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Degradation of cockroach allergen Bla g 2: Does the amount of the allergen increase after partial microbial degradation of faeces?

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Abstract

Microbial degradation of German cockroach (*Blattella germanica* L.) faeces was observed under laboratory conditions at 15 °C and 30 °C during 84 and 168 days. The degree of degradation and microbial growth were visualized by scanning electron microscopy and by biochemical analyses and correlated to the amount of the major allergen Bla g 2. In degraded and control faeces just after defaecation, the amount of Bla g 2 was assayed by commercial ELISA kit. In addition the total proteins, á-amylase, á and â glucosidases activities were compared. The amount of total proteins as well as enzymatic activities decreased in the extracts from degraded faeces. Alltogether with the visualization of fungal decomposers by SEM it indicates the microbial degradation of faeces and denaturation of the protein components depend on temperature. On the contrary, the amount of Bla g 2 after faeces degradation was negatively correlated to the degree of faeces hydrolyses. We hypothesize that microbial enzymes hydrolyze the proteins in the faeces and thus unmask epitopes accessible for binding by a monoclonal antibody in ELISA assay.

Key words: Bla g 2, allergen, degradation, faeces, enzyme.

Introduction

Cockroach allergens are frequent contaminants in flats, farms and food processing factories (Schal and Hamilton, 1990). Up to present time 8 different human IgE binding compounds have been characterized in German cockroach (*Blattella germanica* L.) (www.allergen.org). Among these allergens, there is a high IgE prevalence to Bla g 2 in sensitive patients and this allergen belongs to major species-specific allergens of *B. germanica* (Arruda et al., 1995, Satinover et al., 2005). Bla g 2 shares sequence homology with aspartic proteinases and its high concentrations were observed inside the digestive tract and faecal pellets (Arruda et al., 1995). Oppositely to other allergens of protease nature, this aspartic protease has some changes in catalytic site (Gustchina et al., 2005) and the enzymatic activity is weak. Based on its low activity, the proteolysis is not suggested as the primary allergen function of Bla g 2 (Wunschmann et al., 2005).

While the dynamic of production of the other major allergen Bla g 1 of *B. germanica* has been described recently (Gore and Schal, 2005), the data about degradation of allergens are rare. A recent study has demonstrated that the cysteine protease Der f 1 of house dust-mite *Dermatophagoides farinae* is a very stable allergen in house dust. Natural decay of Der f 1

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was estimated with a half-life of 10 years at housing conditions (Sidenius et al., 2002). It means that the allergens could persist in lower level after population reduction, even or elimination of their producers (cf. Arbes et al., 2004). We tested the temporal stability of the faeces of *B. germanica*, i.e. proteins content, selected enzymes and a model allergen Bla g 2 under laboratory conditions at two different temperature levels.

Material and methods

Faecal pellets were produced by males of *Blattella germanica*. Adult males were transferred from rearing chambers into empty plastic chambers. After 24 hours the faecal pellets were collected. The pellets were stored in deep freezer (-40 °C) until the start of experiment. At the beginning of the experiment, the pellets were remoistened by pre-incubation in a desiccator with distilled water (for 24 hours at 6 °C). The pellets were weighted out into Eppendorf tubes 0.12 ± 0.01 g.

The tubes without lids were covered by muslin and divided into two incubation temperature levels (15 and 30 °C). The tubes were placed into a desiccator containing saturated solution of KCl to keep RH about 85 %. The tubes were removed after 84 and 186 days. Freshly defaecated and unexposed faecal pellets represented control.

After the exposition the moisture content in the pellets was standardized by the same procedure as before the start and pellets were weighted; 0.01 g of pellets were used for quantification of Bla g 2, 0.1 g for enzyme analyzes and estimation of protein content.

The faeces were homogenized in physiological solution, 0.1 g of faeces per 5 ml (IKA Ultra T8 homogenizer). The homogenates were centrifuged (20,000 rpm, 4 °C, Jouan MR23i) for ten minutes and the supernatants analysed to determine their protein contents, á-amylase, á-glucosidase and â-glucosidase activities.

For protein assay, 50 µl of the supernatant was placed on a microtitration plate, 0.25 ml of Bradford reagent (Sigma, B-6916) was added, and after 15 minutes of incubation with shaking the colour was measured at 590 nm using an ELISA reader (Emax®, Molecular Devices). The protein assay was calibrated to bovine serum albumin (protein standard; Sigma, P0914). The protein content was expressed as mg proteins per ml of homogenized faecal pellets.

For á-amylase assay, the starch solution consisted of 0.01 g of soluble potato starch (Sigma, S2630) per 1 ml of 0,05 M Britton-Robinson I (BR-I) buffer was boiled in a water bath for 5 minutes. The pH of starch solution was 5 that corresponded to peak of cockroaches' amylase activity. The assay started by mixing 100 µl of the samples and 400 µl of starch solution in Eppendorf tubes. Then the tubes were placed into a water bath with shaker and incubated at 37 °C for 60 minutes. The incubation was terminated by addition of 700 µl DNS. The DNS solution was prepared as a mixture of 1 % 3,5-dinitrosalicylic acid in 0.4 M NaOH and 30 % Potassium sodium tartrate solution (Rochelle salt, Sigma, S2377), in the ratio 5:2. The absorbance (590 nm) was measured in microplate wells using Emax® reader (Molecular Devices). The method was calibrated using maltose as a standard (Sigma, M-5885).

For cellobiase and maltase activity, substrate solution was prepared from 0.05 g cellobiose (Sigma, C-7252) and 0.05 g maltose (Sigma, M-5885), respectively, both in 10 ml 0.2 M BR-I buffer (pH 5). The samples were incubated at 37 °C for 2 h and 1 h for cellobiose and maltose, respectively. Both were incubated in Thermo-Shaker (PST-60HL, Biosan). After this time, 50 µl of samples was transferred into other microplates wells with 200 µl of solution Glucose GOD-POD 1500 tests (Lachema-Pliva, Brno, Czech R.), in which the colour that developed after 30 minutes of exposure on a microtitration plate was measured at 490 nm using an ELISA reader (Emax®, Molecular Devices). The methods were calibrated using glucose as a standard (Sigma, G6918).

Content of Bla g 2 was assessed in extracts

from faecal pellets of various stages degradation using indirect DAS-ELISA (Indoor Biotechnologies, EL-BG2) according to the manufacture's protocol. Bound immunocomplex was detected by alkaline phosphates conjugated goat anti-rabbit IgG (Sigma, A-3687) and followed by pNPP (Sigma, N4645) in substrate buffer (1M diethanolamine, 0.5 mM $MgCl_2$ x $6H_20$, pH 9.8 adjusted with 36 % HCl). The resulting absorbance was measured on Emax® reader (Molecular Devices) at 405 nm. The assay was corrected on the blank and quantified according to the logarithmic calibration curve obtained for Bla g 2 allergen standard (Figure 2E).

For visualization of faecal pellets degradation, the faecal pellets (from 5 to 10 pellets) were removed from Eppendorf tubes and fixed in 100 % ethanol, drying by CPD method and coated with gold. The observation were done using JEOL 6300 scanning electron microscope (SEM).

Results

At the start of the experiment, spores, mycelium or other morphological structures of microorganisms were not observed on the surfaces of the faecal pellets (Figure 1AB). During degradation of the pellets, their surfaces were overgrown by microscopic fungi. It was remarkable after 84 days in both temperature levels, but the more apparent growth of microscopic fungi appeared after 168 days. Several morphological structures developed on the surface of faecal pellets were observed during the course of the experiment. First, sparse superficial mycelial growth appeared (Figure 1D) with no generative structures. Figure 1C shows individual conidia or clusters of conidia without any mycelium. Subsequently, we observed surface of faecal pellets with abundant growth of microscopic fungi. There were many conidiophores, some with conidial heads, composed of phialides and smooth conidia (Figure 1EG) (cf. genus *Penicillium*). Abundant echinulate conidia were shown in Figure 1F; another type of conidia, lemon-shape (cf. genus *Cladosporium*) was also observed. Apart from vegetative hyphae, germination of echinulate conidia with germination hyphae was recorded (Figure1H).

The amount of total proteins in homogenized faecal pellets decreased with increasing decomposition time; the rate of decrease was stronger at 30 °C in comparison to 15 °C (Figure 2A). The same trends showed specific enzymatic activities; á-amylase (Figure 2B), á-glucosidase (Figure 2C) and â-glucosidase (Figure 2D). On the contrary, after faecal pellets degradation, the amount of Bla g 2 increased as showed ELISA assay (Figure 2F).

Discussion

The amount of total proteins as well as enzymatic activities decreased in the extracts during the course of the experiment. All these effects were stronger at 30 °C. The differences in decreasing trends among the tested enzymes indicated their different stability in the faeces (Figure 2BCD). Alltogether with the visualization of fungal decomposers by SEM it indicated that the microbial degradation of faeces and biochemical denaturation of the protein components depended on temperature.

Although SEM showed partial degradation of faecal membrane by penetration of mycelium and ruptures, the membrane still covered the faecal pellets at the end of the experiment. On the other hand, the allergens were not accessible to be released into respirable fraction of aerosol, because the faeces were still covered by perithrophic membrane. The releasing of the antigen can be enhanced by increasing microbial activity in latter stadia of faecal pellets degradation, i.e. after longer period than 168 days.

Surprisingly, the amount of Bla g 2 in faeces had increasing tendency. We hypothesized, that

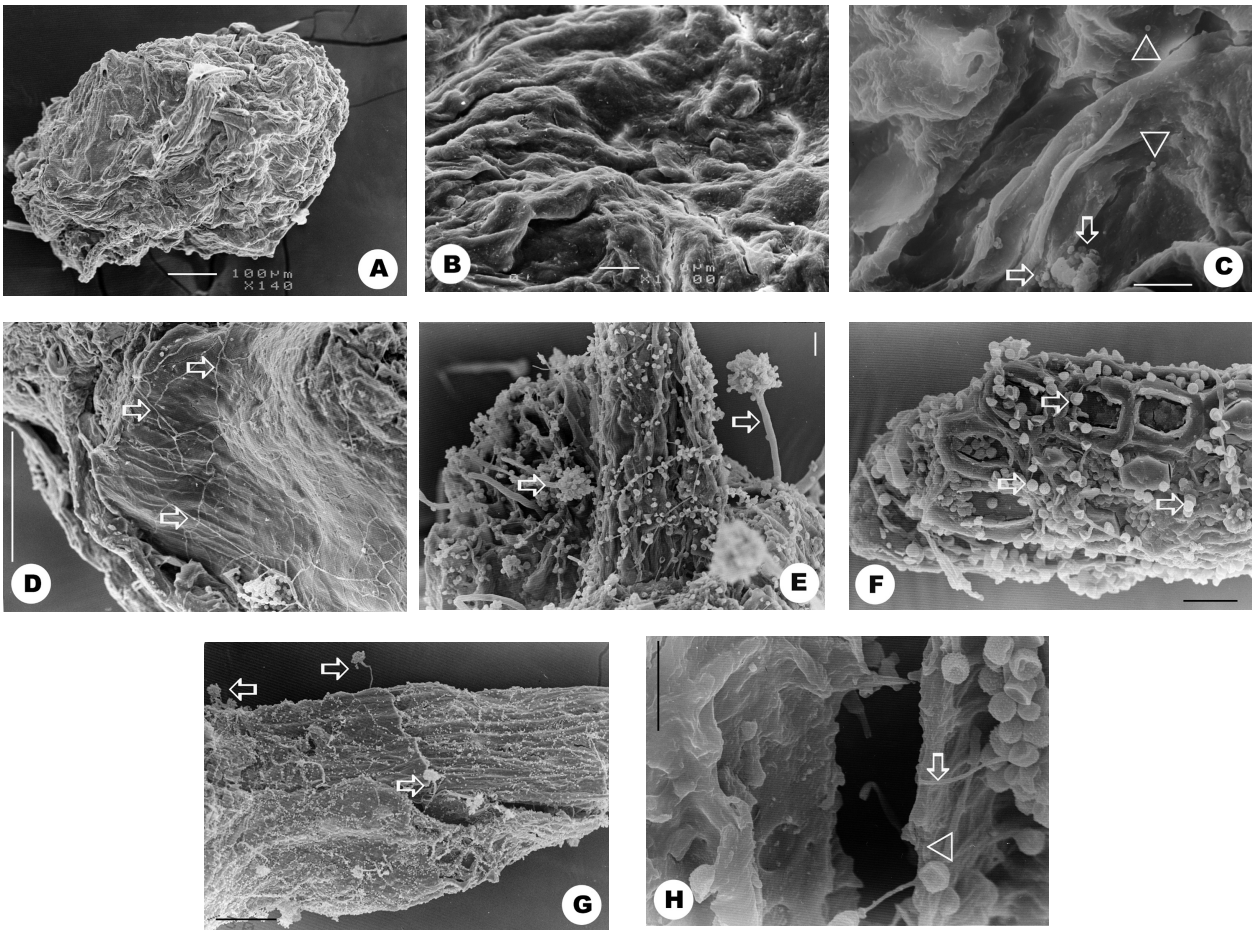

Figure 1. Scanning electron microscopy observation of the faecal pellets of *Blattella germanica* at various stages of degradation. **1AB** - control pellets just after defaecation covered by intact membrane; **1A** - total view, **1B** - detail of faecal membrane. **1C** - detail view of faecal membrane of the pellet after 84 days of degradation at 15 °C, arrows point at single conidia, white arrowheads at the clusters of conidia. **1D** - detail view of faecal membrane of the pellet after 84 days of degradation at 30 °C, the membrane with sparse superficial mycelial growth with no generative structures (arrows). **1EF** – the pellets after 168 days of degradation at 15 °C; **1E** - the faecal membrane is penetrated by mycelium, arrows point at conidiophores with conidial heads, composed of phialides and smooth conidia, **1F** - the pellet with partly penetrated membrane, arrows point at echinulate conidia. **1GH** - the pellets after 168 days of degradation at 30 °C; **1G** -the membrane of the pellet is ruptured, arrows point at conidiophores, **1H** - detail of the rupture and germination of conidia, arrowhead points at vegetative hypha, arrow points at germination hypha.

microbial enzymes hydrolyzed the proteins in the faeces and thus unmasked epitopes accessible for binding by monoclonal antibody in ELISA assay. The similar phenomenon was recorded by Mollet et al. (1997) when they observed that the peaks of Bla g 2 in the studied environment followed the highest cockroach abundance peaks two months later. In addition, there is evidence, that the homes treated by insecticide baits still contained a high level of Bla g 2 allergens in comparison to those that received professional cleaning (McConnell et al., 2003). There is also a possibility that the observed phenomenon is an artifact of used immunochemical assay that can detect peptide fragments derived from the partially degraded allergen.

The question remains opened whether partial hydrolysation of the antigen causes the increase

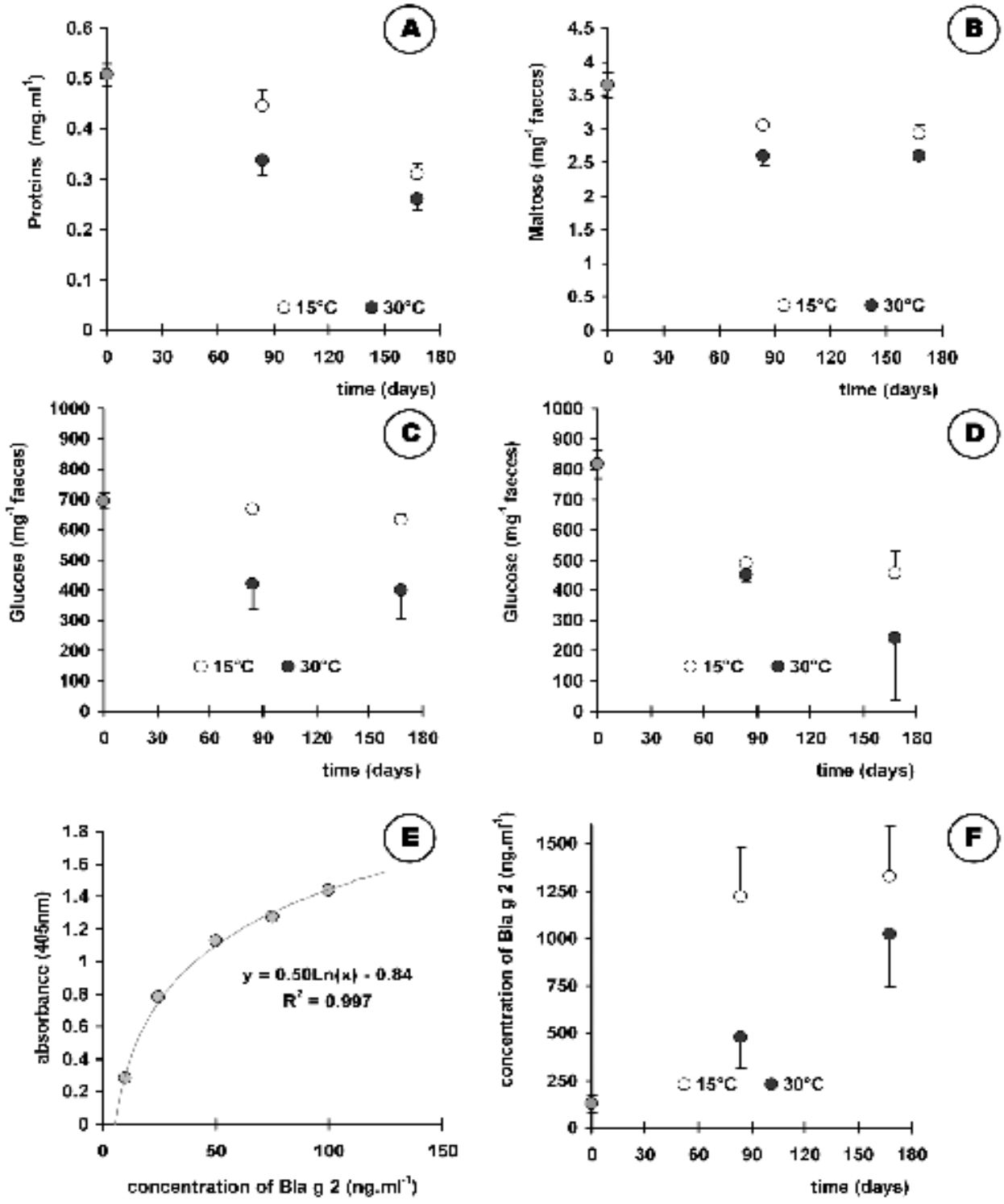

Figure 2. The biochemical features of faeces extracts at various stage of degradation. **2A** - total protein contents; **2B** - á-amylase activity; **2C** - á-glucosidase activity; **2D** - â-glucosidase activity; **2E** - ELISA response to allergen standard fitted by logarithmic curve; **2F** – quantification of Bla g 2.

of its allergenicity to humans. As changes of allergen activity can be critical for the effective allergen-hazard potential of Bla g 2, quantification by immunochemical assays should be correlated with IgE reactivity of the allergen.

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