 16 mg/L at 48 hr (data not shown). The secreted total proteins at 0, 24, 48, 72, 96, 120, 144 hr of induction and purified FfIBP were detected by SDS-PAGE (Fig. 1) and Western blot assay (Supplementary Fig. 2). The FfIBP expressed from *P. pastoris* exhibited a doublet of 24 kDa and ~26 kDa (Fig. 1). Yield of purified FfIBP was 75 mg/L from the supernatant at last 144 hr (Table 1).

Confirmation of protein glycosylation

The potential N-glycosylation site (Asn-X-Ser) of the doublet protein bands of FfIBP on a SDS-PAGE in heterologous expression system was examined by glycosidase digestion. Purified FfIBP was treated with PNGase F, which cleaves the bond N-acetyl-D-glucosamine and an Asn residue. The doublet disappeared following enzymatic digestion, yielding a single protein band of approximately 24 kDa (Fig. 2). According to a web-based tool for identification of N-glycosylation, putative N-glycosylation site was predicted at Asn^{203} (Fig. 3a), located in the β9 strand involved in the B face of FfIBP (Fig. 3b). The glycosylation of FfIBP was determined by PAS staining. PAS-positive glycans appeared as magenta bands on the SDS-PAGE gels (data not shown). Taken together, these results indicate that Asn^{203} was N-glycosylated in FfIBP expressed by *P. pastoris*.

Expression of nonglycosylated FfIBP

To compare the antifreeze activities of glycosylated and nonglycosylated proteins, FfIBP mutants were generated by

Table 1. Comparison of known IBPs.

Specific name	Organism	TH (°C) [AFP conc.]	Mw (kDa)	Expression host	Production level (mg/L)	Reference
Type I	Winter flounder	0.67 [1.5 mM]	3.2425	*E. coli*	16	[18]
Type-II	Sea raven	0.6–1.5	15.8	*P. pastoris*	30	[19]
Type-III	Ocean pout	0.6–1.5	6.5	*E. coli*	13	[20]
LeIBP	*Leucosporidium* sp. AY30	0.43 [400 μM]	25.044	*P. pastoris*	61.2	[9,21]
PgAFP	*Pyramimonas gelidicola*	0.6 [6 mM]	26.4	*E. coli*	30	[3]
Cn-AFP	*Chaetoceros neogracile*	0.8 [40 μM]	26.2	*E. coli*	n.d.	[22]
FcAFP	*Fragilariopsis cylindrus*	0.9 [230 μM]	25.939	*Fragilariopsis cylindrus*	97.1	[23,24]
TisAFP	*Typhula ishikariensis* BRB-1	1 [1 mM]	22	*Typhula ishikariensis*	n.d.	[6]
FfIBP	*Flavobacterium frigoris* PS1	2.5 [50 μM]	25.715	*E. coli*	n.d.	[25]
FfIBP	*Flavobacterium frigoris* PS1	1.7 [104 μM]	24.208	*P. pastoris*	75.0	This study
FfIBP-N203A	*Flavobacterium frigoris* PS1	2.6 [110 μM]	24.165	*P. pastoris*	47.3	This study
FfIBP-N203Q	*Flavobacterium frigoris* PS1	2.2 [108 μM]	24.222	*P. pastoris*	50.5	This study
NagIBP	*Navicula glaciei*	3.22 [1.6 mM]	25	*Navicula glaciei*	n.d.	[23]
ColAFP	*Colwellia sp.* SLW05	3.8 [0.14 mM]	26	*Colwellia sp.* SLW05	n.d.	[26]

n.d., no data; IBPs, ice-binding proteins; TH, thermal hysteresis; AFP, antifreeze protein.

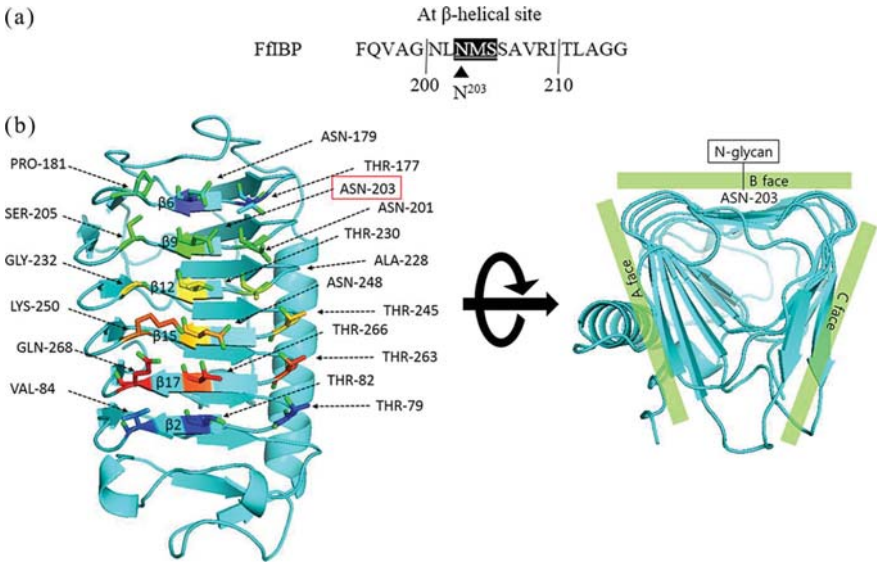

Figure 3. Identification of N-glycosylation site in FfIBP. (a) amino acid sequence of FfIBP with a potential N-glycosylation site (Asn-X-Ser/Thr). Putative N-glycosylation site is underlined and (b) ice-binding residues on the B face of FfIBP. N-glycosylation site was marked with a red box.

site-directed PCR mutagenesis using mutagenic primers. The mutant FfIBPs (FfIBP-N203A and FfIBP-N203Q) were expressed in *P. pastoris* and purified. A single band at 24 kDa on SDS-PAGE was observed for both mutants, similar to that of the deglycosylated FfIBP (Fig. 4a). The production levels of FfIBP-N203A and FfIBP-N203Q after 144 hr of induction were 47.3 and 50.5 mg/L, respectively. Reported production level of LeIBP in 2-L culture was 61.2 mg/L, which is 20% higher than that of recombinant FfIBPs (FfIBP-N203A and FfIBP-N203Q) (Table 1). In comparison with LeIBP, FfIBPs have a competitive advantage showing a higher TH activity at the same molar concentration.[4,11]

Comparison of thermal hysteresis assay

The secreted FfIBP, FfIBP-N203A, and FfIBP-N203Q were collected and concentrated using the supernatant of the culture medium. The soluble FfIBP and mutants were purified

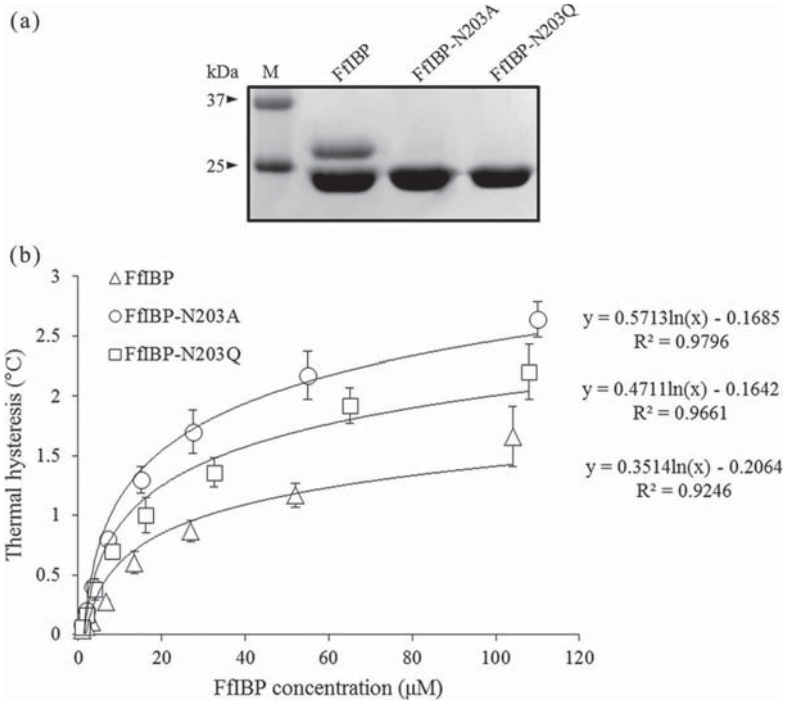

Figure 4. Comparison of expression and activity of glycosylated and nonglycosylated FfIBP. (a) expression of mutant FfIBPs, N203A, and N203Q. The secreted proteins were purified and concentrated from supernatant of culture medium. The mutant FfIBPs showed a single band of protein. Lane M, size marker; lane 1, intact FfIBP; lane 2, mutant FfIBP-N203A; lane 3, mutant FfIBP-N203Q and (b) thermal hysteresis activity of FfIBPs, mutant FfIBP-N203A and N203Q (open triangle, FfIBP; open circle, FfIBP-N203A; open square, FfIBP-N203Q). Each point was measured thrice and averaged. Standard deviations are shown as vertical bars (significant result with $P < 0.05$).

using His-binding resin. The purified proteins were further buffer-exchanged and concentrated using Centriprep filter device (10 kDa cut off) and stored at −20°C before use. The temperature gaps between the melting and freezing points of purified proteins were measured and used to archive a standard curve of TH activity as antifreeze activity. The glycosylated FfIBP showed TH activity of 1.66 ± 0.25°C at 104 μM, which is comparable to other AFPs, such as TisAFP,[6] LpAFP,[27] and LeIBP[4] (Table 1). The mutant FfIBP-N203A exhibited TH activities of 2.64 ± 0.15°C at 110 μM and 2.17 ± 0.2°C at 55 μM, and FfIBP-N203Q exhibited TH activities of 2.2 ± 0.23°C at 108 μM and 1.92 ± 0.15°C at 65 μM. The glycosylated FfIBP showed the lowest activity, and mutant FfIBP-N203A showed the highest antifreeze activity (Fig. 4b). The glycosylation site removal of FfIBP successfully increased the TH activity. We predicted the presence of glycosylated FfIBP secreted from *Pichia* fermentation,[28] and investigated the effect of the glycosylation of FfIBP on the antifreeze activity. The TH activity of deglycosylated FfIBPs (FfIBP-N203A, FfIBP-N203Q) was higher than that of glycosylated FfIBP, however, production efficiency per liter was lower. It could be an adverse effect on protein production, because removal of the glycan from FfIBP could decrease the secretion from yeast.[29]

Previous study reported that recombinant LeIBP was N-glycosylated by yeast expression. Nonglycosylated LeIBP presented ~30% antifreeze activity compared to native LeIBP, but glycosylation does not affect function or cause structural change.[21] However, in the case of FfIBP, N-glycan exists on the B face where the ice-binding site of FfIBP is present, and it may be a significant factor to decrease antifreeze activity by interfering with the adhering of ice crystal and ice-binding motif (Fig. 3b). To increase the TH activity of FfIBP expressed by *Pichia,* we constructed mutant cells expressing Alanine or Glutamine, instead of Asparagine at 203 position of FfIBP. Glutamine was selected because it has the polar side chain as Asparagine, and Alanine has the lowest molecular weight and steric hindrance. The TH activity of mutant FfIBP-N203A was higher than that of FfIBP-N203Q. This suggested that the steric effect was more dominant than polarity. Therefore, residues, such as glycan located at active site, may affect the activity by disturbing the interaction between IBPs and ice molecules. Yeasts including *Saccharomyces cerevisiae* and *P. pastoris* are important cell factories as they are used for pilot-scale production.[30] However, the glycosylated protein secreted from yeast could impair the activity of the protein. When it is utilized for mass production of FfIBP, the present study on how to predict and remove glycosylation would provide the important information on producing protein with an improved activity.

Conclusion

We anticipated that the low activity of FfIBP was due to glycosylation, and demonstrated the glycosylation using enzymatic digestion and site-directed mutagenesis. In this study, the non-glycosylated FfIBP, FfIBP-N203A, showed antifreeze activity (2.64°C;110 μM) very similar to that of FfIBP expressed by *E. coli* (2.8°C; 110 μM).[5] This study clearly demonstrates that FfIBP expressed by *P. pastoris* is glycosylated at Asn^{203}, and elimination of N-linked glycosylation enhances the antifreeze activity of FfIBP. These results could be utilized for the production of hyper-active recombinant FfIBP.

Acknowledgments

This work was supported by grants from the Korea Polar Research Institute (PE16070, PE15330).

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