

ra. The IgE in the patients' sera bound to the 48.3 kDa and the 50 kDa proteins present in the A and B samples, while no IgE binding to the membrane was seen with sample C.

Even though this study was performed on only a small group of cases, it appears to again demonstrate that the allergenic activity of the enzyme is destroyed upon being subjected to the usual baking temperatures, as evidenced in the skin tests. Furthermore, the protein becomes degraded *in toto*, as shown by the SDS-PAGE results, and is most probably reduced to very small peptides lacking any allergenic character.

In conclusion, we may state that, firstly, the dominant allergen in the α -amylase enzyme is a glycoprotein with a molecular weight of approximately 50 kDa. Secondly, its accepted denomination is "Asp o 2". Thirdly, it contains epitopes which are mainly recognised by B-cells. Fourthly, α -amylase does not appear to behave as an allergen of interest upon ingestion after being subjected to the usual baking temperatures.

References

1. Stauffer C. La utilización de enzimas en productos de panadería. Boletín técnico. Ed. Gur Ranhotra 1994; 16: 1-11.
2. Toda H, Kondo K, Narita K. The complete amino acid sequence of taka-amylase A. Proc Japan Acad 1982; 58: 208-212.
3. Swift HJ, Brady L, Erewenda ZS, Dodson EJ, Turkenburg JP, Wilkinson AJ. Structure and molecular model refinement of *Aspergillus oryzae* (TAKA) α -amylase: an application of the simulated-annealing method. Act Crystallogr-B 1991; Aug 1, 47(Pt4): 553-544.
4. Sander I, Baur X. Evidence for continuous B-cell epitopes on α -amylase of *Aspergillus oryzae* (Asp o 2). J Allergy Clin Immunol 1994; 93: 265.
5. Baur X, Chen Z, Sander I. Isolation and denomination of an important allergen in baking additives: α -amylase from *Aspergillus oryzae* (Asp o II). Clin Exp Allergy 1994; 24: 465-470.
6. Alonso L, Blanco J, Juste S, Garcés M, Pérez R, Marcos M, Carretero P. Evaluation of impairment in patients with occupational asthma caused by α -amylase. Allergy 1995; 50: 172.
7. Kanny G, Moneret-Vautrin DA. Alpha amylase contained in bread can induce food allergy. J Allergy Clin Immunol 1995; 95: 132-133.
8. Baur X, Czuppon AB. Allergic reaction after eating alpha-amylase (Aso o 2)-containing bread. Allergy 1995; 50: 85-87.
9. Baur X, Sander I, Jansen A, Czuppon AB. Sind Amylasen (Backmitte-lenzyne) relevante Nahrungsmittelallergene? Schweiz Med Wochenschr 1994; 124: 846-851.
10. Blanco Camona J, Alonso Gil L, Bartolomé Zavala B, Vallverdú A, Palacios R, Juste Picón S, Garcés Sotillos M, García Gonzales F, Pérez Giménez R. Asma por sensibilización a alfa-amilasa. ¿Es este enzima un alérgeno de interés por vía digestiva? Rev Esp Alergol Inmunol Clin 1997; 12: 288-292.

ALLERGY TO CEREAL α -AMYLASE INHIBITORS

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The so-called cereal grains include wheat, maize, rice, barley, sorghum, oat, millet and rye. All these, collectively, represent 72% of the dietary protein in the World.

Wheat, barley and rye are closely related grain (grass) species belonging to the tribe *Triticeae*. A number of different proteins enter into the composition of their seeds: water-soluble albumins, saline-soluble globulins, water-alcohol-soluble prolamines, and insoluble glutenines¹⁻⁴. The soluble proteins represent up to 25% of the protein contents in the seeds, and a number of studies have confirmed their importance in IgE-mediated responses, both because of their role in inhalational sensitizations⁵⁻¹² and because of their role in atopic dermatitis¹³. The former is collectively known as "baker's disease" or "baker's asthma". It is a condition that gives rise to considerable economic and legal problems, quite besides its severity and difficult management when the patient cannot avoid inhaling the causative grain dusts.

Cereal workers (farmers, millers, fodder packagers) and those working in the baking and confectionery environments are exposed to a number of antigenic stimuli²⁻⁴. In our area they mostly work in small family-owned enterprises or in silos and granaries, with the worst imaginable ventilation. Although they are aware of the personal protection measures, they usually do not apply them because of the inconveniences they cause in an environment in which they must carry out considerable efforts under high environmental temperatures. In the case of the dough improvers, solid preparations are being increasingly used instead of powders, and this leads to less particle dispersion. However, this is not possible in the case of flours. Because of the high prevalence of this type of asthma in our region (25% of the individuals exposed suffer occupational asthma), we have been trying since several years ago to isolate, purify and assess the allergenicity of cereals in an effort to find appropriate measures for the environmental and therapeutic control of this condition⁵⁻¹⁸.

We must bear in mind that flour is a complex ecosystem in which a number of elements are represen-



Fig. 1. High-resolution bidimensional electrophoresis of a wheat flour extract, showing (arrows) the most reactive proteins. From Posch et al. (Electrophoresis 1995; 16: 1115-9) and Weiss et al. (Electrophoresis 1993; 14: 804-16).

ted: vegetable elements such as cereal pollens, proteins from the grain endospermium, spores and fungi; a number of additives such as α -amylase, and arthropodal allergens from grain parasites, cockroaches and mites (mainly storage mites, which are themselves potent allergens)^{19,20}. Episodes of asthma caused by the ingestion of cereals contaminated with storage mites have been recently reported²¹. How then are we to identify the allergens that are causative of the allergic symptoms?

The electrophoretic techniques for protein isolation are better than other methods such as chromatography, as they have a better resolution and are able to resolve the complex protein composition of flour in the endeavour to characterise its possible allergens². Posch *et al.*, using high-resolution protein electrophoresis in combination with protein analysis, detected some 700 soluble

proteins in wheat (Electrophoresis 1993; 18:804-816); 70 of these proteins were able to detect IgE, mainly those in the 14-18, 27, 37, 55 and 70-kDa regions. Using amino-acid sequencing, they found homologies in α -amylase-inhibiting proteases (14-18 kDa), acyl-CoA oxidase (26 kDa) and fructose biphosphate aldolase (37 kDa) from wheat, maize and barley (Fig. 1). However, these interesting studies do not fully clarify which proteins might be clinically relevant. We therefore attempted to separate and isolate them so that, once purified and isolated in diagnostic extracts, they may be used for assessment of their reactivity *in vivo*.

To this purpose, we first used a pool of 35 sera from patients with baker's asthma with Class 4 RAST to wheat and barley flour. Our first results allowed us to establish that the most reactive allergens were saline-soluble proteins of 12-15 kDa molecular weight belonging to a single family that includes several heterologous α -amylase inhibitors, with a high level of presence in the cereals and with potential participation in the cereals' defence mechanisms against infesting storage pests¹⁴. Thus, the major allergen in barley was able to inhibit the α -amylase from *Tenebrio molitor*, a common parasite of this cereal⁶. A number of members of this inhibitor family were recognised by our patients' specific IgE; however, they had widely diverging allergenic capacity. Figure 2 shows the bidimensional electrophoresis and the chromatogram of the fractionation through high-resolution reverse liquid chromatography (HPLC) of the tetrameric inhibitor from wheat. We would like to call attention to the positions of the most reactive protein (CM16*), marked by an arrow in the bidimensional map and in the elution profile. The CM16 and CM16* proteins have the same molecular weights, but their immunoblot behaviour against sera from reactive patients is

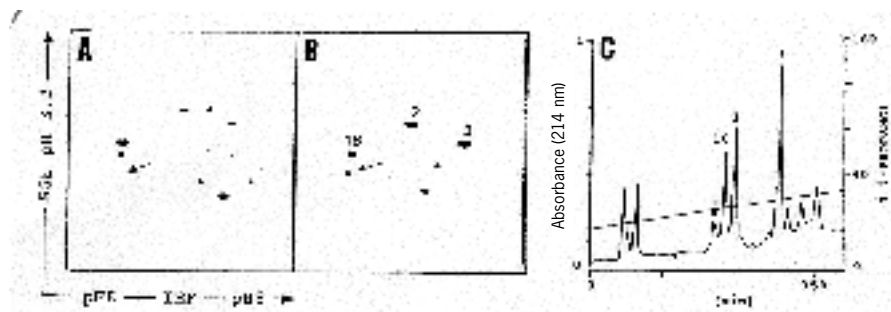


Fig. 2. Isolation of the CM16* protein from wheat dough: bidimensional electrophoresis (IEF x SGE) of the source preparation of the inhibitor (A) and of the gel-filtrated 60-kDa fraction containing tetrameric inhibitors from *Triticum turgidum*, *Senatore Capelli* (pasta wheat). HPLC fractionation of the tetrameric inhibitor (Fig. 2B). The position of the CM16* component is shown (arrows) in the two bidimensional maps and in the HPLC elution profile. Also shown are the positions of the WTAI-CM2 tetrameric inhibitor (2) and of CM16 (16) and CM3B (3).

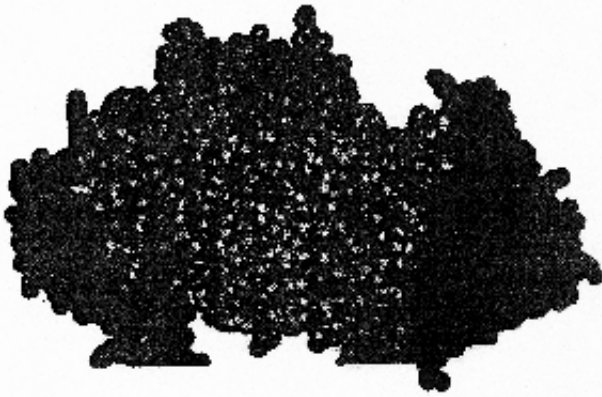


Fig. 3. Molecular structure of *Aspergillus oryzae* α -amylase, highlighting its three domains (Baur et al., Reference 25).

quite different. The same occurred with the CMb and CMb* components from barley. After a number of attempts to find out what was the difference between these proteins as regards their allergenicity (aminoacid sequencing, peptide excision, glycan detection tests) it was shown that the particularity rendering these proteins reactive was the binding to complex xylose and fucose glycans, and that these glycans acted as IgE-binding epitopes⁹. This explained why our cereal workers or bakers mainly reacted to glycosylated purified proteins from barley and wheat, while their skin tests and RASTs with commercial cereal extracts were negative. However, the inhalational challenge tests with these purified proteins were positive, demonstrating their importance as aetiological agents in occupational asthma²². We have recently shown that the complex glycans from other invertebrates and plants have similar epitopes⁹. In this way, the IgE antibodies from our bakers were able to recognise glycoproteins from coleoptera, from pulses and, curiously enough, from bee venom (Fig. 3). This might be the explanation for the curious finding of cases of anaphylaxis after a first sting by these insects, without a prior latency period or sensitization. In this same context, the α -amylase from *Aspergillus oryzae*, Asp o 2, is also a glycoprotein with 478 aminoacyl residues and with three structural domains (Fig. 4).

Recent studies by Sandiford *et al.*¹² have demonstrated that persons sensitized to soluble proteins from wheat are able to generate also specific IgE against the insoluble proteins. Their Western Blot experiences have led to the conclusion that gliadins and glutenins with molecular weights similar to those of the enzyme inhibi-

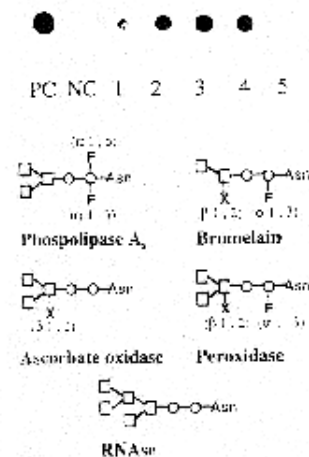


Fig. 4. Glycoprotein immunodetection with hypersensitive sera. Samples: (1) bee venom phospholipase A₂; (2) pineapple bromelain; (3) cucumber ascorbate oxidase; (4) horseradish peroxidase; (5) bovine pancreatic ribonuclease. The membranes were treated with the hypersensitive sera and then with labelled anti-IgE. The wheat allergen CM16* and its non-glycosylated form (CM16) were included as positive (PC) and negative (NC) controls, respectively. The structure of the glycans in these proteins is graphically depicted: L: mannose; O: GlcNAc; X: xylose; F: fucose.

tors are also important allergens (apparently because they share interreactive epitopes with the soluble proteins), so that also they might induce asthma. These results suggest that the number of allergens involved in the development of cereal hypersensitivity is higher than previously believed.

Nevertheless, the albumins and globulins from cereal grains appear to be the most important proteins in the triggering of immediate hypersensitivity reactions²⁻⁵. A number of investigators have confirmed the presence of specific IgE to albumins and globulins in the sera of children with cereal-induced food allergy and with baker's asthma, but not in patients with coeliac sprue^{3,22}. However, while the inhalatory sensitization to cereal flours is one of the better-studied forms of occupational allergy, there are but few investigations of the allergens involved in allergic reactions after the ingestion of these proteins²³. We have been able to show that patients with allergic symptoms after the ingestion of Coca-Cola and Cola Cao might be sensitized to cereals, and particularly to rye proteins in the cases with asthma and to barley proteins in those with anaphylaxis²⁴.

In summary, we believe that the study of the allergenicity of cereal proteins may be highly important, and this importance is mainly based in the fact that baker's

Table I. Characterised allergens in wheat flour

Substance	Molecular weight kDa	Purity	Test	Patients with positive prick test	Reference
α-Amylase inhibitors					
<i>Heterotetramer</i>					
<i>subunits:</i>					
WTAI-CM2	14	e.p. ¹	Skin prick test	11/31	Armentia et al. 1993
WTAI-CM3B	14	e.p. ¹	Skin prick test	11/31	Armentia et al. 1993
WTAI-CM16	16	e.p. ¹	Skin prick test	7/31	Armentia et al. 1993
WTAI-CM16*	16	e.p. ¹	Skin prick test	14/31	Armentia et al. 1993
<i>Homodimer</i>					
<i>subunits:</i>					
WDAI-1	15	e.p. ¹	Skin prick test	5/31	Armentia et al. 1993
WDAI-1 and/or		e.p. ²	Immunoblot	4/8	Fränken et al. 1994
WDAI-2		e.p. ¹	2D-Immunoblot	Sera pool	Posch et al. 1995
		e.p. ²	EAST	13/25	Walsh et al. 1989
<i>Monomer:</i>					
WMAI-1	14		Skin prick test	9/31	Armentia et al. 1993
Aglutinin					
<i>WGA</i>					
<i>homodimer</i>	17	e.p. ²	RAST	5/9	Sutton et al. 1984
<i>subunits</i>					
Acyl-CoA oxidase					
(rice homologue)	27	e.p. ¹	2D-Immunoblot	Sera pool	Posch et al. 1995
Fructose biphosphate					
aldolase	37	e.p.	2D-Immunoblot	Sera pool	Weiss et al. 1997
(rice homologue)					

* = glycosylated subunit; e.p. = electrophoretically pure; ¹one single point in bidimensional electrophoresis; ²one single band in SDS-PAGE.

asthma is the most prevalent occupational respiratory allergy in many countries. According to Baur²⁵, 1800 bakers request compensation because of this condition every year in Germany. In his series of 405 bakers, 60% evidenced sensitization to wheat flour, 57% to rye flour and 27% to α -amylase (Asp o 2). Among the purified allergens causing baker's asthma, a number of saline-soluble proteins stand out as the major allergens: proteins from the endospermium of the cereal grains with molecular weights between 12 and 15 kDa and belonging to the same family of α -amylase and trypsin inhibitors^{1,5-10} (Table I).

These proteins appear to be involved in the defence mechanisms of plants through the inhibition of the digestive α -amylases of different parasites, thus preventing their degradation. A number of observations concur with this consideration, some of them from the field of pest studies and some from the studies of inhibitor specificities against enzymes from a number of insects: the insects that feed on wheat endospermium show very high α -amylase levels^{26,27}; the major allergen in barley is a specific inhibitor for *Tenebrio molitor* α -amylase (*T. molitor* is a common parasite of this cereal⁶), while the inhibitors from rye restrict their activity

to *Omoplus lepturoides* and evidence no activity at all against *T. molitor*.

After the characterisation, isolation and purification of the wheat α -amylase inhibitors involved in baker's disease, our analyses of the allergenic role of proteins from certain foodstuffs (Coca Cola, Cola Cao, certain malted soluble coffee preparations) have pointed out their usefulness in the diagnosis of occult cereal allergy, and lead to the conclusion that one single protein may be reactive through both sensitization pathways. These findings, together with the already-quoted ones of Sandiford¹² and Baur²⁵, might define their behaviour as a multigenic expression of the family of α -amylase inhibitors in different cereals. Thus, the various species in the Tribe *Triticeae* might be considered to represent different manifestations of a single genome, and sensitization to their various proteins might explain the variability in the clinical findings and in the therapeutic response in patients with sensitizations to different cereal flours²⁶.

Finally, and as a practical application of our own studies and of those of other authors, the following suggestions for clinical application may be proposed:

The optimal purification and isolation of the major allergens for use in *in vitro* and *in vivo* tests might be highly important in the diagnosis of these diseases.

The knowledge of these proteins and of their biologic activities in humans might constitute the basis for genetic manipulation of the plants for the cultivation of less-allergenic species that are also more resistant to pests.

The reduction of the allergenicity of these plants through genetic manipulation might lead to the synthesis of less-sensitizing drugs, which represents a serious limitation of present-day plant biotechnology.

Acknowledgements

I am indebted to Dr. Rosa Sánchez Monge, to Dr. Gloria García Casado and to Dr. Gabriel Salcedo, excellent persons at both the scientific and the human levels, for the opportunity to work with them. I should also like to express my acknowledgement to all the members of the Allergy and Biochemistry Sections of the "Rio Hortega" University Hospital in Valladolid, for the same reason.

References

1. Yunginger JW. Food antigens. In Food Allergy: Adverse reactions to foods and food additives. Metcalfe DD, Sampson HA, Simon RA Eds. 2nd Edition 1997, Blackwell Sc.

2. Baur X, Fruhman G, Haug B, Rasche B, Reiher W, Weiss W. Role of *Aspergillus* α -amylase in baker's asthma. Lancet 1986; 1: 43.
3. Baldo BA, Wrigley CW. IgE antibodies to wheat flour components. Studies with sera from subjects with baker's asthma or coeliac condition. Clinical Allergy 1978; 9: 109-124.
4. James JM, Sixbey JP, Helm RM, Bannon GA, Burks AW. Wheat α -amylase inhibitor: A second route of allergic sensitization. J Allergy Clin Immunol 1997; 99: 239-244.
5. García Casado G, Armentia A, Sánchez-Monge R, Malpica JM, Salcedo G. Rye flour allergens associated with baker's asthma. Correlation between *in vivo* and *in vitro* activities and comparison with their wheat and barley homologues. Clin Exp Allergy 1996; 26: 428-435.
6. Barber D, Sánchez-Monge R, Gómez L, Carpizo J, Armentia A, López C, Juan F, Salcedo G. A barley inhibitor of insect alpha-amylase is a major allergen associated with baker's asthma. FEBS Lett 1989; 348: 119-122.
7. Sánchez-Monge R, Gómez L, Barber D, López C, Armentia A, Salcedo G. Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the alpha-amylase inhibitor family have enhanced IgE-binding capacity. Biochem J 1992; 281: 401-405.
8. Armentia A, Sánchez-Monge R, Gómez L, Barber D, Salcedo G. In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. Clin Exp Allergy 1993; 23: 410-415.
9. García Casado G, Sánchez-Monge TR, Chrispeels MJ, Armentia A, Salcedo G. Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. Glycobiology 1996; 6: 471-477.
10. Sánchez-Monge R, García Casado R, López Otín C, Armentia A, Salcedo G. Wheat flour peroxidase is a prominent allergen associated with baker's asthma. Clinical Exp Allergy 1997; 27: 1130-1137.
11. Cockcroft DM. Occupational asthma. Ann Allergy 1990; 65: 169-175.
12. Sandiford CP, Tatham AS, Fido R, Welch JA, Jones MG, Tee RD, Sherry PR, Newman Taylor AJ. Identification of the major water/salt insoluble proteins involved in cereal hypersensitivity. Clinical and Experimental Allergy 1997; 27: 1120-1129.
13. Varjonen E, Savolainen J, Mattila L, Kalimo K. IgE binding components of wheat, rye, barley and oats recognized by immunoblotting analysis with sera from adult atopic dermatitis patients. Clin Exp Allergy 1994; 24: 481-489.
14. García Olmedo F, Salcedo G, Sánchez-Monge R, Gómez L, Rojo J, Carbonero P. Plants proteinaceous inhibitors of proteins and alpha-amylase. Oxford Surv Plant Mol Cell Biol 1987; 4: 275-334.
15. Chai H, Farr R, Froehlich LA, et al. Standardization of bronchial inhalation challenge procedures. J Allergy Clin Immunol 1975; 56: 323-331.
16. Armentia A, Martín J, Quintero A, Fernández A, Barber D, Alonso E, Gil I. Baker's asthma: prevalence and evaluation of immunotherapy with a wheat flour extract. Ann Allergy 1991; 65: 265-272.
17. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M, Bush RK, Metcalfe DD. Double blind placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol 1988; 82: 986-997.
18. Zeiger RS, Heller S, Mellon MH, Halsey JF, Hamburger RN, Sampson HA. Genetic and environmental factor affecting the development of atopy through age 4 in children of atopic parents: a

prospective randomized study of food allergen avoidance. *Pediatr Allergy Immunol* 1992; 3: 110-127.

19. Armentia A, Martínez A, Castrodeza R, Martínez J, Jimeno A, Méndez J, Stolle R. Occupational allergic disease in cereal workers by stored grain pests. *J of Asthma* 1997; 34: 369-378.

20. Armentia A, Tapisa J, Barber D, Martín J, De la Fuente R, Sánchez P, Salcedo G, Carreira J. Sensitization to storage mite *Lepidoglyphus destructor* in wheat flour respiratory allergy. *Ann of Allergy* 1992; 68: 398-430.

21. Blanco C, Castillo R, Ortega N, Álvarez M, Arteaga C, Barber D, Carrillo T. Asthma due to ingestion of contaminated flour. *J Invest Allergol Clin Immunol* 1997; 7: 323-324.

22. Armentia A. Usefulness of specific bronchial challenge in monitoring immunotherapy in occupational asthma. *Allergology and Clinical Immunology* 1997; 7: 325-326.

23. Jones SM, Magnolfi CF, Cooke SK, Sampson HA. Immunologic crossreactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol* 1995; 96: 341-351.

24. Armentia A, Martín Gil FJ, Arranz-Peña ML, Bañueños MC, Navas C, Pardo M. Alergia Alimentaria a Coca-Cola y Cola-Cao y su relación con proteínas purificadas de cereales. *Rev Esp Alergol Inmunol Clin* 1998.

25. Baur X, Posch A. Characterized allergens causing baker's asthma. *Allergy* 1998.

26. Gómez L, Sánchez-Monge R, García Olmedo F, Salcedo G. Wheat tetrameric inhibitors of insect α -amylases: allopoloid heterosis at the molecular level. *Proc Natl Acad Sci USA* 1989; 86: 3242-3246.

27. Gutiérrez C, Sánchez-Monge R, Gómez L, Ruiz Tapiador M, Castañera P, Salcedo G. α -Amylase activities of agricultural insect pests are specifically affected by different inhibitor preparations "Del Río Horteiga".

28. James JM, Sampson HA. An overview of food hypersensitivity. *Pediatr Allergy Immunol* 1992; 3: 67-78.

CROSS REACTIVITY BETWEEN ENZYMES

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Enzymes behave as potent inhalational allergens in the occupational environment and are able to sensitize a large proportion of the workers that manipulate them. Whether this great sensitization capacity is related to their enzymatic activity or simply to the fact that they are glycoprotein macromolecules able to trigger an IgE-mediated immunological response has long been a subject of debate.

A number of additives are used in the manufacture of bread, which are added to the dough with the aim to improve and accelerate the panification process.

Table I. Characteristics of the enzyme additives used in bakery processes

- Mixtures of a number of products
- Scarcely purified industrial enzymes
- Contain impurities
- Unknown composition
- Similar fungal origin
 - Aspergillus oryzae*
 - Aspergillus niger*
- Allergenic potential

Table II. Main allergens responsible for asthma in bakers

- Cereals (wheat and rye): albumins, globulins, gliadins, glutenins, prolamines
- Soybean: trypsin inhibitor, lipo-oxidase
- Fungal enzymes:
 - α -amylase (*Aspergillus oryzae*)
 - Hemicellulase and cellulase (*Aspergillus niger*)
 - Xylanase (*A. niger*)
 - Glycoamylase (*A. niger*)
- Storage mites
- Bread yeast (*Sacharomyces cerevisiae*)
- Moulds (alternaria, aspergillus)
- Grain weevil (*Sitophilus granarius*)

Table III. α -amylase: characteristics of allergologic interest

- Glycolytic enzyme usually derived from *Aspergillus oryzae* (Asp o 2).
- Sensitizes 20-32% of symptomatic bakers.
- Partially thermolabile.
- May induce symptoms upon ingestion.
- Glycoprotein with 478 aminoacid residues.
- Continuous sequential epitopes.
- Exposure/sensitization ratio: 0,25 ng/m³.

Among these additives, glycolytic fungal enzymes are one of the major groups. And yet, the enzymatic additives used are scarcely purified industrial products, so that a number of factors must be kept in mind when a possible sensitization is studied. These factors are summarised in Table I.

Bakers' and confectioners' asthma may be caused not only by cereal flour, but also by any of the additives or contaminants present in those flour and summarised in Table II. The sensitization to a number of