Production of ice binding protein with increased thermal hysteresis activity from recombinant *Pichia pastoris*

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ABSTRACT

Ice binding proteins (IBPs) are found in polar organisms such as fish, plants, and insects. IBPs assist organism to survive in cold environments. The functions of IBPs include adhesion to ice and inhibition of ice recrystallization. The FfIBP was isolated from Antarctic bacterium *Flavobacterium frigoris* PS1. A codon-optimized FfIBP was cloned and produced in *Pichia pastoris* using fed-batch fermentation with methanol feeding. The FfIBP secreted by *P. pastoris* has a glycosylation site, which reduces the thermal hysteresis activity of FfIBP. The FfIBP produced by *P. pastoris* showed a doublet on SDS-PAGE. The results of enzymatic digestion of glycosylated protein suggested that FfIBP has complex N-linked oligosaccharides. The non-glycosylated FfIBP expressed by site-direct mutagenesis exhibited a single band on SDS-PAGE and presented as high thermal hysteresis activity as expressed in *E. coli*. These results indicate that the glycan attached to asparagine at 203 position of FfIBP could disturb the binding of FfIBP to ice molecules. The findings of this study could be utilized to produce IBPs having hyperactivity on a large scale.

MATERIALS & METHODS

The pPICZαA vector (Invitrogen, Carlsbad, CA, USA) and *P. pastoris* expression system (Invitrogen, Carlsbad, CA, USA) were used for FfIBP production. The laboratory-scale production of FfIBP was performed under optimized culture conditions (pH 5, 30˚C) using a 7-L-jar bottom magnet-drive fermenter (KF-7L model, Kobiotech, Korea). 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). Glycosylation sites were predicted using web-based NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc) for N-glycosylation. Plasmids for nonglycosylated FfIBP were used as template DNA for mutagenesis. The TH activity of FfIBP was measured using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand).

RESULTS

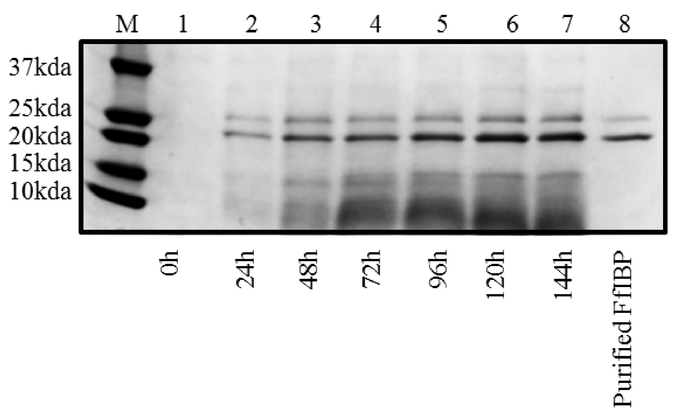

Fig. 1. Expression of FfIBP by *P. pastoris* cultured in a 7-L fermentation. FfIBP expression was visualized using SDS-PAGE by running the supernatant of culture medium. Lane M, size marker; lane 1–7, 0–144 hr postinduction; lane 8, purified FfIBP.

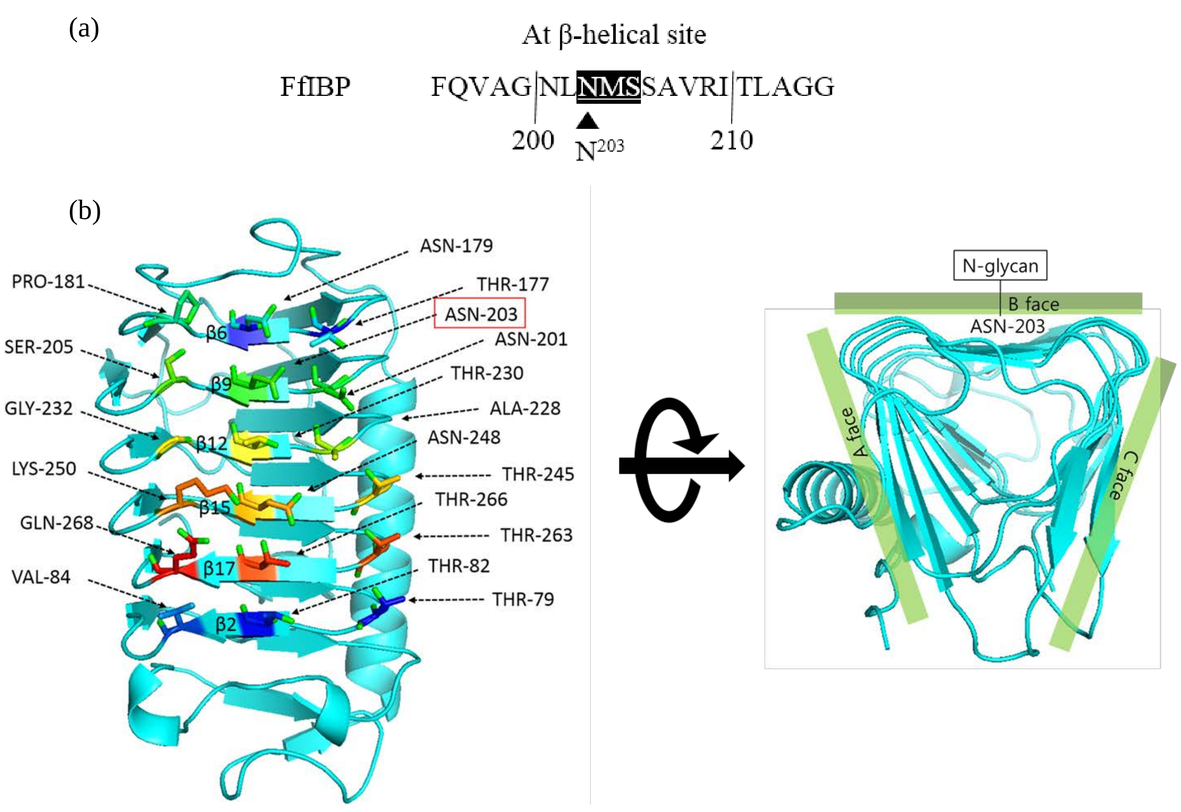

Fig. 2. Identification of N-glycosylation site in FfIBP. (a) amino acid sequence of FfIBP with a potential N-glycosylation site (Asn-X-Ser/Thr). Putative N-glycosylation site is underlined and (b) ice-binding residues on the B face of FfIBP. N-glycosylation site was marked with a red box.

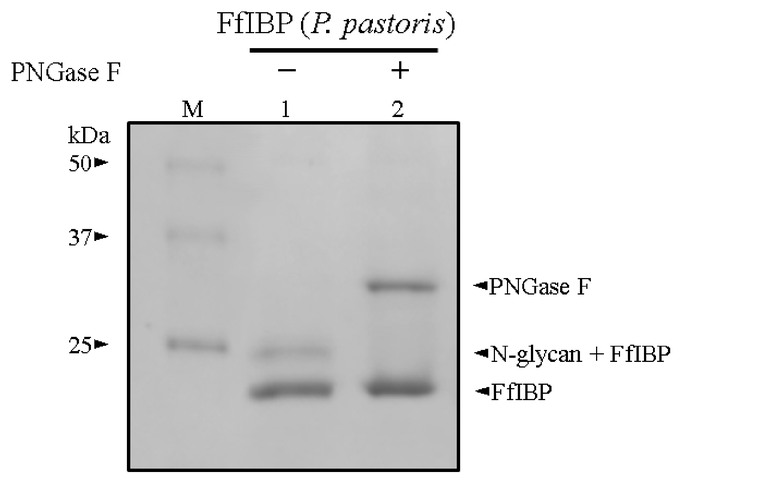

Fig. 3. Enzymatic digestion of N-linked glycosylated FfIBP expressed by *P. pastoris*. Lane M, size marker; lane 1, purified FfIBP; lane 2, purified FfIBP treated with PNGase F. The doublet disappeared following enzymatic digestion, exhibiting a single protein band and enzyme band.

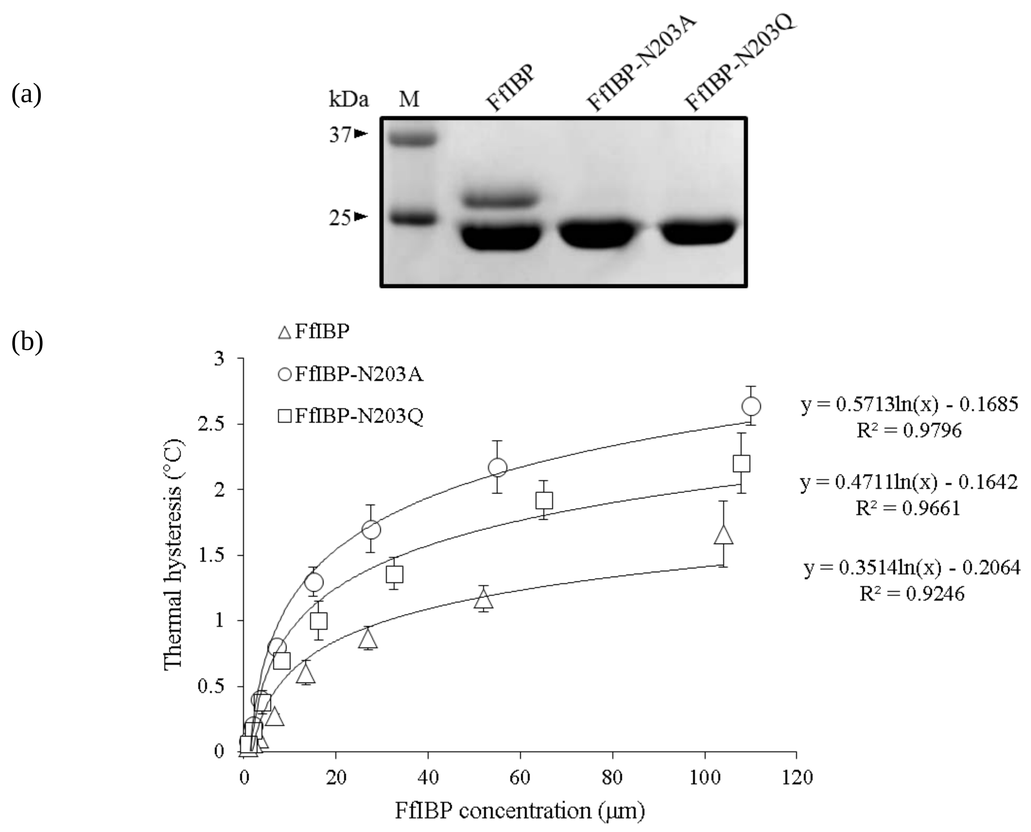

Fig. 4. Comparison of expression and activity of glycosylated and nonglycosylated FfIBP. (a) expression of mutant FfIBPs, N203A, and N203Q. The secreted proteins were purified and concentrated from supernatant of culture medium. The mutant FfIBPs showed a single band of protein. Lane M, size marker; 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 $P < 0.05$).

Table 1. Comparison of known IBPs and AFPs.

specific name	organism	TH (°C) [IBP conc.]	Mw (kDa)	Expression host	Production level (mg/L)	reference
Type Ⅰ	Winter flounder	0.67 [1.5 mM]	3.2425	*E. coli*	16	Tong et al., 2000 Protein Expr. Purif. 18(2):175–181
Type Ⅱ	Sea raven	0.6 – 1.5	15.8	*P. pastoris*	30	Loewen et al., 1997 Appl. Microbiol. Biotechnol. 48(4):480–486
Type Ⅲ	Ocean pout	0.6 – 1.5	6.5	*E. coli*	13	Chao et al., 1993 Protein Sci. 2(9):1411–1428
LeIBP	*Leucosporidium* sp. AY30	0.43 [400 μM]	25.044	*P. pastoris*	61.2	Lee et al., 2013 Appl. Microbiol. Biotechnol. 97, 3383-3393.
PgAFP	*Pyramimonas gelidicola*	0.6 [6 mM]	26.4	*E. coli*	30	Jung et al., 2014 Mar. Biotechnol. 16, 502-512
Cn-AFP	*Chaetoceros neogracile*	0.8 [40 μM]	26.2	*E. coli*	n.d.	Gwak et al., 2010 Mar. Biotechnol. 12, 630–639.
FcAFP	*Fragilariopsis cylindrus*	0.9 [230μM]	25.939	*Fragilariopsis cylindrus*	97.1	Janech et al., 2006 J. Phycol. 42, 410–416.
TisAFP	*Typhula ishikariensis* BRB-1	1 [1 mM]	22	*Typhula ishikariensis*	n.d.	Hoshino et al., 2003 Can. J. Bot. 81, 1175–1181.
FfIBP	*Flavobacterium frigoris* PS1	2.5 [50 μM]	25.715	*E. coli*	n.d.	Do et al., 2012 Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68, 806–809.
FfIBP	*Flavobacterium frigoris* PS1	1.7 [104μM]	24.208	*P. pastoris*	75	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

Abbreviations here; n.d., no data

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 nonglycosylated 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). This study clearly demonstrates that FfIBP expressed by *P. pastoris* is glycosylated at Asn203, 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. [This work was supported by a grant from KOPRI (PE17100)]