

Is fungal α -amylase in bread an allergen?

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Summary

Background The enzyme α -amylase from *Aspergillus oryzae* used in bakeries to improve the bread quality has been identified as an inhalative allergen in baker's asthma. It is doubtful whether this enzyme can induce allergic sensitization in regular bread consumers.

Objective To find out whether fungal α -amylase in bread and rolls retains its antibody-binding capacity and allergenicity after the baking procedure.

Methods Rabbit antibodies directed to fungal α -amylase were used for the development of a two-site enzyme-linked immunosorbent assay (ELISA). This assay was used to analyse different fractions of bread and rolls baked with the usual amounts of α -amylase in comparison with control products without added enzyme. Competitive experiments between bakers' sera containing specific immunoglobulin (Ig) E to α -amylase and the rabbit antibodies were performed. Additionally, specific IgE binding to fungal α -amylase was inhibited by native or heated α -amylase.

Results With the highly specific two-site ELISA for native α -amylase in the crust of bread bottom and sides, 2.3–7 ng antigenic α -amylase per gram crust were measured. No α -amylase could be detected in the crumb fractions. Rabbit antibodies to native α -amylase completely inhibited human IgE binding to α -amylase allergen disks.

Conclusion The results prove residual antibody-binding capacity of α -amylase in bread crusts and in the crust of some rolls. In comparison with the content of α -amylase in dough, between 0.1 and 20% of the antibody-binding capacity remained.

Keywords: α -amylase, antigenic determinants, baker's asthma, bread

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Introduction

Fungal α -amylase used in bakeries to improve the bread quality is a frequently reported cause of occupational asthma, rhinitis and other allergic symptoms [1–6]. This enzyme, usually derived from *Aspergillus oryzae*, could be shown to increase the rate of sensitization depending on airborne exposure levels. α -amylase exposure levels above 0.25 ng/m³ as an average exposure during an 8-h work shift increased the risk of sensitization of bakery workers [7]. Additionally, allergic reactions after ingestion of fungal α -amylase [8] or consumption of α -amylase-containing bread [9,10] have been reported in a few individuals already sensitized through airborne occupational exposure.

In regular bread consumers, fungal α -amylase-induced food allergies have not been reported yet. The risk of sensitization to this enzyme in bread was controversially evaluated. Alday *et al.* [11] stated that this kind of food does after heating not present a health hazard for the population exposed to it because heating α -amylase to 65 °C induced a loss of allergenicity in skin prick tests, histamine release and enzyme-linked immunosorbent assay (ELISA). Baur *et al.* [12] found that heating of α -amylase to 200 °C abolished allergenic activity while heating temperatures between 80 and 99.8 °C partially retained its immunoglobulin (Ig) E-binding capacity. In all these experiments, α -amylase was heated in the absence of flour and yeast and without dough preparation. These conditions are artificial and do not exactly reflect the baking process. In addition to temperature, parameters like the presence of water, substrate (starch) or ions, as it was shown for Ca²⁺ [13], may have great influence on the stability of α -amylase.

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In the present study, we pursued a different strategy. We tried to investigate the antigenic and allergenic binding capacity of α -amylase in bread and rolls baked with usual amounts of α -amylase. Using rabbit antibodies raised specifically for fungal α -amylase, we developed a sensitive two-site antibody-based ELISA and measured antigenic α -amylase in different fractions of bread and rolls. Additionally, the competitive inhibition between these rabbit antibodies and sera of α -amylase-sensitized bakers was investigated.

Materials and methods

Anti- α -amylase antibodies

α -Amylase from *Aspergillus oryzae* (Fungamyl® 1600s, Novo Nordisk, Mainz, Germany) was purified by anion-exchange chromatography [14]. In addition to the purified native α -amylase, α -amylase (4 mg/mL PBS; 0.02 mol/L sodium phosphate buffer, 0.05 mol/L NaCl, pH 7.4) heated for 10 min to 98 °C in a thermocycler (Perkin Elmer, Weiterstadt, Germany) was used for the intradermal immunization of New Zealand white rabbits. For primary injection, 2 mL containing 2 mg protein and 50% Freund's complete adjuvant were given. Two booster injections followed with the same antigen dose in PBS at intervals of 4 weeks.

The polyclonal rabbit antibodies (pAb) directed to native α -amylase were purified by affinity chromatography on Sepharose-coupled Protein G (HiTrap® Protein G, Pharmacia, Freiburg, Germany) following the instructions of the manufacturer.

Parts of the purified pAbs (1–2 mg/mL in 10 mmol/L carbonate buffer, pH 8.5) were biotinylated by mixing with a 15-fold molar excess in dimethylsulphoxide-dissolved biotin-*N*-hydroxysuccinimide-ester (Boehringer Mannheim GmbH, Mannheim, Germany) and by incubating them during continuous agitation for 3 h at 20 °C. The biotinylated antibodies were dialysed extensively and stored in aliquots at –20 °C.

Inhibition of human IgE binding to α -amylase with antigen

Specific human IgE antibodies were measured by the enzyme allergosorbent test (EAST) from Allergopharma (Reinbeck, Germany) with allergen couplings of α -amylase from *Aspergillus oryzae* (Fungamyl® 1600s, Novo Nordisk). For coupling, BrCN-activated paper disks were incubated in 0.1 mol/L NaHCO₃ (pH 8.4) containing 2 mg/mL protein for 5 h at room temperature, saturated by ethanolamine and washed according to the method described by Ceska *et al.* [15].

Heated fungal α -amylase for inhibition was prepared as followed: α -amylase (1 mg/mL PBS) was heated for 10 min to 98 °C in a thermocycler or heated for 50 min to 200 °C in

an incubator (evaporated water was substituted). After centrifugation for 15 min at 4000 *g*, the protein concentration of the supernatants was determined by the Bradford assay [16]. The solution with α -amylase heated to 200 °C showed a loss of 90% of the protein and was concentrated by ultrafiltration with Centriprep-10 centrifugation tubes (Amicon, Witten, Germany).

Eight bakers with workplace-related respiratory symptoms (rhinitis in eight, asthma in six, conjunctivitis in four and eczema in three cases) were selected for serum analysis. These sera contained specific IgE for α -amylase from *Aspergillus oryzae* with the following results in the EAST: 3.0 kU/L, 2.7 kU/L, 2.2 kU/L, 3.3 kU/L, 1.2 kU/L, 12.9 kU/L, 2.4 kU/L and 11.2 kU/L.

Pooled patients' sera (30 μ L) were preincubated for 3 h with 30 μ L diluted antigen (native or heated α -amylase). Subsequently, the sera were incubated overnight with allergen-coated disks, and bound IgE was determined as described by the manufacturer.

Inhibition of human IgE binding to α -amylase with rabbit antisera

Allergen-coated disks were pre-incubated for 30 min with 30 μ L diluted rabbit serum directed to native or heated α -amylase or a control serum directed to the allergen Chi t 1.01 (haemoglobin component III from *Chironomus thummi thummi* [17]). Pooled patients' sera (30 μ L) were added. After incubation for 3 h, bound IgE was determined as described by Allergopharma.

Two-site antibody-based ELISA

MaxiSorp™ StarWell™ (Nunc, Wiesbaden, Germany) microtitre plates were coated with purified pAb (10 μ g protein/mL carbonate/bicarbonate buffer, pH 9.6) overnight at 4 °C. The wells were blocked with 2% (w/v) bovine serum albumin in PBS for 2 h and washed three times with PBS-containing 0.05% Tween 20 (PBST). A standard curve with dilutions containing between 25 pg and 25 ng/mL α -amylase (Fungamyl® Novo Nordisk), and serially diluted wheat flour, rye flour and yeast protein extracts in PBS, and the bread extracts were incubated in 100 μ L/well for 1 h at 37 °C. After washing three times with PBST, the biotinylated pAb (derived from the same animal as the capture antibody) was added. Ninety minutes later, all wells were washed three times with PBST and then incubated for 1 h with 100 μ L 1:4000 in PBST diluted peroxidase-avidin solution (DAKO, Hamburg, Germany). After washing three times with PBST and twice with PBS, the assay was developed with 100 μ L ortho-phenyldiamine substrate solution (DAKO, Hamburg, Germany) which was activated with H₂O₂ according to the instructions of the manufacturer.

The enzyme reaction was stopped after 20–30 min by adding 100 μ L 1 mol/L H_2SO_4 and absorbance measurement at 492 nm. The dose–response curve for the standards was obtained by 4-parameter curve fitting using the software Sigmaplot (Jandel, Erkrath, Germany).

Production and extraction of bread and rolls

For rolls, three different dough samples were prepared containing 0.004% Fungamyl® 1600s, 2.5% of a usual baking additive (Aromalt, Ireks, Kulmbach, Germany) per 100 g wheat flour or without enzyme. Fungamyl® 1600s contains about 20% fungal α -amylase, while the baking additive contains about 15 mg of fungal α -amylase per 100 g [18].

The rolls were baked in an incubator (Heraeus, Osterode, Germany) at 200 °C for 20 min or under conventional baking conditions at 240 °C for 20 min at the 'Bundesanstalt für Getreide-, Kartoffel- und Fettforschung (BAGKF)'. Three different bread samples were also baked there containing 0.00125% Fungamyl® 1600s or 2.5% of the baking additive (Aromalt) per 100 g wheat flour or without enzyme. The bread was baked in metal pans for 40 min at 230 °C. During the initial stage of baking (about 30 s) at the BAGKF, water steam moistened the dough's surface.

Different fractions of baked bread and rolls were selected for the measurements. Dough samples were taken before baking. The crumbs of bread and rolls were taken from the centre and from the area below the crust. The crust of rolls was taken from the upper surface and the bottom. The bread crust was cut from the upper part (outside the pan during baking), bread bottom and bread sides (inside the pan during baking). About 15% of the total bread weight was crust.

For extraction, 200 mg dough or 0.4–5 g of crust or crumb fractions were mixed with 25 mL PBST. Each sample was vortexed for 2 min and sonicated for 3 min. After an interval of 5 min, this procedure was repeated twice. The extracts were centrifuged at 20 000 g for 20 min, and the supernatants were stored at –20 °C. Flour proteins and baker's yeast proteins were extracted in the same way [18]. The protein concentration was determined according to Bradford [16].

Results

Inhibition of IgE-binding to α -amylase by native or heated α -amylase

Binding of human IgE to native α -amylase coupled to cellulose disks was completely inhibited by 0.02 μ g of native α -amylase (Fig. 1). When heated α -amylase was used as inhibitor, about 0.7 μ g of α -amylase heated for 10 min to 98 °C and more than 6 μ g of α -amylase heated

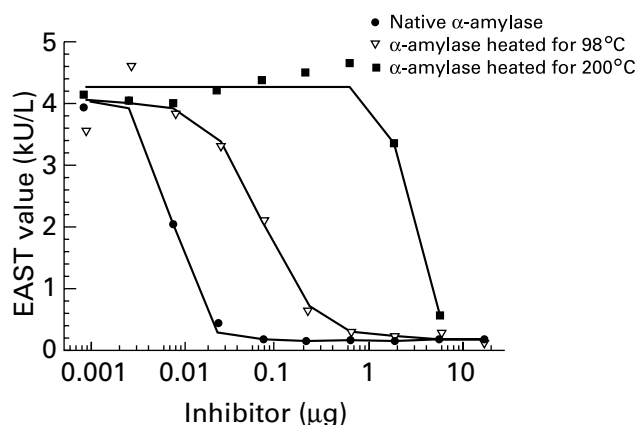


Fig. 1. Inhibition of IgE-binding to fungal α -amylase allergen disks by native or heated α -amylase. In EAST with pooled sera from eight bakers sensitized to α -amylase from *Aspergillus oryzae*, native α -amylase, α -amylase heated to 98 °C or α -amylase heated to 200 °C were used as inhibitor

for 50 min to 200 °C were needed for complete inhibition. Heating of purified α -amylase obviously reduced the antibody-binding capacity of this allergen. This effect cannot be due to aggregation and precipitation of α -amylase during heating because after heating, insoluble α -amylase was discarded and protein concentrations were adjusted according to the results of Bradford assay.

Binding characteristics of rabbit antisera against α -amylase

After immunization, we obtained two rabbit sera directed to native α -amylase and to α -amylase heated to 98 °C. To investigate whether rabbit antibodies to α -amylase compete with anti- α -amylase IgE antibodies of bakers sensitized to this allergen, EAST-inhibition experiments were performed (Fig. 2). Rabbit antiserum to native α -amylase inhibited specific IgE binding to fungal α -amylase completely while rabbit antiserum directed to α -amylase heated to 98 °C gave only partial inhibition. No inhibition occurred with the control serum from a rabbit immunized with the allergen Chi t 1.01 [17]. The inhibition proves competition between human IgE and rabbit antibodies directed to α -amylase.

Two-site ELISA for native α -amylase

With the purified rabbit antibodies directed to native α -amylase, a two-site ELISA was developed. The best results were obtained with 1 μ g capture antibody and 30 ng of biotinylated detection antibody per well. The assay could measure α -amylase in the range of 0.2–1 ng/mL whereas high concentrations of wheat and rye flour (10–300 μ g

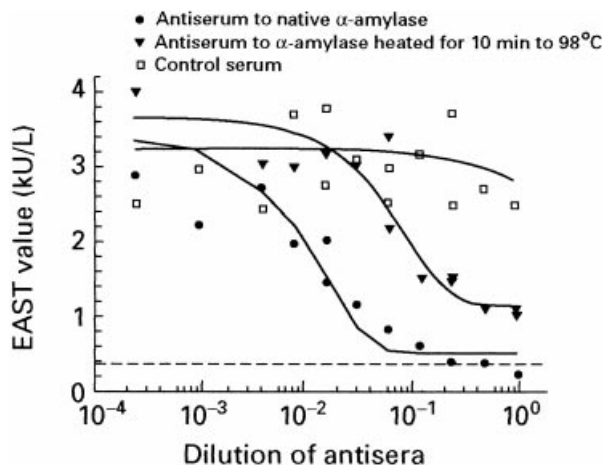


Fig. 2. Competitive inhibition of human IgE-binding to fungal α -amylase by rabbit antisera. Pooled sera from eight bakers sensitized to α -amylase from *Aspergillus oryzae* were incubated with α -amylase allergen disks in the presence of serial dilutions of rabbit antiserum raised to native α -amylase, antiserum to α -amylase heated for 10 min to 98 °C or control serum

protein/mL) and baker's yeast (4–130 μ g protein/mL) were not detected (data not shown).

Antigenicity of α -amylase in bread and rolls

The assay directed to native α -amylase was used to measure antigenic α -amylase in extracts of various fractions from bread and rolls. In rolls baked in our laboratory, we could detect α -amylase in the crust while the crumb fraction did not contain measurable amounts of α -amylase (Table 1).

Table 1. α -amylase in rolls (baked in our laboratory). α -amylase concentrations are arithmetic means $\pm$ standard deviation of repeated measurements (n) with the two-site ELISA for native α -amylase

	Concentration of α -amylase in:		
	Dough (μ g/g)	Crumb centre (μ g/g)	Crust upper part (μ g/g)
Control roll	b.d. ($n = 4$)	b.d. ($n = 8$)	b.d. ($n = 8$)
Roll with α -amylase	3 ± 0.58 ($n = 4$)	b.d. ($n = 8$)	0.79 ± 0.27 ($n = 5$)
Roll with baking additive	2.6 ± 0.19 ($n = 4$)	b.d. ($n = 8$)	0.45 ± 0.12 ($n = 5$)

b.d. below the detection limit for α -amylase.

Compared with the measured amount of antigenic α -amylase in dough which was 60–80% of the theoretically expected α -amylase, about 20% of α -amylase remained antigenic in the crust of these rolls. On the other hand, in rolls baked under conventional baking conditions at the BAGKF, all measurements of crumb and crust fractions were below the detection limit of our assay. The values obtained by repeated measurements of fractions prepared from conventionally baked bread at the BAGKF are presented in Table 2. In the crust from bread bottom and sides, about 2.3–7 ng α -amylase/g were found. In comparison with the measured amount in the dough, only 0.1–0.6% of α -amylase remained antigenic.

Discussion

Enzymes from varying organisms are increasingly used for the preparation of food. It is not known whether this leads to an increased risk of developing food allergies. As an example, we investigated the usage of α -amylase from *Aspergillus oryzae* for the production of bread and rolls and antibody binding after structural modifications of this enzyme during the baking process. The temperatures of dough and bread vary during the baking process. The temperature directly on the surface corresponds to the ambient oven temperature (in general 200–250 °C) while the interior shows after an initial increase a mainly constant temperature of about 98 °C due to water vaporization [19]. Using purified α -amylase heated either for 10 min to 98 °C (simulating the heat conditions of bread and roll interiors) or for 50 min to 200 °C (simulating the heat conditions on the bread surfaces) in inhibition experiments with sera from patients with baker's asthma, the heated allergen showed a reduced IgE-binding capacity when compared with native α -amylase. While only 0.02 μ g of native α -amylase completely inhibited binding to α -amylase allergen disks, about 0.7 μ g of α -amylase heated for 10 min to 98 °C and more than 6 μ g of α -amylase heated for 50 min to 200 °C were needed for inhibition.

However, it was estimated that bread and roll dough contains only about 1 mg of α -amylase per kg flour. High flour quantities including starch (the substrate of α -amylase) may have an influence on the immunological stability of fungal α -amylase. Therefore, we tried to investigate the antibody-binding capacity of fungal α -amylase in bread and rolls.

We produced rabbit antibodies directed to fungal α -amylase in native conformation or to α -amylase after heating for 10 min to 98 °C or 50 min to 200 °C. The rabbit antibodies directed to α -amylase most probably recognized the same or overlapping epitopes as human IgE antibodies to α -amylase as competition experiments between bakers' sera with IgE to α -amylase and rabbit

Table 2. α -amylase in bread (baked on conventional baking conditions). α -amylase concentrations are arithmetic means $\pm$ standard deviation of repeated measurements (n) with the two-site ELISA for native α -amylase

	Concentration of α -amylase in:					
	Bread crumb		Bread crust			
	Dough ($\mu\text{g/g}$)	Centre (ng/g)	Below crust (ng/g)	Upper part (ng/g)	Sides (ng/g)	Bottom (ng/g)
Bread control	b.d. ($n=4$)	b.d. ($n=4$)	b.d. ($n=4$)	b.d. ($n=5$)	b.d. ($n=11$)	b.d. ($n=11$)
Bread with α -amylase	1.13 ± 0.33 ($n=2$)	b.d. ($n=3$)	b.d. ($n=3$)	b.d. ($n=5$)	6.96 ± 1.08 ($n=11$)	5.6 ± 1.13 ($n=11$)
Bread with baking additive	3.35 ± 0.76 ($n=2$)	b.d. ($n=3$)	b.d. ($n=3$)	b.d. ($n=5$)	3.84 ± 1.91 ($n=7$)	2.32 ± 0.93 ($n=7$)

b.d. below the detection limit for α -amylase.

antisera indicate. Binding of human IgE to fungal α -amylase could be inhibited completely with rabbit antiserum directed to native α -amylase and partially with rabbit antiserum directed to α -amylase heated to 98 °C. Using a highly specific and sensitive two-site ELISA based on rabbit antibodies which were raised against native α -amylase, binding to different fractions of bread and rolls was investigated. The fractions were prepared from the crust of the bread bottom, bread sides and upper part or from the crumb below the crust or the centre. No α -amylase could be detected in the crumb fractions while in the crust of the bottom and sides, 2.3–7 ng α -amylase/g crust were measured. These results had not been expected. Our inhibition experiments showed a lower IgE-binding capacity for α -amylase heated to 200 °C for 50 min than for α -amylase heated to 98 °C for 10 min. Baur *et al.* [12] found a complete loss of the antibody-binding capacity of α -amylase heated for 50 min to 200 °C and only a partial loss of the antibody-binding capacity after heating α -amylase to 98 °C.

Obviously, the temperature is not the only important parameter for the modification of epitopes in bread. Probably, the portion of water during heating plays a crucial role. While on the bread's surface the heat is dry, the crumb retains a considerable amount of moisture. This could be the relevant difference between the bread crust from bottom and sides and that from the upper part leading to measurable α -amylase in the first case and to non-detectable α -amylase in the second. Bottom and sides of bread were in the metal pan and thus protected from the water steam moistening dough's surface in the initial stage of baking. A similar, even greater difference was found between the content of antigenic α -amylase in the crust of rolls baked in the laboratory (490–750 ng/g) and the crust of rolls water-steamed in the initial stage of baking as usual. The latter

did not contain antigenic α -amylase; all measurements were below the detection limit of the two-site ELISA.

However, the importance of the heat-stable epitopes on fungal α -amylase in bread and rolls has to be investigated by clinical studies including oral provocation tests with bread baked with and without the enzyme. In a recent study, Cullinan *et al.* [20] performed such challenge tests with 10 *Aspergillus fumigatus*-sensitized patients out of whom only two had specific IgE to α -amylase from *Aspergillus oryzae*. No increase in respiratory or other symptoms was observed after ingestion of the enzyme-containing bread in accordance with anamnestic data reporting that nobody had an 'allergy' to bread. The authors conclude that persons sensitized to *Aspergillus* species do not frequently show important clinical reactions to α -amylase in bread. However, in respect to the selection criteria of the study group (*Aspergillus fumigatus* sensitization) this result cannot answer the question whether consumers generally are at risk to develop α -amylase sensitization and allergy. Patients complaining of oral, gastrointestinal, respiratory or dermatological reactions after ingestion of bread or individuals with sensitization to α -amylase from *Aspergillus oryzae* should be selected for such provocation tests. Some studies detected sensitization to fungal α -amylase in a population without occupational exposure. Baur *et al.* [21] found two out of 98 atopics with specific IgE to α -amylase, Degens *et al.* [22] two out of 52 with specific IgE to α -amylase in a control group without occupational exposure, and Houba *et al.* [7] seven out of 416 laboratory animal workers who had a positive reaction to α -amylase in skin prick tests. It remains to be clarified whether these sensitizations are the result of cross-reactivity between fungal α -amylase and other allergens or, as another explanation, could derive from contact with α -amylase in bread and

pastry. The latter cannot be excluded as we could prove residual antibody-binding capacity of α -amylase in bread crusts and in the crust of some rolls in our study. In comparison with the content of α -amylase in dough, between 0.1 and 20% of the antibody-binding capacity remained.

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