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DIPLOMARBEIT

Molecular and immunological characterization of wheat allergens

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Contents

I. Introduction

The wheat seed[1, 2]

Wheat *Triticum aestivum L*., the most consumed crop in the world is also known as bread wheat. The wheat plant grows from a seed, the wheat kernel or wheat berry. In flour production, the three parts of the kernel split in the milling process. (Figure 1)

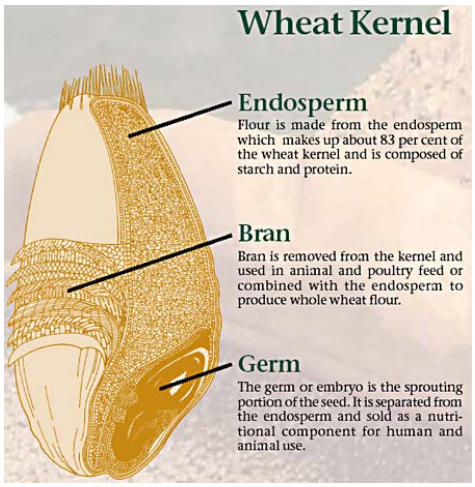

Figure 1. Wheat kernel

- **Endosperm**

The endosperm representing 83% of the kernel weight and comprises the greatest part of protein, carbohydrates, iron as well as many B-complex vitamins (e.g., riboflavin, niacin, and thiamine). It is the origin of soluble fiber and furthermore the source of white flour.

- **Bran**

The wheat bran, representing 14.5% of the kernel weight, is the outer envelope of the kernel. It includes less protein, but larger amounts of B-complex vitamins, trace minerals and indigestible cellulose material also called dietary fiber. Wheat bran is a main component of whole wheat flour.

- **Germ**

  The smallest part (2.5%) of the wheat kernel is the germ, also named embryo or sprouting section. It contains a lot of fat that would limit the keeping-quality, accordingly it is separated from the flour. B-complex vitamins and trace minerals are highly represented but just a small amount of protein is stored. Wheat germ is enclosed in whole wheat flour and can also be acquired separately.

It is obvious that the kernel of wheat is a storehouse of nutrients essential to the human diet. The major storage protein in wheat kernels is gluten, a mixture of various polypeptides which can be separated by their solubility.[3] (Table 1)

Wheat proteins	Protein composition of wheat seed storage proteins
water-soluble albumins	9%
salt-soluble globulins	5%
ethanol-soluble gliadins	40%
urea-, detergent-, KOH-soluble glutenins	46%

Table 1. Classification of wheat proteins

Albumins and globulins are soluble in water and saline buffers, gliadins in ethanol and glutenins in urea-, detergent-, and KOH-solutions. All of them are representative allergens involved in wheat food allergy.[4-6] Gliadins can be classified into α-, β-, γ-, ω- gliadins, whereas ω_5-gliadin acts as a major allergen involved in wheat food allergy.[7]

Triticum aestivum can activate two different hypersensitivity pathways that are specified in the next chapters.

Type I hypersensitivity: Allergy[8]

Allergic diseases are on the rise in industrialised countries, where 25% of the population is affected.[9] In general, allergy can be described as reaction of the immune system against an antigen. Coombs and Gell (1963) described a “classification of allergic reactions which may be deleterious to the tissues and harmful to the host”.[10] This classification includes four types of hypersensitivity reactions which can be distinguished according to the type of immune response and the effector mechanism accountable for cell and tissue damage.[11] Type I-III hypersensitivity reactions are antibody-mediated and are discriminated by the distinct antibodies involved in the antigen-recognition compared to type IV that is T cell-mediated. (Figure 2)[12]

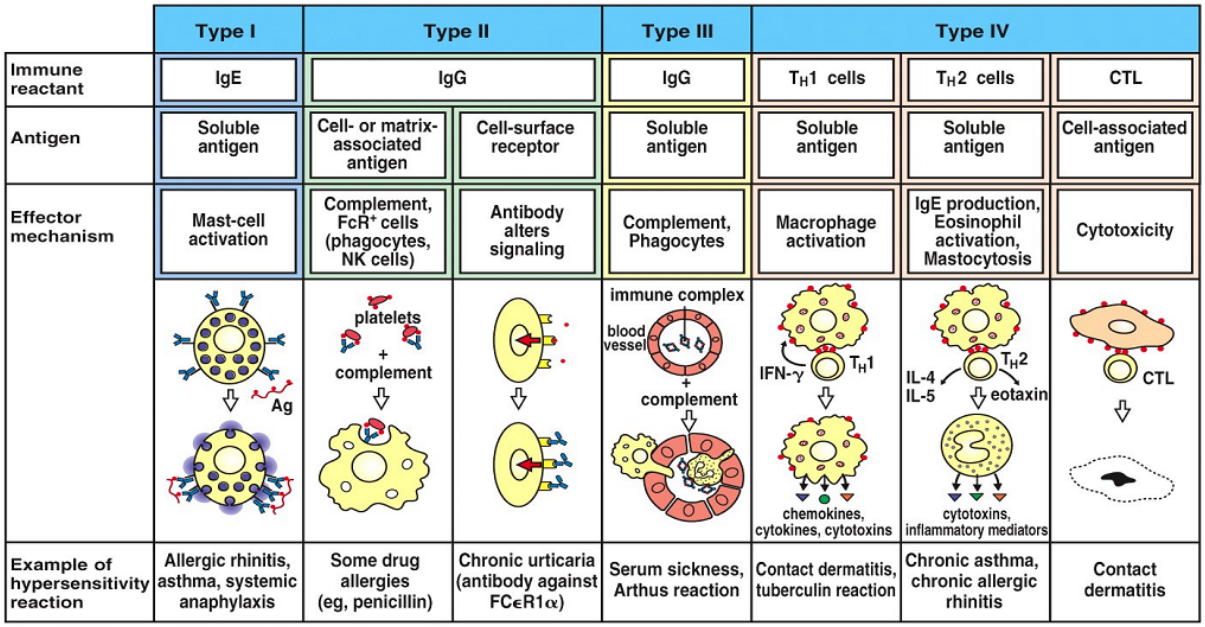

Figure 2. Different types of hypersensitivity

Immunoglobulin E (IgE) plays an essential role in the pathogenesis of type I hypersensitivity diseases. From the point when the allergen gets in contact with the mucosa to the point when IgE antibodies are produced and allergic symptoms can be detected, several intermediate steps are required.

a. Sensitisation and memory

b. Immediate phase reaction

c. Late phase reaction

When an allergen reaches a predisposed person and enters the body which can happen via the respiratory tract by inhalation or via the gut mucosa by ingestion, it is taken up by antigen-presenting cells, processed and presented via MHC class II molecules to T-cells. T_H2-cells are very frequent in allergic persons. When the T_H2-cells are activated they release, e.g.,IL-4, IL-13 which activate B-cells. The next step is the “class switching” of the B-cells to IgE. After the sensitisation, memory T-cells and IgE memory B-cells are generated. (Figure 3)[8]

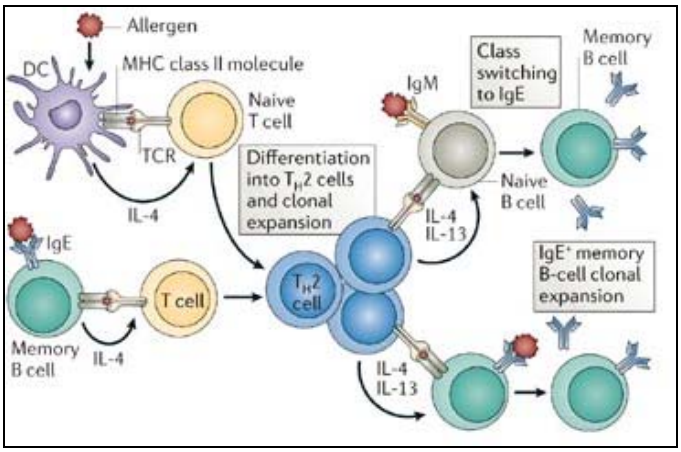

Figure 3. Sensitisation and secondary immune responses

If the person gets exposed repeatedly to the allergen, the allergen forms immune complexes with mast cell bound specific IgE (immediate phase of the allergic reaction).

The high affinity receptor, FcεRI, is cross-linked, inducing release of vasoactive amines (such as histamine), lipid mediators, chemokines and other cytokines (such as IL-4, IL-5, IL-13). (Figure 4)[8]

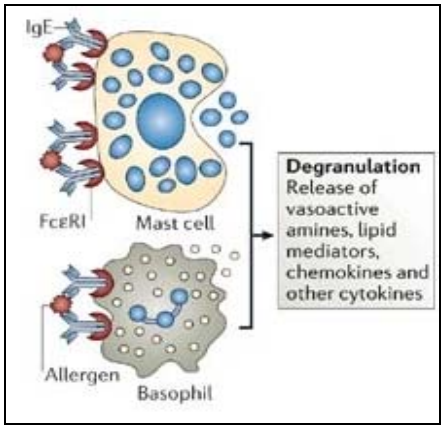

Figure 4. Type I hypersensivity (immediate phase of the allergic reaction)

Chemokines and cytokines lead to migration of allergen-specific T-cells, which are reactivated and clonally expanded, from the blood into the site of allergen exposure. In allergic rhinitis and asthma, local IgE-production can be observed but not in allergic skin inflammation. T_H1-cells producing IFN-γ (interferon-γ) and TNF (tumour-necrosis factor) assist in the activation and apoptosis of keratinocytes (in the skin), bronchial epithelial cells and pulmonary smooth-muscle cells. (Figure 5)[8]

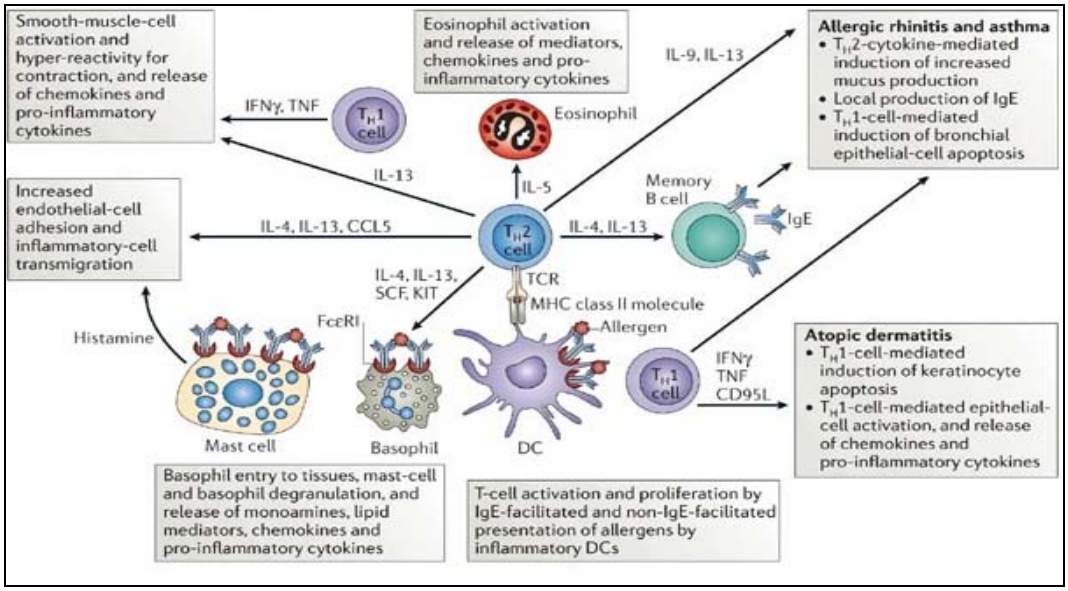

Figure 5. Allergic inflammation (late phase of the allergic reaction)

Via the type I hypersensitivity pathway, wheat can induce food allergy caused by ingestion of wheat products, wheat pollen allergy which is a member of the group of grass pollen allergies and Baker's asthma caused by inhalation of wheat flour. In general wheat allergy can be considered as a serious problem globally.

Wheat food allergy

Review

Wheat (*Triticum aestivum*) and wheat products are a major element in human nutrition but can be responsible for IgE- mediated food allergy. Eight percent of young children (<3 years) and 2% of adolescents and adults (47-50 years) are affected. Fortunately 80% of children "grow out" allergy.[13] The prevalence of wheat food allergy escalated in the last years. Some studies could show that this form of allergy increased in the last years in Japan because people consume higher amounts of western style food.[14, 15] WDEIA is a special form of wheat food allergy in which the patient develops a severe allergic reaction after the ingestion of wheat and subsequent intense exercise.[16, 17]

Recently a group in Japan published data where they could show that exercise and the intake of aspirin advances the wheat allergen absorption from the gastrointestinal tract in patients affected to WDEIA.[18] A recently published paper wants to let us know that WDEIA is induced by aspirin but not by exercise.[19] Eighty percent of the WDEIA-patients have IgE to ω_5-gliadin and the remaining 20% of the patients to high molecular weight glutenin (HMW-glutenin).[20] But at present, FDEIA (food dependent exercise induced anaphylaxis) that includes WDEIA, is still a controversial subject. Food intolerance is a non-immunological reaction to a food component, and therefore no hypersensitivity disease.

But unfortunately there are no authentic tests available for the diagnosis of food intolerance yet.[21]

Allergens

Generally wheat allergens can be allocated in the albumin/globulin and the gluten fraction. α-amylase/trypsin inhibitor subunits, members of the albumin/globulin fraction, are potent allergens involved in baker's asthma as well as in wheat food allergy.[22] Several studies showed that α-, β-, γ-, ω-gliadins which are part of the gluten fraction, are involved in IgE-mediated wheat food allergy. As mentioned above ω_5-gliadin is a major allergen that contributes to WDEIA.

Allergen	Biological function
Tri a 10kD	Prolamin superfamily[23]
Tri a 12	Profilin[24]
Tri a 14	Lipid transfer protein (LTP)[25]
Tri a 18	Hevein-like (Agglutinin)[23]
Tri a 19	Gliadin[26]
Tri a 23kd	Putative Leucine-rich Repeat Protein[23]
Tri a 26	Glutenins[27]
Tri a 30	Alpha-Amylase/Trypsin Inhibitors[28]
Tri a alpha_Gliadin	Alpha gliadin[26]
Tri a beta_Gliadin	Beta gliadin[26]
Tri a Chitinase	Chitinase[29]
Tri a gamma_Gliadin	Gamma-gliadin[26]
Tri a Gliadin	Gliadin[30]
Tri a LMW Glu	LMW glutenin[5]
Tri a omega2_Gliadin	Omega-2-gliadin[26]
Tri a Peroxidase	Peroxidase[31]
Tri a Serpin	Serpin[3]

Table 2. Known allergens involved in wheat food allergy

Symptoms

A difference concerning the clinical symptoms could be observed in children and adults. Skin reactions like atopic dermatitis occur more often in children, on the contrary adults are more often affected by urticaria and wheat-dependent exercise induced anaphylaxis (WDEIA).[32]

Skin	Urticaria Atopic dermatitis
Respiratory tract	Nasal congestion Rhinorrhea Pruritus/Sneezing Laryngeal edema Cough
Gastrointestinal tract	Vomiting Diarrhea Nausea Abdominal pain Intestinal bleeding Constipation Eosinophilic gastroenteritis
Cardiovascular system	Systemic shock reaction

Table 3. Schematic view of wheat-dependent food allergy symptoms

Diagnosis

For IgE-mediated food allergy, several diagnostic tests are available:

- Detailed clinical history of symptoms and reactions to wheat
- Double blind, placebo-controlled food challenge (DBPCFC)
- Skin prick test (SPT)[33]
- RAST (radioallergosorbent test) for the identification of allergen-specific IgE antibodies[34]

Therapy

Today the only possibility to treat a wheat food allergy is to avoid wheat. Immunotherapy or other approaches would require a detailed knowledge and availability of the culprit allergens which is not accomplished so far.

Baker's asthma

Review

Inhalation of wheat flour often causes Baker's asthma, which is together with rhinitis the most frequent occupational respiratory disease in industrialized countries.[35, 36] It is caused by occupational exposure to the antigens from flour and grain dust bakeries.[37] It is known that multiple potential allergens are responsible for the disease.

Sander *et al*. could show in 1998 that in addition to the grain allergens, contaminants of the flour or grain are also important.

Contaminants:[38]

- mold spores
- fungal enzymes
- insect and rodent parts
- pesticides
- pollens
- mineral particles
- bacteria
- mites

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In this study they also determined that other enzymes which have their origin in *Aspergillus* species that are used in bakeries, seem to be important factors in the etiology of baker's asthma. Factors such as too small bakeries, poor ventilation, large quantities of flour in the air are involved in the development of baker's asthma. Four to ten percent of bakery workers are affected in Europe[35], but in Japan a study showed that also people who live near a factory using wheat flour products suffer from baker's asthma.[39]

Pathomechanism

It is already known that two mechanisms are involved in the development of asthma. The first mechanism that causes airway obstruction is type I hypersensitivity which is mediated by immunoglobulin E (IgE). Briefly, circulating IgE binds to FcεRI (receptor for the Fc fragment of IgE) that is expressed on mast cells and eosinophils. Crosslinking of IgE/receptor complexes leads to cell activation and to the release of inflammatory molecules that provoke obstruction.[40]

The second pathway is mediated by IL-4 (interleukin-4) and IL-13 that are T_H2-cytokines. These ligands attach to IL-4Rα, a member of the Janus family of tyrosine kinases (JAKS) and to signal transducer and activator of transcription 6 (STAT6). This cytokines together with their receptor can induce a direct effect on the smooth muscles and the epithelium. The consequences are airway hyper-responsiveness, gobelet cell metaplasia with mucus overproduction and mucosal oedema.[41, 42]

These mechanisms are demonstrated distinctly in Figure 6.[43]

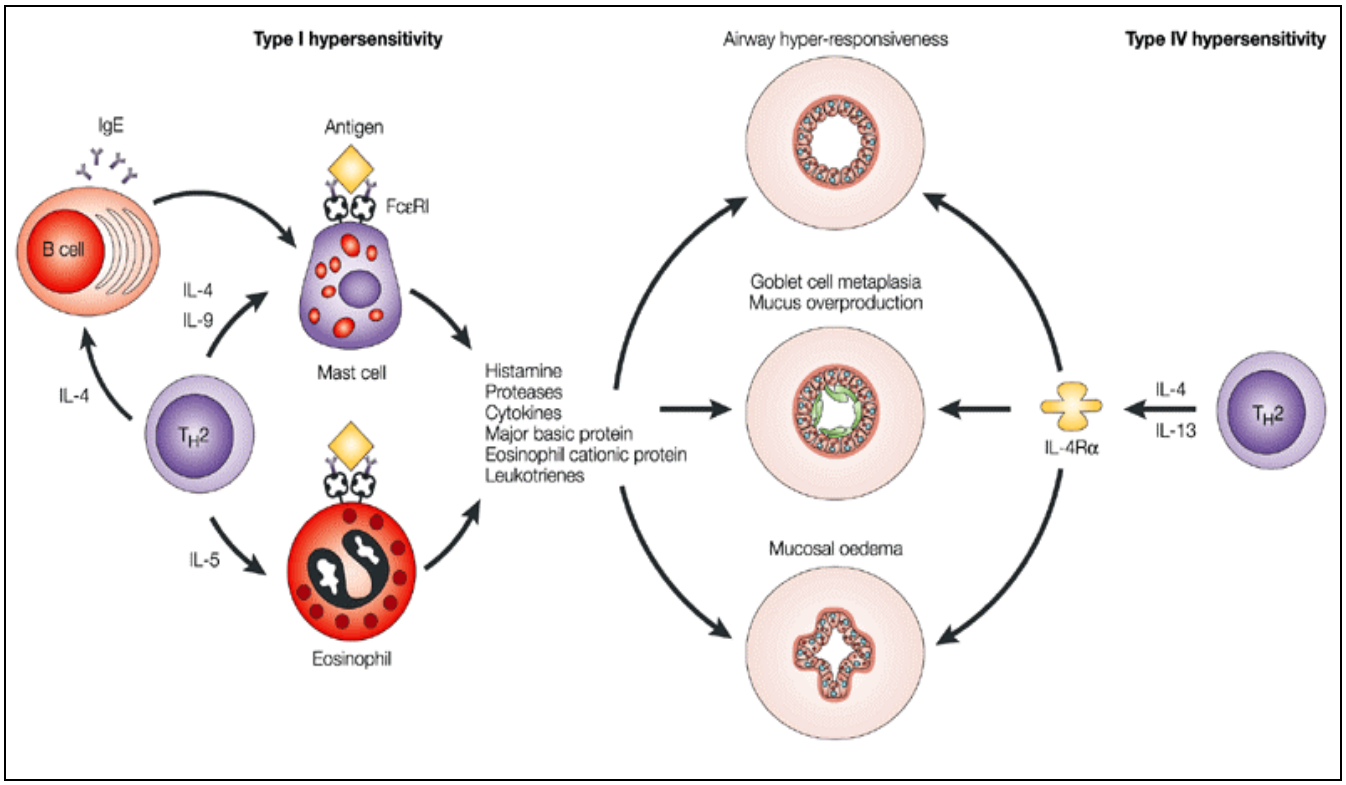

Figure 6. Pathomechanism of Baker's asthma

Asthma is a chronic inflammatory disease of the bronchial tubes. The following cells are involved in the inflammatory process: eosinophil and basophil granulocytes, macrophages, lymphocytes, vascular endothelium and bronchial epithelium cells. The cells are activated, release mediators and agents that are responsible for the pathological-anatomical and functional changes. The epithelium gets destroyed by toxic proteins, released from eosinophils: major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin.[44]

Table 4 shows factors which boost vascular permeability and contraction of smooth bronchial muscle cells:

Lipidmediators	Prostaglandin D2 and F2alpha Leukotrien C4 and D4 PAF = platelet activating factor
Biogenic amines	histamine bradykinine
Neuropeptides	substance P neurokinin A calcitonin gene related peptide

Table 4. Agents boosting the permeability of the vessels and the contraction of the smooth bronchial muscles

It is important to mention that the cholinergic autonomic nervous system induces an excessive mucus and increases the bronchoconstrictive activity.

The major allergens causing Baker's asthma are proteins derived from wheat and rye flours. The strongest IgE reactivity was shown by the registered allergens:

- α-amylase/trypsin inhibitor family[45]
- Thioredoxins[46]
- Peroxidase[47]
- αβ-gliadin[48]
- and other salt-soluble enzymes[49]

Allergen	Biological function
Tri a 1, Tri a 2	Expansin[50]
Tri a 3	[51]
Tri a 4	Berberine Bridge Enzyme[50]
Tri a 5	[52]
Tri a 7	Polcalcin[53]
Tri a 12	Profilin[24]
Tri a 13	Polygalacturonase[54]
Tri a 14	LTP (Lipid transfer protein)[55]
Tri a 25	Thioredoxin h[46]
Tri a 27	Thiol Reductase Homologues[56]
Tri a 28	alpha-Amylase-Inhibitor
Tri a 29	alpha-Amylase-Inhibitor
Tri a 30	alpha-Amylase-Inhibitor[57]
Tri a alphabeta_Gliadin	alphabeta gliadin[48]
Tri a Bd36K	Peroxidase[58]
Tri a DH	Dehydrin[59]
Tri a GST	Glutathione-S-transferase[59]
Tri a PER	Peroxiredoxine[59]
Tri a SPI	Serine Protease Inhibitor[59]
Tri a TPIS	Triosephosphate Isomerase[60]

Table 5. Known allergens involved in Baker's asthma

Description of allergens involved in Baker's asthma desribed in this diploma thesis

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Thioredoxin h

Thioredoxin h is a 12-13kDa ubiqitous protein common in bacteria, unicellular eukaryotes, plant and animal cells.[61] It contains a disulfide-bridge that can be reduced by NADPH-thioredoxin reductase (NTR).[62, 63] This protein acts as a signal to enhance metabolic processes for germination and seedling development during seed germination.[64, 65]

Thioredoxins are known for their highly conserved amino acid sequences that include a canonical, highly conserved active site containing a disulfid-bridge.[66] In addition, thioredoxin (Trx) fold is a common structural fold found in many proteins, also in GST (Glutathione-S-transferase) and 1-Cys-peroxiredoxin.[67, 68]

Glutathione-S-transferase

Glutathione-S-transferases (GST) are cytosolic, dimeric proteins composed of two subunits with a molecular weight of 24-28 kDa each.[69] It was identified as a major allergen involved in house-dust-mite allergy.[70] 40% of house-dust-mite allergics show IgE-reactivity to GST.[70]

GSTs are involved in plant defence-systems against biotic and abiotic stresses, including oxidative stress like drought[71], pathogen attack and xenobiotic toxicity. AOS (activated oxygen species) are important for inter-, and intracellular signalling[72], but if they become highly concentrated, e.g., in case of drought they can cause damaging of the plant.[73] This is the case in the defence-system where GST comes into play to develop protection against AOS. Cell signalling during stress[74] is an important but just one of the many characteristics of GST.[75] The further roles can be summarized as follows: shuttle of toxic

secondary products[76] and detoxification of herbicides by catalyzing the GSH (γ-glutamyl-cysteinyl-glycine) conjugation to hydrophobic, electrophilic and cytotoxic substrates.[77, 78]

Several studies could show that the amount of GST activity correlates with stress tolerance in *Triticum* cultivars.[79]

GST-enzymes are grouped in animals into two classes (zeta, theta) and in plants into four classes (zeta, theta, phi, tau).[80] The GST fold contains an active site between an N-terminal thioredoxin-fold and a C-terminal alpha helical domain.

1-Cys-peroxiredoxin

Peroxiredoxins are proteins catalyzing hydrogen- and alkyl peroxides developed by oxidative stress, mainly by oxidative damage of lipids.[81, 82] They are found in all organisms, in plants they balance hydroperoxide production during photosynthesis. Peroxiredoxins are also involved in antioxidant defence, respiration and the dealing with stress.[82] These proteins use thiol as reductants, 1-Cys-peroxiredoxin utilizes only one cysteine residue in catalysis.[83] Like GST, peroxiredoxins also have a Trx fold (described above). Some studies suggest that thioredoxins and peroxiredoxins have evolved from a common progenitor.[67] Accordingly these proteins feature a canonical Trx fold, an N-terminal extension, a C-terminal extension and an insertion between β2 and α2 of the Trx fold.

Profilin

Wheat profilin is highly cross-reactive with grass-pollen profilin and also with profilins from different plant sources. Because of this characteristic it is responsible for multiple pollen allergies. This small, cytosolic protein occurring in eukaryotic cells, has the capacity of actin-sequestration and is also involved in the regulation of the actin-cytoskeleton.[84] Profilins are allergens recognized by 10-20% of all pollen allergic patients.[85] They are responsible for IgE-autoreactivity in sensitised patients. People with IgE to purified natural or recombinant birch profilin also show IgE reactivity to human profilin, and accordingly recombinant birch profilins could inhibit the binding of human IgE to human profilin.[86] It accounts for IgE-reactivity to pollens from plants that are botanically unrelated[87, 88], cross-reactivity between pollen and food allergens[24] and pollen and latex allergens.[89] Federov *et al.* published in 1997 that current epitopes are arranged in conserved domains and, based on structure homology, the characteristics of the G actin-profilin interaction in all eucaryotic organisms could be found conserved.[90]

Radauer *et al.* could show that there is a high availability of similar epitopes that contain conserved and variable residues. Epitope 177 is conserved and IgE-specific and they assume that this epitope is responsible for the strong cross-reactivity among profilins.[85]

Dehydrin

Dehydrins can be described as highly hydrophobic proteins, common in a broad range of organisms and acting as membrane-, and macromolecule protectors against denaturation. Protein expression starts late in embryogenesis under normal growth conditions and as reply to stresses with a dehydrative component – drough, low temperature, salinity. In winter, dehydrin-clustering near the plasma membrane helps wheat to acclimate to low

temperatures.[91, 92] The precise function of dehydrins was not identified yet, but they suggest that this accumulation stabilizes the plasma membrane during stress exposure.

Symptoms

- Rhinitis
- Respiratory symptoms (wheezing, cough, tachypnoea)
- Skin symptoms
- Conjunctivitis

Diagnosis[35]

- Skin prick test to flour extracts[93]
- Measurement of specific IgE antibodies[33]
- Lung function testing
- Methacholine challenge test
- Inhalative challenge test

Therapy

- **Allergen avoidance**

People who have to manage Baker's asthma can minimize the symptoms by avoiding allergen exposure. Bakers should wear respiratory protection, control the dust, change to a less exposed workstation and keep the bakery very clean.[94]

- **Medication**[95]

There are two major groups of medications to deal with asthma: anti-inflammatory medication (corticosteroids) and bronchodilators. Anti-inflammatory medication reduces inflammatory cells and consequentially the spontaneous spasm of the airway muscle. These drugs are applied to decrease the risk of asthma attacks. Corticosteroids are inhaled or swallowed orally as a tablet. These drugs are used to prevent and reduce symptoms and are prescribed for long-term use.

The second group are the bronchodilators which are used during acute asthma attacks.

- **Specific immunotherapy**

Specific immunotherapy is the application of allergen extracts to an allergic person to induce “desensitisation”.

The combination of the different therapies has to be very specific for every asthma patient. The physician has to arrange an adequate therapy for the patient.

Wheat pollen allergy

Review

Freidhoff *et al*. could show in 1986 that 40% of all allergic individuals show IgE-reactivity to grass pollen allergens.[96] Cross-reactivity between wheat seed allergens and grass pollen allergens is already understood.[97] Several studies confirm the fact that common IgE-epitopes appear in wheat-flour and in grass pollen proteins.[98, 99]

Type IV hypersensitivity: Cell-mediated hypersensitivity

Compared to the above mentioned type of hypersensitivity, type IV is mediated by antigen-specific effector T cells. In this form of immune response, T-cells produce cytokines that lead directly to the manifestations of the disease. (Figure 2)[100]

Celiac disease

Review

This disease is induced by the gliadin fraction of gluten in genetically susceptible individuals.[101] Celiac disease can not be definitely classified into one of the four hypersensitivity diseases. CD patients develop antibodies against ingested wheat proteins, but these antibodies belong to the IgA, IgG and not to the IgE-class. It is also well known that a gluten enteropathy which is a T-cell mediated reaction provokes the disease in 1% of the population in developed countries worldwide.[102-104]

Pathomechanism

In genetically susceptible persons the presence of gluten in the small intestine leads to epithelial damage. Ninety eight percent of people suffering from celiac disease have genetic markers on chromosome 6 called HLA (human lymphocyte antigen) DQ2 and HLA DQ8 compared to 40% of the healthy population.[105] Gliadins come into contact with epithelial cells where the tissue transglutaminase (tTG) becomes involved. The gliadins get deaminated which allows the binding to DQ2 or DQ8. These molecules can activate cytotoxic T-cells, stimulate the damage of the epithelium and production of antibodies to gliadin and tTG. (Figure 7)[106]

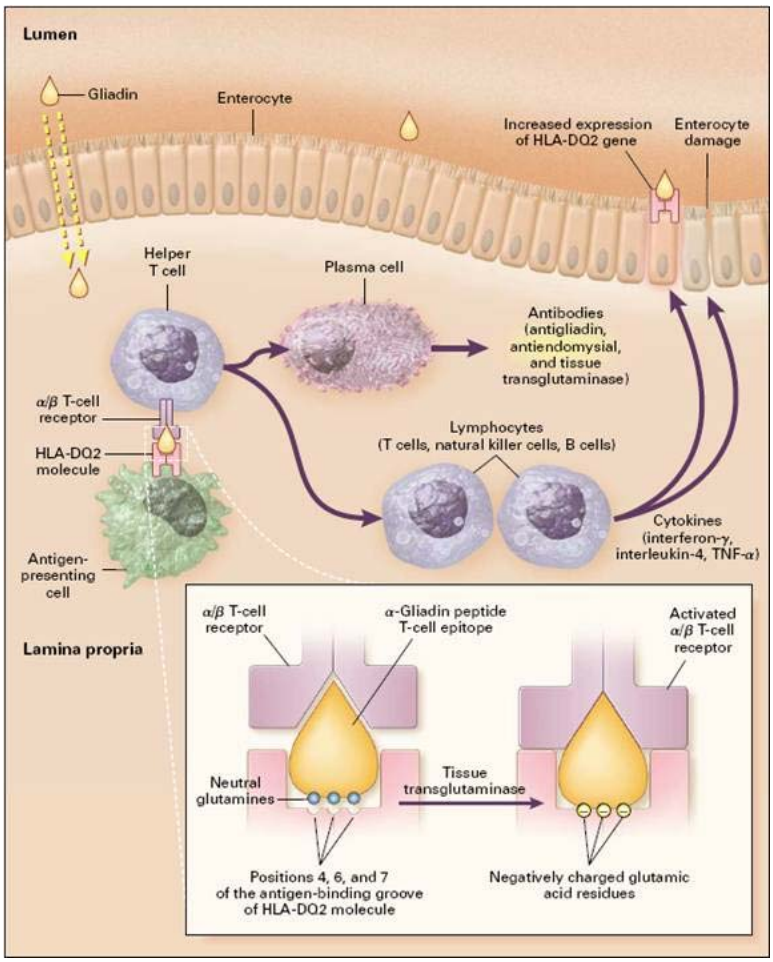

Figure 7. Pathophysiology of Celiac disease (Westerberg *et al*., 2006)

When celiac disease patients eat food containing gluten, it becomes digested, presented to T-cells and an inflammatory reaction is induced. This inflammation causes damage of the villi, which are part of the resorptive epithelium and very important for the uptake of nutrients through the walls of the small intestine into the bloodstream. The consequence is that people get undernourished no matter how much they eat.[107-110]

Destruction of resorptive (villous) epithelium in the small intestine → **Reduction of the absorbtive area** → **Malabsorption of almost all nutrients**

Symptoms[108]

Symptoms	Constipation Fatigue Headache Mild gastrointestinal symptoms Iron deficiency Miscarriage Bone fractures Dermatitis herpetiformis
Special symptoms in children	Weight loss Failure to thrive Abdominal cramping Bloating Flatus Nausea Vomiting Muscle wasting Diarrhoea Steatorrhoea

Table 6. Clinical manifestations of Celiac disease

Diagnosis

To be certain that a patient has celiac disease he has to undergo a food challenge test. Immunological tests including measurement of total IgA, IgA anti-tTG and anti-gliadin IgA and IgG are accomplished subsequently.[102] In case of villous atrophy and a following improvement after gluten free diet detected by biopsy, the diagnosis of celiac disease is confirmed.

The only treatment for celiac disease is a gluten-free diet. Patients have to avoid food containing wheat, rye and barley and also products made from these grains. These patients are definitely limited but today there is still a variety of gluten-free bread, pasta,... from special food companies.

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II. Summary – Zusammenfassung

Summary

Baker's asthma is an IgE-mediated hypersensitivity disease caused by inhalation of wheat flour. The term still indicates the problem specific to a particular profession, from which 4-25% are affected. Identification of the workspace agent could minimize the symptoms by avoiding exposure to the allergen. In the past, many allergens could be isolated, but there is no test available to discriminate between wheat-dependent food allergy and Baker's asthma. Constantin *et al*. presented in a recently published study an allergen microarray based on purified recombinant proteins.

Further isolation, identification and characterization of allergens involved in Baker's asthma will improve this method which could become an effective tool for the diagnosis of the disease in the future.

Based on this idea, the main aim of my diploma thesis was the molecular, structural and immunological characterization of new allergens involved in Baker's asthma.

The isolated IgE-reactive cDNA clones could be identified by sequence analysis as follows: Thioredoxin h (clone 37), Glutathione transferase (clone 38), 1-Cys-peroxiredoxin (clone 112), Profilin (clone 123) and Dehydrin (clone 126). Recombinant proteins were expressed in *E.coli* as C-terminally hexahistidine-tagged proteins and the molecular weight was determined. A dot-blot experiment which is an effective method to study IgE-reactivity of recombinant proteins, was performed. 1-Cys-peroxiredoxin was strongly recognized by serum IgE from 35.7% of patients suffering from Baker's asthma. Furthermore, in histamine release assays, an important method for evaluating allergenicity of allergens, we could show strong allergenic activity in 21% of patients. In contrast, thioredoxin h was

recognized by none of the allergics and only induced a weak degranulation in the histamine release assay.

There is a need of recombinant wheat allergens, specifically recognized by baker’s asthma patients, to use them for diagnosis, reduce provocation testing and possibly immunotherapy of IgE-mediated allergy.

Zusammenfassung

Bäcker Asthma bezeichnet eine IgE-vermittelte Überempfindlichkeitsreaktion, die durch Inhalation von Weizenmehl ausgelöst wird. Der Begriff weist auf eine berufsbezogene Erkrankung hin, von denen 4-25% betroffen sind. Das Erkennen des auslösenden Allergens und die Vermeidung der Allergenbelastung könnte die Symptome verringern. In der Vergangenheit konnten viele Allergene isoliert werden, aber es sind keine Tests verfügbar um zwischen der Weizen-abhängigen Nahrungsmittelallergie und Bäcker Asthma zu unterscheiden. Constantin *et al.* präsentierte in ihrer kürzlich publizierten Studie einen Allergen-Microarray basierend auf gereinigten, rekombinanten Proteinen. Isolierung, Identifizierung und Charakterisierung weiterer Allergene, die in Bäcker Asthma involviert sind, trägt zu einer Verbesserung der Methodik bei, welche sich zu einer effektiven Methode für die Krankheitsdiagnose in der Zukunft entwickeln könnte. Bezogen auf diese Vorstellung, war das Hauptziel meiner Diplomarbeit die molekulare, strukturelle und immunologische Charakterisierung von neuen Allergenen, die an Bäcker Asthma beteiligt sind.

Die isolierten IgE-reaktiven cDNA Klone konnten durch Sequenzanalysen folgendermaßen identifiziert werden: Thioredoxin h (Klon 37), Glutathiontransferase (Klon 38), 1-Cys-peroxiredoxin (Klon 112), Profilin (Klon 123) und Dehydrin (Klon 126). Die rekombinanten Proteine wurden in *E.coli* als C-terminal hexahistidin-markierte Proteine exprimiert und das Molekulargewicht wurde ermittelt. Ein Dot-Blot Experiment wurde durchgeführt um die IgE-Reaktivität der rekombinanten Proteine zu untersuchen. 1-Cys-peroxiredoxin wurde stark von Serum-IgE von 35.7% der Patienten, die an Bäcker Asthma leiden, erkannt. Darüber hinaus konnte mittels Untersuchung der Histaminfreisetzung eine stark allergene Wirkung in 21% der Patienten nachgewiesen werden.

Im Gegensatz dazu wurde Thioredoxin h von keinen der Allergiker erkannt und keine Histaminfreisetzung induziert.

Es gibt einen Bedarf an rekombinanten Weizenallergenen, die speziell von Bäcker Asthma Patienten erkannt werden, um die Diagnose zu verbessern, die Notwendigkeit von Provokationstests zu verringern und sie möglicherweise in der Immuntherapie von IgE-vermittelten Allergie einsetzen zu können.

II. Manuscript

MOLECULAR CHARACTERIZATION OF WHEAT ALLERGENS SPECIFICALLY RECOGNIZED BY BAKER'S ASTHMA PATIENTS

Sandra Pahr, Claudia Constantin, Adriano Mari, Sandra Scheiblhofer, Josef Thalhammer, Susanne Vrtala, Irene Mittermann, Rudolf Valenta

Molecular characterization of wheat allergens specifically recognized by baker´s asthma patients

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Abstract

Background: Wheat (*Triticum aestivum*) is an important allergen source responsible for various clinical manifestations of allergy (i.e., food allergy, pollen allergy, respiratory allergy to flour-Baker's asthma).

Objective: To isolate and characterize cDNAs coding for new wheat allergens.

Methods: A *Triticum aestivum* cDNA library was constructed and screened with serum IgE from patients suffering from wheat allergy to identify cDNAs coding for new wheat allergens. The allergen-encoding cDNAs were expressed in *E.coli* and purified to homogeneity. IgE reactivity of recombinant proteins was analyzed with sera from clinically defined patients and their allergenic activity was assessed in basophil degranulation experiments.

Results: We report the molecular characterization, recombinant expression and purification of five novel wheat allergens, a thioredoxin h isoform, glutathione transferase, 1-Cys-peroxiredoxin, profilin and dehydrin. Testing for IgE reactivity with allergic patients identifies glutathione transferase, 1-Cys-peroxiredoxin and dehydrin as allergens specifically recognized by patients with respiratory allergy to wheat. According to prevalence of IgE recognition and results from basophil degranulation experiments 1-Cys-peroxiredoxin appears to be the most relevant of the newly identified wheat allergens.

Conclusion: The newly characterized recombinant wheat allergens may be useful for the development of serological tests which allow the discrimination of different clinical manifestations of wheat allergy.

Clinical implication: Serological tests based on recombinant wheat allergens may enable the diagnosis of Baker's asthma.

Capsule summary: Our results indicated that recombinant wheat seed allergens facilitate the diagnosis of Baker's asthma and a differentiation to wheat food allergy and wheat pollen allergy.

Key words: *Triticum aesticum, Baker's asthma, Recombinant allergens*

Abbreviations used:

HSA: human serum albumin

Phl p: Phleum pratense

DBPCFC: double-blind placebo-controlled food challenge

RBL: rat basophil leukaemia

LTP: lipid-transfer protein

Introduction

Wheat (*Triticum aestivum*) is a potent allergen source which causes several distinct clinical manifestations of IgE-mediated allergy.[1] These manifestations include wheat food allergy, respiratory allergy to wheat pollen and sensitization to inhaled wheat flour as a major cause for occupational sensitization among bakers and persons processing wheat flour.[2-4] The diagnosis of the various manifestations of wheat allergy is based on a careful anamnesis, the demonstration of allergen-specific IgE antibodies in serum and provocation testing with wheat allergen extracts. The detection of wheat allergen-specific IgE antibodies is not only important for demonstration of the IgE-mediated pathogenesis. It has also been shown that measurement of flour-specific IgE and skin prick testing may predict nasal and bronchial challenge test results in the case of wheat-induced respiratory allergy, mainly in baker´s asthma.[5] Serological testing may therefore be useful to reduce the need of clinical provocation testing.

In the last years many important wheat allergens have been characterized by biochemical, immunological and molecular biological methods. Interestingly, IgE-recognition of certain wheat allergens seems to be associated with defined clinical manifestations of wheat allergy. For example ω_5-gliadin, Tri a 19, has been described as an allergen associated with wheat-dependent, exercise-induced anaphylaxis and wheat food allergy and has been evaluated as a serological marker.[6-10] Wheat gliadins, lipid transfer protein and serine proteinase inhibitor have been described as allergens which may be recognized specifically by patients suffering from respiratory allergy to wheat, mainly in baker´s asthma.[11-13]

In order to search for allergens that could be used as markers for certain clinical manifestations of wheat allergy, we screened a cDNA library prepared from wheat seed RNA with serum IgE from wheat allergic patients. The isolation and characterization of five

cDNAs coding for five novel wheat allergens (thioredoxin h, glutathione transferase, 1-Cys-peroxiredoxin, profilin and dehydrin), their expression and purification as soluble recombinant allergens in *E. coli* is reported. Furthermore, the recombinant allergens were tested with sera from patients suffering from clinically well defined forms of wheat allergy and their allergenic activity was studied in basophil degranulation experiments. Glutathione transferase and in particular 1-Cys-peroxiredoxin were identified as relevant allergens which are specifically recognized by patients suffering from wheat-induced respiratory allergy.

Materials and methods

Biological materials

Wheat seeds (*Triticum aestivum*) were obtained from AGES (Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Vienna, Austria). Wheat pollen was obtained from Allergon (Välinge, Sweden), recombinant Phl p 1, Phl p 5, Phl p 7, Phl p 12 from BIOMAY (Vienna, Austria) and human serum albumin (HSA) from Behring (Marburg, Germany). *E.coli* strain BL21 (DE3): F^- , *omp*$T r_B^- m_B^-$ (DE) was purchased from Stratagene (La Jolla, CA) and plasmid pET17b from Novagen (Madison, WI).

Wheat extracts

Proteins were extracted from pollen (500mg) using 5ml PBS, 2mM EDTA, 1mM PMSF at 4°C over night. The suspension was then centrifuged for 1h at 13.000xg 4°C. A Micro BCA Protein Assay Kit (Pierce, Rockford, IL) was used to determine the concentration of the supernatant and aliquots were stored at –20°C. A wheat seed extract was prepared by grinding wheat seeds in a homogenizer. The powder was extracted in PBS containing 1mM PMSF for 1 hour at 4°C, centrifuged at 4°C at 37.500xg and the supernatant was stored at -20°C until use.

Sera

Sera were from 28 Italian patients (5 females; 23 males; mean age: 40 years; range: 20-61) who suffered from wheat-induced respiratory allergies. The demographic, clinical and serological characterization of the patients is summarized in Table I. Patients were analyzed regarding total serum IgE levels, wheat flour-specific IgE and rTri a 19 (i.e. ω_5-gliadin)-specific IgE by CAP-FEIA testing (Phadia, Uppsala, Sweden). Interestingly, only 92.8% of the patients with wheat-induced respiratory allergy were positive when tested for

IgE reactivity in the wheat flour CAP (Table I). Serum from a non-allergic person was used as negative control in all experiments. Sera were collected on the basis of a study protocol approved by the IDI-IRCCS institutional review board, and the serological analysis was performed on anonymized sera with permission of the Ethics committee of the Vienna General Hospital, Vienna, Austria.

Cloning, characterization, expression and purification of IgE-reactive clones

A wheat seed cDNA library was constructed and screened with a serum pool from patients suffering from wheat allergy as described.[13] IgE-reactive phage clones, designated clones 37, 38, 112, 123, 126 were purified to homogeneity by several rounds of IgE-re-screening. The cDNAs coding for the wheat allergens were obtained by PCR amplification from the purified phages using lambda gt11 forward and reversed primers and directly sequenced (MWG, Ebersberg, Germany).[13] The DNA and deduced amino acid sequences of the clones were compared with the sequences deposited in the GenBank database at the National Center for Biotechnology Information (NCBI). A sequence analysis was performed using the Clustal W multiple alignment tool.

The coding regions of the allergen-encoding cDNAs were PCR amplified using the primers (MWG) listed in supplemental Table I. The primers contained EcoRI (underlined) and NdeI (italics) sites and a sequence coding for a C-terminal hexahistidine tag (bold). The amplified cDNAs were cut with EcoRI and NdeI and subcloned into plasmid pET17b.[13] Recombinant allergens were expressed in *E. coli* BL21 (DE3) and purified by nickel affinity chromatography from the soluble fraction (Quiagen, Hilden, Germany).[13] Proteins were dissolved and stored in 10 mM NaH_2PO4 buffer pH 7.5 at -20°C. The concentrations of the purified allergens were determined by BCA assay (Pierce, Rockford, IL). The purity of the proteins was checked by SDS–PAGE and Coomassie Blue staining (Fling, Bradford) and their identity was confirmed by Western blotting using a monoclonal anti-His tag antibody

(Novagen). Laser desorption mass spectra were acquired for the allergens in a linear mode with a TOF Compact MALDI II instrument (Kratos, Manchester, UK; piCHEM, Research and Development, Graz, Austria).[13]

IgE-reactivity by dot-blotting

Aliquots (1µl containing 0.5 µg) of recombinant wheat proteins, recombinant grass pollen allergens and HSA were dotted onto nitrocellulose strips (Schleicher & Schuell, Dassel, Germany). Wheat pollen extract (3 µg/dot) and wheat seed extract (2 µg/dot) were used as controls. Nitrocellulose membranes were incubated with 1:10 diluted sera from patients with wheat-induced respiratory allergy, and for control purposes, with serum from a non-allergic individual. Bound IgE antibodies were detected with ^{125}I-labeled anti-human IgE antibodies (Demeditec Diagnostics, Kiel, Germany).[14]

Basophil degranulation experiments

Rat basophil leukaemia (RBL) cells transfected with human FcεRI were incubated with sera from those patients with IgE reactivity to the recombinant allergens and wheat extract.[15] For control purposes, serum from a non-allergic person was used. Released ß-hexosaminidase from RBL cells exposed to recombinant allergens, wheat seed extract or buffer was measured as described.[16]

Results

Isolation of cDNAs coding for wheat allergens belonging to distinct protein classes

We used a serum pool from patients suffering from IgE-mediated wheat allergy for screening of a cDNA library prepared from wheat seeds. The comparison of the deduced amino acid sequences of IgE-reactive phage clones with published sequences showed that clone 37 is a wheat thioredoxin h. Thioredoxin h, designated Tri a 25 has been described as wheat allergen earlier.[17, 18] It has a calculated molecular weight of 12.7 kDa, shares the highest degree of sequence identity with thioredoxins from rice (73%) and maize (65%) and also exhibits sequence identities of more than 50% with thioredoxins from several other plant sources (e.g., Balsam poplar 57%; Arabidopsis thaliana 57%) (Supplemental Fig 1). However, the wheat thioredoxin h isolated by us seems to be an isoform which is substantially different from Tri a 25 because it has 55% sequence identity. Wheat thioredoxin contains the highly conserved active site WCGPC (bold letters) and several amino acids which are supposed to maintain the tertiary structure of these proteins (asterisks) .[19, 20]

The cDNA of clone 38 codes for wheat glutathione transferase, a protein of 25 kDa which shares a high degree of sequence identity with glutathione transferases from barley (95%), maize (67%), oil palm (65%), rice (63%) and soy (59%) (Supplemental Fig 2). Clone 112 codes for wheat 1-cys-peroxiredoxin a protein with a deduced molecular weight of 23.9 kDa (Supplemental Fig 3). It shows a more than 80% sequence identity with homologues from barley, rye, rice and maize and a more than 50% sequence identity with homologues in dicotyledonic plants (e.g., sunflower, rape) (Supplemental Fig 3).

Thioredoxin, glutathione transferase and 1-Cys-peroxiredoxin belong to a group of proteins with reducing activity which resemble typical thioredoxin-like folds in their three-dimensional structure (Supplemental Fig 1-3) (Table II).[21, 22]

Clone 123 codes for wheat profilin, a cytoskeletal protein which has been described as highly conserved cross-reactive allergen.[14, 23] It has a deduced molecular weight of 14.1 kDa and shares more that 83% amino acid sequence identity with other plant profilins (Table II).

Clone 126 codes for wheat dehydrin, a protein with a deduced molecular weight of 21.5 kDa which shares a sequence identity of more than 50% with dehydrins from barley, rice and oil palm (Supplemental Fig 4; Table II). Dehydrins represent proteins containing two typical dehydrin sequence motifs (supplemental figure 4) whose expression is up-regulated upon environmental stress such as lack of water.[24]

Expression and purification of soluble recombinant wheat allergens

We expressed the wheat allergens as recombinant proteins with a C-terminal hexahistidine tag in *E. coli*. The recombinant allergens were purified using nickel affinity chromatography from the supernatants of lysed *E. coli* as soluble proteins (Fig 1).

Figure 1 shows a Coomassie brilliant blue-stained 12% SDS-PAGE which demonstrates the purity and migration of the recombinant allergens, thioredoxin h (14 kDa), glutathione transferase (25 kDa), 1-Cys-peroxiredoxin (25 kDa), profilin (15 kDa) and dehydrin (35 kDa). The yields of purified recombinant allergens/L culture were as follows: Thioredoxin: 1.7 mg/L; glutathione transferase: 1mg/L; 1-cys-peroxiredoxin: 44mg/L; profilin: 16.1 mg/L; dehydrin: 34.4 mg/L.

The results of the MALDI-TOF analysis of purified recombinant proteins corresponded with the deduced molecular weights indicating that all recombinant proteins contained a methionine (data not shown).

Identification of recombinant wheat allergens which are specifically recognized by IgE from patients with respiratory wheat allergy

Figure 2 shows the reactivity of dot-blotted recombinant wheat and grass pollen allergens with IgE from 28 patients suffering from wheat-induced respiratory allergy (as reported in Table I). Each of these patients exhibited IgE reactivity to dot-blotted wheat seed extract. Glutathione transferase (14.3%), profilin (10.7%), 1-Cys-peroxiredoxin (35.7%) and dehydrin (14.3%) were recognized to a varying degree. 1-Cys-peroxiredoxin was recognized by serum IgE from 35.7% of the tested patients and thus was the most frequently recognized allergen in our population (Fig 2, lower panel). None of the tested sera showed IgE reactivity to recombinant thioredoxin, which has been identified as allergen recognized by patients with wheat-induced food allergy. Recombinant wheat seed profilin reacted with sera from patients who were also allergic to grass pollen and who exhibited also IgE reactivity to recombinant timothy grass pollen profilin, rPhl p 12. With the combination of recombinant glutathione transferase, profilin, 1-Cys-peroxiredoxin and dehydrin 50% of patients could be detected. Weak IgE reactivity (<1.5 kUA/L) to rTri a 19 (i.e., ω_5-gliadin) was detected in only two out of the 28 sera. (Table I)

Patients suffering also from grass pollen allergy showed IgE reactivity to the recombinant timothy grass pollen allergens and wheat pollen extract (Fig 2, upper panel).

None of the patients exhibited IgE reactivity to dot-blotted human serum albumin and no reactivity to any of the dotted proteins was observed with serum from the non-allergic person (Fig 2).

Recombinant wheat allergens show varying IgE-reactivity and allergenic activity

To investigate the allergenic activity of the individual recombinant allergens, RBL cells expressing the human FcεRI were loaded with serum IgE from the patients with wheat-induced respiratory allergy and exposed to the recombinant allergens and wheat seed

extract (Fig 3). Glutathione transferase induced specific degranulation with sera from those 2 patients (Fig 2 and 3: #2, 10) who had shown strong IgE reactivity in the dot blot but not with the other two (Fig 2: #3, 27). 1-Cys-peroxiredoxin induced also degranulation with those sera which had shown the strongest IgE reactivity to the dot-blotted allergen (Fig 2 and 3: #3, 6, 19, 22) but not with the other IgE-reactive sera (Fig 2: #2, 4, 12, 13, 15, 27). Recombinant wheat dehydrin and profilin did not induce relevant basophil degranulation (data not shown). Each of the sera except serum # 6 induced basophil degranulation with wheat extract (Fig 3; data not shown). However, serum #6 had induced degranulation with recombinant 1-Cys-peroxiredoxin (Fig 3).

It thus appeared that those allergens which exhibited strong IgE reactivity were also more potent in inducing basophil activation than those with lower IgE binding capacity.

Discussion

Here we report the molecular characterization of five new allergens from wheat as well as their production and evaluation as recombinant allergens. According to sequence analysis the allergens belong to three groups of proteins. The first three allergens (i.e., thioredoxin h, glutathione transferase and 1-Cys-peroxiredoxin) form a group of proteins assembling a thioredoxin-like fold. They have general reducing activity and thus act as antioxidants. A thioredoxin h isoform with only moderate (55%) sequence identity has in fact been earlier described as major wheat allergen.[17, 18] Wheat profilin belongs to a family of cytoskeletal proteins which sequester actin and participtate in signal transduction. Profilins have been identified as ubiquitous and cross-reactive allergens in numerous plant species and plant tissues, but have not yet been isolated from wheat seeds. Finally, dehydrin represents a protein which is up-regulated in plants upon lack of water and thus functions as a stress protein which should protect against denaturation.

Each of the five allergens could be expressed with reasonable yield (>1mg/L culture) in *E. coli* as soluble proteins which allowed us to perform a serological evaluation of the frequency of IgE recognition of the allergens in a population of 28 patients suffering from wheat-induced respiratory allergy. We have also tested the frequency of IgE reactivity of the recombinant wheat allergens in a microarray format in 22 more patients suffering from baker´s asthma, 38 patients suffering from wheat food allergy and in 17 grass pollen allergic patients.[25] The combined results demonstrate that thioredoxin h, glutathione transferase, 1-Cys-peroxiredoxin and dehydrin are specifically recognized by sera from patients suffering from wheat-induced respiratory allergy. Profilin represents a cross-reactive allergen which also reacts with IgE antibodies from grass pollen allergic patients. Tri a 25, a thioredoxin which has a 55% sequence homology with the thioredoxin isolated by us has been reported as a major wheat allergen for bakers asthma. However, the recombinant thioredoxin h isolated in our study reacted only with 4.5% of baker´s asthma

patients and only with one serum in the serum pool used for screening but with none of the other 28 tested sera.[25] The latter may be explained by the fact that it represents a distantly related thioredoxin h isoform. According to serology and evaluation of allergenic activity in basophil release assays, we could identify glutathione transferase and in particular 1-Cys-peroxiredoxin as relevant wheat allergens for patients suffering from respiratory allergy to wheat. Using the latter two allergens and a recently described serine protease inhibitor it may be possible to establish serological tests for the identification of patients suffering from wheat allergy on inhalation exposure. In addition, other recently characterized allergens such as the glutenins and the wheat LTP[26] and non-wheat derived allergens may be included in such a test provided that these components are indeed specifically recognized by baker´s asthma patients. Such tests may be of clinical relevance for IgE-based serological screening to identify persons who have developed an occupational sensitization to wheat flour allergens such as bakers and persons working in food industry and restaurants. Current serological tests based on wheat allergen extracts yield clinically irrelevant test results due to the presence of cross-reactive allergens such as profilin and IgE-carbohydrate moieties.[27] Serological tests containing recombinant wheat allergens which are specifically recognized by patients suffering from baker´s asthma may help to reduce the need for provocation testing and thus represent useful diagnostic tools for the diagnosis of occupational allergy to wheat.

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Figure legends

Table I. Demographic, clinical and serological characterisation of patients with respiratory allergy to wheat.

Table II. Groups of wheat allergens and their amino acid sequence identities.

FIG 1. Coomassie brilliant blue-stained SDS-PAGE containing purified recombinant wheat allergens. A protein molecular weight marker has been applied on the left and right side of the gel.

FIG 2. IgE reactivity of patients suffering from respiratory wheat allergy.
Dot-blotted purified recombinant wheat proteins (thioredoxin h, glutathione transferase, profilin, 1-Cys-peroxiredoxin, dehydrin), human serum albumin, recombinant grass pollen allergens (rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12) and wheat pollen and seed extract were incubated with sera from 28 patients suffering from respiratory wheat allergy (1–28) and with serum from one non-allergic individual (neg). Bound IgE Abs were detected with ^{125}I-labeled anti-human IgE Abs and visualized by autoradiography. The numbers and percentages of reactive sera are displayed on the right margin.

FIG 3. Allergenic activity of recombinant allergens. RBL cells transfected with the human FcεRI were exposed to sera from patients with respiratory wheat allergy containing allergen-specific IgE and incubated with wheat extract, recombinant wheat allergens or buffer. β-hexosaminidase releases are displayed as percentages of total β-hexosaminidase on the y-axes.

Supplemental table I. Primers used for PCR amplification and sub-cloning into the expression vector pET17b.

Supplemental Fig 1. Sequence alignment of the clone 37-derived allergen thioredoxin with homologous proteins in other plants. The amino acid sequence (single letter code) of wheat thioredoxin was aligned with thioredoxins from rice (gi|27461140), maize (gi|40287476), poplar (gi|74058514), thale cress (gi|80973754), eucalyptus (gi|116788626), sage (gi|118481453), ricinus (gi|119367477), citrus (gi|157781191), chili (gi|195645418), peach tree (gi|208659912), soy (gi|115470941), clover (gi|186972814), sweet potato (gi|11135312), and greater plantain (gi|255587090). Identical amino acids are indicated by points, gaps (dashes) were introduced to improve the alignment. The conserved active site WCGPC is indicated in bold letters. Amino acids reported to be important for the maintenance of the tertiary structure and function are highlighted with asterisks (*).

Supplemental Fig 2. Sequence alignment of glutathione transferase, the clone 38-derived wheat allergen with homologous plant allergens. Alignment of wheat glutathione transferase sequence (top line) with sequences from barley (gi|6683765), maize (gi|195639794), oil palm (gi|192911948), rice (gi|115471993), soy (gi|2920666), papaya (gi|2853219), mallow (gi|29419702), vine (gi|119633090), pea (gi|110749703), ricinus (gi|255584168), tobacco (gi|19817), camomille (gi|17385642), chili (gi|58578272), thale cress (gi|21592644). Identical amino acids are indicated by points, gaps (dashes) were introduced to improve the alignment.

Supplemental Fig 3. Alignment of 1-Cys-peroxiredoxin, the clone 112-derived wheat allergen with homologous plant proteins. Alignment of the wheat 1-Cys-peroxiredoxin

sequence (top line) with sequences from barley (gi|1710077), rye (gi|1710076), rice (gi|158517776), maize (gi|162460575)m sunflower (gi|109631620), poplar (gi|224101487), ricinus (gi|255556526), thale cress (gi|28393058), barrel clover (gi|75323225), buckwheat (gi|6466096), oil palm (gi|192910660), rape (gi|7381260), bog (gi|1710079), zebra finch (gi|197128384).

Supplemental Fig 4. Alignment of dehydrin, the clone 126-derived wheat allergen with homologous plant proteins. Alignment of the wheat dehydrin sequence (top line) with sequences from barley (gi|6017948), rice (gi|115439431), oil palm (gi|7330252), thale cress (gi|30693389), grass (gi|2970213), grass (gi|121489509), coffee (gi|84314116), ricinus (gi|255561008), vine (gi|57903608), Mexican sunflower (gi|18076154), rape (gi|34539778), sallow thorn (gi|33114013), sunflower (gi|14588999), green tea (gi|215398978). Boxes marked in grey illustrate typical dehydrin sequence motifs.

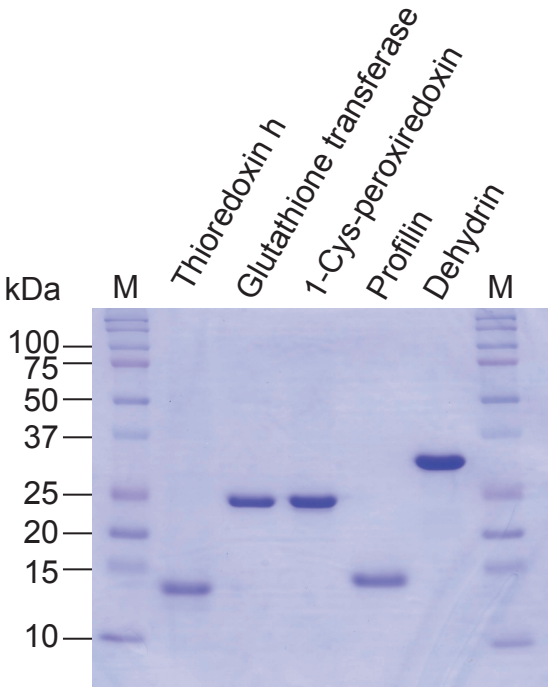
TABLE I. Demographic, clinical and serological characterisation of patients with respiratory allergy to wheat

Patient	Age	Sex	Wheat-indicated symptoms	Wheat SPT (sqmm)	Wheat flour IgE (kUa/l)	omega-5-gliadin (kUa/l)	Other allergies
1	48	m	RC	0.79	10.6	neg	c, hdm, m, mo, w
2	51	m	A, RC	7.07	46.9	neg	b, g, hdm, m
3	23	f	RC	neg	neg	neg	hdm
4	41	m	RC	12.56	neg	neg	hdm
5	33	m	A, RC	3.14	21.3	neg	b, c, g, hdm, o, w
6	61	m	RC	3.14	0.38	neg	g, hdm
7	52	m	A, RC	3.14	7.35	neg	b, hdm
8	34	f	RC	3.14	5.01	neg	b, c, g, hdm, m, o, w
9	33	m	A, RC	3.14	55.5	neg	g, hdm, m
10	58	m	RC	3.14	937	0.95	c, g, hdm, o
11	20	m	RC	12.56	19.2	neg	g, hdm, m, mo
12	22	m	A	3.14	22.8	neg	b, c, g, mo, w, y
13	30	f	A	7.07	3.99	neg	b, g, hdm, o
14	48	m	A, RC	3.14	989	1.13	hdm, o
15	53	m	A	3.14	40.8	neg	b, hdm, m, o
16	50	m	RC	3.14	24.4	neg	b, g, hdm, m, o
17	56	m	RC	7.07	17.6	neg	b, g, hdm, m, mo, o
18	21	m	RC	neg	4.93	neg	b, g, hdm, m, o, w
19	24	f	A, RC	3.14	38.5	neg	g, hdm
20	60	f	A, RC	28.26	6.43	neg	hdm
21	39	m	RC	3.14	5.18	neg	hdm
22	32	m	A	3.14	32.2	neg	b, g, hdm
23	27	m	RC	7.07	9.73	neg	neg
24	32	m	A	28.26	33.9	neg	b, c, g, hdm, m, o
25	61	m	A, RC	7.07	15.8	neg	b, hdm
26	32	m	RC	7.07	3.33	neg	neg
27	26	m	A	7.07	8.88	neg	b, g, hdm
28	52	m	RC	12.56	407	neg	b, hdm, g, mo, y

m: male, f: female, kUa/l: kilounit antigen per liter, A: Asthma, RC: Rhinoconjunctivitis, SPT: skin prick test, b: birch pollen, c: cat, g: grass pollen, hdm: house dust mite, m: mugwort, mo: molds, o: olive pollen, w: weed pollen, y: yeast

TABLE II. Allergen classification

Group	Wheat clone	Allergen name	Homologous proteins/allergens source (%sequence identity)
Thioredoxin-like fold	#37	Thioredoxin h	rice (73%), maize (65%), poplar (57%), thale cress (57%),eucalyptus (56%), sage (55%), ricinus (55%), citrus (55%), chili (54%), peach trea (53%), soy (53%), clover (52%), sweet potatoe (52%). greater plantain (52%)
	#38	Glutathione transferase	barley (95%), maize (67%), oil palm (65%), rice (63%), soy (59%), papaya (58%), mallow (58%), vine (57%), pea (56%), ricinus (56%), tabacco (55%), camomille (55%), chili (55%), thale gress (52%)
	#112	1-Cys-peroxiredoxin	barley (99%), rye (96%), rice (86%), maize (83%), sunflower (74%), poplar (72%), ricinus (72%), thale cress (72%), barrel clover (71%), buckwheat (71%), oil palm (70%), rape (70%), bog (70%), zebra finch (58%)
Cytoskelettal protein	#123	Profilin	maize (83%), cinnamon (80%), oil palm (80%), soy (80%), olive tree (79%), parsley (78%), coconut palm (78%), grass (78%),
Stress-induced protein	#126	Dehydrin	barley (76%), rice (58%), oil palm (54%)


kDa
M
Thioredoxin h
Glutathione transferase
1-Cys-peroxiredoxin
Profilin
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100
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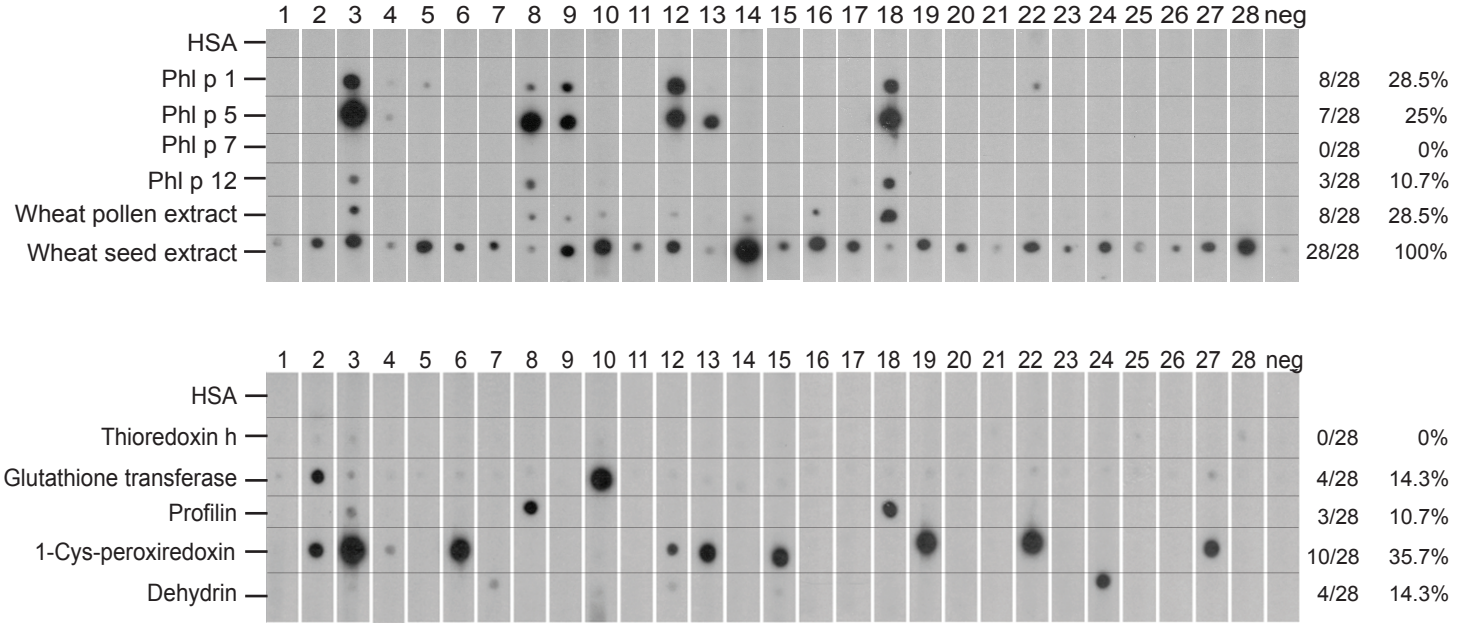
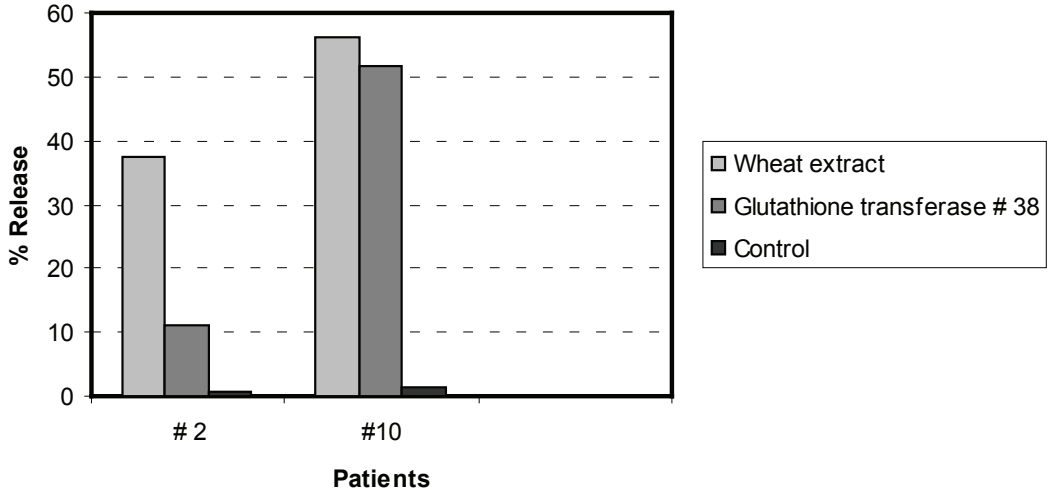
Figure 1


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 neg
HSA
Phl p 1
Phl p 5
Phl p 7
Phl p 12
Wheat pollen extract
Wheat seed extract
8/28 28.5%
7/28 25%
0/28 0%
3/28 10.7%
8/28 28.5%
28/28 100%

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 neg
HSA
Thioredoxin h
Glutathione transferase
Profilin
1-Cys-peroxiredoxin
Dehydrin
0/28 0%
4/28 14.3%
3/28 10.7%
10/28 35.7%
4/28 14.3%

Figure 2


60
50
40
30
20
10
0
% Release
# 2
#10
Patients
Wheat extract
Glutathione transferase # 38
Control

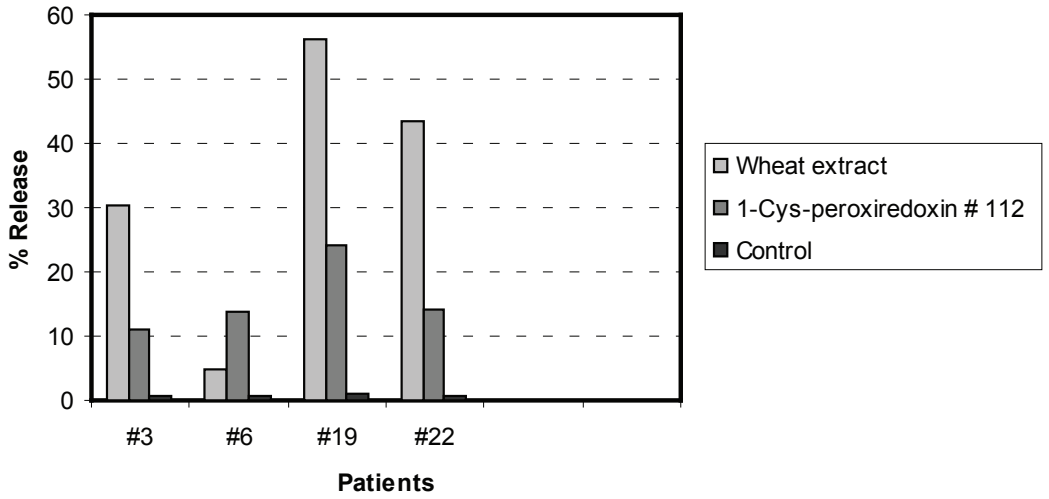
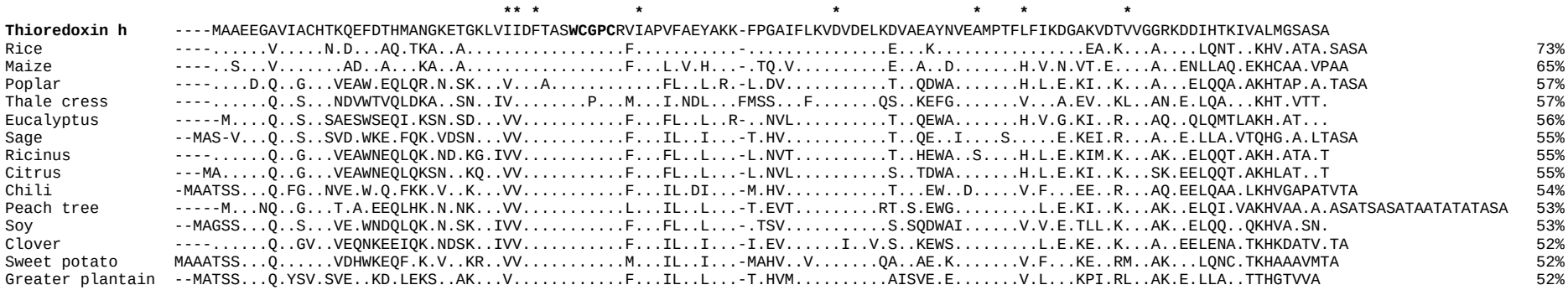

60
50
40
30
20
10
0
% Release
#3
#6
#19
#22
Patients
Wheat extract
1-Cys-peroxiredoxin # 112
Control

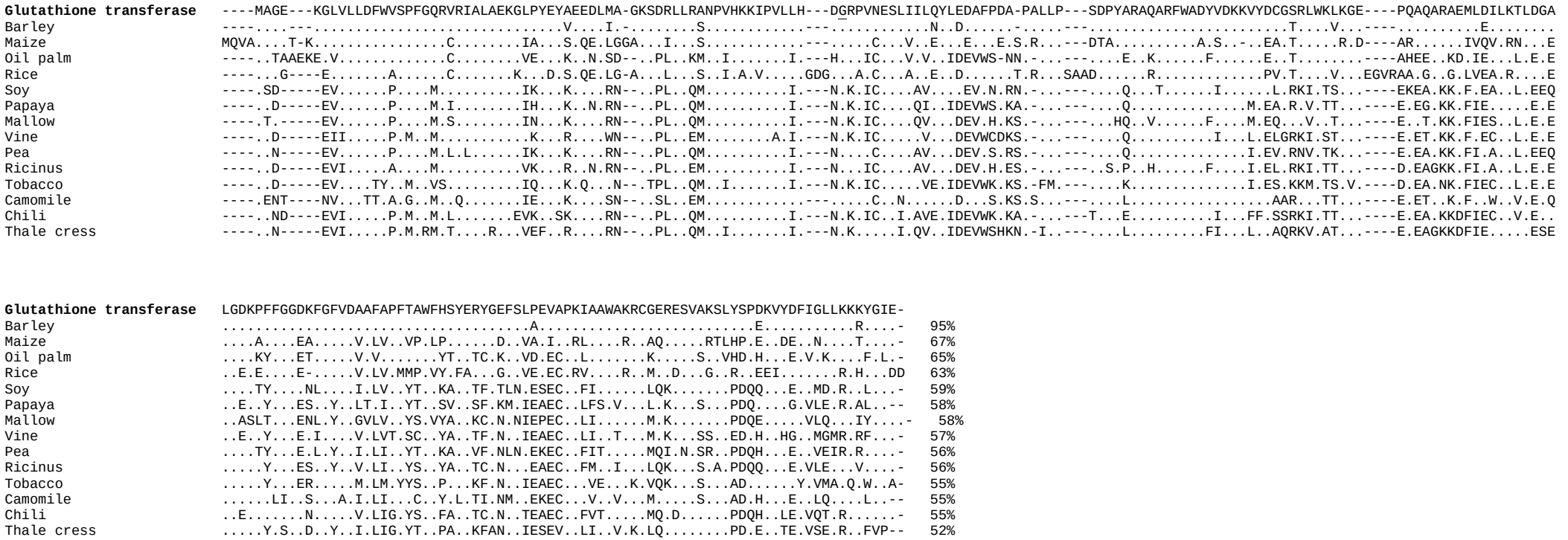
Figure 3

Supplemental table I. Primers used for cDNA synthesis, PCR amplification and subcloning into the expression vector pET17b

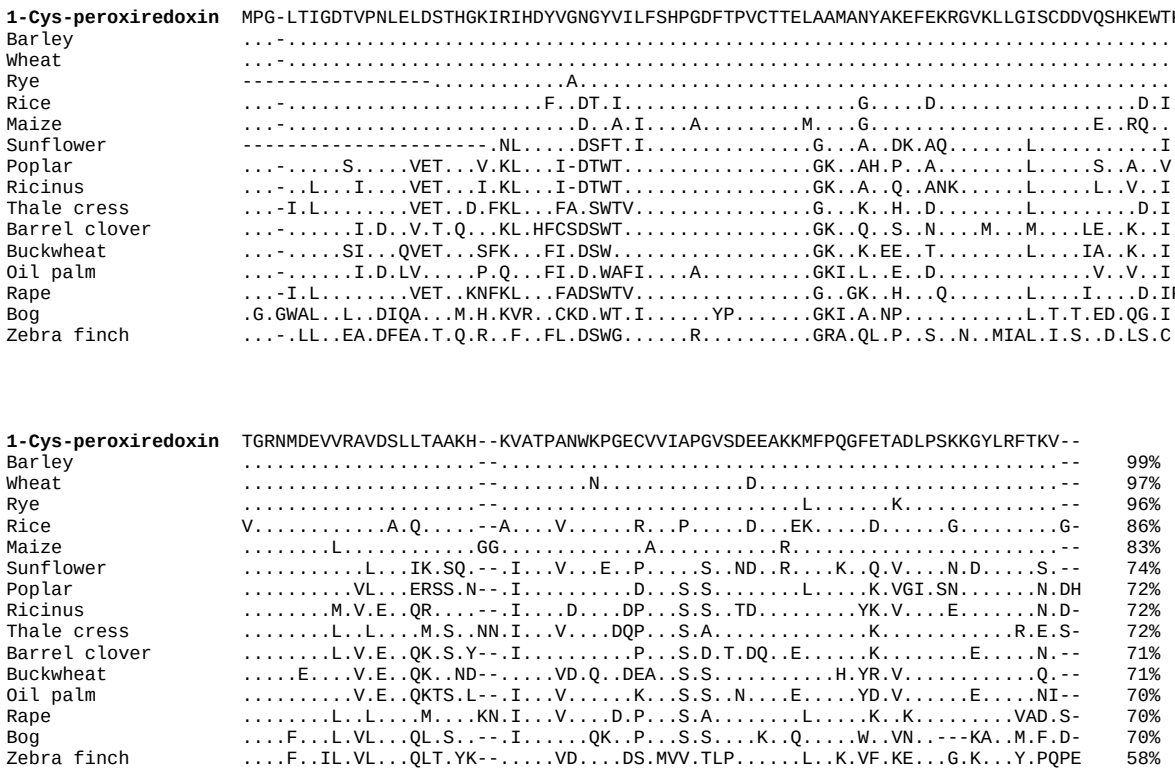
Primer	Sequence
37 fwd	5' *CAT ATG* GCC GCC GAG GAG GGA GCC GTG ATA 3'
37 rev	5' GAATTC TTA **GTG ATG GTG ATG GTG ATG** GGC AGA TGC AGA ACC 3'
38 fwd	5' *CAT ATG* GCG GGC GAG AAG GGC CTG GTG CTG 3'
38 rev	5' GAATTC TTA **GTG ATG GTG ATG GTG ATG** CTC GAT GCC GTA CTT 3'
112 fwd	5' *CAT ATG* CCG GGC CTC ACC ATC GGC GAC ACC GTC 3'
112 rev	5' GAATTC TTA **GTG ATG GTG ATG GTG ATG** GAC CTT GGT GAA GCG 3'
123 fwd	5' *CAT ATG* TCG TGG CAG ACG TAC GTC GAC GAC 3'
123 rev	5' GAATTC TTA **GTG ATG GTG ATG GTG ATG** GAA ACC CTG CTC GAC 3'
126 fwd	5' *CAT ATG* GCG GAC TAC GGT GGA GAG TAC GGG 3'
126 rev	5' GAATTC TTA **GTG ATG GTG ATG GTG GTG** TCC AGG GAG CTT 3'



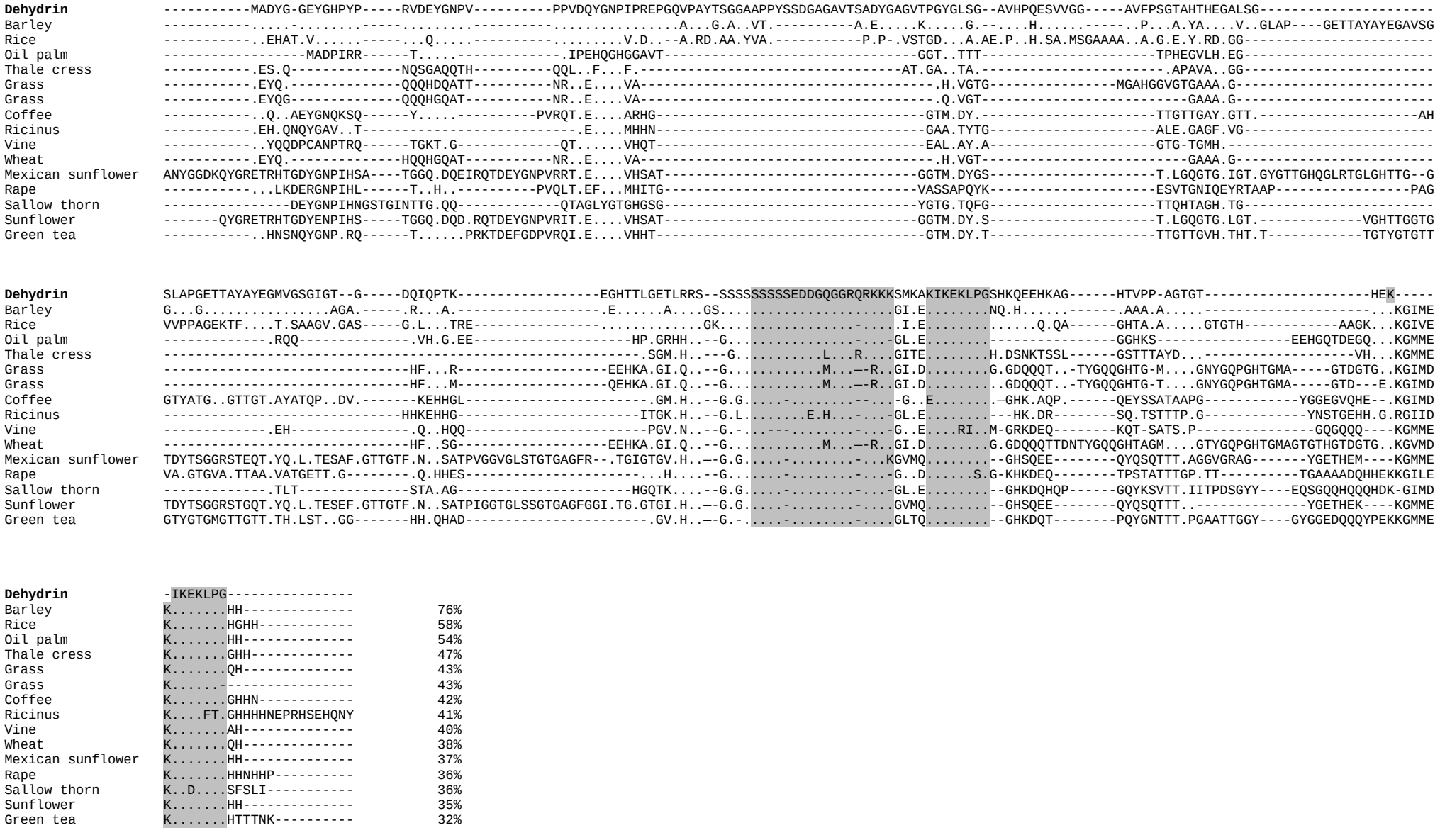
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Supplemental figure 4

Curriculum vitae

Personal Details

Name	Pahr Sandra
Date of Birth	September 8th, 1985
Place of Birth	Oberwart, Austria
Nationality	Austria

Education

Since September 2008	Diploma Thesis at the Department of Pathophysiology, Center of Physiology and Pathophysiology, Medical University of Vienna, under supervision of Prof.Dr.Rudolf Valenta Title: Characterisation of new wheat allergens
Since October 2004	University of Vienna Study of Genetics and Microbiology
2003-2004	Medical University of Vienna Study of Medicine
1995-2003	Grammar school, BG/BRG Oberschützen, Burgenland
1991-1995	Primary school

Memberships

Member of the Austrian Society of Allergology and Immunology (ÖGAI)

Poster presentations

“Molecular cloning and characterisation of a new wheat food allergen”
-International workshop “Current problems in allergen vaccine development and manufacturing”, Varadero, Cuba, October 18-23,2009-10-15

“Recombinant production and characterisation of a new wheat food allergen”
-Karl Landsteiner Meeting, Salzburg, Austria, November 6-7,2009