

Skin-prick tests for hypersensitivity to α -amylase preparations

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Twenty-five asthmatic subjects with suspected α -amylase hypersensitivity were studied by skin-prick tests, a capture ELISA, immunoblotting and bronchial provocation tests. At the same time, different amylases were analysed by SDS–PAGE and immunoblotting using a polyclonal rabbit antiserum. Eight patients showed a positive bronchial response to amylase. Seven of them had positive skin-prick tests, with this method being the most sensitive approach for diagnosis. However, in four cases, skin tests were also positive although the patients had a negative provocation test, thus demonstrating that skin tests are not specific. ELISA and blotting showed similar results in terms of sensitivity and specificity. The enzymes used by the workers included several antigens besides α -amylase. The rabbit antiserum to α -amylase detected a protein in a wheat flour extract. In one case, the IgE antibodies were specific only for a contaminant of lower molecular weight than amylase. These facts suggest that proteins from the culture medium could be responsible for some cases of amylase hypersensitivity, making the diagnosis difficult. The presence of amylase in another enzymatic extract, a protease produced by *Aspergillus oryzae*, was proved by means of skin tests and immunoblotting, thus demonstrating the allergenic properties of this enzymatic preparation.

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Since the first report by Flindt¹ of positive skin tests to the enzyme α -amylase from *Aspergillus oryzae*, several authors have described patients with symptoms caused by sensitization to this antigen^{2–9}. The patients were occupationally exposed to the allergen either in bakeries or the pharmaceutical industries, and showed positive skin-prick tests to aqueous extracts of commercially available amylases. These skin-prick tests were performed using highly concentrated antigen solutions. However, several aspects of this occupational sensitivity remain unclear. In a survey performed in a pharmaceutical company, Losada *et al.*⁴ found that eight out of 14 workers with a positive skin test had a negative bronchial provocation test, and, in another survey of 20 exposed workers, Brisman and Belin² found that three of their six skin-test-positive subjects had a negative nasal provocation test. Furthermore, positive radio-allergosorbent test values were found in an asymptomatic and a non-exposed individual. We recently described a study in which positive skin-prick

tests, ELISAs and immunoblots were found in non-exposed millers⁵, although the percentage of positive skin tests to amylase was considerably higher than for the serological determinations. Recently, Sander *et al.*⁶ have found that low positive amylase enzyme allergosorbent test values can be observed in control sera. Taken together, these facts suggest that some kind of cross-reaction between α -amylase and other allergen(s) might exist. At present, commercially available enzymatic preparations are considered for diagnostic purposes to be pure, so the presence of contaminants has not been considered. If other allergens were contained in the extract used for diagnosis, then the results obtained could be confusing. Bearing in mind these facts, we performed a clinical and immunological study on 25 asthmatic subjects exposed to the enzyme.

MATERIALS AND METHODS

Patients

Twenty-five amylase-exposed symptomatic workers from different bakeries were included in the study. Ten non-exposed subjects served as a control group.

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Antigens

α -amylase was obtained either from the bakeries or purchased from Serva (Heidelberg, Germany). In both cases, the enzymes were diluted in phosphate-buffered saline (PBS) at 20% w/v and extracted for 3 h while shaking. The extract was centrifuged at 1000g for 10 min, passed through a 0.22 μ m filter for sterilization and used without any further manipulations. Protease (type II) from *Aspergillus oryzae* and cellulase from *Aspergillus niger* were purchased from Sigma (St. Louis, Missouri, USA) and were processed in the same way. Barley and wheat flours were extracted in PBS (1/20 w/v) for 60 min while shaking.

Antiserum production

A rabbit antiserum to fungal amylase was obtained by five fortnightly subcutaneous injections of 200 μ g of the antigen in saline. Freund's complete adjuvant was used in the first injection and Freund's incomplete adjuvant was used thereafter.

Skin tests

Skin-prick tests were carried out with the above enzymatic extracts using histamine phosphate at 10 mg/ml as a positive control. An immediate weal of ≥ 3 mm was considered as positive.

Bronchial provocation tests

BPTs were performed using increasing doses of α -amylase ranging from 0.001 to 10 mg/ml as described by Quirce *et al.*⁷. A fall of the FEV₁ higher than 20% or of the airway specific conductance higher than 35% of the basal levels was considered as positive. These basal levels were estimated by previous inhalation of the diluent.

Specific IgE detection by enzyme-linked immunosorbent assay (ELISA)

A new IgE capture assay was used for specific IgE determination. Microtitre plates (Costar 3590, Cambridge, Massachusetts, USA) were coated with 0.1 ml of a 10 μ g/ml solution in PBS of a monoclonal anti-IgE antiserum (Ingenasa, Madrid, Spain) for 18 h at room temperature. Thereafter, the plates were washed three times in PBS and blocked for 1 h with 0.2 ml of 10% fetal calf serum (FCS) in PBS. The sera were diluted 1/2 in PBS–10% FCS and incubated (0.1 ml/well) in duplicate for 18 h at room temperature. After three washes in PBS, the wells were filled with a 1/10000 dilution of the Serva amylase stock solution (diluted in PBS–10% FCS) and incubated for 1 h at room temperature. After three washes in PBS, 0.1 ml of a 1/2000 dilution of the anti-amylase antiserum was incubated for 1 h. The plates were washed and 0.1 ml of a 1/4000 dilution of an alkaline phosphatase-labelled goat anti-rabbit antiserum (Tago Lab, Burlingame, California, USA) was added for 1 h. After further

washes, the substrate was added and the plates were read at 405 nm after 1 h. The substrate was composed of *p*-nitrophenyl-phosphate (2 mg/ml) in a 0.1 M Tris, 1 mM MgCl₂ buffer, pH 9.5. Any result higher than 0.4 absorbance units was considered to be positive.

SDS-PAGE

The discontinuous system described by Laemmli¹⁰ was used. Minigels (12% acrylamide-resolving and 4% stacking) were run on a Mighty Small II system (Hoefer Scientific, San Francisco, California, USA) at 150 V constant voltage until the bromophenol dye reached the front. The different enzymes or antigen extracts (50 μ g) were loaded in each lane. The gel was stained with Coomassie brilliant blue according to standard methods.

Antigen detection by immunoblotting

After SDS-PAGE, the transfer procedure of Petit *et al.*¹¹, with minor modifications, was used. Briefly, the gel was sandwiched between two nitrocellulose sheets and immersed in a transfer medium, while shaking. The proteins migrated out of the gel for 18 h by diffusion, rather than by electric transfer. Thereafter, the membranes were incubated for 30 min in 3% Nonidet P-40 (Sigma) in PBS and blocked in 10 mM Tris, 0.15 M NaCl, 3% bovine serum albumin (BSA), pH 7.4. The membrane was then incubated with a 1/2000 dilution of the anti-amylase rabbit antiserum for 45 min (diluted in the buffer described above, supplemented with 1% Tween-20). After three washes in 2 mM Tris, 0.5 M NaCl, 0.1% Tween-20, pH 7.4 (washing buffer), the membrane was again incubated with a 1/4000 dilution of the goat anti-rabbit antiserum described above. After four 10 min washes, the substrate was added. The substrate was composed of bromo-chloro-indolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT; Bio-Rad Lab, Richmond, California, USA) and the reaction was stopped after approximately 5 min.

Specific IgE detection by immunoblotting

The transfer procedure was the same as described above. Once the membranes were blocked, they were placed in a Mini Protean II (Bio-Rad). The sera were diluted 1/5 in incubation buffer and 0.5 ml of each sample was incubated overnight at room temperature while shaking. After three washes, the membrane was taken out of the device and incubated for 3 h with a 1/1000 dilution of an alkaline phosphatase-labelled monospecific anti-IgE (Caltag Lab, San Francisco, California, USA). Thereafter, the membrane was washed again and finally the substrate (BCIP/NBT, Bio-Rad) was added.

RESULTS

Figure 1 shows the SDS-PAGE analysis results for three enzymatic preparations used by the different

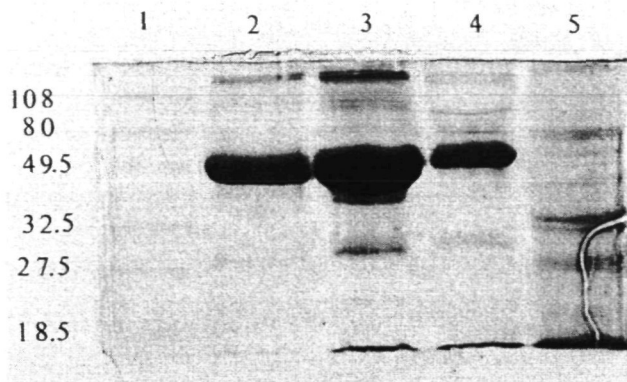


Figure 1. SDS-PAGE. Lane 1: molecular weight markers. Lane 2: α -amylase from Serva. Lanes 3, 4 and 5: different industrial amylases.

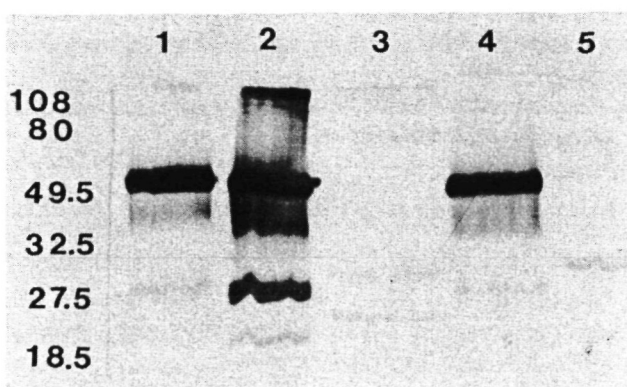


Figure 2. Immunoblotting performed using a polyclonal rabbit antiserum to α -amylase. Lane 1: α -amylase from Serva. Lane 2: industrial α -amylase. Lane 3: cellulase. Lane 4: protease from *Aspergillus oryzae*. Lane 5: wheat flour extract.

workers. One of them was a mixture, where the enzyme had a minor representation. Although α -amylase was the main protein present in the other extracts, several bands of different weights could be detected. This fact was especially evident when immunoblotting was performed using a rabbit anti-amylase antiserum (Figure 2). The antiserum detected the presence of multiple antigens which contaminated the two enzymatic preparations studied (lanes 1 and 2), but with different patterns. These contaminants could be produced by the fungus itself or be derived from the culture medium.

We have previously shown that a protease extract (another enzyme produced by *Aspergillus oryzae*) had amylase enzymatic activity and that this crude enzyme extract in fact contained a significant amount of α -amylase¹². Figure 2 shows that protease undoubtedly contains α -amylase, as well as other antigens as detected by the anti-amylase antiserum. Figure 2 also demonstrates that this antiserum detected several antigens in the cellulase extract, a result that was not found in humans by Quirce *et al.*⁷. Surprisingly, the anti-amylase antiserum detected a protein with lower molecular weight than amylase in the wheat flour extract, while no detection of barley antigens was observed (data not shown). The presence of amylase in the protease extract,

which was probably obtained using a different culture medium, led us to investigate the possibility that this enzyme extract could induce positive skin-prick tests in patients sensitized to amylase, although this was only performed in the recently diagnosed patients.

The clinical and immunological findings obtained in the 25 workers with suspected amylase hypersensitivity are represented in Table 1. As can be seen, four of the five patients with positive amylase and protease skin tests also had a positive BPT to amylase, as well as positive ELISA and blotting values. In contrast, only one patient with a positive skin test and positive BPT to amylase had negative serological values. Taken together, these facts suggest that individuals sensitized to amylase will show positive skin tests to an amylase-containing extract such as protease. This could be the case for patients 3 and 5, where skin tests to protease were not done. On the other hand, we cannot explain why patient 22 had positive skin tests to both extracts despite negative BPT and serological values. Furthermore, patient 1 (negative skin tests and serological values) showed a positive BPT to the enzyme. The rest of the patients showed complete agreement between the different tests. However, the most important finding is that only seven of the 11 skin-test-positive patients had a positive bronchial response. This implies that nearly 40% of the skin tests must be considered false positives as determinants of symptomatic allergy.

Finally, the results for two representative cases (whose sera were referred to us), who were not included

Table 1. Clinical and laboratory findings in 25 exposed workers

Patient	Skin-prick tests		ELISA	Blotting	BPT
	α -amylase	Protease			
1	—	—	0.330	—	+
2	+	ND	0.087	—	+
3	+	ND	1.730	+	+
4	+	+	0.727	+	+
5	+	ND	1.220	+	+
6	+	+	0.977	+	+
7	+	+	2.500	+	+
8	+	+	1.097	+	+
9	—	—	0.023	—	—
10	—	—	0.350	—	—
11	—	—	0.021	—	—
12	—	—	0.049	—	—
13	—	—	0.190	—	—
14	—	—	0.119	—	—
15	—	—	0.181	—	—
16	—	—	0.250	—	—
17	—	—	0.152	—	—
18	—	—	0.176	—	—
19	—	—	0.153	—	—
20	—	—	0.101	—	—
21	+	ND	0.168	—	—
22	+	+	0.084	—	—
23	—	—	0.108	—	—
24	+	ND	0.162	—	—
25	+	ND	0.070	—	—
Controls	—	—	0.143 $\pm$ 0.03	—	—

ND = not done; ELISA = mean of duplicates for absorbance measured at 405 nm; BPT = bronchial provocation test.

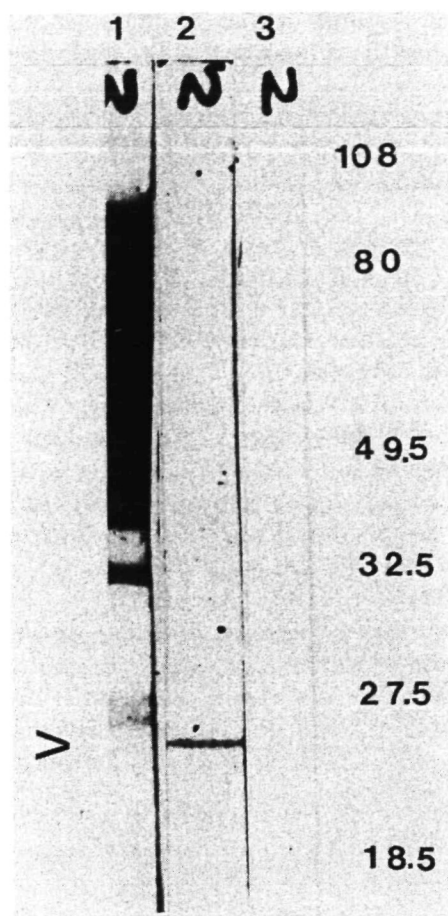


Figure 3. Specific IgE detection in two workers with positive skin tests, histamine release and bronchial provocation test to α -amylase (lanes 1 and 2). Lane 3: control (serum from a patient with negative results).

in the study, are shown in *Figure 3*. Both were diagnosed as allergic to α -amylase due to the fact that they presented bronchial asthma clinically related to use of the enzyme. Both had strong positive skin-prick tests to this allergen, as well as a positive immediate BPT and histamine release. As can be seen, only one of them recognized the 52 kDa antigen on immunoblotting, while the other serum detected an allergen of approximately 25 kDa.

DISCUSSION

The diagnosis of α -amylase hypersensitivity is based at present on positive skin tests to the enzyme and other serological methods such as radio-allergosorbent tests or ELISA. Sometimes BPTs and other challenge tests are also performed. Our study demonstrated that skin-prick tests are sensitive but unspecific tools for the diagnosis of this allergy, in agreement with previous reports^{2,4}. It is usually supposed that α -amylase is the only allergenic fraction contained in the extract. We have frequently found minor bands of lower molecular weight than the enzyme by immunoblotting, although never in the absence of recognition of amylase, with the

exception of the patient in *Figure 3* (data not shown). Our study demonstrated that industrial amylases employed by bakers are not pure allergens, as shown by SDS-PAGE. The immunoblotting performed with a rabbit antiserum indicated that the enzyme extracts were heavily contaminated by antigens of different molecular weight and that the antiserum detected an antigen in the wheat extract. In fact, enzyme preparations used as additives in the food industry only need to be free of toxic effects and may contain proteins of the culture medium^{13,14}. If a given allergen extract contains other contaminating allergens, the results obtained using this extract for *in vivo* as well as *in vitro* tests could be misleading, especially when using highly concentrated antigen extracts. This is the case, in our opinion, in the diagnosis of α -amylase hypersensitivity. We firmly believe that allergen(s) other than the 52 kDa amylase present in the extract can induce positive results in any test, with the exception of immunoblotting. This fact could make specific IgE detection by this method a necessary procedure for correct diagnosis. However, immunoblotting is not an easily available assay for many groups. Therefore, we suggest that the ideal approach should involve the use of highly purified amylase, free of any contaminants.

Knowledge of the composition of the culture medium where the fungus is grown seems to be a key point at present, but this information was not available to us. Perhaps the presence of cereal by-products, soya or other components could be responsible for positive *in vivo* and/or *in vitro* tests or even for clinical symptoms, at least for some patients. This could explain the high percentage of positive skin tests to amylase obtained in non-exposed millers, as described recently by us⁵.

Meanwhile, in our opinion, skin tests should be interpreted with care, due to the strong suspicion that they can be induced by allergens other than α -amylase. On the other hand, if the protease extract is being used for any industrial purpose, new cases of occupational asthma might be discovered in exposed workers, due to the presence of α -amylase at least in the crude extract.

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