Journal of Chromatography, 367 (1986) 247–253
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 851

Note

Isolation of antigen 19/20 from the mite *Dermatophagoides farinae* by chromatography on the Mono S™ column

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(First received May 6th, 1986; revised manuscript received June 4th, 1986)

So-called house dust is an important cause of allergic reactions, especially in children. The mite *Dermatophagoides farinae* and its excretion products are partly responsible for these allergic reactions[1–3].

The isolation of pure allergens is necessary for the determination of their chemical structures which may be the key to understanding why some molecules act as allergens[4]. Purified allergens are also valuable tools in the diagnosis and therapy of allergic disease[5].

Holck *et al.*[6] recently published the isolation of what is described as a slightly basic component of antigen 19/20, denoted Ag 19/20 II a. This component was obtained with a purity of 85% by isoelectric focusing in two steps. Ag 19/20 is one of the most important allergens present in the mite[7]. New separation media have been developed during the last few years and the cation-exchange column Mono S™ based on monodisperse particles has been used with success for purification of insect venoms[8,9] and of a basic glycoprotein allergen from the pollen of timoty[10]. The aim of this work was to develop a procedure based on as few steps as possible to isolate and purify antigen 19/20 from the mite *D. farinae* by means of the Mono S™ column.

EXPERIMENTAL

Materials

Crushed mite (*Dermatophagoides farinae*) batch 009.580.008 was obtained from Allergon (Engelholm, Sweden). Ultrogel AcA-54 was obtained from LKB (Bromma, Sweden). Agarose immunoelectrophoresis tablets with tricine buffer (pH 8.60) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Fuji RX medical X-ray film was obtained from Fuji Photo Film (Tokyo, Japan). Kodak LX 25 developer and AL 4 X-ray fixer were obtained from Kodak (Hemel Hempstead, U.K.). Rabbit antiserum and serum from patients allergic to the mite were provided by Nycomed (Oslo, Norway). Other chemicals used were of analytical reagent grade and obtained from Merck (Darmstadt, F.R.G.).

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Gel permeation chromatography (GPC)

Crushed mites (40 g) were extracted with 0.125 *M* ammonium bicarbonate buffer (pH 8.00) (1600 ml) at 4°C for 20 h with magnetic stirring. After filtration and dialysis against distilled water in a dialysis tube with a cut-off limit of MW 3500, the extract was lyophilized and kept at −20°C; yield 4 g. The extract (1 g) was subjected to GPC on an Ultrogel AcA-54 column (100 × 2.6 cm) pre-equilibrated with 75 m*M* phosphate–sodium chloride buffer pH 7.20 containing 2% *n*-butanol. The column was eluted at a rate of 20 ml/h and fractions of 5 ml were collected. The absorbance at 280 nm was monitored throughout the elution using a LKB Uvicord II detector. Ovalbumin (MW 43 000), chymotrypsinogen-A (MW 25 000), ribonuclease-A (MW 13 700) and cytochrome *c* (MW 12 500) were used to calibrate the column. The fractions (Fig. 1) were also tested for the presence of antigens by fused rocket immunoelectrophoresis (FRIE). Fractions containing antigen 19/20 were pooled, desalted and freeze dried (330 mg). The entire procedure was performed at 4°C.

Chromatography on the Mono S™ column

Chromatography on the Mono S HR 5/5 cation exchanger (Pharmacia, Uppsala, Sweden) was performed in a Pharmacia fast protein liquid chromatography (FPLC) system equipped with two P-500 pumps controlled by a GP-250 gradient programmer. The elution was monitored at 280 nm by a Pharmacia UV-1 monitor fitted with a 10-mm path HR cell. Fractions of 1 ml were collected with a Pharmacia Frac-100 fraction collector. Amounts of 5 mg of the fraction obtained by GPC were injected into the Mono S HR 5/5 column (50 mm × 5 mm, particle size 10 μm). The column was eluted at 1 ml/min using the following buffer systems: (1) (A) 10 or 50 m*M* Tris–HCl (pH 7.50), (B) 10 or 50 m*M* Tris–HCl (pH 7.50) containing 0.5 *M* sodium chloride; (2) (A) 10 or 50 m*M* sodium phosphate (pH 7.50), (B) 10 or 50 m*M* sodium phosphate (pH 7.50) containing 0.5 *M* sodium chloride.

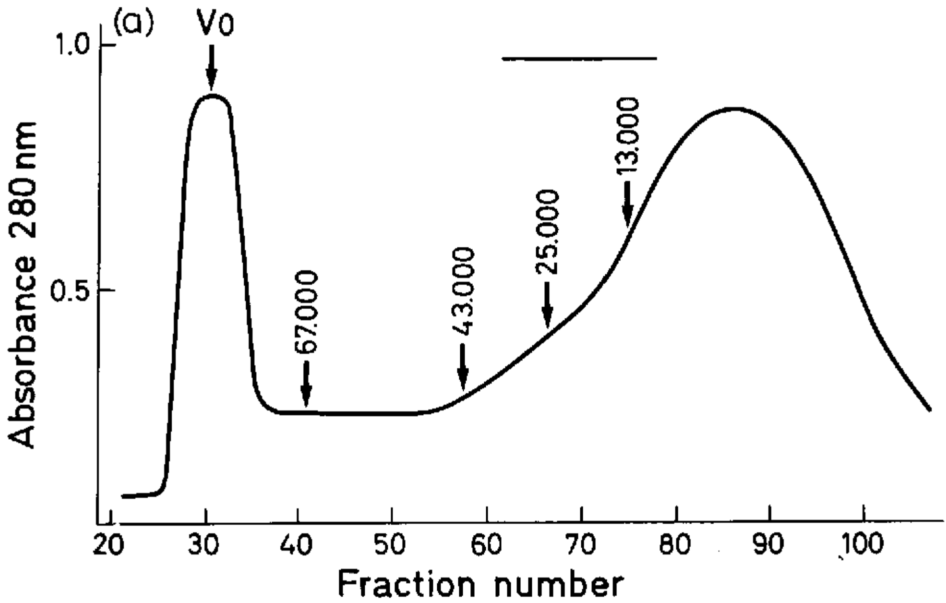
For each buffer at least five different salt gradients were tried in order to obtain optimum resolution. The fractions were tested for the presence of antigen 19/20 by FRIE and for the presence of allergenic activity by the RAST inhibition test. The Mono S column was used 50 times successively using the buffer system and gradient program shown in Fig. 5.

RAST inhibition

RAST inhibition assay of the fractions from the chromatographic columns was performed by a modification[11] of the method of Yman *et al.*[12].

Fused rocket immunoelectrophoresis (FRIE)

FRIE was performed as described by Harboe and Svendsen[13]. Samples of 15 μl obtained from the fractions collected upon GPC and chromatography on the Mono S column respectively were applied in each well and allowed to fuse for 30 min before electrophoresis was started. Electrophoresis was performed at 2 V/cm for 18–20 h in 1% agarose gel containing 25 m*M* tricine buffer (pH 8.60) (Bio-Rad Labs.) and 20–30 μl antiserum against whole mite extract or against a preparation enriched with antigen 19/20. To detect IgE-binding precipitates, the FRIE plates were incubated with a serum pool from mite allegics, followed by incubation with [^{125}I]anti-IgE and autoradiography as described by Weeke *et al.*[14].



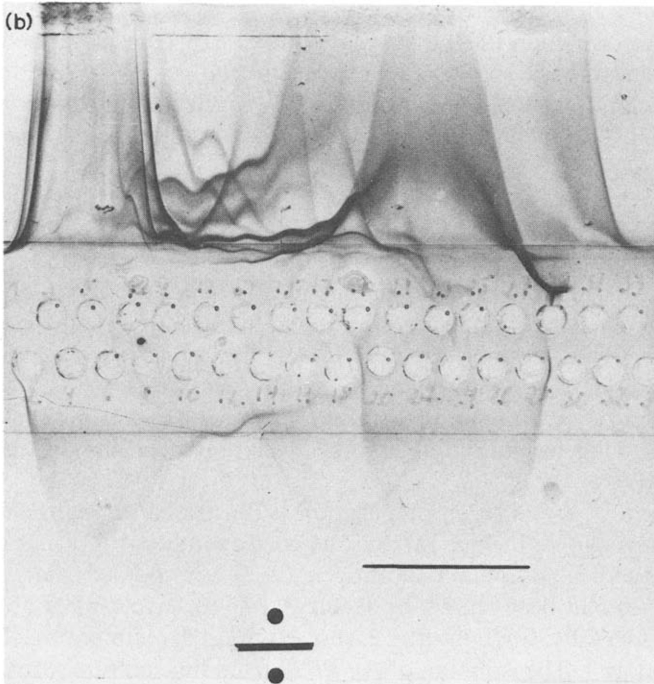


Fig. 1. (a) GPC of the mite extract on an Ultrogel AcA-54 column (100 × 2.6 cm). The fraction size is 5 ml. Absorbance at 280 nm was monitored. (b) FRIE of the fractions collected from the AcA-54 column. Samples of 15 μl were placed in the wells. After 30 min, electrophoresis was performed at 2 V/cm for 18 h in 1% agarose gel (pH 8.60) containing 20 $\mu l/cm^2$ of antibody against whole mite extract. The cathode was positioned as indicated. The gel was stained by Coomassie brilliant blue. The horizontal bar indicates the fractions in which antigen 19/20 was detected.

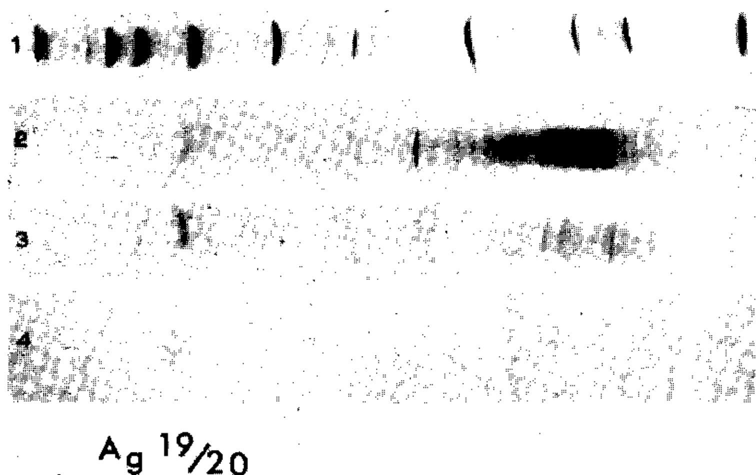


Fig. 2. Flat bed isoelectric focusing on LKB PAG plates at pH 3.5–9.5. The gel was stained with Coomassie brilliant blue. 1, p*I* calibration proteins (pH 3–10, Pharmacia); 2, crude mite extract; 3, GPC fraction containing Ag 19/20; 4, Ag 19/20 as eluted from the Mono S column.

Isoelectric focusing (IEF)

IEF was performed on LKB PAG plates at pH 3.5–9.5 as described in ref. 15. Samples of crude extract, the GPC fraction and the antigen 19/20 fraction isolated on the Mono S column were applied. A mixture of eleven calibration proteins for p*I* determination (pH 3–10) (Pharmacia) was used to determine the pH gradient.

RESULTS AND DISCUSSION

The allergen extract prepared from mite is intensely coloured and the presence of coloured matter, probably of polyphenolic nature, is a problem in certain chromatographic methods. Direct application of the mite extract on the Mono S column was therefore not tested, preliminary experiments having shown that the coloured matter strongly bound to cation-exchange columns. Gel filtration was used as a preliminary purification step, simultaneously also removing allergens, proteins and other material of a molecular weight different from those present in the region where Ag 19/20 was located.

Gel filtration of the allergen preparation is illustrated in Fig. 1. The elution profile at 280 nm is shown in Fig. 1a, and the corresponding FRIE pattern in Fig. 1b. The fractions indicated containing antigen 19/20 were pooled, dialysed and lyophilized. Ag 19/20 had been shown by Holck *et al.*[6] to have a p*I* of about 8, and analytical IEF of the GPC fraction, Fig. 2, showed that the main part of the proteins present had p*I* below 7. The isolation of Ag 19/20 from this mixture seemed possible on a cation-exchange column. As the use of cation exchangers based on monobeads had previously been successful[8–10], purification of Ag 19/20 on the Mono S™ column was tried. Various buffers and ion strengths at pH 7.50 were tested, including sodium phosphate buffer and Tris. Sodium phosphate buffer was not suitable and, in contrast to the general rules that the buffering ion should be different from the counter ion

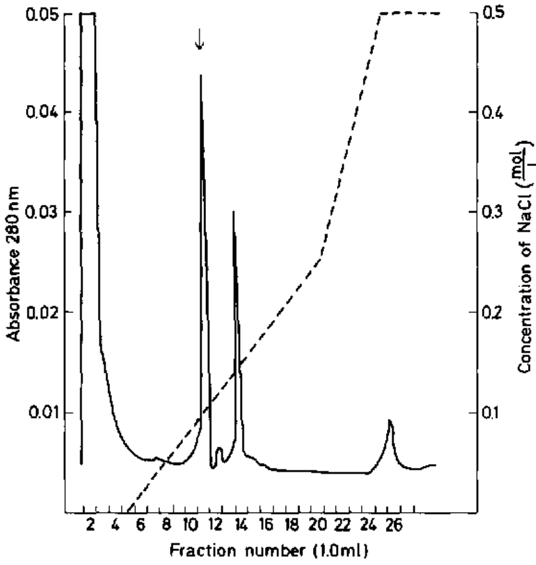


Fig. 3. Chromatography of the GPC fraction (Fig. 1) on the Mono S column with the following buffer system: buffer A, 10 m*M* Tris–HCl (pH 7.50); buffer B, 10 m*M* Tris–HCl (pH 7.50) containing 0.5 *M* sodium chloride. Flow-rate was 1 ml/min. ———, Absorbance at 280nm; – – –, salt concentration. The arrow indicates Ag 19/20 activity.

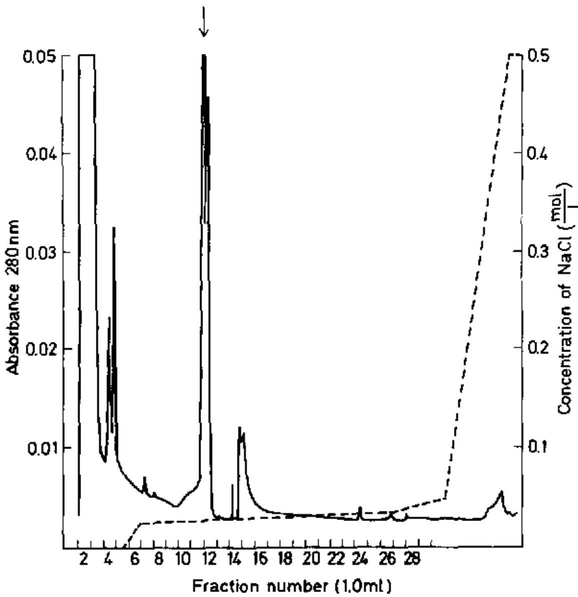


Fig. 4. Chromatography of the GPC fraction (Fig. 1) on the Mono S column. Apart from the change in sodium chloride concentration, the conditions are as described for Fig. 3.

in ion-exchange chromatography, Tris buffer gave the best result. At the recommended ion strength of 50 mM[16], Ag 19/20 was partly bound, while with an ion strength of 10 mM all Ag 19/20 present in the GPC fraction bound to the Mono S column.

In order to elute as pure allergen as possible, various salt gradients were tested. The elution program given in Fig. 3 showed that Ag 19/20 was present in one concentrated peak eluted after 11 min by a salt concentration of *ca.* 0.10 M. To determine whether more than one component was present in the peak containig the Ag 19/20 activity, gradients with lower slopes were tested. The peak containing the Ag 19/20 activity split into two with lower gradients, but the elution position of the allergen peak was the same whether the original slope was used or a different slope with an increase in the salt concentration from 0.02 to 0.035 M between 6 and 26 min of the start of elution (Fig. 4). All salt gradients used eluted the allergen in fraction 11 (or after 11 min). Iscocratic elution of the column with the starting buffer for 18 min before the salt gradient was started (Fig. 5) showed that allergen 19/20 was eluted after 14 min, and the separation of the two components was better than obtained before. FRIE and the RAST inhibition test of the fractions showed that Ag 19/20 was present and still active in fractions 14 and 15 as shown in Fig. 5. In order to get reproducible results a washing period of 20 min (1 ml/min) was necessary.

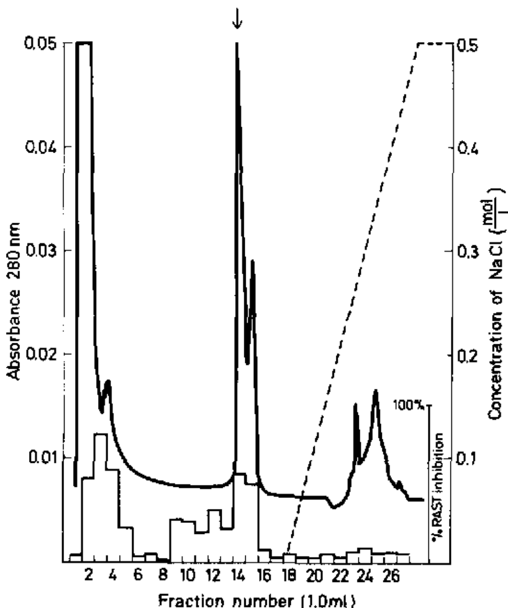


Fig. 5. Chromatography of the GPC fraction of the mite extract on the Mono S column. Buffers as in Fig. 3. ———, Absorbance at 280 nm; – – –, sodium chloride concentration; □, RAST inhibition assay. The arrow indicates the fraction in which Ag 19/20 was detected by FRIE.

It can be concluded that the Mono S column can be used as a separation medium for obtaining pure Ag 19/20 as shown by IEF (Fig. 2). The allergen is eluted with the starting buffer after 14 min, and remaining protein material with a steep salt gradient of up to 0.5 M sodium chloride.

ACKNOWLEDGEMENTS

The authors are indebted to Nycomed., Norway, for providing mite, rabbit antisera, human sera and [^{125}I]anti-IgE. The Norwegian Medical Depot is thanked for financial support. We are also grateful to Svein Haavik and Jens K. Wold for valuable discussions.

REFERENCES

1 T. Miyamoto, S. Oshima, T. Ishizaki and S. Sata, *J. Allergy*, 42 (1968) 14–28.
2 L. Tuft and V. M. Heck, *Ann. Allergy*, 33 (1974) 325–330.
3 S. Romagnani, G. Biliotti, A. Passaleva and M. Ricci, *Clin. Allergy*, 2 (1972) 115–123.
4 K. Aas, *Allergy*, 33 (1978) 3.
5 K. Aas, in C. Steffen and H. Ludwig (Editors) *Clinical Immunology and Allergology*, Elsevier, Amsterdam, 1981, p. 255.
6 A. Holck, S. Dale and K. Sletten, *Allergy* (1986), to be published.
7 S. Dale and E. Landmark, *Allergologia et Immunpathologie*, 8 (1980) 401.
8 R. Einarsson, *Acta Chem. Scand., Sep. B*, 37 (1983) 252.
9 D. R. Hoffman and C. L. Wood, *J. Allergy Clin. Immunol.*, 74 (1984) 93.
10 S. Haavik, B. S. Paulsen and J. K. Wold, *J. Chromatogr.*, 321 (1985) 199.
11 S. Haavik, B. Smestad Paulsen, J. K. Wold and Ø. Grimmer, *Phytochemistry*, 21 (1982) 1913.
12 L. Yman, G. Ponterius and R. Brandt, *Rev. Biol. Stand.*, 29 (1975) 151.
13 N. M. G. Harboe and P. J. Svendsen. *Scand. J. Immunol.*, 17 Suppl. 10 (1983) 107.
14 B. Weeke, I. Sønderdgaard, P. Lind, L. Aukrust and H. Løwenstein, *Scand. J. Immunol.*, 17 Suppl. 10 (1983) 265.
15 A. Winter, K. Ek and V. B. Andersson, *Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels*, LKB, Bromma, 1977, application note 250.
16 J. Bergstrøm, L. Søderberg, L. Whalstrøm, R.-M. Muller, A. Domicelj, G. Hagstrøm, R. Stalberg, I. Kallman and K.A. Hansson, *Protides Biol. Fluids*, 30 (1983) 641.