

ORIGINAL ARTICLE

A new approach to the isolation and characterization of wheat flour allergens

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Summary

Background The incidence of food allergy to wheat is increasing. Its diagnosis depends on the purity of major allergens and their inclusion in tests. Isolation and characterization of wheat allergens are therefore of utmost importance.

Objective To purify and identify wheat flour allergens most frequently recognized by patients' IgE antibodies and to study their allergenicity.

Methods Water/salt-soluble extracts from wheat flour were prepared and separated using a combination of ultrafiltration, isoelectric focusing and liquid chromatography. Purified proteins were analysed by immunoblotting using pooled sera from patients with atopic dermatitis who possessed IgE specific to wheat. Wheat proteins found to bind IgE were subsequently identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. The frequency and intensity of IgE binding of isolated proteins were tested using individual sera from patients and controls.

Results We developed a procedure that allows isolation of wheat allergens from natural sources. Twenty-seven potential wheat allergens have been successfully identified; of these, the following seven are newly reported in food allergy: endogenous α -amylase/subtilisin inhibitor, trypsin/ α -amylase inhibitor (AAI) CMX1/CMX3, thaumatin-like protein (TLP), xylanase inhibitor protein-1, β -glucosidase, class II chitinase and 26 kDa endochitinase. TLP and wheatwin were shown to activate patients' basophils to a similar extent as two well-known allergens, lipid transfer protein (Tri a 14) and AAI 0.19 (Tri a 28.0101).

Conclusion and Clinical Relevance Our new approach enables the isolation of water/salt-soluble wheat allergens in their native form in amounts sufficient both for biological testing (*in vivo* and *in vitro*) and for physicochemical characterization. Such studies will lead to a more detailed knowledge of allergenicity of wheat proteins and to improved accuracy of diagnostic tests.

Keywords basophil activation, IgE antibodies, isolation, purification, wheat allergens

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Introduction

Wheat is an important part of human diet, but its ingestion can trigger IgE-mediated food allergies. The incidence of food allergies is increasing and these allergies are thought to affect 6–8% of children and 1–3% of adults [1, 2]. Depending on the route of allergen exposure, hypersensitivity reactions to wheat may cause a variety of symptoms. For example, ingestion may cause atopic dermatitis, gastrointestinal symptoms or wheat-dependent exercise-induced anaphylaxis; inhalation may cause baker's asthma; and skin contact may occasionally trigger IgE-mediated contact urticaria [3–5].

Diagnosis of food allergy to wheat is not simple. Although oral food challenge tests are considered to be good standard for diagnosis, they are time consuming and can induce severe clinical symptoms. Detection of food-specific IgE antibodies (Abs) by radioimmunoassays or by enzymatic immunoassays, along with a history compatible with IgE-mediated symptoms, are highly sensitive but have low specificity. Moreover, a certain degree of cross-reactivity cannot be excluded [6, 7]. Skin prick tests (SPTs) are diagnostic for baker's asthma, but not for atopic dermatitis [8]. In contrast, basophil activation tests are considered to be a reliable *in vitro* diagnostic technique [9].

Poor predictability and specificity of all currently known diagnostic approaches may be associated with the insufficient purity of wheat extracts used in specific IgE assays or with the lack of inclusion of all major allergens in these extracts. Furthermore, expression of IgE-reactive proteins depends on wheat seed maturation and varies in different wheat sources [10].

On the basis of their differential solubility, wheat proteins can be classified into water/salt-soluble albumins and globulins and water/salt-insoluble gliadins and glutenins. Proteins from all these different groups are responsible for baker's asthma as well as food allergies in adults and children. Wheat food allergy in children and adult patients with multiplicity and diversity of skin symptoms, such as wheat-induced atopic dermatitis, has been investigated by several laboratories [11–13]. Proteomic analysis of wheat flour proteins revealed a number of IgE reactive components in both water/salt-soluble and insoluble fractions [shown by immunoblotting, high-performance liquid chromatography (HPLC) and mass spectrometric analysis] [14–16]. Among the major allergens identified in the water/salt-soluble fraction of wheat flour and shown to be capable of sensitization after both ingestion and inhalation are the cereal α -amylase inhibitors (AAI) and α -amylase/trypsin inhibitors (AATI). This AATI family of inhibitors consists of tetrameric proteins, often called chloroform methanol (CM) proteins on the basis of their selective extraction in chloroform/methanol mixtures (CM1, CM2, CM3, CM16 and CM17). On the other hand, the three AAIs are dimeric (0.19 and 0.53) or monomeric (0.28). Wheat amylase/subtilisin inhibitor is a bifunctional protein capable of simultaneously inhibiting endogenous wheat α -amylase and the proteinase subtilisin [17, 18]. Using IgE from allergic individuals to probe cDNA expression library, AAI 0.19 and CM7, as well as new molecules with high cross-reactive potential, including thioredoxins and β -expansins, have been identified [19].

Cross-reactivity among wheat flour allergens, other cereal allergens (barley, rye, rice and maize) and grass pollen allergens is thought to be because of common IgE-reactive epitopes. However, patients with baker's asthma and those with IgE-mediated food allergy to wheat may be sensitive to different molecules [20–22]. The most important plant panallergens are the non-specific lipid transfer proteins (LTPs), which also act as food allergens, sensitizing individuals by the oral route or by inhalation [6, 23, 24]. Although proteomic analysis has identified other wheat proteins as IgE binding (e.g. β -amylase, peroxidase, thioredoxin and serpins), a proper panel of purified wheat allergens has not yet been developed. The list of these allergens is not complete yet and new allergens are still being discovered. Moreover, the clinical relevance of these proteins remains to be determined [14, 16, 25].

Recombinant allergens may be useful in diagnosing and/or treating allergies [26, 27], but so far only a small number of wheat allergens have been cloned and produced in recombinant form [28, 29]. Many recombinant allergens do not have the same immunological characteristics as their natural counterparts. Alterations of the amino acid sequence of allergens may influence protein folding, resulting in the reduction or total prevention of IgE binding. Several recombinant allergens have been produced in bacterial expression systems as non-glycosylated proteins, although some natural AAI are glycosylated [30]. Thus, highly purified natural allergens are still indispensable in determining the allergenicity of recombinant and natural forms of allergens. Purified allergens are needed not only for the analysis of their structure and function but also in experimental model studies [31–34].

The aim of our study was to isolate, characterize and identify the most important water/salt-soluble wheat allergens from natural sources. Therefore, we developed a three-step isolation protocol, consisting of ultrafiltration, native (liquid-phase) isoelectric focusing (IEF, Rotofor[®], Bio-Rad, Hercules, CA, USA) and affinity chromatography (HPLC). Wheat proteins recognized by IgE of allergic patients were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry. The intensity and frequency of IgE binding of both previously and newly identified potential allergens were determined, as well as – in some cases – their potential to activate basophils.

Materials and methods

Patients and controls

Sera were obtained from 22 patients (13 females, nine males; mean age 23 years; range: 3–63 years) with atopic dermatitis (P1–22). All 22 patients had a suspect history of wheat allergy and elevated specific IgE Abs (mean, 10.24 U/mL; range, 0.4–72 U/mL). An open challenge test was performed with cooked wheat pasta in 14 out of 22 patients and all 14 were positive. In the remaining eight patients, a significant clinical improvement was observed after elimination diet (avoidance of wheat-containing food for 1 month). Total serum IgE and IgE specific for wheat flour, gluten, wheat pollen, timothy grass pollen and birch tree pollen were analysed using an Immulite 2000 (DPC, Siemens Medical Solution Diagnosis, Flanders, NJ, USA). Sera from 10 patients allergic to pollen allergens (D1–10) were used as 'disease controls'. Clinical, demographic and serological characteristics of the 22 patients (P1–22) and 10 disease controls (D1–10) are reported in Table 1. Sera from nine healthy individuals with normal total and wheat-specific IgE Abs were used as negative controls (C1–9).

Table 1. Demographic, clinical and serological characteristics of patients and disease controls

Patient	Sex	Age	sIgE wheat (U/mL)	Total IgE (U/mL)	Symptoms	Open challenge	Other allergies	Disease control	Sex	Age	sIgE wheat (U/mL)	Total IgE (U/mL)
P1	F	5	72.00	355	EA	Pos.	e, m	D1	M	32	<0.10	350
P2	F	6	56.10	1545	EA, RC	Pos.	e, m	D2	M	43	<0.10	345
P3	F	39	4.13	5568	A, EA	ND	bu, f, mo, nu, ri	D3	F	34	0.20	106
P4	F	43	2.60	563	A, EA	Pos.	c, m, po, sh	D4	F	22	<0.10	364
P5	F	10	12.60	1083	A, EA, RC	Pos.	hdm, mo, po	D5	F	32	<0.10	12
P6	M	28	13.30	3590	D, EA, RC	Pos.	ap, nu, po	D6	F	49	<0.10	33
P7	F	29	15.00	21452	A, EA, RC	ND	c, d, hdm	D7	M	36	0.20	634
P8	M	25	1.86	14953	A, EA, RC	ND	hdm	D8	M	24	<0.10	1021
P9	F	63	1.86	45	EA, RC	Pos.	po	D9	F	44	<0.10	54
P10	F	21	3.40	2686	AB, EA, OAS, RC	Pos.	ca, ce, po	D10	F	46	<0.10	49
P11	F	33	3.97	2501	A, EA, OAS, RC	ND	ap, ca, co, nu					
P12	M	8	2.93	2404	EA, RC	Pos.	po					
P13	M	3	1.29	854	EA	Pos.	e, m					
P14	F	20	0.40	272	EA, RC	ND	po					
P15	M	5	3.55	1524	EA	Pos.	e, m					
P16	F	44	1.35	222	EA, IU	ND	co, e, po, sp					
P17	M	6	13.10	482	EA	Pos.	c, e, fr, ho, hon, m, mo, nu, po, se, ve					
P18	F	32	10.80	4331	A, D, EA, OAS, RC	Pos.	fr, hdm, mo, nu, po, se, ve					
P19	M	24	1.14	1757	EA, RC	ND	co, hdm, nu, po					
P20	M	6	0.70	546	A, EA, RC	Pos.	c, hdm, po					
P21	F	4	0.78	147	EA, RC	Pos.	e, m, nu, po					
P22	M	43	2.47	423	OAS, RC	ND	ap, po					

F, female; M, male; ND, not done; pos., positive; sIgE, specific IgE; U/mL, unit antigen per millilitre; A, asthma bronchiale; D dyspepsia; EA, eczema atopicum; IU, idiopathic urticaria; OAS, oral allergic syndrome; RC, rhinoconjunctivitis; a, animals; ap, apple; bu, buckwheat; c, cat; ca, carrot; ce, celery; co, codfish; d, dog; e, egg white; f, fish; fr, fruit; hdm, house dust mite; hon, honey; ho, horse; m, milk; mo, mould; nu, nuts; po, pollen; se, seeds; sh, shrimp; sp, spices; ri, rice; ve, vegetables.

Measurements of total and specific serum immunoglobulin E

The concentrations of total and wheat-specific IgE were determined by fluorescence enzyme immunoassay (Immulite 2000, DPC, Siemens Medical Solution Diagnosis) according to the manufacturer's instructions. Allergen-specific IgE concentrations above 0.35 kU/L were considered positive.

Wheat sample preparation

Unless otherwise specified, all chemicals were from Sigma (Steinheim, Germany). Wheat flour from cultivar Akteur (*Triticum aestivum*) was kindly provided by Professor J. Petr of the Czech University of Agriculture, Prague. Proteins were extracted from wheat flour (4 g) with PBS (40 mL) overnight at 4 °C, ultrasonicated (U50, IKA Laborartortechnik, Staufen, Germany) for 15 min on ice and centrifuged at 20 000 g for 15 min at 4 °C. Extracted proteins were sequentially separated using Amicon Ultra 100k, 30k and 10k ultrafiltration devices (Millipore, Billerica, MA, USA) in this order providing gross separation according to the molecular weight of the proteins.

A 10 mL sample containing 0.35% CHAPS was loaded onto the 100k filter device with a 100 kDa molecular-weight cut-off for 30 min at 2500 g and 20 °C. The concentrated higher molecular-weight fraction was discarded and the filtrate was ultrafiltered using a 30k filter device with a 30 kDa molecular-weight cut-off. The concentrate was stored (fraction I) and the filtrate was purified using a 10k filter device with a 10 kDa molecular-weight cut-off, yielding fraction II (60 min, 2500 g and 20 °C). Protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (BCA, Pierce, IL, USA). Samples were divided into aliquots and stored at −20 °C.

Liquid-phase isoelectric focusing

Proteins in fractions I and II were separated according to their pI using a Rotofor[®] preparative cell (Bio-Rad). Fraction I contained proteins of molecular mass above 30 kDa and below 100 kDa, whereas fraction II contained proteins of molecular mass above 10 kDa and below 30 kDa. Proteins from fraction I (15 mL, containing 45 mg protein) were mixed with 0.5% CHAPS and diluted in

55 mL of distilled water containing 5% glycerol, 5% premixed Bio-Lyte pH 5–8, pH 3–10 (Bio-Rad) and ampholyte pH 4–6 (Fluka, Buchs, Switzerland). Proteins in fraction II were diluted similarly, except that premixed ampholytes pH 4–6 and pH 6–9 were used. Both samples were fractionated using a Rotofor[®] device, equipped with a standard focusing chamber (60 mL total volume) with 20 fractionation compartments. A constant power of 15 W was applied to the system, which was cooled to 4 °C with a water circulator, with fractionation completed within 4 h. A total of 20 fractions were collected, and the pH of each was measured using a microelectrode.

High-performance liquid chromatography

All separations were carried out using an HPLC system Gold 125NM solvent Module (Beckman Coulter, Miami, FL, USA). The sample was centrifuged for 10 min at 12 000 g. The supernatant was applied (containing 2 mg protein) to a polymeric reversed-phase PLRP-S 1000A, 8 µm, 250 × 406 mm column (Varian, Palo Alto, CA, USA) and separated at a flow rate of 1 mL/min using a linear gradient (solvent A, 0.1% TFA/H₂O; solvent B, 0.1% TFA/acetonitrile). The gradient program started with 95% of solvent A and 5% of solvent B, and changed 5–25% B/5 min, 25–45% B/50 min, 45–70% B/5 min and 70–95% B/0.5 min. The column was washed with 95% solvent B (10 min) and equilibrated at initial conditions for 10 min. Protein samples were evaporated using a vacuum centrifuge (SpeedVac, Jouan, Winchester, VA, USA) and dissolved in PBS.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Each of the Rotofor[®] and HPLC fractions was analysed by SDS-PAGE (in concentration 1 mg/mL) using 15% gradient gels under reducing conditions (with 0.5 M DTT). Separated proteins were stained with Coomassie[®] Brilliant Blue R-250 (CBB). Selected fractions were electrotransferred to nitrocellulose membranes (NC2, SERVA, Heidelberg, Germany) for 1 h at room temperature. The membranes were blocked with PBS containing 0.5% Tween 20 and incubated with serum samples diluted 1:10 in blocking buffer overnight at 4 °C. After washing with PBS–0.1% Tween 20, the strips were incubated with peroxidase-labelled sheep anti-human IgE Abs (1:1000; The Binding Site, Birmingham, UK) for 1 h at room temperature. Blots were developed using SuperSignal West Pico Trial kits (Pierce) and luminescence was detected on a Kodak Medical X-ray film (Kodak, Rochester, NY, USA). Data were digitized and subjected to image analysis (AIDA 3.28, Raytest, Straubenhardt, Germany). Relative signal intensities derived from the chemiluminescent signal of IgE reaction with purified proteins were compared and corrected for local background signal (set as value 1). The resultant intensity is

expressed as nine intervals (0–8; corresponding to intensity 1 → 801). Data are expressed as median values and 25% and 75% percentile.

Tryptic digestion and matrix-assisted laser desorption/ionization mass spectrometry

CBB-stained proteins were excised from gels, cut into small pieces and washed several times with 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, the proteins were reduced with 30 mM TCEP at 65 °C for 30 min and alkylated by 30 mM iodoacetamide for 60 min in the dark. The gel pieces were further washed with de-ionized water, shrunk by dehydration in MeCN and reswollen in water. The supernatants were removed and the gels were partly dried in a SpeedVac concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and sequencing-grade trypsin (100 ng; Promega, Madison, WI, USA). After overnight digestion, the resulting peptides were extracted with 40% MeCN/0.5% TFA. A solution of α -cyano-4-hydroxycinnamic acid in aqueous 50% MeCN/0.1% TFA (5 mg/mL) was used as an MALDI matrix. A 0.5 µL aliquot of a sample was deposited onto the MALDI target and allowed to air-dry at room temperature, followed by the addition of 0.5 µL of the matrix solution.

MALDI mass spectra were measured using an Ultraflex III instrument (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam[™] solid-state laser and LIFT[™] technology for MS/MS analysis. The spectra were acquired in the mass range of 700–4000 Da and calibrated internally using monoisotopic $[M+H]^+$ ions of trypsin autoproteolytic fragments (842.5 and 2211.1 Da).

Protein identification

Peak lists in XML data format were created using the flexAnalysis 3.0 program with the SNAP peak detection algorithm. No smoothing was used, and the maximum number of assigned peaks was set at 50. After peak labelling, all known contaminant signals were manually removed. The peak lists were searched using the MASCOT search engine against the SwissProt 57.13 or Trembl 40.13 database subset of green plant proteins with search settings including a peptide tolerance of 30 p.p.m., a missed cleavage site value of 2, and variable carbamidomethylation of cysteine and oxidation of methionine. There were no restrictions on protein molecular weight or pI. We identified proteins with a MOWSE score over a threshold of 57 (SwissProt) or 71 (Trembl). If the score was only slightly higher than the threshold value or the sequence coverage was too low, we confirmed the identity of the protein candidate by MS/MS analysis. In addition to the above MASCOT settings, a fragment mass tolerance of 0.6 Da and instrument-type MALDI-TOF-TOF was applied

to search MS/MS spectra. Amino acid sequences of identified proteins were analysed using the multiple sequence alignment software Clustal W (<http://www.ebi.ac.uk/clustalw>).

Basophil activation test

Flow cytometric basophil activation tests were performed after stimulation of blood cells with four isolated wheat allergens [AAI 0.19, LTP, thaumatin-like protein (TLP) and wheatwin] or control (PBS, anti-IgE), as described previously [14]. The same blood samples were obtained from the patients, disease controls and healthy donors (Table 1). Briefly, 100 µL of heparinized whole blood and 10 µL of stimulation buffer were incubated (30 min at 37 °C) containing commercially available wheat extract (1000 IC/mL Stallergens, Antony Cedex, France) or selected allergens (concentration 1 mg/mL) diluted 1 : 10.

After incubation, the samples were transferred on ice and stained with mAb anti-(cluster differentiation) CD203/PE (Beckman Coulter) and anti-CD63/FITC (Exbio, Prague, Czech Republic) for 15 min. Erythrocytes were lysed using ammonium chloride. After washing the cells, we measured the percentage of activated basophils expressing CD63 and CD203c by flow cytometry (FC500, Beckman Coulter). Before this study, the basophil activation test had been performed in a pilot study in patients (seven per allergen) with AAI 0.19, LTP, TLP and wheatwin for four increasing dilutions (1 : 2, 1 : 10, 1 : 50 and 1 : 100). On the basis of these data, allergen dilution 1 : 10 was selected as optimal. No basophil activation could be induced in the controls. The ROC analysis of basophil activation was performed for all four proteins to determine the optimal cut-off values. Values above the cut-off, 9% of activated basophils for AAI 0.19 and LTP, 11.9% for TLP and 16% for wheatwin, were considered as positive.

Results

Preparation of salt-soluble wheat proteins and fractionation in Rotofor® cells

Salt-soluble proteins of molecular mass ranging from 5 to 120 kDa were extracted from the flour of the wheat cultivar Akeur (*T. aestivum*) were separated using Amicon 100k, 30k and 10k filter devices and were characterized by SDS-PAGE (Fig. 1a; lane 0). Protein aggregates were efficiently removed in the step with Amicon 100k filter device. Fraction 1 consisted mainly of proteins with molecular mass between 100 and 30 kDa, whereas fraction II contained only low-molecular-mass components below 30 kDa.

Proteins in fraction I (Fig. 1a, lane I) were further purified in the Rotofor® cell. Liquid-phase IEF considerably purified wheat proteins and yielded 20 fractions

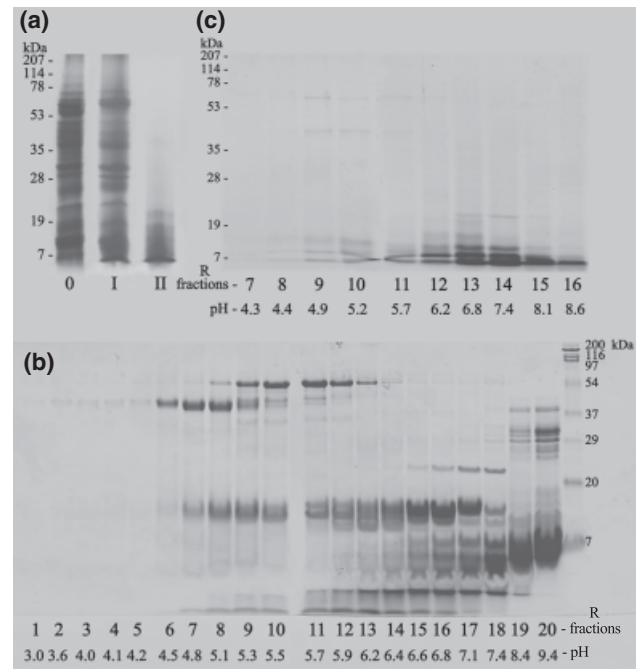


Fig. 1. SDS-PAGE analysis of wheat flour PBS extracts (a) and Rotofor® fractions (b and c). (a) Spectrum of wheat flour proteins extracted with PBS (0) and purified using Amicon 100k and 30k filter devices (fraction I). Fraction I was concentrated using a 10k filter device (fraction II). (b and c) Proteins from fractions (b) I and (c) II were separated by liquid-phase isoelectric focusing in Rotofor® cell into 20 fractions (R1–R20). All gels were stained with Coomassie brilliant blue.

(R1–R20) with pH ranging from 3.0 to 9.4, as documented by SDS-PAGE (Fig. 1b). Fractions R6–R13 contained predominantly protein bands of 50 kDa, fractions R19 and R20 contained proteins of 20–40 kDa, fractions R7–R18 contained proteins of molecular mass below 20 kDa and fractions R11–R20 were enriched in low-molecular-mass components below 10 kDa. Because of their high protein content and their reactivity with IgE Abs from patients' sera (data not shown), eight Rotofor® fractions (R7, R11, R14–R18 and R20) were selected for further purification by HPLC.

Similarly, proteins from fraction II were separated in the Rotofor® cell into 20 fractions with pH ranging from 3.7 to 9.5. Proteins were detected in nine fractions (R8–R16) and were enriched in fractions R13 and R14 (Fig. 1c). Four Rotofor® fractions (R9, R10, R13 and R14) were selected for purification by HPLC.

Purification and identification of major wheat allergens by high-performance liquid chromatography and mass spectrometry

Selected Rotofor® fractions were further purified by HPLC (PLRP-S column) and the resulting fractions were

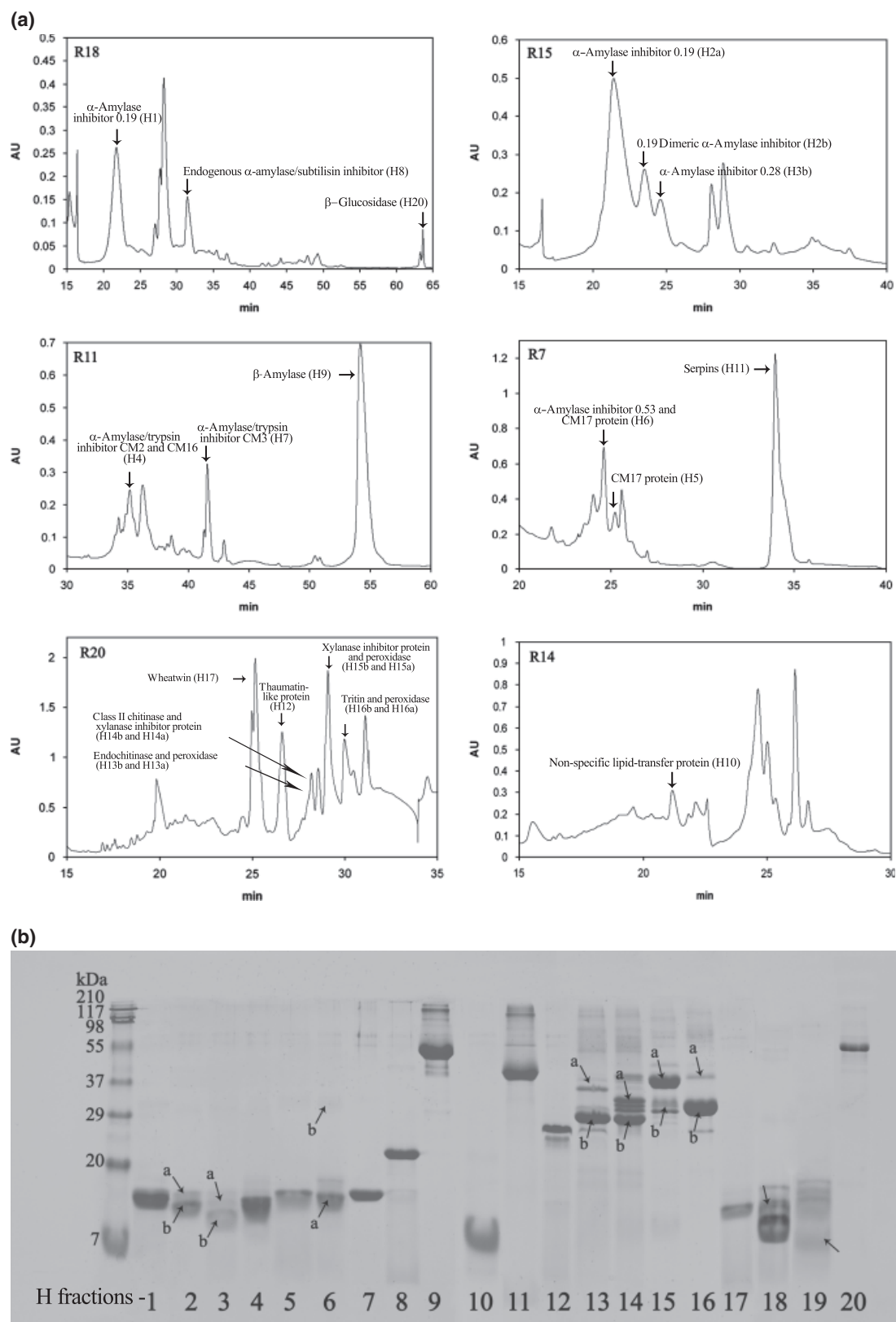


Fig. 2. (a) Examples of the chromatographic profiles of Rotoform[®] fractions R18, R15, R11, R7 and R20 (shown in Fig. 1b) and fraction R14 (shown in Fig. 1c) obtained from reversed-phase HPLC. The HPLC fraction numbers (H1–H20) and the peaks with arrows indicate purified and identified wheat proteins corresponding to those listed in Table 2. (b) Coomassie blue staining of selected wheat proteins purified by HPLC and separated by SDS-PAGE. The HPLC fraction numbers indicate the bands corresponding to the allergens identified in Table 2.

Table 2. Isolated wheat proteins identified by MS analysis (MALDI-TOF)

H fractions	Protein (allergen) name	Accession no.	MW (kDa)	pI	Protein yield (mg)	Peptides matched	Sequence coverage (%)	MS/MS confirmation	R fraction
1	α -Amylase inhibitor 0.19 (Tri a 28.0101)	IAA1_WHEAT	13.30	6.60	1.73	8	74	LAHSEWCR HGAQEGQAGTGAFPR	18
2a	α -Amylase inhibitor 0.19 (Tri a 28.0101)	IAA1_WHEAT	13.30	6.60	0.61	4	44	No	15
2b, 3a	0.19 dimeric α -amylase inhibitor	Q5UHH6_WHEAT	13.20	6.50		8	59	No	15
3b	α -Amylase inhibitor 0.28 (Tri a aAI)	IAA2_WHEAT	16.80	7.40		8	54	No	15
6a	α -Amylase inhibitor 0.53 (Tri a 28)	IAA5_WHEAT	13.20	5.20	0.16	8	54	No	7
6b	α -Amylase inhibitor 0.53 (Tri a 28)	IAA5_WHEAT	13.20	5.20		4	47	LQCNQSQVPEAVLR EHGVSQAGTGAFPSGR	7
6a	CM 17 protein	Q41540_WHEAT	16.00	5.00		5	35	No	7
6b	CM 17 protein	Q41540_WHEAT	16.00	5.00		4	23	SRPDQSLMELPGCPR	7
5	CM 17 protein	Q41540_WHEAT	16.00	5.00	<0.10	8	39	No	7
18	α -Amylase/trypsin inhibitor CM1	IAAC1_WHEAT	15.50	7.50	<0.10	2	24	No	11
4	α -Amylase/trypsin inhibitor CM2 (Tri a 29)	IAAC2_WHEAT	15.50	6.80	0.10	5	29	No	11
4	α -Amylase/trypsin inhibitor CM16 (Tri a CM16)	IAC16_WHEAT	15.80	5.30		10	45	No	11
7	α -Amylase/trypsin inhibitor CM3 (Tri a 30)	IAAC3_WHEAT	18.20	7.40	0.20	10	69	No	11
19	Trypsin/ α -amylase inhibitor CMX1/CMX3	IACX1_WHEAT	13.80	9.20	<0.10	2		EFIAGIVGR EITYESLNACAEYAVR	17
8	Endogenous α -amylase/subtilisin inhibitor	IAAS_WHEAT	19.60	6.70	0.34			WHIDSELVSGR STEWHIDSELVSGR	18
13b	26 kDa endochitinase 1	CHI1_HORVU	33.40	8.50	<0.10	10	44	No	20
14b	Class II chitinase	Q4Z8L8_WHEAT	28.20	8.60	<0.10	13	73	No	20
10	Non-specific lipid-transfer protein (Tri a 14)	NLTP1_WHEAT	11.90	8.50	0.33	3	30	No	14 (Fig. 1c)
17	Wheatw1n-1	WHW1_WHEAT	15.60	7.60	0.51	5	45	No	20
17	Wheatw1n-2	WHW2_WHEAT	15.90	8.20		6	56	No	20
12	Thaumatococcus-like protein	Q8S4P7_WHEAT	23.60	7.80	0.71	12	55	No	20
9	β -Amylase	AMYB_HORVU	60.00	5.60	0.23	7	17	No	11
11	Serpin-Z1A	SPZ1A_WHEAT	43.10	5.60	<0.10	7	27	No	7
	Serpin-Z1B	SPZ1B_WHEAT	43.00	5.40		6	24	No	
	Serpin-Z1C	SPZ1C_WHEAT	42.80	5.60		5	20	No	
	Serpin-Z2A	SPZ2A_WHEAT	43.30	5.40		11	41	No	
	Serpin-Z2B	SPZ2B_WHEAT	43.00	5.20		5	15	No	
13a, 15a, 16a	Peroxidase 1	Q8LK23_WHEAT	38.80	8.10	0.16	23	57	No	20
16b	Tritin (rRNA N-glycosidase)	Q07810_WHEAT	29.60	9.70		15	66	No	20
14a, 15b	Xylanase inhibitor protein 1	XIP1_WHEAT	33.20	8.60		21	61	No	20
20	β -Glucosidase	Q40025_HORVU	57.40	7.20	<0.10			LDFVWYEPHSDSNADQAAQR	18

Protein yield corresponds to the amount of proteins isolated from 15 mL ultrafiltrated sample (containing 45 mg protein), separated by one Rotofor run and purified by HPLC.

The allergenic molecules are named by the International Union of the Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee.

The H fraction numbers refer to Fig. 2 and the R fraction numbers refer to Fig. 1b.

Tri a, *Triticum aestivum*.

analysed by SDS-PAGE. Figure 2a shows the chromatograms of six Rotofor[®] fractions (five obtained from Amicon fraction I – R7, R11, R15, R18 and R20, and one from fraction II – R14). All HPLC fractions were analysed by immunoblotting using patients' pooled sera (data not shown) and fractions containing separated IgE-binding proteins were selected for identification by MALDI-TOF mass spectrometry. Using these methodological approaches and protein database searches, we succeeded in identifying 27 wheat proteins (Table 2). These IgE-binding proteins were detected in 20 HPLC fractions, H1–H20. SDS-PAGE analysis and Coomassie blue staining of these fractions (Fig. 2b) revealed six well-resolved peaks (fractions H1, H7, H8, H10, H12 and H17), each corresponding to a single protein band of sufficient yield (Table 2). The first peak (H1) corresponded to AAI, AAI 0.19, which had been eluted uncontaminated after HPLC separation of Rotofor[®] fraction R18 (Figs 2a and b). Rotofor[®] fractions R14–R17 contained AAI 0.19 with decreasing amounts of 0.19 dimeric AAI (H2b) and AAI 0.28 (H3b) proteins (see R15 Fig. 2a). Other identified proteins, such as wheatwin (H17) and TLP (H12), were observed only in Rotofor[®] fraction R20. Protein AATI CM3 (H7) was identified in all Rotofor[®] fractions, except for R20, separated by HPLC, whereas endogenous protein AATI (H8) was found in Rotofor[®] fractions R14–R18. LTP (H10) was isolated primarily from Rotofor[®] fraction R14, which had been obtained especially from fraction II. The other HPLC fractions (H13, H14, H15 and H16) contained mixtures of proteins, including peroxidase, tritin, xylanase inhibitor protein (XIP), endochitinase and class II chitinase. HPLC fraction H4 contained proteins AATI CM2 and CM16 and fraction H6 contained AAI 0.53 and CM17 protein. HPLC fraction H11 contained a mixture of five members of the serpin family. Fractions H5, H9, H18 and H19 contained one major IgE-binding protein together with small amounts of other proteins. Fraction H20 contained a low amount of β -glucosidase. Out of the 27 identified proteins, the following seven are new potential wheat allergens: endogenous α -amylase/subtilisin inhibitor, trypsin/AAI CMX1/CMX3, TLP, XIP-1, β -glucosidase, class II chitinase and 26 kDa endochitinase.

Reactivity of patients' immunoglobulin E antibodies with isolated proteins

To characterize the intensity and frequency with which IgE Abs bind the individual wheat components, 17 purified HPLC fractions (H1–H17) were immunoblotted with sera from all 22 patients, 10 disease controls and nine healthy controls. An example of the reactivity of IgE from one patient (P1), one disease control (D1) and one healthy control (C8) with isolated wheat proteins is shown in Fig. 3. The data summarized in Table 3 document the individual diversity of IgE response. However, all patients

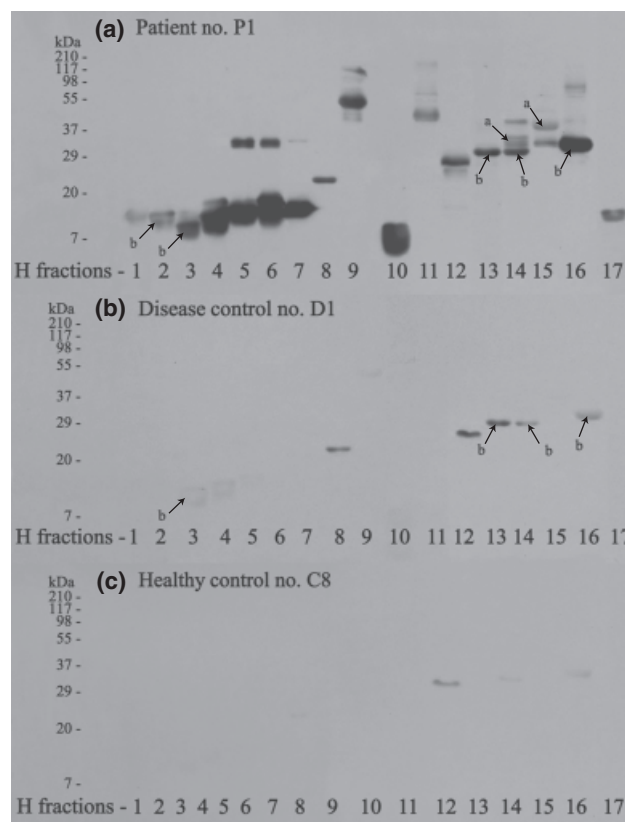


Fig. 3. An example of IgE immunoblotting of purified wheat proteins with the sera of (a) one patient, (b) a disease control and (c) a healthy control. The HPLC fraction numbers indicate the bands corresponding to the allergens identified in Table 2.

sera tested (100%) showed positive IgE reactions with α -amylase inhibitors AAI 0.28 (H3b), AATI CM16 and CM2 (H4), TLP (H12) and tritin (H16b). Of the 22 sera, 20 or 21 (91% or 95%) reacted with CM17 (H5), CM17 and AAI 0.53 (H6), 26 kDa endochitinase (H13b), XIP (H14a) and class II chitinase (H14b). The frequency of recognition of other wheat proteins was also high ranging from 54% to 86%. The densitometric evaluation of intensity of IgE binding on immunoblots documents the differences of binding of individual wheat proteins (Table 3). The IgE of patients reacted with the highest intensity (median 6.0–8.0) with the following already known allergens: AATI CM16 and CM2 (H4), CM17 and AAI 0.53 (H6), CM17 (H5) and tritin (H16b), as well as with the newly identified IgE-binding wheat components TLP (H12), 26 kDa endochitinase (H13b) and class II chitinase (H14b). In contrast, the lowest binding intensity was determined for AATI CM3 (H7) and wheatwin (H17) (median 1 and 2, respectively).

We also performed IgE immunoblotting of purified proteins with sera from 10 disease controls. Endogenous AAI (H8) and tritin (H16b) were recognized by seven sera (70%); other proteins were recognized by <50% sera or did not react. Nevertheless, the intensity of IgE binding

Table 3. Reactivity of IgE antibodies of patients and disease controls on immunoblot analysis with selected proteins

Protein name	0.19		AATI		Endo-genous		LTP		Serpins		TLP		26 kDa endochitinase		XIP		Class II chitinase		Peroxidase		Tritin		Wheatwain	
	AAI 0.19	AAI dimeric	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28	AAI 0.28
H fraction	1	2b	3b	4	5	6	7	8	9	10	11	12	13b	14a	14b	15a	16b	17	17	17	17	17	17	17
Patients																								
P1	1	3	7	8	8	8	8	4	8	8	3	5	5	3	5	3	8	4						
P2	5	4	6	8	6	4	4	6	4	6	0	8	6	1	6	3	6	7						
P3	2	2	3	8	6	8	0	2	5	0	1	6	5	3	5	0	1	1						
P4	1	1	2	8	8	8	0	1	0	0	0	7	6	8	7	4	5	0						
P5	7	5	4	8	6	5	1	4	7	1	5	8	8	8	8	8	8	4						
P6	8	7	5	8	5	8	3	6	8	3	8	8	8	8	8	8	8	7						
P7	8	5	4	8	4	8	0	4	7	4	3	8	8	8	8	8	7	3						
P8	8	7	8	8	8	8	4	6	4	8	ND	8	8	8	8	8	8	3						
P9	3	4	7	8	2	2	2	4	1	4	0	8	7	3	7	3	8	4						
P10	5	5	6	8	3	8	1	3	7	6	5	8	7	8	7	8	6							
P11	3	2	3	8	5	7	0	0	0	0	ND	2	3	8	3	8	5	0						
P12	4	3	5	6	6	5	0	1	0	2	ND	3	2	5	2	1	3	0						
P13	1	1	3	7	7	8	0	2	3	0	ND	7	6	8	6	5	6	0						
P14	6	7	8	8	2	2	1	4	3	2	ND	8	7	3	7	0	8	0						
P15	4	3	8	8	5	5	1	6	5	6	ND	8	8	2	8	3	8	0						
P16	5	4	7	8	0	0	0	3	1	0	ND	5	4	6	4	3	8	1						
P17	0	0	2	ND	8	8	0	0	0	0	ND	4	2	6	2	6	5	0						
P18	0	0	4	ND	0	0	0	1	0	0	ND	1	0	0	0	0	7	0						
P19	0	0	3	4	2	1	0	0	0	0	ND	3	3	0	3	0	4	0						
P20	8	8	8	8	8	8	7	6	8	8	ND	8	8	5	8	8	8	0						
P21	8	7	5	5	8	8	2	5	8	7	ND	8	7	3	7	5	8	3						
P22	8	8	8	8	8	8	7	7	8	8	ND	8	7	7	7	7	8	8						
Median	4.5	4	5	8	6	8	1	4	4	2.5	3	8	6.5	5.5	7	4.5	8	2						
IQR	1-8	1.75-7	3-7.25	8-8	2.75-8	3.5-8	0-3.25	1-6	0-7.25	0-6.25	0-5	4.75-8	3.75-8	3-8	3.75-8	2.5-8	5-8	0-4.5						
Disease controls																								
D1	0	0	0	0	0	0	0	1	0	0	0	2	2	0	1	0	1	0						
D2	0	0	0	0	0	0	0	1	0	0	0	0	ND	0	ND	0	1	0						
D3	0	0	3	3	0	0	0	1	0	0	ND	3	3	0	3	1	3	2						
D4	0	1	3	3	0	0	0	1	0	0	ND	2	1	1	1	1	3	1						
D5	1	1	4	ND	0	0	0	2	1	0	ND	3	3	3	3	0	5	0						
D6	1	1	2	ND	ND	ND	0	2	1	0	ND	3	2	0	2	2	3	0						
D7	1	2	4	ND	ND	ND	0	1	0	0	ND	0	ND	0	ND	0	5	0						
D8	0	0	0	ND	0	0	0	0	0	0	ND	0	0	0	0	1	0	0						
D9	0	0	0	ND	0	0	0	0	0	0	ND	0	0	0	0	1	0	0						
D10	0	0	0	ND	0	0	0	0	0	0	ND	0	0	0	0	0	0	0						
Median	0	0	1	1.5	0	0	0	1	0	0	0	1	1.5	0	1	0.5	2	0						
IQR	0-1	0-1	0-3.25	0-3	0-0	0-0	0-0	0-1.25	0-0.25	0-0	0-0	0-3	0-2.75	0-0.25	0-2.75	0-1	0-3.5	0-0.25						

Range of chemiluminescence intensity: 0, 1-100; 1, 101-200; 2, 201-300; 3, 301-400; 4, 401-500; 5, 501-600; 6, 601-700; 7, 701-800; 8, 801 <.

The resultant chemiluminescence intensity is corrected for local background signal (set as value 1) and expressed as an interval of measured values.

The H fraction numbers refer to Table 1.

AAI, α -amylase inhibitor; AATI, α -amylase/trypsin inhibitor; LTP, non-specific lipid-transfer protein; TLP, thaumatin-like protein; XIP, xylanase inhibitor protein; ND, not done; IQR: interquartile range, 25-75 percentile.

calculated for disease controls was low (median 0–2). None of the sera from the nine healthy donors reacted with any of the isolated proteins, except for low non-

specific reactions of secondary anti-human IgE Ab with TLP (H12) and tritin (H16b) (Fig. 3c).

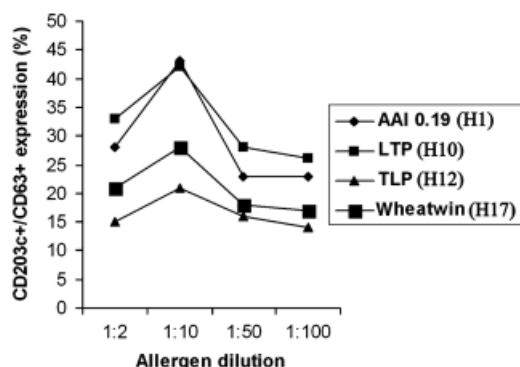


Fig. 4. CD203c/CD63 expression of basophils from seven patients in a dose-response curve after incubation with allergens: AAI 0.19 (H1), LTP (H10), TLP (H12) and wheatwin (H17) (dilution: 1 : 2, 1 : 10, 1 : 50 and 1 : 100).

Table 4. Basophil activation test (%)

Protein name	AAI 0.19	LTP	TLP	Wheatwin	Wash buffer (negative control)	Stimulation with anti-IgE (positive control)
H fraction	1	10	12	17		
Patients						
P1	80	89	ND	ND	6	91
P2	89	57	84	70	10	92
P3	ND	ND	14	45	5	51
P4	32	27	22	22	13	53
P5	17	19	ND	ND	2	85
P6	7.7	30	34	30	5	88
P7	ND	ND	19	31	12	77
P8	ND	ND	11	22	10	87
P9	ND	ND	15	19	11	49
P10	9.1	9.2	3.3	7.2	3	71
P11	ND	ND	15	24	5	96
P12	3.3	6.5	ND	ND	2	93
P13	ND	ND	12	19	10	79
P14	12	9.5	ND	ND	7	76
P15	26	25	16	21	8	62
P16	ND	ND	22	11	10	60
P17	ND	ND	12	27	6	19
P18	ND	ND	2.3	7.9	2	42
P19	ND	ND	1.7	2.1	1.5	95
P20	5.2	4.6	ND	ND	4	62
P21	ND	ND	20	21	3	45
P22	ND	ND	9.4	8.2	4	66
Healthy controls						
C1	6.5	5.9	ND	14	4.5	61
C2	7.3	7.5	10	12	4.4	75
C3	14	6.5	6.7	10	4.1	49
C4	8.7	8.1	10	13	8	41
C5	1.7	12	7.1	9.5	1.5	45
C6	2.4	2.7	1.5	1.7	1	55
C7	ND	ND	11	6.1	6	45
C8	ND	ND	8.2	4.3	3	57
C9	ND	ND	11	14	9	58
cut-off	9	9	11.9	16		

Comparison of the identified α -amylase inhibitors

Of the 27 identified wheat allergens, 11 belonged to the AAI family. These inhibitors could be divided into three groups based on sequence homology obtained from the NCBI database (compared by Clustal W program). The first group consisted of four inhibitors, AAI 0.19, 0.53, 0.28 and 0.19 dimeric; the second consisted of five proteins, AATI CM1, CM2, CM3, CM16 and CM17; and the third one consisted of two inhibitors, endogenous AAI and AATI CMX1/CMX3. Homology among the proteins in the first group ranged from 55% to 94%. Inhibitors from the third group showed very low sequence homologies with other proteins. Independently on the degree of structural homology, each of these inhibitors was found to bind

patients' IgE Abs, but the intensities of their binding varied (Table 3).

Activation of basophils from allergic patients with purified allergens

The biological activity of purified allergens was tested using the basophil activation test. Blood samples were obtained from 22 patients with clinical symptoms of wheat allergy and with positive basophil activation tests to a commercial wheat extract, from 10 disease controls and from nine healthy donors. These blood samples were exposed to four isolated wheat proteins, AAI 0.19 (H1), LTP (H10), TLP (H12) and wheatwin (H17). A bell-shaped dose-response curve for all four allergens was obtained (Fig. 4) and the optimal dilution was selected (1 : 10). Positive cell activation was detected in response to LTP in eight out of 10 patients (80%), to AAI 0.19 in seven out of 10 patients (70%) and to TLP and wheatwin in 12 out of 17 patients (71%) (Table 4).

We compared the number of patients positive for allergens AAI 0.19, LTP, TLP and wheatwin in either IgE immunoblot or in the basophil activation test (analysing both IgE binding and cell activation). We have shown that for all allergens tested in IgE immunoblot, the proportion of positive cases ranged from 60% to 100%. This proportion was somewhat lower in the basophil activation test and the range was more narrow (70–80%).

Discussion

Extensive analysis of allergenic molecules is necessary for allergy diagnosis and treatment. We developed a new, rapid procedure for isolation of wheat flour allergens in amounts allowing subsequent structural and functional analysis. We succeeded in purifying relevant IgE binding proteins from a water/salt-soluble extract of wheat flour using Amicon devices, Rotofor[®] and HPLC techniques.

The advantage of Rotofor[®] (protein separation based on pI value – preparative IEF) is its capacity to optimally fractionate milligrams to grams of total protein. However, the apparent pI of Rotofor[®] fractions may not exactly match the predicted pI of separated proteins; these proteins may be rather spread over two or more fractions [35]. With isolation of natural non-denatured allergen epitopes in mind, we used native (liquid-phase) preparative IEF that resulted in substantial protein enrichment and provided the basis for a successful isolation of native allergens. The purification of individual water/salt-soluble wheat proteins from their mixtures included the preparative reversed-phase HPLC. The purified proteins were subsequently analysed by SDS-PAGE, and IgE-binding proteins (selected by immunoblot) were identified by MALDI-TOF mass spectrometry. This isolation scheme yielded 27 IgE-binding wheat proteins, including the

following previously described proteins: AAI (Tri a 28; Tri a 28.0101; Tri a 29; Tri a 30; Tri a CM16 and Tri a aAI), non-specific LTP (Tri a 14), peroxidase, serpins and β -amylase (identified on 1D or 2D electrophoresis – 14, 16, 30). Some of them were purified here for the first time. In addition, we newly identified the following potential allergens: endogenous α -amylase/subtilisin inhibitor, trypsin/AAI CMX1/CMX3, TLP, XIP-1, β -glucosidase, class II chitinase and 26 kDa endochitinase as associated with food allergy. The fact that some of these proteins do not match wheat components identified so far (such as 26 kDa endochitinase, β -glucosidase) could be explained by the lack of knowledge of the complete genome. The corresponding protein/peptide sequences of wheat may be missing in the current databases.

The findings of the reactivity of patients' IgE with AAI are not unambiguous. For instance, although the endogenous α -amylase/subtilisin inhibitor has not been reported as an allergen [25], we observed a reactivity of this purified protein with IgE Abs from 19 out of the 22 patients, albeit with only medium intensity (median 4). In previous studies, purified AATI CM3 but not CM2 or CM16 reacted with IgE from patients [17], and SPTs were positive with CM3 and CM16 but not CM2 in a patient with anaphylactic reaction to wheat flour [36]. We found that the most frequently recognized AAIs were 0.28 AAI and a mixture of CM16 and CM2, all of them reacting with IgE Abs of our patients. However, the frequency of IgE binding with other inhibitors, such as 0.19 AAI, 0.19 dimeric and CM17 was also high.

We succeeded in identifying and purifying wheat LTP, a food panallergen that is also a major allergen associated with baker's asthma [23]. We found that IgE Abs of 64% patients in our cohort reacted with our purified wheat LTP, which is a higher percentage than previously reported (41% and 28%) [16, 37]. We also purified tritin (a 29.6 kDa ribosome-inactivating protein from *T. aestivum*) previously identified from the water/salt-insoluble fraction of raw wheat [16]; its clinical relevance has not yet been clarified. This wheat component was recognized by all our patients (22/22) with high binding intensity (median 8), and also by some of our disease controls (7/10, median 2).

TLP is one of the successfully isolated new IgE-binding wheat proteins associated with food allergy. In our cohort, TLP was recognized by IgE Abs of all 22 patients and the intensity of IgE binding (median 8) was significantly higher when compared with disease controls (median 1). TLP, as well as wheatwin, are members of the family of pathogenesis-related proteins, which play a role in plant defence. TLP is an important allergen in several fruits, such as apples, cherries, kiwis and grapes [38–40], and wheat TLP has recently been reported as the allergen in baker's respiratory allergy [41]. We showed previously that wheatwin-2 is an IgE immunoreactive protein [14], and the results presented here showed that wheatwin-1

and wheatwin-2 have sequence homology of 97%. Using MALDI-TOF mass spectrometry, we observed at least two unique peptides for each wheatwin sequence (data not shown). Furthermore, we have shown for the first time that newly identified wheat components, such as TLP and wheatwin bind to IgE from patients and can activate patients' basophils to an extent comparable with that of the well-characterized allergens, AAI 0.19 (Tri a 28.0101) and LTP (Tri a 14).

In addition, we purified other new IgE-binding wheat components, such as XIP, class II chitinase and 26 kDa endochitinase, which were not previously identified as allergens in individuals with food allergy (XIP was identified in patients with baker's asthma [41]). These components reacted strongly (median 5.5–7.0) with the vast majority of patients' IgE Abs (20/22, 21/22 and 21/22, respectively) and with a lower intensity (median 0–1.5) and frequency (2/10, 5/8 and 5/9) with IgE of disease controls.

In conclusion, using preparative liquid-phase IEF and HPLC, we have developed a new procedure enabling us to purify native forms of wheat allergens in amounts sufficient for further structural and functional studies. The advantage of this approach is that it can be easily modified for isolation of various allergens (by changing pI range in Rotofor® or HPLC elution profile). We purified 27 IgE-binding wheat components, including seven new ones. We have shown for the first time that purified allergens, such as AAI 0.19, LTP, TLP and wheatwin, can

activate patients' basophils, confirming that our purified proteins maintain their biological activity.

Interestingly, wheat proteins, CM17 (H5), CM17 and AAI 0.53 (H6) as well as newly identified TLP (H12), 26 kDa endochitinase (H13b), XIP (H14a) and class II chitinase (H14b), were shown to belong to those reacting with the highest frequency and intensity with IgE of patients (median 5.5–8) and with low intensity with IgE of disease controls (median 0–1). These proteins may be candidates for clinically relevant wheat allergens.

Our isolation procedure will allow preparation of native allergens for studies of their structural features (linear and conformational epitopes), including the impact of post-translational modifications (e.g. glycosylation) on allergenicity. Better knowledge of the allergenicity of native proteins could lead to a selection of an appropriate vector for recombinant allergen preparation, improving both diagnostic tests and therapy.

Acknowledgements

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