

Immunoblotting analysis of concanavalin A-isolated allergens of *Candida albicans*

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The carbohydrate-containing fraction of *Candida albicans* was isolated from the crude extract with ConA Sepharose affinity chromatography and studied by IgE-immunoblotting with individual and pooled sera from *C. albicans*-allergic subjects. In the ConA-bound fraction there was a diffuse IgE binding in the high molecular weight area which also gave a carbohydrate stain (PAS). A distinct band corresponding to a molecular weight of 70 kD bound specific IgE antibodies. This glycoprotein, presumably a mannoprotein, gave a weak carbohydrate staining and a strong protein staining. Further biochemical studies are needed to reveal the exact nature of the epitopes in the ConA-bound mannose-containing fraction of *C. albicans*.

Key words: allergen; *Candida albicans*; glycoproteins; IgE; immunoblotting; mannans.

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Candida albicans is a dimorphic fungus growing as a yeast in a carbohydrate rich medium. The role of *C. albicans* as a cause of allergy is not clear. There are several publications in the literature suggesting *C. albicans* to be an allergen (1, 11, 12, 14), but because of inadequate knowledge about the allergenic composition and the human immune response to *C. albicans*, allergy towards it is poorly understood.

Mannan, a polysaccharide, is the major constituent of the cell wall of *C. albicans* (5) and also constitutes the major antigenic determinant (27). Edge & Pepys (9) and Longbottom et al. (14) have shown the presence of allergenic activity in both the carbohydrate and the protein fractions. With passive cutaneous anaphylaxis (PCA) tests in guinea pigs it was suggested that the immediate reactivity to purified mannan, prepared with the method of Peat et al. (22), was mediated by short-term

sensitizing antibody (STS) (19). RAST studies showed that IgE antibodies are also involved in the immediate hypersensitivity to mannan (9).

The aim of this study was to perform a detailed analysis of the allergenicity of the carbohydrate fractions of *Candida albicans* with immunoblotting. The carbohydrates and glycoproteins were isolated from *C. albicans* with ConA Sepharose affinity chromatography.

MATERIAL AND METHODS

Yeast cell cultures

C. albicans, CBS 5982, serotype A (Hasenclever), was grown on agar plates in 37°C for 48 h on synthetic medium containing 20.0 g glucose (Merck 8337), 5.0 g (NH₄)₂SO₄ (Merck 1217), 1.0 g KH₂PO₄ (JT Baker 0240), 0.5 g

MgSO₄ · x6H₂O (Merck 5833), 16 µg biotin (Sigma B-4501) and 20 g agar in 1000 ml distilled water.

Preparation of the crude Candida albicans extract (extract C)

The crude *C. albicans* extract was prepared at +4°C according to a modification (16) of the standardized extraction procedure presented by Axelsen (2). Briefly, the yeast cells were washed four times with shaking overnight at the last washing. The washed yeast cells were suspended in ammoniumhydrogencarbonate and were broken in an X-Press chamber (Biox Ab, Järfälla, Sweden). The breakage of the cells was microscopically investigated and found to be 80–90%. The final suspension was ultracentrifuged and the clear supernatant fluid was sterile filtered and thereafter concentrated with ultrafiltration (Amicon, Danvers, MA., USA, nominal cut-off 1000 D). The final dialysate was lyophilised and designated the crude extract (C).

Isolation of the mannan/mannoprotein fraction (M) and the purified protein fraction (P) with ConA Sepharose from the crude extract (C)

ConA Sepharose affinity chromatography was performed according to Ellsworth et al. (10) (Fig. 1). A 600 mg sample of the crude extract (C) was diluted in 15 ml elution buffer containing 0.02 M Tris-HCl (Sigma T-3253), 0.5 M NaCl (Merck 6404), 1 mM MnCl₂·x4H₂O (Merck 5927) and 1 mM CaCl₂ (JT Baker 0072) and was run through a 2.5 × 10 column packed with ConA Sepharose (Pharmacia, Uppsala, Sweden) and equilibrated with the buffer for 30 h, flow rate 20 ml/h. 5 ml fractions were collected from the start of the elution. The protein content was analysed with the Lowry method (20) and the mannan content with a modified Mancini's single radial diffusion method (21) against 0.1 mg/ml ConA (Pharmacia, Uppsala, Sweden). Serial dilutions of man-

nan (Sigma M-3640) were used as standards. Absorbances were measured at 270 nm for proteins and at 206 nm for UV-absorbing material in general, including both carbohydrates and proteins.

Purified protein fraction (P, fr. no. 12–22). The first peak, indicated by both absorbance curves and the Lowry protein determinations, represented proteins eluted through the column without affinity to ConA. These proteins were eluted in fractions 12–22, which were pooled, ultrafiltrated, lyophilised and designated the purified protein fraction (P).

Mannan/mannoprotein fraction (M, fr. no. 68–178). After a long plateau following the purified protein peak 0.3 M alpha-methylmannoside (Sigma M-6882) was used to elute mannan and mannoproteins bound to ConA. These were eluted in fractions 68–178 (Mannan determination, 206 nm absorbance peak), which were pooled, ultrafiltrated, lyophilised and designated the mannan/mannoprotein fraction (M).

Patient sera

Sera obtained from 20 *Candida albicans* RAST-positive patients was pooled in equal proportions and used for specific IgE probing. An individual patient serum (serum A) contained IgE antibodies binding to a diffuse high molecular weight component in immunoblotting with a mannan containing crude extract. Control serum B contained IgE antibodies to a 46 kD protein. Nine non-atopic sera were used as negative controls for the IgE binding.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transfer on to nitrocellulose

Extracts C and fractions P and M and ConA and molecular weight markers (Pharmacia, Uppsala, Sweden) were separated with SDS-PAGE in a large 5–17.5% gradient gel according to Laemmli (17) or in a PhastGel (gradient 10–15) in PhastSystem (Pharmacia, Uppsala,

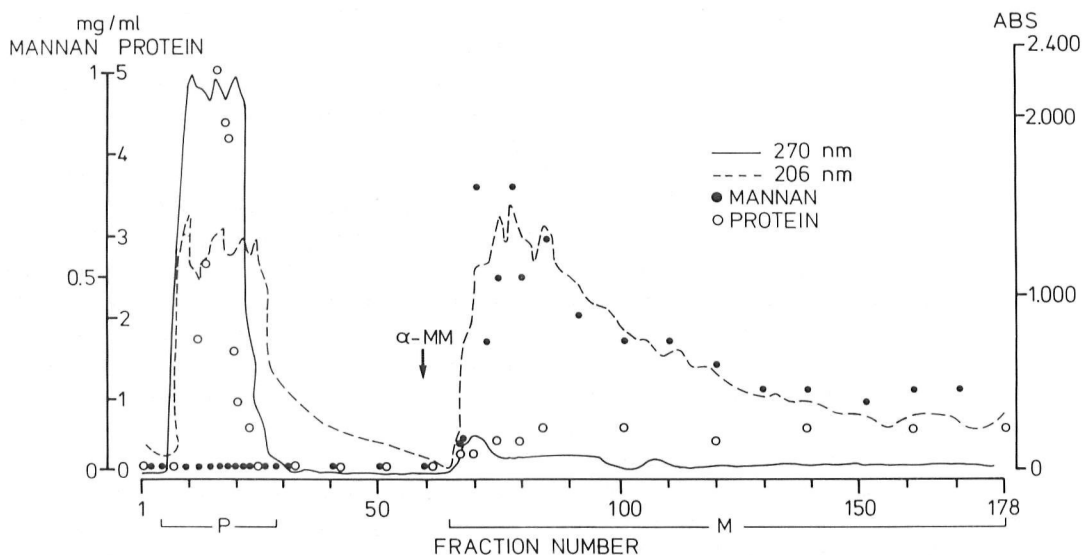


Fig. 1. The elution profile of affinity chromatography with ConA Sepharose. Mannan determinations (filled circle) and M (Mannan) protein (open circle) determinations (mg/ml) of the individual fractions are shown. The solid line is the 270 nm absorbance (ABS) curve for proteins and the dotted line is the 206 nm curve for other UV-absorbing material, including carbohydrates. The desorption by alpha-methyl mannoside (α -MM) was started at fraction 65. Fraction P was pooled from fractions 12–22 and fraction M from fractions 68–178.

Sweden). Separated antigens were transferred to nitrocellulose sheets (Millipore 0.22 μ m, Millipore Co., Bedford, MA., USA) from large gels in a Transphor apparatus (LKB, Bromma, Sweden) according to Towbin et al. (30). The transfer from PhastGels was performed with a vacuum suction through wet filter papers overnight. Proteins were stained with Amido-Black (AB). Glycoproteins and polysaccharides were stained with Periodic Acid Schiff (PAS).

Allergen detection with galactosidase-labelled anti-IgE

The specific IgE binding to allergens on the nitrocellulose was detected according to Bengtson et al. (4). To block unoccupied binding sites, the strips from the large gel or replica of the PhastGels were incubated in PBS containing 0.5% Tween 20 (PBS-T). Thereafter, the strips were incubated in undiluted pooled sera and the replica from the PhastGels in the individual sera diluted 1:4 with PBS-Tween. After washing three times with NaCl-Tween, all nitrocellulose membranes were incubated with

beta-galactosidase-labelled anti-IgE antibodies (Phadezym RAST, Pharmacia Diagnostics, Uppsala, Sweden) and diluted 1:4 with PBS-T. After washing three times with NaCl-Tween and twice with NaCl, the bound IgE was visualised with a chromogenic substrate containing 2-N-beta-D-galactopyranoside monohydrate (Sigma N-2259) and Fast Blue B -Diazonium salt (Dajac 5331, Trevone, PA., USA). The sheet was immersed in the substrate solution and laid on a filter paper pre-wetted with the substrate solution. The paper lay on the filter overnight.

RESULTS

Isolation of mannan/mannoprotein fraction (M) by ConA affinity chromatography

Fig. 1. demonstrates the isolation of the mannan/mannoprotein and protein fractions in crude *C. albicans* extract by ConA affinity chromatography. The first peak of non-ConA binding material, detected by absorbance at 270

nm and Lowry protein assay, was eluted with the starting buffer. This fraction constitutes the major protein part of the crude extract (fraction P). No mannan could be detected in this part of the chromatogram. Mannan was eluted in fractions 68–178 from the ConA matrix by desorption with α -methyl mannoside (α -MM, Fig. 1). The highest amount of mannan could be detected in fractions 68–120 by the specific mannan assay. Small amounts of proteins were also noted in this region, indicating the presence of mannoproteins.

Isolation of the carbohydrates from the proteins in the crude extract is further visualised in Fig. 2, which shows that the crude extract (C) and the mannan/mannoprotein fraction (M) contain material stained with the carbohydrate

stain (PAS) in the high molecular weight area. It was not possible to detect any PAS-active material in the purified protein fraction (P).

IgE-binding analysis of the mannan/mannoprotein fraction (M)

The diffuse high molecular weight fraction isolated with ConA, and stained with PAS, binds weakly specific IgE antibodies when probed with pooled sera from *C. albicans* RAST-positive patients but not from controls (Fig. 2, a row of triangles). IgE-binding is more pronounced when probed with patient serum A (Fig. 3). This serum was previously shown to contain IgE antibodies that bind to high molecular weight components of *C. albicans*. It can be seen that these IgE-binding components can be isolated and condensed by affinity to ConA. Patient serum B was used as a control and shows no detectable IgE-binding to the carbohydrate containing high molecular weight components, although specific binding to a 46 kD protein is shown. In addition, IgE is also bound to a 70 kD protein band in the mannan/mannoprotein fraction (M) (Fig. 2., arrow).

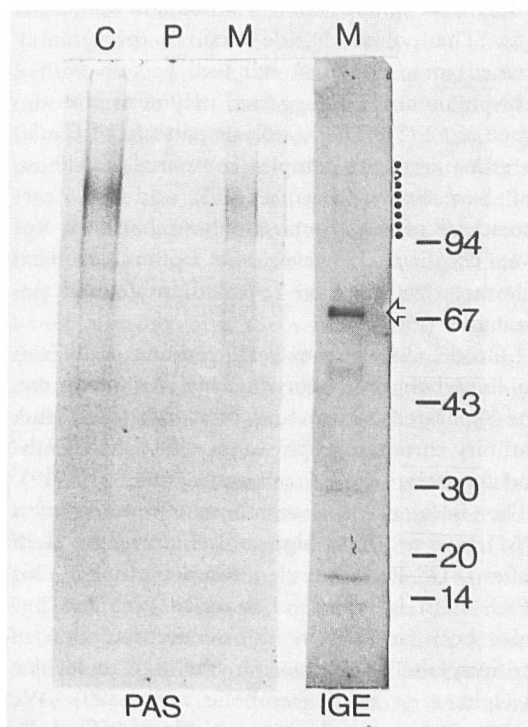


Fig. 2. PAS-staining for carbohydrates of fractions C, P and M on nitrocellulose separated with SDS-PAGE and the detection of IgE bound to fraction M after incubation with *C. albicans* RAST-positive serum pool and galactosidase-labelled anti-IgE. IgE binding to the diffuse high molecular weight region (row of dots) and to a 70 kD glycoprotein band (arrow) is indicated. No binding to these was shown in non-atopic sera.

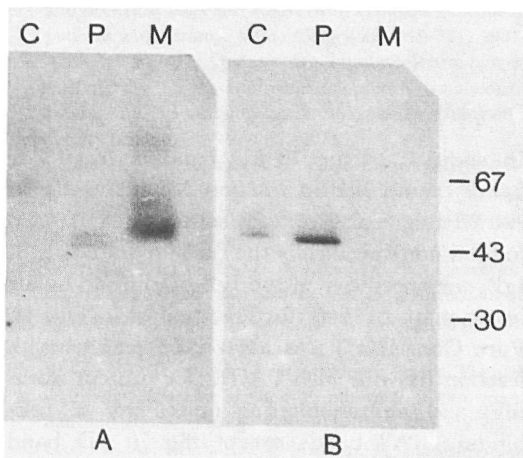


Fig. 3. Binding of IgE to fractions C, P and M, on nitrocellulose separated with Phast SDS-PAGE and probed for IgE with an IgE anti-mannan positive (A) and negative (B) sera and galactosidase-labelled anti-IgE. The IgE anti-mannan negative sera (B) has IgE towards a 46 kD protein.

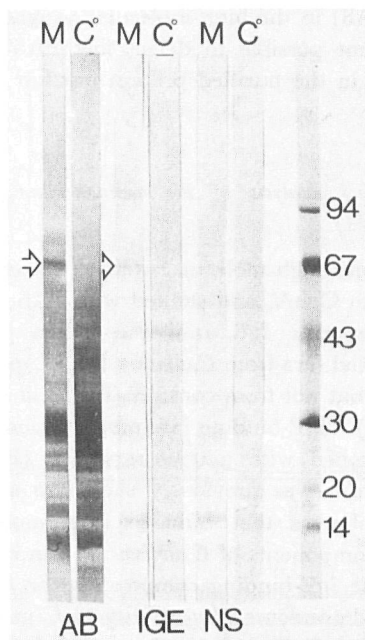


Fig. 4. Comparison of fraction M (M) and pure ConA (C°) on nitrocellulose after separation with SDS-PAGE. Detection was done for proteins with amido-black (AB), for allergens with pool of RAST-positive sera and galactosidase-labelled anti-IgE (IGE) and for non-specific binding with galactosidase-labelled anti-IgE without sera (NS). The 70 kD glycoprotein is indicated with an arrow.

Specificity of the IgE-binding

In addition to the 70 kD band at least four bands bound IgE in fraction M. Since the M extract might also contain some ConA, known to bind non-specifically the carbohydrate part of IgE, the specificity of the IgE-binding to bands in fraction M was further analysed (Fig. 4). Pure ConA (C°) was assayed in parallel with fraction M with SDS-PAGE (for protein staining) and immunoblotting (specificity of IgE-binding). All bands except the 70 kD band (arrow, Fig. 4.) in the M fraction are of the same molecular weight as those with ConA, indicating them to be ConA - related. This was especially noticed in panel AB (Fig. 4.). All bands except the 70 kD band in both fraction

M, as well as pure ConA, bind non-specifically enzyme-labelled rabbit anti-IgE antibodies (panel NS). The 70 kD band is the only band devoid of this non-specific binding but shows specific IgE-binding from the pooled sera. The 70 kD band stains strongly with the protein stain and only weakly with carbohydrate stain, suggesting it to be a glycosylated protein.

DISCUSSION

Mannan is the major constituent of the *Candida albicans* cell wall (5). It is also the major antigenic component (27) towards which almost all individuals have antibodies (7, 13). *C. albicans* mannan consists of mannopyranose molecules linked to make a linear α -1,6 backbone to which side chains with α -1,2 linkages are joined (5). The polysaccharide chain is presumably joined to a structural cell wall protein with a core mannan of a suggested polymerisation degree of 12 (3). The acetolysis patterns of *C. albicans* mannans are complex compared with those of *Saccharomyces cerevisiae* (15), and the exact structure of this complex polysaccharide is not yet clarified. However, its immunochemical characterizations have revealed antigenic determinants (29).

Crude *C. albicans* extract contains easily soluble carbohydrates, of which only mannan can be separated by binding to ConA (6). ConA affinity chromatography is an established method for isolation of *C. albicans* mannan (10, 19). The isolated mannan/mannoprotein fraction (M) gave a diffuse high molecular weight stain after SDS-PAGE and immunoblotting. This PAS stain is typical of mannan (23) and has also been verified by diffuse accumulation of tritium-labelled mannose in the high molecular weight area in a metabolic assay (28). We showed that this mannan binds specific IgE-antibodies from certain *C. albicans* RAST-positive sera. This has been observed before by Edge & Pepys using epichlorohydrin cross-linked modified insoluble mannan (24) as an allergosorbent matrix in RAST (9). We have confirmed their findings by this advanced immunoblotting technique using mannan ab-

sorbed to nitrocellulose, thus avoiding the risk of denaturation.

In the study by Edge & Pepys (9), mannan was prepared with the Fehling precipitation, and the glycopeptide linkages were broken during alkaline conditions (22). These intact glycopeptide structures seem most crucial for the T cell-dependent and HLA-restricted anti-mannan antibody response (8) since the Fehling precipitated mannan is not immunogenic, although serologically active (18). The human IgE response is T cell-dependent and MHC-restricted (31) and the T cell recognition of the intact peptide structures is undoubtedly essential for the initiation of the specific anti-mannan IgE response. However, the polysaccharide part of the mannan/mannoprotein molecule may still act as a hapten and the epitope for the IgE-antibodies might be a carbohydrate structure. This hypothesis is compatible with that of the Fehling precipitated, alkaline degraded mannan bound IgE in the experiments by Edge & Pepys (9). We used a more gentle method for our mannan purification which left the peptide structure intact. The diffuse distribution of the IgE stain in immunoblotting suggests that carbohydrates might be involved in the IgE binding domain. However, small intact peptides would possibly give the same results. Further studies can reveal the exact structure of this IgE epitope although the T cell epitope is certainly more important for the understanding of the human immune response.

The 70 kD band was shown to contain mainly protein and a small amount of carbohydrate. This protein component was not present in pure ConA preparations but still bound human IgE-antibodies. The glycoproteins in the crude extract have molecular weights which differ from the 70 kD component. Similar findings were observed in immunoblotting with the crude and purified protein fractions (25). The reason for the difference between the molecular weights of the glycoproteins in the crude extract and those isolated with ConA might be that the 70 kD glycoprotein contains ConA-related structures, to which the carbohydrate residues in this glycoprotein become attached. The harsh conditions of the SDS-PAGE detach the

ConA bound to allergens, and the detectable parts of the ConA molecules are located between 10 and 25 kD in immunoblotting. These distinct bands bind carbohydrate parts of the conjugate-labelled rabbit anti-IgE antibodies non-specifically and are easily distinguished from the specific IgE-binding in extracts contaminated with ConA.

This study has given more evidence for the allergenicity of the *Candida* polysaccharide, mannan, and in addition revealed a 70 kD glycoprotein, most likely a mannoprotein. Detailed analyses of purified allergens are needed to unravel the exact immunochemical nature of these carbohydrate-containing molecules.

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