

Influence of culture period on the allergenic composition of *Pityrosporum orbiculare* extracts

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Summary

Background Previous characterization studies of *Pityrosporum orbiculare* allergens have led to contradictory results. In immunoblotting studies a range of IgE-binding proteins of 10–100 kDa have been identified. In another study, however, the IgE-binding structures were claimed to be associated with high-molecular-weight polysaccharides or glycoproteins, presumably mannans or mannoproteins.

Objective In the present study the reasons for these discrepancies were investigated.

Methods *P. orbiculare* preparations were compared in IgE ELISA and IgE-inhibition ELISA, as well as in immunoblotting with sera from atopic dermatitis patients.

Results It was inferred that variations in the period of *in vitro* culture of *P. orbiculare* constituted the most important factor determining the different compositions of the resulting yeast cell extracts. After 2 days of culture a wide range of allergenic proteins was present but upon more prolonged culture (>4 days) most proteins of 10–100 kDa were lost. Accordingly, the protein concentration of the extracts gradually declined from 40% to 25% between days 4 and 15 of culture. On the other hand, the carbohydrate content remained fairly constant (approximately 30%). Using inhibition ELISA it was demonstrated that the high-molecular-weight glycoproteins or polysaccharides presumably involved in most of the IgE-binding capacity in extracts from old cultures, were also present in comparable concentrations in all extracts tested, even after culture for only 2 or 4 days.

Conclusion Preparations obtained from the exponential phase of yeast cultures (2–4 days old), should preferably be used in studies of the IgE response to *P. orbiculare*.

Keywords: *Pityrosporum orbiculare*, *Pityrosporum ovale*, atopic dermatitis, IgE, allergen, monoclonal antibody, inhibition ELISA, immunoblotting

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Introduction

Pityrosporum orbiculare (*ovale*), the lipophilic yeast form of *Malassezia furfur*, is a member of the residential skin microflora, colonizing preferentially the sebaceous skin areas [1,2]. Although *P. orbiculare* is usually non-pathogenic, there is evidence that it may induce allergenic

reactions in atopic dermatitis (AD). In 40–65% of AD patients anti-*P. orbiculare* reactivity has been demonstrated with skin tests and/or specific IgE serology [3–8]. These reactions are highly specific for AD, being rarely found in atopic patients with rhinitis and/or asthma but no history of eczema [3,7,9]. Since *P. orbiculare* occurs on the skin, pathogenic involvement would imply local reactions of specific IgE and/or T cells with yeast allergens penetrating the skin, analogously to the reaction mechanism that account for the induction of

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Table 1. *P. orbiculare* preparations

Code	Culture type	Medium	Culture time (days)	Temperature (°C)	Strain no.	Source	Allergen source extraction	Protein concentration* mg/mg extract	CH concentration** mg/mg extract
KH-2	Solid phase	Glycerol monostearate	2	37°	42132	ATCC#	Sonication of freeze-dried yeast cells	0.41	0.21
KH-4		Olive oil medium [15]	4					0.40	0.32
KH-6			6					0.31	0.27
KH-8			8					0.27	0.29
KH-10			10					0.26	0.31
KH-15			15					0.25	0.33
CE	Fluid phase	Glucose neopeptone	10-14	25°	1878	CBSO	Sonication of washed yeast cells	0.23	0.22
		Olive oil medium [16]							
CM	Fluid phase	Glucose neopeptone	10-14	25°	1878	CBSO	Dialysed culture media of CE	0.24	0.14
		Olive oil medium							
D	Solid phase	Unknown	21	25°	unknown	CBSO	X-press (Thurax) of washed yeast cells	0.09	0.26

#ATCC, American Type Culture Collection, CBSO, Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands. *Protein concentrations measured with Pierce bichononic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA) [18]. **Charbohydrate (CH) concentrations were assessed by the phenol-sulfuric acid method of Dubois *et al.* [19].

eczematous lesions after cutaneous application of mite allergens on the healthy skin of AD patients [10–12].

The immunochemical and biochemical characterization of IgE-binding components in *P. orbiculare* extracts has been undertaken by several laboratories [13–16]. In two immunoblotting studies a wide range of IgE-binding proteins in the 10–100 kDa molecular weight range has been demonstrated, and some of the reactive bands could be designated as 'major allergens' — being capable of binding IgE from >50% of the sera in the test panel [13,14]. In another study, however, immunoblotting was completely unsuccessful, and further analysis revealed that the IgE-binding capacity of the extract investigated was associated mainly with polysaccharides or glycoproteins of high molecular weight (HMW), presumably belonging to the mannan or mannoprotein fraction [16]. In an attempt to resolve these apparent discrepancies, different *P. orbiculare* preparations were compared in the present study in IgE ELISA and IgE-inhibition ELISA, as well as in immunoblotting. Since preliminary results suggested that differences in culture period would account for the markedly different allergenic compositions of the extracts, additional experiments were performed with *P. orbiculare* preparations derived from varying culture periods.

Materials and methods

P. orbiculare extracts

The main characteristics of the *P. orbiculare* allergen preparations studied are summarized in Table 1. Partial immunochemical characterization of extract D from Laboratorium Diephuis (Groningen, the Netherlands), a subsidiary of ALK (Allergologisk Laboratorier, Copenhagen, Denmark) [16] and extract KH [13,15] have been reported previously. Essential features of the production of extract D are: culture of the organism at 25°C on a solid 2% agar medium containing 4% (w/v) malt extract, 2% (w/v) bacto oxgall, 1% (w/v) tween-40, and 0.25% (v/v) glycerol. Culture was continued for more than 20 days, until no further growth is observed and a several-mm-thick layer can be harvested. Washed cells were disrupted and extracted by X-press in phosphate-buffered saline (PBS). Several batches of extract D obtained and studied between 1990 and 1992 appeared to behave similarly during the characterization studies (data not shown).

Preparations CE and CM were obtained from fluid phase cultures performed at the University Hospital Utrecht, as described earlier [16]. Briefly, *P. orbiculare* (strain no. 1878, Centraal Bureau voor Schimmelcultures (CBS) Baarn, the Netherlands) was inoculated on a solid

2% agar medium containing 2% glucose, 1% neopeptone (Difco, Detroit, Michigan, USA), and 0.2% (v/v) olive oil, and incubated at room temperature. When substantial colony growth was visible (after 7–14 days), colonies were transferred to 2 L flasks containing 1 L of the same medium without agar. Fluid phase culture was performed at room temperature, with continuous stirring. After 10–14 days, when the medium had become very opaque, cells were harvested, washed with PBS, and sonicated and extracted with PBS. Extracts were dialysed against distilled water and lyophilized. The yields of cell extracts (CE) were 30–50 mg (dry weight after lyophilization)/L culture medium. Cell supernatants from the same cultures were dialysed extensively against tap water, PBS, and distilled water, and then lyophilized. The total yields of the preparations thus obtained from the culture media (CM) were 800–1500 mg/L.

The extract KH-4 was obtained from solid phase cultures of *P. orbiculare* strain no. 42132 (American Type Culture Collection) (ATCC) on Go media containing glycerol monostearate (2.5 g/L), Tween-80 (0.2%) and olive oil (2%), for 4 days at 37°C [13,15]. Whole yeast cells were harvested, freeze-dried, and extracted by sonication in PBS. The extract was desalted and freeze-dried again [13,15]. To assess the influence of culture time on the composition of the extracts, different extracts of strain no. 42132 were also prepared after the yeast cells had been cultured for 2, 6, 8, 10 and 15 days. These extracts according to their culture periods are designated KH-2 to KH-15.

Mouse monoclonal antibodies (MoAbs)

Two MoAbs designated 9G9 and 1D5, both of IgG1 class were produced against *P. orbiculare* by immunizing Balb/c mice with KH-4 extract [15]. Hybridoma cells were cultured in serum-free media and antibodies were isolated from the cell supernatant by precipitation with ammonium sulphate and dialysed against PBS buffer. MoAbs 9G9 and 1D5 are directed to protein components of *P. orbiculare* and recognize a 37 kDa and a 67 kDa major allergen respectively [15].

Sera

Twenty-two sera from patients with AD from the Department of Dermatology, University Hospital, Utrecht, were selected on the basis of a known high, intermediate, or low IgE ELISA reaction with a coating of *P. orbiculare* extract D [9]. In addition, sera from four patients with AD were included from the Department of Dermatology, Karolinska Hospital, Stockholm with high total serum IgE 4500–7000 kU/L and specific IgE

to *P. orbiculare*, RAST class 3–5, measured with RAST-FEIA CAP System (Pharmacia, Uppsala, Sweden).

ELISA and inhibition ELISA

Twenty-two sera from the AD patients of the University Hospital, Utrecht were used in ELISA. Binding of IgE antibodies was assessed in microwells coated with a 1/10 dilution in PBS of stock solutions of the various *P. orbiculare* extracts. The stock solutions were prepared by dissolving lyophilized material at 1 mg/mL in sterile PBS, filtering through 0.45 Millipore to remove non-dissolved materials, and stored at -40°C until use [17]. Frozen stock solutions were used for up to 2 months, during which period no loss of IgE-binding activity was observed.

Crossreactivity of *P. orbiculare* preparations was studied with inhibition ELISA. Appropriately diluted sera were preincubated overnight with the extract to be tested at final concentrations of 0.01 and 0.1 mg/mL, and (residual) IgE reactivity was measured by direct addition of the mixture to coated ELISA wells [17].

Each sample was tested in duplicate, both in ELISA and inhibition ELISA.

SDS-PAGE and immunoblotting

Components of *P. orbiculare* were separated according to molecular size by electrophoresis of SDS-treated and reduced samples in 7.5–20% polyacrylamide gradient gels. All the extracts were run at a concentration of 25 μg of freeze-dried extracts/mm gel. After electrophoresis, one gel was stained with the standard Coomassie blue staining method. The other gels were blotted on the nitrocellulose membrane. The strips of different *P. orbiculare* extracts were incubated overnight with 0.5 mL of the MoAbs 9G9 (concentration 0.0002 $\mu\text{g}/\text{mL}$) and 1D5 (concentration 0.0012 $\mu\text{g}/\text{mL}$) or with the four sera from AD patients from Karolinska Hospital, Stockholm, diluted 1:6 in PBS containing 0.2% bovine serum albumin and 0.1% Tween-20. Blotting and immunological detection of IgE-binding by the blotted components were performed as reported previously [13,15]. In previous studies we have found that reduced and non-reduced conditions have comparable results in immunoblotting of *P. orbiculare* extract using sera from patients with atopic dermatitis (data not shown).

Measurement of protein and carbohydrate contents

The protein concentrations were measured with Pierce bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA), bovine serum albumin

serving as a standard [18]. Sugar concentrations were assessed by the phenol-sulfuric acid method of Dubois *et al.* [19] and were confirmed by sugar analysis [20].

Results

Immunoblotting experiments with sera from four AD patients showed a range of IgE-binding proteins of 10–100 kDa with extract KH-4, whereas extracts CM and D seemed to be completely devoid of IgE-binding components (Fig. 1). Preparation CE showed at most a few IgE-binding components at 20–40 kDa (Fig. 1).

Since extracts D, CM and CE had been successfully used in immunoassays to detect IgE reactivity to *P. orbiculare* [3,9,16,17] a systematic comparison was made of the reactions of 22 AD sera with ELISA microwells coated with these extracts or with extract KH-4. All extracts were coated at 0.1 mg/mL, and KH-4 was also tested at 0.01 mg/mL, since in preliminary tests some sera reacted slightly more strongly with this lower concentration. In contrast to the different patterns showed by immunoblotting, strong correlations were found for the ELISA results obtained with the various extracts. In Table 2, the results of all comparisons are summarized with the r^2 -values found by linear regression analysis of log-transformed OD492 values. The lowest correlations were noted for comparisons of extracts D, CE and CM with KH-4 coated at 0.1 mg/mL (0.742, 0.814 and 0.785 respectively), but even these values were remarkably high, and thus suggested that the immunochemical differences between the four preparations might be much less than would be concluded from the immunoblotting results.

To determine whether these strong correlations were due to crossreactivity, IgE inhibition ELISAs were performed with four AD sera from the University Hospital, Utrecht. In three sera with strong reactions to all extracts, partial (30–60%) inhibition of IgE binding to KH-4 was observed after preincubation with either 0.01 or 0.1 mg/mL of extracts D, CE or CM (shown in Figs 2a, b & c). On the contrary, KH-4 was a much more effective inhibitor, not only of reactions with KH-4 itself, but also of IgE binding to D, CE and CM (Figs 3a–d). When a serum with a strong reaction to KH-4 (OD492 1.75) and relatively weak reactions to the other extracts (OD492 0.3–0.7) was tested in the same way, D, CE and CM did not inhibit (<5%) the reaction with KH-4 (Fig. 2d), whereas the reactions with D, CE and CM were significantly (35–80%) inhibited by KH-4 (Fig. 3d). Thus, most or all of the epitopes involved in IgE binding to any of the *P. orbiculare* extracts seemed to be present in KH-4, while the other extracts contained only a limited proportion of the IgE-binding epitopes of KH-4.

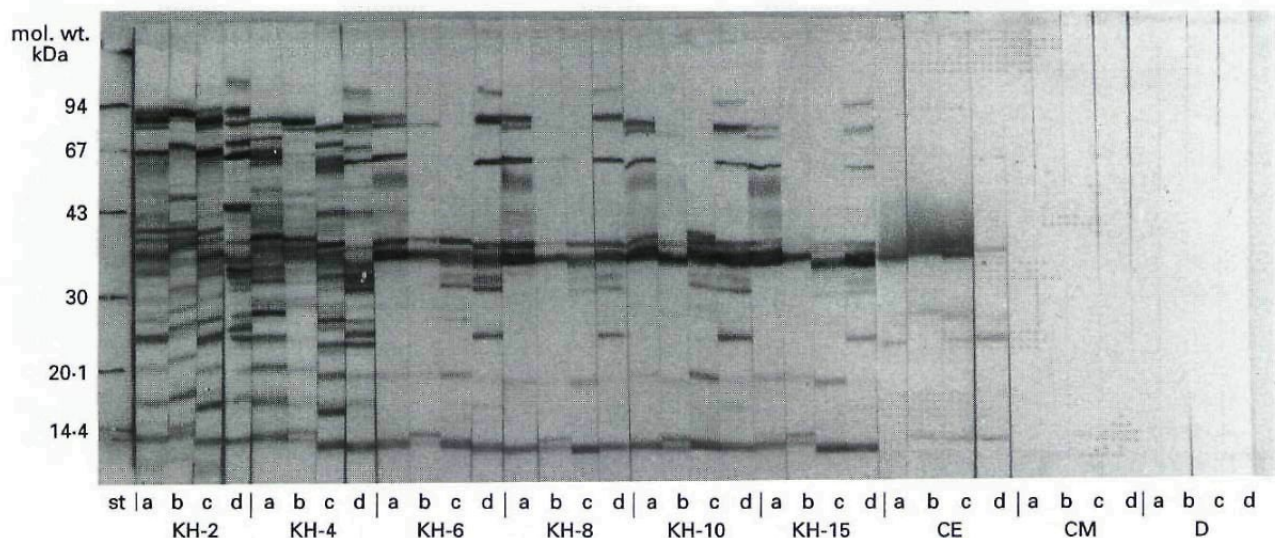


Fig. 1. IgE-binding components in various preparations of *P. orbiculare* (see Table 1) detected by immunoblotting with four AD sera (a, b, c, d) with RAST class to *P. orbiculare* 4, 3, 4 and 5 respectively. Molecular weights of different components in kDa were compared with a low-molecular-weight standard (Pharmacia).

At least one obvious feature in which KH-4 differed from the other extracts was the duration of culture period used for the growth of *P. orbiculare*. To investigate whether this factor might indeed account for the observed differences, the *in vitro* culture system as used for the production of KH-4 was repeated with varying culture periods, ranging from 2 to 15 days. Direct assessment of protein and carbohydrate concentrations revealed a decline in the protein content of the resulting extracts, from approximately 40% at day 2 or 4 to 25% after 15 days (Table 1). For comparison, CE and CM, obtained after 10–14 days of fluid phase culture, were in

this respect similar to KH-10 and KH-15, while extract D, cells harvested from solid phase after more than 20 days, contained only 9% protein. SDS-PAGE confirmed the marked loss of proteins, particularly in the 10–100 kDa range during prolonged culture (Fig. 4). The most dramatic changes seemed to occur between day 4 and day 6, but even between days 2 and 4 some differences in the protein profile could already be noted. Despite their similar total protein concentrations, the SDS-PAGE profiles of KH-10 and KH-15 differed markedly from those of CE and CM, which may indicate that the type of culture system (fluid vs solid phase) and/or strain differences may also influence the biochemical composition. As previously reported [16], extract D showed no protein bands at all in SDS-PAGE (Fig. 4).

The gradual loss of proteins during *in vitro* culture was paralleled by a similar loss of allergenic reactivity in immunoblotting with four AD sera, which were selected on the basis of their strong RAST reactivity with *P. orbiculare* (Fig. 1). One of the best 'surviving' components in the KH extract appeared to be the 37 kDa component which has previously been identified as a major allergen [15]. The concentration of this protein, however, also decreased during culture, as shown by immunoblotting with MoAb 9G9 (Fig. 5). Preliminary immunoblotting of extract KH-4 with MoAbs 9G9 and 1D5 (anti-67-kDa protein) showed that the optimal concentrations for these MoAbs are 0.0002 µg/mL and 0.0012 µg/mL respectively. In these concentrations MoAb 9G9 showed a weak reaction with KH-10 and no reaction with KH-15 while MoAb 1D5 reacted with

Table 2. IgE response to *P. orbiculare* in 22 AD sera measured with ELISA and different *P. orbiculare* extracts

Code*	<i>P. orbiculare</i> extracts			
	KH-4 (0.1)	KH-4 (0.01)	CE	CM
D	0.742**	0.863	0.924	0.903
KH-4 (0.1)		0.927	0.814	0.785
KH-4 (0.01)			0.918	0.885
CE				0.956

* See Table 1 for abbreviations. All extracts were coated at 0.1 mg/mL; extract KH-4 in addition at 0.01 mg/mL.

** Linear regression was performed with log-transformed OD492 values; the figures represent the r^2 -values for each pair-wise comparison.

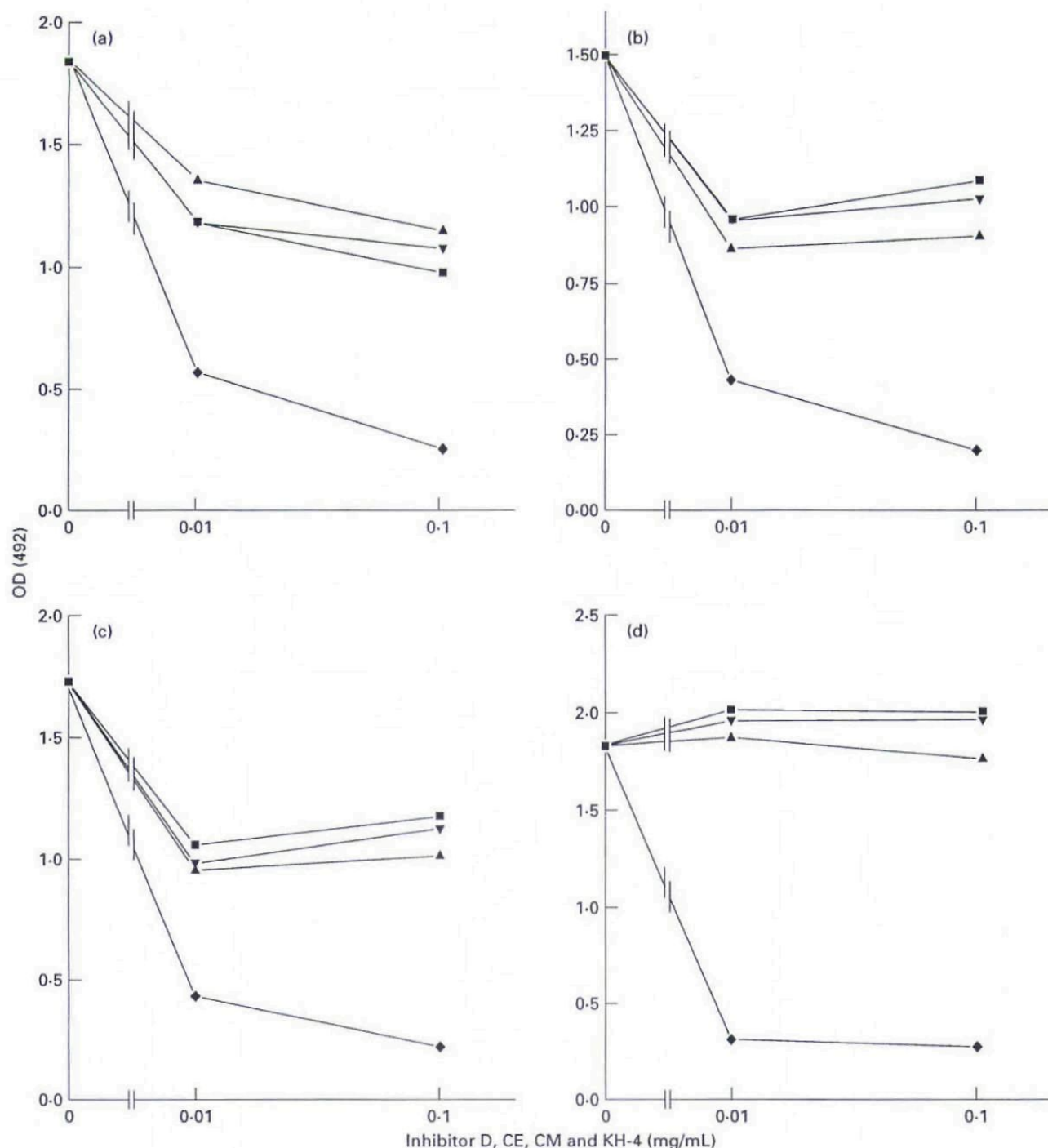


Fig. 2. Crossreactivity of *P. orbiculare* allergen extracts with four AD sera. Inhibition IgE-ELISA with microwells coated with KH-4 extract at 0.1 mg/mL, where the extract D ■, CE ▲, CM ▼ or KH-4 ◆ was used as inhibitor at concentrations of 0.01 and 0.1 mg/mL. Serum no. 1 (a) diluted 1:150, serum no. 2 (b) diluted 1:25, serum no. 3 (c) diluted 1:30 and serum no. 4 (d) diluted 1:30 in PBS buffer.

all KH extracts but also with decreasing intensity (Fig. 5). The MoAbs did not react with extracts CE, CM and D in immunoblotting (Fig. 5).

The total carbohydrate concentrations were apparently

independent of the culture period (Table 1). The only preparation with a lower carbohydrate concentration was CM which is dialysed culture media of CE. Sugar analysis showed that the mannose content of KH-2 was

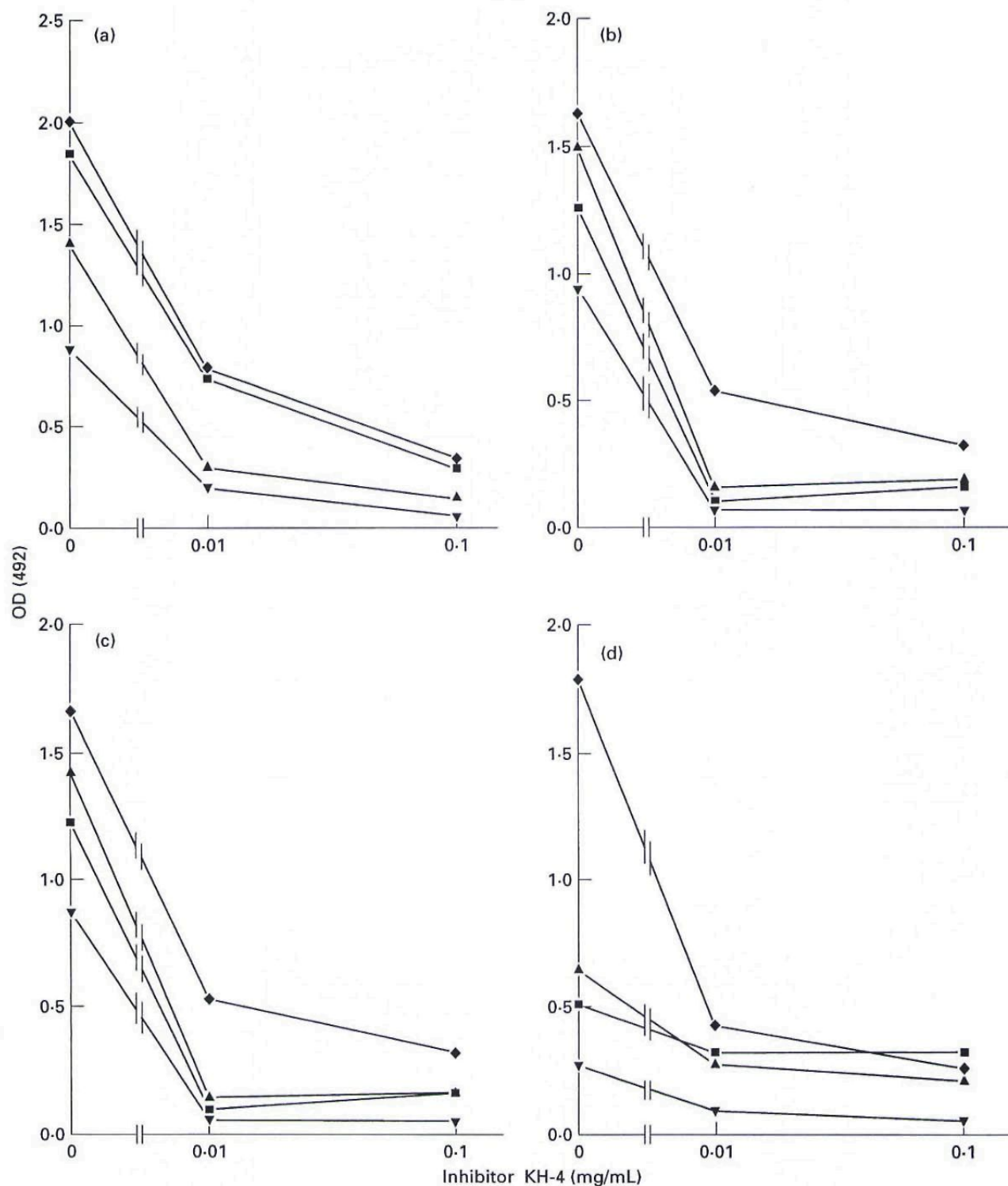


Fig. 3. Crossreactivity of *P. orbiculare* allergen extracts with four AD sera (the same sera as in Fig. 2). Inhibition IgE-ELISA with microwells coated with *P. orbiculare* preparations D ■, CE ▲, CM ▼ or KH-4 ◆, using 4 AD serum no. 1 (a) diluted 1 : 100, serum no. 2 (b) diluted 1 : 25, serum no. 3 (c) diluted 1 : 20 and serum no. 4 (d) diluted 1 : 20. As inhibitor extract KH-4 was used at 0.01 and 0.1 mg/mL.

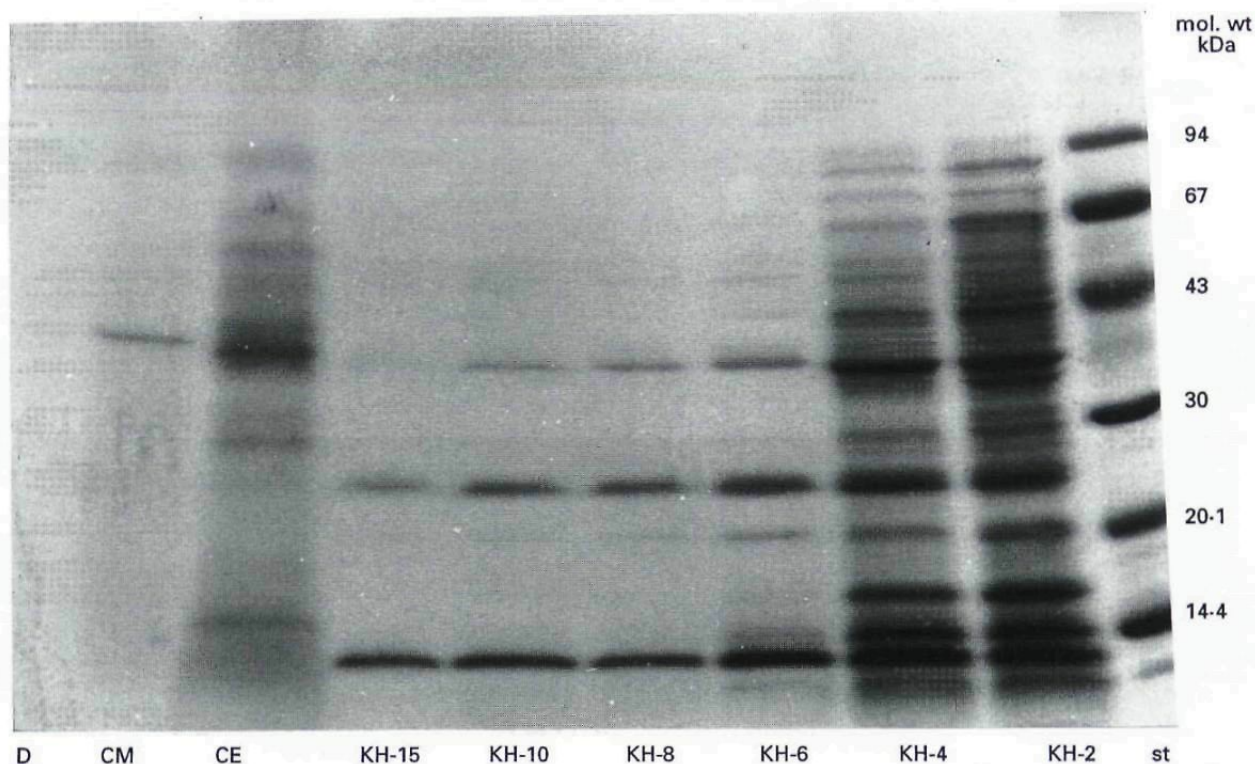


Fig. 4. SDS-PAGE of different preparations of *P. orbiculare* extracts (see Table 1) stained with Coomassie brilliant blue. The last lane is a low-molecular-weight standard (Pharmacia).

about 3.2% (dry weight extract), of KH-4 3.6%, of KH-6 2.8%, of KH-8 2.6% of KH-10 3.1%, of KH-15 2.2%, of CE 3.2%, of CM 2.3% and of D 2.5%. In a previous study we concluded that the major IgE-binding structures in extract D might be HMW glycoproteins or polysaccharides [16]. The results of the inhibition ELISA suggested that such compounds were also present, in at least comparable or higher concentrations, in KH-4 (Fig. 3). We therefore investigated whether this might also be true for the other KH extracts. In inhibition ELISA twofold serially diluted extracts (from 0.1 to 0.003 mg/mL) were tested as inhibitors of the IgE binding to coated extracts D and KH-4. In Table 3 the results obtained with one representative serum are given.

Obviously, all KH-extracts were effective inhibitors of the IgE reactions with extract D. Moreover, the inhibitory capacity appeared to be relatively independent of the culture period (less than twofold difference between days 2, 4 and 15), and was for this serum even three to fivefold higher than that of extract D itself. Similar findings were obtained with two other sera: all KH-extracts inhibited the IgE binding to extract D, with little variation between extracts obtained after different culture periods (data not shown). In the other inhibition ELISA, all the KH-extracts inhibited the reaction with

KH-4, but with strikingly different inhibitory capacities, which in fact decreased four to fivefold between days 4 and 6 (Table 3), in agreement with the similar change in reactivity shown by immunoblotting (Fig. 1). Essentially identical findings were obtained with three other sera; overall, the relative inhibitory capacities in the four tested sera were (range) 100–136% for KH-2, and decreased to 13–32%, 6–24%, 11–29%, and 5–16% for KH-6, -8, -10 and -15, respectively.

Discussion

Characterization of *P. orbiculare* allergens has, until now, been described in only a few reports. Immunoblotting of crude allergen preparations has revealed a range of IgE-binding proteins of 10–100 kDa in extracts of disrupted (freeze-dried and/or sonicated) whole yeast cells, obtained after 2–4 days of solid or fluid phase cultures [13–15]. Although in one of these studies the composition of the extracts was shown to depend on the extraction procedure [14], distinct patterns of IgE-binding components were found in all cases.

Although different extraction procedures might have contributed to the differences in biochemical composition, a more probable explanation might be the

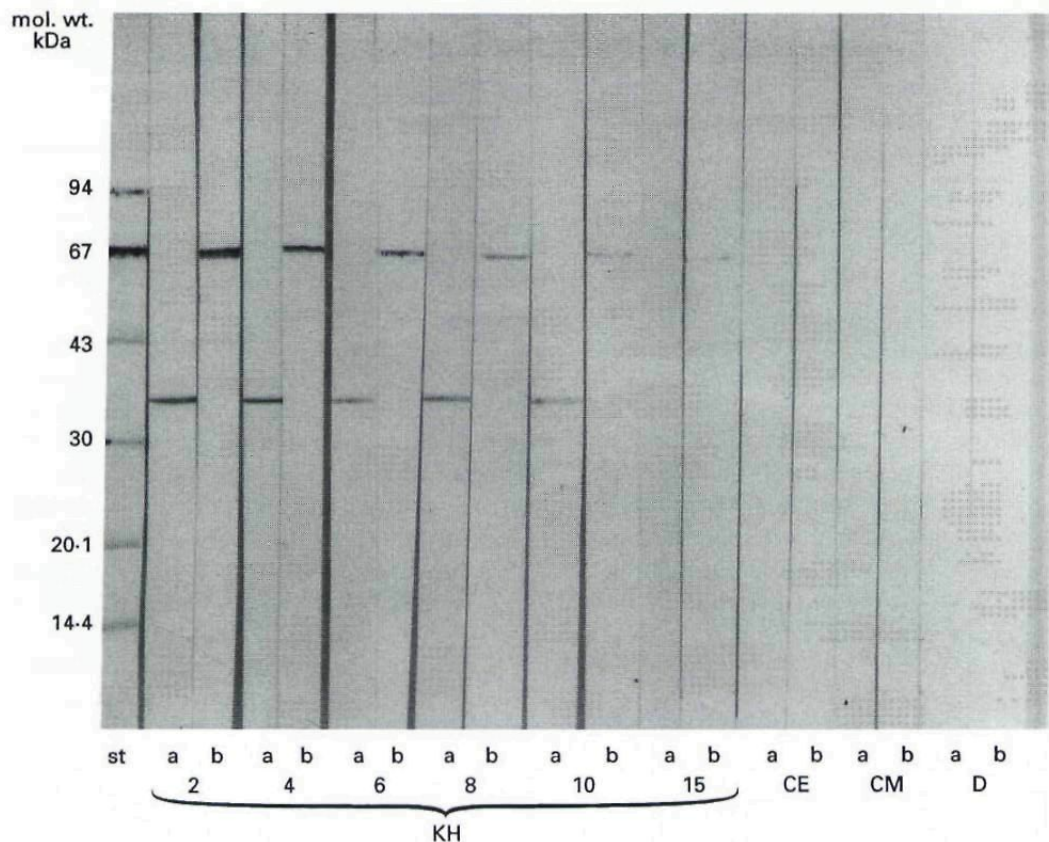


Fig. 5. Identification of 37-kDa and 67-kDa protein components with immunoblotting in different *P. orbiculare* preparations (see Table 1) by MoAbs 9G9 (anti-37kDa) (a) and 1D5 (anti-67-kDa) (b). The first lane is a low-molecular-weight standard (Pharmacia).

Table 3. Inhibition ELISA of IgE binding to *P. orbiculare* extracts D and KH-4 by extracts KH-2 to KH-15

Inhibitor	Inhibition of IgE binding to coating of the wells with	
	D (0.1 mg/mL)	KH-4 (0.01 mg/mL)
KH-2	6.5 (4.5)*	2.5 (1.36)
KH-4	5.8 (5.0)	3.4 (1.0)
KH-6	7.9 (3.7)	16.5 (0.21)
KH-8	8.5 (3.4)	26.5 (0.13)
KH-10	7.6 (3.8)	14.8 (0.23)
KH-15	9.9 (2.9)	33.7 (0.10)
D	29 (1.0)	>200 (<0.02)

* Data from one representative AD serum is given as $C_i(50)$ which is the concentration ($\mu\text{g/mL}$) required for 50% inhibition and (in parentheses) relative inhibitory capacity defined as $C_i(50)$ coated extract/ $C_i(50)$ test extract.

different culture procedures, and in particular the different culture periods. Immunoblotting of the KH-2-15 extracts shows that prolongation of the culture time will lead to loss of the allergen components with protein entity. The use of either a solid (extract D) or a fluid phase culture system (CE and CM) seemed to be of less importance.

In a previous study [16], the investigated *P. orbiculare* extract (preparation D in the present report) remained practically negative in immunoblotting. Chromatographic fractionation — mainly Concanavalin A chromatography and gel filtration — and the results of periodate — and protease treatment led to the conclusion that the IgE-binding components of *P. orbiculare* might be mainly HMW polysaccharides or glycoproteins, presumably mannans or mannoproteins [16]. Our sugar analysis showed that the mannose content of the *P. orbiculare* extracts was nearly constant, regardless of the culture time. The CE and CM preparations, also included in the present study, appeared to have similar properties compared with extract D, and also crossreacted mainly with *C. albicans* prepared in the same way (Doekes and

van Ieperen-van Dijk, unpublished observation). The preparations CE and CM showed few bands in SDS-PAGE and immunoblotting but they correlated with extract KH-4 in ELISA. The present data and the results of several other studies indicate the existence of two types of *P. orbiculare* allergens. First, a number of specific IgE-binding proteins or glycoproteins appear to be readily detectable by immunoblotting as distinct bands in the 10–100 kDa mol. wt. range [13–15]. Optimal production of these components may require the use of relatively short-term cultures, harvested when the yeast cells are still actively growing, and with little accumulation of dead and partially degraded material in the culture. The second type of *P. orbiculare* allergens would be polysaccharides or glycoproteins (probably mannans or mannoproteins) of relatively high, and varying, molecular weight. These components, probably mainly cell wall constituents, are recovered both from short- and long-term cultures, and show extensive crossreactivity with analogous allergenic structures from *C. albicans* [17,21] and possibly also with mannans of other yeasts such as *Saccharomyces cerevisiae* [22].

Recognition of these two types of allergens was due to the very low growth rate of *P. orbiculare* strain no. 1878, even in an optimal, oil-containing medium, which led us and others to allow relatively long culture periods. The present data suggest that despite the relatively low cell numbers in culture there might be extensive cell death and proteolytic degradation of cytoplasmic proteins during this period. On the contrary, the carbohydrate concentration of the short- and long-term cultures seemed to be nearly constant, except for preparation CM which was dialysed culture media of CE (see Table 1). We assume that HMW polysaccharide antigens might be much more stable. The exact nature of the latter components is as yet, however, unknown. Previously, we suggested that they might be mainly cell wall mannans or mannoproteins, which are, e.g. in the case of *C. albicans*, highly immunogenic soluble antigens that can easily be extracted from yeast cells in aqueous solutions without detergents [23,24]. These features also agree with the finding of similar, crossreacting molecules in the culture media (CM).

Another question regarding the nature of the HMW allergens of *P. orbiculare* concerns the possible relationship with extracellular polysaccharides (EPS) of moulds, which are stable polymeric carbohydrates of variable composition, produced and secreted during *in vitro* culture of many mould species [25]. EPS are highly antigenic in rabbits, and recent studies have identified 2-O-methyl-mannoside residues as important epitopes [26]. Anti-EPS IgG can also be found in many human sera [27], but to our knowledge no data have been

reported showing a role for EPS as IgE-binding allergens in, e.g. mould or yeast allergy. The hypothesis is, however, strongly supported by the presence of IgE-binding components, probably HMW polysaccharides, in the culture media (CM) of long-term *P. orbiculare* cultures.

An initially surprising finding was the close correlation between IgE reactions in AD patients towards the different preparations (Table 2) and the apparent immunochemical differences of the allergenic components shown in Fig. 1. The explanation would be that extracts such as KH-4 contain, in addition to the IgE-binding proteins or glycoproteins of 10–100 kDa (Fig. 1), substantial amounts of the IgE-binding HMW polysaccharides that account for the major part of IgE binding activity in preparations such as D, CE and CM. The inhibition ELISA with KH extracts obtained after various culture periods (Table 3) confirmed this hypothesis.

Determination of anti-*P. orbiculare* IgE and/or skin tests have not yet become standard practice in diagnosis of AD patients, and consequently, standardization of *P. orbiculare* allergen preparations has been given little priority. Since the inhibition ELISA and immunoblotting assays show that the majority of IgE-binding components were present in preparations KH-2-4, these preparations might be considered the better choice among those investigated in our study. In conclusion, extracts obtained from log phase yeast cultures (2–4 days old) should be preferred in studies on IgE response to *P. orbiculare*.

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