

Wheat α -amylase inhibitor: A second route of allergic sensitization

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Background: Low molecular weight allergens may be responsible for hypersensitivity reactions after the ingestion of wheat.

Objective: The purpose of this investigation was to identify relevant, low molecular weight allergens after the ingestion of wheat protein.

Methods: Serum samples were collected from seven children with wheat allergy and one adult with baker's asthma. Control serum samples were collected from wheat-tolerant patients. Wheat extracts were prepared and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% gels revealing numerous protein bands. IgE immunoblot analysis of crude wheat extracts identified multiple IgE-binding proteins. Wheat proteins were separated further with two-dimensional gel electrophoresis, which was followed by IgE immunoblotting investigations.

Results: Immunoblot analysis identified a 15 kd wheat protein that bound IgE from all five children with wheat allergy who were evaluated. No IgE binding to this wheat protein was demonstrated in any of the control subjects. Samples representing the 15 kd wheat protein (isoelectric point, 5.85) were selected. The N-terminal peptide sequence of this protein (residues 1 to 20) matched to a wheat α -amylase inhibitor.

Conclusion: These data demonstrate that wheat α -amylase inhibitor is a relevant allergen in patients experiencing hypersensitivity reactions after the ingestion of wheat protein. This wheat protein, which has been implicated as an important allergen in patients with baker's asthma, represents a sensitizing allergen after both ingestion and inhalation. (*J Allergy Clin Immunol* 1997;99:239-44.)

Key words: Wheat, allergen, hypersensitivity, food hypersensitivity, baker's asthma, α -amylase inhibitor, anaphylaxis, grass allergy, cereal grain

Wheat (*Triticum aestivum*) is one of the major cereal grains belonging to the grass family Poaceae (Gramineae) and is a staple food item in most diets.¹ Moreover, wheat is among the six major foods that account for food hypersensitivity reactions in children.²⁻⁴ Although major allergenic proteins from egg,⁵ milk,⁶ and peanuts⁷⁻⁹ have been implicated in specific humoral and cell-mediated immune responses in human beings, much less evidence is available regarding wheat allergens.

Hypersensitivity reactions to ingested wheat protein have been reported by several investigators performing blinded food challenges in children.^{2, 4, 10} These reactions typically occur within an hour after wheat ingestion and include cutaneous, gastrointestinal, and respiratory symptoms. Affected individuals are usually sensitized during infancy,¹¹ and unlike peanut and shellfish allergy, the clinical reactivity typically resolves before adulthood.² These reactions are clinically distinct from other

Abbreviations used

PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

adverse reactions to wheat protein including: (1) baker's asthma,¹² an IgE-mediated reaction to inhaled flour (from wheat and other cereal grains), (2) celiac disease,¹³ a non-IgE-mediated enteropathy caused by wheat gliadin, and (3) wheat-dependent, exercise-induced anaphylaxis.¹⁴⁻¹⁶ Although there are some similarities in these adverse reactions to wheat, differences in the specific allergens, the routes of sensitization, and end-organ responsiveness have not been completely resolved.

Wheat flour consists of water-soluble albumins, salt-soluble globulins, gliadins, and glutenins.¹⁷ Albumins and globulins appear to be the most important proteins contributing to immediate hypersensitivity reactions to wheat protein.^{18, 19} Investigators have confirmed that IgE antibodies specific to wheat albumins and globulins are present in the serum from children with wheat allergy and patients with baker's asthma but not in patients with celiac disease.¹⁸⁻²⁰

Several groups of investigators have reported that some

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of the wheat allergens responsible for baker's asthma are members of a family of wheat α -amylase inhibitors.²¹⁻²⁵ A 16 kd wheat protein, a glycosylated subunit of the wheat tetrameric α -amylase inhibitor, has been identified and confirmed as an important allergen in patients with baker's asthma.^{21, 22} Using IgE immunoblotting, Pfeil et al.²³ identified major wheat allergens with molecular weights of 15 and 17 kd. In similar investigations,^{24, 25} a 15 kd and a 12 kd wheat allergen have been identified in patients with baker's asthma. In contrast, little information is available regarding specific wheat allergens responsible for allergic reactions after the ingestion of this protein. One recent investigation by Jones et al.²⁰ implicated 20 kd and 47 kd wheat proteins as potential allergens in children with confirmed wheat hypersensitivity. The focus of this investigation was to isolate and identify a low-molecular-weight allergen in wheat that may be responsible for hypersensitivity reactions after the ingestion of this protein.

METHODS

Patient recruitment and collection of serum samples

Eligible patients with and without clinical histories of allergic reactions after the ingestion of wheat were recruited from the allergy clinics at the University of Arkansas for Medical Sciences and Johns Hopkins Hospital in Baltimore, Maryland. Patient histories were confirmed by skin prick tests, RASTs, and blinded, oral food challenges. Patients with a convincing history of severe anaphylaxis after wheat ingestion were not subjected to blinded food challenges. Skin testing procedures and blinded food challenges were performed as described previously.²⁶⁻²⁸ Serum samples were collected and stored at -20°C . Control serum samples were collected from wheat-tolerant patients including patients with high serum IgE concentrations, patients with grass allergy, and nonatopic patients. Patients with allergic reactions to common grasses (timothy, Johnson, and Bermuda) were used as a control group because clinically insignificant IgE cross-reactivity among common cereal grains (wheat, rye, oat, and barley) and common grass species has been reported by many investigators.^{1, 15, 20, 29-31} Informed consent was obtained for each subject, and all studies were approved by the Human Research Advisory Board.

Extraction of wheat proteins

Soluble wheat proteins were extracted from nonenriched, whole wheat flour (Arrowhead Mills, Hereford, Texas) in phosphate-buffered saline (PBS), 1:20 (wt/vol), for 24 to 72 hours with constant agitation at 4°C . The mixtures were centrifuged at 3000 rpm for 10 minutes, and the supernatant was decanted and clarified by further centrifugation at 9500 rpm for 10 minutes. The resulting supernatant was dialyzed against deionized, distilled water for 24 hours at 4°C , centrifuged again as described above and lyophilized. Before experimental use, the lyophilized wheat extracts were stored at -70°C until needed in specific assays. Lyophilized extracts were resuspended in either deionized, distilled water or sterile PBS, and the protein content was determined with the BCA protein assay kit (Pierce Chemical Co, Rockford, Ill.).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Crude wheat extracts were separated by using a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system according to a modified method of Laemmli et al.³² In brief, 10 to 20 μl samples of a 1 mg/ml protein solution and 10 μl of molecular weight standards (BioRad, Hercules, Calif.) were resolved in a 3% stacking gel and 12.5% separating gel at 20 mA/gel under nonreducing conditions (25 mmol/L Tris and 192 mmol/L glycine buffer with 1% SDS). Coomassie Blue stain (Sigma Chemical Co., St. Louis, Mo.) was used to stain the separated wheat protein bands.

IgE immunoblotting (Western blotting) studies

Proteins were transferred from SDS-PAGE separating gels to nitrocellulose membranes by using a 25 mmol/L Tris/192 mmol/L glycine/10% SDS/40% methanol transfer buffer³³ in a transblot apparatus (BioRad) for 2 hours at 150 mA. After an overnight blocking step with PBS and 0.05% porcine gelatin at 4°C , the nitrocellulose blot was washed three times with PBS containing 0.05% Tween-20. The blot was probed with patient or control serum samples (1:20 dilution in PBS and 0.05% porcine gelatin) for 2 hours at room temperature with constant rocking. After washing with PBS containing 0.05% Tween-20 three separate times, alkaline phosphatase-labeled goat anti-human IgE (diluted 1:2000 in PBS and 0.05% porcine gelatin; KPL, Gaithersburg, Md.) or iodine 125-labeled equine anti-human IgE (diluted 1:3, vol/vol, in PBS and 0.05% porcine gelatin; Kallestad, Chaska, Minn.) was added and incubated at room temperature with constant rocking for 2 hours. After another wash step, the blot was developed with alkaline phosphatase substrate or exposed to x-ray film at -70°C for 6 to 48 hours, and the autoradiograph was developed.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed by using the Multiphor II electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden). Isoelectric focusing was performed in the first dimension, followed by SDS-PAGE in the second dimension. In brief, 15 μg of the crude wheat extract was loaded onto an Immobiline dry strip (Pharmacia), pH 4 to 7, for the first-dimension electrophoresis. This electrophoresis was carried out in three phases. Phase I: 300 V, 1 mA, 5 W for 3 hours; Phase II: 300 V, 1 mA, 5 W for 5 hours; Phase III: 2000 V, 1 mA, 5 W for 8 hours at 15°C . The dry strips were removed and placed on a horizontal 12.5% homogenous SDS-PAGE gel, and second-dimension electrophoresis was carried out in three phases. Phase I: 600 V, 20 mA, 30 W for 30 minutes; Phase II: 600 V, 50 mA, 30 W for 30 minutes; Phase III: 600 V, 50 mA, 30 W for 70 minutes. The gel was stained with a silver stain (Biorad) or transferred to immobilon-P (Millipore, Bedford, Mass.) transfer membrane by use of the LKB-Multiphor II unit (Pharmacia) with an electrophoretic Novablot transfer kit (Pharmacia).

Sequencing of proteins

Amino-terminal sequencing of the isolated wheat proteins was performed at the Biotechnology Resource Laboratory at the William M. Keck Foundation Biotechnology Research Laboratory (Yale University, New Haven, Conn.). This was accomplished with an Applied Biosystems sequencer with on-line high-performance liquid chromatography for the identification of the resulting phenylthiohydantoin amino acid derivatives.

TABLE I. Clinical characteristics of study patients

Patients with wheat allergy						
Patient	Age (yr)	Wheat allergy history	Atopy history	Skin test wheat	Oral challenge with wheat	Serum IgE concentration (IU/ml)
WW	15	Urticaria	A, AR, AD	Positive	Positive	47,268
EB	6	Anaphylaxis	A, AR, AD	Positive	Not done	Not done
BP	9	Urticaria, vomiting	A, AR, AD	Positive	Positive	75,248
AR	8	Urticaria	A, AR, AD	Positive	Positive	827
DG	4	Vomiting	A, AD	Positive	Positive	52.2
MB	10	Urticaria	A, AR, AD	Positive	Positive	302
ET	5	Urticaria	A, AR, AD	Positive	Positive	653
JV	35	Anaphylaxis, baker's asthma	A, AR	Positive	Not done	191
Wheat-tolerant control patients						
Patient	Age (yr)	Wheat allergy history	Atopy history	Serum IgE concentration (IU/ml)		
Patients with high serum IgE						
VS	50	Wheat tolerant	AR	300		
JN	16	Wheat tolerant	AD	8000		
Patients with grass allergy						
CJ	35	Wheat tolerant	A, AR	Not done		
SC	36	Wheat tolerant	AR	Not done		
Nonatopic patients						
JG	3	Wheat tolerant	nonatopic	Not done		
LD	4	Wheat tolerant	nonatopic	Not done		

A, Asthma; AR, allergic rhinitis; AD, atopic dermatitis.

RESULTS

Description of patients

Several groups of patients were recruited for the identification of low molecular weight allergens in wheat protein extracts. Table I summarizes the clinical characteristics of these patient groups. The first group included seven patients with wheat allergy (ages, 1 to 17 years; median age: 2 years). Six of these patients had positive responses to double-blind placebo-controlled food challenge confirming their clinical reactivity to wheat; the remaining patient had a convincing clinical history of severe anaphylaxis after the ingestion of wheat protein and was not subjected to a blinded food challenge. One adult patient (35 years old) with a long-standing clinical history of baker's asthma and a more recent clinical history of severe anaphylaxis after the ingestion of wheat protein was also included in the investigation. This adult was not subjected to a blinded food challenge. The control groups included wheat-tolerant patients with the following characteristics: (1) two patients with high serum IgE concentrations, (2) two patients with grass allergy, and (3) two nonatopic patients.

Identification of major allergens in wheat

SDS-PAGE (12.5%) analysis of crude wheat extracts revealed multiple protein bands that stained

with Coomassie Blue (Fig. 1). The spectrum of protein-staining bands ranged from 14 kd to 66 kd. To determine which wheat proteins were responsible for specific IgE binding, immunoblotting studies (Western blots) were performed with nitrocellulose blots of separated crude wheat proteins and serum samples from the patient groups. Analysis of the IgE immunoblots with serum samples from the patients with wheat allergy and the patient with baker's asthma identified IgE-binding proteins with apparent molecular weights of 15, 16, 18, 24, and 35 to 47 kd. Similar IgE immunoblotting studies with serum samples from the wheat-tolerant control patients revealed nonspecific IgE binding to wheat proteins of 35 kd and 38 kd, respectively. Fig. 2 shows a representative IgE immunoblot with serum samples from one child with wheat allergy (lane 1) and from three wheat-tolerant control patients (lanes 2, 3, and 4).

On the basis of data of other investigators regarding potential allergens in wheat,²⁵ we focused our investigations on the 15 kd wheat protein. This protein bound IgE from six of the seven patients with wheat allergy, and it appeared that this protein bound IgE in the serum from our adult with baker's asthma. No IgE binding to this low-molecular-weight wheat protein was observed when serum samples from any of the wheat-tolerant control patients were used.

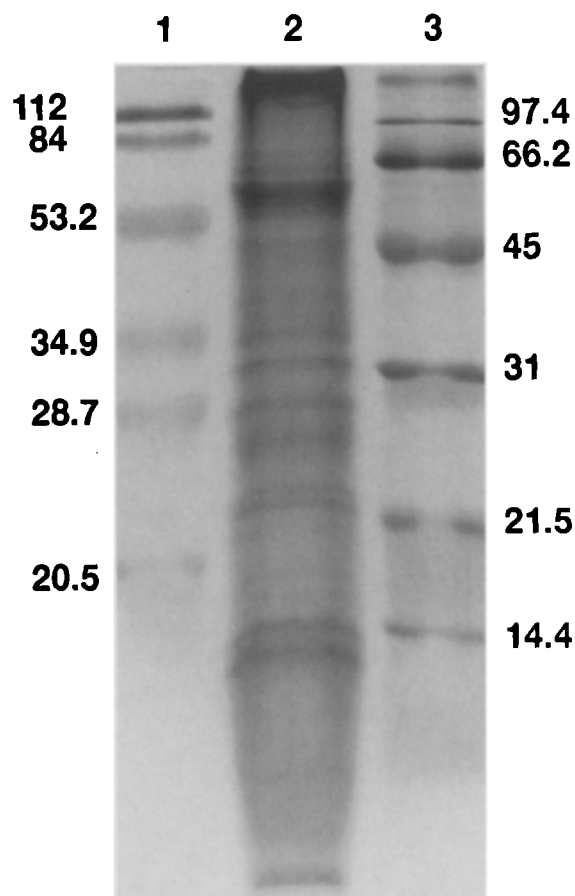


FIG. 1. SDS-PAGE of crude wheat extract and standard molecular-weight markers in a 1.25% gel. *Lanes 1 and 3* represent standard molecular weight markers. *Lane 2* represents multiple Coomassie Blue-stained protein bands (molecular weight range, 14 to 66 kD) that were separated from the crude wheat extract.

Isolation and characterization of the 15 kD wheat allergen

Further isolation and characterization of the 15 kD wheat protein was accomplished with two-dimensional gel electrophoresis and isoelectric focusing, followed by IgE immunoblotting studies. Fig. 3 highlights the results of these investigations. The 15 kD wheat protein, which is identified by the arrows, bound IgE from all five patients with wheat allergy. Samples representing the isolated 15 kD protein were determined to have an isoelectric point of 5.85. No IgE binding to the 15 kD wheat protein was observed when serum samples from two control patients were used (one wheat-tolerant pediatric patient with a high serum IgE concentration and one wheat-tolerant, nonatopic adult patient). Samples representing the 15 kD wheat protein from the two-dimensional gel electrophoresis investigations were individually selected, eluted from nitrocellulose membranes, and subjected to amino acid sequencing. The N-terminal peptide sequence (residues 1 to 20) of the 15 kD wheat protein was compared with protein databases and demonstrated to have 100%

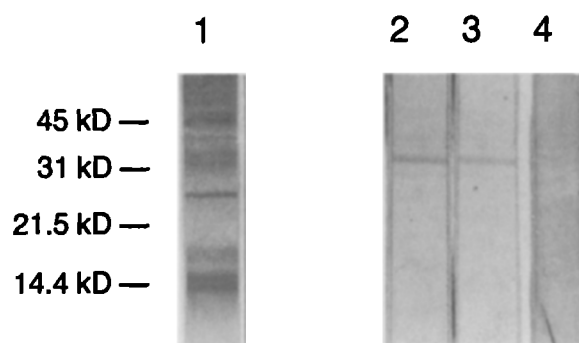


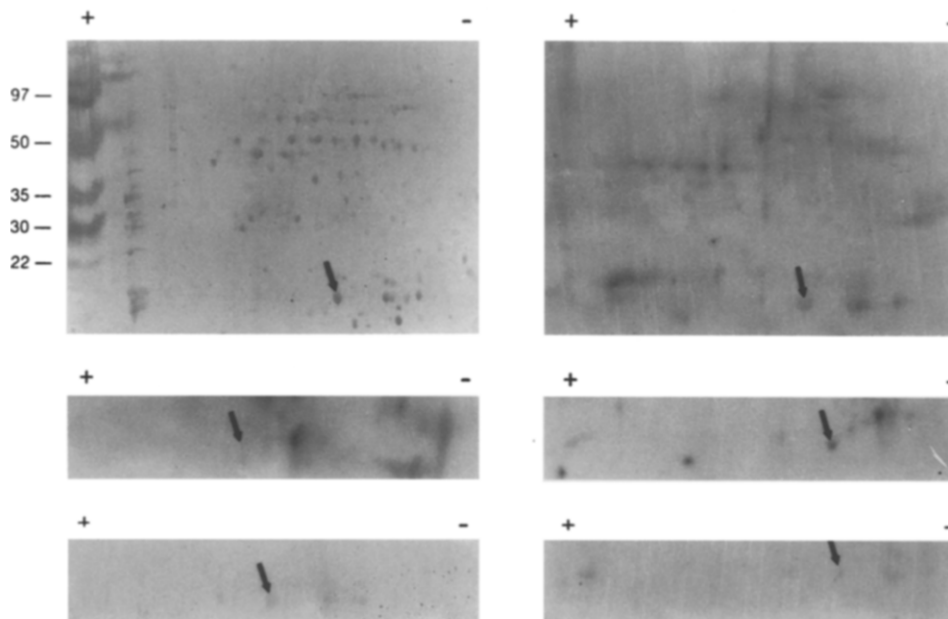
FIG. 2. Representative IgE immunoblot with serum samples from one child with wheat allergy (*lane 1*) and three wheat-tolerant control patients including a patient with a high serum IgE concentration (*lane 2*), an atopic patient with grass allergy (*lane 3*), and a nonatopic patient (*lane 4*). Molecular weight references are labeled on the left side of the figure. In the representative child with wheat allergy, multiple IgE-binding proteins with apparent molecular weights of 15, 18, 24, 35, and 47 kD are evident. IgE immunoblotting studies with serum samples from the control patients revealed only nonspecific IgE binding to wheat proteins of 35 kD and 38 kD in two of the control subjects (*lanes 2 and 3*).

sequence identity to wheat α -amylase inhibitor (Table II). The N-terminal 20 amino acid residues represent 16% of the total 124 amino acid residues in wheat α -amylase inhibitor, which is a member of the cereal trypsin/ α -amylase inhibitor family, found in wheat endosperm.

DISCUSSION

We have identified a 15 kD allergen in wheat flour extracts by using serum samples from patients with well-defined allergic reactions after the ingestion of wheat. In addition, we have demonstrated that this allergen binds specific IgE in serum from an adult with baker's asthma. The N-terminal peptide sequence of this 15 kD wheat protein (residues 1 to 20) showed sequence homology to wheat α -amylase inhibitor. These studies highlight the presence of wheat α -amylase inhibitor as a relevant allergen in patients experiencing hypersensitivity reactions after the ingestion of wheat protein.

The identification of this low-molecular-weight allergen in wheat is consistent with investigations of allergens in patients with baker's asthma. Pfeil et al.²³ used serum samples from 130 bakers with confirmed asthma (42 with wheat allergy and 88 who were wheat-tolerant) in IgE immunoblotting studies with crude wheat extracts. Their results identified wheat allergens with molecular weights of 15, 17, and 47 kD. In related investigations of potential low-molecular-weight allergens in baker's asthma, Gomez et al.²⁴ identified 30, 27, and 15 kD allergens, whereas Sandiford et al.²⁵ demonstrated 33, 26, 21, and 12 kD wheat allergens. These investigators reported that the majority of these allergens were members of a family of wheat α -amylase inhibitors. Moreover, another group of investigators identified and confirmed that a 16 kD wheat protein, a glycosylated subunit of the wheat



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