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Chromatography and generation of specific antisera to synthetic peptides from a protective *Boophilus microplus* antigen

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ABSTRACT

Four oligopeptides corresponding to predicted antigenic regions of the protective Bm86 glycoprotein of the cattle tick *Boophilus microplus* were synthesized and purified. Three were conjugated to carrier proteins and antisera raised in rabbits and cows. All elicited antipeptide antibodies that recognized Bm86 and recombinant derived products in Western blots; however, only one produced antiserum capable of recognizing native Bm86 in an indirect immunofluorescence assay. Ticks fed *in vitro* on this antiserum showed no obvious gut damage.

INTRODUCTION

Antibodies directed against synthetic peptides which react with the intact protein are useful in the detection of gene products, the study of functionally active regions of proteins and the development of synthetic vaccines.

The cattle tick *Boophilus microplus* is a major ectoparasite of cattle in many parts of the world. Bm86 is a gut epithelial cell surface glycoprotein isolated from the tick which, when used to vaccinate cattle, stimulates an immune response which protects cattle against subsequent tick infestation[1]. The DNA sequence of the cloned gene[2], shows that Bm86 has 650 amino acids, 10% of which are cysteines. The cysteine residues are organized into eight domains each containing six cysteines with the spacing characteristic of the epidermal growth factor (EGF) precursor and several other proteins. Previous studies with synthetic peptides have shown that receptor binding of both murine EGF[3] and urokinase-type plasminogen activator (uPA)[4]

occurs within the "B loop" of the 6-Cys units. The intra-chain disulphide bonded peptide of uPA has a higher receptor binding affinity than the reduced form[4]. Since the "B loop" region of these molecules is functionally active it is not unreasonable to speculate that these regions within Bm86 may be also functionally important. Antibodies directed to these regions may inhibit the function of this molecule and confer a protective response.

We report here the synthesis and purification of two "B loop" oligopeptides from Bm86 which were used to generate reagents to help define the epitopes that may elicit protective antibody. We also report the use of two other linear oligopeptides from the NH_2- and COOH-terminal regions of Bm86 in the characterisation of recombinant products.

EXPERIMENTAL

Materials and equipment

All reagents used were of analytical grade. Ovalbumin, bovine serum albumin, keyhole limpet hemocyanin, fish gelatin, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, Coomassie Brilliant Blue R, Freund's complete and incomplete adjuvant were from Sigma (St. Louis, MO, U.S.A.). Glutaraldehyde was from BDH (Kilsyth, Australia). Western blotting reagents were from Promega (Rozelle, Australia). Immulon 2 microtitre plates were purchased from Dynatech (Alexandria, VA, U.S.A.). Peroxidase-conjugated antibodies were from Dakopatts (Glostrup, Denmark). Fluorescein-labelled rabbit anti-bovine Ig was a gift from R. Pearson (CSIRO, Division of Tropical Animal Production). Purified Bm86 (ref. 1) was a gift from CSIRO Division of Tropical Animal Production. Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). N^α-Butyloxycarbonyl (tBoc)-L-amino acid derivatives and 4-methylbenzhydrylamine polystyrene resin were from Applied Biosystems (Foster City, CA, U.S.A.). N-Methylmercaptoacetamide was purchased from Fluka (Buchs, Switzerland).

The high-performance liquid chromatography (HPLC) system consisted of two pumps with extended flow heads (Model 510, Waters Chromatography Division, Millipore, Milford, MA, U.S.A.), a gradient controller and data system (Model 840, Waters), a sample injection valve (Model U6K, Waters) and a multiwavelength detector (Model M-490, Waters). Reversed-phase (RP) HPLC columns were obtained from The Separations Group (Hesperia, CA, U.S.A.).

Peptide synthesis

The protected peptide was assembled by solid-phase synthesis[5] on a cross-linked polystyrene support using an Applied Biosystems 430A automated peptide synthesiser. tBoc-amino acids were coupled sequentially to a 4-methylbenzhydrylamine polystyrene resin. A cysteine was added to the COOH terminal of pep 2 to facilitate coupling to the carrier protein. Internal cysteines present in pep 3 and pep 4 (Cys^{37} and Cys^{307} respectively) were replaced by alanine residues to avoid undesired disulphide-bonded loop formations. After assembly the protected peptide–resin was cleaved and the protecting groups were removed by anhydrous hydrogen fluoride in the presence of scavengers: HF–anisole–dimethylsulphide–*p*-thiocresol (10:1:1:0.2, v/v) for 1 h at $-5°C$ to $0°C$. The cleaved peptide was washed with diethyl ether then dissolved in 10% aqueous acetic acid and lyophilised.

Reduction of methionine sulphoxide in pep 4

After HF treatment the crude pep 4 (5 mg/ml in 10% aqueous acetic acid) was reduced by the addition of 10% (v/v) N-methylmercaptoacetamide (MMA) and the reaction was allowed to proceed for 26 h at 37°C (ref. 6). The reduced peptide was purified by size-exclusion chromatography followed by preparative RP-HPLC.

Chromatography

Analytical RP-HPLC was performed on a wide-pore, octadecyl silica column (Vydac 218TP 54; 250 × 4.6 mm I.D.). Pep 1 and pep 2 were eluted with a linear gradient of 5–60% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) in 30 min. The flow-rate was 1 ml/min and the absorbance was monitored at 220, 254 and 280 nm using a multiwavelength detector. Pep 3 and pep 4 were eluted with a 10–60% gradient of acetonitrile in 0.1% TFA. The separation of the linear and folded forms of pep 3 was achieved using a 17.5–30% gradient.

Preparative RP-HPLC was also performed on an octadecyl column (Vydac 218TP 1022; 250 × 22 mm I.D., 10 μm particle size). Elution gradients were identical to those used for the analytical RP-HPLC using a flow-rate of 16 ml/min. All fractions were lyophilised after collection.

After MMA treatment, pep 2 and pep 4 were chromatographed on Sephadex G-25 gel filtration media to remove reagents and low molecular weight contaminants. The column (100 × 1.6 cm I.D.) was eluted with 5% aqueous acetic acid at a flow-rate of 3 ml/min and monitored at 254 nm.

Disulphide loop formation

RP-HPLC-purified pep 3 was allowed to fold and oxidise by stirring for 20 h at a concentration of 0.1 mg/ml in 100 mM Tris–HCl, pH 7.9. The reaction was monitored by RP-HPLC and Ellman's assay[7]. The reaction products were desalted by preparative RP-HPLC.

Peptide analysis

Amino acid analysis was performed on a Waters Picotag system with precolumn formation of the phenylthiocarbamyl derivatives[8]. NH_2-terminal sequence analysis was carried out on an Applied Biosystems Model 470A gas phase sequencer.

Coupling of peptides to carrier proteins

Pep 1 and pep 2 were coupled to ovalbumin (OVA) and bovine serum albumin (BSA) respectively with *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)[9]. Pep 3 was coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde using a modification of the described method[10]. Carrier proteins and conjugates were subjected to amino acid analysis (Waters Picotag) to monitor coupling efficiency.

Immunization

(i) New Zealand White rabbits were immunized with 0.5 mg of either pep 1–OVA or pep 2–BSA conjugate in Freund's complete adjuvant by subcutaneous injection. Two booster injections were given on days 21 and 38 in Freund's incomplete adjuvant. Venous blood was collected by standard procedures, usually 14 days after each injection.

(ii) Three *Bos taurus* cows were vaccinated on two occasions four weeks apart. A 1-mg amount of a pep 3-KLH conjugate in saline was emulsified with an equal volume of Montanide/Marcol and delivered by intramuscular injection. Blood was collected 14 days after the second vaccination.

Western blotting

Proteins were transfered to nitrocellulose paper as described[11] and blocked for 1 h at room temperature in 0.5% fish gelatin–TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blots were then incubated in antisera diluted in TBST overnight at 4°C, washed four times in TBST, and then incubated in alkaline phosphatase conjugated goat anti-rabbit or rabbit anti-bovine immunoglobulin at room temperature. After washing four times in TBST the blots were developed by incubation in 10 mM Barbital–acetate buffer (pH 10.0) containing 0.35 mg/ml nitro-blue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indoyl phosphate.

Titration of antisera

Antisera were titred by an indirect enzyme-linked immunosorbent assay (ELISA)[12]. Microtiter plates were coated overnight at room temperature with 0.1–1 μg/well of free peptide or protein antigen in 100 mM sodium carbonate buffer (pH 9.6). Pre-immune serum from each animal served as a negative control. Optical density (OD) at 414 and 490 nm was measured after 10–20 min with a multiscan photometer (Bio-Tek Instruments).

Bm86 expression vectors

BTA1696, under control of the pL promoter, encodes a "full-length" protein consisting of 610 amino acids of Bm86 (residues 20–629). An insert encoding amino acids 1–629 (including the leader sequence consisting of residues 1–19 which are post-translationally cleaved) was also transformed into *Aspergillus nidulans* strain VH, under control of the *amdS* promoter[13]. BTA1753, also under control of the pL promoter, encodes a truncated Bm86 protein consisting of 264 amino acids of Bm86 (residues 97–360).

Expression tests

BTA1696, BTA1753 and N4830/pBTA 602 (which is the host–vector combination that lacks an insert) were grown in tryptone soybroth medium (TSB) containing Ampicillin (100 μg/ml) overnight at 28°C, diluted 1/10 in fresh TSB and grown a further 3 h before induction for 2 h at 40°C. Cell culture (0.1 ml) was centrifuged and the cell pellet was resuspended in 20 μl sodium dodecyl sulphate (SDS) boiling buffer (2% SDS, 0.5 M urea and 1% β-mercaptoethanol) and boiled for 5 min. The proteins were resolved on a 10% SDS–polyacrylamide gel and either stained with Coomassie Brilliant Blue or Western blotted.

Culture media from a *A. nidulans* transformant expressing a full-length Bm86 molecule was concentrated by ultrafiltration using a Centricon-30 microconcentrator and secreted proteins were prepared in SDS boiling buffer. The proteins were resolved on a 10% SDS–polyacrylamide gel and either stained with Coomassie Brilliant Blue or Western blotted.

Indirect fluorescent antibody binding to tick gut cells

This was performed by the method of Willadsen et al.[1]. Briefly, semi-engorged adult female ticks were dissected and small pieces of gut everted, reacted with bovine or rabbit antisera for 2 h at 4°C, washed, then reacted with the appropriate second antibody conjugated to fluorescein. The pieces of gut were then examined under a Zeiss fluorescence microscope.

Tick feeding in vitro

Adult ticks were fed in vitro[14] on antisera from cows vaccinated with pep 3 conjugate. Ticks were assumed to have suffered gut damage if they showed evidence of excessive leakage of bovine serum proteins into the haemolymph.

RESULTS

Selection criteria for synthetic peptides

Pep 1 and pep 2 (Table I) were chosen on the basis of hydrophilicity and terminal location. Hydrophilicity analysis[15] allows prediction of protein determinants that are likely to be surface-oriented and, therefore likely to be antigenic. Pep 1 and pep 2 had hydrophilicity indices of $+0.33$ and $+0.94$ respectively. A secondary consideration was the terminal location of both peptides; Walter et al.[16] found that sera against both the NH_2- and COOH-terminal peptides of the large T-antigen of SV40 precipitated the protein from extracts of infected cells. Terminally directed antibodies also reacted strongly with native gp70 of Friend murine leukaemia virus[17]. The flexibility of many chain termini presumably allows a greater chance of a "fit" with an antibody produced to a relatively short peptide that can adopt several confirmations in solution.

Bm86 is similar to EGF precursor and several other extracellular proteins[18]; the homology is mainly due to the conserved cysteine spacing in their growth factor

TABLE 1

PROPERTIES OF SYNTHETIC PEPTIDES

Single-letter amino acid code used.

Peptide	Amino acid sequence[a]	Position[b]	Hydrophilicity[c]	Location
pep 1	E S S I C S D F G N E F C R N A E C	20–37 (1–18)	+ 0.33	NH_2-term.
pep 2	T T T K A K D K D P D P G K S S A A (C)	612–629 (593–610)	+ 0.94	COOH-term.
pep 3	F C R N A E A̲ E V V P G A E D D F V C K	31–50 (12–31)	+ 0.41	B-loop
pep 4	K C H E E F M D A̲ G V Y M N R Q S C Y	299–317 (280–298)	+ 0.08	B-loop

[a] Amino acid sequence predicted from nucleotide sequence of Bm86 cDNA[2]. The residue in parentheses was added to facilitate coupling. Underlined residues are substitutes to prevent formation of unwanted disulphide-bonded products.

[b] Residue numbers in parentheses refer to primary sequence position in mature Bm86 assuming processing of a 19 amino acid leader sequence.

[c] Calculated according to Hopp and Woods[15].

modules. The presence of this conserved module in many proteins may mean that it is a part of a receptor–ligand system responsible for defined biological functions[2]. Synthetic peptide studies[3] showed that murine EGF residues 20–31 (resident in the so-called B-loop) constituted the primary receptor binding region and also contained the predominant EGF antigenic determinant. The homologous region within human uPA is also responsible for specific receptor binding[4]. By analogy with these proteins it is possible that the B-loop region within the growth factor modules present in Bm86 (Fig. 1) also serves a receptor binding function. Pep 3 and pep 4 (Table I) were chosen to represent the putative receptor binding regions in two EGF modules of Bm86. This strategy was based on the observation that the intra-chain disulphide bonded uPA synthetic peptide 12–32 displayed a stronger receptor binding affinity than did the reduced form[4].

Pep 1 and pep 2 were selected to raise antisera primarily for the purpose of characterizing gene products under denaturing conditions. Pep 3 was selected for the purpose of providing reagents useful in the characterization of the native antigen and to investigate the potential for the development of a synthetic peptide vaccine.

Peptide purification and analysis

The quality of the synthetic peptides was assessed by RP-HPLC analysis of the crude peptides using a wide pore C_{18} column. Crude pep 1, pep 2 and pep 3 contained only a single major peak at 220 nm which accounted for greater than an estimated 75–90% of the crude peptide content (data not shown). This supports the conclusion that each of the syntheses proceeded with high yield at each step and few side-reactions had occurred.

Pep 1 and pep 3 were efficiently purified to homogeneity by RP-HPLC using a wide pore C_{18} column and a standard acetonitrile gradient with TFA as the counter-ion. Pep 2, with a single major peak at 220 nm containing greater than an estimated
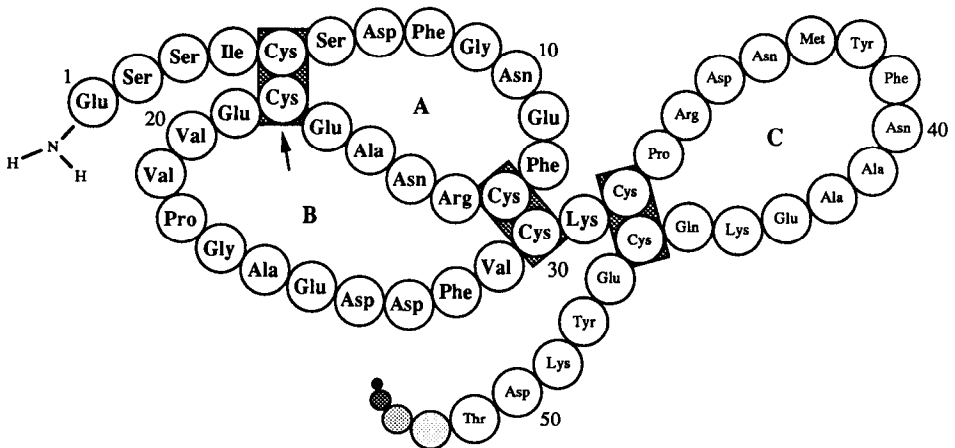

Fig. 1. A working model of the proposed NH$_2$-terminal EGF-like region of Bm86. The three loops designated A to C are analogous to those present in murine EGF (ref. 3). Pep 1 (residues 1–18) and pep 3 (residues 12–31) were chosen for synthesis; the arrow marks the location of the cysteine substituted by alanine in the synthetic peptide to avoid undesired disulphide bond formations.

90% of the crude peptide content, did not require HPLC purification but was desalted on a Sephadex gel filtration column.

In contrast, the crude pep 4 HPLC analysis showed a profile with multiple peaks when monitored at 220 (Fig. 2a), 254 or 280 nm. Further, the ratio of the peak heights at the different wavelengths was the same, indicating that the multiple impurity peaks were not due to partial deprotection or rearrangements of the benzyl protecting groups during the HF cleavage reaction. Pep 4 contained two methionine residues that were protected during synthesis as the sulphoxide form. It was suspected that this group had been incompletely deprotected during the HF cleavage step. This was confirmed by reduction with MMA; upon treatment there was a dramatic change in the chromatogram (Fig. 2b) with only a single major peak now remaining at 14.3 min. The peak at 11.5 min and the doublet at 12.9 min had disappeared. Methionine sulphoxide (Met[O])-containing peptides elute earlier under RP conditions than the peptides containing reduced methionine as illustrated by Fig. 2a. The peak at 11.5 min is most probably the peptide containing two Met[O] residues and the doublet of peaks at 12.9 min are most probably two peptide isomers each with a single Met[O] residue. The reduced peptide was further purified to homogeneity by RP-HPLC (Fig. 2c).

Peptide refolding

Purified pep 3 was air oxidised and folded in dilute basic solution to form the intra-chain disulphide-bonded loop peptide. An analytical RP-HPLC method was developed to separate the linear and the oxidised forms of pep 3 (Fig. 3). The oxidised form of the peptide (as determined by Ellman's assay[7]) elutes ahead of the linear form. This method allowed the progress of the oxidation to be monitored and ensured complete conversion and high recovery of the desired product.
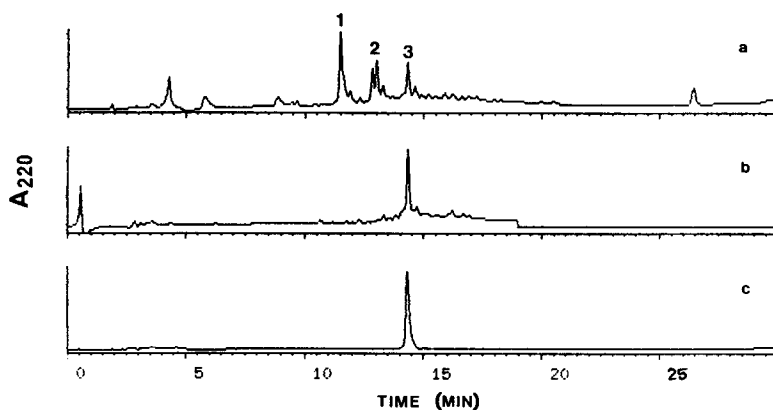

Fig. 2. RP-HPLC analysis of pep 4. (a) Crude pep 4 after HF cleavage from the support resin showing (1) the diMet[O] peptide, (2) two isomer forms of the monoMet[O] peptide and (3) the reduced peptide. (b) Crude pep 4 after MMA reduction. (c) Purified pep 4 after preparative RP-HPLC. Samples were chromatographed on a Vydac 218 TP column (250 × 4.6 mm I.D.) developed with a linear gradient from 10 to 60% acetonitrile containing 0.1% TFA during 30 min. Eluent was monitored at 220 nm and the flow-rate was 1 ml/min.
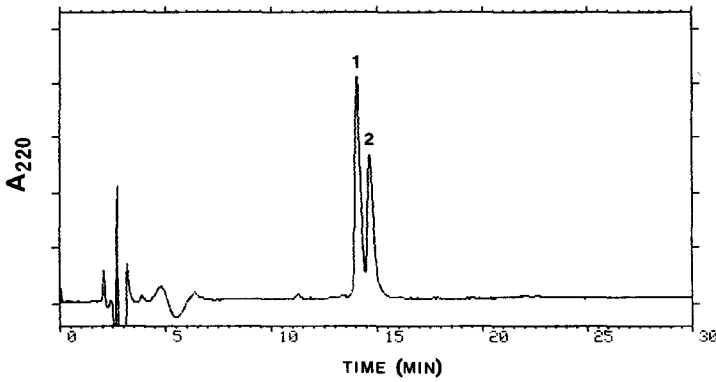
Fig. 3. RP-HPLC analysis of oxidized-refolded pep 3. Purified pep 3 was oxidized and chromatographed to show a shift to a shorter retention time after refolding: (1) oxidized and (2) reduced peptide. Chromatographic conditions were the same as for Fig. 2 with the gradient composition 17.5 to 30% acetonitrile in 0.1% TFA.

Production of peptide antisera

Pep 1 and pep 2 conjugates were analyzed by amino acid analysis and were shown to have coupling efficiencies of 3:1 and 10:1, respectively, on a molar ratio of peptide to carrier protein. The pep 3-KLH conjugate was not analyzed as the carrier had no defined molecular weight and was heterogeneous as judged by SDS–polyacrylamide gel electrophoresis (data not shown). Pep 4 was not conjugated and is the subject of research that will be presented elsewhere.

Rabbits (two per group) were immunized with pep 1 and pep 2 conjugates; three cows were immunized with pep 3 conjugate. Serum obtained 14 days after the second vaccination was titred by ELISA (Table II) and all peptides were shown to be immunogenic. The titres against homologous peptides in rabbits immunized with pep 1 and pep 2 conjugates ranged from 500 to 2500 with the latter conjugate being more immunogenic. Similarly, the titres of the cows immunized with pep 3 conjugate varied significantly with two animals responding relatively poorly and eliciting antibody responses that were an order of magnitude lower than the third animal. At the highest concentration, antisera to the unrelated peptides were not reactive. Antibodies cross-

TABLE II

ANTI-PEPTIDE ANTIBODY TITRES

ELISA titres of the serum from individual animals are defined as the reciprocal of the dilution ($\times 10^{-3}$) providing an OD of 0.5 measured at 414 nm. $-$ = No titre recorded at a 1:10 dilution.

Immunising peptide	*Antiserum titres against*		
	pep 1	*pep 2*	*pep 3*
pep 1	0.5, 2.5	$-$, $-$	$-$, $-$
pep 2	$-$, $-$	1.6, 2.4	$-$, $-$
pep 3	$-$, $-$, 0.05	$-$, $-$, $-$	0.6, 0.8, 10

Fig. 4. Antipeptide antibodies recognise denatured Bm86. Purified Bm86 (ref. 1) was electrophoresed on a 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose. Nitrocellulose replicas were incubated with antiserum directed against pep 1 (a), pep 2 (b) or pep 3 (c). The positions of molecular size markers are given in kilodalton.

reacted poorly with the overlapping pep 1 and pep 3 which suggests that secondary structure may be important. The strongest peptide antiserum of each group was used in subsequent studies.

Recognition of denatured Bm86 by the antipeptide sera

To determine if the antipeptide antibodies were capable of recognising denatured Bm86, sera were diluted 1:1000 and used to probe Western blots. All sera reacted strongly with 100 ng of purified Bm86 (Fig. 4) and this result suggested that the sera would be useful in the characterization of Bm86 and associated recombinant products under denaturing conditions. Pre-immune sera did not react with Bm86.

Characterization of recombinant Bm86 expressed in E. coli

BTA1696 expresses a 70 000-dalton protein at detectable levels upon induction, as shown by Coomassie Blue staining of an SDS–polyacrylamide gel. Uninduced bacteria do not synthesize this protein (data not shown). Two controls were included: (i) the host strain transformed with the expression plasmid without a recombinant insert (N4830/pBTA 602) and (ii) BTA1753 encoding a truncated Bm86 which expresses a detectable protein of 35 000 dalton upon induction (Fig. 5a).

Both controls and the full-length Bm86 were also Western blotted and probed in separate experiments with sera directed against pep 1, pep 2 and pep 3 (Fig. 5b–d). The three antipeptide sera reacted strongly with a band of 70 000 dalton which corresponds to the predicted molecular weight of the full-length Bm86. This band was only present in samples prepared from cells expressing the full length Bm86 (BTA1696). A corresponding band was not detected in samples prepared from cells expressing the truncated Bm86 (BTA1753). This indicates that each antipeptide serum contains
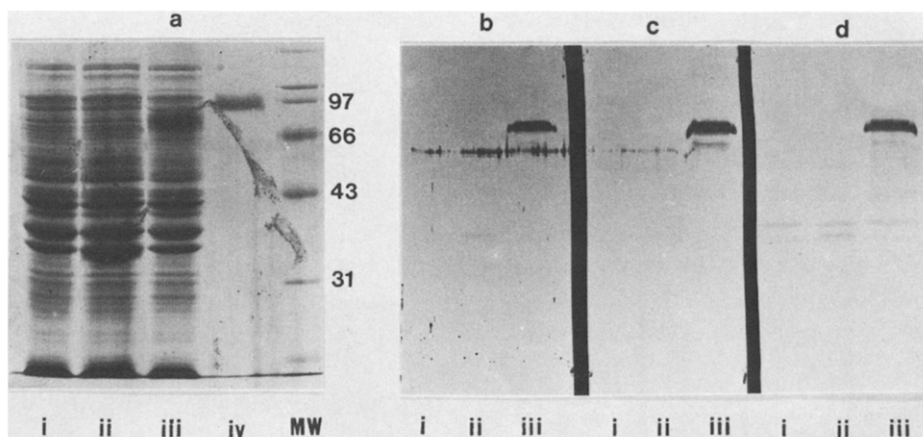
Fig. 5. Characterization of recombinant Bm86 expressed in *E.coli*. Cells were grown in TSB at 28°C and induced at 40°C. Samples were separated on a 10% SDS–polyacrylamide gel and either stained with Coomassie Brilliant Blue (a) or electroblotted onto nitrocellulose (b–d). Samples were from: lane i = host–vector control (N4830/pBTA 602); lane ii = cells expressing a truncated Bm86 (BTA 1753); lane iii = cells expressing full-length Bm86 (BTA1696); lane iv = purified Bm86. Nitrocellulose replicas were incubated with antiserum directed against pep 1 (b) pep 2 (c) and pep 3 (d). Molecular weight (MW) markers' are given in kilodalton.

antibodies that are (a) specific for Bm86 and (b) recognise epitopes in both the NH_2 and COOH termini of the Bm86 and not other regions of the protein. All sera reacted, with varying degree, to several *E. coli* proteins present in all samples however pre-immune sera reacted similarly with these bands (data not shown). The sera should therefore be useful as site-directed reagents for the detection and characterization of Bm86 and proteolytically related products expressed by recombinant organisms.

Characterization of recombinant Bm86 expressed in Aspergillus nidulans

Full-length Bm86 expressed in *E. coli*[2] formed insoluble inclusion bodies and was therefore considered unlikely to posses the same tertiary structure as the glycosylated Bm86 present in ticks. It is more likely that Bm86 expressed and secreted by eukaryotic cells would be correctly folded and glycosylated and, as such, antigenically very similar to native Bm86. The filamentous fungus, *Aspergillus nidulans* has proved to be a useful eukaryotic expression system allowing the expression of a number of heterologous proteins[19–22]. Amino acid residues1–629 of Bm86 were expressed in *A. nidulans* under the *amdS* promotor system[13].

Secreted proteins from one transformant and a control were electrophoresed on polyacrylamide gels and stained with Coomassie Blue. A Bm86 standard was run in addition to molecular weight standards. A faint stained band of approximately 85 000 dalton was visible in the secreted products of the transformant (Fig. 6a). To demonstrate that this protein was Bm86 replicate Western blots were probed with antisera against pep 1, pep 2 and pep 3 and all reacted with the 85 000-dalton diffuse band (Fig. 6a–d). The molecular weight of the *Aspergillus* product is smaller than that of the Bm86 standard isolated from ticks (95 000 dalton); the predicted molecular weight from the DNA sequence is 70 000 dalton with the remainder being carbo-
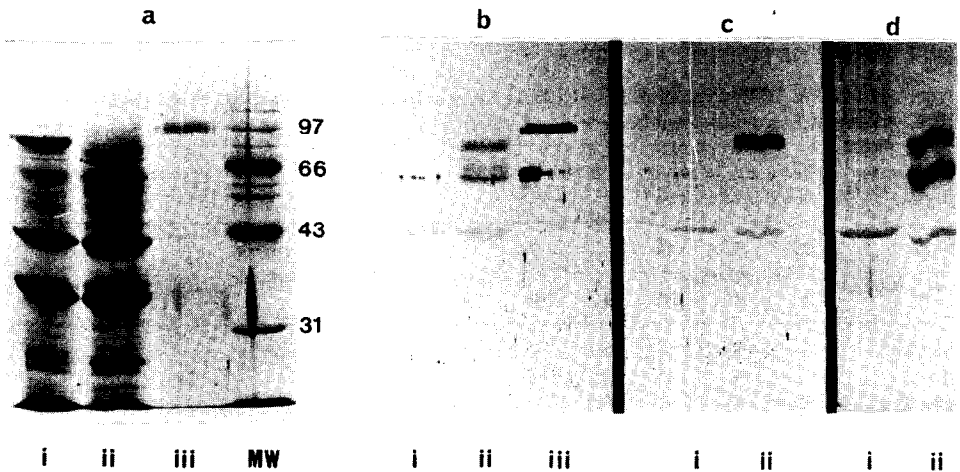
Fig. 6. Characterization of recombinant Bm86 expressed in *A. nidulans*. Culture media from mycelia was concentrated by ultrafiltration and samples were separated on a 10% SDS–polyacrylamide gel and either stained with Coomassie Brilliant Blue (a) or electroblotted onto nitrocellulose (b–d). Samples were from: lane i = wild-type control; lane ii = transformant expressing full length Bm86; lane iii = purified Bm86. Nitrocellulose replicas were incubated with antiserum directed against pep 1 (b), pep 2 (c) and pep 3 (d).

hydrate. As the native Bm86 may have an additional twenty COOH terminal amino acids which are not present in the *A. nidulans* expressed Bm86, it is probable that the latter is glycosylated but perhaps not as extensively as native Bm86. Careful examination of the immunoreactive bands in the *A. nidulans* culture supernatant shows that there are actually three bands bands of nearly identical molecular weight. This is a feature of many glycoproteins and presumably results from a common protein backbone that is differentially glycosylated. An additional band of approximately 55 000 dalton was present in Western blots probed with antiserum to pep 1 and pep 3 but was absent in blots probed with antiserum to pep 2. Such a result suggested the possibility that this band was a COOH terminal degradation product or a product of early termination of translation.

The above data showed that the antipeptide sera were universally reactive with full-length Bm86 when assayed under fully denaturing conditions. The following experiments were carried out to investigate the reactivity of the sera with oxidized recombinant Bm86.

Total secreted protein from cultures of wild-type control and Bm86 transformed *A. nidulans* strains was assayed by ELISA with Bm86 purified from ticks as standard. Antiserum raised against the NH_2-terminal pep 1 failed to react with either the secreted *A. nidulans* Bm86 or the purified, tick-derived Bm86. This may be explained by possible coupling of pep 1 to the carrier protein through the internal cysteines preventing the formation of the appropriate secondary structure. Antisera to pep 2 and pep 3 did recognise both antigens to varying extents (Table III) however the titre of antiserum to pep 2 against recombinant Bm86 was five fold lower than the titre against tick-derived Bm86. This may have been due to some degree of denaturation occurring during the purification of Bm86 from ticks; this was possible since

TABLE III

SPECIFICITY OF PEPTIDE ANTISERA

Abbreviations: W = Western blot; E = ELISA; An = *Aspergillus nidulans*; Bm = *Boophilus microplus*;
IFA = Indirect Fluorescent Antibody test.

Immunising peptide	Reactivity of antiserum with Bm86 produced by[a]						Biological Assay[b]	
	BTA 1753	BTA 1696	An		Bm		Tick gut IFA	In vitro damage
	W	W	W	E	W	E		
pep 1	−	+	+	0	+	0	−	−
pep 2	−	+	+	0.1	+	0.5	−	−
pep 3	−	+	+	0.2	+	0.2	+	−

  [a] Western blot titre defined as reactivity (+ / −) of a 1:1,000 dilution of antiserum with 100 ng Bm86.
ELISA titre defined as the OD measured at 414 nm using a 1:1000 dilution of the antiserum. Only
antiserum from the most productive animal in each group was titred.
  [b] Serum was evaluated for its ability to react in IFA with everted tick gut cells[1] and to elicit gut
damage in ticks fed *in vitro*[14].

the use of a detergent was necessary in each step of the purification[1]. Alternatively,
detergent molecules may bind to the hydrophobic anchor sequence or phosphatidyl-
inositol linkage[2] present in tick-derived Bm86 (but not in the *A. nidulans*-derived
Bm86) and expose epitopes located in the pep 2 region.

Immunofluorescence staining of tick gut cells using antipeptide sera
      A previous study[1] had shown that Bm86 was located on the surface of tick gut
digest cells and could be visualized by an indirect fluorescent antibody test (IFA)
using polyclonal antisera directed against the whole Bm86 isolated from ticks. Small
pieces of everted tick gut were incubated with each of the antipeptide sera and then
stained with a second antibody conjugated to fluorescein. Antibodies directed against
the B-loop pep 3 bound weakly to the gut cell surface (Table III). The distribution of
fluorescence was identical to that previously observed[1] using bovine antiserum direct-
ed against native Bm86. Antisera to pep 1 and pep 2 failed to stain significantly above
pre-immune background levels. The difference in reactivity of the antiserum to pep 2
with Bm86 in ELISA compared with Bm86 *in situ* may possibly be due to steric
interference since the pep 2 region in Bm86 is directly adjacent to the putative hydro-
phobic anchor sequence. Detergent solubilization would remove the Bm86 from the
membrane and expose epitopes that are not accessible when the antigen is bound to
the gut cell.

In vitro feeding
      Antibodies directed against pep 3 were tested for their ability to produce gut
damage in ticks fed *in vitro* on bovine serum. Despite their ability to bind to the
cell-bound Bm86, the antibodies directed to pep 3 did not damage any of the ticks fed
under standard conditions (Table III).

DISCUSSION

We have succesfully synthesized and purified four peptides for use in the immunological study and characterization of Bm86 and related recombinant products. Pep 1, pep 2 and pep 3 were synthesized in high yields and were readily purified to homogeneity by RP-HPLC or size-exclusion chromatography. In contrast, the crude pep 4 preparation recovered after synthesis was heavily contaminated with multiple methionine sulphoxide containing peptides and only after reduction with MMA was it clear that the peptide was the major product. Purified pep 3, with cysteine[37] replaced by an alanine residue, was oxidized and folded in dilute solution to form the intra-chain disulphide-bonded loop peptide.

Pep 1, pep 2 and pep 3 were coupled to carrier proteins and were immunogenic when used to vaccinate both rabbits and cows although titres varied considerably. All antipeptide sera specifically recognized epitopes within Bm86 or recombinant products (expressed in both prokaryotic and eukaryotic cells) as judged by reaction with reduced and denatured proteins in Western blots. The sera could be used in combination to check for the presence of proteolytically related products during expression testing.

Antisera to pep 2 and pep 3 also recognized Bm86 purified from ticks and Bm86 expressed in *A. nidulans* when tested under physiological conditions. This adds weight to the proposal that Bm86 expressed in *A. nidulans* was antigenically similar to the antigen produced by the tick. The failure of antiserum to pep 1 to recognise Bm86 in ELISA may be explained by possible coupling of pep 1 to the carrier protein through the two internal cysteines thus preventing the formation of the appropriate disulphide bonding present within the native Bm86. Alternatively, the pep 1 region within the native molecule may be concealed and unable to react with specific antibodies. The possibility that carbohydrate may be linked to serine and/or threonine within the pep 2 region of Bm86 (ref. 2) may need modification. As antibodies directed against pep 2 were reactive in both Western blot and ELISA, it is unlikely that the serine/threonine residues within this region are glycosylated.

Only antiserum to pep 3 recognised the native, cell-bound Bm86 as judged by IFA. The failure of antiserum to pep 2 to recognize cell-bound Bm86 may be explained by the extreme COOH-terminal location of this peptide within Bm86, abutting the 23 hydrophobic amino acid residues that presumably span the cell membrane. Antibodies directed to epitopes within this region may not bind the cell-bound Bm86 due to steric interference but are capable of binding upon removal of Bm86 from the membrane as is evident from the ELISA results. Alternatively, it is known that Bm86 is in relatively low abundance in the gut cell plasma membrane[1] so the IFA on whole cells is not likely to be a sensitive assay.

The presence of several EGF-like regions in Bm86 suggested that they may play an important role in the function of this molecule. Since pep 3 was the homologue to the B loop region of EGF responsible for cell binding[3], and could be targeted by antibodies *in vitro*, we speculated that antibodies against pep 3 may induce the gut damage evident when ticks are fed on blood from cows vaccinated with Bm86 (ref. 23) or disrupt an essential function of the molecule resulting in a decrease in the viability of the tick. The results of feeding ticks *in vitro* would seem to suggest that this may not be the case; despite the fact that antiserum to pep 3 bound to cell-bound Bm86 this

binding did not lead to any significant gut damage or reduction in tick viability. However, these effects may be titre dependent and it is known that *in vitro* damage is lost when sera are diluted only 1:5 (unpublished data). The immunofluorescent staining of gut cells with the antipeptide serum was considerably weaker than that seen with antiserum from cows vaccinated with native Bm86 (data not shown), so it is possible that this region may be more efficient if higher titre antibodies can be elicited. It is also possible that EGF-like domains other than the NH_2 terminal one are the targets of the protective immune response. In addition, tick viability can be more accurately assessed by *in vivo* feeding which will be undertaken in future studies.

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