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Challenges in the expression of disulfide bonded, threonine-rich antifreeze proteins in bacteria and yeast

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Abstract

Certain freeze-intolerant insects produce antifreeze proteins (AFPs) during overwintering including the spruce budworm (*Choristoneura fumiferana*) and yellow mealworm (*Tenebrio molitor*) AFP gene families. However, only a few of the isoforms, encoded by their multiple-copy gene families, have been characterized. When expressed in bacterial systems the insect AFPs have to be denatured and refolded in vitro, a procedure that is not uniformly successful, presumably due to the β-helix structure and the requirement for disulfide bonds. In an attempt to overcome these difficulties, bacterial vectors and hosts that have been developed to produce soluble, folded proteins, as well as a yeast expression system (*Pichia pastoris*) were employed. Bacterial expression resulted in low quantities of active recombinant protein for certain isoforms. In contrast, both small and large-scale fermentation of recombinant AFP in *Pichia* yielded substantial protein production ($\geqslant 100$ mg/L) but functional ice binding activity of protein produced in three different transformed yeast strains (KM71, X33 or GS115) was low. Inappropriate O-linked glycosylation of the Thr-rich AFPs appeared to be partially reversed by mild chemical deglycosylation, but activity remained low. Substantial quantities, as well as activity were recovered when a fish AFP, with disulfide bonds, but without potential Thr glycosylation sites was expressed in the yeast system.

Keywords: Antifreeze protein; Bacteria; Fermentation; *Pichia pastoris*; Protein expression; Yeast

Some freeze-avoiding insects depend on the presence of hyperactive antifreeze proteins (AFPs)[1] during their overwintering stages. AFPs adsorb to seed ice crystals resulting in a lowering of the freezing point of the hemolymph, thus avoiding mechanical and osmotic shock, damage that would result from generalized ice formation. Spruce budworm AFP is a left-handed helix with 15 amino acids per turn, the repeats form the "ice binding face" along one side of the molecule with a regular, two-dimensional array of Threonine (Thr) residues. The activities of AFPs are assessed by thermal hysteresis (TH) measurements at given AFP concentrations. AFPs lower the non-equilibrium freezing point creating a measurable temperature gap; TH is the difference between the melting point and the freezing point temperatures of a solution [1–3].

AFPs have much potential to improve the rapid freezing and thawing of foods, and techniques are being developed using AFPs that could improve perishable product storage [4]. Furthermore, recombinant AFPs may be potentially important in cancer treatment [5], as well as the low temperature storage of organs, tissues, membranes, and cells [6–9]. The testing, development and use of AFPs

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[1] *Abbreviations used:* AFPs, antifreeze proteins; AOX, alcohol oxidase; YPDS, yeast extract peptone dextrose sorbitol; BMGY, buffered minimal glycerol medium; BMMY, buffered minimal methanol medium; IPTG, isopropyl-β-D-thiogalactopyranoside.

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for various applications requires large amounts of active protein. However, the production, collection, and analysis of insect AFP isoforms are hindered by the small size of the overwintering stages and the large numbers of isoforms (~24 and 12 chromatographic peaks for TmAFP and CfAFP, respectively). To overcome the paucity of protein, AFP isoforms have been subcloned, and a few isoforms have been expressed and purified successfully in *Escherichia coli* [10,11]. However, due to the requirement for disulfide bond formation for the β-helix of these insect AFPs, bacterial expression results in inactive recombinant protein. The protein must then be denatured, refolded in the presence of guanidinium and then repurified to obtain active, correctly folded AFP [12]. This procedure has been successful for only a few isoforms, and potentially useful AFPs remain uncharacterized.

In recent years alternative vectors and mutant *E. coli* hosts have been developed for the production of highly structured proteins. As well, eukaryotic hosts, such as yeast have been used for the production of heterologous proteins containing disulfide bonds [13]. The methylotrophic yeast, *Pichia pastoris*, is noted for its high productivity of recombinant proteins in batch or continuous fermentation protocols [14,15]. The advantage of *P. pastoris* over the budding yeast, *Saccharomyces cerevisiae*, is that it does not ferment sugars to ethanol, which can accumulate and repress protein production. Rather, the methylotrophic yeast utilizes methanol and the first step in methanol metabolism employs alcohol oxidase (AOX). One of the two AOX gene sequences has been developed as a highly expressed promoter for heterologous protein expression; inappropriate glycosylation can occur, but it is reported to be less problematic than with the budding yeast *S. cerevisiae* [16]. As a consequence *P. pastoris* has been used to successfully express high levels of a variety of different proteins such as the production of biomaterials, therapeutic proteins, and vaccines [17,18].

Because of these recent successes with both modified bacterial expression systems and yeast, it was anticipated that one or both of these systems would be appropriate for the expression of "recalcitrant" insect AFPs. We report here our efforts to use these systems for small, tightly folded, β-helical proteins, and show that although activity was recovered for some isoforms in the newer bacterial systems, and high productivity was seen with yeast, neither system provided acceptable levels for both activity and production of insect AFPs, but was successful with a fish AFP.

Materials and methods

Bacteria and yeast strains

Escherichia coli XL1-Blue, TOP10 and JM83, were used for plasmid construction and purification, and *E. coli* BL21-DE3 cells were used for bacterial expression. *E. coli* were grown on standard Luria Broth (LB) or minimal dextrose (MD) media and agar plates. Antibiotics, ampicillin or zeocin, were added at a concentration of 100 μg/mL. Whenever zeocin was used for selection, a low sodium medium (LB low sodium or yeast extract peptone dextrose sorbitol (YPDS) broth, pH 7.5) replaced standard medium as described by Invitrogen [19].

Methylotrophic yeast strains, GS115 (his4), KM71 and X33 (Invitrogen, San Diego, CA) were used for both small and large-scale AFP expression studies. Yeast was cultured as described [19]. Routinely, yeast was grown in buffered minimal glycerol medium (BMGY): 10 g yeast extract, 20 g meat peptone, 13.4 g yeast nitrogen base without amino acids, 0.4 mg biotin, and 10 mL glycerol in 100 mM potassium phosphate buffer (pH 6.0) per liter. Buffered minimal methanol medium (BMMY) contained the same components as BMGY except that methanol (5 mL/L) was used in place of glycerol. Minimal methanol medium (MM) contained 13.4 g yeast nitrogen base without amino acids, 0.4 mg biotin, and 5 mL methanol per liter. Fermentation media used for large-scale growth contained 50 g glycerol, 20 g ammonium sulfate, 12 g KH_2PO_4, 4.7 g $MgSO_4$, 0.36 g $CaCl_2$ per liter with the addition of recommended trace elements.

Escherichia coli plasmid constructs and bacterial expression

AFP sequences for *E. coli* expression were from the spruce budworm, *Choristoneura fumiferana* (Cf). Several AFP cDNA sequences (isoforms 337, 501, 10, and others listed in Results) were cloned into pET20b (Novagen, Madison, Wisconsin) as previously described [20] or into pET102 (Invitrogen) in an analogous manner (Fig. 1A). All inserts were sequenced to confirm their veracity and orientation. These constructs were used to transform *E. coli* stocks, BL21-DE3 and Origami-DE3 (Novagen), using the calcium chloride method, as previously described [21]. Subsequently, a single bacterial colony was used to inoculate a 2 mL culture that was then used to initiate 1 L cultures. Plasmids were maintained during growth by the inclusion of antibiotics in the medium of Ori-DE3 (kanamycin at 30 μg/mL; tetracycline at 12.5 μg/mL and ampicillin at 100 μg/mL) and BL21-DE3 (ampicillin at 100 μg/mL). *E. coli* strains bearing the various plasmids were cultured to log phase at 37 °C and induced for 4–8 h with 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG). The induced *E. coli* cells were then centrifuged and treated with Bugbuster reagent (Novagen), resuspended in the same reagent at 5 mL/g wet cell pellet and incubated at 22 °C for 20 min. The slurry was then centrifuged at 16,000*g* for 20 min at 4 °C. The supernatant, the soluble fraction, was stored at −20 °C.

Purification and folding of bacterially expressed proteins

AFPs from bacterial expression were either present in inclusion bodies or in the soluble fraction. Inclusion bodies were isolated from the remaining pellet by resuspension in Bugbuster reagent containing lysozyme (200 μg/mL final concentration) and incubation at 22 °C for 5 min. After

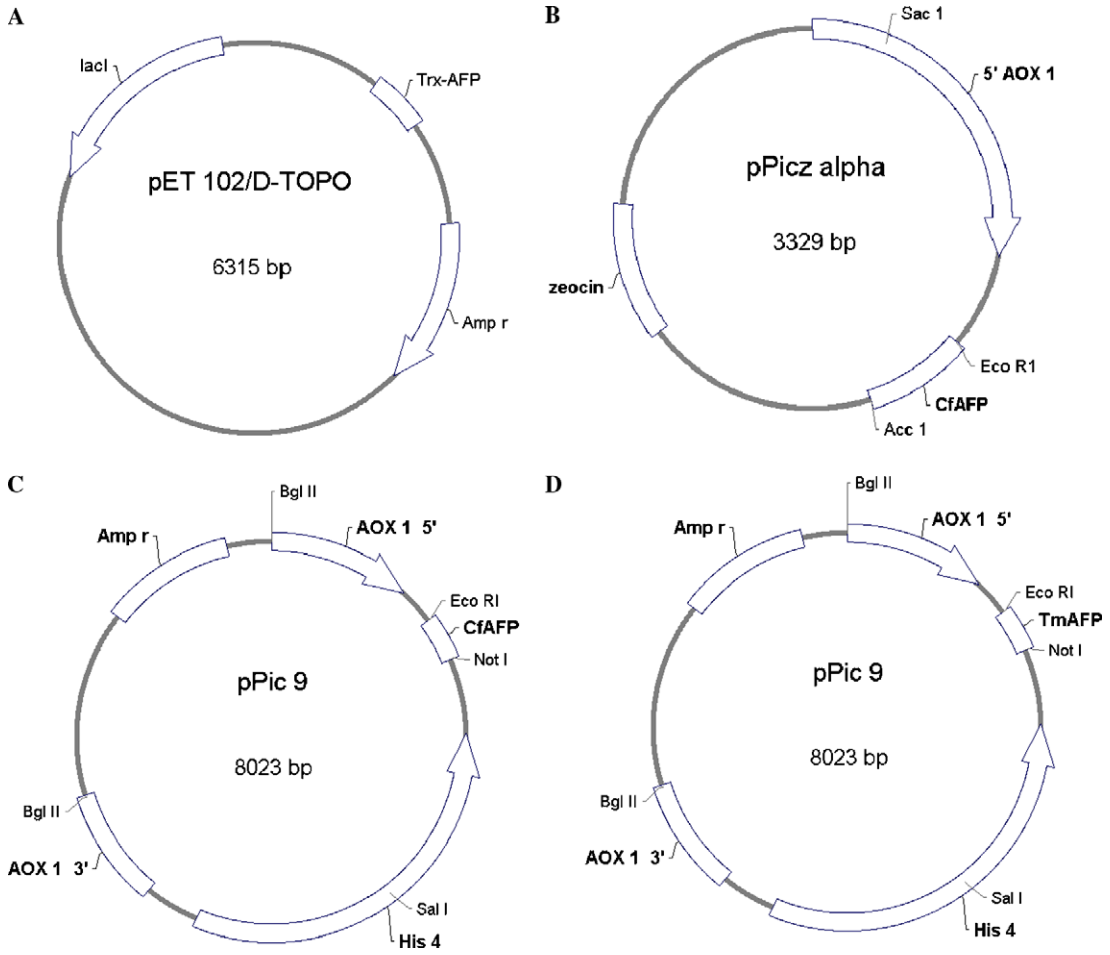

Fig. 1. Schematic diagrams of plasmid constructs used for AFP expression in bacteria with a thioredoxin fusion to CfAFP337 in vector pET102D (A); and yeast using CfAFP expressed in pPICZα (B); or CfAFP and TmAFP sublconed into pPIC9 (C and D).

dilution with 6 volumes of 1:10 Bugbuster reagent/1× phosphate buffered saline (PBS), the sample was vortexed briefly and subsequently centrifuged at 16,000*g*, as above. This wash step was repeated three more times and finally the pellet was suspended in 1–2 mL PBS. They were then denatured by exposure to 8 M guanidine–HCl, 100 mM Tris–HCl, 1 mM EDTA, 12 mM of 2-mercaptoethanol, pH 8 for several hours, and gently refolded over several days at 4 °C as previously described [12]. Western blot analysis was conducted on these samples; if protein aggregation occurred during this refolding, the aggregates were heated at 68 °C for 15 min and allowed to cool slowly and left at 4 °C overnight. Samples were either suspended in 0.1 M ammonium bicarbonate, pH 7.9 or dialyzed against this solution, prior to TH assay to determine activity.

Soluble recombinant proteins were purified by affinity chromatography using Talon resin (Clontech, Palo Alto, CA). Bacterial lysates were diluted 1:15 in binding buffer (20 mM Na-phosphate, 500 mM NaCl, pH 7.8) and incubated with the resin for 20 min at room temperature with gentle mixing. The resin was centrifuged and washed twice (20 mM sodium phosphate, 500 mM NaCl, and 5 mM imidazole, pH 6.0) before elution into the wash buffer, but with 200 mM imidazole. Imidazole was removed by dialysis into 1× PBS. Bacterial lysates from bacteria bearing a pET102D plasmid, and thus producing a thioredoxin-AFP, were further purified by ammonium sulfate precipitation of bacterial proteins at 53% ammonium sulfate. Soluble proteins remaining after precipitation were dialyzed to remove the salt, concentrated with Amicon filtration units and subsequently dialyzed against 100 mM ammonium bicarbonate (3 × 4 L). For a few experiments, ice affinity purification, as described by Kuiper et al. [22] was undertaken. Ice was allowed to grow slowly (−0.5 to −2.5 °C) around a brass "cold finger" immersed in 150 mL bacterial lysate (<1 mg/mL protein) while stirring [22]. When approximately 75 mL of ice were formed, the ice was rinsed briefly and allowed to melt. Proteins incorporated into the ice were concentrated by ultrafiltration.

Escherichia coli plasmid constructs for yeast expression

AFP sequences were derived from the spruce budworm, as described above, the yellow mealworm beetle, *Tenebrio molitor* (Tm) and the polar fish, sea raven (sr), *Hemitripterus americanus*. For yeast expression, the plasmid shuttle vector pPICZα was used to subclone mature CfAFP isoform 337 (GenBank Accession No. AF263009) in-frame

with the Kex2 signal cleavage and ste13 signal cleavage sites. AFP coding sequence was amplified by polymerase chain reaction (PCR) with the appropriate restriction sites and a Met residue at the amino terminus of the protein. AFP fragments were subcloned into the *Eco*RI–*Acc*I restriction site, in-frame, with the C-terminal 6-His tag (Fig. 1B). A second plasmid shuttle vector, pPIC9, was also used to subclone CfAFP isoform 337, TmAFP isoform YL-1 (GenBank Accession No. AF160494) (Figs. 1C and D), and the mature srAFP (GenBank Accession No. J05100). The srAFP was modified to include a C-terminal 6-His tag, as described [23]. AFP isoform CfAFP337 and TmYL-1 were PCR amplified with AFP specific primers containing restriction sites 5′-*Eco*RI and 3′-*Not*I. The pPIC9 vector does not have a downstream poly-His sequence. Inserts were sequenced to confirm sequence and orientation before expression studies. In bacteria, pPICZα transformants were selected by zeocin antibiotic resistance while pPIC9 transformants were selected by ampicillin antibiotic resistance [19]. Plasmid DNA from XL-1 blue bacteria was purified from 5 mL overnight LB cultures by the alkaline lysis SDS method [21]. Plasmid DNA purified for yeast transformation was prepared using 25 mL overnight culture and a midi-prep plasmid purification system (Qiagen, Mississauga, Canada).

Yeast transformation and phenotyping

The pPICZα plasmid constructs (10 μg) were linearized at the *Sac*I restriction site and electroporated into *P. pastoris* yeast strain X33. The plasmid contains a 942-bp fragment of the alcohol oxidase 1 (AOX1) promoter region and targets plasmid integration to the AOX1 locus in the yeast. After a double cross-over homologous recombination event, gene replacement into the AOX1 gene resulted in a Mut s, His+, Zeo+ phenotype (methanol slow utilization, his4 wild type allele, zeocin resistance).

The pPIC 9 vector DNA constructs (10 μg) were linearized at either the *Bgl*II restriction site resulting in GS115 yeast transformants with a Mut s, His+ phenotype or the *Sal*I restriction site resulting in transformants with a Mut+, His+ phenotype (methanol wild type utilization). Transformant yeast obtained by a double cross-over homologous recombination event with gene replacement into the AOX1 promoter and 3′ AOX1 regions of the vector and genome, resulted in a Mut s, His+ phenotype. Single gene cross-over events can occur at either the 3′ AOX1 region or the His 4 gene region resulting in a Mut+, His+ transformant phenotype.

All yeast strains were transformed by electroporation according to published methods for *S. pombe* [24]. Phenotypes were determined by plating on selective media and observing differential colony growth. Methanol utilization phenotyping was performed by one of two methods: (1) strains that were His+, Zeo+ were grown on minimal dextrose and minimal methanol plates in duplicate patterns and incubated for 48–72 h at 30 °C, with control Mut+ and Mut s strains also grown on the same plates for comparison; (2) methanol (10%) as the only available carbon source was added as a hanging drop to the top lid every 24 h. Colonies growing well on minimal dextrose plates but slowly on minimal methanol plates were classified as having a Mut s phenotype. pPICZα yeast transformants were selected by zeocin antibiotic resistance while pPIC9 transformants were chosen by their ability to grow on His-deficient media [19]. After selection Mut s colonies were isolated for growth and AFP production in small-scale shake flasks.

Pichia pastoris small-scale and large-scale cultures

Transformants were grown in standard 250 mL flasks at 30 °C on a flat bed rotary shaker (>250 rpm). Single yeast colonies were grown for 2 days in 25 mL BMGY before being pelleted and resuspended in 25 mL BMMY media and grown for an additional 4–5 days. Methanol was added every day (2 mL of 5% methanol v/v). The shake flask fermentation supernatant was collected after this time and assayed for AFP.

Large-scale fermentation was performed using Chemap FZ-3000 stirred vessel bioreactors (Chemap AG, Volketswil, Switzerland) having a nominal volume of 14 L. Temperature was monitored by means of a thermocouple at the vessel base. Dissolved oxygen was monitored by a sterilizable, polarographic dissolved oxygen electrode (Mettler-Toledo Process Analytical, Wilmington, MA, USA). Fermentation broth pH was controlled by the addition of acid or base by a peristaltic pump and monitored using an Ingold Infit 764–750 sterilizable probe (Xymotech Biosystems, Montreal, Canada).

Fermentation was carried out at 30 °C with aeration at 15 L/min, mixing at 750–900 rpm and dissolved oxygen levels maintained at more than 30% of saturation. Throughout the runs pH was maintained at 5.5 using 5 M KOH added as needed. Prior to inoculation 1.5 mL antifoam 204 (Sigma Chemical, St. Louis, MO, USA) was added to the fermentation media and additional antifoam was added as necessary to prevent excessive foaming. Continuous steady state tank reactor (CSTR) fermentation was carried out by growing the yeast in batch phase until the initial glycerol was consumed as indicated by a sudden increase in the dissolved oxygen level and substrate analysis showed no glycerol remained. Substrate feed with methanol was then added continuously to ensure a steady state methanol concentration at 1–2 mL/L. Excess fermentation broth was siphoned off to maintain a constant volume of 5 L. Steady state was monitored by stable dissolved oxygen readings and maintained for 4–5 days.

A second method, fed batch fermentation was also used. Selected yeast transformants were grown in batch phase until the initial glycerol was consumed. The fermentation culture was then induced for 12 or 24 h to ensure full activity of the AOX1 promoter driving heterologous protein expression by adding 100% methanol using a Gemini Pc-1 pump. Methanol in the fermentation broth was maintained at 1–2 mL/L during the induction phase. After induction, a

mixed fed of 50% glycerol, 0.2% methanol per L (v/v) was added. Fed batch fermentation was carried out until no further cell growth was observed (usually 130–160 h).

AFP analysis using assays and Western blots

The supernatant containing secreted proteins from small or large-scale fermentations were analysed by electrophoresis on 12% polyacrylamide–SDS gels. Gels were stained with Coomassie blue (0.25%) and destained (40% methanol, and 10% acetic acid). For Western analysis proteins were electroblotted to PVDF membrane and incubated with rabbit anti-sbwAFP antiserum and then horseradish peroxidase-linked goat anti-rabbit IgG (Bio-Rad, Mississauga, Canada). Chemiluminescent detection was performed with enhanced chemiluminescence substrate (Amersham–Pharmacia, Baie d'Urfe, Canada) with 5 s to 5 min exposures to Kodak MS film.

Thermal hysteresis (TH) or antifreeze activity was assessed using a nanoliter osmometer (Clifton Technical Physics, New York, USA) as described by Chakrabartty and Hew [25]. Activity was indicated by the presence of distinctive "ice shaping" as viewed under the microscope and quantified by freezing point depression, expressed in milliosmoles (mOsmol); samples were tested in 100 mM ammonium bicarbonate (pH 7.9).

Results

Bacterial expression

Recombinant expression of two CfAFP isoforms in Origami-DE3 cells resulted in the recovery of the thioredoxin-AFP fusion proteins almost exclusively in the soluble fractions. Western blot analysis of CfAFP10 expression (Fig. 2A) and others (not shown) revealed that the AFP fusion protein was of the expected size (24 kDa). Although we did not determine the exact amino terminal sequence of the product in each of the transformants to ensure that the STE13 gene product acted on the cleaved signal sequence, previously we have shown that AFPs with additional residues on either the amino or carboxyl ends retain activity. Contaminating bacterial proteins were precipitated by $\leqslant$53% ammonium sulfate and a single, major single band was detected in silver stained SDS–PAGE gels (Fig. 2B). However, no detectable recombinant protein was incorporated in the frozen fraction after ice affinity fractionation (results not shown) and no TH activity was detected. Since none of the recombinant thioredoxin fusion AFP isoforms were active, various CfAFP sequences were transformed into BL21-DE3. Of all the expression constructs examined, three short isoforms (CfAFP4, CfAFPLu-1, and CfAFP333) showed more recombinant protein in the soluble fraction (Fig. 3A) Since recombinant proteins were expressed almost exclusively in non-soluble inclusion bodies for all of the expressed long isoforms (CfAFP6, CfAFP10, CfAFP18, CfAFP104, and CfAFP501; Fig. 3B),

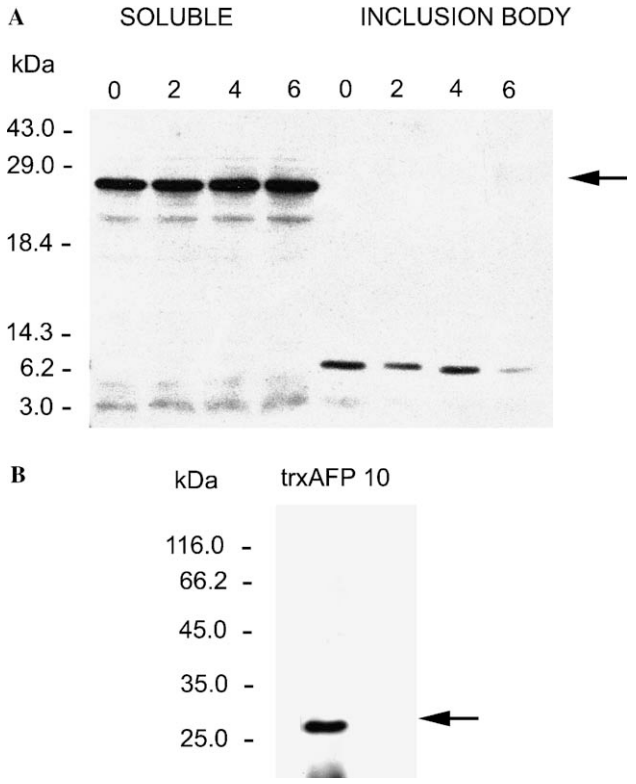

Fig. 2. Representative blots of Western blot time course (0–6 h induction) of thioredoxin AFP fusion protein from Trx CfAFP10 expression in bacterial cells. (A) Expression in soluble and inclusion body fractions. The arrow points to cross-reacting AFP protein. (B) Silver stained TrxCfAFP10 protein sample after purification (see Materials and methods). Similar blots were made to monitor the expression of many other isoforms.

these were subjected to protein refolding procedures. Nine different conditions, including varying the pH, various concentrations of guanidinium and L-arginine, as well as different redox states were employed. In all cases, there was evidence of protein aggregation (results not shown). After heating to 68 °C and then cooling slowly to 4 °C, small amounts of soluble AFP protein could be recovered (~200 μg), which after concentration, showed either no activity or low activity (e.g., CfAFP10 at 0.9 mg/ml with 230 mOsmol).

Yeast expression

Since expression of CfAFPs by various vectors in *E. coli*, resulted in insufficient amounts of active protein and demanded an extended denaturation and refolding procedure [12], yeast expression appeared to be an attractive alternative. Constructs for *P. pastoris* expression were made in two different shuttle vectors, either pPICZα or pPIC9, thus allowing the recovery of various transformant phenotypes depending on the restriction site chosen to linearize the plasmid DNA. When using pPICZα, three different phenotypes were created in KM71 or X33 yeast strains: Mut+, His−; Mut s, His−; and Mut s, His+. All the selected pPIC9 transformants had the same phenotype (Mut s, His+). Mut+ yeast grew well on methanol

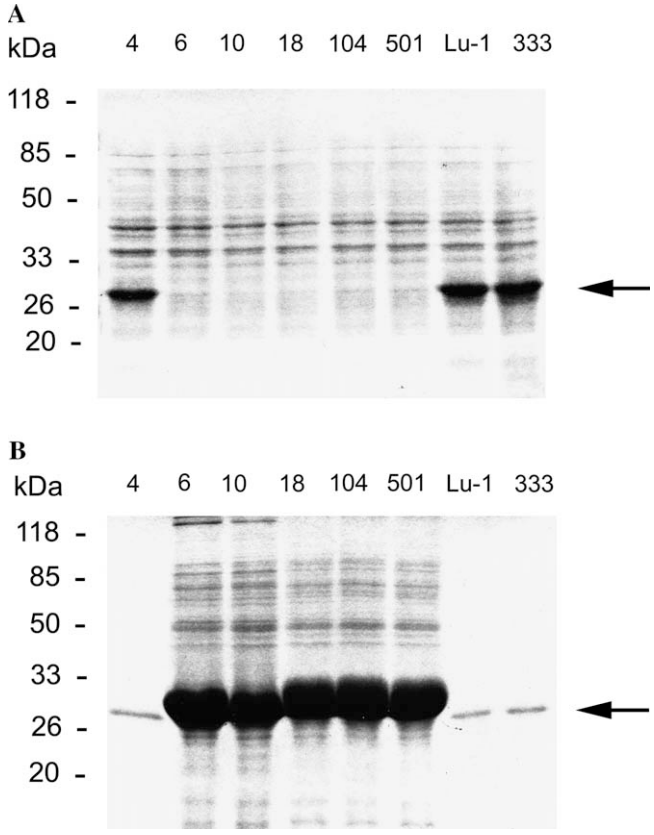

Fig. 3. Representative blots of SDS–PAGE of bacterially expressed thioredoxin fusion CfAFP isoforms from Ori-DE3 cells. Short isoforms 4, Lu-1 and 333 show soluble expression in (A) while in inclusion body fractions show that all long isoforms tested, 6, 10, 18, 104, and 501 accumulate protein in inclusion bodies, shown in (B). Similar blots were made to monitor the expression of many other isoforms.

while Mut s grew slowly, indicating that recombination had inserted the target sequences into the AOX1 gene region.

When tested in small-scale shake flasks, all CfAFP337 pPICZα transformants had TH activity between 10 and 110 mOsmol, regardless of the phenotype (Table 1). Shake flasks of yeast transformants containing CfAFP337 or TmAFPYL-1 in pPIC9 yielded lower activity; CfAFP transformants had TH values of 10–30 mOsmol and there was no detectable TH activity for TmAFP transformants (Table 1). Despite the low TH values, ice crystal morphology was changed in the presence of the TmAFP and CfAFP produced by these pPIC9 transformants, and not in supernatants from control, untransformed yeast, indicating the presence of very low but functional AFP (result not shown).

Individual X33 yeast transformants with CfAFP337 (Mut s, His+) showed variable results when proteins were analyzed using Western blots, with some individual lines showing an accumulation of high molecular weight AFP-cross-reacting material (CRM) (Table 2 transformants 1, 2, and 4). Other transformants appeared to have both high molecular weight protein and a smaller cross-reacting band near the expected protein size (Table 2 transformants 3, 5, and 6). Still other transformants showed very little AFP CRM (Table 2 transformants 7, 8, 10, and 11). One transformant appeared to have correctly sized protein with very little high molecular weight protein and had the highest observed TH activity of 110 mOsmol (Table 2 transformant 9). All other transformants had similar low TH activities of 10–30 mOsmol. Because the conditions in the small-scale flasks were unoptimized but promising, large-scale fermentation production was initiated.

Transformant 9 containing CfAFP in X33 host strain appeared to be a good candidate for large-scale expression studies, producing 478 μg/mL AFP, and thus this line was used for fermentation, either in a continuous steady state tank reactor (CSTR) (Fig. 4C i, lane 1) or in a fed-batch protocol (Fig. 4C i, lane 2). As suggested by d'Anjou and Daugulis [27], dissolved oxygen was used for monitoring. In CSTR there was a methanol consumption rate of 0.030 gM/gX h (g, grams; M, methanol; X, cell density; and h, hour), and for the fed-batch system the methanol consumption rate was three-fold higher at 0.090 gM/gX h. In fed-batch the cell density varied but

Table 1
Expression of CfAFP and TmAFP in several *P. pastoris* host strains

AFP	Shuttle vector	Resulting phenotype	Transformed host strain	Small scale[a] TH mOsmol (μg/mL)	Large scale[b] TH mOsmol (μg/mL)
CfAFP	pPICZα	Mut+ His−	KM 71	10 (n.d.)	—[c] —
CfAFP	pPICZα	Mut s His−	KM 71	20–30 (n.d.)	10–30 (n.d.)
CfAFP	pPICZα	Mut s His+	X33	30–110 Flask (478)	<30 FB (892) CSTR (1318)
CfAFP	pPIC9	Mut s His+	GS115	10–30 (n.d.[d])	— —
TmAFP	pPIC9	Mut s His+	GS115	A[e] (n.d.)	60 (n.d.)

[a] Small scale flasks are 250 mL.
[b] Large scale fermentation used fed batch (FB) or continuous steady state tank reactor (CSTR) strategies.
[c] Dashes indicate those samples that were not tested.
[d] Protein concentrations were not determined (n.d.).
[e] AFP activity present (A) as indicated by ice shaping.

Table 2
AFP production assessed after Western blot analysis of 11 separate transformants in small scale shake flask (X33 with pPICZα)

X33 shake flask transformant	CRM-AFP[a] (whole lane μg/mL)	AFP[b] (single band μg/mL)	% AFP[c] (unglycosylated state)
1	340	n.d. [d]	0
2	590	n.d.	0
3	468	111	23.7
4	423	n.d.	0
5	289	105	36.3
6	130	102	78.4
7	224	n.d.	0
8	215	n.d.	0
9	99	76	76.7
10	182	n.d.	0
11	188	n.d.	0
CfAFP control[e]	n.d.	50	100

[a] Samples (10 μL) were electrophoresed on 12.5% SDS–PAGE gels and proteins were transferred to PVDF membrane and densitometry of the alkaline phosphatase reaction was used to quantify the level of cross-reacting material (CRM) detected by *C. fumiferana* AFP antibody (1:500). All CRM in the lane (less the band at the appropriate molecular weight) is given in this column.
[b] AFP CRM determined by densitometry for the single band of the appropriate molecular weight.
[c] The percent AFP is the amount of AFP detected as a protein band of the correct size, as a percentage of the total CRM.
[d] Samples in which no CRM could be detected are shown as not detected (n.d.).
[e] Purified, recombinant *E. coli* Cf337 protein (500 ng) was used as a control.

was estimated at 60–70 g CDW/L, again approximately three times the cell density observed for CSTR that ranged between 22.2 and 31.0 g CDW/L (Figs. 4A and B). Fed-batch produced 89 mg/L CfAFP protein while CSTR produced more (132 mg/L).

Western blot analysis using CfAFP antibody resulted in detection of large molecular weight CRM, regardless of the large-scale reactor method used (Fig. 4C i). Because high molecular weight CRM could be attributed to glycosylation, alkaline hydrolysis under various conditions of temperature, time, NaOH concentration, and a reducing agent, sodium borohydride were tested [27]. Treatments using: 40–160 mM NaOH, 0.5 M $NaBH_4$ at 50 °C for 3 h were the only conditions that were successful at recovering distinct protein bands that cross-reacted to CfAFP antibody. The protein extracts that yielded AFP CRM at an appropriate size following the mildest denaturing conditions (e.g., treatment with 40 mM NaOH; Fig. 4C ii). After dialysis into 100 mM ammonium bicarbonate, no TH activity was detected.

When disulfide-bonded sea raven AFP was used for recombinant expression in pPIC9 (Mut s His+), the residual methanol concentration was maintained at 1–3 g/L and resulted in an overall methanol consumption rate of 0.02 gM/gX. The cell density was estimated at 45 g CDW/L with the production of 55 mg/L AFP. SDS–PAGE analysis showed a protein band of the expected size, 15.5 kDa and Western blots confirmed the identity of the protein (results not shown). TH activity was detected even with small-scale expression studies medium at expected values corresponding to 50 mOsmol (0.02 °C at a 5 mg/L). Insect AFPs have 10–100-fold higher TH activity than those from fish when compared on a molar basis [28,29]; the srAFP TH activity measured from our yeast expression experiments was consistent with previously published values [23].

Discussion

There is much demand for expression systems that will produce quantities of active recombinant products, and as a result, various "bioreactors" have been developed. The overwintering stage of the spruce budworm is only 2 mm in length, and the purification of its disulfide-bonded AFP involves several laborious steps, including HPLC, and thus, in this case, recombinantly expressed material is crucial for the use of this protein in practical applications. Insect AFPs expressed in *E. coli* tend to accumulate in inclusion bodies and be inappropriately folded and, consequently, inactive [12]. AFP activity can only obtained after reduction of the protein in vitro followed by days or weeks of oxidation at low temperatures followed by the removal of improperly folded forms by HPLC [12,30]. Although this method has been applied to three (including CfAFP337) of the dozens of isoforms of CfAFP, and to only a few of the TmAFPs [30,11] many isoforms cannot be refolded and remain uncharacterized.

In an effort to improve the solubility, ease of purification, and successful recovery of more recalcitrant isoforms, fusions were made to a bacterial thioredoxin containing an engineered metal affinity-binding domain [31]. These were transferred to Origami-DE3 bacteria, bearing a mutated thioredoxin reductase (trxB−; allows disulfide bond formation) and a mutated glutathione reductase gene (*gor*; permits the reduction of disulfide bonds). After purification, either by Talon, metal affinity chromatography or ammonium sulfate precipitation, pure CfAFP was obtained (Fig. 2B). Although essentially all of the CfAFP was made soluble by the thioredoxin fusion, it did not bind to ice and no TH activity could be detected. The yield was increased when the same isoforms were expressed in BL21-DE3 cells but since these cells did not have the mutations found in Origami-DE3 bacteria, most isoforms were sequestered into inclusion bodies and had to be denatured and subsequently refolded. After many

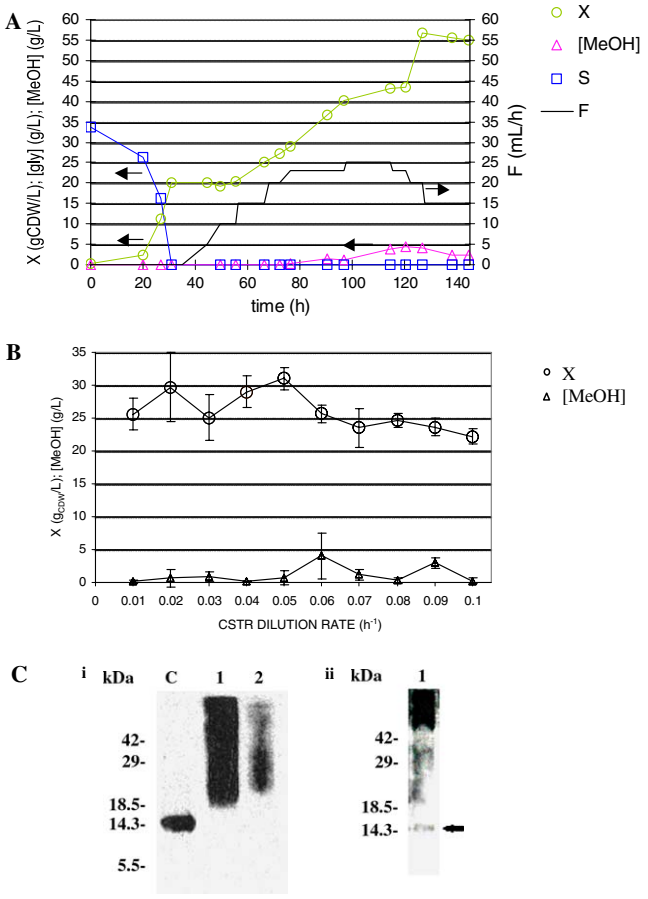

Fig. 4. (A) Fed-batch fermentation of *P. pastoris* X33 pPICZα. Cell density (X), residual methanol (MeOH) and residual glycerol (S) (left axis) monitored over time (h); the flow rate of methanol during the fed batch phase (F) (right axis) over time (h) is also shown. (B) Continuous steady state tank reactor (CSTR) showing the steady state of cell density (X) and methanol (MeOH) consumption maintained by the dilution rate over time. (C) (i) Western blot of large-scale fermentor supernatant from *P. pastoris*. CTSR (1) or fed-batch (2) samples (10 μL) were electrophoresed on SDS–PAGE gels, and proteins were transferred to PVDF membrane and reacted with polyclonal anti-Cf337 antibody using chemiluminescence. Recombinant *E. coli* Cf337 (200 ng) is shown in lane C. (ii) Western blot of soluble fraction after chemically treating samples (in this case, 40 mM NaOH, 0.5 M $NaBH_4$ at 50 °C for 3 h). The arrow points to CfAFP recovered after chemical treatment. Similar blots were made to monitor the results of other deglycosylation regimes.

different protocols were explored, activity was recovered from CfAFP501 and CfAFP10 isoforms. Although these efforts did not result in a protein useful for practical production, it should be noted that sufficient CfAFP10 was obtained so that it has now been classified as a hyperactive AFP (230 mOsmol at 0.9 mg/mL).

Other proteins that are extensively disulfide-bonded have been successfully expressed in eukaryotic systems but the use of moth and fly cell lines, and whole insects (*Drosophila*) did not result in sufficient quantities of insect AFP isoforms, both from spruce budworm and beetles (result not shown). We thus turned our attention to expression in *P. pastoris*, which has been used in large-scale fermentation systems to produce modest and high levels (up to 2.2 mg/L/h for a net production of 120 mg/L) of active sea raven Type II AFP, a cysteine-rich fish AFP [23,26].

After culturing srAFP-transformed yeast in shake flasks, Western blots revealed that the expressed protein had a larger molecular weight than the *E. coli*-produced recombinant protein. This effect has been well documented in other expression studies, attributed to hyperglycosylation, which has been observed in yeast expression systems when cultures are stressed [32,33]. In an effort to reduce this and to maximize AFP production by taking advantage of their higher productivity, large-scale fermentation using both CSTR and fed-batch operations with Mut s transformed strains were tested. The CSTR system resulted in modestly higher productivity compared to the fed-batch system (Table 1). Estimation of heterologous protein production showed CSTR produced 132 mg/L compared to 55 mg/L in fed-batch X33, almost a 1.5-fold increase.

Although the large-scale production of heterologous protein appeared promising in yeast, the produced AFP had very little TH activity and migrated as a higher molecular weight smear in Western blots, similar to those observed in small-scale experiments (Fig. 4C i). We suspected that the yeast had glycosylated the AFPs and this was demonstrated by periodic shift staining of the expressed proteins (not shown) and by removal of the carbohydrate group by alkaline hydrolysis (Fig. 4C ii). O-linked glycosylation can be removed by enzymatic digestion, however this approach proves to be prohibitively expensive for the scale of production of recombinant proteins [34]. The primary sequence of TmAFPYL-1 has one potential N-linked site, which appears to be used in *Tenebrio* [10], CfAFP337 does not contain any Asn-X-Ser/Thr (where X is any amino acid except Pro) motifs characteristic of N-linked glycosylation sites. In contrast, predictive algorithms for O-linked glycosylation, NetOGlyc v3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) at Ser and Thr show that the CfAFP sequence contains five putative O-linked glycosylation sites with their individual, similarly high, potential/threshold values shown in brackets after the amino acid position numbers: Thr 46 (0.9/0.6), Thr 48 (0.7/0.6), Thr 49 (0.8/0.6), Thr 53 (0.8/0.6), and Ser 58 (0.9/0.6) [35]. One of these, Thr 53, is located in the middle of the ice binding face, and thus would be expected to decrease activity. Indeed, CfAFP337 production in yeast resulted in only 30–110 mOsmol activity in shake flask, a decrease of 88–97% in TH activity compared to similar concentrations of properly folded recombinant Cf337 produced in bacteria [28]. Thus, although expression in *Pichia* produced significant quantities of insect AFP, and it was likely appropriately folded, very little TH activity was recovered, and only from a few of the isoforms studied. A recombinant strain of *P. pastoris* (YJN201) has recently been developed that lack endogenous Golgi mannosyl transferase Och1p activity that may prove useful for future protein expression of threonine-rich AFPs [36].

It is possible that AFPs are a class of proteins that are particularly challenging for mass production. Thus, sea raven AFP was chosen and used for recombinant

expression in pPIC9 in a fed batch protocol, mimicking that described for CfAFP, since it is also disulfide-bonded and has a molecular weight similar to that of the insect AFPs. Although less recombinant protein was recovered (55 mg/L for srAFP vs. 89 mg/L CfAFP in fed-batch fermentation), the produced srAFP showed significant AFP activity.

These results demonstrate that there was no problem with the fermentation methodology used. Rather, these results underscore our contention that although great strides have been made in the development of suitable vectors and organisms for the production of recombinant proteins, the sequence of the protein of interest and its three-dimensional conformation remains paramount for success [37]. In the case of insect AFPs, the combination of Cys residues involved in disulfide bonds and the presence of required Thr residues at or on the ice-binding face appears to preclude the production of active insect AFPs; in particular CfAFPs, in the bacterial and *Pichia* systems described here.

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