
Glucoamylase: another fungal enzyme associated with baker's asthma

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Background: *Aspergillus*-derived enzymes are widely used as dough additives in the baking industry. These enzymes may give rise to immunoglobulin (Ig)E-mediated sensitization and occupational asthma. Glucoamylase (or amyloglucosidase) is an important industrial enzyme obtained from *Aspergillus niger* and used to provide fermentable sugars for yeast to improve loaf volume and texture.

Objective: The aim of our study was to investigate the potential allergenic role of glucoamylase in baker's asthma.

Methods: We report four subjects with work-related allergic respiratory symptoms who were exposed to glucoamylase and other starch-cleaving enzymes used as baking additives. The causative role of glucoamylase in work-related asthma was investigated by immunologic tests and specific inhalation challenges (SIC). Glucoamylase allergenic components were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.

Results: Skin prick tests to glucoamylase (10 mg/mL) gave a positive response in all patients. Further, a positive skin prick test to α -amylase was obtained in the four patients and to hemicellulase in two of them. SIC to glucoamylase elicited isolated early asthmatic responses in the three patients tested, and SIC to α -amylase elicited early asthmatic responses in two patients and a dual asthmatic response in another patient. Immunoblotting with glucoamylase showed several IgE-binding bands with molecular masses between 33 and 96 kD. IgE-inhibition assays showed scarce to moderate allergenic cross-reactivity between glucoamylase and α -amylase.

Conclusions: These bakers had developed IgE-mediated occupational asthma to glucoamylase and α -amylase. Fungal glucoamylase is widely used as a baking additive and this enzyme may give rise to allergic respiratory reactions among exposed workers.

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INTRODUCTION

Starch-cleaving enzymes are well known additives in processing of fermented dough products. By hydrolyzing starch molecules to fermentable sugars, these enzymes continuously generate a substrate that can be used by yeast. Inhalation of enzymes is associated with a high risk of developing allergic respiratory diseases.^{1–10} In the baking industry this risk is especially high because of the generalized use of powdered enzymes to hasten and economize the bread-making process.

In a survey conducted in the United Kingdom in 1998, asthma was the most prevalent respiratory illness in the food processing industry, and was noted primarily among bakery workers as a result of flour and enzyme sensitization.¹¹ Overall, the most commonly identified agents causing occupational asthma in this survey were enzymes.¹¹ In 1986 Baur et al¹ reported the first description of allergy to α -amylase from *Aspergillus oryzae* in baker's asthma. They found that 12 of 35 (34%) bakers with asthma had developed type I hypersensitivity to α -amylase. In addition, hemicellulase, cellulase, xylanase, and glucoamylase (or amyloglucosidase) from *Aspergillus niger* have been shown to cause immunoglobulin

(Ig)E-mediated sensitization by means of in vitro and in vivo tests.^{3,4,9,10} The frequency of serum-specific IgE to glucoamylase among symptomatic bakers varies from 5³ to 8%,⁹ indicating that this enzyme may be a relevant allergen in the baking industry. However, it is not known whether sensitization to glucoamylase can merely be attributable to cross-reactivity with other baking enzymes or it may cause true asthmatic reactions in sensitized patients. To the best of our knowledge, the ability of glucoamylase to elicit asthma symptoms has not yet been documented by means of specific inhalation challenges and its actual role in baker's asthma has yet to be defined.

In this paper we report four male employees with work-related allergic respiratory symptoms who were exposed to glucoamylase and other starch-cleaving enzymes used as baking additives. The causative role of glucoamylase was investigated by clinical and immunologic tests and confirmed by specific inhalation challenge in three subjects.

MATERIALS AND METHODS

Subjects

We evaluated three bakers (from three different bakeries) and one employee at an enzyme-processing plant. In this plant, baking enzymes were processed, weighted, packed, and then distributed to bakeries. The four patients were all male and their ages ranged between 27 and 43 years. These subjects had developed work-related symptoms of cough, chest tightness,

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shortness of breath, and wheezing. They had been working in the baking industry between 32 and 240 months. The mean duration of asthma symptoms in these patients was 73 months (range 28 to 120 months). Patients' characteristics and demographic data are presented in Table 1. They were still working at the time of evaluation. Sneezing, itching, and runny nose preceded the development of asthma symptoms in all patients. All of them improved on weekends and particularly during long holidays. These patients handled flour additives containing fungal enzymes (α -amylase, hemicellulase, and glucoamylase) routinely. They all established a causal relationship between handling of these powdered enzymes and immediate worsening of nose and asthma symptoms. All the patients could eat bread and baking products without any symptoms.

They were taking inhaled corticosteroids on a regular basis and short-acting and/or long acting β_2 -agonists at the time of the study. The subjects were told to refrain from taking inhaled bronchodilators for at least 12 hours before inhalation testing and the dose of inhaled corticosteroids was taken 24 hours before bronchial inhalation test.

Preparation of Allergenic Samples

Glucoamylase and hemicellulase from *A. niger* and α -amylase from *A. oryzae* were purchased from Sigma (Sigma Chemical, St. Louis, MO) and prepared at a concentration of 10 mg/mL by adding phosphate-buffered saline (PBS). These samples were filtered through a 0.22- μ m membrane (Millipore, Bedford, MA) and aliquoted. Several twofold dilutions of glucoamylase in PBS were performed for skin and inhalation tests.

Skin Tests

Skin prick tests were performed with commercially available extracts of wheat flour 5% wt/vol (ALK-Abelló, Madrid, Spain) and the aforementioned *Aspergillus*-derived enzymes at 10 mg/mL. Further, patients were tested with a panel of common inhalant allergens (Bial-Aristegui, Bilbao, Spain), including pollens (grass, trees, and weeds), mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), storage mites (*Acarus siro*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*), animal dander (cat and dog), and molds (*Alternaria*, *Aspergillus*, and *Penicillium*).

Histamine phosphate at 10 mg/mL and normal saline were used as positive and negative controls, respectively. The response was read 15 minutes after puncture, and the results were expressed as the largest wheal diameter in millimeters. A positive reaction was defined as a wheal diameter of 3 mm

or larger in the absence of reaction to the saline control and with a positive reaction to histamine.

Serum IgE Determinations and Inhibition Studies

Total serum IgE was measured by Pharmacia CAP system IgE fluoroenzyme immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). The determination of specific IgE antibodies to α -amylase and wheat flour was performed using Pharmacia CAP system fluoroenzyme immunoassay. IgE levels higher than 0.35 kU/L were regarded as positive, as recommended by the manufacturer.

The serum levels of specific IgE against glucoamylase were determined in duplicate by enzyme immunoassay as described elsewhere.⁴ A result was considered positive when a serum bound four times more specific IgE than the mean titer of four control, nonexposed subjects.

IgE inhibition studies were carried out to investigate a possible cross-reactivity among α -amylase, hemicellulase, and glucoamylase. The inhibition of IgE binding to α -amylase (solid phase) was determined using glucoamylase and hemicellulase as liquid-phase allergens. Twenty μ L of four progressive 10-fold dilutions (10, 1, 0.1, and 0.01 mg/mL) of the inhibitor allergens in PBS and 20 μ L of PBS as negative control were preincubated with 50 μ L of patient's serum for 15 minutes and then the assay was continued following the standard CAP technique (Pharmacia CAP System, Uppsala, Sweden). Results were expressed as the percentage of inhibition of CAP obtained with the inhibitor allergen as compared with a control assay with PBS inhibitor.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Peptides from the glucoamylase solution were separated by polyacrylamide gel electrophoresis in the presence of SDS-PAGE according to Laemmli¹² in 12.5% polyacrylamide gel (20 μ g protein applied per lane) under reducing conditions. After running, the separated proteins were electrophoretically transferred to polyvinyl-difluoride membranes (Immobilon-P, Millipore) following the method of Towbin et al.¹³ Unreacted membrane sites were blocked with PBS-Tween 0.05% (vol/vol) for 1 hour at 37° C. After one wash with PBS-Tween 0.1% (vol/vol), blots were incubated with 0.5 mL undiluted human serum (16 hours at 4° C). The blots were washed three times with PBS-Tween 0.1% and incubated with 0.8 mL of 1:10000 peroxidase-conjugated rabbit IgG to human ϵ -chains (Dako, Glostrup, Denmark). After three washes with PBS-Tween 0.1%, bound peroxidase was

Table 1. Clinical Characteristics of the Study Patients

Subject no.	Age yrs.	Smoking (pack-yr.)	Job	Duration of exposure (months)	Duration of symptoms (months)	FVC L (% pred)	FEV ₁ L (% pred)	Total IgE (kU/L)
1	43	Ex-smoker	Baker	240	120	5.32 (112%)	3.35 (86%)	353
2	27	6	Baker	32	28	5.04 (102%)	3.52 (85%)	248
3	30	6	Enzyme handler	108	72	4.25 (96%)	3.03 (81%)	17.7
4	35	No	Baker	156	72	5.05 (94%)	3.91 (88%)	416

detected by the addition of chemiluminescent detection reagents following the manufacturer's recommendations (ECL Plus Western blotting, Amersham Pharmacia Biotech UK, England). Electrophoretic and immunoblotting bands were detected and evaluated by image analysis in the Bio-Image System (Millipore).

Inhalation Challenge Tests

Methacholine inhalation test was performed according to Cockcroft et al¹⁴ with some modifications. The aerosolized particles were generated by a continuous pressurized nebulizer model DeVilbiss 646 (DeVilbiss, Somerset, PA) with an output of 0.28 mL/minute. The result of this test was expressed as the provocative concentration of methacholine causing a 20% fall (PC₂₀) in forced expiratory volume in 1 second (FEV₁) and it was determined by interpolation of the last two concentrations.

Specific inhalation challenge (SIC) to glucoamylase was carried out in three patients who gave informed consent, following the method previously described.⁴ The patient inhaled the aerosolized allergen using the nebulizer method mentioned before (DeVilbiss 646) in progressive concentrations at tidal breathing for 2 minutes. A control challenge with PBS as placebo was carried out before antigen provocation. Increasing concentrations of glucoamylase were given by inhalation starting with a concentration that induced a 2-mm wheal on skin prick testing. The dose was increased in twofold increments at intervals of 10 minutes and FEV₁ was measured at 5 and 10 minutes after inhalation of each concentration. Inhalation challenge test was discontinued when there was a fall in FEV₁ of 20% or greater from the lowest postsaline value or when the highest concentration (10 mg/mL) had been given. At the end of the inhalation test with either allergen or placebo, spirometry was performed at 20, 30, 40, and 60 minutes after challenge, and again the following day. From that moment peak expiratory flow rate and FEV₁ measurements were done hourly with a computerized flowmeter (VM Plus, Clement Clarke, Harlow, UK) for 24 hours after challenge, respecting sleeping time. A fall in FEV₁ of 20% or more from the lowest postsaline within 60 minutes of challenge was considered a positive early asthmatic reaction, and a similar fall between 2 and 24 hours after challenge was considered a positive late reaction if no change

was observed during the control day. PC₂₀ allergen was calculated as described above. The tests were undertaken when the patients were absent from work (for at least 48 hours). The same test was performed in two asthmatic bakers who were not sensitized to this enzyme.

SIC to α -amylase was performed in the same three patients in a close-circuit system for exposure to particles, as previously described.¹⁵ As a control bronchial challenge, the patients were exposed to lactose powder (up to 10 mg/m³). Pulmonary function was monitored as described before, over a 24-hour period after SIC.

Sputum Induction and Processing

Induction of sputum was performed as described by Pin et al.¹⁶ Briefly, peak expiratory flow rate was measured before and after salbutamol inhalation (200 μ g) to prevent airway constriction. Hypertonic saline was then nebulized using an ultrasonic nebulizer (Ne-U10B, Omron, Tokyo, Japan) for up to 21 minutes. The concentration of saline was 3%, followed by 4%, then 5% each for 7 minutes. Subjects were encouraged to cough throughout the procedure, and they regularly interrupted inhalation of hypertonic saline solution to expectorate sputum into a Petri dish. Sputum samples were then processed and homogenized adding 20 mL trypsin 0.001 mg/mL (Merck, Darmstadt, Germany) in normal saline.¹⁷ The samples were then incubated at 60°C for 30 minutes and centrifuged at 2,000 rpm for 10 minutes. The cell pellets were resuspended in saline solution and stained with the Papanicolaou and Giemsa methods for differential cell counts. Two investigators counted at least 200 nonsquamous cells on each sputum slide.

RESULTS

Skin Prick Tests

After skin testing all the patients showed a positive response to both glucoamylase and α -amylase, and two patients (3 and 4) reacted to hemicellulase as well. A positive response to wheat flour extract was observed in two patients (2 and 4). Skin tests results are shown in Table 2. Skin prick tests to these enzymes in 10 atopic, nonexposed subjects elicited no reaction. Skin prick tests to common inhalant allergens were

Table 2. Results of Skin Prick Tests, Specific IgE Measurements, and Inhalation Challenge Tests

Subject no.	Skin prick tests (mm wheal)				IgE determinations (OD* or kU/L)			Specific inhalation challenges			
	Gluco-amylase	Hemi-cellulase	α -amylase	Wheat flour	Gluco-amylase*	α -amylase	Wheat flour	Glucoamylase % fall in FEV ₁ during		α -amylase % fall in FEV ₁ during	
								EAR	LAR	EAR	LAR
1	7	0	14	0	1.58	18.6	<0.35	20	0	20	0
2	9	0	20	10	1.96	32.2	7.86	25	0	40	25
3	6	5	6	0	2.78	2.30	<0.35	ND		ND	
4	10	5	7	7	2.91	2.23	24.3	40	10	21	3

* OD, optical densities at 450 nm; EAR, early asthmatic response; LAR, late asthmatic response; ND, not done.

positive to grass, tree, and weed pollen in patient 2 and negative in the remaining patients.

IgE Measurements and IgE

Inhibition Assays

Total serum IgE levels are shown in Table 1. Specific IgE antibodies against glucoamylase and α -amylase were present in the sera from all patients, and IgE against wheat flour was detected only in two patients (Table 2). In four control, nonexposed subjects, mean specific IgE levels against glucoamylase were 0.058 ± 0.030 optical densities (OD) and against α -amylase <0.35 kU/L.

The results of IgE-inhibition experiments, shown collectively in Figure 1, demonstrate that IgE binding to immobilized α -amylase could be almost completely inhibited with soluble α -amylase in all patients. In contrast, the maximum inhibition of IgE-binding to α -amylase that was achieved with glucoamylase and hemicellulase as liquid phase allergens showed interindividual variability. The maximum inhibition obtained with glucoamylase varied between 17 and 44%, and with hemicellulase between 13 and 43% among the patients.

SDS-PAGE and Immunoblotting

The IgE binding bands of the glucoamylase sample are shown in Figure 2. Serum from all patients reacted to several allergens with apparent molecular masses between 30 and 100 kD. The most frequent IgE-binding bands were at 33.5, 47, and 54 kD. Further, serum from patient 4 showed strong IgE-binding to bands at 68 and 96 kD, which also appeared as faint bands with serum from patients 1 and 3. Immunoblots with a control serum from an atopic patient were negative.

Inhalation Challenge Tests

All patients had nonspecific bronchial hyperresponsiveness, showing PC₂₀ methacholine values of 0.25, 0.47, 1, and 0.25 mg/mL, respectively. SIC to glucoamylase extract elicited isolated early asthmatic responses in the three patients tested. The concentration of glucoamylase extract causing a fall in FEV₁ of 20% (PC₂₀ allergen) was 5, 0.5, and 0.005 mg/mL in

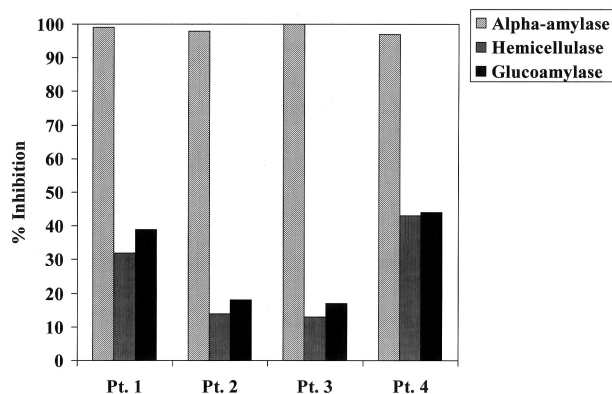


Figure 1. Maximum inhibition of IgE binding to α -amylase (solid phase) with α -amylase, hemicellulase and glucoamylase (inhibitor allergens) in patients 1 to 4.

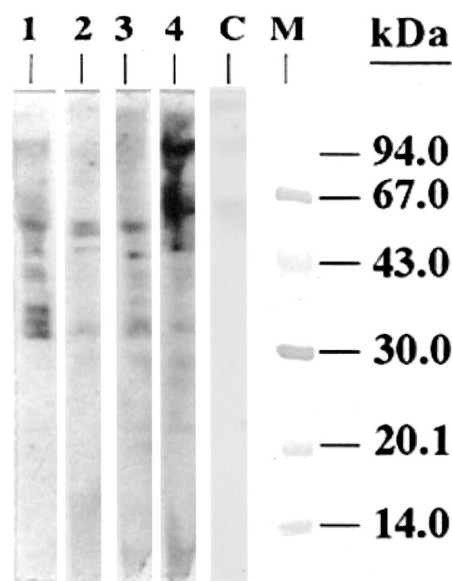


Figure 2. IgE immunoblots of glucoamylase with serum from patients 1 to 4 (lanes 1 to 4) and a negative control serum (C). M, molecular weight markers.

patients 1, 2, and 4, respectively. No late responses were observed over the 24-hour followup period. This test was negative in two asthmatic bakers nonsensitized to glucoamylase. SIC to α -amylase provoked isolated early asthmatic responses in patients 1 and 4, and a dual asthmatic response in patient 2, with concentrations of powdered α -amylase of 0.5, 0.6, and 1.4 mg/m³ for 1 minute, respectively. The results of inhalation challenge tests, with the maximum fall in FEV₁ observed during early and late asthmatic responses, are shown in Table 2.

Induced Sputum

Valid sputum samples were obtained from patients 2 and 4. In patient 2, differential cell counts in induced sputum at baseline showed 60% macrophages, 28% neutrophils, and 12% eosinophils. Twenty-four hours after SIC to glucoamylase, induced sputum revealed 70% macrophages, 12% neutrophils, and 18% eosinophils. In patient 4, differential cell counts in induced sputum at baseline showed 40% macrophages, 35% neutrophils, 15% eosinophils, and 10% bronchial epithelial cells. Twenty-four hours after SIC to glucoamylase induced sputum revealed 40% macrophages, 18% neutrophils, and 42% eosinophils.

DISCUSSION

Baker's asthma is still one of the most prevalent occupational respiratory disorders.¹⁸ Several allergens from cereal flour, soybean, and baking additives have been identified as the main causative agents.¹⁹⁻²¹ The enzyme most frequently reported as a causative agent of IgE-dependent occupational asthma among bakers is α -amylase (EC 3.2.1.1), which has been characterized and designated Asp o 2.⁷ In addition, other

fungus enzymes used as baking additives such as hemicellulase, cellulase (EC 3.2.1.4), glucoamylase (EC 3.2.1.3), and xylanase (3.2.1.8) have been reported to cause IgE-mediated sensitization in bakers.¹⁻¹⁰ The clinical relevance of sensitization to these enzymes, however, has been less investigated and their actual role in baker's asthma has yet to be defined.

Glucoamylase (α -1,4-glucan glucosylhydrolase) was characterized in 1951 as an enzyme that hydrolyzed α -1,4-glucosidic linkages in starch, thereby removing successive glucose units from the nonreducing ends of maltooligosaccharide chains.²² Glucoamylase acts synergistically with α -amylase in raw starch hydrolysis. Although several microorganisms, including bacteria, yeast, and fungi, are capable of producing glucoamylases, *Aspergillus* has most commonly been used for the production of commercial glucoamylases. In 1894 Takamine first used an enzyme mixture from *A. oryzae* for consumption as a digestive aid for Asian populations who had difficulties in digesting rice starch. The real breakthrough in the industrial use of enzymes took place in the mid-1960s, with the development of a glucoamylase which made it possible for starch to be completely hydrolyzed to glucose.²³ Since then, glucoamylase from fungal sources is used in the commercial production of dextrose, and nowadays, this enzyme is one of the most important industrial enzymes with worldwide applications. In the food industry, the largest use of enzymes is in starch processing followed by cheese production, fruit and vegetable juice processing, baking, and brewing. Purified glucoamylases from *A. niger*, designated as GA1 and GA2, were found to have molecular masses of 74 and 96 kD and isoelectric points of 3.8 and 3.95, respectively.²⁴ However, glucoamylases occur in multiple forms. Other authors have reported that the enzyme has a molecular mass between 48 and 80 kD.²² It is thought that the profile of the multiform enzyme depends on growth conditions and composition of the culture medium. Several authors suggest that only one primary glucoamylase form is synthesized, and the remaining ones result from the posttranslational modification.²² Glucoamylases have a catalytic domain and a starch-binding domain connected by an O-glycosylated linker. These enzymes are glycoproteins with a carbohydrate content of 16% and optimal activity at 60°C and pH 4.4.

The allergenicity of glucoamylase among symptomatic bakers was first described by Baur et al³ using serologic assays. In a recent study conducted in 171 bakers with work-place-related allergic respiratory symptoms, it was found that 23% of them had specific IgE to α -amylase, 13% to cellulase, 11% to xylanase, and 8% to glucoamylase.⁹ A case of protein contact dermatitis caused by glucoamylase has been reported in a process operator in a chemical enzyme factory.²⁵ However, the role of this enzyme in baker's asthma has not been previously documented by in vivo tests. The bakers herein reported the causative role of glucoamylase in their asthma symptoms, which was demonstrated by skin, serologic, and inhalation challenges, with evidence of an IgE-dependent mechanism. Induced sputum in two of the patients supported the evidence of eosinophilic bronchial inflammation related to the occurrence of an asthmatic reaction after inhalation

challenge with glucoamylase. In addition, these patients were also allergic to α -amylase and, in two of the cases, to hemicellulase and to cereal flour. Sensitization to fungal enzymes but not to α -amylase seems to be very uncommon among symptomatic bakers.²⁶ The finding of cosensitization to glucoamylase and α -amylase can not be merely attributed to cross-reactivity between these enzymes, because the degree of inhibition of IgE-binding to α -amylase with glucoamylase in our patients varied from insignificant to moderate. However, Sander et al⁹ found that glucoamylase inhibited up to 50% the IgE-binding to xylanase and cellulase, indicating the existence of common epitopes in these enzymes.

We conclude that glucoamylase is another *Aspergillus*-derived enzyme that may cause sensitization and IgE-dependent asthma among bakers and therefore it should be considered in the diagnosis of baker's asthma.

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