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Veterinary Parasitology 105 (2002) 317–325

veterinary
parasitology

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The proteinases of *Psoroptes ovis*, the sheep scab mite—their diversity and substrate specificity

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Received 9 October 2001; accepted 30 January 2002

Abstract

The sheep scab mite, *Psoroptes ovis*, causes severe dermatitis in infected sheep with severe welfare and production implications. The dermatitis has the characteristics of an immediate hypersensitivity type reaction which, by analogy to other mite species, including the house dust mites (*Dermatophagoides* spp.), is likely to be invoked by a variety of allergens including mite-derived proteinases. Here, the proteinases in *P. ovis* extracts were characterised using substrate gel analysis, inhibitor sensitivity and their ability to degrade a variety of potential natural protein substrates. These analyses showed that mites contain several proteinases which could be differentiated on the basis of molecular size and inhibitor sensitivity with cysteine, metalloproteinases and aspartyl proteinases predominating. These proteinases degraded collagen and fibronectin, possibly indicative of a role in lesion initiation, they degraded several blood proteins, a property which may aid mite feeding and they degraded immunoglobulin G, possibly aiding immuno-evasion. Because proteinases, particularly the cysteine class, are demonstrably allergenic in other mite infestations, these proteinases clearly merit further immunological and biochemical definition. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Sheep-arthropoda; *Psoroptes ovis*; Proteinases

1. Introduction

The mite, *Psoroptes ovis*, is an important ectoparasite infecting sheep, goats and cattle being particularly prevalent in sheep in the UK and in cattle in Europe and the USA. *P. ovis* can induce a severe welfare condition and is highly contagious being spread by direct or indirect contact (Lewis, 1997). Infection provokes an extensive dermatitis, scab formation and intense pruritis (Smith et al., 2001). Infected animals show pronounced behaviour responses including rubbing the area of the scab lesion, scratching, wool loss, wounding

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and fitting (Corke and Broom, 1999). Initial focal lesions spread outwards, coalesce and may extend over the whole body. The host response to infestation has all the characteristics of an immediate type hypersensitivity reaction and this response can be experimentally induced by injection of mite extracts intradermally (Wikel, 1984). The individual antigens which invoke this response remain largely undefined although a 16 kDa allergen has, recently, been identified (Pruett, 1999).

Allergens have been extensively studied in the principal house dust mites *Dermatophagoides pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei* with comparative data from storage mites, such as *Lepidoglyphus destructor* and the tropical mite *Blomia tropicalis* becoming available (Stewart, 1995). These analyses show that each species contains several allergens with considerable inter-species similarities. Several allergens are peptidases and include cysteine proteinases (e.g. *Der p1*; Chua et al., 1988) and serine proteinases (Heymann et al., 1989; Stewart et al., 1989). Other peptidases identified include amino- and carboxypeptidases (Stewart et al., 1986, 1991) but their allergenicity remains to be confirmed (Stewart, 1995). These enzymes are commonly found in mite faecal pellets and may facilitate both intracellular and extracellular digestion, in the case of the latter to aid the penetration of collagen and keratin barriers in the skin (Stewart, 1995).

The present study was initiated to define the range of proteinases present in whole extracts of *P. ovis* and to examine their ability to degrade a range of protein substrates which may be encountered in the host environment.

2. Materials and methods

2.1. Mites and mite extracts

P. ovis mites were harvested from skin sections from sheep infested with mites maintained at the Moredun Research Institute, Edinburgh. Mites were extensively washed and water-soluble (S1) and membrane associated (S2) fractions prepared from approximately 500 mg mites as described by Smith and Smith (1993). These fractions were selected for study as they were found to have protective qualities in vaccination trials (Smith, unpublished data).

2.2. Substrate gel analysis

The S1 and S2 fractions (10 µg) were fractionated in 10% SDS-PAGE gel slabs (Laemmli, 1970) containing 0.1% (w/v) protein substrate. The protein substrates used were gelatin, haemoglobin, azocasein, and fibrinogen (all purchased from Sigma, UK). After electrophoresis at 200 V for 45 min in a Mini Protean[®] II Dual Slab Cell (BioRad, UK), the gels were washed in four changes of 2.5% Triton X-100 in 1 h to remove the SDS. The gels were then incubated in 0.1 M buffer (pH 3–6, acetate, pH 7–9, phosphate and pH 10–11 glycine-based buffers) with and without the addition of 2 mM dithiothreitol (DTT), for 16 h at 37 °C. DTT is known to activate cysteine proteases, which are generally found at acidic pH levels. Zones of proteolysis were visualised by 0.25% Coomassie blue staining.

2.3. Degradation assays

Fibrinogen, haemoglobin (each 2 mg/ml), IgG (1 mg/ml, purified from sheep serum using protein-G agarose (Boehringer Mannheim)) and fibronectin (1 mg/ml) were dissolved in distilled water (dH₂O). Substrate (10 μ l) was mixed with 4 μ g of either the S1 or S2 extract or 3 μ l of PBS to act as control. Two microlitres of the appropriate pH buffer as described above but here, all at 0.2 M, was then added and the samples incubated for 16 h at 37 °C. After the addition of an equal volume of reducing sample buffer, electrophoresis was carried out at 200 V for 45 min and gels stained with 0.25% Coomassie blue.

The degradation of azocoll, elastin-orcein and keratin-azure (all from Sigma) was sought using a spectrophotometric assay. Briefly, 100 μ l of azocoll (10 mg/ml in dH₂O), 1 mg elastin-orcein or 1 mg of keratin-azure was mixed with 100 μ l of 0.2 M buffer (as above, pH 3–5 included 2 mM DTT) and either 1.5 μ g of the S1 or S2 extract, or 2 μ l of PBS to act as the control. Samples were incubated at 37 °C for 16 h, spun briefly at 13,000 $\times$ g, and the absorbance of the supernatant determined spectrophotometrically at 520 nm (azocoll), 550 nm (elastin-orcein) or 620 nm (keratin-azure).

2.4. Inhibitor studies

Class-indicative proteinase inhibitors were either added to the overnight incubation buffer for the substrate gels, or included in the degradation assays. Inhibitors used (Sigma, UK) and their final concentrations were: 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF, 2 mM); L-trans-epoxysuccinyl-L-leucylamido- (4-guanidino)-butane (E64, 10 μ M); pepstatin A (10 μ M) and 1,10-phenanthroline (1 mM).

2.5. Antibody inhibition studies

IgG was purified from sera of vaccinated sheep protected against challenge, from sheep with a natural infestation of *P. ovis* or from naive animals by a method described previously (Young et al., 1995). S1 or S2 extract (10 μ g) was mixed with 1 μ l dH₂O to act as a control or 1, 5 or 10 μ g of the purified IgG and incubated at 37 °C for 1 h prior to electrophoresis in 5% native gels containing 0.1% gelatin. The gels were then incubated overnight at 37 °C in pH 5 acetate buffer with or without the addition of 2 mM DTT and stained in 0.25% Coomassie blue.

3. Results

3.1. Substrate gel analysis

Proteinase activity, some of which, at acidic and neutral pH, was enhanced by the presence of DTT, was visible over the pH range 3–11 with gelatin as substrate (Fig. 1). The broad size range where proteolysis was visible (35 to >205 kDa) appeared to comprise several poorly resolved zones (see pH 4 + DTT), the bulk of which were almost completely inhibited by the cysteine proteinase inhibitor, E64 (Fig. 2A). Two sharp zones of proteolysis at

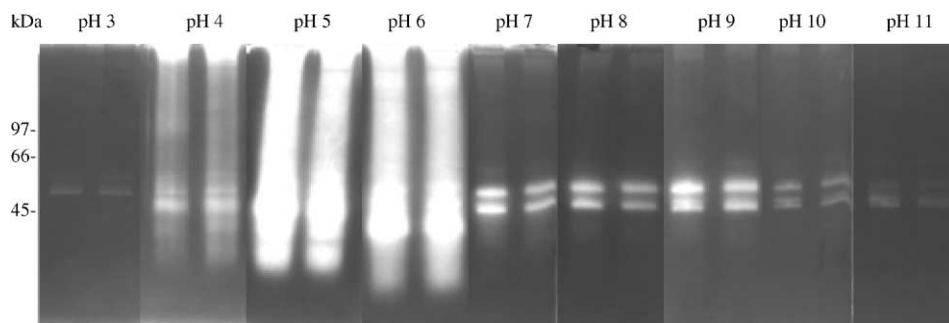


Fig. 1. Gelatin-substrate gel. S1 extract is to the left of each strip, S2 to the right. The pH 3–6 had 2 mM DTT added to overnight incubation buffer.

approximately 50 and 55 kDa were evident over a broad pH range (pH 4–11) with optimal activity at pH 6. These bands were fairly insensitive to the inhibitors tested but activity was reduced by the metalloproteinase inhibitor 1,10-phenanthroline at pH 9 (see Fig. 2B).

When azocasein was used as substrate in the gels, activity was very much reduced compared to gelatin with only faint bands at 45 and 66 kDa being evident, both sensitive to E64, in both extracts at pH 6 and 7 (not shown). No activity was observed with haemoglobin as substrate. Proteolysis of fibrinogen (Fig. 3) was comparatively weak and only visible at pH 5 and 6. However, additional activity, not evident with gelatin as substrate, was observed at 44 and 38 kDa. Activity was strongest in the membrane-associated S2 mite extract and was insensitive to 1,10-phenanthroline.

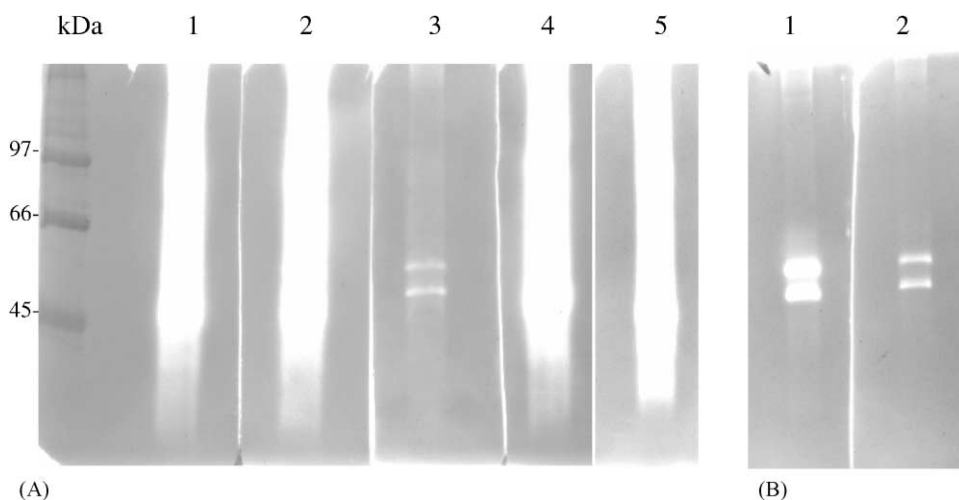


Fig. 2. Gelatin-substrate gel. Panel (A): pH 5 plus 2 mM DTT. Lane 1; control mite extract alone, 2; plus 2 mM AEBSF, 3; plus 10 μ M E64, 4; plus 10 μ M pepstatin, 5; plus 1 mM 1,10-phenanthroline. Panel (B): pH 9. Lane 1: control mite extract alone, 2; plus 1 mM 1,10-phenanthroline.

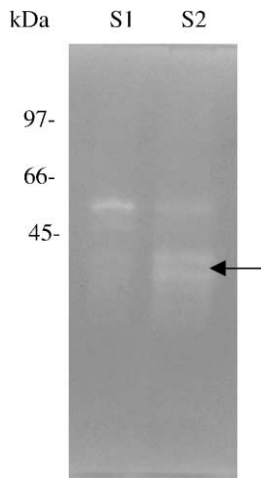


Fig. 3. Fibrinogen-substrate gel, pH 6, of S1 and S2 extracts without DTT, showing the presence of zones of proteolysis (arrow) at approximately 40 and 38 kDa, not observed in gelatin gels.

3.2. Degradation assays

No differences were observed when degradation with S1 and S2 extracts were compared. Results shown were with pooled S1 and S2.

3.2.1. Haemoglobin

Assays with haemoglobin, fibrinogen, IgG and fibronectin are shown in Fig. 4. Haemoglobin degradation was optimal at pH 3 and completely inhibited by the aspartyl proteinase inhibitor, pepstatin. It was unaffected by other class-indicative inhibitors, most notably E64 (Fig. 4A).

3.2.2. Fibrinogen

All three fibrinogen peptide chains were completely degraded at pH 4 and 5 with no degradation evident above pH 5. Degradation was abolished by pepstatin and partially reduced by 1,10-phenanthroline (Fig. 4B).

3.2.3. IgG

IgG degradation was restricted to the heavy chain (~ 55 kDa) and only evident at pH 4 and 5, resulting in a product at about 30 kDa (Fig. 4C). Degradation was completely inhibited by 1,10-phenanthroline and E64 and slightly inhibited by pepstatin and AEBSF.

3.2.4. Fibronectin

Fibronectin was degraded across a broad pH range from pH 3 to 9. At pH 5, proteolysis was inhibited by E64 and 1,10-phenanthroline (Fig. 4D), by pepstatin at pH 3 and slightly inhibited by both E64 and pepstatin at pH 9 (not shown).

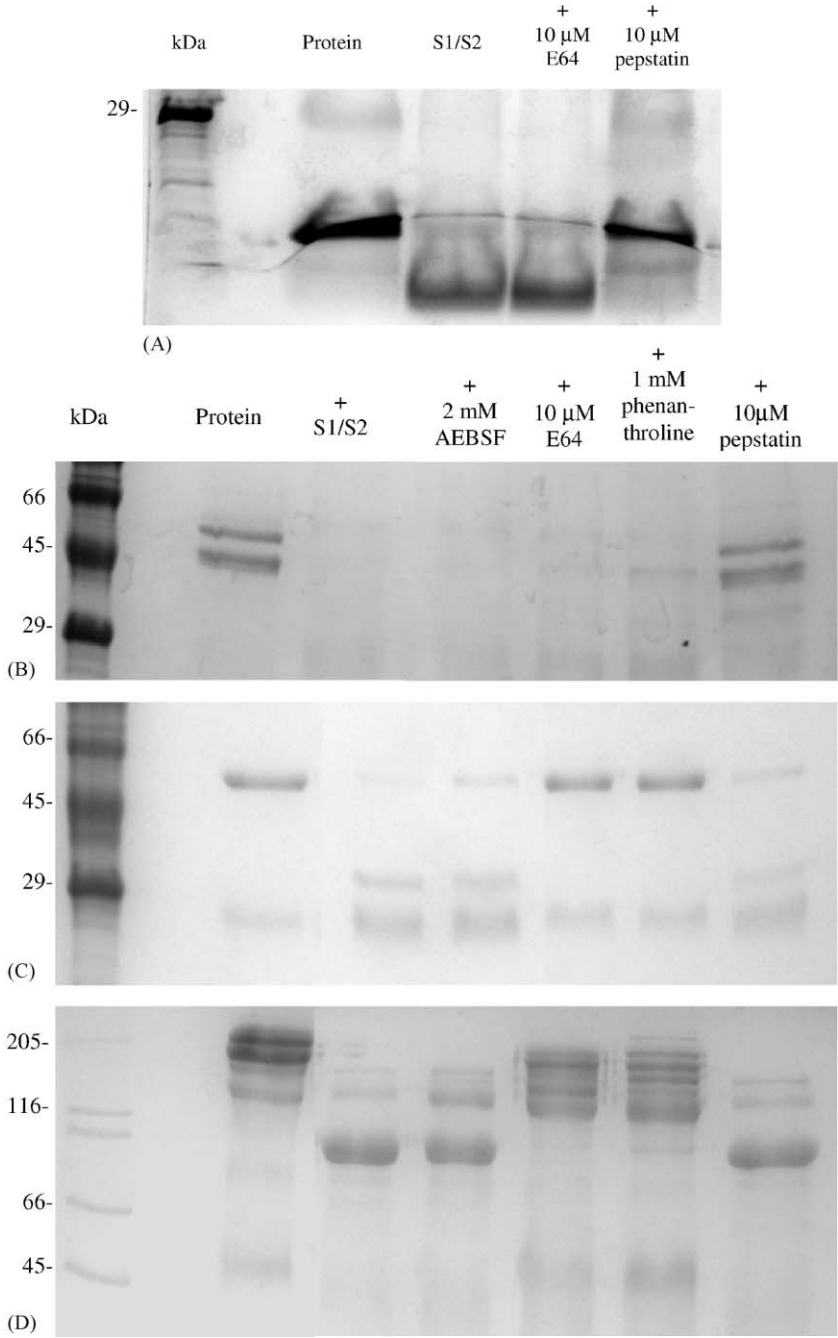


Fig. 4. All samples had 2 mM DTT added. Panel (A): haemoglobin degradation at pH 3. Panel (B): fibrinogen degradation at pH 4. Panel (C): IgG degradation at pH 4. Panel (D): fibronectin degradation at pH 5.

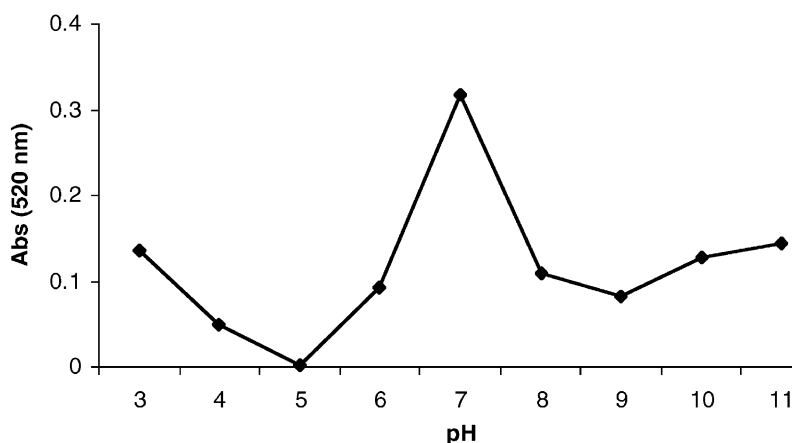


Fig. 5. Azocoll degradation assay showing the pH optima. Each data point on the graph consists of the absorbance at each pH level, minus the absorbance of a PBS control at the equivalent pH level.

3.2.5. Azocoll, elastin-orcein and keratin-azure

Azocoll was degraded by the mite extracts with optimal activity at pH 7 (Fig. 5), this only being inhibited by 1,10-phenanthroline.

Keratin-azure and elastin-orcein were not degraded by the mite extract.

3.3. Antibody inhibition assays

Antibody interactions with the extract proteinases were sought on the basis that antibody binding to the protease would retard migration in gelatin-substrate gels (Young et al., 1995). There were no differences observed between the control S1 and S2 extracts and those that had been pre-incubated with antibody purified from either the vaccinated and protected sera, control sera or the natural infestation sera (results not shown).

4. Discussion

Previous studies (Nisbet and Billingsley, 1999, 2000) suggested that the major endopeptidase activity in *P. ovis* was attributable to an aspartyl proteinase optimally active at low pH and capable of degrading haemoglobin. In addition, Nisbet and Billingsley (2000) showed that *P. ovis* extracts contained a 29 kDa cysteine proteinase using a biotinylated probe. The present study considerably expands these observations showing that extracts of *P. ovis* contain a range of proteinases with the ability to degrade a variety of host proteins. Importantly, we show that *P. ovis* extracts may contain several different cysteine proteinases which may be counterparts of important allergens already identified in house dust and storage mites and may play a central role in lesion development.

At acidic pH, cysteine proteinase activity predominated (Fig. 1), as indicated by E64 inhibition (Fig. 2A). These enzymes degraded the soluble collagen derivative, gelatin,

and fibronectin, an extracellular matrix glycoprotein component of basement membranes (Fig. 4D). They did not degrade keratin or elastin. This suggests that keratin is not a major food source for *Psoroptes* in contrast to house dust mites where skin squames containing keratin are thought to be a major food source (Stewart et al., 1991). Metalloproteinases, indicated by 1,10-phenanthroline inhibition (Fig. 2B), also degraded gelatin. Clearly, proteolysis of these substrates may facilitate penetration of the skin barrier and lesion initiation.

Mite proteinases may perform an anticoagulant function as judged by fibrinogen degradation attributable to aspartyl and metalloproteinases. This action would prolong serous exudation from the lesion and, presumably, aid mite feeding. In agreement with the observations of Nisbet and Billingsley (1999), haemoglobin degradation was solely attributable to aspartyl proteinase activity. Both the cysteine and metalloproteinases played a role in immunoglobulin G degradation with this being confined to the heavy chain, expanding the observations of Pettit et al. (2000). This restricted digestion of the heavy IgG chain suggests that activity is unlikely to be simply the non-specific outcome of mixing protein and proteinases and may indicate that immunoglobulin degradation could enhance mite survival *in vivo*.

The dust mites *Dermatophagoides* spp. produce a range of allergens with different biological activities (Stewart, 1995). In particular, the group 1 allergens *Der p1* and *Der f1* are cysteine proteinases found in mite whole body and faecal extracts, are synthesised by cells lining the gastro-intestinal tract (Thomas et al., 1991) and may degrade skin collagens and keratin (Colloff, 1993). The cysteine proteinases described here may include a *P. ovis* equivalent of the group 1 allergens. A recent expressed sequence tag (EST) survey of genes expressed by *P. ovis* (Kenyon et al., unpublished) indicated that the most abundant cysteine proteinase showed 69% amino acid sequence identity with *Der p1*. This study identified several other distinct cathepsin B and L-type cysteine proteinases encoding transcripts as well as a variety of matrix metalloprotease homologues. The present study draws attention to the potential importance of metalloproteinases in mite physiology and these are clearly worthy of further analysis. Of further note, the group 3, 6 and 9 allergens of *Dermatophagoides* spp. are all serine proteinases and some, especially in the case of *D. pteronyssinus*, show a high frequency of reactivity in affected individuals (Stewart, 1995). Here, no evidence for serine proteinase activity in *P. ovis* extracts was provided despite the use of a variety of substrates and proteinase class-indicative inhibitors.

There is now a requirement to define (1) if these proteinases illicit an immune response in the course of infection, (2) if any invoke an IgE response which would implicate them in the hypersensitivity response to the mite and (3) if any are associated with protective immunity. In the present study, we addressed the latter by seeking antibody/proteinase interactions with a gel-based technique previously used with success in the definition of proteinases in the excretory/secretory components of *Teladorsagia circumcincta*, the ovine abomasal nematode (Young et al., 1995). Here, antibody was harvested from sheep immunised against infection by repeated natural challenge or by vaccination with mite extracts, then reactivity with mite extract proteinases compared with antibody from uninfected controls. No differences were observed in electrophoretic mobility or enzyme activity, suggesting that proteinase inhibition by antibody may not be associated with the protective immune response in *P. ovis* infestation. Work is now in progress to define if recombinant enzyme proteins, expressed in *Escherichia coli*, invoke IgG and/or IgE responses in infected sheep.

Acknowledgements

We would like to thank Dr. W.D. Smith, Mr. D. Pettit and Mrs. J. Machell for provision of mites used in this study and helpful advice throughout. This work was supported by MAFF Welfare grant No. ODO 531 (FK) and the Scottish Executive Environment and Rural Affairs Department (DPK).

References

- Chua, K.Y., Stewart, G.A., Thomas, W.R., Simpson, R.J., Dilworth, R.J., Plozza, T.M., Turner, K.J., 1988. Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p1*. Homology with cysteine proteases. *J. Exp. Med.* 167, 175–182.
- Colloff, M.J., 1993. Differences between the allergen repertoires of house dust mites and stored product mites. *J. Clin. Immunoassay* 16, 114–123.
- Corke, M.J., Broom, D.M., 1999. The behaviour of sheep with sheep scab, *Psoroptes ovis* infestation. *Vet. Parasitol.* 83, 291–300.
- Heymann, P.W., Chapman, M.D., Aalberse, R.C., Fox, J.W., Platts-Mills, T.A.E., 1989. Antigenic and structural analysis of group II allergens (*Der fII* and *Der pII*) from house dust mites (*Dermatophagoides* spp.). *J. Allergy Clin. Immunol.* 83, 1055–1067.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lewis, C., 1997. Update on sheep scab. In *Practice* 19, 558–564.
- Nisbet, A.J., Billingsley, P.F., 1999. Immunological control of scab mites: digestive enzymes as candidate compounds. *Vet. Parasitol.* 83, 231–239.
- Nisbet, A.J., Billingsley, P.F., 2000. A comparative survey of the hydrolytic enzymes of ectoparasitic and free-living mites. *Int. J. Parasitol.* 30, 19–27.
- Pettit, D., Smith, W.D., Richardson, J., Munn, E.A., 2000. Localisation and characterisation of ovine immunoglobulin within the sheep scab mite, *Psoroptes ovis*. *Vet. Parasitol.* 89, 231–239.
- Pruett, J.H., 1999. Identification and purification of a 16 kDa allergen from *Psoroptes ovis* (Acarina: Psoroptidae). *J. Med. Entomol.* 36, 544–550.
- Smith, W.D., Smith, S.K., 1993. Evaluation of aspects of the protection afforded to sheep immunised with a gut membrane protein of *Haemonchus contortus*. *Res. Vet. Sci.* 55, 1–9.
- Smith, W.D., Van Den Broek, A.H.M., Huntley, J.F., Pettit, D., Machell, J., Miller, H.R.P., Bates, P.G., Taylor, M., 2001. Approaches to vaccines for *Psoroptes ovis* (sheep scab). *Res. Vet. Sci.* 70, 87–91.
- Stewart, G.A., 1995. Dust mite allergens. *Clin. Rev. Allergy Immunol.* 13, 135–150.
- Stewart, G.A., Butcher, A., Lees, K., Ackland, J., 1986. Immunochemical and enzymatic analyses of extracts of the house dust mite *Dermatophagoides pteronyssinus*. *J. Allergy Clin. Immunol.* 77, 14–24.
- Stewart, G.A., Thompson, P.J., Simpson, R.J., 1989. Protease antigens from house dust mite. *Lancet* 2, 154–155.
- Stewart, G.A., Lake, F.R., Thompson, P.J., 1991. Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: physicochemical characterisation of potential allergens. *Int. Arch. Allergy Appl. Immunol.* 95, 248–256.
- Thomas, B., Heap, P., Carswell, F., 1991. Ultrastructural localisation of the allergen *Der p1* in the gut of the house dust mite *Dermatophagoides pteronyssinus*. *Int. Arch. Allergy Immunol.* 94, 365–367.
- Wikel, S.K., 1984. Immunomodulation of host responses to ectoparasite infestation—an overview. *Vet. Parasitol.* 14, 321–339.
- Young, C.J., McKeand, J.B., Knox, D.P., 1995. Proteinases released in vitro by the parasitic stages of *Teladorsagia circumcincta*, an ovine abomasal parasite. *Parasitology* 110, 465–471.