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Measurement of Airborne Flour Exposure with a Monoclonal Antibody-Based Immunoassay

Key Words

Baker's asthma
Wheat allergen
Wheat flour α -amylase inhibitor
Exposure, airborne

Abstract

Background: The development of simple and standardised methods to measure airborne levels of workplace biological allergens is an important step in reducing the incidence of occupational asthma. Such a method would be useful for measuring wheat flour allergens which cause asthma in bakers. Measurement of allergen per se rather than total dust enables exposure to be better defined. **Methods:** Monoclonal antibodies were produced, their specificity analysed by immunoblotting and then used to affinity-purify a putative flour allergen. The importance of this protein as an allergen was tested by RAST using sera from allergic bakers and it was identified by N-terminal sequencing. Suitable monoclonal antibodies were chosen to develop an enzyme-linked immunosorbent assay. Commercial baking flours and personal airborne dust samples were analysed using the immunoassay. **Results:** A sensitive and specific monoclonal antibody-based enzyme-linked immunosorbent assay was developed to measure a wheat α -amylase inhibitor. The wheat α -amylase inhibitor content of bulk wheat flours was 0.124% (95% confidence limits 0.083–0.164%) and airborne levels in bakeries had a geometric mean of 744 ng/m³ (95% confidence limits 371–1,496 ng/m³). **Conclusion:** This assay is suitable for widespread use as the monoclonal antibodies and standards are well defined and potentially infinitely available. The assay therefore offers distinct advantages over those exposure assessment methods currently in use. Comparable results would be obtained by different investigators over a prolonged time period. The assessment of flour allergen exposure and the relationship with clinical response could then be investigated using a multi-centered approach.

Introduction

The development of simple and standardised methods to measure airborne levels of workplace respiratory allergens is an important step in helping to reduce the incidence of occupational asthma. This is especially true for occupational biological allergens such as flour where until recently only surrogate measures of exposure such as total dust have been available. In the UK flour and grain dust is suspected

of causing some 8% of new occupational asthma cases per year [1, 2]. Large scale surveys of bakeries and mills have indicated that between 4 and 14% [3–6] of workers report work-related chest symptoms. However, the true incidence of baker's asthma is unclear as many of those reporting symptoms in these studies were not found to have IgE antibodies to flour or other bakery-associated substances and some symptoms may be due to irritation [5, 6]. Baker's asthma may also be untypical often taking 5 years or more

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of exposure to develop possibly due to the tolerance induced by the consumption of flour from an early age [6–8], although it is clear from challenge testing that individual bakers and millers do suffer from asthma induced by flour [7, 9].

Immunoassay of flour antigens and allergens has been undertaken using human serum either in radio-allergosorbent test (RAST) inhibition assays [10] or inhibition enzyme-linked immunosorbent assays using human IgG4 [11] and also using rabbit polyclonal antibody [12]. However, these assays have several disadvantages especially if they are to be widely used for workplace exposure assessment or the results from such measurements are to be used in risk assessment procedures to derive exposure standards. Human sera carry with them a risk of infection and are usually only available in limited quantities and as with animal sera may be difficult to obtain over time with a constant specificity and avidity. In addition, the results obtained with polyclonal-based immunoassays are very dependent on the composition of the standard and these factors make the comparison of results by different investigators difficult or impossible. An answer to these problems is provided by using a monoclonal antibody-based immunoassay in that a constant reagent of single and invariant activity is employed along with a defined and single protein allergen standard. Such an approach requires that the allergen must either be the only or most important allergen or be a suitable marker allergen and be present at a more or less constant ratio in a wide range of flours. The former approach has largely been used to assess house dust mite exposure by measurement of the major allergen *Der p* I although measurements of other major allergens such as *Der p* II are undertaken [13].

In the UK bakers use several cereal flours as ingredients but by far the greatest amount used is wheat flour and this is almost exclusively of European origin. Wheat flour contains both water-soluble (albumins and globulins) and water-insoluble proteins (gliadins and glutenins). The former have been most extensively investigated and considered to be the most important source of allergens although sensitisation to gluteins has been reported [14]. Immunoblotting of the water-soluble fractions of wheat, rye and barley flours with allergic baker's serum has identified several major allergens. These were of the following molecular weights for wheat flour: 47, 17 and 15 kDa [15], 35 and 14 kDa [16], and 69, 33, 26, 21, and 12 kDa [17], for rye flour: 14 kDa [16] and 21 and 12 kDa [17] and for barley: 14.5 kDa [18] and 69, 52 and 10 kDa [17]. The lower molecular weight allergens have been studied in the greatest depth. These are subunits of monomeric, dimeric and tetrameric inhibitors of heterologous α -amylases and belong to the same cereal protein fam-

ily as do the trypsin inhibitors. Measurement of the IgE binding capacity of this family has indicated that all were reactive [19–21]. Armentia et al. [22] investigated the in vivo activities of eleven purified members of the α -amylase/trypsin inhibitory family from wheat and barley and showed that 30/31 flour allergic subjects showed a positive skin prick test to at least one of them. In this study we produced a series of monoclonal antibodies (MAb) to wheat flour-soluble antigens, investigated their specificity using immunoblotting and using one of these antibodies affinity-purified a putative wheat flour allergen.

RAST were used to determine this protein's importance as a flour allergen in baker's asthma and N terminal sequencing identified it as a wheat α -amylase inhibitor (WAAI). We used two MAb to develop a capture immunoassay to measure WAAI and analysed both commercial baking flours and airborne dust in bakeries.

Methods

Flour and Other Allergen Extracts

Twenty percent (w/v) extracts of commercial white and wholemeal wheat flours, barley, oat, rye and soya flours (obtained from commercial bakeries) and fungal α -amylase (Sigma, UK), rye grass, storage mites *L. destructor*, *T. putrescentiae* and *A. siro* (Allergon, Sweden) in 0.15 M NaCl were prepared by overnight end over end mixing at room temperature. After allowing the solids to sediment the supernatant was centrifuged at 10,000 g, passed through a 0.22- μ m filter and stored in aliquots at -20°C . Protein concentration was measured by an automated method of Bradford using human sera as standard [23].

Production of MAB

NS1 myeloma cells were fused with immune cells from mice immunised with either extracts of white or wholemeal wheat flour using polyethylene glycol [24]. Hybridomas secreting antibodies against flour were identified by ELISA screening of culture supernatants. Positive hybridomas were cloned by limiting dilution and expanded into serum-free culture for bulk production of antibody. Antibody was purified from bulk culture supernatants by protein G affinity chromatography, dialysed into phosphate-buffered saline (PBS) and stored in aliquots at -70°C or 0.1% NaN₃ added and stored at 4°C . Antibodies were biotinylated using Biotin (long arm) N-hydroxysuccinimide (Vector Laboratories).

Electrophoresis and Immunoblotting

Flour and other extracts were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions using the Mini-Protean II system (Bio-Rad Laboratories, UK). Gels were stained with Coomassie blue or transferred to nitrocellulose using a semidry electrophoretic method for identification of proteins by antibodies. Immunoblotting using human IgE antibodies was undertaken using a biotinylated antihuman-IgE MAb and streptavidin peroxidase with enhanced chemiluminescent detection. Serum samples were obtained from bakers working at small UK bakeries with allergic respiratory symptoms including asthma.

Purification of Flour Allergen and Confirmation of Allergenicity

Purified MAb HSL-11 was coupled to CNBr-activated Sepharose 4B (Pharmacia & UpJohn, UK) and used to affinity-purify its antigen from white flour extract. Bound antigen was eluted with 0.1 M Tris/glycine HCl pH 2.5, immediately neutralised and dialysed into PBS. Purity was established by SDS-PAGE and the protein concentration measured as described above. To determine whether IgE antibodies from allergic bakers bound to this purified flour protein it was coupled to paper discs and RAST testing undertaken [25]. Briefly, protein and cyanogen bromide-activated paper discs (ratio approximately 1:100) were reacted overnight, unreacted sites were blocked with ethanolamine and the discs washed. RAST using both purified flour protein and wheat flour extract discs were carried out with archived sera from both allergic bakers and unexposed individuals. The inhibitory activity of this flour protein against α -amylase was measured by adapting the method of Gomez et al. [26]. As a source of α -amylase a crude PBS extract of lyophilised *Tenebrio molitor* (Allergen, Sweden) was used along with the Phadebas amylase test tablet (Pharmacia & Upjohn, UK). Inactivation of α -amylase inhibitory activity was demonstrated by boiling test samples for 5 min. N-terminal sequencing of the flour protein was undertaken 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 & PIR Translated Release 88 database using Lasergene software (Lasergene, UK).

Monoclonal Antibody Capture ELISA

The ELISA was first optimised using a chequer board titration technique. The ELISA plates were coated overnight with 0.25 μ g/ml MAb HSL-11, washed and blocked for 120 min (min) with buffered bovine serum albumin, Tween 20. Standards 0–50 ng/ml of WAAI, test or quality control samples were added to the plates and incubated for 60 min when the plates were washed. One hundred nanograms per millilitre biotinylated MAb HSL-8 was added, incubated for 45 min, the plates washed and a 1:1,000 dilution of streptavidin peroxidase conjugate (Amersham) added for 10 min. Following washing of the plates colour was developed by a 15-min incubation with *o*-phenylenediamine. Plates were read at 492 and 690 nm with a Multiskan plus plate reader and data processed with Genesis software (LabSystems, UK). Standard curves were generated using the smoothed cubic spline transformation.

Measurement of Airborne Flour

Personal short- and long-term air samples were obtained using Institute of Occupational Medicine samplers [27] at a medium-sized UK bakery. After equilibration and reweighing of the sample cassettes the GF/A filters were carefully removed, placed into dedicated metal storage cans and stored at -20°C until assayed for WAAI. Prior to extraction air filters were allowed to reach room temperature, then carefully removed from their storage cans with tweezers, cut into approximate quarters and added to 10 ml of 0.15 M NaCl, 1% Tween 20 in plastic 110 $\times$ 16 mm plastic screw top tubes. These were mixed end over end at room temperature for 120 min when the buffer was squeezed from the filters using a syringe. In some cases filters were extracted a second time in an identical manner. Eluates were spun at 600 g for 20 min to remove fine particulate matter prior to immunoassay.

Statistical analysis was by Prism version 2 software (GraphPad Software, USA).

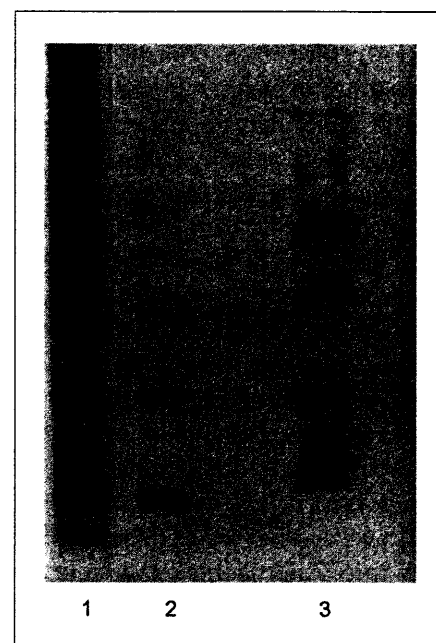


Fig. 1. MAb immunoblot of wheat flour extract separated using 12% SDS-PAGE under non-reducing conditions. Lane 1 represents HSL-8, lane 2 HSL-11, lane 3 molecular weight standards from top of gel 97, 58, 40, 29, 20 and 14 kDa.

Results

Immunoblotting with allergic baker's IgE antibodies showed that all sera bound to wheat flour proteins with molecular weights in the range 10–16 kDa. Binding to other higher molecular weight proteins was found but there was no common pattern (data not shown). Eighteen MABs of the IgG subclass were produced from two fusions. All recognised white and wholemeal wheat flour extracts, many of the MABs recognised more than one flour protein and for some antibodies this reactivity was abolished following disruption of intrachain s-s bonds. The specificities of six of these MABs, which represented the binding patterns of all the MABs, were studied in detail using direct ELISA and immunoblotting. Two of these MABs – HSL-8 and HSL-11 – had properties which suggested they could be used in an immunoassay – they had little cross-reactivity and bound to a similar low molecular weight protein which also appeared to be recognised by IgE antibodies from allergic bakers (fig. 1). Using direct ELISAs HSL-8 was shown to bind slightly to bran extracts and HSL-11 weakly to barley and bran extracts.

The MAB HSL-11 was used to affinity-purify a single flour protein with a molecular weight by SDS-PAGE of

Table 1. Wheat flour extract and WAAI were coupled to paper discs and used for RAST testing with the sera from allergic bakers (sera A–X) and unexposed individuals (individual results not shown)

Sera	Binding, %	
	WAAI disc	wheat flour disc
A	4.93	34.2
B	1.14	4.2
C	1.1	2.5
D	1.22	6.1
E	1.46	14.1
F	2.2	25.7
G	19.36	20.4
H	1.49	10.8
I	0.72	3.3
J	0.71	2.1
K	6.74	20.5
L	1.11	7.4
M	3.54	8.5
N	8.24	29.8
O	9.9	2.7
P	1.06	2.5
Q	29.73	7
R	1.95	3.1
S	2.44	5
T	0.82	2.7
U	6.96	21.7
V	1.72	12.8
W	1.47	4.45
X	1.16	2.56

The mean percentage binding for the unexposed individuals was 0.61 (n = 9) 99% CI 0.41–0.81. Total IgE for the unexposed individuals was in the range 2–1,832 IU/ml.

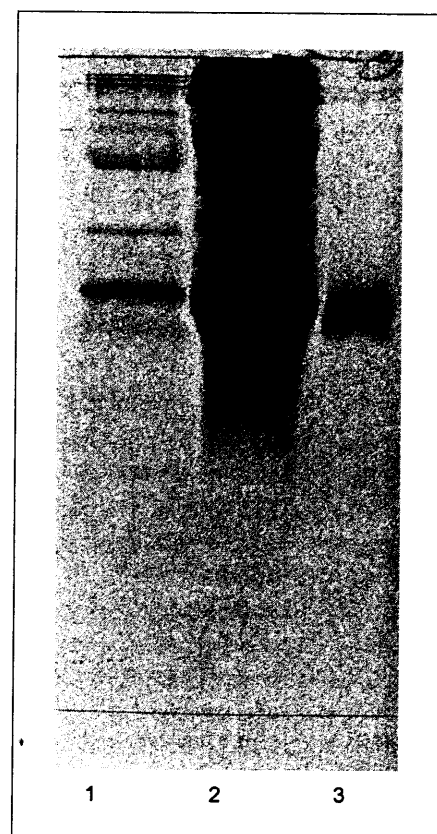


Fig. 2. A non-reducing 17% SDS-PAGE of WAAI stained with Coomassie blue. Lane 1 represents molecular weight standards, prominent bottom marker 14.3 kDa, lane 2 wheat flour extract, lane 3 WAAI.

13 kDa (fig. 2). The protein was coupled to paper discs and used for RAST testing. IgE antibodies from 92% (22/24, 99% confidence interval) of allergic bakers tested gave significantly more binding to the discs than sera from 9 unexposed individuals with total IgE in the range 2–1,832 IU/ml (table 1). The protein had α -amylase inhibitory activity using the *T. molitor* assay and which could be destroyed by heating. N-terminal sequencing gave the sequence PWSWCDPA TGYKVSALTG and this had 100% homology with the sequence given for a WAAI [28] and 95% homology with monomeric WAAI 0.28 (SGPWSWCNPA TGYKVSALTG) [29].

The capture assay for WAAI had a detection range of between 0 and 50 ng/ml and could detect 0.1 ng/ml with 95% confidence. The within-assay coefficient of variation (CV)

7.8% at 9.53 ng/ml (n = 16) and the between-assay CV was 13.4% at 8.6 ng/ml (n = 10). Soya, oat and barley flour, fungal α -amylase, *A. siro* and *L. destructor* extracts were not detected up to 1 mg/ml protein, low cross-reactivity was seen with rye flour (1,724 ng/ml rye flour equivalent to 1 ng/ml of WAAI). Dilution curves for a range of wheat flour extracts and eluates from personal samplers were run and seen to parallel those of the WAAI standard (fig. 3). Sequential 2-hour extractions of wheat flour with 0.15M NaCl with and without 1% Tween 20 showed that 90% of total salt-soluble WAAI was present in the first extraction. The addition of Tween 20 to the salt solution did not change the amount extracted.

Figure 4 shows the amount of WAAI, as a percentage of total weight, in different commercial bread baking and cake making flours (mean 0.124%, 95% confidence limits 0.083–0.164%, CV 54%, n = 13) and in personal airborne dust samples (mean 0.018%, 95% confidence limits 0.007–0.030%, CV 101%, n = 12). Personal airborne dust exposures (n = 12,

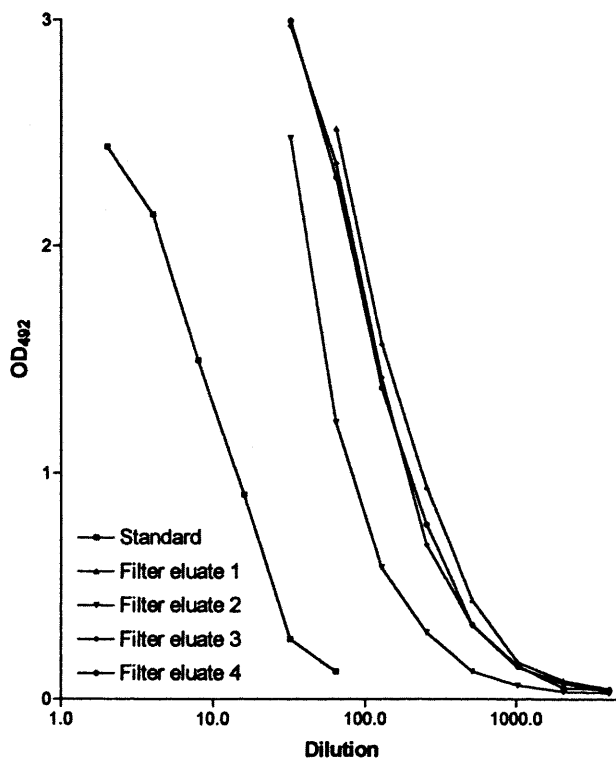


Fig. 3. The WAAI standard curve and dilution curves for personal sample filter eluates.

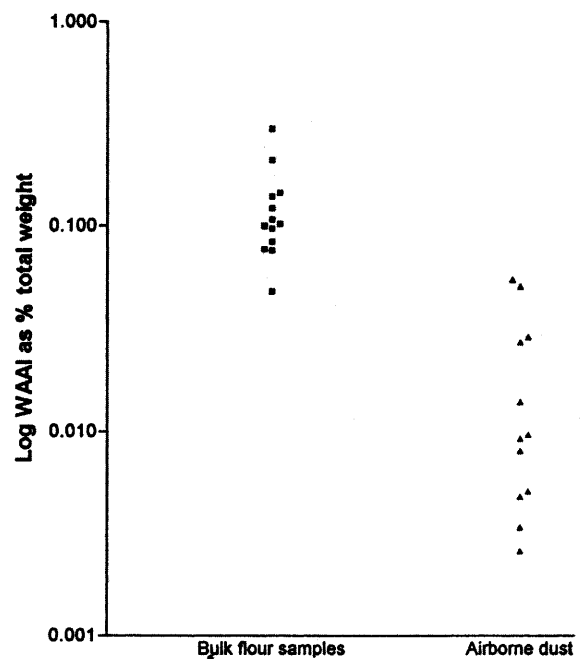


Fig. 4. The amount of WAAI in commercial flours and personal airborne bakery dust samples as a percentage of total weight.

all values geometric mean and confidence limits) were 6.54 mg/m^3 for total inhalable dust (95% confidence limits $4.09\text{--}10.45 \text{ mg/m}^3$), and for WAAI 744 ng/m^3 (95% confidence limits $371\text{--}1,496 \text{ ng/m}^3$). Correlation between log-transformed airborne levels of WAAI and total dust (mg/m^3) for personal samples collected in bakeries was low with a coefficient of determination (R-squared) of 0.269 with a p value (two-tailed) of 0.084. The flours analysed included those milled from solely UK, German, French and Canadian wheat and those milled from a mixture of UK and European wheat as is the normal practice for commercial flour manufacture in the UK.

Discussion

The development of an immunoassay to measure an airborne flour allergen followed an established protocol. Flour proteins were first investigated using SDS-PAGE to deter-

mine the protein profiles, then immunoblotting identified those proteins which were recognised by IgE antibodies from allergic bakers and by murine MAb. Comparison of the immunoblots indicated that MAb HSL-11 bound to a single protein which was also recognised by IgE antibodies from bakers with allergic respiratory symptoms to flour. Purification using affinity chromatography yielded a wheat protein with a molecular weight of some 13 kDa which had α -amylase inhibitory activity. N-terminal sequencing identified it as a WAAI which had 19 of its first 20 amino acids in common with monomeric α -amylase inhibitor I (synonym 0.28). The purified protein was recognised by IgE antibodies from some 92% of allergic bakers tested. This binding was greater than 2% for 42% of sera tested and this proportion rose to 67% if the results for sera with a >10% to wheat flour discs were analysed. This suggests, as perhaps would have been predicted from the immunoblotting data (reviewed in the introduction), that whilst WAAI is recognised by a majority of allergic bakers it is not the only important

wheat flour allergen. The identification of an MAb to pair with HSL-11 enabled a sensitive ELISA to be developed which could measure short-term allergen exposures. This immunoassay had little cross-reactivity with other flours, ingredients or potential contaminants found in bakeries although it measured a member of a family of proteins present in all cereal flours.

The immunoassay was simple to perform, did not use radioactivity and could analyse single or large batches of samples in some 2–4 h whilst having sufficient sensitivity to measure flour allergen in short-term air samples. Analysis of a number of representative commercial white and whole-meal wheat flours showed that WAAI was a small proportion of the total dry weight of flour with a flour to flour CV of 54%. The proportion of WAAI in personal samples of bakery dusts was, as expected, lower and more variable with a CV of 101%. The correlation with total airborne dust was poor, a finding reported by others who have compared total dust and antigen levels. Indeed, flour antigen exposure measurements have been found to show distinct differences between occupational titles in bakeries which are not evident from total dust measurements [11, 30].

The production and binding characteristics of MAbs reacting with flour proteins have been previously described [31, 32]. These MAbs were reported to be mostly of IgM class and bound to multiple proteins (a property shown by some of our MAbs) and therefore were probably unsuitable for incorporation into immunoassays. Thus this report is the first describing measurement of wheat flour exposure in bakeries in terms of a single marker allergen. This approach

of measuring exposure in terms of a single allergen for an allergen source such as flour which contains several allergenic proteins needs careful consideration. The ratio of different allergens and their variability in the source materials is important. In this case, the variability of WAAI in bulk flour samples was lower than that measured in airborne bakery dusts and comparable for that described for *Der p 1* [13]. In addition, the allergen was positive in RAST testing in 92% of the UK bakers we investigated although it is recognised that it is not the only important wheat flour allergen. The assay should therefore be considered for widespread use as it offers distinct advantages to the previously described methods. Its reagents and standards are potentially infinitely available to the same specification and comparable results should be obtained by different investigators and occupational hygienists, although the choice of this single allergen as a measure of flour allergen exposure may need to be re-assessed when the assay has had more widespread usage. Whilst we have been able to define the variability of the immunoassay the overall uncertainty of the complete sampling procedure, along the lines of that described in *prEN 689* [33], is as yet unknown. Indeed this appears to be the case for all the other immunoassays for airborne biological allergens.

More widespread use of immunoassays for occupational exposure assessments of biological allergen is required to move this robust technology from specialist to routine use including exposure assessment. The benefits should be improved risk assessment and in the long term a significant contribution to reducing respiratory ill health in industry.

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