

Purification of a 20 kD allergen from *Aspergillus fumigatus*

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Sera from patients sensitized to *Aspergillus fumigatus* (*A.f.*) were screened for specific IgE using sodiumdodecylsulphate-gradient-polyacrylamidegelelectrophoresis (SDSgPAGE) followed by immunoblotting to nitrocellulose. Approximately 25 IgE-binding components were detected. The components of molecular weight 20, 31, 44, 50, 53, 77 and 90 kD were reacting with more than 50% of the patients. The 90, 77 and 20-kD components showed up as the strongest IgE-binding bands. The 20-kD component, called Ag 20 kD, was purified and further characterized. Ag 20 kD was purified to apparent homogeneity, using a combination of size-exclusion chromatography on a Sephacryl S-200 column, preparative isoelectric focusing in a pI 2.5-6.5 gradient, and a Sephadex G-50 Superfine column. Fractions were characterized with protein and carbohydrate analyses, RAST and SDSgPAGE followed by immunoblotting to nitrocellulose. Ag 20 kD was found to be a glycoprotein as it stained with both Coomassie Brilliant Blue and PAS. However, it did not bind Con A, and thus, did probably not contain any terminal α -D mannopyranosyl end groups. The relation between mannose, galactose and glucose was found to be 2:1:0.5. The isoelectric point was heterogeneous within pH range 5-6, and the molecular weight was estimated to approximately 20 kD. An increased RAST response was shown for the purified component compared with crude extract using patient sera reacting with Ag 20. The antigen was shown not to be identical with the previously described Ag 3. Neither did it fit the description of Ag 5, 7 or 13 earlier described by the same group. The antigen is going to be used for further immunochemical and clinical investigations, and coupling to other systems for antigen characterization.

Key words: *Aspergillus fumigatus*; purification; characterization; size-exclusion chromatography; SDSgPAGE; IEF; immunoblotting.

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The worldwide fungus *Aspergillus fumigatus* (*A.f.*) causes a number of comparatively well-characterized disorders located mainly in the airways. The most extensively studied disorders are inhalation allergy and allergic bronchopulmonary aspergillosis (ABPA). ABPA is a complication of inhalation allergy where the fungus acts as an opportunistic pathogen and grows in mucus in the lungs of atopic asthma or cystic fibrosis patients. (1, 19)

Since Sluyter in 1847 ascribed human disease to this group of fungi (25), many authors have

studied the *Aspergillus* antigens. Among others Kauffman et al. studied the growth conditions and antigenic properties of different preparations in order to improve standardization (9, 10, 11). Kurup et al. (12, 13) and Piechura et al. (20) have studied antigenic variability and cross-reactions with other aspergilli and related fungal taxa as well as fractionation of extracts to obtain better standardized extracts.

Studying inhalation allergy patients, Wallenbeck et al. (29) established a crossed radio-immuno-electrophoresis (CRIE) system where

Table 1.

A.f. specific, and total IgE values obtained for the patients used in the IB screening

Pat. No.	<i>A.f.</i> specific IgE PRU/ml	Total IgE kU/l
A	0.8	245
B	9.9	1010
C	6.3	1165
D	9.3	360
E	5.1	990
F	16.0	1690
G	17.5	1185
H	3.5	1260
I	4.4	180
J	17.0	435
K	3.1	780
L	3.5	420
M	4.0	175
N	2.1	210
O	9.3	360
P	1.2	610
Q	3.8	1980
R	<0.35	10
S	<0.35	1000

the antigens were electrophoresed into rabbit antisera. Two major IgE-binding components were identified, namely Ag 10 and Ag 40.

Longbottom (14) established an antigen characterization system based on crossed radio-immuno-electrophoresis (CRIE) where the antigen was electrophoresed into ABPA patient sera containing high amounts of precipitating antibodies. Four of these components have been further characterized (6, 7, 15, 26): Ag 3, a major IgE-binding component; Ag 13, identical with the C antigen identified by Tran van Ky (27) which binds IgG, and Ag 5 and 7 which bind both IgE and IgG.

A separation system for *A.f.* antigens based on sodiumdodecylsulphate gradient-polyacrylamidegelelectrophoresis (SDSgPAGE) followed by immunoblotting to nitrocellulose (IB) was earlier presented (3).

The aim of the present study was to identify the dominating allergens of *Aspergillus fumigatus* in a sensitized population of atopic allergy patients, using SDSgPAGE followed by immunoblotting, and to purify and further characterize one of these antigens.

MATERIAL AND METHODS

The mould extract was prepared from a single strain of *Aspergillus fumigatus* (*A.f.*) obtained from Allergon AB, Ängelholm, Sweden. *A.f.* was grown on a completely synthetic medium under well-defined culture conditions. The source material contained both conidia and mycelium.

The source material (1/20 w/v) was extracted under agitation (4–8°C) in 0.125 M ammonium bicarbonate buffer pH 7.9 for 24 h. The extract was clarified by centrifugation and filtered through a glass fibre filter. The filtrate was dialysed against distilled water for 24 h and freeze-dried.

The sera used were collected from patients in whom inhalation allergy to *A.f.* was suspected, based on the following criteria: history including asthma or rhinoconjunctivitis caused by moulds, and specific IgE with Phadebas RAST® (Pharmacia AB, Uppsala, Sweden) to *A.f.* (Table 1). None of the sera contained precipitating antibodies as measured by double diffusion test. A serum pool from healthy non-atopic volunteers with or without myeloma IgE added to 10,000 kU/ml was used as control.

RAST discs were prepared by coupling 1 ml of extract to 20 CNBr-activated paper discs (4) using crude extract, gel filtration fraction C, and IEF fraction 7. RAST was performed as described previously (8) for measurement of specific IgE in serum. ¹²⁵I-labelled immunosorbent-purified anti-IgE (Phadebas RAST; Pharmacia Diagnostics AB) was used as a tracer. The count rates obtained were compared with those of the Phadebas RAST Reference System and the results expressed in counts per minute (cpm) or Phadebas RAST units (PRU/ml). Positive results were defined as >0.35 PRU/ml. All assays were run in duplicate. RAST assays were run using 10 patient sera from the initial IB screening. Sera from patients both showing and not showing specific IgE-binding to Ag 20 kD were used.

Immunoblotting was performed as previously described (3). Briefly, the SDSgPAGE gels were moulded in a BIORAD Protean Dual Slab Cell equipment with a polyacrylamide stacking gel

on top of a gradient separation gel (7.5–20%) essentially as described by Neville (18). The separated proteins were transferred to a nitrocellulose membrane (NC) by electrophoretic transfer in a BIORAD Trans-Blot Cell. After transfer unoccupied protein-binding sites were blocked (0.5% Tween 20) before immunological detection with patient sera (diluted 1:4) followed by enzyme-labelled anti-IgE (Phadezym RAST®).

Crossed immuno- and crossed radioimmuno-electrophoresis (CIE/CRIE) was performed as previously described (16). IEF fraction 7 (different amounts ranging between 0.06 mg and 0.6 mg) was electrophoresed into a second dimension gel containing a reference rabbit antiserum (17 µl/cm²). Ag 3 was identified using a single patient serum strongly reactive to Ag 3 (300 µl diluted to 3 ml). Reagents were kindly provided by Dr. J. L. Longbottom.

For size-exclusion chromatography, fractionation on a Sephacryl S-200 Superfine column (2.6 × 100 cm) (Pharmacia-LKB, Bromma, Sweden) was performed by applying 0.65 g of crude, dialysed *A. fumigatus* extract dissolved in 20 ml of elution buffer (0.05 M ammonium bicarbonate, pH 7.9). The flow rate was 17.5 ml/h and fractions of 4.5 ml were collected. Before use, the column was calibrated with standard proteins and dextrans. The elution of proteins was recorded at 280 nm.

Fractionation of preparative IEF fractions on a Sephadex G-50 Superfine column, 2.6 × 38 cm, (Pharmacia-LKB, Bromma, Sweden) was performed by eluting with distilled water at a flow-rate of 12 ml/h. Fractions of 3 ml were collected and the elution of proteins was recorded at 280 nm.

Analytical isoelectric focusing (IEF) was carried out in a polyacrylamide gel according to Winter (30). 450 µg of freeze-dried Sephacryl S-200 fraction was dissolved in distilled water. The electrofocusing in a pH 3–10 gradient (Pharmalyte, pH 3–10, Pharmacia-LKB, Bromma Sweden) proceeded for 1.5–2 h at a constant effect of 25 W. Preparative IEF was performed in a granulated gel (Ultradex, Pharmacia-LKB, Bromma, Sweden) according to Winter (31). 200 mg of a freeze-dried Sephacryl S-200 frac-

tion was dissolved in distilled water. The electrofocusing in a pH 2.5–6.5 gradient prepared by mixing Pharmalyte pH 2.5–5 (4.5 ml) and Pharmalyte pH 4.0–6.5 (3.0 ml) (Pharmacia-LKB, Bromma Sweden) continued for 15 h at a constant effect of 8 W.

Gels were stained for protein with Coomassie Brilliant Blue R-250 or a silver stain reagent (21). Carbohydrate was detected by the periodic acid Schiff (PAS) reagent as described by Zacharius et al. (32).

For the purification procedure, dialysed, crude *A. fumigatus* extract was fractionated with size-exclusion chromatography on a Sephacryl S-200 Superfine column. The fractions containing Ag 20 kD were pooled and freeze-dried. The Ag 20 kD-rich pool was then submitted to preparative IEF in a pH 2.5–6.5 gradient and fractions containing only Ag 20 kD were pooled and freeze-dried. Finally, the IEF pool was cleared from amphotines with size-exclusion chromatography on a Sephadex G-50 column.

The composition and quantity of monosaccharide was established by methanolysis (1 M HCl in dry methanol at 80°C for 24 h), trimethylsilyl (TMSi) derivatization and gas-liquid chromatography (GLC) as described by Reinhold (23). The TMSi ethers of the derived methyl glycosides were chromatographed on a 0.3 mm × 15 mm DB 5 fused silica capillary column with H₂ as carrier gas (0.5 ml/s). The chromatograph (Carlo Erba 4200) was equipped with a flame ionization detection system, and peak areas were determined with a LDC 308/9 Chromatography Integrator. The phenol-sulphuric acid method (5) was used for the detection of carbohydrate in fractions obtained from size-exclusion chromatography or IEF.

Total protein content was estimated according to Lowry (17) and Waddell (28). Bovine serum albumin was used as a reference. The elution of proteins from chromatography columns was recorded by measuring the absorbance at 280 nm.

For affinity chromatography a freeze-dried Sephacryl S-200 fraction, enriched for Ag 20 kD, was fractionated on a Con A-Sepharose 4 B column, 1 × 5 cm (Pharmacia-LKB, Bromma, Sweden). The sample (8 mg) was dissolved in

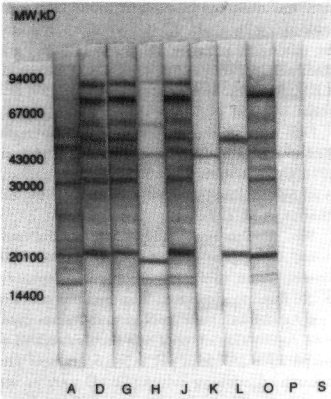


Fig. 1. IgE-binding components detected in a selection of 10 *A.f.* sensitized individuals using immunoblotting technique from SDSgPAGE gels. MW-molecular weight, A-P positive patient sera, S-control serum with myeloma IgE added to 10000 kU/ml. IgE was detected using an enzymelabelled anti-IgE.

0.2 ml of sample buffer (20 mM Tris-HCl, pH 7.4, containing 1 mM MnSO₄ and 1 mM CaCl₂). Elution was started with this buffer (25 ml), followed by adding 1 M NaCl in buffer (15 ml), 20 % ethylene glycol in buffer (15 ml) and finally 0.5 M methyl- α -D-mannopyranoside dissolved in buffer (25 ml).

RESULTS

Patient screening

The IgE-binding patterns of 17 atopic allergy patients, positive in RAST to *Aspergillus fumigatus*, was studied in SDSgPAGE followed by immunoblotting to nitrocellulose (Fig.1). In general, sera giving rise to high RAST scores also gave rise to strong IgE-binding in IB (Table 1). The dominating allergens were defined by counting the number of patient sera with IgE antibodies to each component (Fig. 2).

Approximately 25 IgE-binding components were detected, ranging between 15 and 113 kD in molecular weight. More than 50 % of patient sera showed IgE-binding towards components of molecular weight 90, 77, 53, 50, 44, 31 and 20 kD. In most sera, the strongest IgE-binding components were of molecular weight 90, 77 and 20 kD (Fig.1 and 2). The component of molecular weight 20 kD, was chosen for purification and further characterization.

Size-exclusion chromatography

The crude *A.f.* extract obtained after dialysis and lyophilisation was fractionated with size-exclusion chromatography on a Sephacryl S-200 column. Fig.3a shows the protein and carbohydrate elution profiles. The eluate from the Sephacryl column was divided into 5 fractions; A, B, C, D and E. The yields obtained are shown in Table 2, and the protein and carbohydrate contents of the different fractions are given in Table 3. 92 % of the sample added to the gel was regained after separation, and the largest fractions were fraction E followed by fractions B and C. The protein content was

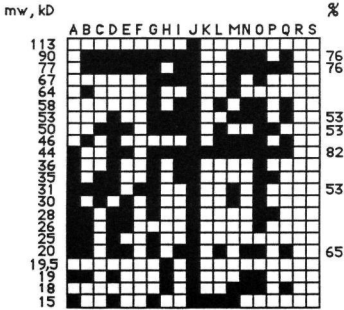


Fig. 2. IgE-binding components detected in 17 *A.f.* sensitized individuals using immunoblotting technique from SDSgPAGE gels. MW-molecular weight, A-Q positive patient sera, R-negative control serum, S-control serum with myeloma IgE added to 10000 kU/ml % - percent of patients reacting with a particular component (only components reacting with more than 50 % of patients indicated)

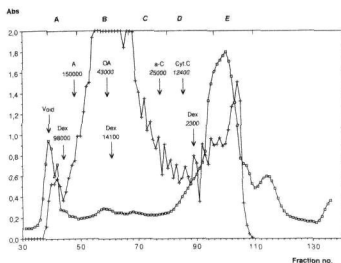


Fig. 3a. Elution profile of an *A.f.* extract after fractionation on a Sephacryl S-200 column. Fractions A-E indicated at top. (+ - + - +)-carbohydrates measured with the phenol-sulphuric acid test. (□-□-□)-protein measured at A_{280} . Molecular weight markers: A-aldolase, OA-ovalbumin, a-C- α -chymotrypsinogen, Cyt.C-cytochrome C. Dex-dextran of molecular weight 2.3, 14.1 and 98 kD respectively.

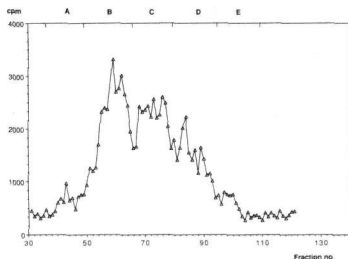


Fig. 3b. Direct RAST analysis of fractions from Sephacryl S-200 column. Fractions A-E indicated at top. (Δ - Δ) - total allergenic activity measured as counts per minute (cpm).

estimated using two different methods, both giving similar results. The highest protein values were obtained for fractions D and E, and a high carbohydrate content characterized all fractions except for E.

All fractions were examined for total allergenic activity with direct RAST using BrCN-activated paper discs (Fig. 3b, Table 2). The maximum allergenic activity was found in fraction B, followed by fraction C.

To identify Ag 20 kD, the crude extract and fractions A - E were submitted to SDSgPAGE followed by immunoblotting (Fig. 4). After im-

muno blotting, detection was performed by using a single broad-spectrum asthma patient serum chosen from the serum screening (serum J, Fig. 2). This serum reacted towards all IgE-binding components detected. The strongest IgE-binding activity was found in fractions B and C. The SDSgPAGE gel and nitrocellulose membrane corresponding to fraction C revealed a strong band in the same position as Ag 20 kD in the crude extract, which stained with both Coomassie Brilliant Blue and PAS, indicating a glycoprotein. Fraction B contained small amounts of Ag 20 kD and a number of high-molecular-weight components. Based on five independent electrophoretic separations, the molecular weight of Ag 20 kD was estimated to approximately 20 kD.

Table 2.

Fractions obtained after gel filtration of *A.f.* extract (650 mg) on Sephacryl S-200 column

Fraction	Yield ¹ mg	% Eluted material	% RAST activity
A	60	10	4.3
B	150	25	39.6
C	140	23	35.1
D	80	13	18.7
E	170	29	2.2
A-E	600	100	99.9

¹ weight after freeze-drying

IEF

Fraction C also contained small amounts of other allergenic components and therefore further purification of Ag 20 kD was necessary. As a second step, fraction C was analysed using analytical IEF in polyacrylamide gel, with subsequent electrophoretic transfer to nitrocellulose membrane. IgE binding components were detected as in the SDSgPAGE immunoblotting experiments. A pH 3-10 gradient was employed; however, all IgE-binding components were

Table 3.

Protein and carbohydrate content of fractions obtained after gel filtration of A.f. extract on Sephacryl S-200

Fraction	Protein (mg)		Carbohydrate (mg)			
	Lowry	Wadell	Mannose	Galactose	Glucose	Gal. A ¹
A	8.1	11.0	15.2	16.3	1.9	1.5
B	11.4	14.9	45.6	38.7	8.6	2.1
C	15.8	18.5	78.4	45.2	2.0	4.2
D	22.6	24.9	13.8	13.4	4.6	5.9
E	43.7	42.7	0.8	4.1	13.1	²

¹ Galacturonic acid² Conclusive results not obtained

detected in the acidic part of the gel. Fraction C focused at pI 4.7–6.8.

Fraction C was then fractionated with preparative IEF in an Ultradex gel using a pH 2.5–6.5 gradient. The gel was cut into 30 equal sections across the pH-gradient. Each section was eluted with water and the eluates were measured for pH, protein and carbohydrate content (Fig. 5). Eluates 1–30 were combined as shown in Fig. 5 to give 7 IEF fractions. Each fraction was dialysed, lyophilised and subjected to SDSgPAGE followed by immunoblotting. Ag 20 kD was present only in IEF fractions 5, 6 and 7, covering pH 4.7–6.8. IEF fraction 7 contained only one component corresponding to Ag 20 kD, which was detected by immunoblotting, Coomassie Brilliant Blue or silver stain (Fig. 6). Fractions 5 and 6 also contained other components in addition to Ag 20 kD, and were not further processed.

The carrier ampholyte was not entirely removed by dialysis, and IEF-fraction 7 was further fractionated on a Sephadex G-50 SF column. This removed the carrier ampholyte.

Carbohydrate analysis

The carbohydrate content of Ag 20 kD was analysed using GLC technique, and the relation between mannose, galactose and glucose was found to be 2:1:0.5. The high carbohydrate content of fraction C and the PAS staining of Ag 20 kD indicated fractionation

on a lectin column to obtain further information on the carbohydrate composition of Ag 20 kD. As mannose was the predominant sugar in the crude extract, separation on a Con A (concanavalin A) column was chosen. To trace

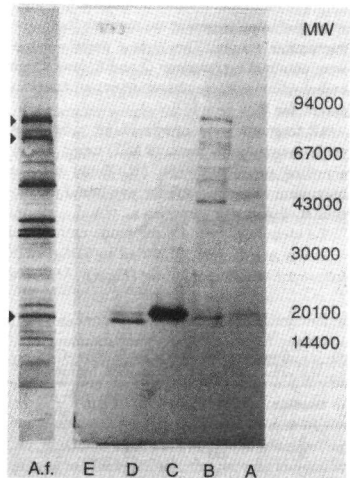


Fig. 4. Immunoblotting from SDSgPAGE of crude A.f. extract, and fractions A-E from Sephacryl S-200. Major components of 20 kD (Ag 20 kD), 77 kD and 90 kD indicated with arrows. Detection with broad-spectrum patient serum J from Fig. 1, and enzyme-labelled anti-IgE.

Ag 20 kD the different fractions obtained were subjected to SDSgPAGE followed by immunoblotting. Many IgE binding components were retained in the column; however, Ag 20 kD was not, and eluted quantitatively with the starting buffer.

RAST-assays using purified components

RAST assays were run using 9 patient sera from the initial IB screening and discs with crude extract, gel filtration fraction C and IEF fraction 7 (Table 4). Sera from patients both showing, and not showing specific IgE-binding to Ag 20 kD were used.

All five patient sera showing strong IgE binding to Ag 20 kD in IB (patients D, G, J, L and O) gave rise to increasing RAST scores when using the gel filtration and IEF fractions. The RAST score of patient A was somewhat lower when using the gel filtration and IEF fractions compared with crude extract. Patients H, K and P who did not react to Ag 20 kD in IB were all negative to IEF fraction 7.

Immunological comparison between Ag 20 and Ag 3

Ag 20 kD shows many similarities to Ag 3 identified by Longbottom (15). An eventual

identity with this antigen was investigated using Dr Longbottom's reference CIE/CRIE system and a patient serum strongly reactive to Ag 3.

No staining of the CIE/CRIE plates was seen either using CBB stain or after incubation with patient serum, and it is concluded that Ag 20 kD is probably not identical with Ag 3.

DISCUSSION

In a screening of sera from patients sensitized to *Aspergillus fumigatus* a 20 kD component was found to react with more than 50% of patients. This component was called Ag 20 kD and was chosen for purification and further characterization.

The first step of the purification was achieved by size-exclusion chromatography. All gel filtration fractions contained Ag 20 in varying amounts as detected by IB, and this was probably due to the different separation principles used. Gel filtration was performed under native conditions while IB was performed under denaturing conditions, and it can not be excluded that Ag 20 is either aggregated during gel filtration or might be part of a larger molecule in

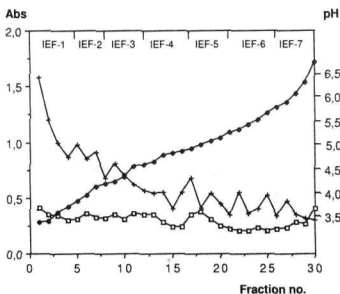


Fig. 5. Fraction C from Sephacryl S-200 separated by preparative IEF. Fractions 1-7 indicated at top. (---) carbohydrates measured with the phenol-sulphuric acid test. (□-□-□) - protein measured at A_{280} (◇-◇-◇) - pH.

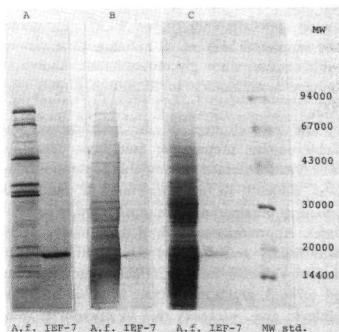


Fig. 6. SDSgPAGE of crude *A.f.* extract, and IEF-fraction 7 from preparative IEF. A. Detection after immunoblotting with broad-spectrum patient serum J from Fig. 1, and an enzyme-labelled anti-IgE. B. Silver stain. C. Coomassie Brilliant Blue.

Table 4.

Specific IgE values obtained with gel filtration fraction C and IEF fraction 7, using 9 patient sera from the IB screening.

Pat. no.	Crude extract	GF C	IEF 7
A	0.9	0.7	0.5
D	9.6	11.1	14.2
G	17.9	23.8	29.6
H	3.2	0.7	>0.35
J	16.6	19.1	23.8
K	3.5	>0.35	>0.35
L	3.5	4.1	4.2
O	9.1	9.8	12.3
P	1.5	>0.35	>0.35

a native system being partly disintegrated during gel filtration. All bands detected in the crude extract could not be seen in the different gel filtration fractions. This was probably due to a combination of different amounts of protein added to the gel and components being retained in the column as only 92% of the sample was recovered. The molecular weight estimations obtained from size-exclusion chromatography are in keeping with the data obtained from SDSgPAGE. SDSgPAGE indicated the molecular weight 20 kD, and in gel filtration, Ag 20 kD was predominant in fraction C ranging between approximately 20 and 40 kD. However, the estimated MW of 20 kD should be viewed with caution since glycoproteins are known to behave anomalously in SDSgPAGE gels (22, 24).

The second step of the purification was achieved by preparative isoelectric focusing. The yield obtained during this separation step was difficult to estimate due to the small amounts obtained and contamination of ampholines. Approximately 1% of the initial material was finally recovered as Ag 20 kD. The pI values obtained for Ag 20 kD ranged between pI 4.7–6.8, and a more exact pI value is impossible to give, probably due to microheterogeneity of the carbohydrate moiety.

Ag 20 kD obtained after the separation procedure described appeared homogeneous. It showed a single band when an SDSgPAGE gel was stained with Coomassie Brilliant Blue or

silver stain also when overloading the gel, and a single band after immunoblotting followed by detection with a broad-spectrum allergic asthma patient serum. The presence of impurities not detected with the reagents used can, however, not be totally excluded.

Ag 20 kD is a glycoprotein as it stained with both Coomassie Brilliant Blue and PAS, and the relation between mannose, galactose and glucose was found to be 2:1:0.5. However, Ag 20 kD does not bind Con A, indicating the absence of α -D mannopyranosyl end groups. Thus, this might be an alternative route for purification as many IgE-binding components were retained in the Con A-column. The role of carbohydrates in forming the allergenic activity is of great interest as many allergens have been shown to be glycoproteins. Few components have been characterized with reference to carbohydrate composition, and it is therefore interesting to continue studying both the carbohydrate and the amino acid composition of Ag 20 kD.

Ag 20 kD does not fit the description of any of the antigens/allergens identified and further characterized by Longbottom, Harvey and Taylor (11, 12, 13, 14). Ag 3 gives approximately the same molecular weight after size-exclusion chromatography and does not bind Con A. However, CIE/CRIE data indicated that Ag 20 kD is not identical with Ag 3. Preliminary data also indicate that Ag 20 kD has a slower electrophoretic mobility than Ag 3. Ag 5, 7 and 13 all indicate higher molecular weights after size-exclusion chromatography: 35 kD, 150–200 kD and 70 kD respectively and, in addition, Ag 7 and 13 bind Con A. Ag 20-kD could possibly be Ag 15, also a major component although not fully characterized (personal communication, J. Longbottom). Wallenbeck's Ag 40 (29) is also a possible candidate, showing approximately the same electrophoretic mobility. Arruda & Chapman (2) have also identified a 20 kD component which might be identical with Ag 20 kD, using immunoblotting technique.

Work is in progress to elucidate the identity and to further study the biochemical characteristics, and the clinical importance of Ag 20 kD in a well-documented group of allergic asthma and ABPA patients.

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