Journal of Chromatography, 398 (1987) 366–371
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 564

Note

Purification of allergens by high-performance liquid chromatography

III. Purification of fungal allergens

NAZARIO RUBIO*
Instituto Cajal, Velazquez 144, 28006 Madrid (Spain)
and
AURORA BRIEVA
Laboratorios Andromaco, S.A., Allergy Division, Torrejón de Ardoz (Spain)
(Received March 16th, 1987)

Fungi imperfecti are of great clinical significance. They are considered to elicit allergy and asthma throughout most of the world. Nevertheless, fungal allergens have been identified only recently and contradictory information is available, mainly due to the complexity of the fungal raw material[1]. In addition, the fungal allergens seem to be very heterogeneous and it is difficult to find sera from patients that react with the same allergens[2].

The separation of a fungal extract can now be performed rapidly by high-performance liquid chromatography (HPLC) and the allergens can be identified by biochemical and immunological assays.

MATERIALS AND METHODS

Fungal extracts

Alternaria tenuis (Centraalbureau voor Schimmelcultures, 103.33), *Aspergillus fumigatus* (CBS 545.65) and *Cladosporium herbarum* (CBS 177.71) were obtained from Allergon (Sweden) as freeze-dried ground samples. Extracts were prepared in phosphate-buffered saline (PBS) over a period of 1 h in an ice-bath at 10% (*Aspergillus* and *Cladosporium*) or 5% (w/v) concentration (*Alternaria*). Subsequent centrifugation and Millipore filtration were done as described previously[3,4].

HPLC

The equipment and methods were described previously[3].

Biochemical techniques

Proteins were determined by the method of Lowry *et al.*[5]. Carbohydrates were quantitated by the phenol–sulphuric acid method of Dubois *et al.*[6] with glucose as a reference. DNA quantification was performed according to the diphenylamine method[7].

0021-9673/87/$03.50

Enzyme digestion

Crude extracts of allergenic fungi were digested with trypsin, chymotrypsin (Worthington) or proteinase K (Merck) at a protein to enzyme ratio of 10:1, at 37°C overnight. The digested extracts were injected and chromatographed in the usual way.

Immunological techniques

Solid-phase radioimmunoassay (SPRIA) was performed according to ref. 3. Human serum pools from eight patients allergic to each fungus [class 2-4 radioallergosorbent test (RAST)] were employed. RAST inhibition was done as described in ref. 4.

RESULTS

Allergen purification

The differences in the distributions of the allergenic material after HPLC is evident. Of the three fungi selected in this study, *Aspergillus fumigatus* is the most homogeneous from the point of view of allergenic activity distributed on the HPLC chromatogram (Fig. 1).

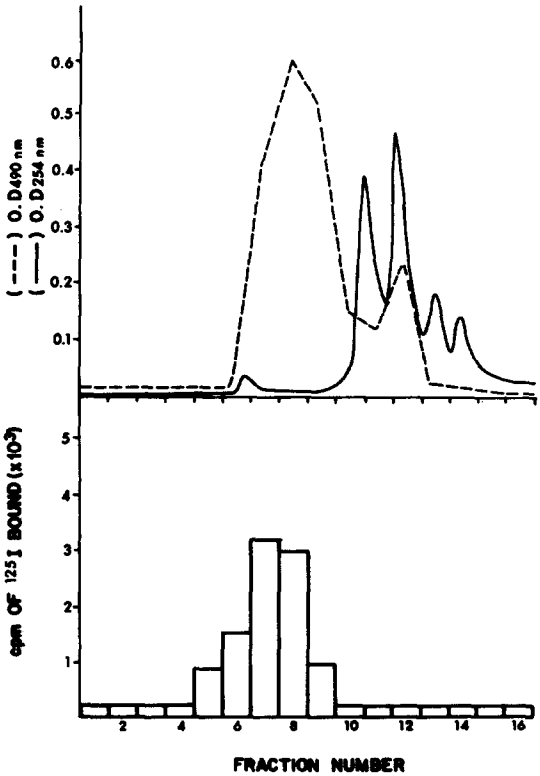

Fig. 1. HPLC of *Aspergillus fumigatus* extract. Conditions as in ref. 3. ---- = Carbohydrate material; —— = protein material. An human serum pool from eight patients (class 2-4 RAST) was utilized in the SPRIA of the different fractions.

The histograms of the distribution of human immunoglobulin E (IgE) binding ability indicate that all the activity corresponds to an high-molecular-weight polysaccharide material, as demonstrated by the absence of absorbance at 254 nm and the positive reaction in the Dubois test. This material is eluted in the void volume of the column. Calibration of the HPLC column with Dextran T (Pharmacia, Sweden) indicated a molecular weight between 500000 and 70000 daltons for the allergenic material.

No allergenic activity associated with protein material was revealed with the pool of human sera used in this study.

In the *Cladosporium herbarum* extract, the IgE binding activity seems to be associated with the material giving a strongly positive phenol–sulphuric acid reaction (Fig. 2). The distribution of allergenic activity (Fig. 2, lower part) parallels that of the carbohydrate material (broken line in upper part). Aukrust and co-workers[8,9] have shown that *C. herbarum* contains allergens mainly in the molecular weight range 10000–30000 daltons which are of a glycoprotein nature. This material must be eluted in fraction 10 or 11 from our column. The corresponding area of the chromatogram still has allergenic activity, but the main activity is associated with fraction 7.

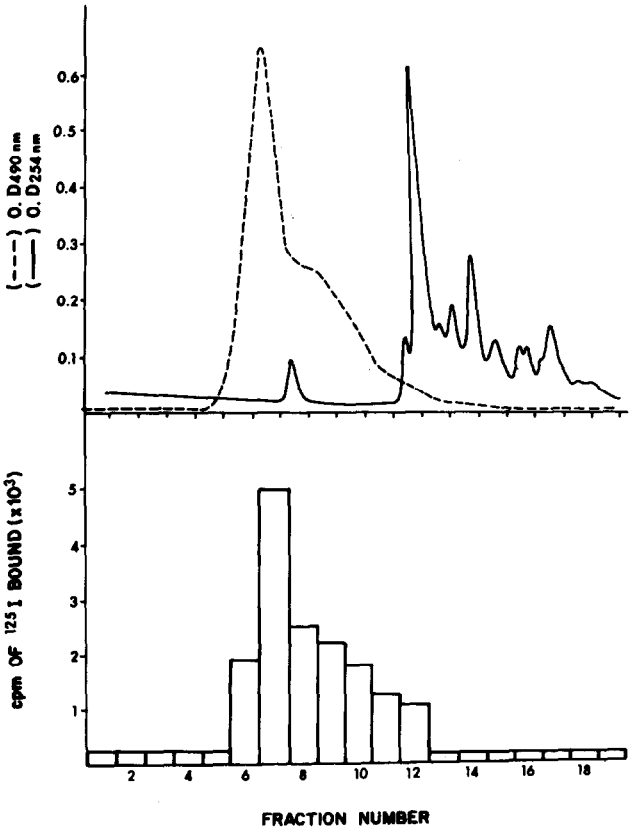

Fig. 2. HPLC of *Cladosporium herbarum* extract. --- = Carbohydrate material; —— = protein material. An human serum pool from eight patients sensitive to *C. herbarum* was utilized in the SPRIA test.

Under the extraction conditions employed by the above mentioned group, 18 h at 4–8°C, an important degradation of the polysaccharide material can take place. According to Swärd-Nordmo *et al.*[10], *C. herbarum* extracts contain (a) β-glucosidase (190 nmol/mg), (b) α-galactosidase (150 nmol/mg), (c) N-acetyl-β-glucosaminidase (150 nmol/mg) and traces of β-glucuronidase, α-glucosidase and α-fucosídase. Similar enzyme activities were found in an *A. tenuis* extract[11].

A. tenuis is more heterogeneous according to the molecular weight distributions of the allergenic activities. Yunginger *et al.*[12] purified and characterized an allergen, termed Alt-I, which shows no absorbance at 280 nm and contains high amounts of carbohydrate. This allergen is the major *Alternaria* allergen. The molecular weight reported is 50000 and its position in the HPLC chromatogram fits very well with the activity peak found in fraction 9 (Fig. 3, lower part).

Nevertheless, other allergens of lower molecular weight and proteic nature have been described in the genus *Alternaria*[13]. The presence of such major and minor allergens in the *A. tenuis* extract explains the broad distribution depicted in Fig. 3. Here, not only the sugar-containing material, but also that absorbing at 254 nm shows an allergenic activity.

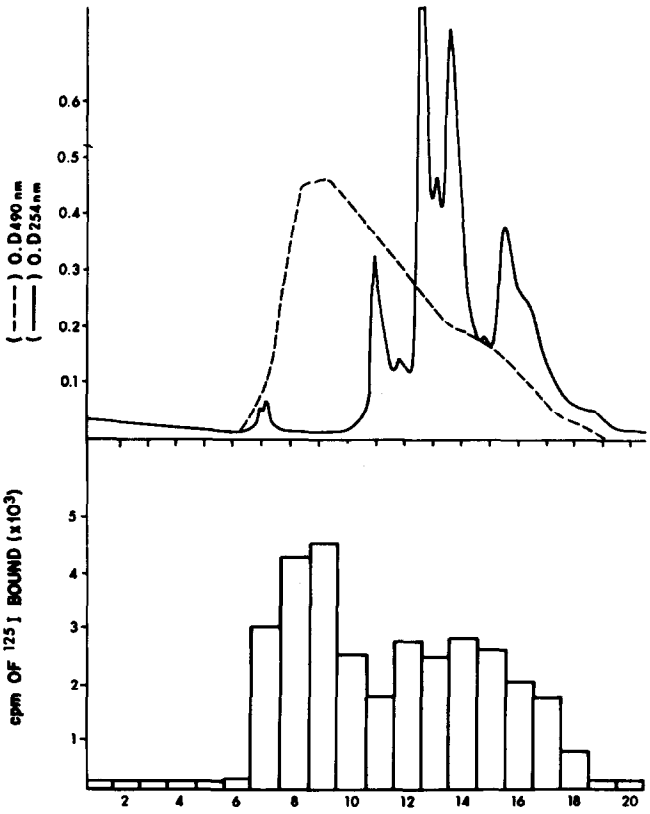

Fig. 3. HPLC of *Alternaria tenuis* extract. - - - = Carbohydrate material; —— = protein material. An serum pool from eight patients was utilized in the SPRIA of the fractions.

Protease treatment

In order to rule out or confirm the involvement of proteins in the allergenicity of *A. tenuis*, digestions with trypsin, chymotrypsin or proteinase K were performed. An high enzyme to substrate ratio and an overnight digestion at 37°C, which must destroy all proteins, was performed. The digested material was further subjected to HPLC separation in the usual way and the eluted fractions analyzed by SPRIA.

The immunological activity of fractions 7–10 (polysaccharide material) remains unchanged, but the three protease treatments completely abolish the IgE binding properties of fractions 11–18 absorbing at 254 nm. This confirms the involvement of protein molecules in the allergenicity of *A. tenuis*. The positive reactions of the above fractions in the phenol-sulphuric acid test seems to indicate a glycoproteic nature of these allergens.

Biochemical characterization of the peaks

The elution profile of the extract after exhaustive protease treatment changes only slightly, with an increase at low molecular weights. This could indicate that material other than proteins contributes to the absorption at 254 nm. The ratios of the absorbances at 254 and 280 nm suggest that most of the material is nucleic acids. This was confirmed by the diphenylamine reaction which demonstrates that most of the UV-absorbing material is DNA. This is not surprising because of the grinding process to which the fungal freeze-dried material is subjected by the suppliers. Nevertheless, as far as we know, no allergenic activity was atributed to DNA in any instance.

Biological activity of HPLC-purified allergens

The allergenic activity of selected purified fractions of the three moulds were compared by RAST inhibition with the *in vivo* standardized extract Pharmalgen®, after dialysis against distilled water and lyophilization. As shown in Table I, the most allergenic species is *A. tenuis*, of which only 0.8 ng are needed to produce 1 Biological Unit (B.U.)*. In this case, due to the heterogeneous distribution of the allergenic activity, all the extracts (fractions 7–18) are tested by RAST inhibition.

A. fumigatus and *C. herbarum* seem less allergenic *in vitro* when purified high-molecular-weight polysaccharides are tested.

TABLE I

BIOLOGICAL ACTIVITIES OF HPLC-PURIFIED FUNGAL FRACTIONS

Fungus	*HPLC fraction number*	*Material equivalent to 1 B.U. (ng)*
Alternaria tenuis	7–18	0.8
Aspergillus fumigatus	5–9	1.2
Cladosporium herbarum	6–12	4.8

* An allergen solution contains 1.000 Biological Unit when it gives a mean weal reaction of the same size as 0.1% histamine hydrochloride in the skin prick test.

The biological activity seems similar to the previously reported 1.4 ng per B.U. in the case of HPLC-purified allergens from *Dermatophagoides pteronyssinus* mite[4].

DISCUSSION

A total molecular size distribution can be obtained when a fungal extract, or any allergenic extract, is subjected to HPLC. This separation, together with an immunological identification, allows us to purify allergens from other molecules present in the crude extract. This purification is extremely important in the case of fungi. Fungi contain a range of toxins, with haemolytic and cardiotoxic activities[14,15], that must be removed from the extracts used in the immunotherapy of allergic processes. In addition, fungal extracts appear to be very unstable[16]. The allergenic activity disappears in a matter of days during storage at 4°C. This is surely due to the presence of enzymes capable of degrading the allergens in the crude extract[10,11], released during long extraction schemes at 4°C or higher temperatures.

The main allergens of *C. herbarum* and *A. fumigatus* are polysaccharidic in nature. Our HPLC method allows us to separate at great speed and with a 100% yield the allergens from toxic and enzymatic proteins. In the case of *A. tenuis* a complete purification could not be obtained due to the heterogeneous distribution of the allergens in the chromatogram. Nevertheless, the leading polysaccharide fractions seem to contain most of the allergenic activity and could be used as a semi-purified fraction for therapeutic purposes.

All the purified material retains its biological activity, as demonstrated in the RAST inhibition experiments.

ACKNOWLEDGEMENT

We thank Miss Mercedes Alonso for technical assistance.

REFERENCES

1 J. Salvaggio and L. Aukrust, *J. Allergy Clin. Immunol.*, 68 (1981) 327.
2 J. W. Yunginger, *Int. Congr. of Allergy, London*, Macmillan, London, 1983, p. 537.
3 A. Brieva and N. Rubio, *J. Chromatogr.*, 370 (1986) 165.
4 N. Rubio, A. Brieva and M. Rubio, *J. Chromatogr.*, 392 (1987) 447.
5 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. L. Randall, *J. Biol. Chem.*, 193 (1951) 265.
6 M. Dubois, K. A. Gilles, J. K. Hamilton, R. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
7 K. Burton, *Biochem. J.*, 62 (1956) 315.
8 L. Aukrust and S. M. Borch, *Int. Arch. Allergy Appl. Immunol.*, 60 (1979) 68.
9 E. Laudmark and L. Aukrust, *Int. Arch. Allergy Appl. Immunol.*, 78 (1985) 71.
10 M. Swärd-Nordmo, B. Smestad Paulsen, J. K. Wold and O. Grimmer, *Int. Arch. Allergy Appl. Immunol.*, 75 (1984) 149.
11 H. M. Vijay, N. M. Young, G. E. D. Jackson, G. P. White and I. L. Berstein, *Int. Arch. Allergy Appl. Immunol.*, 78 (1985) 37.
12 J. W. Yunginger, R. T. Jones, M. E. Nesheim and M. Geller, *J. Allergy Clin. Immunol.*, 66 (1980) 138.
13 H. M. Vijay, H. Huang, N. M. Young and I. L. Bernstein, *Int. Arch. Allergy Appl. Immunol.*, 60 (1979) 229.
14 K. Ebina, S. Ichinowatari and K. Yokota, *Microbiol. Immunol.*, 29 (1985) 91.
15 K. Ebina, K. Yokota and O. Sakaguchi, *Jpn. J. Med. Mycol.*, 24 (1983) 245.
16 *International Allergy Workshop, Funchal, Madeira, May 1982*, Excerpta Medica, Amsterdam, 1983, p. 83.