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Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry

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Abstract

The aim of the study was to investigate the safety to allergic patients of 19 commercially available and authority-approved enzymes used in the food industry. Enzymes produced by genetically modified organisms were included. Four hundred consecutive adult patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp were included. All had at least one positive skin prick test to the above allergens.

Skin prick testing with the 19 enzymes was performed on the forearm and if positive (in 13 patients), in vitro histamine release from blood basophils were performed. Patients with positive results in skin prick test were subsequently reinvestigated with further purified enzymes and finally challenged orally with the enzymes in a double-blind, placebo-controlled protocol. Only one reaction to a placebo challenge was seen.

In some instances a positive skin prick test result or a positive histamine release was seen elicited by the enzymes, but since none of the patients were positive to any of the commercial enzymes in the subsequent oral challenges using exaggerated dosages of the enzymes compared to normal daily intake, the findings are without clinical relevance.

A wide variety of enzyme classes and origins was included in the study. Because there were no allergenic findings of clinical relevance it is concluded that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy.

Keywords: Allergen; Food allergy; Enzyme; Genetically modified; Human; Skin prick test; Basophil histamine release

1. Introduction

Food safety is of major concern worldwide, and one aspect of food safety is avoiding allergic reactions associated with food consumption. Patients with food hypersensitivity always face the risk of developing allergic symptoms after unintentional intake of a non-tolerated food. Such unintentional intake may be due to eating in a restaurant setting, where product labelling is lacking, or be due to insufficient labelling of food compounds in the daily diet.

As a result of the growing awareness and concerns of food allergy, regulatory agencies worldwide are developing allergen evaluation schemes and implementing allergen labelling regulations.

One example of such evaluation scheme is the decision tree as described in the FAO/WHO 2001 report (Aalberse et al., 2001), providing guidance on the assessment of the allergenic potential of foods derived from biotechnology. Such assessment may be required to protect food allergic patients against potential new risks associated with the development of genetically modified foods, whereby

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introduction of foreign allergenic proteins from other foods into a food which the patient has previously tolerated may elicit allergic reactions in the allergic patient (Hansen et al., 2004; Bindslev-Jensen et al., 2003).

For the allergen labelling regulations being implemented, this generally means mandatory declaration of recognized allergenic substances contained in the final foods (EU: Council Directive 2000/13/EC on the labelling, presentation and advertising of foodstuffs as amended by 2003/89/EC of 10 November, 2003. US: 21CFR101.4 and 21CFR101.100. Additionally, the 'Food Allergen Labelling and Consumer Protection Act' (FALCPA), was adopted August 2, 2004, to be effective January 1, 2006. JP: Food Allergen Labelling Guidelines of 2001. AU: Australia New Zealand Food Standards Code of 2000). Such mandatory labelling typically concerns the eight major food allergens (''the Big Eight'': Cow's milk; Hen's egg; Fish; Crustacean/shellfish; Tree nuts; Wheat; Peanuts; and Soybeans), but may also comprise substances, which can cause intolerance only e.g. lactose.

As a result of above developments, all products being produced using modern biotechnology are subject to increased requirements on documenting the safety towards the final consumer from regulatory authorities, including appropriate documentation that ingestion of the products is not a concern for food allergy.

Enzymes constitute a class of products being produced by modern biotechnology. They may be extracted from animal or vegetable sources or may be produced in bacteria, fungi or yeasts. Some enzymes are produced by micro-organisms derived from wild-type strains (non-GMM); others are produced by genetically modified micro-organisms (GMM). Enzymes found in nature have been used since ancient time in the production of foods and in the manufacture of commodities. All these processes relied on either enzymes produced by spontaneously growing microorganisms or enzymes present in added preparations such as calves' rumen or papaya fruit. Industrially produced, food grade enzymes are used as processing aids in the manufacturing of a wide variety of foods such as bread, beer, beverages, dairy products etc.

Enzymes are proteins. Like many other proteins they may have the potential to cause allergic responses. Investigations have demonstrated that workers producing enzymes may develop sensitization to the enzymes after inhalation exposure (Bernstein et al., 1994, 1999; Zober, 2002; Merget et al., 2001; Burstyn et al., 1998; Leser et al., 2001; Quirce et al., 2002; van Kampen et al., 2002). This is today minimized considerable due to better knowledge of safe handling of the enzymes e.g. by making them as free of dust as possible e.g. by developing tough encapsulated granulates. In contrast, no reports on sensitization to these enzyme products in the final commercial food after ingestion exist. This may be due to the difference in exposure pattern of the inhalation route compared to the digestive route, or it may be due to the fact that the enzymes most often are present in the final foods in low amounts and in inactive forms.

The aim of the present study was to investigate the safety to allergic patients of 19 commercially available and authority approved enzymes. The enzymes were selected to reflect a wide range of enzymatic activities as well as enzymes being produced by both non-GMM and GMM.

2. Materials and methods

Four hundred consecutive adult patients (276 female and 124 men, mean age 38 years) with diagnosed allergy to inhalation allergens, food allergens, allergens of bee or wasp, or drugs were included, after informed consent was obtained. All patients had a positive skin prick test (SPT) result towards at least one of the above allergens according to EAACI guidelines (Dreborg et al., 1987) and all were free of symptoms on the day(s) of testing. Patients with a history of severe allergic reaction, pregnant or lactating women and patients treated with drugs known to interfere with the result of skin prick testing were excluded. The sensitizations in the patients are presented in Table 1.

The enzymes investigated are presented in Table 2. The enzyme preparations applied in the SPT and histamine release (HR) testing described in this paper were test batches which all were mixtures of 3–5 separately recovered and fermented sub test batches to secure that the testing material were representative. The test batches were fermented and recovered according to the same procedures as are used for production of commercial enzyme preparations. A part from water and the enzyme protein itself the test batch also contained other soluble organic substances from the fermentation; mainly protein and carbohydrate components. All test batches were analyzed extensively for chemical and microbial content documenting that the test batches complied with the Food and Agriculture Organization/World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA) and Food Chemical Codex (FCC) recommended purity specifications for food grade enzymes, including analyses to show that the test batches did not contain the production strain (FCC, 2001; JECFA, 2004).

The test batches are usually used for all the toxicological investigations done on the enzyme preparations before registration. The same test batches are also used in Novozymes' Occupational Health Service (OHS) for skin prick testing when testing workers for possible occupational allergy

Table 1
The different sensitizations represented and their distribution in the 400 patients included in the study, STEP 1

Positive skin prick test	Female	Male
Birch	149	46
Grass	164	77
Mugworth	62	14
Horse	42	10
Dog	94	21
Cat	94	26
Dust mites	95	47
Moulds	13	4
Vespula vulgaris	9	9
Honey bee	1	3
Hen's egg	4	1
Cow's milk	6	3
Tree nuts	47	14
Penicillin	4	1
Fish	3	8
Latex	1	1
Others (rabbit, tomato, wheat, rye, banana, poultry, peanut, poppy seed, shrimp, crab, guinea pig, chlorohexidine)	5	4

The patients may have a number of different sensitizations.

Table 2
The 19 enzymes tested in the study

Enzyme no.	Enzyme type	PE	Production strain	GMM	Donor organism
1	Maltogenic amylase	−	*Bacillus subtilis*	Yes	*Bacillus* sp. 1
2	Protease	−	*Bacillus amyloliquefaciens*	No	NA
3	Decarboxylase	−	*Bacillus subtilis*	Yes	*Bacillus* sp. 2
4	Alpha-amylase	+	*Bacillus licheniformis*	Yes	*Bacillus* sp. 3
5	Alpha-amylase	+	*Bacillus licheniformis*	Yes	*Bacillus* sp. 1
6	Glucoamylase	−	*Aspergillus niger*	Yes	*Aspergillus* sp. 1
7	Alpha-amylase	−	*Bacillus amyloliquefaciens*	No	NA
8	Pectin lyase	−	*Aspergillus niger*	Yes	*Aspergillus* sp. 1
9	Glucoseoxidase	−	*Aspergillus niger*	No	NA
10	Lipase	+	*Aspergillus oryzae*	Yes	Thermomyces sp. 1
11	Lipase	−	*Aspergillus oryzae*	Yes	Fusarium sp.
12	Xylanase	−	*Aspergillus oryzae*	Yes	Thermomyces sp. 1
13	Pectinesterase	−	*Aspergillus oryzae*	Yes	*Aspergillus* sp. 2
14	Beta-glucanase	−	Humicola insolens	No	NA
15	Glucoseoxidase	−	*Aspergillus oryzae*	Yes	*Aspergillus* sp. 1
16	Laccase	−	*Aspergillus oryzae*	Yes	Myceliopthora sp.
17	Alpha-amylase	−	*Aspergillus oryzae*	No	NA
18	Alpha-amylase	−	*Bacillus licheniformis*	Yes	*Bacillus* sp. 3
19	Protease	−	*Bacillus licheniformis*	No	NA

PE: protein-engineered enzyme, GMM: gene modified microorganism, sp: species, NA: not applicable.

against enzyme preparations. A concentration of 100 μg protein per ml in the SPT has been used by OHS for several years and has also been used for this study. A too high protein concentration will cause many false-positive reactions due to irritation and a concentration of 100 μg protein per ml has been found to have a sensitivity of 100% but a reduced specificity (Bernstein et al., 1994, 1993).

3. Test protocol

After informed consent was obtained the patients were tested using SPT in single determination with all the enzymes using the test batches with a concentration of 100 μg protein per ml and prepared in 50% glycerol (STEP 1). Any positive reaction (wheal > 3 mm larger than the negative control) was repeated in duplicate and if still positive, titrated in 1:10 dilutions in duplicate until the reaction had disappeared. Histamine hydrochloride 10 mg/ml was used as the positive control.

In all patients with a positive initial reaction in skin prick test, blood was drawn for histamine release testing with the enzyme test batch(es) in question.

Histamine release from basophil leukocytes was performed as previously described (Scheurer et al., 2001). Samples of 25 μl heparinized blood was applied to glass fibre coated microtitre wells (HR-Test from RefLab, Copenhagen, Denmark) and incubated with 25 μl of a dose range of the enzymes for 60 min at 37 °C. Each enzyme was tested in 12 concentrations, each in duplicate, from 100 μg/ml to 0.1 ng/ml (dilution factor 1:3.5). All serial dilutions of enzyme were made in PIPES-buffer (RefLab, Denmark). During incubation of patient blood with enzyme, released histamine is adsorbed to the glass microfibre coated microtiter plates followed by a fluorometrically determination of released histamine. A release of 10 ng histamine/ml blood is significant corresponding to 3 × standard deviation of background fluorescence. Unspecific enzyme induced histamine release was examined by incubating each enzyme with blood from two non-allergic healthy individuals and was generally observed down to 10 μg enzyme/ml. A significant histamine release at 1 μg enzyme/ml or less was therefore defined as a specific positive reaction.

In the second phase (STEP 2), all patients positive to one or more enzymes were investigated further. This was done by skin prick testing with the enzymes using the test batch (again) and, if available, the enzyme protein obtained by further purification of the test batch. Furthermore, as a model for everything in the test batch but the enzyme protein itself, fermentation broth from the wild-type *Aspergillus* and *Bacillus* strains grown using standard conditions and standard media were also tested (later referred to as the "wild-type model broth"). Please observe that the strains themselves were removed from the broth before using.

The histamine release testing was also repeated using the relevant skin prick testing material described above.

Subsequently double blind, placebo controlled food challenge (DBPCFC) was performed using commercially available enzyme products on separate days with the culprit enzyme(s) according to EAACI guidelines (Bindslev-Jensen et al., 2004).

4. Challenge material

For the food challenges were in all cases used the individually relevant commercial enzyme products as is. All the commercial enzyme products used have been tested and approved for food use in Denmark with specific usage limits. The enzyme products are generally widely used in different products in Denmark.

The maximum allowed dosages (in Denmark) of each enzyme product in each application have been used for the calculations of the dosages using a fixed intake of the

relevant food/beverage of 250 g in order to illustrate a worst-case situation. To further illustrate a worst-case situation it was in every case assumed that all enzyme activity was retained 100%, even though the enzyme product is largely inactivated and/or removed as a result of the food/beverage production process. The different relevant enzyme products were pooled into one active food challenge prepared especially for each patient, with the exception of the protease enzymes. Proteases may degrade other enzymes and therefore each protease was always given alone. This meant that in some cases a patient was given more than one active food challenge.

To blind the enzyme products they were placed in non-transparent cups with straws and dissolved in water (150 ml) and black currant juice (2 ml), the pH being close to neutral. The cups with content were frozen (−18 °C) immediately after the preparation and de-thawed just before use. Placebo challenges containing water and black currant juice only was used as well.

The protocol was approved by The Ethics Committee for Funen and Vejle Counties (Jr No: VF 20020198).

5. Results

Among the 400 tested allergic individuals, 387 (97%) did not react in the skin prick test when challenged with the selected enzymes using the test batches. Thirteen patients were positive, and their demographic data, sensitizations, skin prick testing and histamine release results obtained in STEP 1 are presented in Table 3. These patients reacted

Table 3
Demographic data, allergic diseases, allergies, skin prick testing and histamine release results of 13 patients having one or more positive skin prick test to an enzyme in STEP 1 and challenge results in STEP 2

Patient no.	Age	Sex	Allergic diseases	Allergies	SPT positive in STEP 1 (dilution)	HR positive in STEP 1 (μg/ml)	Challenge results in STEP 2
23	38	F	A, R, C, AE	Grass, mould, mite, milk	Enzyme 2 [(1:1)]		Negative to both active and placebo
26	37	F	A, R, C	Grass, mould, mite	Enzyme 1 [1:1]		Negative to both active and placebo
28	18	M	R, C, AE	Birch, grass, mugworth, dog, mite	Enzyme 2 [1:1] Enzyme 8 [1:1] Enzyme 11 [1:1]	 Enzyme 11 [1 μg/ml]	Two active challenges, Negative to both active and placebo
30	22	F	A, C, AE	Birch, grass, mugworth, dog, cat	Enzyme 6 [1:10]	Enzyme 6 [1 μg/ml]	Reaction to placebo, negative to active
63	57	F	A, R, C	Birch, grass, dog, mite	Enzyme 6 [1:1]		Negative to both active and placebo
66	42	F	R	Birch, grass, mugworth, cat	Enzyme 6 [1:1] Enzyme 7 [1:1] Enzyme 15 [1:1] Enzyme 19 [1:1]		Two active challenges, Negative to both active and placebo
83	45	F	R	Dog, cat, horse, mite	Enzyme 5 [±] Enzyme 13 [±] Enzyme 16 [±] Enzyme 17 [±]	 Enzyme 16 [0.1 μg/ml] Enzyme 17 [0.3 μg/ml]	Negative to both active and placebo
90	30	F	R, C, AE	Birch, grass, cat, poppy seed	Enzyme 6 [(1:1)]		Negative to both active and placebo
93	34	F	R, C	Grass, cod, shrimp	Enzyme 10 [(1:1)]		Negative to both active and placebo
134	46	M	R, C	Grass	Enzyme 10 [1:1] Enzyme 12 [(1:1)]	Enzyme 10 [1 μg/ml] Enzyme 12 [0.3 μg/ml]	Negative to both active and placebo
185	35	F	A, R	Birch, grass, mite, egg, milk	Enzyme 11 [1:1] Enzyme 12 [(1:1)]	Enzyme 11 [0.3 μg/ml] Enzyme 12 [0.1 μg/ml]	Negative to both active and placebo
367	27	F	A, R, C	Birch, grass, mugworth	Enzyme 1 [(1:1)] Enzyme 3 [(1:1)] Enzyme 6 [(1:1)] Enzyme 10 [1:10] Enzyme 11 [1:1] Enzyme 12 [1:1] Enzyme 13 [(1:1)] Enzyme 14 [1:1] Enzyme 15 [1:1] Enzyme 16 [1:1] Enzyme 17 [1:1]		Negative to both active and placebo
372	29	M	R, C	Birch, grass, dog, cat, mite	Enzyme 15 [(1:1)]		Negative to both active and placebo

F: Female, M: male, A: asthma, R: rhinitis, C: conjunctivitis, AE: a topic eczema. (1:1): The first SPT positive, but negative a repetition. ±: SPT negative (>3 mm), but flare seen in the area of the wheal.

Table 4
Positive skin prick testing and histamine release results in the 13 patients re-tested in STEP 2

Patient no.	Enzyme 6 test batch		Purified Enzyme 6		Enzyme 10 test batch		Purified Enzyme 10		Enzyme 12 test batch		Purified Enzyme 12		Enzyme 16 test batch		Enzyme 17 test batch		Purified Enzyme 17	
	SPT	HR	SPT	HR	SPT	HR	SPT	HR	SPT	HR	SPT	HR	SPT	HR	SPT	HR	SPT	HR
23																		
26																		
28																		
30	12	Neg	9	Neg														
63	7	Pos	5	Pos														
66	3	Neg	3	Neg														
83											0	Pos	0	Pos	3	Pos	0	Neg
90	0	Pos	0	Pos														
93					2	Pos	0	Neg										
134					2	Pos	0	Neg	3	Pos								
185																		
367	0	Neg	0	Neg	3	Pos	0	Neg	0	Neg	2	Pos	2	Pos	2,5	Pos	0	Neg
372			9	Neg														

SPT: skin prick test, all numbers indicate the wheel size in mm. HR: histamine release test. Pos: positive, Neg: negative. Only positive results are presented in the table (if the enzyme test batch elicited one or more positive SPT and/or HR, the results of the purified enzymes are also presented if tested, even if the results were negative in both SPT and HR).

with a positive SPT to various enzymes, most frequent to Enzyme no. 6, a glucoamylase (6 patients) followed by Enzyme nos. 10 and 11, two lipases (each 3 patients). In no cases, we found a positive SPT to dilutions below 1:10 of the stock solution. The patients with positive reactions in SPT and/or HR represented a variety of allergic diseases and all but one (pt 83) were sensitized to pollen(s).

The following enzyme test batches elicited no positive reactions in STEP 1: Enzyme nos. 4, 9, and 18.

In STEP 2, the 13 patients positive in STEP 1 were re-tested with the enzymes causing a positive reaction in the STEP 1 using the test batch(es) again, and, if available, the purified enzyme protein(s) and the wild-type model broth(s) as described in the test protocol.

In STEP 2, the following elicited no positive reactions in any of the patients: Enzyme no. 1 (test batch and purified), Enzyme no. 2 (test batch and purified), Enzyme no. 3 test batch, Enzyme no. 5 (test batch and purified), Enzyme no. 7 (test batch and purified), Enzyme no. 8 (test batch and purified), purified Enzyme no. 10, Enzyme no. 11 test batch, purified Enzyme no. 12, Enzyme no. 13 test batch, Enzyme no. 14 test batch, Enzyme no. 15 (test batch and purified), purified Enzyme no. 17 and Enzyme no. 19 (test batch and purified) (data not shown). Purified Enzyme no. 3, purified Enzyme no. 11, purified Enzyme no. 13, purified Enzyme no. 14 and purified Enzyme no. 16 were not tested as these materials were not available. As can be seen from Table 4, the remaining enzymes elicited positive reactions in SPT and/or HR in some cases; in only one case (Enzyme no. 6, a glucoamylase), however, a positive result was obtained with the purified enzyme proteins (obtained by chromatographic methods).

When testing the wild-type model broths most patients except no. 23, 26 and 63 were positive to the wild-type model broth obtained from *Aspergillus oryzae* and/or *Aspergillus niger* in either SPT and HR, but no patients were positive to wild-type model broth obtained from *Bacilllus* in neither SPT nor HR (data not shown).

The 13 patients were finally challenged in a double blind, placebo controlled protocol with either one or two (patients 28 and 66) different active challenges together with a placebo. One positive reaction (patient 30) to placebo was obtained. Thus, no positive challenges to the enzymes positive in SPT and/or HR in STEP 1 or STEP 2 were found (Table 3).

6. Discussion

Workers exposed to enzymes for use in the food industry may develop allergy to the enzymes via inhalation (Bernstein et al., 1994, 1999; Zober, 2002; Merget et al., 2001; Burstyn et al., 1998; Leser et al., 2001; Quirce et al., 2002; van Kampen et al., 2002), whereas sensitization to the enzymes by oral route in the consumers has not been described.

We investigated a possible clinical allergenicity of 19 enzymes used in the food industry. The enzymes were selected to reflect a wide range of enzymatic activities as well as enzymes being produced by both non-GMM (6 enzymes) and GMM (13 enzymes), see Table 2. The enzymes were tested both in vivo using skin prick testing and in vitrousing histamine release from human basophils. In only 13 of 400 allergic patients investigated (3%) a positive SPT was found, often accompanied by a positive histamine release. These 13 patients were further investigated using the test batch of the enzymes resulting in a positive initial testing again and including purified enzyme protein preparations, if available, and wild-type model broths. These preparations were used for SPT and HR. In 46 out of 55 reactions there was a concordance corresponding to 84% between SPT and HR. For the food challenges (DBPCFC) commercially available enzyme products were used.

The FAO/WHO report on assessment of allergenicity of foods derived from biotechnology (Aalberse et al., 2001) suggests using in vitro techniques such as measurement of specific IgE both when the product arises from an organism known to be allergenic in man (e.g. fish) (Hansen et al., 2004; Bindslev-Jensen et al., 2003) and when the product is produced in organisms not known to be allergenic. In the latter case, a targeted approach is recommended i.e. using sera from patients with a variety of allergies of different nature. We used skin prick testing with the test batches of the enzymes as the initial screening procedure since no commercial IgE methods are available and for practical reasons, since testing of 400 patients with 19 different subjects in histamine release would have been impossible. SPT in workers exposed to enzymes produced in *Bacillus* species has previously been demonstrated to be more sensitive than measurement of specific IgE (Bernstein et al., 1994).

The initial procedure was followed by retesting with crude and purified enzymes in the patients positive in the initial phase. Although some of the enzyme test batches also in the second phase elicited a positive response in SPT or HR, only one of the purified enzyme proteins (Enzyme no. 6, a glucoamylase) was positive in four of the patients (30, 63, 66 and 90). Patients no. 30, 66 and 90 were also positive in SPT or HR to the wild-type model broth from *A. niger* or *A. oryzae*. None of these patients were positive to moulds, where cross reactivity between Cladosporium or Alternaria has been described (Mari et al., 2003). The four patients were all positive to pollens and animal dander, but no data on possible cross reactions between *Aspergillus* and pollen resp. animal dander has been published – and presence of *Aspergillus* antigens in the purified Enzyme no. 6 was not investigated. Only one case report of allergic rhinitis to *Aspergillus* has been reported previously (Taj-Aldeen et al., 2003), whereas Allergic Bronchopulmonary Aspergillosis of course frequently is seen. In a large scale study involving more than 4000 patients, sensitization to *Aspergillus* measured by SPT was seen in 2.4% of the patients, a percentage increasing to 12.6% in patients sensitized to other fungi (Mari et al., 2003).

The reason for the positive findings in SPT and HR remains obscure; one suggestion would be that sensitization (route unknown) may occur in rare cases, but since none of the patients were positive to any of the commercial enzymes in the subsequent oral challenge using exaggerated dosages of the enzymes compared to normal daily intake, the findings are without any clinical relevance.

The allergen labelling regulations and the FAO/WHO decision tree all aim at protecting the allergic consumer – by using oral challenges with the enzymes as the final proof of non-reactivity these criteria has been fulfilled. These results were obtained using enzymes with a wide range of enzymatic activities in active forms, i.e. before they had been degraded by e.g. heat in the final commercial product thus adding a further safety factor to the findings.

There were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in this study.

Considering the wide variety of enzyme classes and origins included in this study it is concluded that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy.

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