

α -Amylase hypersensitivity in non-exposed millers

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Occupational hypersensitivity to α -amylase among bakers and workers in the pharmaceutical industry has been described. We present the results of skin tests and *in vitro* methods used to assess α -amylase sensitivity in 259 millers. There was no occupational contact with the enzyme in this population. Positive skin tests to this allergen were obtained in 16 subjects (6.18 per cent), specific IgE values were found in seven subjects (2.7 per cent), specific IgG was detected on 45 workers (17.37 per cent) and high values for IgG (>2.5 absorbance units) were found in 12 subjects (4.63 per cent). Furthermore, specific IgE and IgG could be determined in several sera by immunoblotting.

All these facts suggested that millers were sensitized to *Aspergillus oryzae* α -amylase, although the allergen was not used by these workers. A cross-reaction cannot be excluded; however, it is the authors' opinion that wheat or wheat flour is a natural substrate for fungi and that the moulds that grow on cereals or cereal-derived by-products need to secrete amylases to support their own development. Hence the workers may be indirectly sensitized to amylases.

INTRODUCTION

Enzymes are increasingly employed in industrial processes, and their allergenic properties and the sensitizing capacity of many of them have been proven¹. The presence of an immediate hypersensitivity has been established in most cases by means of skin and provocation tests, as well as by serological methods¹⁻⁵. The presence of specific IgG to enzymes has never been related to disease, and is considered as an indicator of exposure.

α -Amylase of *Aspergillus oryzae* hydrolyses glucosidic linkages in starch and is thus used in the baking industry to improve the intrinsic enzymatic activity of wheat flour. It is also used in the pharmaceutical industry as a digestive aid. At present, these are the principal industrial uses of this enzyme.

Bakers employ the enzyme mixed with wheat flour and other additives, and positive skin tests to α -amylase have been found for 30 per cent of exposed bakers⁵. However, these workers usually also have positive skin tests to flours and other common allergens. This often makes the clinical evaluation of the skin test results difficult. Therefore, we investigated the frequency of positive skin and serological tests to common allergens in subjects exposed to wheat flour but not to amylase. In this population, α -amylase was included as a control and the frequency of positive results to it was surprising and unexpected. Consequently, an

immunological study was performed which demonstrated that this non-exposed population had specific IgE and IgG to α -amylase and that the immunological findings were similar to those found for exposed workers^{1,5}.

MATERIALS AND METHODS

Subjects

The survey comprised 259 individuals working in the wheat flour-producing industry, from different companies in Spain. In no case were the subjects in contact with α -amylase or other types of cereals or enzymes. All workers underwent skin tests, as described below, and a serum sample was obtained with consent to perform the *in vitro* tests. Thirty-eight volunteers (eight atopics) were used to assess the specificity of the *in vivo* and *in vitro* tests. A clinical questionnaire was distributed among the workers to collect data concerning the existence of any respiratory, nasal or ocular symptoms.

Allergy tests

Skin prick tests were carried out with the common inhalation allergens (moulds, pollens, mites), and with a 50 mg/ml dilution of a fungal α -amylase in saline with 50 per cent glycerol and 0.5 per cent phenol.

Specific IgE determination

The concentration of specific IgE antibodies was measured by a reverse enzyme-linked immunosorbent

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assay (ELISA) as previously described¹. Any result higher than 0.150 absorbance units was considered to be positive. The ELISA positive values were further screened by immunoblotting. Briefly, boiled but not reduced α -amylase was separated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane using the method described by Dunn⁶. Following overnight blocking in 3 per cent bovine serum albumin in phosphate-buffered saline (PBS–BSA), the different sera were incubated at a 1/5 dilution in the same buffer for 3 h at room temperature while constantly shaking. After three 10 min washes in PBS–0.1 per cent Tween 20 (PBS–Tw), the membrane was incubated for 2 h in a 1/2000 dilution of a monospecific anti-human IgE labelled with alkaline phosphatase (Tago Laboratories, Burlingame, California, USA) in PBS–BSA. Thereafter, the membrane was washed as above, and the substrate (bromochloro-indolylphosphate–nitroblue tetrazolium chloride; BCIP–NBT) was added (BioRad Laboratories, Richmond, California, USA).

Specific IgG determination

Specific IgG values were measured by indirect ELISA. Microtitre plates were coated with 0.1 ml of 1 mg/ml α -amylase in PBS overnight. After 1 h blocking in PBS–BSA, the different sera were incubated in duplicate at a 1/500 dilution in PBS–BSA (0.1 ml per well) for 1 h at room temperature. After three washes in PBS–Tw, the wells were incubated with a monospecific anti-human IgG labelled with peroxidase at a 1/4000 dilution (Tago Laboratories) in PBS–BSA for 1 h. Thereafter, the plates were washed and the substrate was added. The substrate was composed of 1,2-phenylenediamine (2 mg/ml) in citrate–phosphate buffer (0.1 mol/l, pH 5, 0.03 per cent hydrogen peroxide). The reaction was stopped after a 10 min incubation period by the addition of 0.1 ml of 2 N sulfuric acid, and absorbance was read at 492 nm.

Samples with IgG ELISA values higher than 2 absorbance units were tested by immunoblotting. The basic procedure described above for IgE determination by immunoblotting was followed with some minor modifications: the sera were diluted 1/200 and incubated for 1 h, and a monospecific anti-human IgG (Tago Laboratories) was employed at a 1/4000 dilution for 1 h.

Statistical analysis

Comparison of the clinical and immunological data was carried out using the χ^2 method (with Yates' correction) and with Fisher's exact test (two-tailed).

RESULTS

α -Amylase skin tests and specific IgE values

Ocular, nasal and respiratory symptoms were reported by 73 workers (28.19 per cent, *Table 1*). Sixteen subjects

Table 1. Clinical and immunological findings

	Exposed subjects		Controls (n = 38)
	With symptoms (n = 73)	Without symptoms (n = 186)	
Positive amylase skin test	12 (12)*	4 (3)	0
Positive ELISA IgE	5 (5)	2 (1)	0

*The numbers of patients with positive skin tests to common allergens are given in brackets.

Table 2. Specific IgG values

Specific IgG (absorbance units)	Exposed subjects		Controls (n = 38)
	With symptoms (n = 73)	Without symptoms (n = 186)	
> 1	13 (8)*	32 (9)	3 (1)
> 1.5	8 (7)	18 (4)	0
> 2	5 (5)	12 (4)	0
> 2.5	4 (4)	8 (2)	0

*The numbers of patients with positive skin tests to common allergens are given in brackets.

belonging to the symptomatic group. All 12 subjects also had positive skin prick tests to common allergens, suggesting that they were atopic. A similar feature was observed for the four asymptomatic workers who displayed a positive skin test to amylase. The correlation between positive skin tests to α -amylase and the presence of symptoms was highly significant ($P < 0.0001$). A positive skin test to α -amylase was also associated with positive skin tests to common allergens ($P < 0.0001$).

Five symptomatic workers had also positive IgE ELISA values, while two asymptomatic subjects had serological results suggestive of hypersensitivity. Once again, this population showed a close relationship between positive serological values and positive skin tests to common allergens ($P = 0.0119$) (*Table 1*).

Specific IgG values

The presence of α -amylase IgG-specific values as measured by indirect ELISA is shown in *Table 2*. Four of the symptomatic subjects had specific IgG values exceeding 2.5 absorbance units, and eight of the asymptomatic population displayed similar antibody concentrations.

The difference between the values obtained for exposed subjects and controls was highly significant ($P < 0.0001$). Once again, a close relationship between positive skin tests to common allergens and specific IgG values was found ($P < 0.0001$). However, no difference between exposed symptomatic and asymptomatic workers was detected ($P = 0.70$).

IgE immunoblotting

Figure 1 shows the results of the immunoblotting performed to detect the presence of specific IgE. It can be seen that two millers showed specific antibodies

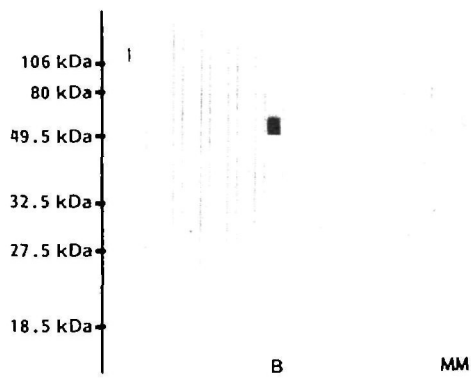


Figure 1. Specific IgE antibodies to α -amylase as detected by immunoblotting. B = serum from a baker with α -amylase hypersensitivity. M = sera from two millers with positive skin tests and IgE ELISA. Negative strips: sera from five millers with positive IgE ELISA and sera from ten workers with negative IgE ELISA.

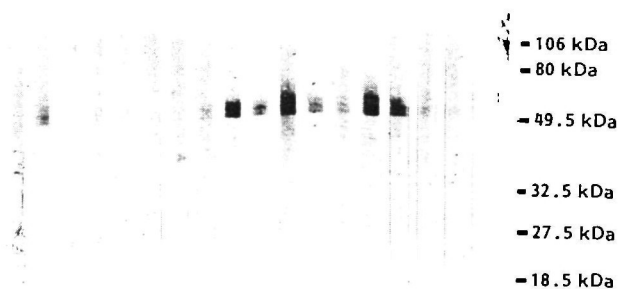


Figure 2. IgG immunoblotting to α -amylase performed with the sera of 17 millers who had IgG ELISA values >2 absorbance units.

to α -amylase, giving a similar pattern to a baker with proven occupational asthma due to α -amylase sensitization.

IgG immunoblotting

All the subjects who showed an IgG ELISA value higher than 2 absorbance units were assayed by immunoblotting in order to test whether they could also recognize the enzyme by this method. As *Figure 2* shows, several sera detected the presence of the 52 kDa protein in the immunoblotting assay.

DISCUSSION

Allergic asthma and rhinitis caused by occupational exposure to α -amylase have been reported in workers in the pharmaceutical and baking industries^{1,3-5}. An immediate type I hypersensitivity has been demonstrated by means of radio-allergosorbent test (RAST) or ELISA methods. In a recent review, Brisman and Belin⁵

found that six out of 20 exposed workers (30 per cent) in a baking company had positive skin tests to α -amylase, although the RAST was positive only in one case (a worker without clinical symptoms). In addition, they found that three out of 20 exposed workers (15 per cent) had detectable specific IgG to this enzyme. Losada *et al.*¹ also found that 31.3 per cent of the subjects studied in a pharmaceutical company had positive skin prick tests to the enzyme. However, only six of the 14 workers who were positive for the skin test also showed a positive bronchial provocation test to α -amylase. Both groups agree that the presence of specific IgG is only a marker of exposure, and no correlations were found between specific IgG levels and disease.

We found that 45 of the workers studied (17.37 per cent) had IgG ELISA values higher than 1.0 absorbance units and that 12 subjects (4.63 per cent) reached levels higher than 2.5 absorbance units. These values were not found in the control group ($P < 0.0001$). Furthermore, our work demonstrated that 10 of the 17 sera with high specific IgG values to the enzyme were also positive when studied by immunoblotting. In our opinion, this suggests that these subjects are in close contact with α -amylase.

The presence of positive skin tests to the enzyme in 16 subjects (6.18 per cent), together with the fact that a positive IgE ELISA value was obtained in seven workers (2.7 per cent), supports this hypothesis. IgE immunoblotting clearly demonstrated that the workers had specific IgE to the 52 kDa protein that corresponds to α -amylase.

The presence of specific antibodies to this enzyme in non-exposed subjects cannot be explained easily, as we are sure that they are not in contact with this enzyme. A cross-reaction cannot be excluded, although the actual antigen remains to be found. We have not detected antigenic identity between fungal amylase and wheat proteins (data not shown). Another possible explanation could be that the workers are in close contact with amylases from moulds that grow spontaneously on wheat and/or flour. Cereal grains or flour can be contaminated by *Aspergillus* and other moulds⁷. In this case, the workers could be sensitized to this antigen. Antigenic cross-reactivity among fungal enzymes has been demonstrated⁸, so that *A. oryzae* need not necessarily be the aetiological agent. Recently, Fakhri⁹ found a high percentage of positive skin test responses to moulds on flour mill workers in Sudan. In that study, *Aspergillus* sp. and *Rhizopus niger* were the predominant fungi in the wheat grains. Further work is necessary to prove this point, but if other groups demonstrate that α -amylase hypersensitivity is also found on millers, then the allergenic relevance of this enzyme will need to be reconsidered.

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REFERENCES

1. Losada E, Hinojosa M, Quirce S, Sanchez-Cano M, Moneo I. Occupational asthma caused by α -amylase inhalation: Clinical and immunological findings and bronchial response patterns. *J Allergy Clin Immunol* 1992; 89: 118-25.
2. Flindt MLH. Allergy to α -amylase and papain. *Lancet* 1979; i: 1407-8.
3. Baur X, Fruhmenn G, Haug B, Rasche B, Reiher W, Weiss W. Role of *Aspergillus* amylase in baker's asthma. *Lancet* 1986; i: 43.
4. Blanco JG, Juste S, Garces M. Occupational asthma in bakeries caused by sensitivity to α -amylase. *Allergy* 1991; 46: 274-6.
5. Brisman J, Belin L. Clinical and immunological responses to occupational exposure to α -amylase in the baking industry. *Br J Ind Med* 1991; 48: 604-8.
6. Dunn SD. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. *Anal Biochem* 1986; 157: 144-53.
7. Scheepers GWH. Allergenic occupational air pollutants. In: Frazier CA, ed. *Occupational Asthma*. New York: Van Nostrand Reinhold, 1981: 307-56.
8. Monod M, Togni G, Rahalison L, Ftrenk E. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. *J Med Microbiol* 1991; 35: 23-8.
9. Fakhri ZI. Causes of hypersensitivity reactions in flour mill workers in Sudan. *Occup Med* 1992; 42: 149-54.

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