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M. C. Loewen · X. Liu · P. L. Davies · A. J. Daugulis

Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*

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Abstract Sea raven type II antifreeze protein (SRAFP) is one of three different fish antifreeze proteins isolated to date. These proteins are known to bind to the surface of ice and inhibit its growth. To solve the three-dimensional structure of SRAFP, study its ice-binding mechanism, and as a basis for engineering these molecules, an efficient system for its biosynthetic production was developed. Several different expression systems have been tested including baculovirus, *Escherichia coli* and yeast. The latter, using the methylotrophic organism *Pichia pastoris* as the host, was the most productive. In shake-flask cultures the levels of SRAFP secreted from *Pichia* were up to 5 mg/l. The recombinant protein has an identical activity to SRAFP from sea raven serum. In order to increase yields further, four different strategies were tested in 10-l fermentation vessels, including: (1) optimization of pH and dissolved oxygen, (2) mixed feeding of methanol and glycerol with Mut^s clones, (3) supplementation of amino acid building blocks, and (4) methanol feeding with Mut^+ clones. The mixed-feeding/Mut^s strategy proved to be the most efficient with SRAFP yields reaching 30 mg/l.

Introduction

Antifreeze proteins (AFP) have been isolated from a number of organisms including fish, insects, plants and bacteria (Davies and Hew 1990; Sun et al. 1995; Griffith et al. 1992; Tomchaney et al. 1982). These molecules protect the freeze-intolerant organisms from freezing in sub-zero conditions by adsorption of the protein to the surface of seed ice-crystals resulting in inhibition of ice-crystal growth (Raymond and DeVries 1977). The viability of freeze-tolerant organisms is helped by the ability of AFP to inhibit ice recrystallization (Carpenter et al. 1992). Type II AFP, one of three different fish AFP identified to date (Davies and Hew 1990), has been isolated from three distantly related fish, including sea raven, herring and smelt. The supply of this AFP from natural sources is scarce, severely limiting biotechnological applications and research into the structure and mechanism of action. Accordingly it is essential to develop a reliable source of defined recombinant type II AFP.

A number of different expression systems have been tested for expression of sea raven type II antifreeze protein (SRAFP). A recombinant baculovirus-vector-infected army worm cell system was initially chosen for its ability to secrete and correctly fold disulfide-bonded proteins (O'Reilly et al. 1992). The T7-promoter-driven expression system in *Escherichia coli* was also tested on the basis of the previous success achieved in expressing mannose-binding protein, a lectin domain homolog to SRAFP (Ewart et al. 1992), in *E. coli* (Weis et al. 1991). Finally the *Pichia pastoris* yeast expression system was tested on the basis of its ability to produce very high yields of foreign proteins (Barr et al. 1992; Digan et al. 1989; Clare et al. 1991; Cregg et al. 1993).

In the *Pichia* system (Invitrogen, San Diego, Calif.) the gene for expression is placed immediately downstream of the strong yeast alcohol oxidase promoter, which is repressed by glycerol and glucose and induced by methanol. The *Pichia* system allows for the genomic integration of foreign genes, thus precluding difficulties associated with plasmid-based expression, such as plasmid instability. In addition, integration of the expression cassette can be directed to two alternative sites in the

This paper is dedicated to the memory of Xiaoting Liu

M. C. Loewen · P. L. Davies
Department of Biochemistry, Queen's University,
Kingston, Ontario, Canada, K7L 3N6

X. Liu · A. J. Daugulis (✉)
Department of Chemical Engineering, Queens University,
Kingston, Ontario, Canada, K7L 3N6
Tel.: (613)545 2784
Fax: (613)545 6637
e-mail: Daugulis@chee.queensu.ca

yeast genome. In one case the methylotrophic phenotype of the yeast is maintained (providing a Mut^+ phenotype), and in the other the methylotrophic phenotype is deleted (Mut^s phenotype). Thus methanol can be used as a carbon source and/or inducer of foreign gene expression. This ability provides a readily controllable system for expression of foreign proteins.

In this work we describe the shake-flask expression of the cystine-rich SRAFP from *Pichia,* and demonstrate that the recombinant protein has identical activity to SRAFP isolated from sea raven serum. This is compared to expression levels obtained from *E. coli* and baculovirus systems. We further describe the development of expression from yeast by fermentation techniques. Through several fermentation strategies, we have developed an efficient system for the biosynthetic expression of wild-type and mutant SRAFP in quantities sufficient for testing biotechnological applications, X-ray crystallography and isotopic labelling for nuclear magnetic resonance (NMR) studies.

Materials and methods

Bacterial and yeast strains

The methylotrophic strain of *P. pastoris*, GS115(*his4*) commercially available from Invitrogen (San Diego, Calif., US) (Cregg et al. 1985), was used for expression of recombinant type II AFP. *E. coli* JM83 was used for all plasmid constructions and propagations.

Media compositions

Buffered minimal glycerol complex medium (BMGY) contained the following (per liter): yeast extract (10 g), meat peptone (20 g), 100 mM potassium phosphate buffer pH 6.0, yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), glycerol (10 ml). Buffered minimal methanol complex medium (BMMY) contained the same components as BMGY except that glycerol was replaced by methanol (5 ml/l). Minimal methanol medium (MM) contained the following (per liter): yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), methanol (5 ml). Minimal glycerol medium (MG) was the same as MM medium except that methanol was replaced by glycerol (10 ml/l). Fermentation medium contained the following (per liter): glycerol (50 g), ammonium sulfate (20 g), KH_2PO_4 (12 g), $MgSO_4 \cdot 7H_2O$ (4.7 g), $CaCl_2 \cdot 2H_2O$ (0.36 g), plus trace elements as follows: $CaSO_4 \cdot 5H_2O$ 0.2 μM, KI 1.25 μM, $MnSO_4 \cdot 4H_2O$ 4.5 μM, $Na_2MoO_4 \cdot 2H_2O$ 2.0 μM, H_3BO_3 0.75 μM, $ZnSO_4 \cdot 7H_2O$ 17.5 μM, $FeCl_3 \cdot 6H_2O$ 44.5 μM. The pH was adjusted to 5.5 using 5 M KOH.

Plasmid construction

The vector pPIC9, which contains the *his4* gene for selection in the $HIS4^-$ strain, GS115(*his4*) and the ampicillin-resistance gene for selection in *E. coli* (Cregg et al. 1985), was used to express the mature sea raven cDNA in *P. pastoris*. The 5′-end primer, SR1 (5′-GAATTCGAATTCCAGAGAGCCCCACCA-3′), which introduced tandem *Eco*RI sites immediately upstream of the Gln codon at the N terminus of mature SRAFP (Hayes et al. 1989), and the 3′ primer, SR2 (5′-ATACGTAAGCGGCCGCCAAACACCCACTAAAGTG-3′), which introduced a *Not*I site 47 base pairs downstream of the stop codon, were used to amplify the mature AFP cDNA by the polymerase chain reaction (PCR) from the clone pT7-7F-SRm. pT7-7F-SRm is a derivative of the *E. coli* expression vector pT7-7 (Tabor 1990), in which the mature cDNA of type II AFP was cloned into the *Nde*I/*Hin*dIII sites of pT7-7F. The pT7-7F vector contains an *oriF* site for production of single-strand DNA as previously described (DeLuca and Elce 1993). A 492-base-pair product was obtained from PCR and was cloned into the *Eco*RI and *Not*I sites of the pPIC9 vector, 24 bases (eight amino acids) downstream from the cleavage site of the α mating factor signal peptide, to give pPIC9-SRm. In addition, the vector pT7-7F-SRm was mutated using a primer-directed mutagenic method (Kunkel et al. 1987) with primer CTHT1 (P5′-TGCGCCATG-ACATTCCACCACCACCACCACTAAGCGGCCGCTGA-GCTAACACAGAGG-3′), which introduced a six-histidine-long His tag at the C-terminal end of the cDNA, immediately prior to the stop codon to allow simplified protein purification by affinity chromatography to a nickel column. The primer also adds a *Not*I site immediately following the stop codon. A fragment, that included the His tag was excised using the *Not*I site and a unique *Bst*EII site further upstream within the cDNA. This was cloned into the corresponding sites within the pPIC9-SRm vector to produce the final expression construct, pPIC9-SRm-CTHT, which differed from pPIC9-SRm by the presence of the C-terminally coded His-tag.

Transformation and induction

Vector DNA (10 μg), produced in *E. coli*, was linearized and transformed into GS115(*his4*). Vector linearized by *Bgl*II was integrated by homologous recombination into the *AOX1* gene site producing clones with a His^+, methanol-utilization-slow (Mut^s) phenotype. Vector linearized at the *Sal*I site integrated into the *his4* gene site of the GS115(*his4*) genome, without disruption of the *his4* plasmid gene, gave a His^+, Mut^+ phenotype. The linearized vector was introduced into *P. pastoris* using the spheroplast transformation procedure essentially as described by Cregg et al. 1985. Transformant cultures (50 ml) were grown in non-baffled shake flasks (250-ml conical flasks) at 250 rpm and 30 °C in a standard New Brunswick Scientific environmental incubator shaker (Edison, N.J.) for 48 h in BMGY medium, which contained glycerol and therefore inhibited the alcohol oxidase promoter. Cells were harvested by centrifugation and resuspended in the same volume of BMMY, which contained methanol for induction of the alcohol oxidase promoter, and again incubated for 48 h at 30 °C with shaking. Cells were removed by centrifugation, leaving recombinant AFP to be purified from the medium.

Analysis of AFP

AFP samples were analyzed by electrophoresis on a 15% polyacrylamide/sodium dodecyl sulfate gel, containing 0.1 M sodium phosphate and 4 M urea, at pH 6.8, and blotted onto a nylon membrane (PVDF, NEN). The membrane was incubated with rabbit anti-(sea raven AFP) antiserum and then horseradish-peroxidase-linked goat anti-(rabbit IgG). Detection was carried out using enhanced chemiluminescence. Quantification was carried out by visual inspection of band intensities compared to intensities of standards.

Measurements of the antifreeze activity were made in 100 mM NH_4HCO_3 (pH 7.9) using a nanoliter osmometer (Clifton Technical Physics, Hartford, New York) as described by Chakrabartty and Hew (1991). Antifreeze activity is defined as thermal hysteresis, which is the difference (°C) in temperatures at which the seed crystal grows and shrinks. Ice crystal growth and morphology were observed using a Panasonic closed-circuit TV camera attached to a Leitz dialux 22 microscope and recorded by a JVC Super VHS video recorder. Still photographs of ice crystals were taken 1 min after the formation of the bipyramidal ice crystal under 0.1 °C supercooling.

Fermentation

Inoculum cultures (600 ml) were grown in MG medium for 48 h at 30 °C, in 2-l conical flasks, at 250 rpm. This 10% inoculum was

used to inoculate a 10-l Chemap bioreactor, containing 6 l medium as described above. Cell culture conditions were set at 30 °C, 800 rpm and 2.3 l air l culture volume^{-1} min^{-1}. The pH was maintained at 5.5 by the automatic addition of 5 M KOH. The level of dissolved oxygen was measured using an Ingold galvanic electrode. Antifoam 204 organic (2 ml; Sigma, St Louis, Mo.) was added prior to inoculation, and as required throughout the fermentation. Methanol and glycerol levels were monitored by HPLC (Waters) on a Sugar-Pac (Waters) column using the Maxima 800-data-acquisition system for peak integration.

Results

Shake-flask expression of recombinant SRAFP

SRAFP produced and secreted by GS115-pPIC9-SRm-CTHT in shake flasks, was detected in the medium by Western analysis at a level of only 5 mg/l. The recombinant protein (including the eight N-terminal additional amino acids and six C-terminal histidines, calculated molecular mass = 15 777 Da) gave rise to a single band that ran at approximately the same position as the wild-type AFP mature standard (calculated molecular mass = 13 999 Da) (Fig. 1A, lanes 2 and 3). The bell-shaped band in lane 2 is the result of overloading. The resolution of this gel system was not sufficient to distinguish the wild-type 14-kDA protein from the 15.5-kDa recombinant protein. A thermal hysteresis reading of approximately 50 mOsm (0.1 °C) was detected in the medium from the pPIC9-SRm-CTHT culture, indicating that the recombinant SRAFP was active as an antifreeze protein. Ice crystals produced a slightly rounded hexagonal bipyramidal morphology characteristic of type II AFP (Chao et al. 1995) (Fig. 1B). Expression levels from the Mut$^+$ clone containing pPIC9-SRm-CTHT were similar to those from the corresponding Mut^s clone (Fig. 1A, lane 4). When Mut^s pPIC9-SRm-CTHT was switched to growth on a minimal medium in order to label SRAFP with ^{15}N for NMR structural studies, the yield of antifreeze decreased by at least half, as detected by Western blot (Fig. 1A, lane 5).

Fermentation

In order to improve the yield of SRAFP, a shift to fermentation techniques was made to allow better control of environmental conditions such as pH and dissolved oxygen. Four different strategies were tested. The first was to improve the yield of SRAFP in defined medium simply by control of pH and dissolved oxygen levels (Fig. 2A). The pH was maintained at 5.5 and the dissolved O_2 at non-limiting (above 10% saturation) values. The fermentation began with a batch growth phase, with 50 g/l glycerol as carbon source and excess (20 g/l) ammonium sulfate in minimal medium. Within 25 h after inoculation the cells had consumed all the glycerol and had reached 22 g/l cell dry weight (CDW). After depletion of the glycerol, induction with methanol was initiated. Methanol was fed at a rate of 1.0 g l^{-1} h^{-1} for a subsequent period of 95 h. Owing to the Mut^s

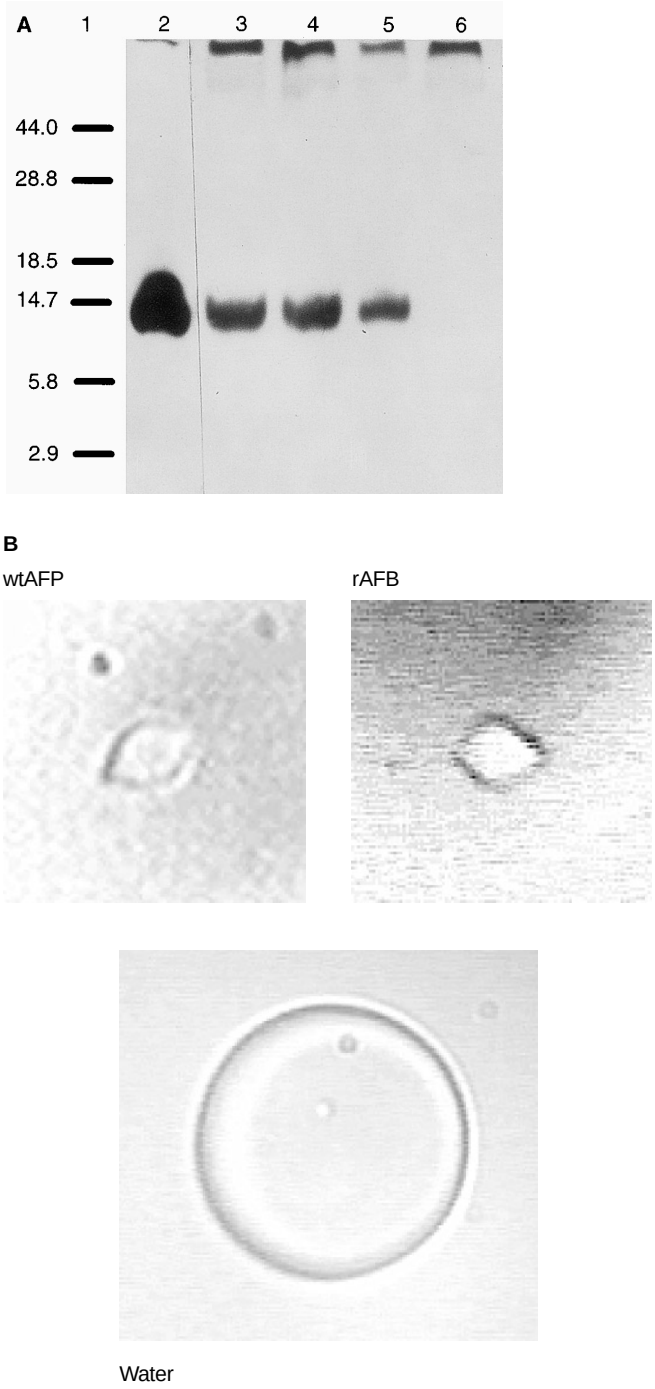

Fig. 1A Western blot analysis of yeast medium (15 µl) from different clones. Lanes: *1* low-molecular-mass prestained markers (kDa) (Gibco BRL), *2* 150 ng sea raven type II antifreeze protein (SRAFP) from sea raven serum, *3* pPIC9-SRm-CTHT (Mut^s), *4* pPIC9-SRm-CTHT (Mut$^+$), *5* pPIC9-SRm-CTHT (Mut^s) from defined medium, *6* GS115 medium. **B** Ice crystal formed in the presence of wild-type SRAFP purified from sea raven serum (*wtAFP*), in the presence of recombinant SRAFP purified from yeast medium (*rAFP*) and in pure water (*water*)

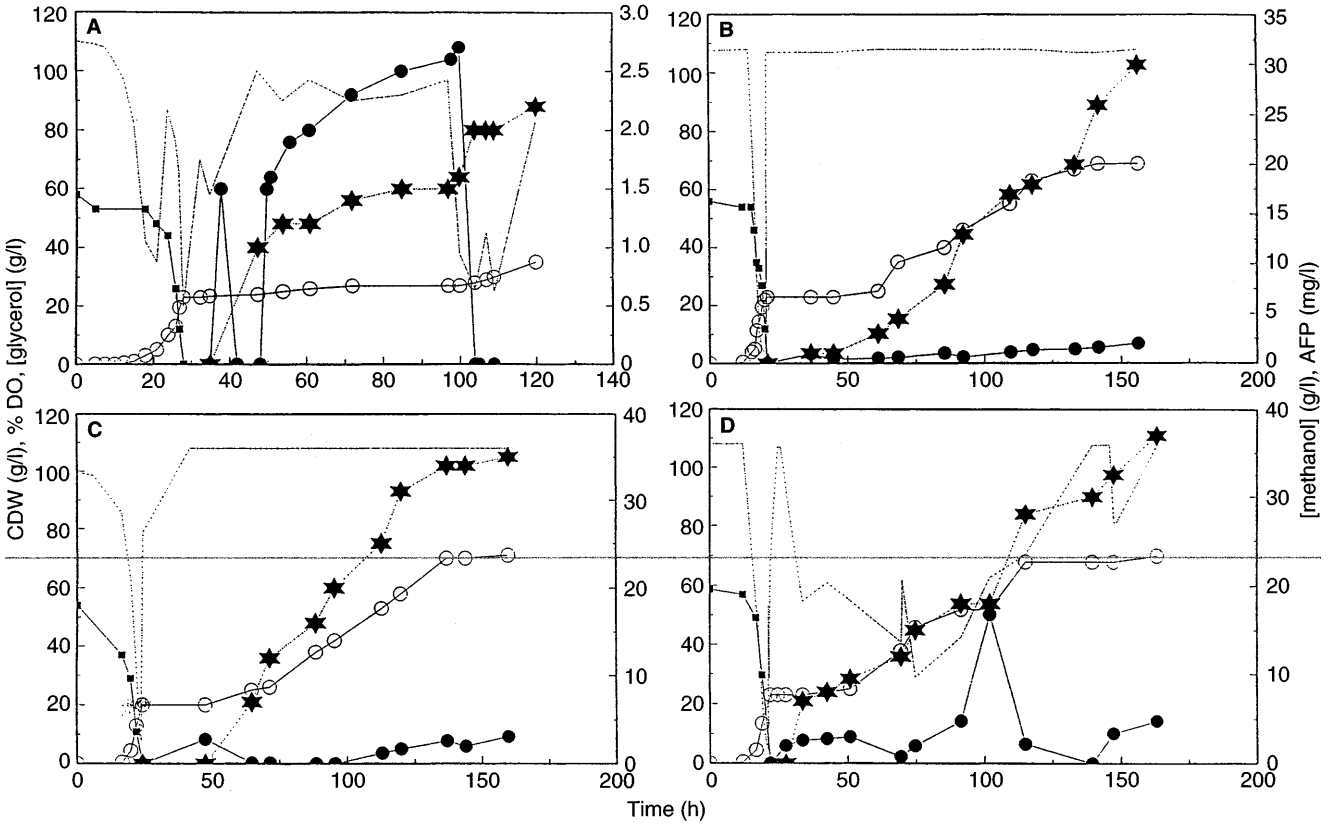

Fig. 2A Fermentation profile of the Mut^s clone with batch phase followed by methanol feed strategy. **B** Fermentation profile of the Mut^s clone with mixed feed strategy. **C** Fermentation profile of the Mut^s clone with mixed feed plus peptone strategy. **D** Fermentation profile of the Mut$^+$ clone with batch phase and methanol feed strategy. ••• Percentage dissolved oxygen (*DO*), ○ cell dry weight (*CDW*), ■ glycerol concentration, ● methanol concentration, ★ antifreeze protein

phenotype (cells that grow poorly on methanol), the CDW increased only slightly to 35 g/l cells by the end of the fed-batch phase. SRAFP accumulated in the medium gradually over time, beginning 24 h after the start of the methanol feed, to a final level of 2.0 mg/l at 120 h, as estimated by Western blotting. This was a slight improvement over shake-flask minimal medium yields, but was still too low for cost-effective ^{15}N labelling and protein production for X-ray crystallographic studies.

Optimization of fermentation

Following the marginal success of the first fermentation profile, attempts were made to increase yields of SRAFP through optimization of the fermentation conditions. The second experiment included a mixed-feed protocol. This strategy of feeding both glycerol and methanol during the induction phase involves providing a readily usable carbon source (glycerol) for the Mut^s cells for biosynthesis, while at the same time providing an inducer for the *AOX1* promoter. Care must be taken in determining the proportion and amounts of each substrate to be fed, since any glycerol accumulation would repress the *AOX1* promoter, and because methanol has to be present, but not at levels that would be inhibitory (above 1%–2%). The cells should then continue to propagate, thereby increasing the cell mass and maintaining an active culture, which might be induced to express the desired protein. The fermentation began with the batch growth on 50 g/l glycerol, which gave 22 g/l CDW (Fig. 2B). The pH was again maintained at 5.5 and dissolved O_2 at non-limiting values. At this time a methanol feed of 1.0 g l^{-1} h^{-1} was started and continued for 24 h, to ensure that the alcohol oxidase promoter was induced. After this methanol feeding period, the feed was switched to the mixed substrate of glycerol/methanol (5:2 volume basis). The feeding rate was started as 1.0 g l^{-1} h^{-1} methanol and 4.0 g l^{-1} h^{-1} glycerol. The rate of feeding was gradually increased to the maximum value permitted without residual glycerol accumulation, and without methanol rising above the 0.5% used in the shake-flask cultures. The latter precaution was to avoid methanol toxicity, as noted. This maximum rate was approximately 2.6 g l^{-1} h^{-1} methanol and 10.4 g l^{-1} h^{-1} glycerol. Fermentation proceeded for 156 h from the time of inoculation. At this point the CDW was approximately 70 g/l cells and the SRAFP, which accumulated gradually over the induction period, starting within 20 h of the methanol feed, had reached 30 mg/l by 156 h. This represents a 15-fold increase of SRAFP over the original fermentation. By the end of the 156 h, a total of 185 g/l glycerol had been consumed. On

the basis of a yield coefficient of 0.42 (estimated from the batch growth phase) this would indicate an expected CDW of 76.8 g/l, very close to our observed value of approximately 70 g/l.

This mixed-feed protocol was repeated to include the addition of peptone as a complex amino acid source (Fig. 2C). This technique had previously been observed to increase yields of an extracellular protein from *Bacillus brevis* 47 dramatically (Wight et al. 1992). In addition, the expression of SRAFP from *Pichia* in complex medium in flasks was shown to produce much higher yields than in minimal medium. The fermentation was spiked with peptone (Gibco BRL) at 48 h (5 g/l), 70 h (5 g/l) and 90 h (10 g/l), with a final spike at 120 h (5 g/l), for a total of 25 g/l peptone. The pH was again maintained at 5.5 and dissolved O_2 at non-limiting values. At the end of 160 h the cell mass had reached 74 g/l CDW and SRAFP had again accumulated uniformly over the induction period to 35 g/l, only marginally better than the previous fermentation.

The final optimization strategy shifted back to the original methanol-feeding profile, using the Mut^+ clone, which is capable of metabolizing methanol as a carbon source (Fig. 2D). This final strategy eliminated the possibility of any inhibition of the *AOX1* promoter by residual glycerol in the mixed-feed profiles, even though none had been detected by HPLC. The conditions were identical to those outlined for the original Mut^s fermentation: a batch growth to 22 g/l CDW on glycerol followed by a methanol feed starting at a rate of 1.0 g l^{-1} h^{-1}. This rate was increased to 2.6 g l^{-1} h^{-1} without the accumulation of methanol above 0.5%. Higher rates of methanol feeding (up to 3.3 g l^{-1} h^{-1}) led to the accumulation of 1.6% methanol in the medium (see peak at about 100 h), which temporarily depressed cell growth and SRAFP production. Upon decreasing the feeding rate, the cells resumed growth with no apparent ill effects. Again pH and dissolved O_2 were maintained as described previously. At 160 h the cells reached 71 g/l CDW and the SRAFP had gradually accumulated to 37 mg/l SRAFP. No significant increase in AFP yield was obtained using Mut^+ over Mut^s. By the end of the fermentation 58.7 g/l glycerol producing 24.6 g/l CDW, and 309.6 g/l methanol (yield coefficient 0.15; Duff and Murray 1988) theoretically producing 46.3 g/l CDW were consumed for a theoretical cell mass of 70.9 g/l CDW, which corresponds to the observed value. The use of oxygen was much greater in the Mut^+ profile, as reflected in lowered dissolved O_2 levels. This reflects the more reduced state of methanol compared to glycerol.

Discussion

Optimization of expression in *Pichia*

A number of different fermentation strategies were tested to optimize the expression of SRAFP from the yeast *Pichia pastoris*. The goal was to reach levels of SRAFP that would provide quantities of (above 100 mg) purified protein sufficient for research and development applications and X-ray crystallography. NMR structural studies, while not requiring large quantities of protein, are facilitated by labelling the protein with ^{15}N. In order to produce labelled proteins, the host system must be grown in a minimal medium with ^{15}N as the sole nitrogen source. It was demonstrated that, at the shake-flask level, yields dropped significantly in minimal media. However, shifting to fermentation with a defined minimal medium produced yields as high as 35 mg/l with a mixed-feed strategy. This appears to be due largely to an increase in cell mass and not to any increased productivity of the yeast. Cell growth levelled-off towards the end of the cultivation period, likely due the depletion of all nitrogen. It was anticipated that the addition of peptone during the induction phase of the fermentation might increase yields dramatically, as was observed in *B. brevis* 47 (Wight et al. 1992). In the bacterial case, the expression of extracellular protein was increased fivefold from 3 g/l to 15 g/l. Unfortunately the peptone did not have the same effect on the expression of foreign protein from *Pichia*. Similarly, the Mut^+ fermentation, with the continuous methanol feed protocol, showed no significant increase in SRAFP yields over the Mut^s mixed feed. Owing to the large amount of methanol consumed by Mut^+ to grow to a CDW of 70 g/l (because of its low yield coefficient of 0.15 compared to 0.42 for glycerol) it is preferable to use the Mut^s with the mixed-feed fermentation profile.

The above results suggest that different medium formulations, feeding strategies and cultivation conditions were largely ineffective in improving individual cell performance in expressing the SRAFP protein; however, these techniques were able to generate much higher final protein levels because of the substantially increased cell concentrations obtained. We believe that the nature of the heterologous protein itself is an important factor in the levels of gene product expressed by *P. pastoris*, and note that expression levels can be one or more orders of magnitude different depending on which protein is expressed, notwithstanding the use of similar expression vectors, strains and cultivation conditions (Cregg et al 1993).

Future optimization will focus on two areas of research, namely the development of more productive genetic variants and the use of modelling and control to improve overall process productivity. In the former case, this would include the isolation of clones with multi-copy insertions of the SRAFP expression cassette into the yeast genome. A second possibility is the production of fusion proteins, where the cDNA of the SRAFP is fused to the gene of a protein known to express high levels in the *Pichia* system (see Cregg et al. 1993). In the area of modelling and control we are developing a model-based algorithm for mixed substrate feeding, which will respond to the predicted instantaneous needs of the changing cell concentration for substrate. Alternatively, a feedback control, based on, for example

dissolved oxygen, could be used to deliver a mixed substrate as it became depleted, as noted by an increase in this metabolic marker. In either of these instances, it is anticipated that the overall time required to reach the final cell density can be reduced relative to the empirical feeding strategies used in this work, resulting in an improvement in overall process productivity.

Comparison of expression systems

Comparison of expression of the cystine-rich SRAFP in three different biosynthetic systems (Table 1) has led to the development of an effective expression/purification scheme. Originally a baculovirus-infected insect cell system was tested for its ability to duplicate many post-translational modifications, including disulfide bond formation (O'Reilly et al. 1992). This system met with only modest success, producing up to 0.8 mg/l (unpublished results) of glycosylated, yet active pro-type-II AFP, secreted into the medium (Duncker et al. 1994). Subsequent attempts at expression from *E. coli*, using both the pT7/pGP1-2 heat-inducible system (Tabor and Richardson 1985) (unpublished results) and the pET isopropyl β-d-thiogalactopyranoside-inducible system (Novogen), produced yields of up to 4.0 mg/l unpurified AFP (unpublished results). At least 50% of this was present in inclusion bodies, and was not inclined to refold into active AFP following solubilization. Expression of the herring type II AFP (40% identical to SRAFP) in a similar *E. coli* system, yielded more than 20 mg/l in the insoluble fraction (V. Ewart, personal communication), which was successfully refolded and purified. Comparison of herring and sea raven AFP expression levels demonstrates the variability in expression that can exist between even closely related proteins. This observation suggests that there exist some characteristics intrinsic to each individual protein that play a role in determining expression levels. Expression strategies for proteins should therefore be developed on a case-by-case basis. Expression of SRAFP in the yeast, *P. pastoris*, as described here, met with the greatest success, achieving levels of up to 5 mg/l in shake-flask culture, which were improved sevenfold through the use of fermentation.

Table 1 Summary of expression levels obtained from different expression systems and different fermentation strategies. Baculovirus host: pro-type II antifreeze protein (AFP) was secreted from fall army worm Sf21 cells by transfected pBlueBac-srAFP vector as previously described (Duncker et al. 1994). *E. coli* host: mature type II AFP was produced intracellularly in *E. coli* JM83 cells by isopropyl β-D-thiogalactopyranoside induction of the T7 promoter on the pet20b+ (Novogen, Madison) expression vector, containing the mature sea raven type II AFP cloned into the *Nde*I and *Bam*HI sites by polymerase chain reaction cloning (unpublished results). *BMMY* buffered minimal methanol complex medium

Host system	Medium	Yield (mg/l)
Pichia pastoris		
Flask: Mut^{s}, pPIC9-SRm CTHT	BMMY	5.0
	Defined	Undetectable
Fermentation:		
Mut^{s} MeOH feed	Defined	2
Mut^{s} Mixed feed	Defined	30
Mut^{s} Peptone	Defined	35
Mut^{+} MeOH feed	Defined	37
Baculovirus	Complex	< 1.0
Escherichia coli	Complex	2.0

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