

A comparison of the binding of IgE in the sera of patients with bakers' asthma to soluble and insoluble wheat-grain proteins

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The IgE-binding proteins from flour, associated with bakers' asthma, have been reassessed by use of a modified RAST suitable for both soluble and insoluble proteins. Nitrocellulose sheet was used for preparing RAST discs, and seven different solvents were compared for their suitability in preparing discs. Dilute alkali (1% potassium hydroxide) was chosen as the best solvent for disc preparation, and its use was compared with that of water as solvent. RAST analyses of sera from 24 allergic bakers demonstrated that the albumin fraction of flour is clearly allergenic (as found in previous studies), but in addition, major IgE-binding proteins were found in the other three fractions (globulin, gliadin, and glutenin) when potassium hydroxide was the solvent (but not with water). We conclude that current RAST procedures, which favor water-soluble allergens, are inadequate because they do not satisfactorily test for water-insoluble allergens. (J ALLERGY CLIN IMMUNOL 76:23-8, 1985.)

Sensitivity to inhaled flour in bakers provides both an excellent model of an occupationally induced disease and an example of allergic asthma in which the allergen source is clearly defined, readily available, and abundant.¹⁻⁴ Despite this model, progress in identifying the important flour allergens has been slow, and the full range of fractions (both water-soluble and insoluble) has never been adequately investigated for their allergenic properties.⁴

Starting with a water-soluble extract of wheat flour, Blands et al.¹ identified 40 different antigens by crossed immunoelectrophoresis. Six of these antigens were demonstrated to be present in a water-soluble extract prepared from a commercial preparation of gliadin, and 18 of the antigens bound IgE in sera from allergic bakers. No attempts were made to isolate and characterize the major allergens. Our studies with wheat-flour fractions and purified proteins also revealed that the albumins are important (and heterogeneous) allergens in bakers' asthma.^{3, 5, 6} However,

Abbreviations used

DTT: Dithiothreitol
SDS: Sodium dodecylsulfate
BSA: Bovine serum albumin
KOH: Potassium hydroxide
PBS: Phosphate-buffered saline

the methodology used in all these studies (in particular the RAST, relying as it does on covalent coupling of water-soluble proteins to a solid support) has been unsuitable for detecting IgE binding to insoluble gluten proteins.³ Proof that gluten antigens do bind IgE antibodies in the sera of allergic subjects has subsequently been obtained after direct binding of these proteins to nitrocellulose by use of stronger solvents capable of dissolving gluten proteins⁷ and also after electrophoretic transfer.⁸

We now describe comparative studies in which the full range of wheat-flour proteins (water-soluble albumins, globulins, gliadins, and glutenins) dissolved in aqueous or organic solvents were adsorbed to nitrocellulose discs and used in IgE-binding studies with sera from allergic bakers. The results further demonstrate the diversity of wheat-flour allergens and clearly demonstrate that important allergens occur in the insoluble gluten as well as in the water-soluble protein fraction.

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TABLE I. Uncorrected values for percent radioactive uptakes by RAST assay

Solvent	Noprotein disc	Albumin	Globulin	Gliadin	Glutenin
Allergic baker (Pal)					
Water	1.5 (1.4)	17.6 (1.6)	7.2 (1.3)	9.2 (1.7)	2.5 (1.4)
4 mol/L Urea 10 mmol/L DTT	2.0 (1.5)	21.9 (3.8)	9.6 (2.2)	3.4 (3.0)	5.9 (2.8)
0.1 mol/L acetic acid	1.4 (1.4)	11.6 (1.5)	13.7 (1.6)	6.6 (1.8)	4.7 (1.7)
1% SDS plus 10 mmol/L DTT	1.5 (1.5)	4.6 (1.8)	9.2 (2.0)	2.3 (1.6)	3.5 (2.0)
70% Ethanol	1.6 (1.6)	10.3 (2.2)	2.2 (1.4)	8.2 (2.1)	8.0 (2.8)
55% isopropanol plus 10 mmol/L DTT	1.5 (1.6)	5.0 (2.3)	2.5 (1.7)	5.4 (2.5)	5.6 (3.5)
1% KOH	2.3 (1.0)	24.5 (3.3)	16.0 (2.6)	5.2 (2.7)	6.2 (3.0)
Nonallergic baker (Pil)					
Water	1.5 (0.6)	2.2 (0.2)	0.4 (0.1)	0.6 (0.3)	0.6 (0.2)
1% KOH	1.5 (1.4)	1.8 (1.7)	2.8 (1.8)	1.3 (1.4)	1.9 (1.7)
Nonallergic baker (Fra)					
Water	1.3 (0.9)	0.1 (0.5)	0.2 (0.3)	0.2 (0.6)	0.3 (0.2)
1% KOH	1.7 (1.6)	1.6 (1.5)	1.5 (1.7)	1.1 (1.1)	1.9 (1.7)

Uncorrected values for percent radioactive uptakes by RAST assay of serum from one allergic baker (Pal, diluted fourfold) and two nonallergic bakers (Pil, diluted twofold, and Fra, undiluted) illustrated in parentheses by use of discs containing no flour protein or discs coupled to a flour fraction.

MATERIAL AND METHODS

Sera

Serum samples were obtained from 24 bakers who experienced asthma and/or rhinitis after inhalation of wheat flour. Nineteen sera were part of a survey of bakers from Western Australia. Sera from three bakers from South Australia were provided by Drs. McMurtrie and Ward, two further serum samples were provided by the Allergy Clinic, Royal Prince Alfred Hospital, Sydney, and cord serum from Royal North Shore Hospital, Sydney.

Reagents

Sources of materials used were KOH and DTT from Merck, Darmstadt, West Germany; ethanol, isopropyl alcohol, acetic acid, and urea from Ajax Chemicals, Sydney, Australia; SDS from BDH, Poole, England; BSA and polyoxyethylene sorbitan monolaurate (Tween 20) from Sigma Chemical Co., St. Louis, Mo.; nitrocellulose membrane (0.45 μ m pore size) from Bio-Rad, Richmond, Calif.; and ^{125} I-labeled anti-IgE from Pharmacia AB, Uppsala, Sweden.

Flour fractions

Flour fractions were prepared as described by Baldo and Wrigley³ according to the classical Osborne procedure of fractional extraction.

Preparation of nitrocellulose discs

The various wheat fractions were dissolved in the appropriate solvents (Table I) at a concentration of 1.75 mg/ml. Nitrocellulose discs (6 mm in diameter) were prepared from sheet nitrocellulose by use of an office punch, and the wheat antigens were applied to the discs by the following

modification of the method of Walsh et al.⁷ Thirty-five discs were soaked in 2 ml of antigen solution for 1 hr with the exception of the fractions dissolved in 1% KOH. When this solvent was used, solutions (20 μ l) were spotted onto the discs individually, since discs lost mechanical strength after prolonged soaking in 1% KOH. All discs were then allowed to dry for at least 1 hr. The discs were blocked overnight in 3% BSA in PBS (50 mmol/L of sodium phosphate, pH 7.2, and 0.9% NaCl) and were washed three times (10 min each) in 2.5 ml of TAPS buffer (0.5% Tween 20, 0.05% BSA in PBS). Blank discs were prepared as described above except that the wheat protein was omitted as a check on nonspecific adsorption of IgE.

RAST method

Each disc was incubated with 25 μ l of either cord or test serum (diluted with PBS if necessary) in a 6 mm diameter tube for 3 hr at room temperature. The discs were washed three times in TAPS (2.5 ml and 10 min per wash). Then 50 μ l of ^{125} I-anti-IgE was placed in each tube and allowed to react overnight. Unbound anti-IgE was removed with three more washes with TAPS. The percentage of ^{125} I-anti-IgE that remained bound to the disc was estimated. Specific IgE binding was calculated by deducting the radioactive uptake (percent) for cord serum (minus the value for its blank disc) from the value for the test serum (minus its blank).

RESULTS

Seven solvents (listed in Table I) were selected for their suitability to dissolve the various protein fractions of wheat flour. All solvents were tested with all

TABLE II. Mean values of specific IgE binding*

Solvent	Albumin	Globulin	Gliadin	Glutenin
Water	7.4 (1.4)	0.8 (0.3)	1.2 (0.4)	0.5 (0.2)
4 mol/L Urea plus 10 mmol/L DTT	9.5 (1.9)	1.7 (0.4)	1.6 (0.5)	2.8 (0.7)
0.1 mol/L Acetic acid	—	1.8 (0.5)	1.9 (0.5)	—
1% SDS plus 10 mmol/L DTT	—	2.0 (0.5)	—	—
70% Ethanol	—	—	4.0 (1.0)	2.8 (0.7)
55% Isopropanol plus 10 mmol/L DTT	—	—	—	3.3 (0.9)
1% KOH	9.4 (1.8)	4.9 (0.9)	2.9 (0.8)	4.8 (1.0)

*Calculated as percent radioactive uptake value for the test serum (minus its blank) minus uptake for cord (minus its blank). Values obtained for wheat-flour protein by use of a range of solvents in RAST disc preparation based on analyses of 24 sera from allergic bakers. Standard errors appear in parentheses.

four protein fractions to observe which combinations provided the best radioactive uptakes by RAST assay by use of nitrocellulose discs as the solid phase. Table I demonstrates the results as percentage uptakes of radioactivity (without any correction for controls) for cord serum and for serum from a baker with asthma. Uptakes were low throughout for cord serum and for discs containing no flour protein. Similarly, low values were obtained when sera was tested from normal subjects or from bakers not sensitive to flour. Table I demonstrates some of the actual results obtained for two nonallergic bakers. In addition, a survey of 156 nonallergic bakers revealed a mean uptake (uncorrected) of 1.18% (SD of $\pm 0.35\%$) for a disc prepared with use of 1% KOH and a recombination of all four flour fractions in their original proportions.

These results, together with similar sets for three other bakers with asthma, were used to select suitable combinations of solvents and fractions (Table II) for evaluation of all 24 bakers' sera. Irrespective of their suitability, three solvents (water, urea-DTT, and KOH) were used for all fractions. Three types of blank analyses were included throughout to assess the significance of the percent uptakes with the test sera. These were the uptakes obtained by use of a nonallergen blank disc with the test serum and cord serum applied to an allergen disc and to a nonallergen disc. In evaluating the full set of results, there was a significant correlation ($p < 0.001$) (0.41 on 429 degrees of freedom) between the serum values (minus their blank) and the corresponding cord values (minus their blank). For this reason it was inadvisable to ignore the cord values in our calculations, and it was decided to use the value of serum (minus its blank) minus cord (minus its blank) as the variable to be considered. Tables I and II demonstrate that there were considerable differences between results obtained for any

particular protein fraction depending on the solvent used for disc preparation.

Allergenicity with water as solvent

With this estimate as the basis for allergenicity, the four flour fractions were compared in a survey of all sera by use of water only as the solvent for preparing nitrocellulose discs. The resulting RAST uptakes are plotted in decreasing order in Fig. 1 (white columns) with respect to the albumin protein fraction. The results clearly demonstrate that the albumins are much more allergenic than any of the other fractions under these conditions. These results confirm previous studies that have also pointed to the water-soluble proteins as being the main allergens responsible for bakers' asthma. Fig. 1 demonstrates that the least allergenic protein fraction was glutenin when water was used in disc preparation.

Other solvents

Table II lists the mean values of allergenicity for all test sera for all flour fractions dissolved in up to seven different solvents for disc preparation. The solvents are arranged roughly in increasing order of their ability to dissolve proteins. There were considerable differences in allergenicity, depending on the solvent used. In general, the highest values were demonstrated with KOH as solvent. In this case the albumins were the most allergenic, but the glutenins and globulins were demonstrated also to be major allergens.

On the other hand, high allergenicity was demonstrated for gliadins with ethanol as solvent, this being the most appropriate solvent to dissolve gliadins. The results of Table II demonstrate that there is a progressive increase in allergenicity with the power of the solvent to dissolve the appropriate protein fraction. KOH was chosen as being the best solvent to use in

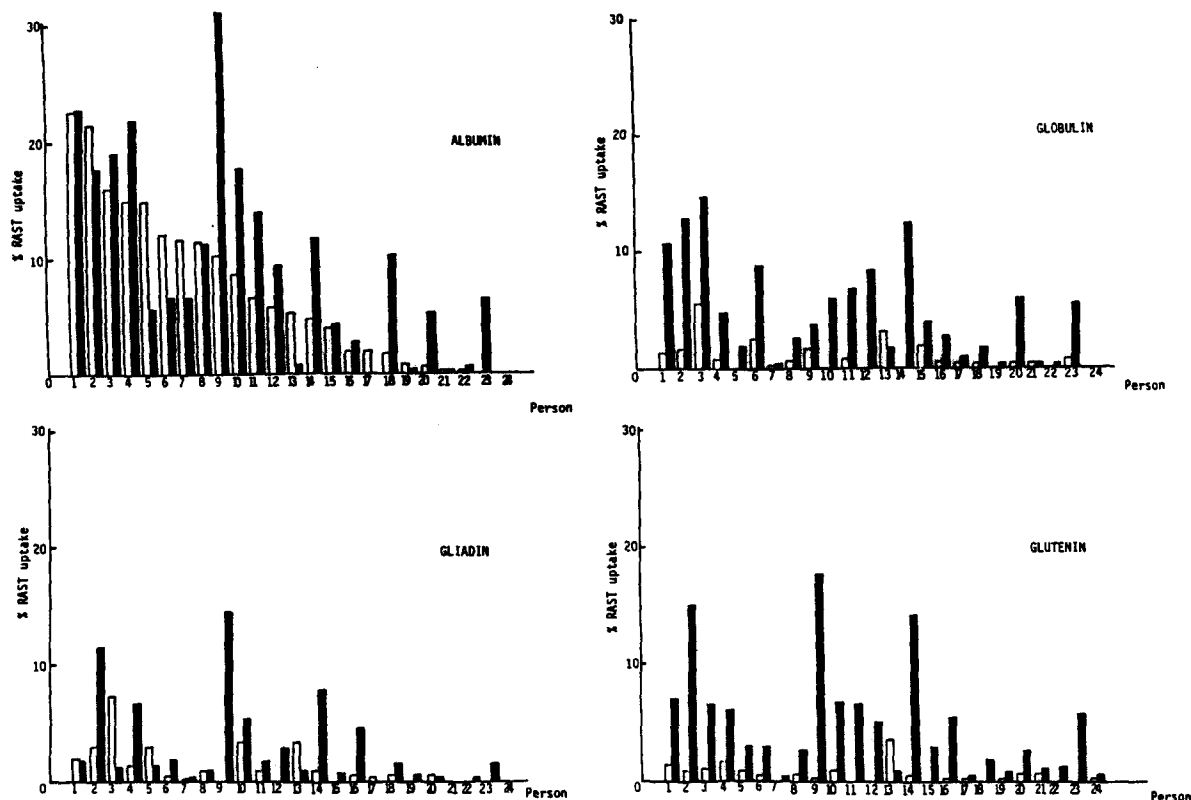


FIG. 1. Specific IgE binding (calculated as in Table II) of 24 bakers to the four main protein fractions of flour (albumin, globulin, gliadin, and glutenin) by use of RAST discs prepared with either water (white columns) or 1% KOH (black columns) as protein solvent.

disc preparation for either soluble or insoluble proteins. The use of KOH in preparing nitrocellulose discs increases the binding of both soluble and insoluble proteins.⁹ However, KOH treatment does not qualitatively modify the IgE binding capacity of individual flour allergens, since similar patterns of IgE binding were obtained for electrophoretically separated flour proteins transferred to a nitrocellulose sheet,⁸ whether or not the sheet had been soaked in 1% KOH solution.

Fig. 1 (black columns) demonstrates the allergenicity for the individual bakers' sera with KOH for disc preparation. Although the albumin proteins are still demonstrated to be important allergens, many individuals are now revealed to be much more allergic to other fractions than when water was used as solvent (Fig. 1).

A correlation matrix (Table III), relating the results illustrated in Fig. 1 for different fractions and solvents, illustrates that the albumin results with water as solvent correlated well to those with use of KOH (0.71), but the associations were progressively poorer with globulin (0.39), gliadin (0.08), and glutenin (-0.03). When results were considered that were obtained with

KOH only, there were significant relationships between the specific IgE bindings to gliadin, glutenin, and albumin. These relationships may be due to cross-contamination between these fractions or, more likely, to a degree of chemical homology particularly between gliadin and glutenin proteins.

DISCUSSION

According to the results in Fig. 1, almost any one of the four fractions of wheat flour may be the major allergen responsible for bakers' asthma depending on the individual. It is thus no longer possible to claim that any single fraction is "the" allergen of flour. The diversity between protein fractions, demonstrated by this procedure, reinforces the findings of allergen diversity at the level of individual protein components, demonstrated in parallel studies involving IgE binding after electrophoretic separation of flour proteins.^{1, 7, 10}

Unfortunately, since there are such considerable differences between individuals in the proteins to which they are sensitized, the tasks of allergen standardization, diagnosis, and hyposensitization are perceived to be much more difficult than they would be if there were fewer allergens and more uniformity

TABLE III. Correlation coefficients relating specific IgE binding values in Fig. 1 for all 24 sera tested

	Albumin H ₂ O	Globulin H ₂ O	Gliadin H ₂ O	Glutenin H ₂ O	Albumin KOH	Globulin KOH	Gliadin KOH
Albumin H ₂ O	1.0						
Globulin H ₂ O	0.45	1.00					
Gliadin H ₂ O	0.58	0.60	1.00				
Glutenin H ₂ O	0.38	0.47	0.58	1.00			
Albumin KOH	<u>0.71</u>	0.29	0.36	0.13	1.00		
Globulin KOH	0.60	0.39	0.56	0.11	0.58	1.00	
Gliadin KOH	0.43	0.11	0.08	0.03	<u>0.72</u>	0.42	1.00
Glutenin KOH	0.46	0.11	0.20	-0.03	<u>0.76</u>	0.64	<u>0.93</u>

between individuals. On the other hand, it is still possible that there is considerable immunologic cross-reactivity⁵ between these allergens and that it will be possible to standardize on a much smaller number of pure allergens representative of the full range of immunologic determinants of clinical importance.

Since there was increased IgE binding by albumin-nitrocellulose discs (KOH-treated) for several patients (4, 9 to 12, 14, 18, 20, 23) in Fig. 1, the improved disc-preparation method may enhance the detection of IgE binding by certain soluble allergens that are not readily bound to the disc in water. This does not appear to be nonspecific binding because it was not evident for control patients.

Perhaps the most important conclusion to be drawn from the results in Fig. 1 is that water-insoluble allergens have not been satisfactorily tested for allergenicity in the past but that such allergens are important, at least in the case of flour allergy. This conclusion, reached on the basis of modifying the RAST procedure, is likely to apply also to skin testing, which is restricted to the use of clear aqueous solutions. Chemical or enzymic modification of insoluble allergens may be needed to suit them for skin testing.

By way of contrast, in the actual situation of allergen attack, the whole particle (of flour, pollen, and mite fragment) is deposited on surfaces of the body tissues affected, e.g., mucosal lining of nose or bronchi or on the skin. There, the allergens most readily soluble in the surface fluids (mucous or sweat) are extracted to produce IgE-mediated reactions. In addition, however, it is likely that some insoluble allergens on the surface of the foreign particle can trigger IgE reactions directly on local mast cells and, importantly, that enzymes (particularly proteases) present on the mucosa, aid the extraction of allergens that are less readily extractable.

Such a mechanism is particularly attractive as an explanation for the delayed reactions^{11, 12} characteristic of bakers' asthma,^{5, 13} grain-dust "fever,"¹⁴⁻¹⁷ and al-

lergy to rice ingestion.¹⁸ The allergens causing each of these examples have high proportions of water-insoluble protein that has previously been ignored in allergy testing. Binding of IgE by the insoluble proteins of wheat grain has been presented in this article, and in a separate study (to be published) we have found that RAST assay of the KOH-extractable proteins of grain dust provides evidence of IgE binding not previously demonstrable.¹⁴⁻¹⁷

Systematic consideration of insoluble as well as soluble allergens may prove to bridge the correlation gaps between RAST, skin testing, and clinical symptoms. Such lack of correlation has been noted for bakers' asthma^{2, 5, 19} and grain-dust fever,¹⁴⁻¹⁷ but it is particularly evident in food allergies. It is also likely that the insoluble nature of many food proteins,^{4, 18} especially after cooking, is a major cause of such problems. These could be solved by suitably modifying the RAST procedure (as described above) and by including the full range of proteins (by partial proteolysis) for skin testing. In particular, there is the need for bronchial testing of allergic patients as the ultimate test of this hypothesis.

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