EXPRESSION IN YEAST (*PICHIA PASTORIS*) OF RECOMBINANT CB-PERITROPHIN-42 AND CB-PERITROPHIN-48 ISOLATED FROM *CHRYSOMYA BEZZIANA* (THE OLD WORLD SCREWWORM FLY)

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ABSTRAK

MUHARSINI, SRI dan TONY VUOCOLO. 2000. Ekspresi protein rekombinan Cb-peritrofin-42 dan Cb-peritrofin-48 yang diisolasi dari lalat *Chrysomya bezziana* (*the Old World Screwworm fly*) pada khamir (*Pichia pastoris*). *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 177-184.

Pichia pastoris telah diteliti untuk mengekspresikan bentuk rekombinan dari dua macam protein membran peritrofik yang diduga sebagai antigen lalat *Chrysomya bezziana*, Cb-peritrofin-42 (Cb42) dan Cb-peritrofin-48 (Cb48). Cb48 rekombinan telah berhasil diekspresikan oleh sel khamir dalam bentuk berikatan dengan glikosilat. Hasil dari ekspresi protein rekombinan ini mencapai 8 mg per liter medium. Sebaliknya Cb42 rekombinan tidak dapat diekspresikan pada *Pichia pastoris* sampai pada level yang dapat dideteksi, hal ini kemungkinan disebabkan oleh banyaknya A + T sekuens yang menyebabkan penghentian transkripsi terlalu awal. Untuk mengatasi hal tersebut, Cb42 dibagi menjadi dua domain: Cb42A dan Cb42B. Cb42B dapat diekspresikan pada *Pichia pastoris* dan menghasilkan 0,4 mg protein rekombinan per liter medium, tetapi Cb42A tidak dapat diekspresikan. Penelitian ini menunjukkan bahwa walaupun *Pichia pastoris* dapat digunakan sebagai sistem untuk menghasilkan protein yang berikatan glikosilat, namun keberhasilan pengekspresian bervariasi dari protein ke protein.

Kata kunci: *Chrysomya bezziana*, peritrofin, *Pichia pastoris*

ABSTRACT

MUHARSINI, SRI and TONY VUOCOLO. 2000. Expression in yeast (*Pichia pastoris*) of recombinant Cb-peritrophin-42 and Cb-peritrophin-48 isolated from *Chrysomya bezziana* (the Old World Screwworm fly). *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus*) 5 (3): 177-184.

Pichia pastoris has been investigated as a means to express recombinant forms of two putative peritrophic membrane antigens from *Chrysomya bezziana*, Cb-peritrophin-42 (Cb42) and Cb-peritrophin-48 (Cb48). Recombinant Cb48 was expressed as a secreted and glycosylated protein. The yield of recombinant protein was 8 mg per litre of culture. In contrast, recombinant Cb42 was not expressed at detectable levels in *Pichia pastoris*, probably due to A + T rich sequence which may cause premature transcriptional termination. To expedite Cb42 expression in yeast, Cb42 was divided into two domains: Cb42A and Cb42B. Cb42B was successfully expressed in *Pichia pastoris*, yielding 0.4 mg per litre of culture. However, Cb42A was not expressed. This work demonstrates that although *Pichia pastoris* offers considerable benefits as an expression system producing high level of glycosylated protein, success may vary from protein to protein.

Key words: *Chrysomya bezziana*, peritrophin, *Pichia pastoris*

INTRODUCTION

The use of eukaryotic expression systems for the production of recombinant proteins has increased in the past few years. Yeast, in particular, has several advantages as a host for the production of heterologous proteins compared to *E. coli* (CREGG *et al*., 1987; BUCKHOLZ and GLEESON, 1991; GLICK and PASTERNAK, 1998). Firstly, yeast allows the formation of correct disulfide bonds that occur at the post-translational stage after synthesis of polypeptide. Properly folded protein may have increased resistance to protease degradation and also a much greater likelihood of possessing biological activity. Secondly, eukaryotic cells such as yeast have the capability to glycosylate proteins through the addition of sugar residues to asparagine (N-linked glycosylation) or serine/threonine (O-linked glyco-sylation). Thirdly, specific constructs in yeast can secrete recombinant proteins in forms, which are biologically active.

P. pastoris is a second generation of yeast expression system, developed after *Saccharomyces cereviciae*. *P. pastoris* is a methylotrophic yeast with the ability to use methanol as a carbon source. The adaptation of *P. pastoris* to use methanol for growing is related to the alcohol oxidase (*AOX*) gene. Its benefits as an expression system over *S. cereviciae* include high levels of expression (RODRIGUEZ *et al*., 1994; KIM *et*

al., 1997REYTOR *et al*., 1998; RICHARD *et al*., 1998; KOCKEN *et al*., 1999) and a lesser tendency to hyperglycosylate as the oligosaccharide chains added post-translationally are relatively short compared to *S. cereviciae* (TSCHOPP *et al*., 1987). Like other eukaryotic systems, yeast has the ability to generate recombinant proteins with biological activity (NILES *et al*., 1998; ZHU *et al*., 1998). A number of commercial products have been produced using this technology (SREEKRISHNA and KROPP, 1996).

In view of the potential benefits of *P. pastoris* as an expression system, we have investigated its ability to express the peritrophic membrane proteins Cb42 and Cb48 from *C. bezziana.*

MATERIALS AND METHODS

The *P. pastoris* system used to express the recombinant proteins was obtained from Invitrogen (San Diego, CA., USA) and the procedure was essentially as outlined by the technical manual accompanying this system. Figure 1 shows a flow-diagram outlining the methods. The following is an outline of the procedures used with four proteins or protein fragments.

Cloning into *P. pastoris* expression vectors

The initial construction of the recombinant constructs was performed by insertion of PCR generated DNA fragments encoding Cb42, Cb42A, Cb42B and Cb48 into an intermediate vector, pGEM-T (Promega, Madison, WI, U.S.A.) and then into the *Pichia* expression vector pPICZα (A or C) (Invitrogen). The *Pichia* expression vectors used in this work were designed for extracellular expression of recombinant protein. These vectors also add a hexaHis tag to the carboxy-terminus of the expressed protein allowing purification on a Ni-NTA affinity column (PEARSON *et al*., 2000). The DNA fragments for expression were.

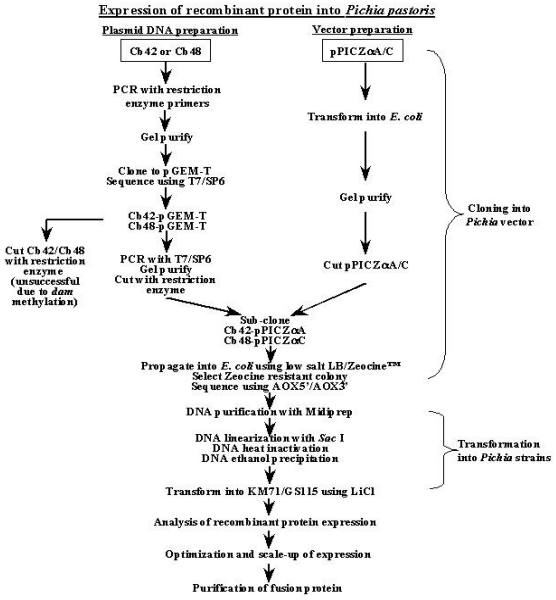

Figure 1. The flow-diagram outlining the methods to produce recombinant Cb42 and Cb48

generated by PCR using oligonucleotide primers to which had been added restriction enzyme sites. The oligonucleotide primers were synthesized using a Gene Assembler 1000 (Beckman) and following cleavage and deprotection were dried using a DNA Speed Vac (Savant) and resuspended in Tris-EDTA (TE) buffer to a concentration of 100 pmol/µl. PCR amplification was performed using 5 ng of plasmid DNA containing the Cb42 or Cb48 cDNA obtained from the cDNA library screens (VUOCOLO *et al*., 2000b). PCR was performed in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.01% gelatin, 200 µM dNTP, 100 pmol of each primer and 2.5 U REDTAQ™ DNA Polymerase (Sigma, Saint Louis, MS, U.S.A.). Amplification was performed for 35 cycles consisting of denaturation for 60 s at 94°C, annealing for 60 s at 52°C and extension for 90 s at 72°C.

The PCR products were run on an agarose gel and the DNA fragments excised and purified using a Bresaclean DNA purification kit (Geneworks, Adelaide, S.A., Australia). These fragments were cloned into the PCR T/A cloning vector, pGEM-T and transformed into *E. coli* (SAMBROOK *et al*., 1989). Plasmid DNA of the Cb42 and Cb48 pGEM-T constructs were prepared by Rapid miniprep kit (Qiagen). The plasmid DNA was digested with the restriction enzymes, *Eco*R I and *Xba* I, for Cb42, *Eco*R I and *Sal* I for Cb42A and Cb42B, and *Cla* I and *Xba* I for Cb48. The digests using *Xba* I or *Cla* I was unsuccessful due to *dam* methylation of these sites in the plasmid DNA (TCTAGATC or ATCGATC). To overcome this problem, the intermediate pGEM-T constructs were subjected to PCR using T7 and SP6 primers under the same amplification conditions as described above. The amplified DNA, which was not methylated, was then digested with the restriction enzymes as described above. The digested DNA was sub-cloned into the appropriately cut pPICZα vector. Cb42, Cb42A and Cb42B DNA fragments were sub-cloned into pPICZαA, while Cb48 was sub-cloned into pPICZαC. These constructs were propagated in *E. coli* by plating onto low salt Luria Bertani (LB) agar (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.5% w/v NaCl and 1.5% w/v bacto agar) containing 25 µg/ml Zeocin™(Invitrogen). The plates were incubated at 37°C, overnight. Five to ten transformants of each construct were selected and inoculated into 3 ml low salt LB medium containing 25 µg/ml Zeocin™ (37°C, overnight) with shaking. Plasmid DNA was isolated by Rapid miniprep kit (Qiagen) and the inserts analysed by automated DNA sequencing (the Australian Genome Research Facility) using 5'AOX1 and 3'AOX1 primers and Big Dye terminator mix (ABI). Clones, which contained correct in frame sequence, were selected and used for transformation into *P. pastoris* strains.

Transformation into *P. pastoris* strains

pPICZαA-Cb42 and pPICZαC-Cb48 constructs were transformed using a LiCl procedure into two *P. pastoris* strains: KM 71 (Mut^s) and GS115 (Mut^+). Briefly, high purity DNA from the constructs was prepared using a plasmid midi kit (Qiagen) and the DNA concentration determined by absorbance at 260nm. The purified plasmid DNA (10 µg) was linearized by digestion with *Sac* I (37°C, overnight), which was then heat inactivated at 70°C, 15 min. The linearized DNA was ethanol-precipitated and resuspended in 50 µl of sterile milli-Q water (MQW).

GS115 and KM71 strains were initially grown in 50 ml of Yeast Peptone Dextrose (YPD: 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) (30°C, overnight) to an OD_{600} of 0.8 - 1.0. The cells were harvested and washed with 25 ml MQW and spun (1,500 *g*, 10 min, RT). The cell pellet was resuspended in 1 ml 100 mM LiCl and transferred to a microfuge tube. The pellet was spun briefly and the LiCl removed. The pellet was resuspended in 400 µl of 100 mM LiCl and dispensed in 50 µl aliquots for immediate use.

The 50 µl cell suspension was centrifuged to remove LiCl. The following reagents were added to the cell pellet in the following order: 240 µl of 50% PEG, 36 µl of 1 M LiCl, 25 µl of 2 mg/ml sheared and denatured herring sperm DNA and 50 µl (10 µg) of the linearized pPICZαA-Cb42 or pPICZαC-Cb48 plasmid DNA. The cell pellet and reagents were vortexed to ensure homogeneity and then incubated at 30°C for 30 min. The cell suspension was then heat shocked (42°C, 25 min), the suspension centrifuged (6,800 *g*, 1 min, RT) and the transformation solution removed. The pellet was resuspended in 1 ml YPD and incubated (30 °C, 3 h) on a rotating culture wheel. The cell suspension (25 µl to 100 µl) was plated on YPD agar containing 100 µg/ml Zeocin™. The plates were incubated (30°C, 3 d) to allow transformed *P. pastoris* colonies to grow.

Transformed *P. pastoris* colonies were analyzed by PCR to ensure they contained the Cb42 or Cb48 DNA fragments. Ten colonies from each transformation were picked and transferred into individual PCR tubes and onto a master YPD agar plate containing Zeocin™. These colonies were subjected to PCR using the same primers and conditions, which had initially been used to amplify the fragments for cloning into pGEM-T vector. The PCR result was visualized by agarose gels to confirm that the colonies contained an appropriate DNA insert.

Analysis for recombinant protein expression

Several positive *P. pastoris* transformants from each transformation were analyzed for expression of the desired recombinant protein. This analysis was per-

formed in 25 ml culture assays. Single colonies of Cb42 or Cb48 in GS115 and KM71, were inoculated into 25 ml of Buffered Glycerol-complex Medium (BMGY: 1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer pH 6.0, $4x10^{-5}$% v/v biotin and 1% v/v glycerol) in a 50 ml tube. The cultures were incubated (30°C, overnight) with shaking (250 rpm) to reach an OD_{600} of approximately 2 - 6. The cells were harvested by centrifugation (1,500 *g*, 15 min, RT). The cell pellet was resuspended in 25 ml Buffered Methanol-complex Medium (BMMY: 1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer pH6.0, $4x10^{-5}$ % biotin and 1% methanol) and transferred to a 100 ml plastic bottle. The culture was incubated (30°C, 4 d) with shaking and supplemented with methanol to a final concentration of 0.5% every 24 h. Samples of the expression culture were taken at time points: 0, 24, 48, 72 and 96 h. These samples were spun (15,300 *g*, 2 min, RT) and the supernatant and pellet collected for analysis.

SDS-PAGE and immuno-blot analyses were performed on the time course samples to determine whether any Cb42 or Cb48 recombinant protein could be detected. The culture media from the different time points was concentrated 10 fold using a Centricon-10™ device (Millipore, Bedford, MA, USA). The cell pellets were resuspended in SDS-PAGE reducing sample buffer and loaded onto the gel. Gels were silver-stained and electrophoresed for immuno-blot analysis using either a Ni-NTA alkaline phosphatase conjugate (Qiagen) or antibodies raised to Cb42 or Cb48 bacterial recombinant protein (WIJFFELS *et al*., 2000).

Optimization and scale-up expression of recombinant protein

The selected clones with highest expression as determined from the small-scale expression experiments, were grown in scaled–up 1 L cultures to optimize recombinant protein expression. Several loops of yeast colonies on a YPD/Zeocin™/agar masterplate which contained the recombinant Cb42 or Cb48, were taken and inoculated into 250 ml BMGY. The culture was incubated (30°C, overnight) with shaking to achieve an OD_{600} ~ 6. The cell pellet was harvested by centrifugation (2,000 *g*, 10 min, RT). The pellet was washed with 100 ml BMMY to remove all traces of glycerol from the starter culture and then resuspended in 1 L BMMY to an initial OD_{600} of ~ 1.5. The culture was incubated (30°C, 4 d) with shaking and supplemented with methanol as previously described. Samples were taken as previously described for gel analysis and the optimum time for harvesting the recombinant protein determined.

Once the optimal conditions for recombinant protein production had been determined, a scale–up to several litres was undertaken. The cultures were treated exactly as outlined previously and grown for the optimal time for recombinant protein production. The culture was then centrifuged (7000 *g*, 4°C, 10 min) and the supernatant collected. The culture supernatant was concentrated approximately 10 fold using an Amicon Y-10 concentrating cell (Millipore, Bedford, MA, USA). This concentrate was subjected to Ni-NTA affinity chromatography to allow purification of the expressed recombinant protein. The purification procedure and resulting analysis of the recombinant proteins are described in PEARSON *et al*. (2000).

RESULTS AND DISCUSSION

Cloning of Cb42 and Cb48

DNA fragments encoding the mature protein sequence of Cb42 and Cb48 were generated by PCR using the primers Cb42F3 and Cb42R3, Cb48PFEX1 and Cb48PREX1, respectively (Table 1). The PCR product size generated was 435 bp for Cb42 and 1088 bp for Cb48 with the restriction linker sites of *Eco*R I and *Xba* I, and *Cla* I and *Xba* I, respectively. These products were initially cloned into the PCR cloning vector, pGEM-T. This vector was to act as an intermediate vector to allow appropriate restriction enzyme cleavage of the amplified DNA products allowing cloning into the identically cut *P. pastoris* transformation vector, pPICZα. The Cb42 and Cb48 intermediate clones were grown and the plasmid DNA isolated. These constructs were sequenced and validated. However, dual restriction digests with the restriction enzymes mentioned previously were unsuccessful. It became evident that only one enzyme was cutting. In the case of these recombinant proteins, *Xba* I and *Cla* I would not cleave the plasmid DNA of Cb42 and Cb48, respectively. Close analysis of the DNA sequence showed that the restriction sites *Xba* I and *Cla* I located in the restriction linkers of Cb42 and Cb48, respectively were affected by *Dam* methylation thus making these sites resistant to cleavage.

Table 1. Oligonucleotide primers and their restriction enzyme linkers used for the generation of DNA fragments encoding Cb48 and three versions of Cb42 for use in expression of recombinant protein in *Pichia pastoris*

Fragment amplified	Size (bp)	Primer designation	Primer linker
Cb42	435	Cb42F3	*EcoR I*
		Cb42R3	*Xba* I
Cb42A	220	Cb42F3A	*EcoR* I
		Cb42R3A	*Sal* I
Cb42B	225	Cb42F3B	*Eco*R I
		Cb42R3B	*Sal* I
Cb48	1088	*CbPFEX1*	*Cla* I
		CbPREX1	*Xba* I

Dam methylation is determined by the *dam* gene, which causes a shift of the methyl group from S-adenosylmethionine to the N6 position of the adenine residue in the sequence 5'….GATC…..3' (MARINUS and MORRIS, 1973; GEIER and MODRICH, 1979). Many strains of *E. coli* contain the site-specific *Dam* methylase gene and indeed, both the *E. coli* strains that have been used in this cloning work, XL1-Blue and DH5α, contain this gene. To circumvent this cleavage problem, direct PCR amplification of the desired sequence and direct restriction cleavage of this product was used or alternatively, a *Dam*$^-$(minus) strain of *E. coli* could be used in the cloning process. The Cb42 and Cb48 intermediate constructs in pGEM-T were amplified by PCR using the T7 and SP6 primers located in the vector. A 600 bp and a 1270 bp PCR fragments were amplified from Cb42 and Cb48, respectively as anticipated (data not shown). These PCR products were purified and cleaved using the restriction enzymes contained within the restriction linkers corresponding to Cb42 and Cb48. These cleaved PCR derived DNA fragments were successfully ligated into the identically cut pPICZαA and pPICZαC vectors respectively and transformed into *E. coli.* Several clones from each transformation were chosen and grown in low salt LB containing Zeocin™. Plasmid DNA was isolated and the clones were sequenced using AOX5' and AOX3' as the forward and reverse primers respectively. The DNA sequence confirmed that the fragments had been cloned in frame into the pPICZα vectors with a secretion signal sequence and a carboxy-terminal hexaHis tag provided by the vector. Figure 2 shows the recombinant Cb42, Cb42A, Cb42B, and Cb48, which were ligated to pPICZαA/C and cut with restriction enzymes. One verified clone for each construct was chosen and processed through to transformation and expression in *P. pastoris*. Figure 3 shows YPD/Zeocin™ plates with transformed *Pichia* colonies containing Cb42 and Cb48.

Expression of recombinant Cb42

The Cb42 transformed *P. pastoris* strains KM71 and GS115 were analysed for recombinant protein expression. These expression studies were initially conducted in small-scale (50 ml) cultures. No expression was evident for the Cb42 transformed *Pichia* cells as determined by SDS-PAGE and immuno-blot using affinity purified antiserum to hexaHis-Cb42 from *E. coli* (WIJFFELS *at al.,* 2000) or anti Ni-NTA conjugates (Qiagen) (data not shown). The expression experiment was optimized to a 1 L culture. However this also returned a negative expression result.

Expression of recombinant Cb42A and Cb42B

The failure of Cb42 expression might be due to the (A+T) rich composition of its DNA sequence, which may cause premature transcriptional termination. Investigation of this problem with regards to the Cb42 construct was undertaken by dividing the Cb42 protein into two putative domains, Cb42A and Cb42B. Cb42A encoded 68 amino acids, consisting of 4 cysteine residues and 1 potential N-linked glycosylation site. Cb42B comprised 73 amino acids with 6 cysteine residues and is similar to Cb-peritrophin-15 (TELLAM *et al*., 1999). The respective A + T content of these two domains is 64% and 59%. Primers Cb42F3A and Cb42R3A, and Cb42F3B and Cb42R3B (Table 1), were used to amplify Cb42A and Cb42B, respectively resulting in a product of 220 bp and 225 bp, respectively (Figure 2). These products were cloned into pPICZαA, sequence validated and transformed into *Pichia* strains, KM71 and GS115. The same protocol for Cb42 expression and analysis were used for Cb42A and Cb42B. There was no detectable recombinant expression for Cb42A when analyzed by SDS-PAGE or immuno-blot analysis. A product of approximately 14 kDa for Cb42B (time course 96 h) was detected by immuno-blot when probed with affinity purified antiserum to recombinant hexaHis-Cb42 from *E. coli* (WIJFFELS *et al*., 2000 and Figure 6). The yield of the recombinant Cb42B expressed in *P. pastoris* was 0.4 mg/l of culture.

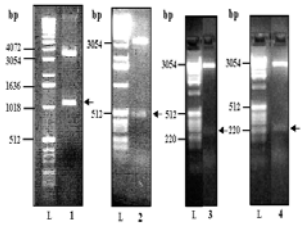

Figure 2. Plasmid DNA encoding recombinant peritrophins Cb48 and Cb42 of *Chrysomya bezziana.* Recombinant DNA was analysed by electrophoresis on 1.3% agarose gel containing ethidium bromide. The plasmid DNA was ligated to pPICZαA/C and digested with restriction enzymes (see Material and Methods). Lane L represents 1 kb ladder. Lane 1, 2, 3, 4 represents recombinant constructs of Cb48, Cb42, Cb42A and Cb42B, respectively. Insert DNA is indicated by an arrow

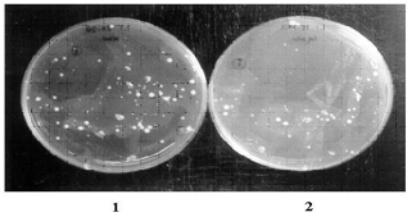

Figure 3. YPD/Zeocin™ plates with transformed *Pichia* colonies containing Cb48 after 4 d incubation. Plates 1 and 2 represent recombinant Cb48 *Pichia* clones in strain GS115 and KM71, respectively

The failure of Cb42A to be expressed in *P. pastoris* is consistent with the hypothesis that the failure of expression of recombinant Cb42 in *Pichia* may be due to the A + T rich stretches of its DNA sequence. Furthermore, genes with a high A+T nucleotide content are not transcribed efficiently due to a propensity for premature terminations (SCORER *et al.*, 1993). A sequence, ATTATTTTATAAA of HIV-1 envelope glycoprotein, has been identified to cause production of truncated mRNA (SCORER *et al.*, 1993). Stretches of sequence similar to this are found in Cb42 and may have the same effect. Conversely, genes which are G + C rich are more efficiently transcribed (ROMANOS *et al.*, 1991). To overcome the transcriptional problem, redesigning the A/T stretches to an A + T content in the range of 30 - 55% could be tried (SREEKRISHNA and KROPP, 1996). Analysis of the A + T content of Cb42A and Cb42B only showed a difference of 5%, suggesting that localized A/T stretches are more likely to explain the failure of expression rather than gross differences in codon usage. Cb48 was expressed successfully and it has an A + T content similar to the whole Cb42 gene, therefore codon usage is probably not a factor in failed expression of recombinant protein.

Expression of recombinant Cb48

Expression of recombinant Cb48 was successful in both strains of *P. pastoris* (KM71 and GS115). Expression of recombinant protein was detected by gel analysis and immuno-blots using antiserum to hexaHis-Cb48 from *E. coli* (VUOCOLO *et al.*, 2000a) or Ni-NTA conjugates (Qiagen). The recombinant protein was soluble and collected from the medium indicating that the recombinant protein was secreted. Recombinant Cb48 was detected in small-scale cultures (50 ml) and was optimized in larger 1 L cultures. KM71 transformed cells appeared to produce significantly more recombinant Cb48, hence this transformed strain was used for recombinant protein production. A time course experiment was undertaken from 0 to 96 h to determine the optimal time for harvesting the recombinant protein. Figure 4 shows gel analysis and an immuno-blot detecting the presence of Cb48 recombinant protein using a Ni-NTA alkaline phosphatase conjugate for the time course investigation. This conjugate detected the expressed protein by binding to the hexaHis tag located at the carboxy-terminus. The optimal time for harvesting of the recombinant Cb48 protein was determined to be 24 – 48 h. The lack of detectable expression for the 72 h and 96 h time points was unexpected and may be due to proteolytic degradation of the protein over extended incubation.

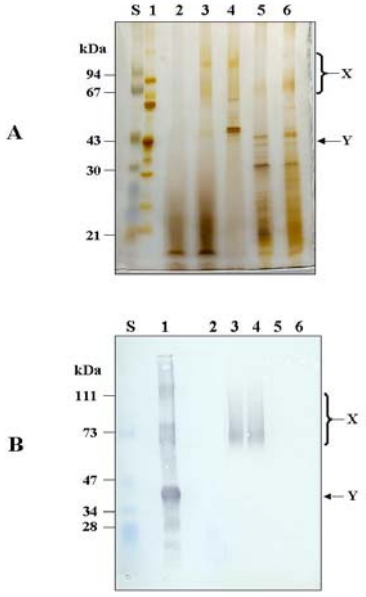

Figure 4. Gel analysis of time course *Pichia*-Cb48 expression. Samples of the culture were taken at nominated time-points, concentrated 10 fold and resolved in 6 - 18% gradient gel. Lane S, molecular weight standard. Lane 1, purified bacterial recombinant hexaHis-Cb48. Lanes 2 – 6, time points 0, 24, 48, 72 and 96 h respectively. X denotes the region of recombinant *Pichia*-Cb48. Y denotes the region of recombinant bacterial Cb48. (A). The gel was silver-stained. (B). The gel was immuno-blotted using a Ni-NTA alkaline posphatase conjugate

Once the parameters for optimal expression of Cb48 were determined, the expression was scaled up to several litres of expression culture. The KM71 transformed Cb48 *Pichia* cells were grown for 48 h after which the culture was centrifuged and the medium collected. The medium was concentrated 10 fold and the expressed protein purified by Ni-NTA affinity chromatography analysis undertaken as described in PEARSON *et al*. (2000). Amino-terminal sequence analysis confirmed that the protein was Cb48 and was correctly processed. The yield of recombinant Cb48 protein was 8 mg/litre of culture. The protein was approximately 75 kDa in size and glycosylated with mannose type sugars. This is consistent with the deduced protein sequence which contains six potential N-linked glycosylation sites. The size difference of the recombinant protein of approximately 75 kDa compared to the size of approximately 40 kDa obtained from the bacterial recombinant protein (WIJFFELS *et al.*, 2000) may also be explained by this glycosylation. Figure 5 shows a comparison of the purified *Pichia* Cb48 and bacterial recombinant protein. This recombinant protein was used for a vaccination trial in sheep (SUKARSIH *et al.*, 2000).

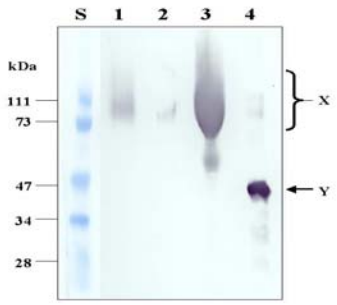

Figure 5. Immuno-blot analysis of purified of *Pichia*-Cb48 in comparison with the purified bacterial recombinant Cb48. The samples were resolved in a 6 - 18% gradient gel. Lane S, molecular weight standard. Lane 1, crude concentrated *Pichia*-Cb48 from culture. Lane 2, unbound material to Ni-NTA column. Lane 3, purified *Pichia*-Cb48 eluted from Ni-NTA column. Lane 4, purified bacterial Cb48. X denotes the region of recombinant Pichia-Cb48, Y denotes the region of recombinant bacterial Cb48. The gel was probed with a Ni-NTA alkaline phosphatase conjugate

CONCLUSION

One of the more important and difficult tasks in the production of recombinant vaccines is the expression of the antigen in a form which retains biological or antigenic activity as close as possible to the native antigen, while potentially allowing cost effective, industrial scale production. The *Pichia pastoris* expression system in principle should have both capabilities although, as this paper shows, not without difficulty in specific cases.

We have expressed recombinant protein of *C. bezziana* larval peritrophins, Cb48 and part of Cb42 (Cb42B). The expression levels of Cb48 and Cb42B proteins were 8 mg and 0.4 mg per liter of culture, respectively. The expression was unsuccessful with two other proteins or fragments of proteins. Thus, whilst use of *P. pastoris* as an expression system may provide benefits over the use of bacteria, not all proteins are expressed efficiently.

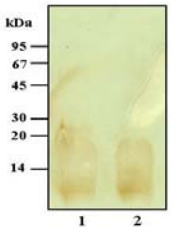

Figure 6. Immuno-blot analysis of recombinant Cb42B at the time course point of 96 h. The samples were resolved in 15% acrylamide gel and the blot was probed with affinity purified antiserum to recombinant hexaHis-Cb42 from *E. coli*. Lanes 1 and 2 represent the positive expression clones

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