

Measurement of airborne proteins involved in Bakers' asthma

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Summary

We have developed a competitive inhibition immunoassay to measure airborne flour, using purified polyclonal rabbit IgG antibodies to cereal flour antigens. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed different flours contained proteins of molecular weight similar to those present in air sample eluates. Western blotting demonstrated that the purified rabbit IgG antibodies bound to proteins whose molecular weights are similar to human allergens. Air sample eluates obtained from a large bakery had slopes of inhibition parallel to cereal flour when compared in the immuno-inhibition assay. The assay identified airborne proteins from several flours with a detection limit of $1 \mu\text{g ml}^{-1}$. Using this immunoassay we have shown that a wide range of airborne flour concentrations occur in flour mills and a smaller range bakeries. This assay which allows measurement of the concentration of flour aeroallergen in the workplace will be valuable both in epidemiological studies and in examining the effectiveness of interventions to reduce the concentration of flour in the air.

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Introduction

Baker's asthma, which was described in Roman times [1] is associated with IgE and IgG antibodies to inhaled flour [2,3]. It remains an important health problem for flour mill and bakery workers. Respiratory symptoms caused by flour dust was reported in 6% of 234 workers in a bakery in Finland [4], in 6% of 176 workers in a bakery in Australia [5] and in 13% of workers in a bakery in the UK. [6]. Understanding the relationship between the development of immunological and airway responses and exposure to inhaled flour depends on the ability to measure flour aeroallergen.

Radio allergosorbent test (RAST) inhibition utilizing human serum pools has previously been used to measure airborne flour antigens and allergens [7]. An important disadvantage of the use of human sera is that IgE in the

serum pools will reflect the allergens to which individuals have responded, e.g. sera from individuals with IgE to fungal α -amylase and ryegrass pollen, will also measure these non-flour components. While human serum pools reflect the exposures of the subjects from which they are derived, monoclonal antibodies are produced to defined proteins [8].

An assay to measure airborne antigens and allergens would entail the use of an immense number of monoclonal antibodies in order to detect the large number of proteins in flour that are potentially allergenic [9]. The use of monoclonal antibodies against a 'marker' protein that is conserved in many different flour producing plant species is problematic, due to the range of crossreacting proteins found in different plants.

In order to obtain a defined assay that is capable of measuring airborne flour proteins we have developed a competitive inhibition immunoassay using purified polyclonal rabbit IgG. We have examined the pattern of inhibition caused by other allergens also present in bakery air and have used Western blotting to compare the binding of purified rabbit IgG and human IgE to flours.

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Materials and methods

Extraction of flours

Canadian Western Red Spring (CWRS) wholemeal, CWRS self-raising, English wholemeal, English self-raising and biscuit flours were obtained additive-free from the Flour Milling and Baking Research Association (Chorleywood, Herts, UK). Standard commercial wholemeal and white flours representative of those used in the UK were obtained from a bakery (Telford's Bakery, Maidstone, UK). The flours were derived from the bread wheat *Triticum aestivum*, which contains similar proteins to *Triticum durum* [10]. Rye flour (Neal's Yard Flours, London, UK), soya flour (Rank Hovis McDougall UK), ryegrass pollen (Allergon, AB, Angelholm, Sweden) and fungal α -amylase (Novo Nordisk, Copenhagen, Denmark) were also extracted. The wheat and rye flours were extracted in 0.1 M Ammonium Hydrogen Carbonate (AHC) (MERCK UK) pH 7.65. The soya flour, ryegrass pollen and fungal α -amylase were extracted in phosphate-buffered saline (PBS). Each sample was shaken for 16 h at 20°C and was then centrifuged at 4000 g for 30 min. The supernatant was dialysed against four changes of distilled water for 16 h at +4°C before being freeze-dried and stored at -20°C.

Rabbit immunization

Female Half-Lop rabbits were initially injected with 1 ml of a 50:50 mix of Freund's Complete Adjuvant (Sigma) and a 20 mg ml⁻¹ solution of the standard commercial wholemeal and white flour in PBS. The rabbits were boosted 14 days later with 1 ml of a 50:50 mix of adjuvant and flour, with Freund's complete adjuvant replaced by the incomplete adjuvant. The rabbits were boosted every 28 days until an antibody titre of 1:1024 against the commercial flours and the additive-free CWRS wholemeal bread flour was achieved. The pooled anti-sera were purified using the caprylic acid and ammonium sulphate precipitation method of McKinney [11], followed by dialysis against PBS diluted 1:10. The resultant dialysis fraction was freeze dried and stored as a powder at -20°C.

Environmental and personal sampling

An Air Sentinel air sampler (Quan-Tec Air, Rochester, USA) was run for 8 h at 140 l/min to sample the air of bakeries. After removal of the polytetrafluoroethylene (PTFE) sample [12] membrane from the polyester backing, the soluble material was eluted by shaking the membrane for 16 h in 20 ml of 0.1 M AHC. The filter eluate was centrifuged for 10 min at 1500 g and the supernatant

dialysed against four changes of distilled water for 16 h at +4°C before being freeze dried. The resulting eluate was compared with CWRS by electrophoresis, Western blotting and in the inhibition immunoassay.

A total of 510 8-h air samples were obtained from a variety of bakeries and mills in the UK using Cassella AFC123 personal sampling pumps running at 2 l/min. The pumps were worn by workers on these sites and the sampling head was located in the respiratory zone; in order to sample material that the workers were likely to inhale. The material was eluted from the PTFE filters using the method of Gordon *et al.* [19] and measured in the assay.

Electrophoresis

The different extracts and an air sample eluate were subjected to non-reduced SDS-PAGE using the Tris-Glycine buffer system of Laemmli [13]. High molecular weight markers (Amersham Ltd, Amersham, UK) were used on all the SDS-PAGE gels. The gels were stained in 0.1% Coomassie blue (Pharmacia, Milton Keynes, UK) overnight and destained until the background was clear.

Western blotting

SDS-PAGE gels (15%) were subjected to electrophoretic transfer onto 0.45 μ M nitrocellulose (Bio-Rad, Hemel Hempstead, UK) for 16 h using the method of Towbin *et al.* [14]. The remaining protein binding sites on the membrane were blocked by incubating in PBS 0.24% detergent containing RAST (Pharmacia, UK) buffer substance for 2 h. The nitrocellulose membrane was probed with the purified rabbit antibody at a concentration of 250 ng ml⁻¹ for 16 h, normal rabbit sera were used as the negative control. Following three washes with PBS-RAST wash the membrane was incubated with a 10 ng ml⁻¹ solution of ¹²⁵I-Protein-A in PBS [15] (Amersham, UK) for 16 h. Following three further washes the membrane was dried and exposed to Hyperfilm MP (Amersham, UK) at -70°C for 24 h.

Rabbit competitive flour inhibition assay

The assay was optimized to produce the best linear range and degree of sensitivity. Immunolon 2 removawell strips (Dynatech, Billingham, UK) were coated with 100 μ l of a 10 mg ml⁻¹ solution in PBS-Glycerol of CWRS wholemeal flour extract diluted 1:100 in 50 mM sodium carbonate-sodium hydrogen carbonate coating buffer pH 8 [16]. The strips were incubated for 16 h at +4°C, then washed four times, using RAST washing buffer (Pharmacia, UK). To produce the standard inhibition

curve 100 μl of CWRS extract in PBS was applied to the plate wells in triplicate using concentrations of 1000, 250, 62.5 $\mu\text{g ml}^{-1}$ and eight doubling dilutions from 31.25 to 0.12205 $\mu\text{g ml}^{-1}$ after being freshly diluted from a 10 mg ml^{-1} PBS-Glycerol stock solution. Triplicate 100 μl volumes of CWRS extract at concentrations of 200, 100, 50, and 1 $\mu\text{g ml}^{-1}$ were applied to the plate as controls in all assays. 100 μl volumes of the rabbit antibody at 250 ng ml^{-1} in PBS containing 10 mg ml^{-1} BSA were added simultaneously. The plates were incubated overnight at +27°C. Following four washes, 100 μl of ^{125}I -Protein-A (Amersham UK) at 10 ng ml^{-1} were applied to each well and the plates incubated overnight at +27°C. The plates were given four final washes, the strips broken into individual wells and counted in a Cobra 5005 gamma counter (Packard). The standard curves were generated using Four Parameter Logistic [17] fitting software.

Comparison of different flours

Eluates of static air samples from bakeries were used to inhibit the binding of CWRS, in order to confirm that the assay would be capable of measuring samples obtained from bakeries and mills. The concentration of each inhibitory test agent was the same as that used in the standard curve.

The percentage inhibition of rabbit IgG binding was

calculated and logit transformed $\log_e(p/[1-p])$; the resulting curves were examined for parallelism as previously described [18].

Assay reproducibility

The slopes and intercepts of the standard curves produced by the four parameter logistic fit were compared with each other after logit transformation using analysis of covariance. Inter-assay variability was assessed using the four controls of 200, 100, 50 and 1 $\mu\text{g ml}^{-1}$.

Measurement of flour aeroallergen

The airborne flour (FA) concentration was calculated as follows:

$$\text{FA } (\mu\text{g m}^{-3}) = \frac{[\text{Flour}] (\mu\text{g ml}^{-1}) * \text{reconstituted volume (ml)}}{\text{Volume of air sampled (m}^3\text{)}}$$

where the flour concentration [Flour] is interpolated from the standard curve. The assay detection limit is between 200 and 1 $\mu\text{g ml}^{-1}$.

Results

SDS-PAGE

Figure 1 shows that the five different wheat flour extracts (tracks 1–5) have very similar protein profiles. Strong

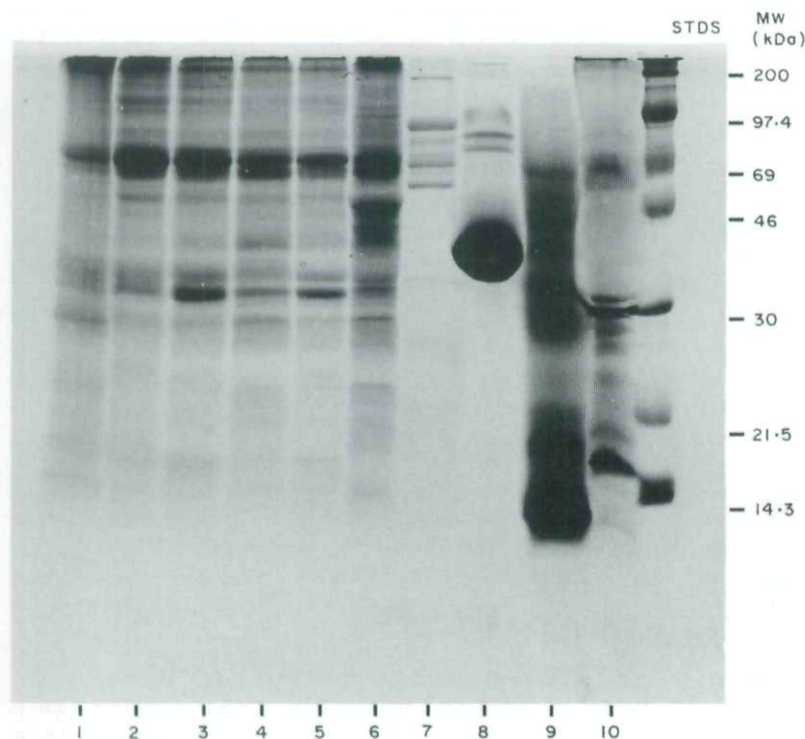


Figure 1. SDS-PAGE gel (15%) 1. CWRS wholemeal. 2. CWRS self-raising (s-r). 3. English wholemeal. 4. English s-r. 5. Biscuit flour. 6. Rye flour. 7. Soya flour. 8. Fungal α -amylase. 9. Ryegrass pollen. 10. Static air sample eluate.

Figure 2. Autoradiograph of immunoblot probed with rabbit IgG against the samples in Figure 1.

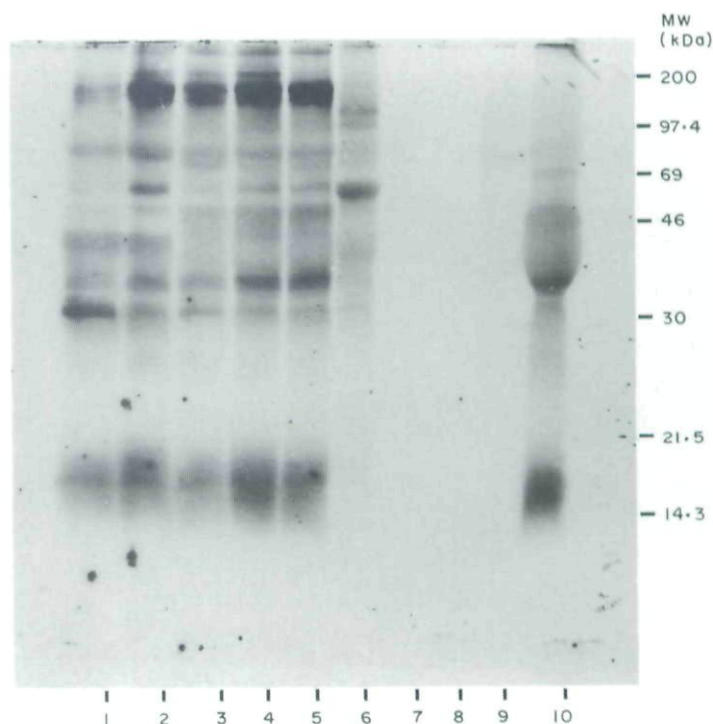


Table 1. Molecular weights (kDa) of proteins detected by Western blotting using rabbit IgG

Wheat flour				Rye flour			Ryegrass pollen		Air sample eluate		
170w		150w	100w	150	130	74	69	46	69	56	46
74	69	56	46	56	38	14	30w	14w	30	14	
38	30	14									

w. Weak binding.

staining (dense distinct bands) was seen with proteins of molecular weight 66, 56, 46, 36, 30, 18 and 14 kDa, weak staining (faint thin bands) was seen with proteins of 98 and 21 kDa. Rye flour (track 6) had a similar protein profile to the wheat flours. Soya flour (track 7) and fungal α -amylase (track 8) were observed to have few bands in common with wheat or rye flour. Ryegrass pollen (track 9) had some proteins bands with similar molecular weights to those seen in wheat flour. The bakery air sample eluate (track 10) had detectable proteins of 66, 56, 46, and 14 kDa.

Western blotting

Figure 2 shows the binding pattern of rabbit IgG to different flours. Table 1 gives the molecular weights of

proteins detected by the rabbit IgG antibodies. Heavy staining protein bands of 74, 56, 38 and 14 kDa were detected in wheat flour, rye flour and the static air sample eluate.

Competitive flour inhibitions

Figure 3a shows that the curves produced by inhibition of CWRS using static air sample eluates were similar to that of CWRS and were found to be parallel after logit transformation (Fig. 3b).

Assay reproducibility

Analysis of co-variance showed that there was no evidence to suggest a significant difference between the

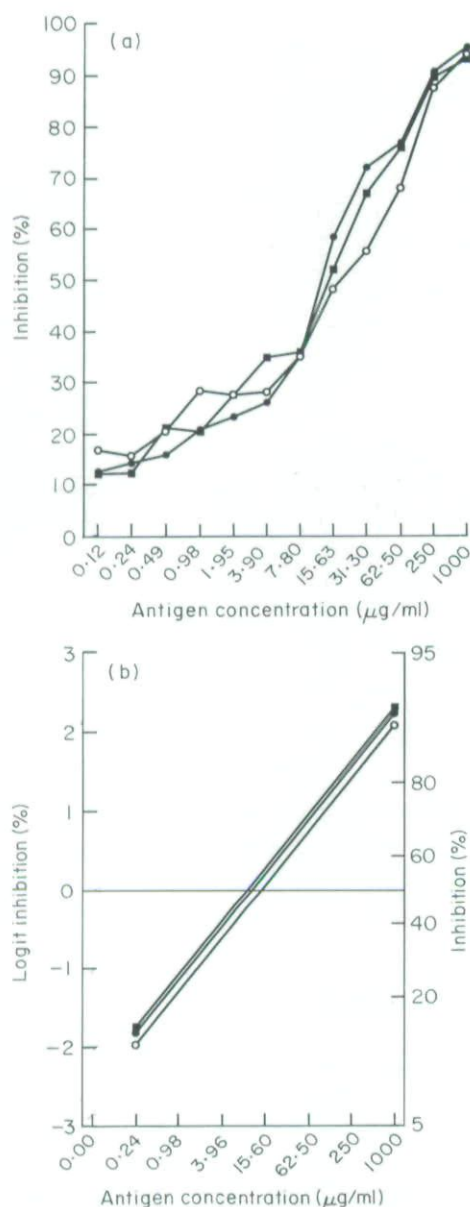


Figure 3a. Inhibition of CWRS standard curve using rabbit IgG with increasing concentrations of (○) CWRS and air sample eluates 1 (■) and 2 (●).

Figure 3b. Logit transformation of CWRS standard curve inhibition by CWRS (○) and air sample eluates 1 (■) and 2 (●) showing common slopes.

slopes and intercepts of the logit transformed curves from 10 different immunoassays ($P > 0.05$). The analysis of covariance would have been strengthened by the use of a greater number of points per curve, however, a maximum number of 12 points per immunoassay curve was imposed by the gamma counter. The results of the analysis of the four control values is shown in Table 2. The analysis of inter-assay co-variance showed that the linear section of

Table 2. Inter-assay coefficient of variation (CV) observed on four standard control values

Target control values $\mu\text{g/ml}^{-1}$	Mean control values $\mu\text{g/ml}^{-1}$	CV (%)	<i>n</i>
200	175.34	36.90	10
100	106.518	11.487	10
50	45.708	25.226	10
1	1.516	49.11	10

the curve produced the most accurate and repeatable results with the sigmoidal sections of the curve showing the greatest variability. The highest level of variance occurred at the lowest concentrations. Measurements outside the linear section of the standard curve were rejected and the sample re-tested after either concentration or dilution.

Airborne flour measurement

Sixty per cent of the samples analysed using the assay were found to have a flour concentration of between $950.06 \mu\text{g m}^{-3}$ and $45.52 \mu\text{g m}^{-3}$. Forty per cent of the samples required dilution and retesting due to the high concentration of flour captured onto the sampling membrane. Figure 4a shows that some of the highest exposures to airborne flour proteins in flour mills occurred in jobs involved in the milling and packing of flour and mill hygiene with geometric mean levels of 468.2, 248.3 and $979.6 \mu\text{g m}^{-3}$ of airborne flour protein respectively. Figure 4b shows that some of the highest exposures to airborne flour proteins in the bakeries occurred in jobs involved in the production of bread rolls and confectionary with geometric mean levels of 228.7 and $252.0 \mu\text{g m}^{-3}$ respectively.

Discussion

SDS-PAGE showed that the different wheat flour and rye flour extracts have very similar protein profiles. When the specificity of the antibodies was examined by Western blotting, the rabbit IgG antibodies bound in a similar manner to wheat and rye flour proteins indicating that the purified rabbit IgG bound to proteins found in a variety of different flours. The 74, 69, 46 and 14 kDa proteins we have detected using rabbit IgG are probably the same as the 74, 69, 47 and 14 kDa wheat allergens reported by Pfeil *et al.* [20]. Fränken *et al.* [21] reported two proteins of 35 and 14 kDa which may be similar to the 38 and 14 kDa salt soluble wheat proteins we have identified.

We have previously identified allergens of molecular

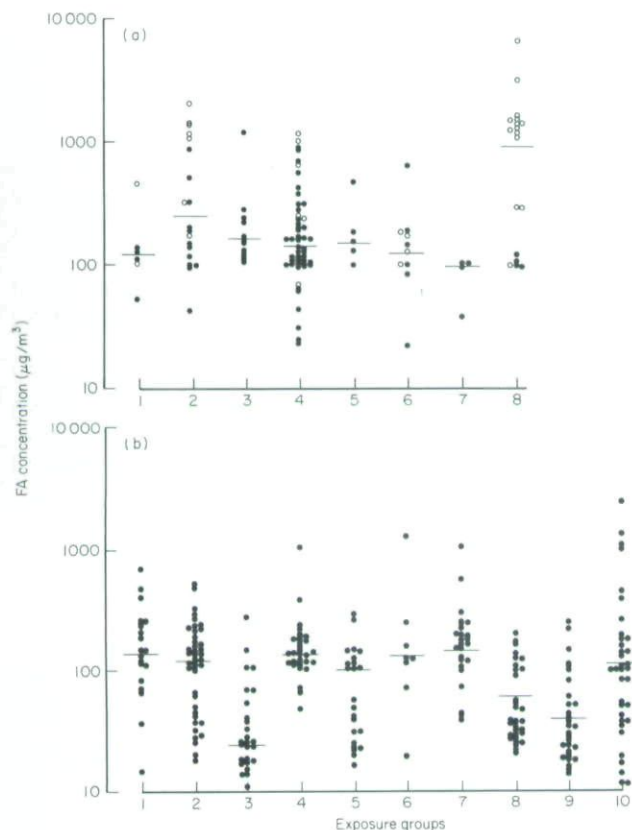


Figure 4a. Airborne flour concentrations in mills by exposure group. 1. Wheat mill. 2. Flour mill. 3. Flour mixing. 4. Packing. 5. Warehouse. 6. Quality control laboratory. 7. Transport. 8. Hygiene. (O. Samples taken at an old flour mill.)

Figure 4b. Airborne flour concentrations in bakeries by exposure group. 1. Dispense. 2. Bread production. 3. Bread wrapping. 4. Roll production. 5. Roll wrapping. 6. Dough brake. 7. Confectionary with flour. 8. Confectionary. 9. Despatch. 10. Hygiene.

weight 74, 56, 46, 30 and 14 kDa [22] by Western blotting. Proteins of similar molecular weight were recognized by the polyclonal rabbit antibodies, suggesting that the rabbit antibodies bound to the proteins which stimulate the human allergic response.

When the air sample eluates were compared with CWRS they showed similar inhibition curves and parallel slopes after logit transformation, implying the air sample eluates were immunologically identical to flour and that the immunoassay would be capable of measuring flour aeroallergen from different sources.

Comparison of airborne flour exposure in different jobs in mills showed that the heaviest exposures occurred in those workers milling and blending flour and in hygiene staff. The highest exposures in the bakeries occurred in the initial stages of bread and confectionary production.

The variation of airborne flour protein concentrations was less in bakeries than in mills.

We have described the production and purification of polyclonal rabbit IgG antibodies which bind to flour proteins, with molecular weights similar to allergens. We have used the rabbit IgG antibodies to produce a reproducible and accurate competitive inhibition immunoassay to measure the concentration of flour proteins in the air of mills and bakeries.

Exposure assessments in bakeries and flour mills are usually based on measurement of total airborne dust which can also include other materials such as sugar in confectionary bakeries. The assay reported here identifies the airborne proteins capable of inducing IgE antibodies relevant to the development of respiratory disease.

Measurement of airborne flour protein concentrations will provide information essential for the assessment of exposure-response relationships and the evaluation of the effectiveness of interventions aimed at reducing exposure to airborne flour.

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