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Molecular and Immunological Characterization of Tri a 36, a Low Molecular Weight Glutenin, as a Novel Major Wheat Food Allergen

Alexandra Baar,^{*,†} Sandra Pahr,^{*,†} Claudia Constantin,^{*} Sandra Scheiblhofer,[‡] Josef Thalhamer,[‡] Stavroula Giavi,[§] Nikolaos G. Papadopoulos,[§] Christof Ebner,[¶] Adriano Mari,^{||} Susanne Vrtala,^{*,†} and Rudolf Valenta^{*,#}

Wheat is an essential element in our nutrition but one of the most important food allergen sources. Wheat allergic patients often suffer from severe gastrointestinal and systemic allergic reactions after wheat ingestion. In this study, we report the molecular and immunological characterization of a new major wheat food allergen, Tri a 36. The cDNA coding for a C-terminal fragment of Tri a 36 was isolated by screening a wheat seed cDNA expression library with serum IgE from wheat food-allergic patients. Tri a 36 is a 369-aa protein with a hydrophobic 25-aa N-terminal leader peptide. According to sequence comparison it belongs to the low m.w. glutenin subunits, which can be found in a variety of cereals. The mature allergen contains an N-terminal domain, a repetitive domain that is rich in glutamine and proline residues, and three C-terminal domains with eight cysteine residues contributing to intra- and intermolecular disulfide bonds. Recombinant Tri a 36 was expressed in *Escherichia coli* and purified as soluble protein. It reacted with IgE Abs of ~80% of wheat food-allergic patients, showed IgE cross-reactivity with related allergens in rye, barley, oat, spelt, and rice, and induced specific and dose-dependent basophil activation. Even after extensive in vitro gastric and duodenal digestion, Tri a 36 released distinct IgE-reactive fragments and was highly resistant against boiling. Thus, recombinant Tri a 36 is a major wheat food allergen that can be used for the molecular diagnosis of, and for the development of specific immunotherapy strategies against, wheat food allergy. *The Journal of Immunology*, 2012, 189: 000–000.

Wheat is one of the most grown cereals worldwide and represents one of the most important components of our nutrition. However, it also contains several Ags that give rise to immunologically mediated hypersensitivity diseases of the gastrointestinal tract, of which celiac disease, a classical type IV hypersensitivity reaction mediated by T cells, was attributed to wheat ingestion already in 1953 (1). Much later, IgE Abs specific for wheat Ags were found in patients with wheat

hypersensitivity (2), and evidence for IgE-mediated allergy was provided for certain clinical cases (3). Today, wheat is established as an important food allergen source, particularly for children (4). After ingestion wheat can give rise to a variety of organ-specific allergic symptoms, including gastrointestinal, oral, cutaneous, and respiratory symptoms but also to severe systemic and life-threatening reactions such as anaphylaxis (5–7). To identify and characterize the disease-causing allergens, attempts were made to separate, by protein chemical techniques, wheat into different fractions and to probe them with IgE Abs from allergic patients (8). Although to date several wheat food allergens have been identified, including α -, β -, γ -, and ω -gliadins, low m.w. and high m.w. glutenin subunits (LMW-GSs and HMW-GSs, respectively) (9, 10), α -amylase inhibitor subunits (11–15), and lipid transfer protein (10, 16), the panel of wheat allergens is still incomplete (17).

The availability of the disease-causing allergens is not only important to study the mechanisms of wheat food allergy, but also to improve diagnosis and therapy (18). The structure of wheat allergens can be either identified by proteomic approaches (19) or by molecular cloning technologies (20). Diagnostic tests based on recombinant wheat allergens may be useful to discriminate between different forms of wheat hypersensitivity (21). Furthermore, similar to approaches used for respiratory forms of allergy, it should be possible to develop immunotherapy strategies for food allergy that are based on recombinant allergens or recombinant hypoallergenic allergen derivatives (18).

To isolate major wheat food allergens, we used a molecular discovery approach. A wheat seed cDNA library was screened with serum IgE from wheat food-allergic patients. One of the isolated IgE-reactive clones represented the C-terminal part of the LMW glutenin GluB3-23, which was expressed as complete recombinant

^{*}Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology, and Immunology, Medical University of Vienna, Vienna General Hospital, 1090 Vienna, Austria; [†]Christian Doppler Laboratory for the Development of Allergen Chips, Medical University of Vienna, 1090 Vienna, Austria; [‡]Department of Molecular Biology, University of Salzburg, 5020 Salzburg, Austria; [§]Allergy and Immunology Research Center, University of Athens, Athens 115 27, Greece; [¶]Ambulatory for Allergy and Clinical Immunology, 1090 Vienna, Austria; ^{||}Centro di Allergologia Molecolare, Istituto Dermatologico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico, 00167 Rome, Italy; and [#]Christian Doppler Laboratory for Allergy Research, Medical University of Vienna, 1090 Vienna, Austria

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Address correspondence and reprint requests to Prof. Rudolf Valenta, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology, and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, 3Q, A-1090 Vienna, Austria. E-mail address: rudolf.valenta@meduniwien.ac.at

Abbreviations used in this article: HMW, high m.w.; HMW-GS, high m.w. glutenin subunit; HSA, human serum albumin; LMW, low m.w.; LMW-GS, low m.w. glutenin subunit; RAST, radioallergosorbent test; RBL, rat basophil leukemia; SPT, skin prick test; WSE, wheat seed extract.

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allergen in *Escherichia coli* and identified as a major wheat food allergen. The characterization of this new major wheat allergen designated Tri a 36, which is a protein resistant to digestion and heating and represents a cross-reactive allergen in various crop species, is reported.

Materials and Methods

Biological materials, patients' sera, and Abs

Wheat seeds were obtained from the Austrian Agency for Health and Food Safety (Vienna, Austria) and planted in a greenhouse. Immature seeds were harvested 7, 10, 15, 20, 25, 30, and 35 d after onset of pollination directly into liquid nitrogen and stored at -80°C until use (20). Rye, barley, oat, spelt, and rice grains produced by Alnatura (Bickenbach, Germany) were bought at a local drugstore. Human serum albumin (HSA) was purchased from Behring (Marburg, Germany) and *E. coli* strain BL21 (DE3) was from Stratagene (La Jolla, CA).

The wheat cDNA library was screened with a serum pool consisting of sera from three patients with confirmed wheat food allergy (i.e., patients showed gastrointestinal and/or systemic symptoms such as diarrhea, urticaria, and asthma that could be directly related to intake of wheat). Sera used for testing the frequency of IgE recognition of rTri a 36 were obtained from 24 patients (nos. 1–7, 9–20, and 22–26) with wheat food allergy clearly attributable to the ingestion of wheat and 2 patients (nos. 8 and 21) who showed only positive IgE serology without proven clinical symptoms to wheat (Table I). Each of the 24 wheat food-allergic patients had presented systemic reactions (anaphylactic shock and/or urticaria) that could be unambiguously attributed to ingestion of wheat or wheat-containing products (Table I). IgE-mediated sensitization to wheat was confirmed by positive skin prick test (SPT) and/or positive IgE serology (ImmunoCAP from Phadia, Uppsala, Sweden, or Immulite from Siemens Medical Solution Diagnostics, Flanders, NJ) to wheat. Data regarding additional sensitizations, symptoms, and serological results are displayed in Table I. Additionally, rTri a 36 was tested for IgE reactivity with sera from 19 grass pollen-allergic patients (Table II) who presented a case history of seasonal grass pollen allergy and positive SPT reactions and/or specific IgE Abs to grass pollen extract (MAST CLA; Hitachi Chemical Diagnostics, Mountain View, CA). For control purposes, sera from patients suffering from

baker's asthma ($n = 60$) and nonallergic persons were included in the experiments as described in Constantin et al. (20).

The serum used for IgE cross-reactivity/IgE inhibition assays and for digested rTri a 36 was from a wheat food-allergic female patients (radioallergosorbent test [RAST] class 6 to wheat) who had suffered from asthma and diarrhea after wheat ingestion and showed equal IgE reactivity to rTri a 36 and rTri a 36_{191–369}. All serum samples represented residual samples from routine diagnosis and were analyzed in a retrospective and anonymized manner with permission from the Ethical Committee of the Medical University of Vienna.

Specific rabbit Abs were raised against purified rTri a 36 and rTri a 36_{191–369} by immunization of rabbits (Charles River, Sulzfeld, Germany) (20).

Isolation and cloning of a wheat cDNA coding for a LMW glutenin, GluB3-23

A wheat cDNA library was screened with sera from three patients with confirmed wheat food allergy symptoms as described (20). The cDNA of the IgE-reactive clone 175 was compared with sequences submitted to the GenBank database at the National Center for Biotechnology Information. A complete sequence identity was found with the DNA coding for the C-terminal portion of a LMW wheat glutenin, GluB3-23. The coding region coding for aa 191–369 of GluB3-23 was amplified by PCR (GoTaq green mix; Promega, Madison, WI) using the following primer pair and the clone 175 cDNA as a template: forward primer, 5'-GGGAGTTCATATGGT-TCTACCGCAACAACAAATACCATTTG-3'; reverse primer, 5'-CGGAAT-TCCATAGTGGTGGTGGTGGTGGTAGGAACCAACTCCGGTGCCA-AC-3' (Eurofins MWG Operon, Ebersberg, Germany).

The PCR product contained the restriction enzyme sites NdeI (bold type) and EcoRI (underlined) and a hexahistidine tag encoding sequence (italics). The amplified DNA sequence was confirmed by sequencing (Eurofins) and then cloned into the expression vector pET17b (Novagen, Madison, WI). A synthetic gene with optimized codon usage for *E. coli* expression coding for the complete mature GluB3-23 protein (GenBank accession number EU369705.1) and a C-terminal hexahistidine tag was cloned into pET 17b by using NdeI and EcoRI restriction enzyme sites (ATG:biosynthetics, Merzhausen, Germany). The sequence of the IgE-reactive GluB3-23 protein was submitted to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee and designated Tri a 36.

Table I. Demographic, clinical, and serological characterization of patients suffering from wheat food allergy and wheat-sensitized individuals (nos. 8 and 21)

ID	DOB	Sex	Symptoms	Allergic Reaction after	SPT Wheat	Other Allergies	IgE Serology					rTri a 36
							Total IgE	Wheat	Gluten	Egg White	Milk	
1	2006	F	a, AD	DBPCFC	4.5	cm	483	66.3	55.6	5.31	10.1	+
2	2004	F	a, AD	DBPCFC	6	cm, e, l	74	7.57	10.1	1.65	17	+
3	2006	M	a, AD	Infant cream	5	e	123	9.2	11.4	0.55	Not done	–
4	1996	M	a, AD, As	Bread	Not done	cm	636	>200	>200	Not done	Not done	+
5	2008	F	u, AD, As	Wheat soup	4	cm	172	6.5	12.9	10.4	>100	+
6	2007	M	a, AD	Infant cream	8	cm, e	165	11.7	11	4.67	Not done	+
7	2009	M	u, AD	Infant cream	2.5	cm	Not done	6.04	Not done	0.72	0.53	+
8	2008	F	AD	No information	0	cm	107	10.2	Not done	6.06	0.39	–
9	2004	F	AD, As	Bread	10	cm, e, f	1818	124	330	114	82.7	+
10	2009	M	a, AD	Wheat soup	3	cm	13.43	0.11	Not done	1.08	0.68	+
11	2004	M	u, g, AD, As	Wheat soup	7	cm, e	189	49	42.3	3.42	4.7	+
12	2009	M	u	Baby cereal	3	no	57.4	3.77	3.32	0	0.37	+
13	2007	M	a, AD	Pasta	4	cm, l	349	8.09	4.67	11.5	42.5	+
14	2005	M	u, AD	Baby cream	7	f	1575	>100	85.1	15.5	Not done	+
15	2007	F	u, AD	Wheat soup	3	cm, e	59.4	3.1	Not done	22.5	4.15	+
16	2007	F	u, AD, As	Pasta	7.5	cm, f	795	165	135	13.6	16.3	+
17	2008	M	a, AD	Pasta	9	no	Not done	Not done	Not done	Not done	Not done	+
18	2008	M	a, AD, As	Pasta	9	no	1994	>100	>100	47	>100	+
19	2005	M	a, AD, As	Yogurt with cereals	7	e, l	2723	695	455	Not done	73.5	+
20	2007	M	u, AD	Bread	4.5	no	1710	8 (i)	8.2 (i)	163 (i)	33.8 (i)	+
21	2005	M	AD, As	No information	Not done	cm, e, f, l	2724	48	Not done	>100	67	–
22	1998	F	u, AD, As	Baby cereal	11	p, n	4539	>100	>100	35.1	Not done	+
23	2007	M	a, AD	Cereals, soups	5	e, l	129	7.95	4.87	8.29	Not done	+
24	2007	M	a, AD, As	Open challenge	5	cm, n	202	9.29	2.8	4.71	61.3	+
25	2000	M	u, AD	Bread	Not done	cm	39	0.36	<0.35	0.39	5.99	–
26	2005	M	u, AD	Bread	5	no	Not done	7.24	0.04	Not done	Not done	+

+, positive; –, positive or negative IgE reactivity to Tri a 36; a, Anaphylaxis, AD, atopic dermatitis; As, asthma; cm, cow's milk; DBPCFC, double-blind placebo-controlled food challenge; DOB, date of birth; e, egg; F, female; f, fish; g, gastrointestinal; (i), immulite; l, other legume; M, male; n, nut/tree nut; p, peanut; u, urticaria.

Table II. Demographic, clinical, and serological characterization of grass pollen-allergic patients

ID	DOB	Sex	Symptoms	IgE Serology MAST CLA			
				Grass Pollen	Wheat Flour Extract	Symptoms after Wheat Ingestion	Other Allergies
1	1985	F	Dry eyes, cough	>300	0	No	bi, b, w, dm
2	1988	M	RC	>300	99	No	dm
3	1978	M	RC	>300	0	No	no
4	1947	F	RC	>300	0	No	bi, a
5	1981	F	AD, asthma	>300	52	No	bi, ah, dm, a, sf, ba
6	1984	M	C	>300	0	No	No information
7	1982	F	RC, asthma	>300	0	No	dm
8	1986	M	RC, asthma	>300	0	No	bi, ah, dm
9	1984	M	RC	>300	0	No	dm
10	1981	M	No information	>300	0	No	No information
11	1982	M	RC, R, itching eyes	>300	>300	Yes	bi, ah, n, ca, ce, a, m, s, wh
12	1984	M	R, asthma	>300	0	No	ah, dm
13	1983	F	Itching eyes and nose	>300	175	No	b, w, ah
14	No information	F	No information	>300	0	No	No information
15	No information	M	No information	>300	98	No	No information
16	1958	F	RC	>300	30	No	bi, dm, n, se
17	1960	F	RC	>300	74	No	No information
18	1971	F	RC, AD	>300	>300	No	dm, n, ah
19	1981	M	RC	>300	242	No	No information

a, Apple; AD, atopic dermatitis; ah, animal hair; b, bee; ba, banana; bi, birch; C, conjunctivitis; ca, carrot; ce, celery; dm, dust mite; F, female; M, male; m, melon; n, nut; R, rhinitis; RC, rhinoconjunctivitis; s, soy; se, sesame; sf, stone fruit; w, wasp; wh, wheat.

Expression, purification, and characterization of clone 175-derived rTri a 36₁₉₁₋₃₆₉ and Tri a 36

The pET 17b-C175 construct and the pET 17b-GluB3-23 construct were transformed into *E. coli* BL21 (DE3). The transformed cells were grown in 1 l Luria-Bertani medium containing 100 mg/l ampicillin at 37°C until an OD₆₀₀ of 0.4–0.6. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (Invitrogen, San Diego, CA) to a final concentration of 0.5 mM. Afterward, the bacteria were grown for 4 additional hours; cells were harvested by centrifugation and frozen over night at –20°C. A cleared cell lysate was prepared and Ni-NTA chromatography was performed under denaturing conditions (Qiagen, Hilden, Germany). The rTri a 36₁₉₁₋₃₆₉-containing fractions were pooled and dialyzed against 10 mM NaH₂PO₄ (pH 5), whereas the rTri a 36-containing fractions were dialyzed against 10 mM CH₃COONa (pH 4). The protein concentration was determined with a BCA assay kit (Novagen). The molecular masses of the recombinant proteins were determined by mass spectrometry as described (22), and the purity of the recombinant proteins was checked by SDS-PAGE under reducing and nonreducing conditions and silver staining (Bio-Rad, Hercules, CA).

Protein extracts

SDS extracts were prepared from wheat (*Triticum aestivum*) seeds obtained 7, 10, 15, 20, 25, 30, and 35 d after pollination and from mature wheat seeds as described (20). Aqueous extracts were prepared from wheat, rye, barley, oat, spelt, and rice. Fifteen grams of seeds of each crop were homogenized in an electric grinder (Clatronic International, Kempen, Germany). Ground material was transferred to 32 ml H₂O containing 32 μl PMSF (10 μg/ml) and stirred for 4 h at 4°C. The extracts were centrifuged at 10,000 × g for 10 min to remove insoluble particles. The protein concentration was determined with a BCA assay kit (Novagen). Aqueous wheat pollen and timothy grass pollen extracts were prepared as described (23).

RAST-based, nondenaturing IgE dot blot experiments and immunoblotting

Aliquots containing 0.5 μg purified recombinant proteins (rTri a 36₁₉₁₋₃₆₉ or rTri a 36), 2 μg aqueous wheat seed extract (WSE), 2 μg wheat pollen extract, 2 μg grass pollen extract, and 0.5 μg HSA were dotted onto nitrocellulose strips (Whatman Protran nitrocellulose membrane; Sigma-Aldrich, St. Louis, MO) and air dried. In experiments testing for heat resistance, recombinant proteins were heated for 10 min at 95°C before dotting. Membranes were then blocked with buffer A (50 mM sodium phosphate buffer [pH 7.4], 0.5% [w/v] BSA, 0.5% [v/v] Tween 20, 0.05% [w/v] Na₂S₂O₅) and incubated with 1:10 diluted sera from patients, nonal-

lergic individuals, or buffer alone. Bound IgE Abs were detected with 1:10 diluted ¹²⁵I-labeled anti-human IgE Abs (BSM Diagnostica, Vienna, Austria) and visualized by autoradiography.

For immunoblotting, protein extracts were separated by preparative 14% SDS-PAGE (24) using a protein m.w. marker (Kaleidoscope/Precision Plus Protein standard from Bio-Rad, or PageRuler Plus prestained protein ladder from Fermentas, St. Leon-Rot, Germany) as standard and then blotted onto nitrocellulose membranes (Whatman Protran nitrocellulose membrane; Sigma-Aldrich) (25). Membranes were blocked with buffer A and then incubated with rabbit sera, diluted 1:10,000 in buffer A or with patients' sera, and diluted 1:10 in buffer A overnight at 4°C. Membranes were then washed three times with buffer A. Bound rabbit Abs were detected with ¹²⁵I-labeled anti-rabbit IgG Abs (PerkinElmer, Waltham, MA), diluted 1:10,000 in buffer A and human IgE Abs with ¹²⁵I-labeled anti-human IgE Abs (BSM Diagnostica) diluted 1:10 in buffer A, and visualized by autoradiography.

IgE immunoblot inhibitions using either 15 μg purified rTri a 36 or rBet v 1 (negative control) as inhibitors were performed as described (26).

Basophil activation assays

Rat basophil leukemia (RBL) assays were performed as described (27). RBL cells (clone RBL-703/21) transfected with the human FcεRI (28) were incubated with sera (diluted 1:20) from two wheat food-allergic patients (nos. 19 and 22) who showed positive dot blot results to rTri a 36 or with serum from grass pollen-allergic patients without wheat food allergy. WSE, rTri a 36₁₉₁₋₃₆₉, or rTri a 36 was added in three different concentrations per allergen per extract (between 1000 ng/ml and 1 ng/ml). Release of β-hexosaminidase from RBL cells was measured as described (27).

In vitro digestion assays

Gastric in vitro digestion of wheat extracts and rTri a 36 was performed as described (29). For the duodenal digestion, Pankreoflat commercial enzyme tablets (Solvay Pharma, Hannover, Germany) were used. Aliquots from samples subjected to gastric digestion were taken 1, 2, 5, 10, 20, 30, 60, and 120 min after reaction start and for samples from duodenal digestion after 1, 2, 5, 10, 20, 30, and 45 min. Digestion was stopped by adding SDS sample buffer and boiling the samples. Aliquots containing 10 μg extract and an equal amount of undigested extract were loaded on SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated either with rabbit Abs raised against rTri a 36 or rTri a 36₁₉₁₋₃₆₉ or with serum from a patient with confirmed wheat food allergy. Bound rabbit Abs were detected with ¹²⁵I-labeled anti-rabbit IgG Abs, bound human IgE Abs were detected with ¹²⁵I-labeled anti-human IgE Abs, and both were visualized by autoradiography.

heterogeneities and varying lengths were found in the N-terminal portions of the proteins.

Expression in E. coli, purification, and biochemical characterization of rTri a 36_{191–369} and rTri a 36

rTri a 36 and rTri a 36_{191–369} (Fig. 1A) were expressed in *E. coli* BL21 (DE3) as C-terminally hexahistidine-tagged proteins. Approximately 6 mg/l liquid culture of each recombinant protein could be purified by Ni-NTA chromatography. For rTri a 36_{191–369} a molecular mass of 20.8 kDa and an isoelectric point of 8.81 were calculated without the N-terminal methionine. For rTri a 36 the calculated molecular mass and isoelectric point were 40.33 kDa and 8.73, respectively. When subjected to SDS-PAGE, we found that Tri a 36_{191–369} and Tri a 36 migrated at a higher molecular mass of ~24 and 45 kDa, respectively, which can be explained by altered migration behavior in SDS-PAGE owing to their basic isoelectric point (Fig. 2). The mass determined by mass spectrometry for rTri a 36 (40,321.094 Da) exactly matched the calculated mass without methionine, and that for Tri a 36_{191–369} (21,021.430 Da) corresponded to the calculated mass with methionine.

Under nonreducing conditions, Tri a 36_{191–369} migrated as monomer, as dimer at ~40 kDa, and as high molecular mass (250 kDa) oligomers, whereas Tri a 36 appeared only as monomeric and HMW oligomeric forms (Fig. 2).

Tri a 36 is a major allergen for patients with wheat food allergy

We tested IgE reactivity of patients with wheat food allergy ($n = 26$) for reactivity to rTri a 36 and rTri a 36_{191–369} in a non-denaturing, RAST-based dot blot assay. We found that 19 (73.1%) and 21 (80.8%) of the food-allergic patients showed detectable (i.e., compared with the negative controls) IgE reactivity to rTri a 36_{191–369} and rTri a 36, respectively (Fig. 3A, Table I). Each of the 26 patients, but not the serum from the nonallergic person, reacted with dot-blotted WSE. Therefore, rTri a 36 is a major allergen for patients with wheat food allergy. No evident differences regarding the clinical phenotypes of Tri a 36-positive and -negative patients were noted (Table I). Interestingly, when we tested a population of 60 patients suffering from baker's asthma only 5% of the patients ($n = 3$) showed IgE reactivity to Tri a 36 (data not shown). At least one of the latter three patients had an allergy to other seeds (i.e., peanut). Next, we tested IgE reactivity in a population of grass pollen-allergic patients ($n = 19$) to Tri a 36 and Tri a 36_{191–369} (Fig. 3B). Forty-seven percent of these patients

gave positive results with WSE in a commercial allergy test but only 10% ($n = 2$) of these patients showed IgE reactivity to Tri a 36_{191–369} and 5% ($n = 1$) to rTri a 36. However, only those who had reacted with rTri a 36 or Tri a 36_{191–369} reported symptoms of food allergy to wheat and/or other seeds (e.g., sesame, peanut), whereas the remaining other grass pollen-allergic patients tolerated wheat.

Next, we tested the allergenic activity of rTri a 36_{191–369} and rTri a 36 in basophil activation assays. A dose-dependent β -hexosaminidase release was observed when basophils were loaded with serum from wheat food-allergic patients (nos. 19 and 22; Table I) and exposed to WSE or the recombinant allergens (Fig. 3C). When basophils were loaded with serum from a grass pollen-allergic patient without wheat food allergy no relevant β -hexosaminidase release was found (Fig. 3C).

Tri a 36 expression increases during wheat seed maturation and Tri a 36 cross-reactive allergens can be detected in various cereal grains

In WSEs prepared 7, 10, 15, 20, 25, 30, and 35 d after pollination and from mature wheat seeds, Tri a 36 was detected with rabbit Abs raised against the recombinant protein already at day 7 and the expression increased strongly during maturation (Fig. 4A). Additional bands below and above 50 kDa, presumably representing degradation or aggregation products, also increased during maturation.

Next, we searched for Tri a 36-related proteins/epitopes in different crop extracts. Tri a 36 cross-reactive proteins could be detected in seed extracts from rye, barley, oat, spelt, and rice (Fig. 4B). To investigate whether the proteins detected by the rabbit Abs indeed may represent cross-reactive allergens, IgE inhibition studies were performed with serum from a Tri a 36-allergic patient suffering from severe manifestations of wheat food allergy (Fig. 4C). This patient showed IgE reactivity to several components in the various crops (Fig. 4C). A reduction of IgE reactivity to several moieties ranging from 20 to 70 kDa in rye, barley, oat, spelt, and rice was observed when the serum was preincubated with purified rTri a 36, demonstrating the presence of cross-reactive IgE epitopes (Fig. 4C).

Domain-specific resistance of Tri a 36 to digestion and resistance to heat

To investigate whether Tri a 36 is resistant to digestion, gastric and duodenal in vitro digestion experiments were performed with WSEs. When wheat extract was subjected to gastric digestion, a fragment of 15 kDa was detected with rabbit anti-Tri a 36 Abs even after 120 min. After 20 min duodenal digestion, bands of ~25 kDa were still detectable (Fig. 5A). When identically prepared blots were incubated with rabbit Abs raised against Tri a 36_{191–369}, most of the immune-reactive bands disappeared after 5 min gastric or duodenal digestion (Fig. 5B). Because only the rabbit anti-Tri a 36 Abs recognize the N-terminal repetitive domain, the experiments indicate that this N-terminal domain is more resistant to gastric digestion than is the C-terminal portion.

Next, we compared the IgE reactivity of rTri a 36_{191–369} and rTri a 36, which had been boiled for 10 min, with the nonheated allergens by dot blot analysis. No differences regarding IgE binding between the boiled and untreated allergens were found (Fig. 6A). After in vitro digestion of the purified recombinant allergens a 15-kDa IgE-reactive fragment remained after 120 min gastric digestion (Fig. 6B). An ~10-kDa IgE-reactive fragment, which was not detected by the rabbit Abs, appeared after 15 min duodenal digestion and remained intact after 45 min (Fig. 6B).

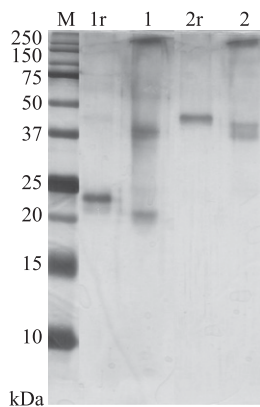


FIGURE 2. Silver-stained SDS-PAGE. Purified recombinant proteins were separated under reducing (rTri a 36_{191–369}, lane 1r; rTri a 36, lane 2r) or nonreducing (rTri a 36_{191–369}, lane 1; rTri a 36, lane 2) conditions. Lane M, Molecular marker.

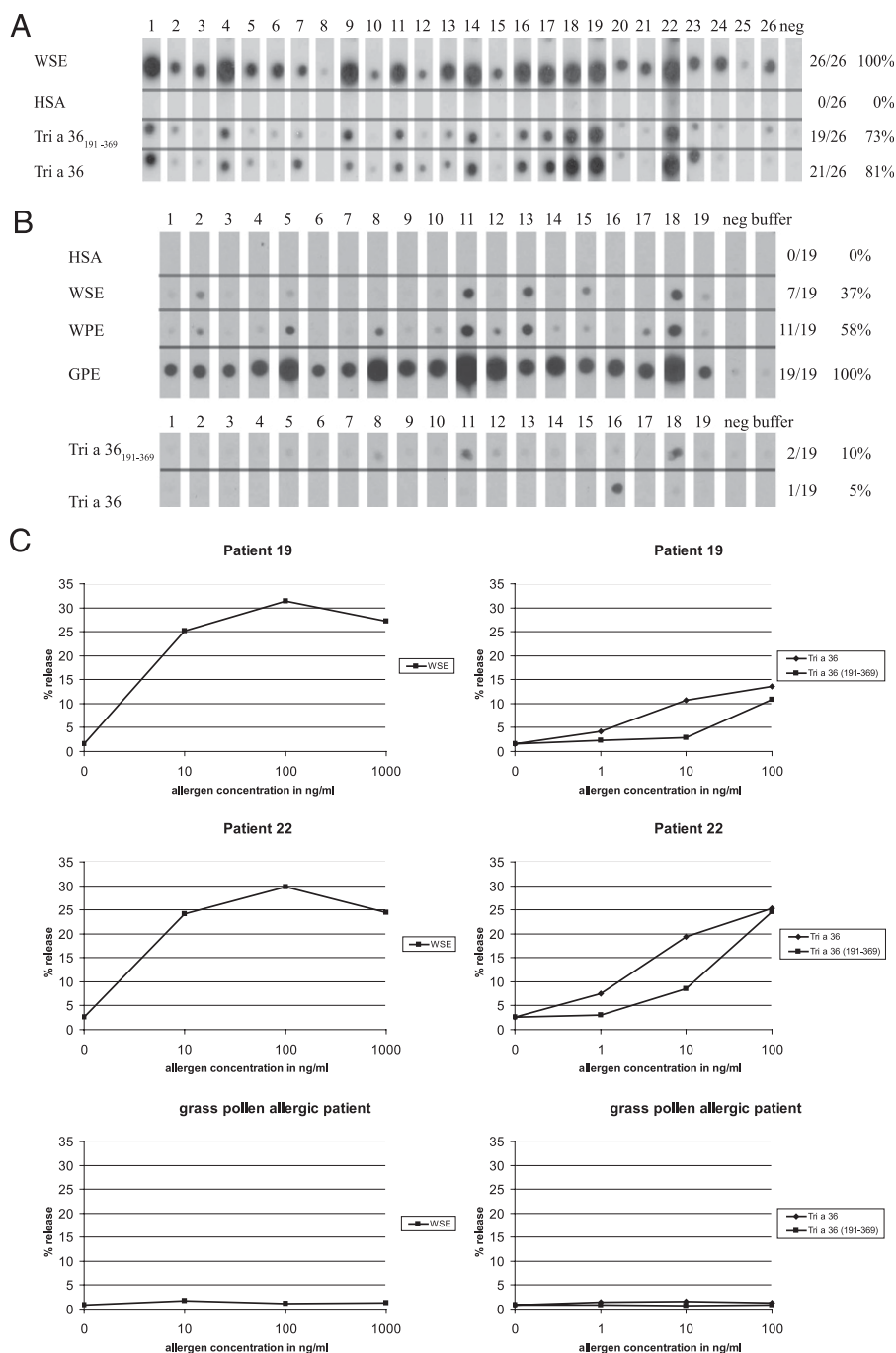


FIGURE 3. IgE reactivity and allergenic activity of rTri a 36 and rTri a 36₁₉₁₋₃₆₉. IgE reactivity of patients with wheat food allergy (1–26) or serum from a nonallergic individual (neg) with dot-blotted rTri a 36, rTri a 36₁₉₁₋₃₆₉, WSE, or HSA (**A**). Reactivity of grass pollen-allergic patients (1–19), serum from a nonallergic individual (neg), or buffer with dot-blotted rTri a 36, rTri a 36₁₉₁₋₃₆₉, WSE, wheat pollen extract (WPE), grass pollen extract (GPE), or HSA (**B**). Bound IgE Abs were detected with ¹²⁵I-labeled anti-human IgE Abs and visualized by autoradiography. (**C**) Allergenic activity of different concentrations (x-axes) of rTri a 36 and rTri a 36₁₉₁₋₃₆₉ or WSE measured by basophil activation tests using sera from wheat-allergic patients (patients 19 and 22) or with serum from a grass pollen-allergic patient without wheat allergy. Percentages of β -hexosaminidase release are shown on the y-axes.

Discussion

In this study the isolation and characterization of a novel major wheat food allergen, Tri a 36, is reported. The cDNA coding for an IgE-reactive fragment comprising the C terminus (aa 191–369) of Tri a 36 was derived from an expression cDNA library that had been constructed from mature wheat seeds using sera from patients with confirmed wheat food allergy. Tri a 36 belongs to the family of proteins known as LMW-GSs, with molecular masses between 20,000 and 65,000 Da, which can be found in various crop species. The IgE-reactive Tri a 36₁₉₁₋₃₆₉ sequence reported in this study seems to be representative for most wheat cultivars because sequence comparisons with homologous genes from other wheat cultivars showed only single base pair differences.

The different molecular weights of these proteins can be mainly explained by variations in their N-terminal repetitive domains.

LMW-GSs belong to the seed storage proteins also called gluten or prolamins in hexaploid wheat (*T. aestivum*) (32) where they account for ~40% of the storage proteins in the wheat kernel (33). They show a high proline and glutamine content and are characterized by a high water absorption capacity, cohesivity, and viscosity and therefore facilitate elasticity of dough, which is important for the extraordinary baking quality of wheat (31).

Approximately 2% of amino acids in LMW-GSs are cysteine residues that form either intrachain disulfide bonds, contributing to the protein stability, or interchain disulfide bonds between different glutenin subunits building up large macropolymers with molecular masses >10 million Da (31). The high amount of the LMW-GSs in the wheat kernel and the stability of these proteins mediated by disulfide bonds may contribute to the allergenicity of Tri a 36. In fact, we found that Tri a 36-immunoreactive fragments

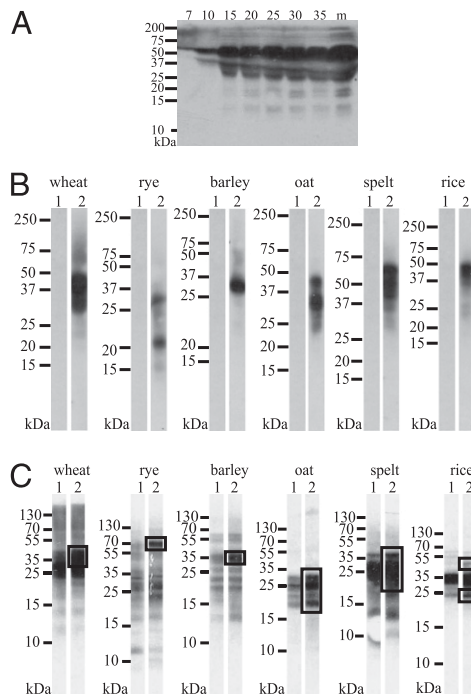


FIGURE 4. Detection of Tri a 36 during wheat seed maturation and of Tri a 36-related allergens in seeds from other cereals. Nitrocellulose-blotted WSEs (7, 10, 15, 20, 25, 30, and 35 d after pollination and from mature seeds [m]) were incubated with rabbit Abs specific for Tri a 36 and detected with ^{125} I-labeled anti-rabbit IgG Abs (**A**). (**B**) Nitrocellulose-blotted seed extracts from different cereals were incubated with rabbit preimmune Abs (lane 1) or Tri a 36-specific immune Abs (lane 2). (**C**) IgE immunoblot inhibitions. Nitrocellulose-blotted seed extracts from different cereals were incubated with serum from an allergic patient who was pre-incubated with rTri a 36 (lane 1) or with the control allergen rBet v 1 (lane 2). Bound IgE Abs were detected with ^{125} I-labeled anti-IgE Abs and visualized by autoradiography.

survived extensive in vitro gastric and duodenal digestion of wheat extracts and, more importantly, rTri a 36 retained its IgE reactivity even after extensive boiling and released IgE-reactive fragments, surviving extensive gastric and duodenal in vitro digestion.

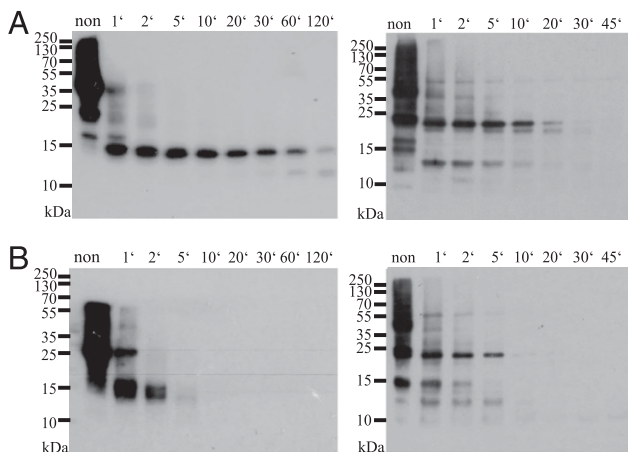


FIGURE 5. Resistance of Tri a 36 to gastric and duodenal digestion. Nitrocellulose-blotted WSE that had been subjected to gastric (left panels) or duodenal (right panels) digestion for different times (1–120 min) were exposed to rabbit anti-Tri a 36 (**A**) or rabbit anti-rTri a 36_{191–369} Abs (**B**). Lanes labeled “non” represent undigested extracts. Bound Abs were detected with ^{125} I-labeled anti-rabbit IgG Abs and visualized by autoradiography.

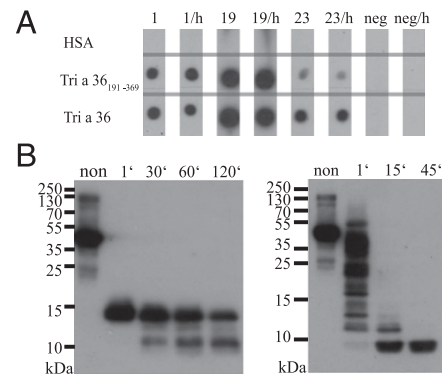


FIGURE 6. Resistance to boiling and digestion of rTri a 36 and rTri a 36_{191–369}. (**A**) Dot-blotted heated (h) or untreated rTri a 36, rTri a 36_{191–369}, or HSA were exposed to sera from three wheat food allergic patients (numbers 1, 19, and 23) and to serum from a nonallergic individual. (**B**) Nitrocellulose-blotted rTri a 36 that had been subjected to gastric (left panel) or duodenal (right panel) digestion for different times (1–120 min) were exposed to serum from a Tri a 36-allergic patient. Lanes labeled “non” represent undigested proteins. Bound Abs were detected with ^{125} I-labeled anti-human IgE Abs and visualized by autoradiography.

Testing of rTri a 36 and its C-terminal portion, rTri a 36_{191–369}, for IgE reactivity with sera from wheat food-allergic patients and from patients suffering from wheat-induced baker’s asthma and grass pollen allergy revealed that Tri a 36 is a major wheat food allergen that is specifically recognized by ~80% of wheat food-allergic patients. Based on the reported prevalence of wheat food allergy, it may therefore be estimated that up to 0.8% of the population is sensitized to Tri a 36 (34).

Only a few of the patients suffering from baker’s asthma (5%) and of the grass pollen-allergic patients (15%) showed IgE reactivity to Tri a 36 or Tri a 36_{191–369}, and a careful analysis of the clinical symptoms of these patients indicated that they suffered also from food allergy. Preliminary analysis with sera from patients with celiac disease showed that certain patients exhibited IgA reactivity to Tri a 36 when compared with sera from individuals without celiac disease (B. Srinivasan, A. Baar, and R. Valenta, unpublished observations).

The allergenic activity of rTri a 36 was demonstrated by its specific and dose-dependent ability to induce the release of β -hexosaminidase from basophils that had been loaded with serum IgE from wheat food-allergic patients. Skin prick test studies also indicated that rTri a 36 alone can induce immediate type skin reactions in sensitized patients, indicating its potential clinical relevance (S. Giavi, A. Baar, N.G. Papadopoulos & R. Valenta, unpublished observations).

Tri a 36 belongs to storage proteins, which are important for the germinating seed (35) and accumulate in protein bodies after their synthesis during the seed maturation (36). Accordingly, we found that the amounts of Tri a 36 increased in wheat seeds during maturation and peaked in mature seeds. As a prolamin, Tri a 36 showed significant sequence identity with related proteins in other cereals (rye, 76%; barley, 64%; oat, 48%; spelt, 46%; rice, 40%). Therefore, we searched for related proteins in these cereals and found by IgE inhibition experiments cross-reactive allergens in these species.

Because Tri a 36 is a major wheat food allergen and primarily recognized by wheat food-allergic patients, it can be used for the diagnosis of wheat food allergy. Furthermore, it may be possible to develop specific immunotherapy strategies based on Tri a 36 for the prevention and treatment of wheat-induced food allergy similar as has been reported for other respiratory allergens (18) and certain food allergens (37).

Disclosures

This study was supported by a research grant from Phadia (Uppsala, Sweden). A patent application has been filed together with the company protecting the recombinant allergen. Furthermore, R.V. serves as a consultant for Phadia. The other authors have no financial conflicts of interest.

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