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Request reprints from: Dr. M. CESKA, Sandoz Research Institute, Brunnerstrasse 59, A-1235 Wien (Austria)

Study of the Nature of the Binding Sites of Some Allergens Interacting with IgE

M. CESKA and G. PONTERIUS

Department of Biochemistry, Pharmacia AB, Uppsala

Abstract. The nature of one of the binding sites of interacting macromolecules was studied by employing a model system consisting of immobilized allergens and specific reaginic antibodies present in the sera of allergen sensitive patients. Prior to their interaction with reaginic antibodies, the immobilized allergens were treated with a variety of enzymes in order to determine their susceptibility to enzymatic hydrolysis.

Introduction

A knowledge of the nature of specifically interacting molecules is perhaps one of the most important prerequisites for an understanding of biological processes. The nature of enzyme binding sites together with their substrates and inhibitors is intensively studied. The size as well as the nature of the binding sites involved in the interaction between antigens and antibodies is one of the primary research objectives of immunochemistry. The interaction and binding of hormones to cell receptor sites and especially the nature of such receptor sites is gaining a great deal of interest and will certainly contribute to an understanding of hormone function at the molecular level.

The present paper aims to describe some experiments which in some cases may be employed to study the nature of the binding sites of interacting macromolecules. Some of the macromolecules, in our case the allergen, is immobilized by attachment to a solid matrix. The immobilized allergens are then subjected to hydrolysis by various enzymes. The remaining allergenic activity of the solidified allergen is then assayed by means of specific reactions using radioactive indicators. In this paper, the allergenic

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activities remaining after the enzymic treatments, were determined by the paper disc radioallergosorbent (RAST) method [CESKA *et al.*, 1972a]. The information gained from such studies may help to establish the nature of those active sites of allergens engaged in interaction with specific reaginic antibodies.

Materials and Methods

Materials

Two different types of allergen extracts (birch and dog) were obtained from a commercial source known for its allergen extract preparation. (The source of allergens will be specified on request.) The following enzymes were used: pepsin, 2,600 U/mg (Worthington); alkaline protease, 2,240 U/mg (Seikagaku, Miles); neutral protease, 12,000 U/mg (Miles); acid protease, 1,800 U/mg (Miles); subtilisin, 1,500 PUN/mg (Mann); pronase, 70 PUK/mg (Merck); bromelin, 2 U/mg (Sigma); elastase, 45 U/mg (Worthington); trypsin, 2 U/mg (Merck); α -chymotrypsin, 45 U/mg (Worthington); Carboxypeptidase A, 66 U/mg (Worthington); carboxypeptidase B, 95 U/mg (Worthington); collagenase, 198 U/mg (Worthington); papain, 3.5 m Anson-E/mg (Merck); ficin, crude – 5–20 times the activity of papain (Mann); α -amylase, 600 E/mg (Serva); β -amylase, 45 U/mg (Serva); amyloglucosidase (1 mg will liberate 3 mg glucose from starch in 3 min at pH 4.5 at 56 °C) (Sigma); dextranase, 21 U/mg (Worthington); cellulase, 15,000 U/mg (Seikagaku); hyaluronidase 300 USP U/mg (Mann); β -galactosidase, 28 U/mg (Sigma); α -neuraminidase, 1 ml liberates 500 mg N-acetyl neuraminic acid in 15 min at 37 °C (Serva). The following buffer systems were used: for pepsin: 0.01 M HCl; for papain: 0.05 M tris-HCl, pH 8.0 containing 0.005 M L-cysteine and 0.002 M EDTA; for ficin: 0.1 M phosphate buffer, pH 7.0 containing 0.001 M EDTA; for alkaline protease: 0.1 M phosphate buffer, pH 7.5; for elastase: 0.2 M tris-HCl buffer, pH 8.8; for pronase E: 0.07 M phosphate buffer, pH 7.4; for bromelin: 0.03 M phosphate buffer, pH 7.2 containing 0.05 M L-cysteine; for trypsin: 0.667 M phosphate buffer, pH 7.0; for α -chymotrypsin: 0.1 M tris-HCl, pH 7.8; for acid protease: 0.05 M lactic acid (pH adjusted to pH 3.1 with 1.0 M NaOH); for neutral protease: 0.01 M NaCl; for subtilisin: 0.03 M phosphate buffer, pH 7.5; for collagenase: 0.05 M tris-HCl, pH 7.5; for carboxypeptidase A: 0.025 M tris-HCl, pH 7.5 containing 0.5 M NaCl; for carboxypeptidase B: 0.05 M tris-HCl, pH 8.0; for α -amylase: 0.02 M phosphate buffer, pH 7.1 containing 0.015 M NaCl; for β -amylase: 0.02 M phosphate buffer, pH 7.1 containing 0.015 M NaCl; for amyloglucosidase: 0.01 M Na-citrate, pH 6.0 containing 0.005 M mercaptoethanol; for dextranase: 0.1 M phosphate buffer, pH 6.0; for cellulase: acetate buffer, 0.1 M, pH 5.0; for hyaluronidase: 0.02 M phosphate buffer, pH 7.0 containing 0.45% NaCl and 0.01% bovine albumine; for α -neuraminidase: 0.1 M acetate buffer, pH 5.0; for β -galactosidase, 0.05 M phosphate buffer, pH 7.4.

The activity of the above enzymes was tested before use.

Methods

Coupling of Allergens to BrCN-Activated Paper Discs and the Paper Disc Radioallergosorbent Test

A detailed account of this method is given elsewhere [CESKA *et al.*, 1972a], but a brief summary of the most important steps are given below: (1) coupling of allergen extracts to BrCN-activated paper discs; (2) incubation of immobilized allergens (both enzymatically digested and undigested) with sera of allergen sensitive patients, and (3) incubation of the paper discs with ^{125} I-anti-Fc IgE(ND) and measurement of the radioactivity retained on the paper discs after extensive washing.

Enzymatic Hydrolyses of Immobilized Allergens

The immobilized allergens were hydrolyzed with the enzymes mentioned under Materials, at the concentrations given below. To each of eight paper discs with immobilized allergens, 0.5 ml of the respective enzyme solution was added. In each case a control containing 0.5 ml of the respective buffer only was prepared. The hydrolysis was allowed to proceed in water-bath with gentle horizontal shaking for 24 h. The temperature of incubation for various enzymes was as follows:

37 °C for: pepsin (0.5 mg/ml), papain (1.0 mg/ml), ficin (2 mg/ml), alkaline protease (1.0 mg/ml), elastase (1.8 mg/ml), pronase E (1.0 mg/ml), bromelin (1.5 mg/ml), collagenase (1.0 mg/ml), α -amylase (0.5 mg/ml), β -amylase (5 mg/ml), cellulase (0.5 mg/ml), hyaluronidase (1.0 mg/ml), α -neuraminidase (dil. 10 times) and β -galactosidase (0.5 mg/ml).

30 °C for: trypsin (0.5 mg/ml), α -chymotrypsin (0.5 mg/ml), acid protease (1.0 mg/ml), neutral protease (1.0 mg/ml) and subtilisin (1.5 mg/ml).

25 °C for: carboxypeptidase A (1 mg/ml), carboxypeptidase B (1 mg/ml) and amyloglucosidase (0.5 mg/ml).

45 °C for: dextranase (1.0 mg/ml).

The enzymatic hydrolyses were terminated by placing the enzyme-assay tubes into an ice-bath. (The hydrolysates are now stored at –20 °C for analyses of peptides and carbohydrates.)

The paper discs were after the enzymatic hydrolyses gently washed 5 times with the incubation buffer (without Tween 20) [CESKA *et al.*, 1972a].

To two paper discs, 100 μ l of the respective patient sera (dil. 2:3) was added (duplicates were run in each case), and the tests were incubated over-night at room temperature. After washing, the paper discs were then incubated with ^{125} I-anti-Fc IgE(ND).

Appropriate controls were run in order to ascertain that after the enzymatic hydrolysis and extensive washing of the paper disc-allergen conjugates that the enzyme had been completely removed. (Their presence could hydrolyze IgE contained in the serum and/or the added ^{125} I-anti-Fc IgE(ND)). Only the enzymatic activities of those enzymes, which showed a higher degree of allergen hydrolysis (pepsin, papain, alkaline protease, pronase E and α -chymotrypsin) were tested. No contaminating enzymatic activities were found.

Results

The experimental system used is represented schematically in figure 1. Allergens were immobilized by covalent linkages to BrCN activated paper discs. They were then either (a) incubated with allergic sera and then with

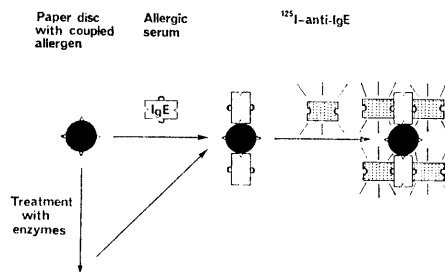


Fig. 1. Schematic representation of the experimental system used. In the control experiment the immobilized allergens were incubated with allergic sera and then with ^{125}I -anti-Fc IgE(ND). In the actual enzymatic degradation experiment, the immobilized allergens were treated with a variety of enzymes prior to the incubation with the respective allergic sera and ^{125}I -anti-Fc IgE(ND).

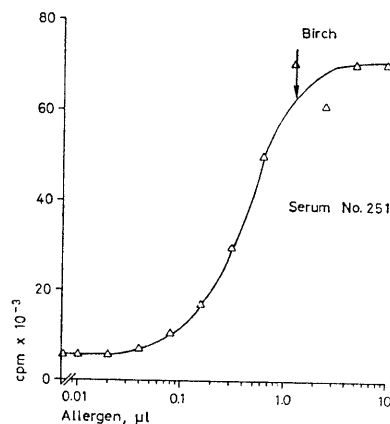


Fig. 2. Dose-response plot. Various concentrations of an immobilized birch allergen extract were tested with serum (serum No. 251) from a birch allergen-sensitive patient. The arrow indicates the immobilized allergen concentration used for the enzyme degradation studies.

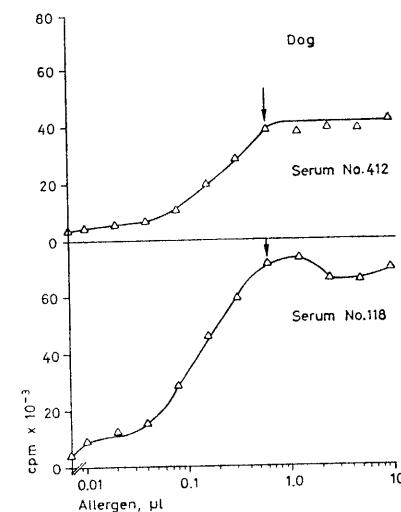


Fig. 3. Dose-response plot. Various concentrations of an immobilized dog allergen extract were tested with two different sera (sera No. 412 and No. 118) from dog allergen sensitive patients. The arrows indicate the immobilized allergen concentration used for the enzyme degradation studies.

^{125}I -anti Fc IgE(ND) – which is a control experiment – or (b) the immobilized allergens were treated with enzymes prior the incubation with allergic sera and ^{125}I -anti-Fc IgE(ND).

Dose-response curves were prepared using the paper-disc RAST procedure [CESKA *et al.*, 1972a] in order to select the appropriate concentration of the allergen extracts necessary for the coupling procedure. The dose-response curve prepared with immobilized birch allergen and using serum No. 251 is shown in fig. 2. (Another birch dose-response curve was also constructed using 2 different sera from birch allergen sensitive patients. These sera unfortunately did not show acceptable levels of reaginic antibodies.) Figure 3 shows two dog dose-response curves obtained with immobilized dog allergen and with sera from two dog sensitive patients, serum No. 412 and No. 118. From these dose-response curves, it was possible to select a suitable allergen extract concentration for the coupling

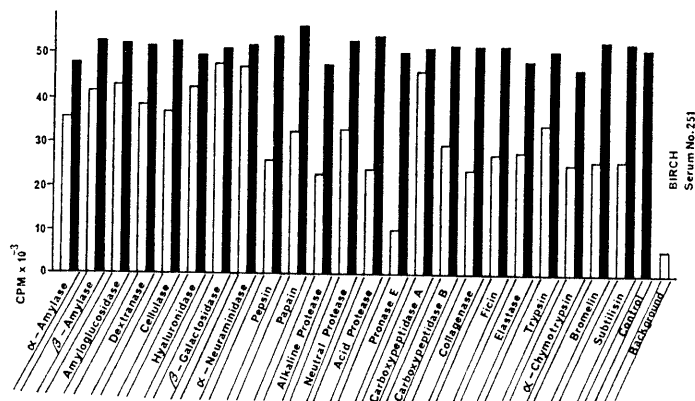


Fig. 4. The enzymatic hydrolysis of immobilized birch allergen extract by a variety of enzymes. The filled bars are the controls (not treated with enzymes), while the empty bars show the extent of enzymatic hydrolysis. The testing was done with birch allergen-sensitive serum No. 251.

to the BrCN-activated paper discs. Birch allergen extract was diluted about 80-fold, while dog allergen extract was diluted about 160-fold, prior the coupling procedure. Such dilutions of allergen extracts ensured that under the experimental conditions employed here for the paper-disc RAST procedure one is working in the 'non-saturation region'.

The immobilized allergens (coupled to BrCN-activated paper discs using the selected allergen extract concentration) were then hydrolyzed with the various enzymes. The results obtained with birch allergen are seen in figure 4. The filled bars are the controls (no enzyme), while the empty bars show the extent of hydrolysis by the various enzymes. The penultimate bar on the right hand side represent a control performed under standard conditions and gives the total uptake of ^{125}I -anti-Fc IgE(ND). In this case the immobilized allergen was measured immediately by the paper-disc RAST procedure in order to determine the total uptake of ^{125}I -anti-Fc IgE(ND). Figure 5 shows the results of enzymatic hydrolysis of immobilized dog allergens. The allergenic activity was assayed by the paper disc RAST procedure using two dog allergen sensitive sera, No. 118 (the top part of the figure) and No. 412 (the lower part of the figure).

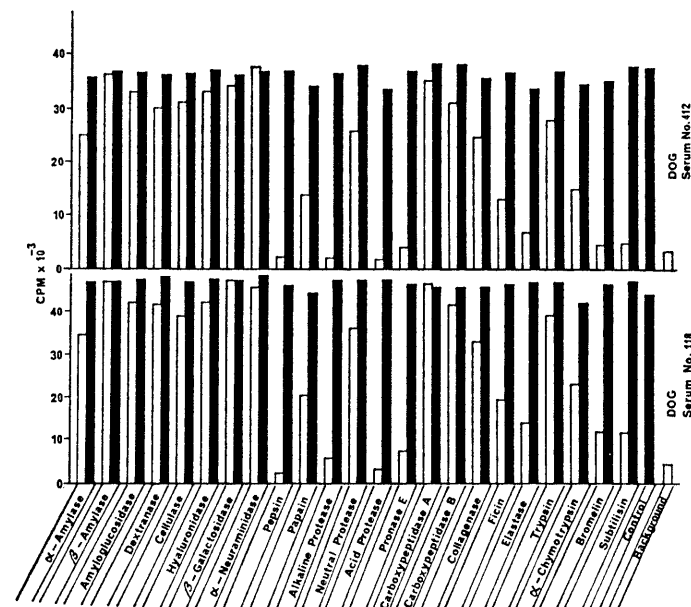


Fig. 5. The enzymatic hydrolysis of immobilized dog allergen extract by a variety of enzymes. The testing was done with two different dog allergen-sensitive sera (sera No. 412 and No. 118).

Discussion

The RAST technique has been used previously with success for measuring the extent of interaction between allergens and specific reaginic antibodies [WIDE *et al.*, 1967]. This technique was then simplified, by using BrCN-activated paper discs as a matrix for the attachment of allergens [CESKA *et al.*, 1972a] and this modified procedure used for assaying the allergenic potencies of various allergen extracts. Combination of the paper disc RAST procedure with isoelectrofocusing of allergen extracts in polyacrylamide gel was employed for characterizing allergen components on the basis of their pI [CESKA *et al.*, 1972b; CESKA and HULTEN, 1972; CESKA, 1972; VARGA and CESKA, 1972a, b]. In this present study, the na-

ture of the immobilized allergens was investigated after exhaustive hydrolysis by various enzymes. Comparing the figure 4 (birch allergen extract) with figure 5 (dog allergen extract) it can be observed that birch allergen is more susceptible to carbohydrases than the dog allergen. However, the dog allergen seem to be more susceptible to proteolytic enzymes compared with birch allergen. The susceptibility to carbohydrases is in case of both allergens less than to proteinases.

The birch allergen is hydrolyzed extensively by pronase. The intermediary hydrolysis values were obtained with pepsin, papain, alkaline protease, neutral protease, acid protease, carboxypeptidase B, collagenase, ficin, elastase, trypsin, α -chymotrypsin, bromelin and subtilisin. The slight hydrolysis by carboxypeptidase B and trypsin is quite evident. Carboxypeptidase A hydrolyzes the allergen at a lower rate than carboxypeptidase B, which may be due to the fact that the birch allergen may have more of the basic amino-acids at the carboxyl terminal end. The comparatively large resistance to pepsin is interesting, as the majority of atopic allergens are shown to be hydrolyzed by pepsin [BLEUMINK and YOUNG, 1968].

The dog allergens are completely hydrolyzed by pepsin, alkaline protease, acid protease, pronase and to a large extent by bromelin and subtilisin. Intermediary hydrolysis values are obtained with papain, ficin, elastase and α -chymotrypsin. The dog allergens are essentially resistant to neutral protease, carboxypeptidase A, carboxypeptidase B and trypsin.

The relatively large resistance of allergens to trypsin digestion is well known and was also observed in this study. The resistance may be related to the extent of substitution of ϵ -amino groups by sugars [BERRENS, 1968]. It is known that there exists an inverse relationship between trypsin digestibility and the average number of sugar substituted lysine residues in for example β -lactoglobulin-lactose conjugates (which exhibits skin-reactivity in milk sensitive atopic patients). The allergenicity of several powerful inhalant allergens is not affected by enzymatic hydrolysis and therefore it was suggested [GOLDOWSKI, 1965] that the atopens must have some special property which enables them to resist proteolysis. Allergens show a higher resistance to trypsin than to α -chymotrypsin which is probably due to the specificities of these enzymes.

Cleavage of the sugar moiety does not diminish the allergenic activity significantly. This fact is well documented also in this study.

This paper describes a preliminary study of the chemistry of immobilized allergens. The *in vitro* assay technique employed and its modifications may be well suited to the study of some aspects of the nature of

binding sites involved in the reaction between specifically interacting molecules.

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