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Faecally Derived Hydrolytic Enzymes from *Dermatophagoides pteronyssinus*: Physicochemical Characterisation of Potential Allergens

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Abstract. The previous findings that the group I and III mite allergens, and amylase present in mite faeces are hydrolytic enzymes has prompted a study to determine whether this material contains other enzymes which could be allergenic. Thus, spent growth medium devoid of whole *Dermatophagoides pteronyssinus* mites was shown to contain glucoamylase, lipase and lysozyme in addition to the cysteine protease, serine protease and amylase activities associated with the above allergens, respectively. All of these enzymes are probably associated with mite digestive processes. They were rapidly solubilised, heterogeneous with regard to charge (pI in the range 4–8) and demonstrated maximum biochemical activity in the neutral pH range. Three serine proteases were detected and comprised a chymotrypsin-like, a trypsin-like and an unclassified enzyme with pI of 4.1 and 5.3, 8.5 and 7.1, respectively. Only one cysteine protease was observed, which paralleled immunochemically identified *Der p* I in a variety of assays. It was shown to cleave at lysyl residues and could be inhibited by the specific cysteine protease inhibitor, E-64. The remaining serine proteases, glucoamylase, lipase and lysozyme represent potential allergens.

Introduction

Allergy to the house dust mite has been shown to be associated with both childhood and adult asthma, and many studies have been devoted to the characterisation of the allergens involved. Recently, a major allergen, designated *Der p* I, from the clinically important mite *Dermatophagoides pteronyssinus* was cloned [1] and homology searches using the sequence data obtained indicated it was a protease belonging to a group of enzymes which have the amino acid residue cysteine as an integral part of their catalytic site. Purified *Der p* I was later shown to be proteolytically active [2] but only after treatment with a reducing reagent such as dithiothreitol (DTT) or cysteine, a characteristic feature of such enzymes. Subsequent analyses have demonstrated the presence of such an enzyme in house dust samples which correlated with immunochemically determined concentrations of *Der p* I [2].

More recently, N-terminal amino acid sequence analyses of the mite allergen *Der f* III from *Dermatophagoides farinae* [3] and its equivalent, *Der p* III, from *D. pteronyssinus* [2] showed that they demonstrated approximately 50% homology with the invertebrate and vertebrate proteases trypsin and chymotrypsin [2]. These enzymes belong to the group of proteases which have serine rather than cysteine in their catalytic site and are, therefore, unrelated. Further biochemical studies have suggested that proteases belonging to this class of enzymes are present not only in extracts of *D. pteronyssinus* and *D. farinae* [2, 4, 5], but also in house dust samples [2].

A variety of enzymes (including proteases) from several species of mites have been previously described. Initially, these data were derived from studies designed either to determine the degree of trophic specialization in the economically important storage mites or to study the mite digestive system per se. In this regard, extracts of *Glycophagus* spp., *Acarus* spp.

and *Tyrophagus* spp. were shown to contain proteases, esterases, lipase, glycosidases, cellulase, amylase, lysozyme and chitinase [6–11], but the determination of the allergenicities of these enzymes has not been vigorously pursued.

Limited enzymatic analyses of mites from the genus *Dermatophagoides* [12–14] have been performed and have revealed a similar array of enzymes. Subsequently, the allergenicity of a sub-group of the enzymes present in *D. pteronyssinus* was evaluated in a mite-sensitive population. However, in this study, the enzyme esterase, acid and alkaline phosphatase, aminopeptidase and a variety of glycosidases appeared to be non-allergenic [14].

Further investigation of other mite enzymes and their allergenicity seem warranted, particularly those involved in mite digestion. Such enzymes are likely to be present in mite faeces and, therefore, respirable. The studies described in this report detail enzymatic analyses of extracts prepared from material devoid of whole mite bodies, but enriched for mite faeces, as a prelude to a study of their potential allergenicity. In addition, the number and properties of the mite proteases, particularly those belonging to the serine class, were assessed, as were the distinguishing properties of mite amylase and glucoamylase.

Materials and Methods

Mite Extracts, Patient Sera and Chemicals

Spent growth medium (SGM), devoid of whole *D. pteronyssinus* mites, and unused growth medium were kindly provided by Commonwealth Serum Laboratories (Parkville, Australia). Extracts of these materials together with *Der p* I were prepared as described previously [15], except where stated. General chemicals, synthetic protease substrates, protease inhibitors and enzymes were purchased from Sigma (St. Louis, USA) except where otherwise stated.

Enzyme and Enzyme Inhibition Analyses

Protease

Non-Specific Substrates. Protease activities were determined using a non-specific, insoluble diazotized collagen substrate (Azocoll; Calbiochem, Sydney, Australia) [16]. Cysteine protease activity was determined after activating the samples with DTT. The results were expressed as OD 520 nm.

Specific Substrates. Specific tryptic and chymotryptic activities were determined using the synthetic arginine substrates N- α -benzoyl-DL-arginine *p*-nitroanalide (BAPNA) and toluenesulphonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTTEE), respectively [17]. In addition, the capacity of mite proteases to cleave at lysyl residues was assessed using N-carboxy-L-lysine nitrophenol ester (CLN) [18].

Protease Inhibitors. A variety of serine protease inhibitors were used to assess the complement of proteases in SGM. The inhibitors included aprotinin (Trasylol; Bayer, Botany, Australia), phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI) and benzamidine (which, in contrast to the former inhibitors, reacts only with trypsin-like enzymes). The capacity of the cysteine protease inhibitor, trans-epoxysuccinyl-L-leucyl amido-(4-guanidino) butane (E-64) [19], to inhibit protease activity was also investigated.

Amylase (EC 3.2.1.1)

Amylase activity was assessed using agarose containing 0.25% (w/v) soluble potato starch [11]. The activity was determined by reference to a standard curve constructed with SGM previously standardised using a commercial assay (Phadebas; Pharmacia Diagnostics, Sydney, Australia) according to the manufacturer's instructions. The results were expressed in U/l of samples or U/mg of protein.

Glucoamylase (EC 3.2.1.3)

Glucoamylase (1,4- α -D-glucan glucohydrolase) activity was determined by measuring the release of glucose from 0.6% (w/v) soluble starch [20]. The glucose was determined by the glucose oxidase method [21] using commercial reagents (Trace Scientific, Baulkham Hills, Australia). The results were expressed in units, where 1 U represented the release of 1 μ mol glucose/min/ml of sample.

Cellulase (EC 3.2.1.4)

Cellulase (1,4- β -D-glucan-4-glucanohydrolase) activity was determined by measuring the release of glucose from 40 mg/ml (w/v) microcrystalline cellulose (Merck, Darmstadt, FRG) at pH 5. Samples were incubated with cellulose and the glucose released determined by the glucose oxidase method. The results were expressed in units where 1 U represented the release of 1 μ mol glucose/min/ml of sample.

Lysozyme (EC 3.2.1.17)

Lysozyme (N-acetylmuramoylhydrolase) activity was determined using agarose gels containing *Micrococcus lysodeikticus* [22]. The concentration of lysozyme was determined by reference to a standard curve constructed using hen egg lysozyme.

Chitinase (EC 3.2.1.14)

Chitinase (poly [1,4- β -(2-acetamido-2-deoxy-D glucoside)]-glucanohydrolase) activity was measured using the insoluble substrate chitin-azure [23] suspended in 0.015 M phosphate buffer (pH 6.2). After incubation at 37 °C, the samples were centrifuged and the OD of the supernatants measured at 510 nm.

Lipase

The glycerol tributyrates-agar plate method [24] was used to determine the presence of lipase. The data were expressed as the diameter of lysis obtained in millimetres.

Determination of Enzyme pH Optima

The pH optima for mite serine and cysteine proteases were determined using the appropriate enzyme fraction isolated by Sephadex G-75 (Pharmacia) gel filtration. The pH optima for mite lysozyme (measured using a commercial assay: Testomar Lyso-

Table 1. Biochemical and physicochemical characteristics of mite enzymes

Enzyme	pH optimum	pI	MW
Cysteine protease (Der p I)	7	5.8–7.5	25,134 [1]
Serine protease (Der p III)	7–8.5	4.1–8.5	28,000–30,000
Chymotrypsin	ND	4.1–5.3	ND
Unclassified	ND	7.1	ND
Trypsin	ND	8.5	ND
Amylase	6.4	4.3–6.8	52,000
Glucoamylase	6–7	4.5–6.9	52,000
Lysozyme	6.2	4.4–>8	10,000
Lipase	ND	5.3–6.3	52,000

MW data obtained from Chua et al. [1] and this study. ND = Not determined. pH and pI data represent either discrete values or ranges.

zyme Mono; Behring, Marburg, FRG) and glucoamylase were determined using SGM.

Kinetics of Enzyme Release from SGM

Aliquots of SGM were incubated with gentle manual agitation for varying periods of time (5 s to 2 h) in the presence of 0.06 M phosphate buffer (pH 6.4) containing 0.003 M CaCl₂. Elution was stopped by immediately centrifuging the sample and removing the supernatant. The supernatants were examined for the enzyme activities described above (with the exception of lipase), and activity plotted against time. The minimum time taken for the appearance of maximum enzymic activity was then determined.

Gel Filtration Studies

SGM was fractionated by gel filtration on Sephadex G-75, and the apparent molecular weights (MW) of the enzymes determined as described [25] using the MW standards; bovine serum albumin (BSA, MW 67,000), ovalbumin (OVA, 43,000), chymotrypsinogen (25,500) and cytochrome C (11,700). Appropriate fractions were pooled, dialysed and freeze-dried.

Analytical Methods

Determination of Isoelectric Point. Isoelectric points (pI) of enzymes were determined either by chromatofocusing or by isoelectric focusing (IEF). Chromatofocusing was performed at room temperature [26, 27] and IEF was performed using a preparative electrophoresis cell (Rotaphor; Bio Rad, Richmond, USA) at 0 °C according to the manufacturer's instructions. Appropriate fractions were pooled, dialysed and freeze-dried.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-Page). SDS-Page analysis was used to determine the apparent MW of the radiolabelled proteases and was performed essentially as described [28]. The apparent MW of the proteins were determined by reference to a standard curve constructed using: BSA, OVA, glyceraldehyde-3-dehydrogenase (36,000), carbonic anhy-

dase (29,000), trypsinogen (24,000), SBTI (20,100) and lactalbumin (14,200).

Protease Labelling with 1,3-³H Di-Isopropyl Phosphofluoridate (³H-DFP). The number and apparent MW of serine proteases present in SGM were determined using ³H-DFP (Amersham, Amersham, UK), as described [29]. SGM was fractionated on Sephadex G-75 and three fractions were each labelled with 1.87 mBq namely: a sample from the ascending region of the serine protease peak, a sample from the descending region of the cysteine protease peak and a sample from a region containing both types of protease activity. The labelled proteins in each fraction were assessed by SDS-PAGE and autoradiography after processing using fluor (Amplify; Amersham).

Organomercurial Affinity Chromatography

The capacity of the enzymes to bind to an organomercurial coupled to Sepharose 4B via accessible sulphhydryl groups was determined as described [30] using SGM. Bound material was desorbed with 20 mM cysteine-HCl. All fractions were examined for enzyme activity as described above.

Immunochemical Methods

Der p I concentrations in column fractions were determined either by fused rocket immunoelectrophoresis or single radial immunodiffusion using a rabbit anti-Der p I antiserum (15).

Results

Proteases

Demonstration of Proteolytic Activity

Cysteine proteases generally require activation with a reducing reagent such as DTT before enzymic activity can be detected. The optimum concentration of DTT required for maximum cysteine protease activity expression in SGM was found to be 1.5 mM. Both cysteine and serine protease activities were demonstrable in SGM using a non-specific substrate, but neither activity was detected in unused growth medium. The pH optima for the serine and cysteine protease activities using SGM were found to be 7–8, and 7, respectively (table 1).

Characterisation of Individual Proteases

Gel Filtration Studies. SGM was subjected to Sephadex G-75 gel filtration chromatography and fractions examined for non-specific protease activity as well as specific, trypsin- and chymotrypsin-like activities in the presence or absence of DTT. In the absence of DTT, a single, gaussian peak of general protease activity was demonstrated which eluted with an apparent MW of approximately 25,000 (fig. 1). This peak corresponded with the proteolytic activity de-

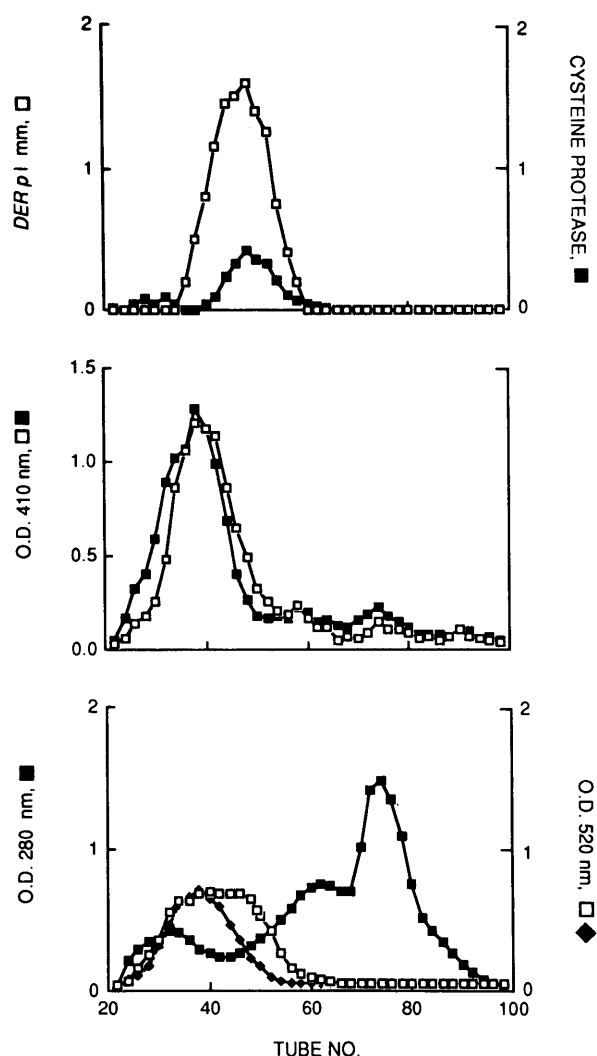


Fig. 1. Sephadex G-75 gel filtration fractionation of proteases present in SGM performed in 0.05 M sodium acetate buffer (pH 5.5). The bottom panel shows the protein elution profile determined at 280 nm and the non-specific proteolytic activity profiles determined before (◆) and after (□) activation with DTT. The middle panel illustrates the proteolytic activity determined using the synthetic arginine substrate BAPNA before (■) and after (□) activation with DTT. The top panel shows the location of *Der p* I as determined by fused rocket immunoelectrophoresis and cysteine-dependent proteolytic activity determined by subtraction of the non-cysteine-dependent activity from the combined activity.

tected when using both a trypsin substrate, BAPNA (fig. 1) and a chymotrypsin substrate, BTEE (data not shown). In the presence of DTT, a broader peak of protease activity was observed which encompassed not only that previously detected in the absence of DTT, but also activity associated with material eluting

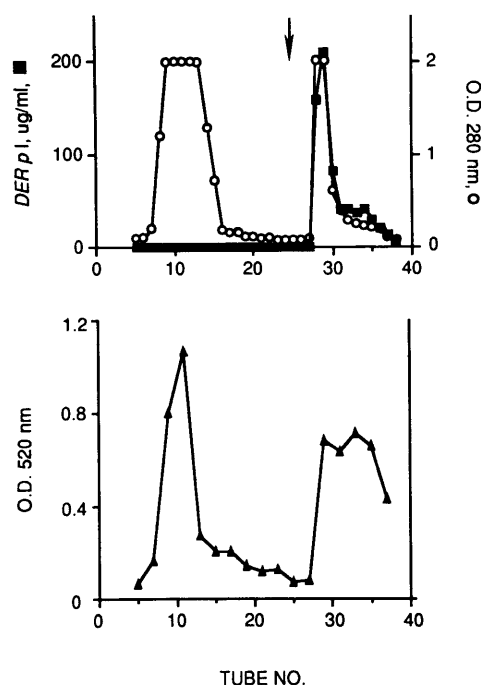


Fig. 2. Organomercurial Sepharose 4B affinity chromatography of proteases from SGM. The arrow indicates the application of 20 mM cysteine to elute bound material. The top panel shows the protein absorption at 280 nm and the concentration of *Der p* I measured by single radial immunodiffusion. The lower panel shows the protease activity determined using the insoluble substrate, Azocoll. The protease activity in the unbound fractions was determined without prior activation whereas that in the bound fractions represents activated protease due to the cysteine in the elution buffer.

with an apparent lower MW (approximately 12,000). This extension to the initial peak of protease activity was not detected using the specific trypsin and chymotrypsin substrates. Immunochemical studies showed the cysteine protease activity in each fraction paralleled the presence of *Der p* I (fig. 1).

Organomercurial Affinity Chromatography. An organomercurial affinity matrix was used to isolate the cysteine protease(s) from SGM. A protease(s) bound to the column and was only eluted in the presence of a reducing reagent (fig. 2). Immunochemical studies showed that this activity paralleled the presence of *Der p* I (fig. 2). The activity in the unbound fraction was found to be associated with serine proteases.

Protease Inhibitor Studies. The specific serine protease inhibitors aprotonin, PMSF and SBTI were shown to completely inhibit the proteolytic activity in SGM with the exception of the cysteine protease, but the maximum inhibition achieved with the specific trypsin inhibitor benzamidine was only half that achieved with the more general serine protease inhibitors. The minimum concentrations of each inhibitor required to effect maximum inhibition were 0.2 μ M, 4 mM, 0.3 μ M and 5 mM, respectively. The specific cysteine protease inhibitor, E-64, completely inhibited the cysteine protease activity associated with purified *Der p* I (at 1 μ M), but had no effect on serine protease activity.

IEF Studies. IEF of the proteases over the pH range 3–10 demonstrated four peaks of protease activity under non-reducing conditions, with pI ranging from 4.1 to 8.5 (fig. 3, table 1). When specific substrates were used, chymotrypsin- and trypsin-like activities with pI of 4.1 (major) and 5.3 (minor) and 8.5, respectively, were found (fig. 3, table 1). In addition to these proteases, a further serine protease was detected which was neither chymotrypsin- nor trypsin-like (pI 7.1, table 1). When cysteine protease assays were performed, three peaks of protease activity were detected with pI in the range of 5.8–7.5. The major cysteine protease activity was found to have a pI of 6.4 (fig. 3). Further assays showed that the cysteine protease cleaved at lysyl residues (fig. 3) and immunochemical studies showed that the cysteine protease activity identified using either substrate paralleled the presence of *Der p* I (fig. 3).

Protease Labelling with ^3H -DFP

Equal volumes of serine and cysteine protease fractions (with comparable protease activity), together with a mixture of both, were labelled with ^3H -DFP. The radioactivities (cpm) for a 5- μ l aliquot of each fraction were 2920, 3 and 437, respectively. The apparent MW of the radiolabelled serine proteases were determined following SDS-PAGE and autoradiography. Five bands with apparent MW of 15,000; 18,000;

28,000; 30,000 and 31,000 were observed, although the first two bands were broad and faint. A similar distribution was seen for the mixture of proteases, but bands were absent from the cysteine protease fraction.

Carbohydrases

Amylase, Glucoamylase and Cellulase. Of these enzymes, only amylase and glucoamylase activities were found, but since it is known that both enzymes can release glucose from starch substrates, it was possible that the glucoamylase activity was due to amylase activity per se. Physicochemical studies were performed to distinguish between the two enzymes.

Both enzymes were similar with regard to pH optima (6–7; table 1) and apparent MW (52,000; table 1), but other physicochemical studies indicated that the two enzymes were separate entities. Thus, the bulk of amylase activity was precipitated in the 0–60% saturated ammonium sulphate fraction, whereas the bulk of the glucoamylase activity was precipitated in the 60–80% saturated fraction. Similarly, chromatofocusing (table 1) showed that whilst both enzymes were heterogeneous with regard to charge, the pI of the major peaks of amylase and glucoamylase activity were 4.8 and 5.4, and 6.3 and 6.9, respectively. Further studies showed that amylase, in contrast to glucoamylase, bound to an organomercurial matrix (fig. 4), a finding in accord with the known properties of other amylases [31].

Lysozyme and Chitinase. Of these enzymes, only lysozyme was detected, and it was shown to have a pH optimum of 6.2 (table 1). It was precipitable with ammonium sulphate in the range 50–80% saturation and was shown to have an apparent MW of 10,000 (table 1). The pI was determined by chromatofocusing, and the enzyme was found to be heterogeneous with regard to charge (range 4–>8; table 1) with the major peaks of enzymatic activity eluting with pI of 7.9 and >8. The majority of the enzyme did not bind to an organomercurial affinity matrix (fig. 4).

Lipase

Lipase was found in SGM and shown to have an apparent MW of 52,000 (table 1) and demonstrated charge heterogeneity by chromatofocusing (range 5–7; table 1). The pI of the major peaks of enzymatic activity were found to be 5.3, 5.7 and 6.1. The ability of lipase to interact with the organomercurial affinity matrix was not investigated.

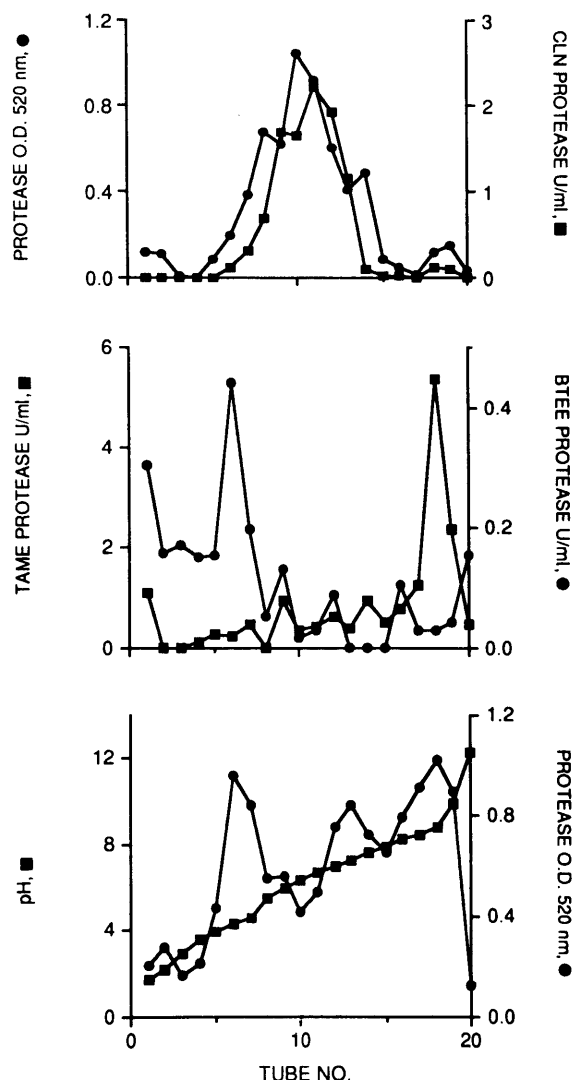


Fig. 3. IEF analysis of the proteases present in SGM over the pH range 3–10. The bottom panel shows the non-cysteine-dependent proteolytic activity obtained using the non-specific substrate Azocoll and the pH profile. The middle panel shows the non-cysteine-dependent proteolytic activity detected using the trypsin arginine substrate TAME and the chymotrypsin tyrosine substrate BTEE. The top panel shows the cysteine-dependent proteases detected using Azocoll determined by subtracting the OD values obtained under non-reducing conditions from those obtained under reducing conditions. This panel also shows the proteolytic activity obtained using the synthetic lysine substrate CLN under reducing conditions.

Release of Enzymes from Mite Faecal Pellets

Experiments were performed to determine the time taken for cysteine and serine protease, amylase, glucoamylase and lysozyme to be eluted from unex-

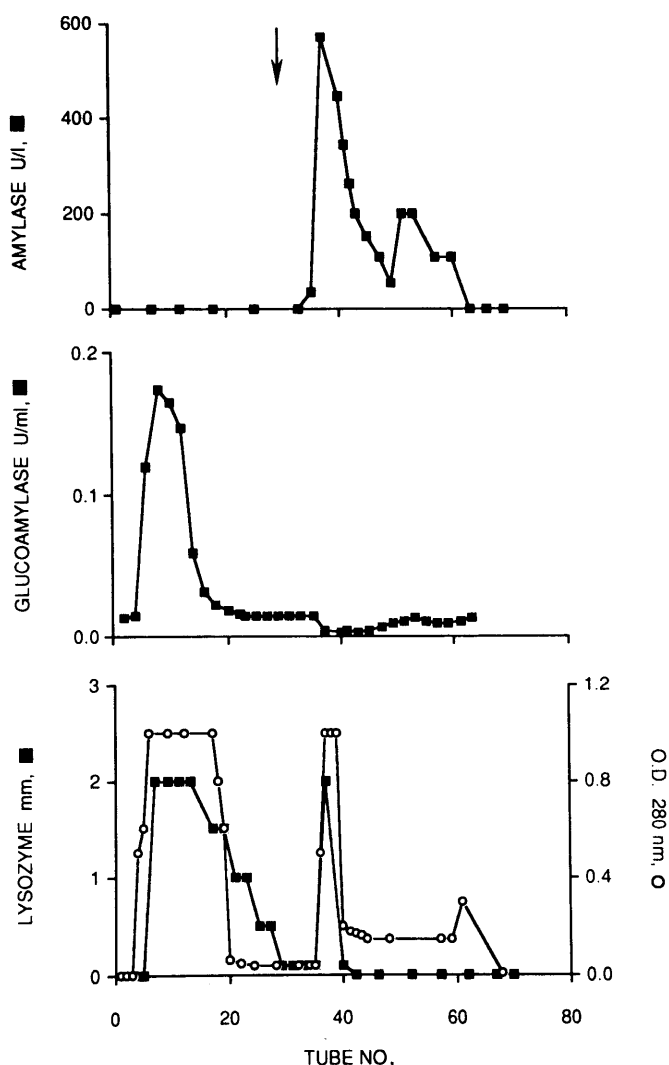


Fig. 4. Organomercurial Sepharose 4B affinity chromatography of amylase (top panel), glucoamylase (middle panel) and lysozyme (bottom panel) from SGM. The arrow indicates the application of 20 mM cysteine to elute bound material. Fractions were examined for enzyme activity and protein as described in the text.

tracted SGM on the assumption that these enzymes were present solely in the faecal pellets. The data obtained indicated that all of the enzymes were released rapidly and that maximum activity was observed after 40–60 s extraction.

Discussion

Analysis of *D. pteronyssinus* SGM revealed the presence of a wide range of enzymes including amylase, glucoamylase, cysteine and serine proteases, lipase and lysozyme. Similar findings have been reported for a variety of other mites [4–14], but the present study extends these observations by demonstrating that these enzymes are also present in mite faeces and are, presumably, of gut origin. As faecal pellets represent a significant source of allergen including the cysteine protease *Der p* I [1, 2, 14, 32, 33] and are thought to be the vehicle via which most mite allergens enter the respiratory system [32, 33], the detection of other rapidly eluting enzymes in such particles suggests these too may be allergenic in susceptible individuals.

The proteases present in SGM were studied in some detail in an attempt to determine their diversity and specificity prior to assessing their allergenicity. In this regard, only one MW species of cysteine protease was detected, and this paralleled immunochemically detected *Der p* I with regard to MW and pI. The synthetic substrate data indicated that *Der p* I cleaves proteins or peptides at lysyl residues, but not at arginyl or tyrosyl residues, findings consistent with the known behaviour of other cysteine proteases from diverse sources [18]. It is probable that *Der p* I represents a digestive enzyme although the presence of cysteine proteases in the alimentary tract of invertebrates is rare [34]. Teleologically, however, it may reflect the presence of a reducing environment in the gut which not only contributes to the disruption of disulphide bonds in the keratin present in skin squames, a major food source for this arachnid, thus facilitating digestion but also to the activation of the enzyme per se.

At least three serine proteases were detected comprising a trypsin- and chymotrypsin-like enzyme, and a, thus far, unclassified serine protease with pI of 8.5, 4.1 and 7.1, respectively. Although the actual apparent MW of the individual proteases were not ascertained, SDS-PAGE and gel filtration studies indicated that they were in the region of 30,000, findings consistent with those described for the *D. farinae* allergen, *Der f* III [3] which, on the basis of N-terminal sequence studies, was shown to be a serine protease showing approximately 50% homology with both trypsin and chymotrypsin [2, 3].

SGM was also examined for the presence of carbohydrases, and in this regard, amylase, glucoamylase

and lysozyme were detected. The finding that amylase was present in mite extracts is consistent with earlier observations which showed that it was present in several diverse species of mites [6, 8, 11, 12], although to our knowledge, glucoamylase has not been previously reported. Both amylase and glucoamylase are known to hydrolyse starch to release glucose but data from several physicochemical studies indicated that they were separate entities.

The demonstration of lysozyme in SGM is consistent with data reported for extracts prepared from storage mites [10]. In many vertebrate and invertebrate species, this enzyme is associated with defence against gram-positive bacteria where it hydrolyses β -(1-4) glycosidic bonds between N-acetyl-D-glucosamine and N-acetyl-muramic acid in bacterial cell wall peptidoglycan. Recently, however, it has been implicated in invertebrate digestion [35], and it is feasible that the enzyme performs a similar function in mites. In *D. pteronyssinus*, lysozyme was shown to be of similar size to lysozymes from both insects, mammals and birds, but chromatofocusing data showed that the mite enzyme was not as negatively charged. In addition, and in contrast to other lysozymes, the mite enzyme did not possess chitinase activity.

Of particular importance to the study of mite allergy is to determine which of the enzymes present in mite faeces are allergenic. Studies from our laboratory indicate that not all enzymes are allergens [14] but it has been clearly shown that the cysteine protease and at least one of the serine proteases are allergenic and, in addition, recent immunoblotting studies showed that purified mite amylase is also allergenic [36]. The allergenicities of the remaining serine proteases, glucoamylase, lipase and lysozyme is unclear, but empirical analyses of data from mite immunoblotting studies indicate the existence of allergens with MW [37, 38] similar to those reported for these enzymes. In addition, preliminary, unpublished studies using specific affinity matrices suggest that both mite trypsin and a mite lysozyme rich fraction are allergenic in radioallergosorbent assays. Interestingly, mite lysozyme shares several properties with the allergen *Der p* II, such as MW, basic pI and precipitation between 50–80% ammonium sulphate saturation, and further investigations are being pursued to see if the two components are related.

It has been shown that cysteine and serine proteases, amylase, lysozyme and amylase from animal, bacterial and fungal sources are important allergens

in the context of occupational asthma. It is not known whether this reflects increased exposure to these agents or, alternatively, reflects both increased exposure to agents which are not only allergenic, but also biochemically active. It would now appear that mite faecal pellets contain a similar array of enzymes, a significant number of which are allergenic, and it is feasible that other enzymes contained therein will be shown to be allergenic. Combined, these data raise the possibility that, in certain circumstances, there may be an association between enzyme activity and allergenicity.

The possible nature of this association is unclear but recent *in vitro* studies from our laboratory have shown that the cysteine protease activity of the allergen *Der p* I can induce epithelial permeability in a bovine lung epithelial model [39] indicating that, for example, the proteolytic nature of an allergen could contribute to the sensitisation process *in vivo* by facilitating passage of the allergen (and perhaps bystander molecules) across epithelial barriers. Further investigation of the allergenic activity of enzymes and the enzymatic activity of particular allergens seems warranted.

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References

- Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM: Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p* I. Homology with cysteine proteases. *J Exp Med* 1988;167:175-182.
- Stewart GA, Thompson PJ, Simpson RJ: Protease antigens from house dust mite. *Lancet* 1989;ii:154-155 and correction p 462.
- Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TAE: Antigenic and structural analysis of group II allergens (*Der f* II and *Der p* II) house dust mites (*Dermatophagoides* spp). *J Allergy Clin Immunol* 1989;83:1055-1067.
- Ino Y, Ando T, Haida M, Nakamura K, Iwaki M, Okudaira H, Miyamoto T: Characterisation of the proteases in the crude mite extract. *Int Arch Allergy Appl Immunol* 1989;89:321-326.
- Takahashi K, Aoki T, Kohmoto S, Nishimura H, Koderia Y, Matsushima A, Inada Y: Activation of kallikrein system in human plasma with purified serine protease from *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* 1990;91:80-85.
- Hughes TE: The physiology of the alimentary canal of *Tyroglyphus farinae*. *Q J Microsc Sci* 1950;91:45-61.
- Matsumoto K, Shirasaka R, Anbe K: Digestive enzymes in grain mites. *Jpn J Sanit Zool* 1964;15:118.
- Akimov IA, Barabanova VV: Deyaki osoblyvosti travlennya klitsiv *Cosmoglyphusabsoloni* Samsinar, 1961 i *Coleochaeta molitor* Volgin et Akimov, 1975. *Dopov Akad Nauk Ukr RSR Ser B* 1976;7:643-644.
- Bowman CE: Hide protease in stored produce mites (Astigmata: Acaridae). *Comp Biochem Physiol* 1981;70B:803-805.
- Childs M, Bowman CE: Lysozyme activity in six species of economically important Astigmatid mites. *Comp Biochem Physiol* 1981;70B:615-617.
- Bowman CE, Childs M: Polysaccharidases in Astigmatid mites (Arthropoda: Acari). *Comp Biochem Physiol* 1982;72B:551-557.
- Barabanova VV, Zheltikova TM: Digestive enzymes of house dust mites *Dermatophagoides pteronyssinus* Trouessart, 1897 and *D. farinae* Hughes, 1961 (Acariformes: Pyroglyphidae). *Dokl Akad Nauk SSSR* 1985;283:225-227.
- Bousquet J, Hale R, Guerin B, Michel FB: Enzymatic activities of house dust extracts. *Ann Allergy* 1980;45:316-321.
- Stewart GA, Butcher A, Lees K, Ackland J: Immunochemical and enzymatic analyses of extracts of the house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1986;77:14-24.
- Stewart GA: Isolation and characterization of the allergen Dpt 12 from *Dermatophagoides pteronyssinus* by chromatofocusing. *Int Arch Allergy Appl Immunol* 1982;69:224-230.
- Chavira R, Burnett TJ, Hageman JH: Assaying proteinases with Azocoll. *Anal Biochem* 1984;136:446-450.
- Hummel BCW: A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. *Can J Biochem* 1959; 37:1393-1399.
- Bajkowski AS, Frankfater A: Specific spectrophotometric assays for cathepsin B1. *Anal Biochem* 1975;68:119-127.
- Hanada K, Tamai M, Yamagishi M, Omura S, Sawada J, Tanaka I: Isolation and characterization of E-64, a new thiol protease inhibitor. *Agric Biol Chem* 1978;42:523-536.
- Bhella RS, Altosaar I: Large scale purification of fungal glucoamylase using ion-exchange resin chromatography. *Anal Biochem* 1984;140:200-202.
- Trinder P: Determination of blood glucose using 4-aminophenazone as oxygen acceptor. *J Clin Pathol* 1969;22:246.
- Osserman EF, Lawlor DP: Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J Exp Med* 1966;124:921-951.
- Hackman RH, Goldberg M: New substrates for use with chitinases. *Anal Biochem* 1964;8:397-401.
- Willis AT: The lipolytic activity of some Clostridia. *J Pathol* 1960;80:379-390.
- Andrews P: Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem J* 1964;91:222-233.
- Hearn MTW, Lyttle DT: Buffer-focusing chromatography using multicomponent electrolyte elution systems. *J Chromatogr Biomed* 1981;218:483-495.

- 27 Stewart GA, Holt PG: Immunogenicity and tolerogenicity of a major house dust mite allergen, *Der p* I from *Dermatophagoides pteronyssinus*, in mice and rats. *Int Arch Allergy Appl Immunol* 1987;83:44-51.
- 28 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
- 29 Verwaerde CV, Auriault C, Neyrinck JL, Capron A: Properties of serine proteases of *Schistosoma mansoni* schistosomula involved in the regulation of IgE synthetase. *Scand J Immunol* 1988;27:17-24.
- 30 Sluyterman LAA, Widjenes J: An agarose mercurial column for the separation of mercaptopyrin and non-mercaptopyrin. *Bochim Biophys Acta* 1970;200:593-595.
- 31 Karn RC, Malacinski GM: The comparative biochemistry, physiology and genetics of animal alpha-amylases. *Adv Comp Physiol Biochem* 1978;7:1-103.
- 32 Tovey ER, Chapman MD, Platts-Mills TAE: Mite faeces are a major source of house dust allergens. *Nature* 1981;289:592-593.
- 33 Platts-Mills TAE, Chapman MD: Dust mite: Immunology, allergic disease and environmental control. *J Allergy Clin Immunol* 1987;80:755-775.
- 34 Houseman JG, Downe AER: Identification and partial characterisation of digestive proteinases from two species of bedbug (Hemiptera: Cimicidae). *Can J Zool* 1982;60:1837-1840.
- 35 Terra WR, Espinoza-Fuentes FP, Ferreira C: Midgut amylase, lysozyme, aminopeptidase and trehalase from larvae and adults of *Musca domestica*. *Arch Insect Biochem Physiol* 1988;9:283-297.
- 36 Lake FR, Thompson PJ, Ward LD, Simpson RJ, Stewart GA: House dust mite derived amylase: Allergenicity and physico-chemical characterisation. *J Allergy Clin Immunol* 1991, in press.
- 37 McWilliam AS, Stewart GA: Production of multilamellar, small unilamellar and reverse-phase liposomes containing house dust mite allergens. Potential adjuvants in the immunotherapy of allergic disease. *J Immunol Methods* 1989;121:53-60.
- 38 Tovey ER, Baldo BA: Comparison by electroblotting of IgE-binding components in extracts of house dust mite bodies and spent mite culture. *J Allergy Clin Immunol* 1987;79:93-102.
- 39 Herbert CA, Holgate ST, Robinson C, Thompson PJ, Stewart GA: The effect of mite allergen on the permeability of the bronchial mucosa. *Lancet* 1990;ii:1132.

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