



Development and Validation of a Monoclonal Based Immunoassay for the Measurement of Fungal Alpha-Amylase: Focus on Peak Exposures

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The inhalation of flour dust has been implicated in the induction of sensitisation and elicitation of respiratory symptoms, such as asthma in bakers. In addition to the cereal allergens present in wheat flour, enzymes in flour improvers, in particular fungal alpha-amylase, are now known to be a significant cause of respiratory allergy in the baking industry.

A monoclonal antibody based enzyme-linked immunoassay (ELISA) was developed using two monoclonal antibodies that recognised two distinct epitopes of the fungal alpha-amylase enzyme. The ELISA had an inter-assay variation of 12.0% at 1360 pg/ml and 12.8% at 564 pg/ml and intra-assay variation of 4.9% at 1340 pg/ml and 6.1% at 504 pg/ml. The assay had a sensitivity of 200 pg/ml. Competitive inhibition assays confirmed that the monoclonal antibodies had no cross reactivity with other enzymes used in the baking industry and could distinguish added fungal alpha-amylase from cereal amylase.

We assessed the levels of exposure to dust, total protein and fungal alpha-amylase in four UK bakeries ranging in size and technical capabilities. Within the bakeries we surveyed, workers were exposed to variable levels of inhalable dust (0.8–39.8 mg/m³), total protein (0–5.7 mg/m³) and fungal alpha-amylase (0–29.8 ng/m³). Consecutive 15 min personal samples taken over a 1 h period demonstrated that the ELISA could measure fungal alpha-amylase exposure in such a 15 min period. Short term peak exposures to fungal alpha-amylase could be identified which may contribute to the sensitisation in individuals who appear to have low exposure levels if measured over a full shift period. Crown Copyright © 2001 Published by Elsevier Science Ltd on behalf of British Occupational Hygiene Society. All rights reserved.

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INTRODUCTION

Occupational respiratory disease is common amongst bakers, with occupational asthma frequently reported (Sallie *et al.*, 1994; Ross *et al.*, 1995). The inhalation of cereal allergens has been implicated in the induction of sensitisation and elicitation of respiratory symptoms in bakers. However, the list of causative agents has now increased to include flours from other

sources, such as soya; and other agents such as storage mites, insects, and fungi. Throughout the 1980s, the use of enzymes in flour improvers increased. Many are derived from *Aspergillus*, such as cellulase/hemicellulase, glucoamylase and especially fungal alpha-amylase. These agents are now known to be a significant cause of respiratory allergy in the baking industry (Baur *et al.*, 1988).

Fungal alpha-amylase (1,4- α -D-glucanohydrolase) is a glycoprotein derived from *Aspergillus oryzae*. It is added to flour to compensate for the low natural amylase content of cereal flour, improve the quality of bread and economise the baking process. The fungal alpha-amylase content of flour is usually less than 0.01%, and is often added to the flour at the mill, but may also be added at the bakery as part of the flour improver which is added to the bread mix.

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The potential of fungal alpha-amylase to cause asthma has been verified with reports of patients with immediate-type hypersensitivity to this allergen and resulting respiratory symptoms (Brisman and Belin, 1991; Quirce *et al.*, 1992). Baur *et al.* (1986) demonstrated that up to 34% of bakers with respiratory symptoms were sensitised to alpha-amylase. Further studies assessing alpha-amylase sensitisation with the use of skin prick and radioallergosorbent tests, have suggested that between 8–15% of all bakers are sensitised to alpha-amylase (Smith *et al.*, 1997; Houba *et al.*, 1996; Jeffrey *et al.*, 1999).

These findings have led to a focus on the determination of fungal alpha-amylase exposure levels in bakeries, and the identification of workplace categories of individuals most at risk of sensitisation. Previous methods for the detection of airborne fungal alpha-amylase have included both enzymic techniques and immunoassays, but as yet there is no recommended method for the quantification of this enzyme. Therefore, it is difficult to compare data obtained from different investigations. The enzymic methods for detecting inhalable fungal alpha-amylase measures all active alpha-amylase, whereas immunoassays detect immunoreactive amylase. Enzymic methods have been employed by investigators (Brisman and Belin, 1991; Jauhiainen *et al.*, 1993) which were primarily developed for measuring fungal alpha-amylase in sera such as Phadebas® Amylase test (Pharmacia and Upjohn), and colorimetric assays that measure the fungal alpha-amylase activity using 2-chloro-4-nitrophenyl- β -D-maltoheptaoside as a substrate. The measurement of fungal alpha-amylase by a sandwich EIA assay, has been described (Houba *et al.*, 1996), with the use of affinity purified polyclonal rabbit anti-alpha-amylase antibodies. A monoclonal antibody based immunoassay has been developed in our laboratory which has no cross reactivity with other allergens tested and distinguished added fungal alpha-amylase from cereal amylase.

With the use of our monoclonal antibody based immunoassay for the detection of fungal alpha-amylase, airborne levels of this enzyme were quantified in a pilot study to investigate the effect of sampling times on inhalable dust and fungal α -amylase measurements and to identify whether peak exposures to this enzyme could be quantified. Personal exposures to inhalable dust, total protein and fungal alpha-amylase were also identified for a limited number of bakers.

METHOD

Purification of fungal alpha-amylase

Crude fungal alpha-amylase from *Aspergillus oryzae* (Sigma, Dorset, UK) or Fungamyl, a gift from Novo Nordisk, Copenhagen, Denmark, was purified by anion-exchange chromatography using a Mono Q column on the FPLC system (Pharmacia Biotech,

Upsala, Sweden) as described by Swift *et al.* (1991). Fractions containing pure alpha-amylase were identified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF) and by enzyme activity using the Phadebas Amylase Test (Pharmacia Biotech, Upsala, Sweden). The fungal alpha-amylase purified from the Sigma reagent was used for all immunisations and was suitably diluted to provide standards for the ELISAs.

N-terminal amino acid sequencing

The purified fungal alpha-amylase was N-terminal sequenced using an automated protein sequencer (Model 473A, Applied Biosystems, UK) in order to further confirm its identity. The sequence was then compared with the Swiss and PIR Translated Release 88 database using Lasergene software (Lasergene, UK).

Production of monoclonal antibodies

NS1 myeloma cells were fused with immune cells from mice immunised with purified fungal alpha-amylase (Brown *et al.*, 1988). Hybridomas secreting antibodies against fungal alpha-amylase were identified by ELISA screening of the culture supernatants. Positive hybridomas were cloned by limiting dilution and finally expanded into serum free culture for bulk production of antibody, which was purified by Protein G affinity chromatography. The purified antibody was dialysed against PBS and stored in aliquots at -70°C or 0.1% NaN_3 added and stored at 4°C . Antibodies were biotinylated using Biotin (long arm) N-hydroxysulfosuccinimide (Vector Laboratories, CA, USA) as described by Hudson and Hay (1989).

Preparation of extracts

Twenty percent (w/v) extracts of flours, flour improvers, bakery enzymes (Sigma, Dorset, UK) and environmental allergens (Swann Technology, Hertfordshire, UK) in phosphate buffered saline (PBS) were prepared by overnight end-over-end mixing at room temperature. The extracts were then centrifuged at 3000 rpm for 20 min and the supernatant filtered through a $0.45\text{ }\mu\text{m}$ filter. The protein concentration of each extract was measured by an automated method (Bradford, 1976) using human serum albumin as the standard.

Electrophoresis and immunoblotting

Fungal alpha-amylase and protein extracts were separated by SDS-PAGE (12.5% homogenous gels) as described by Laemmli (1970), under reducing and non-reducing conditions using the Mini-Protein II system (BioRad Laboratories, Ltd, Hertfordshire, UK). Gels were stained with Coomassie blue or transferred to nitro-cellulose using a semi-dry electrophoretic method for identification of proteins by mono-

clonal antibodies. Molecular weight standards (BioRad Laboratories Ltd, Hertfordshire, UK) were included on all gels so that the molecular weights of bands could be calculated.

Monoclonal antibody inhibition assay

The ELISA plates (Nunc maxisorb) were coated with purified fungal alpha-amylase overnight at 4°C (100 µl per well) at a concentration of 2 µg/ml diluted in coupling buffer. The plates were washed and 50 µl per well of fungal alpha-amylase (Sigma, Dorset, UK), flour improver, purified fungal alpha-amylase, Canada best flour (which does not contain fungal alpha-amylase) hemicellulase and cellulase, were added with the monoclonal antibodies HSL-19 or HSL-20 (50 µl per well) at a concentration of 2 µg/ml diluted in PBS/1% BSA/0.1% Tween 20 for one hour at room temperature. After washing, 100 µl per well of anti-mouse IgG peroxidase conjugate (Sigma, Dorset, UK) diluted in PBS/1% BSA/0.1% Tween 20 was added and incubated for 30 min. After the final washing step, colour was developed by a 15 min incubation in the dark with O-phenylenediamine dihydrochloride (Sigma, Dorset, UK) and the reaction stopped by the addition of 2M H₂SO₄. The plates were read on a Multiskan plus reader at 492 nm with correction at 600 nm, and the data processed using Genesis software (LabSystems, UK).

Monoclonal enzyme-linked immunosorbent assay for the determination of airborne fungal alpha-amylase

The ELISA plates (Nunc maxisorb) were coated with Mab HSL-19 overnight at 4°C (100 µl per well) at a concentration of 1 µg/ml diluted in coupling buffer. The alpha-amylase standards (at concentrations of 0–5000 pg/ml), quality controls, and test samples were prepared in PBS/1% BSA/0.1% Tween 20, with the test samples initially diluted at concentrations of 1/2, 1/4 and at a higher dilution if necessary. The plates were washed and 100 µl per well of the standards, QC's and diluted test samples were added and incubated for 1 h at room temperature. After washing, 100 µl per well of biotinylated Mab HSL-20, at concentration of 1 µg/ml diluted in PBS/1% BSA/0.1% Tween 20 was added and again incubated for 1 h. The plates were then washed and 1 µg/ml Avidin horseradish peroxidase (Vector Laboratories, CA, USA) in PBS was added and the plates incubated for 10 min. After the final washing step, colour was developed by a 15 min incubation in the dark with O-phenylenediamine dihydrochloride (Sigma, Dorset, UK) and the reaction stopped by the addition of 2M H₂SO₄. The plates were read on a Multiskan plus reader at 492 nm with correction at 600 nm, and the data processed using Genesis software (LabSystems, UK).

Total protein estimation

Total protein estimation was performed using a Bicinchoninic acid protein assay, (BCA) (Smith *et al.*, 1985). The BCA method has the advantage of being tolerant to detergents used in protein solubilisation, such as Tween 20. The protein standard used was bovine serum albumin (1 mg/ml, Sigma, Dorset, UK.) and quality control samples were prepared from human serum albumin (Sigma, Dorset, UK) in distilled H₂O. The assay had a between assay coefficient of variation of 3.0% at 528.9 µg/ml, and of 2.0% at 267.5 µg/ml.

Sampling methods

Four bakeries from South Yorkshire, England were surveyed in this study. They were randomly chosen from a local phone directory, with sizes ranging from a workforce of 7 to 600. They also differed in the type of products produced, and the technical capabilities employed (Table 1). Each bakery was visited once in January 1998, but at this time of year the workload in the bakeries was not at its peak. Production workers were classified into eight main task categories, flour mixers, dough cutters and flour mixers, packer, loader, confectionery maker, cleaner, ovens, and end of line workers (Table 2).

Personal short and long term samples were collected in the workers breathing zone using IOM sampling heads with glass fibre filters (GF/A, 1.6 mm, Millipore) at a flow rate of 2 l/min. Eight bakers were randomly chosen for personal short term sampling. Each baker was observed, while four consecutive 15 min samples were taken to examine peak exposures to fungal alpha-amylase. The remaining bakers involved in this study were sampled once for 1–8 h depending on how long they were involved in their job task. The filters were weighed twice in a preconditioned room before and after sampling was undertaken and the personal dust exposure (mg/m³) calculated. Total protein and fungal alpha-amylase allergens were recovered by elution of the filters into PBS/0.1% Tween 20 overnight by end-over-end mixing. Short term samples and controls were eluted in 2 ml, whereas long term samples were eluted in 5 ml. The filters and supernatant were squeezed through a 2 ml syringe and centrifuged at 600g for 5 min to remove any fine particle matter. The supernatant was removed and stored in bijoux at –20°C until analysis.

RESULTS

Hybridoma production

The purified fungal alpha-amylase had a molecular weight of some 50 kD and a pI of ≈4 which is similar to that reported by Baur *et al.* (1994). N-terminal sequencing gave the sequence ATPADWRSQSIYFLLTDRFA which had 100% homology with the sequence listed in the Swiss and Pir and Translated Release 88 datab-

Table 1. Comparison of the different types of bakeries studied

Bakery no.	No. of employees	Products made	Flour improver added to dough mix	Shared job tasks
1	≈600	Bread, morning goods, doughnuts	Yes	No
2	21	Bread and confectionery	Yes	Yes
3	≈120	Bread, morning goods, confectionery	Yes	Yes
4	7	Bread and confectionery	No	Yes

Table 2. Definition of different occupations in the bakeries surveyed

Occupation	Description
Flour mixer (mixing room)	Operates mixer in a closed environment, mixes flour with other ingredients such as flour improver
Flour mixer (bread products)	Operates mixer on factory floor, combines flour with other ingredients such as flour improver, at times will not be performing tasks
Dough cutter and flour mixer	Uses automated dough cutting machinery or cuts dough manually, may combine some flour ingredients
Packer	Packs bread products, at times will not be in the main production area
Loader	Loads bread products for transportation and moves flour sacks, at times will not be in the main production area
Confectionery and pastries	Produces confectionery and pastry products, generally not exposed to flour dust unless "dusting surfaces"
Cleaner (mixing room)	Cleaning the equipment and production area in the mixing room in bakery no. 1
Cleaner	Cleaning the equipment, production area, and non-production areas
Ovens	Operates the ovens
End of line	Dusts bread products, moves bread products to packing area

ase. The purified fungal alpha-amylase was subsequently used in all immunizations and as a standard for the ELISA. Seven stable hybridomas secreting MAbs were produced from one fusion and screened using ELISAs and immunoblotting. Two of these monoclonal antibodies were chosen which bound to purified alpha-amylase and to fungal alpha-amylase in flour but did not bind to components of wheat flour without added amylase (Fig. 1). The specificity's of these MAbs were studied in detail and immunoblots

are shown in Figs 2(A) and (B). HSL-19 bound to a single band corresponding to the fungal alpha-amylase, but HSL-20 also bound faintly to bands of 28 and 38 kD. Neither antibody inhibited the binding of the other antibody to alpha-amylase, suggesting that the antibodies bound to different epitopes of amylase.

ELISA assay

The capture assay had a detection range of between 200 and 5000 pg/ml. The within assay coefficient of

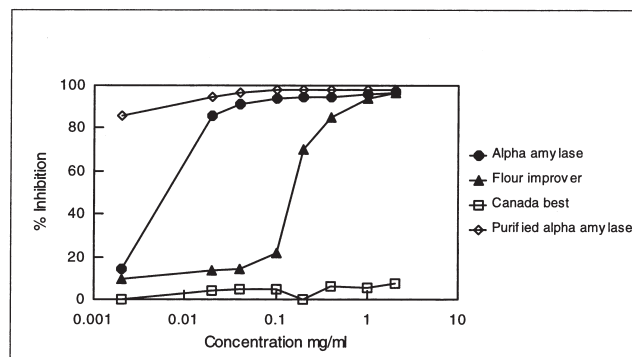


Fig. 1. Inhibition of antibody binding of HSL-19 using flour improver containing fungal alpha-amylase, but not with flour with no added fungal alpha-amylase.

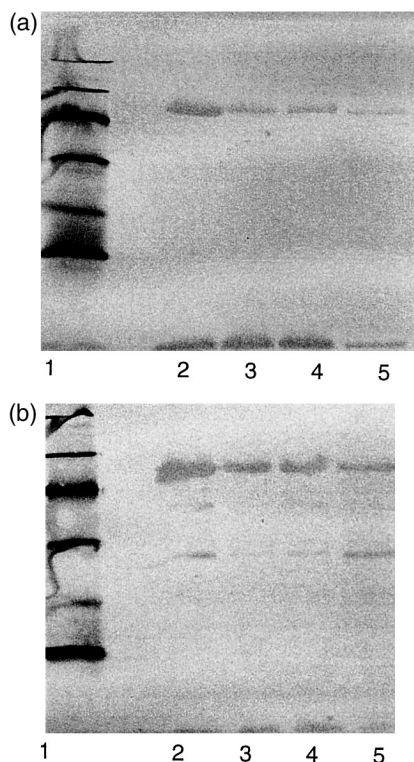


Fig. 2. (A and B) Gel A—monoclonal antibody HSL-19 immunoblot of samples separated using reduced 12% SDS-PAGE. Lane 1, molecular weight standards, from top of gel 97.4, 58.1, 39.8, 29, 20.1, and 14.3 kD; lane 2, Fungamyl; lane 3, purified fungal alpha-amylase; lane 4, crude fungal alpha-amylase; and lane 5, flour improver. The bands seen at the very bottom of the gel is a marker dye. Gel B as above but using monoclonal antibody HSL-20.

variation (CV) was 4.9% at 1340 pg/ml and 6.1% at 504 pg/ml ($n=20$). The between assay CV was 12.0% at 1360 pg/ml and 12.8 at 564 pg/ml ($n=16$). Wheat, barley, soya flour and *Aspergillus fumigatus* soluble protein were not detected up to 1 mg/ml and the common allergens Rye grass, milk, oat, *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae* and *Dermatophagoides pteronyssinus* were equally not detected at the same concentration of soluble protein. In addition, the enzymes hemicellulase or cellulase, which are commonly used in the bakery industry, did not inhibit the assay, up to concentrations of 1 mg/ml (Fig. 3). The dilution curves of eluates from personal samplers were run and seen to parallel that of the fungal alpha-amylase standard.

Bakery details

During this investigation a range of different sized bakery premises were sampled, from small 'craft' bakeries where workers performed several production tasks, to a large factory where workers undertook single tasks usually in separate areas. In the small bakeries the flour was usually delivered in bags, manually weighed and mixed, and also often shaped and dusted

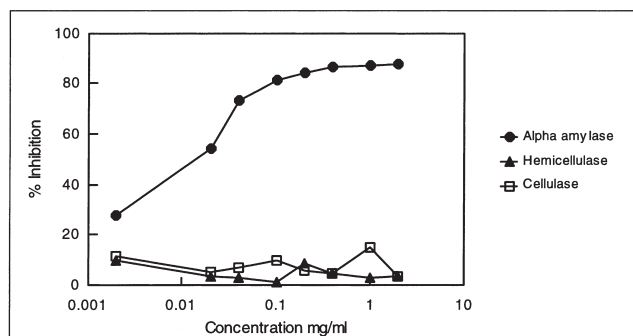


Fig. 3. High concentrations of enzymes used in the baking industry did not inhibit the binding of HSL-20 to fungal alpha-amylase.

by hand. These operations were potentially very dusty, and could be a significant source of direct exposure to flour dust. In these small bakeries, exposure to flour could also arise from the general contamination of the workplace. In the large bakery the flour was held in silos prior to use, and mixing with flour improvers occurred generally in one area. The flour was delivered to individual mixers in different areas of the factory, where additional amounts of flour improvers were added depending on what products were being produced. Confectionery and pastry making differs from bread baking in that the addition of flour improvers is not an essential part of the process. However, small amounts of fungal amylase are still present in the flour and exposure may occur during the manual throwing of flour to 'dust down' work-surfaces.

Airborne dust total protein and fungal alpha-amylase exposure

In the work places sampled, the levels of inhalable dust were undetectable below a 1 h sampling time and therefore gravimetric analysis for the determination of personal dust exposure was only appropriate for samples taken for longer than 1 h. Each bakery demonstrated variation in exposure levels, to inhalable dust, total protein and alpha-amylase (Table 3).

Sampling times

Four consecutive 15 min personal samples were taken for three mixers, one end of line worker, two confectionery workers and two dough cutters and mixers. The other job tasks were not included as the workers were not stationary in the bakery which hindered observation. During the hour of sampling, the mixers were not constantly weighing and handling flour improver, which is reflected in the exposure data in Table 3. The data demonstrated that the total inhalable dust exposure over 1 h did not give a reliable estimate of average fungal alpha-amylase exposure measurements over that time. ($r=0.1039$ at $P=0.8066$). As 15 min dust exposures could not be

Table 3. Fungal alpha-amylase exposure during four consecutive 15 min sample. The inhalable dust levels could not be measured over 15 minutes and therefore measurement was taken for 1 h. (–) none detected

Occupation	Inhalable fungal α -amylase (ng/mg ³)					Inhalable dust (mg/m ³)
	1	2	3	4	Average	
Mixer bakery 1	95.4	–	131.8	190.9	104.5	7.9
Mixer bakery 2	–	38.4	–	–	9.6	12.3
Mixer bakery 3	38.7	–	–	29.5	17.1	7.5
Dough cutter and mixer bakery 4	–	–	11.2	–	2.8	13.5
Dough cutter and mixer bakery 2	–	–	–	–	–	0.9
End of line bakery 1	–	24.1	–	–	6	5.1
Pastries bakery 3	–	–	50.7	–	12.7	–
Pastries bakery 3	–	–	70.5	–	17.6	3.5

accurately determined, dust measurements would not give any indications of peak exposures to fungal alpha-amylase. The pattern of highly varied exposure levels to fungal alpha-amylase was observed for this method of sampling for the other occupational categories (Table 4).

Fungal alpha-amylase is not often used in the confectionery production process in the smaller bakeries and accordingly workers in these sections were exposed to levels of alpha-amylase that were usually below the detection limit of the ELISA assay. This situation altered dramatically when the workers were in the process of 'dusting surfaces'. In this short period of time these staff were exposed to relatively high

levels of inhalable fungal alpha-amylase which raised their mean exposure significantly. The flour used for dusting surfaces is often the cheapest flour available and may contain fungal alpha-amylase.

DISCUSSION

Purified alpha-amylase was prepared from a commercially available crude preparation derived from *Aspergillus oryzae* and shown to be identical with that previously described in the literature. This preparation was used to produce monoclonal antibodies which were screened for specificity using ELISAs and immunoblotting. The two MAbS HSL-19 and 20,

Table 4. Comparison of inhalable fungal alpha-amylase and total protein concentrations for baking occupations, in the different bakeries

Activity	Bakery no.	Inhalable fungal α -amylase (ng/m ³)	Total inhalable protein (mg/m ³)	Inhalable dust (mg/m ³)	Sampling time (h)
Flour mixer (mixing room)	1	16.7	0.5	7.9	1
Flour mixer (bread products)	1	0	0.4	10.4	1
		2.7	0.6	36.7	4
		18.3	4.1	21.4	1
	2	26.5	1.7	12.3	1
	3	8.7	1.2	12.2	8
Dough mixer and cutter		3	0.9	7.5	1
	2	10.9	1.4	2.2	4
	3	0	3.3	68.1	4
	4	0	0.1	9	4
		19.2	1	13.5	1
Packer	1	4	1.6	3.1	6
		2.2	0.8	1.3	3.5
	2	0	0	1.2	4
		0	2.9	39.8	1
	3	0	0.3	3	1
Loader	4	0	0.4	3.8	1
	1	29.8	0.2	0.8	6
		0	1	3.6	1
	2	10.9	0.5	7.8	1
	1	0	0.1	2.3	1
Confectionery and pastries	3	0	0.2	3.5	1
Ovens	2	0	0.8	5.2	1
End of line	3	0.2	0.2	2.8	8
		4.6	0.7	20.4	4
		0	1.1	18.6	1

chosen for incorporation into a capture immunoassay, had high specificity to the purified fungal α -amylase. HSL-19 was used as the capture antibody, as it did not bind to fragments of α -amylase. Both antibodies were specific for fungal α -amylase, which ensured that only fungal α -amylase and not cereal amylase, or other enzymes used in the baking industry were measured using this assay. The assay has not been compared to other polyclonal (Houba *et al.*, 1996) or monoclonal based assays (Sander *et al.*, 1997) although it would be useful to compare results. During this study, personal exposure to total dust, protein and α -amylase were assessed in workers in bakeries of different sizes and capacities, ranging from small craft bakeries to a large industrialised bakery. This sampling protocol was used to establish the usefulness of the assay in measuring personal exposure to fungal α -amylase in a variety of workplace situations. Our results showed the range of inhalable dust levels that can be found in bakeries. It is possible that overall high levels of dust, are due to very high transient levels of exposure during certain operations. The levels of personal total inhalable protein were also measured during this study. The content of this inhalable protein is likely to consist of other established allergens such as cereal allergens. Although total airborne protein estimation cannot quantify individual allergens it may give an indication of total potential allergen exposure.

Many studies have measured fungal α -amylase exposure levels in bakers over full shift periods using various assays. Consecutive 15 min sampling revealed peak exposures to total protein and fungal α -amylase, which would have been overlooked if only full shift sampling was undertaken. We also demonstrated that total dust measurements could not be accurately determined over a 15 min period and consequently could not be used to identify peak exposures. These peak exposures may contribute to sensitisation in individuals who appear to have low exposure levels if measured over a full shift period.

In conclusion, the assay for the measurement of fungal α -amylase has the potential to become a powerful tool for the risk management of sensitisation to fungal α -amylase in bakers. The identification of short term peak exposures to fungal α -amylase will give the industrial hygienist a tool to identify the sources of exposure and to advise on appropriate control measures to reduce exposure levels.

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