

The role of cereal and fungal amylases in cereal flour hypersensitivity

C. P. SANDIFORD, R. D. TEE and A. J. NEWMAN TAYLOR

Department of Occupational and Environmental Medicine, National Heart and Lung Institute,
Royal Brompton and National Heart Hospital, London, UK

Summary

To investigate the role of cereal α and β -amylase in bakers' asthma, we have compared the IgE response of 30 wheat-flour-allergic individuals to barley α and β -amylases with that of fungal α -amylase using radioallergosorbent test (RAST), RAST inhibition assays and Western blotting. RAST analysis showed 29 of the 30 subjects with inhalant induced cereal allergy had positive IgE to cereal amylases, but only 16 were positive to fungal α -amylase. Regression analysis showed an association between specific IgE to wheat-flour and to barley α -amylase ($r=0.70$) and barley β -amylase ($r=0.92$) but a poor association with fungal α -amylase ($r=0.34$). RAST inhibition showed minimal crossreactivity between barley α or β -amylase and barley and fungal α -amylase. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting showed that non-reduced barley α -amylase had a molecular weight of 54 kDa and barley β -amylase a molecular weight of 64 kDa. Reduced fungal α -amylase had a molecular weight of 54 kDa. Cereal α and β -amylase appear to be important allergens in patients with allergy to flour.

Clinical and Experimental Allergy, Vol. 24, pp. 549–557. Submitted 27 September 1993; revised 15 November 1993; accepted 15 January 1994.

Introduction

Baker's asthma is an allergic occupational lung disease of millers and bakers which is primarily caused by the inhalation of cereal flour [1].

The working environment in bakeries contains many additives in addition to cereal flour. It has been reported that one additive, α -amylase from *Aspergillus oryzae*, is allergenic [2]. Brisman and Belin found that six of 20 individuals who had been exposed to fungal α -amylase had a positive skin-prick test [3]. The cereals from which flour is produced contain both α -amylase (α -1,4-glucan 4-glucanohydrolase) and β -amylase (α -1,4-glucan maltohydrolase) [4,5]. These cereal α and β -amylases may also be allergenic and stimulate specific IgE production.

We have examined the IgE response of subjects with bakers' asthma to barley α and β -amylase and fungal α -amylase using RAST and competitive RAST inhibitions.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used to identify the molecular weight of these allergens.

Materials and methods

Patient selection

Sera were obtained from 30 wheat-flour-exposed individuals referred to an occupational asthma clinic because of suspected hypersensitivity to wheat flour. Clinical data were unavailable for two of the 30 referrals. Evidence of occupational asthma was based on history in 10 cases, history supported by peak expiratory flow rate records in 13 cases and history supported by bronchial provocation test in two cases. One individual had a history of work-related respiratory symptoms and anaphylactic reactions to flour. One individual had a history of work-related respiratory symptoms to fungal α -amylase. One individual was not diagnosed as having work related occupational asthma.

RAST was undertaken on each sample to barley α -amylase, barley β -amylase, fungal α -amylase, Canadian Western Red Spring (CWRS) and barley flour. Sera from

Correspondence: Mr C. P. Sandiford, Department of Occupational and Environmental Medicine, National Heart and Lung Institute, 1B Manresa Road, London SW3 6LR, UK.

11 of these individuals were used in a pool to undertake RAST inhibitions and individually to probe Western blots of non-reduced and reduced barley α -amylase, barley β -amylase and fungal α -amylase.

Extraction of flours and enzymes

Canadian Western Red Spring (CWRS) flour and pearled barley flour free from fungal α -amylase were obtained from the Flour Milling and Baking Research Association (Chorleywood, Herts, UK). Fungamyl α -amylase 1600s (derived from *Aspergillus oryzae*) was obtained from Novo Nordisk Bioindustries (Surrey UK). Barley α -amylase (A-2771) and barley β -amylase (A-7130) were obtained from Sigma Chemicals (Dorset UK). We were unable to obtain purified wheat α and β -amylase and have therefore used barley α and β -amylase enzymes as surrogates for wheat α and β -amylase in this study, as these enzymes have similar structures and function to wheat α and β -amylase [5]. The flours and the fungamyl α -amylase 1600s were extracted in 0.1 M ammonium hydrogen carbonate (Merck, UK) overnight by shaking. The extracts were dialysed against distilled deionized water for 16 h and freeze-dried.

RAST

Barley α -amylase, barley β -amylase, fungal α -amylase, CWRS and barley flour were coupled to CNBr activated paper discs using the method of Ceska *et al.* [6]. The level of specific IgE to each allergen was measured by RAST for 30 sera obtained from wheat flour hypersensitive individuals [7]. Linear regression analysis was conducted using the SAS statistical package to investigate the relationship between the responses to the different allergens.

Competitive RAST inhibitions

Competitive RAST inhibition assays were conducted using pooled sera from 11 wheat flour hypersensitive individuals. Barley α and β -amylase, fungal α -amylase, CWRS flour and barley flour were used to inhibit the binding of IgE to (i) barley α -amylase, (ii) barley β -amylase and (iii) fungal α -amylase discs. Competitive inhibitions were carried out using fourfold dilutions of the inhibiting antigen (with values from 1000 $\mu\text{g ml}^{-1}$ to 0.015 $\mu\text{g ml}^{-1}$) extract in phosphate-buffered saline (PBS). The percentage inhibition of IgE binding was calculated and the resulting curves were examined for parallelism as previously described [8].

Electrophoresis

The CWRS and barley flours, barley α and β -amylase, fungal α -amylase and electrophoretic markers of known

molecular weight (Amersham, UK) were subjected to reduced and non-reduced SDS-PAGE in a Protean II system (Bio-Rad) using the method of Laemmli [9]. The flours were reduced by heating for 5 min at 100°C in 0.3% SDS 62.5 mM Tris-HCl buffer containing 5 mM DTT. The enzymes were reduced by heating for 5 min at 100°C in 0.3% SDS 62.5 mM Tris-HCl buffer containing 25 mM DTT.

Western blotting

Non-reduced and reduced samples of barley α -amylase, barley β -amylase and fungamyl 1600s α -amylase were subjected to electrophoretic transfer on to 0.45 μm nitrocellulose membranes (Bio-Rad UK) using the method of Towbin *et al.* [10]. The remaining protein binding sites on the membrane were blocked by incubating in PBS with 0.24% RAST washing substance which contains detergent (Kabi Pharmacia, UK) for 2 h. The membranes were cut into strips and probed with the 11 individual wheat flour hypersensitive human sera that had comprised the serum pool used for the RAST inhibitions. A strip was incubated with PBS as a negative control. After washing three times with PBS-RAST wash the strips were incubated with ^{125}I anti-IgE for 16 h. Following a further three washes the strips were air-dried and exposed to Hyperfilm MP (Amersham, UK) for 24 h at -70°C .

Results

RAST

A specific IgE binding of $\geq 1\%$ was detected in 100% of the sera to CWRS flour, barley flour and barley β -amylase. Specific IgE was found in 29 of the 30 sera to barley α -amylase and in 16 to fungal α -amylase.

The level of IgE to fungal α -amylase was independent of the level of IgE to the cereal allergens. Table 1 shows the results of the linear regression analysis using the IgE data from the 30 wheat allergic individuals. A good association was observed between the CWRS flour and both barley α -amylase ($r=0.74$) and barley β -amylase ($r=0.92$) but a poor association between CWRS flour and fungal α -amylase ($r=0.29$). The levels of specific IgE for the individual sera used in RAST inhibitions and Western blotting to CWRS flour, barley flour, barley α -amylase, barley β -amylase and fungal α -amylase are given in Table 2.

Competitive RAST inhibitions

Low crossreactivity was found between the barley amylases and fungal α -amylase (Fig. 1a, b and c). After logit transformation of the curves, a common slope was not

Table 1. Results of specific IgE regression analysis in 30 sera from wheat allergic individuals

Allergen disc	CWRS flour	Barley flour	Barley α -amylase	Barley β -amylase	Fungal α -amylase
CWRS flour	1.000	0.844	0.742	0.922	0.290
Barley flour		1.000	0.723	0.848	0.221
Barley α -amylase			1.000	0.841	0.159
Barley β -amylase				1.000	0.202
Fungal α -amylase					1.000

$P \leq 0.01$ except for the following: CWRS vs fungal α -amylase $P = 0.08$; barley flour vs fungal α -amylase $P = 0.14$; barley α -amylase vs fungal α -amylase $P = 0.32$; barley β -amylase vs fungal α -amylase $P = 0.18$.

Table 2. RAST binding (%) of 11 sera from wheat-flour-hypersensitive individuals used in RAST inhibition assays and Western blotting

Sera	Specific allergen disc				
	CWRS flour	Barley flour	Barley α -amylase	Barley β -amylase	Fungal α -amylase
1	43.12	39.83	29.87	35.70	51.42
2	30.94	22.92	12.04	21.39	0.55
3	49.98	42.22	35.26	43.74	5.96
4	14.01	8.81	8.13	11.11	2.29
5	11.59	3.06	1.83	1.98	0.75
6	36.16	ND	23.65	32.07	14.46
7	27.16	8.65	6.88	12.03	27.18
8	20.57	13.42	11.87	17.40	0.64
9	8.25	3.56	3.52	3.41	28.70
10	22.76	13.07	4.56	4.85	14.78
11	24.57	13.54	5.50	8.79	0.87

ND. Not determined.

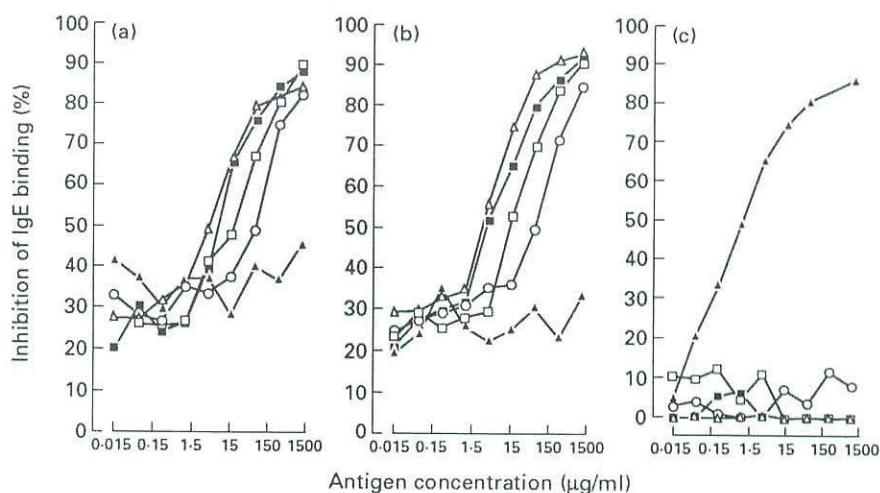


Fig. 1. Rast inhibitions of pooled sera from 11 wheat flour hypersensitive individuals of (a) barley α -amylase RAST; (b) barley β -amylase RAST and (c) fungal α -amylase RAST. Inhibitors: barley α -amylase (○), barley β -amylase (△), fungal α -amylase (▲), CWRS (■), and barley flour (□).

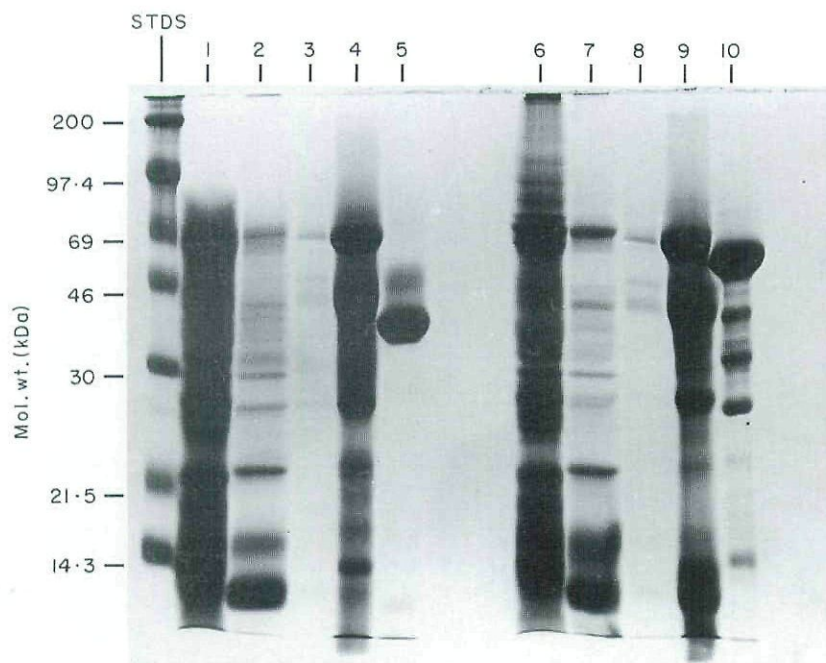


Fig. 2. Coomassie blue stain of a 15% homogenous SDS-PAGE. Tracks 1 to 5 non-reduced samples: 1 CWRS flour, 2 barley flour, 3 barley α -amylase, 4 barley β -amylase, 5 fungal α -amylase. Tracks 6 to 10 reduced samples of 6 CWRS flour, 7 barley flour, 8 barley α -amylase, 9 barley β -amylase, 10 fungal α -amylase.

observed between the three enzymes, indicating the absence of antigenic identity between the barley amylases and fungal α -amylase. A common slope was observed between barley α and β -amylase indicating immunological identity.

Gel electrophoresis

Non-reduced and reduced SDS-PAGE were used to compare CWRS and barley flour with the cereal α and β -amylases and fungal α -amylase (Fig. 2). The wheat and barley flour samples (non-reduced tracks 1 and 2, reduced tracks 6 and 7) were observed to be complex mixtures of proteins with molecular weights ranging from 70 and 10 kDa. Proteins of molecular weight 68, 54, 39 and 27 kDa were observed in both the non-reduced and reduced barley α -amylase samples (tracks 3 and 8). These proteins were detected in both the reduced wheat flour (track 6) and reduced barley flour (track 7) samples. The crude barley β -amylase (tracks 4 and 9) was seen to be a complex mixture of proteins which resembled the non-reduced and reduced barley flour. Proteins of molecular weight 64, 45, 27, 21 and 14 kDa were observed in the non-reduced sample and of molecular weight 58, 44, 27,

14, and 12.5 kDa in the reduced sample. Electrophoresis showed that non-reduced fungal α -amylase (track 5) had a single band of 37 kDa. Reduction of fungal α -amylase (track 10) produced proteins of molecular weights 54, 40, 34, 27 and 14 kDa.

Western blotting

Immunoblotting showed that non-reduced barley α -amylase had a strong IgE-binding band of molecular weight 54 kDa (Fig. 3a). Minor bands of molecular weight 30 kDa were seen in tracks 2, 3, 6, and 9 respectively. Reduced barley α -amylase was also shown to have an intense IgE binding band of molecular weight 54 kDa (Fig. 3b). Bands of molecular weight 42 and 32 kDa were observed in tracks 3 and 6 while a band of 12 kDa was observed in tracks 3, 4 and 6; this band was also weakly staining in tracks 7 and 10.

Non-reduced barley β -amylase (Fig. 4a) was observed to have an intense IgE binding band of 64 kDa in all tracks. A protein of 36 kDa was detected in tracks 1, 2, 3, 6, 7, 8, 10 and 11. A further band of 30 kDa was found in tracks 1, 2, 3, 6, 7, 8, 10, and 11 with a protein of 24 kDa seen in tracks 1 to 4, 6, and 8. A protein of 14 kDa was also

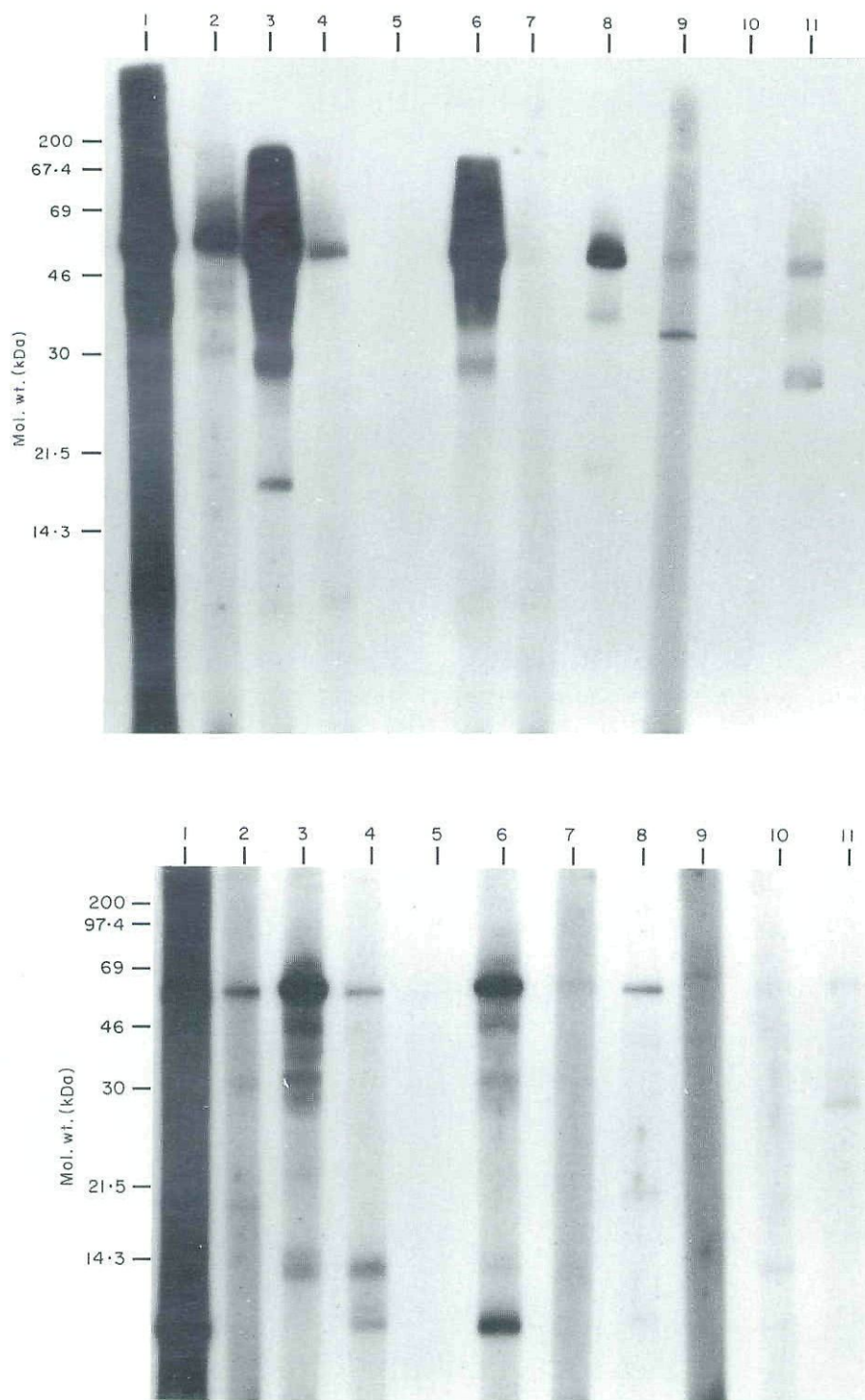


Fig. 3. Western blot: IgE response of 11 wheat-flour-hypersensitive individuals against (a) non-reduced barley α -amylase or (b) reduced barley α -amylase.

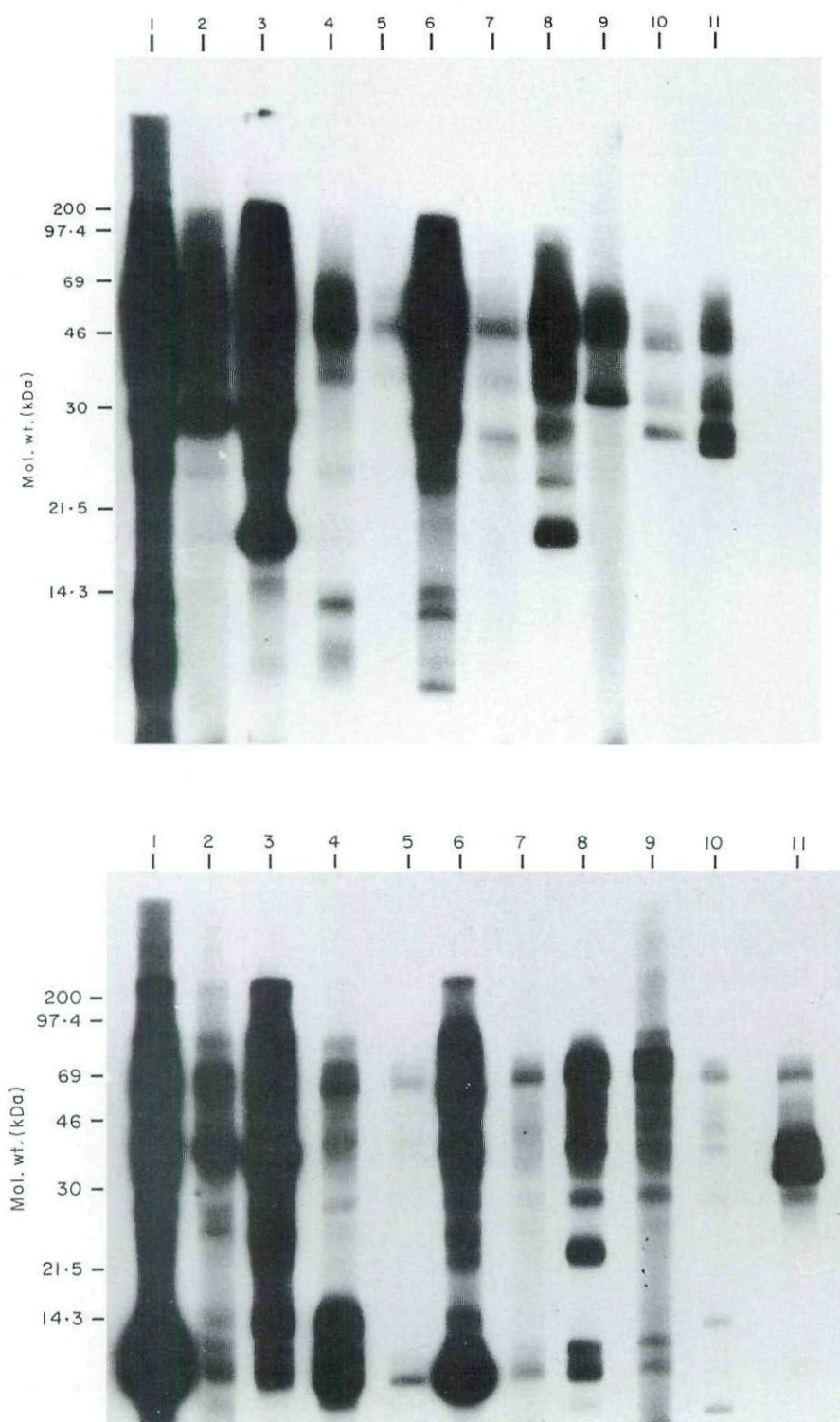


Fig. 4. Western blot: IgE response of 11 wheat-flour-hypersensitive individuals against (a) non-reduced barley β -amylase or (b) reduced barley β -amylase.

observed in tracks 1, 3, 4, and 6. Reduced barley β -amylase (Fig. 4b) was observed to have an intense IgE-binding band of 60 kDa in all tracks. A band of 28 kDa was further found in tracks 1, 2, 3, 4, 6, 7, 8, 9, and 11. This protein was also faintly visible in track 10. Proteins of molecular weight 14–12 kDa were detectable in all tracks.

Non-reduced fungal α -amylase had a molecular weight of 37 kDa by Western blotting (Fig. 5a). Reduced fungal α -amylase (Fig. 5b) was found to have a main allergen of 52 kDa. Two other bands of 40 and 27 kDa were also seen in tracks 6, 7 and 9.

Discussion

Bakers' asthma is an allergic occupational lung disease whose primary causes are thought to be water and salt soluble wheat flour proteins [1]. Bakers are also exposed to flour improvers such as fungal α -amylase, which has been reported to cause allergic reactions. Using a fungal α -amylase skin-prick test solution at a concentration of 50 mg ml⁻¹, Belin and Brisman [3] demonstrated that six of 20 individuals exposed in a bakery to fungal α -amylase had an immediate skin-prick test reaction to this enzyme. The prevalence of skin-prick test responses to fungal α -amylase in a longitudinal examination within a cross-sectional study increased with increasing exposure to flour dust [11].

In the present study we have examined the IgE to cereal α and β -amylase enzymes in a group of flour allergic individuals occupationally exposed to cereal flour, the great majority of whom had been diagnosed as having occupational asthma. We also compared the IgE responses of these individuals to cereal α and β -amylases with their IgE response to fungal α -amylase. SDS-PAGE and Western blotting have been used to identify the molecular weights of these proteins.

RAST analysis showed that the level of specific IgE to either cereal α and β -amylase was correlated with the level of specific IgE to wheat or barley flour. Kruger *et al.* found that wheat and barley α -amylase occurred only in trace amounts [12] and that between 50–60% of cereal α -amylase was removed by the milling process [13]. We have therefore concluded that the high prevalence of specific IgE to both cereal enzymes, coupled with their low occurrence in flour indicates that they are potent allergens.

Competitive RAST inhibition using a human serum pool showed little crossreactivity between the cereal amylases and fungal α -amylase. This may be explained by the low amino acid sequence homology observed between cereal α -amylase and fungal α -amylase. Nakajima *et al.* found that only 33 of the 438 (7.53%) amino acids that comprise barley α -amylase are homologous with 33 of 512

(6.44%) amino acids of fungal α -amylase from *Aspergillus oryzae* [14].

Blands *et al.* found that over 40 proteins were present in an ammonium acetate extract of flour [15], indicating the large number of proteins released from flour following water/salt extraction. Barley α -amylase has been shown by non-reduced and reduced SDS-PAGE to be a water soluble protein. The preparation that we have used in this study was observed to be contaminated with proteins of molecular weights 68, 39 and 27 kDa. The contaminating proteins were seen to bind Coomassie blue with an intensity equal to, or greater than that of the barley α -amylase suggesting that this enzyme may be present in low concentrations in barley flour. When the Barley α -amylase sample was compared with barley flour, it was observed that the enzyme was one of a large number of water soluble proteins visible following electrophoretic separation, further indicating that this protein is present in low concentrations in flour. The 54 kDa we have observed as the molecular weight of barley α -amylase is similar to the molecular weight 52 kDa found by Kruger *et al.* [13] for wheat and barley α -amylase. The molecular weight of this protein did not change with reduction suggesting that it is a monomer without internal disulphide bridges. The crude barley β -amylase sample is water soluble and was shown to be a mixture of proteins. The 45, 27 and 14 kDa proteins were observed to have the same molecular weights following reduction suggesting that they are also monomers without internal disulphide bridges. The main staining band of barley β -amylase sample changed from a molecular weight of 64 kDa to 58 kDa following reduction with DTT. This molecular weight is similar to the 57.2 kDa found by Visuri and Nummi [5]. Our electrophoresis data support previous observations that α -amylase occurs in low concentrations in cereal flour [12,13]. We observed a non-reduced molecular weight of 37 kDa for fungal α -amylase which increased to 54 kDa upon reduction. This molecular weight is similar to the 52 kDa observed by Baur *et al.* [2].

Western blotting has been used to identify these proteins as allergens and has shown that barley α -amylase is an allergen with a molecular weight of 54 kDa which did not change under reducing conditions. The 14/12 kDa protein detected by Western blotting is probably the barley α -amylase inhibitor, which is known to be associated with barley α -amylase [16,17]. The 64 kDa barley β -amylase protein was shown to be an allergen by immunoblotting. When reduced barley β -amylase was examined by Western blotting, we could not clearly observe a band of 58 kDa in all tracks. This is probably caused by using crude barley β -amylase which produced a large number of allergenic bands after reduction with DTT. The increased number of bands observed in both

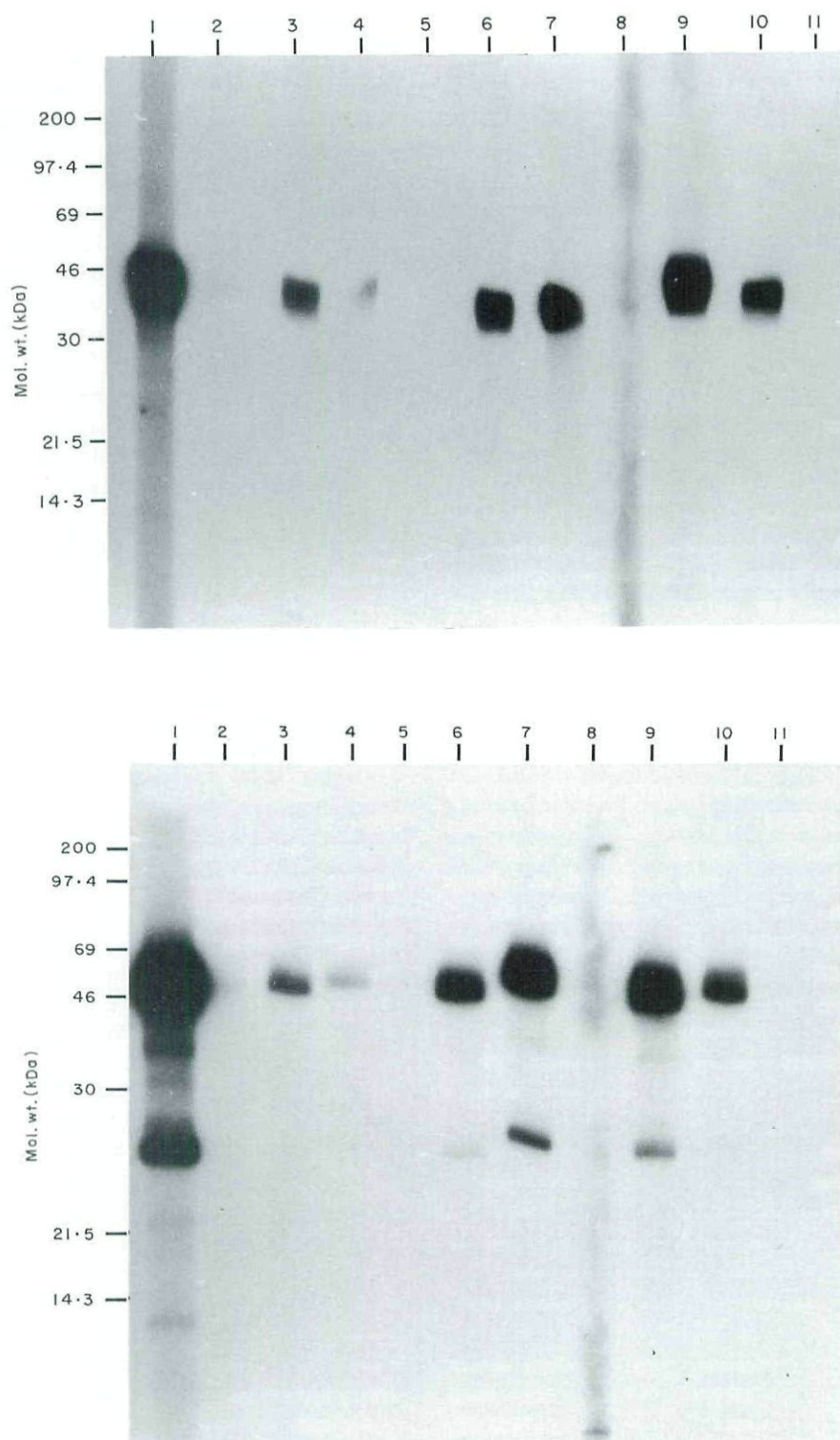


Fig. 5. Western blot: IgE response of 11 wheat-flour-hypersensitive individuals against (a) non-reduced fungal α -amylase or (b) reduced fungal α -amylase.

the non-reduced and reduced samples is probably due to immuno-staining being of greater sensitivity than Coomassie blue staining and by the large number of contaminating proteins present within the sample. Fungal α -amylase was shown to be allergenic with a molecular weight of 37 kDa in the non-reduced form and 52 kDa in the reduced state. Western blotting has shown that the cereal α and β -amylase and fungal α -amylase enzymes are allergens in both their non-reduced and reduced conformations.

Enzymes used commercially such as subtilisin and papain as well as fungal α -amylase [18] are well described causes of allergy and asthma amongst those exposed to them at work. More recently the major allergens of the house dust mite *D. pteronyssinus* [19,20] and *A. fumigatus* [21] have also been shown to have enzyme activity. The relationship of allergenicity to enzyme activity remains unclear.

We have found that cereal α and β -amylase are water-soluble allergens of different molecular weights, which have little immunological crossreactivity with fungal α -amylase. These observations show that cereal α and β -amylase can stimulate specific IgE antibodies, independently of IgE antibodies stimulated by fungal α -amylase. Cereal α and β -amylase appear to be important allergens in inducing occupational allergy and asthma in bakers and millers.

Acknowledgments

We gratefully thank Dr P. Sherwood Burge for his help and assistance in providing us with clinical data and sera from several wheat hypersensitive subjects. We thank Dr R. D. Barker for his help in the collection of sera from several of the patients. We also acknowledge the secretarial assistance of Mrs A. Addison.

References

- Sutton R, Skerrett J, Baldo B, Wrigley C. The diversity of allergens involved in Bakers asthma. *Clin Allergy* 1984; 14:93-107.
- Baur X, Fruhmant G, Haug B *et al.* *Lancet*, 1986; 1:43.
- Brisman J, Belin L. Clinical and immunological responses to occupational exposure to α -amylase in the baking industry. *Brit J Ind Med* 1991; 48:604-8.
- Akaeva M, Fursov O. Synthesis, Activation and Secretion of α -amylase in the Aleurone layer and Scutellum of the Wheat Caryopsis. *Sov Plant P* 1990; 37:908-12.
- Visuri K, Nummi M. Purification and Characterization of Crystalline β -amylase from Barley. *Eur J Biochem* 1972; 28:555-65.
- Ceska M, Lundkvist U. A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry* 1972; 9:1021-30.
- Tee RD, Gordon DJ, Hawkins ER *et al.* Occupational allergy to Locusts: an investigation of the sources of the allergen. *J Allergy Clin Immunol* 1988; 81:517-25.
- Tee RD, Gordon DJ, Lacey J *et al.* Occupational allergy to the common house fly (*Musca domestica*): Use of immunologic response to identify atmospheric allergen. *J Allergy Clin Immunol* 1985; 76:826-31.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970; 227:680-5.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Nat Acad Sci* 1979; 76:4350-4.
- Cullinan P, Gordon S, Lowson D *et al.* Exposure-Response Relationships in Occupational Asthma. *Am Rev Resp Dis* 1993; 147 (suppl.): A113.
- Wrigley CW, Bietz JA. Proteins and Amino acids. In Pomeranz Y, ed. *Wheat: Chemistry and Technology*. St Paul Minnesota: American Association of Cereal Chemists 1988;159-275.
- Kruger JE, Lineback DR. Carbohydrate-degrading enzymes in cereals. In Kruger JE, Lineback DR, Stauffer CE, eds. *Enzymes and their role in cereal technology*. St Paul Minnesota: American Association of Cereal Chemists. 1987;117-39.
- Nakajima R, Imanaka T, Aiba S. Comparison of amino acid sequences of eleven different α -amylases. *Appl Microbiol Biotechnol* 1986; 23:355-60.
- Blands J, Diamant B, Kallos P, Kallos-Defner, Lowenstein H. Flour allergy in Bakers: Identification of allergenic fractions in flour and comparison of diagnostic methods. *Int Arch Allergy Appl Immunol* 1976; 52:392-406.
- Petrucci T, Tomasi M, Cantagalli P, Silano V. Comparison of wheat albumin inhibitors of α -amylase and trypsin. *Phytochemistry* 1974; 13:2487-95.
- Weselake RJ, MacGregor AW, Hill RD. Endogenous Alpha-Amylase Inhibitor in Various Cereals. *Cereal Chem* 1985; 62:120-3.
- Chan-Yeung M, Lam S. Occupational Asthma. *Am Rev Respir Dis* 1986; 133:686-703.
- Thomas WR. Mite allergens groups I-VII. A catalogue of enzymes. *Clin Exp Allergy* 1993; 23:350-3.
- Hill MR, Newton MR, Hart BJ. Comparative IgE responses to extracts of five species of house dust mites, using Western blotting. *Clin Exp Allergy* 1993; 23:110-6.
- Quirce S, Cuevas M, Diez-Gomez M *et al.* Respiratory allergy to *Aspergillus*-derived enzymes in bakers' asthma. *J Allergy Clin Immunol* 1992; 90:970-8.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.