

Airborne levels of α -amylase allergens in bakeries

Remko Houba, PhD, Paula van Run, Gert Doekes, PhD, Dick Heederik, PhD, and Jack Spithoven Wageningen, The Netherlands

Background: In the baking industry the use of enzymes has increased throughout the 1980s. Several studies have reported sensitization and respiratory disorders among bakery workers caused by enzymes in dough improvers. Fungal α -amylase is the most frequently reported cause of allergy. α -Amylase allergen exposure levels in the bakery industry, however, have not yet been reported.

Objective: The main objective of this study was to quantify personal α -amylase exposure levels of bakery workers.

Methods: α -Amylase allergens were measured in 507 personal samples of airborne dust taken in bakeries by using a newly developed sandwich enzyme immunoassay with affinity-purified polyclonal rabbit IgG antibodies. A cascade impactor was used to estimate the size of dust particles carrying α -amylase allergens.

Results: The rabbit IgG antibodies used in the assay showed, in immunoblotting with commercially available α -amylase, a reaction profile very similar to that of IgE from sensitized bakers. The enzyme immunoassay appeared to be highly specific for fungal amylase. Allergen exposure levels varied considerably among bakery workers, depending on the type of bakery and job category (range, 0 to 40 ng/m³). In confectioneries no α -amylase allergens were detected. In other bakeries α -amylase exposure was only found for workers directly involved in dough making. Measurements of the particle size distribution in these bakeries showed that α -amylase allergens are most likely to be deposited in the nose and ciliated airways.

Conclusion: This study shows that personal monitoring of fungal amylase allergen exposure in bakeries is possible. This permits the identification of high-risk tasks and allergen sources, as well as the study of exposure-response relationships. (*J Allergy Clin Immunol* 1997;99:286-92.)

Key words: Occupational asthma, bakery workers, enzymes, α -amylase, allergy, exposure assessment

The production of enzymes with biotechnical methods and the commercial use of these enzymes have been associated with the occurrence of allergic sensitization

Abbreviations used

EIA:	Enzyme immunoassay
OD:	Optical density
PBS:	Phosphate-buffered saline
PBTG:	PBS containing 0.1% Tween-20 and 0.2% gelatin

and occupational asthma in exposed individuals in a wide variety of industries.^{1,2} In the baking industry the use of enzymes has increased throughout the 1980s, and since then, several studies have reported sensitization and respiratory disorders among bakery workers caused by enzymes in dough improvers.

The following enzymes have been reported to cause sensitization in bakery workers: α -amylase, amyloglucosidase, cellulase, glucoamylase, hemicellulase, lipoxigenase, papain, pectinase, and xylanase.³⁻⁶ Of these enzymes, mostly derived from *Aspergillus* spp., α -amylase is the most frequently reported cause of allergy. Several individual case reports of baker's asthma caused by this starch-cleaving enzyme have been published.⁷⁻¹⁰ Other reports show that 24% to 34% of bakers with respiratory symptoms are sensitized to α -amylase.^{3,11} Recently, epidemiologic studies have been performed in which α -amylase sensitization was assessed in open populations of bakery workers. In these studies it appeared that 5% to 15% of all bakers were sensitized to α -amylase, as shown by either skin prick testing or specific IgE analysis.¹²⁻¹⁵ These studies convincingly showed that occupational exposure to fungal α -amylase increases the risk of respiratory allergy in bakery workers.

This increased risk results in an obvious need to quantify personal α -amylase exposure levels in this type of industry. Personal monitoring of enzyme allergens in other industries has been developed before for the proteolytic enzymes Esperase and papain.^{16,17} To our knowledge, however, levels of personal exposure to airborne α -amylase allergens have not yet been reported. We have therefore developed a sensitive sandwich enzyme immunoassay (EIA), which uses affinity-purified polyclonal rabbit IgG antibodies to measure α -amylase allergens. The EIA was used to assess personal α -amylase allergen exposure levels of workers in various bakeries.

The presented results are part of a large prospective epidemiologic study among a few hundred bakery workers. The primary aim of the whole project is to study

From the Department of Epidemiology and Public Health, Agricultural University Wageningen.

Supported by grants from the Netherlands Organization for Scientific Research (NWO) and the Directorate General of Labor from the Ministry of Social Affairs and Employment. The study was also part of the European concerted action "Epidemiology of Occupational Asthma and Exposure to Bioaerosols" (contract no. BMH1-CT94-1446).

Received for publication Dec. 6, 1995; revised July 31, 1996; accepted for publication Aug. 2, 1996.

Reprint requests: Gert Doekes, PhD, Department of Epidemiology and Public Health, Agricultural University Wageningen, P.O. Box 238, 6700 AE Wageningen, The Netherlands.

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0091-6749/97 \$5.00 + 0 1/177350

exposure-incidence relationships between allergen exposure and baker's asthma.

METHODS

α -Amylase allergen standard

A standard α -amylase allergen preparation was made from lyophilized Fungamyl 1600S (NOVO Nordisk, Denmark). This fungal α -amylase (derived from *Aspergillus oryzae*) was dissolved in phosphate-buffered saline (PBS) and filtered through a 0.22 μ m filter (Millipore). Its protein content was 44% (wt/wt), as measured with the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.).

Anti- α -amylase antibodies

Anti- α -amylase antibodies were raised in a New Zealand White rabbit (Broekman Institute, Someren, The Netherlands), by subcutaneous injection of 1 ml of Fungamyl 1600S (0.5 mg protein/ml) mixed 1:1 with Freund's complete adjuvant. Booster injections were given at 4-week intervals with the same amount of antigen in incomplete Freund's adjuvant. Serum was collected at 2-week intervals after booster injections and stored at -20° C.

Immunospecific antibodies were isolated by affinity chromatography. Ammonium sulfate-precipitated immunoglobulins from 60 ml of pooled antiserum were applied to an immune adsorbent of immobilized α -amylase, prepared by coupling 20 ml of a Fungamyl 1600S solution (10 mg/ml) to 40 ml of CNBr-Sepharose-4B (Pharmacia LKB, Uppsala, Sweden), according to the instructions of the manufacturer. Bound antibodies were eluted with 0.1 mol/L glycine-HCl (pH 2.8); fractions with an optical density at 280 nm (OD_{280}) greater than 0.1 were pooled, neutralized with 1 mol/L Tris, dialyzed, concentrated by lyophilization, redissolved in 5 ml of PBS, and stored at -20° C, at a protein concentration of 2.35 mg/ml.

Some of the antibodies were biotinylated by mixing 1 ml of a 1 mg/ml solution with 10 μ l of 1 mol/L carbonate buffer (pH 8.6) and 180 μ l of biotin-N-hydroxysuccinimide ester (1 mg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) in dimethylsulfoxide and incubating them for 4 hours at 20° C. After extensive dialysis, the biotinylated antibodies were stored at 4° C.

Immunoblotting

The specificity of EIA reagents was tested by immunoblotting. The α -amylase preparation (0.3 ml with 1 mg protein/ml) was subjected to sodium dodecylsulfate electrophoresis in a $10 \times 7.5 \times 0.05$ cm slab gel of 12% polyacrylamide and transferred to nitrocellulose as described previously for immunoblotting of wheat antigens.¹⁸ After blocking of unoccupied sites with PBS containing 0.1% Tween-20 and 0.2% gelatin (PBTG), strips of the nitrocellulose sheet were incubated either with undiluted sera from bakers with a high anti- α -amylase IgE titer or with 1:50 or 1:100 dilutions of the affinity-purified and biotinylated rabbit anti- α -amylase antibodies. Bound human IgE was detected with mouse-anti-human IgE (1:500), biotinylated rabbit anti-mouse immunoglobulin (1:500), and avidin-peroxidase (1:200); whereas binding of biotinylated rabbit antibodies was shown directly with avidin-peroxidase (1:200). Bound peroxidase was visualized by incubation for 30 minutes at room temperature with tetramethylbenzidine (Promega Corp., Madison, Wis.).

Sandwich EIA

The α -amylase concentration in dust samples was measured by sandwich EIA. Microtiter plates (Greiner, Nuertingen, Germany) were coated overnight at 4° C with affinity-purified anti-amylase antibodies at 2.35 μ g/ml in PBS. Free coating sites were blocked with PBTG (30 minutes), after which test samples and serial dilutions of the amylase standard (ranging from 47 to 2000 pg/ml) were added in PBTG and incubated for 1 hour at 37° C. Bound amylase was measured by subsequent incubations of 1 hour at 37° C with biotinylated immunospecific anti-amylase antibodies (1:500 in PBTG) and avidin-peroxidase (DAKO; 1:2000 in PBTG), and finally 30 minutes with *o*-phenylenediamine at 2 mg/ml in 0.05 mol/L phosphate-citrate buffer (pH 5.6) with 0.015% H_2O_2 . All incubations were performed with 200 μ l per well, and between incubations the plates were washed extensively with PBS-Tween. The reaction was stopped with 2 N HCl (50 μ l/well), and the OD was read at 492 nm.

Test samples were regularly first screened at a 1:10 dilution; positive samples were retested at several dilutions, and negative samples were retested undiluted. The concentrations were determined by interpolation of OD_{492} values on the calibration curve obtained in the same test plate with the standard preparation, by using four-parameter curve fitting, as in the wheat antigen inhibition EIA.¹⁸

Materials for specificity tests

The specificity of the assay was investigated by testing the following allergen extracts (all from ALK Benelux, Houten, The Netherlands): *A. flavus* (22.11), *A. niger* (22.02), *A. oryzae* (22.12), baker's yeast (*Saccharomyces cerevisiae*, 52.01), barley flour (52.02), house dust mite (mixture of *Dermatophagoides farinae* and *D. pteronyssinus*, SQ510), *Penicillium expansum* (25.02), *Penicillium mix* (*P. brevicompactum*, *P. expansum*, *P. notatum*, and *P. commune*; 25.00), rye flour (52.05), and storage mite mix (35.00). A wheat antigen preparation was obtained by extraction of a batch of wheat flour from one of the bakeries.¹⁸

Personal dust sampling and filter extraction

Personal inhalable dust was sampled in the workers' breathing zones during full-shift periods of 6 to 8 hours, by using polytetrafluoroethylene (Teflon) filters (Millipore; pore size 1.0 μ m) and PAS-6 sampling heads at a flow rate of 2 L/min.¹⁹ Production workers in 21 bakeries participated for 1 to 6 days. These bakeries differed in a number of characteristics, such as number of employees, type of products, and technology level. All bakery workers were classified into nine job categories. More information on bakery characteristics, job categories, and dust and wheat flour allergen exposure levels in each job category can be found elsewhere.¹⁸ Dust samples were stored at -20° C, and α -amylase allergens were recovered from the filters with PBS, as described previously.¹⁸

Particle size determination

Particle size distribution was measured in small bakeries and dough-making areas of large bakeries by ambient dust sampling with an Andersen 1 CFM Ambient Fractionating Sampler (Andersen, Atlanta, Ga.) at a flow rate of 28.3 L/min, by using glass fiber filters (Nenimij BV, Zoetermeer, The Netherlands). This cascade impactor separates the airborne particles into nine fractions from diameters of 10 μ m or greater down to 0.4 μ m. The sampler was located at about 1 m from the working areas of the bakers. Allergens were recovered from the filters in 15 ml of PBS, in the same way as described for the personal samples.

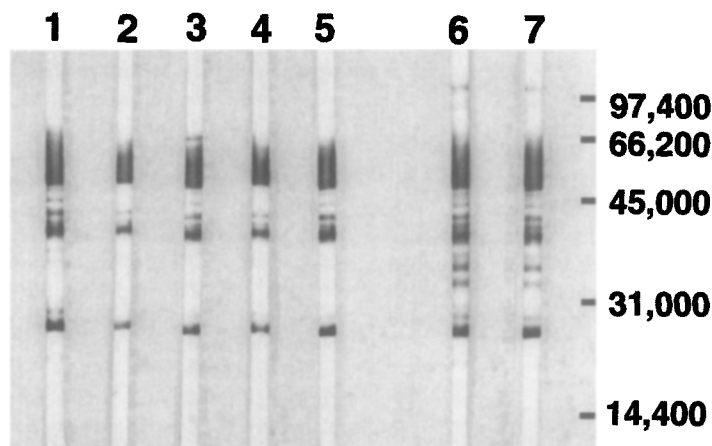


FIG. 1. Immunoblotting of a commercially available α -amylase preparation. Reactions with human IgE from sensitized bakers (lanes 1 to 5), and with affinity-purified rabbit polyclonal antibodies (lanes 6 and 7).

Statistical analyses

Differences in mean α -amylase exposure levels between groups of workers were tested by using a *t* test (SAS software; PROC ANOVA). Differences with *p* values less than 0.05 (two-sided) were considered significant. Because of the log-normal distribution of exposure data within groups, these tests were applied to log-transformed allergen concentrations.

RESULTS

Assay characteristics

The validity of the assay was assessed by immunoblotting experiments, in which the reaction profile of the polyclonal rabbit IgG antibodies was compared with that of IgE antibodies in the sera of α -amylase-sensitized bakery workers (Fig. 1). Electrophoresis showed that the fungal α -amylase preparation contains several proteins, of which most appeared to be immunogenic and/or allergenic. Both human IgE and rabbit IgG antibodies showed a strong reaction with a protein of approximately 52 kD, and at least two other important bands of 40 and 27 kD also reacted with both rabbit IgG and human IgE antibodies. In addition to these three prominent bands, other reactive proteins were detected at 29, 33, 35, 42, and 44 kD, all binding rabbit antibodies and several also binding human IgE; but the intensity of these bands was much lower. In general, and especially for the three main bands, the reaction profiles of human IgE and rabbit IgG antibodies were similar (Fig. 1). An identical reaction profile was also observed for the nonbiotinylated rabbit antibodies used for coating in the sandwich EIA and for the preparation of the biotin conjugate (not shown). No reaction was found for any of the control strips incubated with, for example, PBTG instead of serum, and subsequently with all reagents used for human IgE detection or with the avidin-peroxidase conjugate only.

In the sandwich EIA, a steep dose-response curve was obtained with the standard α -amylase preparation at low protein concentrations. At concentrations of 100 pg/ml,

the measured OD_{492} was in all assays significantly higher than the OD_{492} of the reagent blank (no α -amylase). An essentially linear relation was found between amylase concentration and OD up to 2.5, which was reached at approximately 2000 pg/ml. In practice, only samples demonstrating an OD_{492} greater than ($OD_{492,blank} + 5$ SD) were considered positive, which implied a mean detection limit of 100 pg/ml.

The reproducibility of the assay was estimated by computing coefficients of variation for the duplicate analysis of α -amylase allergens in personal samples. Of the 93 samples with a detectable concentration of α -amylase, 75% were analyzed in duplicate on 2 different days. The between-day coefficient of variation in the analysis was 22% and was independent of the allergen concentration.

The specificity of the assay was extensively tested, particularly by titrating (a) a series of extracts from common mold species and (b) other compounds, which are regularly present in airborne dust in bakeries, and thus are a potential source of false-positive reactions (e.g., like wheat antigens). Substantial reactivity, with linear dose-response relations, was noted only for extracts of *Aspergillus* spp.; and the strongest reactivity was found for *A. oryzae*, from which the α -amylase is derived. However, reactivity of *A. oryzae* extract was about 50 times less per microgram of protein when compared with the α -amylase standard. For other *Aspergillus* species, the reactivity ranged from 1/100 to 1/10,000 when compared with the α -amylase standard. Some reactivity in the EIA was found for extracts of baker's yeast and storage mites, but only at protein concentrations greater than 10 μ g/ml. For extracts of wheat, barley, and rye flour, hardly any reactivity was found; and the measured OD_{492} was not different from the background at protein concentrations of these grain species up to 100 μ g/ml. Finally, no reactivity in our EIA was found for extracts of *Penicillium* species and house dust mite.

Dust samples from bakeries usually contain large amounts of wheat antigen.¹⁸ We therefore also investi-

TABLE I. Personal α -amylase exposure levels of bakery workers classified by job category and, if relevant, by type of bakery ($n = 507$)

Job category	α -Amylase (ng/m ³)		
	<i>n</i>	GM	GSD
Large industrialized bakeries (>50 workers)			
1. Dough makers			
Wheat bread*	32	0.8	5.14
Pastry	32	All < detection limit	
Crispbakes*	27	18.1	4.56
Rye bread	7	0.2	1.48
2. All-round staff			
Wheat bread	35	0.2	2.35
Pastry	4	All < detection limit	
Crispbakes*	26	1.3	11.02
3. Oven staff	77	0.2	1.45
4. Slicers, packers, & transport workers	119	0.2	1.44
5. Production managers	20	All < detection limit	
6. Maintenance & cleaning workers	27	0.2	1.33
Small traditional bakeries (1-6 workers)			
7. Bread bakers	31	0.3	3.09
8. Mixed bakers†	54	0.2	1.81
9. Pastry cooks	16	All < detection limit	

n, Number of samples; *GM*, geometric mean; *GSD*, geometric standard deviation.

*Exposure significantly different from all other groups within job category ($p < 0.05$).

†Bakers involved in both bread and pastry production.

gated whether the simultaneous presence of wheat compounds might affect the measurement of α -amylase allergens in dust samples. Titration curves were produced for the standard α -amylase after dilution in either PBTG or PBTG containing various concentrations of wheat flour extract. Wheat flour protein concentrations up to 10 to 25 μ g/ml had no influence on the α -amylase standard curve. Measured OD₄₉₂ values were slightly higher (0.1 to 0.4 OD units) when the amylase standard was dissolved in solutions containing 100 μ g/ml wheat flour proteins.

Personal airborne α -amylase allergen measurements

Five hundred fifty personal measurements were available from 224 workers in all bakeries. Results of dust exposure levels are described in detail elsewhere.¹⁸ Briefly, on the basis of dust exposure levels, production workers could be classified into nine job categories (Table I). The highest airborne dust levels were found for dough makers in large industrialized bakeries (geometric mean = 3.0 mg/m³) and bread bakers in small bakeries (geometric mean = 3.3 mg/m³), and the lowest levels were found for slicers, packers, and transport workers (geometric mean = 0.4 mg/m³).

α -Amylase allergens were measured in 507 personal samples from 21 Dutch bakeries. Because all filters were extracted in 2.5 ml and the sample volume for one 8-hour work shift is around 1 m,³ the detection limit for personal allergen measurements was 250 pg/m.³ In the statistical analysis, a value of two thirds of this limit was

assigned to all samples with concentrations below this value ($n = 414$).²⁰ Samples with high concentrations (>1.5 ng/ml, $n = 43$) were retested at three or four higher dilutions, and for each dilution the allergen concentration was determined. These tests showed parallelism between the standard curve and sample curves.

Ninety-three samples (18%) were definitely positive in the sandwich EIA. Amylase allergens could only be measured in samples of bakers working in or close to the dough-making areas, where bread improvers are added to the dough (Table I). Dough makers in large industrialized bakeries had the highest α -amylase exposure, but not all dough makers had equal exposure levels. Amylase is not used in pastry production, and no α -amylase allergens were detected in samples from those bakeries that made only pastry, although dust exposure levels were comparable to those of other dough makers.¹⁸ In other bakeries α -amylase exposure levels varied greatly. Highest exposure levels were found for dough makers producing crispbakes (a kind of toast), followed by dough makers in wheat-bread production sites, and bread bakers and bakers of mixed products in small traditional bakeries.

α -Amylase allergens were also detected in personal samples from all-round staff workers, who have no specific job, but sometimes work as dough makers, packers, etc. In all-round workers, full-shift personal α -amylase allergen exposure levels varied greatly, depending on the type of work performed on the day of sampling. For some job categories (production managers in large bakeries and pastry cooks in small bakeries),

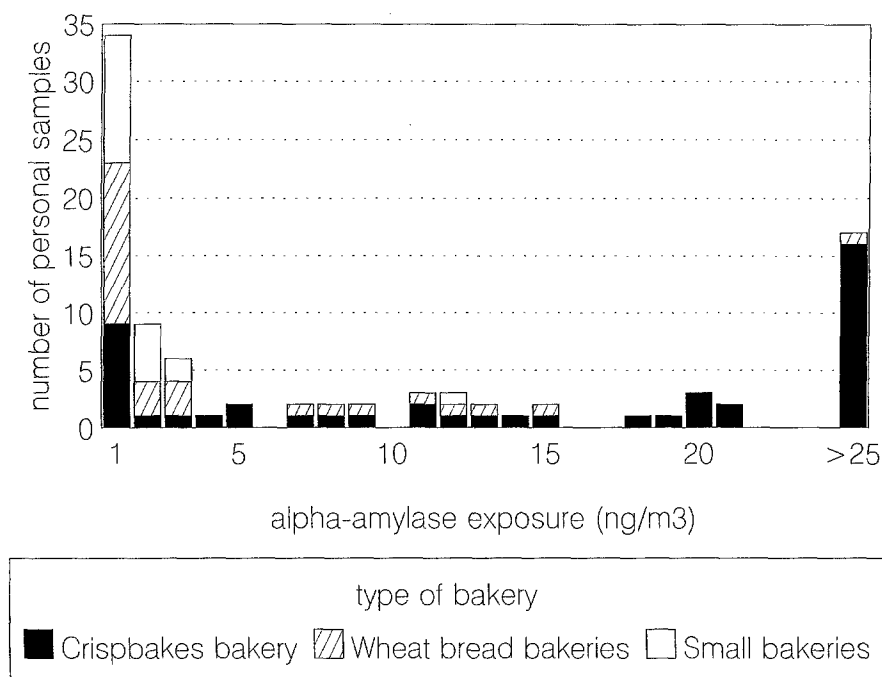


FIG. 2. Personal α -amylase allergen exposure levels measured in three types of bakeries. Only samples with detectable α -amylase concentrations are presented ($n = 93$). The last column (>25) represents concentrations of more than 25 ng/m^3 α -amylase.

TABLE II. Distribution of α -amylase allergens in different stages of the cascade impactor

Type of bakery	Total dust concentration (mg/m^3)	α -Amylase concentration (ng/m^3)	Particle size fraction of the dust (μm)*				
			>9.0	5.8-9.0	4.7-5.8	3.3-4.7	<0.4-3.3
Wheat bread	0.24	1.2	14%	11%	75%	—	—
Crispbakes	1.45	229.3	62%	24%	11%	1%	1%
	0.50	3.4	73%	18%	7%	3%	—
	0.76	17.2	90%	9%	—	—	—
Small bakery	1.13	4.5	70%	13%	9%	8%	—
	1.03	0.3	51%	49%	—	—	—
	2.39	59.1	64%	33%	—	—	4%

All samples were taken in dough-making areas from several types of bakeries ($n = 7$). Sampling time varied from 7 to 28 hours.

*In some samples the sum of percentages of all stages is not exactly 100% because individual percentages were rounded.

all measurements were below the detection limit; and in oven staff and slicers and packers, 96% and 98% of all samples were below the detection limit.

Fig. 2 shows a histogram of all 93 samples with α -amylase levels above the detection limit. This distribution suggests a distinction of three groups of exposure levels: 5 ng/m^3 or less, 6 to 15 ng/m^3 , and 15 ng/m^3 or greater. In the last category, all samples but one were from the facility producing crispbakes (the other is from a dough maker in a large bakery). Most of the samples from bread bakers or bakers of mixed products in small traditional bakeries showed levels below 5 ng/m^3 ; only one sample had a higher α -amylase concentration. Samples from large (wheat bread-producing) bakeries were distributed among the first ($\leq 5 \text{ ng/m}^3$) and second (6 to 15 ng/m^3) groups.

Particle size distribution

In Table II results of the measurements with the Andersen sampler are presented. In each stage of the cascade impactor, α -amylase allergens were determined. As can be seen in Table II, α -amylase allergens are predominantly present in particles larger than $5 \mu\text{m}$. In general, more than half of the α -amylase allergens were found in the dust fraction with a particle size greater than $9 \mu\text{m}$.

DISCUSSION

In our study personal α -amylase allergen exposure levels in bakeries were measured with a sandwich EIA by using affinity-purified polyclonal rabbit IgG antibodies. Several tests were performed to determine the validity and specificity of this method. Immunoblotting revealed

a very similar reaction profile for IgE from sensitized bakery workers and the rabbit antibodies that were used in the sandwich assay. Three major bands were found, a very strong band around 52 kd and two other important bands around 40 and 27 kd. Several other studies also demonstrated a dominant IgE-binding band in immunoblots with molecular weights varying from 51 to 54 kd.^{4, 21-23} Baur et al.²² have demonstrated that this band represents the active α -amylase. In addition to this major allergen, commercially available extracts of fungal α -amylase appear to contain several other allergenic components, and as a result, other bands can also be detected with IgE from sensitized bakery workers.²¹⁻²³ Although the exact nature of these other components, which also reacted with our EIA reagents, is not known, their allergenicity justifies inclusion in the allergen exposure assay.

The polyclonal rabbit antibodies appeared to be sufficiently specific for use in a bakery environment. Some reactivity was detected against extracts of *Aspergillus* spp., baker's yeast, and storage mites; but it is very unlikely that airborne exposure to these agents reaches such levels in bakeries that false-positive results in the sandwich assay are to be expected. Wheat flour proteins are present in personal samples in considerable amounts and therefore may be an important confounding or modifying factor. It is, however, not very likely that the presence of wheat flour would affect the measured amylase exposure levels. Most of the personal dust samples in bakeries (98%) have flour protein levels below 60 $\mu\text{g}/\text{m}^3$, which is equivalent to protein levels in extracted samples of approximately 25 $\mu\text{g}/\text{ml}$ ¹⁸. At this concentration in our amylase assay, the wheat flour extract produced OD₄₉₂ values that hardly differed from the background and did not interfere with the measured α -amylase levels.

In some previous studies the enzyme activity of α -amylase was measured in airborne dust samples to evaluate α -amylase exposure levels in a protein-processing factory²⁴ and in bakeries.^{25, 26} For the exposure assessment of fungal amylase allergens, however, these methods have two important limitations. First, inactive or denatured enzymes are not measured but can still act as allergens. Sander and Baur²⁷ showed that digested fragments of α -amylase were still able to bind IgE antibodies. In this case the amylase allergen exposure may be underestimated if enzyme activity is measured. Second, these methods measure the amylase activity of both fungal and cereal origin. Amylase (β more than α) is already present in flour, but there appeared to be only minimal immunologic cross-reactivity between cereal and fungal amylases.²¹ Therefore measurements of enzyme activity may have limited validity in quantifying exposure to allergens of fungal amylase, and methods based on immunochemical techniques may be preferred.

For bakery workers, large differences in α -amylase allergen exposure were found, with mean exposure levels of occupational titles varying from 0 to 40 ng/m^3 . The type of bakery in which the bakers work seemed to

be very important for the level of α -amylase exposure, and in some types of bakeries no airborne α -amylase exposure was found at all. Amylase exposure was higher in large industrialized bakeries than in small traditional bakeries. In all bakeries α -amylase exposure was only found for workers directly involved in dough making or for workers present in the dough-making area. In these groups there is a high day-to-day variability in α -amylase allergen exposure.

Measurements with the cascade impactor showed that α -amylase allergens are present in particles of 5 μm or larger. In most bakeries more than half of the α -amylase is in the dust fraction with particle sizes larger than 9 μm . This means that α -amylase allergens are most likely to be deposited in the nose and the ciliated airways (bronchi and bronchioles).²⁸ This is in agreement with effects of α -amylase allergen exposure (rhinitis and asthma) described in the literature.

This study shows that personal monitoring of airborne exposure to fungal amylase allergens in bakeries is possible. The dust sampling procedure and sandwich EIA for α -amylase allergens permit the identification of allergen sources, as well as the study of exposure-response relationships. Analyses with data from our epidemiologic study have shown that the measured α -amylase exposure levels are strongly associated with enhanced risk of α -amylase sensitization of the bakery workers.¹⁵ The availability of an assay and the (first) indications for an exposure-response relationship open a way to standard setting, as for proteolytic enzymes of *Bacillus subtilis*.²⁹ With the possibility of identifying allergen sources, the assay can also assist in identifying high-risk activities to be targeted for modification and prevention strategies.

We thank the bakery workers for their cooperation. We also thank Anke Boumans, Trienke Jongedijk, Monique Leblanc, Marijn Meijler, Pauline Oosterveld, Susan Peelen, Isabella van Schothorst, Femke Steenstra, Heidi Timmermans, Marianne Veerman, Dick Zutt, and Mariëlle van Zuylen for their assistance in different stages of the project.

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