

Longterm persistence of proteolytic activities in frass of *Blattella germanica* increases its allergenic potential

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Abstract. Chromogenic microplate assays in 96 wells were used to determine the stability of enzyme activity in frass of *Blattella germanica* (Blattodea: Blattellidae). Frass samples were exposed to controlled conditions [temperature 15–35 °C and/or 53–100% relative humidity (RH)] and to household conditions (apartment). Exposure times were 0 (control), 90, 183 and 276 days. Starch digestion and cellulolytic activities decreased during exposure. Non-specific proteolytic activities were affected by changes in selective proteolytic activities. Activities towards AAPpNA and SA₃pNA strongly increased at 100% RH, indicating the possible influence of microorganisms growing on frass. Activities towards BApNA and ArgpNA decreased with increasing decomposition time, whereas activity towards ZRRpNA was not influenced by exposure time. The largest decrease in activities towards ArgpNA and BApNA occurred at temperatures of 15 °C, 30 °C and 35 °C and at 100% RH. Activities towards BApNA and ZRRpNA were very stable under different temperature and RH conditions; this was confirmed by findings showing that these activities were stable in the experimental apartment. In comparison with the control, activities towards ZRRpNA and BApNA after 276 days decreased by 1% and 19%, respectively. The longterm persistence of proteolytic activities in cockroach frass increases their allergenic hazard potential.

Key words. Allergen stability, amylase, cellulase, cockroach frass, protease.

Introduction

The cockroach allergens, like allergens from mites, domestic animals and synanthropic rodents, are highly abundant in the domestic environment (Wilson *et al.*, 2010). The allergens of *Blattella germanica* and *Periplaneta americana* (Blattaria: Blattellidae) are important risk factors for the development of asthma and other allergic diseases (Pomés, 2008). During infestation, cockroaches produce a wide spectrum of compounds with high allergenic potential. To date, six and seven different biochemical compounds with immunoglobulin E (IgE) reactivity have been reported in *P. americana* and *B. germanica*, respectively (<http://www.allergen.org>). Extracts of cockroach allergens showed high proteolytic activity (Roelandt *et al.*, 2008) and a serine protease (Per a 10) has been identified as a major allergen of *P. americana* (Sudha *et al.*, 2008). The Bla g 2 allergen of *B. germanica* is an unusual aspartate protease present in frass with remnant proteolytic activity 1000 times lower than that of pepsin (Gustchina *et al.*, 2005;

Wünschmann *et al.*, 2005). In addition to the characterized allergens, recent progress on the immunology of cockroach and mite allergens has clearly demonstrated the importance of proteolytic activity in the sensitization process (Jeong *et al.*, 2008). The proteolytic activities of cockroach proteins influence epidermal permeability and polarize the immune response by receptor activation (Page *et al.*, 2006; Roelandt *et al.*, 2008). Cockroach frass is a source of proteases in the environment and has been shown to include significant allergen content (Musmand *et al.*, 1995; Page *et al.*, 2009). The main proteases in frass are thought to be serine proteases (Page *et al.*, 2006; Hughes & Page, 2007). However, to date none of the allergens from *B. germanica* described in the International Union of Immunological Societies (IUIS) list of allergens (<http://www.allergen.org/>) has a standard proteolytic activity (serine, aspartate, cysteine).

In a previous study, we demonstrated that Bla g 1 and Bla g 2 allergens remained very stable in mite frass when

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the frass was exposed to controlled (constant temperature or humidity) conditions or to household conditions in Eppendorf tubes covered by muslin (Erban *et al.*, 2010). In this study, we characterize the degree and nature of enzyme activity in frass of *B. germanica* just after defecation and after exposure. Highly stable allergens are expected to persist for longer periods in the environment and to correspond to higher risks for allergen sensitization. The aims of the current study were to determine the stability of selected proteolytic, α -amylase/ α -glucosidase and cellulase activities and to compare these activities during frass exposure.

Materials and methods

Experimental design

The experimental design included an initial sample of frass obtained immediately after defecation (control) and three samples exposed for 90, 183 and 276 days, respectively. The experiment was carried out using 10 replicates per treatment and per decomposition period except for the control (Erban *et al.*, 2010). When the exposure time had concluded, tubes were sealed and stored in the freezer until analysis. To measure enzyme activities, the replicates for each treatment were mixed into one sample and three independent measurements were obtained. The control included 16 replicates mixed to one sample and nine independent measurements. The control is defined as the composition of enzyme activities just after defecation (i.e. exposure time: 0 days). All samples consisted of frass collected ≤ 24 h after defecation (see below). The effect of temperature was observed under five conditions (15 ± 0.5 °C, 20 ± 0.5 °C, 25 ± 0.5 °C, 30 ± 0.5 °C, 35 ± 0.5 °C) using thermostats with forced air circulation. For all temperature conditions, experimental tubes were placed into bags in desiccators maintained at 85% relative humidity (RH). The effects of variations in RH were observed at a controlled temperature of 25 ± 0.5 °C. The desiccators contained distilled water to maintain 100% RH. A saturated solution of NaCl was used to achieve 75% RH and a solution of $\text{Mg}(\text{NO}_3)_2$ was used to achieve 53% RH. Exposure under natural, household conditions was observed in a family house in Tuchomerice, 10 km west of Prague. The experiment began on 18 July 2006 and was terminated on 20 April 2007. Temperature and RH were monitored by Tinytalk temperature and humidity sensors (Gemini Data Loggers UK Ltd, Chichester, U.K.). The mean $\pm$ standard deviation (SD) temperature in the model house was 20 ± 1.6 °C (minimum 16 °C, median 20 °C, maximum 28 °C), and the mean $\pm$ SD RH was $48 \pm 9\%$ (minimum 26%, median 47%, maximum 73%).

Frass collection

The German cockroach, *B. germanica* Linnaeus 1767, was used in these experiments. Specimens originated from the Crop Research Institute, Prague. The cockroaches were reared in an environmental chamber maintained at light : dark 12 : 12 h, 27 ± 1 °C and 65% RH (Stejskal, 1997). The diet consisted

of dehydrated food for rabbits (ZZN Rakovník AS, Rakovník, Czech Republic) and dogs (Provini Pet Food, SRO, Prague, Czech Republic) at a ratio of 1 : 4 (dry weight). For the experiment, males aged 7–18 days were removed from rearing chambers and transferred into empty 1-L plastic chambers (Alliachem SRO, Chropyně, Czech Republic). Faeces were collected at 24-h intervals and cockroaches were moved to a new empty chamber and fresh food was added. The whole procedure was repeated five times. Collected faeces were stored in 50-mL Greiner centrifuge tubes (Sigma-Aldrich Corp., Saint Louis, MI, U.S.A.) in a deepfreeze (-40 °C) until used. Just before the experiment, the faeces were mixed together to obtain a large homogeneous sample which was then lyophilized in a PowerDry LL3000 apparatus (Thermo Fisher Scientific, Shanghai, China). When the faeces had been lyophilized, the material was divided into samples weighing 50 ± 0.5 mg using a Mettler AE 240 microbalance (Mettler-Toledo, Inc., Columbus, OH, U.S.A.) and placed in 600- μL Eppendorf tubes. The unsealed Eppendorf tubes were covered by muslin fixed with an elastic ring. The muslins protected the frass against contamination by synanthropic arthropods or mechanical losses during manipulation.

Preparation of the samples for biochemical analyses

A 500- μL sample of cold physiological solution containing 0.1% Triton X-100 was added to each Eppendorf tube containing frass. Each sample was mixed for 1 min using an MS1 Minishaker (IKA Werke GmbH, Staufen, Germany), after which the mixtures were kept in a rack on ice. The Eppendorf tubes were shaken for 12 h in a rack on a PST-60 HL shaker (Biosan Ltd, Riga, Latvia) at 4 °C using an ES500 thermostat (Nüve AS, Ankara, Turkey). The extracts were centrifuged (14 000 g, 4 °C) using a Jouan MR23i centrifuge (Jouan Ltd, Ilkeston, U.K.), and 200 μL of the homogenate were transferred into Eppendorf tubes. The homogenates were diluted 1 : 5 by the addition of 800 μL of cold physiological solution. The protein content of each sample was determined using Bradford reagent (Sigma-Aldrich Corp.) detected at 595 nm (Multiskan Ascent; Thermo Fisher Scientific). A protein standard (Sigma-Aldrich Corp.) was used for calibration.

Enzyme assays

Non-specific and specific proteolytic activity were compared at pH 6 in KH_2PO_4 - Na_2HPO_4 (Sørensen) buffer containing NaCl. The buffer pH was selected according to the pH of the digestive tract of *B. germanica* reported in the literature (Vinokurov *et al.*, 2007). Having been mixed with extracts, the buffer yielded a final concentration of 0.1 mol/L and 0.15 mol/L of NaCl in each reaction. Non-protease assays contained protease inhibitors (TLCK and TPCK) in buffers and the assay for starch digestion contained 0.5 mmol/L CaCl_2 in the buffer (Erban *et al.*, 2009). Azocasein (Sigma-Aldrich Corp.) was used as a non-specific substrate of proteases.

The microplate assays were adopted to detect selective proteolytic activity (Erban & Hubert, 2010). These were based on the following p-nitroaniline-bound specific substrates: BApNA

Table 1. Substrate specificity of various proteases according to the manufacturer's instructions.

Substrate	Code	Target protease
Azocasein	(A2765; Sigma-Aldrich Corp.)	General
BAPNA	(B4875; Sigma-Aldrich Corp.)	Trypsin, amidase, balterobin (thrombin-like)
SA ₃ pNA	(S4760; Sigma-Aldrich Corp.)	Elastase
AAPpNA	(A9148; Sigma-Aldrich Corp.)	Chymotrypsin, tripeptidyl peptidase I
ZRRpNA	(P-138; Biomol International)	Cathepsin B
ArgpNA	(P-136; Biomol International)	Cathepsin H, aminopeptidases

(N α -benzoyl-D,L-arginine 4-nitroanilide hydrochloride; Sigma-Aldrich Corp.); SA₃pNA (N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide; Sigma-Aldrich Corp.); AAPpNA (N-succinyl-L-alanyl-L-alanyl-L-phenylalanine 4-nitroanilide; Sigma-Aldrich Corp.); ZRRpNA (benzyloxycarbonyl-L-arginine-L-arginyl 4-nitroanilide; Biomol International, Exeter, U.K.), and ArgpNA (L-arginyl 4-nitroanilide; Biomol International) (Table 1). The p-nitroaniline-bound specific substrates were prepared to obtain a final concentration of 1 mM in each reaction. Azocasein, the non-specific protease substrate, was prepared at a concentration of 0.2% w/v. Cellulose azure (Sigma-Aldrich Corp.) was used as a cellulose substrate. Finally, 0.1% w/v potato starch (Sigma-Aldrich Corp.) was used as a starch substrate. A sample of 200 μ L of each substrate was applied to each of the 300- μ L wells on the microplates (Gama Group a.s., České Budějovice, Czech Republic). After pre-incubation for 10 min at 37 °C, each reaction was started by the addition of 50 μ L of each frass homogenate in the protease and cellulase assays and 25 μ L of each frass homogenate in the starch digestion assay. Measurements were performed in triplicate and included blank measurements. The assays were incubated and measured as described above. BAPNA, azocasein, ZRRpNA and AAPpNA were incubated for 45 min, ArgpNA and SA₃pNA for 90 min, cellulose azure for 20 h and potato starch for 30 min for enzymatic analyses. The reactions were stopped by the addition of 50 μ L of 0.3 M trichloroacetic acid. The plates were then centrifuged at 1500 g for 20 min at 2 °C in an MR 23i centrifuge (Jouan Ltd). A sample of 70 μ L of each supernatant was transferred in duplicate from each well to a 384-well microplate and absorbance was read at 405 nm for the substrates containing p-nitroaniline and azocasein (Erban & Hubert, 2010). The absorbance of cellulose azure was read at 595 nm. The starch digestion assay was visualized as described in Erban *et al.* (2009). Finally, in the appropriate wavelengths and assays, measured activity was corrected for any change in absorbance caused by the extracts. Activities were expressed as relative activities where 1 unit = change in absorbance/ μ g protein/hour of reaction (Δ A/h).

The correlations between enzyme activity and environmental variables (decomposition time, RH, temperature) were compared using Pearson's correlation coefficients in XLStat 2007 (Addinsoft, Inc., New York, NY, U.S.A.).

Results

Baseline enzyme activity in frass

The enzyme activities measured in these experiments were detected in control samples. Non-specific proteolytic activity, cellulase and starch-degrading enzymes were present in *B. germanica* frass after defecation. Analysis of specific proteolytic activities in frass on p-nitroaniline-bound substrates showed high activity towards ZRRpNA under most conditions. High activity was also detected on AAPpNA and BAPNA. To a lesser extent, activities towards SA₃pNA and ArgpNA were also present. Mean $\pm$ SD enzyme activities were: starch, 2.64 ± 0.11 Δ A/h; cellulase, 0.69 ± 0.05 Δ A/h; azocasein, 0.18 ± 0.01 Δ A/h; AAPpNA, 0.98 ± 0.02 Δ A/h; ArgpNA, 0.32 ± 0.01 Δ A/h; BAPNA, 0.97 ± 0.02 Δ A/h; SA₃pNA, 0.14 ± 0.01 Δ A/h, and ZRRpNA, 2.20 ± 0.02 Δ A/h.

Changes in enzyme activity in frass during decomposition

The exposure of frass to various temperature and humidity regimes and to household conditions changed the levels of enzyme activities (Table 2). Generally, activity decreased, increased or disappeared during exposure to other conditions compared with baseline (Figs 1 and 2). The significance of the effects of time, temperature and RH on total activity was determined by calculation of Pearson's correlation coefficients (Table 3). Significant correlations were observed between exposure to moisture and activities towards BAPNA, AAPpNA, ZRRpNA, ArgpNA and SA₃pNA, as well as length of exposure and activities towards BAPNA, ArgpNA and cellulase and starch digestion. There were no significant correlations between controlled temperature conditions and any enzyme activity. However, all the non-specific enzyme activities measured showed a thermolabile trend and decreased stability after exposure to 15 °C and 35 °C conditions (Figs 1 and 2), except activity towards ZRRpNA. Activities towards AAPpNA and SA₃pNA were highly increased at RH 100% compared with control samples (Figs 1 and 2).

When enzyme activities were compared between frass immediately after defecation (Fig. 3A) and frass samples after 276 days of degradation, three different patterns of enzyme activity emerged: (a) under household conditions (Fig. 3B) and at temperatures of 20 °C and 25 °C, as well as under RH of 53%, 75% and 85%, the ranking of enzyme activities was similar to that in the baseline sample; (b) at temperatures of 15 °C, 30 °C and 35 °C, the ranking of major enzyme activities changed (Fig. 3C), and (c) under 100% RH, a different spectrum of major enzyme activity was observed and activity towards AAPpNA, cellulose azure and SA₃pNA dominated (Fig. 3D).

Changes in starch digestion and cellulase activities

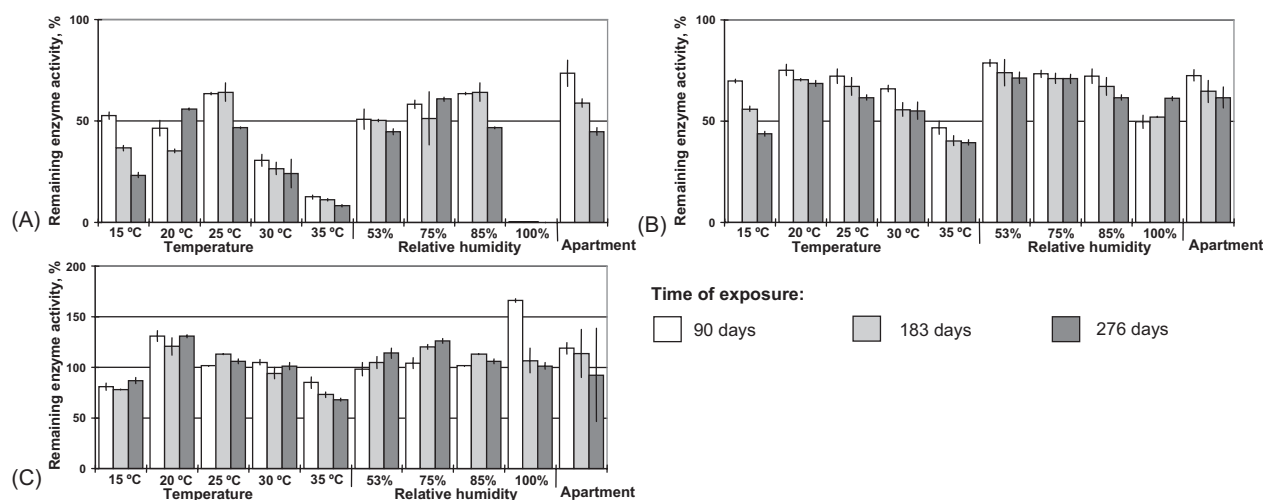
The starch-degrading enzymes found in frass included α -amylase and α -glucosidase. In comparison with baseline activity in frass, starch hydrolytic activity decreased with increasing time of exposure (Table 2). The thermostability

Table 2. Changes in total enzyme activity in frass of *Blattella germanica* under controlled temperature and humidity regimes and under household conditions.

Substrate	Temperature, °C		Relative humidity		Apartment
Starch*	15, 30, 35	Large decrease	100%	Degraded	Decrease
	20, 25	Decrease	53–85%	Decrease	
Cellulose	15, 35	Decrease	Decrease		Decrease
	20–30	Small decrease			
Azocasein†	20	Increase	100%	Increase	No change
	15, 35	Decrease	53–85%	No change	
	25, 30	No change			
ZRRpNA	No change		100%	Large decrease	No change
			53–85%	No change	
ArgpNA	15, 30, 35	Decrease	100%	Large decrease	Decrease
	20, 25	Small decrease	53–85%	Decrease	
BApNA	Decrease		100%	Large decrease	Low decrease
			85%	Decrease	
			53%, 75%	No change	
AAPpNA	15, 35	Decrease	100%	Large increase	No change
	20–30	No change	53–85%	No change	
SA ₃ pNA	15, 35	Decrease	100%	Large increase	No changes
	20–30	No change	53–85%	No change	

* α -amylase/ α -glucosidase activity.

†Non-specific protease activity.

**Fig. 1.** Enzyme activity (%) in samples of frass of *Blattella germanica* at various durations of exposure: (A) starch hydrolytic activity; (B) cellulase activity; and (C) azocaseinolytic activity. Bars represent standard deviations.

of the enzymes was lowest under the 15 °C, 30 °C and 35 °C temperature conditions (Fig. 1). Under 100% RH, starch hydrolytic enzymes disappeared; however, lower degradation was observed under RH of 75%, 53% and 85%. Cellulase activity decreased with increasing length of exposure and was lower than in the control sample. The largest decrease in cellulase activity was observed in frass exposed to temperatures of 15 °C and 35 °C.

Changes in protease activities

The variability in non-specific proteolytic activity can be interpreted as reflecting the influence of the activities of

specific proteases; azocasein was degraded with different degrees of efficiency depending on the level of activity of various specific proteases and without any significant correlation to environmental variables (Table 3). Total proteolytic activity varied among the different regimes.

The selective proteolytic activities examined included activity on ZRRpNA (cathepsin B), ArgpNA (cathepsin H or amidopeptidase), BApNA (trypsin-like protease), AAPpNA (chymotrypsin-like protease) and SA₃pNA (elastase). Activities towards BApNA, ZRRpNA and ArgpNA were negatively correlated to RH, whereas activities towards AAPpNA and SA₃pNA were positively correlated to RH (Table 3). There was no correlation between selective proteolytic activity and temperature. The thermolabile trend and decrease in activity

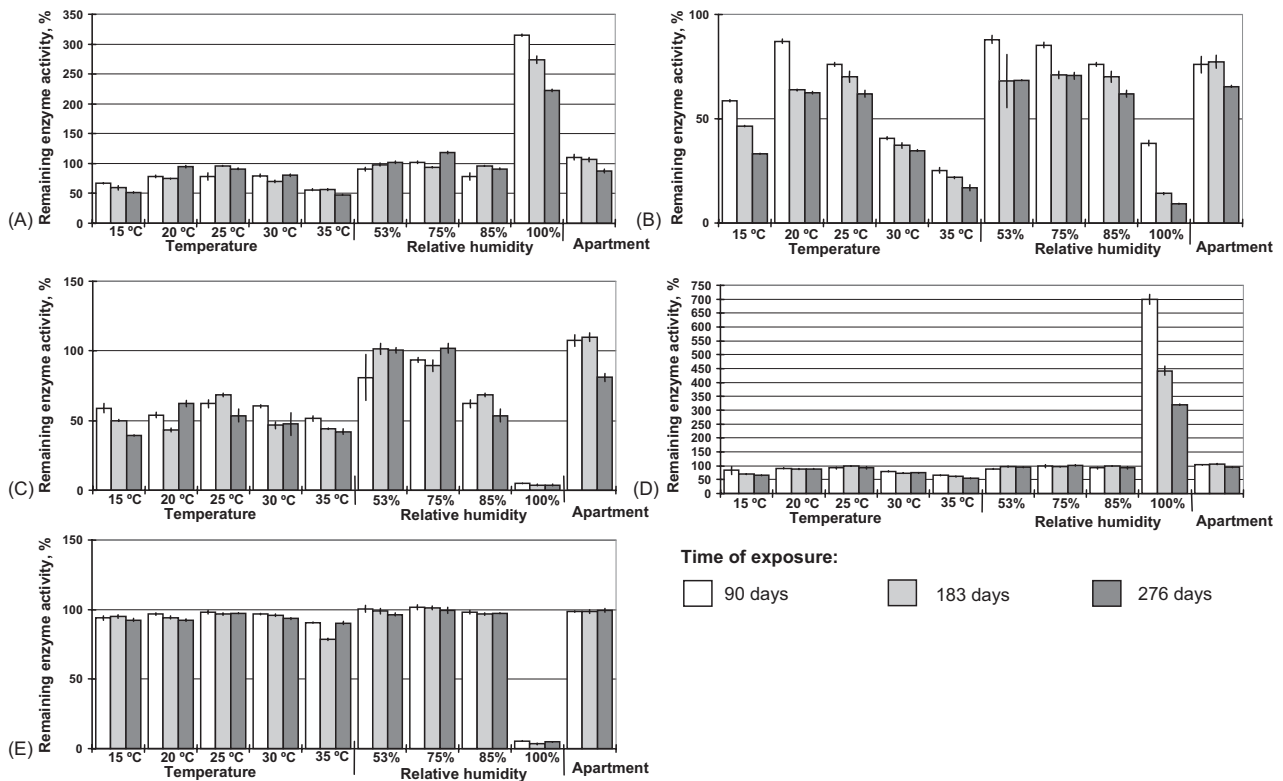


Fig. 2. Enzyme activity (%) in samples of frass of *Blattella germanica* during exposure on specific substrates for proteases: (A) AAPpNA; (B) ArgpNA; (C) BApNA; (D) SA₃pNA, and (E) ZRRpNA. Bars represent standard deviations.

Table 3. Correlations between environmental factors and total enzyme activity during decomposition of frass of *Blattella germanica*.

Variables	Relative humidity	Temperature	Time	Azocasein*	BApNA	AAPpNA	ZRRpNA	ArgpNA	SA ₃ pNA	Cellulose	Starch†
Relative humidity		0.00	0.00	0.06	-0.58	0.35	-0.50	-0.37	0.41	-0.24	-0.26
Temperature	0.00		0.00	-0.18	-0.02	-0.03	-0.04	-0.24	-0.03	-0.18	-0.18
Time	0.00	0.00		0.03	-0.49	-0.01	-0.22	-0.68	0.00	-0.74	-0.69
Azocasein*	0.00	0.03	0.00		-0.06	0.58	-0.30	0.19	0.57	0.11	-0.01
BApNA	0.34	0.00	0.24	0.00		-0.43	0.75	0.85	-0.55	0.78	0.86
AAPpNA	0.12	0.00	0.00	0.34	0.18		-0.88	-0.21	0.96	-0.06	-0.27
ZRRpNA	0.25	0.00	0.05	0.09	0.56	0.78		0.59	-0.89	0.40	0.59
ArgpNA	0.14	0.06	0.47	0.03	0.72	0.05	0.34		-0.31	0.92	0.94
SA ₃ pNA	0.17	0.00	0.00	0.33	0.30	0.92	0.79	0.09		-0.21	-0.38
Cellulose	0.06	0.03	0.55	0.01	0.61	0.00	0.16	0.85	0.04		0.94
Starch†	0.07	0.03	0.47	0.00	0.74	0.07	0.35	0.89	0.15	0.89	

*Non-specific protease activity.

† α -amylase/ α -glucosidase activity.

Pearson's correlation coefficients are above the diagonal; R^2 values are below.

Values in bold are significantly different from 0 ($\alpha = 0.05$).

towards ArgpNA, BApNA, AAPpNA and SA₃pNA at 15 °C and 35 °C corresponded well with the trends of non-specific activities of proteases on azocasein.

Activities towards BApNA and ArgpNA were negatively correlated to exposure time, but this correlation was not significant for ZRRpNA (Table 3). Activity towards ZRRpNA under all tested temperature and RH conditions except 100% RH demonstrated the extreme stability of this enzyme (Fig. 2).

Activity towards ArgpNA decreased with increasing time of exposure and after exposure to each of the environmental regimes. The effects of RH on activities towards ZRRpNA and ArgpNA were similar at 85%, 75% and 53% RH. Both activities decreased markedly at 100% RH. The stability of activity on ArgpNA under different temperature regimes was highest at 25 °C and 20 °C. This level of activity corresponded well with the samples incubated under household conditions

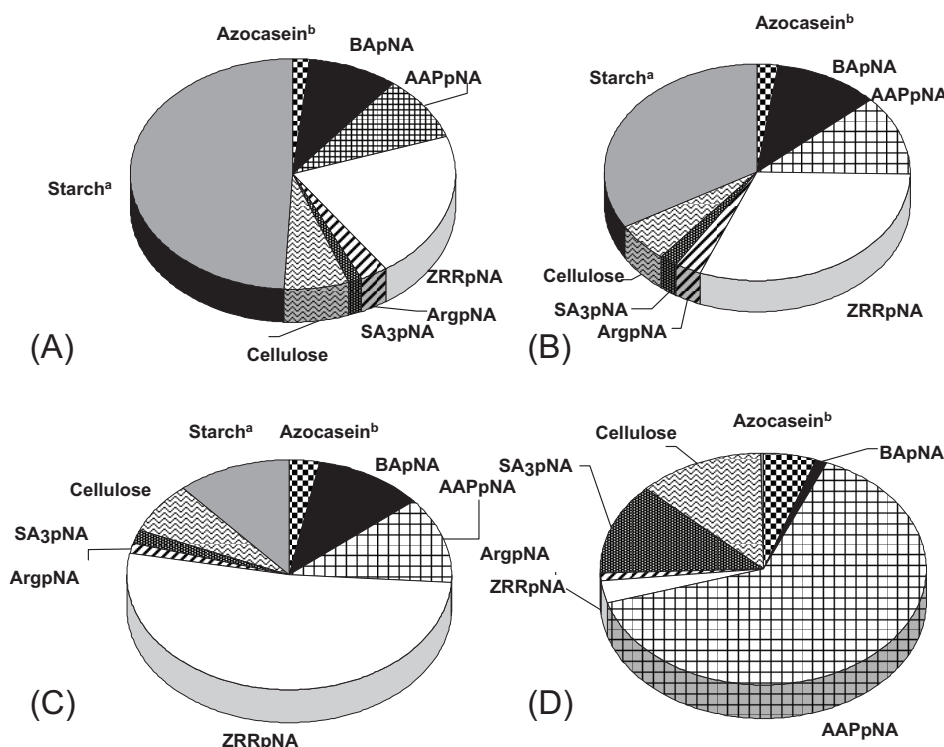


Fig. 3. Breakdown of enzyme activity and protein-related activity in samples of frass of *Blattella germanica* in: (A) faeces immediately after defecation (control); (B) faeces exposed to household (apartment) conditions at 276 days; (C) faeces exposed to a temperature of 35 °C at 276 days; and (D) faeces exposed to 100% RH at 276 days. ^a α -amylase/ α -glucosidase activity; ^bnon-specific protease activity.

and was similar to activity at 85%, 75% and 53% RH. The observed decrease in activity towards ArgpNA at 30 °C and 35 °C indicated thermolabile properties, which were also evident in decreased stability at 15 °C. Activity towards BApNA decreased with increasing length of exposure in most conditions, but not after exposure to RH of 53% and 75%. The most significant decrease in activity towards BApNA occurred when the frass was exposed to 100% RH. By contrast, activity towards AAPpNA increased at 100% RH. A decrease in activity towards AAPpNA was observed under temperatures of 15 °C and 35 °C, but no remarkable changes from baseline (control) occurred under the other conditions. Activity towards SA₃pNA showed a pattern similar to that towards AAPpNA.

Discussion

Composition of enzymes in frass just after defecation

The majority of studies describe enzyme activities in the gut of cockroaches (Elpidina *et al.*, 2001; Genta *et al.*, 2003; Hivrale *et al.*, 2005; Vinokurov *et al.*, 2007). However, the presence of enzymes and their activities in the gut can differ from those in frass as a result of enzyme recycling systems (Terra *et al.*, 1996) and the contribution of gut microbes (Cruden & Markovetz, 1987). Frass is suggested to represent a complex system, in which some enzymes are degraded and others are produced by microflora that colonize the frass.

Enzymes are attached to the digested food in gut lumen and are concentrated in cockroach frass. We confirmed the presence of starch-degrading enzymes as well as some cellulolytic activity in frass after defecation. α -Amylase (Vinokurov *et al.*, 2007), α - and β -glucosidases (Genta *et al.*, 2003), trypsin and chymotrypsin (Hivrale *et al.*, 2005; Vinokurov *et al.*, 2007) are known digestive enzymes in cockroaches and therefore are expected to be present in the frass of *B. germanica*. In *Nauphoeta cinerea* (Dictyoptera: Blaberidae), cysteine proteases, subtilisin-like proteases and serine proteases have been detected in the gut (Elpidina *et al.*, 2001). In this study, hydrolytic activity towards AAPpNA and BApNA indicates chymotrypsin-like and trypsin-like activities, respectively. Activities towards ZRRpNA indicate possible cathepsin B-like activity and activities towards SA₃pNA possible elastase-like and/or chymotrypsin-like activities. However, ZRRpNA can be partly hydrolyzed by trypsin and activity towards BApNA may be influenced by the activity of cysteine proteases. Activity towards ArgpNA may indicate possible aminopeptidase or cathepsin H activities, but aminopeptidase activity is more likely than cathepsin H based on activity in synanthropic acaridid mites (Erban & Hubert, 2010). Such activities were detected in the frass of *B. germanica* for the first time. Cysteine proteases (cathepsins B and L) have also been shown to be present in yolk granules of *B. germanica* (Liu *et al.*, 1996). The enzyme activities detected in the control samples indicate, as well as their allergenic impact, the importance of these enzymes in the digestion processes of the cockroach.

Effects of exposure under controlled conditions on enzyme activities

During exposure, frass has been shown to host various microorganisms (Krizkova-Kudlikova *et al.*, 2007). It is predicted that endogenous enzymes are degraded by enzymes of microbial origin and endogenous cockroach proteases. Generally, the enzymes in this study differed in their stability in frass after exposure in different conditions. Based on their activities, the enzymes could be designated as: (a) stable (activities on ZRRpNA and BApNA), and (b) less stable [α -amylase/ α -glucosidase (starch-degrading enzymes), cellulolytic activity and activity on ArgpNA]. The less stable enzymes showed a significant decrease in activity during exposure because they were thermolabile at 15 °C, 30 °C and 35 °C. However, less stable enzymes were still active in frass after exposure and the activity of these enzymes after exposure to household conditions ranged from 45% to 65% of initial activity. These levels of stability are analogous to those previously found in the allergens Bla g 1 and Bla g 2 (Erban *et al.*, 2010). Bla g 1 was more stable than Bla g 2, but the content of Bla g 2 after 276 days of exposure to household conditions remained at 67% of that of the initial sample (Erban *et al.*, 2010). By comparison with the control, activities towards AAPpNA and SA₃pNA increased; it has been suggested that enzyme activity may be increased by the growth of microorganisms that use the frass as substrate (Krizkova-Kudlikova *et al.*, 2007). This was clearly illustrated under 100% RH, when the majority of enzymes were degraded (α -amylase/ α -glucosidase, decrease of hydrolytic activity towards BApNA, ArgpNA and ZRRpNA), but activity towards AAPpNA and SA₃pNA increased.

Hazard levels of proteolytic activities in frass

The importance of cockroach proteases in allergy is determined by the proteolytic activity of serine proteases (Sudha *et al.*, 2008, 2009; Page *et al.*, 2009). In the present study, we found novel hazardous compounds in *B. germanica* frass, as indicated by activities towards ZRRpNA, ArgpNA and ZRRpNA. In general, proteases are important allergens released into the human environment by mites, the most important of which are suggested to be Group 1 mite allergens, which contain both cysteine and serine protease components (Brown *et al.*, 2003). Group 1 mite allergens can modulate the adaptive immune system by the cleavage of CD23 and CD25 (Hewitt *et al.*, 1995; Schulz *et al.*, 1995, 1998) and by disrupting intercellular tight junctions to facilitate the transport of allergens in the lung epithelium (Wan *et al.*, 1999, 2001). Similar activities can be expected in cockroach protease. Based on the pH of airway surface liquid in the lung (~pH 6.6) (Ng *et al.*, 2004) and protease activities, cockroach proteases with maximal activities in low acid or neutral pH conditions should be regarded as having the greatest allergenic potential, similarly to proteases in synanthropic acaridid mites (Erban & Hubert 2010). This may include proteases in *B. germanica* frass because the proteolytic enzymes are highly active at pH 6 and because the pH in the gut of *B. germanica* ranges from pH 5.7 to pH 6.8 (Vinokurov *et al.*, 2007). In addition, proteolytic activity can synergize with

Bla g 1 and Bla g 2 and increase the allergenic response. The isolation and characterization of proteases in frass with allergenic potential represents a challenge for future study.

Recent research confirmed a reduction in the risk for asthma in sensitive people in response to decreasing exposure time. Pest control and cleaning regimes against cockroaches were found to decrease the levels of cockroach allergens in most conditions (Gore & Schal, 2007). However, other studies have shown that the eradication of cockroaches does not lead to a reduction in allergens in the environment (Eggleston *et al.*, 1999; Gergen *et al.*, 1999; Williams *et al.*, 1999). Recently, Bla g 1 and Bla g 2 allergens were shown to persist in the environment for long periods under household conditions (Erban *et al.*, 2010). Similarly, in this study we found all the enzyme activities measured to be very stable, especially under typical household conditions. A similarly high level of stability has been reported for Group 1 mite allergens (Kort & Knies, 1994; Sidenius *et al.*, 2002). The stability of the enzyme activities of proteases present in cockroach frass increases their allergenic potential. Thus, we propose that allergens that are stable in the environment are cumulated during arthropod infestation.

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