

ACARI 641

A comparative study of allergenic and potentially allergenic enzymes from *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*

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ABSTRACT

Stewart, G.A., Bird, C.H., Krska, K.D., Colloff, M.J. and Thompson, P.J., 1992. A comparative study of allergenic and potentially allergenic enzymes from *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*. *Exp. Appl. Acarol.*, 16: 165–180.

The presence of the enzymatically active allergens equivalent to *Der p* I (cysteine protease), *Der p* III (serine protease) and amylase in extracts of *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei* was determined using appropriate enzymatic techniques. Biochemical equivalents of all three allergens were present in each extract studied. Studies also showed that the mite extracts contained a variety of other biochemically active enzymes including trypsin, chymotrypsin, carboxypeptidase A and B, glucoamylase and lysozyme. Marked differences in the relative concentrations of some of these enzymes in different mite extracts were observed, particularly trypsin and carboxypeptidase A. The enzymes were physicochemically similar to equivalent enzymes from vertebrate and invertebrate sources. Chromatofocusing studies of faecal extracts derived from *D. pteronyssinus* and *D. farinae* showed that several isoforms of each enzyme were present. The data indicated that there were more trypsin isoforms, with pI over a wider range, in extracts prepared from *D. pteronyssinus*. Proteases and carbohydrases were also found in extracts prepared from faecally enriched material suggesting that they were endoperitrophic and associated with mite digestion. The data suggest that not only are the group I, III and amylase allergens a consistent feature of most pyroglyphid dust mites but also that other proteases and carbohydrases present in mite faeces are allergenic.

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INTRODUCTION

Much effort has recently been devoted to the characterisation of the allergens of the house dust mite since it has been clearly shown that they are associated with both childhood and adult asthma (reviewed in International Workshop Report, 1988). These studies have culminated in the molecular cloning of the immunologically cross-reacting allergens designated group I (e.g. *Der p* I, *Der f* I) and group II (e.g. *Der p* II, *Der f* II) from mites of the genus *Dermatophagoides* (Chua et al., 1988; Simpson et al., 1989; Yuuki et al., 1990; Dilworth et al., 1991; Trudinger et al., 1991) and the group I allergen from *Euroglyphus maynei* (Kent et al., 1990). They have also shown that the group I allergens are proteases belonging to a family of enzymes which have a cysteine residue as an integral part of their catalytic site (Chua et al., 1988; Stewart et al., 1989).

The presence of a proteolytically active allergen in mite faeces at high concentration (Tovey et al., 1981; Stewart et al., 1988) suggests that it is intimately involved in mite digestive processes, the corollary of which is that other faecal enzymes may also be allergenic. In support of this, amino acid sequence data indicate that the mite group III allergens (e.g. *Der p* III and *Der f* III) may be analogous to the serine proteases trypsin or chymotrypsin (Heymann et al., 1989; Stewart et al., 1989). In this regard, biochemical studies have shown that mite extracts contain an array of serine proteases including trypsin- and chymotrypsin-like enzymes (Stewart et al., 1988, 1990; Ino et al., 1989; Takahashi et al., 1990) and an, as yet, uncharacterised protease (Stewart et al., 1991). Recent studies have shown that mite amylase from faecal extracts of *D. pteronyssinus* is also allergenic (Lake et al., 1991).

In addition to the above allergenic enzymes, faecally enriched extracts from *D. pteronyssinus* have also been shown to contain a variety of other digestive enzymes including glucoamylase, lipase and lysozyme (Stewart et al., 1990, 1991). The allergenicity of these enzymes remains to be determined but they must be considered potentially allergenic since they are present in mite faeces which are respirable and they have all been shown to be similar to the allergenic mite enzymes previously described with regard to solubility and isoenzyme complexity (Stewart et al., 1991). In addition, Western blotting studies indicate that allergens with molecular weights similar to the above enzymes are present in mite extracts (Tovey and Baldo, 1987; Baldo et al., 1989).

On the basis of the above observations, comparative enzymatic analyses of some of the major, clinically important mites *D. pteronyssinus*, *D. farinae* and *Euroglyphus maynei* were performed, with particular emphasis on allergens which have previously been shown to be enzymatically active. In addition, the physicochemical properties and the range of other potentially allergenic proteases and carbohydrases which are present in mite extracts were assessed.

MATERIALS AND METHODS

Mite extracts and chemicals

Extracts of *D. pteronyssinus* were prepared from both whole mites (WME) and spent growth medium devoid of whole mites (SGM), *D. farinae* from SGM and *E. maynei* from medium and mites combined. An extract of fresh medium (UGM) used in the culture of *D. pteronyssinus* and *D. farinae* was also examined as a control. The medium used in the preparation of *E. maynei* was unavailable. SGM and *E. maynei* were extracted using 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl; and *D. pteronyssinus* WME was prepared using 0.05 M ammonium carbonate. General chemicals and synthetic protease substrates were purchased from Sigma Chemical Co. Ltd. (St Louis, USA) except where otherwise stated.

*Protease analyses**Non-specific substrates*

Serine and cysteine protease activities were determined using a non-specific, insoluble diazotized collagen substrate (Azocoll) as described (Stewart et al., 1989) or azocasein (Tomarelli et al., 1949). The results were expressed in units (U), where 1 U represented the optical density (520 and 405 nm respectively) $\times 1000$ /incubation time in min $\times$ protein concentration in mg. In addition to these assays, the casein clotting activity of the extracts was also determined (Foltmann et al., 1985). In brief, samples were placed in wells cut into agarose containing 1% (w/v) commercial skimmed milk powder and the plates incubated at room temperature overnight. The diameters of the zones of clotting were measured and activity expressed in U/mg protein after reference to a standard curve constructed using a *D. pteronyssinus* SGM extract containing a previously defined number of casein clotting U. Cysteine protease activity was also determined using both Azocoll and azocasein. The mite samples were activated with 5 mM dithiothreitol as described (Stewart et al., 1991) and the cysteine protease activity determined by subtracting the U of activity obtained in the absence of reducing reagent from the U obtained in its presence.

Specific substrates

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) esterase activities were determined using toluene sulphonyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) respectively (Hummel, 1959). Trypsin amidase activity was measured using *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) (Erlanger et al., 1961) in 96-well microtitre plates. Carboxypeptidase A and B activities were determined using hippuryl-L-phenyl-

alanine and hippuryl-L-arginine respectively as described in Worthington (1982). Data obtained using all substrates were expressed in milliU, where 1 mU represented the amount of substrate in nmoles hydrolysed/min/mg protein.

Carbohydrase analyses

Amylase (EC 3.2.1.1)

Amylase activity was assessed using agarose containing 0.25% (w/v) soluble potato starch as described (Bowman and Childs, 1982; Lake et al., 1991). The activity was determined by reference to a standard curve constructed with *D. pteronyssinus* SGM previously standardised using a commercial assay (Phadebas; Pharmacia Diagnostics, Sydney, Australia) according to the manufacturer's instructions. The results were expressed in U/mg of protein.

Glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3)

Glucoamylase activity was determined by measuring the release of glucose from 0.6% (w/v) soluble starch as described by Stewart et al. (1991). The glucose was determined by the glucose oxidase method (Trinder, 1969) using commercial reagents (Trace Scientific, Baulkham Hills, Australia). The results were expressed in U, where 1 U represented the release of 1 μ mole of glucose/min/mg of protein.

Lysozyme (N-acetylmuramoylhydrolase, EC 3.2.1.17)

Lysozyme activity was determined using a modification of the lysis of *Micrococcus lysodeikticus* assay (Osserman and Lawlor, 1966; Stewart et al., 1991). In brief, 50 μ l of test sample were added to 200 μ l of bacterial suspension (200 μ g/ml in 0.01 mol/l phosphate buffer, pH 6.2, containing 500 μ g/ml bovine serum albumin) in 96-well microtitre plates. The OD at 450 nm was determined at the beginning of the assay and the plate incubated at room temperature for 12 min. The decrease in OD was then determined and the data expressed in U, where 1 U represented the decrease in OD $\times$ 1000/min/mg protein.

Physicochemical characterisation of enzymes

Determination of isoelectric point

The isoelectric points (pI) of individual enzymes in SGM extracts of the mites from the genus *Dermatophagoides* were determined by chromatofocusing at room temperature as described (Hearn and Lyttle, 1981; Lake et al., 1991). Insufficient material precluded a similar analysis of *E. maynei*. Appropriate fractions were collected and analysed for enzyme activity as described above.

Protein determination

The protein concentrations of individual samples were determined using the Lowry modification of the Folin assay as described (Peterson, 1983) using bovine serum albumin as the standard.

Gel-filtration studies

These studies were performed only on SGM extracts from mites of the genus *Dermatophagoides* due to insufficient *E. maynei* material. The extracts were fractionated on a column (1 × 52 cm) of Sephadex G-75 equilibrated with 0.1 M acetate buffer, pH 4.5. The apparent molecular weights (mol wt) of the enzymes were determined as described (Andrews, 1964) using the mol wt standards: ovalbumin (mol wt 43 000), chymotrypsinogen (25 500) and cytochrome C (11 700).

RESULTS

General proteolytic activity

Protease activity was demonstrable in the four mite extracts studied using non-specific substrates derived from collagen and casein (Table 1). In each

TABLE 1

Proteases detected in mite extracts

Enzyme or substrate hydrolysed	Enzyme activity in Units ^a /mg protein			
	<i>D. pteronyssinus</i> ^b	<i>D. pteronyssinus</i> ^c	<i>D. farinae</i> ^c	<i>E. maynei</i> ^d
<i>Serine proteases</i>				
Non-specific substrates				
Azocoll	3.67	7.97	4.80	0.72
Azocasein	13.80	108.80	51.70	5.90
Casein (clotting)	0.30	11.39	6.41	0.83
Specific substrates				
TAME (trypsin)	0	390	90	20
BAPNA (trypsin)	0	5.23	4.19	0
BTEE (chymotrypsin)	210	220	90	190
<i>Cysteine protease</i>				
Azocoll	3.25	3.14	1.48	0.91
Azocasein ^e	13.25	-35.2	-13.0	1.02
<i>Carboxypeptidases</i>				
A	50	0	0	0
B	40	130	240	0

^aSee Methods Section for the definition of units. ^bWhole-mite extract. ^cSpent growth medium extract.

^dGrowth medium and whole-mite extract. ^eThe negative numbers obtained indicate a diminution of protease activity on activation with reducing reagent. Unused growth medium was used as a control in all enzymatic assays.

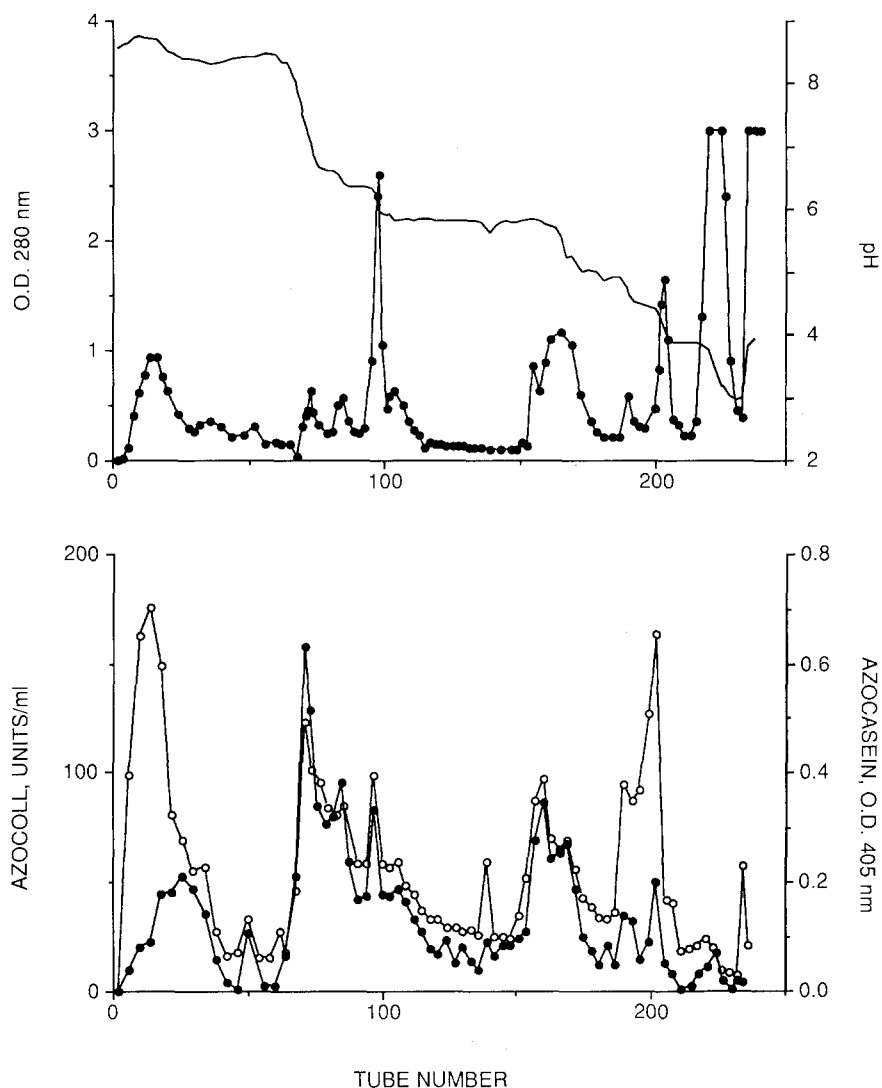


Fig. 1. Chromatofocusing of *D. pteronyssinus* spent growth medium extract on an anion exchange matrix over the pH range 3 to 8.5. The top panel shows the protein elution profile determined at 280 nm (●) and the pH gradient (—) obtained. The bottom panel shows the protease elution profile obtained using the non-specific protease substrates Azocoll (●) and azocasein (○).

case, the rank order of activity was *D. pteronyssinus* SGM > *D. farinae* SGM > *D. pteronyssinus* WME > *E. maynei*. In addition to the degradative processes seen with Azocoll and azocasein, each extract was shown to possess casein clotting activity with a rank order of potency of *D. pteronyssinus* SGM > *D. farinae* SGM > *E. maynei* > *D. pteronyssinus* WME.

Chromatofocusing studies using *D. pteronyssinus* and *D. farinae* SGM showed that some of the proteases present demonstrated preferential activity towards Azocoll and azocasein substrates. An alkaline (pI > 8.5) and an acid protease (pI 4.0) demonstrated greater relative reactivity towards azocasein than to Azocoll (Fig. 1). In addition, the alkaline protease appeared to be the only enzyme showing casein clotting activity (data not shown). The alkaline protease detected did not correspond to either trypsin or chymotrypsin.

All four mite extracts were shown to contain cysteine protease activity using Azocoll in that augmentation of protease activity was observed on addition of dithiothreitol to the reaction mixture, a characteristic feature of such proteases. The rank order of potency was *D. pteronyssinus* WME > *D. pteronyssinus* SGM > *D. farinae* SGM > *E. maynei*, and the increases seen were 1.9-, 1.4-, 1.3- and 2.3-fold higher than in the absence of reducing reagent respectively. When similar studies were performed with azocasein, 2- and 1.2-fold increases in protease activity were observed for *D. pteronyssinus* WME and *E. maynei* but treatment of *D. pteronyssinus* SGM and *D. farinae* SGM with reducing reagent resulted in a decrease in protease activity (both 0.7-fold increases) rather than the anticipated augmentation.

Specific proteolytic activity

All extracts were shown to contain chymotryptic activity, with a rank order of specific BTEE esterase activity of *D. pteronyssinus* SGM > *D. pteronyssinus* WME > *E. maynei* > *D. farinae* SGM. Tryptic TAME esterase activity was found in both SGM and the *E. maynei* extracts, but not in *D. pteronyssinus* WME. The pH optimum for this activity in *D. pteronyssinus* SGM was pH 7–9 (Table 2). Tryptic BAPNA amidase activity was found only with the SGM extracts of *D. pteronyssinus* and *D. farinae*. The rank order of specific TAME

TABLE 2

Some biochemical and physicochemical characteristics of proteases and carbohydrases from *D. pteronyssinus*

Enzyme	Allergenicity/ nomenclature	pH optimum ^a	pI ^a	Molecular weight ^a
Cysteine protease	<i>Der p I</i>	7	5.8–7.5	25 134
Serine protease				
Chymotrypsin	<i>Der p II?</i>	ND	4.1, 5.3	30 000 ^b
Trypsin	<i>Der p III?</i>	7–9	8.5	30 000 ^b
Amylase	Allergenic	6.4	4.3–6.8	52 000
Glucoamylase	?	6–7	4.5–6.9	52 000
Lysozyme	?	6.2	4.4–> 8	13 000 ^b

^aMolecular weight, pH optima and pI data obtained from Chua et al. (1988, 1990), Lake et al. (1991) and Stewart et al. (1991) except where indicated. ^bMolecular weight data obtained from this study. ND=not determined. pH and pI data represent either discrete values or ranges.

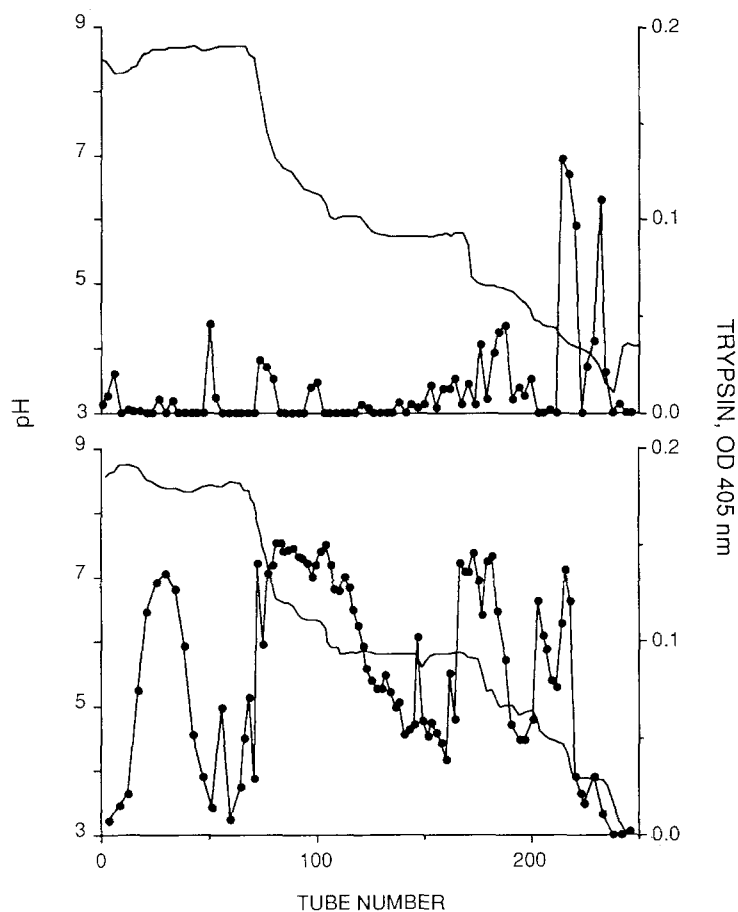


Fig. 2. Chromatofocusing of trypsin in *D. farinae* spent growth medium extract (top panel) and the equivalent extract from *D. pteronyssinus* (bottom panel) on an anion exchange matrix over the pH range 3 to 8.5. The panels show the tryptic activity determined using BAPNA (●) and the pH gradient (—) obtained.

TABLE 3

Protease isoforms from faecal extracts of *D. pteronyssinus* and *D. farinae*

Enzyme	No. of major isoforms		pI of major isoforms	
	<i>D. pteronyssinus</i>	<i>D. farinae</i>	<i>D. pteronyssinus</i>	<i>D. farinae</i>
Trypsin	9(8)	2(14)	4 to > 8	4 to 4.5
Chymotrypsin	2(4)	2(0)	3.8, 4.4	3.7, 4.1

esterase activity was *D. pteronyssinus* SGM > *D. farinae* SGM > *E. maynei*. The rates of hydrolysis of the ester substrate in the *D. pteronyssinus* and *D. farinae* SGM extracts were 21- and 75-fold higher than the rates obtained with the nitroanilide substrate. Carboxypeptidase A activity was found only in *D. pteronyssinus* WME, and carboxypeptidase B was found in *D. pteronyssinus* WME, *D. pteronyssinus* SGM and *D. farinae* SGM extracts, with a rank order of potency of *D. farinae* SGM > *D. pteronyssinus* SGM > *D. pteronyssinus* WME.

The physicochemical characteristics of mite trypsin and chymotrypsin in the SGM extracts were determined using gel-filtration and chromatofocusing. The apparent mol wt of both trypsin and chymotrypsin from *D. pteronyssinus* and *D. farinae* were found to be 30 000 as judged by gel filtration on Sephadex G75 (Table 2). In certain runs, however, there was evidence of high mol wt tryptic activity (data not shown). Further characterisation of this enzyme was not pursued and attempts to differentiate it from the major 30-kDa component in the chromatofocusing studies described below were not pursued.

Figure 2 shows the chromatofocusing elution profiles for trypsin and chymotrypsin obtained from *D. pteronyssinus* and *D. farinae*. These studies showed that both *D. pteronyssinus* and *D. farinae* contained multiple isoforms of trypsin (Table 3), which were empirically classified as either major or minor isoforms based on their relative activities towards BAPNA (Table 3). Of the two mite species, the trypsin from *D. pteronyssinus* was the most complex with nine major isoforms detected and amidase activity was associated with components with pI over the range 4 to >8. In contrast, only two major isoforms were detected in *D. farinae* and these components had pI of 4.2 and 4.4. *D. pteronyssinus* and *D. farinae* contained two isoforms of chymotrypsin with pI of 3.8 and 4.4, and 3.7 and 4.1 respectively.

Amylase, glucoamylase and lysozyme activities

These enzymes were found in all mite extracts (Table 4). The rank orders of specific amylase, glucoamylase and lysozyme activities were: for amylase, *D. farinae* SGM > *D. pteronyssinus* WME > *D. pteronyssinus* SGM > *E. maynei*.

TABLE 4

Carbohydrases detected in mite extracts

Enzyme	Enzyme activity in Units ^a /mg protein			
	<i>D. pteronyssinus</i> ^b	<i>D. pteronyssinus</i> ^c	<i>D. farinae</i> ^c	<i>E. maynei</i> ^d
Amylase	0.20	0.17	0.57	0.05
Glucoamylase	0.13	0.17	0.10	0.13
Lysozyme	17.31	13.9	6.2	12.9

See footnotes to Table 1 for explanatory information.

nei; for glucoamylase, *D. pteronyssinus* SGM > *D. pteronyssinus* WME > *E. maynei* > *D. farinae* SGM and for lysozyme, *D. pteronyssinus* WME > *D. pteronyssinus* SGM > *E. maynei* > *D. farinae*. The above enzymes from *D. farinae* and *D. pteronyssinus* SGM were examined by gel-filtration and chromatofocusing (Table 2). One mol wt species of lysozyme was detected in each species. In contrast, the amylase and glucoamylase from both mite species demonstrated apparent mol wt heterogeneity but this was most evident with glucoamylase (range approx. 10 000 to > 60 000). Multiple isoforms of all three enzymes from the two species were detected (Table 5).

DISCUSSION

Previous data from *D. pteronyssinus* and/or *D. farinae* showed that some of the enzymes present in mite faecal extracts are allergenic, in particular the cysteine (group I) and serine protease (group III), and amylase (tentatively designated *Der p* IV, see Table 2). The biochemical data from this study are consistent with the immunochemical and molecular biology data accumulated thus far and show that the presence of each enzyme or class of enzyme (in the case of the group III allergen), is a consistent feature of all three mite species studied.

Although studies have shown that the cysteine and serine proteases and amylase are allergenic in *D. pteronyssinus* and the former two in *D. farinae*, formal proof showing that, for example, the above three enzymes from *E. maynei* and amylase from *D. farinae* are allergenic is, at present, lacking. However, it is probable that they are allergenic in environments where these mites predominate. In this regard, recent immunoblotting studies performed by one of us (Colloff et al., 1991) indicate that an extract of *E. maynei* contains allergens with mol wt similar to those reported for both the proteases and amylase.

Biochemical analyses of *D. pteronyssinus*, *D. farinae* and *E. maynei* described in this study and those previously reported from our laboratory, re-

TABLE 5

Carbohydrase isoforms from faecal extracts of *D. pteronyssinus* and *D. farinae*

Enzyme	No. of major isoforms		pI of major isoforms	
	<i>D. pteronyssinus</i>	<i>D. farinae</i>	<i>D. pteronyssinus</i>	<i>D. farinae</i>
Amylase	2(5)	3(4)	5.6, 5.8	5.6 to 6.2
Glucoamylase	3(1)	4(7)	6 to 6.9	4 to 6.2
Lysozyme	3(6)	2(6)	6.5 to > 8	> 8, > 8

Isoforms detected by chromatofocusing. Figures in parentheses represent the number of minor isoforms detected.

veal that dust mites contain a range of enzymes in addition to those listed above (Table 6). The concentrations of individual enzymes in each extract, as judged by specific activities, varied with the extract studied. For example, trypsin was shown to be absent from the whole-mite extract of *D. pteronyssinus* and present at low concentrations in *E. maynei*, whereas the concentrations of chymotrypsin were, in general, similar in each extract. The reason(s) for the low values of trypsin in the extracts prepared from whole mites is unknown but may reflect complete absence, autolysis or the presence of specific endogenous inhibitors.

Carboxypeptidase A and B were detected in the whole-mite extract, only B in the spent growth medium extracts, and both forms were absent from *E. maynei* but the reasons for these observations are unclear. Although it may reflect species differences, it is known that mammalian carboxypeptidase A is very susceptible to inhibition by both substrate and hydrolysis products (Hartsuck and Lipscomb, 1971). All extracts were shown to contain similar concentrations of carbohydrases but the highest concentration of lysozyme was found in the *D. pteronyssinus* whole-mite extract.

Physicochemical characteristics of the mite enzymes described in this study, such as isoelectric point and apparent mol wt, were similar to those reported for both vertebrate and invertebrate equivalents. All enzymes showed heterogeneity with regard to charge and several isoforms were detected for each. Marked species differences were observed between faecal extracts of *D. pteronyssinus* and *D. farinae* for trypsin but the reasons for this are unclear. Mite enzyme polymorphisms have previously been reported for esterases and amylase (Bowman and Lessiter, 1985; Hart et al., 1990; Stewart et al., 1991). Interestingly, the pH optima reported for the carbohydrases, although in the neutral range, appear to be slightly lower than the pH optimum seen with

TABLE 6

Allergenic and non-allergenic enzymes present in dust mite faecal extracts

Enzymes of unknown allergenicity	Non-allergenic enzymes	Allergenic enzymes
Glucoamylase	Esterase	Cysteine protease (<i>Der p</i> I)
Lysozyme	Acid phosphatase	Serine protease (<i>Der p</i> III)
Lipase		(Trypsin/motrypsin)
Chymotrypsin		Amylase
Aminopeptidase		
Carboxypeptidase B		
Alkaline phosphatase		
Phosphoamidase		
β -Galactosidase		

Data taken from this study, Stewart et al. (1986, 1991) and Lake et al. (1991).

trypsin. These differences may reflect the conditions seen within the gut of the mite since it has been shown that the pH in the individual component parts vary (Fig. 3) (Hughes, 1950).

In invertebrates other than mites, lysozyme has more often been described in association 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 rather than with digestion. It is not clear whether the equivalent enzyme in mites, particularly in faecal extracts, plays a defensive function or, as recent data from insect studies suggest, a role in digestion (Terra et al., 1988).

Only limited studies of the allergenicity of mite enzymes have been performed. Other than the cysteine and serine protease, and amylase (Table 6), only esterase and acid phosphatase have been examined and these were shown to be non-allergenic on the basis of crossed radioimmuno-electrophoretic studies using a human mite-allergic serum pool (Stewart et al., 1986). However, the allergenicity of the remaining enzymes is unknown, although previous studies showed that the group III allergens may correspond to either trypsin or chymotrypsin (Stewart et al., 1989). Unpublished amino acid sequence data from our laboratory, however, suggest that the group III allergens correspond to trypsin. In addition, we have recently highlighted the many similarities between lysozyme and the group II allergens (Stewart et al., 1991). If confirmed, the group II allergens will also be a consistent feature of dust mites. These enzymes, together with the others listed in Table 6, are currently being isolated to determine their allergenicity.

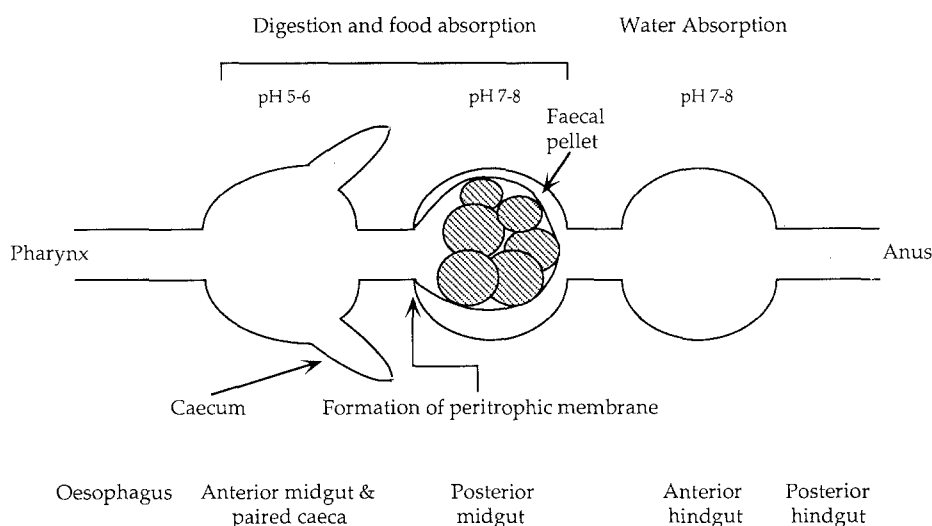


Fig. 3. Diagrammatic representation of the alimentary tract of a dust mite. The pH and physiological data were obtained from Hughes (1950) and Brody et al. (1972).

The presence of all of the above enzymes in those extracts devoid of whole mites indicates that the enzymes are associated with mite faeces and are, presumably, of gut and salivary gland origin. Indeed, all of the enzymes thus far found in mite faecal extracts correspond to hydrolytic enzymes often associated with digestive processes in both invertebrates and vertebrates. Studies on insects have clearly shown that there is a compartmentalisation of digestive enzymes within the midgut, with the major proportion of the enzyme activity present in the material enclosed by the peritrophic membrane rather than outside (Terra et al., 1979; Moritz and Crailsheim, 1987).

Dust mites are also known to produce a peritrophic membrane around food particles in the posterior midgut (Brody et al., 1972; Wharton and Brody, 1972) and, on this basis, the enzymes detected in mite faecal extracts are likely to be present within material enclosed by the membrane (Fig. 3) or attached to it (Hughes, 1950; Wharton and Brody, 1972). However, data from studies on insect digestion indicate that all digestive enzymes, apart from leucine aminopeptidase which is found in association with the membrane (Peters and Kalnins, 1985), are endoperitrophic. In this regard, SGM from *D. pteronyssinus* has also been shown to contain high concentrations of this enzyme (Stewart et al., 1986) suggesting that this enzyme might also be membrane bound.

CONCLUSIONS

In conclusion, biochemical equivalents of the enzymatically active allergens originally described for *D. pteronyssinus* were found to be present in *D. farinae* and *Euroglyphus maynei*. In particular, the cysteine protease group I, the serine protease group III (on the assumption that it is either trypsin or chymotrypsin) and amylase (tentatively designated group IV) were detected suggesting that these enzymatic allergens are common to mites which inhabit human domestic dwellings and use skin as a food source. The mites were also found to contain other enzymes including an alkaline protease, carboxypeptidase A and B, glucoamylase and lysozyme. The enzymes were physicochemically similar to their counterparts in both vertebrates and invertebrates. The finding that all enzymes were present in extracts enriched for mite faeces suggests that they are endoperitrophic and, in addition, that each is potentially allergenic in susceptible individuals.

ACKNOWLEDGEMENTS

This study was supported by the Australian National Health and Medical Research Council and the Asthma Foundation of Western Australia Inc. M.J.C. was supported by a grant from the Wellcome Trust. We thank Commonwealth Serum Laboratories (Parkville, Australia) for the unused growth

medium, spent growth medium from both *D. pteronyssinus* and *D. farinae*, and whole *D. pteronyssinus* mites, and Dr. I. Spadolini, Anallergo (Florence, Italy) for the *E. maynei*.

REFERENCES

- Andrews, P., 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.*, 91: 222–233.
- Baldo, B.A., Ford, S.A. and Tovey, E.R., 1989. Towards a definition of the complete spectrum and rank order of importance of the allergens from the house dust mite, *Dermatophagoides pteronyssinus*. In: *Allergy and Molecular Biology, Proceedings of the DPC First International Symposium on Allergy and Molecular Biology*. Adv. Biosci., 74: 13–31.
- Bowman, C.E. and Childs, M., 1982. Polysaccharidases in astigmatid mites (Arthropoda: Acari). *Comp. Biochem. Physiol.*, 72B: 551–557.
- Bowman, C.E. and Lessiter, M.J., 1985. Amylase and esterase polymorphisms in economically important stored product mites (Acari: Astigmata). *Comp. Biochem. Physiol.*, 81B: 353–360.
- Brody, A.R., McGrath, J.C. and Wharton, G.W., 1972. *Dermatophagoides farinae*: The digestive system. *J.N.Y. Entomol. Soc.*, 80: 152–177.
- Chua, K.Y., Stewart, G.A., Thomas, W.R., Simpson, R.J., Dilworth, R.J. and Plozza, T.M., 1988. Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p I*. Homology with cysteine proteases. *J. Exp. Med.*, 167: 175–182.
- Chua, K.Y., Doyle, C.R., Simpson, R.J., Turner, K.J., Stewart, G.A. and Thomas, W.R., 1990. Isolation of cDNA coding for the major mite allergen *Der p II* by IgE plaque immunoassay. *Int. Arch. Allergy Appl. Immunol.*, 91: 118–123.
- Colloff, M.J., Howe, C.W. and Smith, H.V., 1991. IgE binding profiles of atopic sera to house dust mite allergens, using enhanced chemiluminescent immunoblotting. *Int. In: Dust Mite Allergens and Asthma*. UCB Press, Brussels, pp. 102–106.
- Dilworth, R.J., Chua, K.Y. and Thomas, W.R., 1991. Sequence analysis of cDNA coding for a major house dust mite allergen, *Der f I*. *Clin. Exp. Allergy*, 21: 25–32.
- Erlanger B.F., Kokowsky, N. and Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.*, 95: 217–278.
- Foltmann, B., Szeci, P.B. and Tarasova, N.I., 1985. Detection of proteases by clotting of casein after gel electrophoresis. *Anal. Biochem.*, 146: 353–360.
- Hart, B.J., Fain, A. and Hart, D.T., 1990. Differentiation of mite species by using cellulose acetate and equilibrium polyacrylamide gel electrophoresis of isoenzymes. *Exp. Appl. Acarol.*, 8: 97–104.
- Hartsuck, J.A. and Lipscomb, W.N., 1971. Carboxypeptidase A. In: P.D. Boyer (Editor), *The Enzymes*, Vol. III. Academic Press, New York, NY, pp. 1–46.
- Hearn, M.T.W. and Lyttle, D.T., 1981. Buffer-focusing chromatography using multicomponent electrolyte elution systems. *J. Chromatogr. Biomed. Appl.*, 218: 483–495.
- Heymann, P.W., Chapman, M.D., Aalberse, R.C., Fox, J.W. and Platts-Mills, T.A.E., 1989. Antigenic and structural analysis of group II allergens (*Der f II* and *Der p II*) from house dust mites (*Dermatophagoides* spp.). *J. Allergy Clin. Immunol.*, 83: 1055–1067.
- Hughes, T.E., 1950. The physiology of the alimentary canal of *Tyroglyphus farinae*. *Q.J. Microscopical Sci.*, 91: 45–61.
- Hummel, B.C.W., 1959. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. *Can. J. Biochem.*, 37: 1393–1399.
- Ino, Y., Ando, T., Haida, M., Nakamura, K., Iwaki, M., Okudaira, H. and Miyamoto, T., 1989.

- Characterization of the proteases in the crude mite extract. *Int. Arch. Allergy Appl. Immunol.*, 89: 321–326.
- International Workshop Report, 1988. Dust mite allergens and asthma: A worldwide problem. *Bull. W.H.O.*, 66: 769–780.
- Kent, N.A., Hay, D.B., Holland, P.W.H. and Hart, B.J., 1990. The molecular characterisation of house dust mite allergens. *Clin. Exp. Allergy*, 20 Suppl. 1: 116.
- Lake, F.R., Ward, L.D., Simpson, R.J., Thompson, P.J. and Stewart, G.A., 1991. House dust mite derived amylase: Allergenicity and physicochemical characterisation. *J. Allergy Clin. Immunol.*, 87: 1035–1042.
- Moritz, B. and Crailsheim, K., 1987. Physiology of protein digestion in the midgut of the honeybee (*Apis mellifera* L.). *J. Insect Physiol.*, 33: 923–931.
- Osserman, E.F. and Lawlor, D.P., 1966. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J. Exp. Med.*, 124: 921–951.
- Peters, W. and Kalnins, M., 1985. Amino-peptidases as immobilised enzymes on the peritrophic membranes of insects. *Entomol. Genet.*, 11: 25–32.
- Peterson, G.L., 1983. Determination of total protein. *Methods in Enzymology*, 91: 95–119.
- Simpson, R.J., Nice, E.C., Moritz, R.L. and Stewart, G.A., 1989. Structural studies on the allergen *Der p* I from the house dust mite *Dermatophagoides pteronyssinus*: similarity with cysteine proteinases. *Protein Seq. Data Anal.*, 2: 17–21.
- Stewart, G.A., Butcher, A., Lees, K. and 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., Nisbet, A., Thomas, W.R., Chua, K.Y. and Steinkrug, S., 1988. Immunobiology of proteases from the house dust mite. *N. Engl. Allergy Proc.*, 9: 415.
- Stewart, G.A., Thompson, P.J. and Simpson, R.J., 1989. Protease antigens from house dust mite. *Lancet*, 2: 154–155 and correction, *Lancet*, 2: 462.
- Stewart, G.A., Lake, F.R., Bird, C.H. and Thompson, P.J., 1990. Mite allergens as digestive enzymes. In: A. Sehon, D. Kraft and G. Kunkel (Editors), *Epitopes of Atopic Allergens, Workshop Proceedings, XIVth Congress of the European Academy of Allergology and Clinical Immunology*, Berlin. The UCB Institute of Allergy, Brussels, Belgium, pp. 83–86.
- Stewart, G.A., Lake, F.R. and Thompson, P.J., 1991. Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: Physicochemical characterisation of potential allergens. *Int. Arch. Allergy Appl. Immunol.*, 95: 248–256.
- Takahashi, K., Aoki, T., Kohmoto, S., Nishimura, H., Kodera, Y., Matsushima, A. and Inada, Y., 1990. Activation of kallikrein system in human plasma with purified serine protease from *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.*, 91: 80–85.
- Terra, W.R., Ferreira, C. and De Bianchi, A.G., 1979. Distribution of digestive enzymes among the endo- and ectoperitrophic spaces and midgut cells of *Rhynchosciara* and its physiological significance. *J. Insect Physiol.*, 25: 487–494.
- Terra, W.R., Espinoza-Fuentes, F.P. and Ferreira, C., 1988. Midgut amylase, lysozyme, aminopeptidase and trehalase from larvae and adults of *Musca domestica*. *Arch. Insect Biochem. Physiol.*, 9: 283–297.
- Tomarelli, R.M., Charney, J. and Harding, M.L., 1949. The use of azoalbumin as a substrate in the colourimetric determination of peptic and tryptic activity. *J. Lab. Clin. Med.*, 34: 428–433.
- Tovey, E.R. and Baldo, B.A., 1987. Comparison by electroblotting of IgE-binding components in extracts of house dust mite bodies and spent mite culture. *J. Allergy Clin. Immunol.*, 79: 93–102.
- Tovey, E.R., Chapman, M.D. and Platts-Mills, T.A.E., 1981. Mite faeces are a major source of house dust allergens. *Nature*, 289: 592–593.

- Trinder, P., 1969. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J. Clin. Pathol.*, 22: 246.
- Trudinger, M., Chua, K.Y. and Thomas, W.R., 1991. cDNA encoding the major mite allergen *Der f*II. *Clin. Exp. Allergy*, 21: 33–37.
- Wharton, G.W. and Brody, A.R., 1972. The peritrophic membrane of the mite *Dermatophagoides farinae*: Acariformes. *J. Parasitol.*, 58: 801–804.
- Worthington, 1982. *Enzymes and Related Biochemicals*. Worthington Diagnostic Systems, Freehold, NJ, pp. 28–29.
- Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M. and Okudaira, H., 1990. Cloning and sequencing of cDNAs corresponding to major mite allergen *Der f*II. *Jpn. J. Allergol.*, 39: 557–561.