

Identification of an antigen from the sheep scab mite, *Psoroptes ovis*, homologous with house dust mite group I allergens

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SUMMARY

Infestation of sheep with the ectoparasitic mite Psoroptes ovis, results in a severe allergic dermatitis. Currently, little is known about the allergens/antigens that stimulate the allergic response. We have isolated an 836-bp cDNA from a P. ovis cDNA library which displays strong homology to cysteine proteases and, in particular, to the group I house dust mite allergens Der p 1, Der f 1 and Eur m 1. The cDNA was expressed in Escherichia coli, fused to a hexahistidine tag and the recombinant protein (Pso o 1) purified using a nickel-affinity column. The recombinant Pso o 1 was tested for recognition by immunoglobulin (Ig)G and IgE in serum from P. ovis naïve and P. ovis infested sheep. Using Western blots, both classes of antibody to Pso o 1 were detected in postinfestation serum. In enzyme-linked immunosorbent assays, a pronounced IgG-antibody response to Pso o 1 was detected in five of five sheep 3 weeks postinfestation. The IgE-antibody response to whole mite extract was poor in four of five animals. However, a marked IgE response occurred in the fifth animal, and IgE anti Pso o 1 was detected in the serum.

Keywords *Psoroptes ovis*, sheep-arthropoda, sheep scab, cysteine protease, allergen, antigen

INTRODUCTION

Sheep scab is a highly contagious disease caused by the nonburrowing, obligate ectoparasitic mite *Psoroptes ovis*. Infestation is characterized by exuberant scab formation and intense pruritis, leading to fleece loss and severe debilitation. The disease is a serious animal welfare issue and an important cause of economic loss to the sheep industry (1,2).

The clinical signs of sheep scab result from a severe allergic dermatitis (3), but little is known about the allergens responsible for the hypersensitive reaction. However, the allergens from the dust mites such as *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Euroglyphus maynei* and *Lepidoglyphus destructor* have been extensively studied and characterized (4,5). Allergens from these mites are contributing factors in allergic disorders such as perennial rhinitis, asthma and atopic dermatitis (5–8). Pruett *et al.* (9) recently reported the identification of a 16-kDa polypeptide from *P. ovis* that elicits an immediate hypersensitive response in cattle and displays sequence homology to the group II mite allergens *Lep d 2* (10) and *Der f 2* (11) from *L. destructor* and *D. farinae*, respectively. This leads us to speculate on the existence of more *P. ovis* allergens or antigens homologous to the house dust mite allergens. By analogy with the house dust mite, it is expected that the *P. ovis* proteins responsible for the pathology of sheep scab are located in the gut and are sequestered in the faecal pellets (12,13).

Approximately 90% of dust mite allergic patients have immunoglobulin (Ig)E directed against a group of 25–30 kDa protein allergens that display significant homology to the papain-superfamily of cysteine proteases (14). These are collectively known as the group I allergens and include *Der p 1*, *Der f 1* and *Eur m 1* from *D. pteronyssinus*, *D. farinae* and *E. maynei* (15–17). *Der p 1* is currently the best characterized. The mature *Der p 1* protein has 222 amino acid residues, but is synthesized as a precursor of 320 amino acids, including an 18-amino acid signal peptide and 80-amino acid *N*-terminal pro-sequence (16). It is thought that the enzyme is activated

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by removal of the pro-region by autocatalytic processing. The protease activity of *Der p 1* may enhance its allergenicity (4,18).

We describe the cloning of a *P. ovis* cDNA homologous to the house dust mite group I allergens and expression in *Escherichia coli*. The purified recombinant protein was used in Western blots and enzyme-linked immunosorbent assays (ELISAs) to investigate whether IgG and IgE antibodies to native *P. ovis* cysteine protease are generated in sheep experimentally infested with *P. ovis*.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) amplification of a *P. ovis* partial cDNA encoding a putative cysteine protease

A *P. ovis* cDNA library (19) was screened by PCR using primers that were designed according to the nucleotide sequence of the house dust mite cysteine protease *Der p 1* (GenBank accession no. U11695). The sequence of the sense primer was 5'-TTGGCCATCGCCTCATTGTTGG-3' and the antisense, 5'-TTACCAACACGTGTATAAACACCTGG-3'. The amplification reaction mixture (100 µl) contained 20 mM Tris-HCl buffer, pH 8.4, 50 mM KCl, 5 mM MgCl₂, 0.2 mM dNTPs, 20 pmol of each primer, 2 U Taq DNA polymerase (Life Technologies, Paisley, UK) and 1 µl *P. ovis* cDNA library. PCR was performed in a Hybaid Omnigene thermal cycler (Hybaid, Ashford, UK) with an initial denaturation step of 95°C for 10 min, followed by 30 cycles of amplification, each consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. A final extension cycle was performed at 72°C for 10 min. An approximately 0.85 kb PCR product was purified with a GeneClean spin kit (BIO 101 Inc., Vista, CA, USA) and ligated into the shuttle vector pGEM T-Easy (Promega, Southampton, UK) according to the manufacturer's instructions. Potential positive recombinant clones were identified by PCR with the sense and antisense primers and the identity of the clones confirmed by sequence analysis using the BLAST facility at the NCBI database. An 836-bp insert that exhibited homology to *Der p 1* was designated clone *Pso o 1*.

Preparation of the pBAD/*Pso o 1* expression construct

To subclone the *Pso o 1* insert in-frame into the expression vector pBAD/gIII A (Life Technologies, Groningen, the Netherlands), *Pso o 1* was amplified by PCR using the sense primer 5'-CCATGGAAATTCGATTGCC-3' and antisense primer 5'-TCTAGATTGGCAGCAAATAACC-3' to introduce *NcoI* and *XbaI* restriction sites at the 5' and 3' ends of the insert, respectively. The amplification reaction mixture (100 µl) contained 20 mM Tris-HCl buffer, pH 8.4, 50 mM KCl, 5 mM MgCl₂, 0.2 mM dNTPs, 100 pmol of each

primer, 1 U Taq DNA polymerase and 0.2 ng template DNA. PCR was performed in a Hybaid Omnigene thermal cycler with an initial denaturation step of 95°C for 5 min, followed by 30 cycles of amplification, each consisting of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min. A final extension cycle was performed at 72°C for 10 min.

The resulting PCR product was purified using a Strata-Prep PCR purification kit (Stratagene, La Jolla, CA, USA) and ligated into the shuttle vector pCR-script Amp SK(+) (Stratagene) according to the manufacturer's instructions. Recombinant clones were screened by restriction enzyme analysis. Positive clones were digested with *NcoI* (Life Technologies) and *XbaI* (Promega) and the modified *Pso o 1* insert subcloned into *NcoI*-*XbaI* digested expression vector pBAD/gIII A. Ligation products were transformed into LMG194 *E. coli* by standard methods. Recombinant clones were identified by restriction digestion and sequenced to confirm an in-frame fusion at the 5' and 3' ends and the fidelity of the PCR reactions.

Expression and purification of recombinant protein (*Pso o 1*)

One millilitre of an overnight culture was used to inoculate 100 ml RM medium containing glucose and 50 µg/ml ampicillin and grown at 37°C to an OD₆₀₀ of 0.5. Expression of the recombinant protein was induced by the addition of 0.2% arabinose and the culture incubated at 37°C for a further 4 h. Cells were harvested by centrifugation and the pellet resuspended in 20 ml phosphate-buffered saline (PBS: 8.4 mM Na₂HPO₄, 1.9 mM NaH₂PO₄, pH 7.4). The cell suspension was sonicated on ice using a 1-cm diameter probe (four separate bursts of 1 min, with an interval of 1 min on ice) and the insoluble fraction was pelleted by centrifugation (2500 r.p.m., 30 min, Jouan, Tring, UK, centrifuge).

Recombinant *Pso o 1* protein was purified using Ni-NTA resin (Qiagen, Crawley, UK) under denaturing conditions. The supernatant was discarded and the pellet resuspended in 5 ml lysis buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0). The sample was agitated at room temperature for 60–90 min, before centrifugation at 13 000 r.p.m. for 10 min to pellet the cellular debris. The supernatant was retained and mixed with 0.4 ml of 50% Ni-NTA slurry and the lysate-resin mix agitated gently for 2 h, at room temperature, by rocking or shaking. This mix was loaded into an empty 5 ml column that was allowed to empty by gravity flow. The flow-through was collected and recycled through the column twice. The column was then washed sequentially with 10 × 1 ml aliquots of wash buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 6.3) and the recombinant protein specifically eluted in 4 × 0.5 ml aliquots of elution buffer 1 (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 5.9) and 8 × 0.5 ml aliquots elution buffer 2 (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris HCl, pH 4.5).

Nucleotide sequencing

Plasmid mini-preps (Promega) were used as template for sequencing. pGEM T-Easy and pCR-script clones were sequenced using M13-20 and M13Rev primers and pBAD/gIII A clones were sequenced using the pBAD Forward primer (Life Technologies). The complete sequence of *Pso o* 1 was obtained by primer-walking. Sequencing reactions were performed in a LEP scientific PREM thermal cycler using Amplitaq DNA polymerase FS dye terminator cycle sequencing (Applied Biosystems, Foster City, CA, USA) and the reaction products were analysed on an ABI-prism 373 A automated DNA sequencer (Applied Biosystems). Alternatively, samples were sequenced by the Sequencing Facility, Department of Biochemistry, University of Oxford, Oxford, UK.

SDS-PAGE and Western Immunoblot analysis

Western blots using anti-His (C-terminal) antibody

SDS-PAGE was performed by standard methods (20) using a Bio-Rad (Herts, UK) Mini protean II electrophoresis system. Samples were heat denatured at 95°C in 4 × loading buffer (250 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 30% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 2% (v/v) bromophenol blue) and a 10-μl aliquot of each sample applied to a 3% (w/v) stacking, 12% (w/v) resolving polyacrylamide gel. Protein bands were visualized with Coomassie brilliant blue (21).

For the Western blots, 10 μl sample was loaded per lane and electrophoretic transfer onto Hybond-P membrane was performed by standard methods (22) using the Bio-Rad mini blot system. After incubation in blocking buffer [5% (w/v) dried skimmed-milk powder and 0.1% (v/v) Tween-20 in PBS], the filter was probed with mouse monoclonal anti-His (C-terminal) antibody (Novagen, Madison, WI, USA) diluted 1 : 3000 in blocking buffer (for 1 h at room temperature or overnight at 4°C), followed by peroxidase conjugated goat anti-mouse IgG (Sigma, Poole, UK) diluted 1 : 5000 in blocking buffer for 1 h at room temperature. The filter was washed several times with PBS and PBS-Tween following each incubation. Specific antibody binding was detected using the Enhanced Chemiluminescence Plus system (Amersham, Bucks, UK).

SDS-PAGE and Western blotting with isotype-specific antibodies

Proteins in crude extracts of *E. coli* containing *Pso o* 1 and whole *P. ovis* antigen, or preparations of purified *Pso o* 1, were separated by SDS-PAGE under nonreducing conditions employing 4%–12% (w/v) gradient NuPage Bis-Tris gels, as described by the manufacturers (Novex, San Diego, CA, USA). Samples were subjected to 200 V constant current

for 40 min, before the separated proteins were transferred to nitrocellulose membranes at 30 V constant current for 1 h, as described by the manufacturer (Novex). The nitrocellulose membranes were blocked by preincubation in PBS/Tween 80 (PBS/T80) for 30 min at room temperature, before being incubated for 1 h with pooled sera collected from five animals infected 12 weeks previously with *P. ovis* (diluted 1 : 10 in PBS/T80), or a similar preparation of sera from the same animals collected prior to infection. Membranes were washed six times in PBS/T80, and then incubated for 1 h sequentially with monoclonal anti-ovine IgG (VPM6) (23), or anti-ovine IgE (2F1) (24) (diluted 1 : 1000 in PBS/T80), biotinylated sheep antimouse immunoglobulin (diluted 1 : 1000 in PBS/T80), and a 30-min incubation with Streptavidin-HRP (diluted 1 : 2000 in PBS/T80). Each incubation was separated by six washes in PBS/T80 and after the final wash, peroxidase activity was revealed for (i) IgE antibody by chemiluminescence detection using ECL reagent (Amersham Life Sciences, Bucks, UK) as described by the manufacturer, or (ii) IgG antibody with di amino-benzidine (DAB)/urea/H₂O₂ substrate (Sigma Ltd, Poole, UK). Protein molecular weight markers were employed (SeeBlue, Plus2 Pre-stained Standards, Novex) to estimate the molecular weight of reactive bands. Controls consisted of blots incubated with the omission of either primary serum, monoclonal antibody or biotinylated secondary antibody.

ELISA

ELISA assay plates (M129B, Dynex Technologies, Ashford, Middlesex, UK) were incubated for 1 h at room temperature with purified *Pso o* 1 (50 μl/well) at a concentration of 1 μg/ml in carbonate/bicarbonate buffer pH 9.6. Wells were washed twice with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween20), before adding 50 μl of a 1 : 10 dilution of sera in PBS containing 0.5% (v/v) Tween 80 and 0.5 M NaCl (PBS/T80) and incubating for 1 h. The sera assayed were collected from a previous study (3), where blood was taken at intervals 0–12 weeks following a primary infection with 25 ovigerous female mites, and sera stored at –20°C. After washing the wells six times in PBS/Tween 20, plates were sequentially incubated for 1 h with 50 μl of a 1 : 2000 dilution of either monoclonal antibody to ovine IgG (VPM6) or ovine IgE clone 2F1 in PBS/T80, biotinylated goat anti-mouse immunoglobulin conjugate (Dako, Ely, UK) and 50 μl Streptavidin-HRP (Dako). Each incubation was for 1 h at room temperature and plates were washed six times in PBS/Tween 20 after each incubation. Colour reaction was developed by incubation with 50 μl 3,3',5,5'-tetramethylbenzidine substrate for 30 min at room temperature and stopped by the addition of 50 μl 0.18 M H₂SO₄ (LabSystems Life Sciences International, Hants, UK) (25). In order to

compare results between assays, aliquots of a standard pool of sera, collected from animals previously infected with *P. ovis* and stored at -20°C , were included on each plate and the absorbance at 450 nm (OD) of unknown samples were adjusted relative to this. In practice, between-test variability was minimal. Controls consisted of (i) performing the reactions on uncoated wells and (ii) omitting the primary sera. The results with *Pso o 1* were compared with OD readings obtained for antibodies against *Psoroptes cuniculi* whole-mite antigen from sera collected from five rats in a previous study, and assayed for IgG and IgE antibody as described above and previously (3).

RESULTS

Sequence analysis of clone *Pso o 1*

The PCR screen of the *P. ovis* cDNA library generated several products ranging from approximately 150 bp to 850 bp. The 850 bp product was the most intense and, based on the sequence of the house dust mite group I allergens, was the correct size. This product was cloned, sequenced and designated *Pso o 1*.

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TTGGCCATCGCCTCATTGTTGGTGTGAGCGTTGTTTACGCTTACCCATCAGAAATCAGA 60
L A I A S L L V L S V V Y A Y P S E I R

ACATCGAAGAATTCAAAAAGCATTCACAAACACTATGTAACACCTGAAGCAGAAACAA 120
T F E E F K K A F N K H Y V T P E A E Q

GAAGCTCGTCAAAATTTCTTAGCTTCATTGGAACACATTGAAAAGCTGGAAGAGTGC 180
E A R Q N F L A S L E H I E K A G K G R

ATCAATCAATCTCAGATATGTCATTGGAAGAAATCAAAACCAATATTTGTGAGTGATA 240
I N Q F S D M S L E E F K N Q Y L M S D

CAAGCATACGAAGCTTTAAAAAAGAAATTTGATTAGATGCTGGAGCTCAAGCTTGCCAA 300
Q A Y E A L K K E F D L D A G A Q A C Q

ATCGGCGCCGTAACATTCCAATGAAATTTGATTACGTGCTTTGGGTTATGTAACAAA 360
I G A V N I P N E I D L R A L G Y V T K

ATCAAGAATCAAGTTGCCTGTGGTTCATGCTGGGCTTTCTGTGTTGCTACAGTCGAA 420
I K N Q V A C G S C W A F S G V A T V E

TCAAAATATTATATACAGATAATGTATCATAGATCTTTCTGAACAGAATTTGGTTGAC 480
S N Y L S Y D N V S L D L S E Q E L V D

TGTGCTTCACAAACGGTTGCGGTGGTGATACAGTTCTAAACGGTTTACGTTATATTCAA 540
C A S Q H G C G G D T V L N G L R Y I Q

AAGAATGGTGTGGTTGAAGACAAAGCTATCCATACAAAGCTCGCGAAGGACGATGCCAA 600
K N G V V E E Q S Y P Y K A R E G R C Q

AGACCAATGCTAAACGATACGGTATCAAGATTTATGTCAAATTTATCCACCAACGGT 660
R P N A K R Y G I K D L C Q I Y P P N G

GATAAAATCCGTACCTATCTTGAACAAAACAGCAGCCCTTTCAGTCATCATTGGTATC 720
D K I R T Y L A T K Q A A L S V I I G I

CGAGATTTAGATTCTTTCCGTCATTATGATGGCCGAACAATTTGCAAGTGATAATGGT 780
R D L D S F R H Y D G R T I L Q S D N G

GGCAACGAAATTTCCATGCAATCAATATTGTTGGTTACGGTTATTTTGCTGCCAAC 836
G K R N F H A I N I V G Y G Y F A A N

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Figure 1 Nucleotide and deduced amino acid sequence of *Pso o 1*. The nucleotide residues of the cDNA are numbered at the end of each row. The PCR primers used to amplify the cDNA are underlined. These sequence data are available from the NCBI database under accession number AF495854.

The cDNA sequence and deduced amino acid sequence of *Pso o 1* is shown in Figure 1. The insert corresponds to a 279-amino acid polypeptide with a calculated molecular mass of 31.2 kDa. A homology search against current databases revealed that the sequence exhibits homology with cysteine proteases and significant homology to the sequences of the mite cysteine proteases *Der p 1*, *Der f 1* and *Eur m 1* (Figure 2). The deduced amino acid sequence of *Pso o 1* displays 54% identity and 69% similarity (i.e. including conserved amino acid substitutions) to *Der f 1*, 53% identity and 66% similarity to *Der p 1* and 53% identity and 68% similarity to *Eur m 1*.

Expression and purification of recombinant *Pso o 1*

Recombinant *Pso o 1* was expressed in LMG194 *E. coli*, fused to C-terminal hexahistidine tag and c-myc epitope to facilitate purification and detection. SDS-PAGE analysis indicated that a predicted 36 kDa protein was detected in arabinose-induced cultures and in insoluble inclusion bodies following sonication. A single, discrete band at 36 kDa, corresponding to the protein band visualized by Coomassie staining, was detected by Western blot analysis with anti-His (C-terminal) antibody, confirming the expression of recombinant *Pso o 1*.

The recombinant protein was purified by Ni^{2+} -affinity chromatography under denaturing conditions from the insoluble fraction of *E. coli* expressing the pBAD/*Pso o 1* construct. Analysis of fractions eluted from the column by SDS-PAGE indicated a major protein band of apparent molecular mass 36 kDa (Figure 3). This was identified as the recombinant *Pso o 1* using immunoblotting with anti-His (C-terminal) antibody.

Western blots

Western blot analysis of whole mite antigen, and crude and purified preparations of *Pso o 1* are shown in Figures 4 and 5. When membranes were probed with sera from previously infected animals for IgG antibody, several bands were observed in both the whole *P. ovis* extract, and in the crude extract of *E. coli* expressing *Pso o 1*. However, a prominent band was observed at a molecular mass of 36 kDa in the *E. coli* extract, and this corresponded to the strong reactive band for purified *Pso o 1* (Figure 4). Similarly, numerous IgE reactive bands were observed following the chemiluminescence reaction in blots probed with immune sera in tracks containing crude *P. ovis* antigen. In crude extracts of *E. coli* expressing *Pso o 1*, three reactive bands were observed. A particularly prominent band was observed at an approximate molecular mass of 36 kDa, and a similar but less intense band was demonstrated in the lane containing

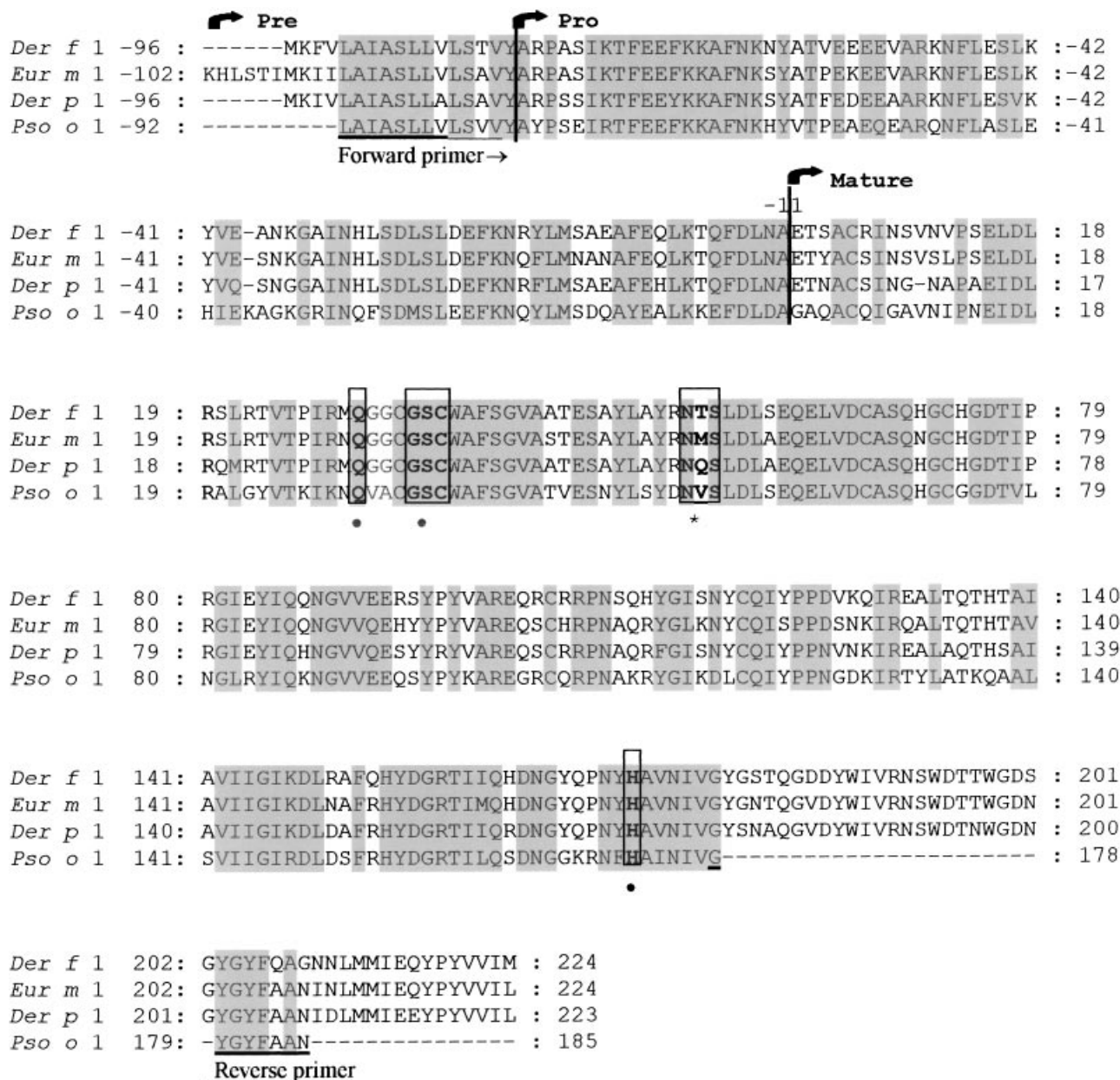


Figure 2 Multiple sequence alignment of the mite group one allergens *Der p 1*, *Der f 1* and *Eur m 1* with *Pso o 1*. Identical or similar amino acids within the four sequences are highlighted in grey. Amino acids that are conserved cysteine protease active site residues are in **bold**, boxed and indicated by '●'. A potential *N*-glycosylation site is marked in **bold**, boxed and indicated by '*'. The starting positions of the pre and propeptide as well as the mature peptide are indicated by the arrows. For consistency, the *Pso o 1* sequence has been numbered using the same convention as the other sequences, although it may not be processed in the same way as the other peptides. The amino acids coded by the PCR primers used to clone *Pso o 1* are underlined.

purified *Pso o 1* (Figure 5). For both the IgG and IgE blots, only faint bands were visible in the *P. ovis* antigen at an equivalent molecular weight to the *Pso o 1*. In control blots for IgG and IgE antibody reacted with naïve (noninfected) sera, little or no reactive bands were observed (Figures 4 and 5).

ELISA

In order to investigate further the putative antibody response to *Pso o 1*, an ELISA was performed to investigate the generation of IgG and IgE anti-*Pso o 1* during a primary infection of *P. ovis*. The results for IgG antibody to *Pso o 1*

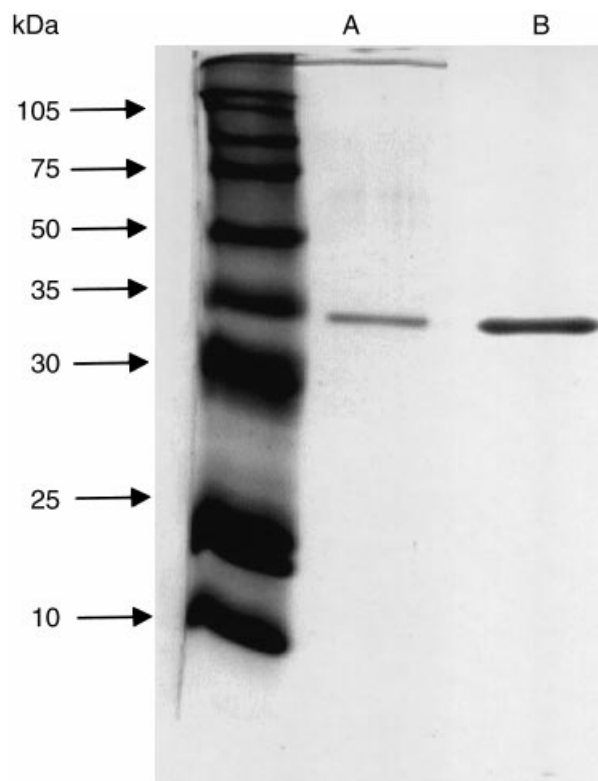


Figure 3 Purification of recombinant *Pso o 1*. Proteins denatured in the presence of β -mercaptoethanol were separated on a 12% (w/v) SDS-PAGE gel and visualized by staining with Coomassie blue R-250. Molecular sizes of protein standards are indicated on the left. (a) Recombinant *Pso o 1* purified using a Ni^{2+} -affinity column. (b) Immunoblot analysis of purified recombinant *Pso o 1* using 6 $\times$ His (C-terminal) antibody.

are shown in Figure 6 (a). After 20 days post primary infection, the levels of anti-*Pso o 1* IgG increased dramatically, with high levels detected from day 40–80. In three animals, the levels remained high until the termination of the experiment (day 120), while the response in two animals declined from day 60 to reach base levels by day 110. The same samples were also assayed for IgG antibody to whole mite extract antigen (PBS soluble fraction), and the response was similar to that observed for *Pso o 1* (Figure 6b). However, IgG antibody was detected earlier in two animals when whole *P. cuniculi* antigen was employed, and, in two different sheep, the antibody levels remained higher and did not show the relatively rapid decline observed with the anti-*Pso o 1* assay. In general, assays for IgG *Pso o 1* antibody tended to produce higher OD 450 nm, and lower 'background' OD readings, than those demonstrated against whole mite antigen.

The IgE response to whole mite extract and *Pso o 1* were also compared. Only one animal (2296B) showed a marked

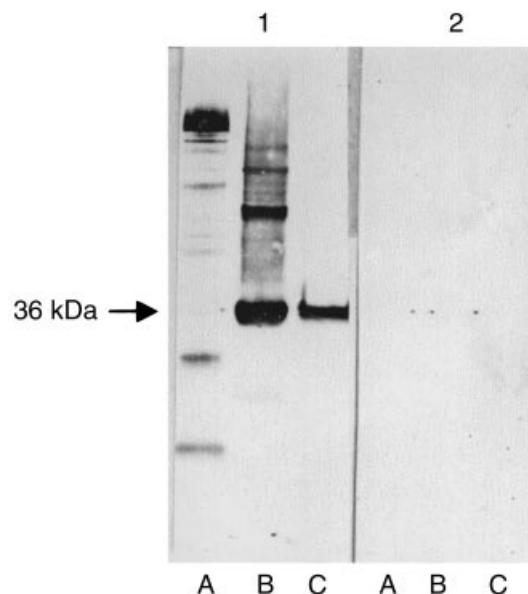


Figure 4 Western blot analysis for IgG antibody of (a) whole *P. ovis* antigens (10 $\mu\text{g}/\text{lane}$), (b) crude lysate of *E. coli* expressing *Pso o 1* (10 $\mu\text{g}/\text{lane}$) and (c) purified recombinant *Pso o 1* (1 $\mu\text{g}/\text{lane}$), reacted with a pool of (i) sheep anti-*P. ovis* sera and (ii) preimmune sera. Reaction product was developed following incubation with monoclonal anti-ovine IgG, sheep anti-mouse IgG-biotin and Streptavidin-HRP with DAB substrate.

IgE antibody response to *Pso o 1*, with a peak OD observed on day 90 postinfection (Figure 7a). An IgE anti-*Pso o 1* response was also detected in one other animal (2417B), although the OD readings were very low. In the other three sheep, only trace IgE antibody was detected with OD readings either at, or marginally above, control values. The IgE response to whole mite extract was essentially similar to that observed for *Pso o 1* (Figure 7b), although the background values were markedly higher than in the *Pso o 1* assay. Interestingly, the same animal (2296B) in which IgE anti-*Pso o 1* was readily detected, also produced the highest OD readings for IgE anti-whole mite extract.

DISCUSSION

Although several studies have used electrophoretic and immunoblotting techniques to detect *P. ovis* antigens/allergens that stimulate an immune response in sheep and cattle (9,26–29), these protein remain largely unidentified. To our knowledge, only one *P. ovis* allergen has been characterized (9). The first 30 amino acid residues of this 16 kDa protein have been reported and the sequence displays significant homology to the mite allergens *Lep d II* and *Der f 2* (10,11). In this study, we have demonstrated that *P. ovis* contains a homologue of the house dust mite group 1 allergens, which

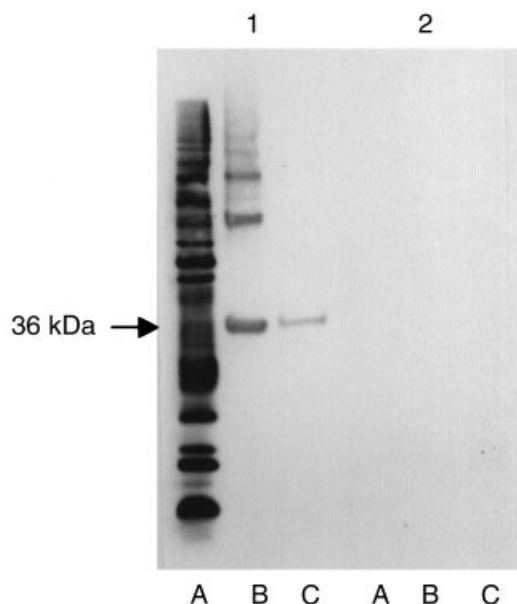


Figure 5 Western blot analysis for IgE antibody of (a) whole *P. ovis* antigens (10 µg/lane), (b) crude lysate of *E. coli* expressing *Pso o 1* (10 µg/lane) and (c) purified recombinant *Pso o 1* (1 µg/lane), reacted with a pool of (i) sheep anti-*P. ovis* sera and (ii) preimmune sera. Reaction product was developed following incubation with monoclonal anti-ovine IgE, sheep anti-mouse IgG-biotin and Streptavidin-HRP, with chemiluminescence detection using ECLTM reagent.

induces high levels of IgG antibody during experimental infections.

A multiple sequence alignment of the house dust mite allergens *Der p 1*, *Der f 1* and *Eur m 1* sequences (15–17) with that of *Pso o 1* (Figure 2) indicates that amino acids known to participate in enzyme activity (4,30) are conserved in the *Pso o 1* sequence (Glu³⁰, Cys³⁶ and His¹⁷²). In addition, a putative *N*-glycosylation site (Asn-X-Ser, where X is any amino acid except proline) (15–17) is also conserved. Comparative molecular modelling suggests that an immunodominant IgE epitope of *Der p 1* (amino acid residues Leu¹⁴⁷-Gln¹⁶⁰) is highly conserved in cysteine proteases from several sources (31), and this is also conserved in the *Pso o 1* sequence.

Der p 1, *Der f 1* and *Eur m 1* are initially synthesized in pre-pro forms (15–17) which undergo enzymatic processing, and examination of the *Pso o 1* sequence suggests that the native *P. ovis* protein may be processed in a similar manner. Sequence analysis suggests that the signal peptide cleavage site (between Tyr⁻⁷⁹ and Ala⁻⁷⁸) is conserved. However, because the forward PCR primer used to amplify the *Pso o 1* cDNA encodes the first eight amino acid residues, we can only speculate that the native protein contains a

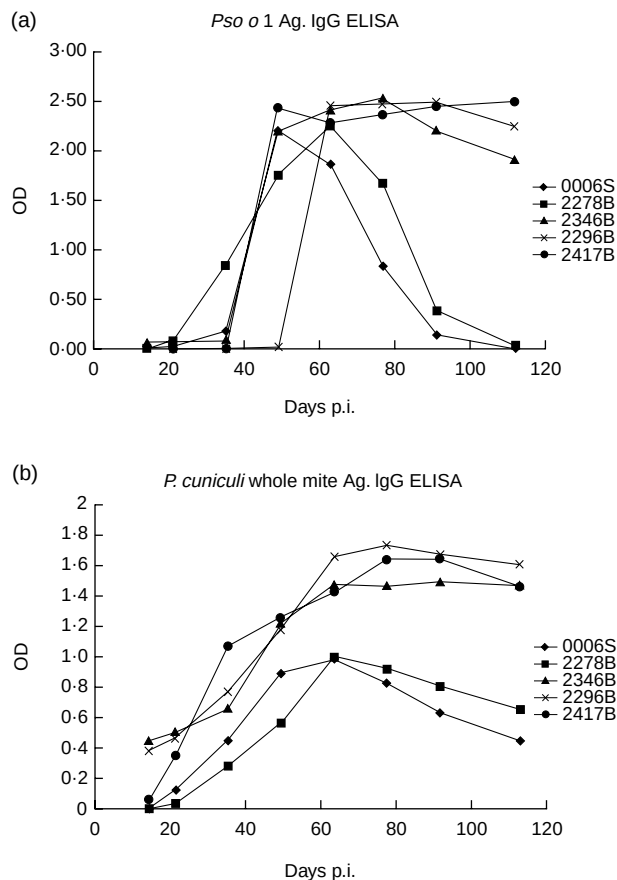


Figure 6 Evaluation of serum IgG titres by ELISA in five animals postinfestation with 25 ovigerous female *P. ovis* mites, to (a) *Pso o 1* and (b) whole *P. cuniculi* mite antigen.

signal peptide. The *Pso o 1* sequence also displays a high degree of homology to the residues that surround the *Der p 1*, *Der f 1* and *Eur m 1* propeptide cleavage site, suggesting that the mature *P. ovis* protein begins with a glycine residue.

We did not attempt to refold the purified protein because the *C*-terminus is truncated. By comparison with the house dust mite group I allergen sequences, we predict that the full-length *Pso o 1* would possess 46 additional amino acids at the *C*-terminus. Since this region of the house dust mite cysteine protease contains one of the catalytic site residues (4,30) (Asn¹⁹¹ in the *Der p 1* sequence), it is unlikely that the truncated *Pso o 1* would be enzymatically active.

The dust mite allergen *Der p 1* is considered to be the most immunodominant allergen contributing to IgE-mediated dust mite hypersensitivity, and was the first allergen to be fully characterized (32). Thompson *et al.* (33) and O'Brian & Thomas (34) have shown that asthmatic children have a strong IgE reactivity to *Der p 1*.

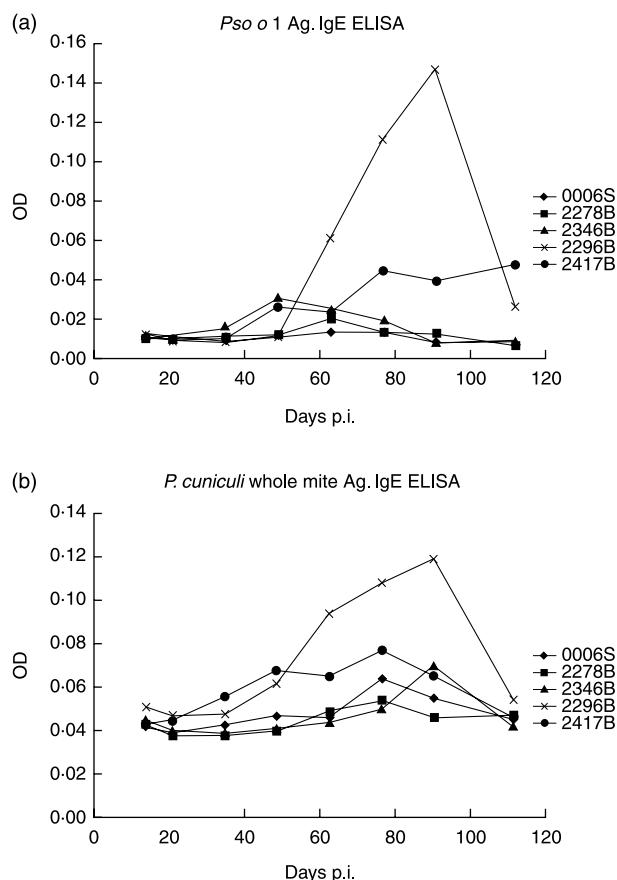


Figure 7 Evaluation of serum IgE titres by ELISA in five animals postinfestation with 25 ovigerous female *P. ovis* mites, to (a) *Pso o 1* and (b) whole *P. cuniculi* mite antigen.

Lesion development in sheep scab has been characterized as an immediate type 1 hypersensitive reaction (3). In this study, western-blot analysis of whole *P. ovis* extracts revealed that three major high molecular weight (100–180 kDa) and several minor and lower molecular weight (14–100 kDa) IgE reactive antigens were recognized by postinfestation sheep sera. These observations are similar to those of Matthes *et al.* (27) where *P. ovis* specific IgE recognized 13 proteins in whole *P. ovis* extracts with molecular weights ranging from 19 to 170 kDa.

The Western blot results suggest that *Pso o 1* binds IgE, whereas in the ELISAs, the IgE response was poor or non-existent in four of five animals. This apparent discrepancy may be due to the fact that we used pooled sera for the Western blots. The IgE response to whole mite extract of the same four animals was also poor. Competition by excess IgG may have contributed to this. From the current ELISA results, it would seem likely that *Pso o 1* is represented amongst the IgE reactive antigens in significant or

immunodominant proportions, although this remains to be confirmed.

There are several other possible reasons for the weak IgE-reactivity to *Pso o 1*. Western blot studies of house-dust mite allergens have indicated that *Der p 1* does not react strongly to IgE antibody following transfer to nitrocellulose, being susceptible to heat and acidification (35), and it is possible that a similar situation exists for *Pso o 1*. Second, the tertiary structure of the mature protein is probably very important and, in this study, *Pso o 1* was purified from insoluble inclusion bodies under denaturing conditions. Also, the protein is lacking amino acids from the C-terminus and the pro-region was not removed. Shoji *et al.* (36) observed that removal of the pro sequence from recombinant *Der f 1* produced by baculovirus infected *Spodoptera fugiperda* Sf9 cells was essential for IgE reactivity. The choice of expression system and the resulting glycosylation pattern (or lack of) may also affect the IgE reactivity. Recombinant *Der f 1* and *Der p 1* produced in *E. coli* and *Saccharomyces cerevisiae* were less immunoreactive than the native allergen (37–39). However, the pro-form of *Der p 1* expressed in *Drosophila* S2 Schneider cells displayed a similar immunoreactivity towards IgE as the native *Der p 1* antigen (40), while Best *et al.* (41) observed that the IgE-reactivity of mature recombinant *Der f 1* produced in *Pichia pastoris* was similar to the native allergen and that glycosylation was not essential for IgE binding *in vitro*.

In this study, a pronounced IgG response to *P. ovis* antigen was detected within a few weeks of infestation, which is in agreement with previous observations (42). Western blot analysis showed that IgG reacted with numerous protein bands (> 15) in the whole mite extract. This is more than the number of antigens found by Jayawardena *et al.* (29). These differences could be due to the methods employed and the mite strain.

The antibody response during experimental infection would suggest that *Pso o 1*, similar to its closely related house dust mite homologue, is an important antigen that may be involved in the pathogenesis and/or the immune response to the mite. Studies with house dust mites have shown that 50–70% of the IgE to a *D. pteronyssinus* extract was directed to *Der p 1* and the anti *D. pteronyssinus* specificities comprised 6–21% of the total IgE (32). In our studies, comparison of the response with whole extracts of mite antigen and to *Pso o 1* showed that the preinfection OD values were lower, and that the IgG antibody reached higher levels for *Pso o 1* than for the whole mite extract. This also suggests that *Pso o 1* is a major and/or immunodominant antigen.

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