

Wheat flour peroxidase is a prominent allergen associated with baker's asthma

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Summary

Background Putative wheat allergens with molecular sizes around 35 kDa have been repeatedly detected in immunoblots using sera from patients with baker's asthma. However, none of these allergens had been previously isolated.

Objective To purify and characterize a major component of the 35 kDa allergenic band which seems to be present in flour from diploid, tetraploid (pasta) and hexaploid (bread) wheats.

Methods Gel-filtration chromatography followed by RP-HPLC have allowed the purification of a new wheat allergen. Immunodetection with sera from allergic patients and with a specific serum for asparagine-linked complex glycans, as well as amino acid sequencing, were used to identify the isolated protein.

Results A 36 kDa allergen has been purified from wheat flour. Its N-terminal and internal sequences, N-linked glycans, and enzymatic activity indicated that this allergen is a seed-specific peroxidase. Sera from six out of 10 patients hypersensitive to wheat flour displayed positive reactions when assayed against the purified dot-blotted allergen.

Conclusion Seed-specific peroxidases from cereals (flours) seem to be important allergens in hypersensitive diseases associated with the manipulation of these plant materials. A new enzyme has thus been involved in IgE-mediated diseases.

Keywords: allergen, baker's asthma, cereal, complex glycans, seed peroxidase, wheat

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Introduction

Occupational asthma and rhinitis induced by the inhalation of cereal flour dust is an IgE-mediated disease, known as baker's asthma, which affects a significant proportion of workers in the baking and milling industries [1–3]. Cereal flour proteins extracted by salt solutions have been identified as the major allergens, although flour proteins of other classes, as well as additives, storage mites and pests proteins, can also elicit an IgE response [3–5].

Several components of salt extracts from wheat flour with molecular sizes in the range of 10–100 kDa have been

previously identified as major IgE-binding proteins [3]. Among them, an uncharacterized protein band of approximately 35 kDa has been frequently reported [6–8], being one of the most reactive with some sera [6,7].

The isolation and chemical characterization of potential allergens detected by immunoblotting is still very limited, both in wheat and in other cereal flours. Only a group of 12–16 kDa allergens, which belong to the α -amylase inhibitor family has been extensively studied in wheat, barley and rye [9–11]. The most reactive members of this protein family are glycoproteins whose sequences have been determined [12]. The role of their complex asparagine-linked glycans in IgE-binding properties have been recently established [13]. Amino acid sequences of grain amylases, which have been recently implicated in flour hyper-sensitivity [14],

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and of a 27 kDa protein group (N-terminal) [6], are the only ones reported for wheat allergens associated with baker's asthma, besides those of the inhibitors mentioned above.

In addition to bread (*T. aestivum*, genomes AABBDD) and pasta wheat (*T. turgidum*, genomes AABB), some Einkorn wheats (genomes AA), particularly *T. monococcum*, are extensively cultivated in certain areas and used in wheat-breeding programs. These Einkorn wheats show bread-making characteristics comparable to conventional bread wheats, have high carotene and protein contents, and are suspected to be less toxic than bread and pasta wheats to coeliac disease patients [15].

Most lines of Einkorn wheats lack the highly-allergenic components of the α -amylase inhibitor family, but their flour salt extracts show levels of IgE-binding similar to those of bread and pasta wheats [16]. Putative major allergens with molecular sizes in the range of 20–60 kDa detected in Einkorn wheats are responsible of the latter result.

We report here the isolation and characterization of a major IgE-binding protein from *T. monococcum* flour, that appears to be present also in pasta and bread wheat. The new allergen has a molecular size of 36 kDa and has been identified as a seed-specific peroxidase. No allergens with this enzymatic activity had been previously purified from plant or animal sources.

Materials and methods

Plant material

Flour from *Triticum monococcum* (Einkorn wheat) accession UP-1 was used to purify the 36 kDa allergen, and from *Triticum aestivum* (bread wheat cv. Chinese Spring) and *Hordeum vulgare* (barley cv. Betzes) to confirm the presence of equivalent allergenic components in other cereal species. Additionally, thirty-six lines of Einkorn wheats (kindly supplied by Drs F. Salamini and M. Heun, Max

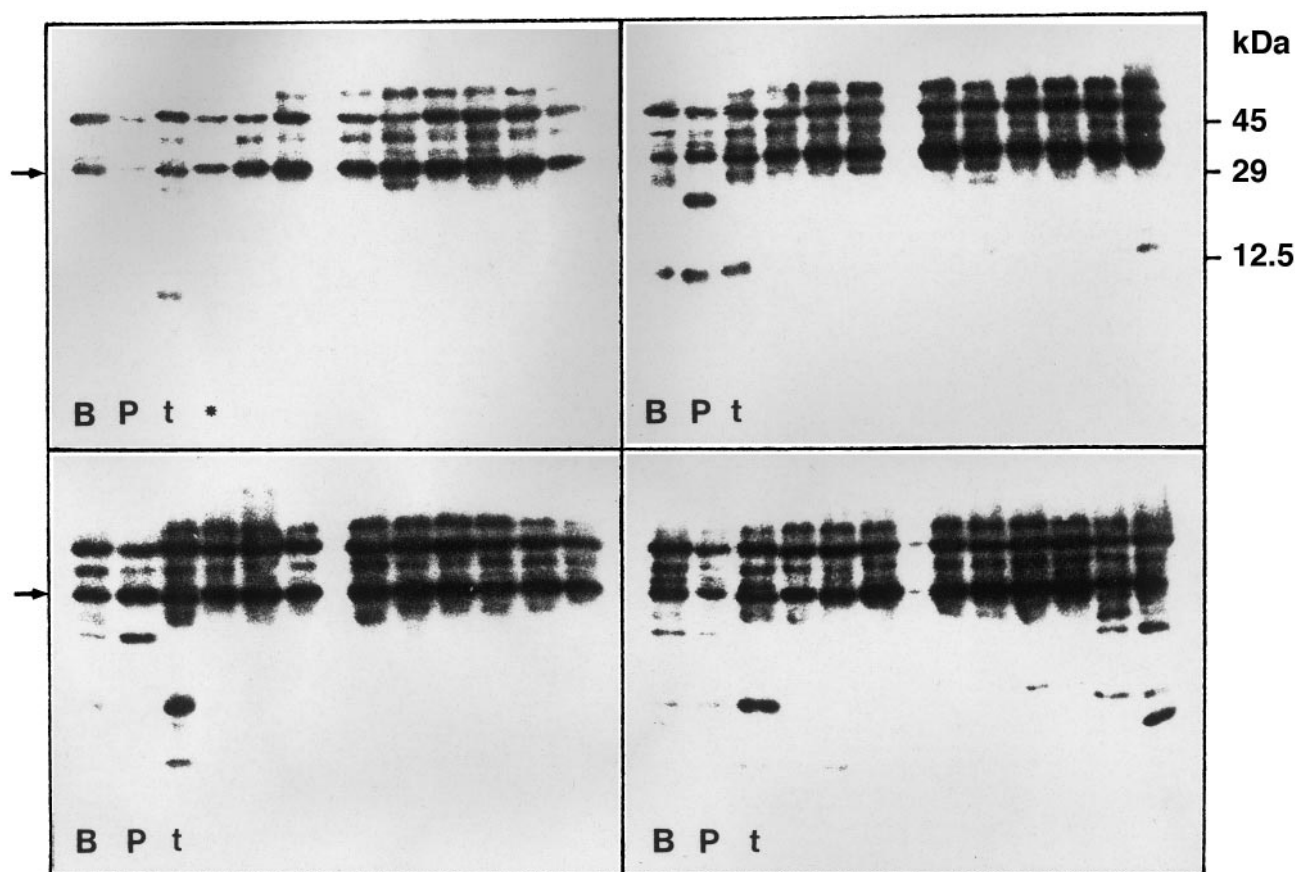


Fig. 1. IgE immunodetection after SDS-PAGE of salt extracts from different wheat samples of Einkorn wheats (no mark), bread wheats (B), pasta wheats (P) and *T. tauschii* (t). An asterisk indicates the Einkorn wheat line used to purify the allergen. The band under study is indicated by an arrow.

Planck Institut, Köln, Germany), three cultivars of bread wheat, three of pasta wheat and one accession of *Triticum tauschii* (diploid, genomes DD) were used in the initial immunodetection survey (see Fig. 1 and ref. 16).

Protein extraction

Flour samples were extracted with 0.5 M NaCl (2×; 1×10 w/v; 1 h). The salt extracts were dialysed against 0.1 M ammonium acetate and lyophilized.

Isolation of the 36 kDa allergen

The 0.5 M NaCl extract from *T. monococcum* was fractionated by gel-filtration on Sephadex G-100 (80 cm×2.5 cm column; 25 mL/h) using 0.1 M ammonium acetate, pH 6.8, as elution buffer. Fractions showing the highest level of allergen by immunodetection were subjected to reverse-phase high-performance-liquid-chromatography (RP-HPLC). Separation was carried out on a preparative Vydac-C4 column (22 mm×250 mm; particle size 10 µm), eluting with a three-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) (10–20% in 30 min, 20–50% in 160 min, 50–85% in 60 min; 1.5 mL/min). The peak corresponding to the allergen was identified by immunodetection after dot-blotting the HPLC fractions.

Characterization of the 36 kDa allergen

SDS-PAGE was performed according to Laemmli [17] on Bio-Rad Miniprotein II system gels. Two-dimensional electrophoresis, ∇ pH PAGE×SDS-PAGE, was carried out on the Miniprotein II 2-D cell system (Bio-Rad). Three micrograms of purified allergen were inserted at the acidic end of preformed pH gradients (ampholines pH 3–10, Serva) in the first-dimension gel, and subjected to 1500 V for 30 min after a pre-run of 10 min at 750 V.

Enzymatic hydrolysis of the purified allergen was carried out by endoproteinase Lys-C (EC 3.4.99.30), after reduction and carboxymethylation of the protein. A relation 1:15 of enzyme:allergen was used during 18 h at 37°C in a 25 mM Tris-HCl (pH 8.5), 1 mM EDTA buffer. The resulting peptides were fractionated by RP-HPLC on a semi-preparative Nucleosil C4 column (4.6 mm×250 mm, particle size 5 µm), using a two-step linear gradient of 0.1% TFA in 70% acetonitrile (0–50% in 20 min, 50–100% in 50 min; 0.5 mL/min), after a previous elution with 0.1% TFA during 15 min. The three major peaks obtained were subjected to amino acid sequencing.

N-terminal amino acid sequences were determined by standard methods using an Applied Biosystems 477A gas-phase sequencer. Peroxidase activity was assayed using the OPD tablets kit (Sigma), according to the manufacturer's

instructions. Protein concentration was quantified in all samples by the bicinchoninic acid test [18].

Glycoprotein detection

The presence of complex N-linked glycans attached to the proteins under study was determined as in Garcia-Casado *et al.* [13], using a specific serum previously described [19] (kindly provided by Dr M. J. Chrispeels, University of California, San Diego, USA) and goat anti-rabbit IgG coupled to alkaline phosphatase.

IgE immunodetection

A pool of sera from five patients with baker's asthma was used. All sera were class 4 when tested by the Phadebas-RAST kit (Pharmacia).

Samples were subjected to SDS-PAGE, electrotransferred to PVDF membranes, and then treated with 1:3 dilutions of the pool of sera and ¹²⁵I-labelled anti-human IgE (Pharmacia), as reported in [20].

Ten individual sera (RAST classes 4–2) from wheat-induced asthmatic patients were also used to determine the IgE-binding capacity of the purified allergen after slot-blotting. Samples (1 µg of protein) in 20 mM Tris-HCl, 150 mM NaCl, pH 8.3 buffer, containing 0.001% (w/v) SDS and 2% (v/v) 2-mercaptoethanol, were adsorbed to PVDF membranes. After drying, membranes were treated with the ten individual sera separately (1:3 dilutions) and ¹²⁵I-labelled anti-human IgE. Responses obtained were quantified by densitometry of the autoradiograms, using an image analyzer program (NIH Image version 1.55 of W. Rasband; scanner model VC840 Max vision). A negative control (buffer) and poorly-reactive samples (BSA and the wheat α -amylase inhibitor subunit-CM3) were also included. Sera from a healthy individual and an atopic non-flour sensitive patient showed no reaction with either the isolated allergen or the other cereal samples tested.

Results

Bread, pasta and Einkorn wheats share IgE-binding protein(s) of around 35 kDa

Flour salt extracts from several cultivars of bread and pasta wheat, as well as from accessions of Einkorn wheat and *T. tauschii* (a bread wheat parental species), were separated by SDS-PAGE and immunoblotted with a pool of sera from baker's asthma patients (Fig. 1). For most Einkorn wheat lines, no IgE-binding components were detected in the 12–16 kDa molecular size range. In addition, very similar patterns of reactive bands were observed in all the samples tested above 29 kDa. Particularly, a protein band of around

35 kDa was among those displaying the strongest response in all wheat species (Fig. 1).

The most reactive protein of the 35 kDa band is glycosylated

To isolate highly reactive 35 kDa proteins, the salt extract from *T. monococcum* accession UP-1 (a wheat line with a prominent 35 kDa band in its immunodetection pattern; see Fig. 1) was fractionated by gel-filtration on Sephadex G-100 (Fig. 2a & b). IgE immunodetection of the seven fractions obtained showed that the most reactive IgE-binding component appeared in fraction 5 and corresponded to the 35 kDa band (Fig. 2C). This gel-filtration fraction was further separated by RP-HPLC (Fig. 3a). The highest response with the pool of sera after dot-blotting the eluted proteins was concentrated in peak 36 (Fig. 3b).

Analysis of this chromatographic peak by SDS-PAGE showed a single band of 36.6 kDa, which strongly reacted with a serum specific to plant complex glycans (Figs 4a & b), indicating that the allergen was glycosylated. Homogeneity of this glycoprotein was confirmed by two-dimensional electrophoresis (Fig. 4c).

To investigate the role of its complex glycan(s) in the IgE-binding capacity of the purified allergen, enzymatic

deglycosylation was attempted using PNGase A (EC 3.5.1.51, Boehringer) under conditions previously described [13]. However, the glycan(s) of this allergen could not be removed even after incubation for 24 h. Under the same conditions about 50% of BMAI-1, a glycosylated allergen from barley [13] used as a positive control, had lost its single glycan.

The purified 36 kDa allergen is a seed specific peroxidase

The N-terminal amino acid sequence (10 residues) of the purified allergen was determined (Fig. 5). Interestingly, three out of four batches of the isolated protein showed no N-terminal residues, indicating that this protein terminus was blocked.

To extend the above data the isolated allergen was digested by endoproteinase Lys-C. The resulting peptides were separated by RP-HPLC and the three major ones subjected to automatic sequencing (Fig. 5). One of them, corresponding to the N-terminus of the native protein, fully confirmed the sequence determined for the uncleaved protein. No heterogeneity was detected at any point of the sequences, except at position 7 (D/V) of the first internal peptide (Fig. 5).

Comparison of the three partial amino acid sequences

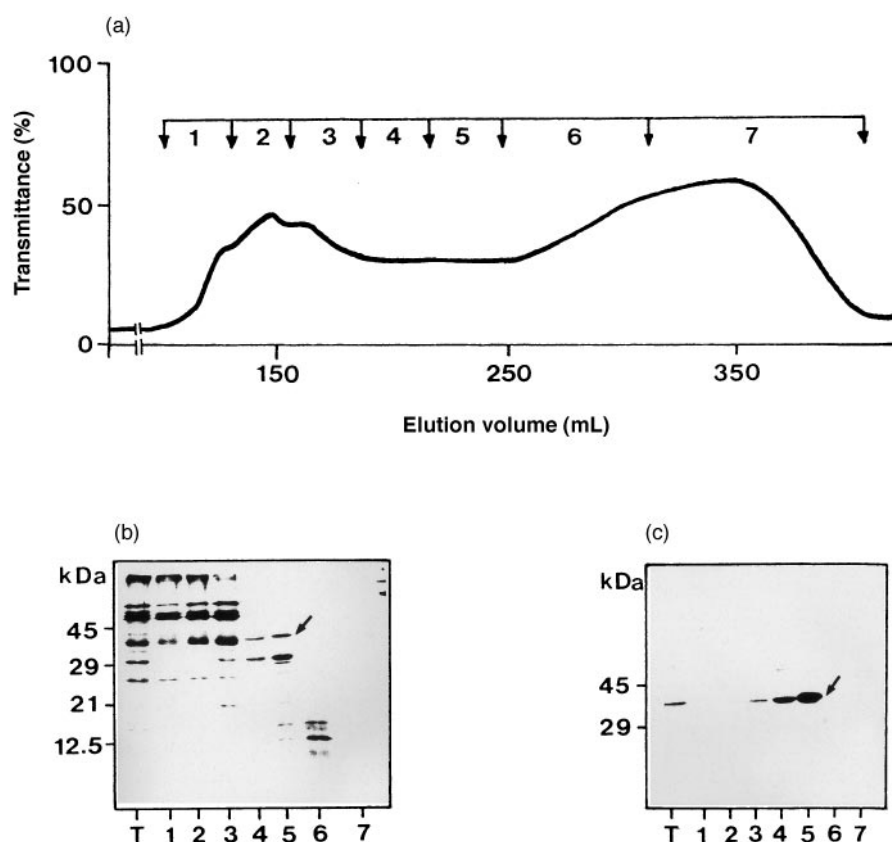


Fig. 2. (a) Gel-filtration on Sephadex G-100 of the salt extract from *T. monococcum* flour. Fractions are indicated (1–7). (b), (c) SDS-PAGE of the salt extract (T) and gel-filtration fractions indicated in (a) (1–7). Proteins were stained with Coomassie Blue (b) or IgE immunodetected (c). The band under study is indicated by an arrow.

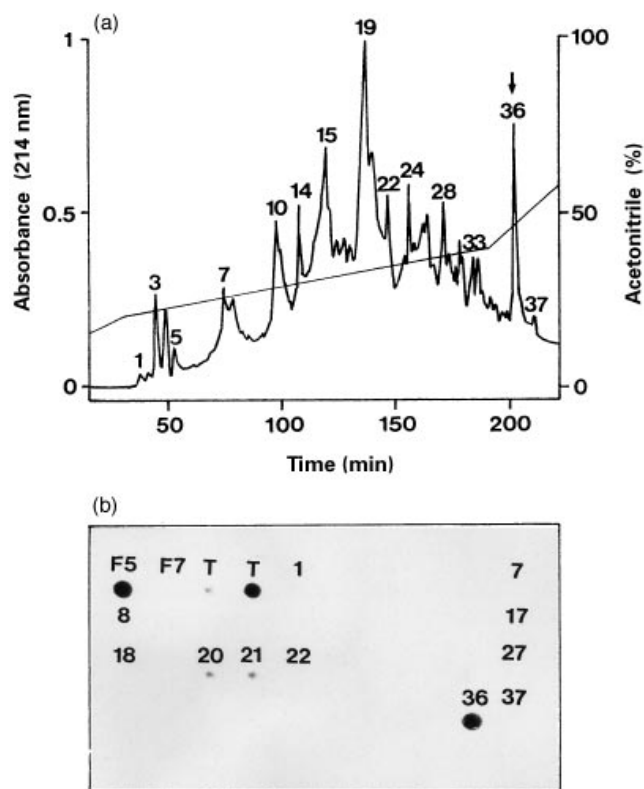


Fig. 3. A. Reverse-phase HPLC separation of the gel-filtration fraction 5 of Fig. 2(b). IgE immunodetection after dot-blotting (1 μ g of protein) fractions 1–37 of the RP-HPLC profile. Gel-filtration fractions 5 (F5) and 7 (F7), as well as the salt-extract (T; 0.5 and 1 μ g) are also included.

obtained with those included in the plant sequence data banks uncovered a high degree of sequence identity (20/35 residues) with the barley seed-specific peroxidase BP1 [21]. Joint consideration of the sequence alignment showed in Fig. 5 and the molecular size of both proteins, strongly suggested that the similarity extends to the whole sequence. Moreover, BP1 is also a glycoprotein with asparagine-linked complex glycans [21]. Therefore, the 36 kDa allergen

Table 1. Peroxidase activity of the 36 kDa allergen. Horseradish peroxidase (positive) and bovine serum albumin and wheat inhibitor subunit CM3 (negative) were used as controls. Conditions of the assay are described in Materials and methods. Values are means $\pm$ SE ($n = 3$)

Sample	μ g	Δ Absorbance (450 nm)
Horseradish peroxidase	1	3.447 ± 0.117
Bovine serum albumin	1	0.008 ± 0.001
Wheat tetrameric α -amylase inhibitor-CM3	1	0.004 ± 0.001
Allergen	1	0.210 ± 0.002
0.5 M NaCl Crude extract <i>T. monococcum</i>	10	1.812 ± 0.075

was identified as the wheat equivalent to barley peroxidase BP1.

To further confirm these results, the peroxidase activity of the purified allergen was tested (Table 1). The protein displayed a clear enzymatic activity when compared with the negative controls used, but it was considerably lower than that of the horseradish peroxidase. These data agree with the low specific activity previously reported for its barley equivalent BP1 using classical peroxidase substrates [22].

IgE-binding capacity of the 36 kDa allergen

To study the IgE-binding capacity of the 36 kDa allergen, the isolated protein was slot-blotted and then probed with individual sera from baker's asthma patients and ^{125} I-labelled anti-human IgE. The intensity of the responses obtained was quantified by densitometry of the corresponding autoradiograms (Table 2).

The purified allergen gave positive responses with 6/10 sera, while the total salt extracts from bread and *monococcum* wheat reacted with 8/10 sera. The mean intensity of the responses to the allergen and the bread wheat extract was very similar (460 and 454 arbitrary units, respectively),

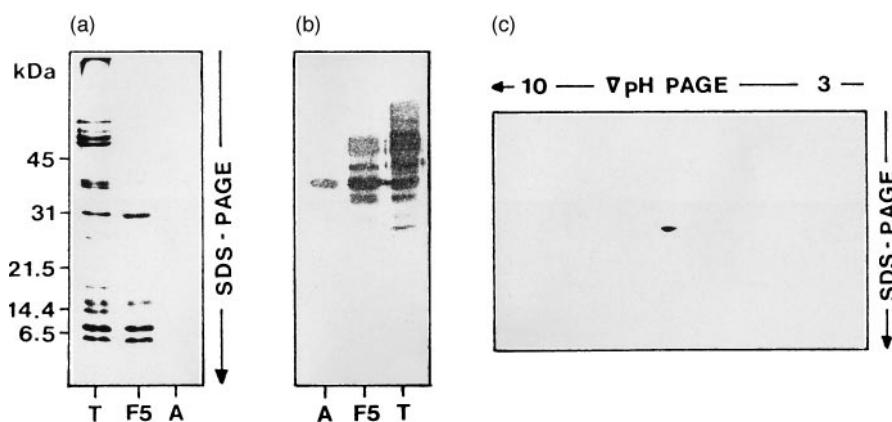


Fig. 4. (a), (b) SDS-PAGE of the purified allergen (A), the gel-filtration fraction 5 (F5) and the salt extract (T) from *T. monococcum*. Samples were stained with Coomassie Blue (a) or immunodetected using a serum specific for asparagine-linked complex glycans (b). (c) Two-dimensional electrophoresis (VpH PAGE, pH 3–10 $\times$ SDS-PAGE) of the purified allergen (3 μ g).

Table 2. Intensities of IgE-binding responses in immunoblots with the purified 36 kDa allergen. Samples: bovine serum albumin (BSA), wheat tetrameric inhibitor subunit-CM3 (CM3), horseradish peroxidase (HPX), 0.5 M NaCl extract from bread (T bw) and *T. monococcum* wheat (T mw), and purified 36 kDa allergen (A). One microgram of protein was adsorbed onto PVDF membranes and treated with individual sera from baker's asthma patients and ¹²⁵I-labelled anti-human IgE. Intensities of each slot in the autoradiograms were quantified by densitometry. Values under 20 arbitrary units were considered as negative, according to the readings for negative controls (buffer)

	Serum no									
	1	2	3	4	5	6	7	8	9	10
RAST	3	3	4	3	4	2	3	3	3	3
BSA	–	–	77	–	32	–	45	29	28	–
CM3	–	–	96	–	42	–	22	26	27	–
HPX	–	–	1085	37	336	38	915	21	194	–
T bw	–	103	952	56	501	67	797	–	96	312
T mw	–	31	675	27	381	42	556	–	106	304
A	–	20	1012	–	684	–	799	–	85	124

and higher than that of the *monococcum* extract (342 units), when only the six sera reacting with all three samples are considered (sera no 2, 3, 5, 7, 9 and 10).

Taking into account the enzymatic activity of the purified allergen, commercial horseradish peroxidase was also included in the immunoblots. This protein has also attached complex glycans, but shows less than 50% of sequence

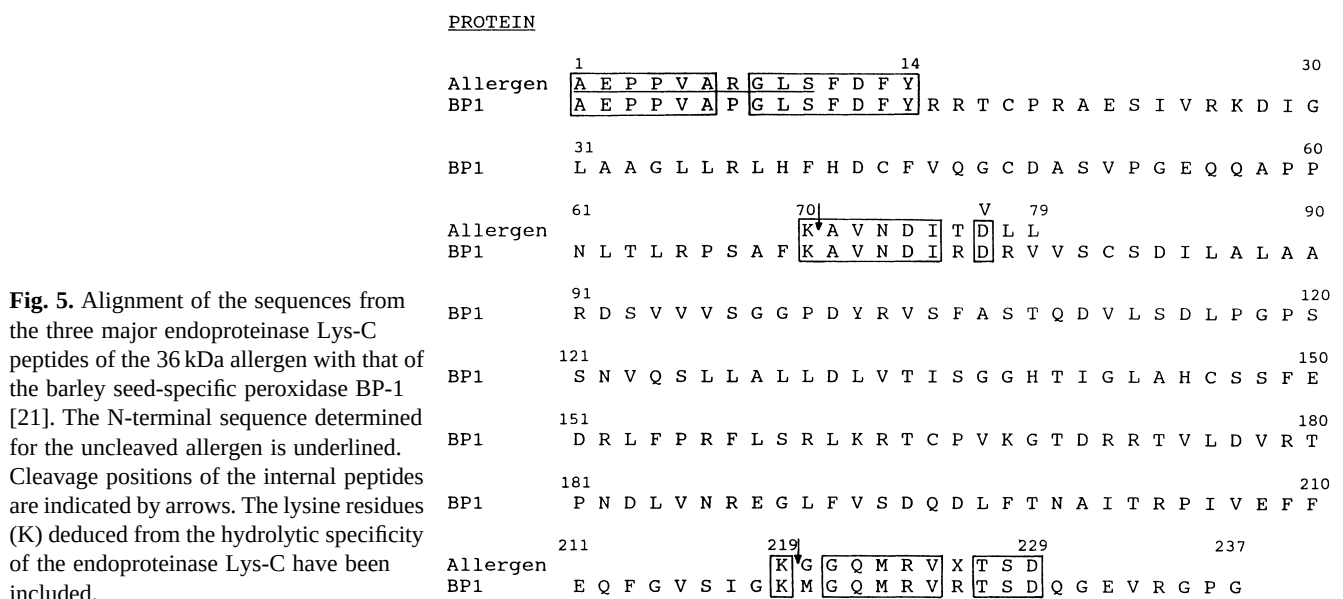
identity with the barley peroxidase BP1 [21]. The horseradish enzyme reacted with 7/10 sera, showing strong responses with some of them. IgE-binding by this peroxidase have been previously reported using sera from wheat-induced [13] and olive pollen-induced [23] asthmatic patients.

IgE-binding glycoproteins of 36 kDa, presumable peroxidases, are also present in bread wheat and barley flour

To test whether other cereal species had IgE-binding glycoproteins of around 35 kDa (putative homologues of the purified allergen), salt extracts of barley and bread wheat were fractionated by SDS-PAGE. Separated bands were then immunodetected with the serum specific for complex glycans and assayed for IgE-binding with the pool of sera from wheat-induced asthmatic patients (Fig. 6). A band of the expected molecular size was recognized by the two sera, both in barley and bread wheat. These results suggest that the flour peroxidases from both cereals are also active IgE-binding components.

Discussion

A survey (SDS-PAGE followed by immunodetection) of more than 40 flour samples from different wheat species has shown very similar IgE-binding patterns, with major bands between 30 and 60 kDa. A putative allergen of around 35 kDa, previously detected by several groups [6–8], is very prominent in most samples and the main one in several Einkorn wheats, such as *T. monococcum* UP-1 (Figs 1, 2 and 6). Consequently, we used flour of this line to isolate



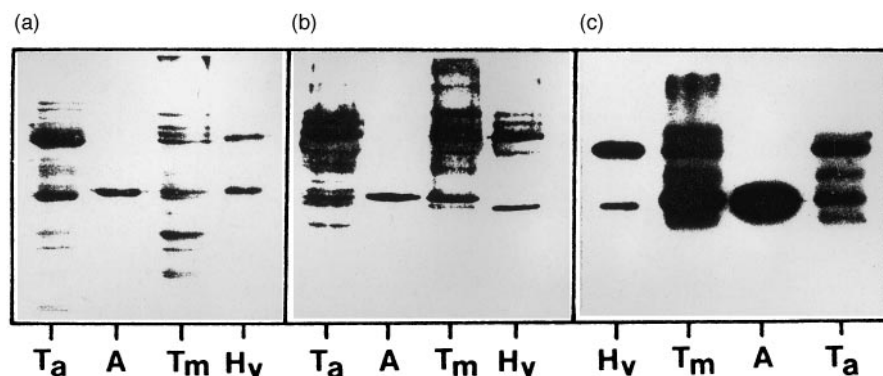


Fig. 6. SDS-PAGE fractionation of salt extracts from barley (H_v), bread (T_a) and *monococcum* (T_m) wheats, and the purified allergen (A). Separated bands were stained with Coomassie blue (a) and immunoblotted with a serum specific for complex glycans (b) or with a pool of sera from baker's asthma patients and ^{125}I -labelled antihuman IgE (c). Samples correspond to 1 μg (allergen) and 10 μg (salt extracts). Only the appropriate portions of the gels are shown.

the allergenic protein(s) included in the 35 kDa band. Moreover, this diploid species (genomes AA), has a significantly lower genetic complexity than bread wheat (genomes AABBDD), and, therefore, a lesser level of protein redundancy. For these reasons, Einkorn wheat flour should be, in principle, a suitable starting material to purify proteins that are widely distributed among diploid, tetraploid (pasta) and hexaploid (bread) wheats. Furthermore, the diploid state of its genome, could support higher genetic gains during selection to lower the concentrations of allergens present in flour, either by conventional breeding or by genetic engineering.

The purified allergen is a 36 kDa protein with complex glycan(s) attached to asparagine residues. Most likely, it includes a single molecular species (or a very limited number of isoforms with highly similar sequences), as judged by two-dimensional analysis (Fig. 4c). Several preparations of the purified protein had its N-terminus blocked, a common observation for plant peroxidases that may explain why Posch *et al.* [6] have failed to sequence 37 kDa IgE-binding components from bread wheat.

Enzymatic cleavage of the isolated allergen and amino acid sequencing of the major peptides obtained, have provided enough information to identify it as a seed-specific peroxidase. This was further confirmed by testing the allergen enzymatic activity. BP1, a well characterized homologue from barley flour [21,22,24], is similar in molecular size (37 kDa; 309 amino acid residues), the nature of its asparagine-linked glycan, and its low specific activity with various peroxidase substrates. Although BP1 equivalents (homologues) have been suggested in wheat [24], the 36 kDa allergen purified here is the only wheat homologue so far characterized.

The allergenic capacity of the purified protein has been further tested using ten individual sera from baker's asthma patients. More than 50% showed a positive IgE-binding response with the allergen, supporting the strong reaction obtained with the pool of sera used to follow the protein through the isolation process. Preliminary *in vivo* studies

by skin-prick tests have also shown a high proportion (around 60%) of positive reactions using both the isolated allergen and the whole salt extract from *monococcum* wheat (Armentia, Sanchez-Monge and Salcedo; unpublished results).

IgE-mediated crossreactions among cereal flours are well known [7,8,25], but very few of the involved allergens have been characterized (an example are homologous members of the α -amylase inhibitor family present in wheat, barley and rye) [10,11]. Seed (flour)-specific peroxidases, such as the 36 kDa allergen described here, are candidates to explain, in part, these cross-reactions. In fact, IgE-binding glycoproteins with similar size have been detected in barley and bread wheat with the pool of sera used throughout this study (Fig. 6). Furthermore, a commercial horseradish peroxidase, which has the same type of attached glycans than BP1 and the purified allergen, but less than 50% of sequence identity and very different enzymatic specificity, displayed positive reactions with most of the individual sera tested. This result is in agreement with recent findings from our laboratory [13] indicating that certain N-linked sugar residues present in plant and insect, but not mammalian, complex glycans are largely responsible for the allergenicity of the proteins which carry them. Most likely, the complex glycan(s) attached to the wheat allergen, BP1 and horseradish peroxidase are involved in the crossreactions presented in these study (Table 2 and Fig. 6). To test this hypothesis, the wheat allergen was treated with PNGase A, an enzyme that is able to remove at least some $\alpha 1 \rightarrow 3$ fucose-containing complex glycans such as those commonly found in plant glycoproteins [26]. However, this experiment was not conclusive since no deglycosylation of the purified protein was observed under conditions in which the single complex glycans of BMAI-1, a well characterized barley allergen [13,20], was partially removed. On the other hand, chemical deglycosylation methods may alter the peptide backbone as well, thus leading to spurious results [13].

The data presented here indicate that cereal flour peroxidases are relevant in IgE-mediated hypersensitive

reactions, and suggested that proteins with this enzymatic activity should be considered as potential allergens in other plant materials.

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References

- Baldo BA, Wrigley CW. Allergies to cereals. *Adv Cereal Sci Technol* 1984; 6:289–356.
- Chang-Yeung M, Lam S. Occupational asthma: state of art. *Am Rev Resp Dis* 1986; 133:686–703.
- Sandiford CP. Cereal-induced occupational asthma. *Clin Exp Allergy* 1996; 26:128–31.
- Blands J, Diamant W, Kallos P, Kallos-Deffner L, Løwenstein H. Flour allergy in baker's. Identification of allergenic fractions in flour and comparison of diagnostic method. *Int Arch Allergy Appl Immunol* 1976; 52:392–406.
- Baldo BA, Wrigley CW. IgE antibodies to wheat flour components. *Clin Allergy* 1978; 8:109–24.
- Posch A, Weiss W, Wheeler C, Dunn MJ, Görg A. Sequence analysis of wheat grain allergens separated by two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1995; 16:1115–9.
- Sandiford CP, Tee RD, Newman-Taylor AJ. Identification of crossreacting wheat, rye, barley and soya flour allergens using sera from individuals with wheat-induced asthma. *Clin Exp Allergy* 1995; 25:340–9.
- Varjonen E, Savolainen J, Mattila L, Kalimo K. IgE-binding components of wheat, rye, barley and oats recognized by immunoblotting analysis with sera from adult atopic dermatitis patients. *Clin Exp Allergy* 1994; 22:481–9.
- Walsh BJ, Howden MEH. A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping. *J Immunol Methods* 1989; 121:275–80.
- Armentia A, Sanchez-Monge R, Gomez L, Barber D, Salcedo G. In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin Exp Allergy* 1993; 23:410–5.
- García-Casado G, Armentia A, Sanchez-Monge R, Malpica JM, Salcedo G. Rye flour allergens associated with baker's asthma. Correlation between in vivo and in vitro activities and comparison with their wheat and barley homologues. *Clin Exp Allergy* 1996; 26:428–35.
- Carbonero P, Salcedo G, Sanchez-Monge R et al. A multigene family from cereals which encodes inhibitors of trypsin and heterologous α -amylase. In: Aviles FX, ed. *Innovations on proteases and their inhibitors*. Berlin: Walter de Gruyter, 1993: 333–48.
- García-Casado G, Sanchez-Monge R, Chrispeels MJ et al. Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* 1996; 6:471–7.
- Sandiford CP, Tee RD, Newman-Taylor AJ. The role of cereal and fungal amylases in cereal flour hypersensitivity. *Clin Exp Allergy* 1994; 24:549–57.
- Borghi B, Castagna R, Corbellini M, Heun M, Salamini F. Breadmaking quality of Einkorn wheat (*Triticum monococcum* ssp *monococcum*). *Cereal Chem* 1996; 73:208–14.
- Sanchez-Monge R, García-Casado G, Malpica JM, Salcedo G. Inhibitory activities against heterologous α -amylases and in vitro allergenic reactivity of Einkorn wheats. *Theor Appl Genet* 1996; 93:745–50.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Smith PK, Krohn RI, Hermanson GT et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985; 150:76–85.
- Lauriere M, Lauriere C, Chrispeels MJ, Johnson KD, Sturm A. Characterization of a xylose-specific antiserum that reacts with the complex asparagine-linked glycans of extracellular and vacuolar glycoproteins. *Plant Physiol* 1989; 90:1182–8.
- Sanchez-Monge R, Gomez L, Barber D et al. Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the α -amylase inhibitor family have enhanced IgE-binding capacity. *Biochem J* 1992; 281:401–5.
- Johansson A, Rasmussen SK, Harthill JE, Welinder KG. cDNA, amino acid and carbohydrate sequence of barley seed-specific peroxidase BP1. *Plant Mol Biol* 1992; 18:1151–61.
- Andersen MB, Johansson T, Nyman PO, Welinder KG. Substrate specificity and manganese stimulation of plant and fungal peroxidases. In: Lowarzewski J, Greppin H, Gastar T, Penel C, eds. *Biochemical, molecular and physiological aspects of plant peroxidases*. University of Geneva, Geneva, 1991:169–73.
- Batanero E, Villalba M, Monsalve RI, Rodriguez R. Cross reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J Allergy Clin Immunol* 1996; 97:1264–71.
- Rasmussen SK, Welinder KG, Hejgaard J. cDNA cloning, characterization and expression of an endosperm-specific barley peroxidase. *Plant Mol Biol* 1991; 16:317–27.
- Baldo BA, Krilis S, Wrigley CW. Hypersensitivity to inhaled flour allergens. Comparison between cereals. *Allergy* 1980; 35:45–56.
- Faye L, Johnson KD, Sturm A, Chrispeels MJ. Structure, biosynthesis, and function of asparagine-linked glycans on plant glycoproteins. *Physiol Plant* 1989; 75:309–14.