

The high molecular weight glutenin subunit Bx7 allergen from wheat contains repetitive IgE epitopes

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Abstract

Background: Wheat is one of the most common food allergen sources for children and adults. The aim of this study was to characterize new wheat allergens using an IgE discovery approach and to investigate their IgE epitopes.

Methods: A cDNA expression library representing the wheat transcriptome was constructed in phage lambda gt11 and screened with IgE antibodies from wheat food allergic patients. IgE-reactive cDNA clones coding for portions of high molecular weight (HMW) glutenin subunits were identified by sequence analysis of positive clones. IgE epitopes were characterized using recombinant fragments from the HMW Bx7 and synthetic peptides thereof for testing of allergic patients' sera and in basophil degranulation assays.

Results: We found that the major IgE-reactive areas of HMW glutenins are located in the repetitive regions of the protein and could show that two independent IgE-reactive fragments from HMW Bx7 contained repetitive IgE epitopes.

Conclusions: Our results demonstrate that IgE antibodies from wheat food allergic patients can recognize repetitive epitopes in one of the important wheat food allergens. Recombinant HMW Bx7 may be included into the panel of allergens for component-resolved diagnosis of wheat food allergy.

Wheat food allergy may be induced by direct sensitization involving class I food allergens via the gastrointestinal tract (1) involving often linear IgE epitopes, whereas respiratory allergens often contain discontinuous and/or conformational IgE epitopes (2). Sequential IgE-reactive peptide epitopes have been reported for the major peanut allergens, Ara h 1 and Ara h 2 (3, 4), for cow's milk allergens (5, 6), certain tree nut allergens (7), nonspecific lipid transfer proteins in fruits (8), and wheat allergens (9). However, for certain class I food allergens such as the major fish allergen, parvalbumin, and certain milk allergens (e.g., alpha-lactalbumin), also conformational IgE epitopes have been described (10, 11).

To identify IgE epitopes in wheat, we used a discovery approach. For this purpose, a wheat seed cDNA library

containing the wheat transcriptome was constructed and screened with serum IgE from wheat food allergic patients with the aim to identify new wheat allergens (12). Seven independent IgE-reactive cDNA clones coded for fragments of high molecular weight (HMW) glutenin subunits, which typically consists of a signal peptide, an N-terminal nonrepetitive region, a large repetitive region, and a C-terminal nonrepetitive region (13). Interestingly, each of the IgE-reactive clones was located in the repetitive regions of the HMW subunits. A detailed analysis with recombinant fragments and synthetic peptides revealed the presence of repetitive IgE epitopes in the HMW glutenin subunit Bx7. Our finding that allergens can contain repetitive IgE epitopes provides a novel mechanism for the cross-linking of allergic effector cells and may explain the allergenic potency of wheat.

Materials and methods

Biological materials, patients' sera and antibodies

Wheat seeds were obtained from AGES (Austrian Agency for Health and Food Safety, Vienna, Austria). Immature seeds were harvested 7, 10, 15, 20, 25, 30, and 35 days after onset of pollination directly into liquid nitrogen and stored at -80°C until use (12). Rye and barley seeds produced by Alnatura GmbH (Bickenbach, Germany) were bought at a local drugstore. Human serum albumin (HSA) was purchased from Behring (Marburg, Germany), *Escherichia coli* strain BL21 (DE3) from Stratagene (La Jolla, CA, USA), and maltose-binding protein (MBP) from New England Biolabs (Ipswich, MA, USA). The wheat cDNA library (12) was screened with a serum pool from patients with confirmed wheat food allergy (14).

Sera from patients with wheat food allergy were from the Allergy and Immunology Research Centre, University of Athens, Greece, the Ambulatory for Allergy and Clinical Immunology, Vienna, Austria, and from the Skin and Allergy Hospital, Helsinki University Central Hospital, Finland, as described (14). The diagnosis of wheat food allergy was based either on clinical symptoms of food allergy (e.g., gastrointestinal symptoms, airway symptoms, skin symptoms, systemic anaphylaxis) which could be unambiguously attributed to the ingestion of wheat-containing food and/or open food challenge. In addition, skin prick testing with wheat extracts was performed and the presence of IgE antibodies specific for wheat was demonstrated by serology using the ImmunoCAP system (Phadia, Uppsala, Sweden). For control purposes, sera from 15 nonallergic individuals and sera from 18 grass pollen allergic patients were used as described (14). 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 antibodies were raised against a purified recombinant fragment containing the clone 43-defined sequence without MBP by immunization of rabbits (Charles River, Sulzfeld, Germany) (12).

Isolation and characterization of wheat cDNAs coding for portions of HMW glutenins, expression, and purification of recombinant HMW Bx7 allergen fragments

A cDNA library was screened with sera from wheat food allergic patients as described (12). Seven IgE-reactive cDNA clones showing sequence homology with high molecular weight glutenin subunits were identified. The cDNA sequences of two clones (i.e., clones 43 and 82) were codon-optimized for *E. coli* expression and cloned with an additional sequence coding for a hexahistidine tag at their 3' end into the expression vector pMAL-c4X using the restriction enzyme sites EcoRI and HindIII (GenScript, Piscataway, NJ, USA). The clone 43-defined sequence with a 3' hexahistidine-encoding sequence was also inserted into EcoRI and HindIII sites of pET17b.

The pET17b-43, pMAL-c4X-43, and pMAL-c4X-82 constructs were transformed into *E. coli* BL21 (DE3). Recombinant

proteins were expressed and purified as described (12). The protein concentration was determined with a BCA Assay Kit (Merck EMD, Novagen, Darmstadt, Germany). The molecular masses and the purity of the recombinant proteins were checked by SDS-PAGE Coomassie Brilliant Blue staining (15) using protein molecular weight marker (Bio-Rad or PageRuler Plus; Prestained Protein Ladder, Thermo Fisher Scientific, Fermentas, MA, USA).

Protein extracts and chemical synthesis of HMW Bx7 peptides

SDS-protein extracts were prepared from immature wheat seeds and from mature wheat seeds, as described (12). Aqueous seed extracts were prepared from wheat, rye, and barley as described (16). Bread extracts were prepared by milling 3 g of frozen bread crust (roll, brown, and rye bread) or inner part of the bread (roll, brown-, rye-, and gluten-free bread) in an electric grinder (CLATRONIC, Kempen, Germany). The homogenized bread was added to 32 ml SDS sample buffer, mixed, and boiled for 10 min. The suspension was centrifuged for 30 min at 3220 g, and the supernatant was stored at -20°C .

Two peptides, Pep1 (QQGYPTSPQQSGQGQPGQGQ) and Pep2 (YYPTSPQQPGQLQPPAQGGQ), were synthesized (Microwave peptide synthesizer liberty CEM GmbH, Germany) using a Fmoc (9-fluorenylmethoxycarbonyl) strategy with 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphate activation as described (17) with preloaded Fmoc-Wang (polyethyleneglycol polystyrene) resins (AAPP-Tec llc, Louisville, KY, USA). Peptides were identified by mass spectrometry and purified by HPLC to a purity $>90\%$ (17).

Dot blotting and immunoblotting experiments

Dot blot experiments were performed as described (16). Immunoblotting experiments with rabbit antibodies were carried out as described (18).

Basophil degranulation assays

Rat basophil leukemia cells (RBL-2H3) transfected with human high-affinity IgE receptor chains (Fc ϵ RI) (19) were sensitized with human sera (diluted 1/20) (20). Recombinant proteins m43 and m82 (100, 10, 1 ng/ml) or wheat seed extract (1000, 100, 10 ng/ml) was added. Release of β -hexosaminidase was measured as described (20).

Inhibition ELISA

ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 μl of recombinant m43, m82, or Bet v 1 ($c = 2.5 \mu\text{g/ml}$). Patients sera were diluted 1/5 in 0.5% (w/v) BSA in PBS, 0.05% (v/v) Tween (PBST), and preincubated with 15 μg m43, m82, Bet v 1 or BSA per 100 μl serum. After blocking plates with 3% (w/v) BSA, PBST, preincubated sera were added over night at 4°C . Detection was done as described (21), and measurements were performed at optical density

405–550 nm. Results are shown as mean values of duplicate determinations with <5% deviation.

Results

Isolation and characterization of cDNA clones coding for IgE-reactive fragments of the HMW Bx7 allergen

Using serum IgE from wheat food allergic patients, seven partial cDNA clones, showing sequence identities to different high molecular weight glutenin subunits, were isolated. Three clones (clone 50: 165 aa; clone 72: 64 aa; and clone 91: 56 aa) showed significant sequence homology with HMW 1Dx (GenBank accession number: ABF14401) although clones 72 and 91 contained only relative short fragments. One clone (clone 116: 79 aa) was homologous to HMW R1y (GenBank accession number: AAF23507.1). Three additional clones (clones 43, 82, and 118) showed complete sequence identity to the HMW Bx7 precursor (GenBank accession number AAZ23584.1). The calculated molecular weight of the HMW Bx7 protein was 86 055 Da. Figure S1 provides the deduced amino acid sequences of the IgE-reactive clones. Each of these fragments was derived from the repetitive region of the corresponding HMW glutenin subunits.

Clone 43 coded for the fragment aa 344–618, a part of the long repetitive region. Clone 82 coded for a C-terminal fragment of HMW Bx7 comprising aa 600–795 representing a portion of the repetitive- and of the C-terminal nonrepetitive region (Fig. 1A), which included the fragment 696–795 defined by clone 118 (Fig. 1A). Thus, the sequences of clone 43 and clone 82 comprised together the 452 C-terminal amino acids of the full length HMW Bx7 allergen (795 aa). The HMW Bx7 allergen showed a 70.8% sequence identity with the Tri a 26 allergen (Fig. S2) (22). The sequence analysis of the HMW Bx7 allergen revealed that it contained two sequence motifs (Fig. 1B: Pep1: green; Pep2: red) which occurred in completely identical form several times in the protein. Pep1 was found once in the N-terminal repetitive region, once in the clone 43-defined fragment and once in the clone 82-defined fragment. Pep2 was found twice in the clone 43-defined fragment (Fig. 1B). Pep1 and Pep2 were absent from the Tri a 26 sequence (Fig. S1) and from the HMW Bx7-related proteins in rye and barley (Fig. 1B) which otherwise were of similar length and architecture as HMW Bx7.

Expression and purification of IgE-reactive and allergenic HMW Bx7 fragments

The HMW Bx7 fragments defined by clones 43 and 82 were expressed as MBP fusion proteins (m43, m82) with MBP at the N-terminus and a C-terminal hexahistidine tag in *E. coli* BL21 (DE3). M43 and m82 could be purified to homogeneity, yielding approximately 0.5–1 mg per liter culture. The clone 43-defined fragment was also expressed as C-terminal hexahistidine-tagged protein without MBP, but the yields were low (data not shown).

The theoretical pI and molecular masses were calculated for the recombinant proteins, m43 pI: 5.81, MW:

73324.41 Da and m82 pI: 5.99, and MW: 64610.58 Da, and each of them migrated in SDS-PAGE approximately at their calculated masses (Fig. 2A).

Five of the 24 Greek patients (i.e., 21%) showed IgE reactivity to the recombinant fragments (Fig. 2A), whereas 15% of the Finnish patients reacted with m43 and m82 (data not shown). Interestingly, all but two patients showed IgE reactivity always to both fragments (i.e., m43, m82) although the fragments represented different portions of the C-terminal part of HMW Bx7. For control purposes, we also tested 18 grass pollen allergic patients because they usually show IgE reactivity to wheat seed extract although they do not suffer from wheat food allergy due to recognition of cross-reactive carbohydrates (18) (Fig. 2C), and 15 nonallergic individuals (data not shown). In fact, none of them recognized m43 or m82 as allergen, but 33% of grass pollen allergic patients showed IgE reactivity to the wheat seed extract (Fig. 2C).

Although m43 and m82 contained only portions of the HMW Bx7 allergen, both fragments induced a dose-dependent β -hexosaminidase release after loading RBL cells with serum from an IgE-reactive wheat food allergic patient which was even stronger as that induced with a complete wheat seed extract (Fig. 2D). The latter may be due to the exposure of cryptic IgE epitopes on the fragments which are hidden in the complete allergen. When basophils were loaded with serum from a nonallergic individual, no relevant β -hexosaminidase release could be observed (Fig. 2D).

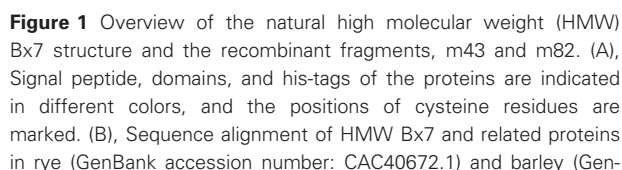
HMW Bx7 fragments contain repetitive IgE epitopes

As almost each of the HMW Bx7-reactive patients showed IgE reactivity to both fragments (m43, m82; Fig. 2B) and repetitive peptides (Pep1, Pep2) were found in their sequences (Fig. 1B), we searched for repetitive IgE epitopes. We found that both Pep1 and Pep2 showed IgE reactivity and hence represent repetitive IgE epitopes (Fig. 3A, left panel). Rabbit antibodies specific for m43 also reacted with m82 (Fig. 3A, right panel), confirming the presence of repetitive epitopes in the HMW Bx7 allergen because m43 and m82 represent different portions of the allergen. Interestingly, Pep1 and Pep2 did not react with the rabbit antibodies (Fig. 3A, right panel). When the possible surface exposure of Pep1 and Pep2 within the complete HMW Bx7 sequence was analyzed using the ExPASy ProtScale accessibility program (23), they appeared largely surface-exposed (Fig. S3).

In IgE-inhibition ELISA experiments, we observed that preincubation of patients' sera with m43 inhibited IgE binding to m82 as well as preincubation with m82 itself and *vice versa*. Preincubation with unrelated proteins (birch pollen allergen Bet v 1 or BSA) did not affect IgE binding to m43 or m82 (Fig. 3B).

The HMW Bx7 allergen and related proteins can be detected during wheat maturation and in different cereals

The expression of HMW Bx7 during wheat seed maturation was studied (Fig. 4A). A band of approximately



170 000 Da became detectable at day 10 after pollination, and full expression was observed from day 15 after pollination until complete maturation of wheat seeds (Fig. 4A). As the calculated molecular weight of HMW Bx7 is 86 055 Da, the 170 000 Da band most likely represents a dimeric form of the protein. Using an identically prepared blot and the preimmune serum, no signals were observed (data not shown). Figure 4B shows that HMW Bx7-related proteins can be detected in rye and barley, but the molecular weights of the antibody-reactive bands were smaller than those detected in wheat (<100 000 Da). No bands were detected with the rabbit preimmune serum in these extracts (Fig. 4B).

Using antibodies specific for the clone 43-defined HMW Bx7 fragment, immune-reactive bands of 250 000 Da and less were found in the inner part as well as in the crust of the rolls, brown bread, and rye bread (Fig. 5, lanes 2). Interestingly, even in gluten-free bread, bands were detected. No reactivity was observed with the preimmune IgG of the rabbit (Fig. 5, lanes 1).

Using serum IgE from wheat food allergic patients, we isolated seven independent cDNA clones coding for IgE-reactive

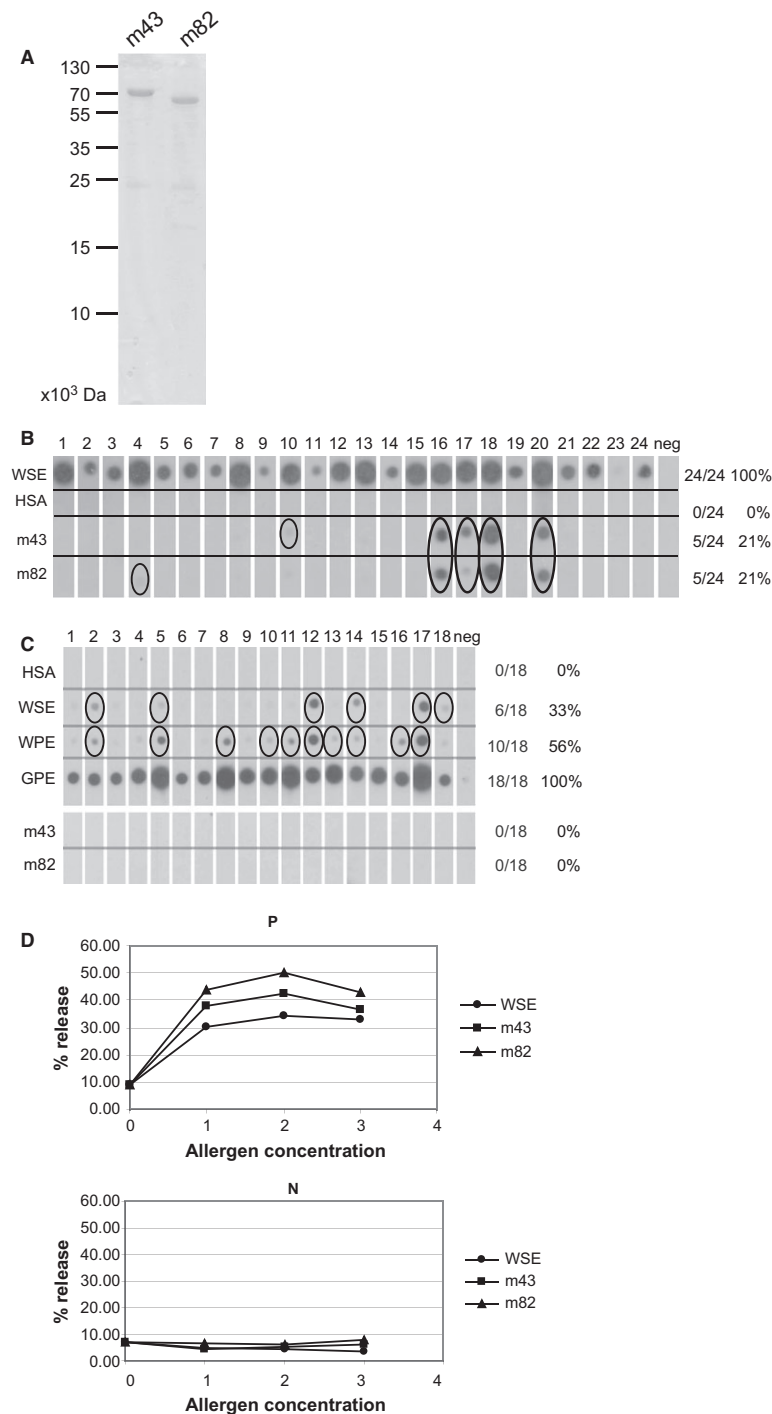


Figure 2 IgE reactivity and allergenic activity of the high molecular weight (HMW) Bx7 fragments, m43 and m82. (A), Coomassie Brilliant Blue-stained SDS-PAGE containing purified recombinant fusion proteins. Molecular weights are shown on the left margin in dalton. (B), Representative IgE reactivity profile of wheat food allergic patients (1–24) and a nonallergic person (neg) with dot-blotted m43, m82, wheat seed extract (WSE), and human serum albumin (HSA). (C), IgE reactivity of grass pollen allergic patients (1–18) and serum from a nonallergic individual (neg) with the same proteins,

wheat pollen extract (WPE) and grass pollen extract (GPE). Bound IgE Abs were detected with ^{125}I -labeled anti-human IgE Abs and visualized by autoradiography. (D), Allergenic activity of different concentrations (x-axes) of m43, m82 (0: buffer, 1: 1 ng/ml, 2: 10 ng/ml, 3: 100 ng/ml), or WSE (0: buffer, 1: 10 ng/ml, 2: 100 ng/ml, 3: 1 $\mu\text{g/ml}$) measured by basophil degranulation tests using sera from a wheat food allergic patient (P) and serum from a nonallergic individual (N). Percentages of β -hexosaminidase release are shown on the y-axes.

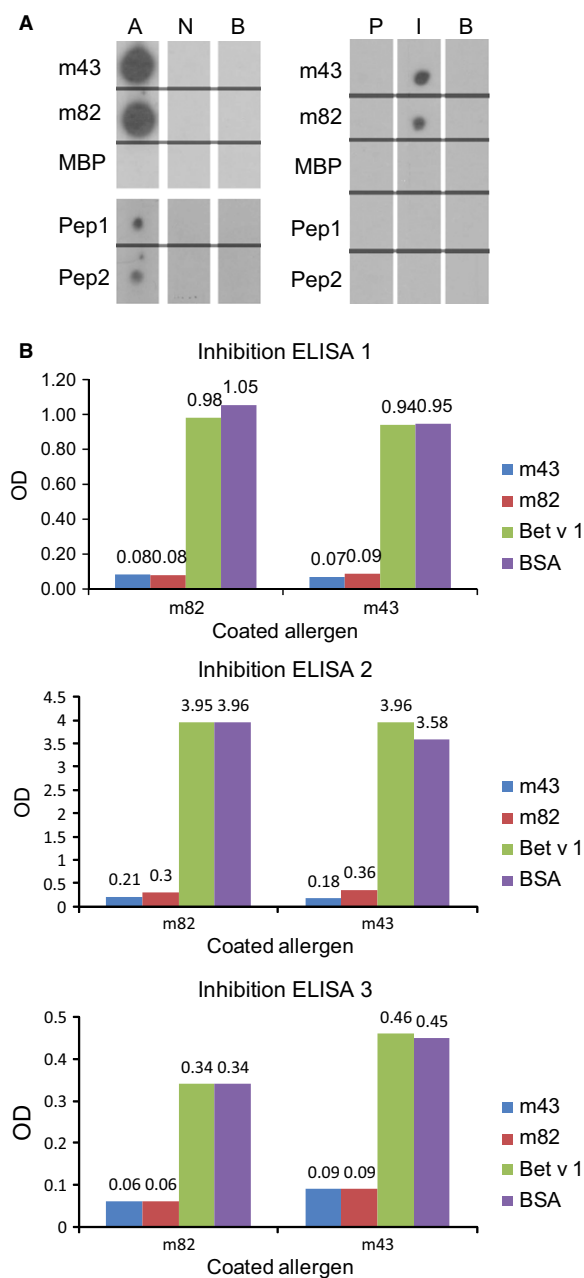


Figure 3 Demonstration of repetitive epitopes in the high molecular weight (HMW) Bx7 allergen. (A), Antibody reactivity of recombinant HMW Bx7 fragments and repetitive peptides Pep1 and Pep2. Dot-blotted m43, m82, maltose-binding protein, Pep1, and Pep2 were incubated with serum from a wheat food allergic patient (A), serum from a nonallergic individual (N), rabbit preimmune serum (P), and rabbit anti-43-immune serum (I) or with buffer alone (B). Bound IgE/IgG Abs were detected with ^{125} I-labeled anti-human IgE Abs or ^{125} I-labeled anti-rabbit-IgG Abs and visualized by autoradiography. (B), Repetitive cross-reactive IgE epitopes in m43 and m82. Coated HMW Bx7 fragments (x-axis: m43 and m82) were reacted with sera from three wheat food allergic patients which were preincubated with m43 (blue), m82 (red), Bet v 1 (green), or BSA (lilac). OD-values corresponding to bound IgE are shown on the y-axis.

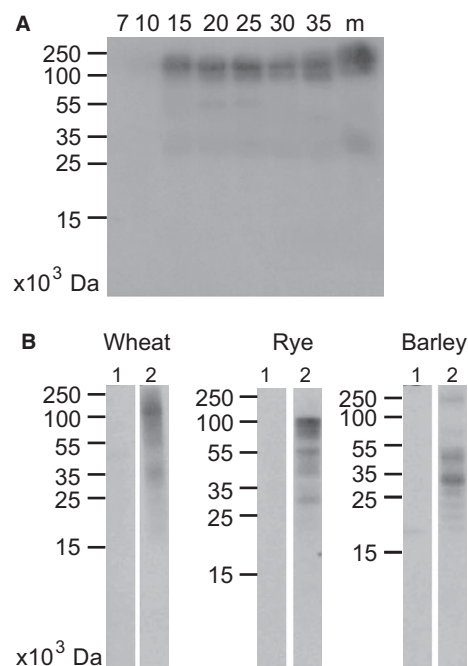


Figure 4 Detection of high molecular weight (HMW) Bx7 during wheat seed maturation and in other cereals. (A), Nitrocellulose-blotted wheat seed extracts (7, 10, 15, 20, 25, 30, 35 days after pollination and from mature seeds m) were incubated with rabbit Abs specific for fragment 43; bound Abs were detected with ^{125} I-labeled anti-rabbit IgG Abs. (B), Nitrocellulose-blotted seed extracts from different cereals (wheat, rye, barley) were incubated with rabbit preimmune serum (lanes 1) or HMW Bx7-specific Abs (lanes 2). Bound IgG Abs were detected with ^{125} I-labeled anti-rabbit IgG Abs and visualized by autoradiography. Molecular masses are displayed in dalton (Da).

fragments of HMW glutenin subunits from a gene library representing the wheat transcriptome. HMW glutenin subunits belong to the gluten proteins of wheat which are characterized by a high amount of glutamine and proline residues (24). Six of the isolated IgE-reactive clones (i.e., 43, 50, 72, 82, 91, and 118) seemed to belong to the x-type and one (i.e., 116) to the y-type HMW glutenin subunits, respectively. In fact, HMW glutenin subunits are cereal storage proteins functioning in the germinating seed as supply for carbon, nitrogen, and sulfur (25). Accordingly, we found that full expression of HMW Bx7 can be detected already at day 15 after pollination in the wheat and the amount of protein remained stable during the maturation to serve the demand of carbon, nitrogen, and sulfur of the growing seed.

High molecular weight glutenin Bx7 fragments m43 and m82 were recognized by approximately 20% of wheat food allergic children, indicating that the HMW Bx7 may be important in children suffering from wheat food allergy. It may therefore be used as a relevant allergen for molecular diagnosis of wheat food allergy. Interestingly, almost every patient who showed IgE reactivity to m43 also recognized the independent fragment m82, indicating that the similar and repetitive IgE epitopes are present in two different portions of

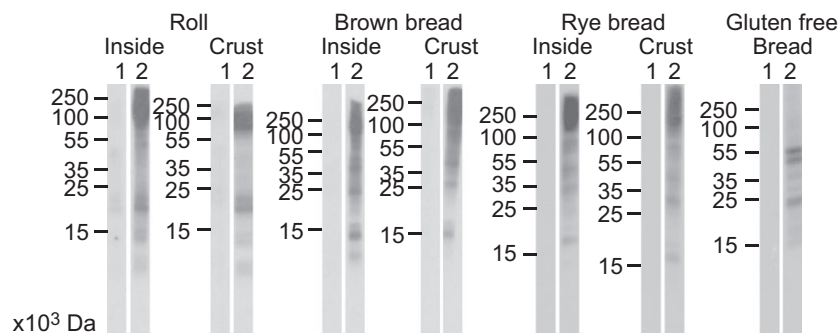


Figure 5 Detection of high molecular weight (HMW) Bx7-related proteins in different kinds of bread. Nitrocellulose-blotted bread extracts from roll, brown bread, rye bread, and gluten-free bread were incubated with rabbit preimmune Ig (lanes 1) or HMW Bx7-

specific Abs (lanes 2). Bound IgG Abs were detected with ^{125}I -labeled anti-rabbit IgG Abs and visualized by autoradiography. Molecular masses are displayed in dalton (Da).

the allergen. The presence of a duplicated IgE-binding area was confirmed by an IgE-inhibition ELISA. In fact, each of the seven IgE-reactive HMW glutenin subunit fragments was derived from the central repetitive domain (26) of the protein. As duplication of blocks of repeat motifs and gene conversion lead to an unequal length of the repetitive domain within the high molecular weight glutenin subunits (26), major differences among the HMW glutenin subunits are observed in this region. Also, a previously identified HMW glutenin (i.e., Tri a 26) showed only 70.8% sequence identity to the HMW Bx7 glutenin subunit identified by us (27). The lower frequency of IgE recognition of the recombinant HMW glutenin subunit fragments described by us and a high molecular weight glutenin reported recently (28) may be due to low sequence identity of the proteins, the fact that we tested recombinant fragments which may have less IgE reactivity than the complete protein or due to the patients tested.

Although the most conserved domains between the different wheat HMW glutenin subunits and between different cereals are found in the nonrepetitive regions, the major IgE epitopes are located in the less conserved repetitive domain (Fig. 1B, Fig. S1). A more detailed analysis of IgE epitopes using synthetic peptides representing two fully identical and repetitive sequences which occur twice (i.e., peptide 2) and three-times (i.e., peptide 1) within the repetitive domain demonstrated also the presence of repetitive peptide IgE epitopes in the HMW Bx7 allergen. The IgE epitopes mapped for clinically relevant allergens are usually independent epitopes, and the occurrence of repetitive sequential IgE epitopes is a novel and unexpected finding. In fact, the presence of repetitive IgE epitopes in HMW Bx7 explains why each of the fragments could induce basophil degranulation. In fact, basophil degranulation induced with each of the fragments was stronger than that induced with wheat allergen extract which may be explained by the presence of cryptic IgE epitopes which may become available only after allergen fragmentation as it may occur upon digestion. As several wheat allergens contain repetitive domains (e.g., omega-5-gliadin) (29), it is quite possible that wheat is rich in repetitive IgE epitopes which may be one of the reasons why it is a frequent and important food allergen source.

Author contributions

Alexandra Baar was involved in the concept and design, acquisition of data, analysis, and interpretation of data, drafted the article, and finally approved the version to be published. Sandra Pahr, Stavroula Giavi, Nikolaos G. Papadopoulos, Anna S. Pelkonen, Mika Mäkelä, Sandra Scheibelhofer, Josef Thalhamer, Milena Weber, Christof Ebner and Adriano Mari were involved in acquisition of data, analysis, and interpretation of data, critically revised the article, and finally approved the version to be published. Claudia Constantin was involved in acquisition of data, analysis, and interpretation of data critically revised the article, and finally approved the version to be published. Susanne Vrtala was involved in acquisition of data, analysis, and interpretation of data, critically revised of the article, and finally approved the version to be published. Rudolf Valenta was involved in study concept and design, analysis, and interpretation of data, drafted and critically revised the article, and gave final approval of the version to be published.

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Conflicts of interest

Rudolf Valenta and Susanne Vrtala have received research grant support from ThermoFisher, Uppsala, Sweden, and Rudolf Valenta serves as a consultant for ThermoFisher.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Deduced amino acid sequences of IgE-reactive wheat clones. Clones are indicated in an overview of the HMW structure as in Fig. 1A.

Figure S2. Sequence alignment of Tri a 26 and HMW Bx7. The signal peptide, domain structure, and peptides are colored as in Fig. 1, and numbers of amino acids are shown on the right margin.

Figure S3. Accessibility plot of the HMW Bx7 sequence. The number of amino acids, signal peptide and domain structure (colored as in Fig. 1) of the full length protein, the protein fragments, and the repetitive peptides is shown on the x-axis. The accessibility of the residues is indicated on the y-axis; scores above 5.5 represent regions of high surface accessibility.

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[illegible]

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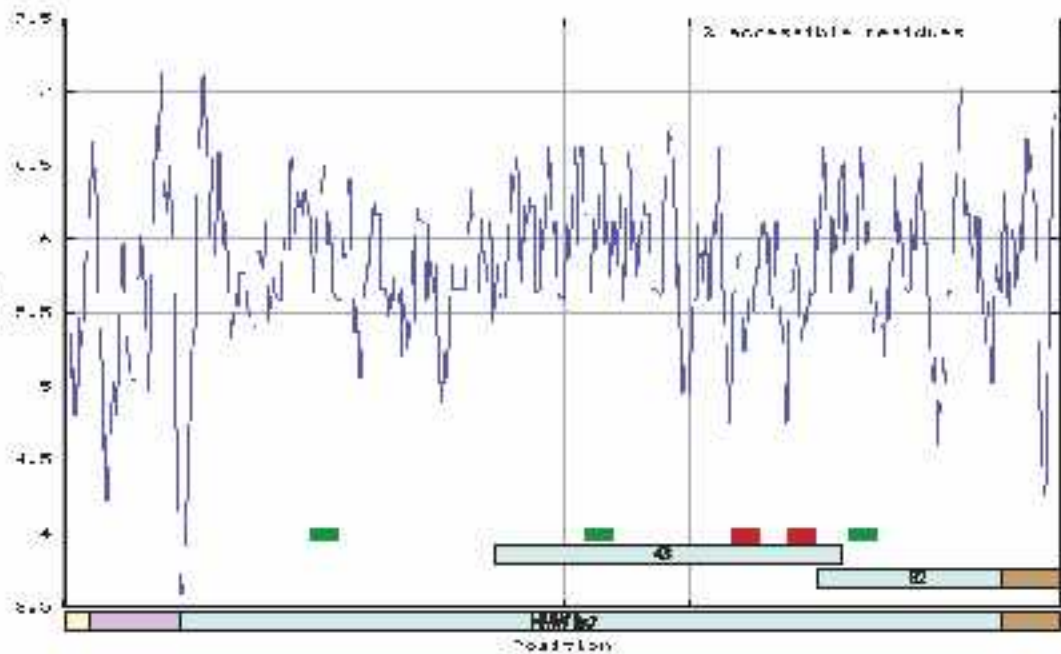
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FIGURE S1

**FIGURE S3**