

Allergens of *Pityrosporum ovale* and *Candida albicans*

II. Physicochemical characterization

Doekes G, Kaal MJH, van Ieperen-van Dijk AG. Allergens of *Pityrosporum ovale* and *Candida albicans*. II. Physicochemical characterization. Allergy 1993; 48: 401-408. © Munksgaard 1993.

Pityrosporum ovale has recently been recognized as a source of allergens to which many patients with atopic dermatitis (AD) show type I skin reactions and specific IgE antibodies. In this study the IgE-binding components and/or epitopes in *P. ovale* extract were shown to be partially sensitive to pronase or trypsin treatment, whereas periodate oxidation resulted in a complete loss of IgE-binding capacity, thus suggesting the involvement of carbohydrate structures. In Con A affinity chromatography most of the IgE-binding capacity of crude *P. ovale* extract bound to the column, and could be eluted with mannoside. Gel filtration on Sephacryl S-400 revealed a marked heterogeneity with respect to molecular mass, with most of the IgE-binding activity associated with high-mol.-mass fractions (from 5×10^4 up to 2×10^6 Da). A similar heterogeneity was found after chromatofocusing, with IgE-binding in the whole pI-range from 7.0 to 4.0. Essentially identical results were obtained with extracts of *Candida albicans*, in agreement with the previously shown cross-reactivity of IgE-binding components in the two yeast extracts. In inhibition ELISA, gel filtration and chromatofocusing fractions containing components with widely different mol. mass or pI showed complete reciprocal cross-inhibition, and were all capable of inhibiting the binding of IgE to unfractionated extracts. We therefore conclude that the cross-reacting anti-*P. ovale*/anti-*C. albicans* IgE antibodies in the sera of AD patients are mainly directed at a restricted number of carbohydrate epitopes that are expressed on a heterodisperse range of high-mol.-mass components, probably mannans or mannoproteins.

Clinical aspects

Anti-*Pityrosporum ovale* IgE can be found in a large proportion of patients with atopic dermatitis (AD), and in $\pm 50\%$ of the anti-*P. ovale*, IgE-positive sera these IgE antibodies cross-react with extracts of *Candida albicans*. The results of this biochemical characterization study indicate that a major proportion of the cross-reacting IgE antibodies reacts with carbohydrate epitopes expressed on a wide range of high-mol.-mass glycoproteins and/or polysaccharides - possibly mannans or mannoproteins, which are well-known antigenic structures of many yeast species.

G. Doekes, M. J. H. Kaal, A. G. van Ieperen-van Dijk

Department of Dermatology, University Hospital Utrecht, The Netherlands

Key words: allergens; atopic dermatitis; *Candida*; IgE; *Pityrosporum*.

Dr G. Doekes
Department of Human Epidemiology and Public Health
Agricultural University of Wageningen
P.O. Box 238
6700 AE Wageningen
The Netherlands

Accepted for publication 5 November 1992

Pityrosporum ovale is a lipophilic yeast inhabiting the normal skin of most healthy adult persons (21). *P. ovale* is usually nonpathogenic, but excessive growth, differentiation of the yeast, and/or inappropriate host immunoreactivity may cause pityriasis versicolor or pityrosporum folliculitis, while *P. ovale* may also be involved in seborrheic dermatitis and dandruff (9). A

role for anti-*P. ovale* allergy in the pathogenesis of atopic dermatitis (AD) has been suggested by the high prevalence of anti-*P. ovale* type I skin reactions and anti-*P. ovale* IgE antibodies in AD patients (4, 13, 14, 17, 22, 26, 31, 33, 35). These reactions are highly specific for AD, and are rarely found in patients with seborrheic eczema (14, 22) or atopic

patients with asthma or rhinitis, but not eczema (17, 33, 35).

The molecular properties of the IgE-binding components of *P. ovale* are largely unknown. In contrast, several studies have reported on the characteristics of IgE-binding allergens from another common yeast, *Candida albicans* (7, 16, 24, 25, 27). In previous studies, a strong correlation between levels of anti-*P. ovale* and anti-*C. albicans* IgE was noted (17, 33), and in an accompanying paper we have demonstrated that this correlation is due to a marked cross-reactivity (5). We therefore studied in more detail the biochemical properties of allergens in both yeast extracts, using protease and periodate treatment, Con A affinity chromatography, S-400 gel filtration, and chromatofocusing.

Material and methods

Reagents

Sephacrose-concanavalin A, Sephacryl S-400, anion exchanger PBE 94 and Polybuffer 74 (Pharmacia LKB), trypsin (bovine pancreas, type III; Sigma), pronase (from *Streptomyces griseus*; Boehringer, Mannheim, Germany), and sodium periodate (Merck) were purchased as indicated. Lyophilized extracts of *P. ovale* and *C. albicans* were kindly provided by Laboratorium Diephuis, Groningen, The Netherlands (5).

ELISA and inhibition ELISA

The IgE-binding activity of test samples was determined by coating appropriate dilutions in phosphate-buffered saline (PBS) in microwells, and monitoring the reaction with specific IgE from diluted AD sera (5, 33). For very strong reactions, extrapolated OD₄₉₂ values (> 2.0) were obtained by multiplication of the OD₄₀₅ read directly before the addition of H₂SO₄. For a Flow Titertek Mk II ELISA reader, the multiplication factor – derived from the OD₄₉₂/OD₄₀₅ in wells with an OD₄₉₂ of 1.5–2.0 – appeared to be 4.0.

Enzyme- and periodate-sensitivity

Standard solutions of yeast allergens (1 mg dry, lyophilized extract per ml PBS) were preincubated for 1 h at 37°C with trypsin (0.2 mg/ml) or pronase (0.2 mg/ml), and subsequently coated at 0.1 mg/ml in microwells and tested for their IgE-binding capacity. For periodate oxidation, which preferentially alters carbohydrate structures while leaving peptides and proteins intact (19, 34), allergen (1 mg/ml) was incubated (20 min; 37°C) with 20 mM NaIO₄ in 0.1 M acetate, pH 5.5. The reaction was stopped by

neutralization with 1 M ethanolamine, which also blocks the newly formed aldehyde groups. Trypsin-, pronase-, and periodate-treated extracts were also tested for their capacity to react with IgE antibodies in the fluid phase by the inhibition ELISA method (5). In that experiment, suboptimal amounts of pretreated *P. ovale* and *C. albicans* extracts were used as fluid-phase inhibitors, to ensure that any significant decrease of IgE-binding capacity would be detected.

Con A chromatography

Con A affinity chromatography was performed at room temperature in 0.01 M Tris HCl, 0.5 M NaCl, 0.01% NaN₃, pH 7.4. An amount of 50–100 mg of lyophilized yeast extract was dissolved in 5–10 ml of this buffer, filtered, and applied to a 1.0 × 15 cm Con A-Sepharose column. After passage of nonbinding material, and washing with at least 30 ml of the starting buffer, elution was performed with two 25-ml batches of 0.3 and 0.5 M mannoside in starting buffer. Fall-through and mannoside-eluted fractions were extensively dialyzed against PBS-azide, concentrated to 3–5 ml, and stored in aliquots at –40°C.

Gel filtration

An amount of 50 mg extract was dissolved in 5 ml phosphate-buffered 0.5 M NaCl, 0.01% NaN₃, pH 7.4; microfiltered; applied to a 2.5 × 90 cm column of Sephacryl S-400; and eluted at 15–20 ml/h. Calibration was based on the V_e for IgM, IgA, IgG, and albumin from normal human serum. The calibration results were in agreement with the manufacturer's indications for globular proteins; estimation of polysaccharide mol. masses was based on the manufacturer's data.

Chromatofocusing

We performed chromatofocusing on a 1 × 15 cm column of PBE 94, using 0.025 M imidazole/HCl, pH 7.4, as starting buffer, elution with a linear pH gradient (pH 7.0–4.0) of Polybuffer 74, and 1 M NaCl to elute components that were still bound at pH 4.0.

Protein and sugar determinations

Protein was estimated by measuring OD₂₈₀ values, and determined more accurately with the bicinchoninic acid (BCA) reagent (Micro BCA protein reagent; Pierce, Rockford, IL, USA), bovine serum albumin serving as a standard. Sugar concentrations were assessed by the phenol-sulfuric acid method of Dubois et al. (6).

Table 1. Fluid-phase IgE-binding capacity of trypsin-, pronase-, and periodate-treated *P. ovale* and *C. albicans* extracts. Percent inhibition of the IgE reactions of two AD sera with ELISA wells coated with nontreated extracts was used as a measure of IgE binding in the fluid phase. The first four lines show the positive controls: sera preincubated in buffer alone or with pretreatment reagents, without allergen

Preincubation +/- allergen*/allergen pretreatment	Serum MB (1/10)				Serum 038 (1/10)			
	<i>P. ovale</i>		<i>C. albicans</i>		<i>P. ovale</i>		<i>C. albicans</i>	
	OD ₄₉₂	(inh)*	OD ₄₉₂	(inh)	OD ₄₉₂	(inh)	OD ₄₉₂	(inh)
-/-	3.385		0.466		1.431		0.302	
-/trypsin	3.260		0.478		1.403		0.304	
-/pronase	3.330		0.416		1.465		0.354	
-/periodate	3.515		0.452		1.508		0.306	
+/-	1.125	(67%)	0.196	(58%)	0.269	(81%)	0.051	(83%)
+ /trypsin	1.761	(48%)	0.202	(57%)	0.314	(78%)	0.059	(80%)
+ /pronase	1.836	(46%)	0.237	(49%)	0.837	(42%)	0.114	(62%)
+ /HAc-ethanolamine [#]	1.739	(49%)	0.200	(57%)	0.268	(81%)	0.052	(83%)
+ /periodate	3.230	(5%)	0.498	(-7%)	1.516	(-6%)	0.323	(-7%)

* *P. ovale* and *C. albicans* extract used as inhibitors at 0.01 and 0.1 mg/ml, respectively. Only inhibition of the reaction with the same extract was studied (no cross-inhibition).

** % decrease of OD₄₉₂ values compared with the positive control (first line).

Buffer and stop reagent of the periodate treatment.

Results

Protease- and periodate-sensitivity

Preincubation of *P. ovale* and *C. albicans* extracts with trypsin, pronase, or periodate, before coating in ELISA wells, resulted in a complete or partial loss of IgE binding (not shown). Since the decrease in IgE binding by coated allergens in this experiment could also be due to a diminished adherence to the microwells, the effects of enzyme- and periodate-treatment were also assessed in the inhibition ELISA, in which the binding of IgE by soluble allergens is assessed as the percent inhibition of subsequent IgE binding to coated allergens (Table 1). In three of the four tested combinations, trypsin had only a marginal effect, and in one case it caused partial reduction from 67 to 48% inhibition. The effect of pronase was more pronounced, but also only partial. In contrast, periodate oxidation completely eliminated the fluid-phase IgE-binding capacity of both yeast extracts. Inhibition by control extracts "pretreated" with acetate buffer and the stop reagent ethanolamine was unchanged in three cases, and partially diminished in the fourth (line 8). Preincubation of AD sera with trypsin, pronase, or periodate at the concentrations used in the experiment, but in the absence of allergens, had no significant effect on the IgE reactions with coated *P. ovale* and *C. albicans* (lines 2-4).

Con A chromatography

P. ovale and *C. albicans* extracts were fractionated three to five times by Con A chromatography, different AD sera being used to assess IgE-binding activity; Fig. 1 shows a representative Con A-profile

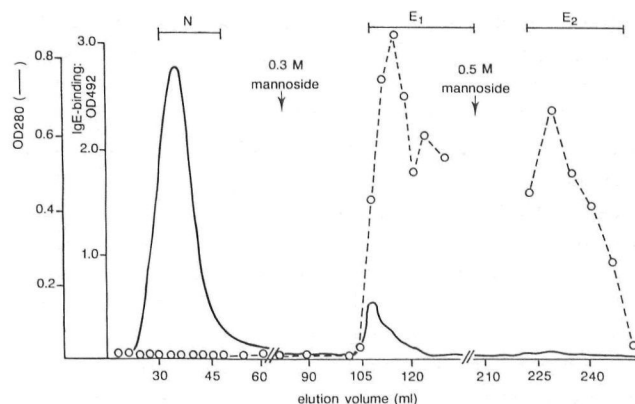


Fig. 1. Con A chromatography of *P. ovale* extract. Fifty milligrams of lyophilized extract was applied to 1 × 15 cm column of Sepharose-concanavalin A. Bound components were eluted with 0.3 M and 0.5 M mannoside. IgE-binding capacity was determined by coating 1/10 dilutions of fractions in ELISA wells and monitoring subsequent reaction with 1/20 diluted AD serum (○ - - - ○). Horizontal bars indicate pools of nonbound components (N), and 0.3 M (E1) and 0.5 M eluates (E2) used in further experiments.

of *P. ovale* extract. Most of the material did not bind to the column, and did not exhibit IgE-binding activity. Strong IgE binding was found in a small OD₂₈₀ peak eluted with 0.3 M mannose, and a second peak of IgE-binding activity was found after elution with 0.5 M mannose, which resulted in a hardly visible rise of OD₂₈₀ values above the base line (Fig. 1). Similar profiles were obtained after Con A chromatography of *C. albicans* extract, with, however, a relatively high IgE-binding capacity in the 0.5 M, as compared with the 0.3 M, eluate.

The preferential IgE-binding activity of Con A eluates was confirmed in an experiment in which the IgE reactions of 10 AD sera were compared in wells

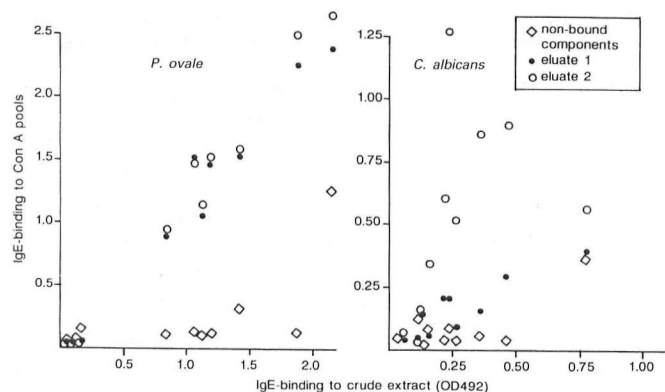


Fig. 2. IgE binding from 10 AD sera to Con A pools of *P. ovale* (panel a) and *C. albicans* (panel b) extracts. Sera were simultaneously tested in microwells coated with whole extracts (0.1 mg/ml), or pools N (0.1 mg protein and carbohydrate/ml), E1 (4 μ g/ml), or E2 (2 μ g/ml). OD₄₉₂ values after reaction with the three Con A pools are plotted against OD₄₉₂ in wells coated with whole extract.

coated either with whole extracts or with the various Con A pools. For *P. ovale*, IgE binding to both the 0.3 M and the 0.5 M mannoside eluates was strongly correlated with IgE binding to the whole extract, while only one serum showed, in addition to its reaction with the eluates, reactivity with the Con A fall-through pool (Fig. 2a). The reaction pattern of the sera with *C. albicans* pools was similar, although less pronounced (Fig. 2b). Most sera had a stronger reaction with the 0.5 M than the 0.3 M mannoside eluate of *C. albicans* extract.

Table 2 summarizes the Con A chromatography results. Only small amounts (<10%) of the starting material were recovered in the Con A eluates. Their protein/carbohydrate ratio did not differ from that of the fall-through pools, a fact which might be explained by the highly selective binding to Con A of

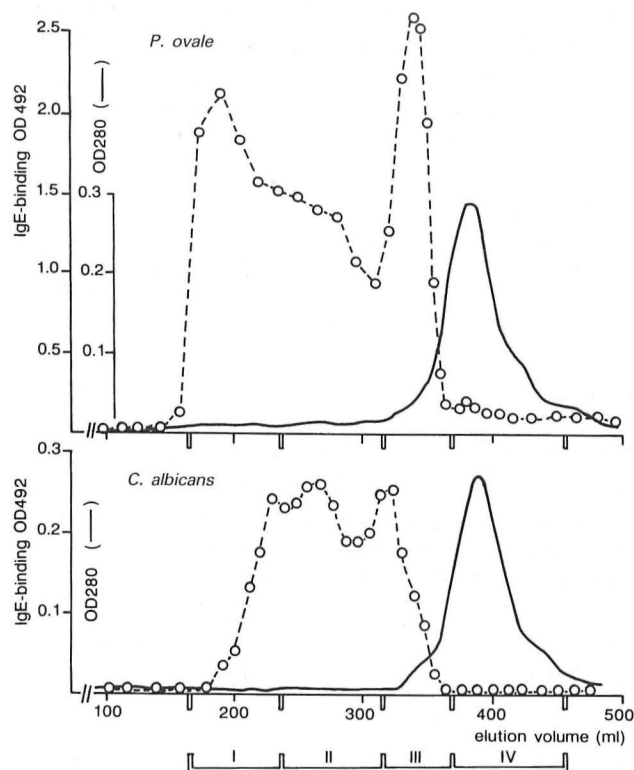


Fig. 3. Gel filtration of *P. ovale* and *C. albicans* extracts. Fifty milligrams of each extract was fractionated on a 2.5 $\times$ 90 cm column of Sephacryl S-400. IgE-binding capacity of the fractions (5 ml) was assessed as described in Fig. 2. Fractions were pooled as indicated on the x-axis, concentrated, and used for further analysis.

glycoproteins or polysaccharides with terminal D-mannopyranoside or D-glucopyranoside residues. The yeast extracts apparently also contain relatively large amounts of other carbohydrate-containing molecules that do not bind to Con A and that have no allergenic activity. Titration experiments in

Table 2. Composition and IgE-binding capacity of Con A isolated pools of *P. ovale* and *C. albicans* extracts

Pool*	Volume		Protein (mg/ml)	Carbohydrate (mg/ml)	Titer in IgE ELISA	Specific IgE-binding capacity	
	(ml)	OD ₂₈₀					
P (w)	10	3.85	2.04	2.62	1250	269	(1.00)
P (N)	5	0.38	0.20	0.88	<5	<5	(0.02)
P (E1)	5	0.14	0.06	0.15	1500	7143	(26.7)
P (E2)	5	0.06	0.03	0.01	230	5500	(20.4)
C (w)	7	3.75	3.95	3.20	70	9.8	(1.00)
C (N)	5	0.52	0.69	1.30	<1	<0.5	(0.05)
C (E1)	5	0.16	0.07	0.55	15	24.2	(2.48)
C (E2)	5	0.20	0.06	0.10	120	730	(74.5)

* P: *P. ovale*; C: *C. albicans*; w: whole extract (starting material applied to the Con A column); N: nonbound components; E1 and E2: 0.3 M and 0.5 M mannoside eluate, respectively.

** Serial dilutions of the pools were coated, and made to react with specific IgE from 1/20 diluted AD serum. The titer was arbitrarily defined as the coating dilution that gave an OD₄₉₂ of 0.6, read by interpolation from the curve obtained.

Calculated as (titer)/(concentration protein and carbohydrate). (Italics): specific activity relative to that of the whole extract.

which serially diluted Con A pools were used as coated allergens revealed that a great part of the total IgE-binding capacity was recovered in the eluates, and calculation of the specific activity (titer/concentration) indicated a 25–75-fold enrichment.

S-400 gel filtration

Gel filtration was also performed two to four times with both extracts. Examples of OD₂₈₀ and IgE binding-profiles on S-400 are shown in Fig. 3. A major OD₂₈₀ peak at mol. mass 10–100 kDa or 5–50 kDa (for globular proteins or polysaccharides, respectively), followed a long range of low concentrations of high-mol.-mass components (Fig. 3). IgE-binding capacity was almost completely absent from the major OD₂₈₀ peak, whereas most of the earlier fractions reacted strongly as coated allergens with patients' IgE. Although the profile for *P. ovale* suggested two peaks of predominant IgE-binding activity, separate pooling of these peaks and the intermediate fractions – as indicated in Fig. 3 – resulted

in three pools with only small differences in their IgE-binding activity: half-maximal OD₄₉₂ values were obtained at coatings of 100, 180, and 210 ng protein and carbohydrate per well, respectively. In contrast, the corresponding value for pool IV was > 10 000 ng (not shown). The S-400 profile for *C. albicans* extract was very similar (Fig. 3; lower panel), except for a low IgE-binding capacity in the void volume and early high-mol.-mass fractions ($V_e < 200$ ml). Moreover, this pool contained hardly any detectable protein or carbohydrate after concentration.

Chromatofocusing

Chromatofocusing at pH 7.0–4.0 indicated the presence of a wide variety of components with different pI, with substantial IgE binding in most of the fractions, but no peaks of IgE-binding activity (not shown). A large proportion ($\pm 30\%$) of the crude material bound very firmly to the column, and was recovered, after completion of the pH gradient, by elution with 1 M NaCl. These apparently acidic components (pI < 4.0) also contained a major part of the IgE-binding capacity.

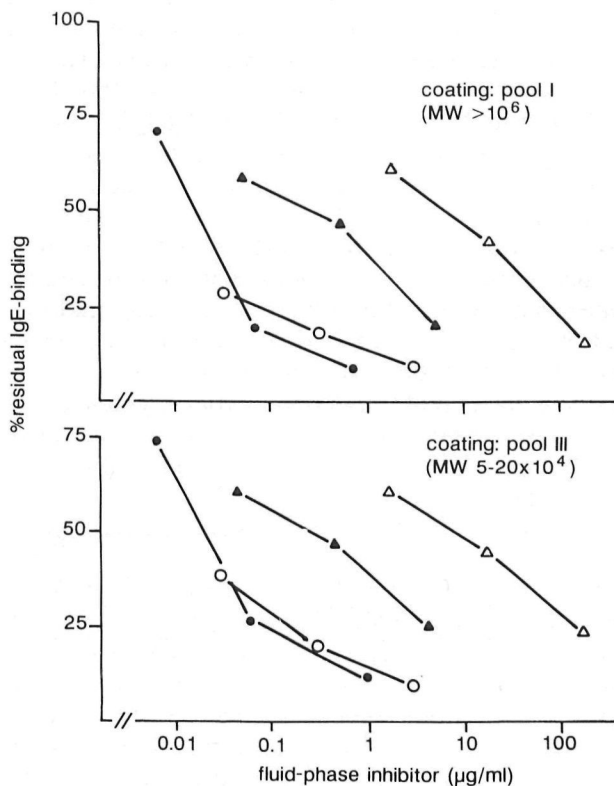


Fig. 4. Cross-inhibition of IgE-binding *P. ovale* components of different size. Diluted AD serum was preincubated with three dilutions of the S-400 pools I (○), II (●), III (▲), and IV (△), and then tested for residual IgE binding to wells coated with either pool I (0.65 µg/ml; upper panel) or pool III (4.8 µg/ml; lower panel). "100%" represents OD₄₉₂ obtained with corresponding positive controls. Mol. wt. (insert) are estimated values for polysaccharides.

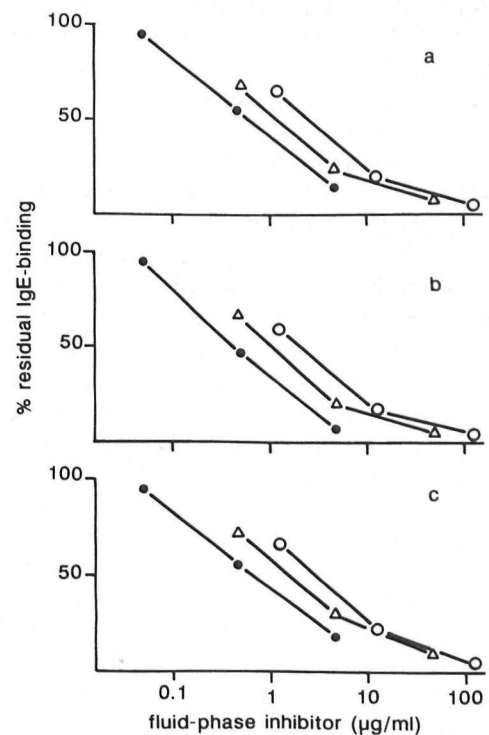


Fig. 5. Cross-inhibition of *P. ovale* components of different pI. Diluted AD serum was preincubated with three dilutions of whole extract (△), or of two chromatofocusing fractions with pI = 7.0 (●) or pI ≤ 4.0 (○). After preincubation, residual IgE binding was assessed in wells coated with the whole extract (0.1 mg/ml; panel a), the pI 7.0 fraction (5 µg/ml; panel b), or the pI ≤ 4.0 fraction (125 µg/ml; panel c).

Inhibition of IgE binding by S-400 and chromatofocusing fractions

The heterogeneity of IgE-binding components with respect to mol. mass and pI was not reflected by a comparable heterogeneity of their IgE epitopes. In cross-inhibition experiments, S-400 pools of different mol. mass showed a remarkable capacity to inhibit the IgE reactions with other pools (Fig. 4), and also showed a dose-dependent, and at high concentrations complete, inhibition of the IgE binding to the unfractionated extract (not shown). Chromatofocusing fractions of widely different pI also showed complete cross-inhibition and inhibition of the reactions with whole extract (Fig. 5). This indicates that components of different mol. mass or pI reacted with the same population of IgE antibodies, and that the complete set of IgE-binding epitopes of the whole extract was expressed in each of the S-400 or chromatofocusing fractions.

Discussion

Characterization of allergenic extracts usually aims at the identification of "major allergens", i.e. molecules with well-defined properties, against which most persons with IgE-reactivity against the crude extract have detectable IgE antibodies. A wide range of major allergens has thus far been identified in extracts from, for example, dust mites, foods, animal dander, and many pollen species (2, 3), and the defined characteristics may include mol. mass, pI, amino acid composition, and carbohydrate content and composition. Most of these components are highly soluble glycoproteins of 10–50 kDa, and often a relatively low pI. The attached carbohydrates may vary considerably in amount and composition, and in most instances seem to be of no importance to the IgE-binding capacity. However, IgE-binding epitopes on carbohydrate moieties of glycoproteins have also been reported, as, for example, in mold extracts (29, 30), tunicate allergens (18, 28), pollens, and Hymenoptera venom (1, 15, 32).

Allergens of microbial origin, particularly from yeasts, are relatively poorly characterized. Early studies on *C. albicans* have shown that both cytoplasmic proteins and cell-wall polysaccharides – mainly mannans – may be involved in IgE-mediated allergy (7, 16). In recent immunoblotting studies a number of IgE-binding proteins with mol. mass of 10–100 kDa have been described (24, 25, 27), and in one study the involvement of mannans was also reported (25). However, immunoblotting may not be suitable for the detection of antimannan reactions, because of their high mol. mass and heterodisperse nature (9). Thus, either mannans might not reach the running gel during SDS–PAGE, or the variance in

mol. mass might preclude detection of distinct bands. This might also be of relevance for the recently reported immunoblotting studies with *P. ovale* extracts (12, 13). Both studies have demonstrated the presence of a wide range of IgE-binding proteins in the 10–100 kDa range. However, relatively large amounts of allergen extract were used in SDS–PAGE (0.1 and 0.2 mg/mm gel, respectively), and part of the IgE-binding components might escape detection in such immunoblotting experiments. Initially, we also tried immunoblotting, using up to 0.2 mg extract/mm gel, and were unable to find any IgE-binding bands with the commercial extracts used in the present study. Degradation of components and/or epitopes during SDS–PAGE seems improbable, since we used the same SDS-preincubation methods as the other investigators. We suspect that the apparent discrepancy with the other studies may be primarily due to a different molecular composition of the various extracts. This conclusion was recently confirmed in our laboratory by the comparison of *P. ovale* extracts from different sources (Doekes et al., paper in preparation).

The chromatography experiments indicated that most IgE epitopes in the *P. ovale* and *C. albicans* extracts were expressed on a heterodisperse range of Con A-binding glycoproteins or polysaccharides of varying, but usually high mol. mass (> 50–100 kDa), and a pI ranging from below 4 to 7. The IgE epitopes of coated allergens were sensitive to protease treatment and periodate oxidation, whereas IgE binding in the fluid phase was also completely lost after periodate treatment, but only partially reduced by proteases (Table 1). The effects of proteases in the first experiment might therefore be due to an impaired adherence to the ELISA wells, and not to a direct modification of IgE epitopes. Differences in the expression of peptide and carbohydrate epitopes on coated and fluid-phase allergens might be an alternative explanation; however, this explanation seems very improbable, since soluble allergens did completely block the IgE reactions with coated allergens.

A surprising finding was the capability of components in a restricted mol.-mass or pI range to block the IgE reactions with other components and with the whole extracts (Figs. 4 and 5). These findings were not due to a very poor resolution of the gel filtration or chromatofocusing methods, because rechromatography of selected fractions showed recovery of the IgE-binding activity at the original elution volume or pH of the applied fractions (not shown). It thus appears that very similar or identical IgE epitopes are expressed on a range of components of different mol. mass and pI. Together with the affinity for Con A, and the periodate-sensitivity of the epitopes, the findings are quite compatible with the identification of the IgE-binding components as

mannans. Mannans are known as mannose-rich, highly antigenic, heterodisperse, high-mol.-mass polysaccharides containing small (<10%) amounts of peptides, with marked differences in pI because of variations in the amounts of glucuronic acid, phosphate, and sulfate, and they are major constituents of the cell walls of most fungal species (9, 11, 12, 20, 23). The mannans of *C. albicans* have been studied thoroughly, although not with respect to their possible IgE epitopes. To our knowledge, the biochemical composition of the *P. ovale* cell wall has not yet been determined, but the predominant presence of mannans, as in other yeast species, may be expected. Because of the polymeric nature, their epitopes presumably are repeating structures. However, the exact definition of epitopes on carbohydrates requires extensive and rather complicated (bio)chemical and immunochemical procedures, since they might consist of 5–10 monosaccharide units; thus, a wide range of primary and conformational structures is possible. In preliminary experiments we observed no inhibition with mono- or disaccharides – glucose, mannoside, lactose, or saccharose – nor with purified *Saccharomyces* mannan (not shown). Treatment with glycosidic enzymes might be another way to confirm the carbohydrate nature of the IgE epitopes, and to delineate their primary structure. Such an analytic approach, however, requires the use of a panel of carefully selected enzymes. Partial degradation of carbohydrate by exoglycosidases or by cleavage of peptide-carbohydrate bonds by endoglycosidases might leave epitopes on relatively large polysaccharide structures intact, and, thus, easily lead to false-negative results. Therefore, the isolation and characterization of mannans from both *P. ovale* and *C. albicans* are required to confirm the conclusion from our present results that these cell-wall components may be important IgE-binding allergens, and are primarily responsible for the allergenic cross-reactivity of the two yeasts.

Acknowledgments

We thank Dr W. J. Koers and colleagues of the Department of Dermato-Allergy of University Hospital Utrecht for providing us with atopic dermatitis sera, Mr C. L. H. Guikers for technical assistance, and Prof. Dr C. A. F. M. Bruijnzeel-Koomen for critically reading the manuscript.

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