

Identification of *Candida albicans* Antigens Reactive with Immunoglobulin E Antibody of Human Sera

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Candida albicans antigens which reacted with immunoglobulin E (IgE) antibodies of 57 allergic patients were detected by immunoblotting. Of the various antigens, the 175-, 125-, 46-, 43-, and 37-kDa antigenic components reacted most frequently with the patient sera. To purify the major antigens, *C. albicans* cells were fractionated. The 46-, 43-, and 37-kDa antigens were recovered in cytoplasmic fractions, but the 175- and 125-kDa antigens were not recovered in any fraction. The 46-, 43-, and 37-kDa antigens were purified from cytoplasmic fractions by DEAE and P11 ion-exchange chromatography. Antigens were isolated by cutting bands out of sodium dodecyl sulfate-polyacrylamide gels. The purified components confirmed by immunoblotting were next processed for amino acid sequencing. Parts of the sequences of the 46-, 43-, and 37-kDa antigens had significant levels of homology with *Saccharomyces cerevisiae* glycolytic enzyme enolase, phosphoglycerate kinase, and aldolase, respectively. Rabbit IgG antibodies prepared against the 46- and 43-kDa antigens strongly cross-reacted with the homologous proteins of *S. cerevisiae*. However, *S. cerevisiae* enolase and phosphoglycerate kinase did not cross-react with IgE of patient sera. This result suggests that IgE antibodies against only small parts of their epitopes are elevated in the allergic patients. Since enolase is reported to be a major antigen for systemic candidiasis, this enzyme may be the immunodominant protein in both allergies and fungal infections.

The yeast *Candida albicans* is a common commensal of the human oral and vaginal mucosae and gastrointestinal tract and causes not only opportunistic infections in immunocompromised patients but also allergic reactions in people sensitized to *C. albicans*.

A high immunoglobulin E (IgE) titer is thought to be a risk factor for type 1 responses in humans (11). For example, a high titer of IgE antibodies against *C. albicans* antigenic components tended to cause *Candida* allergy (20). For many studies, the IgE titer was measured in a radioallergosorbent test (RAST) or enzyme-linked immunosorbent assay. Because standard antigens have not been established, only overall titers against mixed antigens including proteins and polysaccharides were obtained. However, it has been shown that antigenic components of *C. albicans* can stimulate immediate hypersensitivity responses in animals (1, 20). The antigenic components, identified as polysaccharides and proteins, differ in their abilities to provoke responses. Polysaccharides tend to be more common and provoke more intense antigen responses than proteins. However, it is thought that proteins and glycoproteins also play an important role allergies to *C. albicans* (14, 22, 23).

Recently, some major protein allergens of *C. albicans* were identified by immunoblotting with antibodies specific for human IgE. Shen et al. (25) and Savolainen et al. (24) identified at least 16 allergenic components with molecular masses ranging from 20 to 94 and 16 to 135 kDa, respectively, reacting with antibodies of serum samples from about 30 asthmatic patients. In the study done by Shen et al. (25), IgE antibodies of 77% of the tested serum samples reacted with the 40-kDa antigenic component. In the study done by Savolainen et al. (24), 77% of the serum samples reacted

with the fractions containing the 46-kDa protein. The major antigens have not been isolated and identified yet.

To extend these allergen studies and to study *Candida* allergy, identification and characterization of IgE-binding *Candida* antigen are essential. Therefore, the present study focused on the identification and characterization of IgE-binding *Candida* antigens.

MATERIALS AND METHODS

Strain. *C. albicans* C9 (9, 12) was used for antigen preparation. It was grown in yeast extract-peptone-dextrose (YPD) broth or agar (10).

Enzymes. Enolase (catalog no. E6126) and phosphoglyceric phosphokinase (PGK) (catalog no. P7634) from *Saccharomyces cerevisiae* or enolase (catalog no. E0379) and PGK (catalog no. P2399) from rabbit muscles were all purchased from Sigma Co.

RAST. Antibody titer of IgE was estimated by RAST with a Phadebas RAST kit (Pharmacia Diagnostics).

Human sera. Serum samples with RAST scores over 1 for *C. albicans* antigen from 50 bronchial asthmatic patients (aged 2 to 26 years) and 7 atopic dermatitis patients (aged 7 to 78 years) were supplied by nine hospitals in Nagoya district. For controls, sera were also collected from 34 healthy females (aged 20 to 22 years).

Cell culture and preparation of crude antigens. *C. albicans* cells were cultured in YPD broth at 37°C with shaking, and yeast cells were harvested at the logarithmic phase. After being washed three times in distilled water and centrifuged, the cells were suspended in water to give an A_{660} of 30. A 50- μ l sample of this suspension was mixed with 10 μ l of 6 \times TDG buffer (6 \times TDG buffer is 0.2 M Tris-HCl [pH 6.8], 6% sodium dodecyl sulfate (SDS), 38% glycerol, 0.006% bromophenol blue) and 5 μ l of 2-mercaptoethanol. The mixture

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was heated at 100°C for 5 min and used as *C. albicans* crude antigens for immunoblotting.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (13). A 12 or 10% polyacrylamide gel was used. A 25- μ l sample of this suspension was mixed with 5 μ l of 6 \times TDG buffer and 2.5 μ l of 2-mercaptoethanol and then heated. The molecular mass markers used were bovine serum albumin (BSA) (68 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (14 kDa).

Proteins in the gel were stained with 0.1% Coomassie brilliant blue R250 (CBB-R250) in methanol-acetic acid-water (5:1:4) and destained in 7% acetic acid.

Immunoblotting. Antigenic components separated in a SDS-polyacrylamide gel were transferred onto a nitrocellulose sheet (Schleicher & Schnell) by the method of Howe and Hershey (8) and blotted. Nonspecific sites of the nitrocellulose sheet were blocked by incubating in 3% skim milk in TBS (20 mM Tris-HCl [pH 7.8], 0.5 M NaCl) for 15 min at room temperature and blocked. The nitrocellulose sheet was then immersed overnight with gentle shaking in the serum solution diluted with 1% skim milk in TBS. After being washed four times with 1% skim milk in TBS, the sheet was incubated with an affinity-purified alkaline phosphatase (AP)-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories Inc.) diluted 1:2,500 with 1% skim milk in TBS for 5 h and washed again four times with 1% skim milk in TBS. The nitrocellulose sheet was then developed for AP by incubation in a mixture of 5 ml of AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM MgCl₂) containing 0.033 ml of NBT solution (50 mg of nitroblue tetrazolium per ml in 70% dimethylformamide) and 0.0165 ml of BCIP solution (50 mg of 5-bromo-4-chloro-3-indolylphosphate per ml in dimethylformamide).

Lectin blotting. The nitrocellulose sheets transferred from SDS-polyacrylamide gels were treated with 3% BSA in 0.5 \times TBS for 30 min and incubated for 1 h with 1% BSA in 0.5 \times TBS containing lectin conjugated with horseradish peroxidase (0.02 mg/ml) (Seikagaku Kogyo Co., Tokyo, Japan). Six kinds of lectins were tested; concanavalin A, lentil lectin, wheat germ lectin, *Phaseolus vulgaris* E4, castor bean lectin (RCA-120), and peanut lectin. After being washed with 1% BSA in 0.5 \times TBS four times for a total of 20 min, the sheet was developed for horseradish peroxidase with a mixture of 50 ml of TBS containing 0.03 ml of ice-cold 30% H₂O₂ and 10 ml of methanol containing 30 mg of 4-chloro-1-naphthol (Nakarai Tesque Co., Kyoto, Japan).

Purification of antigenic components. (i) **Cell fractionation.** Cell fractionation was carried out by the method of Goud et al. (5) with minor modifications. Cells grown at 37°C in YPD broth to the logarithmic phase were harvested by centrifugation at 4,000 \times g for 5 min. After being washed twice in cold 10 mM NaNO₃, the cells were converted to spheroplasts by suspension in 40 ml of 50 mM phosphate-buffered saline (pH 7.5) containing 1.4 M sorbitol, 20 mg of Zymolyase 100T (Seikagaku Kogyo Co., Tokyo, Japan), and 0.24 ml of 2-mercaptoethanol for 30 min at 37°C and then centrifuged at 10,000 \times g for 20 min. The supernatant (S1) was refrigerated before SDS-PAGE was performed. The pellet was homogenized in 10 ml of 10 mM triethanolamine (pH 7.2) containing 0.8 M sorbitol and 1 mM EDTA by 20 strokes with a glass tissue homogenizer and centrifuged at 10,000 \times g for 20 min to remove unbroken spheroplasts and cell debris. The pellet was again homogenized and centrifuged; this procedure was repeated three times. The resulting pellet (P2) was reserved for SDS-PAGE, and the pooled supernatant (S2) was further

centrifuged at 100,000 \times g for 90 min. The pellet consisted of two layers, an upper softly packed layer (P3-a) and a lower tightly packed layer (P3-b). The supernatant also consisted of two layers, an upper cloudy layer (S3-a) and a lower clear layer (S3-b). All four layers were recovered and analyzed by SDS-PAGE.

(ii) **DEAE-Sephacel ion-exchange chromatography.** The supernatant (S3b) was applied to a DEAE-Sephacel (Pharmacia) column (2 by 7 cm) and eluted with 300 ml of a linear NaCl gradient of 0 to 0.15 M in 10 mM Tris-HCl (pH 7.5). UV absorbance of fractions was monitored by using an UV detector (model 115; Gilson) at 280 nm. Tightly bound material was washed from the column with 0.3 M NaCl in 10 mM Tris-HCl (pH 7.5).

(iii) **Cellulose phosphate chromatography.** Fractions separated by DEAE-Sephacel chromatography were diluted three times with 10 mM Tris-HCl (pH 7.5), applied to a cation-exchanger cellulose phosphate P11 column (Whatman) (1.5 by 5.2 cm), washed with 10 mM Tris-HCl (pH 7.5), and eluted with 200 ml of a linear salt gradient, 0.05 to 0.5 M NaCl in 10 mM Tris-HCl (pH 7.5). Tightly bound material was washed from the column with 1 M NaCl in 10 mM Tris-HCl (pH 7.5).

Protein sequencing. Proteins purified by column chromatography were separated in SDS-polyacrylamide gels and transferred to Immobilon sheets (Millipore) by using a Tris-borate buffer system by the method of Matsudaira (16) with a semi-dry electroblotter (Sartorius). The blotted sheets were washed with distilled water. Protein bands detected by brief staining with CBB-R250 were cut out of the gels and destained in 90% methanol. The bands were analyzed with a peptide sequencer (ABI 473A; Applied Biosystems).

Polypeptide fragmentation by cyanogen bromide (CNBr). Protein bands in SDS-polyacrylamide gels were cut out and blended in distilled water with a Teflon homogenizer. Formic acid and CNBr were added to the homogenate to 70% and 1/300 M in final concentrations, respectively. After incubation at 30°C for 24 h, the resulting homogenate was centrifuged. The pellets were washed with distilled water and centrifuged again. The pooled supernatant fluid was lyophilized and resuspended in a small amount of distilled water. The cleaved-polypeptide sample was separated again in a 12% polyacrylamide-SDS gel for amino acid sequencing as described above.

Search for homology in amino acid sequence. Computer search for homologous sequences was done by DNASIS program (Hitachi SK Co., Yokohama, Japan) from the data bases of NBRF-PDB and SWISS-PROT.

Preparation of antiserum against protein. Proteins were separated in SDS-polyacrylamide gels, and protein bands were cut out. Pieces of the gel were homogenized and emulsified in Freund complete adjuvant for intramuscular injection into rabbits. Two weeks later, the rabbits were given booster injections of the homogenate without adjuvant subcutaneously. After an additional 2 weeks, the sera were obtained.

RESULTS

Immunoblot profiles of the crude antigens with human sera. The crude *C. albicans* antigens from the whole-cell extract, which were separated in SDS-polyacrylamide gels and stained with CBB-R250, showed at least 24 bands with molecular masses ranging from 14 to 85 kDa. Eight protein bands, 85-, 75-, 43-, 41-, 36-, 32-, 25-, and 14-kDa bands, were most strongly stained (Fig. 1A). As we showed previ-

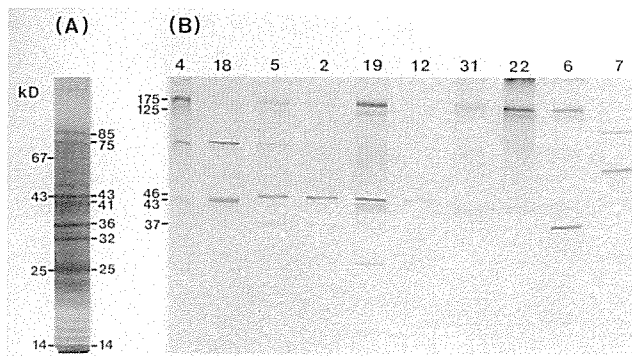


FIG. 1. SDS-PAGE and immunoblot analysis of the whole-cell antigens of *C. albicans* C9. (A) Protein profile of SDS-polyacrylamide gel stained with CBB-R250. The molecular masses (in kilodaltons) of the markers are shown to the left of the gel and those of major antigens are shown to the right of the gel. (B) Immunoblotting with the sera from allergic patients for anti-*C. albicans* IgE antibody detected by using AP-conjugated secondary antibody. Numbers above the gel indicate the patients whose sera were used for blotting.

ously (9), polysaccharides are present in the high-molecular-mass region not stained by CBB-R250. The antigens reacting with IgE antibody were examined by immunoblotting. Human sera diluted 1:10 were applied to nitrocellulose sheets, which were then treated with the second antibody specific for human IgE. IgE-binding bands were obtained in 32 of the 57 patient serum samples (56%) (Fig. 1B and Table 1). The band patterns of the patient sera were varied. For example, patients 4, 19, 31, and 22 (Fig. 1B) showed diffuse staining in the high-molecular-mass regions. We could distinguish 24 IgE-binding components with molecular masses ranging from 14 to 175 kDa (Table 1). Among the IgE-positive patients, the antigenic bands most frequently encountered were the 175- (56%), 125- (41%), 46- (47%), 43- (41%), and 37-kDa (25%) bands. RAST scores of the sera revealed no correlation with the intensity and the occurrence of IgE-binding bands (Table 1). In the control sera from healthy females, no bands were stained. IgG-, IgA-, and IgM-binding patterns of the allergic patients (data not shown) were similar to the patterns of healthy females tested in this study and of patients with vaginal candidiasis, as we showed previously (9).

Purification of major antigens. (i) Cell fractionation. The spheroplasts were homogenized, and crude fractionation

TABLE 1. Antigen components of *C. albicans* reacting with IgE antibodies from sera of allergic patients^a

| Patient | Presence of IgE-binding components of the following size (kDa): | | | | | | | | | | | | | | | | | | | | | | | |
|---------|---|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| | 175 | 125 | 83 | 78 | 65 | 64 | 63 | 61 | 56 | 52 | 51 | 48 | 46 | 43 | 41 | 40 | 37 | 32 | 31 | 29 | 27 | 25 | 22 | 14 |
| 1 | + | | | | | | | | | | | | + | | | | + | | | | | | + | + |
| 2 | | | | | | | | | | | + | | + | | | | + | | | | | | | |
| 3 | + | + | | + | | | | | | | + | | + | | | + | + | | + | | | | | |
| 4 | + | + | | | + | | | | | + | | | + | | | + | | + | | | | | + | |
| 5 | + | + | | | + | + | | | | | | | + | | | | + | | | | | | | |
| 6 | + | + | | | | | | | | | | | + | + | | | + | | | | | | | |
| 7 | | | + | | | | | | + | | | | + | | | | + | | | | | | | |
| 8 | + | + | | | | | | | | | + | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | + | | | | | | | | | | + | |
| 10 | | | | | | | | + | | | | | + | | | | | | | | | | + | |
| 11 | | | | | | | | | | | | | | | | | | | | | | | + | |
| 12 | | | | | | | | | | | | | + | | | | | | | | | | + | |
| 13 | | | | | | | | | | | | | | | | | | | | | | | + | |
| 14 | | | | | | | | | | | | | | | | + | | | | | | | + | |
| 15 | + | | | | | | | | | | | | | | + | | | | | | | | | |
| 16 | + | | | | | | | | | | | | | | | | | | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | | | | + | |
| 18 | | | | | + | + | + | | | | + | + | + | + | + | | + | | + | + | + | | | |
| 19 | + | + | | | | | | | | | | | + | + | | | + | | | | | + | | |
| 20 | + | + | | | | | | | | | | | + | + | | | | | | | | | + | |
| 21 | + | + | | | + | | | + | | | | | + | | | | | | + | | | | | |
| 22 | + | | + | | | | | | | | | | | | | | | | | | | | | |
| 23 | + | + | | | | | | | | | | | | | | | | | | | | | | |
| 24 | + | + | | | | | | | | | | | | | | | | | | | | | | |
| 25 | + | + | | | | | | | | | | | | | | + | | | | | | | | |
| 26 | | | | | | | | | | | | | + | | | | | | + | | | | + | |
| 27 | + | | | | | | | | | | | | | | | | | + | | | | | | |
| 28 | | | | | | | | + | | | | | + | | | | | | | | | | | |
| 29 | | | | | | | | | | | | | | | | + | | | | | | | + | |
| 30 | + | | | | | | | | | | | | | | | | | | | | | | | |
| 31 | + | + | | | | | | | | | | | | | | + | | | | | | | | |
| 32 | + | + | | | | | | | | | | | | | | + | | | | | | | | |

^a The RAST scores for *C. albicans* were as follows: 4 for sera from patients 1 and 2, 3 for sera from patients 3 to 17, 2 for patients 18 to 30, and 1 for patients 31 and 32. Patients 4, 21, 25, 26, and 28 were atopic dermatitis patients; the other patients were bronchial asthmatic patients. The most common IgE-binding antigen components were the 175-, 125-, 46-, 43-, and 37-kDa antigens found in 18, 13, 15, 13, and 8 patients, respectively.

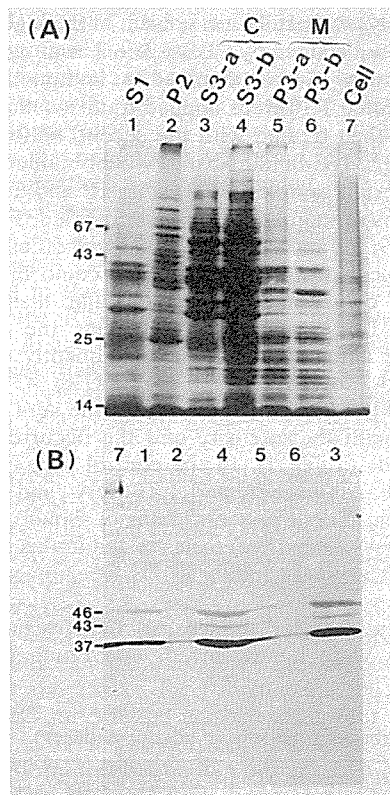


FIG. 2. SDS-PAGE and immunoblot profiles of the fractions obtained by cell fractionation. (A) CBB-R250 staining of the fractions separated by differential centrifugation. C, cytoplasm; M, membrane. (B) Immunoblot analysis using serum from patient 6. Numbers to the left of the gels show the molecular mass (in kilodaltons). Lane: 1, S1, the supernatant after centrifugation of the spheroplast suspension at $10,000 \times g$ for 20 min; lane 2, P2, the pellet after centrifugation of the homogenized spheroplasts at $10,000 \times g$ for 20 min. The resulting supernatant (S2) was spun down at $100,000 \times g$ for 90 min and separated into four fractions, S3-a (lane 3), S3-b (lane 4), P3-a (lane 5), and P3-b (lane 6). Lane 7, whole-cell extract from log-phase cells. See Materials and Methods for the details.

was done by ultracentrifugation. The supernatant fluid and the cell lysate pellet were thought to contain mainly membrane and cytoplasmic components, respectively (Fig. 2A). To detect where the major allergens were fractionated, IgE-specific immunoblotting was performed by using sera from patient 6 which recognized the 175-, 125-, 46-, 43-, and 37-kDa bands from the crude antigens (Fig. 2B). Three major allergens of 46, 43, and 37 kDa were recovered in cytoplasmic fractions S3-a and S3-b, while the 175- and 125-kDa bands were not detectable in any of the fractions separated. CBB-R250-stained profiles of cytoplasmic fractions S3-a and S3-b were similar. Thus, we tried to purify each of the three 46-, 43-, and 37-kDa antigenic components from the S3-b fraction.

(ii) **DEAE-Sephacel ion-exchange chromatography.** S3-b fractions were applied to DEAE-Sephacel columns. Six peak fractions detected with UV detector at 280 nm were identified on the gradient elution of NaCl (0 to 0.15 M) in the 71 fractions. The fraction containing peak 7 was obtained by elution with 0.3 M NaCl washing buffer. The seven peak fractions were run on SDS-polyacrylamide gels, transferred to nitrocellulose sheets, and blotted with the patient sera to

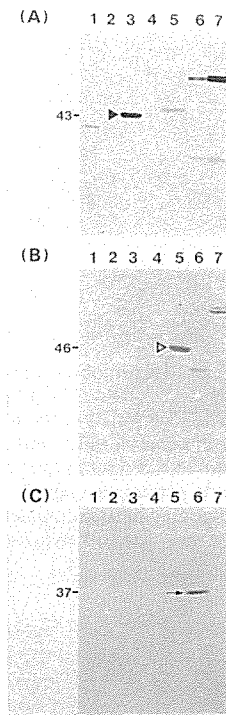


FIG. 3. Immunoblot analysis for IgE-binding proteins of the peak fractions on DEAE-Sephacel chromatography. Peaks 1 to 6 were eluted with linear salt gradient, and peak 7 was tightly bound material eliminated with salt washing buffer. The fractions were blotted with sera from patients 12 (A), 6 (B), and 5 (C). The bands with IgE-binding proteins of 43 (▶), 46 (▷), and 37 (→) kDa are shown.

detect IgE-binding bands (Fig. 3). The IgE blotting results of sera from several patients showed that fractions containing peaks 3, 5, and 6 mainly contained the 43- (Fig. 3A), 46- (Fig. 3B), and 37-kDa (Fig. 3C) allergens, respectively. Column fractions around the peaks were run on SDS-polyacrylamide gels, which were stained with CBB-R250 (Fig. 4). The bands corresponding to the three major allergens were distributed in the different fractions. Their profiles completely correlated with IgE binding profiles (data not shown). On the basis of the profiles, fractions 50 to 57, 33 to 40, and 63 to 71 were combined for the 46-, 43-, and 37-kDa allergens, respectively.

(iii) **P11 cellulose phosphate chromatography.** Each of the three allergens fractionated by DEAE-Sephacel column chromatography was then subjected to P11 cellulose phosphate chromatography. The fractions eluted with a linear gradient (0.05 to 0.5 M NaCl) were analyzed by SDS-PAGE and immunoblotting for antibodies specific for IgE, and CBB-R250 staining was performed.

The 46-kDa band was fractionated into flowthrough fractions and a major contaminating protein with a molecular mass slightly higher than 46 kDa was eluted by using a salt gradient (A2 [Fig. 5]). The 46-kDa component in the flowthrough fractions was confirmed as the 46-kDa allergen by immunoblotting (A1 [Fig. 5]).

The 43-kDa band was eluted into fractions 24 to 31 of from 43 fractions, and two major contaminating proteins were recovered from flowthrough fractions and separated from the 43-kDa band (B2 [Fig. 5]). The 43-kDa component was

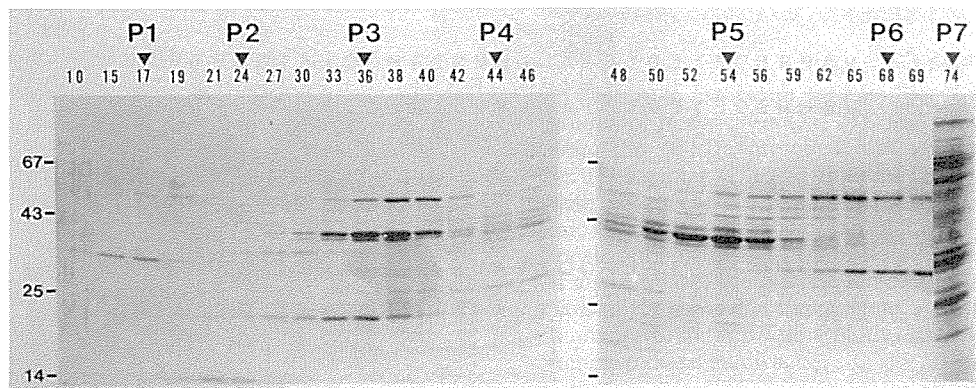


FIG. 4. SDS-polyacrylamide gel stained with CBB-R250 of the proteins separated by DEAE-Sephacel chromatography. Numbers 10 to 74 show the fractions separated, and P1 to P6 are peaks eluted with linear salt gradient buffer detected by A_{280} . P7 was the tightly bound material removed with salt buffer. The numbers to the left of the gel are molecular mass markers (in kilodaltons).

confirmed as the 43-kDa allergen by immunoblotting (B1 [Fig. 5]).

The 37-kDa band was fractionated into flowthrough fractions, and there was no detectable protein in eluted fractions analyzed by SDS-PAGE and CBB-R250 staining. A small peak was detected at fractions 14 to 18 from 44 fractions with UV absorbance (C2 [Fig. 5]). The 37-kDa component was confirmed as the 37-kDa allergen by immunoblotting (C1 [Fig. 5]).

The fractions containing the 46- (103-ml), 43- (27-ml), and 37-kDa (88-ml) allergens were concentrated 41-, 15-, and 44-fold, respectively, by ultrafiltration. The purified samples were run in SDS-polyacrylamide gels. Some other contaminating proteins were still in the concentrated samples of the

46- and 37-kDa allergens (Fig. 6). However, the allergens seemed to be a sufficiently pure single component to cut out the respective bands from the SDS-polyacrylamide gel. The samples were tested for lectin blotting, and no band or area was stained (data not shown).

Amino acid sequence. The purified allergens from bands cut out from SDS-polyacrylamide gels were processed for amino acid sequencing. The 40-amino-acid sequence was determined from the intact N terminus of the 37-kDa component. Both the 46- and 43-kDa antigenic components were found to be blocked at N termini and were subjected to polypeptide cleavage with CNBr. The cleaved polypeptides were each separated into three fragments on 12% polyacrylamide-SDS gels. Separated polypeptides were transferred onto Immobilon sheets and used for amino acid sequencing. A 34-amino-acid sequence starting from the N terminus from the smallest polypeptide of the 46-kDa component (46 kDa-C) was examined. The largest polypeptide was not examined. The medium one was probably blocked at the N terminus. A 27-amino-acid sequence was examined for the smallest polypeptide of the 43-kDa protein (43 kDa-C). The largest and medium polypeptides could not be determined, probably because of blocking at the N termini. The amino acid sequences of the three polypeptides are shown in Fig. 7. A search for homology found that the 46-, 43-, and 37-kDa antigens have high degrees of homology with *S. cerevisiae* enzymes: 82% with enolase (7), 70% with PGK (3), and 63% with aldolase (NBRF-PDB accession number P14540).

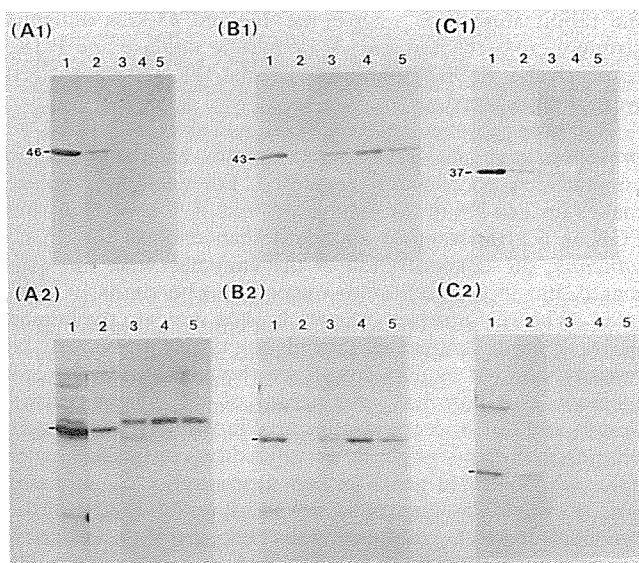


FIG. 5. Purification of IgE-binding antigens by P11 cellulose phosphate chromatography. Panels A1, B1, and C1 represent the IgE-binding bands by immunoblot analysis with the sera from patients 12, 18, and 3, respectively. The numbers to the left of the gels indicate the molecular mass (in kilodaltons). Panels A2, B2, and C2 are the corresponding SDS-polyacrylamide gels stained with CBB-R250. Lane 1, starting material loaded on the column; lane 2, flowthrough fraction; lanes 3, 4, and 5, peak fractions eluted with salt gradient.

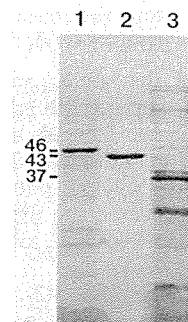


FIG. 6. SDS-PAGE profile of the three antigens purified by P11 chromatography. Lane 1, 46-kDa antigen; lane 2, 43-kDa antigen; lane 3, 37-kDa antigen. The gel was stained with CBB-R250, and molecular mass (in kilodaltons) is shown to the left of the gel.

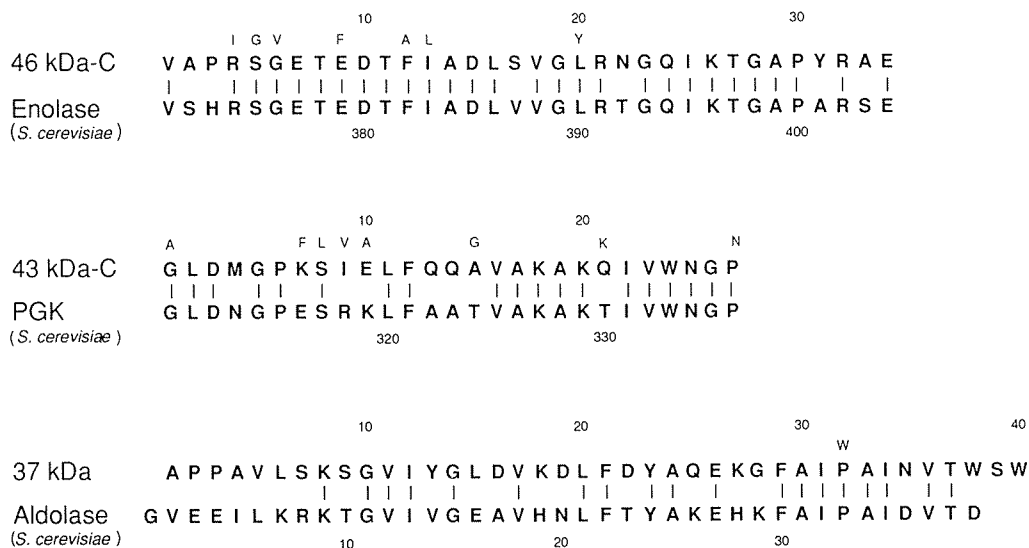


FIG. 7. Amino acid sequences of the IgE-binding *C. albicans* antigens in comparison with homologous proteins of *S. cerevisiae*. The third polypeptide fragment of the 46-kDa antigen obtained with CNB treatment (46 kDa-C), *S. cerevisiae* enolase from amino acid 371 to 404, the third polypeptide fragment of the 43-kDa antigen after CNBr cleavage (43 kDa-C), *S. cerevisiae* PGK from amino acid 310 to 336, N-terminal region of the 37-kDa protein, and *S. cerevisiae* aldolase are shown. Small letters above the sequences show second probable amino acids when a clear peak was not found by sequencing.

Cross-reactivity with homologous proteins. On the basis of the sequence homologies, immunological homologies were studied between the 46-kDa allergen and enolase or between the 43-kDa allergen and PGK. Both enolase and PGK derived from the yeast *S. cerevisiae* and from rabbit muscle were commercially obtained and run with our preparations by SDS-PAGE (Fig. 8A). CBB-R250-stained gels showed that the enolase from the two organisms had a molecular mass slightly higher than 46 kDa and that the yeast PGK had a molecular mass somewhat higher than 43 kDa.

Rabbit antibody against the 46-kDa allergen strongly cross-reacted with enolase from *S. cerevisiae* and weakly cross-reacted with enolase from rabbit muscle (Fig. 8B, lanes 1 to 3). Rabbit antibody against the 43-kDa allergen strongly cross-reacted with PGK from both *S. cerevisiae* and rabbit muscle (Fig. 8B, lanes 4 to 6). IgG antibodies to the 46- and 43-kDa allergens were not detected in preimmune rabbit sera (data not shown). On the other hand, immunoblotting with the patient sera showed IgE binding with only *C. albicans* antigens and with neither enolase nor PGK from other organisms (Fig. 8C).

DISCUSSION

At the beginning of this study, we identified up to 24 antigenic components reactive with IgE of sera from allergic patients in SDS-polyacrylamide gels of crude antigens from whole-cell extracts of *C. albicans*. The IgE-positive sera were detected in 56% of allergic patients and none of the healthy subjects. We selected the five most frequently occurring antigens 175-, 125-, 46-, 43-, and 37-kDa antigens, for purification.

The IgE-binding antigens, 46-, 43-, and 37-kDa antigens, were found to be present mainly in cytoplasmic fractions (Fig. 2). Two antigens, 175- and 125-kDa antigens, were not recovered in any fractions. Antigenicities may have been lost in the fractionation process. In the purification procedure, the cytoplasmic 46-, 43-, and 37-kDa antigens were

always monitored by IgE immunoblotting using patient sera, and the immunoblot profiles and CBB-R250-stained profiles were compared. Finally, each antigen could be purified as a single protein from a SDS-polyacrylamide gel.

Parts of the amino acid sequence were identified, and the cytoplasmic 46-, 43-, and 37-kDa antigens were found to be homologous to *S. cerevisiae* enolase, PGK, and aldolase, respectively. This was also confirmed immunologically when the rabbit antibodies against the 46- and 43-kDa antigens strongly cross-reacted with *S. cerevisiae* enolase and PGK, respectively. Rabbit antibodies against the 37-kDa antigen were not prepared.

The three proteins, enolase, PGK, and aldolase, which are present in large amounts in the cytoplasm, and the glycolytic enzymes are coordinately regulated. For example, enolase makes up 2 to 5% of the soluble protein of *S. cerevisiae* and PGK is 6.5 times more abundant than enolase (6). In *C. albicans*, the concentration of the enzymes were not estimated, but their synthesis is expected to be similarly regulated. When *C. albicans* cells are broken down in the human body, a large amount of the glycolytic enzymes will be released and act as antigens. Even though *C. albicans* is a common and harmless commensal of the mucous membranes and digestive tracts of normal individuals (19), atopic dermatitis patients might be sensitized by continuous exposure of these antigens during saprophytic *C. albicans* growth. It is difficult to explain the high antibody titer of the specific IgE only by the continuous exposure of the antigens, because there are many other abundant antigens in cells. Consequently, we conclude that the antigens might have a high immunogenicity to produce the antibody specific for IgE.

Baldo and Baker (2) tested 47 subjects diagnosed as having inhalant allergies to fungi for allergic sensitivity to *S. cerevisiae*. They found that the purified enolase from *S. cerevisiae* was a major allergic component. They also suggested cross-reactivity between the allergenic proteins of *S. cere-*

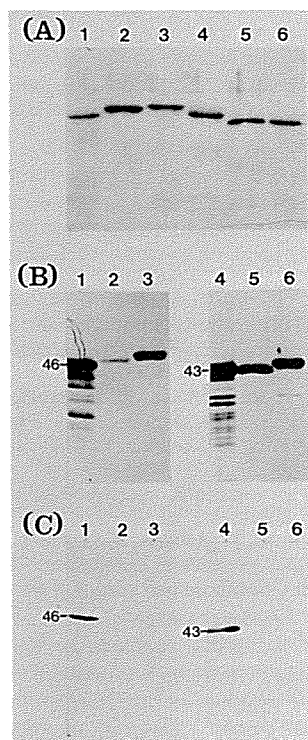


FIG. 8. Immunoblot analysis of the rabbit and human sera. (A) CBB-R250-stained SDS-polyacrylamide gel of the purified antigens and commercially available enzymes. Lanes: 1, 46-kDa protein from *C. albicans*; 2, enolase from rabbit muscle; 3, enolase from *S. cerevisiae*; 4, PGK from *S. cerevisiae*; 5, PGK from rabbit muscle; 6, 43-kDa protein of *C. albicans*. (B) Reactivity of the rabbit serum against the 46- and 43-kDa proteins. Lanes 1 to 3 and 4 to 6 were blotted with anti-46-kDa rabbit serum and anti-43-kDa rabbit serum, respectively. Lane 1, 46-kDa component from *C. albicans*; 2, enolase from rabbit muscle; 3, enolase from *S. cerevisiae*; 4, 43-kDa component from *C. albicans*; 5, PGK from rabbit muscle; 6, PGK from *S. cerevisiae*. Numbers to the left of the gels show molecular mass (in kilodaltons). (C) Reactivity of the sera from allergic patients. Lanes 1 to 3 and 4 to 6 were blotted with the sera from patients 12 and 18, respectively. The samples in the lanes are the same as in panel B. Numbers to the left of the gel show molecular mass (in kilodaltons).

visiae and *C. albicans*. On the other hand, we showed that IgE from the patient sera did not react with the *S. cerevisiae* enzymes, enolase and PGK, only with the enzymes from *C. albicans*, although the enzymes from *S. cerevisiae* reacted with IgG of rabbit antiserum against the enzymes from *C. albicans*. Our results suggest that IgE antibodies are raised against only small parts of their epitopes in the allergic patients. The determination of epitopes is important for the detection of cross-antigenicity common to other fungi. Furthermore, it would be very interesting to know whether IgE epitopes are shared or different among the patients.

Many *C. albicans* antigens reacting with antibodies in human sera were detected by Western blotting (immunoblotting) and their binding profiles varied among patients (17, 18, 21, 24–26, 28). Some of the antigens were most commonly detected as an immunodominant antigen for IgG and thought to be useful as a marker for systemic infection. One antigenic component was reported to be homologous to the glycolytic enzyme enolase (4, 15, 28). Thus, *Candida* enolase seems to

be an immunodominant protein that is responsible for both allergy and candidiasis.

The major antigens which showed the highest IgE-binding frequency in asthmatic patients have been reported by two groups of researchers (24, 25). The major antigens reported were the 40-kDa protein which reacted with 77% of the serum samples (25) and the 46-kDa protein which reacted with 77% of the serum samples and was located in cytoplasmic fractions (24). The molecular size and cytoplasmic localization of the 46-kDa protein leads us to believe that it is also enolase. We did not detect the 40-kDa major antigen. This might show that the antigens vary in immunogenicity in humans. Standardization of the antigens is imperative to improve these studies.

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REFERENCES

- Ashman, R. B., J. M. Papadimitriou, A. K. Ott, and J. R. Warmington. 1990. Antigens and immune responses in *Candida albicans* infection. *Immunol. Cell Biol.* **68**:1–13.
- Baldo, B. A., and R. S. Baker. 1988. Inhalant allergies to fungi: reactions to bakers' yeast (*Saccharomyces cerevisiae*) and identification of bakers' yeast enolase as an important allergen. *Int. Arch. Allergy Appl. Immunol.* **86**:201–208.
- Dobson, M. J., M. F. Tuite, N. A. Roberts, A. J. Kingsman, and S. M. Kingsman. 1982. Conservation of high efficiency promoter sequences in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **10**:2625–2637.
- Franklyn, K. M., J. R. Warmington, A. K. Ott, and R. B. Ashman. 1990. An immunodominant antigen of *Candida albicans* shows homology to the enzyme enolase. *Immunol. Cell Biol.* **68**:173–178.
- Goud, B., A. Salminen, N. C. Walworth, and P. J. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell* **53**:753–768.
- Holland, M. J., and J. P. Holland. 1978. Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. *Biochemistry* **17**:4900–4907.
- Holland, M. J., J. P. Holland, G. P. Thill, and K. A. Jackson. 1981. The primary structures of two yeast enolase genes. *J. Biol. Chem.* **256**:1385–1395.
- Howe, J. G., and W. B. Hershey. 1985. A sensitive immunoblotting method for measuring protein synthesis initiation factor levels in lysates of *Escherichia coli*. *J. Biol. Chem.* **256**:12836–12839.
- Ishiguro, A., M. Homma, T. Sukai, K. Higashide, S. Torii, and K. Tanaka. *J. Med. Vet. Mycol.*, in press.
- Iwaguchi, S.-I., M. Homma, and K. Tanaka. 1990. Variation of electrophoretic karyotype analysed by assignment of DNA probes in *Candida albicans*. *J. Gen. Microbiol.* **136**:2433–2442.
- Knauer, K. A., and N. F. Adkinson, Jr. 1983. Clinical significance of IgE, p. 673–688. In E. Middleton, Jr., C. E. Reed, and D. F. Ellis (ed.), *Allergy*, 2nd ed. The C.V. Mosby Company, St. Louis.
- Kwon-Chung, K. J., D. Lehman, C. Good, and P. T. Magee. 1985. Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. *Infect. Immun.* **49**:571–575.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.

14. Longbottom, J. L., W. D. Brighton, G. Edge, and J. Pepys. 1976. Antibodies mediating type I skin test reactions to polysaccharide and protein antigens of *Candida albicans*. Clin. Allergy 6:41-49.
15. Mason, A. B., M. E. Brandt, and H. R. Buckley. 1989. Enolase activity associated with a *C. albicans* cytoplasmic antigen. Yeast 5:S231-S239.
16. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035-10038.
17. Matthews, R. C., J. P. Burnie, and S. Tabaqchali. 1984. Immunoblot analysis of the serological response in systemic candidiasis. Lancet ii:1415-1418.
18. Matthews, R. C., J. P. Burnie, and S. Tabaqchali. 1987. Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to *Candida albicans*. J. Clin. Microbiol. 25:230-237.
19. Odds, F. C. 1988. *Candida* and candidiasis, p. 68-92. Bailliere Tindall, London.
20. Odds, F. C. 1988. *Candida* and candidiasis, p. 231-234. Bailliere Tindall, London.
21. Paul, T. R., S. N. Smith, and M. R. W. Brown. 1989. Effect of iron depletion on cell-wall antigens of *Candida albicans*. J. Med. Microbiol. 28:93-100.
22. Savolainen, J., M. Viander, R. Einarsson, and A. Koivikko. 1990. Immunoblotting analysis of concanavalin A-isolated allergens of *Candida albicans*. Allergy 45:40-46.
23. Savolainen, J., M. Viander, and A. Koivikko. 1990. Distribution of watersoluble antigens and allergens of *Candida albicans* in blastospore cell extract fractions. Allergy 45:47-53.
24. Savolainen, J., M. Viander, and A. Koivikko. 1990. IgE-, IgA- and IgG-antibody responses to carbohydrate and protein antigens of *Candida albicans* in asthmatic children. Allergy 45:54-63.
25. Shen, H.-D., K. B. Choo, R. B. Tang, C. F. Lee, J.-Y. Yeh, and S. H. Han. 1989. Allergenic components of *Candida albicans* identified by immunoblot analysis. Clin. Exp. Allergy 19:191-196.
26. Strockbine, N. A., M. T. Lergen, S. M. Zweibel, and H. R. Buckley. 1984. Identification and molecular weight characterization of antigens from *Candida albicans* that are recognized by human sera. Infect. Immun. 43:715-721.
27. Walsh, T. J., J. W. Hathorn, J. D. Sobel, W. G. Merz, V. Sanchez, M. Maret, H. R. Buckley, M. A. Pfaller, R. Schaufele, C. Sliva, E. Navarro, J. Lecciones, P. Chandrasekar, and J. Lee. 1991. Detection of circulating *Candida* enolase by immunoassay in patients with cancer and invasive candidiasis. N. Engl. J. Med. 324:1026-1031.
28. Zweerink, M. M., C. S. Maloney, T. G. Mitchell, and H. Weston. 1986. Immunoblot analyses of *Candida albicans*-associated antigens and antibodies in human sera. J. Clin. Microbiol. 23:46-52.