

Allergy to *Aspergillus*-derived enzymes in the baking industry: Identification of β -xylosidase from *Aspergillus niger* as a new allergen (Asp n 14)

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Abstract

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Background: *Aspergillus*-derived enzymes are used in dough improvers in bakeries. Some of these enzymes are identified as causing IgE-mediated sensitization in up to 25% of bakers with workplace-related symptoms.

Objective: The aim of this study was to compare the frequency of sensitization to *Aspergillus* xylanase, cellulase, and glucoamylase with the sensitization to α -amylase (Asp o 2) and to identify IgE-reactive proteins in enzyme preparations.

Methods: Sensitization to *Aspergillus*-derived enzymes and cross-reactivity were retrospectively studied by enzyme allergosorbent test (EAST) and EAST-inhibition experiments. IgE-reactive proteins were detected by electrophoretic separation and immunoblotting. Liquid chromatography with electrospray ionization mass spectrometry and Edman degradation of tryptic protein fragments were used for the biochemical identification of an unknown IgE-binding protein.

Results: Twenty-three percent of 171 tested bakers had specific IgE to α -amylase, 8% reacted to glucoamylase, 13% reacted to cellulase, and 11% reacted to xylanase. Xylanase and cellulase preparations, each containing at least 6 different proteins, showed

cross-reactivity in the range of 80%. The main IgE-binding protein in the xylanase preparation recognized in 7 of 8 xylanase-positive subjects was a protein of about 105 kd. This protein was identified as β -xylosidase by peptide mass spectrometric fingerprinting. The identification was confirmed by matching 12 peptide sequences obtained by N-terminal and mass spectrometric sequencing to this protein

Conclusions: β -Xylosidase from *Aspergillus niger* is an occupational allergen present in currently used baking additives, which causes sensitization in at least 4% of symptomatic bakers. According to the International Union of Immunological Societies nomenclature, we suggest the term Asp n 14 for this allergen. (J Allergy Clin Immunol 1998; 102:256-64.)

BSA:	Bovine serum albumin
EAST:	Enzyme allergosorbent test
ESI:	Electrospray ionization
HPLC:	High-performance liquid chromatography
LC:	Liquid chromatography
MALDI-MS:	Matrix-assisted laser desorption ionization mass spectrometry
MS:	Mass spectrometry
PBS:	Phosphate-buffered saline
pI:	Isoelectric point
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBST:	Tris-buffered saline plus 0.05% Tween-20

Key words: [Baker's asthma](#), [endoxylanase](#), [\$\beta\$ -xylosidase](#), [cellulase](#), [glucoamylase](#), [\$\alpha\$ -amylase](#), [Aspergillus](#), [enzyme](#)

Baker's asthma and rhinitis are among the most commonly reported occupational disorders in European countries. In the United Kingdom the high relative risk for development of asthma symptoms is reflected by an annual rate of 334 new cases per million bakers.¹ In Germany, about 1800 bakers claim for compensation because of these diseases every year.²

Several enzymes, most of them derived from genus *Aspergillus*, are used in the baking industry to improve the quality of bread and to reduce production costs. In addition to the cereal flour allergens, these baking additives are considered to play an important role in the cause of baker's asthma. The first recognized^{3,4} and most frequently reported allergen of these fungal enzymes used in bakeries is α -amylase (EC 3.2.1.1) from *A. oryzae*. It has been proven that α -amylase itself was the main allergen in commercial preparations; the characterized and purified allergen was designated Asp o 5 according to the allergen nomenclature.⁶ The importance of this allergen was confirmed by numerous European

studies.^{7,9} A recent overview of 405 bakers with work-related respiratory symptoms seeking medical services in Germany showed 22% (89) to have specific IgE to α -amylase from *A. oryzae*.²

In addition, glucoamylase (EC 3.2.1.3), cellulase (EC 3.2.1.4), and hemicellulase preparations from *A. niger* were shown to contain allergens by skin prick tests, detection of specific IgE, and immunoblotting.^{10,11} Five percent of 261 people who were employed in the baking industry and who suffered from workplace-related asthma, rhinitis, and/or conjunctivitis had specific IgE to glucoamylase, and 10% had specific IgE to a hemicellulase preparation, which also revealed cellulase activity.¹² Although the allergenic potency of these enzymes seems to be lower than that of α -amylase, their actual role has yet to be defined. Because the industrial grades of enzymes are poorly purified, they usually contain, in addition to the enzyme after which they are named, varying amounts of other enzymes produced by the microorganism and other impurities. Biochemical identification of the IgE-binding proteins has not been performed yet.¹³

German suppliers of baking additives most frequently use (next to α -amylase) enzyme preparations containing xylanase (results of our own survey). Xylan is the major hemicellulosic polysaccharide in the cell walls of land plants representing more than 30% of the dry weight. Endo-1, 4- β -D-xylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) show the most important activities among the enzymes involved in the xylan hydrolysis.¹³

Xylanases from *A. niger* have been shown to efficiently increase bread volume. The increase is further enhanced if xylanase is used in combination with α -amylase.¹⁴ The presence of xylanase in bakeries was measured by a Finnish group using an immunochemical method.¹⁵ The authors supposed, however, that their measurements indicated the natural xylanase activity of wheat. Tarvainen et al.¹⁶ reported on four workers in biotechnical laboratories with allergic respiratory symptoms and contact urticaria who had specific IgE antibodies to *A. niger*-derived xylanase and cellulase preparations. Cross-reactivity between cellulase and xylanase up to 92% was shown by RAST inhibition with sera from 2 patients.

In our study we present the first investigation on bakers' sensitization to xylanolytic enzymes in comparison with other *Aspergillus*-derived enzymes and the immunochemical and biochemical identification of a new allergen present in baking additives.

METHODS

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Patients' sera and case reports

About 171 sera from German bakers complaining of workplace-related allergic respiratory symptoms were screened retrospectively (1992 to 1997) for specific IgE to enzymes used in the baking industry. For further investigation, 16 of these bakers were selected who had positive serum IgE antibody levels (enzyme allergosorbent test [EAST] class 1 or higher) to xylanase and cellulase from *A. niger*. Patients' characteristics, including diagnoses by a trained physician, are summarized in Table I.

Table I. Patients' characteristics and diagnoses

Patient no.	Sex	Age	Occupation	Years of employment	Work-related symptoms
1	M	24	Baker	7	A,R,C
2	F	25	Baker	6	R,C,E
3	M	31	Baker	11	A,R,C
4	M	30	Baker (doughmaker)	15	A,R,C,E
5	M	26	Baker	8	A,R,C
6	M	24	Baker	9	A,R
7	M	26	Baker (doughmaker)	9	A,R,C
8	M	41	Baker	17	A,R,C
9	M	30	Baker	13	A
10	F	24	Baker	9	A,R
11	M	21	Baker	4	A,R,C
12	M	52	Baker	38	A,R,C
13	M	28	Baker	4	A,R
14	M	55	Baker	41	R,C,E
15	M	25	Baker	10	A,R
16	M	26	Baker	9	A,R,C

A, Asthma; R, rhinitis; C, conjunctivitis; E, eczema.

Specific IgE determination

Human IgE antibodies were measured by EAST with the following allergen couplings with xylanase (BASF, Ludwigshafen, Germany), cellulase (Sigma, Deisenhofen, Germany), and glucoamylase (synonymously named amyloglucosidase; Serva, Heidelberg, Germany), all 3 derived from *A niger*, and α -amylase derived from *Oryzae* (Fungamyl 1600s; Novo, Denmark). For coupling, BrCN-activated paper disks were incubated in 0.1 mol/L NaHCO₃ (pH 8.4) containing 1 mg/mL protein for 5 hours at room temperature, saturated by ethanolamine and washed according to the method described by Ceska et al.¹⁷ The allergen-coated disks were incubated with patients' sera, and bound IgE was determined as described by the manufacturer. The EAST reference curve (Pharmacia), always run in parallel, was used for calculation. EAST values less than 0.35 kU/L (Phadezym RAST units) represent absent or undetectable levels of allergen-specific IgE antibodies. Values of 0.35 to 0.69 kU/L correspond to RAST class 1, values of 0.7 to 3.49 kU/L to RAST class 2, values between 3.5 and 17.49 kU/L to RAST class 3, and values of 17.5 kU/L or greater to RAST class 4.

EAST inhibition experiments

Patients' sera with high levels of specific IgE according to EAST results were diluted with phosphate-buffered saline (PBS) to fit the linear range of the EAST reference curve. Sera with EAST values below 0.7 kU/L were not suitable for reproducible inhibition experiments. EAST inhibition was performed by preincubating 50 μ L of the diluted serum overnight at 4° C with 10 μ L of inhibition solution or PBS for control. The solutions used for inhibition had

a protein concentration of 1 mg/mL according to the Bradford assay,¹⁸ with bovine serum albumin (BSA) used as standard. On the next day, the allergen disks were added, and bound IgE was measured as described previously.

The inhibition percentage was calculated by the following formula: % inhibition = [(EAST value of PBS control) - (EAST value in the presence of inhibition solution)]/[(EAST value of PBS control - 0.35)]×100

When the EAST value of the inhibition experiment was as high or higher than the control value, the inhibition percentage was defined as 0; when the EAST value of inhibition was below 0.35 kU/L (detection limit of defined IgE antibody levels), the inhibition was defined as being 100%.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting

Briefly, 12 µg protein per lane, or 180 to 360 µg per gel, was mixed with sample buffer containing 2.3% sodium dodecylsulfate (SDS) and 33.5 mmol/L dithiothreitol, boiled for 3 minutes, and electrophoresed in 12% polyacrylamide separation gels with 5% stacking gels according to the Laemmli procedure.¹⁹ Molecular weight markers used were phosphorylase B (M_r 97,400), BSA (M_r 67,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,000), cytochrome c (M_r 12,500), and bovine lung trypsin inhibitor (M_r 6,500) from Serva (Heidelberg, Germany). The separated proteins were stained with Coomassie brilliant blue R-250 or transferred by semi-dry blotting²⁰ to polyvinylidene difluoride membrane (Pall, Dreieich, Germany). Blotted membrane strips were stained by Coomassie brilliant blue R-250 or blocked with 1% BSA and 1% polyvinylpyrrolidone-40 in Tris-buffered saline buffer (15 mmol/L Tris, 150 mol/L NaCl, and 1 mmol/L ethylenediamine tertaacetic acid, pH 7.8) for 7 hours. Sera from bakers and other subjects diluted with 2% BSA-TBS plus 0.05% Tween-20 (TBST) according to the EAST values were incubated overnight with the blot strips at room temperature and washed 3 times with TBST. Bound IgE was detected after incubation for 4 hours with anti-human-IgE/alkaline phosphatase (Sigma) diluted 1:2000 in TBST with 2% BSA by NBT/BCIP as a substrate (0.03% 4-nitro tetrazolium-chlodirblue and 0.015% 5-bromo-4-chloro-3-indolyl-phosphate).

For inhibition experiments before the overnight incubation, the sera were preincubated for 5 hours with 10% (vol/vol) inhibition solutions (1 mg protein/mL).

Two-dimensional gel electrophoresis

Sample preparation and two-dimensional electrophoresis with immobilized pH gradient (IPG-Dalt) were performed as described by Posch et al.²¹ For the first dimension, an immobilized pH gradient of 4 to 9 was used, and for the second dimension, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% T, 4% C) was used. Thirty micrograms of xylanase (BASF) were applied at the cathode.

In situ gel digestion for mass spectrometry

Tryptic digestions were performed in the gel matrix according to the procedure of Gevaert et al.,²² with certain modifications. The excised gel plugs were divided into 2 parts, each containing 180 µg protein. The gel was destained by several washing procedures with water and a solution of 50% acetonitrile. After a further washing with water for 10 minutes,

the protein was reduced by a solution containing 25 mmol/L dithiothreitol, 0.8 mmol/L ethylenediamine tetraacetic acid, 4 mol/L guanidine hydrochloride, and 100 mmol/L Tris-HCl, pH 8.2 (1 hour, 37° C). Alkylation was performed by incubation with 0.5 mol/L iodoacetamide in 100 mmol/L Tris-HCl (pH 8.2) for 30 minutes in the dark. After addition of 5 µL mercaptoethanol, the gel plugs were washed several times with water and shrunk by acetonitrile. Subsequently, the acetonitrile was removed, and digestion was done with 3 µg trypsin (1/50) in 50 mmol/L ammonium hydrogen carbonate (pH 7.8) overnight at 37° C. Elution was achieved with 70% formic acid and 50% formic acid in acetonitrile, and finally, acetonitrile was added to shrink the gel plugs. All supernatants were combined and evaporated to dryness. For high-performance liquid chromatography (HPLC) analysis, peptides were dissolved in 0.1% trifluoroacetic acid (TFA). One sample was used for electrospray ionization mass spectrometry (MS)/MS (ESI-MS/MS), and the other was used for matrix-assisted laser desorption ionization MS (MALDI-MS) and Edman degradation.

LC-ESI-MS/MS

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Peptides were separated on a C18-reversed phase column (150 mm × 0.18 mm, 5 µm particle size, 300 Å pore size; LC Packings, The Netherlands) by using an ABI 140 B solvent delivery system (Applied Biosystems). The HPLC flow rate of 50 µL/min was reduced to a final flow rate of 2 µL/min through a titanium tee piece. After injection of a 5-µL sample, the peptides were eluted beginning at 95% of a solution containing 0.025% TFA (by volume) (solution A) and 5% of a solution of acetonitrile/water/TFA (84/16/0.02; vol/vol/vol) (solution B). The proportion of solution B was elevated to 75% in 35 minutes and from 75% to 100% of solution B in 2 minutes. Mass spectrometry was done on a TSQ 7000 (Finnigan) equipped with an electrospray ion source. The mass spectra were acquired in the positive ion mode in the mass range (mass-to-charge ratio) 200 to 2500, with a scan duration of 2 seconds. The electrospray voltage was held at 3.5 kV, and 2-propanol was used as sheath liquid. Collision-activated decomposition spectra were obtained by using argon as collision gas, and a gas pressure of approximately 3×10^{-3} mbar was used. The collision energy was set to 15 to 46 eV depending on the parent ion mass-to-charge ratio. During HPLC-MS/MS analysis, the collision-activated decomposition of peptides was automatically performed by an instrument control language procedure.

LC-MALDI-MS and Edman degradation

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For subsequent Edman degradation, a 15-µL sample was subjected to a reversed-phase C18 column (5-µm particle size, 300 Å pore size; LC Packings) with a length of 250 mm and an inner diameter of 300 µm. The ABI 140 B solvent delivery system (Applied Biosystems) produced a flow of 15 µL/min, which was reduced to 5 µL/min. Peptides were eluted with a gradient of 0.1% TFA (solution A) versus 0.08% TFA and 84% acetonitrile (solution B) running from 5% to 50% of solution B in 90 minutes and from 50% to 100% of solution B in 15 minutes. The detector was a Shimadzu SPD-10A model (Shimadzu, Germany) set to 0.5 absorbance units at full scale (AUFS) at a wavelength of 215 nm and to 0.05 AUFS at a wavelength of 295 nm. Peak fractions were collected manually and analyzed by MALDI-MS. The spectra were recorded with a linear-mode Finnigan Lasermat 2000 (Finnigan MAT) equipped with a nitrogen laser (337 nm, 3 ns pulse duration). The analyzer operated in positive ion mode with an acceleration voltage of 20 kV. The spectra

were obtained by summing 5 scans. A solution of 10 mg/mL α -cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% TFA was used as the matrix. Part of the matrix (0.5 μ L) was mixed with a 0.5 μ L sample on the target and then analyzed. Fractions containing only 1 peptide were subjected to Edman degradation with an Applied Biosystems model 476A pulse-liquid sequenator (Applied Biosystems).>

Protein identification

Peptide mass fingerprint database search was performed with 50 masses obtained by HPLC-MS analysis with the program PeptideSearch/WWW(http://www.mann.embl-heidelberg.de/Services/PeptideSearch/FR_PeptideSearchForm.html) from the EMBL Protein and Peptide Group in Heidelberg.^{23,24} The nonredundant protein database (version 07/08/97) was used, and the expected protein mass range was adjusted from 50 to 200 kd. Peptide masses were assumed to be monoisotopic, with a mass accuracy of 0.1%.

RESULTS

TOP

Specific IgE to *Aspergillus*-derived enzymes

About 171 sera from bakers examined for occupational allergic respiratory symptoms were tested for specific IgE to cellulase, xylanase, α -amylase, and glucoamylase. Thirty-nine sera (22.8%) had specific IgE to α -amylase, 13 (7.6%) reacted to glucoamylase, 22 (12.9%) reacted to cellulase, and 18 (10.5%) reacted to xylanase. The EAST data on 16 xylanase-positive sera are shown in Table II.>

Table II. EAST results of 16 selected bakers

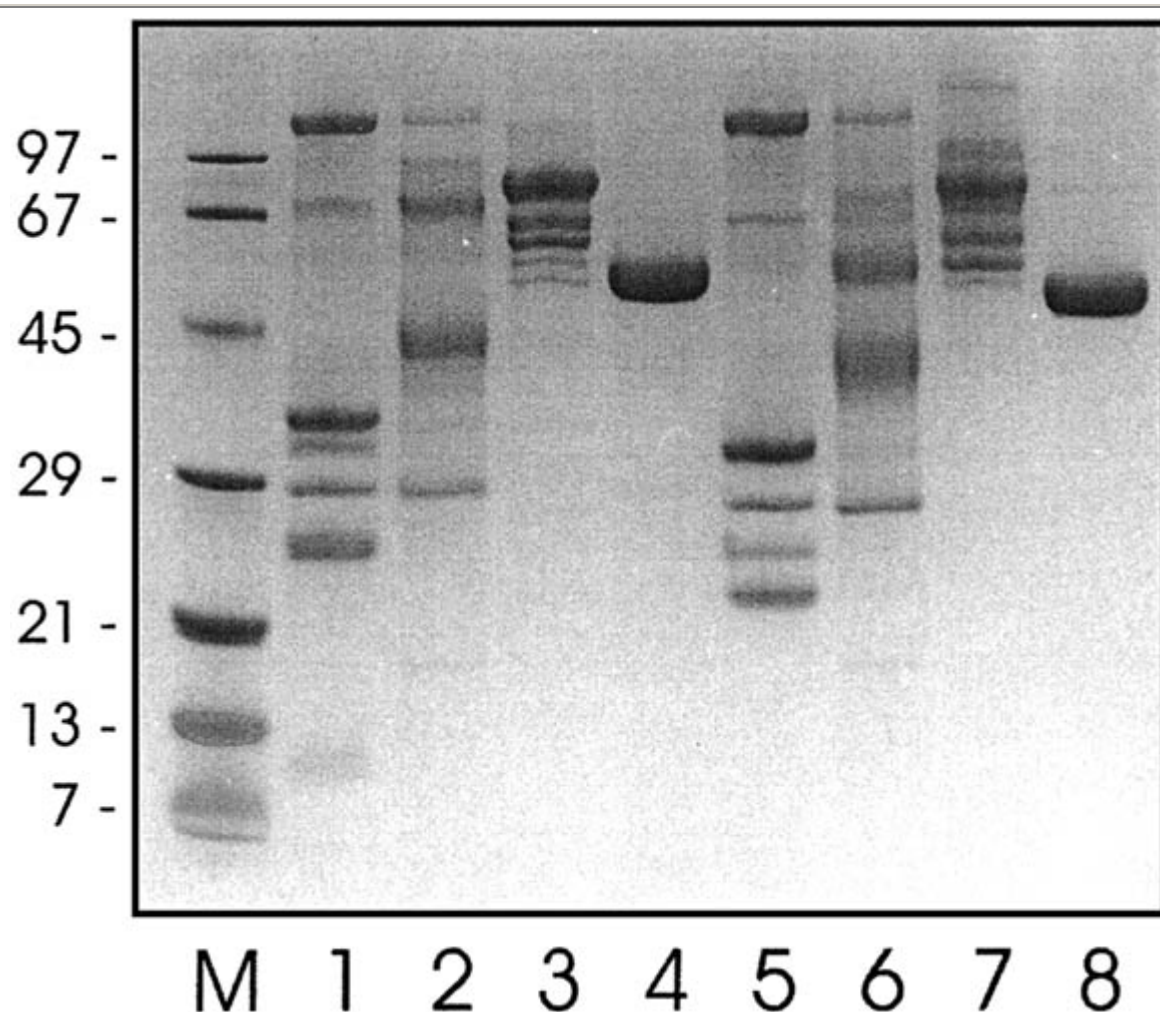
Patient no.	Xylanase, kU/L (RAST class)	Cellulase, kU/L (RAST class)	α -Amylase, kU/L (RAST class>	Glucoamylase, kU/L (RAST class)
1	3.94 (3)	3.44 (2)	<0.35 (0)	2.46 (2)
2	11.39 (3)	3.34 (3)	<0.35 (0)	0.89 (2)
3	6.74 (3)	5.82 (3)	12.88 (3)	11.74 (3)
4	8.23 (3)	9.20 (3)	21.85 (4)	26.85 (4)
5	1.12 (2)	1.04 (2)	0.67 (1)	1.39 (2)
6	4.12 (3)	7.45 (3)	1.42 (2)	<0.35 (0)
7	19.20 (4)	9.53 (3)	1.67 (2)	4.53 (3)
8	4.30 (3)	9.53 (3)	1.17 (2)	<0.35 (0)
9	1.13 (2)	0.90 (2)	<0.35 (0)	0.75 (2)
10	0.61 (1)	0.63 (1)	0.53 (1)	0.56 (1)
11	0.77 (2)	0.67 (1)	1.28 (2)	<0.35 (0)
12	0.58 (1)	0.64 (1)	2.16 (2)	2.90 (2)
13	0.70 (2)	0.73 (2)	2.31 (2)	<0.35 (0)
14	0.41 (1)	0.42 (1)	<0.35 (0)	0.47 (1)
15	0.65 (1)	0.63 (1)	0.44 (1)	<0.35 (0)

All 16 selected sera had positive IgE levels to xylanase and cellulase allergen disks. Twelve of the sera additionally reacted to α -amylase, and 10 reacted to glucoamylase. EAST classes of xylanase and cellulase reactions are identical in 13 of the 16 cases. >

SDS-PAGE of enzyme preparations

The enzyme preparations used for EAST were analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 1).

Fig. 1. SDS-PAGE of dithiothreitol-treated (*lanes 1 to 4*) and nonreduced (*lanes 5 and 6*) enzyme preparations. Xylanase (*lanes 1 and 5*), cellulase (*lanes 2 and 6*), glucoamylase (*lanes 3 and 7*), and α -amylase >*lanes 4 and 8*) were electrophoresed and stained with Coomassie brilliant blue.



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With the exception of α -amylase, all enzyme samples were mixtures of at least 6 proteins with molecular weights from 8 to 105 kd. Glucoamylase mainly consists of proteins in the range of 55 to 70 kd. The xylanase and cellulase mixtures contained 2 proteins with the

same apparent molecular masses. (105 and 26 kd), both running identically under reducing and nonreducing conditions. >

Cross-reactivity of *Aspergillus* enzyme preparations

Cross-reactivity between *Aspergillus* enzyme preparations was investigated by EAST inhibition experiments (Table III).

Table III. EAST inhibition experiments

Patient no.	Xylanase allergen disks				Cellulase allergen disks			
	Xylanase*	Cellulase*	α -Amylas>*	Glucoamylase*	Cellulase*	Xylanase*	α -Amylas>*	Glucoamylase*
1	74	70	8	26	100	48	14	48
2	100	100	0	48	98	64	0	17
3	81	86	0	40	100	40	0	19
4	50	64	0	47	89	68	21	84
5	100	45	0	47	98	100	36	85
6	84	96	0	73	100	88	7	80
7	100	83	16	64	100	92	8	100
8	100	100	24	40	100	69	1	32
9	100	100	ND	38	98	92	ND	6
AM $\pm$ SD	88 $\pm$ 17	83 $\pm$ 19	6 $\pm$ 9	47 $\pm$ 14	98 $\pm$ 4	73 $\pm$ 21	11 $\pm$ 13	52 $\pm$ 35

The inhibition of IgE-binding to xylanase or cellulase allergen disks by enzymes (10 μ g protein) was calculated in percentages.

AM, Arithmetic mean; SD, standard deviation; ND, not done.

*Percent inhibition.

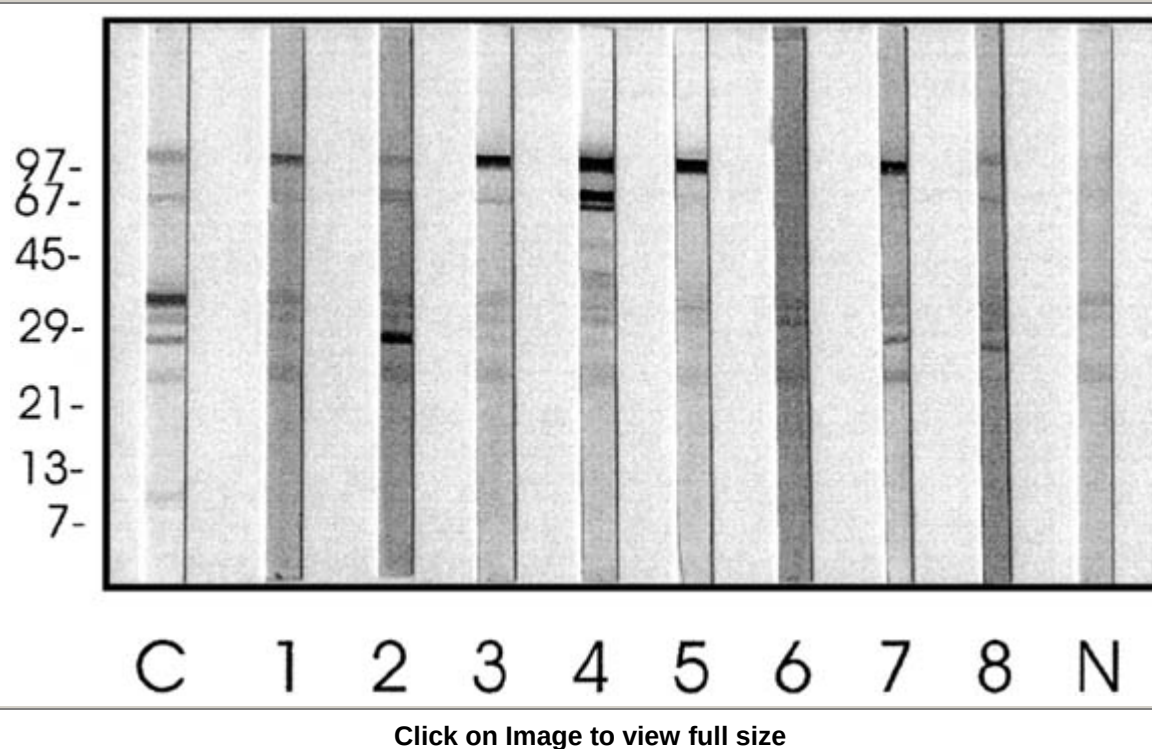
For inhibition, 10 μ g of protein used was found to be sufficient for complete inhibition.²⁵ Whereas α -amylase showed no significant inhibition of xylanase and cellulase allergen disks, glucoamylase showed a mean of about 50% cross-reactivity to xylanase as well as to cellulase, with a great interindividual variability. Cross-reactivity between xylanase and cellulase preparations was very high. Cellulase inhibited IgE binding to xylanase by 83% on the average, and xylanase inhibited binding to cellulase by about 73% >

Identification of IgE-binding proteins by immunoblotting

To identify allergenic proteins, the xylanase preparation was separated electrophoretically, electroblotted, and immunostained with bakers' sera and a secondary alkaline phosphatase-labeled anti-human IgE antibody (Fig. 2).

Fig. 2. Xylanase proteins (180 μ g) were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane. Membrane strips were stained with Coomassie brilliant blue (Lane C) or reacted to bakers' sera with positive EAST to xylanase (lanes 1 to 8) and secondary alkaline phosphatase-labeled anti-human IgE. Serum of health care worker with latex allergy (lane

N) was used as negative control.

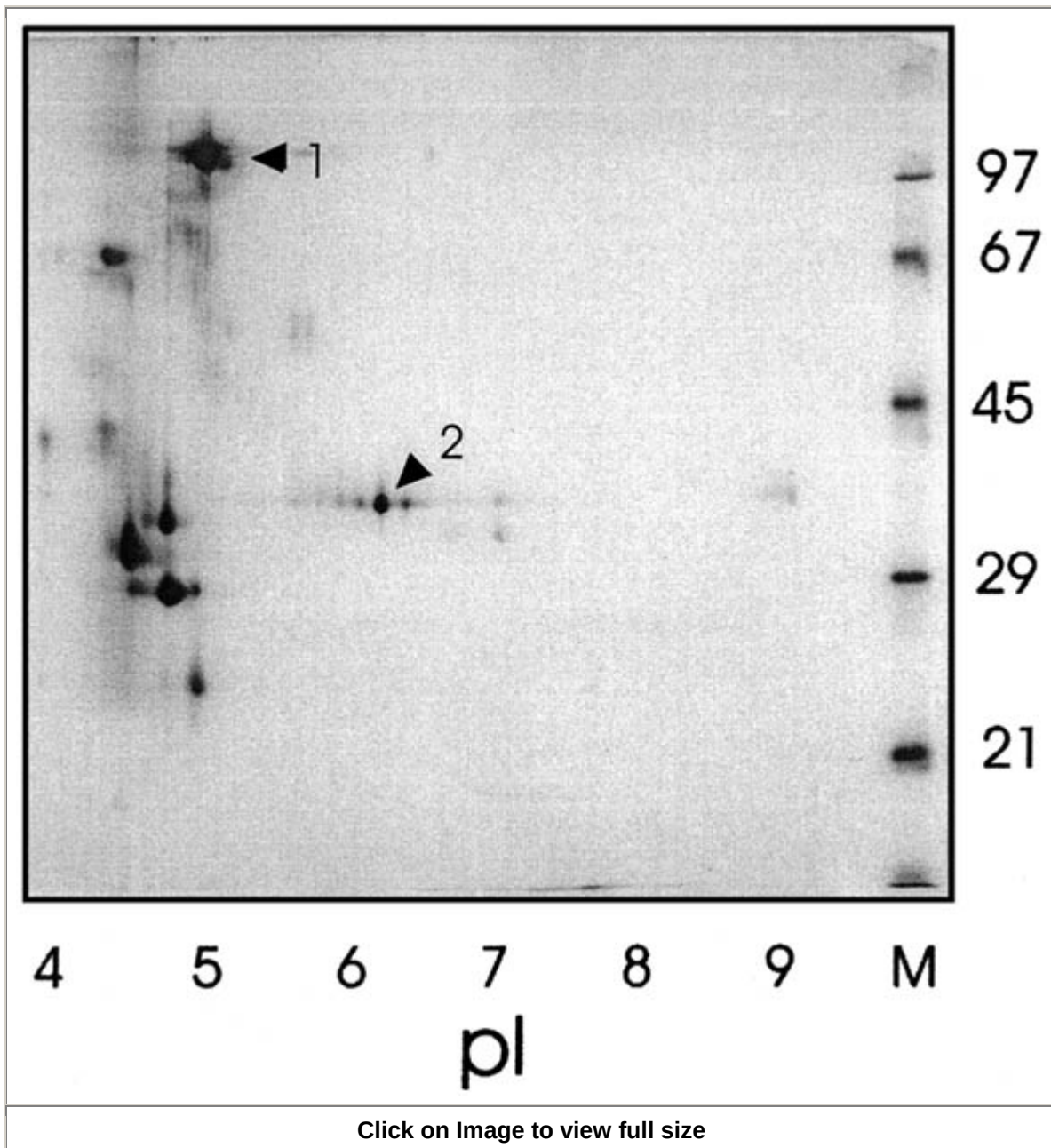


Sera with the highest EAST results to xylanase (patients no. 1 to 8 from Tables I to III) were selected for this experiment. All these patients had workplace-related asthma and rhinitis, and most of them also had conjunctivitis. With the exception of sera from patient number 6 (lane 6), all sera reacted to the protein with the highest apparent molecular weight (105 kd). Furthermore, a protein band (or double band) of about 75 kd was detected by sera from patients number 2 to 8, with a very strong reaction seen in lane 4. No binding to these high molecular weight proteins could be seen in the control sera (lane N). Four protein bands in the range of 25 to 38 kd were weakly visible after reacting to the control serum and all patients' sera, probably indicating unspecific binding. Only the protein of about 29 kd strongly reacted to the serum of patient number 2. The most important IgE-binding protein was that of about 105 kd. Known *Aspergillus*-derived xylanase had molecular weights of 22 to 46.5 kd.¹³ Therefore biochemical identification of the 105 kd protein was intended.

Two-dimensional electrophoresis of xylanase preparation

Proteins with the same apparent molecular weight cannot be separated by SDS-PAGE and often appear as one band in the gel. To check whether the IgE-reactive band at 105 kd consists of more than 1 protein, two-dimensional electrophoresis with an immobilized pH gradient in the first dimension was applied (Fig 3).

Fig. 3. IPG-Dalt of xylanase (20 µg protein was applied at cathode). First dimension IPG 4 to 9; second dimension SDS-PAGE, T = 10% (silver stain).



Seven prominent spots could be clearly detected by silver staining. Spot 1 represents the 105 kd protein with an isoelectric point (pI) of 5. No other spot was detectable at this molecular weight position. Spot 2 and the neighboring 5 weaker spots (with the same molecular weight but different pIs, pH 5.5 to 6.7) probably represent xylanase I from *A. niger* described to have a molecular weight of 39 kd and pI of 5.7 to 6.7.²⁶

Biochemical identification of the IgE-binding 105 kd protein

The xylanase preparation seems to contain only one 105 kd protein. Therefore SDS-PAGE was sufficient to separate this IgE-reactive component from the other proteins. At first, a preparative SDS-polyacrylamide gel with xylanase was electroblotted, and the membrane was rapidly stained with Coomassie brilliant blue. The 105 kd band was excised from the blotting matrix and subjected to Edman degradation. Because of blockage of the N-terminus, no sequence information was obtained. Therefore a second preparative SDS-PAGE with rapid Coomassie Blue staining and destaining was performed. The 105 kd

protein was digested with trypsin in the gel matrix, and the peptides were extracted and analyzed by LC-ESI-MS and LC-MALDI-MS and Edman degradation. Fifty monoisotopic masses obtained from HPLC-ESI-MS of the tryptic fragments were used for a peptide mass fingerprint database search. Initially, 19 masses could be matched to the translated EMBL DNA sequence of the xlnD gene of *A. niger* with the accession number Z84377.²⁷ The belonging mature protein, with a predicted length of 778 amino acids, 15 potential N-glycosylation sites, and a pI of 4.5, had β -xylosidase activity. This extracellular β -xylosidase had a predicted molecular mass of 85 kd but an apparent molecular weight of 110 kd in SDS-PAGE.>

Nine sequences of these 19 peptides with matching masses were confirmed by mass spectrometric sequencing. Furthermore, 3 HPLC fractions containing single peptides controlled by MALDI-MS were sequenced by Edman degradation. All matching peptide masses and sequence data are summarized in Table IV.

Table IV. Protonated monoisotopic masses determined by HPLC-ESI-MS matching the calculated m from β -xylosidase>(*A. niger*)

No.	Measured (d)	Calculated (d)	Sequence confirmation	Start residue	End residue	Sequence
1	646.209	646.378	*	570	575	(R)DIITGK(K)
2	749.309	749.370	—	139	145	(R)AFNNAGR(Y)
3	751.309	751.400	*	160	165	(R)HPVWGR(G)
4	774.409	774.473	—	570	576	(R)DIITGKK(N)
5	931.509	931.479	—	705	711	(K)WLVGWDR(L)
6	985.409	985.463	—	450	458	(R)AAFEEAGYK(V)
7	1044.409	1044.533	*	342	350	(R)DDIEQGVIR(L)
8	1061.009	1060.579	*	725	734	(R)VPVEVGSFAR(V)
9	1459.209	1458.807	*	722	734	(R)ELRVPVEVGSFAR(V)
10	1638.209	1637.934	*	124	138	(R)TLIHQIASIISTQGR(A)
11	1642.809	1642.823	*	146	159	(R)YGLDVYAPNINTFR(H)
12	1664.109	1676.746	*†	39	52	(R)SHLICDESATPYDR(A)
13	1899.209	1898.832	—	205	219	(K)HYAGYDIENWHNHSR(L)
14	2026.009	2025.056	*	79	96	(R)LGLPAYQVWSEALHGLDR(A)
15	2375.509	2375.205	—	146	165	(R)YGLDVYAPNINTFRHPVWGR(G)
16	2572.009	2573.223	—	681	704	(K)LES DY TAM VFANTSDAGPAPY P KK
17	2633.909	2633.415	*	53	78	(R)AASLISLFTLDELIANTGNTGLGVSR(L)
18	3116.409	2912.400	†	97	123	(R)AXFSDSGAYNWATSFPQPILTTAALXR(T)
19	3499.609	3501.679	—	681	711	(K)LES DY TAM VFANTSDAGPAPY P KKWL VGWDR(L)
20	3705.709	3703.723	†	582	614	(R)LVTTQYPASYAE EFPATDMNLRPEGDNPGQTYK(W)
21	5561.209	5558.943	—	521	575	(K)LASAAGKKPLIVLQMG G GQVDSSSLKNNNTNVSALLW

Differences from the sequence described in the data base are indicated by underlined boldface letters. Start residue is the suspected mature protein without the potential signal according to van Peij et al.²⁷

*Sequence confirmed by ESI-MS/MS.

†Sequence determined by Edman degradation.

One sequence (number 20) exactly matched the amino acids from position 582 to 614 in the β -xylosidase sequence. In the sequence of number 12 (residue 39 to 52 in β -xylosidase), the threonine residue in position 46 is replaced by serine, thus resulting in a mass that was 12 d lower in the measured peptide than in the calculated fragment. This sequence difference was additionally confirmed by mass spectrometric sequencing. The third sequence (number 18, matching residue 97 to 123 in β -xylosidase) showed 2 potential N-glycosylation sites. The asparagine residues in position 98 and 122 could not be identified by Edman degradation. Furthermore, the measured peptide mass was 204 d higher than the calculated one. This difference in molecular weight corresponds to the mass of N-acetyl hexosamine.>

Altogether, 21 of 50 determined masses could be identified as part of β -xylosidase from *A. niger*, thus amounting to 39% (303 amino acid residues) of the predicted mature enzyme.

DISCUSSION

TOP

In our study we investigated the frequency of sensitization to *Aspergillus*-derived enzymes, including α -amylase, glucoamylase, cellulase, and xylanase in a group of bakers seeking medical services. In accordance with previous investigations¹² the highest frequency of sensitization (23%) was found for α -amylase from *A. oryzae*. This was followed by a sensitization frequency of 13% to cellulase and 11% to xylanase.

To our knowledge, bakers' sensitization to xylanase has only been documented as a case report by our group (Baur X, Sander I, Posch A, Raulf-Heimsoth M, submitted). One baker showing sensitization in skin prick tests and EAST (data included in this study as number 7 in Tables I■ to III■) reacted to xylanase in a bronchial provocation test, which proves the clinical relevance of the sensitization to xylanase. Vanhanen et al.¹⁵ did not find any sensitization by using xylanase in skin prick tests. The source of this xylanase remains unclear, however, and no information on protein composition and purity of their test solution was given. We found the lowest frequency of sensitization to glucoamylase (8%) being somewhat higher than that previously described.¹²

Altogether, about 29% of the tested bakers reacted to baking enzymes. Most bakers were sensitized to more than 1 baking enzyme. One explanation for this fact is that baking additives in general contain enzyme mixtures. Thus coexposure to more than 1 enzyme occurs. Another explanation could be the cross-reactivity between enzyme preparations. Most probably, both explanations complement each other; coexposure and cross-reactivity are responsible for the high number of cosensitized bakers.

We investigated the cross-reactivity by EAST inhibition experiments and detected a very high cross-reactivity between the *Aspergillus*-derived cellulase and xylanase preparations. Cellulase inhibited IgE binding to xylanase by 83% on the average, and xylanase inhibited binding to cellulase by about 73%. From a theoretical point of view, in such experiments the more efficient inhibitor corresponds better to the sensitizer. In some cases inhibition experiments were helpful to identify the source of sensitization.²⁵ But in the case described above, the difference of inhibition by xylanase and cellulase was too small to serve as a basis of interpretation. The high cross-reactivity confirms the results of Tarvainen et al.,¹⁶

who found more than 90% RAST inhibition between *Aspergillus*-derived xylanase and cellulase by using sera of 2 workers from biotechnical laboratories. No cross-reactivity between α -amylase from *A. oryzae* and xylanase or cellulase was observed, the latter being in line with the results from Quirce et al.¹¹ who performed reverse enzyme-immunoassay inhibition experiments with α -amylase and cellulase. With glucoamylase, an inhibition of about 50% of IgE-binding to xylanase and cellulase was obtained, indicating common epitopes in all three preparations.>

To identify IgE-binding proteins, the xylanase preparation was analyzed by immunoblotting. The main IgE-reactive protein was a 105 kd protein binding to 7 of 8 sera from patients with workplace-related rhinitis, asthma, and/or conjunctivitis and positive EAST results to xylanase. Two-dimensional gel electrophoresis of the xylanase preparation showed only 1 protein spot in this molecular weight range with a pI of 5.0.

Because the 105 kd protein was N-terminally blocked, successful identification was achieved by mass spectrometric peptide mapping after complete tryptic digestion. In total, 21 of 50 determined masses could be matched to β -xylosidase from *A. niger*, the cloning and sequencing of which was published recently by van Peij et al.²⁷ Additionally, 3 peptide sequences were obtained by Edman degradation, and 10 sequences could be confirmed by mass spectrometric sequencing. All sequences were identical to β -xylosidase with the exception of one single exchange of threonine to serine. Furthermore, 2 of the 15 potential N-glycosylation sites were confirmed by Edman degradation because no phenylthiohydantoin-asparagine was obtained at those positions, and the peptide mass was elevated by 204 d, matching the molecular weight of N-acetyl hexosamine. The remaining 29 peptide masses could not be matched. This could be due to incomplete digestion, partial peptides, sequence modifications, or posttranscriptional modifications.> β -Xylosidase from *A. niger* is a glycoprotein as has been concluded from the reduction of apparent molecular weight in SDS-PAGE from 110 kd to 85 kd after N-glycanase treatment.²⁷ However, the identification of the IgE-reactive high molecular weight protein appears to be correct. The theoretical pI of β -xylosidase (4.5) is close to that determined by means of two-dimensional gel electrophoresis (pI 5.0), and the apparent molecular weight in SDS-PAGE was nearly the same in our experiment (105 kd) as that described by van Peij et al.²⁷ (110 kd). An additional coincidence shows the suspected N-terminal blockage.

β -Xylosidase from *A. niger* is the main IgE-binding protein in the xylanase preparation; 7 of 8 tested sera reacted to this protein. According to the International Union of Immunological Societies nomenclature,⁶ we suggest the term Asp n 14 for this allergen.*[■] Hitherto, no allergens from *A. niger* were named, but referring to the characterized allergen Asp o 2 from *A. oryzae* and the number of characterized allergens from *A. fumigatus*,^{28>31} the new allergen's number should be 14.

The high cross-reactivity between xylanase and cellulase preparations and preliminary results with immunoblot inhibition experiments (data not shown) indicate that commercial cellulase mixtures also contain β -xylosidase or at least a highly homologous protein.>

We conclude that β -xylosidase from *A. niger* is an occupational allergen present in currently used baking additives. One possibility of prevention would be the use of xylanase extracts with reduced side activities. For example, endoxylanase was produced in transformed strains of *A. awamori*, with multiple gene copies of the endoxylanase II gene resulting in an up to 20-fold over-expression of the endoxylanase compared with wild-type

strains.³² In such preparations the amount of other enzymes, including β -xylosidase, is drastically reduced compared with endoxylanase.>

However, our results show that the problem of sensitization to enzymes in the baking industry has not been solved yet. With the introduction of new additives, new cases of sensitization can be observed. Preventive measures to reduce airborne allergens in the workplace, for example by the use of granulated baking enzymes, are highly recommended.

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