

Identification of an abundant allergen from the sheep louse, *Bovicola ovis* [☆]

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ABSTRACT

Infestation of sheep with the louse *Bovicola ovis* is common worldwide and leads to an allergic dermatitis referred to as 'scatter cockle'. IgE from an infested lamb was used in immunoaffinity chromatography to purify allergens from crude preparations of whole *B. ovis* and its faeces. SDS–PAGE of the affinity-purified eluates from both preparations showed a dominant band with *M_r* of 28.5 kDa. Spleen cells from a mouse immunised with *B. ovis* faecal antigens were used to produce hybridomas which were screened by ELISA to identify those producing monoclonal antibodies (mAb) to the allergens purified by IgE immunoaffinity chromatography. Western blotting demonstrated that all of the mAbs examined recognised the 28.5 kDa allergen. The allergen, purified using immunoaffinity columns constructed with one of the specific mAbs, was shown to cause immediate and late-phase responses on intradermal skin testing in *B. ovis*-infested but not in naïve lambs. Levels of serum IgE specific for the purified allergen were significantly higher in infested than in naïve lambs ($P \leq 0.0025$). N-terminal and internal amino acid (aa) sequences obtained from the purified 28.5 kDa allergen were used to design primers to amplify a partial cDNA probe from *B. ovis* cDNA by PCR. The amplified probe was radiolabeled and used to screen a *B. ovis* cDNA library. The complete nucleotide sequence of the allergen was determined from the sequences of the positive clones from the library. The full-length cDNA encodes a 255 aa protein including a secretory leader sequence of 26 aas and a mature protein of 229 aas. The encoded protein showed strong homology to several hypothetical proteins of unknown function from diverse species and weak homology with lipid-binding proteins of *Xenopus tropicalis* and *Galleria mellonella*. This is the first allergen to be identified from a louse and it has been designated Bov o 1 in accordance with the criteria of the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee.

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1. Introduction

Hypersensitivity responses to numerous arthropods have been recognised in human beings and animals however the allergens involved have been identified and characterised in only a limited number of species (Hill and DeBoer, 2001; Sousa and Halliwell, 2001; Arlian, 2002). The louse, *Bovicola ovis*, which parasitizes sheep, has been shown to be associated with an allergic dermatitis, commonly referred to as scatter cockle, which devalues the pelts and leather produced from these animals (Heath et al., 1995a,b). The immunological and inflammatory responses of sheep to infestation with this louse show a strong Th-2 bias and many characteristics of immediate hypersensitivity (Pfeffer et al., 2007; Shu et al.,

2008). However, the origin and the nature of the allergens from *B. ovis* have not been described.

Sources of allergens from arthropods include saliva, venom of stinging insects, body secretions, excreta and exoskeleton (Arlian, 2002). In the case of ectoparasites, saliva is a potential source of allergens especially in those species that inject the saliva into the host tissues to facilitate feeding (McDermott et al., 2000; Arlian, 2002). Another potential source of allergens from ectoparasites is the faeces where these are deposited on the skin. In support of the latter is the demonstration that sucking lice excrete immunogenic proteins in their faeces which can elicit specific immunological responses in the host (Volf, 1994). The immunological reaction occurring naturally in the host was thought to occur because the faeces were scratched into the skin by the infested animal. While the allergenicity of these immunogenic proteins was not investigated, the possibility that some may be allergens is suggested by the presence of allergens in the faeces of house dust mites and cockroaches (Tovey et al., 1981; Musmand et al., 1995; Park et al., 2000; Gore and Schal, 2004). Indeed, the faeces of the latter arthropods are regarded as major sources of inhaled allergens for

[☆] Patents covering this molecule and applications using it have been obtained (NZ, 504096; Aus., 2001252799) or applied for. Nucleotide sequence data reported in this paper are available in the GenBank™ database under Accession No. BD404785.

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patients with atopic disease. Further, allergens derived from house dust mites have been shown to cross the skin barrier in human atopic dermatitis which indicates that allergens derived from ectoparasites also may access the host by this route (Maeda et al., 1992; Kapp, 1995). This has been suggested in the case of the parasitic mite *Psoroptes ovis*, the causative agent of sheep scab (Lee et al., 2002; van den Broek and Huntley, 2003). *Psoroptes ovis* is a surface feeder and has been shown to produce a number of allergens with homology to those of house dust mites as well as other factors which are postulated to play a role in the development of the scab lesions in sheep (Kenyon et al., 2003; Lee et al., 2002; Huntley et al., 2004).

Bovicola ovis is an obligate ectoparasite of sheep and the entire lifecycle is spent on the skin and in the wool of the host. This louse is regarded as a surface feeder and the route by which it immunologically sensitises sheep has not been demonstrated (Sinclair et al., 1989). However, it seems likely that the faeces of the louse, which accumulate on the sheep, contain antigens and allergens as demonstrated for house dust mites, *P. ovis*, cockroaches and sucking lice. Further, the demonstration that proliferative responses to crude *B. ovis* antigen were confined to cells from skin-draining lymph nodes of infested lambs suggests that antigenic molecules from the louse penetrate the skin (Bany et al., 1995). In these respects, the sensitisation of the host may be analogous to that of house dust mites and *P. ovis* in the elicitation of atopic dermatitis and sheep scab, respectively. In this paper we describe the identification, purification and partial characterisation of a novel allergen which is abundant in extracts from whole body and the faeces of *B. ovis*. The allergen has previously been referred to as Bo1 but, according to the criteria of the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee, may be designated Bov o 1 (King et al., 1995).

2. Materials and methods

2.1. Preparation of soluble antigens from whole *B. ovis* and its faeces

Live nymph and adult *B. ovis* were collected and soluble antigen extracted as previously described (Bany et al., 1995) except that the ground up lice were suspended in PBS/Pefabloc (PBS containing 1 mM Pefabloc SC, (Roche Diagnostics, Castle Hill, NSW, Australia)). For short-term storage, the soluble antigen was held at 4 °C. For long-term storage, the soluble antigen was mixed 1–1 (v/v) with glycerol (AnalaR®, BDH, Poole, England) and stored at –20 °C. Protein concentrations of the soluble antigen were measured using the BCA Protein Assay (Pierce, Rockford, IL, USA) following precipitation with 5% trichloroacetic acid.

To obtain louse faeces, lice separated from wool and debris were maintained overnight in clean glass petri dishes under conditions of controlled temperature and relative humidity (Hopkins, 1970). Lice were then poured off the dish and dead lice, parts of lice or any other debris on the surface of the dish were removed. The faecal pellets remaining were suspended in 10 ml PBS/Pefabloc and homogenised in a glass homogeniser on ice. This homogenate was then centrifuged and the supernatant was passed through sterile 0.2 µm filters and stored at 4 °C. Protein levels in soluble *B. ovis* faecal antigen preparations were measured by absorbance at 280 nm or by the BCA Protein Assay.

2.2. Isolation of ovine IgE and coupling to affinity chromatography columns

Sera collected from sheep infested with *B. ovis* were screened by ELISA to identify those with higher levels of IgE specifically binding to whole *B. ovis* antigen as described below. IgE was separated from a

selected serum using an immunoaffinity column constructed by coupling the ovine IgE-specific monoclonal antibody (mAb), XB6, to a HiTrap N-hydroxysuccinimide (NHS) activated column (Amersham Bioscience, Uppsala, Sweden) as described by Shaw et al. (1996). The eluates from the IgE-specific affinity column were dialysed against wash buffer (50 mM phosphate buffer, 500 mM NaCl buffer, pH 7.0) and further purified by passing the preparation over affinity columns to which were coupled a non-specific mAb or protein G. Analysis of the resultant preparations by SDS–PAGE under reducing conditions showed bands typical of IgE heavy and light chain at high levels of purity (>90%). Approximately 10 mg of the purified ovine IgE were coupled to 1 ml HiTrap NHS-activated columns as per the manufacturer's recommendations.

2.3. Isolation of native allergens from *B. ovis* using ovine-IgE affinity chromatography

Crude *B. ovis* whole body and faecal antigen preparations were prepared as above except that the diluent was wash buffer. The diluted antigen preparations were loaded onto an ovine IgE immunoaffinity column as described above. The column was then washed with wash buffer, and the native allergens were eluted with 100 mM glycine at pH 3.0. The eluates were returned to neutral pH by adding 1 M Tris, 1.5 M NaCl, pH 8.0 at a ratio of 1–10 (v/v). The allergen eluates were concentrated by ultrafiltration (Microsep™ Centrifugal Concentrators, Pall Filtron Corporation, cut-off 3,000 kDa).

2.4. SDS–PAGE and Western blotting

Protein samples were mixed with SDS sample buffer containing DTT and brought to 100 °C for 4 min. The samples were run on 12% polyacrylamide gels, 0.75 mm thick, and either 13 cm × 16 cm or 8.0 cm × 8.5 cm in dimension (Laemmli, 1970). For Western blots, the gel-separated proteins were electrophoretically transferred to nitrocellulose membranes in Tris–glycine buffer (pH 8.3). Membranes were blocked with Blotto buffer (0.65 M NaCl, 10 mM phosphate buffer, pH 7.2, 0.05% Tween 20) containing 5% skim milk powder and then washed in PBS. A strip containing biotinylated mol. wt markers (Bio-Rad, Hercules, CA, USA) and strips in the regions of the blotted proteins were cut from the nitrocellulose membranes. The mol. wt marker strips were exposed to Streptavidin-horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL, USA). Strips blotted with proteins were reacted with mouse-derived primary antibodies and then with goat anti-mouse γ chain-HRP (Sigma–Aldrich, St. Louis, MD, USA). In both cases the colour reaction was developed with amino-ethyl carbazole (AEC).

2.5. Production of mAbs

BALB/c mice were injected s.c. with crude *B. ovis* faecal antigen (up to 1 mg total protein) mixed 1–1 (v/v) with FCA (Sigma–Aldrich) and boosted i.p. with similar amounts of the antigen in Freund's Incomplete Adjuvant on two occasions. Splenic lymphocytes from one mouse with a high antibody response to *B. ovis* faecal antigen as determined by ELISA were used to produce mAbs essentially as described by Shaw et al. (1996). Supernatants from hybridoma cultures were screened by ELISAs for murine IgG mAb recognising, firstly, *B. ovis* crude faecal antigen and, secondly, IgE affinity-purified native *B. ovis* allergens. Supernatants from a selection of wells positive by both criteria were then reacted in Western blots with crude whole *B. ovis* antigen. The hybridomas in wells selected on all these criteria were then subjected to limiting dilution culture in 96 well plates at mean concentrations of 0.5, one and two cells per well. The hybridomas derived were again screened for IgG recognising crude *B. ovis* faecal antigen. Positive

wells containing single hybridomas were subjected to limiting dilution culture a second time to produce clones. For bulk mAb production, cryopreserved cloned hybridoma aliquots were thawed and expanded by standard culture techniques, cultures were overgrown and the supernatant collected.

2.6. Purification of the Bov o 1 allergen

A selected mAb (designated Bo1-2b) specific for Bov o 1 was purified from bulk culture supernatants using a Protein G affinity chromatography column and was coupled to HiTrap NHS-activated columns in a similar manner to that described above. The latter columns were used to purify Bov o 1 from soluble whole *B. ovis* antigen by the same methodology used to purify ovine IgE.

2.7. Amino acid (aa) sequencing of the Bov o 1 allergen

Native Bov o 1 purified using the Bo1-2b mAb affinity column was subjected to SDS-PAGE under reducing conditions and electroblotted to polyvinylidene fluoride (PVDF) membrane (Problott™, Applied Biosystems) using standard techniques for preparing proteins for sequencing. The PVDF membrane was stained with 0.1% Ponceau S and the major protein band was excised. The membrane pieces were briefly washed with methyl alcohol containing 0.1% triethylamine followed by two washes with methyl alcohol alone. Automated microsequencing was carried out on a gas phase instrument (470AR/120A/920A/610A, Applied Biosystems) and the N-terminal aa sequence was obtained.

The major Bov o 1 protein band was also excised from gels following SDS-PAGE under reducing conditions for in-gel trypsin digests according to the method of Rosenfeld et al. (1992). Peptides eluted from the gel were separated on a Phenomenex Jupiter C18 column (300 angstrom, 5 µm, 2 × 250 mm) attached to a microbore HPLC (PE Biosystems, 140A delivery system and 1000S Diode array detector). Selected peptides were sequenced (PE Biosystems Procise protein sequencer, model 492) using chemicals and methods supplied by the manufacturer.

2.8. RNA isolation and cDNA library construction

Bovicola ovis (100 mg), snap frozen in liquid nitrogen, were ground with a pestle and mortar over liquid nitrogen. Total RNA was isolated from the ground *B. ovis* essentially as described by Frenkel et al. (1989) utilizing two rounds of guanidine-HCl extraction and ethanol precipitation followed by separation with water-saturated phenol:chloroform. The RNA in the aqueous layer was ethanol precipitated, dried and resuspended in 50 µl of double distilled water. A cDNA library was synthesized from the *B. ovis* mRNA using a ZAP-cDNA® Synthesis Kit as recommended by the manufacturer (Stratagene, Cedar Creek, TX, USA). The cDNA was ligated into the bacteriophage lambda Uni-ZAP™ XR vector arms with T4 DNA ligase and packaged with GIGAPACK® II Packaging Extract. The library had a complexity of about 4.45×10^5 and an average insert size of 817 bp (range of 169 bp–2.4 kbp).

2.9. Cloning and characterisation of the coding DNA for Bov o 1

Based on the Bov o 1 aa sequences obtained, an oligonucleotide primer (BoP14A- CATGCTGGATATAATGAAGT(A/T)AA(C/T)CC) was designed to hybridise to Bov o 1 cDNA encoding the amino-terminal region and a second oligonucleotide (BoP14B- TTAACAACTTCA GGAACCTC(A/T)GT(A/G) TC(A/G)AA) was designed to hybridise to Bov o 1 cDNA encoding an internal peptide. The oligonucleotides, designed to hybridise to opposing strands of the cDNA, were used in a PCR to amplify the intervening cDNA derived from *B. ovis* mRNA. The conditions for the PCR reaction were primers at 0.3 µM, dNTPs at

200 µM and 20 ng of template, three cycles of 94 °C for 30 s, 45 °C for 60 s and 72 °C for 60 s; 30 cycles of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 60 s; and then held at 72 °C for 10 min.

Following PCR, an amplified DNA fragment of approximately 390 bp was identified by agarose gel electrophoresis. This fragment was purified using the Wizard® PCR Preps DNA Purification System (Promega, San Luis Obispo, CA, USA) and then radiolabeled with [α -³²P]dCTP using the RTS RadPrime DNA Labelling System (Invitrogen, Carlsbad, CA, USA). The radiolabeled Bov o 1 cDNA probe was used to screen the *B. ovis* cDNA library to identify cDNA clones homologous to the probe essentially as described in Sambrook et al. (1989). Approximately 45,000 phage clones from the library were plated onto agar plates with *Escherichia coli* XL1-Blue MRF' cells and incubated to produce phage plaques. The plaques were replicated onto Hybond-N + nylon membranes (Amersham) and processed for hybridisation with the probe. Positive hybridising clones were plaque purified until they were clonal. The *B. ovis* cDNA inserts from these were amplified by PCR using primers that hybridised to the DNA surrounding the cDNA cloning site and the PCR products were sequenced. The complete coding sequence of Bov o 1 could be discerned from the sequences. From this nucleotide sequence, the aa sequence was predicted using codon preferences recognised for *B. ovis* and the N-terminal and internal aa sequences determined previously. Homology comparisons with known sequences were made using BLASTx on the NCBI database (www.ncbi.nlm.nih.gov/).

2.10. In situ immunohistochemical location of Bov o 1

Bovicola ovis were collected from sheep and were briefly washed in 5% Tween 20 in PBS and then twice in PBS to reduce surface contamination. The lice were then imbedded in optimal cutting temperature compound (Tissue-Tek®, Miles Inc., Elkhart, USA), frozen over liquid nitrogen and stored at –20 °C until used. Sections were cut at 6 µm and were processed and immunohistochemically stained by the indirect peroxidase method described in Pfeffer et al. (1996). The primary mAb was Bo1-2b described above and the secondary antibody was goat anti-mouse γ chain-HRP (Sigma-Aldrich). Control sections were treated identically except that the primary antibody was omitted.

2.11. Experimental animals, intradermal skin testing and blood sampling

The derivation of louse-naïve and -infested lambs and intradermal skin testing in these animals have been previously described (Pfeffer et al., 2007). The skin test solutions in this study were injected in volumes of 0.1 ml over the lateral shoulder regions of the lambs. Native Bov o 1 purified using a Bo1-2b mAb affinity chromatography column was at approximately 6.0 µg protein per ml (determined by absorbance at 280 nm) in PBS. Crude whole *B. ovis* antigen was diluted to 100 µg protein per ml in PBS. Appropriate diluent solutions without antigen were also injected as negative controls. Histamine HCl (1 in 250,000, w/v) in PBS was used in some cases as a positive control. The longest diameter and the diameter perpendicular to this were measured for each skin reaction at 0.5 or 1, 5, 24 and 48 h after injection. The product of the diameters for each skin reaction was taken to represent the area (mm²) of the reactions. Blood samples were taken from the lambs by jugular venipuncture into serum separation tubes (Becton Dickinson, Plymouth, UK), and the sera were collected after centrifugation and stored at –20 °C until assayed. The use of the lambs in these experiments was approved by the AgResearch Ltd., Wallaceville Animal Ethics Committee and the care and maintenance of the animals was in accordance with AgResearch Ltd. guidelines.

2.12. ELISA methodology for detection of *B. ovis*-specific ovine IgE

The ELISA method described by Shaw et al. (1998) were used to detect IgE specific to *B. ovis* antigens in sheep sera. The serum samples were treated with 70% saturated ammonium sulphate solution to reduce levels of competing IgG and the supernatants obtained were diluted to give a final dilution of 1 in 16. The plates were coated with Bo1-2b mAb affinity-purified Bov o 1 or whole *B. ovis* antigen at predetermined optimal dilutions in PBS.

2.13. Statistical analyses

Differences in levels of absorbance for specific IgE in ELISA between the louse-infested and -naïve lambs were tested for statistical significance using the Student's *t*-test. Correlations were examined using the Pearson method. Statistical significance was set at $P \leq 0.05$. The statistical analyses were undertaken using Minitab® (Minitab Pty Ltd., Sydney, Australia).

3. Results

3.1. Characterisation of *B. ovis* allergens isolated by ovine-IgE affinity chromatography

Allergens were purified from crude whole *B. ovis* antigen preparations using IgE purified from a *B. ovis* sensitised lamb and bound to an affinity chromatography column. Intradermal skin tests were performed to examine the responses elicited with the IgE affinity-purified allergens or with crude whole *B. ovis* antigen in either louse-infested or naïve lambs (Fig. 1). The estimated protein concentration of the crude *B. ovis* antigen solution was 100 µg per ml but was not determined for the eluate. Both preparations caused a flare in the infested but not in the naïve lambs and thickening of the skin was more substantial in the former lambs by 1 h after injection. After this time, the responses in the infested lambs to both preparations increased in size, indicative of a late-phase response, while the responses in the naïve lambs decreased to baseline by 24 h. Responses in all lambs to the diluent control

injections were negligible (data not shown). Although the responses to the purified allergens were less than those to the crude *B. ovis* antigen, the results indicated allergic responses to both preparations in the infested lambs.

Eluates of whole *B. ovis* and faecal antigens purified by IgE affinity chromatography contained a prominent protein band at 28.5 kDa M_r on SDS-PAGE under reducing conditions (Fig. 2). Both crude whole *B. ovis* and faecal antigen preparations showed a substantial band corresponding to the 28.5 kDa band in the IgE affinity chromatography eluates which suggested that this protein was abundant both in the louse and its faeces (Figs. 2 and 3). Additional weaker bands were observed in both eluates at 27.8 and 29.8 kDa (Fig. 2). Several higher mol. wt bands also were observed in the IgE eluate of whole *B. ovis* antigen in positions corresponding to bands in the crude *B. ovis* faecal antigen. Evidence that the bands at 42 and 83 kDa are related to the 28.5 kDa protein is presented below.

3.2. mAbs recognising Bov o 1

The supernatants from numerous master wells containing hybridomas derived from the fusion of B cells from a mouse immunised with crude *B. ovis* faecal antigen were demonstrated to contain antibody to the faecal antigen in ELISA. Forty-two of the supernatants showing the highest absorbances in the ELISA were further investigated. All of these supernatants contained IgG that bound to the IgE affinity-purified allergens in ELISA and recognised a 28.5 kDa M_r protein in Western blots with crude

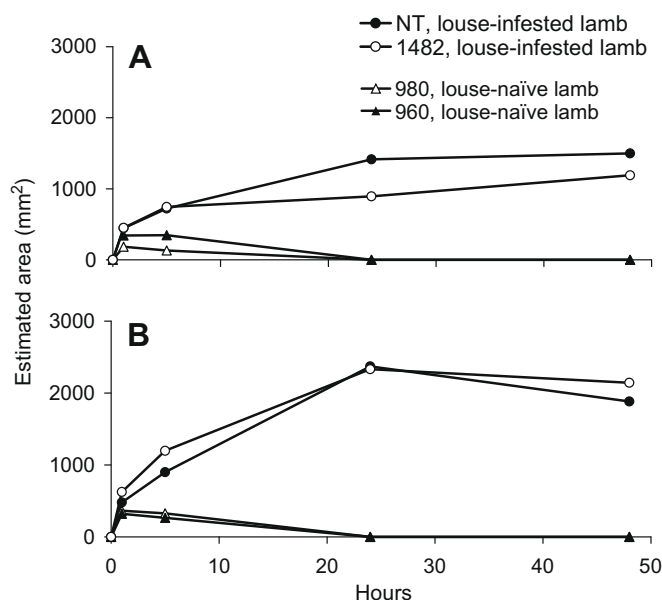


Fig. 1. Intradermal skin test results from two louse-infested and two louse-naïve lambs using (A) allergens purified from crude whole *Bovicola ovis* antigen on an affinity chromatography column constructed with IgE from a lamb sensitised to *B. ovis* and (B) antigen from whole *B. ovis*. NT, no tag.

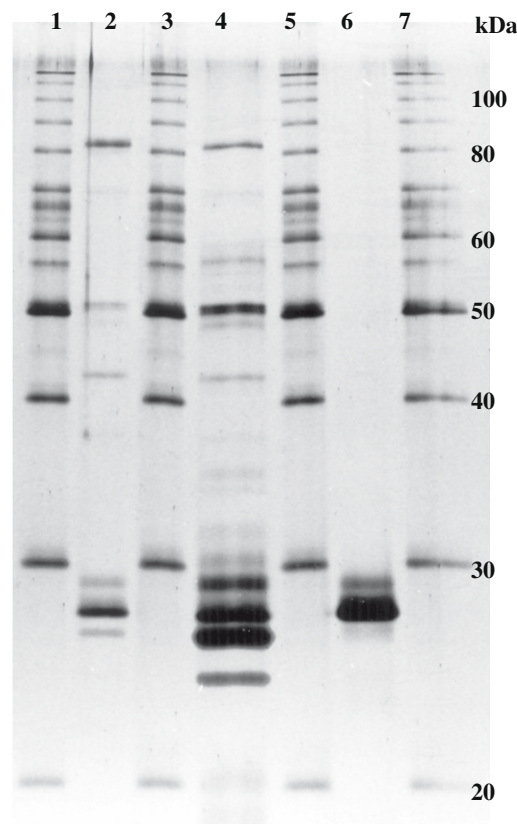


Fig. 2. SDS-PAGE under reducing conditions of eluates of antigens from *Bovicola ovis* obtained from an affinity chromatography column constructed with IgE from a lamb sensitised to *B. ovis* and compared with crude *B. ovis* faecal antigen. Lanes 1, 3, 5 and 7, mol. wt marker; lanes 2 and 6, IgE column eluates of soluble antigen from whole *B. ovis* and *B. ovis* faeces, respectively; lane 4, crude antigen from *B. ovis* faeces. The M_r of the major band in the eluates in lanes 2 and 6 was 28.5 kDa. A corresponding band in the faecal antigen (lane 4) suggests this is an abundant protein in the faeces of the louse. Section of a 13 cm × 16 cm gel, 12% acrylamide, stained by the silver method.

whole *B. ovis* antigen. The hybridomas from 11 of these master wells with supernatants showing strong positive results were cloned by limiting dilution. The mAbs produced recognised the 28.5 kDa band on Western blots of crude *B. ovis* antigen and minor bands were also sometimes observed at approximately 14, 42 and 83 kDa M_r (Fig. 4). One of these hybridoma clones, designated Bo1-2b, was used for bulk mAb production.

3.3. Purification of Bov o 1 by affinity chromatography

The eluates from affinity chromatography columns constructed using the Bo1-2b mAb contained a protein at high levels of purity and having the same SDS–PAGE mobility as the major protein in allergens purified by IgE affinity chromatography from crude *B. ovis* antigens (Fig. 3). Minor bands at 14, 42 and 83 kDa corresponded to those variably observed on Western blots probed with specific mAb and in allergen preparations purified by IgE affinity chromatography from *B. ovis* and its faeces (Figs. 2–4). A major band from the crude whole *B. ovis* antigen was clearly removed following multiple passages over the Bo1-2b mAb affinity column, supporting the conclusion that the allergen, Bov o 1, is abundant in the crude *B. ovis* preparation (Fig. 3).

3.4. Nucleotide and aa sequences of Bov o 1

Purified Bov o 1 protein, and tryptic fragments of this protein, were subjected to aa sequencing (Fig. 5). Degenerate PCR primers

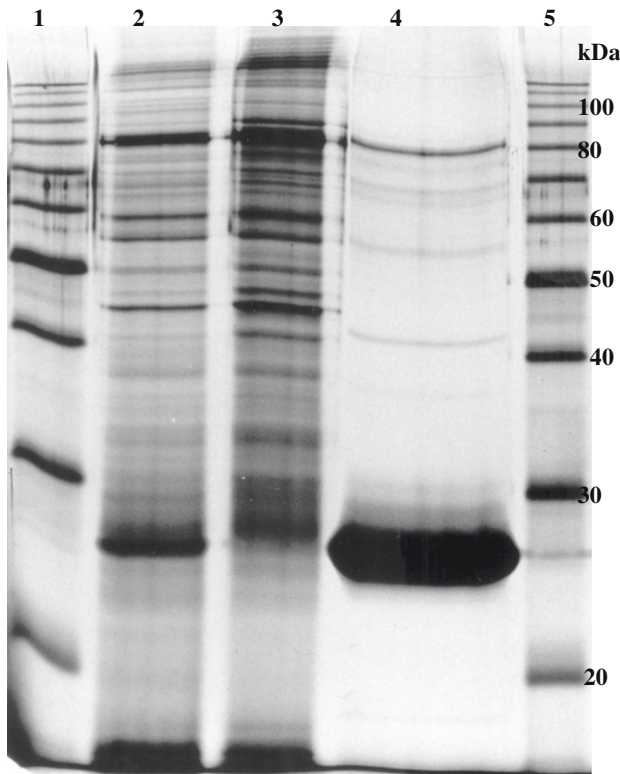


Fig. 3. SDS–PAGE under reducing conditions of crude whole *Bovicola ovis* antigen before and following removal of the allergen Bov o 1 by affinity chromatography using a column constructed with specific monoclonal antibody (mAb) and purified Bov o 1. Lanes 1 and 5, mol. wt marker; lane 2, crude antigen from whole *B. ovis*; lane 3, crude antigen from whole *B. ovis* following extraction of Bov o 1 over a mAb affinity chromatography column; lane 4, Bov o 1 purified on the mAb affinity chromatography column. The Bov o 1 shows a high level of purity with a major band at 28.5 kDa. Several of the higher mol. wt bands may represent multimers of the allergen (see text). A major band obvious at 28.5 kDa in the crude antigen (lane 2) has been removed following extraction over the affinity column (lane 3). 8.0 cm × 8.5 cm gel, 12% acrylamide, stained by the silver method.

were designed based on two of these aa sequences (see Section 2) and used to amplify a portion of the Bov o 1 coding DNA. This amplified fragment was then used as a probe to identify cDNA clones that together encoded the full-length Bov o 1 protein (GenBank™ Accession No. BD404785). The deduced sequence of Bov o 1 predicts a protein of 255 aas with a mol. wt of approximately 30 kDa (Fig. 5). The sequence indicates an amino-terminal secretory leader sequence of 26 aas and a mature protein of 229 aas. The sequences obtained by aa sequencing of Bov o 1 protein were each found within the coding DNA (underlined in Fig. 5). A search of the NCBI database revealed the strongest matches to be hypothetical proteins of unknown function from diverse species including honey bee (XP001121093) and malaria parasites (e.g. XP001347878). The closest homologies to proteins of known function were to apolipoproteins from *Xenopus tropicalis* (NP001088004) and *Galleria mellonella* (wax moth, AAT76806).

3.5. In situ location of Bov o 1 in B. ovis

Immunohistochemical staining with the Bo1-2b mAb demonstrated that Bov o 1 was present throughout the alimentary tract of the louse including the crop, midgut and hindgut with staining concentrated in the two latter locations (Fig. 6). In the midgut, the most intense staining often occurred at the luminal surface of the epithelium and in spherical bodies in close proximity to this.

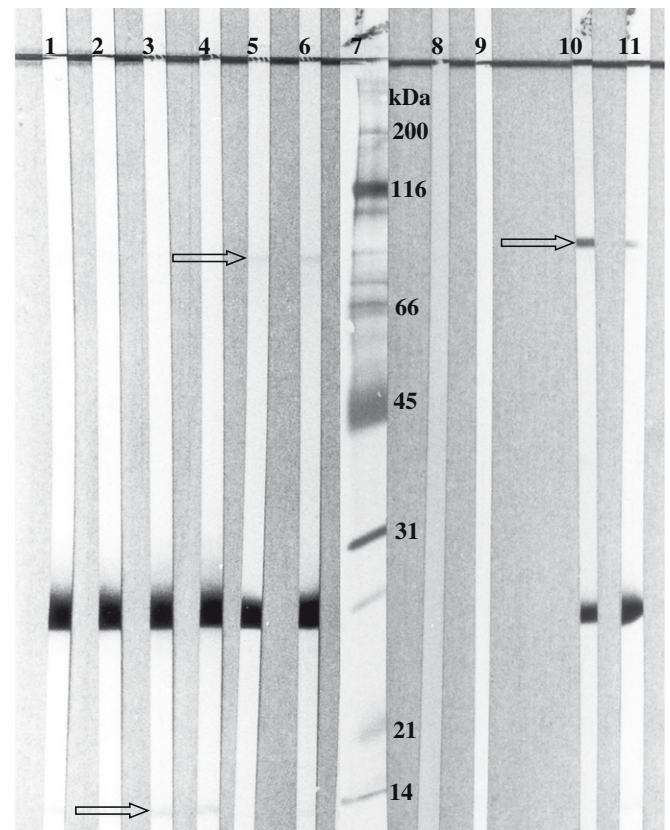


Fig. 4. Western blot of crude whole *Bovicola ovis* antigen reacted with monoclonal antibodies (mAbs) derived from a Balb/c mouse immunised with crude faecal antigen from *B. ovis*. Strips 1–4, mAbs from hybridomas cloned from a single master well; strips 5–6, mAbs from hybridomas cloned from a second master well; strip 7, mol. wt marker; strips 8 and 9, negative controls using normal mouse serum and hybridoma culture medium, respectively; strips 10 and 11, positive controls using serum from a mouse immunized with crude *B. ovis* faecal antigen and a known positive hybridoma culture supernatant, respectively. The major band in all positive preparations has a M_r of 28.5 kDa while minor bands are apparent on some strips at approximately 14 and 83 kDa (arrows).

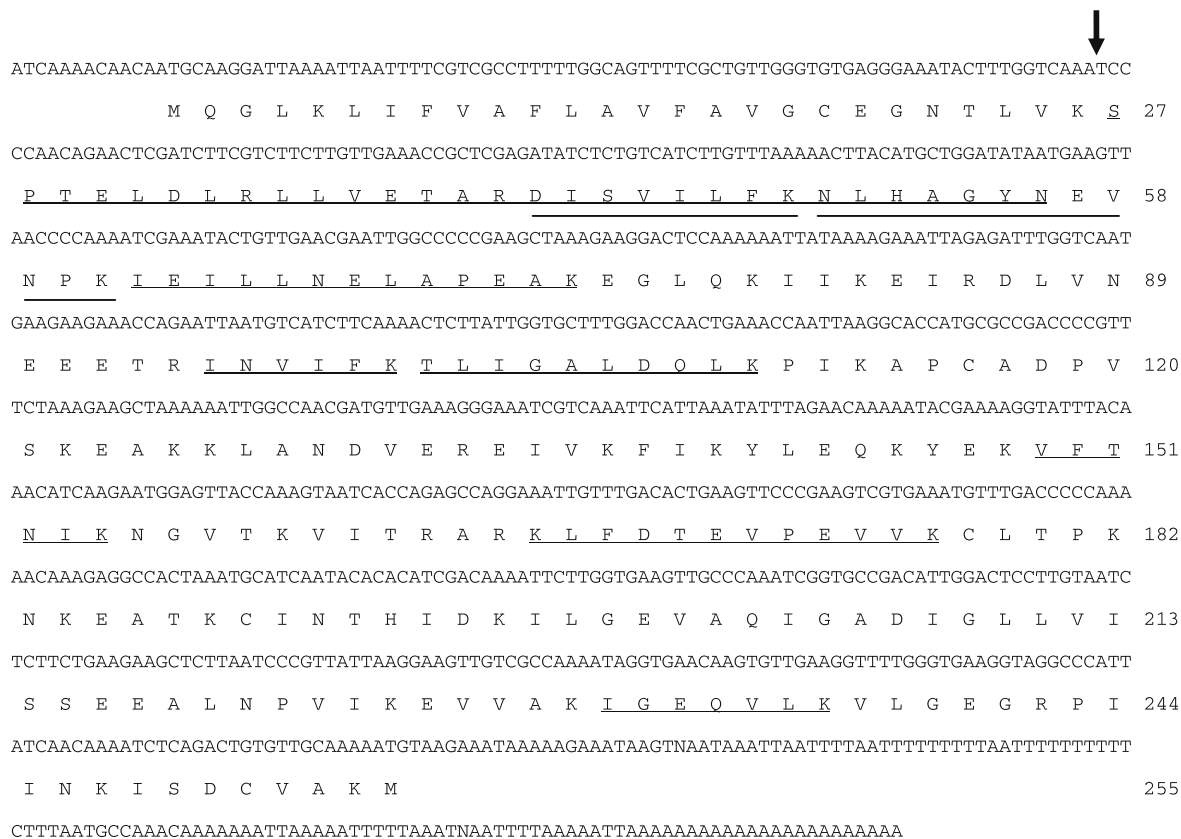


Fig. 5. The nucleotide and deduced amino acid (aa) sequences of the allergen Bov o 1. The arrow indicates the beginning of the mature protein. The underlined aas were determined by N-terminal and internal sequencing of the native protein.

Salivary glands did not stain. The epithelium of the mid- and hind-gut often appeared to be faintly stained and patchy staining was sometimes evident in areas of the body cavity. These latter findings were possibly artefacts due to dispersion of Bov o 1 through leaching, damage to the walls of the alimentary organs or carry-over on the sectioning knife.

3.6. Intradermal skin test responses to purified Bov o 1

Intradermal injection of Bov o 1 purified by Bo1-2b mAb affinity chromatography led to flares and moderate swelling of the skin within 30 min in the louse-infested lambs but little or no response in the naïve lambs (Fig. 7A). Skin swelling persisted in all the infested lambs to 5 h and to 24 h in two of them. The responses to the purified Bov o 1 were substantial but were of lesser magnitude and shorter duration than observed following injection of crude *B. ovis* antigen (Fig. 7B). Estimated protein concentrations of the purified allergen and the crude antigen solutions were 6.0 and 100 µg per ml, respectively. Responses to the diluent and histamine control injections were similar in the two groups of lambs (data not shown).

3.7. Detection of *B. ovis*- and Bov o 1-specific IgE in lamb sera

Mean (SEM) absorbances for specific IgE in the infested and naïve lambs to crude *B. ovis* antigen were 0.053 (0.009) and 0.011 (0.002), respectively and to purified Bov o 1 were 0.031 (0.006) and 0.010 (0.002), respectively (Fig. 8). For both the crude *B. ovis* antigen and Bov o 1, the mean absorbances in the infested lambs were significantly higher than in the naïve lambs ($P \leq 0.0001$ and 0.0025, respectively). Sixty-three percent (19/30) of the infested

lambs showed *B. ovis*-specific IgE absorbances greater than the mean plus 2 SD of absorbances obtained using sera from louse-naïve lambs. The sera of 50% (15/30) of the louse-infested lambs showed elevated IgE absorbance to purified Bov o 1 based on the same criteria. In 13 of the serum samples, elevated IgE responses to both antigen preparations were detected. However, within the infested lambs, only moderate correlation between the IgE responses to both antigen preparations was observed ($r = 0.49$, $P \leq 0.006$).

4. Discussion

A 28.5 kDa band on SDS-PAGE represented the major protein purified from soluble extracts of *B. ovis* faeces and whole lice by immunoaffinity chromatography using IgE from a louse-sensitised lamb (Fig. 2). Immunisation of mice with *B. ovis* faeces for mAb production led to the production of numerous hybridoma clones producing antibodies against an immunodominant molecule of the same M_r (Fig. 4). One of these mAb, Bo1-2b, was used to affinity purify the molecule, designated Bov o 1, from soluble extracts of crude *B. ovis* antigen (Fig. 3). Western blots using this mAb indicated that additional minor bands of 14, 42 and 83 kDa may be related to the 28.5 kDa protein (Figs. 2–4). The 14 kDa band may arise from cleavage of the 28.5 kDa molecule while the higher mol. wt bands may represent polymers of these cleaved or intact molecules, for example, 3×14 or $28.5 + 14 \approx 42$ kDa and 6×14 or $3 \times 28.5 \approx 83$ kDa.

The pattern of immunohistochemical staining for Bov o 1 in the louse was suggestive of production in midgut epithelium (Fig. 6) and accords with the origin of antigens and allergens previously identified from the faeces of sucking lice, mites and cockroaches

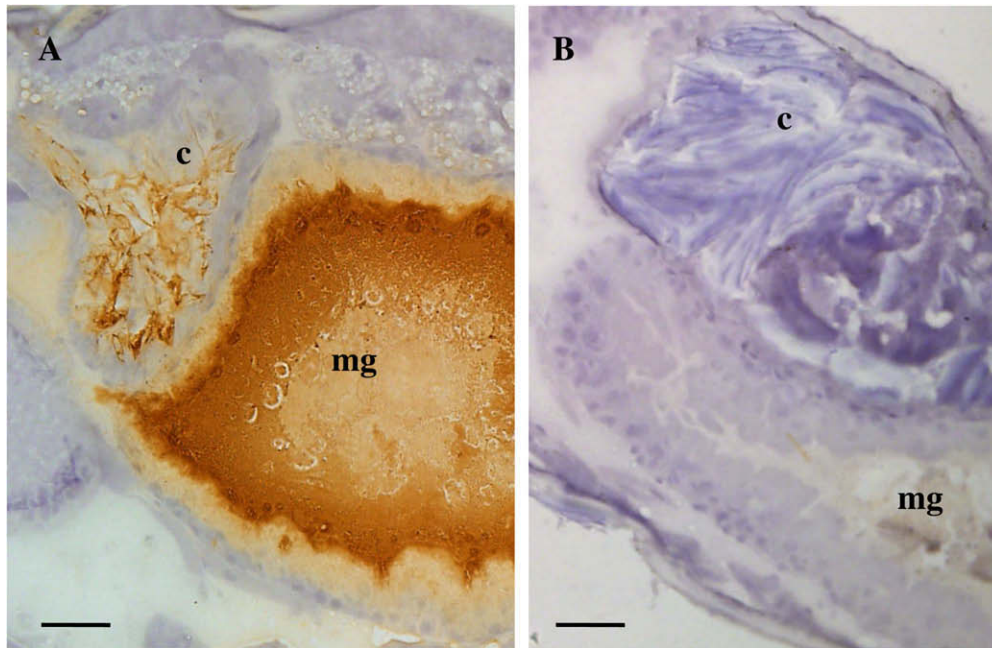


Fig. 6. Transverse sections of *Bovicola ovis*. (A) Indirect peroxidase immunohistochemical stain for the allergen Bov o 1 using the Bo1-2b monoclonal antibody (mAb) as primary antibody. (B) Control section in which the Bo1-2 mAb was omitted during immunohistochemical staining. mg, midgut; c, crop; bar = 25 µm, counter-stained with haematoxylin.

(Volf, 1994; Mumcuoglu et al., 1996; Jeong et al., 2002; Wu et al., 2007). The presence of Bov o 1 in the crop of the louse may be due to contamination of the louse's diet with its faeces which accumulate on the skin and in the wool where it browses. However, this possible contamination of the alimentary tract with ingested faecal antigens, which would not be expected in sucking lice, prevents a definitive statement on the site of production of Bov o 1 in *B. ovis* without further investigation.

In ELISAs using mAb affinity-purified Bov o 1, binding by serum IgE was significantly higher in louse-infested compared with louse-naïve lambs and the elevation of the IgE response in at least 50% of infested lambs indicated that the allergen commonly led to allergic sensitisation (Fig. 8). However, while all lambs in the infested group would have been exposed to the louse, individual levels of infestation and other measures of sensitisation to the louse were not determined. Thus the percentage of lambs actually sensitised to the louse may have been less than the number exposed and the percentage of sensitised lambs showing serum IgE responses to Bov o 1 may have been higher than 50%. It is noteworthy, however, that levels of absorbance in the IgE ELISA were generally low. This low systemic IgE response to Bov o 1 in sheep, and *B. ovis* allergens generally, contrasts with skin test responsiveness suggesting that specific IgE production is largely confined to the skin compartment or the IgE is sequestered there. Low ELISA absorbances have also been reported for serum IgE responses to *P. ovis* in sheep (van den Broek et al., 2000). On intradermal skin testing both the IgE affinity-purified allergens and the mAb affinity-purified Bov o 1 led to early flares and swelling in louse-infested lambs which is indicative of immediate hypersensitivity (Figs. 1 and 7). Further, persistent skin swelling at 5 h following injection and extending to 24 or 48 h in most of the infested lambs indicated late-phase responses. Because the crude antigen may contain numerous unidentified allergens, the total allergen challenge administered with this preparation could not be quantified. Thus the lesser responses observed with the purified louse allergens may have been due to overall lower total allergen challenge. Nonetheless, both the immediate and late-phase responses observed with the purified allergens were typical of those observed in allergic patients following

intradermal challenge with appropriate allergens and are regarded as being dependent on the presence of allergen-specific IgE (Solley et al., 1976). Altogether, these data indicate that the 28.5 kDa protein represents a major allergen from *B. ovis* according to the criteria of the WHO/IUIS Allergen Nomenclature Subcommittee (King et al., 1995).

The homology of the Bov o 1 sequence with several lipid-binding proteins is intriguing because an apolipophorin/vitellogenin is an immunodominant allergen of mites (Epton et al., 1999; Kawamoto et al., 2002; Huntley et al., 2004). However, substantial homology specifically with the mite allergens was not observed and these allergens have higher mol. wts on SDS-PAGE (177 kDa for Der p 14 and 180 kDa for the homologue in *P. ovis*) compared with the dominant band observed for Bov o 1

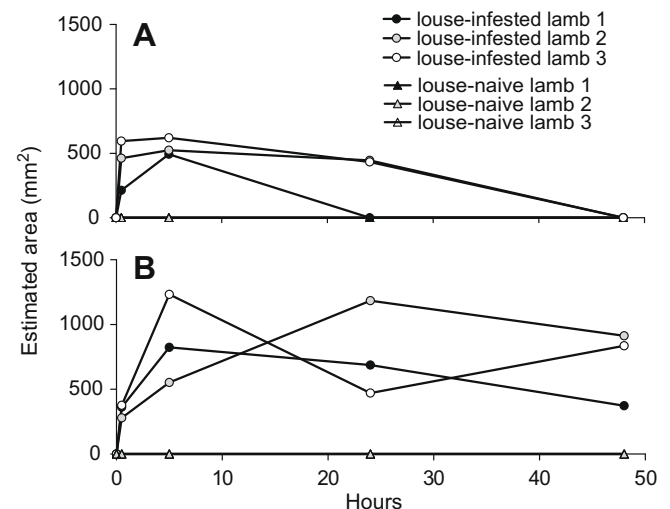


Fig. 7. Intradermal skin test results from three louse-infested and three louse-naïve lambs using (A) the allergen Bov o 1 purified by monoclonal antibody affinity chromatography and (B) crude antigen from whole *Bovicola ovis*.

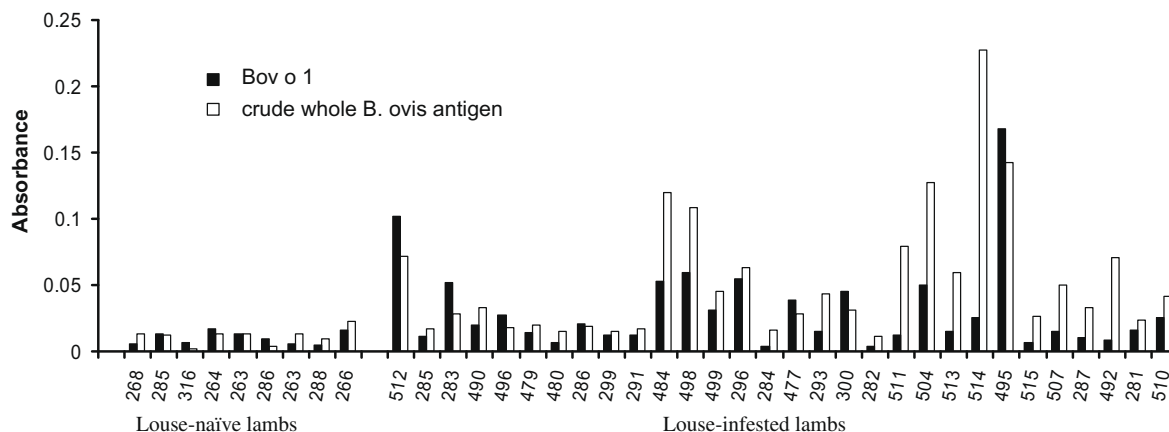


Fig. 8. Absorbances in ELISA for IgE specific for the allergen Bov o 1 and crude whole *Bovicola ovis* antigen in the sera of nine louse-naïve and 30 louse-infested lambs. Lamb tag numbers are shown below the x-axis.

(28.5 kDa). Smaller mol. wt apolipoproteins have been reported in several insects (Ryan and van der Horst, 2000). Functional characterisation of Bov o 1 remains to be undertaken and the possibility that the molecule may be lipid-binding is one avenue of investigation.

The present study has identified and partially characterised a novel allergen, Bov o 1, from the ectoparasitic louse *B. ovis*. Bov o 1 appears to be a major allergen and likely has a role in eliciting the skin disease, scatter cockle, in lambs infested with the louse. Given the abundance of this allergen in louse faeces, it is probable that the faeces accumulated on the skin surface are the major source of this allergen leading to sensitisation of the sheep. Further research is required to determine the biological function of this molecule in the louse and whether such function influences the pathogenesis of the skin disease in sheep and the allergenicity of the molecule. Further work is also required to determine whether other allergens are produced by the louse and are involved in eliciting scatter cockle in sheep.

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