PURIFICATION OF RECOMBINANT PERITROPHIC MEMBRANE PROTEINS OF THE OLD WORLD SCREWWORM FLY, *CHRYSOMYA BEZZIANA*

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ABSTRAK

PEARSON, ROGER, SRI MUHARSINI, GENE WIJFFELS, dan TONY VUOCOLO. 2000. Pemurnian beberapa protein membran peritrofik rekombinan lalat *the Old World Screwworm*, *Chrysomya bezziana*. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 185-191.

Untuk mengevaluasi kelayakan vaksinasi domba terhadap lalat *the Old World Screwworm Chrysomya bezziana*, beberapa protein peritrofin rekombinan telah diekspresikan baik pada *Pichia pastoris* dalam keadaan denaturasi maupun pada *Escherichia coli* dalam bentuk natif. Pemurnian protein dengan label hexaHis tersebut dilakukan dengan *immobilized metal affinity chromatography*. Protein yang dimurnikan dalam keadaan reduksi dinetralkan kembali dengan glutation. Pemurnian protein fusi S-transferase glutation dilakukan dengan menggunakan *glutathione affinity chromatography* dilanjutkan dengan *anion exchange chromatography*. Keaslian dari protein yang diekskresikan dievaluasi kembali dengan teknik perunutan asam amino dari asam amino terminal. Analisis karbohidrat dengan *biotinylated lectin* menunjukkan bahwa Cb-peritrophin-48 yang diekspresikan pada *Pichia pastoris* mengalami proses glikosilasi dengan gugus gula manosa dengan jumlah banyak. Empat protein rekombinan yang telah dimurnikan digunakan untuk mengevaluasi tingkat imunogenisitas protektifnya pada domba terhadap investasi larva lalat *Chrysomya bezziana*.

Kata kunci: Lalat *screwworm*, *Chrysomya bezziana*, protein rekombinan, *immobilized metal affinity chromatography*

ABSTRACT

PEARSON, ROGER., SRI MUHARSINI, GENE WIJFFELS, TONY VUOCOLO, and GEORGE RIDING. 2000. Purification of recombinant peritrophic membrane proteins of the Old World Screwworm fly, *Chrysomya bezziana*. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 185-191.

To evaluate the feasibility of vaccinating sheep against the Old World Screwworm fly *Chrysomya bezziana* several recombinant peritrophin proteins were expressed in either a denatured form in *Escherichia coli* or a native-like form in *Pichia pastoris* cultures. Purification of the hexaHis tagged proteins was achieved by immobilized metal affinity chromatography. Proteins purified under reducing conditions were refolded using a glutathione shuffle procedure. Purification of a glutathione-S-transferase fusion protein was attempted using glutathione affinity chromatography in conjunction with anion exchange chromatography. The authenticity of the expressed proteins was verified by amino terminal amino acid sequencing. Carbohydrate analysis using biotinylated lectins revealed that Cb-peritrophin-48 expressed in *Pichia pastoris* was glycosylated with high mannose-type sugars. Four of the purified recombinant proteins were used to evaluate their protective immunogenicity in sheep against *Chrysomya bezziana* strike.

Key words: Screwworm fly, *Chrysomya bezziana,* recombinant proteins, immobilized metal affinity chromatography

INTRODUCTION

This paper describes the purification of several *C. bezziana* recombinant peritrophin proteins prior to their evaluation as immunogens potentially able to protect sheep against *C. bezziana* fly strike. Native peritrophin proteins were isolated from third instar larval peritrophic membrane, an insoluble matrix that lines the midgut, and comprises proteins, proteoglycans and polysaccharides (TELLAM *et al*., 1999). These purified proteins were characterized by their apparent molecular weight as determined by SDS-PAGE and their amino (N) terminal and internal peptide amino acid sequences (RIDING *et al*., 2000). Recombinant versions of these proteins were expressed in either *E. coli* or in the yeast *P. pastoris*. The cDNAs encoding two of these proteins, Cb-peritrophin-15 (Cb15) and Cb-peritrophin-42 (Cb42) were cloned from a Screwworm fly cDNA library using peptide sequence information, whilst the cDNA encoding the protein Cb-peritrophin-48 (Cb48)

was cloned from the library after screening with the PM48 gene from *L. cuprina* (VUOCOLO *et al*., 2000a). All three proteins were expressed in *E. coli,* with N-terminal or carboxy (C) terminal hexaHis tags. Due to problems of solubility with the full length Cb42, this protein was split into 2 domains and expressed as Cb42 domain A and B in *E. coli* and in *P. pastoris* (WIJFFLES *et al*., 2000, MUHARSINI and VUOCOLO, 2000). A glutathione-S-transferase (GST) Cb-peritrophin-15 (GST-Cb15) was also expressed in *E. coli* as a fusion protein with the GST fused to the N-terminal end of the protein to facilitate purification by glutathione agarose affinity chromatography.

Cb48 and Cb42 domains A and B were expressed as C-terminal tagged hexaHis proteins in the yeast *P. pastoris* (MUHARSINI and VUOCOLO, 2000).

The fusion of a hexaHis tag to these recombinant proteins aided in their detection during expression and facilitated their purification by immobilized metal affinity chromatography (IMAC) using nickel-nitrilotriacetic acid agarose (Ni-NTA), (Qiagen, Germany).

MATERIALS AND METHODS

Detection and localization of expressed recombinant proteins

In *E. coli* cultures, cells from 10 ml of culture were lysed by sonication on ice for 3 × 30 s (A180G Rapidis Sonicator at power setting 10 with a 3 mm probe) in 2 ml of 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, (TBS) containing 1.0 mg/ml lysozyme. The lysed material was centrifuged at 14,000 g for 20 min and the supernatant and pellet collected for analysis. *P. pastoris* cultures were centrifuged at 5000 g for 20 min and the pelleted yeast cells from 10 ml of culture, lysed and centrifuged as described previously. Lysed cell pellet and supernatant from *E. coli* and *P. pastoris* cells and supernatant from *P. pastoris* cultures were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. All gels were stained either by Coomassie Blue or by silver staining to identify samples that contained recombinant protein. When expression levels of recombinant proteins were low, samples were analysed by Western blots and probed with either Ni-NTA Conjugate (QIAGEN, 1999) or when available, antibodies specific to the mature protein or to the hexaHis or GST tag.

Purification of insoluble hexaHis tagged proteins expressed in *E. coli*

Recombinant proteins expressed as insoluble inclusion bodies were purified under denaturing conditions as recommended (QIAGEN, 1997) with several modifications. All buffers used contained Complete® EDTA free protease inhibitors (Boehringer Mannheim, Germany) and all manipulations unless stated otherwise, were performed at 4^{o}C. Essentially the procedure was as follows:

E. coli cells from 1 L cultures were harvested by centrifugation at 5,000 g for 20 min and washed once in TBS. The pellet was resuspended using a glass tissue homogenizer in 50 ml of TBS containing 1.0 mg/ml lysozyme, 0.1% Triton X-100. The suspension was sonicated on ice for 3 × 30 s (180 W Rapidis Sonicator at power setting 12 with a 9 mm probe) and centrifuged at 12,000 g for a further 20 min. The pellet was resuspended in TBS containing 0.5% Triton X-100, sonicated for a further 3 × 30 s and centrifuged at 12,000 g for 20 min. The cell membrane/inclusion body pellet was solubilized for 1 h in 50 ml of 8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0, 2 mM 2-mercaptoethanol. The solution was clarified by centrifugation at 100,000 g for 30 min and the supernatant containing solubilized recombinant protein mixed with 3 ml of Ni-NTA resin and gently rolled for 1 h. Non-bound proteins were removed by washing the resin twice with solubilizing buffer and centrifuging at 1,000 g for 2 min.

In preparation for chromatography, the Ni-NTA resin was loaded into an open glass chromatography column (10 mm × 100 mm) connected to an FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden) for gradient elution of the bound protein. Weakly bound proteins were removed by washing the resin with 8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 6.3. Recombinant protein was eluted with a 0 – 1.0 M imidazole gradient in pH 6.3 buffer at a flow rate of 1.0 ml/min over 40 min. The chromatography was performed at room temperature and 1.0 ml fractions collected. Fractions were analysed by SDS-PAGE gel under reducing conditions to determine the location and purity of the recombinant protein. A 10 µl sample of the Ni-NTA resin after elution of bound protein was analysed in the same manner to ensure that all recombinant protein had been recovered.

Recombinant proteins purified under denaturing conditions were in an unfolded state. Prior to use in vaccination trials, they were renatured using a reduced/oxidised glutathione shuffle procedure to allow refolding. Pure protein fractions were pooled, dialysed against 8 M urea, 20 mM Tris–HCl, pH 8.5, 500 mM NaCl, to remove imidazole and the protein concentration determined using a BCA kit (Pierce, Rockford, IL.). The denatured recombinant protein was pumped at a flow rate of 2.0 ml/h into a pre-determined volume of folding buffer (3 M urea, 20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 10 mM reduced glutathione, 1.0 mM oxidised glutathione, 1.0 mM EDTA, 0.01% v/v Tween-20) to give a final protein concentration of 50

μg/ml. The solution was stirred in a beaker open to the air for 48 h. On completion of folding the recombinant protein was concentrated to 1.0 mg/ml by ultra-filtration in an Amicon stirring cell (Millipore Corporation, USA) fitted with a YM 10 (10 kDa cut-off) filter. Dialysis, folding and ultrafiltration were performed at 4°C. Any aggregates of protein were removed by centrifugation at 100,000 g for 30 min.

Purification of soluble hexaHis tagged proteins expressed in *E. coli*

Soluble recombinant proteins located in the cytoplasm of *E. coli* were purified under native conditions. All buffers contained Complete EDTA free protease inhibitors and all manipulations were performed at 4°C. *E. coli* cells from 1 L cultures were harvested by centrifugation at 5,000 g for 20 min and washed once in TBS. The pellet was resuspended using a glass tissue homogenizer, in 50 ml of lysis/binding buffer (10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM imidazole, 1.0 mg/ml lysozyme, 0.1% v/v Triton X-100). The suspension was sonicated on ice for 3 × 30 s and centrifuged at 100,000 g for 30 min. The supernatant was collected, mixed with 3.0 ml of Ni-NTA resin, gently rolled for 1 h and non-bound proteins removed by washing the resin twice with lysis/binding buffer and centrifuging at 1,000 g for 2 min in preparation for chromatography.

The chromatography, concentration and SDS-PAGE analysis were performed as described above with the following alterations. Gradient buffers used to elute the bound recombinant protein comprised buffer A (10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM imidazole) and buffer B (10 mM Tris-HCl, pH 8.0, 100 mM NaH_2PO_4, 500 mM NaCl, 1.0 M imidazole).

Purification of soluble GST fusion proteins expressed in *E. coli*

E. coli cells from 1 L culture were collected by centrifugation at 5,000 g 20 min and washed in TBS. The pellet was resuspended using a glass tissue homogenizer in 60 ml of lysis buffer (50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM EDTA, 5 mM benzamidine HCl, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), (ICN Biomedicals Irvine, CA.) 0.1% v/v Triton X-100) containing 2.0 mg/ml lysozyme. The suspension was sonicated as described above and the homogenate centrifuged at 12,000 g for a further 20 min. The pellet was frozen at –20°C and the supernatant containing recombinant protein, clarified by centrifugation at 100,000 g for 30 min. All manipulations were performed at 4°C.

Affinity chromatography was performed using glutathione agarose. The glutathione agarose (reduced, 1.5g), (Sigma-Aldrich, Australia) was rehydrated according to the manufacturer's instructions and equilibrated with TBS pH 8.2 (containing 5 mM EDTA, 5 mM benzamidine HCl) and added to 60 ml of cell lysate supernatant prepared as described above. The mixture was rolled for 30 min and the supernatant removed following centrifugation at 1,000 g for 2 min. The agarose was loaded into an open glass chromatography column (10 mm × 100 mm), connected to an FPLC and non-bound proteins removed by washing the agarose with equilibration buffer. Bound GST fusion protein was recovered with a pulse of equilibration buffer containing 5 mM reduced glutathione and collected fractions analyzed by SDS-PAGE. All chromatography was performed at 4°C.

Pooled fractions of GST recombinant protein from the glutathione agarose affinity chromatography were dialysed against 2 L buffer A (20 mM Tris-HCl, pH 8.0) containing 0.1% Zwittergent 3-14 (Boehringer Mannheim) and loaded onto a 1.0 ml anion exchange column (Mono-Q), (Amersham Pharmacia Biotech, Uppsala, Sweden). Non-bound proteins were removed by washing the column with buffer A. Proteins bound to the resin under these conditions were eluted with buffer B (buffer A + 1.0 M NaCl) delivered at 1% buffer B/min at 0.5 ml/min. Fractions were collected and analysed by SDS-PAGE gel. The anion exchange chromatography was performed at room temperature.

Purification of soluble hexaHis proteins expressed in *P. pastoris*

Media (1.25 L) from 62 h *P. pastoris* cultures was clarified by centrifugation at 5,000 g for 20 min and the supernatant fluid concentrated to 50 ml by ultra-filtration using an Amicon stirring cell fitted with a YM 10 kDa cut-off, filter. The concentrated retentate was dialysed against Ni-NTA equilibration buffer (10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM imidazole and any aggregates removed by centrifugation at 100,000 g for 30 min. Recombinant proteins were purified under non-denaturing conditions and the chromatography performed as described above (soluble hexaHis tagged proteins expressed in *E. coli*).

Amino-terminal amino acid sequencing

All purified recombinant proteins were analysed by SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membrane for authentication by N-terminal amino acid sequencing. Proteins were visualized by staining the membrane for 5 min with 0.05% Commassie Blue R-250, 40% methanol, 1% acetic acid, 59% distilled water. The membrane was destained in a

solution of 50% distilled water, 50% methanol for 5 min or until the protein bands were clearly visible, rinsed in distilled water and dried at 37°C for 2 h. Protein bands for N-terminal amino acid analysis were excised from the membrane and sequenced by an Applied Biosystems 471A sequencer (PE Applied Biosystems, Foster City, CA.).

Carbohydrate analysis

The glycosylation analysis of recombinant Cb48 expressed in *P. pastoris* (*Pichia*-Cb48) was performed using lectin blots (ELVIN *et al*., 1996). Essentially the method involved transferring 5 µg samples of the protein from SDS-PAGE to a nitrocellulose membrane which was subsequently blocked with a solution of TBS containing 5% skimmed milk powder, 0.1% v/v Tween-20, for 1 h. Individual lanes containing recombinant protein were probed with a solution of 20 µg/ml biotinylated lectin (Sigma-Aldrich) in TBS-Tween containing 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$ for 1 h. After washing with TBS-Tween the membrane strips were reacted with streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech) diluted 1:1,000 in TBS-Tween for a further 1 h. After washing, the strips were developed in a solution of TBS containing 0.6 mg/ml 4-chloro-1-naphthol (Bio-Rad), 0.6 µl/ml hydrogen peroxide (30% v/v). The lectins used in this investigation were wheat germ agglutinin, lentil lectin, concanavalin A and *Glycine max*. All reactions were performed at room temperature.

RESULTS AND DISCUSSION

Vaccination trials with recombinant antigens demand the production of protein in either bacteria or a eukaryotic system in a quantity sufficient for large animal trials, in high purity and with structure as close as possible to the native structure. Papers elsewhere in this series have described the production of the recombinant polypeptides themselves. Equally critical, is the downstream processing of expressed proteins. A particular problem is the generation of near-native structure in proteins that have varying degrees of disulphide bonding and glycosylation and whose gross three dimensional structure is unknown. This and the accompanying papers describe three approaches to this problem. The preferred approach is expression in an organism like *P. pastoris*, where the full cellular machinery for the post-translational formation of disulphide bonds is found. However, for reasons of ease of expression and expression efficiency, bacteria are often preferred. In this case, proteins may be found in the cell cytoplasm and so the disulphide bonding is likely to be largely intermolecular. Alternatively, they may occur as insoluble inclusion bodies, with substantial intramolecular disulphide bonding. Examples of all three are given, with the strategies for downstream processing that they imply. These strategies involve three stages. Firstly, the solubility and location of the various recombinant proteins must be determined, whether secreted, soluble though intracellular or insoluble. The polypeptide must then be purified from contaminating cellular material, almost always using, as one step in the process, an affinity tag derived from the expression vector. Finally, where appropriate, intermolecular disulphide bonds must be generated.

When expression levels were greater than 1.0 mg/L, SDS-PAGE directly stained with Coomassie or silver proved a reliable method of detecting the recombinant protein in lysate samples. Figure 1 a, arrow A shows the presence of recombinant protein Cb42 in the insoluble (pellet) fraction of lysed *E. coli* cells. Arrow B indicates the lysozyme used in the cell lysing process and was observed in both the cell lysate supernatant and in the insoluble pellet following centrifugation. Positive identification of recombinant proteins with molecular weights similar to lysozyme, (14.3 kDa) was difficult when using these staining methods. Where expression levels were less than 1.0 mg/L, or when the expressed protein could not be positively identified by staining, samples were analyzed by Western blots. The recombinant proteins were identified by probing with either horseradish peroxidase labeled Ni-NTA conjugate specific to the hexaHis tag or with antibodies raised against the mature protein, or the GST component of the fusion protein. Figure 1b shows recombinant protein *Pichia*-Cb42, secreted into the culture medium and identified by probing with Ni-NTA conjugate. The Ni-NTA conjugate proved a very reliable and cost effective means of identifying poorly expressed recombinant proteins.

In all, 7 hexaHis tagged recombinants proteins were successfully purified to homogeneity using Ni-NTA affinity resin. Protein yields per litre of culture, the position of the hexaHis tag and N-terminal amino acid sequence data are presented in Table 1. Figure 2, shows the various purified recombinant proteins after SDS-PAGE run under reducing conditions.

Recombinant Cb48, Cb42 and Cb42 domain A and B (Figure 2a, b, and c) were expressed in *E. coli* cells as insoluble inclusion bodies. Purification was carried out under denaturing conditions and each protein folded using an oxidized/reduced glutathione shuffle strategy. HexaHis-Cb15 (Figure 2 d) was expressed as a soluble protein located in the cytoplasm of *E. coli* cells. Purification was achieved under non-denaturing conditions and as a result additional folding of this protein was not required.

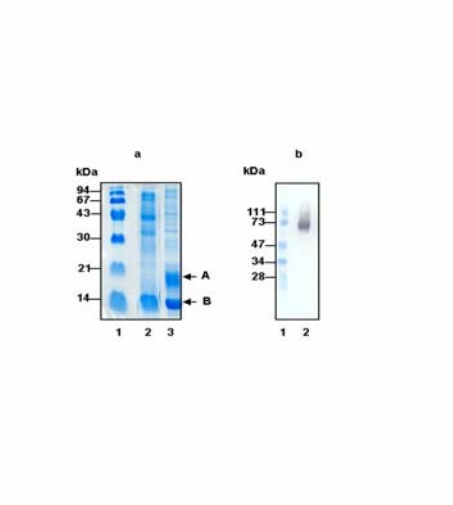

Figure 1. Detection of recombinant protein in *E. coli* lysate and *P. pastoris* culture fluid. (a) Coomassie stained SDS-PAGE (reducing). lanes 1, molecular weight standards; 2, lysed *E. coli* cells, supernatant; 3, lysed *E. coli* cells, pellet; arrow A depicts Cb42; arrow B depicts lysozyme from lysis buffer. (b) lanes 1, molecular weight standards; 2, *P. pastoris*-Cb48 concentrated culture media blotted from SDS-PAGE (reducing) to nitrocellulose and probed with Ni-NTA conjugate

GST-Cb15 (Figure 2f) was initially expressed as a soluble protein in the cytoplasm of *E. coli* cells and purification was attempted under non-denaturing conditions. The purification of this recombinant to homogeneity using glutathione agarose affinity chromatography was unsuccessful possibly due to co-elution of host glutathione-S-transferase or complexing of the recombinant with itself or with host cell proteins. Examination, by western blots of the material recovered from the glutathione agarose, gave a strong reaction between sheep anti-glutathione-S-transferase antiserum and several proteins in the eluted fractions (data not shown). Further fractionation of the affinity enriched recombinant by anion exchange chromatography in the presence of the detergent Zwittergent 3-14 only marginally improved the purity of the protein. An alternative strategy such as preparative SDS-PAGE maybe required to achieve a homogeneous level of purity for this type of fusion protein. As a result of the difficulties associated with purifying GST fusion proteins and the immunological implications of the GST tag when these recombinant proteins are used as vaccines, many researchers now favor hexaHis tagged fusion proteins as an alternative to GST recombinants (see hexaHis-Cb15. above).

Expression of *Pichia*-Cb48 and *Pichia*-Cb42B in *P. pastoris* (Figure 2a and e respectively) offered the advantage of producing recombinant proteins with eukaryotic post-translational modifications such as glycosylation, combined with secretion in a soluble native form, into a relatively uncomplicated medium. Purification of these proteins was achieved under non-denaturing conditions. Dialysis of the concentrated media against the Ni-NTA binding buffer before chromatography was necessary to remove any agents that might interfere with protein-nickel resin binding and was an essential part of this purification strategy.

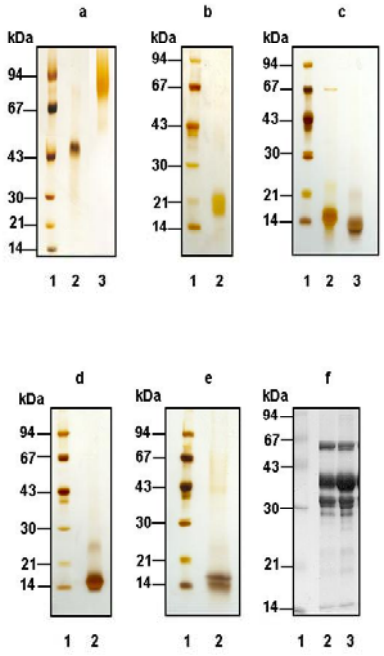

Figure 2. Silver-stained SDS-PAGE (reducing) of purified recombinant peritrophins. (a) lanes 1, molecular weight standards; 2, *E. coli*-Cb48 (5 µg); 3, *P. pastoris*-Cb48 (25 µg); (b) lanes 1, molecular weight standards; 2, *E. coli*-Cb42 (5 µg); (c) lanes 1 molecular weight standards; 2, *E. coli*-Cb42 domain A (5 µg); 3, *E. coli*-Cb42 domain B (5 µg); (d) lanes 1, molecular weight standards; 2, *E. coli*-Cb15 (15 µg); (e) lanes 1, molecular weight standards; 2, *P. pastoris*-Cb42 domain B (25 µg); (f) lanes 1, molecular weight standards; 2 and 3 *E. coli*-GST Cb15 (anion exchange purified pool, 5 µl)

Table 1. Purified recombinant peritrophin proteins, N-terminal amino acid sequence (letters underlined denote mature protein), terminal position of hexaHis tag and protein yield per litre culture

Peritrophin	N-terminal sequence	Tag position	Yield mg/L
E. coli Cb48	MGGSDYDVAS	C	1.0
Pichia Cb48	EAEASIDYDVAS	C	8.0
E. coli Cb42 (full length)	MRGSHHHHHHGSVINVKDER	N	8.0
E. coli Cb42 (domain A)	MRGSHHHHHHGSVINVKDER	N	17.5
E. coli Cb42 (domain B)	MRGSHHHHHHGSCVYHVKAP	N	16.0
Pichia Cb42B (domain B)	EAEAEFCVYHVKAP	C	0.4
E. coli Cb15	MRGSHHHHHHGSVDLQCDPDG	N	10.0

SDS-PAGE analysis of eluted hexaHis protein fractions and a sample of the Ni-NTA resin after 1 M imidazole stripping, showed that all proteins were recovered with imidazole concentrations between 60 – 300 mM. Experience has shown that higher concentrations of imidazole (up to 1.0 M) are occasionally required to completely elute hexaHis tagged proteins from Ni-NTA resin. This applied particularly to dimeric and trimeric forms of the recombinant protein (data not shown).

Glycosylation analysis of *Pichia*-Cb48 (Figure 3a) indicated a strong reaction with this protein and concanavalin-A and only a very weak reaction with lentil lectin. The specific reaction between concanavalin-A and the recombinant protein was blocked by pre-incubating the lectin with 50 mg/ml methyl α-D-mannopyranoside before probing the blotted protein (Figure 3b). The different staining and migration characteristics of *E. coli*-Cb48 (~ 46 kDa) compared to *Pichia*-Cb48 (~70 kDa) in SDS-PAGE (Figure 2a) are consistent with glycosylation of the latter protein. This result suggested that *Pichia*-Cb48 was glycosylated with high mannose type sugars. The cDNA sequence of Cb48 contains 6 potential N-linked glycosylation sites (VUOCOLO *et al.,* 2000 a,b).

CONCLUSION

The intrinsic peritrophic membrane proteins or peritrophins as a group show significant structural similarities, probably due to similarities of tissue location and potential function (TELLAM *et al*., 1999). It is interesting therefore that their preparation as recombinant proteins in quantity sufficient for vaccination trials has required a range of expression strategies and, as illustrated in this paper, a range of isolation and purification procedures. Nevertheless, once the location and solubility of the expressed polypeptide has been determined, the 7 examples described here show that a combination of conventional protein purification steps, affinity chromatography, particularly with the hexaHis ligand, and controlled reduction/reoxidation is able to yield material satisfactory for vaccination trials.

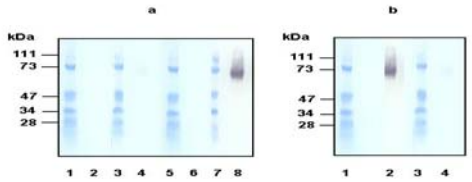

Figure 3. Western-blotted purified *P. pastoris*-Cb48 probed with biotinylated lectins. (a) Lanes 1,3,5 and 7, molecular weight standards (pre-stained, 5 µl); 2,4,6 and 8, *P. pastoris*-Cb48 (5 µg/lane). Biotinylated lectin probes: lanes 2, wheat germ agglutinin; 4, lentil lectin; 6, glycine max; 8, concanavalin-A; (b) lanes 1 and 3, molecular weight standards (pre-stained, 5 µl); lanes 2, and 4 *P. pastoris*-Cb48 (5 µg/lane). Lanes 2, probed with Concanavalin-A; and 4, probed with Concanavalin-A pre-incubated with 50 mg/ml Methyl α-D-Mannopyranoside

ACKNOWLEDGEMENTS

This work was made possible by the financial support from the Australian Centre for International Agricultural Research. (ACIAR). S. Muharsini holds a John Allwright fellowship (ACIAR).

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