

Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen *Der p I*

C. R. A. HEWITT, H. HORTON, R. M. JONES* & D. I. PRITCHARD†

Immunotoxicology Laboratory and *Biomonitoring and Molecular Interactions Section, MRC Toxicology Unit, Centre For Mechanisms Of Human Toxicity, University Of Leicester and †Department Of Life Science, University Of Nottingham, Nottingham, UK

Summary

Background Exposure of the skin or respiratory tract to proteinases is frequently associated with allergic sensitization. This is of particular significance in the domestic indoor environment where the proteolytic activity of *Der p I*, the group I allergen of the house dust mite *Dermatophagoides pteronyssinus*, may influence the allergenicity of mites. Using class-specific proteinase inhibitors and active-site affinity chromatography, we have previously shown that *Der p I* exhibits a mixed cysteine-serine proteinase activity. Measurement of the amount of cleavage, however, did not determine whether the inhibitors used were targeting exactly the same proteolytic mechanism.

Objective To resolve this issue, we have examined whether the cleavage specificity of the cysteine and serine proteinase activities of *Der p I* was the same.

Methods HPLC and mass spectrometry were used to analyse and identify the products of a *Der p I*-digested peptide substrate and thus identify the peptide bonds cleaved.

Results *Der p I* cleaves different peptide bonds, depending upon the class of proteolytic mechanism used. In the model peptide substrate insulin B chain, the cysteine and serine proteinase activities of *Der p I* showed preference for glutamic acid and arginine respectively in the P1 position.

Conclusion These data suggest the existence of more than one mechanistic form of the allergen immunologically identified as *Der p I*. If proteolytic activity is indeed a function of allergenicity, this information may have important implications for the pathogenicity of *Der p I* and the ability of innate antiproteinase defences in the respiratory tract to prevent immune sensitization.

Keywords: house dust mite, *Der p I*, *Dermatophagoides pteronyssinus*, cysteine proteinase, serine proteinase, cleavage specificity, antiproteinase

Clinical and Experimental Allergy, Vol. 27, pp. 201–207. Submitted 22 December 1995, revised 16 July 1996, accepted 24 August 1996.

Introduction

Proteolytic enzymes are well-established occupational allergens [1–5]. The hazards of exposure to proteinases are, however, not confined to the work place, and immune sensitization to proteinases contaminating the domestic indoor environment is critical in the development of allergy to house dust mites [6].

Correspondence: Colin R. A. Hewitt, Immunotoxicology Laboratory, Centre for Mechanisms for Human Toxicity, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester LE1 9HN, UK.

The causal relationship between exposure to domestic house dust mites and allergic asthma, rhinitis, conjunctivitis and atopic dermatitis is now undisputed. Most mite-allergic patients in the UK possess IgE antibodies specific for at least one of the group I mite allergens, *Der p I*, *Der f I* or *Eur m I*. All three allergens contain sequences conserved in the active sites of cysteine proteinases [7–9], and based on the predicted amino acid sequence of *Der p I* and its homology to known cysteine proteinases, a molecular model of *Der p I* has been constructed [10]. This suggests that *Der p I* adopts a two-domain structure similar to that of papain, with

a single active site region involving a thiolate/imidazolium ion pair at C34/H170.

Several groups suggest that the proteolytic activity of *Der p* I may contribute to its potent allergenicity [11–13]. Two mechanisms have been tested experimentally, damage to the integrity of the respiratory epithelium [12] and disruption of immune regulation within the IgE network [13]. Our own investigations [13], were the first to demonstrate that *Der p* I cleaves selectively from the surface of human B cells the low affinity IgE Fc receptor (CD23). Based on this study we hypothesized that loss of CD23 from IgE-secreting B cells, and the subsequent generation of soluble CD23, might enhance IgE synthesis and contribute to the potent allergenicity of mites.

In our characterization of purified *Der p* I proteinase, we found that the enzyme exhibited an unusual, mixed cysteine-serine proteinase activity [13]. Here, we have further characterized this activity, and now show that purified *Der p* I cleaves model peptide substrates at sites dependent upon the catalytic mechanism used. This suggests the existence of more than one mechanistic form of the proteinase immunologically identified as *Der p* I. This raises the clinically important possibilities that certain forms of *Der p* I may be more or less able to perturb the immune system, or that innate antiproteinase defences of the respiratory tract are more or less able to prevent immune sensitization [13]. Detailed knowledge of the active sites, and specificity of all the forms of *Der p* I may also have important implications for the design of therapeutics targeted to the active site of the allergen.

Materials and methods

Purification of *Der p* I

Der p I was purified from non-cloned laboratory cultures of mites containing only *Dermatophagoides pteronyssinus* [14], using immunoaffinity chromatography with a well-established and characterized anti-group I mite allergen monoclonal antibody, 4C1 [15] (generously donated by Dr Martin Chapman, University of Virginia Health Sciences Centre, MD, USA). Whole mite culture (15 g) was stirred for 16 h at 4°C in 200 ml PBS pH 8.0. Debris and insoluble material was removed by centrifugation at 13 000 *g* and 0.2 µm filtration. The extract was applied to the 4C1 immunoaffinity column, which was washed extensively with PBS pH 8.0. *Der p* I was eluted with 50 mM glycine 50% ethylene glycol, pH 10.0. After dialysis against PBS pH 8.0, *Der p* I was concentrated to 1 mg ml⁻¹ using a Centriprep 10 ultrafiltration unit (Amicon, Stonehouse, Gloucester, UK) and stored at -80°C. Purity was assessed by silver stained 15% SDS PAGE and casein zymography (see [13], data not shown).

Substrates and inhibitors

The oxidized B chain of insulin (insulin B chain) (Sigma, Poole, Dorset, UK) was used as a model peptide substrate of *Der p* I. This essentially linear peptide of amino acid sequence FVNQHLCGSHLVEALYLVCGERGFFYTPKA, is particularly suitable and used frequently for this type of study as it contains a wide range of amino acid combinations for determining the cleavage site specificity of proteinases [16]. The irreversible inhibitors of cysteine-, serine-, aspartic- and metalloproteinases, L-trans-epoxysuccinyl-leucylamide-[4-guanidino]-butane, (E64) (Novabiochem, Nottingham, UK), 4-[amidinophenyl] methanesulphonyl fluoride (APMSF) (Novabiochem), pepstatin-A (Novabiochem) and 1,10-phenanthroline (Novabiochem), were reconstituted as recommended by the manufacturers and used at their optimally effective concentrations of 5 µM, 50 µM, 1 µM and 1 mM respectively (data not shown).

Digestion of oxidised insulin B chain by *Der p* I

Immunoaffinity purified *Der p* I (0.125 µg) was incubated with 4.25 nmol of insulin B chain at 37°C in a final volume of 50 µl PBS at pH 8.0. At various time intervals reactions were terminated by the addition of 10 µl of 0.1% trifluoroacetic acid (TFA) in acetonitrile. Samples were stored at -20°C until analysed by high performance liquid chromatography (HPLC). In experiments using class-specific inhibitors of proteinases, *Der p* I was pre-incubated at 37°C with the inhibitors for 30 min prior to addition of the substrate.

Reverse-phase HPLC

Digested substrates were analysed by reverse-phase HPLC using an OSD2 C18 column (Vydac, Hesperia, CA, USA) and a Varian 9012 pump controller, (Varian Ltd, Walton Upon Thames, Surrey, UK) run at a flow rate of 0.75 ml min⁻¹. Peptides were eluted over 30 min in a 0–60% buffer A–B gradient of buffer A, 0.1% TFA in water to buffer B, 0.1% TFA in acetonitrile (Fisons, Loughborough, UK). Peaks were detected at OD 214 nm using a Varian 9050 UV-VIS detector.

Electrospray mass spectrometry

The mass spectra of each HPLC fraction were acquired on a Quattro BQ tandem quadrupole mass spectrometer (VG Organic, Manchester, Lancashire, UK) fitted with an atmospheric pressure ionisation electrospray source. Samples were introduced into the spectrometer by continuous infusion using a Harvard 22 syringe pump (Harvard Apparatus, Edenbridge, Kent, UK) at a flow rate of 30 µl min⁻¹. The source temperature was 90°C, and ion spectra were acquired

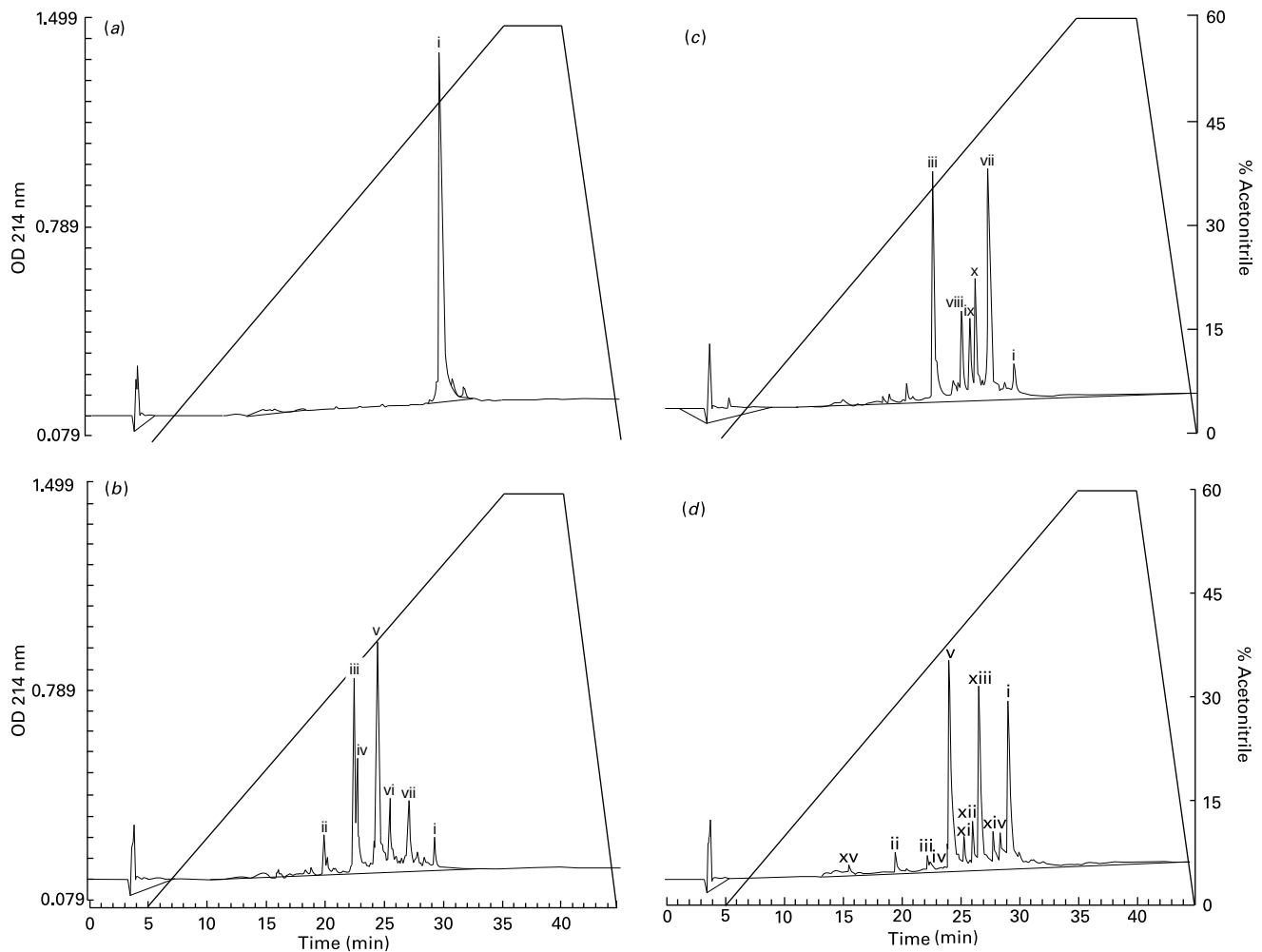


Fig. 1. *Der p I* digestion products of insulin B chain in the presence and absence of proteinase inhibitors. Reactions were incubated for 4 h under the conditions described in the Materials and methods section. Digests were analysed by reverse-phase HPLC using a 0–60% acetonitrile, 0.1% TFA gradient as described (right hand y axis). Peaks were detected at OD214 nm (left hand y axis), and those labelled i to xv were collected for analysis by mass spectrometry. Panel (a) insulin B chain alone; panel (b) insulin B chain incubated with *Der p I*; panel (c) insulin B chain incubated with *Der p I* and the serine proteinase inhibitor APMSF; panel (d) insulin B chain incubated with *Der p I* and the cysteine proteinase inhibitor E64.

in the positive ion mode, over the m/z range of 200 to 2000 at a rate of one scan every 4 s. The mass of the protonated species observed for each peptide was then used to unambiguously distinguish and identify the peptide of that mass in insulin B chain using Biolynx software (VG Organic, Manchester, Lancashire, UK).

Results

Our finding that *Der p I* exhibits an unusual mixed cysteine-serine protease activity [13] could be interpreted as meaning that *Der p I* represents a novel class of proteinase, has more than one active site, or is conformationally altered by inhibitors binding outside the active

site. To resolve this issue we have determined the cleavage site of *Der p I* in a model peptide substrate in the presence of irreversible inhibitors of cysteine and serine proteinases to determine if the location of the scissile peptide bond was inhibited equally by each of the class-specific inhibitors.

When incubated in the absence of *Der p I* and analysed by reverse-phase HPLC, insulin B chain appeared as a single major peak, peptide **i** (Fig. 1a) with a protonated molecular weight of 3497. When 0.125 μg of *Der p I* alone was incubated in parallel with substrate digestions, then analysed under the same HPLC conditions, no peaks that differed from the HPLC trace obtained with PBS alone were observed (data not shown).

Table 1. Summary of mass spectrometric analysis of the *Der p* I digestion products of insulin B chain. Predicted sequences of peptides isolated from the reverse-phase HPLC analysis of the uninhibited digestion of insulin B chain with *Der p* I as determined from masses obtained by mass spectrometry as described in the Materials and methods section

Peak	Mass*	Sequence
i	3497	1 5 10 15 20 25 30 FVNQHLCGSHLVEALYLVCGERGFFYTPKA
iii	1532	FVNQHLCGSHLVE
vii	1984	ALYLVCGERGFFYTPKA
iv	1073	ALYLVCGER
v	931	GFFYTPKA
vi†	1713	LYLVCGERGFFYTP
ii†	888	YLVCGER

* Mass of the protonated peptide species $[M + H]^+$. † Potential degradation products.

Der p I activity in the absence of inhibitors

In the absence of inhibitors, *Der p* I digested insulin B chain into six peptides (and one peak representing undigested insulin B chain) (Fig. 1b, Table 1). *Der p* I cleaved insulin B chain at two sites, between amino acids E13-A14 and R22-G23 (Table 1). As there is no evidence for a peptide with the predicted mass of amino acids from F1 to R22, it is most likely that *Der p* I cleaves first between E13 and A14 to generate peptides **iii** and **vii**. A subsequent cleavage event may then occur between R22 and G23, within peptide **vii**, to generate the peptides **iv** and **v**. Additional material resolving as peptides **vi** and **ii** were found to have masses consistent with truncated peptides derived from **vii** and **iv** respectively and may be degradation products.

Der p I activity in the presence of inhibitors of aspartic and metalloproteinases

When *Der p* I was pre-incubated with the class-specific inhibitors of aspartic and metalloproteinases, pepstatin A and 1,10-phenanthroline, prior to addition of the substrate insulin B chain, no alterations in the diversity or retention times of peptides produced by *Der p* I in the absence of inhibitors was noted (data not shown). This is consistent with our published findings that aspartic and metalloproteinases have no effect on the cleavage of substrates by *Der p* I [13].

Inhibition of *Der p* I serine proteinase activity

Pre-incubation of *Der p* I with APMSF, an irreversible inhibitor of serine proteinases, prior to the addition of

Table 2. Summary of mass spectrometric analysis of the *Der p* I digestion products of insulin B chain in the presence of APMSF. Predicted sequences of peptides isolated from the reverse-phase HPLC analysis of the digestion of insulin B chain with *Der p* I in the presence of the serine proteinase inhibitor APMSF. Sequences are determined from masses obtained by mass spectrometry as described in the Materials and methods section. The predominant cysteine proteinase cleavage site is located between E13 and A14

Peak	Mass*	Sequence
i	3497	1 5 10 15 20 25 30 FVNQHLCGSHLVEALYLVCGERGFFYTPKA
iii	1532	FVNQHLCGSHLVE
vii	1984	ALYLVCGERGFFYTPKA
ix	1716	FVNQHLCGSHLVEAL
x	1800	YLVCGERGFFYTPKA
viii†	1637	LVCGERGFFYTPKA

* Mass of the protonated peptide species $[M + H]^+$. † Potential degradation products.

Table 3. Summary of mass spectrometric analysis of the *Der p* I digestion products of insulin B chain in the presence of E64. Predicted sequences of peptides isolated from the reverse-phase HPLC analysis of the digestion of insulin B chain with *Der p* I in the presence of the cysteine proteinase inhibitor E64. Sequences are determined from masses obtained by mass spectrometry as described in the Materials and methods section. The predominant serine proteinase cleavage site is located between R22 and G23

Peak	Mass*	Sequence
i	3497	1 5 10 15 20 25 30 FVNQHLCGSHLVEALYLVCGERGFFYTPKA
xiii	2585	FVNQHLCGSHLVEALYLVCGER
v	931	GFFYTPKA
xii	1879	FVNQHLCGSHLVEALY
xv	725	LVCGER
iii‡	1532	FVNQHLCGSHLVE
xiv†	1285	NQHLCGSHLVE
iv‡	1072	ALYLVCGER
xi†	1714	LYLVCGERGFFYTP
ii†	888	YLVCGER

* Mass of the protonated peptide species $[M + H]^+$. † Potential degradation products. ‡ Produced at low efficiency when compared with the production of the same peptides in the presence of APMSF and the absence of inhibitors (compare Fig. 1d with 1b and 1c), thus showing that in the presence of E64, cleavage between E13 and A14 is very inefficient.

insulin B chain allowed the remaining cysteine proteinase activity of *Der p I* to generate five identifiable peptides (and one peak representing undigested insulin B chain) (Fig. 1c, Table 2), three of which were not present in the digestion products of the reaction with no inhibitors.

Cleavage of the peptide bond between amino acids E13 and A14 generated two peptides, **iii** and **vii**, that dominated the HPLC trace (Fig. 1c, Table 2). These peptides were also produced by the uninhibited *Der p I* (Fig. 1b, Table 1). Since this bond is cleaved by APMSF-inhibited *Der p I*, it is most likely that the cysteine proteinase activity of APMSF-inhibited *Der p I* is responsible for the cleavage of the bond between amino acids E13 and A14.

In the presence of APMSF-inhibited *Der p I*, no peptide digestion product of insulin B chain could be detected that had a mass consistent with cleavage of the peptide bond between amino acids R22 and G23. This bond was identified as a *Der p I* cleavage site in the absence of inhibitors (Fig. 1b, Table 1), thus strongly suggesting that the serine proteinase activity of *Der p I* is responsible for the cleavage of insulin B chain between R22 and G23.

The peak with a retention time of 20.4 min in Figure 1c was not identifiable by mass spectrometry but is unlikely to be identical to peptide **ii** present in Figure 1b due to significantly different retention times.

Inhibition of Der p I cysteine proteinase activity

Pre-incubation of *Der p I* with E64, an irreversible inhibitor of cysteine proteinases, prior to the addition of insulin B chain, allowed the remaining serine proteinase activity of *Der p I* to generate nine identifiable peptides (and one peak representing undigested insulin B chain) (Fig. 1d, Table 3), five of which were present in the uninhibited digest (Fig. 1b, Table 1). The HPLC trace is dominated by peptides **xiii** and **v** cleaved from insulin B chain (**i**), between amino acids R22 and G23. The presence of peptides with these termini in digests containing E64-inhibited *Der p I*, and their absence in APMSF-inhibited digests, confirms that the cleavage site between amino acids R22 and G23 is due to the serine proteinase activity of *Der p I*.

Although E64 inhibits cleavage of insulin B chain between amino acids E13 and A14, inhibition is clearly not complete. However, examination of the relative sizes of the peaks representing peptides **iii** and **iv** in the presence of E64 with those produced in the absence of inhibitors and in the presence of APMSF (compare Fig. 1d with 1b and 1c), clearly demonstrates that in the presence of E64, cleavage between E13 and A14 is very inefficient. When combined with data showing very efficient cleavage between E13 and A14 in the presence of APMSF, this confirms that it is the cysteine proteinase activity of *Der p I* that cleaves insulin B chain between amino acids E13 and A14.

Inefficient cleavage by E64-inhibited *Der p I* between amino acids E13 and A14, generated three additional peptides **xiv**, **xi** and **ii**. In a similar manner to the additional peptides found in the uninhibited reaction products, these peptides appeared to have truncated termini and may represent degradation products of other peptides.

Additional cleavage sites

In the E64-inhibited digestion, an additional pair of peptides were produced by cleavage between Y16 and L17 (peptides **xii** and **xv**); whilst in the APMSF-inhibited digestion, an additional cleavage took place between L17 and Y16 to generate peptides **ix** and **x**. All four additional peptides, however, were not present in the uninhibited digest, and their presence in the digestion products is not simple to interpret.

In the APMSF-inhibited digest, a peptide of insulin B chain with L17 at the amino terminus was observed (peptide **viii**). This peptide was unpaired, however, and it most likely represents a degradation product of peptide **x**. It is intriguing to suggest that the presence of potentially degraded peptides, particularly in the uninhibited and E64-inhibited digests (peptides **ii**, **vi**, **xi** and **xiv**), may indicate that *Der p I* might also exhibit an additional serine-like exopeptidase activity upon some of the fragments generated by the endopeptidase activities.

Discussion

Studies using class-specific proteinase inhibitors and active-site affinity chromatography show that *Der p I* exhibits an unusual mixed cysteine-serine protease activity. Several other groups have purified *Der p I* and demonstrated sensitivity to cysteine proteinase inhibitors but none have sought evidence for additional mechanistic classes of activity. In addition to our observations with *Der p I*, a fungal proteinase [17] and a proteasome [18] have been reported to have mixed mechanistic class. However, unlike the multi-subunit proteinases, which possess more than one active site, there is considerable evidence to suggest that *Der p I* adopts an essentially conventional, papain-like structure with a single active site [10]. The gene encoding *Der p I*, however, is polymorphic [19], and although the polymorphisms identified do not directly affect the thiolate/imidazolium ion pair at C34/H170, it is possible that they may conformationally alter, and thus influence the function of the enzyme.

Our earlier studies showed that cleavage of a fluorogenic substrate, FITC-casein, by *Der p I* was only partially inhibited by the cysteine and serine proteinase inhibitors E64 and APMSF. By only measuring the *amount* of cleavage, however, this approach was unable to determine whether the inhibitors used were targeting exactly the same proteolytic

mechanism. The current investigation has resolved this problem by analysing the cleavage site specificity of the two mechanistic classes of proteinase observed, and finding them to be different. Partial inhibition of *Der p*I by E64 and APMSF may be due to there being two separate proteinase activities associated with enzymes identified immunologically as *Der p*I.

This analysis has also identified that the P1 position specificity [20], of the cysteine and serine proteinase activity of *Der p*I is for glutamic acid (E13 in insulin B chain), and arginine (R22 in insulin B chain) respectively. Interestingly, two other cysteine proteinases, papain and cathepsin B, also cleave insulin B chain between E13 and A14, and trypsin, a serine proteinase, cleaves between R22 and G23 [16].

Knowledge of the cleavage site specificity of *Der p*I, almost certainly excludes the possibility that *Der p*I represents a novel mechanistic class of proteinase. Given the structural evidence that *Der p*I has only one active site, our results may be explained by the *Der p*I proteinase existing in more than one isoform, as reported for other allergens [21] with the variant amino acids affecting proteolytic activity. If, as several authors suggest, the proteinase activity of *Der p*I is important in the allergenicity of the protein, this may have important implications in determining how hazardous each form of *Der p*I may be as an indoor pollutant. The possibility that *Der p*I exists in more than one form means it is now important to characterize each of the forms and determine their ability to disrupt respiratory epithelial surfaces the IgE network, and provoke allergic IgE responses.

Acknowledgements

We thank Dr Barbara J. Hart of the Royal Agricultural College, Cirencester, UK, for helpful discussions and cultured mites, Dr Chang Kee Lim of the Medical Research Council Toxicology Unit at the University of Leicester, for advice concerning mass spectrometry, and Dr Rose A. Maciewicz of ZENECA Pharmaceuticals, for discussions on the mechanisms of cysteine proteinases.

C. R. A. Hewitt and H. Horton are grateful to the Medical Research Council and the Centre for Mechanisms of Human Toxicity for funding this work. R.M. Jones is supported by the Medical Research Council Toxicology Unit and D. I. Pritchard is funded by the Wellcome Trust.

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