Proteinase and gelatinolytic properties of a bat feces extract

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**ABSTRACT**

It was previously demonstrated that a bat feces extract (BAT) was able to produce a specific IgG in animals, a specific IgE in respiratory atopic humans and a hypersensitivity pneumonitis in guinea pigs. As numerous allergens (such as house-dust mite, cockroaches and pollens) revealed an enzymatic activity measured by different assays we decided to study the proteinase and the gelatinolytic activities of the BAT.

Several protease inhibitors such as E-64, TLCK, TPCK, PMSF, leupeptin, o-phenantroline and pepstatin-A were applied to establish the chemical properties of the enzymatic activity. These assays revealed a serine-trypsin-like proteolytic and gelatinolytic activities specially at pH 8,5.

On the other hand, two bands of 21 and 40 kDa reacted with the human atopic sera suggesting a possible correlation between allergenicity and proteinase activity.

Their role in the etiology of perennial rhinitis and asthma requires further investigations.

**Key words:** Bat feces. Proteases. Serine-like. IgG. IgE immunoreactivity.

**INTRODUCTION**

In previous papers it was demonstrated that a bat feces extract (BAT) was capable to elicit a specific IgG response in rabbits, a specific IgE in atopics suffering perennial rhinitis and asthma and a hypersensitivity pneumonitis in an animal experimental model.1,2,3.

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Peptidases are classified in serine, cysteine, aspartyl and metalloproteinases, according to 1): the reaction that they catalyze;²: the chemical nature of the catalytic site and³ the evolutionary relationship revealed by their structures⁴,⁵.

In this paper, we present evidence indicating that BAT contain serine-like proteinases with gelatinolytic properties which might be involved in their immunogenicity.

**MATERIAL AND METHODS**

**Antigen**

BAT was collected as previously described ¹,² and the extract was prepared with these modifications. Three grams of the feces were defatted with sulphuric ether and after its total evaporation at room temperature the BAT was treated with 100 ml of buffer containing 70 mM NaCl, 20 mM CO₃HNa at pH 8.5 with gentle shaking during 48 hs at 4°C.⁶

The extract was centrifuged and the supernatant was dialysed against distilled water 3 times during 24 hs. Then 5 % glycerol was added and the extract was sterilized with Millipore filters and stored at –20°C. The Bradford method was applied to establish the protein content of the BAT⁷.

**Enzymatic activity assay**

Minigels of 10 × 10 cm each and 1.5 mm thick composed of 12 % acrylamide were made as described by Laemmli⁸ with gelatin at a final concentration of 0,2 %. They were run at 130 V for 2 hs. When the bromophenol blue used as a marker reached the bottom, the run was stopped and the gels were washed twice in distilled water with Triton-X-100, 0,1 % for 15 min each, was stopped and the gels were washed twice in distilled water with Triton-X-100, 0.15 % for 15 min each, then incubated at 37°C in 0,1 % 2-[N-morpholino] ethane sulfonic acid (MES) buffer at pH 6, in Tris AcH 100 mM pH 3,5 and Tris ClH 100 mM pH 8,5 containing 0,5 mM dithiothreitol (DTT).

The reaction was stopped and the remaining protein was stained by incubation at room temperature with 0.25 Coomassie brilliant blue R-250 in methanol-acetic acid-water 5:1.5 (v/v/v).

After destaining in methanol 20 % and acetic acid 10 %, the active bands were observed as colorless over a blue background.

**Inhibitory assays**

The washing and incubation of the gels were done with and without the protease inhibitors.

The solutions employed were E 64 (L-trans-epoxy-succinylleucylamido [4-guanidino]-butane) 20 μM; tosyl-lysyl-chloro-methyl-ketone (TLCK) 100 μM; tosyl-phenyl-alanyl-chloro-methyl-ketone (TPCK) 1 mM; phenyl-methyl-sulphonyl-fluoride (PMSF) 10 mM; leupeptin 100 μM; o-phenantroline 1 mM and pepstatin-A 2 μM⁹,¹⁰.

The molecular weight markers were phosphorylase-b (97,4 kDa), bovine serum albumin (BSA) (66,2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21,5 kDa) and lysozyme (14,4 kDa). For activity gels, the samples were not reduced nor boiled before loading.

**Western blots**

The samples with or without β-mercaptoethanol and boiling were run in 15 % standard polyacrylamide gel in the presence of SDS (SDS-PAGE), electrotransferred to nitrocellulose sheets, blocked for 2 h with a solution containing 2 % fatty acid-free BSA, 0.01 % v/v Tween-20, PBS pH 7,2 and then incubated overnight with rabbit polyclonal anti-BAT serum 1/400 and human sera 1/20. After overnight incubation with the rabbit or human antisera, respectively, and repeated washing the sheets were treated with 1/3000 goat anti-rabbit IgG horseradish peroxidase conjugate or 1/500 rabbit anti-human IgE specific for e-chains peroxidase conjugate at room temperature during 2 hs.. The chromagenic detection was developed using α-chloronaphtol and hydrogen peroxide¹¹.

**RESULTS**

BAT in SDS-PAGE showed 6 to 8 bands between 21 to 97 kDa (fig. 1).

The gelatinolytic activity of the BAT in SDS-PAGE with co-polymerized gelatin as substrate was recorded. The proteolytic activity pattern of BAT was preliminarily analyzed at three different pH levels, 3,5, 6 and 8.5. The highest enzyme activity was at pH 8,5 with less activity at pH 6 and almost no activity at pH 3,5 (fig. 2).

Total activity pattern at pH 8,5 was highly sensitive to TLCK and PMSF while the major and broad band (65 kDa) and the minor (31 kDa) showed the same inhibition pattern. Hence, we tentatively characterized this enzyme as a trypsin-like serine protease (fig. 3).

When the BAT was separated by SDS-PAGE, transferred to nitrocellulose and incubated with a polyclonal rabbit anti-BAT serum, the 6 to 8 bands of apparent molecular weights between 21 to 97 kDa...
showed immunoreactivity (fig. 4). The same results were obtained with or without DTT as a reductor agent in the sample suggesting that disulfide bonds must be absent in these immunogenic bands. On the other hand, when a human anti-BAT serum was employed only the bands of 21 and 40 kDa with gelatinolytic protease activity seemed to be associated with immunoreactivity although many different proteins may be present in each broad band.

**DISCUSSION**

Several major allergens in the extracts of insects possess protein hydrolase properties. Also, acid phosphatase activities correlate well with allergenic potency in pollen extracts. Previous studies showed the identification of a novel serin-protease with allergenic activity from Dermatophagoides pteronyssinus.

Elsewhere, we described the correlation between some proteases with gelatinolytic activity and the allergenicity of house-dust mite and cockroach extracts.

We suppose that this is the first report about the trypsin-like serine protease with gelatinolytic activity of a extract of BAT feces of mammalian origin and not
from an avian source\textsuperscript{14}. Meanwhile all the proteins separated by SDS-PAGE showed immunoreactivity in the Western blots with a polyclonal rabbit anti BAT serum only 2 bands (21 and 40 kDa) reacted with the human atopic sera suggesting a correlation between allergenicity and gelatinolytic protease activity.

The presence of serine-like proteases with gelatinolytic activity in the bat feces is intriguing and led us to suspect the existence of enzymatic products coming from the digestive tract of the bat, or exuded serum proteins or metabolic products deriving from the daily ingestion of the bat. It was also taking into account the relevant importance that tropomiosin had gained in the last decade as a common molecule present in several arthropods, molluscs and mammals although the physicochemical properties are different. It is very important to define the proteases present in all these extracts and to determine their ability to degrade other proteins of the same extract or in mixtures of allergen extracts.

Their role in the immunopathological aspects of rhinitis and asthma requires further investigation.

REFERENCES