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Low-Temperature Increases the Yield of Biologically Active Herring Antifreeze Protein in *Pichia pastoris*

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Antifreeze proteins and antifreeze glycoproteins are structurally diverse molecules that share a common property in binding to ice crystals and inhibiting ice crystal growth. Type II fish antifreeze protein of Atlantic herring (*Clupea harengus harengus*) is unique in its requirement of Ca^{2+} for antifreeze activity. In this study, we utilized the secretion vector pGAPZα A to express recombinant herring antifreeze protein (WT) and a fusion protein with a C-terminal six-histidine tag (WT-6H) in yeast *Pichia pastoris* wild-type strain X-33 or protease-deficient strain SMD1168H. Both recombinant proteins were secreted into the culture medium and properly folded and functioned as the native herring antifreeze protein. Furthermore, our studies demonstrated that expression at a lower temperature increased the yield of the recombinant protein dramatically, which might be due to the enhanced protein folding pathway, as well as increased cell viability at lower temperature. These data suggested that *P. pastoris* is a useful system for the production of soluble and biologically active herring antifreeze protein required for structural and functional studies.

Antifreeze proteins (AFPs)[2] and antifreeze glycoproteins (AFGPs) play an important role in protecting many marine fishes from freezing in icy seawater. They are capable of binding to ice crystals, inhibiting ice recrystallization, and exhibiting freezing point depression. One of the most attractive applications of AFPs is to introduce them into other organisms that originally do not have natural cold protection so that they can survive in a colder environment. Transgenic expression of AFPs in fish and plants has been performed for this purpose (1–3). In addition, AFPs can also be used in cryopreservation of material of biomedical interest and in cryosurgery (4–6). Five types of AFPs/AFGPs have been reported and characterized. Despite their similar function to bind ice crystal and inhibit ice crystal growth, these proteins are structurally diverse (7). Type II AFPs, isolated from herring (*Clupea harengus*

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[2] Abbreviations used: AFP, antifreeze protein; AFGPs, antifreeze glycoproteins; BYPD, buffered yeast extract peptone dextrose medium; C-type, calcium-dependent; DNA, deoxyribonucleic acid; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; hAFP, herring antifreeze protein; HIC, hydrophobic interaction chromatography; HPLC, high-pressure liquid chromatography; Ni–NTA, nickel–nitrilotriacetic acid; OD, optical density; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetate; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, tris-(hydroxymethyl)aminomethane; YPD, yeast extract peptone dextrose medium; WT, recombinant wild-type herring antifreeze protein; WT-6H, recombinant wild-type herring antifreeze protein with a six-histidine tag at C-terminus.

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harengus), smelt (*Osmerus mordax*), and sea raven (*Hemitripterus americanus*), are cystine-rich proteins that have significant amino acid sequence similarity to the carbohydrate-recognition domain of calcium-dependent (C-type) lectins. Herring AFP (hAFP) consists of 130 amino acids with a molecular mass of 14.6 kDa. This single-chain protein contains five intrachain disulfide bonds and its antifreeze activity and conformation are Ca^{2+} dependent (8–10).

Previously, expression of hAFP in *Escherichia coli* has resulted in the formation of inactive inclusion bodies, which required further manipulation to gain biological activity (10). The goal in developing an efficient expression system for hAFP is to facilitate the biochemical and biophysical characterization of hAFP and its mutants to further our understanding of its mode of action, including the elucidation of its ice-binding mechanism. Since *E. coli* expression systems are generally not suitable for disulfide-rich proteins due to the reducing environment of the cytoplasm, we explored whether a properly folded and functional hAFP could be successfully expressed in yeast *P. pastoris*. It is known that *P. pastoris* has been successfully utilized to produce large quantities of disulfide-containing proteins (11–13). It has the potential for high expression levels, efficient secretion, proper protein folding, disulfide bond formation, and glycosylation. Furthermore, it is a robust fermentation organism capable of high cell density in simple inorganic media.

The vector we chose was pGAPZα A, which allows recombinant protein to be expressed constitutively using the promoter of the gene GAP, which encodes the glyceraldehyde-3-phosphate dehydrogenase (14). The recombinant protein will also be secreted into the medium directly using the *Saccharomyces cerevisiae* α-factor secretion signal peptide (15, 16). It is known that *P. pastoris* itself secretes very few proteins to the medium (17, 18). Thus, secretion of the recombinant protein into the culture medium facilitates protein purification.

In this study, we described the successful expression of hAFP in *P. pastoris* and demonstrated that the recombinant hAFPs can be secreted into the culture medium and exhibit full antifreeze activities comparable with the native protein. Furthermore, we found that expression of recombinant hAFP at a lower temperature increased the production dramatically.

MATERIALS AND METHODS

Materials. *P. pastoris* expression vector pGAPZα A as well as yeast strains X-33 and SMD1168H were from Invitrogen (San Diego, CA) (19). *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). Restriction enzymes and ligase were from New England Biolabs Ltd. (Beverly, MA) and Gibco BRL (Gaithersburg, MD). Media components were purchased from Difco (Detroit, MI). *E. coli* strain DH5α was used for DNA subcloning. Nickel–nitrilotriacetic acid (Ni–NTA) agarose was purchased from Qiagen Inc. (Valencia, CA). Prestained protein molecular weight standards (low range) were from Gibco BRL. Ruthenium red was from Fluka (St. Louis, MO). Endoproteinase Glu-C was obtained from Boehringer Mannheim (Laval, Quebec, Canada). All other chemicals were reagent grade.

Construction of yeast expression systems. The hAFP gene excluding the putative N-terminal signal sequence was PCR amplified from hAFP cDNA (9). The primers used for WT were 5′GGCCGAATTCGCTGATGAATGTCCCACTGATTGGAAG3′ as the sense primer and 5′GCGCTCTAGATCATTTCAGTGGCTTGGCGCAGATTGA3′ as the antisense primer. *Eco*RI and *Xba*I endonuclease sites were included in these two primers respectively to facilitate subsequent cloning steps. For construction of WT-6H, a construct (WT-myc6H) with C-terminal tag containing a *myc* epitope and six histidine residues presented in the vector was generated first. Similar to the construction of WT, WT-myc6H was PCR amplified by using the same sense primer as WT and 5′GCGCTCTAGAGCTTTCAGTGGCTTGGCGCAGATTGA3′ as antisense primer. The PCR products were inserted in frame with an upstream yeast α-factor secretion signal sequence between *Eco*RI and *Xba*I cloning sites in the vector pGAPZα A. In order to further generate the construct of WT-6H, two complementary oligonucleotides containing nucleic acid sequence coding for a six-histidine tag and a stop codon with *Xba*I and *Sal*I sites were designed. The two primers used were 5′CTAGACCATCACCATCACCATCACTGAG3′ and 5′TCGACTCAGTGATGGTGATGGTGATGGT3′, respectively. After phosphorylation, complementary oligonucleotides were annealed and ligated into the construct of WT-myc6H, which had been cut by *Xba*I and *Sal*I restriction enzymes. All constructs were transformed into *E. coli* DH5α and selected with 25 μg/ml Zeocin. Plasmid DNAs from these constructs were isolated and hAFP genes were confirmed by DNA sequencing.

Transformation of P. pastoris. Plasmids prepared from the Qiagen plasmid midi kit were linearized with *Avr*II and integrated into pretreated *P. pastoris* strain X-33 (wild type) or a protease-deficient strain SMD1168H by electroporation using a gene pulser apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions from Invitrogen. Transformed colonies were selected on YPD agar plates (1% yeast extract, 2% each of peptone, dextrose, and agar) containing 100 μg/ml Zeocin and incubated at 30°C for 3–4 days. Colonies containing hAFP inserts were identified by PCR.

Analysis of P. pastoris transformants. Direct PCR

was used to screen genomic integration of the expression constructs in *P. pastoris* following the procedure of Linder *et al.* (20) with modification (F. Xiong, unpublished result). A colony was picked and suspended in 10 μl of water. Five microliters of a 5 U/liter solution of lyticase (Sigma, St. Louis, MO) was added, mixed, and incubated at 30°C for 10 min. The samples were frozen at −80°C for an additional 10 min and then diluted with 30 μl of water. Five microliters of each sample was used for PCR.

Comparison of hAFP expression in different host strains. Positive colonies of WT in strains X-33 and SMD1168H were chosen to inoculate 5 ml of YPD and grew at 30°C in a shaking incubator at 250 rpm overnight. One-hundred microliters of the overnight culture was used to inoculate 50 ml of buffered YPD (BYPD, YPD with 0.1 M potassium phosphate buffer, pH 6.0) in a 250-ml shake flask and incubated at 30°C. At different time intervals, 1 ml of the expression culture was transferred to a 1.5-ml microcentrifuge tube and the supernatant was frozen. These samples were used to analyze protein expression levels and the optimal time to harvest.

Comparison of hAFP expression at different temperatures. Positive colonies of WT-6H in SMD1168H strain were selected to inoculate 5 ml of YPD and grew at 23 or 30°C overnight in a shaking incubator, respectively. These overnight cultures were adjusted to the same OD_{600} and 100 μl of each culture was used to express hAFP in 50 ml of BYPD at 30 and 23°C, respectively. Samples were collected at different time intervals and analyzed. Cell density was monitored spectrophotometrically at 600 nm. For viable cell analysis, the media collected were diluted and plated on the YPD agar plates with 100 μg/ml Zeocin. All samples were performed in triplicate and the viable cells were counted after colony formation. Expression levels of hAFP were detected by Western blot using the culture media after removal of the cells. Densitometry analysis of the Western blot results was performed with Scion Image software (www.scioncorp.com).

Scale-up of hAFP expression. Positive colonies of the two constructs in SMD1168H strain were used to inoculate 5 ml of YPD and were grown overnight. One milliliter each of these overnight cultures was used to inoculate a 1-L volume of BYPD. The cultures were grown at 23°C for both recombinant proteins. After 2 days, cells were harvested by centrifugation at 6000*g* for 10 min at 4°C and the supernatant fractions were saved.

Total protein analysis. Total protein was measured by using the Bio-Rad protein assay based on the Bradford method (Bio-Rad).

Tricine SDS–PAGE and Western blot analysis. The recombinant hAFPs were analyzed by tricine SDS–PAGE, using 4% acrylamide stacking gel and 16% separating gel, and detected by silver staining or Coomassie blue staining.

Western blot analysis was performed as the manufacturer's instructions using ECL Western blotting analysis system (Amersham Pharmacia Biotech, Quebec, Canada). Anti-hAFP antibody raised in rabbit was used as the primary antibody followed by an anti-rabbit secondary antibody conjugated to horseradish peroxidase. The proteins were detected using ECL detection reagents.

Purification of recombinant hAFPs. The supernatants containing hAFP were precipitated by incubation with 31.3 g ammonium sulfate per 100 ml of culture medium at 4°C overnight. For purification of WT, proteins were desalted with a Sephadex G-25 column first. Further purification was performed with FPLC hydrophobic interaction chromatography (HIC), using a Phenyl–Superose HR5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was performed using a linear gradient of 1.7–0 M ammonium sulfate in 20 mM potassium phosphate, pH 6.0. Final purification was achieved by reverse-phase HPLC, using a Jupiter 10-μm C4 300-Å column, 250 × 21.20 mm (Phenomenex, Torrance, CA) with a gradient of 40–46% acetonitrile in 0.06% TFA.

As for WT-6H, the ammonium sulfate precipitate was resuspended in 20 mM Tris–HCl, pH 8.0, and purified by Ni–NTA chromatography as described by the QIAexpressionist (Qiagen). After binding and washing, the bound protein was eluted with 100 mM imidazole in 20 mM Tris–HCl, containing 0.5 M NaCl, pH 8.0. These products were desalted and further purified by reverse-phase HPLC C4 column using a gradient of 40–43% acetonitrile in 0.06% TFA.

Purified proteins were characterized by tricine SDS–PAGE or matrix-assisted laser desorption/ionization mass spectrometry (Carbohydrate Analysis Facility, University of Toronto).

Ruthenium red staining of recombinant hAFPs. Both purified recombinant hAFPs were run on tricine SDS–PAGE under nonreduced conditions and electrophoretically transferred to a 0.2-μm nitrocellulose membrane and stained with ruthenium red (10).

Proteolysis protection assay. A proteolysis protection assay was performed on both recombinant hAFPs in 20 mM Tris–HCl, pH 8.0, with 1 mM $CaCl_2$ or 5 mM EDTA, containing 0.2 mg/ml endoproteinase Glu-C for 1 h at 21°C (10). The reaction mixtures were resolved on nonreduced tricine SDS–PAGE and stained with Coomassie blue.

Protein N-terminal sequencing. Purified WT-6H and its Glu-C digested product in the presence of Ca^{2+} were transferred to a PVDF membrane after tricine

SDS–PAGE. After staining the membrane with Coomassie blue, the bands containing hAFP and the Glu-C-digested hAFP were cut out and subjected to automated Edman degradation (Biotech Service Center, Hospital for Sick Children, Toronto).

Assay of antifreeze activity. Antifreeze activities of the recombinant and native hAFPs were measured by using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) as described by Chakrabartty *et al.* (21). Measurements for each sample were performed in triplicate from three different sample wells.

RESULTS AND DISCUSSION

Construction of plasmids for expression of hAFP in P. pastoris. Two constructs (WT and WT-myc6H) encoding mature hAFPs were generated by PCR amplification using hAFP cDNA as a template. WT-myc6H contained a *myc* epitope and six-histidine tag at its C-terminus. The construction of WT-6H was created based on WT-myc6H by inserting a six-histidine tag and a stop codon between the *Xba*I and *Sal*I sites. The final constructs generated in the *P. pastoris* vector pGAPZα A are shown in Fig. 1. Two strains of *P. pastoris*, wild-type X-33 and the protease-deficient strain SMD1168H,

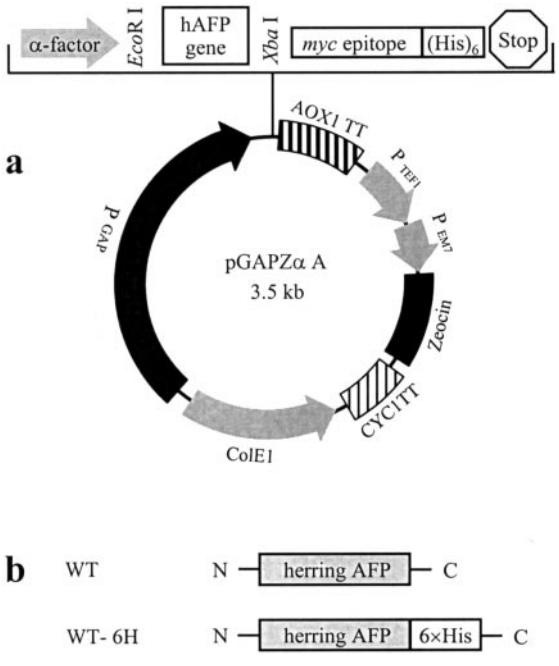

FIG. 1. (a) pGAPZα A expression construct. P_{GAP}, GAP promoter region. AOX1 TT, alcohol oxidase transcription termination region. P_{TEF1}, promoter region of transcription elongation factor 1. P_{EM7}, promoter conferring Zeocin resistance. Zeocin, a selectable marker. CYC1 TT, CYC1 transcription termination region. ColE1 origin, allowing replication and maintenance of the plasmid in *E. coli*. (b) Construction of the two recombinant hAFPs. WT and WT-6H were designed to express mature hAFP and mature hAFP with a six-histidine tag, respectively.

were used. *P. pastoris* transformants were selected with Zeocin and analyzed for the presence of the inserts by PCR (data not shown).

Time course studies of hAFP expression in P. pastoris. The time courses of the WT expressed in both wild-type strain X-33 and the protease-deficient strain SMD1168H were examined by tricine SDS–PAGE and Western blot (Fig. 2). These results demonstrated the recombinant WT was expressed successfully in both *P. pastoris* strains as secreted protein. The secretion of recombinant WT was detectable on day 1 and reached maximal level after 2 days when expressed in the X-33 strain. Expression of the WT in SMD1168H strain was not detectable on day 1, yet also reached maximum after 2 days. Both strains had similar expression levels except that less partially degraded proteins were detected in the medium of the protease-deficient strain SMD1168H as indicated by the smear bands below those of the intact proteins in strain X-33. Proteases presented in the culture medium might be released by the dead cells. As SMD1168H strain is deficient in protease A which further causes a deficiency in carboxypeptidase Y and protease B, the proteases released into the medium were less compared with the wild-type strain; thus less degradation of the recombinant hAFP was observed.

It was also noticed that the recombinant hAFP ran faster than 14.3-kDa molecular weight marker although its molecular mass was predicted to be 15,293 Da. This was because the markers we used were prestained and their mobilities were not consistent with the nonstained ones, especially in tricine SDS–PAGE. Similar phenomena were also observed on WT-6H as shown in Figs. 3 and 6.

Effects of temperature on expression of hAFP. In order to facilitate the purification process, hAFP with a six-histidine tag (WT-6H) in strain SMD1168H was generated. Temperature effect was tested to optimize the expression of WT-6H. First of all, the time courses

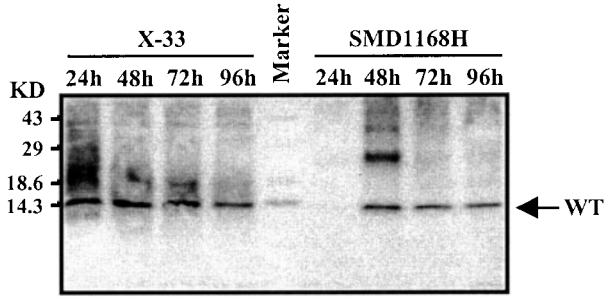

FIG. 2. Time course of expression of WT in *P. pastoris* strains X-33 and SMD1168H as detected by Western blot. The cells were cultured at 30°C and 40 μl each of the supernatants of the culture media were loaded to the gel. Anti-hAFP antibody was used as described under Materials and Methods. The arrow indicates the recombinant WT.

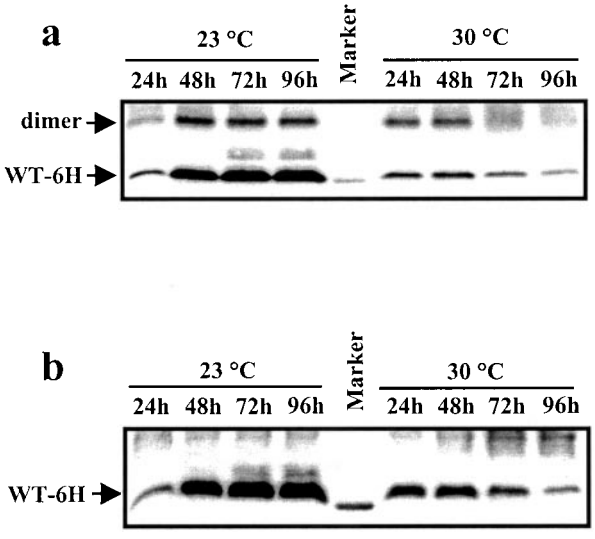

FIG. 3. Time course of WT-6H expression in *P. pastoris* strain SMD1168H as detected by Western blot after (a) nonreduced tricine SDS–PAGE and (b) reduced tricine SDS–PAGE. The cells were cultured at 30 or 23°C and 20 μl each of the supernatants of the culture media was loaded to the gel. The monomer and dimer of recombinant hAFPs were indicated. The marker shown is 14.3-kDa protein standard.

of expression of WT-6H at 23 and 30°C were compared (Fig. 3). In the nonreduced tricine SDS–PAGE (Fig. 3a), both monomer and dimer of recombinant hAFPs could be detected. However, only monomer could be detected after reducing the samples by β-mercaptoethanol (Fig. 3b), indicating that the dimer was caused by the formation of intermolecular disulfide bonds between the hAFPs. These results demonstrated that expression at a lower temperature increased the yield of hAFP significantly and reduced dimer formation simultaneously. It also showed that WT-6H was degraded after 2 days when expressed at 30°C, but not at 23°C. Moreover, expression of WT-6H at 23°C could be detected directly by Coomassie blue staining, while its expression at 30°C could only be detected by silver staining (data not shown), further confirming the dramatic difference in expression levels between these two temperatures.

In order to investigate whether the degradation of WT-6H at a higher temperature was caused by the release of proteases from the dead cells, the viability of cells cultured at 23 and 30°C was compared. As shown in Fig. 4, cells grew faster at 30°C and entered the stationary phase after 60 h. Generally, cells grown at 23°C were more viable compared to those with the same cell densities at 30°C. At 30°C, the amount of viable cells dropped after 3 days and the expression of hAFP was less that 5 mg/liter. As for 23°C, the amount of viable cells kept increasing even after 4 days with increased production of hAFP. Densitometry assay of the Western blot result indicated that the yield of WT-6H

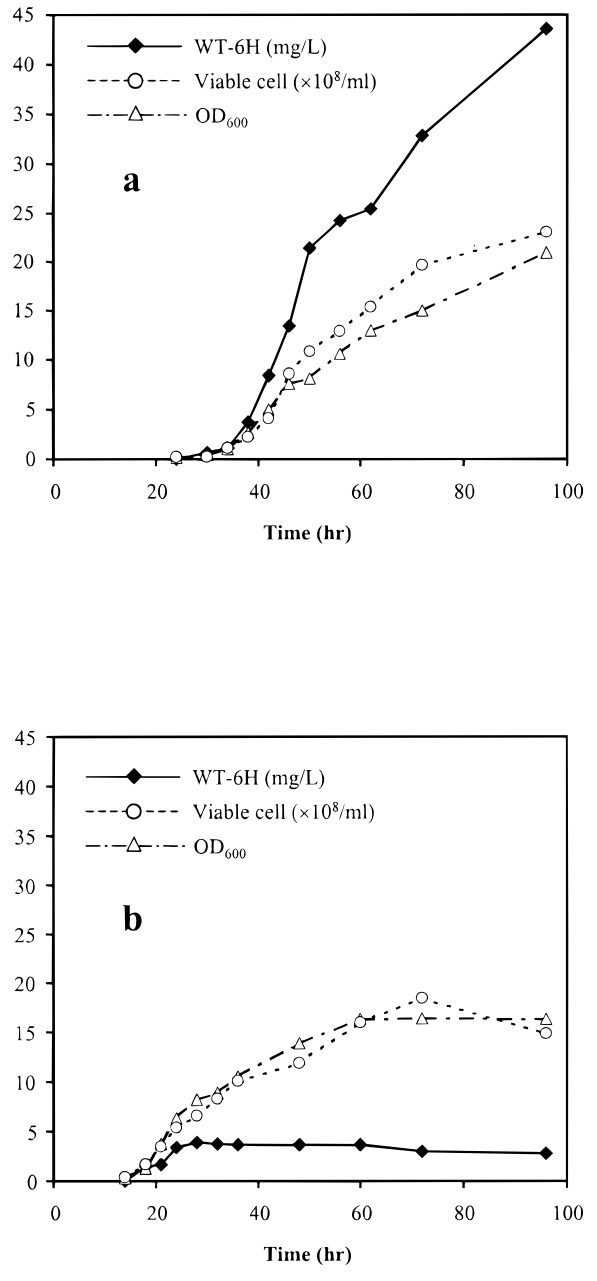

FIG. 4. Effects of temperature on cell growth and hAFP production. Cell densities (△), viable cell amount (○), and expression of recombinant WT-6H (◆) were detected at (a) 23°C and (b) 30°C, respectively.

expressed at 23°C increased about 10 times compared with that expressed at 30°C after 4 days.

The significant increase of hAFP expression level at a lower temperature might benefit from better protein folding. Low temperatures have been shown to improve the solubility of heterologous proteins in *E. coli* (22–25). Cultivating the cells at lower growth temperatures reduces the rate of protein synthesis and thus may allow more time for the nascent peptide chains to fold properly. hAFP may be susceptible to misfolding for a variety

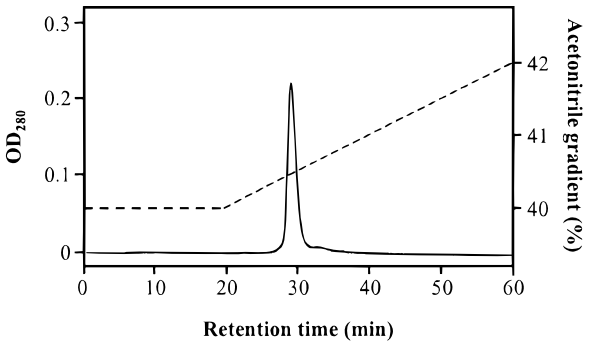

FIG. 5. Purification of WT-6H using reverse-phase HPLC C4 column. The gradient was 40–43% acetonitrile in 0.06% TFA with a flow rate of 4 ml/min. Solid line represents UV absorbance at 280 nm and dashed line indicates the acetonitrile gradient.

of reasons including the formation of intermolecular disulfide bonds and exposure of its hydrophobic surfaces. It has been reported that intermolecular disulfide bonds are preferentially formed at higher temperatures when protein is expressed in *E. coli* (26, 27). The involvement of sulfhydryl group shuffling will result in aggregate formation. This would at least partially explain why expression of WT-6H at 23°C showed a much higher yield of monomer than that of dimer compared with the expression at 30°C. Moreover, higher temperature may also lead to exposure of more hydrophobic surfaces during peptide folding and favor hydrophobic interaction and thus may predispose hAFP to aggregate formation. And the misfolded and aggregated proteins are more susceptible to the intracellular proteolytic degradation (28, 29). Therefore, expressing at a lower temperature may help reduce protein misfolding and produce more properly folded protein to be secreted into the culture medium.

Besides enhancing the proper folding pathway, an added benefit of lowering expression temperature is to reduce the proteolytic degradation of the recombinant hAFP in the culture medium. Our experiment indicated that cell viability of the culture at 23°C was higher compared with that of the same cell density at 30°C, probably due to the stabilization of cell membrane at lower temperature. This would reduce the amount of proteases released from the dead cells and thus reduce the degradation of the secreted protein. This information will be useful to optimize the production of hAFP by fermentation as one needs to consider cell viability besides cell density to achieve higher production of proteolytically sensitive proteins. In general, low-temperature expression may be widely applicable to increase the yields of aggregation-prone and/or unstable gene products in *P. pastoris*.

Purification of recombinant hAFPs. Proteins precipitated by ammonium sulfate from the culture medium were purified by HIC or Ni–NTA agarose column and further purified by reverse-phase HPLC C4 column. A single peak with a retention time of 29.65 min of WT-6H was obtained after reverse-phase HPLC

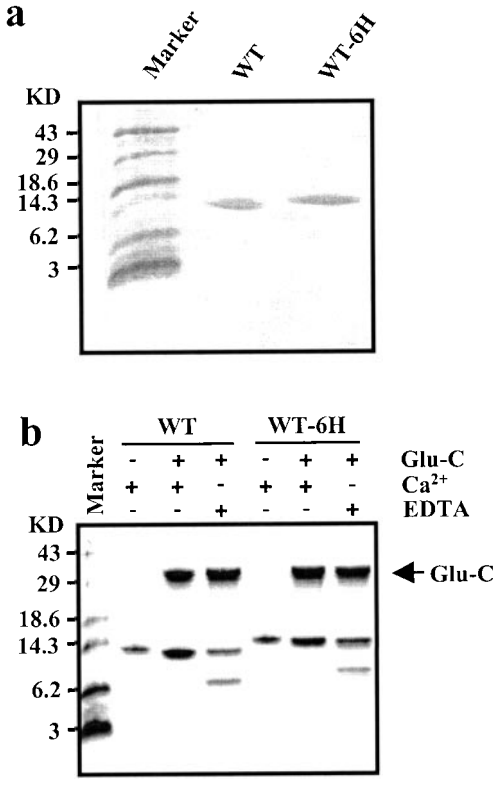

FIG. 6. Ca^{2+} dependence of recombinant hAFPs. (a) Staining of hAFPs with ruthenium red. hAFPs (1.5 μg) were run on tricine SDS–PAGE, transferred to a nitrocellulose membrane, and stained with ruthenium red. (b) Proteolysis of recombinant hAFPs in the presence and absence of Ca^{2+}. Glu-C was added to 1.5 μg of recombinant hAFPs in the presence of Ca^{2+} or EDTA. Proteins were resolved by tricine SDS–PAGE and stained with Coomassie blue.

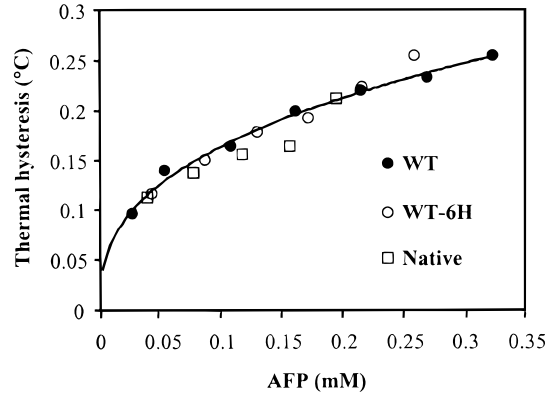

FIG. 7. Antifreeze activities of WT and WT-6H. Concentration-dependent antifreeze activities were measured as described under Materials and Methods using a Clifton nanoliter osmometer. Values shown represent means of triplicate measurements done on each single sample.

TABLE 1

Purification of Recombinant hAFPs Secreted by *P. pastoris*

Purification steps	Total protein (mg)		hAFP (mg)		Purity (%)		Fold purification		Yield (%)	
	WT	WT-6H	WT	WT-6H	WT	WT-6H	WT	WT-6H	WT	WT-6H
Culture medium	57	64	5.3	18	9.3	28.1	1	1	100	100
Ammonium sulfate precipitate	8.7	9.6	1.9	6.7	21.9	69.8	2.4	2.5	35.8	37.2
HIC/Ni–NTA	1.75	4.6	0.88	4.2	50.3	91.3	5.4	3.3	17	23.3
HPLC C4	0.22	2.3	0.22	2.3	~100%	~100%	10.8	3.6	4	12.8

(Fig. 5). The purity of the recombinant proteins demonstrated by tricine SDS–PAGE is also provided in the lanes of WT and WT-6H with Ca^{2+} only in Fig. 6b. The purification procedures of WT and WT-6H are depicted in Table 1. The molecular mass of WT-6H was determined by mass spectrometry as 16,404 Da (data not shown), which was not fully consistent with the predicted mass of 16,005 Da. However, it agreed with the addition of four amino acid residues EAEA at the N-terminus of WT-6H, which was further confirmed by N-terminal sequencing. This result indicated the cleavage by the STE13 gene product, which is used to remove the Glu-Ala repeats, was not efficient (30). Among other possibilities, it might be due to the fact that the amino terminus of hAFP was not readily accessible by the STE13 protein or the expression level of STE13 protein was low.

Characterization of recombinant hAFPs. The recombinant hAFPs were tested whether they were properly folded and functioned like the native protein. First of all, the Ca^{2+}-binding ability of purified recombinant hAFPs was analyzed by using ruthenium red, which can stain Ca^{2+}-binding protein specifically (10). Ruthenium red staining result demonstrated that both WT and WT-6H also bind Ca^{2+}, as the native protein does (Fig. 6a).

Native hAFP has been shown to resist to Glu-C proteolysis upon Ca^{2+} binding (10). Tricine SDS–PAGE analysis showed that both WT and WT-6H were also resistant to be extensively digested in the presence of protease Glu-C and Ca^{2+} just like the native protein (Fig. 6b). However, the mobility of these bands appeared to be faster compared with the undigested ones. N-terminal sequencing revealed that the nature of the faster mobility was due to the removal of three amino acids (EAE) of WT-6H at its N-terminus. And it also showed heterogeneity of recombinant hAFP at the N-terminus with a minor fraction containing only two additional amino acid residues (EA) at its N-terminus caused by the partial removal of the first two amino acid residues by the STE13 gene product.

Antifreeze activity assay demonstrated that both recombinant hAFPs exhibited antifreeze activity comparable to the native hAFP (Fig. 7). This result indicated that the C-terminal histidine tag has a minimal effect on the antifreeze activity of hAFP.

In conclusion, the successful secretion of fully active recombinant hAFP in *P. pastoris* should allow large-scale production of the recombinant protein by fermentation technology and should facilitate studies aimed at deciphering its protein structure and biological functions.

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