FRACTIONATION, IDENTIFICATION AND VACCINATION EFFICACY OF NATIVE ANTIGENS FROM THE SCREWWORM FLY, *CHRYSOMYA BEZZIANA*

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ABSTRAK

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Beberapa protease yang diisolasi dari bahan hasil eksresi/sekresi larva dan protein membran peritrofik adalah produk biologik yang paling intensif dipelajari sebagai sumber antigen protektif yang potensial pada myiasis yang disebabkan oleh *Lucilia cuprina*. Peranan produk biologik sejenis sebagai antigen protektif potensial pada lalat *Chrysomya bezziana* sudah diteliti dan dilaporkan dalam tulisan ini. Protease serin yang telah dimurnikan diuji kapasitasnya sebagai antigen protektif dengan menyuntikkan pada domba, kemudian efekasinya sebagai vaksin dievaluasi dengan menggunakan teknik uji *in vitro* dan *in vivo* dengan larva lalat *Chrysomya bezziana*. Pada uji *in vitro* dengan menggunakan serum domba yang telah divaksinasi tidak berpengaruh terhadap pertumbuhan larva, tetapi pada uji *in vivo* didapatkan penurunan bobot larva pada serum domba yang divaksinasi walaupun jumlah larva yang ditemukan sedikit meningkat. Vaksinasi dengan membran peritrofik dari *Chrysomya bezziana* memberikan respon imunologik yang efektif terhadap parasit yang ditunjukkan dengan penurunan bobot larva dan peningkatan mortalitas larva secara nyata. Fraksinasi secara bertahap dari membran peritrofik dengan berbagai *surfactants* dan agen *chaotrophic* untuk meningkatkan daya larut berhasil memisahkan grup-grup protein yang mempunyai ciri yang berbeda-beda. Grup-grup protein hasil fraksinasi tersebut dibuat vaksin, kemudian dicoba pada domba percobaan, untuk kemudian diuji efekasinya secara *in vitro*. Ekstrak urea, ekstrak guanidin-HCl dan fraksi yang larut di dalam SDS masing-masing merangsang terjadinya proteksi terhadap larva *Chrysomya bezziana* secara nyata, tetapi daya proteksinya masih lebih rendah dibandingkan dengan vaksin membran peritrofik natif yang utuh. Sebagian besar protein yang dipilih dari tiga fraksi yang paling protektif adalah yang dimurnikan dengan SDS *polyacrylamide gel electrophoresis*. Karena secara kuantitas tidak cukup banyak jumlah protein ini sehingga tidak mungkin digunakan untuk uji vaksin pada domba percobaan, maka protein tersebut kemudian disekuensi secara langsung dari terminal-amino atau didigesti dengan endoproteinase Lys-C, yang selanjutnya diikuti dengan pemurnian peptida dan sekuensing asam amino. Hal ini memberi informasi yang berguna untuk ekspresi beberapa di antara protein tersebut sebagai protein rekombinan dalam bentuk yang cocok untuk studi vaksinasi.

Kata kunci: *Chrysomya bezziana*, membran peritrofik, vaksinasi, sekuensi asam amino, protease serin

ABSTRACT

RIDING GEORGE, SRI MUHARSINI, ROGER PEARSON, SUKARSIH, EDY SATRIA, GENE WIJFFELS and PETER WILLADSEN. 2000. Fractionation, identification and vaccination efficacy of native antigens from the screwworm fly, *Chrysomya bezziana. Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5(3): 150-159.

Proteases of larval excretory/secretory material and proteins of the peritrophic membrane are the most intensively studied sources of potential protective antigens from the sheep blowfly *Lucilia cuprina*. Their importance in the screwworm fly *Chrysomya bezziana* has now been investigated. Purified serine proteases from *Chrysomya bezziana* were tested for their potential as vaccine antigens in sheep, efficacy being assessed by *in vitro* and *in vivo* assays with larval *Chrysomya bezziana*. No effect of vaccination was observed by the *in vitro* assay. However, in the *in vivo* challenge, larval weights were diminished in the vaccinated sheep, although larval recoveries increased marginally. Vaccination with *Chrysomya bezziana* peritrophic membrane does induce an effective immune response against the parasite resulting in a significant reduction in larval growth and considerable larval mortality in the *in vitro* assay. Sequential fractionation of the peritrophic membrane with various surfactants and chaotrophic agents of increasing solubilisation capacity resulted in the separation of discrete groups of proteins. The groups of fractionated proteins were tested in a vaccination trial in sheep with vaccine efficacy assessed by *in vitro* assays. The urea extract, guanidine-HCl extract and SDS soluble fraction each induced significant levels of protection against *Chrysomya bezziana* larvae but the effects were poorer than those obtained from vaccination with whole, native peritrophic membrane. Several major proteins selected from the three most protective fractions were purified by SDS polyacrylamide gel electrophoresis. Since insufficient quantities of these proteins were available for vaccination trials, they were either sequenced directly from the N-terminus or subjected to endoproteinase Lys-C digestion, followed by peptide purification and amino acid

sequencing. This gave the information necessary for the expression of several of these proteins as recombinants in a form suitable for vaccination studies.

Key words: *Chrysomya bezziana,* peritrophic membrane, vaccination, amino acid sequence, serine protease

INTRODUCTION

Successful vaccination against an ectoparasite would be an attractive alternative to pesticide usage. This approach to parasite control has been shown to be a promising alternative in controlling myiases flies and other ectoparasites (WILLADSEN *et al.,* 1989; KAY and KEMP, 1994; TELLAM and BOWLES, 1997). Central to the development of a vaccine is the ability to isolate and identify protective antigens from the parasite. This task is often a multi-faceted and lengthy procedure. Firstly, a complex mixture of proteins, derived from either the whole organism or selected tissues, must be fractionated. Secondly, the isolated fractions must then be evaluated for protective efficacy in vaccination and parasite challenge experiments. The isolation and purification of just one protective antigen can take many fractionation steps and a number of vaccination trials before the efficacious protein is identified. Furthermore, antigen purification can be even more challenging, when the target proteins (*eg.* peritrophic membrane proteins) are membrane bound, often heavily glycosylated, and soluble only in surfactants and strong denaturants. The complexity and time-consuming nature of antigen identification is well illustrated in the development of a vaccine against the cattle tick, *B. microplus* (WILLADSEN *et al.,* 1988; 1989) and the investigation of vaccination against the sheep blowfly, *L. cuprina* (EAST *et al.,* 1993; ELVIN *et al*, 1996).

The identification of protective antigens can be enhanced if the process begins, not with whole and therefore very complex parasite extracts, but with selected tissues or extracts. Two such sources of antigens have been extensively investigated with *L. cuprina*, the peritrophic membrane (PM) and the excretory/secretory (E/S) material (EAST *et al.,* 1993; ELVIN *et al.*, 1996; TELLAM and BOWLES, 1997). To date, the use of PM proteins as candidate vaccine antigens in other parasites has been somewhat limited, whereas proteases have been studied more comprehensively. For example, several antigens from E/S material of *F. hepatica* (liver fluke) have been reviewed (SPITHILL and DALTON, 1998). Vaccination with purified cysteine proteases (cathepsin L_1 and L_2) of *F. hepatica* decreases worm fecundity and provides 38 – 69% protection (WIJFFELS *et al*., 1994, DALTON *et al*., 1996). Serine proteases, as major proteolytic enzymes in insects, have been reviewed (APPLEBAUM, 1985; TERRA and FERREIRA, 1994; TERRA *et al*., 1996). These enzymes are thought to have an important role in wound formation and also to provide digestion of nutrients for *L. cuprina* larvae (SANDEMAN *et al*., 1990). Their use as possible vaccine antigens has been discussed (TELLAM and BOWLES, 1997). An *in vitro* feeding assay using several serine protease inhibitors caused the inhibition of larval growth and larval death (BOWLES *et al*., 1990; CASU *et al*., 1994), though vaccination with several enzymes individually has been unsuccessful (TELLAM *et al*., 1994).

While *L. cuprina* larvae cause a cutaneous myaisis, *C. bezziana* larvae penetrate deeply into host muscle tissue. In view of the more invasive tissue penetration of *C. bezziana* larvae, the role of *C. bezziana* proteases might be more significant than those of *L. cuprina*. A preliminary biochemical investigation of E/S material of *C. bezziana* larvae has been performed (MUHARSINI *et al*., 2000). This material contains serine proteases as a major protein component. Both trypsin- and chymotrypsin-like enzymes are present. The *C. bezziana* serine proteases showed typical characteristics of insect proteases. However, their efficacy as an experimental vaccine in *C. bezziana* has not been described previously.

In this paper, following the evidence that native *C. bezziana* PM material does induce a significant protective immune response against the parasite (SUKARSIH *et al.,* 2000a), fractionation of this tissue has been performed and the protein components tested for vaccination efficacy. Results of the vaccination of sheep with *C. bezziana* protein extracts are presented. The purification and partial characterisation of some individual PM proteins from these extracts is also included. In addition, we describe the purification of several *C. bezziana* proteases from the larval E/S material and present the results of vaccination of these proteins in sheep.

MATERIALS AND METHODS

Production and harvest of larval peritrophic membrane and excretory/secretory material

The establishment and maintenance of *C. bezziana* in laboratory culture is described in detail by SUKARSIH *et al.* (2000b). The procedures for growing *C. bezziana* in sterile larval culture, the harvesting of larval PM and the collection of larval E/S is similar to that described for *L. cuprina (*EAST *et al.,* 1993) with some minor modifications for *C. bezziana* described by MUHARSINI *et al.* (2000). Following production, both the larval PM and E/S material were stored at –70°C until required.

Fractionation of peritrophic membrane proteins

The extraction of proteins from *C. bezziana* PM was achieved by progressive homogenisation and treatment of the PM with various surfactants and chaotrophic agents of increasing solublization capacity, as previously described for the extraction of proteins from the PM of *L. cuprina* (EAST *et al.,* 1993; ELVIN *et al.,* 1996). Some modifications of the *L. cuprina* protocol were developed over time with at least six batches (7.6 g to 16.9 g wet wt.) of *C. bezziana* PM being processed during the course of the project. Typically, proteins were extracted sequentially from PM by homogenisation and incubation in the presence of 40 ml (final volume) of the following solutions: (1) water, (2) 100 mM Tris-HCl, pH 7.2 containing 140 mM NaCl, 4 mM EDTA, 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine (AEBSF) and Complete® Protease Inhibitor (used according to the manufacturer's instructions), (3) 20 mM Tris-HCl, pH 7.2, 140 mM NaCl, 4 mM EDTA, 50 μM AEBSF and Complete® Protease Inhibitor (TBS^{+++}) containing 2% SB 3-14 (N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate), (4) TBS^{+++} containing 6 M urea, and (5) TBS^{+++} containing 6 M guanidine-HCl. Following each extraction, homogenates were subject to centrifugation (48,000 g, 30 min, 4°C) followed by resuspension of the pellet in the next extraction solution. Extract incubation times were: water, 5 min; TBS, 30 min; 2% SB 3-14, 2 h; 6 M urea, 16 h; and 6 M guanidine-HCl, 16 h. All extractions were performed at 4°C. All buffers, extraction solutions, media etc. mentioned in this paper were made using MilliQ water (MQW).

To produce the sodium dodecyl sulfate (SDS) soluble fraction, the guanidine-HCl insoluble pellet was first washed twice with MQW followed by two washes with 50 mM Tris-HCl, pH 7.5. Following each wash the suspension was pelleted by centrifugation (14,000 g, for 10 min at room temperature (RT) and the pellet resuspended in fresh buffer. The pellet was finally resuspended in 50 mM Tris-HCl, pH 7.5 containing 10 mM dithiothreitol (DTT) and 5% SDS. The suspension was incubated for 30 min at 95°C, followed by centrifugation (14,000 g, 15 min at RT) and the SDS soluble supernatant was collected (SDS soluble material).

Individual proteins present in various fractions that were chosen for further purification and characterisation, were named *C. bezziana* peritrophins followed by their apparent molecular weight (MW) on SDS-PAGE (*eg.* Cb-peritrophin 15, for convenience Cb15).

Anion exchange high performance liquid chromatography

Anion exchange high performance liquid chromatography (AE-HPLC) was used to further purify proteins present in the complex mixture of proteins isolated in the urea and guanidine-HCl soluble fractions. The AE-HPLC was performed on a Waters 650E Advanced Protein Purification System using a Brownlee AX-300 anion exchange column (Applied Biosystems). Proteins were loaded onto the column in 20 mM Tris-HCl buffer pH 8.4 containing 6 M urea, and eluted in a 0 to 1.0 M NaCl gradient in the same buffer. Cb68 was further purified on the same column and buffer system with 6M urea being replaced with 0.1% SB 3-14.

Purification of serine proteases from excretory/secretory material

The purification of serine proteases from the E/S material of *C. bezziana* larvae was performed as previously described (MUHARSINI *et al*., 2000). Briefly, the serine proteases were purified using soybean trypsin inhibitor-Sepharose (SBTI-Sepharose) affinity chromatography. The proteins bound were eluted using 100 mM acetic acid (pH 2.5) containing 0.1% SB 3-14 (Boehringer). Fractions containing high protein concentrations were pooled for vaccination.

SDS-PAGE

Analytical gel electrophoresis of PM extracts and preparative gels for blotting and N-terminal sequencing were routinely conducted on 6-18% gradient SDS-PAGE gels using the discontinuous buffer system described by LAEMMLI (1970). The *in situ* endoproteinase digestion of individual proteins was conducted on either 10 or 12% SDS-PAGE gels, depending on the MW of the target protein. Silver staining of gels was performed using the modified method of RABILLOUD *et al.* (1988). The apparent MW of target proteins was determined by a linear regression plot of the $\log_{10}$ MW verses relative electrophoretic mobility.

Protein estimations

Protein concentrations were estimated using BCA protein assay reagent (Pierce) and/or dye-binding protein assay reagent (Bio-Rad) (both used according to the manufacturer's instructions). Where amounts of

protein were too low or in short supply, a semi-quantitative estimate was obtained by a comparison of selected stained protein bands on SDS-PAGE gels and MW standards of a known amount, run on the same gel.

Purification of proteins for *in situ* digestion on SDS-PAGE

Four PM proteins were identified for processing. Individual aliquots of (1) SDS soluble material (containing approximately 400 µg of Cb15), (2) AE-HPLC purified guanidine-HCl soluble fraction (containing approximately 75 µg of Cb23 and 150 µg of Cb42) and (3) AE-HPLC purified urea soluble fraction (containing approximately 100 µg of Cb68) were first precipitated with methanol (1:10) and incubated at –20°C overnight. Following centrifugation (3000 g, 30 min, 4°C) the pellet was resuspended in sample buffer containing 100 mM Tris-HCl pH 6.8, 10 mM DTT, 4% SDS, 10% glycerol and bromophenol blue. The protein samples were resolved by SDS-PAGE on a 12% analytical gel (Mini-PROTEAN II Electrophoresis System, Bio-Rad) at 200 V for 40 min and visualised with Coomassie Brilliant Blue R-250 stain (Biorad).

***In situ* endoproteinase digestion of PM proteins and the isolation of peptides**

Protein bands corresponding to Cb15, Cb23, Cb42 and Cb68 were excised from SDS-PAGE gels and cut into 1 mm^2 pieces. Gel pieces were washed twice for 30 min at RT in a solution containing 0.2 M ammonium bicarbonate and 50% acetonitrile (HPLC grade) and then dried under vacuum for 4 h. The desiccated gel pieces were rehydrated in 200 µl of digestion buffer containing 0.2 M ammonium bicarbonate, 1 mM EDTA and 5 µg of endoproteinase Lys-C (Boehringer Mannheim). On rehydration, gel pieces were sonicated for 5 min followed by incubation at 37°C for 16 h. Following enzymatic digestion, gel pieces were pelleted by centrifugation (14,000 g, 10 min, RT) and the supernatant containing cleaved peptides was collected. Gel pieces were washed with 200 µl of 1.0% trifluoroacetic acid (TFA) in MQW, sonicated for 30 min at RT and centrifuged as before and the supernatant removed. A second wash contained 0.1% TFA and 60% acetonitrile. All supernatants were combined and the acetonitrile removed under vacuum. Peptides contained in the supernatant were isolated by reverse phase HPLC on a Brownlee RP-300 C-8 column. Sequential chromatographic separations of the peptides were performed, first in a 0.1% heptafluorobutyric acid-acetonitrile gradient, then selected groups of peptides rechromatographed on a 0.1% TFA-acetonitrile gradient (WILLADSEN *et al.,* 1989). Peptide sequencing was performed on an Applied Biosystems 471A protein sequencer.

N-terminal sequencing

Two independent preparations of Cb15 were sequenced, one containing approximately 100 pmol and the other, 500 pmol of protein (the second preparation was identical to that used for the generation of internal peptides described above). An aliquot of TBS extract containing approximately 200 pmol of Cb22 and 300 pmol of Cb27 was also processed. Fractions were first separated by SDS-PAGE on 6 to 18% gradient gels. The proteins were then transferred to polyvinylidene difluoride (PDVF) membrane on a LKB Instruments Multiphor II Novablot semidry system according to the manufacturer's instructions, using a transfer buffer containing 25 mM ethanolamine, 40 mM glycine and 0.2% SDS. The PVDF membranes were stained in a solution containing 0.05% coomassie stain, 40% methanol, 1% acetic acid and 59% water for 5 min and then destained in methanol/water (1:1) and finally washed with MQW then air dried. Protein bands were excised and sequenced using an Applied Biosystems Blott cartridge.

Sheep vaccination

The protocol used in *C. bezziana* PM vaccination trials has been described previously (SUKARSIH *et al.,* 2000b). Briefly, for each fraction, 6 ml of extract was homogenized with 14 ml of Montanide ISA 70 adjuvant (Seppic, Paris, France) in the presence of antibiotics, 20.000 IU of penicillin and 25 mg of streptomycin.

In the vaccination trial assessing PM fractions, merino sheep (six animals per group) were used and were vaccinated twice, four weeks apart. The amount of protein injected per sheep per vaccination was: water wash, 0.37 mg; TBS extract, 0.36 mg; SB 3-14, 1.09 mg; urea extract, 0.45 mg; guanidine-HCl extract, 0.85 mg and SDS soluble material, 1.65 mg.

The vaccination trial for SBTI purified serine proteases was carried out using Javanese Thin Tail sheep (six animals per group). Animals were vaccinated three times. The second and third vaccinations were carried out four weeks and six weeks respectively, after the first vaccination. Each animal was injected with 40 µg of purified serine proteases per vaccination.

***In vitro* and *in vivo* larval assays**

The procedure used for *C. bezziana in vitro* larval assays was essentially that described for *L. cuprina* (EISEMANN *et al.,* 1989) with some minor modifications (SUKARSIH *et al.,* 2000a). The assay for each serum was carried out with five-fold replication and the larval

recoveries and weights averaged for each serum. The procedure used for *C. bezziana in vivo* larval assays has been described (PARTOUTOMO *et al.,* 1998; SUKARSIH *et al.,* 2000a) and was similar to that described for the measurement of growth and survival of *L. cuprina* on sheep (EISEMANN *et al.,* 1989). The parameters of larval weight and larval recovery were compared between control and vaccinated groups. Statistical analyses of the *in vitro* assay data were performed using a student's T-test. *In vivo* data was analysed using a two way ANOVA analysis of variance with treatment and site of infestation as factors.

ELISA assays

ELISA assays were performed as described by SUKARSIH *et al.* (2000a). Titres for the sheep vaccinated with PM fractions were standardized against a pool of antisera from sheep vaccinated with whole PM and assigned an arbitrary titre of 20,000, which lay within the linear portion of a calibration curve (SUKARSIH *et al.,* 2000a). For the E/S proteases, the coating antigen was purified proteases (same as the vaccination antigen), at a concentration of 20 μg/ml.

RESULTS

Fractionation of PM proteins

Vaccination of sheep with homogenised whole *C. bezziana* PM induced a significant immune response against the parasite (SUKARSIH *et al.,* 2000a). *In vitro* assay results showed a reduction in larval weights of 73% and a reduction in the number of larvae recovered of 32% giving an overall reduction in the mass of larvae recovered of 82%. *In vivo* results showed a 41% reduction in larval weights and 13% reduction in larval recovery giving an overall reduction in the mass of larvae recovered of 49%. These results provided strong evidence that components of PM can induce a significant protective response and justified the fractionation and purification of PM components with the intention of identifying and characterizing the antigens responsible for protection.

The cultured PM was fractionated in batches ranging from between 7.6 g and 16.9 g (wet wt.). In early fractionations, the amount of extraneous material co-pelleting with the PM on harvest was evident in the first water wash (preparation 2, Table 1). In later preparations, more attention was given to PM cleanliness, with larval growth medium, exudate and faecal contamination being thoroughly washed away from the PM prior to homogenisation (preparation 4, Table 1). The low proportion of total weight extractable as protein reflects, in part, the large amount of water entrapped in the PM but also the amount of material remaining insoluble in the residue of the PM even after very vigorous extraction.

Table 1. Protein yield from four PM protein fractionations

	Preparation			
	1	2	3	4
PM (g wet wt.)	7.60	8.54	13.08	13.22
Water wash (mg)	2.90	7.18	3.19	1.84
TBS extract (mg)	1.18	1.02	1.18	3.65
SB 3-14 extract (mg)	3.46	2.95	4.36	10.12
Urea extract (mg)	2.44	2.03	1.22	4.78
Guanidine-HCl extract (mg)	2.90	2.18	3.89	7.52
SDS soluble material (mg)				12.17

A flow diagram showing the protein extracts removed from the whole PM following progressive treatment with agents of increasing solublisation capacity is presented in Figure 1. With each successive extraction step, different groups of proteins were dislodged from the PM membrane. The extracted proteins present in each fraction are visualised on the SDS-PAGE gradient gel shown in Figure 2. The amount of protein recovered at each extraction step from four independent preparations is shown in Table 1. The PM used in these extractions was stored wet prior to use and contained varying amounts of buffer, which is included in the wet weight shown in Table 1. Preparations 3 and 4 were used in the sheep vaccination trial, the results of which are presented below.

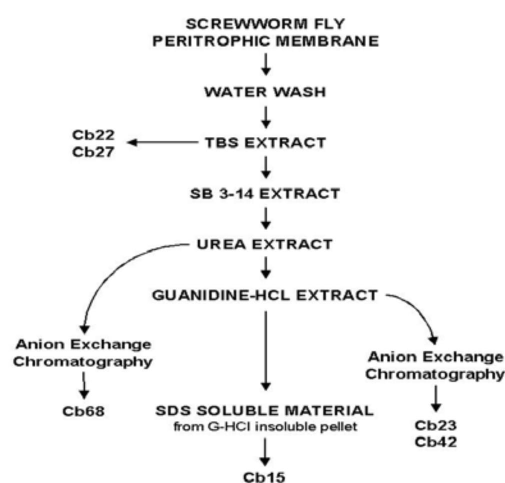

Figure 1. Flow diagram of the sequential extraction of proteins from *C. bezziana* PM. The origins of specific major PM proteins that were subsequently purified and identified by amino acid sequencing are indicated

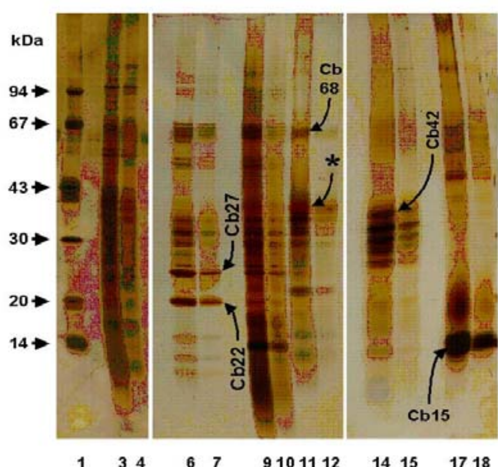

Figure2. Silver stained SDS-PAGE gels of proteins extracted from *C. bezziana* PM. The gel was run under reducing conditions (10 mM DTT). Low MW markers (Pharmacia) were run in lane 1. Two aliquots (30 µl and 10 µl) of each extract were run; PM water wash, lane 3 and 4; TBS extract, lanes 6 and 7; SB 3-14 extract, lanes 9 and 10; urea extract, lanes 11 and 12; guanidine-HCl extract, lanes 14 and 15 and the SDS soluble material, lanes 17 and 18. Selected PM proteins that were subsequently characterised by N-terminal and internal amino acid sequencing are labelled. The asterisk indicates an unnamed major protein of approximately 40 kDa MW on which N-terminal sequencing was attempted but was unsuccessful

Vaccination with PM fractions

A vaccination trial was carried out in sheep and the vaccination efficacy determined with *in vitro* assays. The results are listed in Table 2. The effect of vaccination with the PM water wash, TBS extract and SB 3-14 extract were not statistically significant. The most effective fraction was the urea extract with a reduction in larval recovery of 11% (p=0.05) and a reduction in larval weight of 30% (p=<0.05). To a lesser extent the guanidine-HCl extract and the SDS soluble material gave some protection with a reduction in larval weight of 15% (p<0.05) and 19% (p<0.01) respectively. Reduction in larval recovery for the guanidine-HCl extract was 9% (p≤0.05) but was not significant with the SDS soluble material.

ELISAs were conducted on the same sera as those used for *in vitro* assays. With the exception of the SB 3-14 group, all vaccinated animals showed a good immune response. Antibody titres ranged from 32,000 to greater than 250,000. In contrast, the titres of the SB 3-14 vaccinates were 0-16,000. The absence of comparable high antibody titres in this group may have been due to the presence of the SB 3-14 detergent in the antigen preparation. We observed poor emulsion formation with the adjuvant in the presence of the detergent. However, in a repeat of the SB 3-14 vaccination trial, the detergent was removed from the fraction by methanol precipitation prior to mixing with adjuvant. Even though a significant immune response was obtained, protection as measured by *in vitro* assay was still not significant (data not shown).

Table 2. Vaccination with PM fractions: *in vitro* assays

Fraction	Reduction in larval recovery (%)	Reduction in larval weight (%)
Water wash	N.S.	24 (N.S.)
TBS extract	N.S.	N.S.
SB 3-14 extract	N.S.	N.S.
Urea extract	11 (p = 0.05)	30 (p<0.05)
Guanidine-HCl extract	9 (p<0.05)	15 (p<0.05)
SDS soluble material	N.S.	19 (p<0.01)

N.S. = not significant

Identification of major proteins extracted from PM

The vaccination results with whole PM and with PM extracts, together with the suggested mechanism of vaccination effects in *L. cuprina* (WILLADSEN *et al*., 1993) indicated it was appropriate to focus on the major proteins present in protective PM fractions. Some of the major proteins present in protective *C. bezziana* fractions (Figure 2) were selected for further purification and characterization by amino acid sequencing. Proteins nominated for further characterization included Cb22 and Cb27 from the TBS extract, Cb23 and Cb68 from the urea extract, Cb42 from the guanidine-HCl extract and Cb15 from the SDS soluble material. The migration of these proteins on 6 to 18% gradient SDS-PAGE gels are indicated in Figure 2. The apparent molecular weights of selected proteins, determined by a linear regression plot of the $\log_{10}$ MW verses relative electrophoretic mobility, are presented in Table 3.

Table 3. Apparent molecular weight of selected PM proteins

PM protein	Molecular weight (daltons)
Cb15	15,300
Cb22	20,900
Cb27	25,700
Cb42	41,000
Cb68	70,000

In situ endoproteinase Lys-C digestion of Cb15, Cb23, Cb42 and Cb68 proteins was successful (Table 4). Cb68 peptide sequences are presented in Table 4. The N-terminal sequences of Cb15 (WIJFFELS *et al.,* in preparation), Cb22, Cb27 and some other proteins sourced from anion exchange fractions (Table 4), were obtained after blotting directly from SDS-PAGE gels onto PVDF membrane. Cb22 and Cb27 had identical N-terminal sequences and were identical to that of *C. bezziana* excreted/secreted serine proteases isolated from *C. bezziana* growth media (MUHARSINI *et al.,* 2000). The four proteases are probably very closely related proteins or in fact truly identical. Attempts to N-terminally sequence Cb42 and Cb68 and the major protein (approximately 40 kDa) present in the urea extract (marked * in Figure 2) were unsuccessful and it was assumed they were N-terminally blocked. Chemical modification of protein N-termini can often occur during protein purification.

Amino acid sequence information obtained from native Cb15 and Cb42 has been used to facilitate the identification of the genes encoding each protein (WIJFFELS *et al.,* in preparation; MUHARSINI *et al.,* in preparation). Attempts have been made to isolate the Cb68 gene, but without success. Cb23 peptides have near identical sequences to Cb15 and the protein species isolated from the urea fraction is assumed to be an aggregate of Cb15.

Vaccination with excretory/secretory proteases

Vaccination with affinity purified serine proteases were assessed with *in vitro* and *in vivo* assays. Table 5 shows a 3% reduction on larval recovery and no increase on larval weight from the *in vitro* assays. Statistically, there was no significant difference between vaccinated and control animals for either of these parameters. In contrast, *in vivo* assays showed significant effects of vaccination on these parameters (Table 5). The *in vivo* results showed an average reduction in larval weight of 37% in the larvae returned from the vaccinated sheep. The results were analyzed in terms of two effects which potentially contributed to these differences: the effect of vaccination with *C. bezziana* larval serine proteases and the site of the larval infestation (ring position) which was replicated on each animal. The reduction in larval weights on vaccination was highly significant ($F_{1,47} = 22$, $p<0.001$). There was no effect of ring position.

Table 4. Amino acid sequences of PM protein N-termini and endoproteinase Lys-C generated peptides

Peptide	Amino acid sequence
N-terminal sequences	
Cb15	(WIJFFELS *et al.,* in preparation)
Cb22	IVGGVATTIGNFPWQVSLQR
Cb27	IVGGVATTIGNFPWQVSLQR
SWAXU41	NYGMEQA
SWAXU42	DEEAKKEEXGNK
SWAXU70	VXNKXQPNGV
Cb15 Peptides	(WIJFFELS *et al.,* in preparation)
Five peptides sequenced	
Cb23 Peptides	(WIJFFELS *et al.,* in preparation)
Two peptides sequenced	
Cb42 Peptides	(MUHARSINI *et al.,* in preparation)
Three peptides sequenced	
Cb68 Peptides	
SWAX68U03	(K)NYIYMYRK
SWAX68U06	(K)QTVAGRYPYPNDTXH
SWAX68U94	(K)ANXGEYPN

[1] The one letter code for amino acids has been used. The initial (K) of a peptide sequence is assumed to be present because of the specificity of the endoproteinase Lys-C. Residues that could not be assigned with confidence are represented with an X.

Interestingly, the average larval recovery increased 21% for vaccinated animal as compared to the controls. This was significant ($F_{1,47} = 10$, $p<0.05$). As with the larval weights, ring position did not influence larval recovery.

Table 5. Vaccination with serine proteases: *in vitro* and *in vivo* assays

Antigen	*In vitro*		*In vivo*	
	Larval recovery (%)	Larval weight (mg)	Larval recovery (%)	Larval weight (mg)
Serine protease	81.3 ± 3.3	2.2 ± 0.3	72	17.3
Control	84.3 ± 6.1	2.2 ± 0.4	60	27.5

DISCUSSION

The results presented in this paper show that the isolation and characterisation of potential antigens, even those as difficult to study as membrane bound glycoproteins, is possible using a range of techniques which are potentially available to any protein biochemistry laboratory. In purifying proteins it is usually advantageous and frequently essential to develop initial separation steps which can be carried out on relatively large amounts of material but which nevertheless lead to a workable number of fractions containing discrete protein species. This is obviously possible with proteases extracted from excretory/ secretory material by affinity chromatography on SBTI-Sepharose. However, it applies equally to the peritrophic membrane, where a series of sequential extraction procedures using surfactants and denaturants of increasing severity is able to separate discrete classes of proteins even from a difficult tissue like the peritrophic membrane.

It is interesting to note that the N-terminal protein sequence obtained from some of the secretory/ excretory proteases (MUHARSINI *et al.,* 2000) is identical to two of the proteins extracted from the peritrophic membrane in TBS. There are two possible explanations for this. The family of serine proteases shows regions of high sequence conservation. Using PCR primers designed around these regions, a large number of serine protease genes have been identified in *L. cuprina* (ELVIN *et al.,* 1994). Using a statistical argument, this sequence information was used to further predict that the probable size of the family of serine proteases in this parasite was about 200 (ELVIN *et al.,* 1994). Therefore, it may not be surprising if an identical N-terminal sequence is obtained for a number of these proteases, with varying molecular weight and fraction origin. Certainly, it is reasonable to expect serine proteases in both the secretory/excretory material and in the peritrophic membrane, that is, involved in protein digestion in the wound and in the parasite's digestive system. The second explanation appears, at first sight, less likely, namely that the same enzymes could be both membrane bound then secreted. However this has been shown to be the case with another protein from a pertirophic membrane, PM 95 from *L. cuprina* (TELLAM *et al.,* 2000).

The two vaccine assay systems used in this work again are likely to measure different components of the host-parasite interaction. The *in vitro* assay system uses serum from vaccinated animals in an agar medium. As such, factors controlling wound formation are unlikely to be important, but those affecting digestion relatively important. By contrast, the *in vivo* assay system, since it involves penetration of first instar larvae into a minimal wound in sheep skin, would clearly be dependent on the efficacy of wound formation as well as on subsequent feeding performance. The use of these two assay systems jointly can begin to give some information about the relevance of the target antigens to these two processes.

It is therefore interesting that vaccination of sheep with serine proteases followed by *in vitro* feeding showed no significant effect of the serum. The very marginal effects on larval weight were not statistically significant and very much less than the dramatic effects on larval weight in the same feeding system shown after feeding larvae on serum from sheep vaccinated with larval peritrophic membrane. In contrast, effects *in vivo* showed a significant reduction in larval weight after a standardised feeding interval.

There are two obvious possible explanations for this observation. The first is that the proteases used in vaccination are important in wound formation and establishment and feeding of larvae in sheep, but not critical in the artificial system of the *in vitro* feeding. The second is that some component of the immune system is present in the *in vivo* feeding but absent or in reduced quantities in the *in vitro* system. This second possibility can certainly not be excluded. For example, even the availability of complement will be much less after preparation of the *in vitro* feeding medium.

The observation that there is a slight though statistically significant increase in larval recovery after *in vivo* feeding on sheep vaccinated with proteinases is surprising. Nevertheless, there is also evidence from vaccination with peritrophic membrane as well as vaccination with some recombinant peritrophic membrane proteins, that the production of antibody to these leads not to inhibition of feeding, but to some significant increases in the recovery of larvae *in vivo* in the absence of any detectable effect *in vitro.* One possible explanation of this is that formation of antigen-antibody complexes in the developing wound may, by causing localised inflammation, facilitate larval feeding. This, if true, complicates the interpretation of vaccination effects but is also an important consideration in vaccine development.

In addition to the serine proteases, *C. bezziana* E/S material contains a number of gelatin-degrading enzymes not inhibited by the classic serine protease inhibitors (MUHARSINI *et al.,* 2000). These too are potential vaccine antigens, but their efficacy remains to be investigated.

For the peritrophic membrane, the results in this paper extend the observations with whole peritrophic membrane reported elsewhere (SUKARSIH *et al*., 2000a). Several fractions have significant effects *in vitro*, though the effects are markedly less than that of the whole membrane. It has also been shown that these proteins once separated on SDS-PAGE can yield both N-terminal and internal amino acid sequences. These

can be obtained working with amounts of proteins in the low µg range. As will be described in subsequent papers (this issue), such peptide sequence is usually essential for the identification of the gene coding for the protein of interest.

CONCLUSIONS

Fractions derived from the peritrophic membrane and affinity purified serine proteases are both able to induce immune responses in vaccinated sheep which confer a degree of immune protection against larval screwworm fly. Techniques exist for the characterization of individual antigens in these relatively simple antigenic mixtures in terms of unique amino acid sequences, obtained on amounts of antigen in the low microgram range. This characterization, in turn, permits the expression of such antigens as recombinant proteins. These are key steps in the investigation of the true feasibility of vaccine development.

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