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Inhalant Allergies to Fungi: Reactions to Bakers' Yeast (*Saccharomyces cerevisiae*) and Identification of Bakers' Yeast Enolase as an Important Allergen

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Abstract. Forty-seven subjects diagnosed as having inhalant allergies to fungi were tested for allergic sensitivity to bakers' yeast. Skin prick tests with yeast extract showed that 35 subjects responded with wheal reactions that were at least 3 mm while 32 subjects were regarded as clearly RAST-positive to bakers' yeast antigens. Skin and RAST testing with purified enolase from bakers' yeast and comparisons with the whole yeast extract showed that the enzyme is a major allergenic component of the extract. This conclusion was supported by results of electroblotting studies. RAST inhibition experiments demonstrated allergenic cross-reactivity between bakers' yeast, bakers' yeast enolase and *Candida albicans*.

Introduction

Allergies due to fungi remain one of the most neglected areas of study in the field of allergic diseases. Consequently, we often have little knowledge of the fungal species involved in an allergic reaction and their relationship to clinical symptoms [1-3]. Attempts to identify fungal allergens are also preliminary and the extent of allergenic cross-reactivity for even the common airborne species remains to be determined [4-6].

In a large group of patients with suspected inhalant allergy to airborne fungi, skin prick testing showed that positive reactions to an extract of *Saccharomyces cerevisiae* (bakers' yeast) occurred in a high proportion of the subjects. Further studies employing skin prick tests and radioallergosorbent tests (RASTs) revealed that bakers' yeast enolase was an important and potent allergen in the subjects examined.

Data supporting this conclusion are presented together with results suggesting that allergenic cross-reactivity exists between bakers' yeast extract and enolase on the one hand and the imperfect 'yeast' *Candida albicans* extract on the other.

Materials and Methods

Subjects

In a large Sydney clinical allergy practice, the sera of 76 subjects with multiple inhalant 'mould' allergies were selected for inclusion in a Pharmacia RAST trial of fungal allergen discs [Baldo and Baker, unpubl. data]. Subjects were classified as allergic to fungi on the basis of their case histories and skin prick test reactions to a panel of fungal extracts which included *Alternaria tenuis*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Botrytis cinerea*, *C. albicans*, *Cladosporium herbarum*, *Curvularia lunata*, *Epicoccum purpurascens*, *Fusarium moniliforme*, *Helminthosporium halodes*, *Mucor racemosus*, *Penicillium notatum*, *Phoma betae*, *Rhizopus nigricans*, *Stemphylium botryosum* and *Trichoderma viride*, and to a panel of common inhalant allergenic extracts including grasses, mites and foods. Following some preliminary findings on a few patients which showed that many of the subjects responded with strong skin prick test reactions to bakers' yeast extract, an attempt was made to systematically examine the patients' IgE antibody and skin prick test responses to this possible allergen source. Accordingly, we set out to skin test all 76 of the selected mould-allergic subjects but this did not prove possible and a complete set of results including skin prick tests and RASTs could be obtained for only 47 subjects. Each of the 47 subjects had symptoms of rhinitis and/or asthma and, in a few patients, these conditions were accompanied by eczema and/or urticaria (see below).

Bakers' Yeast and Enolase

Bakers' yeast was obtained from Société Industrielle Lesaffre (Marcq-en-Baroeul, France). Purified enolase from bakers' yeast

(EC 4.2.1.11) [7, 8] was provided by Prof. J.M. Brewer (Department of Biochemistry, The University of Georgia, Athens, Ga.).

Bakers' Yeast Extract

Dry bakers' yeast (50 g) was ground in a mortar, stirred overnight at 4°C with 100 ml of water, centrifuged at 12,000 rpm at 4°C for 30 min, filtered, dialysed against 6 changes of water and freeze-dried. The yield was approximately 1 g.

C. albicans Extract

C. albicans aqueous extract, labelled as Candida Immunodiffusion Antigen, was purchased from Immuno-Mycologics Inc., (Norman, Okla.). The protein content of the extract, using the method of Bradford [9], was found to be 324 µg/ml.

Skin Tests

For prick testing, yeast extract and enolase were each formulated at concentrations of 1 and 10 mg/ml in a diluent of 50% glycerin in sterile saline. Diluent was added to the dry powders immediately before use and the reconstituted solutions were stored frozen. Solutions were tested for non-specific irritant effects by skin prick testing non-allergic, healthy individuals. Prick testing was performed as described previously [10]. Wheal sizes were recorded 15 min after testing as the average diameter $(D + d)/2$, where D is the largest diameter of the wheal and d the diameter perpendicular to this.

RAST and RAST Inhibition

Direct RASTs were carried out with paper discs [11] using ^{125} I-anti-human IgE (Kallestad Laboratories Inc., Austin, Tex.). For covalent coupling to CNBr-activated paper discs, proteins were used at concentrations equivalent to 10–200 µg/disc. RAST inhibition studies were carried out as previously described [12].

Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out using the PhastSystem™ (Pharmacia, Uppsala, Sweden) and according to the PhastSystem Separation Technique File numbers 110 (for SDS-PAGE) and 200 (for staining). Proteins were separated on 8–25% gradient PhastGels with the aid of PhastGel SDS Buffer Strips (Pharmacia). Alkaline gradient PAGE was carried out in 3–40% highly cross-linked (GG-HX) acrylamide gels (Gradient Pty. Ltd, Pyrmont, Australia) as described by Tovey and Baldo [13]. Gradient gels were stained with Coomassie brilliant blue G250 (Sigma Chemical Co., St. Louis, Mo.) by the method of Neuhoof and Stamm [14].

Electroblotting and Detection of IgE-Binding Proteins

The methods used have been described in detail [13, 15–17].

Results

Skin Prick Test and RAST Findings

In almost every one of the 47 subjects, clear wheal and flare skin reactions to bakers' yeast extract (10 mg/ml) were obtained. Subjects showing strong reactions also invariably responded with clear wheal

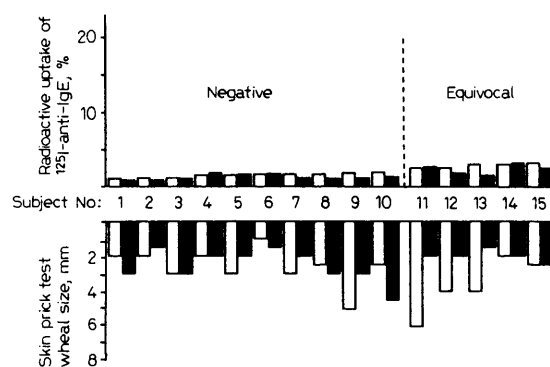


Fig. 1. RAST and skin prick test results for 10 individuals whose sera were classified as RAST-negative (radioactive uptakes 2.5 times the cord serum/normal serum value) to bakers' yeast allergens and for 5 individuals whose sera were classified as equivocal reactions in the RAST (radioactive uptakes 2.5–3.5 times the cord serum/normal serum value). Open bars show RAST and skin prick test results with bakers' yeast extract, black bars the corresponding results with purified bakers' yeast enolase. Results with each subject are arranged in increasing order of RAST reactivity with bakers' yeast extract.

and flare reactions to the extract when it was used at a concentration of 1 mg/ml. By contrast, no skin test reactions were seen when the same extract at a concentration of 10 mg/ml was used to test 10 non-allergic subjects and 10 grass-pollen- and/or mite-allergic subjects who were not allergic to any fungi.

Only 1 of the 47 subjects prick-tested with bakers' yeast extract responded with a wheal size of less than 2 mm while 35 subjects responded with wheals of at least 3 mm and almost half (23) of the subjects produced wheals of 4 mm or more. Nine subjects responded with wheals of at least 6 mm. These results suggested that IgE antibodies that react with antigens of bakers' yeast might be found in the sera of many of the subjects. The likelihood of this seemed to be increased by results of patients' skin test reactions to enolase, a purified protein from bakers' yeast. Twenty-three subjects responded to enolase with wheals of at least 4 mm and 17 subjects produced wheals of 5 mm or more.

Sera from all 47 subjects were subjected to RAST testing using both bakers' yeast extract and enolase. A negative RAST was taken as a radioactive uptake (of ^{125} I-anti-human IgE) of less than 2.5 times the cord/normal serum value [13, 14]. Uptakes of 2.5–3.5 times the cord serum value were recorded as equivocal while uptakes of 3.6 times and over were grouped as

Fig. 2. RAST and skin prick test results for 32 individuals whose sera were classified as RAST-positive to bakers' yeast allergens. Subjects are grouped as weak (3.6–5 times the cord serum/normal serum radioactive RAST uptake), medium (5.1–10 times) and strong (> 10 times) RAST reactors. Open bars show RAST and skin prick test results with bakers' yeast extract, black bars the corresponding results with purified bakers' yeast enolase. Results with each subject are arranged in increasing order of RAST reactivity with bakers' yeast extract.

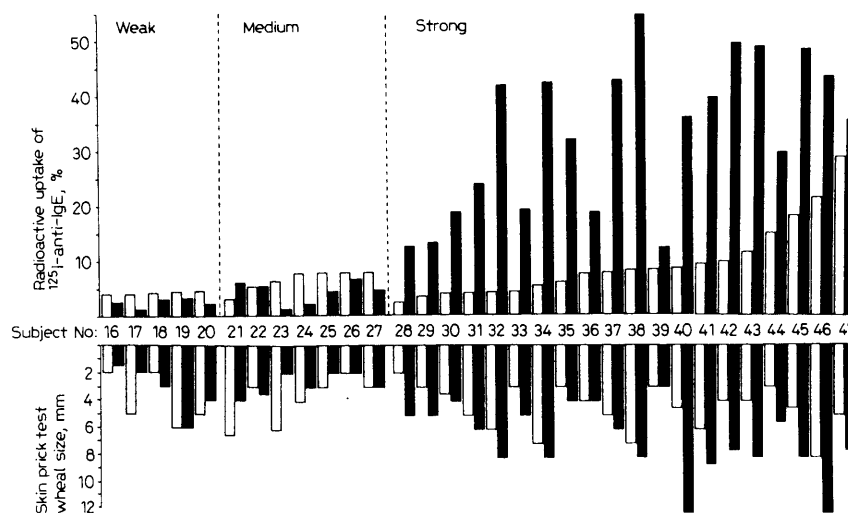


Table I. Relationships between RAST reactions to bakers' yeast (*S. cerevisiae*) extract and skin prick test responses to bakers' yeast and bakers' yeast enolase in 47 subjects with suspected inhalant allergy to fungi

RAST classification ¹	Number of sera	Number of subjects showing a prick test reaction to					
		bakers' yeast			bakers' yeast enolase		
		wheal size: < 2 mm	< 3 mm	< 4 mm	< 2 mm	< 3 mm	< 4 mm
Negative	10	1	6	9	2	5	9
Equivocal	5	0	2	2	1	5	5
		wheal size: ≥ 2 mm	≥ 3 mm	≥ 4 mm	≥ 2 mm	≥ 3 mm	≥ 4 mm
Positive							
Weak	5	5	3	3	4	3	2
Medium	7	7	6	3	7	4	1
Strong	20	20	19	13	20	20	19
Total	32	32	28	19	31	27	22

Subjects were thought, on the basis of case histories, to have inhalant allergy to fungi. Symptoms included rhinitis and/or asthma, and sometimes eczema and/or urticaria. Bakers' yeast extract and bakers' yeast enolase were used at 10 mg/ml in glycerin-saline 50:50.

¹ RAST negative, <2.5 times uptake of ¹²⁵I-anti-human IgE obtained with cord serum; equivocal, 2.5–3.5 times; weak positive, 3.6–5.0 times; medium positive, 5.1–10.0 times; strong positive, > 10.0 times radioactive uptake with cord serum.

clear positive reactions. The positive group was further subdivided into weak (3.6–5.0 times cord serum), medium (5.1–10.0 times cord serum) and strong (> 10.0 times cord serum) reactions. The results are summarized in figures 1 and 2, where reactions of individual sera within each section are arranged in increasing order of RAST reactivity with bakers' yeast extract, and matched RAST enolase reactions and skin prick test responses are included. The relation-

ships between the skin prick test responses to both bakers' yeast and enolase and the RAST results with baker's yeast for each of the different RAST classifications (negative, equivocal, positive) are set out in table I. Sera from 10 subjects proved to be RAST-negative to bakers' yeast extract and enolase and 5 other sera were regarded as doubtful positives (that is, equivocal). Agreement between the RAST results for the negative and equivocal groups and skin prick test

Table II. Relationships between symptoms and RAST reactions to bakers' yeast (*S. cerevisiae*) antigens in 47 subjects with suspected inhalant allergy to fungi

RAST classification ¹	Number of sera	Number and percentage of subjects with			
		asthma	rhinitis	eczema	urticaria
Negative	10	3 (30)	9 (90)	1 (10)	2 (20)
Equivocal	5	3 (60)	5 (100)	2 (40)	1 (20)
Negative and equivocal	15	6 (40)	14 (93)	3 (20)	3 (20)
Positive					
Weak	5	4 (80)	5 (100)	1 (20)	0 (0)
Medium	7	4 (57)	6 (86)	2 (29)	0 (0)
Weak and medium	12	8 (67)	11 (92)	3 (25)	0 (0)
Strong	20	14 (70)	18 (90)	3 (15)	2 (10)
Total	32	22 (69)	29 (91)	6 (19)	2 (6)

Numbers in parentheses are percentages.

¹ See footnote 1 in table I.

wheel sizes of <2 mm were poor for both bakers' yeast extract and enolase but considerably better for wheel sizes of <3 and <4 mm. For the positive RAST group which comprised 32 subjects, skin test wheel sizes were in much better agreement. For example, when ≥ 2 and ≥ 3 mm were taken as the cut-off points for a positive skin prick test reaction, the number of subjects recorded as positive were 32 and 28, respectively, for bakers' yeast extract, and 31 and 27, respectively, for enolase. For a positive cut-off point of ≥ 4 mm, agreements were not as good although 19 of the 20 strongly RAST-positive subjects for enolase recorded wheel sizes of this order. Overall, results showed that the best agreement between the RAST results for all 3 groups (negative, equivocal, positive) and skin prick test reactions were obtained when a wheel size of 3 mm was used as the positive cut-off figure (table I).

Of the 32 sera from RAST-positive patients, 22 showed RAST uptakes with enolase that were equal to, or higher than, the uptakes recorded with bakers' yeast extract. Likewise, skin prick tests on these 32 subjects revealed that in 25 subjects wheel sizes to enolase were equal to, or greater than, wheel sizes recorded for bakers' yeast extract (fig. 2). For almost all of these 25 subjects the superior biological potency of enolase, on a weight for weight basis, was maintained

even when it was used at a concentration equal to one tenth of the concentration of the bakers' yeast extract prick test solution.

Relationships between Patients' Symptoms and the Presence of IgE Antibodies to Bakers' Yeast Antigens

The numerical relationships between symptoms and RAST reactions in the 47 subjects are summarized in table II. Major differences within the RAST-positive groups (weak, medium and strong) and between RAST-positive and RAST-negative groups for subjects with asthma, rhinitis, eczema and urticaria were not detected.

Demonstration by Electrophoretic of IgE-Binding by Purified Enolase and Enolase in Bakers' Yeast Extract

SDS-PAGE of purified enolase revealed a single band (fig. 3c, e) running at a position in the gradient gel corresponding to a molecular weight of approximately 51,000 daltons which is close to the published figure of 52,000 daltons for the bakers' yeast enolase subunit [18]. A clearly stained band at a point in the gel corresponding to the mobility of the enolase standard was observed in the electrophoretically separated pattern of bakers' yeast proteins (fig. 3d). Fol-

lowing gradient gel electrophoresis under non-dissociating conditions of bakers' yeast extract and purified bakers' yeast enolase, the separated bands were electrophoretically transferred to nitrocellulose membrane and probed with sera from subjects who gave strong RAST reactions to bakers' yeast and enolase discs. Protein staining of the gels (fig. 4A) revealed a complex array of bands in the whole extract (fig. 4A, b) and two bands following electrophoresis of the highly purified enolase preparation (fig. 4A, a). The appearance of more than one enolase band on gradient gels under non-dissociating conditions may be due to deamidation or partial denaturation which can produce electrophoretically distinct forms. IgE antibodies in the sera used for probing the transferred proteins reacted strongly with the transferred enolase bands (fig. 4B, a) and with bands in the extract that had the same electrophoretic mobility as enolase (fig. 4B, b-d).

Investigation of Possible Cross-Reactions between Bakers' Yeast Extract and Enolase and *C. albicans* Extract

The 47 sera were subjected to further RAST studies with discs prepared from extracts of 15 other fungi. These examinations, to be reported in detail elsewhere [Baldo and Baker, in preparation], revealed a number of interesting findings with one in particular being relevant to the present study. Sera positive to bakers' yeast and enolase were almost invariably also positive to *C. albicans* discs. Ten of the forty-seven sera were classified as RAST-negative or equivocal to *C. albicans* (that is, they produced radioactive uptakes less than 3.5 times the value obtained with cord serum) and each of these sera belonged to the bakers' yeast/enolase RAST-negative/equivocal group.

To further investigate a possible allergenic relationship between these two yeasts, RAST inhibition studies were undertaken. These studies employed discs coupled to bakers' yeast and *C. albicans* proteins and serum pools prepared from sera classified as strong RAST reactors to both fungi (that is, radioactive uptakes greater than 10%). Sera were pre-incubated with bakers' yeast extract, enolase or *C. albicans* extract before adding the solid-phase antigens. With both solid supports enolase was clearly the most potent inhibitor although the bakers' yeast and *Candida* extracts were also active in both the homologous (bakers' yeast extract as inhibitor - bakers' yeast solid support; *C. albicans* extract as inhibitor - *C. albicans*

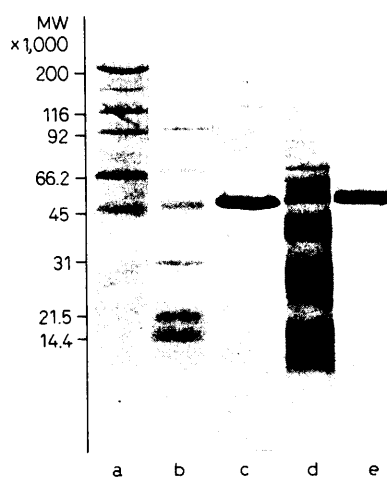


Fig. 3. Examination of purified bakers' yeast enolase and unfractionated bakers' yeast extract on 8-25% PhastGels (Pharmacia) under dissociating conditions in the presence of SDS. a, b = high- and low-molecular-weight (MW) markers (Bio-Rad); c, e = purified enolase (0.5 mg/ml, 1 μ l); d = bakers' yeast extract (20 mg/ml, 1 μ l).

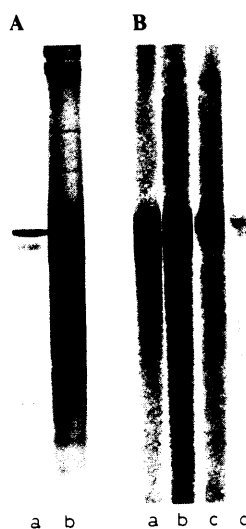


Fig. 4. A Electrophoretograms of purified bakers' yeast enolase (a, 2.5 μ g) and bakers' yeast crude extract (b, 25 μ g) run on 3-40% polyacrylamide gradient gels under non-dissociating conditions for 1,000 Vh. B Purified bakers' yeast enolase (a, 15 μ g) and bakers' yeast crude extract (b-d, 130 μ g) separated on 3-40% polyacrylamide gradient gels under non-dissociating conditions, electroblotted onto nitrocellulose and reacted with sera from subjects strongly RAST- and skin-test-positive to enolase and bakers' yeast extract. IgE antibodies that bound to the enolase bands were detected with ^{125} I-anti-human IgE followed by autoradiography. Strips a and b were incubated with serum from the same subject; strips c and d with sera from 2 different subjects.

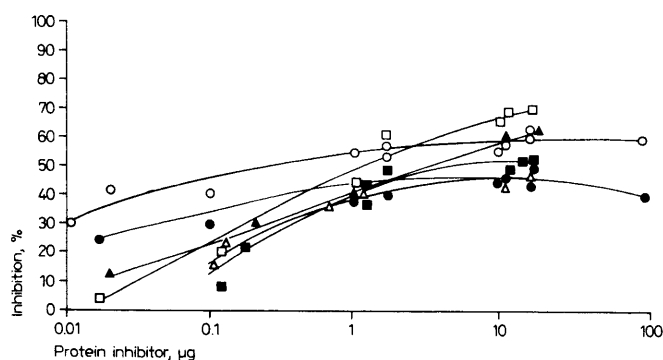


Fig. 5. RAST inhibition results using paper discs coupled to bakers' yeast or *C. albicans* antigens together with serum from subjects classified as strong RAST reactors to both fungi. Uptake of IgE antibodies by the discs was inhibited by pre-incubation of the serum with bakers' yeast enolase or *C. albicans* extract. IgE antibodies that bound to the discs were estimated by the addition of ^{125} I-labelled anti-human IgE. ○ = Enolase inhibitor with bakers' yeast discs; ● = enolase with *C. albicans* discs; □ = bakers' yeast extract with bakers' yeast discs; ■ = bakers' yeast extract with *C. albicans* discs; △ = *C. albicans* extract with bakers' yeast discs; ▲ = *C. albicans* extract with *C. albicans* discs.

Table III. Allergenic cross-reactivity¹ between IgE-binding components from bakers' yeast (*S. cerevisiae*) and *C. albicans*: relative inhibitory potencies of bakers' yeast enolase and extracts of the fungi

Inhibitor	Protein (µg) needed for inhibition ² using discs coupled to proteins from	
	Bakers' yeast extract ³	<i>C. albicans</i> extract ⁴
Bakers' yeast enolase	0.01	0.09
Bakers' yeast extract	0.50	0.80
<i>C. albicans</i> extract	0.75	0.70

¹ Detected with serum pool prepared from sera classified as strong RAST reactors to both fungi.

² Detected as 30% inhibition of the uptake of ^{125} I-labelled anti-human IgE.

³ Discs used with serum diluted 1:10.

⁴ Discs used with serum diluted 1:15.

solid support) and non-homologous (bakers' yeast extract - *C. albicans* solid support; *C. albicans* extract - bakers' yeast solid support) systems. Figure 5 shows typical results obtained with one serum pool and the relative inhibitory potencies of the enolase and extracts used are shown in table III.

Discussion

Although yeasts including *S. cerevisiae* have been implicated in chronic urticaria [19], there appears to be no reports implicating this species in inhalant allergies to fungi. Consequently, data on the allergenic nature and content of bakers' yeast are also lacking. RAST, skin prick tests and IgE binding following electroblotting all showed the allergenic nature of bakers' yeast and demonstrated the importance and potency of the enzyme enolase as a major allergenic component. Skin prick test and RAST responses to enolase that were equal to, or greater than, the responses to bakers' yeast extract suggested that the purified protein is a major contributor to the total allergenicity of the mixed proteins in the crude extract. This conclusion seems justified when one also considers that IgE antibodies in patients' sera reacted only with the enolase bands on nitrocellulose blots of whole bakers' yeast extract. This is not to say that enolase is the only allergen of any significance in *S. cerevisiae*. Further studies, including electroblotting experiments with crude bakers' yeast and more sera from allergic subjects, will almost certainly lead to the identification of other allergenic components in bakers' yeast.

It should be emphasized that even though subjects in this study showed a high incidence of positive skin test and RAST reactions to bakers' yeast enolase this does not necessarily mean that the subjects were allergically sensitized by *S. cerevisiae*. Sensitization may have occurred via another species of fungus and the cross-reactivities detected by RAST inhibition between bakers' yeast extract and bakers' yeast enolase on the one hand and *C. albicans* extract on the other are consistent with this possibility. The allergenic cross-reactivity detected may, of course, extend more widely than *C. albicans* which, like *S. cerevisiae*, may also not be the sensitizing source. Depending on the availability of extracts from other fungal species, it will be interesting to examine allergic populations such as the one studied here for sensitivity to a wide range of different fungi, particularly yeasts, and for allergenic cross-reactivity between species.

The term 'yeast' is of no taxonomic significance and is useful only to describe a morphologic form of a fungus [20]. Yeasts, which may be defined as unicellular fungi reproducing by budding or fission, are taxonomically diverse and include Ascomycetes and Basidiomycetes. A third group, the imperfect yeasts or

Deuteromycetes which have not yet been shown to have a sexual stage, has both ascomycetous and basidiomycetous affinities [21].

The two fungal species studied here, *S. cerevisiae*, an ascomycete, and *C. albicans*, generally considered to be an imperfect yeast, are both freely accessible to man. *S. cerevisiae* is economically important and is very widely used. *C. albicans* co-exists with man as a harmless commensal in the digestive system and vaginal tract and less frequently as an opportunistic pathogen [22].

Although a minimum wheal size of 2 mm is often taken as a positive skin prick test, a number of factors related to the nature of the extract and the patient population should influence [23, 24] any decision on the relationship between wheal size and diagnosis, that is, whether the subject is classified as skin-test-positive or -negative. On the basis of bakers' yeast extract and enolase skin prick test findings with both allergic and normal non-allergic subjects, and comparisons with the RAST results, we selected a skin prick test wheal size of ≥ 3 mm as probably the most likely diagnostic indicator of the presence or absence of immediate hypersensitivity to the fungus *S. cerevisiae*.

Regardless of the source of the fungal hypersensitivity we have studied here, it seems that bakers' yeast and purified bakers' yeast enolase may prove to be useful materials for the diagnosis of fungal hypersensitivity in at least a population of subjects showing symptoms of inhalant allergy to moulds. In addition, bakers' yeast enolase, and perhaps even its cross-reacting protein(s) in other fungal species, promises to be a valuable agent for the standardization of yeast allergenic extracts. A further important point to be kept in mind in relation to bakers' yeast enolase is that this protein has been well characterized and its amino acid sequence is known [8, 25]. Since so few allergens have been purified and sequenced [26], bakers' yeast enolase is therefore also a potentially important molecule for structure-activity studies directed at defining the physicochemical basis of allergenicity.

Acknowledgements

We thank Gail Knowland for technical assistance. This work was funded by the National Health and Medical Research Council of Australia.

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Received: November 16, 1987

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