

Enzymatic analysis of *Blomia tropicalis* and *Blomia kulagini* (Acari: Echimyopodidae) allergenic extracts obtained from different phases of culture growth

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Received: 5 April 2004 / Accepted: 5 May 2006
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Abstract The majority of important allergenic extracts from arthropods present enzymatic activity. This activity has been studied particularly in *Dermatophagoides* house dust mites because of its implication in the stability and immunogenicity of extracts used as tools for the diagnosis and specific treatment of allergic diseases. Extracts from cultures of *Blomia tropicalis* [van Bronswijk (1973a, b). *Acarologia* 15:477–489, 490–505] and *Blomia kulagini* (Zakhvatkin 1936) were used to study enzymatic profiles during three growth periods of the mite population: latency phase, maximum mite concentration during exponential growth, and drop stage. The activities of 19 enzymes were analyzed using the Api Zym system. The results show a large variety of enzymes. Some enzymatic activity was found to be (almost) exclusively attributable to mites. The activity levels of proteases, glycosidases and lipases overlapped with the growth curve. Only phosphatase activity showed no significant change during mite growth when compared with the culture medium. We suggest that the glycosidases (β -galactosidase, β -glucuronidase, β -*N*-acetylglucosaminidase, α -mannosidase and α -fucosidase) and proteases (leucine aminopeptidase and trypsin) may constitute suitable parameters for inclusion in the quality control process for the production of allergenic mite extracts, and may help define a new index for conducting environmental controls.

Keywords *Blomia tropicalis* · *Blomia kulagini* · Enzyme activity · Allergenic extracts · Mites

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Introduction

In recent years, studies of mite sensitization prevalence and mite fauna in house dust samples in non-tropical and subtropical regions, have demonstrated the increasing importance of *Blomia* as a cause of sensitization and allergy (Arias and García del Hoyo 2002; Thomas et al. 2003). Most allergenic extracts from clinically important mite species display enzymatic activity (Mori and Sajiki 1997; Hewitt et al. 1998; Iraneta et al. 1999; Nisbet and Billingsley 2000; Martínez et al. 1999; Ortego et al. 2000; Montealegre et al. 2000). The main relevant allergens seem to be associated with digestion by these arthropods, since they have been identified both in the body and feces of mites (Voorhorst et al. 1969; Tovey et al. 1981; Thompson and Carswell 1988; Stewart et al. 1991; Ortego et al. 2000).

The enzymatic activity of allergenic mites may form a reliable tool for evaluating mite growth in vitro (Montealegre et al. 2002; Martínez et al. 1999), and may prove a useful means for standardizing optimum growth material for the production of allergenic extracts (Martínez et al. 1999) and for measuring environmental exposure to such mites (Montealegre et al. 2002).

A link between mite allergen enzymatic activity and immunogenicity has been suggested (Robinson et al. 1997). Allergens with proteolytic activity act as proallergenic adjuvants (Hewitt et al. 1998), and some allergens—such as the major *Dermatophagoides pteronyssinus* (*Der p* 1) allergen, which shows cysteine protease activity—selectively enhance IgE-specific and non-specific immune responses. This *Der p* 1 enzymatic activity seems to be a major contributor to allergenicity (Gough et al. 1999). Previous studies with *Dermatophagoides* spp. have demonstrated qualitative and quantitative differences between enzymatic activities, protein profiles and allergenic components (including major allergens) depending on the stage of culture growth (Eraso et al. 1997; Martínez et al. 1999). Few studies have conducted enzyme characterization for *Blomia* spp., and all these studies are limited to the study of *Blomia tropicalis* proteolytic enzymes or extracts from its cultures (Montealegre et al. 2002; Mora et al. 2003; Flores et al. 2003).

Here, we screened enzymatic activities of *B. tropicalis* and *B. kulagini* culture allergenic extracts in different growth phases, and considered possible uses in quality and environmental control procedures.

Materials and methods

Mite cultures

Details of mite culturing are described by Martínez et al. (1999). *B. tropicalis* (Central Science Laboratory, UK) and *B. kulagini* (Gómez and Portús 1977) (Department of Parasitology, Faculty of Pharmacy, University of Barcelona, Spain) were cultured in a 1:1 (wt:wt) autoclaved mixture of commercial mouse meal (A.04 Panlab, Barcelona) and dried yeast powder. Cultures (in triplicate) were prepared simultaneously by inoculating with 3,600 mites in 30 mg of medium, and were later incubated at 24–26°C and 75–80% RH. The cultures were examined regularly (every 2 weeks) to assess mite growth and to ensure that no cross-contamination had occurred by checking the identity of mites according to the keys proposed by Colloff and Spieksma (1992). Cultures were handled in a biological safety cabinet. At 2-week intervals, a small

culture sample (50 mg) was used to determine mite concentration (live and dead mites) by microscope count. Cultures were then processed to obtain the extracts.

Preparation of extracts

Extraction from whole cultures was performed by shaking for 6 h at 4–8°C in a saline phosphate buffer, pH 7.2 (1 g raw culture/10 ml buffer). Extract suspension was clarified by centrifugation at 3,000g for 30 min, and the supernatant was filtered using cellulose AP20 filters (Millipore, Bedford, USA). The extracts were thoroughly dialyzed against distilled water at 4°C during 24 h, using membranes with a 5,000 Da cut-off. Dialyzed material was centrifuged at 12,000g for 30 min at 4°C and the supernatant was freeze-dried. An extract from the culture medium without mites was also obtained using the same protocol (Eraso et al. 1997).

A latent phase (20 and 30 days incubation for *B. tropicalis* and *B. kulagini*, respectively), an exponential phase (130 and 58 days incubation, respectively), and a drop stage (150 days incubation each) were defined according to Cardona et al. (2004).

Enzymatic activity

The enzyme activity of mite extracts was determined using the Api Zym System (Bio Mérieux, France), in accordance with the manufacturer's instructions, and as described in Martínez et al. (1999). This system has been used by other authors to study mite extracts (Bousquet et al. 1980; Stewart et al. 1986; Martínez et al. 1999; Nisbet and Billingsley 2000), as well as extracts of grass pollen (Bousquet et al. 1978), moulds (Sáenz de Santamaría et al. 2006) or bee venom (Bousquet et al. 1979).

Nineteen enzymes were assayed (see Table 1). Three samples of each extract were tested in each assay. The results can only be evaluated as semiquantitative data based on the manufacturer's scale: 5, ≥ 40 nmol of hydrolyzed substrate; 4, 30–40 nmol, 3, 20–30 nmol, 2, 10–20 nmol, 1, 5–10 nmol.

Enzymatic profiles of both species were compared by calculating the coincidence index $[CI = N_A(N_A - 1)/N(N - 1) + \dots + N_Z(N_Z - 1)/N(N - 1)]$.

The relationship between growth phases and enzymatic activities (classes) was analyzed by non-parametric χ^2 Pearson's test with 95% confidence intervals using GraphPad Prism v4.0 for Windows (GraphPad Prism software, San Diego, CA, USA).

Results

Table 1 shows enzymatic activity for the extract from the non-inoculated culture medium and its separate components. The values for most of the enzymes were low (5–10 nmol of hydrolyzed substrate) or not detectable. Activity of acid phosphatase, phosphoamidase, alkaline phosphatase and β -galactosidase was higher (> 20 nmol hydrolyzed substrate), in all cases attributable to the mouse meal component.

Blomia tropicalis

Enzymatic activity for *B. tropicalis* correlated well with mite growth, with the highest activity during the exponential phase, with maximum growth rate. With the exception of esterase lipase, α -galactosidase, α -glucosidase and α -fucosidase, no changes were detected between the exponential growth and drop stages.

Table 1 Enzymatic activities of extracts of culture medium and its components

	Enzyme	Mouse meal	Dried yeast powder	Culture medium
Proteases	Leucine aminopeptidase	1	0	1
	Valine aminopeptidase	0	0	0
	Cystine aminopeptidase	0	0	0
	Trypsin	0	0	0
	Chymotrypsin	0	0	0
Lipases	Esterase	2	0	2
	Esterase lipase	0	0	0
	Lipase	0	0	0
Glycosidases	α -Galactosidase	1	0	1
	β -Galactosidase	3	0	3
	β -Glucuronidase	0	0	0
	α -Glucosidase	0	0	0
	β -Glucosidase	0	0	0
	β - <i>N</i> -Acetylglucosaminidase	1	1	1
	α -Mannosidase	1	1	1
	α -Fucosidase	1	1	1
Phosphatases	Alkaline phosphatase	3	0	3
	Acid phosphatase	5	1	5
	Phosphoamidase	5	1	5

For scale definitions see 'Materials and methods'

Leucine aminopeptidase, trypsin, β -glucuronidase, β -*N*-acetylglucosaminidase and α -mannosidase showed values of ≥ 30 nmol of hydrolyzed substrate, coinciding with the maximum mite growth and drop stages.

Esterase, esterase lipase, α -galactosidase, α -glucosidase and α -fucosidase showed maximum activity during the maximum growth phase (5–30 nmol of hydrolyzed substrate). There was no enzymatic activity for the lipases and phosphatases in any of the growth phases.

Blomia kulagini

Activity of most enzymes correlated well with *B. kulagini* growth, with the greatest activity occurring during the maximum growth phase (Table 2). Activity of esterase lipase, valine aminopeptidase, α -glucosidase and α -fucosidase was higher during exponential growth than during the drop stage, activity of the other enzymes was the same in these phases, or lowest during exponential growth. Eight of the 19 enzymes showed values of ≥ 30 nmol of hydrolyzed substrate, coinciding with the maximum mite growth and drop stages. α -Glucosidase, α -fucosidase, esterase lipase, alkaline phosphatase and valine aminopeptidase showed maximum activity during the exponential phase. For cystine aminopeptidase, chymotrypsin, lipase, α -galactosidase and β -glucosidase maximum values occurred at the end of the life cycle.

As for *B. tropicalis*, acid phosphatase and phosphoamidase showed no activity for any of the growth phases.

Coincidence

Comparison of the enzymatic profiles of the two species yielded coincidence indices of 0.813 (latent phase), 0.543 (exponential phase) and 0.483 (drop stage). This

Table 2 Enzymatic activities of *B. tropicalis* and *B. kulagini* extracts for three growth phases, after subtraction of the culture medium values (see Table 1)

Enzyme	Latent phase		Exponential phase		Drop stage	
	<i>B. tropicalis</i>	<i>B. kulagini</i>	<i>B. tropicalis</i>	<i>B. kulagini</i>	<i>B. tropicalis</i>	<i>B. kulagini</i>
Proteases	Leucine aminopeptidase	2	4	4	4	4
	Valine aminopeptidase	1	1	4	1	3
	Cystine aminopeptidase	0	1	1	1	2
	Trypsin	0	5	5	5	5
Lipases	Chymotrypsin	0	1	1	1	4
	Esterase	1	1	2	0	2
	Esterase lipase	2	3	5	2	4
	Lipase	0	0	1	0	2
Glycosidases	α -Galactosidase	0	1	3	0	5
	β -Galactosidase	0	2	2	2	2
	β -Gucuronidase	0	5	5	5	5
	α -Glucosidase	0	1	3	0	2
	β -Glucosidase	0	1	3	1	4
	β -N-acetylglucosaminidase	0	4	4	4	4
Phosphatases	α -Mannosidase	0	1	4	4	4
	α -Fucosidase	0	3	4	1	1
	Alkaline phosphatase	0	0	2	0	1
	Acid phosphatase	0	0	0	0	0
	Phosphamidase	0	0	0	0	0

For scale definitions, see ‘Materials and methods’

illustrates a decrease of similarity among the species profiles throughout the growth cycle.

Pearson χ^2 was 19.6 ($P < 0.05$) for *B. tropicalis* and 29.8 ($P < 0.001$) for *B. kulagini*, demonstrating the association of mite growth and enzyme profiles for both species.

Discussion

The activity of only some of the enzymes in the mite culture medium made it difficult to evaluate the complete enzymatic profile. The phosphatases exhibited greatest activity in the medium.

The literature on *Blomia* enzymes affords few data, and practically all studies refer to proteases obtained in *B. tropicalis* extracts. Montealegre et al. (2002) demonstrated enzyme activity associated with *B. tropicalis*, corresponding to trypsin, elastase, chymotrypsin, kallikrein, convertase, serine protease and mast cell protease. Mora et al. (2003) published the first description of a Group 1 cysteine peptidase allergen from *B. tropicalis*. Flores et al. (2003) and Cheong et al. (2003) associated serine proteases (trypsin-like) with Group 3 mite allergens. Thomas et al. (2003) reported other *Blomia* enzymatic activities, matching amylase and chymotrypsin, associated with *Blo t 4* and *Blo t 6* allergens, respectively.

In our study, proteolytic activity for valine aminopeptidase, cystine aminopeptidase and chymotrypsin from *B. kulagini* was greater than from *B. tropicalis*. Leucine aminopeptidase and trypsin activities were the same for both species, and these enzymes seemed to be the most active proteases in the study. Similar results were reported by Martinez et al. (1999) for *Dermatophagoides* spp., while Nisbet and Billingsley (2000) identified no trypsin activity for either *Acarus siro* or *Tetranychus urticae*. Bearing in mind that proteolytic enzymes appear to be particularly potent allergens in susceptible individuals (Stewart and Robinson 2003), and also that this category of molecules can enhance the role of such allergens as immunogens, leucine aminopeptidase and serine proteases may constitute a suitable tool for monitoring the quality of *Blomia* allergenic extracts.

Few papers provide data on non-proteolytic enzymes obtained from allergenic mites. Szlendak et al. (2000), Mori et al. (2000) and Anazawa et al. (2003) described esterase activity in *A. siro* and *T. urticae*, respectively, and suggested that these activities are implicated in the resistance of these species to organophosphates. Martinez et al. (1999) and Nisbet and Billingsley (2000) studied a broad range of enzyme activities in soluble fractions of extracts from *Dermatophagoides* and from *A. siro* and *T. urticae*, respectively, and they found high activity levels due to esterases and esterase lipases, with low activity levels for lipases.

The two species of *Blomia* had qualitatively similar profiles for the glycosidases, with a high level of activity during the maximum growth phase. Similar profiles have also been described for *Dermatophagoides* spp. (Martinez et al. 1999) and for *A. siro* and *T. urticae* (Nisbet and Billingsley 2000). The latter found significant differences between glycosidase-class enzyme profiles of ectoparasitic and free-living mites, and suggested that the intense activity of these enzymes may be related to the polyphagic characteristics of some species, as well as to the different gut symbionts and the need to digest polysaccharides or cleave carbohydrate moieties.

As has been demonstrated, first for *Dermatophagoides* spp. (Martinez et al. 1999) and second for *Blomia* (Cardona et al. 2004), the mite growth phase is a determining

factor in preparing optimized allergenic extracts, and exponential growth is key for obtaining row material. The present results reinforce these findings, because some proteolytic enzymes revealed equal or superior activities during the drop stage, which could reduce the stability of extracts obtained from mite cultures during the death phase.

During the latent and exponential mite growth phases, the similarity between the enzymatic profiles of both species is quite high (from 69 to 89% of coincident values, and CI values 0.5–0.8), but it is lower during the drop stage (56% of coincident values, CI value 0.483). These data together with those reported by Cardona et al. (2005), would indicate the need to further explore the taxonomical separation of both species. Our current understanding of the taxonomy of *Blomia* is based largely on the work of van Bronswijk et al. (1973a, b) and the criteria used by Colloff and Spieksma (1992). However authors such as Fain et al. (1977) and Cardona et al. (2005) recommend a review of the taxonomical position of the two *Blomia* species, given the confusion within the *Blomia* genus and the great similarity in the allergenic composition.

As a general conclusion, we indicate that the activity of the glycosidases (β -galactosidase, β -glucuronidase, β -*N*-acetylglucosaminidase, α -mannosidase and α -fucosidase) and the proteases (leucine aminopeptidase and trypsin) constitute excellent parameters for monitoring the size of mite populations in cultures, and could be useful tools for inclusion as a quality control parameter in the production of allergenic mite extracts, or for developing a new index for conducting environmental controls.

Acknowledgements This study was supported by a grant from Sweden Diagnostics Spain S.L., with funding from the University of the Basque Country, Project No. 093.123.

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