# Improvement of thermal hysteresis activity of ice-binding protein from recombinant *Pichia pastoris* by removing of N-glycosylation

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## ABSTRACT

Polar organisms produce ice-binding proteins (IBPs) to survive in a sub-zero environment. These IBPs prevent the formation of large intracellular ice crystals, which may be fatal to the organism. The FfIBP having a hyper-thermal hysteresis activity was isolated from Antarctic bacterium *Flavobacterium frigoris* PS1. Recombinant FfIBP was cloned and produced in *Pichia pastoris* using fed-batch fermentation with methanol feeding. The FfIBP produced by *P. pastoris* has a glycosylation site, which reduces the thermal hysteresis (TH) 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. The non-glycosylated FfIBP produced by site-direct mutagenesis exhibited a single band on SDS-PAGE and showed as high TH activity as expressed in *E. coli*. 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 ice-binding proteins 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

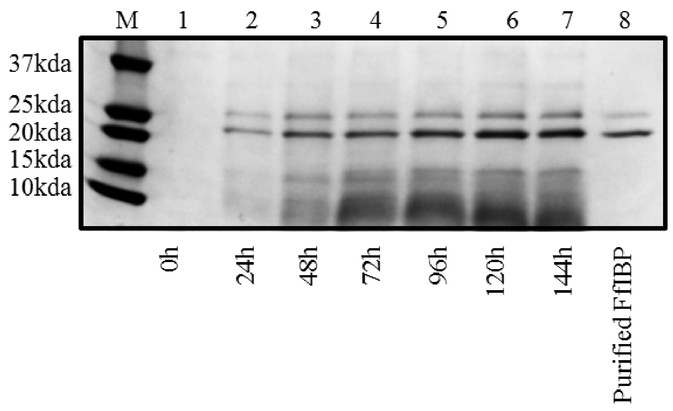



**Fig. 1.** Expression of FfIBP by *P. pastoris* cultured in a 7-L fermentation. FfIBP expression was visualized using SDS-PAGE by running the supernatant of culture medium. Lane M, size marker; lane 1–7, 0–144 hr postinduction; lane 8, purified FfIBP.

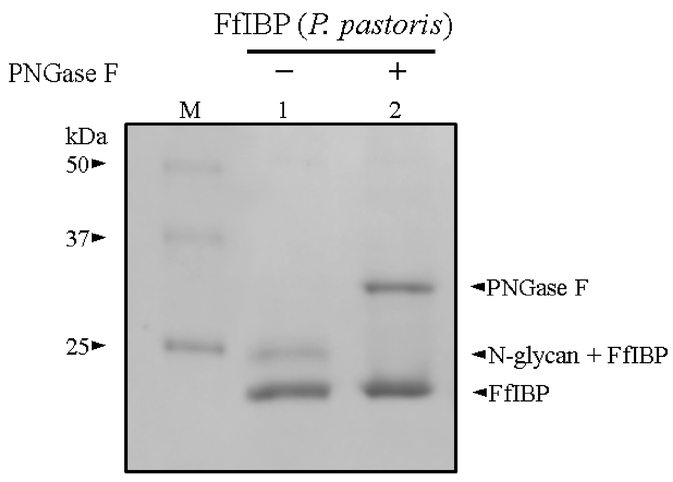


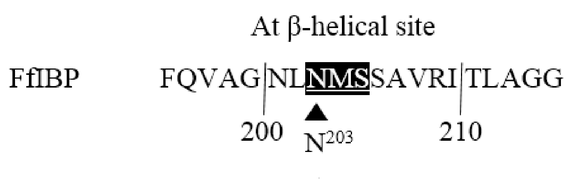

**Fig. 2.** Enzymatic digestion of N-linked glycosylated FfIBP expressed by *P. pastoris*. Lane M, size marker; lane 1, purified FfIBP; lane 2, purified FfIBP treated with PNGase F. The doublet disappeared following enzymatic digestion, exhibiting a single protein band and enzyme band.

**Table 1.** Comparison of known IBPs and AFPs.

| Specific name | Organism | TH (°C) [AFP conc.] | Mw (kDa) | Expression host | Production level (mg/L) | |
|---|---|---|---|---|---|---|
| Type I | Winter flounder | 0.67 [1.5 mM] | 3.2425 | *E. coli* | 16 | |
| Type-II | Sea raven | 0.6–1.5 | 15.8 | *P. pastoris* | 30 | |
| Type-III | Ocean pout | 0.6–1.5 | 6.5 | *E. coli* | 13 | |
| LeIBP | *Leucosporidium* sp. AY30 | 0.43 [400 μM] | 25.044 | *P. pastoris* | 61.2 | |
| PgAFP | *Pyramimonas gelidicola* | 0.6 [6 mM] | 26.4 | *E. coli* | 30 | |
| Cn-AFP | *Chaetoceros neogracile* | 0.8 [40 μM] | 26.2 | *E. coli* | n.d. | |
| FcAFP | *Fragilariopsis cylindrus* | 0.9 [230 μM] | 25.939 | *Fragilariopsis cylindrus* | 97.1 | |
| TisAFP | *Typhula ishikariensis* BRB-1 | 1 [1 mM] | 22 | *Typhula ishikariensis* | n.d. | |
| FfIBP | *Flavobacterium frigoris* PS1 | 2.5 [50 μM] | 25.715 | *E. coli* | n.d. | |
| 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. | |
| ColAFP | *Colwellia sp.* SLW05 | 3.8 [0.14 mM] | 26 | *Colwellia sp.* SLW05 | n.d. | |

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

(a)




(b)

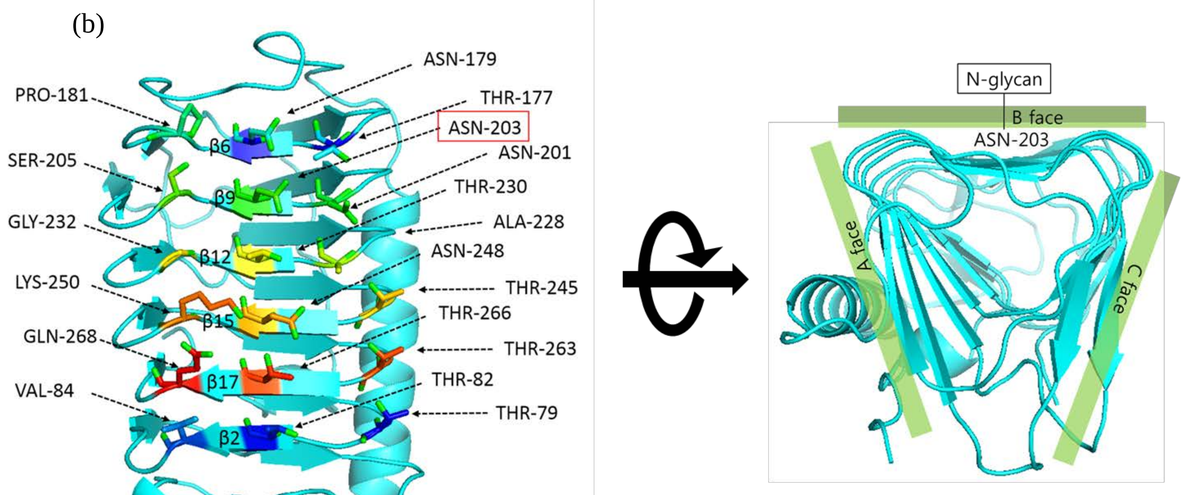
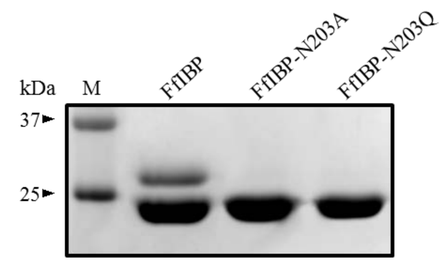



**Fig. 3.** Identification of N-glycosylation site in FfIBP. (a) amino acid sequence of FfIBP with a potential N-glycosylation site (Asn-X-Ser/Thr). Putative N-glycosylation site is underlined and (b) ice-binding residues on the B face of FfIBP. N-glycosylation site was marked with a red box.

(a)




(b)

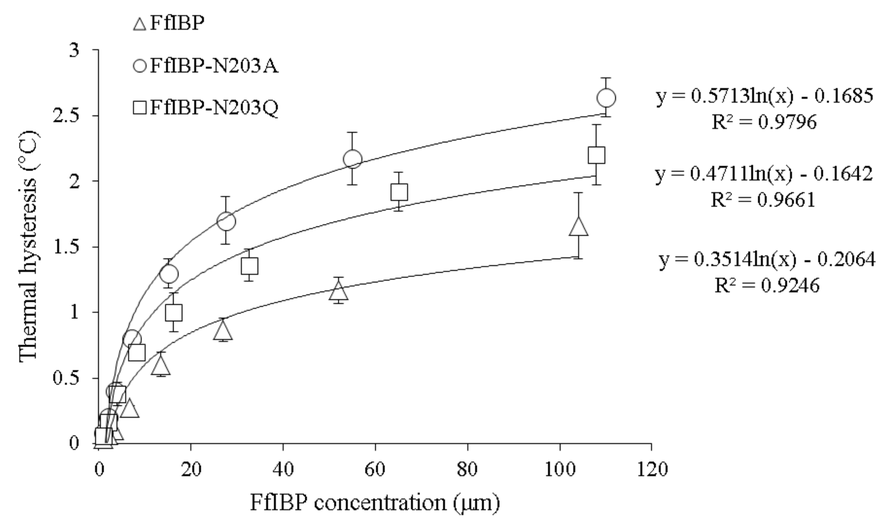



**Fig. 4.** Comparison of expression and activity of glycosylated and nonglycosylated FfIBP. (a) expression of mutant FfIBPs, N203A, and N203Q. The secreted proteins were purified and concentrated from supernatant of culture medium. The mutant FfIBPs showed a single band of protein. Lane M, size marker;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

## 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.

## Acknowledgement

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