ALLERGENIC ACTIVITY OF AN ESTERASE FROM *BOOPHILUS MICROPLUS*

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1. Introduction

Cattle exposed to the tick *B. microplus* show an immediate hypersensitivity reaction to tick extracts which is small or absent in animals which have not been exposed to the parasite. Substances producing this reaction will be referred to as allergens. The isolation and partial characterization of one tick allergen has been reported [1]. It was suggested that the allergen was a DFP-sensitive esterase because the esterase and allergenic activities could not be separated by a variety of chromatographic and electrophoretic techniques. Such evidence must remain inconclusive, since the possibility cannot be excluded that allergen and esterase are physically very similar, but still distinct, proteins. A convincing demonstration that both activities are due to a single protein must depend on a unique property of one of the activities. Fortunately, the sensitivity of the enzyme to inhibition by organophosphates provides such a property. This inhibition occurs by formation of a phosphorylated enzyme, a reaction which is normally irreversible, but which can be reversed under appropriate conditions by the addition of nucleophiles such as hydroxylamine [2]. This paper reports the preparation of a reactive phosphoramidate coupled to cellulose as an insoluble support and the use of this material to demonstrate the allergenic activity of the tick esterase.

2. Materials and methods

The preparation of tick esterase, the assay of esterase activity towards fluorescein dibutyrate as substrate and the assay of allergenic activity have been described previously [1]. Purified allergen was used as the reference material in the estimation of allergen concentration and the results are given on a logarithmic scale in arbitrary units.

2.1. *Preparation of aminocellulose*

Twenty millimoles periodic acid was diluted to 25 ml of solution and the pH adjusted to 1.9. Two grams of Whatman cellulose powder, CF1, was stirred in this solution for 20 h at room temperature, the mixture filtered and washed with water. The cellulose was suspended in 50 ml water, 3.1 mmol of 1,6-diaminohexane dihydrochloride was added and the pH adjusted to 9.0. Eleven millimoles of potassium borohydride was added over 1.5 h and the suspension stirred for a further 1 h, maintaining pH at 9.0–9.5 throughout. The pH was reduced to 2.0, the suspension filtered, washed in water, then resuspended, adjusted to pH 10.0, again filtered and washed, before drying to yield 1.44 g.

Titration of samples of the aminocellulose showed 0.22 mmol/g of ionizable groups with a pK_a of approx. 7.7 and 1.74 mmol/g of groups titrating between pH 5.0 and pH 2.6. The former can be attributed to the primary or secondary amino groups and the latter to the mixture of tertiary amine species expected from the reaction.

2.2. *Preparation of the ethyl* p*-nitrophenyl phosphoramidate derivative of aminocellulose*

Dry aminocellulose, 1.0 g, was suspended in 20 ml chloroform with 1 mmol *p*-nitrophenyl phosphorodichloridate and 1 mmol 2,6-lutidine. This was refluxed 2.5 h, then 1 ml dry ethanol and a further 2 mmol 2,6-lutidine were added. After an additional 2 h under reflux, the suspension was filtered and washed thoroughly with absolute ethanol, to yield

0.96 g after drying. This material will be referred to as 'activated cellulose'.

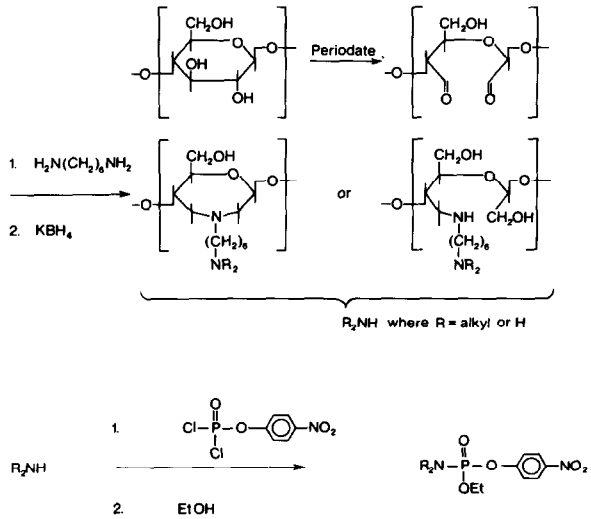
Cellulose-bound *p*-nitrophenol amount was estimated spectrophotometrically after hydrolyzing samples of about 10 mg in 5 ml 5 N hydrochloric acid at 100°C for 5–68 h. The product contained 1.6×10^{-2} mmol *p*-nitrophenol/g cellulose.

Chymotrypsin was found to react very slowly with the activated cellulose. However, 54 mg α-chymotrypsin and 8.6 mg cellulose, in 1 ml phosphate buffer, pH 7.5, after 20 h at 37°C, released *p*-nitrophenol equivalent to 5.9×10^{-3} mmol/g cellulose, while in a control lacking enzyme there was no significant release. This would be a minimum estimate of reactive phosphoramidate concentration. However, for an esterase of mol. wt 60 000, it still corresponds to a binding capacity of 0.36 g protein/g support.

The synthesis is summarized in Scheme 1, but is simplified to the extent that it does not show possible side reactions.

2.3. *Inhibition of esterase by activated cellulose*

Activated cellulose, 6.8 mg, was added to 8 μg of esterase in 1.05 ml 0.1 M phosphate buffer, pH 7.5, 1 mM in 2-mercaptoethanol and kept in a water bath at 37°C with occasional shaking. After 9 min, the residual enzyme activity was less than 1%.



Scheme 1

2.4. *Chromatography of esterase on activated cellulose*

Two buffers were used in the chromatography. Buffer A contained 0.1 M phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 0.5 M sodium chloride, 1 mg/ml bovine serum albumin and had a final pH of 7.1. Buffer B was identical, except for the substitution of 0.5 M hydroxylamine hydrochloride for sodium chloride. A 0.9 × 0.6 cm column containing 50 mg activated cellulose was prepared in buffer A. Then 240 μg of antigen in this buffer, in a total volume of 1.4 ml, was washed onto this column at a flow rate of 5 ml/h. The calculated time for reaction between solution and solid support was less than 2.7 min. The column was at 24°C, but fractions were collected at 4°C. After the column had been washed thoroughly, buffer A was replaced by buffer B.

The concentration of hydroxylamine in the fractions was measured by adding 20 μl aliquots to 2 ml of phosphate buffer, pH 7.8, containing 10^{-4} M *p*-nitrophenyl butyrate. The initial rates of *p*-nitrophenol release were measured at 400 nm using a Varian 635 M spectrophotometer and compared with those for a series of known hydroxylamine concentrations.

After completion of chromatography, all fractions were dialyzed against 0.05 M phosphate buffer, pH 7.5, containing 1 mM 2-mercaptoethanol, and allergenic activities measured subsequently.

DIP-esterase was prepared as described previously [1]. It was chromatographed on activated cellulose exactly as described for the unmodified esterase.

3. Results and discussion

If allergenic and esterase activities are due to the same protein, attachment of esterase to the cellulose by phosphorylation of the enzyme's active site serine residue would result in the attachment of allergenic activity, subsequently released by treatment with hydroxylamine. The test of the hypothesis, that a single protein possesses these two biological activities, is whether or not a hydroxylamine-catalyzed release of allergen occurs. The chromatography of esterase on the column of activated cellulose is shown in fig.1. Of the esterase applied to the column, 16% was eluted immediately. It was accompanied by allergenic activity, which could be due to unbound esterase and

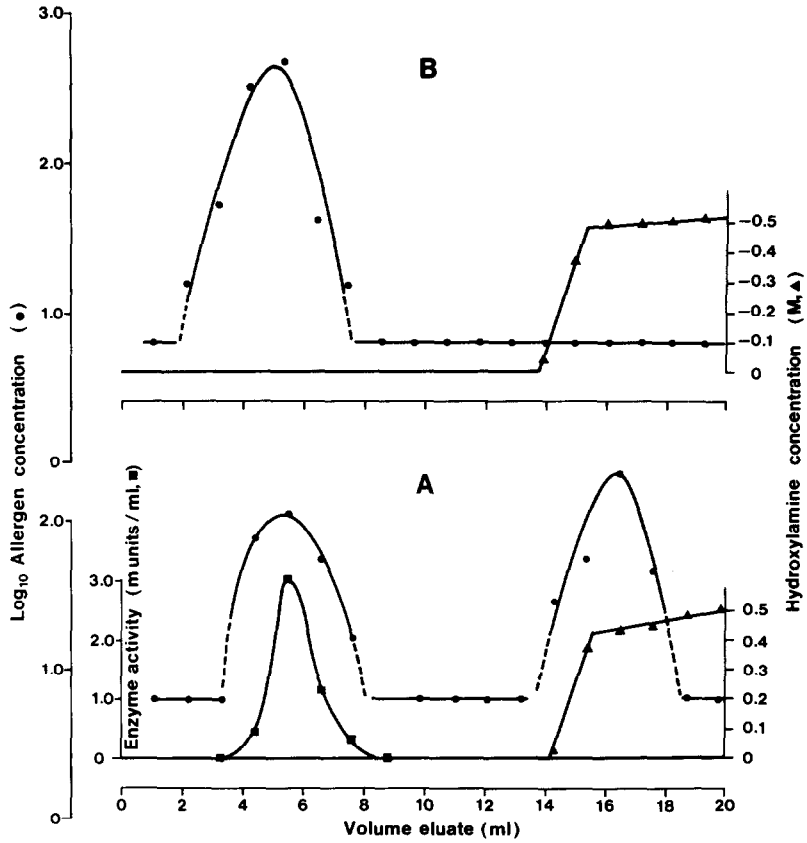


Fig.1. Chromatography of tick esterase (Graph A) and DFP-treated esterase (Graph B) on activated cellulose. The experimental points for allergen concentration with a $\log_{10}$ concentration of less than 1.0 gave no detectible dermal reaction.

any enzymatically inactive, esterase protein present. It has been shown that the cellulose is capable of completely inhibiting the esterase activity when the two are mixed in a tube. However, under the conditions of the chromatography, with a short reaction time at 24°C, incomplete reaction may be expected. The significant result is that allergenic activity is eluted with hydroxylamine. The result seems unambiguous. Although the cellulose will have some ion-exchange capacity due to attached amino groups, elution of any ionically-bound protein by the hydroxylamine-containing buffer is extremely unlikely since the pH of the solution does not alter during buffer change and ionic strength is, if anything, reduced.

As a further check, DIP-esterase was chromatographed under the same conditions. Here, the reactive serine of esterase was phosphorylated before loading onto the column. All allergenic activity should be eluted immediately and none should appear in the hydroxylamine-containing fractions. That this occurred is shown in fig.1. These two experiments show that the tick serine esterase possesses allergenic activity.

It might still be argued that the allergen could be a separate protein, non-covalently associated with the esterase. The association would then have to be stable not only to the separation procedures described previously [1] but also to the relatively high salt concentrations used in these experiments. In addition, the molecular weight of the esterase, 60 000 [1], is typical of such enzymes and does not suggest the association of two different proteins.

The release of esterase protein from the cellulose could occur in two ways, either by cleavage of the P–O bond to the enzyme or the P–N bond to the

cellulose support. Reactions analogous to the former have been thoroughly investigated, since they result in regeneration of enzymatic activity. Reactions analogous to the latter have been observed [3,4] but have been studied far less. In the present case, control experiments showed that both esterase and allergenic activities were unaffected by incubation in buffer B at 24°C for up to 64 min before dialysis. However, in the fractions eluted from the cellulose column by hydroxylamine, only about 0.3% of bound enzymatic activity was recovered, though it accompanied allergenic activity. This suggests that the esterase has been mainly released by cleavage of the P–N bond. A possible model for this is the acid-catalyzed hydrolysis of phosphoramidates [5].

Antigens producing immediate hypersensitivity reactions have been investigated, almost without exception as immunologically active materials, without regard to the possibility of additional biochemical functions. That the tick allergen is an esterase suggests an important aspect of the biochemistry of such antigens may have been neglected. One other allergen has been identified with an enzyme. Evidence has been given that phospholipase A is one of the allergens of honey bee venom [6,7].

Acknowledgement

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