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PARTIAL CHARACTERIZATION OF AN ANTIGENIC SITE OF HIGH MOLECULAR WEIGHT BASIC ANTIGEN, A RYEGRASS POLLEN ALLERGEN, USING A MONOCLONAL ANTIBODY*

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(First received 30 January, 1985; accepted in revised form 23 July 1985)

Abstract—We have previously reported on the production of murine monoclonal antibodies (Mabs) to the retentate fraction of the aqueous extract of Kentucky bluegrass pollen (KBG-R) [Kisil *et al.* (1980) *Fedn Proc.* 39, 3479]. In the present study, the ability of one of these Mabs (Mab 12) to recognize various antigenic components present in KBG-R and the corresponding fraction of ryegrass (rye-R) was evaluated by the Western and immunoblot methods. Thus, KBG-R and rye-R were resolved electrophoretically into a large number of components. Remarkably, the concurrent immunoblot analysis with Mab 12 detected only a single antigenic component in each of the retentate fractions. The position of the antigenic component observed on these immunoblots was identical to that obtained with the rye allergen high mol. wt basic antigen (mol. wt 56,800). To characterize the antigenic site recognized by the Mab, the size of HMBA was reduced by cleavage with CNBr; the resulting fragments separated by high-performance liquid chromatography on a reverse-phase column and their antigenic activity analyzed by immunoblot. Two peptides, CB-1 (mol. wt = 17,400) and CB-2 (mol. wt = 22,000), retained the capacity to react with Mab 12 and also IgE antibodies present in a pool of sera from grass allergic individuals.

INTRODUCTION

Studies to identify and elucidate the structural moieties of allergens, which can trigger IgE-mediated allergic reactions, require that highly purified allergens be available in sufficient amounts. The complex and heterogeneous nature of the constituents of pollen which are extractable with water necessitates that a combination of several different methods be employed to isolate an allergen with a high degree of purity. However, in spite of painstaking measures for antigen purification, the preparation of an antiserum capable of recognizing a single antigenic specificity is a difficult, if not an impossible task to achieve. The production of monoclonal antibodies by hybridoma technology (Köhler and Milstein, 1975) has surmounted this limitation associated with the conventional method of preparing a monospecific antiserum. Thus, clones of hybridoma cells, each capable of producing antibodies of a single specificity, were prepared with spleen cells from mice immunized with

the heterogeneous and complex mixture of components present in the retentate fraction of the dialyzed preparation of the aqueous extract of KBG[‡] pollen (Kisil *et al.*, 1980). In the process of characterizing the specificity of a monoclonal antibody, designated as Mab 12, it was found to recognize not only an antigen present in KBG-R but also HMBA, isolated from the retentate fraction of ryegrass pollen (Ekramoddoullah *et al.*, 1983). The importance of HMBA was demonstrated by the finding that all sera from individuals allergic to ryegrass pollen contained IgE antibodies to this allergen.

In the present study, the analysis of KBG-R and rye-R by a combination of Western and immunoblot methods employing Mab 12 revealed the presence, in both pollen extracts, of an antigenic component that was similar, if not identical, in size and immunoreactivity to that of HMBA. Since there was a high incidence of an allergic response to this allergen and it was available in a pure form, HMBA was judged to be a good candidate for attempts to characterize the antigenic site recognized by Mab 12.

MATERIALS AND METHODS

Preparation of KBG-R, rye-R and HMBA

The non-dialyzable components of KBG (*Poa pratensis*) and ryegrass (*Lolium perenne*) pollen (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) were prepared by extracting defatted pollen with distilled water at room temp followed by dialysis

*This work was supported by grants from the Medical Research Council of Canada.

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‡Abbreviations: HSA, human serum albumin; KBG, Kentucky bluegrass; KBG-R, KBG retentate; rye-R, rye retentate; HMBA, high mol. wt basic allergen; TBS, Tris-buffered saline; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunoassay; RAST, radioallergen sorbent test; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IB, incubation buffer.

through Spectrapor membrane tubing (mol. wt cutoff = 6000–8000, Spectrum Medical Industries Inc., Los Angeles, CA). The retentate fractions were designated as KBG-R and rye-R, respectively. HMBA was isolated from rye-R as described previously (Ekramoddoullah *et al.*, 1983).

Briefly, rye-R was first fractionated on Sephadex G-50 (Pharmacia AB, Uppsala, Sweden) with 0.05 M Tris-HCl, pH 8.6, as the eluant. Fraction rye-R-1 was rechromatographed on Sephadex G-50 and was next separated by preparative isoelectrofocusing using ampholyte buffer, pH range 3.5–10 (LKB Products AB, Bromma, Sweden). The extreme cathodal fraction was further separated by gel filtration on Ultrogel AcA 44 (LKB) equilibrated with 0.05 M phosphate buffer, pH 7.2, containing 0.15 M Na₂SO₄. Fraction 2 was rechromatographed on AcA 44 to obtain HMBA.

Production and purification of Mab 12

Mab 12 was one of several murine monoclonal antibodies produced against KBG-R (Kisil *et al.*, 1980). The methods used for the fusion and screenings were described in detail elsewhere (Ekramoddoullah *et al.*, 1984). Briefly, BALB/c female mice (Timco Breeding Laboratories, Houston, TX) were immunized with KBG-R. The immune spleen cells were fused with P3 × 20 myeloma cells. Clones of hybridoma cells secreting anti-KBG-R antibody were expanded *in vitro* and then propagated in the peritoneum of C2B6F1/J (BALB/cJ × C57BL/6J) female mice (Jackson Laboratory, Bar Harbor, ME). The ascitic fluid containing the monoclonal antibody was collected by puncturing the abdominal wall with a 20-gauge needle and allowing the fluid to leak out.

The monoclonal antibody was purified by the method of Bruck *et al.* (1982). For this purpose, DEAE-Affi-Gel blue (Bio-Rad Laboratories, Richmond, CA) was washed according to the manufacturer's instructions and packed into a glass column (1 × 50 cm) and equilibrated with 0.02 M Tris-HCl, pH 7.2. The ascitic fluid was dialyzed against the Tris-HCl buffer, then clarified by centrifugation before applying to the column. The column was washed with three bed vols of the equilibrating buffer, followed by three bed vols of equilibrating buffer containing 25 mM NaCl. The monoclonal antibody was then eluted with the equilibrating buffer containing 50 mM NaCl. The isotypicity of the monoclonal antibody was determined by ELISA as outlined elsewhere (Ekramoddoullah *et al.*, 1984).

RAST

The RAST was performed with disposable flexible polyvinyl chloride microtiter plates as described before (Ekramoddoullah *et al.*, 1983).

Briefly, the wells coated with a solution of HMBA, CB-1 or CB-2 consisting of 1 µg in 100 µl of coating buffer (1.59 g of Na₂CO₃, 2.93 g of NaHCO₃ and 0.2 g of Na₂S₂O₅ made up to 1 liter with distilled water, pH

9.6) by maintaining the plates at 4°C in a moist chamber for 18 hr. The solutions of allergen were then withdrawn and the plates washed with PBS (0.9% NaCl, 0.05 M phosphate buffer, pH 7.4, 0.05% sodium azide) (200 µl/well). The capacity of the wells to further adsorb proteins was blocked by coating with an unrelated protein such as HSA using an IB (PBS containing 0.3% HSA) according to the conditions described for the coating procedure. Finally, the plates were washed 3 times with PBS (200 µl/well). Volumes of 100 µl of a pool of 14 sera obtained from grass-allergic patients (which had been diluted two-fold with IB) were added to the allergen-coated wells and kept overnight at room temp; the serum samples were then withdrawn and the wells washed 3 times with IB containing 1% Tween 20. ¹²⁵I-labelled rabbit anti-human IgE antibodies (Pharmacia AB, Uppsala, Sweden, 5 µCi/0.8 µg) were added to the wells (100,000 cpm/well) and incubated overnight at room temp. The wells were washed 3 times with PBS containing 1% Tween 20 and cut out from the plates. The levels of radioactivity were measured in a Beckman gamma counter.

Polyacrylamide gel electrophoresis

SDS-PAGE. This was carried out in the Protean slab cell apparatus (Bio-Rad) with 1.5 mm thick polyacrylamide gels (12%) utilizing the Laemmli buffer system (Laemmli, 1970). Samples were incubated in the sample buffer overnight at room temp prior to electrophoresis. The gel was stained according to the method of Fairbanks *et al.* (1971) or with a silver stain according to the procedure of Marshall (1984). Low mol. wt protein standards (Bio-Rad Laboratories) were used to calibrate the gels for the determination of the mol. wts of components according to their relative electrophoretic mobilities.

Western blotting. Proteins separated by SDS-PAGE were electrophoretically transferred from the gel onto nitrocellulose membrane (Towbin *et al.*, 1979) with the Bio-Rad transblot cell according to the manufacturer's instructions. To determine the mol. wts of the bands developed on the immunoprint, a strip of the membrane to which the standard proteins were transferred was cut out and stained with 0.1% amido black in 45% methanol–10% acetic acid for 1 min. The strip was destained in 5% methanol–7% acetic acid. To block any further binding of proteins to unreacted sites, the rest of the membrane was incubated overnight with a solution of gelatin [3% in TBS (20 mM Tris, 500 mM NaCl, pH 7.5)].

Immunoblotting. Detection of proteins and peptides on the nitrocellulose was achieved by immunoblotting as follows. After the blocking step, the membrane was immersed either in a solution of a purified murine monoclonal antibody preparation (13 µg protein/ml) in TBS containing gelatin (1%) or in human sera (a pool of 14 sera obtained from grass-allergic patients) diluted five-fold with IB and

gently agitated overnight at room temp. The membrane was washed with TBS, then incubated with either goat anti-mouse IgG-horseradish peroxidase (Bio-Rad), diluted 3000-fold with TBS containing gelatin (1%) for 1 hr at room temp or with goat anti-human IgE-horseradish peroxidase (Tago Inc., Burlingame, CA) diluted 250-fold with TBS containing gelatin (1%) for 18 hr at room temp and then washed with TBS. Solutions of the substrate 4-chloronaphthol and H₂O₂ were added to initiate the reactions which developed as a colored band(s) on the membrane. The immunoprint of the immobilized antigen–monoclonal antibody complex thus obtained was immediately photographed for a permanent record.

Cleavage of HMBA with CNBr

HMBA (2 mg) was dissolved in 1.963 ml of 70% formic acid; 37.3 µl of a solution of CNBr (100 mg/ml of 70% HCOOH) was added to achieve a 1000-fold molar excess of CNBr over HMBA. The reaction was carried out in a glass-stoppered round bottom flask for 18 hr at room temp in the dark; 28 ml of distilled water was then added to the reaction flask and the mixture was lyophilized in the same vessel.

HPLC

The peptides generated by CNBr cleavage of HMBA were separated on a Vydac C-18 reverse-phase column (4.6 mm × 25 cm) (218 TP 54.6 C-18, Vydac, Hesperia, CA) utilizing the Waters HPLC system (Waters Associates, Inc., Milford, MA).

The ability of various fractions obtained by HPLC to bind Mab 12 was analyzed by a direct immunoblotting procedure. In this test, 2 µl of each HPLC fraction was directly applied onto the nitrocellulose membrane (Bio-Rad). The membrane was blocked with gelatin (3% in TBS) and processed as described under Immunoblotting.

Enzymatic digestion of the immunoreactive CNBr peptide of HMBA

The immunoreactive CNBr-peptide HPLC fraction (see Results), was digested with trypsin TPCK (Worthington Biochemical Corporation, Freehold, NJ) or TLCK-treated α-chymotrypsin (Sigma Chemical Co., St. Louis, MO) as described previously (Ekramoddoullah *et al.*, 1984).

RESULTS

Characterization and specificity of Mab 12

The Mab 12 isolated from ascitic fluid by the one-step purification procedure of Bruck *et al.* (1982) was shown by SDS-PAGE to be homogeneous. Since the clone producing Mab 12 was obtained by the limiting dilution method, the possibility that Mab 12 might be the product of more than one clone is unlikely. Mab 12 was of the IgG-1 isotype and possessed kappa light chains.

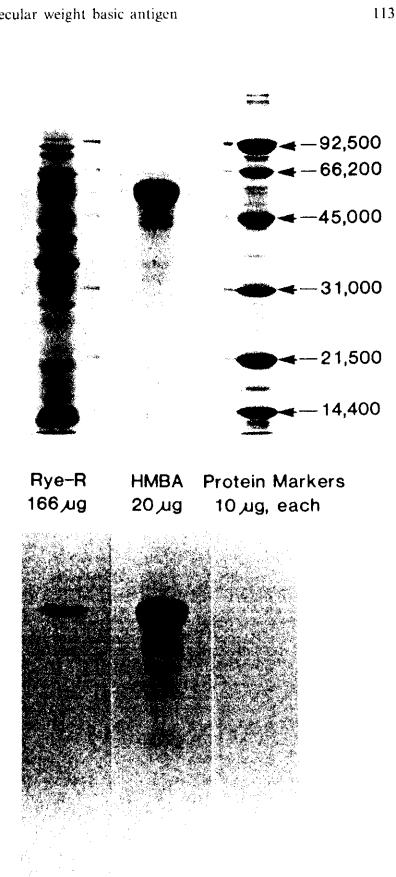


Fig. 1. Specificity of monoclonal antibody Mab 12 as demonstrated by Western and immunoblot analysis. *Top panel:* gels stained with Coomassie brilliant blue following SDS-PAGE of the indicated samples. *Bottom panel:* the corresponding immunoprint obtained by electrotransfer of the separated proteins from the unstained gels onto nitrocellulose membranes; components which bound Mab 12 were visualized by an immunoenzyme reaction as described in Materials and Methods.

The specificity of Mab 12 was evaluated in terms of its reactivity towards the various antigens present in KBG-R or rye-R by a combination of SDS-PAGE and immunoblot methods. KBG-R was resolved by SDS-PAGE into multiple components; however, only a single band was visualized on the immunoprint (data not shown). Similarly, rye-R was resolved into at least 20 components as demonstrated on the

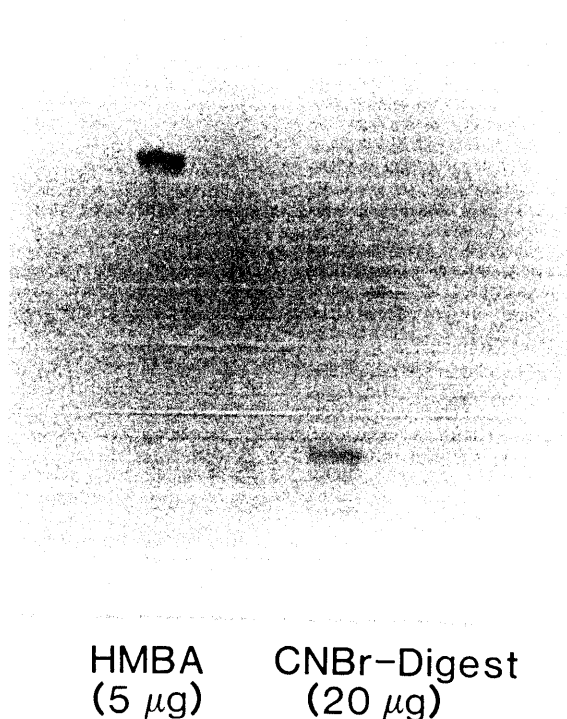


Fig. 2. Analysis of HMBA and the CNBr digest of HMBA by Western and immunoblot procedures employing Mab 12.

stained gels (shown in the top panel of Fig. 1), whereas, in the corresponding immunoprint (bottom panel), only one component was found to have bound Mab 12. Moreover, a comparison of the immunoprints of KBG-R and rye-R developed with Mab 12 indicated that the positions of the bands were identical (data not shown). As can be seen in Fig. 1, the positions of the antigenic component detected on the immunoprints of rye-R and HMBA were identical, and corresponded to a mol. wt of 56,800. This observation indicated that the components detected in KBG-R and rye-R were antigenically and physico-chemically related to HMBA.

It is interesting to note that, with the preparation of rye-R (from which HMBA was isolated), a single antigenic component was detected on Western and immunoblot analysis with Mab 12, whereas, with the purified preparation of HMBA, the presence of this main antigenic component and several additional components with molecular sizes below 56,800 daltons were detected. A single antigenic component was also detected in rye-R when the amount of rye-R used

for analysis was increased to 332 µg. The multiple banding obtained with the purified HMBA but not rye-R may reflect the presence of other proteins with related antigenic determinations or the presence of degradation products of HMBA. It was noted that none of the size reference proteins bound Mab 12.

Antigenicity of the CNBr digest of HMBA

SDS-PAGE analysis of the products of the CNBr digest revealed the presence of two peptides with mol. wts of 17,400 and 22,000. The concurrent Western and immunoblot analysis with Mab 12 revealed that only one of these peptides bound Mab 12 (Fig. 2).

The peptides were isolated by fractionating 2 mg of the CNBr digest of HMBA by HPLC on a C-18 column (Fig. 3). The direct-immunoblotting procedure revealed that HPLC fractions 62-65 and 68-76 [indicated within the shaded area (Fig. 3)] reacted with Mab 12. These HPLC fractions, i.e. 62-65 and 68-76, were pooled separately and designated as CB-1 and CB-2 respectively. CB-1 and CB-2 were analysed by SDS-PAGE (Fig. 4). CB-1 was

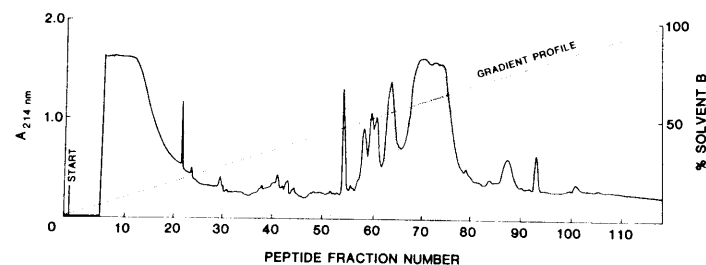


Fig. 3. Fractionation of the CNBr-digest mixture of HMBA (2 mg) by HPLC on the reverse-phase Vydac C-18 column equilibrated with solvent A (0.1% TFA). A linear gradient was generated with solvent B (70% propanol, 0.1% TFA) at a flow rate of 0.5 ml/min over a period of 2 hr. Fractions were collected at 1-min intervals. The shaded area indicates fractions that bound Mab 12 in a direct immunoblotting procedure as described in Materials and Methods.

shown to be homogeneous and had a mol. wt of 17,400. The major staining band detected in CB-2 had a mol. wt of 22,000. On further analysis by the Western and immunoblot methods it was revealed that the 17.4-kDa component of CB-1 and the 22-kDa component of CB-2 bound Mab 12 (Fig. 5). Analysis by Western and immunoblot methods em-

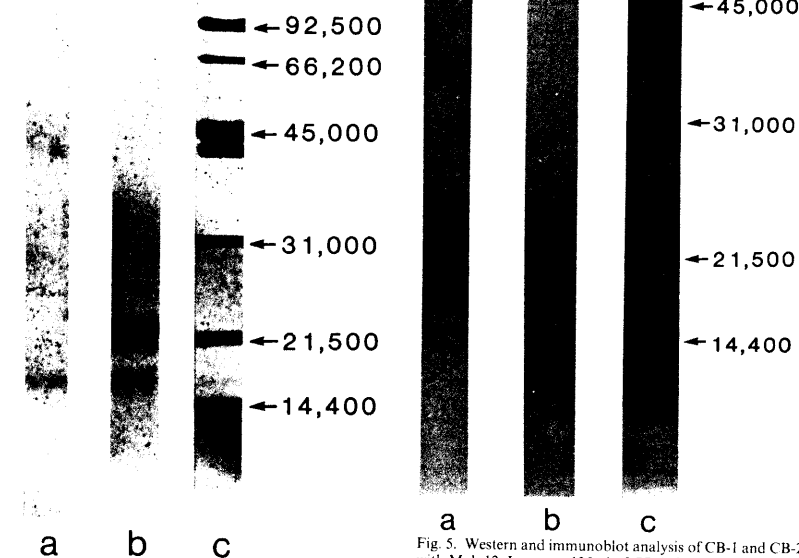


Fig. 4. SDS-PAGE analysis of CB-1 and CB-2. Gels were stained with silver stain. Lane a = 125 µl of CB-1 (i.e. the sample pool of HPLC fractions 62-65 within the shaded area in Fig. 3). Lane b = 50 µl of CB-2 (i.e. the sample pool of HPLC fractions 68-76 as indicated within the shaded area in Fig. 3). Lane c = protein markers (2.5 µg each) visualized on the membrane by staining with amido black.

Fig. 5. Western and immunoblot analysis of CB-1 and CB-2 with Mab 12. Lane a = 125 µl of CB-1 (i.e. the sample pool of HPLC fractions 62-65 within the shaded area in Fig. 3). Lane b = 50 µl of CB-2 (i.e. sample pool of HPLC fractions 68-76 as indicated within the shaded area in Fig. 3). Lane c = protein markers (2.5 µg each) visualized on the membrane by staining with amido black.

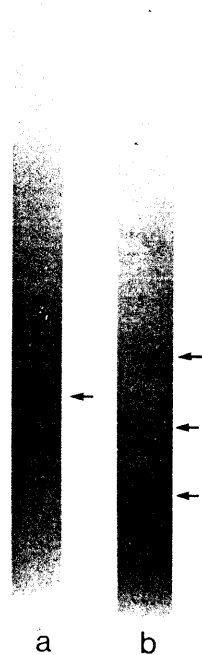


Fig. 6. Western and immunoblot analysis of CB-1 and CB-2 with human IgE antibody. Lane a = 125 μ l of CB-1 (i.e. the sample pool of HPLC fractions 62–65 within the shaded area in Fig. 3). Lane b = 50 μ l of CB-2 (i.e. sample pool of HPLC fractions 68–76 as indicated within the shaded area in Fig. 3).

playing human allergic sera showed that CB-1 had one band corresponding to the mol. wt of 17,400 and CB-2 had three bands, one of which corresponded to

the mol. wt of 22,000 (Fig. 6). From these results, it may be concluded that the two peptides, 17.4- and 22-kDa, derived from the CNBr cleavage of HMBA carry an antigenic site capable of binding Mab 12. Moreover, these two peptides also carry some allergenic site(s) capable of binding human IgE antibodies.

HMBA and its immunoreactive CNBr peptides were examined by a RAST for their ability to combine with the human IgE antibodies present in a pool of sera from individuals allergic to grasses. CB-1 bound IgE antibodies to the extent of 10% of the binding obtained with the parent molecule, HMBA and CB-2 bound human IgE antibodies to the extent of 25% of the binding obtained with HMBA. These results indicated that CB-1 and CB-2 possessed some allergenic determinants recognized by human IgE antibodies.

In further attempts to prepare still smaller immunoreactive peptides, CB-2 was cleaved either with trypsin or chymotrypsin and the resulting enzymatic digests were separated by HPLC (Figs 7 and 8, respectively). The treatment of CB-2 with either of the enzymes generated many peptides which could be resolved on HPLC. However, on direct immunoblotting, none of the tryptic or chymotryptic peptides reacted with Mab 12.

DISCUSSION

Previous studies in this laboratory, using antisera produced in rabbits and sheep, have demonstrated the presence of cross-reactive antigens (Chakrabarty *et al.*, 1981) and allergens (Ekramoddoullah *et al.*, 1982) in KBG and ryegrass pollen extracts. In the present study, the use of a monospecific monoclonal antibody probe has identified an antigenic component, present in both grass pollen extracts, that possessed a similar, if not identical, antigenic determinant in common with the other. Moreover, it was established that this antigenic component was related also to the rye allergen HMBA, in that the immunoprints obtained on Western and immunoblot analysis of KBG-R, rye-R and HMBA with Mab 12 were virtually identical.

One of the important findings of the present study is that Mab 12, which was produced by utilizing a

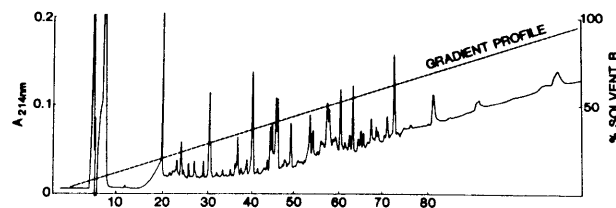


Fig. 7. Separation of the tryptic digest of CB-2 (18 μ g) by HPLC on the reverse-phase Vydac C-18 column (conditions as described in Fig. 3).

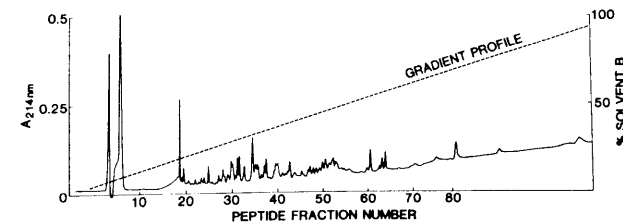


Fig. 8. Separation of the chymotryptic digest of CB-2 (18 μ g) by HPLC on the reverse-phase Vydac C-18 column (conditions as described in Fig. 3).

heterogeneous extract of KBG pollen as the immunogen, recognized an important ryegrass pollen allergen, HMBA. In a separate study (Albuquerque *et al.*, 1985), this monoclonal antibody has been used as a reversed immunosorbent to isolate allergens from both ryegrass and KBG pollen. Moreover, the affinity-purified antigen was shown to be allergenically as active as HMBA.

The elucidation of the structural features of an allergen can be greatly facilitated by the availability of fragments of the allergen which retain the capacity of the parent molecule to be recognized by antibodies. With this in mind, an attempt was made to degrade HMBA into smaller fragments. The choice of using rye allergen HMBA for further studies to characterize the nature of an antigenic site was made on the basis of the following considerations: (i) the finding that HMBA was an important allergen, (ii) it was available in a pure state, and (iii) a monoclonal antibody capable of binding to this allergen was available.

Cleavage of HMBA with CNBr generated peptides, of which two peptides retained the capacity to react with Mab 12. In the initial fractionation of 0.8 mg of the CNBr digest of HMBA only CB-2 was shown to bind Mab 12 in the immunoblot. However, when the amount of CNBr digest was increased to 2 mg for fractionation (see Fig. 3), CB-1 was also shown to bind Mab 12. This observation indicated that CB-2 is present in the CNBr digest of HMBA in larger amounts than CB-1. This may also explain the detection of only one immunoreactive peptide by the Western and immunoblot analysis of the CNBr digest of HMBA. The immunoreactive peptides, which were isolated by means of HPLC, had mol. wts of the order of 17,400 and 22,000 and were demonstrated to have also retained the capacity to bind human IgE antibodies. The question whether the monoclonal and IgE antibodies recognize the same or different determinants was not addressed in this study. Further attempts to reduce the size of this immunoreactive peptide by enzymatic digestion were not successful. This observation contrasts with our previous finding that immunoreactive peptides of ryegrass pollen cytochrome *c* could be readily produced by enzymatic

degradation with either trypsin or chymotrypsin (Ekramoddoullah *et al.*, 1984).

Acknowledgements—The capable technical assistance of Tom Cook and Peter Warkentin is gratefully acknowledged.

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