

Allergens in *Aspergillus fumigatus*

I. Characterization of two different allergen extracts and evaluation of their stability and the importance of carbohydrate for IgE binding

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Aspergillus fumigatus grown in submerged and surface cultures was extracted, and the extracts were analyzed separately. The submerged extract contained 31.9% protein and 8.3% carbohydrate, while the corresponding values were 17.0% and 33.3% for the surface material. With individual sera from patients with allergic asthma, SDS–PAGE combined with immunoblotting revealed that the submerged extract contained at least six strong IgE-binding components (20, 30, 38, 50, 68, and 90 kDa) in addition to several weak to medium IgE-binding components. The surface extract contained about the same number of IgE-binding components, but only one gave a strong reaction (20 kDa). The allergens present were shown to have pI between 4.5 and 5.6 as demonstrated by isoelectric focusing (IEF) combined with immunoblotting. For identification of *A. fumigatus* glycoprotein allergens, both extracts were treated with periodate under mild conditions. Two allergens of the submerged extract (90 and 38 kDa) partly lost their IgE-binding ability by this treatment, indicating that these components are glycoproteins and that the carbohydrate moiety is involved in the IgE binding. The IgE-binding ability of the 20-kDa allergen was not influenced by periodate. For assessment of the stability of the two allergen extracts, aqueous solutions were kept at 4°C for 2, 7, and 21 d and then analyzed by SDS–PAGE and immunoblotting. The results showed that most allergens of the submerged extract were partly inactivated after 2 d. After 21 d, only the 20-kDa and 30-kDa components were still able to bind IgE. Similar results were obtained by analyzing the surface extract. When the same experiment was performed on samples in a 50% glycerol solution, the results strongly indicated that glycerol had a stabilizing effect on allergens in both extracts. The enzyme content, estimated by the API ZYM-test, showed that both extracts contained several protein- and carbohydrate-degrading enzymes. The presence of these enzymes may explain the lability of the extracts.

Key words: *Aspergillus fumigatus* allergens; enzyme activity; glycoprotein allergens; IEF; SDS–PAGE; stability.

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The mold *Aspergillus fumigatus* is able to cause several forms of severe disease in man (30). This pathogenicity has led to several immunochemical as well as clinical studies. Many studies have investigated allergen and antigen contents of this mold (8, 13, 14, 16, 17, 19, 25), but the results differ concerning the total number and mol. mass of allergens and antigens. This may be due to differences in *A. fumigatus* source material. Several authors have reported large

strain (7, 15, 29) and batch variations (7, 18). Karlsson-Borgå et al. (11) have studied a number of *A. fumigatus* strains and the reproducibility among batches of a given strain of this fungus. In addition, different source materials obtained from submerged-grown culture, surface-grown culture, culture filtrate, and conidia of *A. fumigatus* were investigated.

The results from two groups of patient sera, atopic allergy and allergic bronchopulmonary aspergillosis

(ABPA), showed that atopic allergy patient sera reacted more strongly to the submerged extract than to the surface extract, while the ABPA patient sera reacted similarly to both extracts. The culture filtrate preparation gave a positive reaction only to the ABPA patient sera. The response of the conidia extract was weak with both groups of patient sera, and conidia were considered to be unsuitable as raw material for allergen preparations.

The present work has further compared submerged culture and surface culture allergens and antigens by investigation of their stability in solution and identification of glycoprotein allergens present.

Material and methods

Allergen extracts

The *A. fumigatus* source material was prepared from a single strain of the mold (CBS 54565.13) grown by Allergon AB, Ängelholm, Sweden, under well-defined conditions. The mold was grown with the same synthetic medium both in a submerged culture and on a surface culture. The growth conditions were optimized with respect to IgE-binding capacity. Extracts were prepared from 5.19 g and 13.00 g of submerged and surface-grown material, respectively, with extraction time of 1 h, as described by Samuelsen et al. (24). The yield was 587 mg (11.3%) and 573 mg (4.4%) from submerged and surface-grown source material, respectively.

Patient sera

Thirty-four individual sera (1–34) collected from patients with serum having specific IgE against *A. fumigatus*, as measured by Phadebas RAST (Pharmacia AB, Uppsala, Sweden), were investigated. All patients had atopic allergy to *A. fumigatus*. The broad-spectrum patient serum used by Samuelsen et al. (24) was included as number 34. Serum from a healthy, nonatopic subject was used as control.

SDS-PAGE

Homogeneous SDS-PAGE gels were molded in a ProteanTM mini-dual slab cell apparatus (Bio-Rad) with a 6.0% C and 2.67% T stacking gel on top of a 12.0% C and 2.67% T separation gel. Electrophoresis was performed at 200 V for 1 h with the discontinuous buffer system described by Neville (21). The samples were prepared for SDS-PAGE analysis by heating for 5 min at 100°C with SDS and 2-mercaptoethanol. Gels were stained for proteins with Coomassie brilliant blue R-250 or transferred to nitrocellulose 0.45 µm (Bio-Rad) or ImmobilonTM PVDF (Millipore) transfer membrane.

Immunoblotting from SDS-PAGE gels

Protein transfer by a Mini-Trans-Blot[®] electrode module and a Mini-Protean II cell (Bio-Rad) was performed as described by Nilsen & Smestad Paulsen (22), and the unoccupied protein-binding seats were blocked by 0.5% Tween in PBS (3). The IgE-binding components were identified by β-galactosidase-marked anti-IgE (4).

Analytic isoelectric focusing (IEF)

Electrofocusing was performed in a polyacrylamide gel in a pH gradient of 3.5–10, as described by Nilsen & Smestad Paulsen (22). IEF reference proteins, pH 3–10 (Pharmacia), were included for pI determination. The proteins were fixed and stained with Coomassie brilliant blue R-250 according to Winter et al. (31).

Immunoblotting of isoelectrofocusing gels

The separated proteins were electrophoretically transferred to a nitro-cellulose membrane in 0.7% acetic acid at 100 V for 3.5 h (Trans-Blot cell, Bio-Rad). The unoccupied protein-binding sites were blocked, after which immunologic detection was done, as described above.

Oxidation with periodate

Samples of both extracts corresponding to 4–7 mg carbohydrate were oxidized by sodium metaperiodate (3 ml 0.01 mol/l) in the dark at 4°C. The oxidation was stopped after 6.5 h by adding two drops of ethylene glycol, followed by 2–3 mg sodium borohydride. After 17–20 h, acetic acid was added to give pH 5–6. The solution was dialyzed against distilled water at 4°C and then lyophilized. Control experiments were performed with both extracts, omitting sodium metaperiodate.

Carbohydrate analysis

The sample (about 1 mg) was methanolized (1 mol/l HCL in MeOH, 80°C, 20–24 h) and the resulting methylglycosides were trimethylsilylated, as described by Reinhold (23). Gas chromatography was performed on a Carlo Erba 4200 gas chromatograph equipped with a flame ionization detection system, a splitless injector (6:1), and a fused silica capillary column (15 × 0.32 mm). Helium was used as carrier gas at a rate of 5 ml/min.

The gas chromatography was done with a column temperature gradient starting at 140°C at injection, followed by an increase of 1°C/min to 160°C and 6°C/min from 160 to 250°C.

Protein content

The crude extracts were hydrolyzed under vacuum in 6 mol/l HCL at 110°C for 24 h. The HCl was removed under reduced pressure, and the amino acid composition was determined with a Biotronic LC 5000 automatic amino acid analyzer. These results were used for calculation of total protein present.

Enzyme content

The API ZYM-test (Api System, France) was performed to investigate the presence of certain enzymes in the submerged and surface extracts. A 65-μl sample solution corresponding to 65 μg lyophilized crude extract was applied in each well. The API ZYM strips were incubated at 4, 20, and 37°C, respectively.

Stability studies

Crude allergen extract was dissolved in 0.02% NaN₃ with or without 50% glycerol, and aliquots were kept at 4°C in the dark for 2, 7, and 21 d, respectively. One control sample was stored at -20°C for 21 d. SDS-PAGE and IEF were performed on all the samples. Corresponding gels were stained for protein or carbohydrate, or they were subjected to electrophoretic blotting to nitrocellulose.

Results

Protein and carbohydrate composition

The carbohydrate content and composition as well as total protein content of submerged and surface extracts are shown in Table 1. The submerged extract had a higher content of protein but a lower carbohydrate content than the surface extract. It appears from Table 1 that the proportions of individual sugars in the two extracts were different.

Patient screening

The IgE-binding properties of the two extracts were studied by SDS-PAGE in combination with immunoblotting to Immobilon transfer membrane with sera from 34 patients with allergic asthma. The respective amounts applied of submerged and surface extract were 3.6 and 7.2 mg.

All patient sera included in this investigation were shown to contain IgE against at least one component in the submerged extract (Fig. 1a). But the number of bands appearing on the strips ranged from one to about 10. Twenty patient sera gave rise to a band of about 90 kDa (15 strong, four medium, and one weak), while three patient sera gave a strong response to a 68-kDa component. Of the investi-

Table 1. Carbohydrate composition and total carbohydrate and protein content of submerged and surface extracts

Monosaccharide composition	% Dried extract	
	Submerged	Surface
Mannose	0.8	15.3
Galactose	0.4	13.1
Glucose	5.1	3.6
N-acetylglucosamine	0.7	1.8
N-acetylgalactosamine	1.3	—
Total carbohydrate	8.3	33.7
Total protein	31.9	17.0

gated patient sera, 16 seemed to contain IgE against a 50-kDa component (two strong, 13 medium, and one weak). A band in the 38-kDa area was seen on seven strips, while only patient serum no. 34 gave a response to a 30-kDa component. Most patient sera (25 of 34) gave rise to a broad band in the 20-kDa area. As expected, the broad-spectrum patient serum (no. 34) seemed to contain IgE against most allergen components of both extracts.

The investigated sera gave very variable binding patterns with the surface extract (Fig. 1b), making further discussion complicated. However, a band appeared in the 20-kDa region on 17 of 34 strips (nine strong and eight weak).

Immunoblotting from IEF gels revealed several IgE-binding components characterized by pI between 4.5 and 5.6. In addition, the submerged and surface extracts both contained one allergen with pI of about 8.8 (results not shown).

Enzyme content

The enzyme contents of the two extracts were investigated by the API-ZYM test. Both extracts contained most of the enzymes included. The submerged and surface extracts had similar enzyme composition, apart from quantitative differences (Table 2). Corresponding API ZYM strips were incubated at 4, 20, and 37°C. The results (not shown) indicate that the enzymes are highly active at all these temperatures.

Stability

The stability of allergens in solution with or without 50% glycerol was studied with SDS-PAGE and IEF in combination with immunoblotting. Coomassie brilliant blue gel staining after SDS-PAGE (Fig. 2a) indicated that several proteins in the submerged extract (150 μg/well) are labile in solution at 4°C in the absence of 50% glycerol as stabilizing agent, giving bands with decreasing intensity with time. The immunoblotting results from patient serum

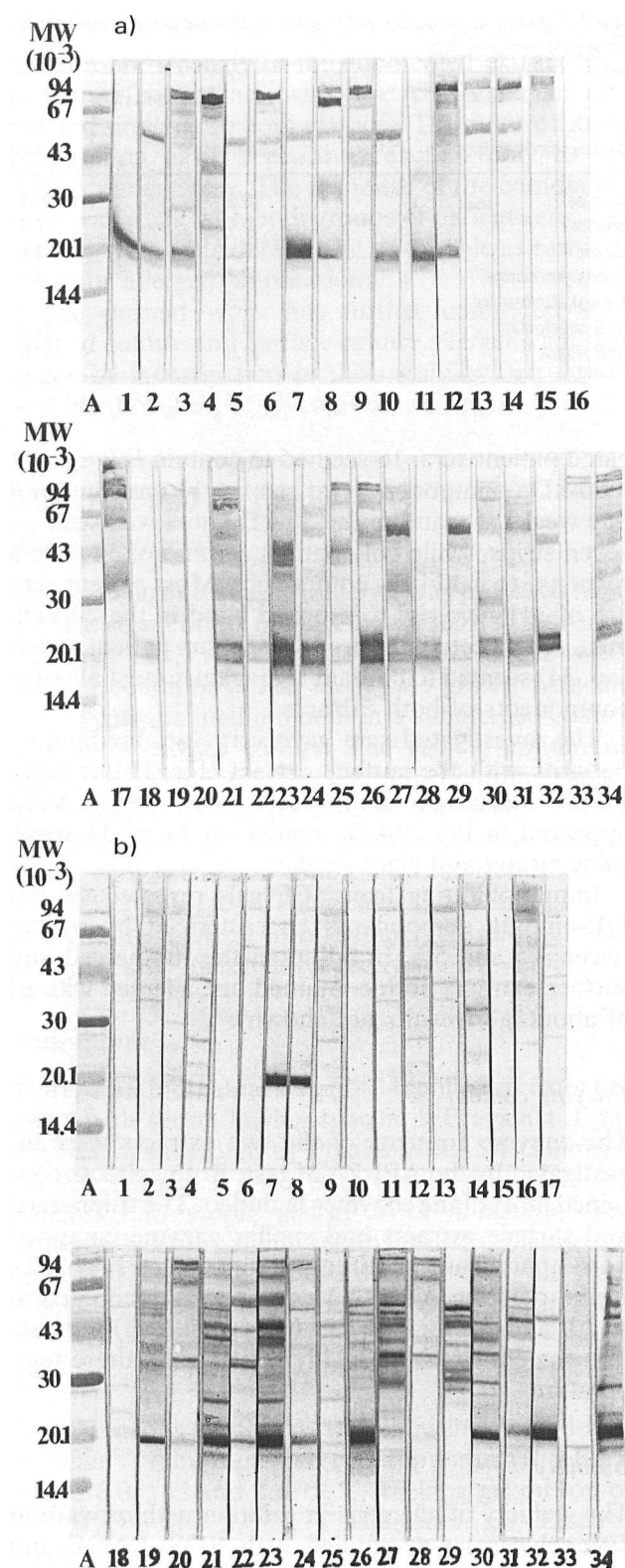


Fig. 1. IgE-binding components detected in 34 *A. fumigatus*-sensitized subjects (lanes 1–34) with immunoblotting from SDS-PAGE gel to PVDF transfer membrane. a) submerged extract, 3.6 mg/gel; b) surface extract 7.2 mg/gel. Lane A: MW marker stained with Coomassie brilliant blue R-250.

Table 2. Enzyme content of submerged extract and surface extract estimated by API ZYM-test

Enzyme assayed for	Submerged (nmol/mg)	Surface (nmol/mg)
1 Control	—	—
2 Phosphatase alkaline	> 650	615
3 Esterase (C4)	310	310
4 Esterase lipase (C8)	310	310
5 Lipase (C14)	< 75	—
6 Leucine arylamidase	460	150
7 Valine arylamidase	75	< 75
8 Cystine arylamidase	75	< 75
9 Trypsin	—	—
10 Chymotrypsin	75	—
11 Phosphatase acid	> 650	310
12 Naphthol-AS-BI-phosphohydrolase	460	150
13 α -galactosidase	150	615
14 β -galactosidase	—	—
15 β -glucuronidase	—	—
16 β -glucosidase	< 75	310
17 α -glucosidase	75	> 650
18 <i>N</i> -acetyl- β -glucosaminidase	75	615
19 α -mannosidase	< 75	—
20 α -fucosidase	—	—

no. 34 further indicated that the IgE-binding ability of several allergens of the submerged extract decreases with time in solution (Fig. 2b). This was observed already after 48 h and became more pronounced after several days. Examination of the 21-d sample in SDS-PAGE showed that only the 30- and 20-kDa components were still able to react with IgE. The 38-, 50-, 68-, and 90-kDa components were not visible. However, 50% glycerol in the sample solution did stabilize most allergens in this extract, except for the 68- and 38-kDa allergens that became weaker IgE binders with time.

The surface extract (300 μ g applied/well) showed comparable results (Fig. 2c and d). Dissolved in water, all the allergens except the 20-kDa allergen were rapidly inactivated in solution. Again, the stabilizing effect of 50% glycerol was pronounced.

Similar results were obtained by analysis of submerged and surface extracts in IEF (results not shown).

Periodate oxidation

Mild periodate oxidation was performed on submerged and surface extracts, followed by SDS-PAGE and IEF combined with immunoblotting, to investigate the significance of the carbohydrate for allergen activity. The results are given in Fig. 3a and b.

Comparison of the periodate-oxidized sample and control suggests that at least two allergens (90 and 38 kDa) in the submerged extract (150 μ g/well) partly lost their IgE-binding ability after periodate treatment, while the 20-kDa component was not affected.

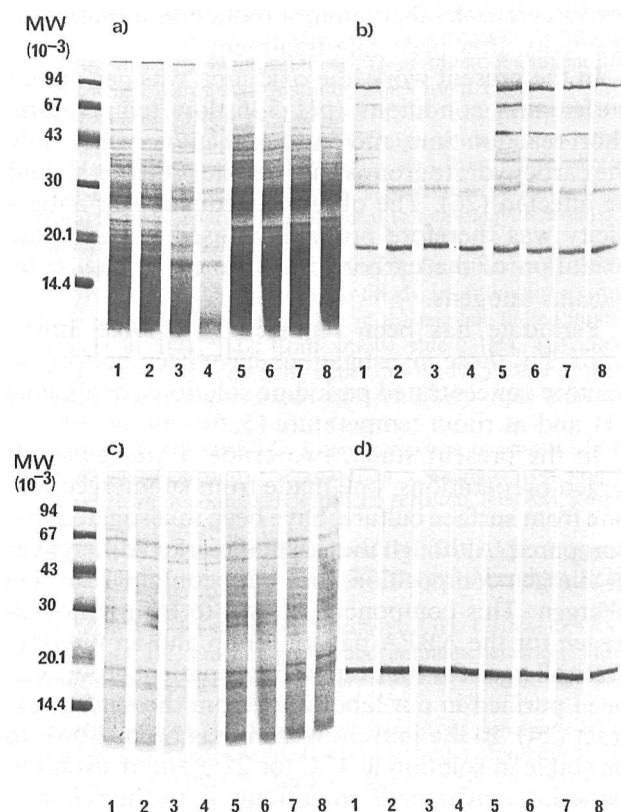


Fig. 2. Stability at 4°C of allergen preparations of *A. fumigatus* with or without 50% glycerol, as shown by SDS-PAGE and immunoblotting. a) and b) submerged extract 150 µg/well; c) and d) surface extract, 300 µg/well. a) and c) Coomassie brilliant blue R-250 stained gels; b) and d) IgE-binding components shown by patient serum no. 34 from patient screening. Lane 1: -20°C, 21 d; lane 2: 4°C, 2 d; lane 3: 4°C, 7 d; lane 4: 4°C, 21 d; lane 5: 50% glycerol, -20°C, 21 d; lane 6: 50% glycerol, 4°C, 2 d; lane 7: 50% glycerol, 4°C, 7 d; lane 8: 50% glycerol, 4°C, 21 d.

IEF immunoblotting of periodate-oxidized submerged extract showed that some allergens within the pI range 4.5–5.2 and the allergen with pI of about 8.8 were destroyed by the oxidation. After analysis of the surface extract in SDS-PAGE (300 µg/well), only the 20-kDa component of this extract appeared to be unchanged on both sample and control and thus seemed to be periodate-resistant. However, it is not possible to draw any conclusions on the other allergens of the surface extract by this method. IEF revealed that the allergen with pI 8.8 in the surface extract was destroyed by periodate.

Discussion

Patient screening

Karlsson-Borgå et al. (12) investigated sera from 18 patients with atopic allergy using submerged extract and immunoblotting from SDS-PAGE gels. Thirty-five IgE-binding components were detected. Two

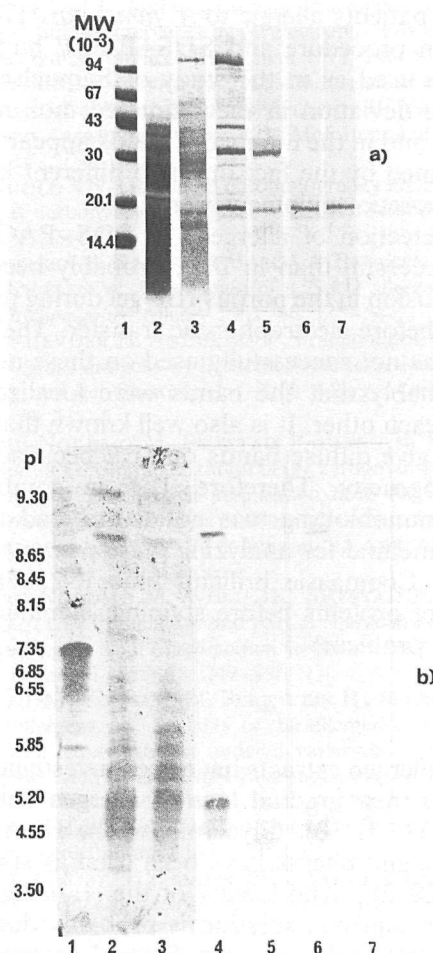


Fig. 3. Detection of IgE-binding components in periodate-oxidized extract shown by patient serum no. 34 from patient screening in a) SDS-PAGE and b) IEF. Lanes 1–3: stain with Coomassie brilliant blue R-250; lanes 4–7: IgE binding; lane 1: MW and pI marker, respectively; lane 2: native submerged extract (150 µg); lane 3: native surface extract (300 µg); lane 4: submerged extract, control (150 µg); lane 5: periodate-oxidized submerged extract (150 µg); lane 6: surface extract (300 µg); lane 7: periodate-oxidized surface extract (300 µg).

compounds of 50 and 90 kDa, respectively, reacted with more than 50% of the patient sera tested and were considered to be major allergens. In the present work, 17 and 20 of the 34 sera showed IgE-binding to the 50- and the 90-kDa components, respectively. These are probably identical with the earlier identified allergens in the submerged extract. Twenty-five patient sera reacted with a 20-kDa component, probably the allergen previously shown to be a major allergen for ABPA patients by Karlsson-Borgå et al. (12). By investigation of the surface extract, Samuelsen et al. (24) found seven components (20, 31, 44, 50, 53, 77, and 90 kDa) which were bound by four of seven atopic allergy patient sera.

The patient sera investigated in the present work yielded a representative type of IgE-binding pattern

found in patients allergic to *A. fumigatus*. The same extraction procedure and SDS-PAGE buffer system were used as in the study of Samuelsen et al. (24). The deviation in the estimated mol. mass of allergens and in the number of bands appearing may be explained by the fact that two different kinds of electrophoretic equipment were used.

The detection of allergens in SDS-PAGE was more successful than in IEF, probably because of band diffusion in the porous IEF gel during preequilibration before electrophoretic transfer. The reason that it was not successfully used on these materials was probably that the bands were localized very close to each other. It is also well known that glycoproteins give diffuse bands on IEF because of microheterogeneity. Therefore, IEF in combination with immunoblotting was not considered to be a suitable method for analyzing these extracts. Staining with Coomassie brilliant blue R-250 allowed fixation of proteins before staining, eliminating the diffusion problem.

Stability

Several allergen extracts have been investigated with regard to their gradual loss of allergen activity in solution (1, 2, 10). Glycerol, tween, HSA, buffer solutions, and phenol have been tried as stabilizing agents (20, 28). The lability of the *A. fumigatus* allergens in aqueous solution is probably due to the presence of active enzymes. Several proteases and glycosidases were detected in both extracts by the API ZYM system (Table 2). The enzymes were highly active at all temperatures tested, indicating that nonstabilized solutions of these allergen extracts should not be stored for more than a few days at 4°C.

Periodate oxidation

A number of allergens have been shown to be glycoproteins. Ag-54 (*Cla* h II), an important allergen of the mold *Cladosporium herbarum*, contains 19.5% protein and 80.2% carbohydrate (27). The protein-to-carbohydrate ratio of an important *Artemisia vulgaris* allergen (*Art* v II) was found to be 10 to 1 (22). The allergen extracts investigated in the present work contained 8.3% and 33.7% carbohydrate, respectively.

By periodate oxidation, vicinal diol groups of carbohydrates are oxidized and destroyed. Glycoprotein allergens in which the carbohydrate part of the molecule is involved in IgE binding may thus be inactivated. In the present work, allergens in both extracts were affected by periodate treatment. Another group (9) who analyzed the 200-kDa glycoprotein antigen isolated from the mold *Saccharo-*

myces cerevisiae also found a reduction in immunoreactivity after periodate treatment.

In the present work, the oxidation was performed under mild conditions (pH 5–6, low temperature, short reaction time, and darkness), under which only the carbohydrate groups, and not the proteins, should be affected (26). The observed reduction in antigenicity was therefore probably caused by periodate oxidation of the carbohydrate moiety of the *A. fumigatus* antigens.

Periodate has been reported to oxidize amino acids, but these investigations have been performed in more concentrated periodate solutions, at alkaline pH and at room temperature (5, 6).

In the present study, two crude *A. fumigatus* allergen preparations, one made from submerged and one from surface culture, have been investigated and compared. Although they exhibit marked differences in allergen composition, they both contain a 20-kDa allergen. This component, shown to be a major allergen for the ABPA patient group and an intermediate allergen for the atopic allergy patient group, has been purified in our laboratory from the surface extract (24). In the present work it has been shown to be stable in solution at 4°C for 21 d and resistant to periodate under mild conditions. A further characterization of this allergen is in progress.

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