
Thaumatococcus-like protein and baker's respiratory allergy

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Background: Baker's asthma and rhinitis are among the most common occupational diseases. Inhaled cereal flours, such as wheat, especially cause this disease.

Objective: To identify and test in vivo clinically important wheat allergens in baker's respiratory allergy in a Finnish population.

Methods: Potential wheat allergens were purified using chromatographic methods from salt-soluble protein extracts of wheat flour and were used in skin prick tests with serial 10-fold dilutions (0.5–0.005 mg/mL). Twenty patients with baker's rhinitis, asthma, or both participated in this study. All the patients had positive skin prick test reactions and specific IgE antibodies to wheat flour. The control group consisted of 10 healthy individuals. Molecular identities of purified wheat allergens were characterized using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and tandem mass spectrometry.

Results: Allergen concentrations of 0.3–0.5 mg/mL revealed that 12 patients reacted to α -amylase inhibitor (α -AI), 9 to peroxidase I (PI), 9 to thaumatococcus-like protein (TLP), and 6 to lipid transfer protein 2G (LTP2G). Conversely, with allergen concentrations of 0.05 mg/mL, 5 patients responded to α -AI, 3 to PI, 4 to LTP2G, and 6 to TLP. Of these, TLP and LTP2G are now observed to be new allergens associated with baker's asthma.

Conclusions: In addition to the earlier-described α -AI and PI, TLP and LTP2G are important in vivo wheat allergens in baker's allergies in Finland. Further studies are needed to elucidate the role of these novel wheat allergens in respiratory disorders.

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INTRODUCTION

Baker's asthma and rhinitis are among the most common causes of occupational diseases.¹ They share a common etiology and are closely associated.² In most cases, rhinitis precedes asthma, and baker's rhinitis can be considered a prestage of baker's asthma.² However, it has been shown that asthma and rhinitis can, in some cases, develop simultaneously.³ Finally, respiratory symptoms are occasionally accompanied by conjunctivitis⁴ and, rarely, contact urticaria. Baker's asthma and rhinitis are IgE-mediated diseases, and the main agents that cause these disorders are inhaled cereal flours, especially wheat, although other baking ingredients, additives, and sometimes contaminant insects or molds may cause allergy.^{2,5}

Water- and salt-soluble proteins of wheat grains are considered to be major allergens in patients with baker's asthma.^{6–10} In

general, wheat-allergic IgE responses of patients with baker's asthma and rhinitis are especially directed toward α -amylase inhibitors (α -AI), thioredoxins (cross-reactive with grass allergens), a wheat lipid transfer protein (LTP) (Tri a 14), and a wheat serine proteinase inhibitor.^{7,11–13} Furthermore, some studies^{8,10,14,15} demonstrate that non-water-soluble gliadins and glutenins also are important allergens in this disorder.

Knowledge of relevant allergens would be advantageous for diagnostic in vitro testing and possible desensitization treatments for this disease. To characterize relevant in vivo wheat flour allergens in patients with baker's respiratory allergy, immunoreactive water-soluble proteins of wheat grain extracts were identified and purified, and eventually their role as allergens was determined. Various abbreviations and acronyms, such as abbreviations of wheat allergens, are explained in Table 1.

METHODS

Study Patients

Twenty patients with baker's rhinitis, asthma, or both diagnosed in the Finnish Institute of Occupational Health between February 4, 2004, and December 31, 2007, were recruited (Table 2). The inclusion criteria included (1) verified exposure to flours at work, (2) symptoms in relation to wheat exposure, and (3) elevated specific IgE antibodies to wheat flour (>0.35 kU/L) (ImmunoCap; Phadia, Uppsala, Sweden) or positive skin prick test (SPT) wheals (≥ 3 mm) to wheat

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Table 1. Abbreviations

Abbreviation	Full name
α -AI	α -Amylase inhibitor
chit II	Class II chitinase
GST	Glutathione S-transferase
HEP	Histamine equivalent in prick testing
LTP1	Lipid transfer protein 1
NCBI nr	Nonredundant (nr) protein database compiled by the National Center for Biotechnology Information
nsLTP1	Nonspecific lipid transfer protein 1
nsLTP2G	Nonspecific lipid transfer protein 2G
PER1	1-Cys peroxiredoxin
PI	Peroxidase I
RT	Room temperature
SPT	Skin prick test
TLP	Thaumatococcus-like protein
XIP-1	Xylanase inhibitor protein 1

flour (100 mg/mL mixed with 0.1M phosphate-buffered saline [PBS], pH 7.4).¹⁶ Nineteen of 20 cases of rhinitis or asthma were verified by means of nasal or inhalation challenge tests with grains to which patients were exposed at their workplaces, performed as previously described.^{17,18} However, poor lung function did not allow an inhalation challenge test to be performed for 1 patient. Simultaneous sensitization to rye, barley, or oats was detected in most patients. Seven of 20 patients had sensitization to at least 1 of 15 common envi-

ronmental allergens in SPT, marked as atopy in Table 2. The tested allergens were birch, alder, meadow fescue, timothy, mugwort, latex, horse, dog, cat, cow, house dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, 10 HEP), and molds (*Alternaria alternata* and *Cladosporium herbarum*, 1:20 wt/vol). They were obtained from ALK-Abello (Madrid, Spain), except for natural rubber latex (Stallergenes, Antony Cedex, France) (100 IR/mL) and in-house extract of powdered latex gloves (340 mg/10 mL PBS). The control group consisted of 10 healthy individuals who had negative SPT reactions to wheat and the environmental allergens mentioned previously herein. This study was approved by the coordinating ethics committee of the Hospital District of Helsinki and Uusimaa, and written informed consent was obtained from each participant.

Challenge Tests

The nasal provocation test was performed to confirm occupational rhinitis without asthma. On average, 0.025 g of wheat flour was applied on both of the inferior conchae using a nasal elevator. The degree of rhinorrhea and nasal blockage in the nasal provocation test was scored after 15 and 30 minutes as described elsewhere.¹⁷ Acoustic rhinometry was performed before and after the provocation but was considered to be mainly supportive to the diagnosis.

If lower airway reactions were suspected, inhalation challenge tests with wheat flours were performed.¹⁸ The compressed air blew flour into the 6-m³ exposure chamber every

Table 2. Demographic and Clinical Data for 20 Patients With Baker's Asthma and Rhinitis

Patient No./sex/age, y	SPT wheat to grains ≥ 3 mm	Atopy	Challenge test		IgE total, kU/L	IgE wheat ImmunoCAP, kU/L
			Rhinitis	Asthma		
1/F/46	WROB	Yes	W+	W+	13	9.1
2/M/30	WROB	No	ND	W+	77	7.9
3/F/62	WRB	No	W+	ND	42	5.9
4/F/44	WROB	No	W+	W+	104	5.8
5/F/23	WROB	Yes	W+	ND	821	27.6
6/F/35	WROB	No	WR+	ND	12	0.6
7/F/35	WROB	Yes	W+	ND	157	9.4
8/F/49	WROB	No	W+	W+	220	61.2
9/M/23	W	Yes	W+	ND	307	0.4
10/F/32	B	Yes	R+	R+	165	4.1
11/F/33	WOB	No	WR+	ND	58	2.7
12/M/41	WRB	No	ND	R+	120	3.8
13/M/31	WROB	Yes	R+	R+	533	13.4
14/F/43	WROB	No	W+	ND	73	3.8
15/F/27	WRB	No	W+	ND	34	0.8
16/M/27	WROB	No	R+	R+	72	4.1
17/F/46	WRB	No	WR+	ND	127	1.1
18/M/57	WRB	Yes	W-	ND	105	0.5
19/M/34	WROB	No	W+	ND	115	11.9
20/F/40	WRB	No	W+	ND	55	0.5
Overall		Yes (n = 7)	+ (n = 17)	+ (n = 8)	160.5 ^a	8.7 ^a

Abbreviations: B, barley; ND, not done; O, oat; R, rye; SPT, skin prick test; W, wheat.

^a Mean.

minute for up to 30 minutes. The air concentrations were adjusted to mimic exposure levels in the workplace. Rhinitis was evaluated before inhalation challenge testing in the morning and approximately 20 minutes after the challenge using the same criteria as in nasal provocation testing. In addition, the symptoms and signs of rhinitis and asthma during inhalation challenge testing were recorded. A peak expiratory flow rate or forced expiratory volume in 1 second decrease of 20% was diagnostically significant. Peak expiratory flow rate or forced expiratory volume in 1 second decreases of 15% to 20% with simultaneous expiratory wheezing or dyspnea within 1 hour of the occupational exposure was also considered to be a positive asthmatic reaction. Control tests with lactose were performed with each patient on a different day, ruling out nonspecific reactions.

Wheat Protein Extraction and Purification

Water-soluble wheat (*Triticum aestivum*, cultivar Tjalve) proteins were extracted from grounded grains in 25 mL of 20-mmol/L ammonium bicarbonate (pH 8.4) or 20-mmol/L ethanolamine (pH 9.5) (Merck, Darmstadt, Germany), with constant stirring on a reciprocating shaker (Edmund Bühler GmbH, Hechingen, Germany) for 4 hours at 4°C. After centrifugation (30,000g for 30 minutes at 4°C), supernatants were collected, filtered (0.45 μ m) (Gelman Sciences, Ann Arbor, Michigan), and frozen as aliquots. Protein assays were made using a Lowry method (BioRad, Hercules, California). Ammonium bicarbonate extracts were used to analyze IgE reactivity of wheat water-soluble proteins by means of immunoblotting after 2-dimensional (2-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (see later herein), and ethanolamine extracts were used for isolation of water-soluble proteins from wheat flour.

Purification of proteins from ethanolamine extracts involved gel filtration and reversed-phase chromatography. The first gel filtration was made using a Superdex 75 HighLoad 16/60 column (GE Healthcare, Uppsala) at a flow rate of 1 mL/min in 20-mmol/L ethanolamine (pH 9.5). Monitoring was performed at 280 nm, and fractions (1.5 mL) were collected and analyzed by means of 1-dimensional (1-D) SDS-PAGE, Coomassie blue staining, and immunoblotting (see later herein). Fractions containing immunogenic proteins (identified by means of tandem mass spectrometry and shown to be allergenic by means of immunoblotting) were pooled, concentrated under nitrogen stream (TurboVap LV Evaporator; Zymark Corp, Hopkinton, Massachusetts), and further purified by means of another gel filtration step using a Superdex 200 HR 10/30 column (GE Healthcare) similarly collecting 1.0-mL fractions. The final purification of different protein pools was performed by means of reversed-phase chromatography using a Resource RPC 3-mL column (GE Healthcare) and a linear gradient of acetonitrile (0% to 100% in 40 minutes) in 0.1% trifluoroacetic acid.

1-D and 2-D SDS-PAGE

Protein samples were evaporated using a vacuum centrifuge (SpeedVac; Savant Instruments Inc, Farmingdale, New York), and a reducing buffer (50-mmol/L Tris, 5% 2- β -mercaptoethanol, 2% SDS, 10% glycerol, and 0.05% bromophenol blue) was added for 1-D SDS-PAGE. To perform 2-D SDS-PAGE, 125 μ L of rehydration buffer (8M urea, 2% CHAPS [3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate], 0.5% immobilized pH gradient buffer 3 to 10, 65-mmol/L dithiothreitol, and 0.002% bromophenol blue) was added to 100 μ g of crude wheat extract, and the mixture was absorbed into 7-cm, 3- to 10-L 0.5% immobilized pH gradient strips (GE Healthcare) at room temperature for 24 hours. Isoelectric focusing was performed using Ettan IPGPhorII (GE Healthcare) to a total of 6.5 kVh, after which proteins were separated using 12% SDS-PAGE according to the manufacturer's instructions (BioRad). After 2-D SDS-PAGE, 1 of 2 parallel gels was stained with silver stain, and the other was exposed to immunoblotting with sera from patients with baker's asthma and rhinitis.

Immunodetection of Wheat Allergens From 2-D SDS-PAGE Gels

Immunoblotting was performed as described previously with modifications.¹⁹ Briefly, proteins were transferred by means of electrophoresis (MiniTransBlot; BioRad) from 2-D SDS-PAGE gels onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp, Bedford, Massachusetts). Membranes were then incubated with diluted (1:10 or 1:5) patient's sera overnight at 4°C. Biotinylated goat anti-human IgE (Vector Laboratories Inc, Burlingame, California), diluted 1:1,000, was added, followed by streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, California), diluted 1:240,000, and the color development solution (BioRad).

Identification of Allergens Using Tandem Mass Spectrometry

Protein spots were cut out from the gels, reduced with dithiothreitol, and alkylated with iodoacetamide before in-gel digestion with modified sequencing grade porcine trypsin (Promega, Madison, Wisconsin) for 16 hours at 37°C. After removal of the supernatants to fresh tubes, the remaining peptides in gel pieces were extracted twice with 100 μ L of 5% formic acid in 50% acetonitrile. Combined peptide extracts were dried in a vacuum centrifuge. Each peptide mixture was analyzed by means of automated nanoflow capillary liquid chromatography–tandem mass spectrometry using a CapLC system (Waters, Milford, Massachusetts) coupled with an electron spray ionization quadrupole time-of-flight mass spectrometer (Q-TOF Global; Waters). Reversed-phase separation of peptides was performed using a 75 μ m $\times$ 15 cm Atlantis dC₁₈ NanoEase column (Waters) at a flow rate of 250 nL/min. Solvent A was 0.1% formic acid in 5% acetonitrile and solvent B was 0.1% formic acid in 95% acetonitrile.

Peptides were eluted from the column with a linear gradient of 0% to 60% solvent B in 30 minutes.

The obtained mass fragment spectra were searched in the NCBI database against all entries using in-house Mascot v.2.1 (Matrix Science Ltd, London, England) with the following variables: trypsin specificity, 1 missed cleavage, precursor ion mass accuracy of 0.2 Da, and 0.5-Da mass tolerance for fragment ions; the fixed modification was carbamidomethylation of cysteine, and oxidations of methionine, histidine, and tryptophan were accepted as variable modifications.

SPTs for Isolated Wheat Proteins

Purified wheat allergen proteins were diluted serially before use with PBS (5, 50, and 500 $\mu\text{g/mL}$, except for peroxidase I (PI): 5, 50, and 300 $\mu\text{g/mL}$) for SPT. The SPT was performed using a lancet (ALK-Abello) and purified allergen liquid drop sizes of 2 μL . The SPTs were performed immediately after the dropping so that drops did not dry. Histamine (10 mg/mL) (ALK-Abello) was used as a positive control and solvents as negative controls. Histamine responses were examined using a normal-sized drop and a 2- μL drop, and mean values were 6.5 and 6.1 mm, respectively. Averages of these histamine responses were used as results. The SPTs were also performed with Finnish commercial wheat flour (100 mg/mL in PBS), wheat (Tjalve) extract (500 $\mu\text{g/mL}$), gliadin (1 mg/mL) (crude from wheat gluten) (Sigma-Aldrich Corp, St Louis, Missouri), and a commercial $\alpha\text{-AI}$ (Sigma-Aldrich Corp). Criteria for positive SPT reactions were skin wheal reactions of 3 mm or greater.

ELISA for Specific IgE Antibodies to Purified Wheat Proteins

IgE ELISA was performed as previously described with some modifications.²⁰ Briefly, the concentration of purified proteins was adjusted to 5 $\mu\text{g/mL}$ in PBS (except PI, 2 $\mu\text{g/mL}$), and microtiter plates were incubated for 1 hour at 37°C. The wells were then postcoated with 1% human serum albumin (Red Cross Finland Blood Service, Helsinki) in PBS for 1 hour at room temperature. Patient or control serum (diluted 1:5) was added and incubated overnight at room temperature. Biotinylated goat anti-human IgE (Vector Laboratories Inc) (diluted 1:1,000) was added for 1 hour at room temperature. After that, streptavidin-conjugated alkaline phosphatase (Bio-Rad) (diluted 1:3,000) followed by substrate (p-nitrophenyl phosphate) (ICN Biomedicals Inc, Aurora, Ohio) was used to detect the bound antibody levels with the ELISA plate reader (optical density, 405 nm) (Labsystems Multiscan; Thermo Electron, Turku, Finland).

RESULTS

Identification of IgE-Binding Wheat Proteins in Baker's Respiratory Allergy

Water-soluble extract of wheat grains is protein rich, as can be seen in the silver-stained 2-D SDS-PAGE from ammonium bicarbonate extraction (Fig 1A). Western blotting with

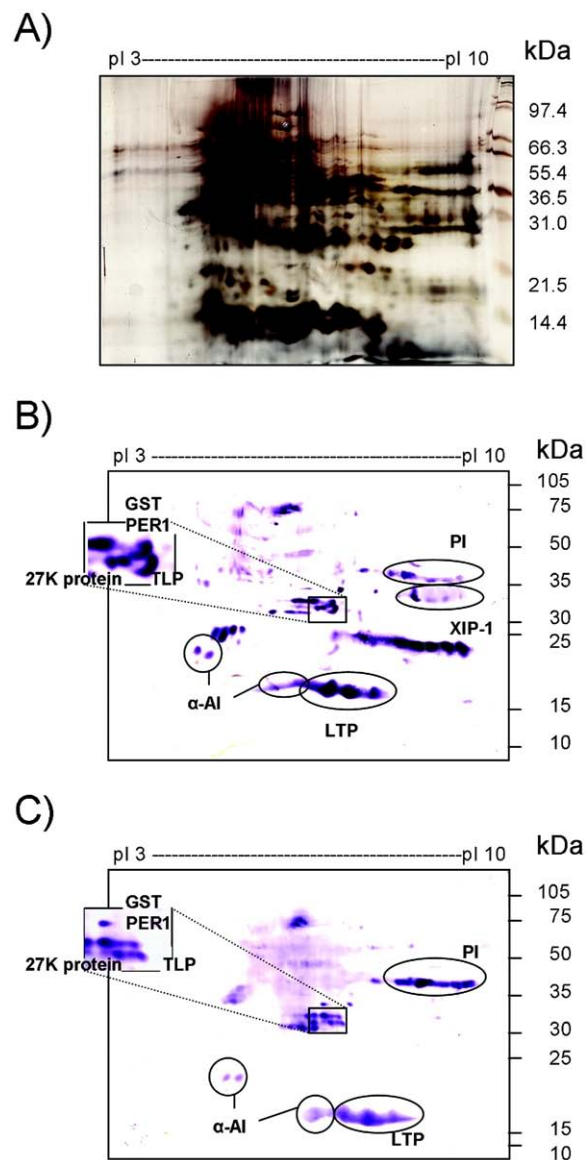


Figure 1. Identification of water-soluble allergens in wheat extract. A, Silver-stained 2-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. B and C, Western blots of 2-dimensional SDS-PAGE-separated wheat extract proteins detected using 2 individual serum samples from patients with baker's asthma and rhinitis. Marked proteins were identified by means of tandem mass spectrometry. $\alpha\text{-AI}$ indicates α -amylase inhibitor; GST, glutathione S-transferase; LTP, lipid transfer protein; PER1, 1-Cys peroxiredoxin; PI, peroxidase I; TLP, thaumatin-like protein; XIP-1, xylanase inhibitor protein 1.

serum samples from patients with baker's asthma and rhinitis indicated that many of these proteins in these patients bind IgE, ie, are allergens (Fig 1B and C). However, IgE-binding responses of individual patients to these proteins varied. Some of their sera recognized only a few proteins, whereas others reacted to several protein spots. Tandem mass spec-

Table 3. Wheat Allergens Identified Using Tandem Mass Spectrometry

Swiss-Prot accession No.	Identification	MW, kDa	pI	Mascot score ^a	Matched peptides, No. ^b /sequence coverage, % ^c
P82900	nsLTP2G	7	8.2	281	4/92
P24296	nsLTP1	12	8.1–8.5	106	2/16
P01083	α -AI	12–16	5.0–5.3	260	6/54
P01085	α -AI	13–17	6.3–7.4	312	5/34
P27357	TLP	23	7.8	352	7/39
Q7Y1Z2	27K protein	23	6.0	338	9/56
Q6W8Q2	PER1	24	6.1	425	8/43
Q8RW03, Q8LGN5	GST	25	6.0–6.3	363	9/35
AAX83263	chit II	28	8.7	334	5/34
Q8L5C6	XIP-1	34	8.6	127	2/6
Q05855	PI	39	8.1	823	13/29

Abbreviations: α -AI, α -amylase inhibitor; chit II, class II chitinase; GST, glutathione S-transferase; MW, molecular weight; nsLTP1, nonspecific lipid transfer protein 1; nsLTP2G, nonspecific lipid transfer protein 2G; PER1, 1-cys peroxiredoxin; pI, isoelectric point; PI, peroxidase I; TLP, thaumatin-like protein; XIP-1, xylanase inhibitor protein 1.

^a Mascot score = $10 \times \log(P)$, where P is the probability that the observed match is a random event.

^b Number of peptides from digested gel spots that have sequence similarity with the identified protein.

^c Percentage of matching sequences with the whole protein sequence.

trometry analysis of IgE-binding protein bands suggested that especially α -AI (molecular weight [MW] = 12–17 kDa), LTP1 (MW = 12 kDa) and 2G (MW = 7 kDa), thaumatin-like protein (TLP) (MW = 23 kDa), 1-Cys-peroxiredoxin (PER1) (MW = 24 kDa), glutathione S-transferase (GST) (MW = 25 kDa), 27K protein, xylanase inhibitor protein 1 (XIP-1) (MW = 34 kDa), and PI (MW = 39 kDa) could be important allergens in patients with Finnish baker's asthma and rhinitis (Table 3).

Purification of Water-Soluble Wheat Allergens

Two gel filtration steps and reversed-phase chromatography were used to separate IgE-reactive water-soluble wheat proteins from each other. We purified LTP, α -AI, PI, and TLP, which are shown on 1-D SDS-PAGE gels after Coomassie brilliant blue staining (Fig 2). The LTP fractions contained mainly 1 protein form (2G), whereas α -AI and TLP contained different isomorphs. On the other hand, semipurified PI fractions contained small amounts of class II chitinase (chit II) and LTP2G besides PI.

SPT and ELISA Reactivity to Purified Wheat Proteins

All the patients had positive SPT reactions to a total wheat flour preparation and to the pool of water-soluble wheat proteins (Fig 3A). Dose-dependent SPT responses to purified water-soluble wheat allergens were found, and results are shown for 2 concentrations (50 μ g/mL [Fig 3B] and 300–500 μ g/mL [Fig 3C]). A higher concentration of proteins revealed that even 60% of patients with baker's respiratory allergy (12 of 20) recognized α -AI as an allergen, and 75% of patients (15 of 20) recognized at least 1 tested wheat allergen in SPT, on average 2 of 5 (range, 0–5). Both TLP and PI were equally important wheat allergens in patients: 45% (9 of 20) had

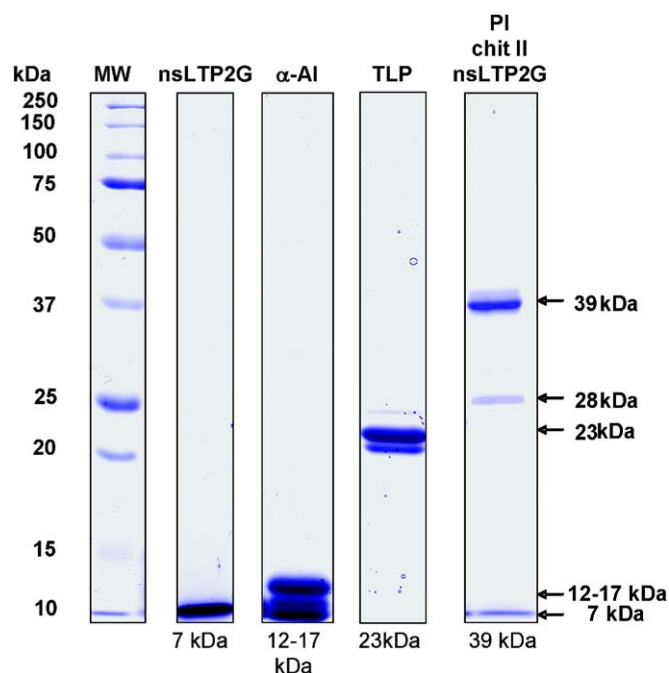


Figure 2. Purified wheat allergens on 1-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels after Coomassie brilliant blue staining. Proteins were purified from ethanol amine extracts of grained wheat in 2 gel filter chromatography steps and using reversed-phase chromatography. α -AI indicates α -amylase inhibitor; chit II, class II chitinase; MW, molecular weight; nsLTP2G, nonspecific lipid transfer protein 2G; PI, peroxidase I; and TLP, thaumatin-like protein.

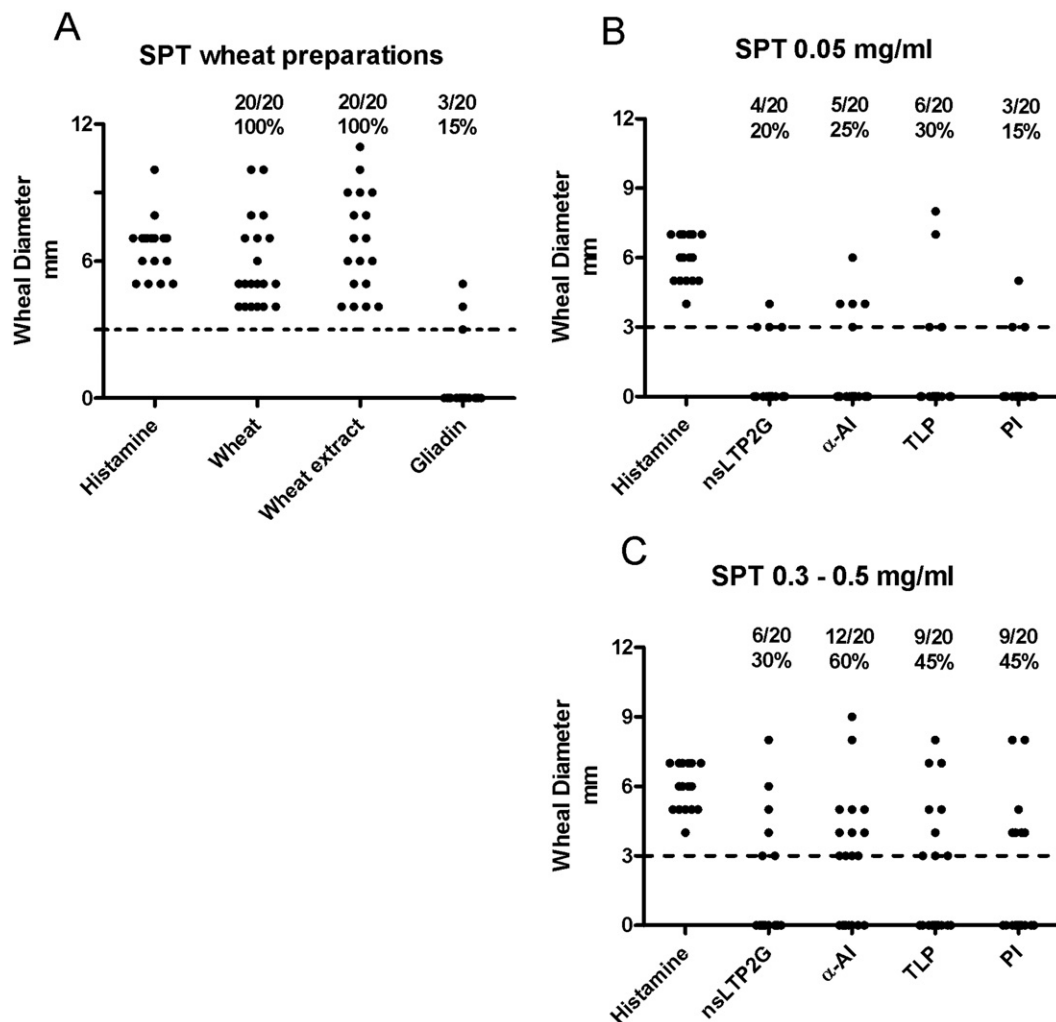


Figure 3. Skin prick test (SPT) responses of patients with baker's asthma and rhinitis ($n = 20$). A, Wheat components (concentrations: wheat, 1 mg/mL; ethanol amine extract of wheat, 5 mg/mL; and gliadin, 1 mg/mL). B, Purified allergens at a concentration of 50 μ g/mL. C, Purified allergens as at a concentration of 500 μ g/mL (except peroxidase I [PI], 300 μ g/mL). Dashed cutoff lines are shown (positive responses: wheal diameter ≥ 3 mm). α -AI indicates α -amylase inhibitor; nsLTP2G, nonspecific lipid transfer protein 2G; and TLP, thaumatin-like protein.

positive test results. The LTP is also an important allergen because 30% of patients (6 of 20) had positivity toward it. At the lower concentration, the role of TLP (30% positivity) and α -AI (25% positivity) as major allergens is emphasized compared with other purified proteins (LTP2G, 20% positivity; PI, 15% positivity). Three patients (15%) were SPT positive to crude gliadin, but all the patients had larger SPT wheals to some other tested allergen at the same time. All 10 control subjects had negative SPT reactions to the tested wheat allergens.

In vitro IgE reactivity to purified allergens was, in general, low when analyzed by means of ELISA. When we used cutoff levels (averages + 3 SDs) obtained from control patients, 12 of 20 patients had a positive response to α -AI, whereas only 1 patient showed positive IgE reactivity to LTP2G, 1 to PI preparation, and 2 to purified TLP.

DISCUSSION

Recognition of relevant allergens is important for correct diagnosis and treatment of baker's respiratory allergy. Wheat flour is the leading cause of this disease. These investigations were directed to discover relevant allergens from water-soluble extracts of grained wheat. Several water-soluble allergens from wheat flour were identified and purified, after which their in vivo reactivity by SPTs were studied in Finnish individuals. However, sensitivity of the ELISA methods was not sufficient in this study (data not shown), emphasizing the importance of SPT in the diagnosis of baker's respiratory allergy to water-soluble wheat proteins.

Water-soluble TLP purified from wheat extracts showed SPT reactivity in approximately 45% of these patients. The

TLP belongs to the plant defense proteins, which are important allergens in several fruits, such as apples and cherries,²¹ whereas in grains they prevent fungal diseases. This is the first study to show that TLP is an allergen in baker's respiratory allergy.

The LTPs belong to a family of plant food panallergens.²² A recent study¹² showed that even 62% of patients with wheat-sensitized baker's asthma have positive SPT reactions to nsLTP1 (Tri a 14). We also found that many patients had IgE antibodies to nsLTP1 in their sera. However, we purified another LTP (MW = 7 kDa), ie, nsLTP2G, from water extracts of wheat. One-third of the patients (30%) had reactivity to nsLTP2G. None of the earlier studies had noticed a significance of nsLTP2G in allergies. Altogether, it seems that several LTPs can be important allergens in baker's asthma and rhinitis.

Another earlier-found wheat allergen in patients with baker's asthma is PI.^{6,23,24} We used semipurified PI for SPT where PI was a main component and chit II and nsLTP2G were minor components. Immunoblotting results confirmed that sera of tested patients recognized PI and not the other proteins from this protein mixture (data not shown). This conclusion is supported by the fact that 4 patients had positive reactivity to this protein and no reactivity to purified nsLTP2G. The SPT results showed that 45% of patients with baker's respiratory allergy were sensitized to this allergen.

One of the purified allergens in this study was α -AI, which has been considered to be a major allergen in patients with baker's asthma.^{6,7,25-27} In an earlier study with a recombinant protein of α -AI, 65% of patients with baker's asthma were sensitized to this protein.¹³ This protein was a major allergen also in this study of baker's respiratory allergy because 60% of patients had positive SPT reactions to this protein.

In addition, we identified but did not purify other proteins from water-soluble wheat extract that were recognized by IgE antibodies by various sera from Finnish patients with baker's asthma and rhinitis, including XIP-1, PER1, GST, and 27K protein. XIP-1 has been isolated from wheat, and its amino acid sequence has a significant homology with chit III.²⁸⁻³⁰ Thus, XIP-1 is also a new putative allergen in baker's respiratory allergy, and its exact significance awaits further studies.

A recent study³¹ using recombinant proteins suggests an important role for dehydrin (IgE antibody recognition of 32%) and PER1 (18% of patients were positive), whereas antioxidant GST (4.5% reactivity) has only a minor role. 27K protein, identified in this study, is also considered to be a prominent allergen in wheat allergies.^{32,33} In addition, earlier studies have found that, for example, acyl-CoA oxidase,³⁴ triosephosphate isomerase,⁹ thioredoxin (h) (Tri a 25),¹³ and serine proteinase inhibitor (serpin) (27% reactivity in patients with respiratory allergy to wheat)^{9,11} are involved in baker's respiratory allergy.

Although we did not purify non-water-soluble proteins, we tested crude commercial gliadin in SPT as well. Fifteen percent of patients showed some positivity in SPT, but other tested allergens had stronger reactions at the same time in

those patients. We find this to be in line on the presumption that these proteins are stronger allergens for wheat allergy from oral ingestion rather than from respiratory exposure.^{35,36}

Diagnosis of wheat-dependent allergies has been complicated with the fact that wheat flour contains many different possible allergens, responses to which vary greatly among patients. Another confusing factor is a mutual cross-reactivity of taxonomically related grains, such as wheat, rye, and barley,^{19,37} and cross-reactivity of inhaled flour allergens with grass pollens, eg, thioredoxins¹³ and profilins.³¹ In line with this, many patients with baker's respiratory allergy had positive SPT reactivity to several grains (eg, wheat, rye, and barley).

In conclusion, these results confirm that patients with baker's asthma and rhinitis recognize in vivo several different water- and salt-soluble proteins from wheat grains, and most of these belong to plant defense proteins.^{38,39} We found that α -AI, TLP, LTP, and PI are relevant allergens in Finnish patients with baker's respiratory allergy. Notably, TLP and LTP2G have not earlier been connected to baker's respiratory allergy. In the future, when trying to produce better clinical tests for the diagnosis of baker's respiratory allergy, these proteins should be included in test preparations. Because purification is a complex process, their production as recombinant proteins could be helpful. However, recombinant proteins often lack immunologically important posttranslational modifications. Also, the existence of different isomorphous forms of allergens, as shown by α -AI and LTP, also complicates planning of relevant recombinant allergens. For these reasons, the use of native purified proteins from wheat extracts is justified for testing and possibly also treatment materials in allergies. Finally, knowledge of relevant allergens can help to resolve mechanisms of sensitization in allergic diseases.

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REFERENCES

1. van Kampen V, Merget R, Baur X. Occupational airway sensitizers: an overview on the respective literature. *Am J Ind Med.* 2000;38:164-218.
2. Baur X, Degens PO, Sander I. Baker's asthma: still among the most frequent occupational respiratory disorders. *J Allergy Clin Immunol.* 1998;102:984-997.
3. Walusiak J, Hanke W, Gorski P, Palczynski C. Respiratory allergy in apprentice bakers: do occupational allergies follow the allergic march? *Allergy.* 2004;59:442-450.
4. Gautrin D, Ghezzi H, Infante-Rivard C, Malo JL. Incidence and host determinants of work-related rhinoconjunctivitis in apprentice pastry-makers. *Allergy.* 2002;57:913-918.
5. Brisman J. Baker's asthma. *Occup Environ Med.* 2002;59:498-502; quiz, 426.
6. Baur X, Posch A. Characterized allergens causing bakers' asthma. *Allergy.* 1998;53:562-566.
7. Brant A. Baker's asthma. *Curr Opin Allergy Clin Immunol.* 2007;7:152-155.

8. Mittag D, Niggemann B, Sander I, et al. Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheat-dependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility. *Mol Nutr Food Res.* 2004;48:380–389.
9. Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J Allergy Clin Immunol.* 2001;107:907–913.
10. Weiss W, Vogelmeier C, Gorg A. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. *Electrophoresis.* 1993;14:805–816.
11. Constantin C, Quirce S, Grote M, et al. Molecular and immunological characterization of a wheat serine proteinase inhibitor as a novel allergen in baker's asthma. *J Immunol.* 2008;180:7451–7460.
12. Palacin A, Quirce S, Armentia A, et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. *J Allergy Clin Immunol.* 2007;120:1132–1138.
13. Weichel M, Glaser AG, Ballmer-Weber BK, Schmid-Grendelmeier P, Cramer R. Wheat and maize thioredoxins: a novel cross-reactive cereal allergen family related to baker's asthma. *J Allergy Clin Immunol.* 2006;117:676–681.
14. Bittner C, Grassau B, Frenzel K, Baur X. Identification of wheat gliadins as an allergen family related to baker's asthma. *J Allergy Clin Immunol.* 2008;121:744–749.
15. Sandiford CP, Tatham AS, Fido R, et al. Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. *Clin Exp Allergy.* 1997;27:1120–1129.
16. Tasman AJ, Witzel A, Simonis B, Sohmen R, Grieshaber R, Heppt W. The skin prick test and nasal provocation test with individually prepared flour extracts in patients with bakers' rhinitis and asthma [in German]. *HNO.* 1999;47:718–722.
17. Airaksinen L, Tuomi T, Vanhanen M, Voutilainen R, Toskala E. Use of nasal provocation test in the diagnostics of occupational rhinitis. *Rhinology.* 2007;45:40–46.
18. Airaksinen LK, Tuomi TO, Tuppurainen MO, Lauerma AI, Toskala EM. Inhalation challenge test in the diagnosis of occupational rhinitis. *Am J Rhinol.* 2008;22:38–46.
19. Palosuo K, Alenius H, Varjonen E, Kalkkinen N, Reunala T. Rye γ -70 and γ -35 secalins and barley γ -3 hordein cross-react with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis. *Clin Exp Allergy.* 2001;31:466–473.
20. Lehto M, Palosuo K, Varjonen E, et al. Humoral and cellular responses to gliadin in wheat-dependent, exercise-induced anaphylaxis. *Clin Exp Allergy.* 2003;33:90–95.
21. Breiteneder H. Thaumatin-like proteins: a new family of pollen and fruit allergens. *Allergy.* 2004;59:479–481.
22. Breiteneder H, Mills C. Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. *Curr Opin Allergy Clin Immunol.* 2005;5:275–279.
23. Sanchez-Monge R, Garcia-Casado G, Lopez-Otin C, Armentia A, Salcedo G. Wheat flour peroxidase is a prominent allergen associated with baker's asthma. *Clin Exp Allergy.* 1997;27:1130–1137.
24. Yamashita H, Nanba Y, Onishi M, Kimoto M, Hiemori M, Tsuji H. Identification of a wheat allergen, Tri a Bd 36K, as a peroxidase. *Biosci Biotechnol Biochem.* 2002;66:2487–2490.
25. Amano M, Ogawa H, Kojima K, et al. Identification of the major allergens in wheat flour responsible for baker's asthma. *Biochem J.* 1998;330(pt 3):1229–1234.
26. Armentia A, Sanchez-Monge R, Gomez L, Barber D, Salcedo G. In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin Exp Allergy.* 1993;23:410–415.
27. Sanchez-Monge R, Gomez L, Barber D, Lopez-Otin C, Armentia A, Salcedo G. Wheat and barley allergens associated with baker's asthma: glycosylated subunits of the α -amylase-inhibitor family have enhanced IgE-binding capacity. *Biochem J.* 1992;281(pt 2):401–405.
28. Elliott GO, Hughes RK, Juge N, Kroon PA, Williamson G. Functional identification of the cDNA coding for a wheat endo-1,4- β -D-xylanase inhibitor. *FEBS Lett.* 2002;519:66–70.
29. Payan F, Flatman R, Porciero S, Williamson G, Juge N, Roussel A. Structural analysis of xylanase inhibitor protein I (XIP-I), a proteinaceous xylanase inhibitor from wheat (*Triticum aestivum*, var. *Soisson*). *Biochem J.* 2003;372:399–405.
30. Payan F, Leone P, Porciero S, et al. The dual nature of the wheat xylanase protein inhibitor XIP-I: structural basis for the inhibition of family 10 and family 11 xylanases. *J Biol Chem.* 2004;279:36029–36037.
31. Constantin C, Quirce S, Poorafshar M, et al. Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis. *Allergy.* 2009;64:1030–1037.
32. Kimoto M, Suzuki M, Komiyama N, et al. Isolation and molecular cloning of a major wheat allergen, Tri a Bd 27K. *Biosci Biotechnol Biochem.* 2009;73:85–92.
33. Posch A, Weiss W, Wheeler C, Dunn MJ, Gorg A. Sequence analysis of wheat grain allergens separated by two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis.* 1995;16:1115–1119.
34. Weiss W, Huber G, Engel KH, et al. Identification and characterization of wheat grain albumin/globulin allergens. *Electrophoresis.* 1997;18:826–833.
35. Palosuo K, Alenius H, Varjonen E, et al. A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol.* 1999;103:912–917.
36. Palosuo K, Varjonen E, Kekki OM, et al. Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J Allergy Clin Immunol.* 2001;108:634–638.
37. Sandiford CP, Tee RD, Newman-Taylor AJ. Identification of crossreacting wheat, rye, barley and soya flour allergens using sera from individuals with wheat-induced asthma. *Clin Exp Allergy.* 1995;25:340–349.
38. Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol.* 2000;106:27–36.
39. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol.* 2008;121:847–852.e7.

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