

Wheat and maize thioredoxins: A novel cross-reactive cereal allergen family related to baker's asthma

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Background: Baker's asthma is a serious problem for a significant proportion of workers in bakeries, confectionaries, and the food industry. Although several wheat allergens related to baker's asthma have been described, standardized reagents for a reliable diagnosis are not yet available.

Objective: To clone novel wheat allergens related to baker's asthma and investigate the cross-reactive potential of their maize and human homologues.

Methods: A wheat cDNA phage display library was screened with sera from bakers with occupational asthma for IgE-binding structures. Homologous sequences from maize and human thioredoxins were amplified from corresponding cDNA libraries.

Results: Within the enriched wheat cDNA repertoire we identified, among others, the sequence encoding wheat thioredoxin-hB (*Triticum aestivum* allergen 25 [Tri a 25]). The recombinant protein displayed enzymatic activity, and we observed a sensitization rate of 47% among bakers with occupational asthma and of 35% among patients with grass pollen allergy, but without a clinical history of cereal allergy. Furthermore, the previously characterized maize thioredoxin-h1 (*Zea mays* allergen 25 [Zea m 25]), sharing 74% identity with Tri a 25, exhibited distinct IgE cross-reactivity with its wheat homologue. Two bakers also showed sensitization to human thioredoxin, which shares 29% identity with Tri a 25. In a comparative study, we included recombinant α -amylase inhibitor 0.19, showing a sensitization rate of 65% in individuals with baker's asthma.

Conclusion: Thioredoxins represent a novel family of cross-reactive allergens that might contribute to the symptoms of baker's asthma and might in addition be related to grass pollen allergy, as indicated by the reactivity of grass pollen allergic patients to cereal thioredoxins.

Clinical implications: The recombinant cereal thioredoxins will, together with the already reported wheat allergens, contribute to a more reliable diagnosis of baker's asthma and, perhaps, become a tool for the development of component-resolved immunotherapy. (J Allergy Clin Immunol 2006;117:1101-1107.)

Key words: Baker's asthma, *Triticum aestivum*, *Zea mays*, recombinant allergens, cross-reactivity, cDNA phage display

Baker's asthma is an occupational disease affecting 4% to 10% of bakery workers in European countries.¹ Causative allergens related to baker's asthma are proteins derived from wheat flour, rye flour, and soy bean flour, and baking additives like fungal α -amylase (Asp o 21).^{2,3} Several wheat proteins have been described as allergens on the molecular level: α -amylase inhibitors,⁴ acyl-coenzyme A oxidase,⁵ fructose-biphosphate aldolase,⁵ and wheat-flour peroxidase.⁶ ω -5 Gliadin from wheat (*Triticum aestivum* allergen 19 [Tri a 19]) has been described as major allergen in children with food allergy to wheat⁷ and in adults with wheat-dependent exercise-induced anaphylaxis,⁸ but has not been related to baker's asthma. Although there is consensus in the literature that the albumin/globulin fraction of wheat flour contains most of the IgE-binding proteins involved in baker's asthma, there is much more disagreement about which proteins can be termed major allergens. Several studies report that members of the α -amylase family constitute the major allergens of wheat,^{4,9,10} whereas proteins in the 26 to 28 kd, 30 to 38 kd, and 69 to 70 kd range are also considered potential allergens.^{5,11-13} In contrast, other studies demonstrate very heterogeneous reactivity of bakers' sera against wheat proteins in Western blot or 2-dimensional immunoblot analyses.^{14,15}

Commercially available wheat extracts routinely used for skin prick tests differ greatly in antigen concentration and composition.¹⁶ Consequently, there is a need for improvement and standardization of the diagnostic procedures for baker's asthma.

The pJuFo (phagemid containing the Jun and Fos leucine zippers used for the construction of cDNA libraries displayed on phage surface) technology has proven to be a versatile tool for the selection and characterization of naturally occurring allergens of fungi,¹⁷⁻²⁰ mites,²¹ and peanut,²² and recombinant allergens of *Aspergillus fumigatus* are nowadays routinely used for the diagnosis of

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Supported by the Swiss Federal Office for Education and Science, Grant BBW #01.0159-2, as a part of the 5th European Union framework programme FAREDAT—Quality of Life and Management of Living Resources, QLRT-2001-00301, and by the Swiss National Science Foundation Grants #31-63381.00/2 and #310000-112540.

Received for publication September 5, 2005; revised November 14, 2005; accepted for publication November 29, 2005.

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0091-6749/\$32.00

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doi:10.1016/j.jaci.2005.11.040

Abbreviations used

AI: Amylase inhibitor
 Tri a 19: *Triticum aestivum* allergen 19
 Tri a 25: *Triticum aestivum* allergen 25
 TRX: Thioredoxin
 Zea m 25: *Zea mays* allergen 25

allergic bronchopulmonary aspergillosis.^{18,23} To identify proteins related to baker's asthma, we screened a wheat cDNA phage display library against immobilized serum IgE from 8 bakers with occupational asthma. Among the enriched sequences, we identified an open reading frame encoding wheat thioredoxin *hB* (*Triticum aestivum* allergen 25 [Tri a 25]; EMBL acc no AJ404845). The sequence exhibited 74% identity with a thioredoxin from maize, *Zea m 25* (*ZmTRXh1*), which was originally identified among sequences related to food allergy.²⁴ Together with a second thioredoxin of maize, *ZmTRXh2*, and human thioredoxin, we demonstrate that the thioredoxin family includes cross-reactive members that might be of relevance for patients occupationally exposed to inhalant allergens.

METHODS**Construction of wheat and maize phage display cDNA libraries, patients' sera**

The construction of the wheat cDNA phage display cDNA library is described elsewhere.²⁴ For the enrichment of IgE-binding sequences, we used sera of 8 patients with a well documented history of baker's asthma and with positive CAP-RAST (Pharmacia, Uppsala, Sweden) against wheat flour (Table I). Diagnoses were confirmed by pre-past work spirometry. ELISA was performed with sera of 17 patients with baker's asthma. The patients met the American Thoracic Society criteria for the diagnosis of asthma²⁵ and were included if they had a methacholine-induced $\geq 20\%$ decrease in FEV₁ after inhalation and a $\geq 15\%$ increase in FEV₁ after 2 inhalations of albuterol. These sera were compared with 20 sera of individuals with grass allergy proven by positive clinical history, skin prick tests, and serum IgE to grass pollen, without clinical signs of wheat or maize allergy. As control, we used 17 sera of healthy individuals. An ethical approval for taking blood was obtained from the ethics committee of the University of Zürich.

Cloning of thioredoxins and α -amylase inhibitor 0.19

The coding sequence of thioredoxin *hB* from wheat (Tri a 25; EMBL acc no AJ404845) was amplified from the TaTRXhB cDNA and subcloned into pQE30 expression vector (Qiagen, Hilden, Germany) as described.²⁶ The coding sequences of *Zea m 25* (EMBL acc no AJ890020), *ZmTRXh2* (EMBL acc no AJ890021), and *HsTRX* (EMBL acc no X77584²⁷) were amplified using the following oligonucleotides: *Zea m 25*: 5'-primer: 5'-CG GGATCC ATG GCG GCT TCG GAG GCG GCG GCG GCG GCG GCG-3', 3'-primer: 5'-CC AAGCTT A CGA GGC CAT GTG TAG TTC CAG CTT CC-3'; *ZmTRXh2*: 5'-primer: 5'-CG GGATCC ATG GCG TCC GAG CAG GG-3', 3'-primer: 5'-CCT AAGCTT A GGC AGA CGC AGA CGC-3'; *HsTRX*: 5'-primer: 5'-CG GGATCC ATG GTG AAG CAG ATC GAG AGC-3', 3'-primer:

TABLE I. Characteristics of the patients' sera selected for affinity enrichment of the wheat cDNA phage surface display library

Patient #	Age, y	Sex	CAP-RAST wheat flour (F4), kU/L
1	23	F	4.29
2	22	M	24.1
3	16	F	45.5
4	24	M	45.4
5	25	F	4.77
6	28	M	2.35
7	42	M	18.7
8	42	F	1.08

5'-CCC AAGCTT A GAC TAA TTC ATT AAT GGT GGC TTC CAG CTT TTC C-3'.

The oligonucleotides used for the amplification of α -amylase inhibitor 0.19 were designed on the basis of the cDNA (EMBL acc no AY729672): 5'-primer: 5'-CG GGATCC AGT GGT CCT TGG ATG TGC-3', 3' primer: 5'-CCC AAGCTT A GGC GTC TGG GTA TGC GGC-3'.

The respective cDNAs were used as target for amplification reactions. After *Bam*HI/*Hind*III hydrolysis, the amplified DNA fragments were subcloned into likewise restricted vector pQE30 and used for transformation of *Escherichia coli* M15/pREP4. Correctness of the sequences was confirmed by DNA sequencing (Microsynth, Balgach, Switzerland).

Production of recombinant proteins and Western blot

For the production of the recombinant proteins, gene expression was induced at 37°C in 1 l cultures, with a final concentration of 1 mmol/L isopropyl thiogalactoside (IPTG) for 5 hours as soon as the exponentially growing cultures had reached an OD₅₅₀ of 0.5. Cells were harvested and recombinant protein was extracted and purified under denaturing conditions as described elsewhere.²⁰ The yield of recombinant protein was ≈ 10 mg/L of the thioredoxins and 0.25 mg/L of the 0.19 amylase inhibitor (AI).

For Western blot, 0.5 μ g recombinant protein was subjected to SDS-PAGE (NuPAGE; 12% Bis-Tris, Invitrogen, Groningen, The Netherlands), electrotransferred onto Hybond-P PVDF membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) and processed as described.²⁴

Thioredoxin catalyzed reduction of insulin

The catalytic reduction of insulin was performed as described by Holmgren.²⁸ Briefly, the reaction mixtures contained, in a final volume of 800 μ L, 0.1 mol/L potassium phosphate pH 6.5, 2 mmol/L EDTA, 0.13 mmol/L bovine insulin (Sigma, St Louis, Mo), 0.33 mmol/L dithiothreitol, and 3.9 μ mol/L of the respective thioredoxin. The precipitation of insulin was monitored with a Uvikon XL spectrophotometer (Bio-Tek Instruments, Winooski, Vt) at a wavelength of 650 nm.

Titration of allergen-specific serum IgE and competition ELISA

For allergen-specific IgE titrations, Nunc-Immuno Plates (Maxisorp Surface; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μ L 5 μ g/mL antigen in PBS, and washed and blocked with 2% (wt/vol) skimmed milk powder in PBS containing 0.5% Tween-20 (buffer A). After washing with PBS containing

Tri a 25	MAASAATATAA---AVGAGEVISVHSLEQWTMOIEEANAARKLVVDFATSWCGPCORIMA	57
Zea m 25	MAASEAANAATTPVAPTEGTVAIAHSLEEWSTOIEEANSARKLVVDFATWCPORAMA	60
ZmTRXh2	-MASEFGTRSEQ-----GVVIACHSKAEFDAHMTKQAEAGKLVVDFATWCGPCORAI	53
HsTRX	-----MVKQIESKTAFAQEALDAAG--DKLVVDFSAWCGPCCKMIK	39

Tri a 25	PIFADLAKKFPAAVFLKVDVDELKSIAEQFSVEAMPTFLFMKEGDKDRVVGAKKEELTN	117
Zea m 25	PIFADMAKKSINNVFLKVDVDEMKTIAEQFSVEAMPTFLFMKEGDKDRVVGAKKEELAR	120
ZmTRXh2	PIEVEHAKKFTQVFLKVDVDEVEKVTAAEVEAMPTFHEVKNCKTVATIVGAKKEELLA	113
HsTRX	PIFHSLSEKYSNVIFLEVDVDDCQDVASECEVVKCMPTFOFFKKGQKVGEFSGANKEKLEA	99

Tri a 25	KVGLHAAQ-----	125
Zea m 25	KLELHMAS-----	128
ZmTRXh2	LIEKHAAAPAPASASA	128
HsTRX	TINELV-----	105

FIG 1. Sequence alignment of cereal and human thioredoxins. Residues shared by all thioredoxins are in black, among wheat and maize thioredoxin in grey, and identical residues of wheat and maize thioredoxin are framed. Amino acid identities: Tri a 25/Zea m 25, 74%; Tri a 25/ZmTRXh2, 44%; Tri a 25/HsTRX, 29%; Zea m 25/ZmTRXh2, 48%; Zea m 25/HsTRX, 30%; ZmTRXh2/HsTRX, 32%.

0.1% Tween-20 (buffer B), patients' IgE was titrated starting with a serum dilution of 1:8 in buffer A, diluting serially 2-fold throughout the plate, and incubated for 2 hours at 37°C. IgE binding was detected by incubation with mAb TN-142 mouse antihuman IgE mAb (1 mg/mL) diluted 1:6500 in buffer A and visualized with alkaline-phosphatase-conjugated goat-antimouse IgG H + L chain (#31320, 1:6500; Pierce, Rockford, Ill) in buffer A. The enzymatic hydrolysis of 4-nitrophenylphosphate was monitored at a wavelength of 405 nm (A_{405}). Competition assays between the different antigens were performed on ELISA plates coated as described. The antigens were titrated starting with a concentration of 50 μ mol/L, keeping the serum concentration constant (1:10 in buffer A). Detection and visualization were performed as described. Percentage of competition was calculated from the background-corrected absorbency of samples where serum was incubated without antigen in the liquid phase (0%). For graphic display, the measured absorbencies were plotted against the antigen concentration in the fluid phase.

RESULTS

Wheat thioredoxin *hB* (Tri a 25) was identified in an enriched allergen repertoire after affinity selection against serum IgE of patients with baker's asthma. In an earlier study, we had identified Tri a 25 also in a repertoire affinity-selected against serum IgE from patients with wheat-dependent food allergy.²⁴ The maize thioredoxins identified in that study, Zea m 25 and ZmTRXh2, exhibit an amino acid identity of 48% showing that these proteins do not represent isoforms according to the definition of the International Union of Immunological Societies Allergen Nomenclature Sub-Committee.²⁹ In contrast, Tri a 25 and Zea m 25, although from different plants, share an amino acid identity of 74%, with a difference in the dipeptide between the 2 active site cysteines, which is -Pro-Pro- in Zea m 25, compared with -Gly-Pro- present in the other thioredoxins used in this study (Fig 1).

The thioredoxins were produced in *E coli* M15/pREP4, and purification under denaturing conditions yielded about 10 mg/L culture volume. SDS-PAGE was used to validate the purity of the recombinant proteins (Fig 2, A). The biological activity of the recombinant thioredoxins was demonstrated by the enzymatic reduction of insulin shown in Fig 2, B, indicating a natively folded of the proteins.

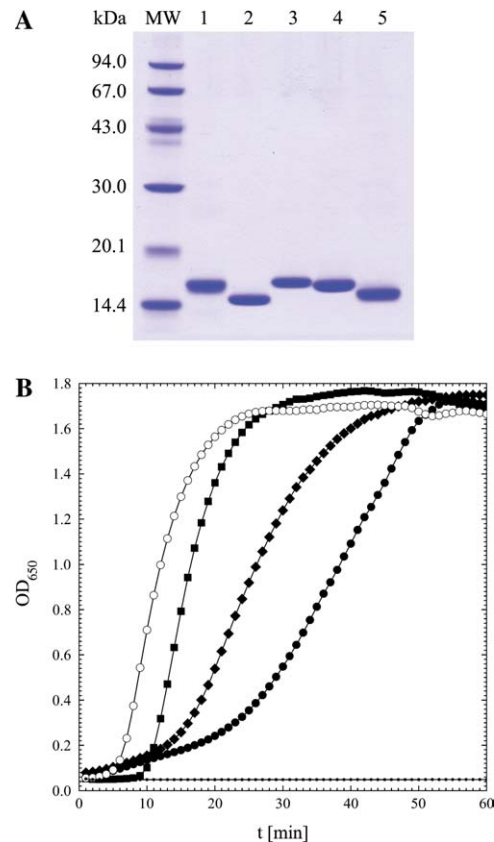


FIG 2. A, SDS-PAGE analysis of purified recombinant proteins. (MW) molecular mass standard, (1) 0.19 α -amylase inhibitor, (2) Tri a 25, (3) Zea m 25, (4) ZmTRXh2, (5) human thioredoxin. B, Thioredoxin-catalyzed reduction of bovine insulin by dithioerythrol. (●) Tri a 25; (◆) Zea m 25; (■) ZmTRXh2; (○) HsTRX; (●) uncatalyzed reaction mixture without dithioerythrol.

IgE binding of the thioredoxins was surveyed by ELISA using sera of 17 patients with baker's asthma, 20 sera with grass pollen allergy without history of wheat or maize allergy, and 17 sera of healthy individuals. For comparison, we used recombinant α -amylase/trypsin inhibitor 0.19 (0.19 AI) from wheat (Fig 3). Signals were

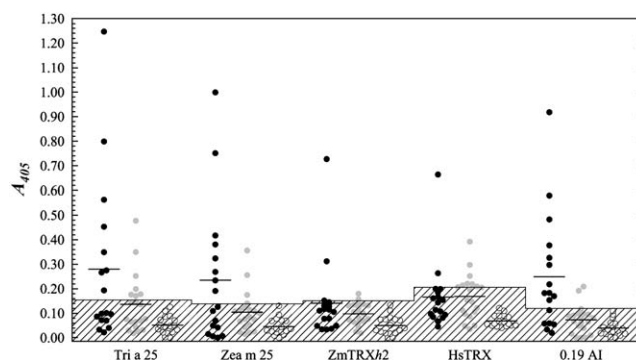


FIG 3. IgE-ELISA data for recombinant thioredoxins as well as 0.19 α -amylase inhibitor. (●) Bakers with a history of occupational asthma; (◐) grass-pollen allergic individuals without allergy to wheat and maize; (○) healthy controls. The median of each patient group for the respective protein is indicated by (—).

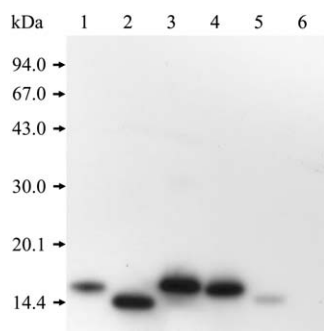


FIG 4. IgE binding of recombinant proteins demonstrated by Western blot analysis using serum of a baker with occupational asthma. (1) 0.19 α -amylase inhibitor, (2) Tri a 25, (3) Zea m 25, (4) ZmTRXh2, (5) human thioredoxin, (6) phospholipase A2 (PLA₂) from honey bee venom.

assigned positive when the A_{405} value was more than 3-fold higher than the mean A_{405} value of the healthy controls for the respective antigen. Among the bakers' sera, we thus identified 8 individuals with IgE reactivity to Tri a 25. Eight sera also reacted with Zea m 25, whereas only 2 reacted with ZmTRXh2 or with the human HsTRX. IgE binding against α -amylase inhibitor 0.19 (0.19 AI) was found in 11 bakers' sera, confirming that the protein is a major allergen related to baker's asthma. However, there was no correlation between IgE binding to thioredoxin and 0.19 AI.

Together with grasses, wheat and maize belong to the Poaceae family. We therefore investigated whether patients with grass pollen allergy, but without a clinical history of wheat or maize allergy, would show sensitization to any of the cereal thioredoxins. Among the 20 sera tested, 7 reacted with Tri a 25 and 4 with Zea m 25, and 2 showed moderate sensitization against ZmTRXh2 and 0.19 AI. Surprisingly, the level of sensitization against the human thioredoxin, although in an overall higher background, was comparable to that against wheat thioredoxin, with 4 to 9 sera showing IgE binding, but without significant correlation.

IgE binding was also confirmed by Western blot analysis for a patient with baker's asthma (Fig 4). To

explore whether IgE of patients with baker's asthma cross-reacts with the different thioredoxins, we performed competition ELISA. Distinct cross-reactivity was observed between Tri a 25 and Zea m 25, with the thioredoxins sharing 74% sequence identity (Fig 5, A), whereas cross-reactivity between Tri a 25 and ZmTRXh2 was less pronounced (Fig 5, A and B). Moderate cross-reactivity was also found between Tri a 25 and the human thioredoxin (Fig 5, B), and no cross-reactivity was observed between Tri a 25 and 0.19 AI.

DISCUSSION

Baker's asthma is an occupational disease caused by the job-related inhalation of cereal flour, mainly wheat and, to a minor extent, baking additives such as fungal α -amylase (for a review, see Houba et al³⁰). We have presented cloning and characterization of wheat thioredoxin hB (Tri a 25) as a further allergen related to baker's asthma. Tri a 25 is a 13.4-kD protein and has been reported to reduce intramolecular disulfide bridges of wheat storage proteins such as gliadins and glutenins. In this way, Tri a 25 has been proposed to enhance the mobilization of storage proteins in germinating wheat in a NADPH-dependent process.³¹ Here we show that 8 out of 17 sera from patients with baker's asthma showed IgE reactivity against Tri a 25 as well as against its homologue from maize, Zea m 25, sharing 74% amino acid identity. The degree of sensitization against these 2 thioredoxins is in the range of sensitization to α -amylase inhibitor 0.19, an allergen reported to be associated with baker's asthma, which was recognized by 11 of the 17 tested sera. By performing competition ELISA, we demonstrated the marked IgE cross-reactivity between Tri a 25 and Zea m 25. IgE reactivity against a second h-type thioredoxin from maize, ZmTRXh2, sharing only 46% identity with Tri a 25, was found in only 2 bakers' sera. The extent of IgE cross-reactivity between ZmTRXh2 and Tri a 25 was also relatively low, as shown by a maximum IgE-binding inhibition of 40% to 50% (Fig 5, A and B). In 2 of the bakers' sera, we also detected sensitization against human thioredoxin (Fig 3), and

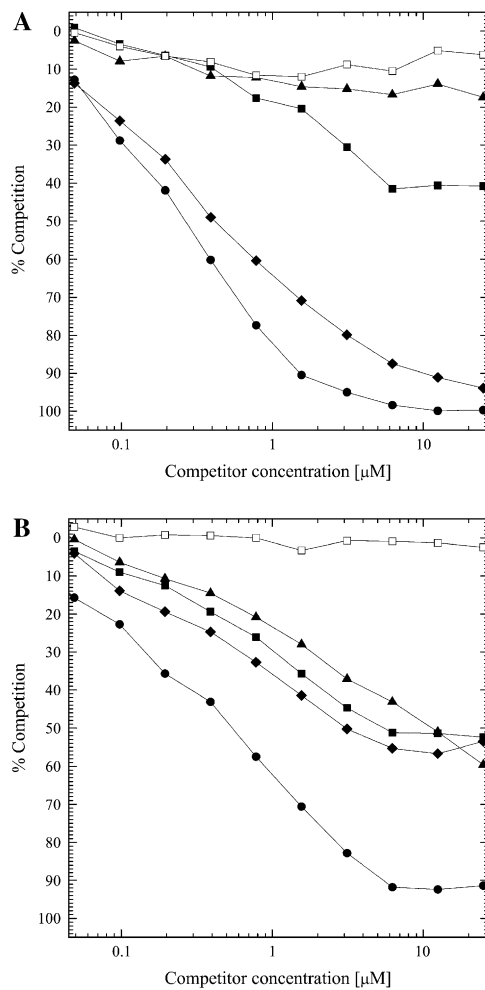


FIG 5. Competition of IgE binding to Tri a 25 in 2 different bakers with occupational asthma (*A and B*) with itself (●), with Zea m 25 (◆), with ZmTRXh2 (■), and human TRX (▲). Recombinant 0.19 AI (□) was used as control.

cross-reactivity could be demonstrated by IgE-binding inhibition of 40% to 50% (Fig 5, *B*), indicating that cereal and human thioredoxins share common B-cell epitopes. This condition could lead to maintenance and deterioration of an allergic inflammation. Endogenous human thioredoxin, also described to play a protective role in anti-inflammatory processes in the lungs and other tissues (for a review, see Nakamura et al³²), can be recognized by IgE raised against cereal thioredoxins caused by cross-reactivity based on molecular mimicry. Therefore, cross-linking of thioredoxin-specific IgE immobilized on the surface of effector cells by human thioredoxin can be expected to induce mediator release and thus inflammatory processes also in the absence of external exposure. As expected, cross-reactivity between wheat thioredoxin and 0.19 α -amylase inhibitor could not be observed. Sharing a molecular mass of ≈ 13 kd but differing in almost 2 pH units (Table II), Tri a 25 and 0.19 α -amylase inhibitor represent allergens in the often observed IgE-reactive 12 to 18 kd range of water-soluble wheat extracts.⁴

TABLE II. Sequence and physical data of cereal and human thioredoxins and 0.19 α -amylase inhibitor

Protein	EMBL acc no	pl	Molecular mass, kd	Reference
Tri a 25 (TaTRX/hB)	AJ404845	4.900	13.354	26
Zea m 25 (ZmTRX/h1)	AJ890020	4.672	13.881	24
ZmTRX/h2	AJ890021	6.209	13.039	24
HsTRX	X77584	4.564	11.709	27
0.19 AI	AY729672	6.720	13.337	9

Sensitization to cereal thioredoxins could also be detected in sera from patients with grass pollen allergy without allergy to wheat or maize. This finding indicates that occupational asthma could also have an underlying component of sensitization against wheat or maize pollen, which could contribute to the symptoms of baker's asthma. In fact, an earlier study based on wheat and grass pollen extracts shows that individuals with baker's asthma are sensitized against wheat as well as grass pollen proteins, whereas individuals with grass pollen allergy are exclusively sensitized to those wheat proteins that share common B-cell epitopes with grass pollen allergens.³³

The finding that thioredoxins have allergenic potential is furthermore corroborated by the observation that patients with allergic bronchopulmonary aspergillosis or atopic dermatitis show sensitization against *A fumigatus* thioredoxins (Asp f 28 and 29) or thioredoxin from the skin-colonizing yeast *Malassezia sympodialis* (Mala s 13), respectively (Limacher A, Personal communication, August 2005). However, the extent of cross-reactivity between plant and fungal thioredoxins as well as their clinical relevance in occupationally affected patients remains to be elucidated.

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