BACTERIAL EXPRESSION OF LARVAL PERITROPHINS OF *CHRYSOMYA BEZZIANA*

GENE WIJFFELS[1], TONY VUOCOLO[1], SRI MUHARSINI[2], and FLORENTINA SUPRIYANTI[3]

[1]*CSIRO Livestock Industries, Molecular Animal Genetics Centre, Level 3, Gehrmann Laboratories, University of Queensland, 4072, Australia*
[2] *Research Institute for Veterinary Science*
Jalan R.E. Martadinata 30, P.O. Box 151, Bogor 16114, Indonesia
[3]*Inter-University Centre on Biotechnology, Institute of Technology Bandung*
P.O. Box 1361, Bandung 40132, Indonesia

ABSTRAK

WIJFFELS, GENE., TONY VUOCOLO, SRI MUHARSINI, dan FLORENTINA SUPRIYANTI. 2000. Ekspresi bakterial dari peritrofin larva *Chrysomya bezziana*. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 170-176.

Tiga antigen kandidat lalat *Chrysomya bezziana*, Cb-peritrofin-48, -15, dan -42 disiapkan untuk produksi protein rekombinan pada *Escherichia coli* dengan menggunakan berbagai vektor ekspresi. Cb48 telah berhasil diekspresikan sebagai protein rekombinan yang mempunyai sebuah terminal-karboksi dengan label *hexaHis*. Cb15 diekspresikan baik sebagai fusi protein S-transferase glutation maupun sebagai protein yang berlabel *hexaHis* pada terminal-amino. Konstruksi S-tranferase *glutation* Cb15 menghasilkan sebuah grup fusi protein yang heterogen. Cb42 juga diekspresikan sebagai protein yang berlabel *hexaHis* pada terminal-amino. Kultur protein yang berlabel *hexaHis* dapat membentuk Cb48, Cb15 dan Cb42, hal ini menunjukkan bahwa cara tersebut berguna untuk memproduksi dan memurnikan antigen protein dalam jumlah besar untuk keperluan uji vaksin dan karakterisasi protein.

Kata kunci: *Chrysomya*, membran peritrofik, peritrofin, protein rekombinan, *hexaHis tag*, GST

ABSTRACT

WIJFFELS, GENE., TONY VUOCOLO, SRI MUHARSINI, and FLORENTINA SUPRIYANTI. 2000. Bacterial expression of larval peritrophins of *Chryosomya bezziana*. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 170-176.

Three candidate antigens, *Chrysomya bezziana* peritrophin-48, *Chrysomya bezziana* peritrophin-15 and *Chrysomya bezziana* peritrophin-42, were prepared for recombinant protein production in *Escherichia coli* using a variety of expression vectors. Cb peritrophin-48 was expressed as a recombinant protein possessing a carboxy-terminal hexaHis tag. Cb peritrophin-15 was expressed as both a glutathione S-transferase fusion protein and as an amino-terminal hexaHis tagged protein. The glutathione S-transferase Cb peritrophin-15 construct produced a heterogeneous group of fusion proteins. Cb peritrophin-42 was also expressed as an amino-terminal hexaHis tagged protein. The two putative domains of Cb peritrophin-42 were also separately expressed, again with amino-terminal hexaHis tags. Cultures of the hexaHis constructs Cb peritrophin-48, -15 and –42 were demonstrated to be useful for the production and purification of these protein antigens and were scaled-up for vaccine trials and protein characterization studies.

Key words: *Chrysomya*, peritrophic membrane, peritrophin, recombinant protein, hexaHis tag, GST

INTRODUCTION

Previous studies in another myiasis fly, *Lucilia cuprina,* have shown that intrinsic peritrophic membrane proteins (termed peritrophins) are potential vaccine antigens against the invasive larval stage. Three antigens, *Chrysomya bezziana* peritrophin-48, *C. bezziana* peritrophin-15 and *C. bezziana* peritrophin–42 (abbreviated to Cb48, Cb15 and Cb42) were identified as peritrophins of the larval stage of the Old World Screwworm fly, *C. bezziana* (RIDING *et al.,* 2000; VUOCOLO *et al*., 2000b). All three candidate antigens were successfully cloned from a *C. bezziana* larval cDNA library (VUOCOLO *et al.,* 2000b). We set out to find means of expressing these three candidate vaccine antigens in *Escherichia coli*, in order to generate suitable quantities of the proteins for further studies.

Development of any subunit vaccine requires access to reasonable quantities of pure antigen. It can be a difficult task producing sufficient quantities of pure native antigen to conduct all the protein characterization and vaccine trials required in the development of a vaccine and the native antigen will rarely be available in commercial quantities. This problem can usually be overcome by developing a recombinant vaccine typically using a bacterial and/or yeast protein expression system. This approach requires cloning of the gene encoding the candidate antigen into suitable expression vectors. Typically DNA is resynthesized by

PCR to have compatible ends (usually restriction enzyme sites) to allow ligation into a vector.

Expression vectors are characterized by the presence of a strong inducible promoter to drive the transcription of the gene, and thus lead to good expression of the candidate antigen. Many expression vectors will generate a fusion protein with the antigen protein. The fused protein will contain either a peptide or protein that will assist with the identification and purification of the recombinant protein. Ideally, the introduced component should not interfere with the folding, antigenicity or behavior of the candidate vaccine protein. However, it is not uncommon for recombinant proteins to be produced as insoluble aggregates within cells. Processing of these aggregates into a form suitable for vaccination trials is the subject of another publication in this issue (PEARSON *et al.,* 2000).

MATERIALS AND METHODS

Preparation and manipulation of DNA

Oligonucleotide primers were designed to incorporate restriction enzyme cleavage sites (linkers) at their ends to allow cloning into expression vectors. The primers were synthesized on an Oligo 1000 DNA Synthesizer (Beckman Instruments), and then cleaved from the resin as instructed by the manufacturer. The cleaved oligonucleotides were dried by vacuum centrifugation (Savant), and then resolubilized in sterile water or a Tris-EDTA buffer. PCR was conducted in 50 or 100 µl volumes using Taq polymerase and buffers from Promega and 100 pmol oligonucleotide primers (in an Omnigene Temperature Cycler (Hybaid)). PCR products were isolated from agarose gels and purified using DNA clean up kits (Bresaclean (Geneworks, Adelaide, Australia) and Qiagen (Germany). Purification was monitored by agarose gel electrophoresis run in TAE buffer (0.04M Tris-acetate, 1mM EDTA).

Vectors used in this work were pGEM-T and pGEM-T Easy (Promega, USA) for the cloning of PCR products. Vectors used for recombinant protein expression were pQE9 and pQE60 (Qiagen) and pGEX-2T (Amrad-Pharmacia, Australia). These expression vectors are inducible with IPTG. All restriction enzymes used were obtained from Promega. DNA of the cloned PCR fragments were digested with restriction enzymes corresponding to their restriction linkers and gel purified. The expression vectors were cleaved at their cloning sites with the same restriction enzymes, phosphorylated with Calf Intestinal Phosphatase (Promega), and gel purified. The purified DNA fragments were ligated into the appropriately digested and purified expression vector with T4 DNA ligase (Promega). pGEM-T Easy recombinant plasmids were transformed into competent XL1-Blue cells. Recombinant expression vectors were transformed into competent XL1-Blue or M15 *E. coli* cells

Expression trials

Cultures of 3 ml Luria Bertani (LB) broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.5% w/v NaCl, pH 7.5) containing 200 µg/ml ampicillin were inoculated from DMSO stocks of the expression clones and grown overnight (37°C) with shaking (250 rpm). The cultures were then added to 20 ml of prewarmed Superbroth (2.5% w/v bacto-tryptone, 1.5% w/v bacto-yeast extract, 0.5% w/v NaCl, pH 7.5) containing 200 µg/ml ampicillin and grown at 37°C with shaking (250 rpm). When the OD_{600} reached 0.8, IPTG was added to a final concentration of 1 or 2 mM. Samples (1 ml) were taken at the time of induction and then at hourly intervals for 5 h. After determining the optical density at 600 nm (OD_{600}), the samples were immediately pelleted (12000 *g*, 5 min, 4°C) and all supernatants removed. Pellets were stored at –20°C prior to analysis. On thawing, the pellets were resuspended in 2x reducing SDS-PAGE sample buffer at a theoretical density of OD_{600} = 10. The suspension was vortexed, heated to 100°C for 5 min and vortexed again. The homogenates were spun (12000 *g*, 2 min) to remove any insoluble material before loading on SDS-PAGE.

Large scale expression experiments

In preparation for 1-4 L broth cultures, 2 to 4 flasks of 100 ml Superbroth containing 200 µg/ml ampicillin were inoculated with 10 µl DMSO stock of the relevant plasmid in XL1-Blue or M15 cells. After overnight culture (37°C) on a shaker (250 rpm), an appropriate volume from each 100 ml culture was added to a 900 ml volume of prewarmed Superbroth containing 200 µg/ml ampicillin (in a 2 L flask) to give a starting OD_{600} of 0.2. The 1 L broth cultures were mounted on a shaker and OD_{600} monitored. When the OD_{600} reached 0.6–0.8, IPTG was added to a final concentration of 1 or 2 mM. To monitor bacterial growth and induction of protein expression, 1.5 ml samples of the culture were removed at hourly intervals. After the final time point, the cells were pelleted (1500 *g*, 20 min, 4°C) and the supernatant removed. The pelleted cells were resuspended in cold TBS and transferred to 50 ml centrifuge tubes. The cells were washed twice in cold TBS containing EDTA-free Complete™ protease inhibitors (Boehringer Mannheim, Germany) prior to being stored at –20°C.

SDS-PAGE and Immuno-blots

Minigels were prepared and run using the Bio-Rad (USA) Minigel systems. Midi-gradient gels (6–18%

acrylamide) were assembled and run with a Bio-Rad system. All gels were run under reducing conditions. Gels were either stained with Coomassie Brilliant Blue or silver stained (RABILLOUD *et al*., 1988), and occasionally electro-transfered for immuno-blot analysis (as described by WIJFFELS *et al*., 1996 for minigels, and EAST *et al*., 1993 for midi-gels).

Immuno-blot analyses employed various probes to identify the recombinant proteins. The hexaHis tag produced as a consequence of expression in the Qiagen vectors was detected by two means: a Ni-NTA alkaline phosphatase conjugate (Qiagen) and specific antibody reagents. An affinity purified anti-hexaHis reagent was prepared by passing a 100 ml pool of sheep sera raised against a hexaHis tagged version of *L. cuprina* peritrophin-48 (SCHORDERET *et al*., 1998) down a Sepharose column conjugated with a *H. irritians exigua* aquaporin possessing an amino-terminal hexaHis tag (ELVIN *et al*., 1999). The eluted antibodies were specific for the hexaHis tag and the four preceding residues Met-Arg-Gly-Ser. An affinity purified reagent to GST was prepared in a similar manner. A pool of sheep sera against *L. cuprina* peritrophin-44 fused to GST was passed down a GST-Sepharose column. The eluted antibodies were specific for GST.

If possible, serum raised to the antigen was also used. In the case of hexaHis-Cb15, a pool of sheep serum to the Cb-peritrophic membrane (PM) SDS extract, which was highly enriched for Cb15, was prepared. Six sheep were immunized subcutaneously with 3 mg Cb PM SDS extract. The protein extract was emulsified in the adjuvant, Montanide ISA70 (Seppic, Paris, France) with a hand held motorized pestle (Kontes, Vineland, NJ). The sheep received two subcutaneous injections, 4 weeks apart in the right hind leg. Two weeks after the final immunization, blood was collected by venepuncture. The sera were stored at –20°C until required. The pooled sera were absorbed against a lysate of *E. coli* expressing *L. cuprina* hexaHis-PM55 to ensure that the pooled sera did not possess specificities to *E. coli* proteins that would confound immuno-blot analyses of lysates. The sera were analyzed to determine which sera reacted to Cb15 by immuno-blot analyses. The 3 most reactive sera were pooled (1.5 ml each). A pool was also prepared from the corresponding pre-vaccination sera. Bacterial lysate (3.5 ml) was added to both pools, and the mixtures were rotated for 2 h (4°C) and then clarified (10000 *g*, 15 min, 4°C). The supernatants were further absorbed against the *E. coli* with another 2 ml lysate overnight with rotation (4°C). The mixtures were clarified again before addition of a further 1.5 ml lysate and absorption for another 2 h (4°C). The mixture was finally clarified (20000 g, 30 min, 4°C). The absorbed sera pools (now a 9.5 ml volume) were aliquoted and stored at –20°C.

An affinity purified antibody reagent was also prepared for the detection of hexaHis-Cb42 and its domains. HexaHis-Cb42 produced from *E. coli* was purified as described (PEARSON *et al.,* 2000). The protein was homogenized into Montanide ISA 70 (Seppic) as described above. Two rabbits each received three subcutaneous immunizations of 30 μg hexaHis-Cb42 over 12 weeks. Their sera were collected two weeks after the final boost. After verification of immunoreactivity by immuno-blot analysis and ELISA, the hexaHis-Cb42 specific antibodies were affinity purified. A Cb42–Sepharose conjugate was produced by conjugating 3 mg purified hexaHis-Cb42 to 1.5 g cyanogen bromide activated-Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The Cb42–Sepharose conjugate was poured into a glass column (Bio-Rad). The rabbit serum was dialyzed against 100 mM Tris, pH 8.0 containing 0.5 M NaCl (4°C). The serum was loaded onto the column, washed in the Tris buffer and the specifically bound antibodies eluted with 0.2 M glycine-HCl (pH 2.2). The eluant was neutralized by addition of saturated Tris and then was dialyzed against PBS pH 7.2 (4°C). The eluted antibody solution was concentrated using a Centricon 30 device (Millipore, Bedford, MA).

RESULTS AND DISCUSSION

Cloning and expression of Cb48

A DNA fragment encoding the mature Cb48 protein sequence, 345 amino acids, was amplified by PCR using *Bam*H I linkered primers as described in VUOCOLO *et al*. (2000a), purified and cloned into pQE60. This vector fuses a hexaHis tag to the carboxy-terminus of the expressed recombinant protein. The recombinant plasmids were transformed into *E. coli* M15 cells. Twelve individual colonies were picked, plasmid DNA isolated and digested with *Bam*H I to verify the presence of the Cb48 insert DNA. Pilot cultures of twelve Cb48 *E. coli* clones were assayed for recombinant protein expression. These clones were all shown to contain the expression plasmid and the Cb48 DNA (Figure1, Panel A). Only clones 5, 7 and 12 were found to contain inserts in the correct orientation (data not shown). All 12 clones with insert and a vector only clone were used in pilot expression studies. SDS-PAGE run under reducing conditions and stained with Coomassie Blue, (Figure1, Panel B) shows the protein profile produced by these clones. No Cb48 recombinant protein was apparent from this gel. Immuno-blot analysis using a Ni-NTA conjugate was used to detect expression of recombinant Cb48 (Figure1, Panel C). Only clones in the correct orientation (5, 7 and 12) were expected to produce recombinant Cb48 protein. Indeed, it was shown that only clones 7 and 12 produced

recombinant Cb48 with no apparent expression in clone 5. An unstained standard containing carbonic anhydrase (30 kDa) served as a positive control for the Ni-NTA conjugate. Carbonic anhydrase binds directly to the nickel conjugate. Clone 12 was DNA sequenced to verify that it accurately encoded Cb48. A 4 L culture of clone 12 was grown and recombinant Cb48 protein was found to be present in the form of inclusion bodies in the *E. coli* cells. These inclusion bodies were solubilized and purified as outlined by PEARSON *et al*. (2000).

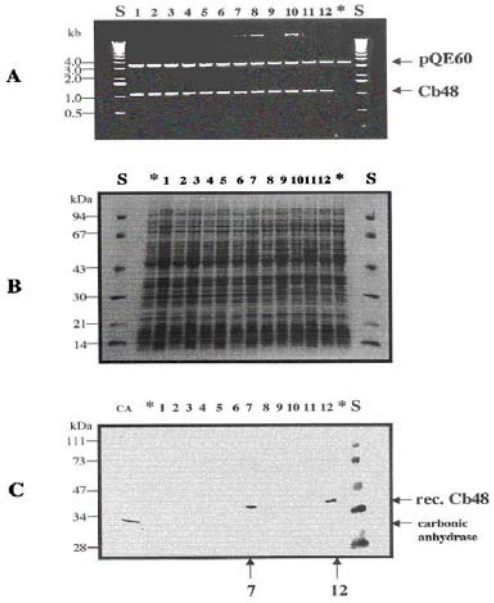

Figure 1. Analysis of twelve Cb48 recombinant bacterial clones for expression of hexaHis-Cb48. Lanes 1 to 12, Cb48 recombinant clones; lane S, DNA standards; lane *, pQE60 vector only (control). Panel A: 1% agarose gel with clones digested with *Bam*H I. Panel B: Coomassie Blue stained SDS-PAGE gel of pilot expression cultures run under reducing conditions. Panel C: Immuno-blot analysis of expression cultures probed with a Ni-NTA alkaline phosphatase conjugate. Unstained carbonic anhydrase was a positive reaction control for the Ni-NTA conjugate reaction in lane CA

Cloning and expression of Cb15

The DNA encoding Cb15 was cloned into the expression vectors pQE9 and pGEX-2T. The pQE9 vector will yield a recombinant protein with an amino-terminal hexaHis tag, whereas the pGEX-2T vector will produce a fusion protein with an amino-terminal GST domain. To simplify preparation, the inserts and vectors were prepared for bi-directional cloning. pQE9 and pGEX-2T were digested with *Hind* III and *Eco*R I, respectively. Forward and reverse primers containing the *Hind* III and EcoR I restriction sites were prepared and used in a PCR to generate the inserts. The inserts were first cloned into pGEM-T, an intermediate vector and transformed into competent XL1-Blue cells. Inserts from pGEM-T vector were digested with *Hind* III and *Eco*R I and were ligated into the relevant expression vector. Only 7 pGEX-2T and a single pQE9 transformant resulted. DNA sequencing demonstrated that each transformant contained the correct insert in the correct orientation.

Pilot expression experiments of 2 pGEX-2T transformants and the pQE9 transformant were conducted in 20 ml cultures, with samples of the culture removed hourly for 5 h after induction with 2 mM IPTG. SDS-PAGE and immuno-blots were used to analyse the samples for Cb15 recombinant protein expression (data not shown). The hexaHis-Cb15 protein was just discernible in the Coomassie Blue stained gel, but clearly evident in a Western blot probed with a hexaHis antibody (Figure 2). The GST-Cb15 fusion protein was not detected in the Coomassie stained gel, but a 32–38 kDa band was detected in a Western blot probed with a GST antibody. Scale up to 2 L culture expression experiments were undertaken.

As with the pilot expression experiments, the 2 L pGEX-2T culture was sampled hourly before and after induction. There were three forms of expressed protein: 26, 32 and 38 kDa, evident in the Coomassie Blue stained gel and immuno-blot analysis using the anti-GST serum. The expression pattern did not change in quantity or profile with time. The minor 26 kDa band is likely to be GST alone and the fusion proteins are probably represented by the two more slowly migrating forms. The predicted size of the Cb15 only polypeptide is 8 kDa. However native Cb15 protein has only been seen to migrate at 15 kDa in SDS-PAGE (RIDING *et al*., 2000). The 38 kDa protein is therefore reasonably consistent with a GST-Cb15 protein comprising 26 kDa and 15 kDa for the GST and Cb15 component respectively. The 32 kDa protein may represent the product of a partial mRNA of the gene, or a GST-Cb15 misfolded protein.

Analysis of time points of the hexaHis-Cb15 2 L culture gave very clear results (Figure 3). The Cb15 recombinant protein was evident in the Coomassie blue stained gel and both immuno-blot analyses. Extended growth after the 55 min time point (post-induction) did not improve the amount of the 15 kDa recombinant protein.

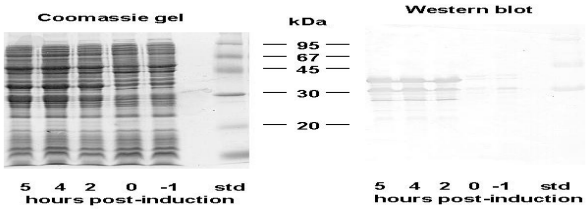

Figure 2. Analyses of the induction of GST-Cb15 expression in 2 L culture. Time point samples of a 2 L culture of a pGEX-2T transformant were analyzed for expression of GST-Cb15 by SDS-PAGE and immuno-blot analyses using the antibody to GST. Time points are as indicated. The 12% acrylamide gels were loaded as described in Material and Methods, and Coomassie stained or processed for immuno-blotting

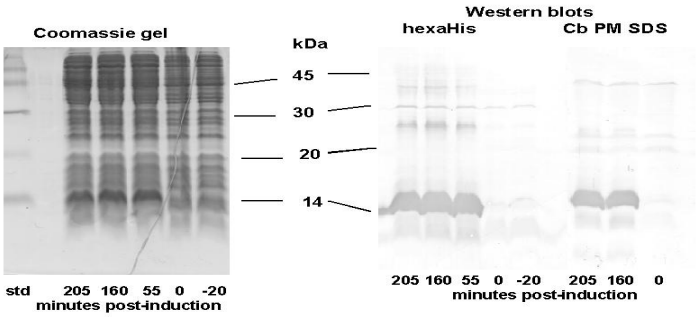

Figure 3. Analyses of the induction of hexaHis-Cb15 expression in 2 L culture. Time point samples of a 2 L culture of the pQE9 transformant were analyzed for expression of hexaHis-Cb15 by SDS-PAGE and immuno-blot analyses using an anti-hexaHis serum and an *E. coli* absorbed anti-Cb PM SDS extract. Time points are as indicated. The 20% acrylamide gels were loaded as described in Material and Methods, and Coomassie stained or processed for immuno-blotting

Cloning and expression of Cb42 and its two domains

Recombinant expression of Cb42 was performed in the pQE9 expression vector. A sense primer containing a *Bam*H I linker and an anti-sense primer containing a *Hind* III linker were prepared and successfully used in PCR on the K1.1 phagemid (VUOCOLO *et al.*, 2000b). The 500 bp product was purified and ligated into pGEM-T Easy for sequencing. The verified fragment was then ligated into *Bam*H I/*Hind* III cut and phosphatased pQE9. The ligated plasmid was transformed into competent XL1-Blue cells, and 3 clones were picked and shown to possess the fragment encoding Cb42 (Figure 4, lane 1). Pilot expression trials were conducted on all three transformants. Two of the three clones expressed hexaHis-Cb42 and one clone was grown in a large scale (8 L) culture. HexaHis-Cb42 was expressed after induction with 1 mM IPTG, but SDS-PAGE analysis of the cell lysate showed that the protein was insoluble in the form of inclusion bodies (Figure 5, lane 1).

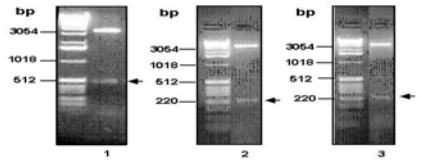

Figure 4. Insert sizes of Cb42, Cb42A and Cb42B pQE9 clones. Purified pQE9 DNA of a full length Cb42 clone (lane 1), a domain A clone (lane 2) and a domain B clone (lane 2) were digested with *Bam*H I and *Hind* III to release the inserts (as indicated by the arrows). Lanes L are DNA marker ladders (Gibco-BRL). The DNA products were resolved in a 1% agarose gel and stained with ethidium bromide

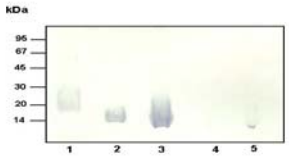

Figure 5. Immuno-blot analysis of the expression trial of pQE9 constructs of full length hexaHis-Cb42, or hexaHis tagged Cb42 domain A or B. The insoluble cellular material of a bacterial cell lysate of cells induced with 1 mM IPTG, was solubilized in SDS-PAGE reducing sample buffer. The solubilized pellet was analyzed for the presence of the recombinant protein by immuno-blot analysis using the Ni-NTA alkaline phosphatase conjugate. Lane 1, 10 µl full length Cb42 solubilized pellet; lanes 2 and 3, 10 and 20 µl domain A solubilized pellet respectively, and lanes 4 and 5, 10 and 20 µl domain B solubilized pellet respectively

A soluble recombinant protein is generally more useful in vaccine trial work and is essential for protein function and characterization studies. In the hope of generating soluble protein, the Cb42 protein was divided into 2 recognizable domains implied from its primary sequence. Domains A and B each represent approximately one half of Cb42. Domain B has sequence similarities to Cb15. The same method used in constructing the full length Cb42 expression plasmid was applied to assembling the domain A and B constructs. Each construct was engineered to contain an amino-terminal hexaHis tag. The PCR fragments containing *Bam*H I/*Hin*d III restriction sites at their ends were 200 and 220 bp for Cb42 domain A and B respectively. These fragments were directionally cloned into pQE9 expression vector cut with B*am*H I and *Hin*d III after shuttling through pGEM-T Easy vector. Transformants in XL1-Blue cells containing the correct fragment are shown in Figure 4 (lanes 2 and 3). The DNA from 2 clones for each construct was sequenced and found to be correct. One of each of these clones was taken on to pilot expression trials. Both constructs produced insoluble proteins of approximately 14 kDa, although the domain B construct appeared to be expressing at very low levels (Figure 5, lanes 2 - 5). In a larger volume culture (750 ml) induced with 1 mM IPTG, both constructs produced similar amounts of insoluble recombinant protein (data not shown).

CONCLUSION

The results of the attempted expression of five different recombinant antigens in three different expression vectors are summarized in Table 1. Clearly, all five antigens, representing all or part of three candidate native antigens, were produced in reasonable quantity as recombinant proteins in *E. coli*. Nevertheless, the results also show the variability of the ease with which expression is achieved in different protein/vector combinations, despite the fact that the three antigens of interest have a number of structural similarities. This demonstrates the need to adopt a flexible approach to the expression of any protein needed in quantity and good purity for further biochemical or veterinary research.

Table 1. Summary of bacterial expression constructs engineered for recombinant protein production of Cb15, Cb42 and Cb48

Protein	Expression Vector	Intended Recombinant Protein	Result
Cb15	pQE9	N-terminus hexaHis tagged Cb15.	Successful expression of a single soluble product. High yields.
	pGEX-2T	N-terminus GST fused Cb15.	Successful expression/multiple soluble products obtained.
Cb42	pQE9	N-terminus hexaHis tagged Cb42.	Successful expression of a single product. High yields. Insoluble product.
Cb42A (Domain A)	pQE9	N-terminus hexaHis tagged Cb42 Domain A.	Successful expression of a single product. High yields. Insoluble product.
Cb42B (Domain B)	pQE9	N-terminus hexaHis tagged Cb42 Domain B.	Successful expression of a single product. Low yields. Insoluble product.
Cb48	pQE60	C-terminus hexaHis tagged Cb48.	Successful expression of a single product. Satisfactory yields. Insoluble product.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge ACIAR (Australian Centre for International Agricultural Research) for funding this research. S. Muharsini holds a John Allwright Fellowship (ACIAR).

REFERENCES

EAST, I.J., C.J. FITZGERALD, R.D. PEARSON, R.A. DONALDSON, T. VUOCOLO, L.C. CADOGAN, R.L. TELLAM, and C.H. EISEMANN. 1993. *Lucilia cuprina*: inhibition of larval growth induced by immunization of host sheep with extracts of larval peritrophic membrane. *Int. J. Parasitol.* 23: 221-229.

ELVIN, C.M., R. PEARSON, N.E. LIYOU, J. GOUGH, and R.D. DRINKWATER. 1999. Molecular cloning and expression in *Escherichia coli* of an aquaporin-like gene from adult buffalo fly *(Haematobia irritans exigua*). *Insect Mol. Biol.* 8: 369-380.

PEARSON, R., S. MUHARSINI, G. WIJFFELS, and T. VUOCOLO. 2000. Purification of recombinant peritrophic membrane proteins of the Old World Screwworm fly, *Chrysomya bezziana*. *J. Ilmu Ternak Vet.* (Edisi Khusus) 5 (3): 185-191.

RABILLOUD, T., G. CARPENTIER, and P. TARROUX. 1988. Improvement and simplification of low background silver staining of proteins by using sodium dithionate. *Electrophoresis* 9: 288-291.

RIDING, G., S. MUHARSINI, R. PEARSON, SUKARSIH, E. SATRIA, G. WIJFFELS, and P. WILLADSEN. 2000. Fractionation, identification and vaccination efficacy of native antigens from the screwworm fly, *Chrysomya bezziana. J. Ilmu Ternak Vet.* (Edisi Khusus) 5 (3): 150-159.

SCHORDERET, S., R.D. PEARSON, T. VUOCOLO, C. EISEMANN, G.A. RIDING, and R. TELLAM. 1998. cDNA and deduced amino acid sequences of a peritrophic membrane glycoprotein, peritrophin-48, from the larvae of *Lucilia cuprina. Insect Biochem. Molec. Biol.* 28: 99-111.

VUOCOLO, T., C.H. EISEMANN, R.D PEARSON, P. WILLADSEN, and R.L. TELLAM. 2000a. Identification and molecular characterization of a peritrophin gene, peritrophin-48, from the myaisis fly *Chrysomya bezziana*. *Insect Biochem. Molec. Biol.* (Submitted).

VUOCOLO, T., F. SUPRIYANTI, S. MUHARSINI, and G. WIJFFELS. 2000b. cDNA library construction and isolation of genes for candidate vaccine antigens from *Chrysomya bezziana* (the Old World Screwworm fly). *J. Ilmu Ternak Vet.* (Edisi Khusus) 5(3): 160-169.

WIJFFELS, G., C. FITZGERALD, J. GOUGH, G. RIDING, C. ELVIN, D. KEMP, and P. WILLADSEN. 1996. The cloning and characterization of angiotensin-converting enzyme from the dipteran species, *Haematobia irritans exigua,* and its expression in the maturing male reproductive system. *Eur. J. Biochem.* 273: 414-423.