# CHARLES UNIVERSITY IN PRAGUE

**Faculty of Science**

**Study program: Immunology**

**Dissertation**

# Food allergy to wheat flour proteins

by

RNDr. Petr Šotkovský

Supervisor:

Doc. RNDr. Ludmila Tučková, DrSc.

Department of Immunology and Gnotobiology

Institute of Microbiology, v.v.i.

Academy of Sciences of the Czech Republic

Prague, 2012

ŠOTKOVSKÝ, Petr. Potravinová alergie na proteiny pšeničné mouky. *[Food allergy to wheat flour proteins]*. Praha, 2012, Disertační práce (Ph.D.). Univerzita Karlova v Praze, Přírodovědecká fakulta. Vedoucí závěrečné práce: Doc. RNDr. Ludmila Tučková, DrSc.

**Statement of originality**

The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, the thesis is the product of my own work and contains no material previously published or written by another person, except where due references are made.

Prague, 11th December 2012 Petr Šotkovský

**ACKNOWLEDGMENTS**

I would like to thank to my supervisor Ludmila Tučková for her continuous advice, encouragement and instructions throughout this study.

I wish to thank all colleagues in the laboratory for their kind help and support and for pleasant work environment.

My thanks belong also to my family for supporting me and for the tolerance they have for my work.

**TABLE OF CONTENTS:**

ABBREVIATIONS .................................................................................. 7

THESIS SUMMARY .................................................................................. 9

SOURHN V ČEŠTINĚ .................................................................................. 10

HYPOTHESIS AND AIMS .................................................................................. 11

1. INTRODUCTION .................................................................................. 12

1.1. Food allergy .................................................................................. 12

1.2. Wheat allergy .................................................................................. 16

1.3. Diagnosis .................................................................................. 17

1.4. Important aspects of allergen components.................................................. 19

1.5. Classification of wheat flour proteins .................................................. 21

1.6. Allergens in wheat.................................................................................. 22

1.7. Allergen databases .................................................................................. 26

1.8. Effects of processing on allergenicity .................................................. 27

1.9. Models of allergic diseases .................................................................................. 29

2. RESULTS .................................................................................. 31

2.1. Identification of wheat flour proteins recognized by IgE Abs of allergic patiens .................................................................................. 31

2.2. Isolation and purification of wheat flour allergens .................................................. 34

2.3. Mouse model of food allergy .................................................................................. 37

3. CONCLUSIONS AND DISCUSSION .................................................................................. 40

4. ORIGINAL PUBLICATIONS .................................................................................. 49

5. CONFERENCE ABSTRACTS .................................................................................. 50

6. ORAL PRESENTATIONS .......................................................................................... 53

7. BIBLIOGRAPHY.................................................................................................... 54

# ABBREVIATIONS

| | |
|---|---|
| 1-DE | one-dimensional electrophoresis |
| 2-DE | two-dimensional electrophoresis |
| Abs | antibodies |
| CD | cluster of differentiation |
| CM | chloroform methanol |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| HPLC | high performance liquid chromatography |
| IEF | isoelectric focusing |
| IFN | interferon |
| IgE | immunoglobulin class E |
| IL | enterleukin |
| LC | liquid chromatography |
| MALDI | matrix-assisted laser desorption/ionization |
| MHC | major histocompatibility complex |
| MLN | mesenteric lymph nodes |
| MMCP-1 | mouse mast cell protease-1 |
| MS | mass spectrometry |
| OVA | ovalbumin |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| Q | quadrupole |
| ROC | receiver operating characteristic |
| SDS | sodium dodecyl sulphate |
| sIgE | specific IgE |
| tTG | tissue transglutaminase |
| TGF | transforming growth factor |
| TLP | thaumatin-like protein |
| TLR | toll-like receptor |

| | |
|---|---|
| TNF | tumor necrosis factor |
| TOF | time of flight |
| WDEIA | wheat-dependent exercise induced anaphylaxis |
| XIP | xylanase inhibitor protein |

# THESIS SUMMARY

Food allergy is one of the frequent disorders and its incidence in paediatric as well as adult population is continuously rising, having doubled in the last two decades. Although wheat belongs to major food allergens and is a staple food in most diets, we have only little knowledge of wheat proteins causing IgE mediated hypersensitivity reaction. Diagnostic approaches of food allergy to wheat have a high sensitivity, but low specificity. Poor predictability and specificity may be associated with the insufficient purity of wheat extracts used in sIgE assays or with the lack of major allergens in these extracts.

In the first step, we characterized 19 potential allergens recognised by IgE Abs of allergic patients, using proteomic techniques (1-DE, 2-DE, MALDI-TOF, QTOF and $LCQ^{DECA}$ nLC-MS/MS ion trap technique). We identified these IgE-binding molecules such as: α-amylase inhibitors, β-amylase, profilin, serpin, β-D-glucan exohydrolase and 27K protein. To quantify sIgE in patient's sera we developed ELISA using the whole wheat extract and two commercially available α-amylase inhibitors.

Second, we developed a procedure that allows isolation of wheat allergens from natural sources using Rotofor cell and HPLC. Twenty-seven potential wheat allergens have been successfully identified; of these, the following seven are newly reported in food allergy: endogenous α-amylase/subtilisin inhibitor, trypsin/α-amylase inhibitor CMX1/CMX3, TLP, XIP, β-glucosidase, class II chitinase and 26 kDa endochitinase. The biological activity of purified allergens was tested using the basophil activation test. We have shown for the first time that purified allergens, such as α-amylase inhibitor 0.19, lipid transfer protein, TLP and wheatwin, can activate patients' basophils, confirming that our purified proteins maintain their biological activity.

Third, we investigated how thermal processing influences the ability of OVA to induce allergic symptoms and immune responses in mouse model of food allergy.

The aim of our studies is to identify the most important wheat allergens in IgE mediated hypersensitivity reaction. We developed new procedures of identification and isolation of allergens in their native form in amounts sufficient both for biological testing (*in vivo* and *in vitro*) and for physicochemical characterization. Such studies will lead to a more detailed knowledge of allergenicity of wheat proteins and to improved specificity of diagnostic tests.

# SOUHRN V ČEŠTINĚ

Potravinová alergie je jedním z nejčastějších onemocnění a postihuje jak dětskou, tak dospělou populaci. Její výskyt neustále roste, v posledních dvaceti letech se zdvojnásobil. Ačkoliv pšenice patří mezi hlavní potravinové alergeny a je základní složkou většiny jídel, naše znalosti pšeničných proteinů způsobujících IgE hypersensitivní reakci je nedostatečná. Diagnostika potravinové alergie na pšenici se vyznačuje vysokou sensitivitou, ale nízkou specificitou. Ta může být spojena s nedostatečnou čistotou používaných extraktů anebo absencí hlavních alergenů v těchto extraktech.

V prvním kroku jsme charakterizovali 19 potenciálních alergenů rozpoznávaných IgE protilátkami ze sér pacientů. Díky proteomickým metodám (1-DE, 2-DE, MALDI-TOF, QTOF a $LCQ^{DECA}$ nLC-MS/MS iontová past) jsme identifikovali např. tyto IgE vázající molekuly: inhibitory α-amylázy, β-amyláza, profilin, serpin, β-D-glukan exohydroláza a 27K protein. Pro kvantifikaci IgE protilátek jsme vyvinuli metodu ELISA, ve které byl použit jak pšeničný extrakt, tak komerčně dostupné inhibitory α-amylázy.

V dalším kroku jsme vyvinuli metodu postupné izolace pšeničných alergenů z přírodních zdrojů, která využívá zařízení Rotofor a HPLC. Tímto přístupem bylo identifikováno 27 potenciálních alergenů, z nichž 7 bylo nově popsáno u potravinové alergie: endogenní inhibitor α-amylázy, inhibitor α-amylázy CMX1/CMX3, TLP, XIP, β-glucosidáza, chitináza třídy II a 26 kDa endochitináza. Pro testování biologické aktivity purifikovaných alergenů byl použit test aktivace bazofilů. Jako první jsme ukázali, že purifikované alergeny jako inhibitor α-amylázy 0.19, lipid transfer protein, TLP a wheatwin aktivují pacientské bazofily. Tímto se potvrdilo, že naše purifikované proteiny si zachovávají svou biologickou aktivitu.

Na myším modelu jsme také zkoumali vliv tepelného opracování vaječného ovalbuminu na jeho schopnost vyvolat odpověď imunitního systému.

Hlavním přínosem těchto prací je identifikace nejdůležitějších pšeničných alergenů schopných vyvolat IgE hypersenzitivní reakci. Vyvinuli jsme nové metody pro identifikaci a izolaci alergenů v jejich přírodní formě a to v množství, které je dostatečné jak pro biologické testy (*in vivo, in vitro*), tak pro fyzikálně-chemickou charakterizaci. Tato studie povede ke zpřesnění znalostí o alergenicitě pšeničných alergenů a ke zlepšení specificity diagnostických testů.

# HYPOTHESIS AND AIMS

The general aim of this thesis was to identify and characterise the most frequently recognised wheat allergens and to analyse their allergenicity in an attempt to improve the understanding of hypersensitivity reactions and the diagnosis of wheat allergy.

**Specific aims:**

- to identify wheat flour allergens most frequently recognized by patients' IgE antibodies using proteomic techniques (1-DE, 2-DE, MALDI-TOF, QTOF and $LCQ^{DECA}$ nLC-MS/MS ion trap technique)

- to developed a new procedure for isolation and purification of identified IgE-binding wheat allergens in their natural form in amounts that allowed subsequent structural and functional analyses as well as to find new potential allergens using ultrafiltration, isoelectric focusing (Rotofor), and liquid chromatography.

- to investigate the clinical relevance of the newly identified and purified allergens using *in vitro* specific IgE detection (ELISA, Immunoblot) in correlation with the results of skin prick test.

- to test the cellular response of patient's basophils against the extracted proteins and identified allergens in the basophil activation test.

- To analyse the effect of pepsin on the imunogenicity of the extracted wheat proteins to mimic the digestion of wheat containing food.

- to investigate how thermal processing influence ability of ovalbumin to induce allergic symptoms and immune responses in mouse model of food allergy.

# 1. INTRODUCTION

## *1.1. Food allergy*

Food allergy is an important health problem affecting 6 to 8% of children and 2 to 3% of adults, and is increasing in prevalence [Poole et al., 2006; Ostblom et al., 2008; Zicari et al., 2012]. Under physiological conditions, immune system develops oral tolerance, a state of active inhibition of systemic response to the huge amount of antigens regularly ingested and to the commensal organisms developing an active immune suppression of digestive pathogens. Oral tolerance depends on an intact and immunologically active gastrointestinal mucosal barrier. This barrier includes immunity components, which are innate (e.g. neutrophils, macrophages, toll-like receptors, natural killer and epithelial cells) or adaptive (e.g. intraepithelial and lamina propria lymphocytes, Peyer's patches, secretory IgA and cytokines). These components play central role in the tolerance of foreign antigens that are not harmful to the body and cooperate with a number of immune cells (e.g. antigen-presenting cells: intestinal epithelial cells, dendritic cells and regulatory T cells) [Strobel et al., 2006; Dupont, 2011]. Despite high acidity in the stomach and enzyme activity, around 2% of ingested food antigens still cross the gastrointestinal barrier and are transported throughout the body in a form that is immunologically intact enough to produce a food allergy [Sampson,1999]. The development of oral tolerance might also be influenced by several non-host factors, such as the physical properties of the antigen, and amount and frequency of exposure [Chehade et al., 2005]. Lastly, the commensal gut flora is likely to play a role in oral tolerance, as suggested by studies in germ-free mice [Bashir et al., 2004]. However, in some genetically susceptible individuals, food allergy can develop. An inappropriate immune response is thought to be consequence of the failure to establish or maintain tolerance towards harmless antigens [Perrier et al., 2010].

Based on the pathophysiological mechanism of the reaction, there are differences between food allergy and food intolerance. Food intolerance may be due to properties of the food (i.e. toxic contaminant, pharmacologic active component) or to characteristics of the host (i.e. metabolic disorders, idiosyncratic responses, psychological disorder). Food allergies may be further classified in a) IgE-mediated, which are mediated by IgE

antibodies and are the best-characterized food allergy reactions; b) cell mediated when the cell component of the immune system is responsible of the food allergy and mostly involve the gastrointestinal tract; c) mixed IgE mediated-cell mediated when both IgE and immune cells are involved in the reaction [Sampson, 2003; Cianferoni et al., 2009; Sicherer at al., 2009] (Fig. 1).

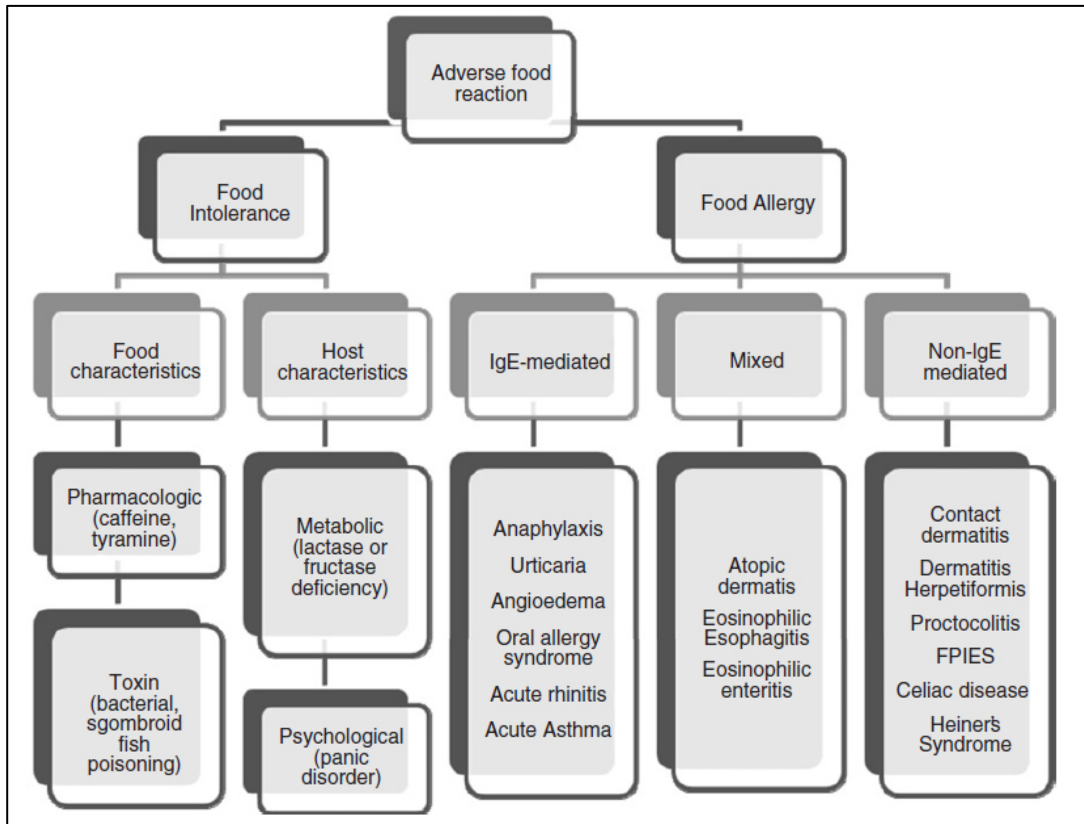



**Figure 1.** Classification of adverse food reaction as food intolerance and food allergy. Patients with food allergy have IgE-mediated, non-IgE mediated symptoms or mix of both. Adapted from Cianferoni et al, 2009.

In general, the immunological mechanism in food allergies is IgE-mediated Type-I response characterized by high serum total IgE concentration and by the presence of IgE Abs specific to ordinarily harmless environmental antigens. However, following studies reveal that food allergies may also involve Type-II and Type-IV responses [Johansson et al., 2004]. The IgE mediated response is divided into two phases: a) allergic sensitization

and b) elicitation. The sensitization to an allergen is the induction phase during which Th2 cells express IL-4, IL-5, IL-6 and IL-13 and this Th2 response leads to B-cell differentiation into IgE producing cells. IgE Abs bind to its high-affinity receptor on the surface of mast cells in the skin, gut, respiratory, and cardiovascular systems. The elicitation is the effector phase during which classic allergic symptoms occur within minutes after allergen exposure, when IgE-bound mast cells recognize the allergen and become activated. On the other hand, mechanism controlled by Th1 cells express IL-2, IL-12, IL-18, IFN α and γ, as well as TNF α and β (Fig. 2) [Angelis et al., 2010, Vickery at al., 2011].

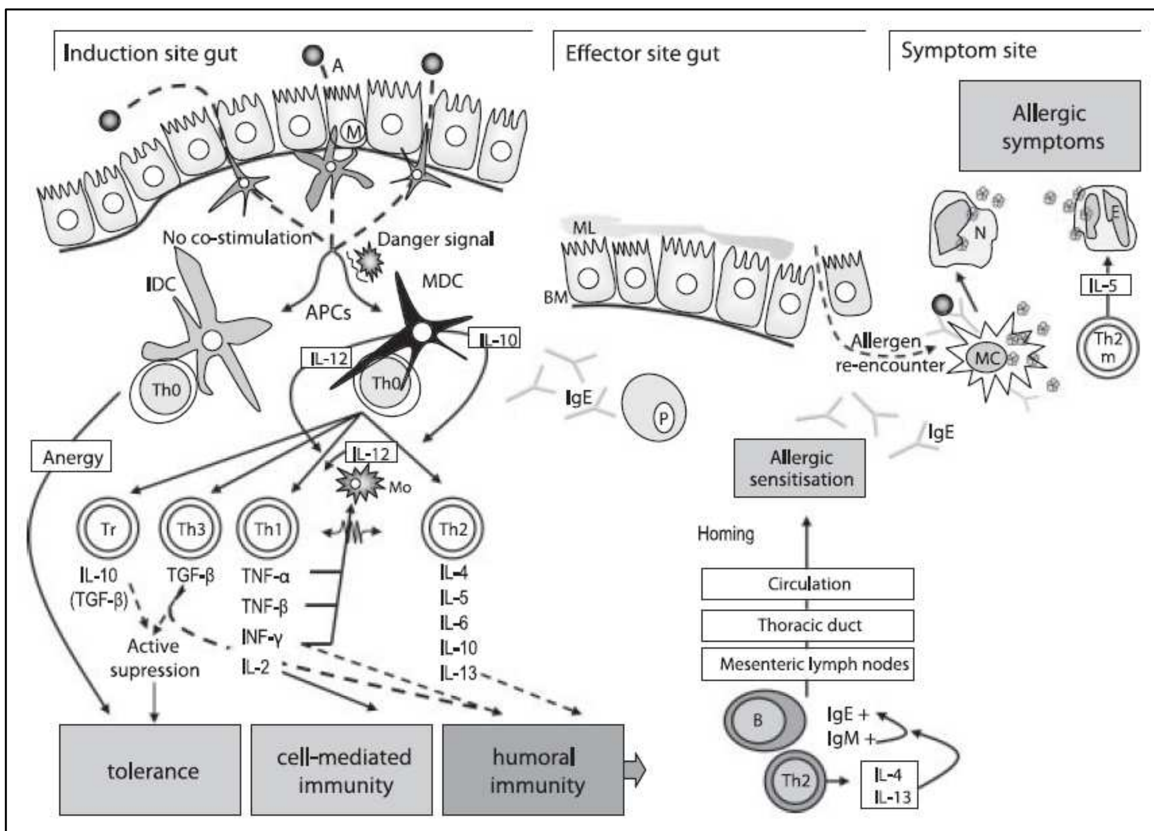



**Figure 2.** Schematic depiction of the mechanism of food allergy. A: allergen, M: M cell, ML: mucous layer, BM: basement membrane, APCs: antigen presenting cells, IDC: immature dendritic cell, MDC: mature dendritic cell, IL: interleukin, TGF: transforming growth factor, Th: T-helper, Tr: T-regulatory cell, Mo: macrophage, TNF: tumour necrosis factor, B: B cell, P: plasma cell, MC: mast cell, E: eosinophil, N: neutrophil, m: memory cell. Adapted from Angelis et al, 2010.

Food allergens can enter from the luminal side of the epithelium through damaged epithelial cell layer or via active uptake by enterocytes or so-called M cells that are specialized to actively sample the gut contents by transporting macromolecules and particles from the lumen into organized lymphoid follicles known as Peyer's patches. Tissue dendritic cells, the most important antigen-presenting cells, ingest and present allergens at sites of exposure and activate naïve T cells. Activation of dendritic cells induces their migration to local lymphoid tissue and their maturation into highly effective antigen-presenting cells. Allergens that enter the immature dendritic cell via phagocytosis are processed in the endocytic compartment for presentation by MHC class II molecules and they activate TLRs. TLR signalling causes the dendritic cells to become licensed and begin to undergo maturation. Mature conventional dendritic cells have become powerful activators of naïve T cells but are no longer phagocytic [Murphy, 2012].

In food allergic individuals the majority of acute allergic reactions to foods are due to the engagement of allergen sIgE Abs with its high-affinity receptor (FcεRI) that is expressed on mast cells and basophils, and low affinity receptor (FcεRII), which is present on macrophages, monocyte, lymphocytes and platelets. When a specific antigen binds the IgE linked to the FcεRI it determines a receptor cross-linking and consequent release of mediators [Ebo et al., 2001; Lee et al., 2006; Sicherer et al., 2009]. The major factors for mast-cell growth and development include stem-cell factor, IL-3, IL-4 and IL-9. Mast cells are activated and degranulated when antigens cross-link IgE to FcεRI receptor. Mast-cell degranulation releases inflammatory mediators such as histamine, serine esterases and proteases (chymase and tryptase). Histamine acts via $H_1$ receptors on local blood vessels to cause an immediate increase in local blood flow and vessel permeability. Histamine can increase antigen-presenting capacity of dendritic cells and Th1 priming (through the $H_1$ receptor). On T cells, it can enhance Th1 proliferation and IFN-γ production (also through the $H_1$ receptor). The proteases released by the mast cells activate matrix metalloproteinases, which break down extracellular matrix proteins, causing tissue disintegration and damage [Murphy, 2012]. The activation of T cell subset, which secretes inflammatory factors, including IL-4, IL-5 and IL-13, is a key event in the pathogenesis of allergy. At the initiation of allergic responses, IL-4 and IL-13 are essential for allergen-specific IgE production and IL-5 is required for survival of eosinophilic granulocytes. Together with histamine these cytokines initiate immediate hypersensitivity against

allergens (symptom phase), activate inflammatory tissue and effector cells, and induce various allergic symptoms including skin, respiratory or digestive system.

The activity of immune system in healthy individuals appears to be controlled by regulatory T (Tr) cells, which secrete suppressive cytokines such as IL-10 and TGF-β. An imbalance between Th2 and Tr cells in allergic patients leads to insufficient tolerance to the allergen. This was recently shown for healthy and allergic responses to various allergens by using isolated peripheral $CD4^+$ T cells secreting IL-10 (Tr1), IL-4 (Th2) or IFN-γ (Th1) [Akdis et al., 2004; Schmidt-Weber et al., 2004].

## *1.2. Wheat allergy*

Wheat is the most widely consumed food grain in the world and belongs to six major food allergens (milk, egg, wheat, peanut, soya and fish) that account for over 80% of the reaction in food allergies [Ramesh, 2008]. Although cereals are essential in daily nutrition for the majority of people, only little information is available on wheat allergens causing hypersensitivity reactions. Hypersensitivity reactions to wheat proteins are clinically distinct, depending on the route of allergen exposure, target organs, and immunological mechanisms involved. One of the main structural protein complexes of wheat is gluten. Human diseases related to gluten exposure can be classified into wheat allergy and celiac disease. Wheat allergy is defined as an adverse immunologic reaction to wheat and may manifest as a classical food allergy to ingested wheat proteins including cutaneous, gastrointestinal, and respiratory symptoms; contact urticaria; and wheat-dependent exercise induced anaphylaxis (WDEIA) [Johansson et al., 2004]. Occupational asthma (so-called baker's asthma) and rhinitis are reactions to inhaled flour, therefore, many workers in the milling, baking and food processing industries are exposed to wheat flour in the atmosphere, raising the risk of respiratory allergy. IgE antibodies and chemical mediators such as histamine from basophils and mast cells play a central role in the pathogenesis of these diseases. In contrast, ingestion of wheat gluten may cause dermatitis herpetiformis, a blistering skin eruption; gluten ataxia; and celiac disease, T cell-mediated intestinal inflammation demonstrated by specific serologic autoantibodies, most notably serum anti-tissue transglutaminase (tTG) and anti-endomysial antibodies (EMA). In addition, there are cases of gluten reactions in which neither allergic nor autoimmune

mechanisms are involved. These are generally defined as gluten sensitivity (Fig. 3) [Palosuo, 2003; Sapone et al., 2012].

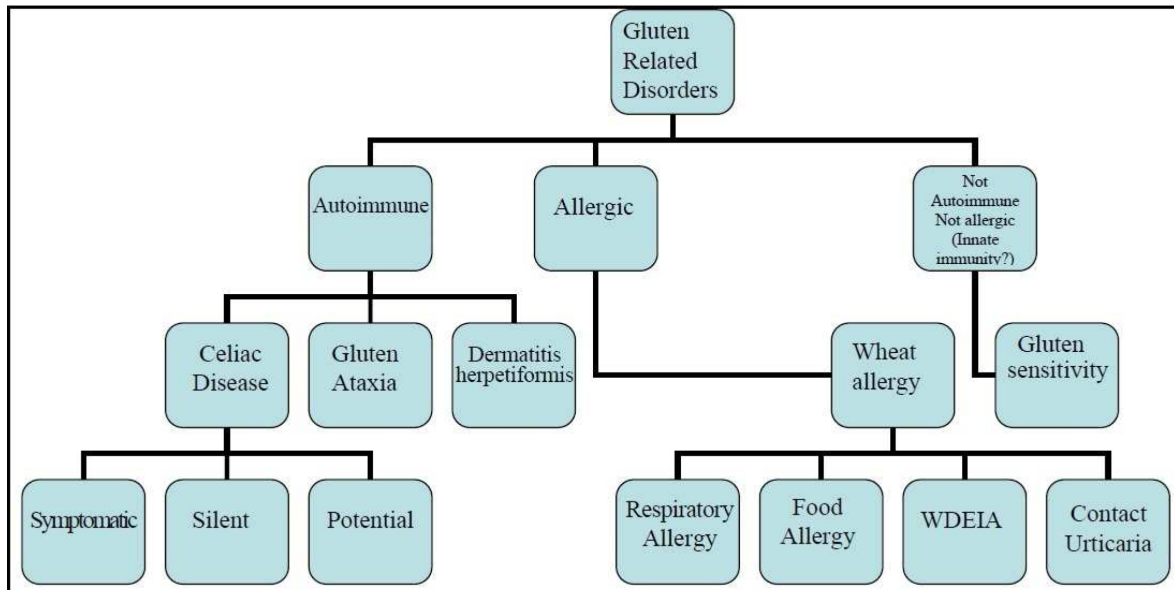



**Figure 3.** Proposed new nomenclature and classification of gluten-related disorders. Adapted from Sapone et al., 2012.

### *1.3. Diagnosis*

Diagnosis of food allergy to wheat is, however, not simple. The main available *in vivo* methods for the assessment of food allergens are double-blind placebo-controlled food challenge and skin prick tests. Although oral food challenge tests are considered to be the gold standard for the diagnosis, they are time consuming and can induce severe clinical symptoms. Skin prick tests are diagnostic for baker's asthma but not for atopic dermatitis [van Kampen et al., 2009]. In contrast, basophil activation tests are considered a reliable *in vitro* diagnostic technique [Tokuda et al., 2009]. These tests are based on quantitative determination on a flow cytometric measurement of CD203c up-regulation and assessment of CD63 membrane expression by basophils, in response to FceRI-bound IgE cross-linking by specific allergens. Blood samples are obtained from patients with clinical symptoms and exposed to selected allergens. The optimal cut-off values of basophil activation are determined by the ROC analysis. The clinical benefit of such functional techniques was already demonstrated in different types of IgE-mediated food allergy [Moneret-Vautrin et

al., 1999; Eberlein-Konig et al., 2006; Shreffler, 2006; Ocmant et al., 2007]. *In vitro* detection of food-specific IgE Abs by radioimmunoassays (RIAs) or enzymatic immunoassays (ELISA) were designed to detect and even quantify sIgE, either free in the serum or bounded to peripheral blood cell surface. Even though these immunological methods are highly sensitive they have low specificity, and a certain degree of cross-reactivity cannot be excluded [Sampson 2001; Tordesillas et al., 2009].

At the beginning, crude natural and non-standardized extracts were used, although, the use of standardized extracts with a more precise content of allergenic components can improve the diagnostic value of the techniques. The poor predictability and specificity of all currently known diagnostic approaches may be associated with the purity of the wheat extracts used in the specific IgE assays or to their lack of inclusion of all major allergens. Furthermore, the expression of IgE-reactive proteins depends on wheat seed maturation and varies in different wheat sources [Constantin et al., 2009]. The absence of potentially allergenic impurities is a major quality criterion, as even minor contamination with a highly IgE reactive protein may produce false positive results. Moreover, allergens can be present in natural sources as a mixture of isoforms differing only in a few amino acid residues and showing different IgE-binding activity as has been shown for the 36 different isoforms of the birch pollen allergen Bet v 1 [Zaborsky et al., 2010]. Other allergens, such as profilins or nonspecific lipid transfer proteins have been identified as either single protein sequences or as a limited number of isoforms [Gao et al., 2005]. Whereas for immunotherapy the hypoallergenic variant might be the ideal candidate, for diagnostic approaches the variant showing the highest IgE-binding activity would be the isoform of choice [Sancho et al., 2010].

Advances in the field of molecular allergology led to the development of a new concept in allergy diagnosis, component-resolved diagnosis, allowing the identification of the potential disease-eliciting molecules. Recombinant allergens have also been used in a mixture with natural extracts to increase test sensitivity [Lidholm et al., 2006; Ferrer et al., 2009]. The extract gives the overall answer to whether the patient is sensitized to the particular allergen source and the individual components adds vital information on risk, specificity and cross-reactivity. With the ability to prepare recombinant or native proteins, it is possible to investigate the contribution of individual components to the allergenicity of the food. This capability of component-resolved diagnostics allows for more focused

diagnostic and prognostic abilities than do the current methods of assaying for the presence of serum food-specific IgE [Lieberman, 2011].

### *1.4. Important aspects of allergen components*

In order to properly interpret test results in the clinic, it is important to understand some properties of allergen components and their clinical implications. Each allergen source contains many different allergenic proteins (allergen components). On each allergen component, there are commonly several different epitopes that can be divided into sequential or conformational. A sequential or linear epitopes are recognized by antibodies by its linear sequence of amino acids, or primary structure. In contrast, a conformational epitopes have a specific three-dimensional shape and protein structure. Allergenicity and biological activity of proteins relate to their sequence and structure and some conformational epitopes are only present when the protein is correctly folded. Antibodies that recognize these epitopes can be used to monitor protein folding. An example of this is the Durotest$^{TM}$ antibody, which only binds to the folded (i.e. disulphide-bonded) forms of the puroindoline proteins of wheat [Wiley et al., 2007]. However, when epitopes are present as short sequences then antibodies to these will recognize unfolded proteins. Therefore, these antibodies can be used to confirm the identities of purified proteins but not to monitor their authenticity [Sancho et al., 2010].

Currently, there is no known structure that is common to all allergen components or epitopes (*i.e.* no common feature that makes a substance an allergen or not). On the one hand, every species contain species-specific allergen epitopes, and antibodies formed to these structures bind only to the allergen epitopes in this particular species. Species-specific allergen components are unique markers for its allergen source. The value of identifying species-specific allergen components is to single out the primary sensitizer that induces restricted reactions only to one specific source *e.g.* wheat [Sastre J, 2010]. On the other hand, proteins with similar structures are often present in biologically related species. Antibodies formed against such protein structures, can of course bind to the same or similar structure on a protein from several different species and these allergen components are classified as markers for cross-reactivity. They can be present in many different allergen sources, sometimes not even closely related. Identifying markers for cross-

reactivity, on the contrary, gives valuable information on possible sensitizations to several different sources *e.g.* profilin from wheat, apple, timothy grass or celery. One of the most important clinical utility in allergy is its ability to reveal the allergens to which patients are sensitized, including primary or species-specific allergens and markers of cross-reactivity or panallergens. The panallergens families encompass of related proteins, which are involved in general vital functions and can be widely found throughout nature. The Greek prefix "pan" means "all", emphasizing the ubiquitous distribution of these allergenic molecules. Plan panallergens share highly conserved sequence regions, structure, and function. They are responsible for many IgE cross-reactions even between unrelated pollen and plant food allergen sources. Sensitization to panallergens might be problematic as it bears the risk of developing multiple sensitizations [Hauser et al., 2010]. Identifying whether the sensitization is genuine in nature (primary, species-specific) or if it is due to cross-reactivity to proteins with similar protein structures may help to evaluate the risk of reaction on exposure to different allergen sources. For example, profilins are proteins with a highly conserved structure and highly similar in their primary sequence not only present in virtually all pollens and plants contributing to cross-reactions between botanically unrelated species but also present in all eukaryotic cells [Hed, 2009].

Protein stability in the gastrointestinal tract is another important aspect for the clinical expression in food allergy. Consequently, it is important to know the protein structure and to which protein family the allergen belongs, because these features may affect the tolerance of different foods and the degree of severity of clinical reactions. There is a great variability between allergen components with respect to stability to heat and protease digestion mimicking the gastric environment. Stable allergens such as lipid transfer proteins and storage proteins are still functional in the gastrointestinal tract and may result in systemic reactions (urticaria, dyspnea and anaphylaxis) in contrast to pathogenesis-related proteins and profilins, which are easily destroyed and mainly give rise to the oral allergy syndrome [Salcedo, 2004, Mills, 2004].

Post-translational modifications such as glycosylation, proteolysis and phosphorylation of allergens and their impact on the allergenicity should not be underestimated. In general, sIgE binding to carbohydrate moieties is associated with low clinical significance for clinical allergy as shown for grass pollen allergens. However, in some cases carbohydrates appear to contribute to the stability of the protein, as observed

for Api g 5, thus enabling IgE binding to epitopes [Bublin et al., 2003]. In the case of recombinant allergen production, a suitable heterologous expression system has to be selected to obtain a pattern equivalent to that of the natural protein.

### *1.5. Classification of wheat flour proteins*

The wheat grain comprises three major components: starch, protein and fibre with proteins accounting for about 10–15% of the dry weight. The analysis of the proteins of wheat and other cereals has a long and distinguished history, dating back over 250 years. This early work established a classification based on extraction in a series of solvents (formalized by the American protein chemist T. B. Osborne). He defined four wheat protein fractions (often called 'Osborne fractions'): water-soluble albumins (about 15% of the total proteins), salt, *e.g.* 0.5 M NaCl-soluble globulins (5% of the total proteins), water/salt insoluble monomeric gliadins (about 40 % of the total proteins) and the polymeric glutenins. The terms 'albumin' and 'globulin' are also more widely used for proteins with similar solubility properties from other organisms while the prolamins (characterised by a high glutamine and proline content) are given specific names in different cereals: gliadins in wheat, hordeins in barley, secalins in rye and zeins in maize. According to amino acid sequence and electrophoretic mobility gliadins are classified into α-, β-, γ-, and ω-gliadins, all rich in non-polar amino acids and glutamine. The ω-gliadins, consisting to about 80 % of the amino acids: glutamine, proline and phenylalanine, are almost completely repetitive in sequence. The ω-gliadins are further classified as slowly-migrating ω-1 and ω-2, ω-3 and fast-migrating ω-4 and ω-5. The ω-5 gliadin, a component of the fast ω–gliadin fraction, is a major allergen among water/salt-insoluble proteins. The glutenins are polymers of individual proteins linked by interchain disulphide (S-S) bonds. On reduction, the component subunits are classified into high molecular weight (HMW) and low molecular weight (LMW) groups after separation by SDS-PAGE [Tatham et al., 2008].

## *1.6. Allergens in wheat*

Since cereals are essential in daily nutrition, not all of the wheat allergens have been identified. However, in recent years proteomic analysis of wheat flour proteins revealed a number of IgE reactive components in both water/salt-soluble and insoluble fractions. Proteins from wheat flour were extracted, separated by 1-DE and 2-DE, analysed by immunoblotting using patients IgE Abs, purified by HPLC and identified by mass spectrometric analysis (Fig. 4) [Görg et al., 2004; Akagawa et al., 2007]. Many of these proteins are presumed to be protective in function, contributing to a broad spectrum resistance to fungal pathogens and invertebrate pests.

Among the major allergens identified in the water/salt-soluble fraction of wheat flour and capable of sensitization after both ingestion and inhalation are the cereal α-amylase inhibitors and α-amylase/trypsin inhibitors. A variety of α-amylase inhibitors of endogenous and exogenous origins that inhibit α-amylases from plants, microorganisms, mammalian and insect have been found in wheat endosperm [Buonocore et al., 1977]. The physiological role of the α-amylase enzymes family is the hydrolysis of α-D-(1,4)-glucan linkages in amylose, amylopectin, glycogen, and phytoglycogen, important in the carbohydrate metabolism of many autotrophic and heterotrophic organisms. Heterotrophic organisms use α-amylases mainly to digest starch in their food sources. Interestingly, by inhibiting activities of digestive hydrolases, α-amylases, or proteinases, the α-amylase inhibitors may be involved in plant defense against insect pests and pathogens [Hejgaard, 2005; Wang et al., 2006]. The α-amylase/trypsin inhibitor family could be divided into groups based on sequence homology obtained from the NCBI database (compared by Clustal W program) and displayed as Phylogram (Fig. 5). These inhibitors consists of tetrameric proteins often called chloroform methanol proteins based on their selective extraction in chloroform/methanol mixtures (CM1, CM2, CM3, CM16 and CM17), whereas the three α-amylase inhibitors are dimeric (0.19 and 0.53) or monomeric (0.28). Endogenous and CMX1/CMX3 α-amylase inhibitors differ from the previous groups. The α-amylase/trypsin inhibitors (approximately 12-19 kDa in size) are implicated as a major allergen associated with baker's asthma [Armentia A, 1993] and also with food allergy [James JM, 1997]. In particular, the subunits of the tetrameric α-amylase inhibitor, CM2, CM3 and CM16, are known to be major allergens for baker's asthma, but also play an

important role in food allergy. Wheat amylase/subtilisin inhibitor is a bifunctional protein capable of simultaneously inhibiting endogenous wheat α-amylase and the proteinase subtilisin [Kusaba-Nakayama et al., 2000 and 2001]. Using IgE from allergic individuals to probe a cDNA expression library identified the α-amylase inhibitors 0.19 and CM7, as well as new molecules with high cross-reactive potential, including thioredoxins and beta expansins [Weichel et al., 2006]. Protein inhibitors of α-amylase such as CM proteins have received considerable attention concerning their possible role in plant protection, human nutrition and quality improvement of cooked wheat products.

The another most important plant panallergens are non-specific lipid transfer proteins, which also act as food allergens, sensitizing individuals by the oral route or inhalation [Lauer et al., 2007; Palacin et al., 2007; Tordesillas et al., 2009]. Plant lipid transfer proteins are ubiquitous lipid binding proteins of the plant kingdom. They are of great technological importance because they are involved in beer foam formation [Jones et al., 1997; Jégou et al., 2000]. They can exhibit posttranslational modification such as phosphorylation and that different isoforms coexist in a given plant [Neumann et al., 1997].

Peroxidase is a 36 kDa seed-specific protein found specifically in T. monococcum, but also present in flour from diploid, tetraploid (pasta) and hexaploid (bread) wheats. Sensitization occurs via inhalation. Sera from 6 out of 10 patients hypersensitive to wheat flour were shown to have sIgE directed to this allergen [Sanchez-Monge R, 1997]. This allergen is one of the most reactive with majority patients' sera [Varjonen E,, 1994].

Although proteomic analysis has identified other water/salt soluble wheat proteins binding to IgE, including β-amylase, thioredoxin and serpins, a proper panel of all purified wheat allergens has not yet been developed and the clinical relevance of these proteins remains to be determined [Pastorello et al., 2007; Tatham et al., 2008].

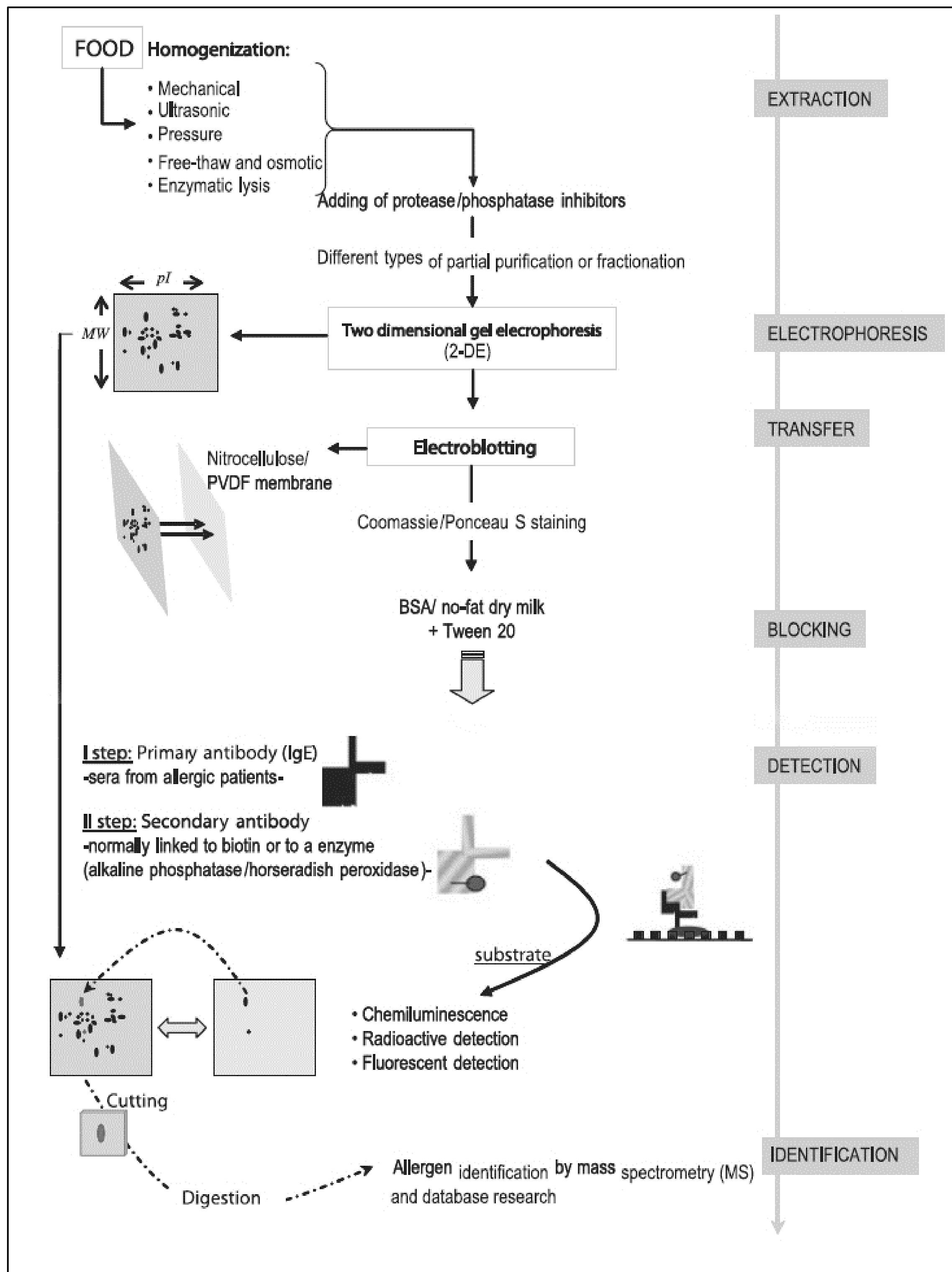



**Figure 4.** Schematic representation of the strategies for allergenomic analysis of wheat proteins. Adapted from Angelis, 2010.

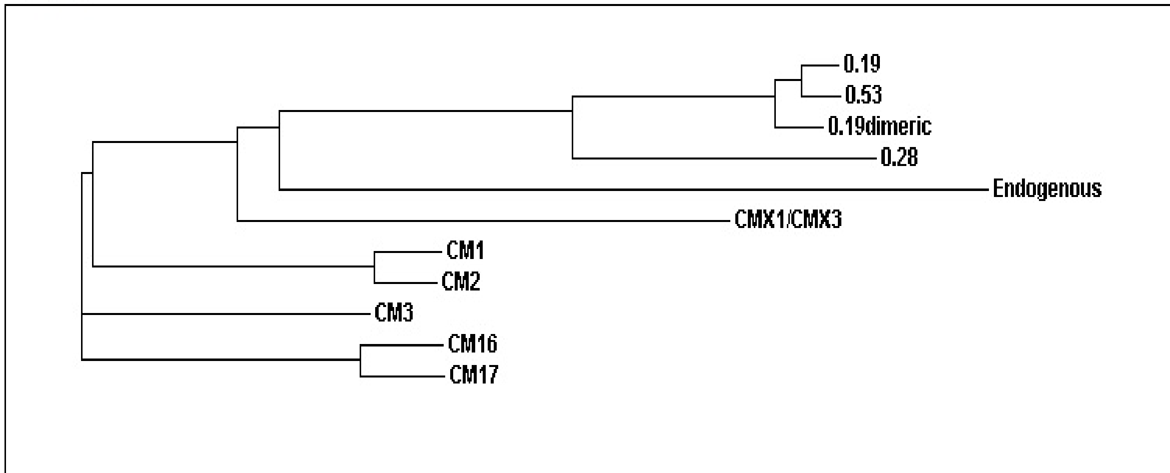



**Figure 5.** Phylogenetic tree showing the relationships of the eleven α-amylase inhibitors compared according to their sequences.

In water/salt-insoluble gluten, gliadins are involved in WDEIA (ϖ-5 gliadin was identified as a major allergen), in some cases of atopic dermatitis and also in anaphylaxis in adults and children [Palosuo et al., 1999; Morita et al., 2003]. Four of seven B-cell epitopes of ϖ-5 gliadin identified in WDEIA were found to be dominant [Matsuo et al., 2004]. Some authors reported that IgE Abs of patients with WDEIA and classic wheat allergy recognized sequential epitopes of repetitive domains of ϖ-5 gliadin [Battais et al., 2005]. Furthermore, a study reports that ω-5 gliadin-derived peptides are cross-linked by tTG, which causes a marked increase in IgE antibody binding both *in vitro* and *in vivo*. Activation of tTG in the intestinal mucosa during exercise in patients with WDEIA may lead to the formation of large allergen complexes capable of eliciting anaphylactic reactions [Palosuo K et al., 2003]. The B-cell epitopes of gliadin were found to be different from those involved in not only celiac disease but also contact urticaria. These results indicated that the protein state and the route of exposure to very similar gluten structures probably orient the pattern of epitope reactivity and wheat food allergy manifestations [Matsuo et al., 2005].

### *1.7. Allergen databases*

It is important to know the allergen names including their scientific acronym (*e.g.* Tri a 1 means allergen 1 from *Triticum aestivum* or expansin). There are several allergen databases that contain exhaustive information on allergens and protein families, such as the official International Union of Immunological Societies allergen nomenclature database – http://www.allergen.org, or the allergen literature database allergome – http://www.allergome.org or the allergen database grouping the allergens into protein families, allfam – http://www.meduniwien.ac.at/allergens/allfam/. Identified and described wheat allergens at allergome database are listed at Table 1.

**Table 1.** Identified wheat allergens (source: www.allergome.org).

| **Name** | **Common Names** | **Allergome Code** |
|---|---|---|
| Tri a 1 | expansin | 769 |
| Tri a 10kD | 5a2 protein | 5862 |
| Tri a 12 | profilin | 767 |
| Tri a 13 | polygalacturonase | 2529 |
| Tri a 14 | lipid-transfer protein | 1059 |
| Tri a 15 | α-amylase inhibitor 0.28 | 8725 |
| Tri a 18 | aglutinin | 650 |
| Tri a 19 | ω-5 gliadin/gluten | 3677 |
| Tri a 21 | gliadin | 3994 |
| Tri a 25 | thioredoxin | 2683 |
| Tri a 26 | glutenin subunit | 2898 |
| Tri a 28 | α-amylase inhibitor 0.53 | 8186 |
| Tri a 28.0101 | α-amylase inhibitor 0.19 | 8187 |
| Tri a 29 | α-amylase inhibitor CM2 | 8188 |
| Tri a 30 | α-amylase inhibitor CM3 | 1051 |
| Tri a 32 | peroxiredoxine | 6327 |
| Tri a 33 | serpin | 5724 |
| Tri a 34 | glyceraldehyde-3-phosphate dehydrogenases | 9501 |
| Tri a 35 | dehydrin | 9503 |
| Tri a 36 | LMW glutenin | 9534 |
| Tri a 37 | α-purothionin | 9834 |
| Tri a 7 | polcalcin | 1396 |
| Tri a aA_SI | α-amylase/subtilisin inhibitor | 9593 |
| Tri a alpha_Gliadin | α-gliadin | 3682 |

**Table 1. con´t**

| Name | Common Names | Allergome Code |
|---|---|---|
| Tri a bA | β-amylase | 9594 |
| Tri a Bd36K | peroxidases | 985 |
| Tri a beta_Gliadin | β-gliadin | 3683 |
| Tri a Chitinase | class 1 chitinase | 1274 |
| Tri a CM16 | α-amylase/trypsin inhibitors CM16 | 8777 |
| Tri a DH | dehydrin | 6328 |
| Tri a Endochitinase | endochitinase | 9595 |
| Tri a gamma_Gliadin | γ-gliadin | 3678 |
| Tri a Germin | germin | 778 |
| Tri a Gliadin | gliadin | 651 |
| Tri a GST | glutathione-S-transferases | 6326 |
| Tri a LMW Glu | glutenin subunit | 2674 |
| Tri a LTP2 | lipid transfer protein 2 | 8724 |
| Tri a omega2_Gliadin | ω2-gliadin | 3681 |
| Tri a Peroxidase 1 | peroxidases | 9597 |
| Tri a SPI | serine protease inhibitor | 4075 |
| Tri a TLP | thaumatin-like protein | 8726 |
| Tri a Tritin | tritin | 9598 |
| Tri a Trx | thioredoxin | 9967 |
| Tri a XI | xylanase inhibitor | 9596 |
| Tri td 14 | lipid transfer protein | 3675 |
| Tri td aA_TI | α-amylase/trypsin inhibitors | 3676 |

### *1.8. Effects of processing on allergenicity*

Wheat and related cereals are consumed by humans as a raw food (in bioproducts), but mainly after processing. Thermal processing may be accomplished by dry heat (e.g., oven roasting, baking, oil roasting, infrared heating, and ohmic heating) or may use wet heating conditions such as the ones encountered in cooking in aqueous media, microwave cooking, pressure cooking (autoclaving), extrusion, blanching, boiling, and steaming. Non-thermal processing methods include soaking, germination, milling, fermentation (beer, spirits), high-pressure processing, dehulling and dehusking, and grinding. These processes clearly have impacts on the food components, resulting in both chemical and physical modifications, which may affect the allergenicity of protein components. Processing can result in a reduction, no change or an increase in the number

of protein epitopes as mentioned earlier. Conformational epitopes are typically expected to be more susceptible to processing induced destruction than the linear epitopes on the same allergen. Linear epitopes are more likely to be altered if the linear epitopes are hydrolyzed. Alternatively, linear epitopes may be chemically modified during food processing or be intentionally changed by introducing mutations through genetic engineering. Thermal treatment of proteins in processed foods may result in a few of modifications including denaturation, hydrolysis of peptide bonds, aggregation by non-covalent and disulphide bonding, and reaction with other food molecules, such as sugars in Maillard reactions, lipids and carbohydrates. Biochemical processes can also occur including a range of enzyme-mediated reactions including proteolysis, oxidation or reactions with transglutaminases. The potential degree of complexity of the reactions in a food material is, therefore, enormous.

Additionally, gastrointestinal digestion could hydrolyze potential flour allergens. As described by Mittag et al., gastrointestinal digestion of wheat flour protein extracts shows that most of the salt-soluble and glutenin allergens were rapidly digested and not detected in immunodetection with a serum pool from wheat food allergic patients [Mittag et al., 2004]. Gliadins were stable to gastric enzymes but digested by simulated duodenal fluid. However, heat treatment (for instance during baking) can modify the digestibility of wheat proteins due to aggregation or Maillard reactions with sugars [Gerrad, 2002]. In this context, a simulated gastrointestinal digestion assay has shown a persistent IgE-binding capacity of bread crumb and crust proteins, compared with those of bread dough, which were readily digested [Simonato et al., 2001]. In similar study using boiled pasta, most of the wheat proteins were hydrolyzed, but the resulting peptides still bound IgE from wheat food allergic patients [De Zorzi et al., 2007].

However, because of its complex nature the impact of food processing on allergenicity of proteins has only recently become a subject of research. Such investigations are difficult, not least the fact that food processing often renders food proteins insoluble in the simple salt solutions frequently employed in serological or clinical studies. As a consequence, our understanding of the impact of food processing on allergenicity is limited to the more soluble and extractable residues in foods and the allergenic potential of insoluble protein complexes are virtually not studied. They could represent the vast bulk of food proteins consumed [Pasini et al., 2001; Simonato et al.,

2001; Tatham et al., 2008; Mills et al., 2009]. Future research should aim at giving a better description of how food allergens are digested, absorbed and further processed by the immune system, and in particular how this is influenced by factors such as age, health, eating habits, digestive system, nonallergenic components of the food, medication, and disease itself, *i.e.* whether s (subclinical) allergic reaction will change the absorption of undigested proteins.

### *1.9. Models of allergic diseases*

The prevalence of food allergy has increased significantly in recent decades, including reports of life-threatening anaphylactic reactions. As studies in allergic individuals are necessarily limited, animal models of food allergy have helped understand the underlying mechanisms of the disease, the potential for new therapeutic targets. In the light of these considerations there has been a growing interest in the design and development of appropriate animal models and their potential integration into safety assessment paradigms [Kimber I, 2003]. Investigators have explored the use of various species for the assessment of allergenic potential, including rats, dogs and swine [Knippels et al., 2003; Helm RM et al., 2002]. An alternative approach is the development of mouse models of sensitization to food proteins [Dearman RJ et al., 2003; Li XM et al., 2000]. Mouse models have been developed for almost all of the allergic diseases. In addition to the many important advances gained from investigation of these models, there are several limitations that preclude simple translation of the findings in mice to human disease. There are many important differences between humans and mice in terms of the anatomical, physiological, and immunological contributors [Finkelman FD, 2008, Wenzel S, 2006]. Caution in interpretation of the results of the studies in mice is necessary and knowledge and limitations of the various approaches used is essential [Takeda K, 2009]. The mouse, especially the BALB/c strain, has a number of advantages compared with other animal models, particularly with respect to the availability of inbred high IgE responder strains and of various immunological and molecular reagents. Experience to date suggests that the measurement of antibody (IgE) responses in BALB/c mice serves to identify food allergens accurately, and to distinguish them from those materials that apparently lack significant allergenic potential.

Depending on the route of exposure, dose of allergen and the presence of suitable adjuvant, the immune response can result in either sensitization or oral (mucosal) tolerance induction [Repa A et al., 2008; Perrier C, Thierry AC et al., 2010]. In mouse models of food allergy, oral administration of allergen usually results in oral tolerance induction, but its co-administration with strong mucosal adjuvant such as cholera toxin or with anti-acid drugs (increasing gastric pH) could be used for allergic sensitization [Brunner et al., 2009; Pali-Scholl I et al., 2010]. Another reliable and effective approach to overcome the oral tolerance induction is pretreatment of mice by systemic intraperitoneal (i.p.) administration of allergen with aluminium hydroxide (alum) as adjuvant followed by repeated intra-gastric treatments. This experimental model mimics a mild form of human allergy with IgE–mediated mast cell degranulation causing increased small intestine permeability [Dearman et al., 2007; Brandt EB et al., 2009] with diarrhea as one of the symptoms of anaphylaxis. Histological examination of small intestine revealed changes of epithelium, e.g. alteration in number of goblet cells and mucin production and the damage of tips of villi, as well as changes of lamina propria, e.g. increased cell infiltration and/or activation [van der Ventel ML et al., 2011].

The mucosa of small intestine is an actively metabolizing, rapidly proliferating, absorptive epithelium with nutritional and homeostatic functions. The activity of brush border enzymes is sensitive marker of intestinal cell differentiation and postnatal development, reflecting both dietary changes and microbial colonization [Hudcovic T et al., 2009]. Partial and subtotal atrophy of the villous apparatus was shown to correlate with the activity and expression of alkaline phosphatase. Moreover, this enzyme may be also involved in host’s defence against pathological stress-induced damage, such as during inflammation and infection [Lalle`s JP, 2010].

# 2. RESULTS

## *2.1. Identification of wheat flour proteins recognized by IgE Abs of allergic patients*

In routine settings, natural allergen extracts are used for *in vivo* (skin-prick) and *in vitro* (sIgE Abs) testing. However, most patients do not raise IgE Abs in response to all extracted wheat components, but only to some. Our study has contributed to the identification of frequently recognized wheat allergens and to the analysis of factors affecting immunogenicity of these molecules. This is a starting point in the development of diagnostic tests with higher sensitivity and specificity.

Publication:

**Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients**

Petr Šotkovský, Martin Hubálek, Lenka Hernychová, Petr Novák, Marie Havranová, Ivana Šetinová, Andrea Kitanovičová, Martin Fuchs, Jiří Stulík and Ludmila Tučková

Proteomics 2008; 8: 1677-91

The salt-soluble proteins from the flour of six frequently used wheat cultivars (Sulamit, Nela, Alana, Ebi, Banquet and Mladka) and one ancient cultivar (Spalda) were extracted, separated by 1-DE and detected by IgE immunoblotting using sera of patients with allergy to ingested wheat. Using this approach we have demonstrated a large spectrum of wheat IgE-binding proteins of molecular mass ranging from 10 to 100 kDa. Initially, we documented the impact of various wheat cultivars, extraction procedure, and of the technique of separation on the detectable number of IgE-binding wheat components. The cultivar Sulamit was selected for further experiments because of its most frequent use in food industry (due to the high quality for bakery) and its large spectrum of IgE-binding

proteins. Separation by 1-DE and immunoblot technique indicated the existence of three groups of reactive sera from allergic patients. IgE Abs of the first group recognized predominantly proteins of molecular mass above 30 kDa, those of the second group low-molecular mass components below 30 kDa, and IgE of the third group recognized the whole spectrum of wheat molecules. When the wheat extract was separated under reducing conditions, IgE-binding proteins ranging from 10 to 70 kDa were detected in the sera of all patients tested.

The complexity of the proteins extracted from wheat was documented after resolving the samples by 2-DE. IgE binding molecules were detected after a transfer of separated proteins onto nitrocellulose membrane and subsequent incubation with the patient's sera. Most frequently recognized protein spots of Sulamit extract were identified by MALDI-TOF and QTOF analysis. The technique combining 1-DE separation, immunoblotting, and ion-trap mass spectrometer was employed to complete the spectrum of the wheat components recognized by IgE Abs. These methodological approaches revealed 19 IgE binding proteins including previously reported α-amylase inhibitors (CM16, CM17 and 0.19 dimeric), profilin, and Tri a Bd 27K protein [Kitta et al., 2006; Weichel et al., 2006]. Moreover, we succeeded in identifying new IgE-binding wheat proteins such as β-amylase, β-D-glucan exohydrolase, serpin, α-amylase/trypsin inhibitor CM 3, α-amylase inhibitor CIII and three protein species of 27K protein.

To mimic the digestion of wheat containing food, we have tested the effect of pepsin on the imunogenicity of the extracted wheat proteins. After this treatment the number of IgE binding wheat components was significantly reduced and four proteins were identified as pepsin resistant: 0.19 dimeric α-amylase inhibitor, peroxidase 1, cytosolic cyclophilin and glyceraldehyde-3-phosphate dehydrogenase.

All techniques used in our study detected members of α-amylase inhibitor family and confirmed this enzyme as one of the major wheat allergens. Therefore, we developed an ELISA assay for the detection and quantification of sIgE Abs using our wheat extract and/or commercially available α-amylase inhibitors type 1 and 3 as the coupling antigens. The difference between sIgE mean values in healthy donors and allergic patients was statistically significant for all wheat allergens tested. Moreover, in pilot experiments blood cells, obtained from six patients with clinical symptoms of wheat allergy and positive basophil activation test to a commercial wheat extract, were exposed to the salt extract

from wheat cultivar Sulamit and to α-amylase inhibitors type 1 and 3, for comparison. Basophil activation induced by the Sulamit extract was similar to that induced by the commercial wheat extract, ranging from 31 to 97% of activated cells. Cell activation in response to α-amylase inhibitor type 1 was detected in three out of six patients tested (leading to 23, 30, and 97% of activated cells). The activation by α-amylase inhibitor type 3 was not detected using any of the patient's blood cells. These results suggest that not only the α-amylase inhibitor type 1, but also other extracted wheat components are involved in basophil activation

In conclusion, our findings contribute to the identification of wheat allergens aimed to increase the specificity of serum IgE and cell activation diagnostic assays.

Research Article

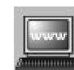
# Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients

*Petr Šotkovský*[1], *Martin Hubálek*[2], *Lenka Hernychová*[2], *Petr Novák*[3], *Marie Havranová*[4], *Iva Šetinová*[4], *Andrea Kitanovičová*[1], *Martin Fuchs*[5], *Jiří Stulík*[2] *and Ludmila Tučková*[1]

[1] Department of Immunology and Gnotobiology, Institute of Microbiology v.v.i, Czech Academy of Sciences, Prague, Czech Republic
[2] Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Hradec Kralové, Czech Republic
[3] Department of Biogenesis and Biotechnology of Natural Compounds, Institute of Microbiology v.v.i, Czech Academy of Sciences, Prague, Czech Republic
[4] Imumed, Prague, Czech Republic
[5] Department of Pediatrics, Hospital Bulovka, Prague, Czech Republic

Wheat belongs to six major food allergens inducing IgE-mediated hypersensitivity reaction manifesting as cutaneous, gastrointestinal, and respiratory symptoms. Although cereals are a staple food item in most diets, only a few wheat proteins causing hypersensitivity have been identified. To characterize wheat allergens, salt-soluble wheat extracts were separated by 1-DE and 2-DE and IgE-binding proteins were detected by immunoblotting using sera of patients with allergy to ingested wheat. Proteins, frequently recognized by IgE on 2-DE were analyzed by MALDI-TOF and QTOF and their spectrum was completed by 1-DE and LCQ$^{DECA}$ nLC-MS/MS IT technique. Using all three techniques we identified 19 potential wheat allergens such as α-amylase inhibitors, β-amylase, profilin, serpin, β-D-glucan exohydrolase, and 27K protein. Employing newly developed ELISA, levels of IgE Abs against Sulamit wheat extract and α-amylase inhibitors type 1 and 3 were quantified and shown to be significantly elevated in sera of allergic patients compared to those of healthy controls. The level of IgE Abs against α-amylase inhibitor type 3 was lower, slightly above the cut-off value in the majority of patients' sera. Our findings contribute to the identification of wheat allergens aimed to increase the specificity of serum IgE and cell activation diagnostic assays.

Received: April 10, 2007
Revised: November 28, 2007
Accepted: December 11, 2007




**Keywords:**
Basophil activation / IgE antibodies / Wheat allergens

## 1 Introduction

Food allergy, one of the frequent disorders, is characterized by an adverse reaction to food components or additives, with an underlying immunological mechanism. It appears mainly in early childhood and affects 6–8% of the pediatric population and 1–3% of adult population. The incidence appears to be continuously rising and has doubled in the last two decades. Six major foods, milk, egg, wheat, peanut, soya, and fish account for approximately 90% of food hypersensitivity. Although cereals are essential in daily nutrition for the majority of people, only little information is available on wheat allergens causing hypersensitivity reactions.

Hypersensitivity reactions to wheat proteins are clinically distinct, depending on the route of allergen exposure, target organs, and immunological mechanisms involved. They may

**Correspondence:** Dr Petr Šotkovský, Department of Immunology and Gnotobiology, Institute of Microbiology v.v.i, Vídeňská 1083, 142 20 Prague 4, Czech Republic
**E-mail:** psotkovsky@gmail.com
**Fax:** +420-241-721-143

**Abbreviation: Ab**, antibody

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

manifest as a classical food allergy to ingested wheat proteins including cutaneous, gastrointestinal, and respiratory symptoms or as a rare form of allergy wheat-dependent exercise-induced anaphylaxis. Occupational (baker's) asthma and rhinitis are reactions to inhaled flour. The most recognized food hypersensitivity reactions are IgE antibody mediated [1–7]. In general, the diagnosis of food allergy is difficult. Detection of food-specific IgE antibodies (Abs), history compatible with IgE-mediated symptoms as well as skin tests have a high sensitivity, but low specificity. In RIAs or enzymatic immunoassays, a certain extent of cross-reactivity cannot be excluded (depending on the purity of the allergens and/or their structural homologies). The diagnosis is more reliable when confirmed by double-blind, placebo controlled challenge. However, oral food challenges are time consuming and can induce severe clinical symptoms. A useful *in vitro* technique to confirm the diagnosis seems to be blood basophil activation test [8, 9]. Since the specificity of all diagnostic approaches depends on the purity of the allergens applied, the attention of investigators has focused on the identification and employment of defined allergens in diagnosis [10].

Based on differential solubility, wheat proteins can be classified into water/salt-soluble albumins and globulins and water/salt-insoluble gliadins and glutenins. IgE Abs specific to wheat albumins and globulins were found in the sera of patients with wheat allergy and with baker's asthma. Several groups of investigators reported some members of wheat α-amylase inhibitor family (Tri a Bd 17K, CM16) as sensitizing allergens after both ingestion and inhalation [4, 11–16]. Nonspecific lipid-transfer proteins (nsLTPS) have also been recognized as allergens in several plant species among which maize, barley, and fruits (peach and olives) are important in human nutrition [17, 18]. Various modifications of 1-DE and 2-DE were used for analysis; among them acetic acid/urea-PAGE (AU-PAGE) revealed low-molecular weight allergens that bind to IgE Abs of patients with food allergy [19–21].

In recent studies, the repertoire of IgE-binding components of wheat and maize was investigated employing cDNA libraries for allergens displayed on phage surfaces. Wheat components detected by this approach included newly identified molecules with high cross-reactive potential, including thioredoxins and beta expansins and proteins with sequences as yet not related to cereal allergy, such as 14 kDa high mobility protein, serine carboxypeptidase II, and 47 kDa leucine-rich repeat protein [22].

As recently demonstrated, low pH and treatment with enzymes such as pepsin may have a significant impact on solubility, imunogenicity, IgE binding, and *in vivo* activities of various ingested allergens. Allergens resistant to these enzymes may be responsible for clinical symptoms [23–27]. A comparative study evaluating the use of defined allergens in serological and biological assays and their standardization is therefore required.

Our study was aimed at characterizing salt-soluble wheat allergens. Extracted wheat proteins were separated using 1-DE and high efficacy 2-DE gel resolution techniques and IgE Abs-binding components were detected by immunoblotting. The effect of the digestive enzyme pepsin on the immunogenicity of wheat allergens was also analyzed. Proteins, most frequently recognized by IgE Abs of allergic patients, were identified using MS (MALDI-TOF, QTOF, and LCQ$^{DECA}$ nLC-MS/MS IT, ESI). To evaluate the diagnostic value of specific IgEs new ELISA using wheat extract and selected allergens was developed and the results of the two methods were compared. The serological data were complemented by results of an *in vitro* blood basophil activation assay using the same wheat allergens.

## 2 Materials and methods

### 2.1 Wheat sample preparation

Wheat flour from six most frequently used cultivars (*Triticum aestivum*): Sulamit, Nela, Alana, Ebi, Banquet, Mladka, and cultivar Spalda (*Triticum spelta*) were kindly provided by Professor J. Petr from the Czech University of Agriculture, Prague. Proteins were extracted with PBS overnight at 4°C, ultrasonicated (U50, Kika Laborator Technik, Germany) 15 min on ice and centrifuged at 20 000 × *g* for 15 min at 4°C. Extracted proteins from cultivar Sulamit were also treated with pepsin bound to agarose gel (ICN, Biomedicals, OH). One mililiter of protein extract (3 mg/mL) was incubated with 1 mL pepsin–agarose in 0.1 M HCl, pH 1.8 (30 min, 37°C). Removing the gel by centrifugation (1500 × *g*, 10 min) stopped enzymatic cleavage.

Protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (BCA) (Pierce, IL, USA). Samples were divided into aliquots and stored at −20°C.

### 2.2 Patients

Sera were obtained from 50 patients (19 children and 31 adults) with allergy to wheat, suffering from various symptoms (atopic dermatitis, asthma, urticaria eczema, and/or digestive symptoms). Food allergy was established by means of a positive skin prick test and specific IgE and/or positive basophil activation test. Sera from 42 healthy blood donors were used as controls. Skin prick tests were performed with commercial allergen extracts (Alyostal, Stallergenes, France). A positive response was defined as a wheal measuring 3 mm and more in diameter compared with a negative control. Sera from 18 patients allergic to pollen allergens were used as "disease controls."

### 2.3 Measurement of total and specific serum IgE

The concentrations of total and wheat-specific IgE were determined by fluorescence enzyme immunoassay (UniCAP, Pharmacia, Sweden or Immulite 2000, DPC, USA) according to manufacturer's instructions. Values of allergen-specific IgE above 0.35 kU/L, were considered as positive.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

### 2.4 SDS-PAGE and immunoblotting

Isolated wheat proteins (in concentration 3 mg/mL) were separated by SDS-PAGE as described by Laemmli [28] using a 5–20% gradient gel under nonreducing or reducing conditions (with 5% mercaptoethanol). Separated proteins were stained with Coomassie® Brilliant Blue R-250 or electrotransferred on NC membranes (NC2, SERVA) for 1 h at room temperature. The membranes were blocked with PBS containing 0.5% Tween 20 and NC strips were incubated with sera from all patients with wheat allergy, healthy and disease controls diluted 1:10 or 1:50 in blocking buffer overnight at 4°C. After washing with PBS-0.1% Tween 20 the strips were incubated with peroxidase-labeled sheep anti-human IgE antibodies (1:1000; The Binding Site, UK) for 1 h at room temperature. Development was carried out using SuperSignal West Pico Trial kit (Pierce).

### 2.5 2-DE

Wheat proteins were precipitated overnight in 20% TCA in acetone (−18°C) containing 0.2% DTT and then solubilized in IEF buffer containing 9 M urea, 4% w/v CHAPS, 70 mM DTT, 40 mM Tris-base, and 5% v/v carrier ampholytes pH 9–11 (Sigma, St. Louis, MO, USA). Protein concentration in IEF buffer was determined by modified BCA assay. Immobiline DryStrips, 18 cm, with nonlinear pH 3–10 gradient (Amersham Biosciences, Uppsala, Sweden) were used. Protein samples (100 μg) were applied to analytical gel, or 300 μg protein samples were loaded on IPG strip for micropreparative gel. The strips were swollen in samples diluted to total volume 350 μL with rehydration buffer containing 2 M thiourea, 6 M urea, 4% w/v CHAPS, 40 mM Tris-base, 12 μL/mL DeStreak (Amersham Biosciences), 0.003% w/v bromophenol blue, 1% v/v pharmalyte pH 3–10 (Amersham Pharmacia Biotech), and 0.5% v/v pharmalytes pH 8–10.5 (Sigma) overnight.

Separation was performed with Multiphor II (Amersham Biosciences). The following running conditions were used: 300 V for 30 min, 600 V for 30 min, 1000 V for 30 min, 2000 V for 30 min, 2500 V for 30 min, 3000 V for 30 min, 3500 V for 3 h, and 5000 V for 12 h. In the second dimension, 9–16% SDS-PAGE gradient was used [29, 30]. Electrophoresis was done on Protean II xi Multi Cell (BioRad, Hercules, CA, USA), at a constant current of 5 mA/gel for 1.5 h, and then at 40 mA/gel for 5 h. Proteins on analytical gels were visualized by silver staining [31]. The micropreparative gels were stained either with Colloidal Blue stain kit (Invitrogen, San Diego, CA, USA) or silver according to Schevchenko *et al.* [32] (compatible with MS analysis). The analytical and micropreparative gels were digitalized by laser densitometer Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Image analysis was carried out using PDQuest (BioRad). Separated proteins were also electrotransferred onto NC membranes and their reactivity with two samples (mixture of five patients' sera), five individual patients' sera, and sera of two healthy controls was tested as described above (Section 2.4).

### 2.6 In-gel digestion

For the micropreparative 2-DE gels, selected spots stained with Colloidal Blue Staining Kit were excised and covered with 500 μL of 100 mM Tris-HCl, pH 8.5 in 50% ACN for 20 min at 30°C. Then, 500 μL of equilibration buffer (50 mM ammonium bicarbonate, pH 7.8 in 5% ACN) was added to the gel pieces. After that, the gel pieces were vacuum dried, covered with 0.1 μg of sequencing grade trypsin (Promega, Madison, WI, USA) in 30 μL of 50 mM ammonium bicarbonate, pH 7.8 and 5% ACN, and mildly shaken overnight at 37°C [33]. Protein digests were removed from gel pieces by sequential extraction with 2% TFA and 60% ACN mixed 3:2, 2:3 to give 50 μL, and finally with 60% ACN alone. The volume of the extract was reduced to approximately 10 μL and frozen at −20°C.

The more sensitive silver staining was applied for colloidal blue staining undetected spots. The micropreparative gels were stained according to Schevchenko *et al.* [32] except the concentration of formaldehyde in the developer which was reduced to 0.02%. Formaldehyde was found to be the major compound in the silver staining protocol that negatively affects MALDI-TOF analysis [34]. Excised protein spots were destained with a mixture of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate for 30 min at 20°C. The gel pieces were then washed four times in 50% methanol/40% water/10% acetic acid for 10 min and the protocol for CBB stained gels or immunoblotting, as described above followed.

### 2.7 MALDI-TOF analysis and database searching

The samples were mixed in an Eppendorf tube with the same volume of the matrix solution – 2,5-dihydroxybenzoic acid (10 mg/mL in 20% ACN in water with 0.5% TFA) or CHCA (10 mg/mL in 50% ACN in water with 0.5% TFA). One microliter of each mixture was applied to the MALDI sample plate, allowed to air-dry and introduced into the mass spectrometer. PMF spectra were recorded on MALDI-TOF mass spectrometer Voyager DE STR (Perseptive Biosystems, Farmingham, MA, USA) equipped with delayed extraction. The spectra were obtained in reflectron mode with 20 kV acceleration voltage, 75% grid voltage, 0.02 wire voltage, 100 ns delay time, and low mass gate at 500 $m/z$. An external calibration with five individual peptides (angiotensin II, angiotensin I, neurotensin, ACTH clip (1–17), and ACTH clip (18–39)) covering the 1000–2500 Da mass range was used during measurement. Each mass spectrum was obtained by averaging 100 laser shots. Proteins were identified by PMF using ProteinProspector and MASCOT programs.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

### 2.8 Hybrid quadrupole (Q)/TOF analysis

Proteins not identified by MALDI-TOF MS were analyzed by nLC ESI-MS/MS. The peptide extracts were concentrated in a vacuum centrifuge and diluted in buffer containing 2% ACN and 0.1% v/v formic acid in water. The samples were separated on Atlantis™ C18 column, 75 μm × 150 mm (Waters), mounted on the CapLC system (Waters, UK) interfaced through nanospray with QTOF Ultima™ API (Waters). Data were subtracted, smoothed, and centroided using the ProteinLynx script in MassLynx software (Waters), and saved as a.pkl file. Searching was performed in Phenyx 2.1 (GeneBio, Switzerland) against all green plant proteins in NCBInr. A minimum peptide *z*-score 6 and maximum peptide *p*-value 1E −7 was required for correct sequence assignment. A minimum of two peptides *per* protein were required for the successful identification.

### 2.9 Proteolytic digestion and sample preparation for LCQ^DECA nLC-MS/MS on IT mass spectrometer

CBB-R 250 stained protein bands were cut from the gel and decolorized in sonic bath at 60°C several times with 0.05 M 4-ethylmorpholine acetate (pH 8.1) in 50% ACN. After complete destaining, the gel was shrunk by dehydration with ACN and reswollen in 0.05 mM 4-ethylmorpholine acetate (pH 8.1) and 40 mM TCEP (Tris(2-carboxyethyl)phosphine), and reduced at 60°C for 10 min. After the reduction step, the gel was shrunk by dehydration with ACN and reswollen in 0.05 mM 4-ethylmorpholine acetate (pH 8.1) and 100 mM iodoacedamide, and alkylated at 60°C for 30 min. When the alkylation was completed, the gel was washed with water, shrunk by dehydration with ACN and reswollen in water. The rehydration and dehydration of the gel was repeated one more time. Next, the gel was reswollen in 0.05 M 4-ethylmorpholine acetate (pH 8.1) in 50% ACN and then it was partly dried using a SpeedVac concentrator (Savant, Holbrook, NY, USA). Finally, the gel was reconstituted with cleavage buffer containing 0.01% 2-mercaptoethanol, 0.05 M 4-ethylmorpholine acetate, 10% ACN, and sequencing grade trypsin (Promega, 10 ng/μL). Digestion was carried out overnight at 37°C, the resulting peptides were extracted with 30% ACN/1% HAc and subjected to mass spectrometric analysis.

Tryptic peptide mixture was applied on the Magic-C18 column, 0.180 × 150 mm$^2$, 200 Å, 5 m (Michrom Bioresources, Auburn, CA) and separated using gradient elution. The column was connected to a LCQ$^{DECA}$ IT mass spectrometer (ThermoQuest, San Jose, CA) equipped with a nanoelectrospray ion source. Spray voltage was held at 1.8 kV, tube lens voltage was 10 V. The heated capillary was kept at 150°C with a voltage of 30 V. The LCQ$^{DECA}$ was set to acquire a full MS scan between 350 and 1600 *m/z* followed by full MS/MS scans of the top two ions from the preceding full MS scan. Activation time for CID was 30 ms and the relative collision energy was set to 42%. Dynamic exclusion was enabled with two repeat counts, repeat duration of 30 s and 3 min exclusion duration window. Spectra were searched with the SEQUEST™ software against the Swiss-Prot database. For spectra from a multiply charged peptide, an independent search was performed on both the 2+ and 3+ mass of the parent ion. The search parameters were as follows: trypsin specificity; mass errors 2 Da for precursor and 1.0 Da for fragment ions; possible modifications: +16 Da for Met and Trp and +57 Da for Cys. SEQUEST results were automatically processed with the DTA Select and Contrast software [35] using the following criteria: $X_{Corr}$ values were 1.8 for singly charged, 2.2 and 2.8 for doubly and triply charged peptides, respectively.

### 2.10 Basophil activation test

Flow cytometric basophil activation tests [8, 9, 36] were performed after stimulation of blood cells with wheat allergen for prick test (Stallergenes, Paris, France), with Sulamit wheat extract or with α-amylase inhibitor type 1 and type 3 (Sigma). In brief, 100 μL of whole blood and 10 μL of stimulation buffer containing IL-3 were incubated with 10 μL of wheat allergens and 100 μL PBS for 30 min at 37°C. The samples were then transferred on ice and stained with mAb anti-(cluster differentiation) CD203/PE (Beckman Coulter, Miami, USA) and anti-CD63/FITC (Exbio, Prague, Czech Republic) for 15 min. Erythrocytes were lysed using ammonium chloride. After cell washing the percentage of activated bashophils expressing CD63 and CD203c was measured by flow cytometer FC500 (Beckman Coulter). Samples containing>15% basophils expressing CD63 (CD203c+, CD63+) were considered as positive.

### 2.11 ELISA

For allergen-specific IgE assay Nunc-Immuno Plates (Maxisorp Surface; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μL of various antigens (20 μg/mL) in PBS and blocked with 1% BSA in PBS containing 0.1% Tween 20. After washing with PBS and PBS containing 0.05% Tween 20, patient's IgE was titrated in serum diluted serially two-fold throughout the plate (in blocking buffer), and incubated overnight at 4°C. The plates were washed as described above and IgE binding was detected after incubation with peroxidase-labeled sheep antihuman IgE Abs (1:500) for 1 h at room temperature. The reaction was visualized after washing by adding 50 μL of TMB (Sigma) with $H_2O_2$ substrate *per* well for 15 min. The reaction was stopped by 50 μL of 2 M sulfuric acid, the resulting yellow color was measured at 450 nm and the results were expressed as corresponding OD.

### 2.12 Statistical analysis of the data

The statistical significance was determined by the nonparametric Mann–Whitney *t*-test and correlation coefficient

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

($r^2$) was calculated using Prism™ program version 2.01. Program MedCalc (version 8.1.1.0) was used to perform ROC analysis.

## 3 Results

### 3.1 Salt-soluble allergens in various wheat cultivars

The salt-soluble proteins were extracted from the flour of six frequently used wheat cultivars (*T. aestivum*): Sulamit, Nela, Alana, Ebi, Banquet, Mladka, and cultivar Spalda (*T. spelta*). The extracted proteins were analyzed by 1-DE and their reactivity with IgE Abs was compared by immunoblotting using the same patient's sera (Fig. 1). The spectrum of IgE-binding wheat proteins was similar in all cultivars and the molecular mass of corresponding proteins ranged from 10 to 110 kDa. The strongest IgE binding was detected in the extract from the wheat cultivar Mladka and the lowest in that from the Ebi cultivar. However, the number of IgE recognized proteins and the intensity of binding differed to some extent for various patients' sera used. The cultivar Sulamit was selected for further experiments because of its most frequent use in food industry (due to the high quality for bakery) and its large spectrum of IgE-binding proteins.

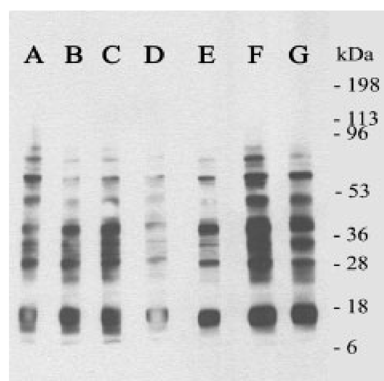



**Figure 1.** An example of IgE reactivity of one allergic patient's serum with proteins in salt extracts from wheat cultivars: A, Sulamit; B, Nela; C, Alana; D, Ebi; E, Banquet; F, Mladka; and G, Spalda. 1-DE separated wheat extracts were transferred onto NC membranes, incubated with the sera of patients with wheat allergy, followed by the secondary sheep antihuman IgE antibodies. The binding was visualized by ECL reagents and autoradiography.

### 3.2 Heterogeneity of wheat components recognized by patient's IgE

IgE-binding proteins in the Sulamit extract were analyzed in the sera of 50 patients with food allergy using 1-DE separation and immunoblot technique. This analysis indicated the existence of three groups of reactive sera from allergic patients, according to the spectrum of recognized wheat components separated under nonreducing conditions. IgE Abs of the first group (26% of sera) recognized predominantly proteins of molecular mass above 30 kDa, those of the second group (8%) low-molecular mass components below 30 kDa, and IgE of the third group (50%) recognized the whole spectrum of wheat molecules. The remaining 16% of sera have shown only a weak reactivity, however, out of these 16% of samples 10% exhibited clear IgE reactivity, when the film was exposed for longer time (Table S1 of Supporting Information). When the wheat extract was separated by SDS-PAGE under reducing conditions, IgE-binding proteins ranging from 10 to 70 kDa were detected in the sera of all (100%) patients tested (Fig. 2b). The IgE immunoblotting with the sera from healthy donors revealed only one nonspecific Ab binding to a 32 kDa wheat component (visible in all samples including secondary antihuman IgE Ab control – in Fig. 2). Similar reactivity of IgE Abs was seen in the majority of sera from patients with pollen allergy (78%). In the remaining sera, however, the IgE binding up to three wheat components was detected (Fig. 2b). Birch allergens, separated by 1-DE, were used in the immunoblot analysis for comparison. The reactivity to birch allergens of sera from 60% of patients with wheat allergy was not detectable, 35% recognized a 20 kDa protein and 5% an additional 40 kDa protein (data not shown).

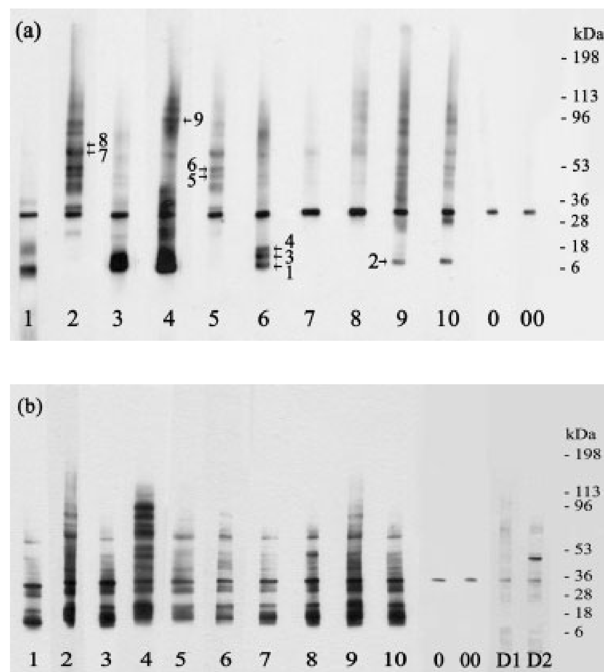
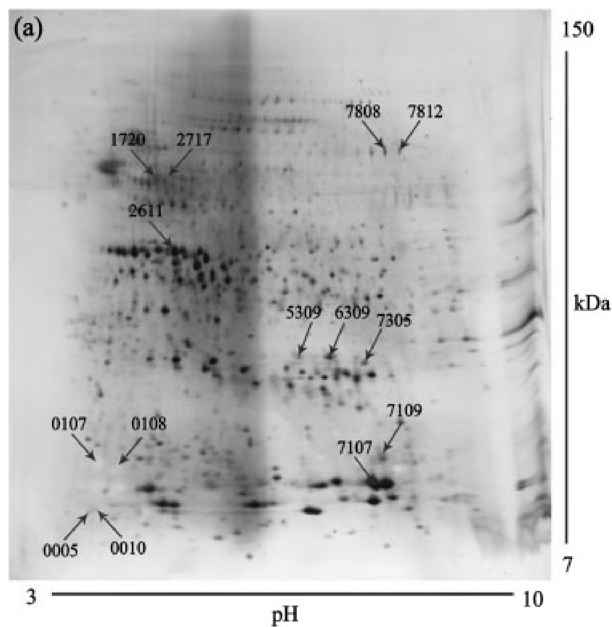
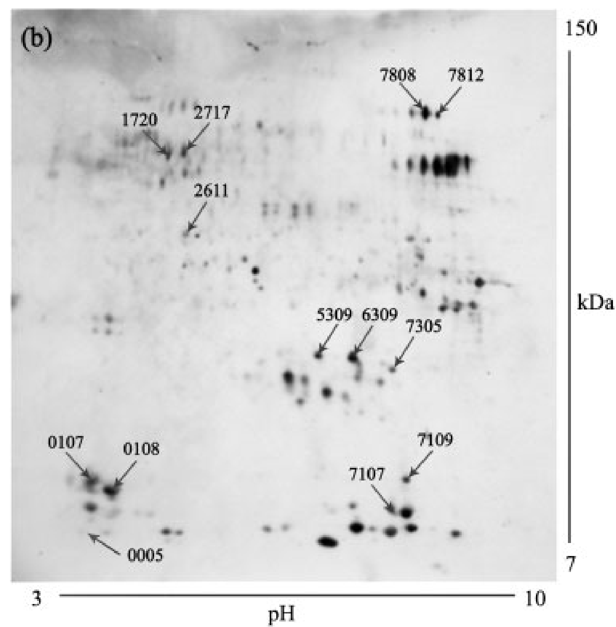
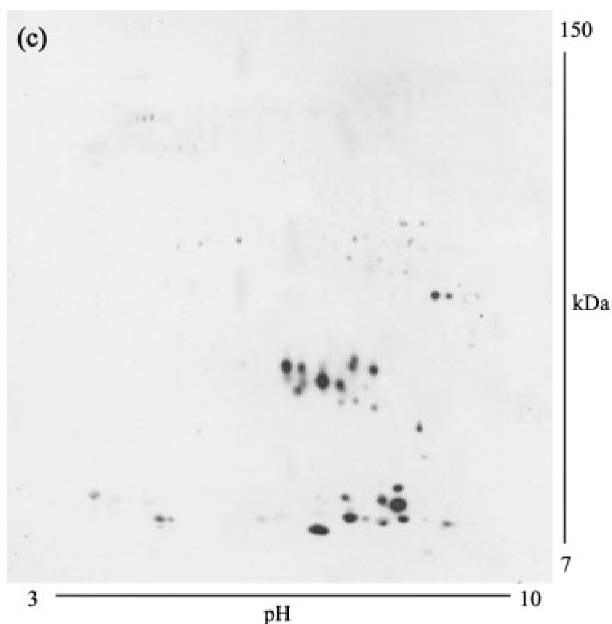


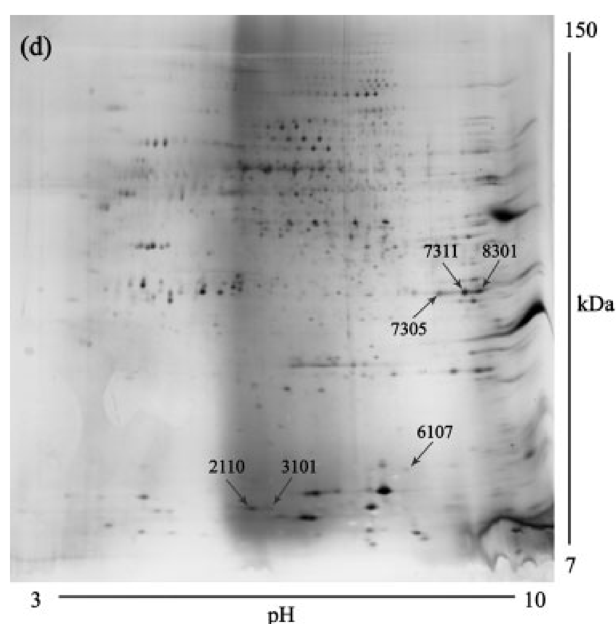

**Figure 2.** IgE binding to wheat components in the Sulamit extract separated by 1-DE under nonreducing (a) and reducing (b) conditions; an example of immunoblot using the same set of sera from patients with wheat allergy (numbered 1–10), healthy control (no. 0) and disease control (no. D1 and D2) and the secondary antihuman IgE antibody (no. 00). The bands selected for MS analysis are marked by arrows and numbered. The numbering refers to Fig. 4 and Table 2.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 




## 3.3 Identification of major allergens in wheat

The complexity of the proteins extracted from wheat was documented after resolving the samples by 2-DE and silver staining. About 800 spots with p*I* from 3 to 10 and molecular mass from 10 to 120 kDa were visualized (Fig. 3a). IgE-binding molecules were detected after a transfer of separated proteins onto NC membrane and subsequent treatment with the patient's sera. IgE Abs in serum samples reacted with sets of about 80 wheat components; 60 of them were recognized by 80–100% of patient's sera tested (example is shown in Fig. 3b: here 88 components were reactive). To mimic the digestion of wheat containing food, we have tested the effect of pepsin on the imunogenicity of the extracted wheat proteins. The spectrum of proteins in the pepsin digest is documented in Fig. 3d. After this treatment the number of IgE-binding wheat components was significantly reduced to approximately 20 spots visualized by staining (Fig. 3e).

Most frequently recognized protein spots, both before and after pepsin treatment of Sulamit extract, were analyzed by MS. The spots reacting with sera of healthy donors and secondary antihuman IgE Abs were not included in this analysis. MALDI-TOF analysis was used in an attempt to characterize 21 spots before and eight spots after pepsin

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

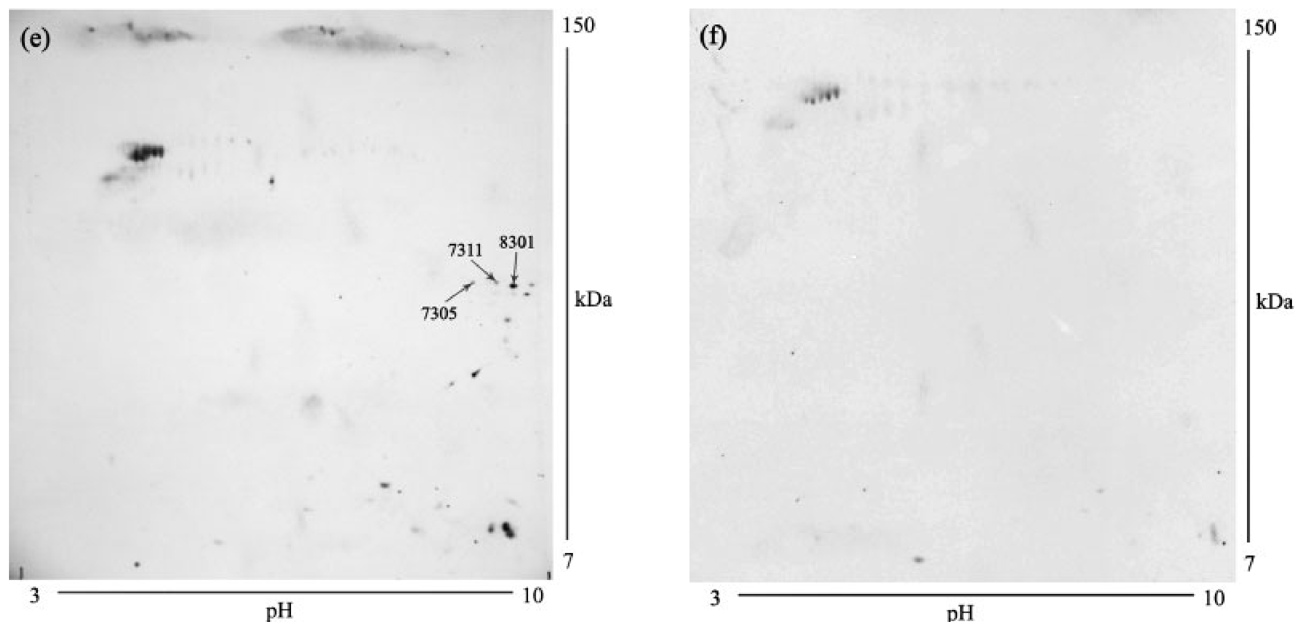



**Figure 3.** Spectrum of proteins in the wheat Sulamit extract separated by 2-DE: (a) visualized by silver staining, and (b) recognized by IgE antibodies of patients with wheat allergy, and (c) reactivity of secondary antihuman IgE antibody. Extracted proteins were digested by pepsin, separated by 2-DE and: (d) visualized by silver staining, (e) recognized by IgE antibodies of patients with wheat allergy, and (f) reactivity of secondary antibody. The spots selected for MS analysis are indicated by numbers. The numbering refers to Table 1.

digestion. Four nondigested and two spots after pepsin digestion were identified using this approach. The spots not identified by MALDI-TOF MS were further analyzed by QTOF. This technique enlarged the number of identified IgE-binding spots by further ten before and another four after pepsin treatment of the wheat extract. Nine spots binding IgE remained nonidentified. The list of all identified IgE-binding wheat components from 2-DE is shown in Table 1.

The technique combining 1-DE separation, immunoblotting, and LCQ$^{DECA}$ (IT, ESI) was employed to complete the spectrum of the wheat components recognized by IgE Abs. Ten IgE-binding protein bands were analyzed and eight proteins were identified. Three of them corresponded to the formerly identified spots from 2-DE and five were newly described (Table 2). Using these three methodological approaches and the data from various protein databases, we succeeded in identifying 19 potential wheat allergens including 15 of wheat, two of barley, one of maize, and one of *Arabidopsis thaliana* origin (Tables 1 and 2 and Fig. 4).

We confirmed the reactivity of the IgE Abs in the sera of allergic patients with the wheat protein α-amylase inhibitors by Western blot analysis. IgE Abs recognized molecules of about 10, 15, 35, and 55 kDa in the commercially available α-amylase inhibitor type 1 separated by 1-DE under nonreducing conditions. Reactivity with protein band corresponding to molecular mass of about 10 kDa was observed after separation under reducing conditions, in all patients' sera. In some of these sera, the IgE reactivity with higher molecular weight components was also documented (Figs. 5a and b).

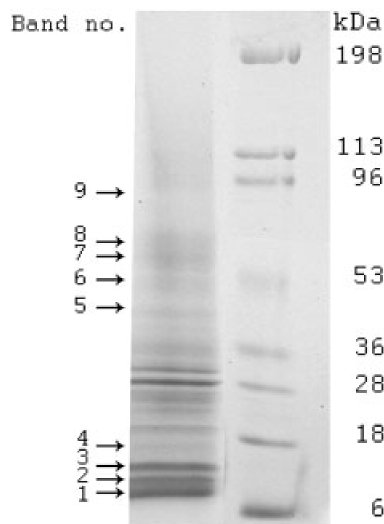



**Figure 4.** Proteins from the Sulamit extract separated by 1-DE under nonreducing conditions and stained by CBB. The bands selected for MS analysis are marked by arrows and numbered.

### 3.4 The level of IgE Abs against α-amylase inhibitors in sera of allergic patients

All techniques used in our study detected members of α-amylase inhibitor family and confirmed this enzyme as one of the major wheat allergens [4, 11–13].

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

**Table 1.** A list of wheat proteins identified by MS analysis (MALDI-TOF and QTOF) of spots from 2-DE, selected by IgE Abs in immunoblotting

| MS | Spot no. | Protein name, organism | Accession no. | $M_r$ (kDa)/p*I* | | Sequence coverage (%) | Peptides matched | Peptide sequence | Charge | Expectation | *p*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Theor | Measured | | | | | | |
| **(a) IgE recognized wheat proteins** | | | | | | | | | | | |
| Q | 7107 | 0.19 Dimeric α-amylase inhibitor, *T. aestivum* | AAV39515 | 13.3/7.5 | 17.1/6.8 | | 4 | SGPWMCYPGQAFQVPALPACR | 3 | | 1.24E − 24 |
| | | | | | | | | EHGAQEGQAGTGAFPR | 3 | | 3.83E − 18 |
| | | | | | | | | QEGQAGTGAFPR | 2 | | 3.59E − 15 |
| | | | | | | | | DCCQQLAHISEWCR | 3 | | 4.97E − 10 |
| Q | 7109 | | | | 20.8/6.9 | | 2 | LPIVVDASGDGAYVCK | 2 | | 2.13E − 18 |
| | | | | | | | | DCCQQLAHISEWCR | 3 | | 2E − 15 |
| Q | 7107 | α-Amylase/trypsin inhibitor CM3 precursor, *T. aestivum* | P17314 | 18.2/8.0 | 17.1/6.8 | | 6 | PGQCNLATIHNVR | 2 | | 5.42E − 21 |
| | | | | | | | | LPEWMTSASIYSPGKPYLAK | 3 | | 4.98E − 20 |
| | | | | | | | | LATIHNVR | 2 | | 2.71E − 18 |
| | | | | | | | | LLVAPGQCNLATIHNVR | 3 | | 9.42E − 08 |
| | | | | | | | | YFIALPVPSQPVD | 2 | | 5.49E − 08 |
| | | | | | | | | YFIALPVPSQPVDPR | 2 | | 4.05E − 07 |
| Q | 7109 | | | | 20.8/6.9 | | 3 | SGNVGESGLIDLPGCPR | 2 | | 1.18E − 21 |
| | | | | | | | | SGSCVPGVAFR | 2 | | 1.21E − 10 |
| | | | | | | | | YFIALPVPSQPVDPR | 2 | | 6.31E − 10 |
| M | 0107 | α-Amylase/trypsin inhibitor CM16 precursor, wheat | P16159 | 15.8/5.3 | 19.2/4.6 | 45 | 9 | | | $2.8 \times 10^{-5}$ | |
| Q | 0108 | | | | 18.7/4.7 | | 6 | SRPDQSGLMELPGCPR | 2 | | 6.74E − 24 |
| | | | | | | | | MSTLITPLPSCR | 2 | | 1.66E − 19 |
| | | | | | | | | SRPDQSGLM*ELPGCPR | 2, 3 | | 7.23E − 18 |
| | | | | | | | | KQQCCGELANIPQQCR | 3 | | 7.83E − 14 |
| | | | | | | | | QQCCGELANIPQQCR | 2, 3 | | 2.29E − 09 |
| | | | | | | | | EDCTPWMSTLITPLPSCR | 2 | | 1.18E − 07 |
| M | 0107 | CM 17 protein precursor, *T. aestivum* | CAA42453 | 15.9/5.1 | 19.2/4.6 | 40 | 7 | | | $5.3 \times 10^{-4}$ | |
| Q | 0005 | α-Amylase inhibitor CIII, *T. aestivum* | 0810252A | 13.3/7.4 | 15.4/4.5 | | 2 | LQCVGSQVPEAVLR | 2 | | 3.08E − 17 |
| | | | | | | | | LTAASVPEVCK | 2 | | 2.83E − 10 |
| Q | 0005 | Profilin, *T. aestivum* | CAA61945 | 15.2/5.9 | 15.4/4.5 | | 3 | YMVIQGEPGVVIR | 2 | | 4.1E − 16 |
| | | | | | | | | YM*VIQGEPGVVIR | 2 | | 7.65E − 15 |
| | | | | | | | | DFEEPGHLAPTGLFLGGTK | 3 | | 4.05E − 11 |
| Q | 0010 | | | | 15.4/4.6 | | 3 | YMVIQGEPGVVIR | 2 | | 3.09E − 14 |
| | | | | | | | | YM*VIQGEPGVVIR | 2 | | 1.07E − 12 |
| | | | | | | | | DFEEPGHLAPTGLFLGGTK | 3 | | 3.11E − 10 |
| M | 6309 | 27K protein, *T. aestivum* | BAC76688 | 23.3/6.1 | 28.8/6.2 | 48 | 8 | | | $4.1 \times 10^{-3}$ | |
| M | 7305 | | | | 27.6/6.6 | 33 | 7 | | | $5.9 \times 10^{-3}$ | |
| Q | 5309 | | | | 28.6/5.8 | | 8 | VHVAIYYESLCPYSVR | 3 | | 6.96E − 19 |
| | | | | | | | | GHNLSLEYGR | 2 | | 4.41E − 17 |
| | | | | | | | | PLYNDYGNFK | 2 | | 4.89E − 16 |
| | | | | | | | | HREWESCFQK | 3 | | 4.32E − 13 |
| | | | | | | | | PVTECYKGER | 3 | | 2.33E − 12 |
| | | | | | | | | QGLDPKPVTECYK | 2 | | 3.82E − 11 |
| | | | | | | | | EWESCFQK | 2 | | 8.46E − 10 |
| | | | | | | | | IYCVSDLVLK | 2 | | 3.21E − 08 |
| Q | 1720 | β-Amylase, *T. aestivum* | AAP80614 | 30.9/8.6 | 54.3/5.1 | | 11 | FFVDNGTYLTEQGR | 2 | | 2.18E − 18 |
| | | | | | | | | ASLNFTCAEMR | 2 | | 6.92E − 18 |
| | | | | | | | | YDPTAYNTILR | 2 | | 1.76E − 13 |
| | | | | | | | | EGLNM*ACENALPR | 2 | | 2.07E − 12 |
| | | | | | | | | SAPEELVQQVLSAGWR | 3 | | 9.26E − 12 |
| | | | | | | | | ASLNFTCAEM*R | 2 | | 7.95E − 12 |

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

**Table 1.** Continued

| MS | Spot no. | Protein name, organism | Accession no. | $M_r$ (kDa)/p*I* | | Sequence coverage (%) | Peptides matched | Peptide sequence | Charge | Expectation | *p*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Theor | Measured | | | | | | |
| | | | | | | | | FFLAWYSNNLIK | 2 | | 3.86E − 11 |
| | | | | | | | | DAGQYNDAPQR | 2 | | 1.89E − 11 |
| | | | | | | | | SFPGIGEFICYDK | 2 | | 3.88E − 10 |
| | | | | | | | | AAAAMVGHPEWEFPR | 3 | | 3.65E − 10 |
| | | | | | | | | AAAAM*VGHPEWEFPR | 3 | | 1.67E − 08 |
| Q | 2717 | | | | 54.0/5.1 | | 7 | ASLNFTCAEMR | 2 | | 1.76E − 15 |
| | | | | | | | | YDPTAYNTILR | 2 | | 1.25E − 13 |
| | | | | | | | | EGLNMACENALPR | 2 | | 9.26E − 14 |
| | | | | | | | | ISGIHWWYK | 2 | | 1.58E − 11 |
| | | | | | | | | AAAAMVGHPEWEFPR | 3 | | 6.37E − 09 |
| | | | | | | | | ASLNFTCAEMR | 2 | | 1.94E − 07 |
| | | | | | | | | AAAAM*VGHPEWEFPR | 3 | | 1.7E − 07 |
| M | 2611 | Serpin, *T. aestivum* | CAA72273 | 43.1/5.4 | 40.5/5.2 | 44 | 12 | | | $2.4 \times 10^{-8}$ | |
| Q | 7808 | β-D-Glucan exohydrolase, *T. aestivum* | AAM13694 | 67.3/7.2 | 61.0/6.9 | | 4 | TAGTTILSAIK | 2 | | 7.86E − 18 |
| | | | | | | | | SVDQLPM*NVGDK | 2 | | 2.02E − 13 |
| | | | | | | | | GFVISDWQGIDR | 2 | | 2.68E − 13 |
| | | | | | | | | SASAPLLPLPK | 2 | | 2.03E − 09 |
| Q | 7812 | | | | 61.0/7.2 | | 4 | TAGTTILSAIK | 2 | | 8.37E − 13 |
| | | | | | | | | GFVISDWQGIDR | 2 | | 9.39E − 13 |
| | | | | | | | | SVDQLPM*NVGDK | 2 | | 1.47E − 10 |
| | | | | | | | | SASAPLLPLPK | 2 | | 2.99E − 08 |
| **(b) Wheat proteins identified after pepsin digestion** | | | | | | | | | | | |
| M | 2110 | 0.19 Dimeric α-amylase inhibitor, *T. aestivum* | AAV39519 | 13.2/6.5 | 16.1/5.4 | 70 | 7 | | | $3.4 \times 10^{-4}$ | |
| M | 3101 | | | | 16.1/5.5 | 79 | 8 | | | $8.6 \times 10^{-6}$ | |
| Q | 6107 | Cytosolic cyclophilin, *A. thaliana* | AAB96833 | 18.9/7.7 | 19.1/7.6 | | 2 | IIPGFMCQGGDFTR | 2 | | 3.98E − 15 |
| | | | | | | | | IIPGFM*CQGGDFTR | 2 | | 9.87E − 14 |
| Q | 7305 | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1, *Zea mays* | CAA33620 | 36.5/6.7 | 38.0/7.0 | | 2 | VIHDNFGIVEGLM*TTVH | 3 | | 3.56E − 19 |
| | | | | | | | | VIHDNFGIVEGLM* | 2 | | 1.16E − 15 |
| Q | 7311 | | | | 38.0/7.2 | | 4 | VIHDNFGIVEGLMTTVHAITATQK | 3 | | 1.05E − 23 |
| | | | | | | | | VIHDNFGIVEGLM*TTVH | 3 | | 8.44E − 19 |
| | | | | | | | | VIHDNFGIVEGLMTTVH | 3 | | 2.55E − 15 |
| | | | | | | | | VIHDNFGIVEGLM*TTVHAITATQK | 4 | | 6.93E − 10 |
| Q | 8301 | Peroxidase 1, *T. aestivum* | AAM88383 | 38.8/8.5 | 38.2/8.2 | | 2 | GAVVSCADILALAAR | 2 | | 2.37E − 16 |
| | | | | | | | | DSVVVSGGPDYR | 2 | | 3.56E − 12 |

*, Oxidized methionine; M, proteins analyzed by MALDI-TOF; Q, proteins analyzed by QTOF.

Despite the fact that this allergen was already known, a clinical study of quantitative estimation of IgE antibodies against α-amylase inhibitors in the sera of allergic patients is missing. We have now developed an ELISA assay for the detection of specific IgE Abs using Sulamit wheat extract and/or α-amylase inhibitors type 1 and 3 as the coupling antigens. Individual levels of specific serum IgE Abs against wheat allergens in the sera of healthy donors (42) and patients with wheat allergy (50) or pollen allergy (18) are shown in Fig. 6. Using either Sulamit extract or α-amylase inhibitors type 1 and 3, ELISA has shown that the median of IgE Abs (0.266, 0.261, and 0.177, respectively) in the sera of allergic patients was significantly higher ($p<0.0001$) than that in the sera of healthy donors (0.068, 0.051, and 0.020, respectively). The highest level of IgE Abs was detected with the Sulamit extract, a slightly lower level with the α-amylase inhibitor type 1. When the α-amylase inhibitor type 3 was used in ELISA, the OD corresponding to the median value of

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

**Table 2.** A list of identified proteins from 1-DE, selected by immunoblotting and analyzed by LCQ$^{DECA}$ nLC-MS/MS (IT, ESI)

| Band no. | Protein name, organism | Accession no. | $M_r$(kDa)/p*I* Theor | Peptides matched | Peptide sequence | Charge | XC Score |
|---|---|---|---|---|---|---|---|
| 3 | α-Amylase inhibitor 0.53, *T. aestivum* | P01084 | 13.2/5.2 | 7 | DVAAYPDA | 1 | 1.80 |
| | | | | | LTAASITAVCR | 2 | 2.90 |
| | | | | | CGALYSMLDSMYK | 2 | 3.40 |
| | | | | | CGALYSM*LDSMYK | 2 | 2.39 |
| | | | | | LQCNGSQVPEAVLR | 2 | 2.96 |
| | | | | | CGALYSM*LDSM*YK | 2 | 3.20 |
| | | | | | LPIVVDASGDGAYVCK | 3 | 3.20 |
| 1 | | | | 3 | LQCNGSQVPEAVLR | 2 | 2.98 |
| | | | | | CGALYSM*LDSM*YK | 2 | 2.41 |
| | | | | | LPIVVDASGDGAYVCK | 2 | 3.64 |
| 4 | | | | 1 | LPIVVDASGDGAYVCK | 2 | 2.54 |
| 3 | α-Amylase inhibitor 0.19, *T. aestivum* | P01085 | 13.3/6.6 | 4 | EHGAQEGQAGTGAFPR | 2 | 3.33 |
| | | | | | DCCQQLAHISEWCR | 3 | 3.66 |
| | | | | | SGPWMCYPGQAFQVPALPACR | 2 | 2.78 |
| | | | | | SGPWM*CYPGQAFQVPALPACR | 2 | 3.53 |
| 3 | α-Amylase/trypsin inhibitor CM3 precursor, *T. aestivum* | P17314 | 18.2/7.4 | 2 | SGNVGESGLIDLPGCPR | 2 | 3.69 |
| | | | | | DYVLQQTCGTFTPGSK | 2 | 3.88 |
| 4 | | | | 3 | EMQWDFVR | 2 | 2.15 |
| | | | | | SGNVGESGLIDLPGCPR | 2 | 3.96 |
| | | | | | DYVLQQTCGTFTPGSK | 2 | 3.29 |
| 3 | Wheatwin-2 precursor (pathogenesis-related protein 4b), *T. aestivum* | O64393 | 15.9/8.2 | 1 | VTNPATGAQITAR | 2 | 2.48 |
| 2 | | | | 1 | VTNPATGAQITAR | 2 | 2.35 |
| 4 | α-Amylase/trypsin inhibitor CMd precursor, *Hordeum vulgare* | P11643 | 18.5/6.1 | 1 | LLVAPGQCNLATIHNVR | 2 | 2.67 |
| 7 | β-Amylase, *T. aestivum* | P93594 | 56.6/5.2 | 4 | ILDEANK | 1 | 1.75 |
| | | | | | SAPEELVQQVLSAGWR | 3 | 3.79 |
| | | | | | NIEYLTLGVDDQPLFHGR | 2 | 4.51 |
| | | | | | LTEAGVDGVMIDVWWGLVEGK | 2 | 2.69 |
| 6 | | | | 1 | NIEYLTLGVDDQPLFHGR | 3 | 3.33 |
| 8 | | | | 3 | SAPEELVQQVLSAGWR | 2, 3 | 3.35 |
| | | | | | NIEYLTLGVDDQPLFHGR | 2, 3 | 3.50 |
| | | | | | LTEAGVDGVMIDVWWGLVEGK | 2 | 3.28 |
| 7 | β-Amylase, *H. vulgare* | P16098 | 59.6/5.6 | 9 | ASINFTCAEMR | 2 | 2.64 |
| | | | | | ASINFTCAEM*R | 2 | 2.11 |
| | | | | | EGLNVACENALPR | 2 | 2.29 |
| | | | | | FFLAWYSNNLIK | 2 | 3.28 |
| | | | | | LSNQLVEGQNYVNFK | 2, 3 | 4.33 |
| | | | | | VPSHAAELTAGYYNLHDR | 3 | 4.45 |
| | | | | | NIEYLTLGVDNQPLFHGR | 2 | 5.06 |
| | | | | | LVEAGVDGVMVDVWWGLVEGK | 2, 3 | 4.46 |
| | | | | | LVEAGVDGVM*VDVWWGLVEGK | 2 | 2.72 |
| 9 | | | | 1 | LVEAGVDGVMVDVWWGLVEGK | 2 | 2.02 |
| 8 | | | | 7 | EGLNVACENALPR | 2 | 3.22 |
| | | | | | FFLAWYSNNLIK | 2 | 4.56 |
| | | | | | LSNQLVEGQNYVNFK | 2, 3 | 4.21 |
| | | | | | NIEYLTLGVDNQPLFHGR | 2, 3 | 4.16 |
| | | | | | LVEAGVDGVMVDVWWGLVEGK | 2, 3 | 5.01 |
| | | | | | LVEAGVDGVM*VDVWWGLVEGK | 2 | 3.05 |
| | | | | | YPSYPQSHGWSFPGIGEFICYDK | 3 | 4.41 |
| 5 | Phosphoglycerate kinase, cytosolic, *T. aestivum* | P12783 | 42.1/5.6 | 1 | LASVADLYVNDAFGTAHR | 3 | 3.68 |

*, Oxidized methionine.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

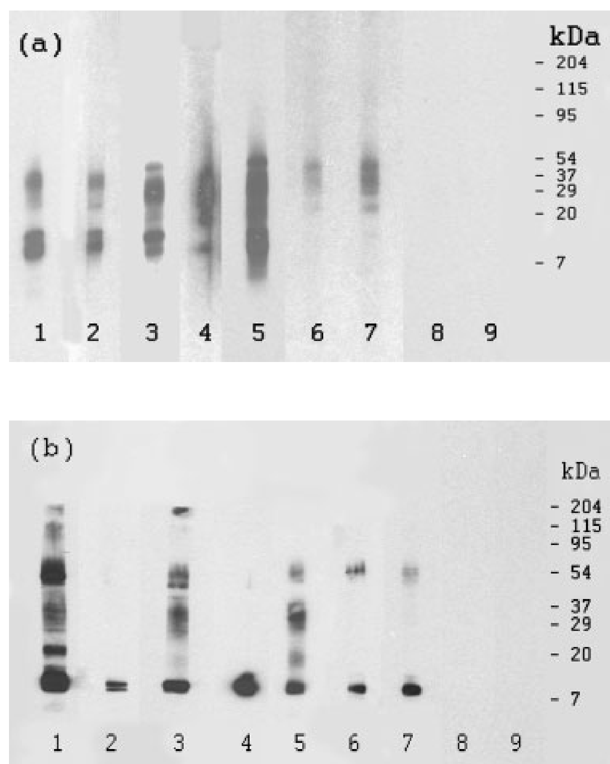



**Figure 5.** An example of immunoblot analysis confirming the binding of patients' IgE Abs (numbered 1–7), to the components in α-amylase inhibitor type I separated (a) under nonreducing and (b) reducing conditions; reactivity with healthy control (8) and secondary antihuman IgE antibody (9).

specific IgE reached only half of the value obtained with the former two allergens. The sensitivity, specificity, and ROC analysis of ELISA data were calculated for all three allergens and were found to be above 90%; the highest value was estimated for α-amylase inhibitor type 1 (Table 3). When sera of patients with pollen allergy were tested as a disease control, the median of OD reached 0.178 using Sulamit extract, 0.166 for α-amylase inhibitor type 1 and 0.113 for α-amylase inhibitor type 3. The differences in reactivity of serum IgE in patients with wheat or pollen allergy and healthy donors were statistically significant (Fig. 6).

**Table 3.** Statistical evaluation of IgE Abs against wheat allergens measured by ELISA

| Alergens | Sensitivity (%) | Specificity (%) | AuROC (%) | $r^{2a)}$ |
|---|---|---|---|---|
| Sulamit | 90 | 95 | 99 | 0.71 |
| α-Amylase inhibitor, type 1 | 96 | 95 | 99.6 | 0.75 |
| α-Amylase inhibitor, type 3 | 90 | 93 | 97.7 | 0.5 |

a) Correlation between specific IgE measured by CAP system and ELISA.

### 3.5 Activation of basophils from allergic patients with wheat allergens

In pilot experiments blood cells, obtained from six patients with clinical symptoms of wheat allergy and positive basophil activation test to a commercial wheat extract, were exposed to the salt extract from wheat cultivar Sulamit and to α-amylase inhibitors type 1 and 3, for comparison. An example of FACS analysis evaluating the activation marker (CD203c) expression on blood basophils (bearing CD63 marker) after *in vitro* exposure to wheat allergens is documented in Fig. 7. Basophil activation induced by the Sulamit extract was similar to that induced by the commercial wheat extract, ranging from 31 to 97% of activated cells. Cell activation in response to α-amylase inhibitor type 1 was detected in three out of six patients tested (leading to 23, 30, and 97% of activated cells). The activation by α-amylase inhibitor type 3 was not detected using any of the patient's blood cells. These results suggest that not only the α-amylase inhibitor type 1, but also other extracted wheat components are involved in basophil activation and further analysis of the stimulatory (biological) activity of wheat allergens is necessary.

## 4 Discussion

Proteomic analysis has proved to be a useful tool for the identification of the causative food allergens and for increasing the specificity of diagnostic tests. Using this approach we have demonstrated a large spectrum of wheat proteins recognized by IgE Abs in the sera of allergic patients. Initially, we documented the impact of various wheat cultivars, extraction procedure, and of the technique of separation on the detectable number of IgE-binding wheat components. In accordance with previously published data [5, 20], separation of salt-soluble wheat extracts by 1-DE under nonreducing conditions and immunoblotting revealed IgE-binding proteins of molecular mass ranging from 10 to 100 kDa. However, separation under reducing conditions revealed predominantly low-molecular mass components (ranging from 10 to 30 kDa) recognized by the majority of patient's sera, irrespective of how they reacted under the nonreducing conditions. These changes in the IgE-binding pattern could be a consequence of conformational changes, reduction of interchain disulfide bridges and uncovering or generation of new antigenic structures (epitopes).

To identify IgE reactive wheat components we employed MALDI-TOF and QTOF analysis. Using both methodological approaches we have identified 14 IgE-binding proteins (including those digested by pepsin). Discrepancy between the number of identified spots and the number of identified proteins can be caused by the fact that two proteins were found in some spots (for example, spot number 0005 contained both α-amylase inhibitor CIII and profilin). In some cases one gene product was matched up with three spots

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

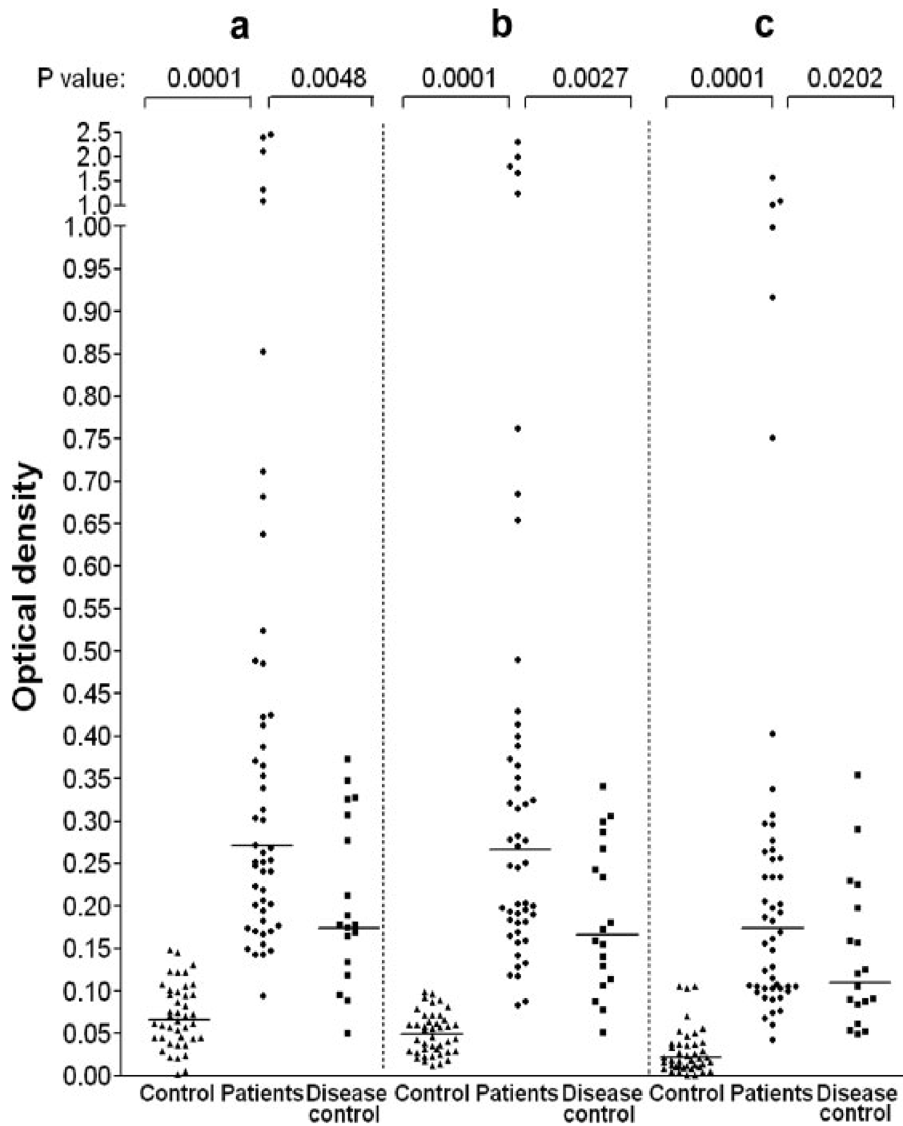



**Figure 6.** Scattergram of IgE Abs to the wheat Sulamit extract (a), the α-amylase inhibitor type 1 (b), and the α-amylase inhibitor type 3 (c) in the sera from 42 healthy controls, 50 patients with diagnosed allergy to wheat components, and from 18 patients allergic to pollen (disease control). The mean level was significantly increased ($p<0.001$) in the patient group for all three coupled antigens (a–c). The solid line represents the median level; values above cut-off (0.145 for a, 0.099 for b, and 0.082 for c are considered as positive.

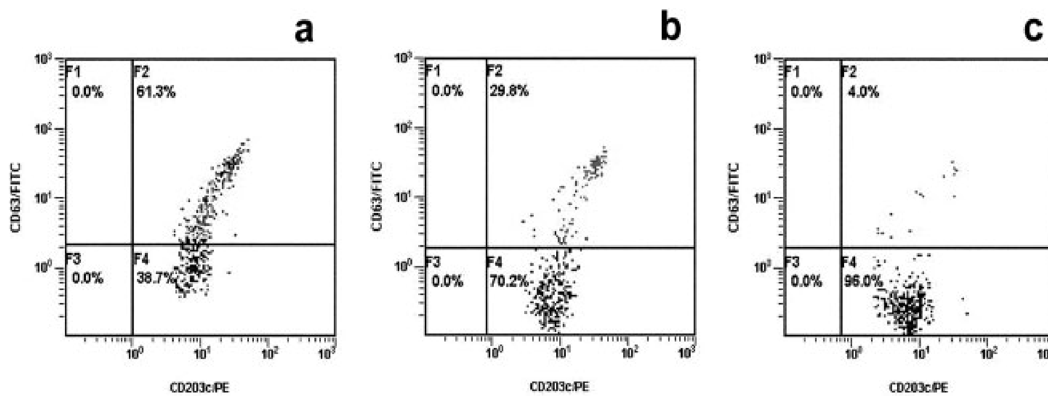



**Figure 7.** Basophil activation test of blood samples from allergic patients measured by flow cytometry as expression of CD63 and CD203c using (a) Sulamit extract, (b) α-amylase inhibitor type 1, and (c) α-amylase inhibitor type 3. Samples containing >15% basophils were considered as positive.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

with the same molecular mass and different p*I* (for example spots number 5309, 6309, and 7305) that refer to different protein species. The spectrum of identified proteins was supplemented with further five molecules using 1-DE separation and LCQ$^{DECA}$ (IT, ESI) detection technique.

These three methodological approaches revealed IgE-binding proteins including previously reported α-amylase inhibitors (CM16, CM17 precursors, and 0.19 dimeric form) [21, 22], profilin (of high sequence identity with birch pollen allergen) [37], and Tri a Bd 27K protein [16]. Moreover, we succeeded in identifying new IgE-binding wheat proteins such as β-amylase, β-D-glucan exohydrolase, three protein species of 27K protein, serpin, α-amylase/trypsin inhibitor CM 3 precursor, and α-amylase inhibitor CIII. The following IgE-binding proteins were identified after pepsin digestion: 0.19 dimeric α-amylase inhibitor, peroxidase 1, cytosolic cyclophilin (matching sequence from *A. thaliana*), and glyceraldehyde-3-phosphate dehydrogenase (matching corn sequence). The fact that these proteins do not match wheat components could be explained by the lack of knowledge of the complete genome and the corresponding protein/peptide sequences of wheat may be missing in the current databases. Furthermore, we are aware of the fact that two or more proteins identified in one band (1-DE separation, band number 3) or one spot (2-DE separation, spot number 7107) may include not only IgE Ab-binding proteins but also other molecules. Their reactivity with patients' IgE Abs is to be confirmed in further studies.

Using newly developed ELISA we have quantified IgE Abs against Sulamit extract and some frequently recognized allergens, *e.g.*, α-amylase inhibitors type 1 and 3. The difference between specific IgE mean values in healthy donors and allergic patients was statistically significant for all wheat allergens tested. The highest levels of IgE and the highest number of positive patient's sera were detected using the whole Sulamit extract. Using the α-amylase inhibitor type 1 as an antigen, the mean level of IgE Abs was similar to that obtained with the whole extract. The level of IgE Abs against the α-amylase inhibitor type 3 was low in the majority of patients' sera tested. However, the difference between the mean value of IgE Abs in healthy donors and patients with wheat allergy was statistically significant even for this allergen, probably due to the few highly positive sera in the patients' group. Data obtained by testing disease controls, *i.e.*, patients with pollen allergy using wheat extract, reflect the presence of the so-called "pan-allergens" such as nsLTPS or profilins recognized also by IgE of these patients. Interestingly, cross-reactive epitopes in pollen and food allergens have been recently demonstrated and similar epitopes may exist also in pollen and wheat allergens (including α-amylase inhibitors) [38–41].

The physiological role of the α-amylase enzymes family is the hydrolysis of α-D-(1,4)-glucan linkages in amylose, amylopectin, glycogen, and phytoglycogen, important in the carbohydrate metabolism of many autotrophic and heterotrophic organisms. Heterophilic organisms use α-amylases mainly to digest starch in their food sources. Interestingly, by inhibiting activities of digestive hydrolases, α-amylases, or proteinases, the α-amylase inhibitors may be involved in plant defense against insect pests and pathogens [42, 43].

In routine settings, natural allergen extracts are used for *in vivo* (skin-prick) and *in vitro* (specific IgE Abs) testing. As was demonstrated by us and other authors, most patients do not raise IgE Abs in response to all extracted wheat components, but only to some. Our study has contributed to the identification of frequently recognized wheat allergens and to the analysis of factors affecting immunogenicity of these molecules. This is a starting point and a necessary step in the development of diagnostic tests with higher sensitivity and specificity [44, 45]. The potential application of these defined wheat allergens will be their use in specific IgE Ab testing, cell activation assays as well as in complex allergen-microarrays (biochip technology).

*This work was supported by the Grant Agency of the Czech Republic (grants 310/07/0414, 310/05/2245 and 310/03/H147); Grant Agency of the Czech Academy of Science (grants A500200801 and KJB5020407); Institutional Research Concept AV0Z50200510; Grant Agency of Charles University (grant 162/2006/B/BIO); grant MOOFVZ0000501 from the Czech Ministry of Defence; Grant Agency of Ministry of Education (grants 2B06155 and LC7017); grant S500200572 of Academy of Sciences of the Czech Republic.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Sampson, H., Food allergy. *J. Allergy Clin. Immunol.* 2003, *111*, 540–547.

[2] Prioult, G., Nagler-Anderson, C., Mucosal immunity and allergic responses: Lack of regulation and/or lack of microbial stimulation. *Immunol. Rev.* 2005, *206*, 204–218.

[3] ESPGAN Working group for the Diagnostic Criteria for Food Allergy, *J. Pediatr. Gastroenterol. Nutr.* 1992, *14*, 108–112.

[4] James, J. M., Sixbey, J. P., Helm, R. M., Bannon, G. A. *et al.*, Wheat α-amylase inhibitor: A second route of allergic sensitization. *J. Allergy Clin. Immunol.* 1997, *99*, 239–244.

[5] Simonato, B., De Layyari, F., Pasini, G., Polato, F. *et al.*, IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin. Exp. Allergy* 2001, *31*, 1771–1778.

[6] Pourpak, Z., Mansouri, M., Mesdaghi, M., Kazemnejad, A. *et al.*, Wheat allergy: Clinical and laboratory findings. *Int. Arch. Immunol.* 2004, *13*, 168–173.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

[7] Pourpak, Z., Mesdaghi, M., Kazemnejad, A., Toosi, S. B. *et al.*, Which cereals is a suitable substitute for wheat in children with wheat allergy? *Pediatr. Allergy Immunol.* 2005, *16*, 262–266.

[8] Boumiza, R., Monneret, G., Forissier, M. F., Savoye, J. *et al.*, Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin. Exp. Allergy* 2003, *33*, 259–265.

[9] Eberlein-Koning, B., Varga, R., Nemlel, M., Darsow, U. *et al.*, Comparison of basophil activation using CD63 or CD203c expression in patients with insect venom allergy. *Allergy* 2006, *61*, 1084–1086.

[10] Valenta, R., Lindholm, J., Niedeberger, V., Hayek, B. *et al.*, The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin. Exp. Allergy* 1999, *29*, 896–904.

[11] Sánchez-Monge, R., Gomez, I., Barber, D., López, C. *et al.*, Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the alpha-amylase inhibitor family have enhanced IgE-binding capacity. *Biochem. J.* 1992, *281*, 401–405.

[12] Zapatero, M., Martínez, M. I., Alonso, E., Salcedo, G. *et al.*, Oral wheat flour anaphylaxis related to wheat $\alpha$-amylase inhibitor subunits CM3 and CM16. *Allergy* 2003, *58*, 956.

[13] Kusaba-Nakayama, M., Ki, M., Kawada, E., Sato, M. *et al.*, Intestinal absorbability of wheat allergens, subunits of wheat $\alpha$-amylase inhibitor, expressed by bacteria. *Biosci. Biotechnol. Biochem.* 2001, *65*, 2448–2455.

[14] Sander, I., Rauf-Heimsoth, M., Van Kampen, V., Baur, X., Is fungal $\alpha$-amylase in bread an allergen? *Clin. Exp. Allergy* 2000, *30*, 560–565.

[15] Sánchez-Monge, R., García-Casado, G., López-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.

[16] Yamashita, H., Nanba, Y., Onishi, M., Kimoto, M. *et al.*, Identification of a wheat allergen, Tri a BD 36K, as a peroxidase. *Biosci. Biotechnol. Biochem.* 2002, *66*, 2487–2490.

[17] Salcedo, G., Sanchez-Monge, R., Diaz-Perales, G., Garcia-Casado, G. *et al.*, Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin. Exp. Allergy* 2004, *34*, 1336–1341.

[18] Capocchi, A., Fontanini, D., Muccilli, V., Cunsolo, V. *et al.*, Tetraploid and hexaploid wheats express identical isoforms of nsLTP1. *J. Agric. Food Chem.* 2006, *54*, 2372–2377.

[19] Sander, I., Flagge, A., Merget, R., Halder, T. M. *et al.*, Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J. Allergy Clin. Immunol.* 2001, *107*, 907–913.

[20] Constantin, C., Huber, W. D., Granditsch, G., Weghofer, M. *et al.*, Different profiles of wheat antigens are recognized by patients suffering from coeliac disease and IgE-mediated food allergy. *Int. Arch. Allergy Immunol.* 2005, *138*, 257–266.

[21] Kitta, K., Ohmishi-Kameyama, M., Moriyama, T., Ogawa, T. *et al.*, Detection of low-molecular weight allergens resolved on two-dimensional electrophoresis with acid-urea polyacrylamide gel. *Anal. Biochem.* 2006, *351*, 290–297.

[22] Weichel, M., Vergoossen, N. J., Bonomi, S., Scibilia, J. *et al.*, Screening the allergenic repertoire of wheat and maize with sera from double-blind, placebo-controlled food challenge positive patients. *Allergy* 2006, *61*, 128–135.

[23] Mesa-del-Castillo, M., Martinez-Cocera, C., Caballero, M. L., Vazquez, L. *et al.*, IgE to pepsin-digeted water soluble and insoluble wheat proteins. *Allergy* 2004, *39*, 1229–1232.

[24] Yong, Y. H., Yamaguchi, S., Matsumura, Y., Effect of enzymatic deamidation by protein-glutaminase on structure and functional properties of wheat gluten. *J. Agric. Food Chem.* 2006, *54*, 6034–6040.

[25] Tesaki, S., Watanabe, J., Tanabe, S., Sonozama, K. *et al.*, An active compound against absorption in hypoallergenic wheat four produced by enzymatic modification. *Biosci. Biotechn. Biochem.* 2002, *66*, 1930–1935.

[26] Leduc, V., Morenet-Vautrin, D. A., Guerin, L., Morisset, M. *et al.*, Anaphylaxis to wheat isolates: Immunochemical study of a case proved by means of double-blind, placebo-controlled food challenge. *J. Allergy Clin. Immunol.* 2003, *111*, 897–899.

[27] Palosuo, K., Update on wheat hypersensitivity. *Curr. Opin. Allergy Clin. Immunol.* 2003, *3*, 205–209.

[28] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680–685.

[29] Görg, A., Obermaier, C., Boguth, G., Csordas, A. *et al.*, Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electrophoresis* 1997, *18*, 328–337.

[30] Brown, R. E., Jarvis, K. L., Hyland, K. J., Protein measurement using bicinchoninic acid: Elimination of interfering substances. *Anal. Biochem.* 1989, *180*, 136–139.

[31] Hochstrasser, D. F., Merril, C. R., 'Catalysts' for polyacrylamide gel polymerization and detection of proteins by silver staining. *Appl. Theor. Electrophor.* 1988, *1*, 35–40.

[32] Schevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 1996, *68*, 850–858.

[33] Lamer, S., Jungblut, P. R., Matrix-assisted laser desorption-ionization mass spectrometry peptide mass fingerprinting for proteome analysis: Identification efficiency after on-blot or in-gel digestion with and without desalting procedures. *J. Chromatogr. B Biomed. Sci. Appl.* 2001, *752*, 311–322.

[34] Richter, S., Luche, S., Chevallet, M., Van Dorsselaer, A. *et al.*, About the mechanism of interference of silver staining with peptide mass spectrometry. *Proteomics* 2004, *4*, 909–916.

[35] Tabb, D. L., McDonald, W. H., Yates, J. R., DTA select and contrast: Tools for assembling and comparing protein identifications from shotgun proteomics. *J. Proteome Res.* 2002, *1*, 21–26.

[36] Setinova, I., Havranova, M., Honzova, S., The basophil activation test with recombinant allergens in diagnosis of the birch pollen allergy. *Alergie (Prague)* 2005, *7*, 104–108.

[37] Rihs, H. P., Rozynek, P., May-Taube, K., Welticke, B. *et al.*, Polymerase chain reaction based cDNA cloning of wheat profilin: A potential plant allergen. *Int. Arch. Allergy Immunol.* 1994, *105*, 190–194.

[38] Weber, R. W., Cross-reactivity of pollen allergens: Impact on allergen immunotherapy. *Ann. Allergy Asthma Immunol.* 2007, *99*, 203–11.

[39] Soeria-Atmadja, D., Onell, A., Kober, A., Matsson, P. *et al.*, Multivariate statistical analysis of large-scale IgE antibody measurements reveals allergen extract relationships in sen-

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

sitized individuals. *J. Allergy Clin. Immunol.* 2007, *120*, 1433–1440.

[40] Asero, R., Mistrello, G., Roncarolo, D., Amato, S., Detection of some safe plant-derived foods for LTP-allergic patients. *Int. Arch. Allergy Immunol.* 2007, *144*, 57–63.

[41] Pastorello, E. A., Farioli, L., Conti, A., Pravettoni, V. *et al.*, Wheat IgE-mediated food allergy in European patients: Alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int. Arch. Allergy Immunol.* 2007, *144*, 10–22.

[42] Wang, J. R., Yan, Y. H., Wei, Y. M., Nevo, E. *et al.*, Molecular characterization of dimeric alpha-amylase genes in wheat and development of genome allele-specific primers for the genes located on chromosome 3BS and 3DS. *J. Cereal Sci.* 2006, *43*, 360–368.

[43] Hejgaard, J., Inhibitory plant serpins with a sequence of three glutamine residues in the reactive centre. *Biol. Chem.* 2005, *386*, 1319–1323.

[44] Wohrl, S., Vigl, K., Zehetmayer, S., Hiller, R. *et al.*, The performance of a component-based allergen-microarray in clinical practice. *Allergy 206*, *61*, 633–639.

[45] Jahn-Schmid, B., Harwanegg, C., Hiller, R., Bohle, B. *et al.*, Allergen microarray: Comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. *Clin. Exp. Allergy* 2003, *33*, 1443–1449.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

## *2.2. Isolation and purification of wheat flour allergens*

The aim of this study was to isolate and purify previously identified wheat allergens in their natural form as well as to find new potential allergens. The poor predictability and specificity of all currently known diagnostic approaches may be associated with the purity of the wheat extracts used in the specific IgE assays or to their lack of inclusion of all major allergens. Therefore, we developed a new procedure for isolating and purifying relevant IgE-binding proteins from a water/salt-soluble extract of wheat flour in amounts that allowed subsequent structural and functional analyses.

Publication:

**A new approach to the isolation and characterization of wheat flour allergens**

Šotkovský P, Sklenář J, Halada P, Cinová J, Šetinová I, Kainarová A, Goliáš J, Havlásková K, Honzová S and Tučková L.

Clin Exp Allergy 2011; 41(7): 1031-43

Isolation protocol consists of a three steps: ultrafiltration, isoelectric focusing (Rotofor), and liquid chromatography. Extracted salt soluble wheat proteins were sequentially separated on an Amicon ultrafiltration devices (providing gross separation according to molecular weight of proteins) and purified on the Rotofor cell (based on pI gradient in liquid phase) into 20 fractions. Sufficient quantities of proteins were obtained to enable their further fractionation by HPLC. Preparative reverse-phase HPLC was the main step in the purification of relatively large quantities of several water/salt soluble wheat proteins. Purified proteins were analyzed by immunoblotting using sera from 22 patients with atopic dermatitis and food allergy to wheat; and components binding to IgE were identified by MALDI-TOF mass spectrometry. This purification scheme yielded 27 IgE binding proteins, including several previously described α-amylase inhibitors, non-specific lipid-transfer proteins, peroxidase, serpins and β-amylase identified by two-dimensional electrophoresis [Pastorello et al., 2007; Sotkovsky et al., 2008], as well as several new

wheat proteins, such as endogenous α-amylase/subtilisin inhibitor, wheatwin, TLP, tritin, XIP, β-glucosidase, class II chitinase and 26 kDa endochitinase.

TLP and wheatwin are wheat flour allergens associated with food allergy. TLP is an important allergen in several fruits, such as apples, cherries, kiwis and grapes [Gavrovic-Jankulovic et al., 2002; Pastorello et al., 2003; Breiteneder et al., 2004]. Moreover, this protein isolated from wheat has been shown to be an allergen in patients with baker's respiratory allergy [Lehto et al., 2010]. We previously showed that wheatwin-2 is an IgE immunoreactive protein, and the results presented here showed that wheatwin-1 and wheatwin-2 have sequence homology of 97%. We were able to isolate them as a mixture of both isoforms with high purity. Furthermore, we have shown for the first time that both thaumatin-like protein and wheatwin bind to IgE from patient's sera and can activate patients' basophils to an extent comparable to that of the well characterized allergens, α-amylase inhibitor 0.19 and lipid-transfer protein. Moreover, our yield of purified natural α-amylase inhibitor 0.19 was higher than that of other purified proteins and we confirmed its reactivity with patients' IgE Abs. This protein has been described as one of the major allergens of patients with baker's asthma those with allergy to wheat [Amano et al., 1998; Weichel et al., 2006; Sotkovsky et al., 2008], but it had not been isolated in its natural form in sufficient amount and purity.

To characterize the intensity and frequency with which IgE Abs bind the individual wheat components, 17 purified allergens were immunoblotted with sera from all 22 patients, 10 disease controls and nine healthy controls. All patients sera tested (100%) showed positive IgE reactions with α-amylase inhibitors (0.28, CM16 and CM2), TLP and tritin. Of the 22 sera, 20 or 21 (91% or 95%) reacted with CM17, 0.53, 26 kDa endochitinase, XIP and class II chitinase. The frequency of recognition of other wheat proteins was also high ranging from 54% to 86%. The densitometric evaluation of intensity of IgE binding on immunoblots documented the differences of binding of individual wheat proteins. The IgE of patients reacted with the highest intensity (median 6.0–8.0) with the following already known allergens: α-amylase inhibitors 0.53, CM16 and CM2, CM17 and tritin, as well as with the newly identified IgE-binding wheat components TLP, 26 kDa endochitinase and class II chitinase. In contrast, the lowest binding intensity was determined for α-amylase inhibitors CM3 and wheatwin (median 1 and 2, respectively). Moreover, seven sera of disease controls (70%) recognized endogenous α-amylase

inhibitor and tritin. Other proteins were recognized by < 50% sera or did not react. Nevertheless, the intensity of IgE binding calculated for disease controls was low (median 0–2). None of the sera from the nine healthy donors reacted with any of the isolated proteins.

In conclusion, we have identified and purified several IgE reactive components of wheat flour using liquid-phase IEF and HPLC in sufficient quantities. This evaluation allows us to study the function of native allergens, the effects of their tertiary structure and post translational modifications (*e.g.* glycosylation) on allergenicity, as well as the supplementation in to diagnostic assays. Moreover, purified allergens are suitable for a selection of an appropriate vector for recombinant allergen preparation, improving both diagnostic tests and therapy.

doi: 10.1111/j.1365-2222.2011.03766.x
*Clinical & Experimental Allergy*, **41**, 1031–1043
© 2011 Blackwell Publishing Ltd

ORIGINAL ARTICLE Allergens

# A new approach to the isolation and characterization of wheat flour allergens

**P. Šotkovský[1], J. Sklenář[1], P. Halada[1], J. Cinová[1], I. Šetinová[2], A. Kainarová[1], J. Goliáš[1], K. Pavlásková[1], S. Honzová[2] and L. Tučková[1]**
[1]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic and* [2]*SYNLAB (IMUMED), Prague, Czech Republic*

Clinical & Experimental Allergy

**Summary**

*Background* The incidence of food allergy to wheat is increasing. Its diagnosis depends on the purity of major allergens and their inclusion in tests. Isolation and characterization of wheat allergens are therefore of utmost importance.
*Objective* To purify and identify wheat flour allergens most frequently recognized by patients' IgE antibodies and to study their allergenicity.
*Methods* Water/salt-soluble extracts from wheat flour were prepared and separated using a combination of ultrafiltration, isoelectric focusing and liquid chromatography. Purified proteins were analysed by immunoblotting using pooled sera from patients with atopic dermatitis who possessed IgE specific to wheat. Wheat proteins found to bind IgE were subsequently identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. The frequency and intensity of IgE binding of isolated proteins were tested using individual sera from patients and controls.
*Results* We developed a procedure that allows isolation of wheat allergens from natural sources. Twenty-seven potential wheat allergens have been successfully identified; of these, the following seven are newly reported in food allergy: endogenous α-amylase/subtilisin inhibitor, trypsin/α-amylase inhibitor (AAI) CMX1/CMX3, thaumatin-like protein (TLP), xylanase inhibitor protein-1, β-glucosidase, class II chitinase and 26 kDa endochitinase. TLP and wheatwin were shown to activate patients' basophils to a similar extent as two well-known allergens, lipid transfer protein (Tri a 14) and AAI 0.19 (Tri a 28.0101).
*Conclusion and Clinical Relevance* Our new approach enables the isolation of water/salt-soluble wheat allergens in their native form in amounts sufficient both for biological testing (*in vivo* and *in vitro*) and for physicochemical characterization. Such studies will lead to a more detailed knowledge of allergenicity of wheat proteins and to improved accuracy of diagnostic tests.

**Keywords** basophil activation, IgE antibodies, isolation, purification, wheat allergens
*Submitted 28 July 2010; revised 5 February 2011; accepted 21 March 2011*

*Correspondence:*
Petr Šotkovský, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, 142 20 Prague, Czech Republic.
E-mail: psotkovsky@gmail.com
*Cite this as*: P. Šotkovský, J. Sklenář, P. Halada, J. Cinová, I. Šetinová, A. Kainarová, J. Goliáš, K. Pavlásková, S. Honzová and L. Tučková, *Clinical & Experimental Allergy*, 2011 (41) 1031–1043.

## Introduction

Wheat is an important part of human diet, but its ingestion can trigger IgE-mediated food allergies. The incidence of food allergies is increasing and these allergies are thought to affect 6–8% of children and 1–3% of adults [1, 2]. Depending on the route of allergen exposure, hypersensitivity reactions to wheat may cause a variety of symptoms. For example, ingestion may cause atopic dermatitis, gastrointestinal symptoms or wheat-dependent exercise-induced anaphylaxis; inhalation may cause baker's asthma; and skin contact may occasionally trigger IgE-mediated contact urticaria [3–5].

Diagnosis of food allergy to wheat is not simple. Although oral food challenge tests are considered to be good standard for diagnosis, they are time consuming and can induce severe clinical symptoms. Detection of food-specific IgE antibodies (Abs) by radioimmunoassays or by enzymatic immunoassays, along with a history compatible with IgE-mediated symptoms, are highly sensitive but have low specificity. Moreover, a certain degree of cross-reactivity cannot be excluded [6, 7]. Skin prick tests (SPTs) are diagnostic for baker's asthma, but not for atopic dermatitis [8]. In contrast, basophil activation tests are considered to be a reliable *in vitro* diagnostic technique [9].

Poor predictability and specificity of all currently known diagnostic approaches may be associated with the insufficient purity of wheat extracts used in specific IgE assays or with the lack of inclusion of all major allergens in these extracts. Furthermore, expression of IgE-reactive proteins depends on wheat seed maturation and varies in different wheat sources [10].

On the basis of their differential solubility, wheat proteins can be classified into water/salt-soluble albumins and globulins and water/salt-insoluble gliadins and glutenins. Proteins from all these different groups are responsible for baker's asthma as well as food allergies in adults and children. Wheat food allergy in children and adult patients with multiplicity and diversity of skin symptoms, such as wheat-induced atopic dermatitis, has been investigated by several laboratories [11–13]. Proteomic analysis of wheat flour proteins revealed a number of IgE reactive components in both water/salt-soluble and insoluble fractions [shown by immunoblotting, high-performance liquid chromatography (HPLC) and mass spectrometric analysis] [14–16]. Among the major allergens identified in the water/salt-soluble fraction of wheat flour and shown to be capable of sensitization after both ingestion and inhalation are the cereal α-amylase inhibitors (AAI) and α-amylase/trypsin inhibitors (AATI). This AATI family of inhibitors consists of tetrameric proteins, often called chloroform methanol (CM) proteins on the basis of their selective extraction in chloroform/methanol mixtures (CM1, CM2, CM3, CM16 and CM17). On the other hand, the three AAIs are dimeric (0.19 and 0.53) or monomeric (0.28). Wheat amylase/subtilisin inhibitor is a bifunctional protein capable of simultaneously inhibiting endogenous wheat α-amylase and the proteinase subtilisin [17, 18]. Using IgE from allergic individuals to probe cDNA expression library, AAI 0.19 and CM7, as well as new molecules with high cross-reactive potential, including thioredoxins and β-expansins, have been identified [19].

Cross-reactivity among wheat flour allergens, other cereal allergens (barley, rye, rice and maize) and grass pollen allergens is thought to be because of common IgE-reactive epitopes. However, patients with baker's asthma and those with IgE-mediated food allergy to wheat may be sensitive to different molecules [20–22]. The most important plant panallergens are the non-specific lipid transfer proteins (LTPs), which also act as food allergens, sensitizing individuals by the oral route or by inhalation [6, 23, 24]. Although proteomic analysis has identified other wheat proteins as IgE binding (e.g. β-amylase, peroxidase, thioredoxin and serpins), a proper panel of purified wheat allergens has not yet been developed. The list of these allergens is not complete yet and new allergens are still being discovered. Moreover, the clinical relevance of these proteins remains to be determined [14, 16, 25].

Recombinant allergens may be useful in diagnosing and/or treating allergies [26, 27], but so far only a small number of wheat allergens have been cloned and produced in recombinant form [28, 29]. Many recombinant allergens do not have the same immunological characteristics as their natural counterparts. Alterations of the amino acid sequence of allergens may influence protein folding, resulting in the reduction or total prevention of IgE binding. Several recombinant allergens have been produced in bacterial expression systems as non-glycosylated proteins, although some natural AAIs are glycosylated [30]. Thus, highly purified natural allergens are still indispensable in determining the allergenicity of recombinant and natural forms of allergens. Purified allergens are needed not only for the analysis of their structure and function but also in experimental model studies [31–34].

The aim of our study was to isolate, characterize and identify the most important water/salt-soluble wheat allergens from natural sources. Therefore, we developed a three-step isolation protocol, consisting of ultrafiltration, native (liquid-phase) isoelectric focusing (IEF, Rotofor®, Bio-Rad, Hercules, CA, USA) and affinity chromatography (HPLC). Wheat proteins recognized by IgE of allergic patients were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry. The intensity and frequency of IgE binding of both previously and newly identified potential allergens were determined, as well as – in some cases – their potential to activate basophils.

## Materials and methods

### *Patients and controls*

Sera were obtained from 22 patients (13 females, nine males; mean age 23 years; range: 3–63 years) with atopic dermatitis (P1–22). All 22 patients had a suspect history of wheat allergy and elevated specific IgE Abs (mean, 10.24 U/mL; range, 0.4–72 U/mL). An open challenge test was performed with cooked wheat pasta in 14 out of 22 patients and all 14 were positive. In the remaining eight patients, a significant clinical improvement was observed after elimination diet (avoidance of wheat-containing food for 1 month). Total serum IgE and IgE specific for wheat flour, gluten, wheat pollen, timothy grass pollen and birch tree pollen were analysed using an Immulite 2000 (DPC, Siemens Medial Solution Diagnosis, Flanders, NJ, USA). Sera from 10 patients allergic to pollen allergens (D1–10) were used as 'disease controls'. Clinical, demographic and serological characteristics of the 22 patients (P1–22) and 10 disease controls (D1–10) are reported in Table 1. Sera from nine healthy individuals with normal total and wheat-specific IgE Abs were used as negative controls (C1–9).

Table 1. Demographic, clinical and serological characteristics of patients and disease controls

| Patient | Sex | Age | sIgE wheat (U/mL) | Total IgE (U/mL) | Symptoms | Open challenge | Other allergies | Disease control | Sex | Age | sIgE wheat (U/mL) | Total IgE (U/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | F | 5 | 72.00 | 355 | EA | Pos. | e, m | D1 | M | 32 | < 0.10 | 350 |
| P2 | F | 6 | 56.10 | 1545 | EA, RC | Pos. | e, m | D2 | M | 43 | < 0.10 | 345 |
| P3 | F | 39 | 4.13 | 5568 | A, EA | ND | bu, f, mo, nu, ri | D3 | F | 34 | 0.20 | 106 |
| P4 | F | 43 | 2.60 | 563 | A, EA | Pos. | c, m, po, sh | D4 | F | 22 | < 0.10 | 364 |
| P5 | F | 10 | 12.60 | 1083 | A, EA, RC | Pos. | hdm, mo, po | D5 | F | 32 | < 0.10 | 12 |
| P6 | M | 28 | 13.30 | 3590 | D, EA, RC | Pos. | ap, nu, po | D6 | F | 49 | < 0.10 | 33 |
| P7 | F | 29 | 15.00 | 21452 | A, EA, RC | ND | c, d, hdm | D7 | M | 36 | 0.20 | 634 |
| P8 | M | 25 | 1.86 | 14953 | A, EA, RC | ND | hdm | D8 | M | 24 | < 0.10 | 1021 |
| P9 | F | 63 | 1.86 | 45 | EA, RC | Pos. | po | D9 | F | 44 | < 0.10 | 54 |
| P10 | F | 21 | 3.40 | 2686 | AB, EA, OAS, RC | Pos. | ca, ce, po | D10 | F | 46 | < 0.10 | 49 |
| P11 | F | 33 | 3.97 | 2501 | A, EA, OAS, RC | ND | ap, ca, co, nu | | | | | |
| P12 | M | 8 | 2.93 | 2404 | EA, RC | Pos. | po | | | | | |
| P13 | M | 3 | 1.29 | 854 | EA | Pos. | e, m | | | | | |
| P14 | F | 20 | 0.40 | 272 | EA, RC | ND | po | | | | | |
| P15 | M | 5 | 3.55 | 1524 | EA | Pos. | e, m | | | | | |
| P16 | F | 44 | 1.35 | 222 | EA, IU | ND | co, e, po, sp | | | | | |
| P17 | M | 6 | 13.10 | 482 | EA | Pos. | c, e, fr, ho, hon, m, mo, nu, po, se, ve | | | | | |
| P18 | F | 32 | 10.80 | 4331 | A, D, EA, OAS, RC | Pos. | fr, hdm, mo, nu, po, se, ve | | | | | |
| P19 | M | 24 | 1.14 | 1757 | EA, RC | ND | co, hdm, nu, po | | | | | |
| P20 | M | 6 | 0.70 | 546 | A, EA, RC | Pos. | c, hdm, po | | | | | |
| P21 | F | 4 | 0.78 | 147 | EA, RC | Pos. | e, m, nu, po | | | | | |
| P22 | M | 43 | 2.47 | 423 | OAS, RC | ND | ap, po | | | | | |

F, female; M, male; ND, not done; pos., positive; sIgE, specific IgE; U/mL, unit antigen per millilitre; A, asthma bronchiale; D dyspepsia; EA, eczema atopicum; IU, idiopathic urticaria; OAS, oral allergic syndrome; RC, rhinoconjunctivitis; a, animals; ap, apple; bu, buckwheat; c, cat; ca, carrot; ce, celery; co, codfish; d, dog; e, egg white; f, fish; fr, fruit; hdm, house dust mite; hon, honey; ho, horse; m, milk; mo, mould; nu, nuts; po, pollen; se, seeds; sh, shrimp; sp, spices; ri, rice; ve, vegetables.

### *Measurements of total and specific serum immunoglobulin E*

The concentrations of total and wheat-specific IgE were determined by fluorescence enzyme immunoassay (Immulite 2000, DPC, Siemens Medical Solution Diagnosis) according to the manufacturer's instructions. Allergen-specific IgE concentrations above 0.35 kU/L were considered positive.

### *Wheat sample preparation*

Unless otherwise specified, all chemicals were from Sigma (Steinheim, Germany). Wheat flour from cultivar Akteur (*Tritium aestivum*) was kindly provided by Professor J. Petr of the Czech University of Agriculture, Prague. Proteins were extracted from wheat flour (4 g) with PBS (40 mL) overnight at 4 °C, ultrasonicated (U50, IKA Laboratortechnik, Staufen, Germany) for 15 min on ice and centrifuged at 20 000 g for 15 min at 4 °C. Extracted proteins were sequentially separated using Amicon Ultra 100k, 30k and 10k ultrafiltration devices (Millipore, Billerica, MA, USA) in this order providing gross separation according to the molecular weight of the proteins. A 10 mL sample containing 0.35% CHAPS was loaded onto the 100k filter device with a 100 kDa molecular-weight cut-off for 30 min at 2500 g and 20 °C. The concentrated higher molecular-weight fraction was discarded and the filtrate was ultrafiltered using a 30k filter device with a 30 kDa molecular-weight cut-off. The concentrate was stored (fraction I) and the filtrate was purified using a 10k filter device with a 10 kDa molecular-weight cut-off, yielding fraction II (60 min, 2500 g and 20 °C). Protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (BCA, Pierce, IL, USA). Samples were divided into aliquots and stored at −20 °C.

### *Liquid-phase isoelectric focusing*

Proteins in fractions I and II were separated according to their pI using a Rotofor® preparative cell (Bio-Rad). Fraction I contained proteins of molecular mass above 30 kDa and below 100 kDa, whereas fraction II contained proteins of molecular mass above 10 kDa and below 30 kDa. Proteins from fraction I (15 mL, containing 45 mg protein) were mixed with 0.5% CHAPS and diluted in

55 mL of distilled water containing 5% glycerol, 5% premixed Bio-Lyte pH 5–8, pH 3–10 (Bio-Rad) and ampholyte pH 4–6 (Fluka, Buchs, Switzerland). Proteins in fraction II were diluted similarly, except that premixed ampholytes pH 4–6 and pH 6–9 were used. Both samples were fractionated using a Rotofor® device, equipped with a standard focusing chamber (60 mL total volume) with 20 fractionation compartments. A constant power of 15 W was applied to the system, which was cooled to 4 °C with a water circulator, with fractionation completed within 4 h. A total of 20 fractions were collected, and the pH of each was measured using a microelectrode.

### *High-performance liquid chromatography*

All separations were carried out using an HPLC system Gold 125NM solvent Module (Beckman Coulter, Miami, FL, USA). The sample was centrifuged for 10 min at 12 000 g. The supernatant was applied (containing 2 mg protein) to a polymeric reversed-phase PLRP-S 1000A, 8 μm, 250× 406 mm column (Varian, Palo Alto, CA, USA) and separated at a flow rate of 1 mL/min using a linear gradient (solvent A, 0.1% TFA/$H_2O$; solvent B, 0.1% TFA/acetonitrile). The gradient program started with 95% of solvent A and 5% of solvent B, and changed 5–25% B/5 min, 25–45% B/50 min, 45–70% B/5 min and 70–95% B/0.5 min. The column was washed with 95% solvent B (10 min) and equilibrated at initial conditions for 10 min. Protein samples were evaporated using a vacuum centrifuge (SpeedVac, Jouan, Winchester, VA, USA) and dissolved in PBS.

### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting*

Each of the Rotofor® and HPLC fractions was analysed by SDS-PAGE (in concentration 1 mg/mL) using 15% gradient gels under reducing conditions (with 0.5 M DTT). Separated proteins were stained with Coomassie® Brilliant Blue R-250 (CBB). Selected fractions were electrotransferred to nitrocellulose membranes (NC2, SERVA, Heidelberg, Germany) for 1 h at room temperature. The membranes were blocked with PBS containing 0.5% Tween 20 and incubated with serum samples diluted 1 : 10 in blocking buffer overnight at 4 °C. After washing with PBS–0.1% Tween 20, the strips were incubated with peroxidase-labelled sheep anti-human IgE Abs (1 : 1000; The Binding Site, Birmingham, UK) for 1 h at room temperature. Blots were developed using SuperSignal West Pico Trial kits (Pierce) and luminescence was detected on a Kodak Medical X-ray film (Kodak, Rochester, NY, USA). Data were digitized and subjected to image analysis (AIDA 3.28, Raytest, Straubenhardt, Germany). Relative signal intensities derived from the chemiluminescent signal of IgE reaction with purified proteins were compared and corrected for local background signal (set as value 1). The resultant intensity is expressed as nine intervals (0–8; corresponding to intensity 1 → 801). Data are expressed as median values and 25% and 75% percentile.

### *Tryptic digestion and matrix-assisted laser desorption/ionization mass spectrometry*

CBB-stained proteins were excised from gels, cut into small pieces and washed several times with 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, the proteins were reduced with 30 mM TCEP at 65 °C for 30 min and alkylated by 30 mM iodoacetamide for 60 min in the dark. The gel pieces were further washed with de-ionized water, shrunk by dehydration in MeCN and reswollen in water. The supernatants were removed and the gels were partly dried in a SpeedVac concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and sequencing-grade trypsin (100 ng; Promega, Madison, WI, USA). After overnight digestion, the resulting peptides were extracted with 40% MeCN/0.5% TFA. A solution of α-cyano-4-hydroxycinnamic acid in aqueous 50% MeCN/0.1% TFA (5 mg/mL) was used as an MALDI matrix. A 0.5 μL aliquot of a sample was deposited onto the MALDI target and allowed to air-dry at room temperature, followed by the addition of 0.5 μL of the matrix solution.

MALDI mass spectra were measured using an Ultraflex III instrument (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam™ solid-state laser and LIFT™ technology for MS/MS analysis. The spectra were acquired in the mass range of 700–4000 Da and calibrated internally using monoisotopic $[M+H]^+$ ions of trypsin autoproteolytic fragments (842.5 and 2211.1 Da).

### *Protein identification*

Peak lists in XML data format were created using the flexAnalysis 3.0 program with the SNAP peak detection algorithm. No smoothing was used, and the maximum number of assigned peaks was set at 50. After peak labelling, all known contaminant signals were manually removed. The peak lists were searched using the MASCOT search engine against the SwissProt 57.13 or Trembl 40.13 database subset of green plant proteins with search settings including a peptide tolerance of 30 p.p.m., a missed cleavage site value of 2, and variable carbamidomethylation of cysteine and oxidation of methionine. There were no restrictions on protein molecular weight or pI. We identified proteins with a MOWSE score over a threshold of 57 (SwissProt) or 71 (Trembl). If the score was only slightly higher than the threshold value or the sequence coverage was too low, we confirmed the identity of the protein candidate by MS/MS analysis. In addition to the above MASCOT settings, a fragment mass tolerance of 0.6 Da and instrument-type MALDI-TOF-TOF was applied

to search MS/MS spectra. Amino acid sequences of identified proteins were analysed using the multiple sequence alignment software Clustal W (http://www.ebi.ac.uk/clustalw).

### *Basophil activation test*

Flow cytometric basophil activation tests were performed after stimulation of blood cells with four isolated wheat allergens [AAI 0.19, LTP, thaumatin-like protein (TLP) and wheatwin] or control (PBS, anti-IgE), as described previously [14]. The same blood samples were obtained from the patients, disease controls and healthy donors (Table 1). Briefly, 100 μL of heparinized whole blood and 10 μL of stimulation buffer were incubated (30 min at 37 °C) containing commercially available wheat extract (1000 IC/mL Stallergens, Antony Cedex, France) or selected allergens (concentration 1 mg/mL) diluted 1 : 10.

After incubation, the samples were transferred on ice and stained with mAb anti-(cluster differentiation) CD203/PE (Beckman Coulter) and anti-CD63/FITC (Exbio, Prague, Czech Republic) for 15 min. Erythrocytes were lysed using ammonium chloride. After washing the cells, we measured the percentage of activated basophils expressing CD63 and CD203c by flow cytometry (FC500, Beckman Coulter). Before this study, the basophil activation test had been performed in a pilot study in patients (seven per allergen) with AAI 0.19, LTP, TLP and wheatwin for four increasing dilutions (1 : 2, 1 : 10, 1 : 50 and 1 : 100). On the basis of these data, allergen dilution 1 : 10 was selected as optimal. No basophil activation could be induced in the controls. The ROC analysis of basophil activation was performed for all four proteins to determine the optimal cut-off values. Values above the cut-off, 9% of activated basophils for AAI 0.19 and LTP, 11.9% for TLP and 16% for wheatwin, were considered as positive.

## Results

### *Preparation of salt-soluble wheat proteins and fractionation in Rotofor® cells*

Salt-soluble proteins of molecular mass ranging from 5 to 120 kDa were extracted from the flour of the wheat cultivar Akteur (*T. aestivum*) were separated using Amicon 100k, 30k and 10k filter devices and were characterized by SDS-PAGE (Fig. 1a; lane 0). Protein aggregates were efficiently removed in the step with Amicon 100k filter device. Fraction 1 consisted mainly of proteins with molecular mass between 100 and 30 kDa, whereas fraction II contained only low-molecular-mass components below 30 kDa.

Proteins in fraction I (Fig. 1a, lane I) were further purified in the Rotofor® cell. Liquid-phase IEF considerably purified wheat proteins and yielded 20 fractions

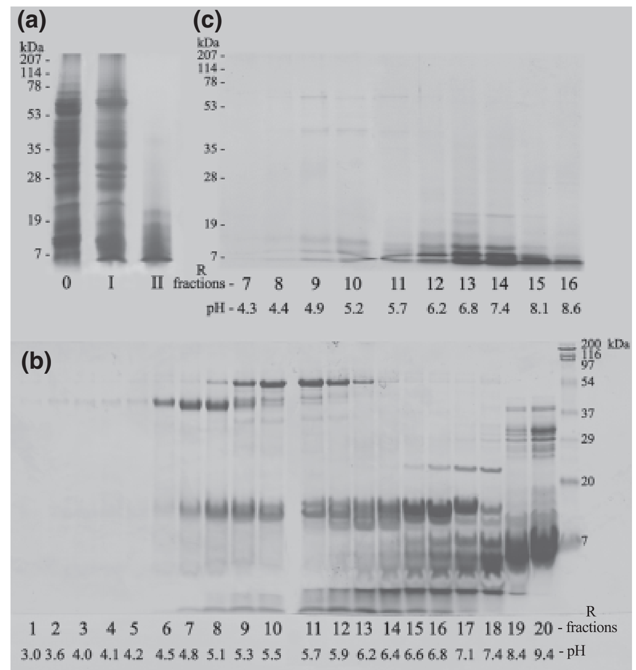



Fig. 1. SDS-PAGE analysis of wheat flour PBS extracts (a) and Rotofor® fractions (b and c). (a) Spectrum of wheat flour proteins extracted with PBS (0) and purified using Amicon 100k and 30k filter devices (fraction I). Fraction I was concentrated using a 10k filter device (fraction II). (b and c) Proteins from fractions (b) I and (c) II were separated by liquid-phase isoelectric focusing in Rotofor® cell into 20 fractions (R1–R20). All gels were stained with Coomassie brilliant blue.

(R1–R20) with pH ranging from 3.0 to 9.4, as documented by SDS-PAGE (Fig. 1b). Fractions R6–R13 contained predominantly protein bands of 50 kDa, fractions R19 and R20 contained proteins of 20–40 kDa, fractions R7–R18 contained proteins of molecular mass below 20 kDa and fractions R11–R20 were enriched in low-molecular-mass components below 10 kDa. Because of their high protein content and their reactivity with IgE Abs from patients' sera (data not shown), eight Rotofor® fractions (R7, R11, R14–R18 and R20) were selected for further purification by HPLC.

Similarly, proteins from fraction II were separated in the Rotofor® cell into 20 fractions with pH ranging from 3.7 to 9.5. Proteins were detected in nine fractions (R8–R16) and were enriched in fractions R13 and R14 (Fig. 1c). Four Rotofor® fractions (R9, R10, R13 and R14) were selected for purification by HPLC.

### *Purification and identification of major wheat allergens by high-performance liquid chromatography and mass spectrometry*

Selected Rotofor® fractions were further purified by HPLC (PLRP-S column) and the resulting fractions were

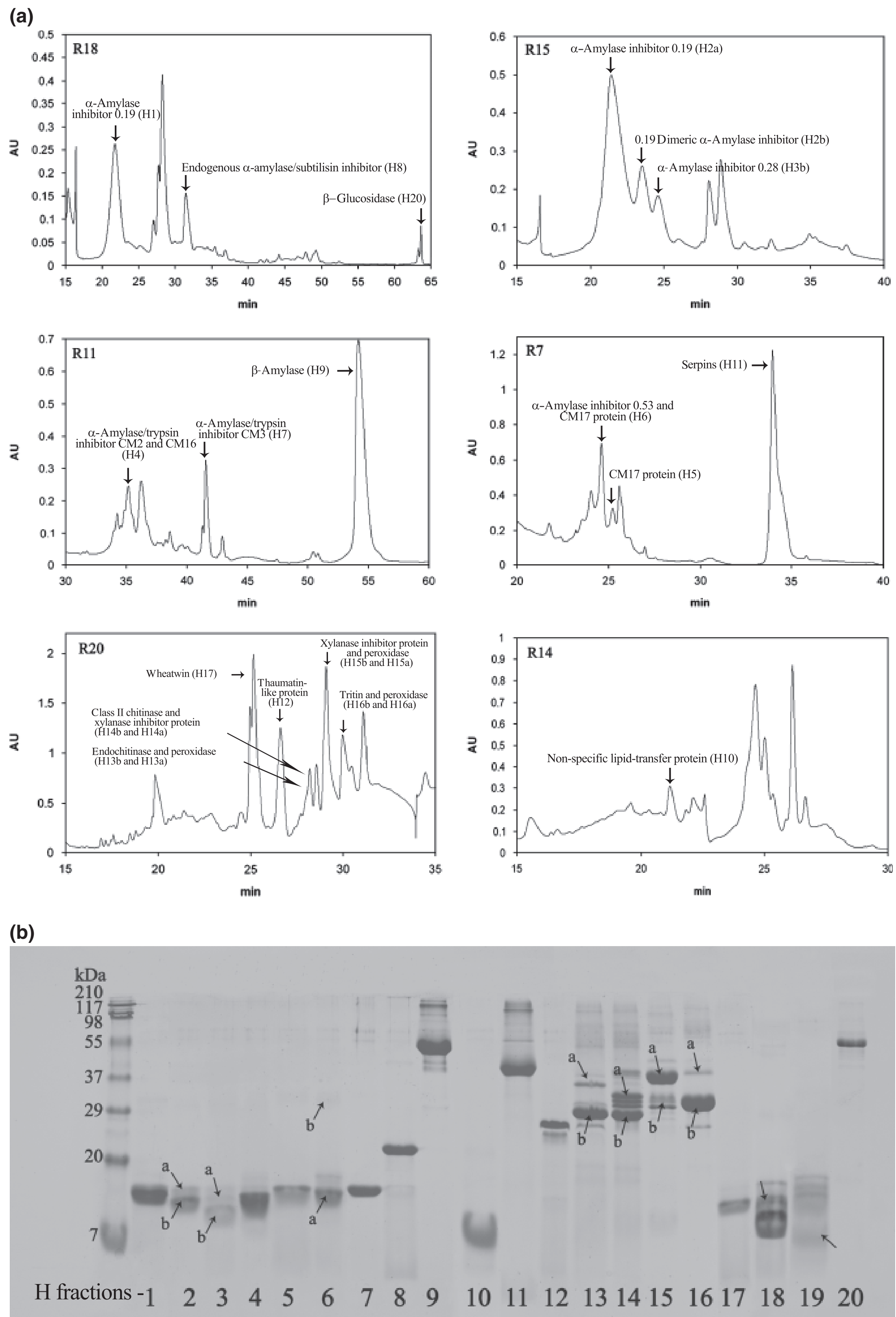



Fig. 2. (a) Examples of the chromatographic profiles of Rotofor® fractions R18, R15, R11, R7 and R20 (shown in Fig. 1b) and fraction R14 (shown in Fig. 1c) obtained from reversed-phase HPLC. The HPLC fraction numbers (H1–H20) and the peaks with arrows indicate purified and identified wheat proteins corresponding to those listed in Table 2. (b) Coomassie blue staining of selected wheat proteins purified by HPLC and separated by SDS-PAGE. The HPLC fraction numbers indicate the bands corresponding to the allergens identified in Table 2.

**Table 2.** Isolated wheat proteins identified by MS analysis (MALDI-TOF)

| H fractions | Protein (allergen) name | Accession no. | MW (kDa) | pI | Protein yield (mg) | Peptides matched | Sequence coverage (%) | MS/MS confirmation | R fraction |
|---|---|---|---|---|---|---|---|---|---|
| 1 | α-Amylase inhibitor 0.19 (Tri a 28.0101) | IAA1_WHEAT | 13.30 | 6.60 | 1.73 | 8 | 74 | LAHISEWCR HGAQEGQAGTGAFPR | 18 |
| 2a | α-Amylase inhibitor 0.19 (Tri a 28.0101) | IAA1_WHEAT | 13.30 | 6.60 | 0.61 | 4 | 44 | No | 15 |
| 2b, 3a | 0.19 dimeric α-amylase inhibitor | Q5UHH6_WHEAT | 13.20 | 6.50 | | 8 | 59 | No | 15 |
| 3b | α-Amylase inhibitor 0.28 (Tri a aAI) | IAA2_WHEAT | 16.80 | 7.40 | | 8 | 54 | No | 15 |
| 6a | α-Amylase inhibitor 0.53 (Tri a 28) | IAA5_WHEAT | 13.20 | 5.20 | 0.16 | 8 | 54 | No | 7 |
| 6b | α-Amylase inhibitor 0.53 (Tri a 28) | IAA5_WHEAT | 13.20 | 5.20 | | 4 | 47 | LQCNGSQVPEAVLR EHGVSEGQAGTGAFPSCR | 7 |
| 6a | CM 17 protein | Q41540_WHEAT | 16.00 | 5.00 | | 5 | 35 | No | 7 |
| 6b | CM 17 protein | Q41540_WHEAT | 16.00 | 5.00 | | 4 | 23 | SRPDQSGLMELPGCPR | 7 |
| 5 | CM 17 protein | Q41540_WHEAT | 16.00 | 5.00 | < 0.10 | 8 | 39 | No | 7 |
| 18 | α-Amylase/trypsin inhibitor CM1 | IAAC1_WHEAT | 15.50 | 7.50 | < 0.10 | 2 | 24 | No | 11 |
| 4 | α-Amylase/trypsin inhibitor CM2 (Tri a 29) | IAAC2_WHEAT | 15.50 | 6.80 | 0.10 | 5 | 29 | No | 11 |
| 4 | α-Amylase/trypsin inhibitor CM16 (Tri a CM16) | IAC16_WHEAT | 15.80 | 5.30 | | 10 | 45 | No | 11 |
| 7 | α-Amylase/trypsin inhibitor CM3 (Tri a 30) | IAAC3_WHEAT | 18.20 | 7.40 | 0.20 | 10 | 69 | No | 11 |
| 19 | Trypsin/α-amylase inhibitor CMX1/CMX3 | IACX1_WHEAT | 13.80 | 9.20 | < 0.10 | 2 | | EFIAGIVGR EITYESLNACAEYAVR | 17 |
| 8 | Endogenous α-amylase/subtilisin inhibitor | IAAS_WHEAT | 19.60 | 6.70 | 0.34 | | | WHIDSELVSGR STEWHIDSELVSGR | 18 |
| 13b | 26 kDa endochitinase 1 | CHI1_HORVU | 33.40 | 8.50 | < 0.10 | 10 | 44 | No | 20 |
| 14b | Class II chitinase | Q4Z8L8_WHEAT | 28.20 | 8.60 | < 0.10 | 13 | 73 | No | 20 |
| 10 | Non-specific lipid-transfer protein (Tri a 14) | NLTP1_WHEAT | 11.90 | 8.50 | 0.33 | 3 | 30 | No | 14 (Fig. 1c) |
| 17 | Wheatwin-1 | WHW1_WHEAT | 15.60 | 7.60 | 0.51 | 5 | 45 | No | 20 |
| 17 | Wheatwin-2 | WHW2_WHEAT | 15.90 | 8.20 | | 6 | 56 | No | 20 |
| 12 | Thaumatin-like protein | Q8S4P7_WHEAT | 23.60 | 7.80 | 0.71 | 12 | 55 | No | 20 |
| 9 | β-Amylase | AMYB_HORVU | 60.00 | 5.60 | 0.23 | 7 | 17 | No | 11 |
| 11 | Serpin-Z1A | SPZ1A_WHEAT | 43.10 | 5.60 | < 0.10 | 7 | 27 | No | 7 |
| | Serpin-Z1B | SPZ1B_WHEAT | 43.00 | 5.40 | | 6 | 24 | No | |
| | Serpin-Z1C | SPZ1C_WHEAT | 42.80 | 5.60 | | 5 | 20 | No | |
| | Serpin-Z2A | SPZ2A_WHEAT | 43.30 | 5.40 | | 11 | 41 | No | |
| | Serpin-Z2B | SPZ2B_WHEAT | 43.00 | 5.20 | | 5 | 15 | No | |
| 13a, 15a, 16a | Peroxidase 1 | Q8LK23_WHEAT | 38.80 | 8.10 | 0.16 | 23 | 57 | No | 20 |
| 16b | Tritin (rRNA N-glycosidase) | Q07810_WHEAT | 29.60 | 9.70 | | 15 | 66 | No | 20 |
| 14a, 15b | Xylanase inhibitor protein 1 | XIP1_WHEAT | 33.20 | 8.60 | | 21 | 61 | No | 20 |
| 20 | β-Glucosidase | Q40025_HORVU | 57.40 | 7.20 | < 0.10 | | | LDFVWYEPHSDSNADQAAAQR | 18 |

Protein yield corresponds to the amount of proteins isolated from 15 mL ultrafiltrated sample (containing 45 mg protein), separated by one Rotofor run and purified by HPLC.
The allergenic molecules are named by the International Union of the Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee.
The H fraction numbers refer to Fig. 2 and the R fraction numbers refer to Fig. 1b.
Tri a, *Triticum aestivum*.

analysed by SDS-PAGE. Figure 2a shows the chromatograms of six Rotofor® fractions (five obtained from Amicon fraction I – R7, R11, R15, R18 and R20, and one from fraction II – R14). All HPLC fractions were analysed by immunoblotting using patients' pooled sera (data not shown) and fractions containing separated IgE-binding proteins were selected for identification by MALDI-TOF mass spectrometry. Using these methodological approaches and protein database searches, we succeeded in identifying 27 wheat proteins (Table 2). These IgE-binding proteins were detected in 20 HPLC fractions, H1–H20. SDS-PAGE analysis and Coomassie blue staining of these fractions (Fig. 2b) revealed six well-resolved peaks (fractions H1, H7, H8, H10, H12 and H17), each corresponding to a single protein band of sufficient yield (Table 2). The first peak (H1) corresponded to AAI, AAI 0.19, which had been eluted uncontaminated after HPLC separation of Rotofor® fraction R18 (Figs 2a and b). Rotofor® fractions R14–R17 contained AAI 0.19 with decreasing amounts of 0.19 dimeric AAI (H2b) and AAI 0.28 (H3b) proteins (see R15 Fig. 2a). Other identified proteins, such as wheatwin (H17) and TLP (H12), were observed only in Rotofor® fraction R20. Protein AATI CM3 (H7) was identified in all Rotofor® fractions, except for R20, separated by HPLC, whereas endogenous protein AATI (H8) was found in Rotofor® fractions R14–R18. LTP (H10) was isolated primarily from Rotofor® fraction R14, which had been obtained especially from fraction II. The other HPLC fractions (H13, H14, H15 and H16) contained mixtures of proteins, including peroxidase, tritin, xylanase inhibitor protein (XIP), endochitinase and class II chitinase. HPLC fraction H4 contained proteins AATI CM2 and CM16 and fraction H6 contained AAI 0.53 and CM17 protein. HPLC fraction H11 contained a mixture of five members of the serpin family. Fractions H5, H9, H18 and H19 contained one major IgE-binding protein together with small amounts of other proteins. Fraction H20 contained a low amount of β-glucosidase. Out of the 27 identified proteins, the following seven are new potential wheat allergens: endogenous α-amylase/subtilisin inhibitor, trypsin/AAI CMX1/CMX3, TLP, XIP-1, β-glucosidase, class II chitinase and 26 kDa endochitinase.

### *Reactivity of patients' immunoglobulin E antibodies with isolated proteins*

To characterize the intensity and frequency with which IgE Abs bind the individual wheat components, 17 purified HPLC fractions (H1–H17) were immunoblotted with sera from all 22 patients, 10 disease controls and nine healthy controls. An example of the reactivity of IgE from one patient (P1), one disease control (D1) and one healthy control (C8) with isolated wheat proteins is shown in Fig. 3. The data summarized in Table 3 document the individual diversity of IgE response. However, all patients

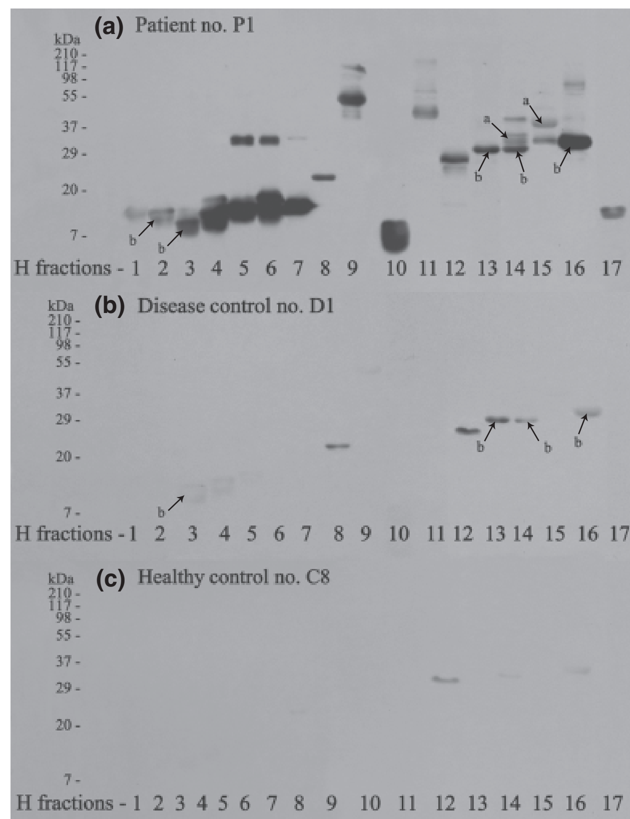



Fig. 3. An example of IgE immunoblotting of purified wheat proteins with the sera of (a) one patient, (b) a disease control and (c) a healthy control. The HPLC fraction numbers indicate the bands corresponding to the allergens identified in Table 2.

sera tested (100%) showed positive IgE reactions with α-amylase inhibitors AAI 0.28 (H3b), AATI CM16 and CM2 (H4), TLP (H12) and tritin (H16b). Of the 22 sera, 20 or 21 (91% or 95%) reacted with CM17 (H5), CM17 and AAI 0.53 (H6), 26 kDa endochitinase (H13b), XIP (H14a) and class II chitinase (H14b). The frequency of recognition of other wheat proteins was also high ranging from 54% to 86%. The densitometric evaluation of intensity of IgE binding on immunoblots documents the differences of binding of individual wheat proteins (Table 3). The IgE of patients reacted with the highest intensity (median 6.0–8.0) with the following already known allergens: AATI CM16 and CM2 (H4), CM17 and AAI 0.53 (H6), CM17 (H5) and tritin (H16b), as well as with the newly identified IgE-binding wheat components TLP (H12), 26 kDa endochitinase (H13b) and class II chitinase (H14b). In contrast, the lowest binding intensity was determined for AATI CM3 (H7) and wheatwin (H17) (median 1 and 2, respectively).

We also performed IgE immunoblotting of purified proteins with sera from 10 disease controls. Endogenous AAI (H8) and tritin (H16b) were recognized by seven sera (70%); other proteins were recognized by $<50\%$ sera or did not react. Nevertheless, the intensity of IgE binding

**Table 3.** Reactivity of IgE antibodies of patients and disease controls on immunoblot analysis with selected proteins

| Protein name | AAI 0.19 | 0.19 dimeric AAI | AAI 0.28 | AATI CM16 & CM2 | CM17 | CM17 & AAI 0.53 | AATI CM3 | Endo-genous AAI | β-Amylase | LTP | Serpins | TLP | 26 kDa endochitinase | XIP | Class II chitinase | Peroxidase | Tritin | Wheatwin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H fraction | 1 | 2b | 3b | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13b | 14a | 14b | 15a | 16b | 17 |
| Patients | | | | | | | | | | | | | | | | | | |
| P1 | 1 | 3 | 7 | 8 | 8 | 8 | 8 | 4 | 8 | 8 | 3 | 5 | 5 | 3 | 5 | 3 | 8 | 4 |
| P2 | 5 | 4 | 6 | 8 | 6 | 4 | 4 | 6 | 4 | 6 | 0 | 8 | 6 | 1 | 6 | 3 | 6 | 7 |
| P3 | 2 | 2 | 3 | 8 | 6 | 8 | 0 | 2 | 5 | 0 | 1 | 6 | 5 | 3 | 5 | 0 | 1 | 1 |
| P4 | 1 | 1 | 2 | 8 | 8 | 8 | 0 | 1 | 0 | 0 | 0 | 7 | 6 | 8 | 7 | 4 | 5 | 0 |
| P5 | 7 | 5 | 4 | 8 | 6 | 5 | 1 | 4 | 7 | 1 | 5 | 8 | 8 | 8 | 8 | 8 | 8 | 4 |
| P6 | 8 | 7 | 5 | 8 | 5 | 8 | 3 | 6 | 8 | 3 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 7 |
| P7 | 8 | 5 | 4 | 8 | 4 | 8 | 0 | 4 | 7 | 4 | 3 | 8 | 8 | 8 | 8 | 8 | 7 | 3 |
| P8 | 8 | 7 | 8 | 8 | 8 | 8 | 4 | 6 | 4 | 8 | ND | 8 | 8 | 8 | 8 | 8 | 8 | 3 |
| P9 | 3 | 4 | 7 | 8 | 2 | 2 | 2 | 4 | 1 | 4 | 0 | 8 | 7 | 3 | 7 | 3 | 8 | 4 |
| P10 | 5 | 5 | 6 | 8 | 3 | 8 | 1 | 3 | 7 | 6 | 5 | 8 | 7 | 8 | 7 | 8 | 8 | 6 |
| P11 | 3 | 2 | 3 | 8 | 5 | 7 | 0 | 0 | 0 | 0 | ND | 2 | 3 | 8 | 3 | 8 | 5 | 0 |
| P12 | 4 | 3 | 5 | 6 | 6 | 5 | 0 | 1 | 0 | 2 | ND | 3 | 2 | 5 | 2 | 1 | 3 | 0 |
| P13 | 1 | 1 | 3 | 7 | 7 | 8 | 0 | 2 | 3 | 0 | ND | 7 | 6 | 8 | 6 | 5 | 6 | 0 |
| P14 | 6 | 7 | 8 | 8 | 2 | 2 | 1 | 4 | 3 | 2 | ND | 8 | 7 | 3 | 7 | 0 | 8 | 0 |
| P15 | 4 | 3 | 8 | 8 | 5 | 5 | 1 | 6 | 5 | 6 | ND | 8 | 8 | 2 | 8 | 3 | 8 | 0 |
| P16 | 5 | 4 | 7 | 8 | 0 | 0 | 0 | 3 | 1 | 0 | ND | 5 | 4 | 6 | 4 | 3 | 8 | 1 |
| P17 | 0 | 0 | 2 | ND | 8 | 8 | 0 | 0 | 0 | 0 | ND | 4 | 2 | 6 | 2 | 6 | 5 | 0 |
| P18 | 0 | 0 | 4 | ND | 0 | 0 | 0 | 1 | 0 | 0 | ND | 1 | 0 | 0 | 0 | 0 | 7 | 0 |
| P19 | 0 | 0 | 3 | 4 | 2 | 1 | 0 | 0 | 0 | 0 | ND | 3 | 3 | 0 | 3 | 0 | 4 | 0 |
| P20 | 8 | 8 | 8 | 8 | 8 | 8 | 7 | 6 | 8 | 8 | ND | 8 | 8 | 5 | 8 | 8 | 8 | 8 |
| P21 | 8 | 7 | 5 | 5 | 8 | 8 | 2 | 5 | 8 | 7 | ND | 8 | 7 | 3 | 7 | 5 | 8 | 3 |
| P22 | 8 | 8 | 8 | 8 | 8 | 8 | 7 | 7 | 8 | 8 | ND | 8 | 7 | 7 | 7 | 7 | 8 | 8 |
| Median | 4.5 | 4 | 5 | 8 | 6 | 8 | 1 | 4 | 4 | 2.5 | 3 | 8 | 6.5 | 5.5 | 7 | 4.5 | 8 | 2 |
| IQR | 1–8 | 1.75–7 | 3–7.25 | 8–8 | 2.75–8 | 3.5–8 | 0–3.25 | 1–6 | 0–7.25 | 0–6.25 | 0–5 | 4.75–8 | 3.75–8 | 3–8 | 3.75–8 | 2.5–8 | 5–8 | 0–4.5 |
| Disease controls | | | | | | | | | | | | | | | | | | |
| D1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 1 | 0 |
| D2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | ND | 0 | ND | 0 | 1 | 0 |
| D3 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 1 | 0 | 0 | ND | 3 | 3 | 0 | 3 | 1 | 3 | 2 |
| D4 | 0 | 1 | 3 | 3 | 0 | 0 | 0 | 1 | 0 | 0 | ND | 2 | 1 | 1 | 1 | 1 | 3 | 1 |
| D5 | 1 | 1 | 4 | ND | 0 | 0 | 0 | 2 | 1 | 0 | ND | 3 | 3 | 3 | 3 | 0 | 5 | 0 |
| D6 | 1 | 1 | 2 | ND | ND | ND | 0 | 2 | 1 | 0 | ND | 3 | 2 | 0 | 2 | 2 | 3 | 0 |
| D7 | 1 | 2 | 4 | ND | ND | ND | 0 | 1 | 0 | 0 | ND | 0 | ND | 0 | ND | 0 | 5 | 0 |
| D8 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| D9 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| D10 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Median | 0 | 0 | 1 | 1.5 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1.5 | 0 | 1 | 0.5 | 2 | 0 |
| IQR | 0–1 | 0–1 | 0–3.25 | 0–3 | 0–0 | 0–0 | 0–0 | 0–1.25 | 0–0.25 | 0–0 | 0–0 | 0–3 | 0–2.75 | 0–0.25 | 0–2.75 | 0–1 | 0–3.5 | 0–0.25 |

Range of chemiluminescence intensity: 0, 1–100; 1, 101–200; 2, 201–300; 3, 301–400; 4, 401–500; 5, 501–600; 6, 601–700; 7, 701–800; 8, 801 <.

The resultant chemiluminescence intensity is corrected for local background signal (set as value 1) and expressed as an interval of measured values.

The H fraction numbers refer to Table 1.

AAI, α-amylase inhibitor; AATI, α-amylase/trypsin inhibitor; LTP, non-specific lipid-transfer protein; TLP, thaumatin-like protein; XIP, xylanase inhibitor protein; ND, not done; IQR: interquartile range, 25–75 percentile.

calculated for disease controls was low (median 0–2). None of the sera from the nine healthy donors reacted with any of the isolated proteins, except for low non-specific reactions of secondary anti-human IgE Ab with TLP (H12) and tritin (H16b) (Fig. 3c).

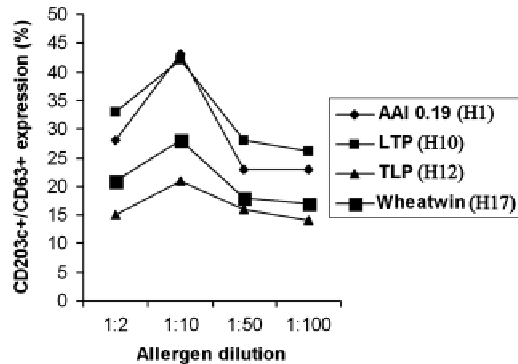



**Fig. 4.** CD203c/CD63 expression of basophils from seven patients in a dose–response curve after incubation with allergens: AAI 0.19 (H1), LTP (H10), TLP (H12) and wheatwin (H17) (dilution: 1 : 2, 1 : 10, 1 : 50 and 1 : 100).

### *Comparison of the identified α-amylase inhibitors*

Of the 27 identified wheat allergens, 11 belonged to the AAI family. These inhibitors could be divided into three groups based on sequence homology obtained from the NCBI database (compared by Clustal W program). The first group consisted of four inhibitors, AAI 0.19, 0.53, 0.28 and 0.19 dimeric; the second consisted of five proteins, AATI CM1, CM2, CM3, CM16 and CM17; and the third one consisted of two inhibitors, endogenous AAI and AATI CMX1/CMX3. Homology among the proteins in the first group ranged from 55% to 94%. Inhibitors from the third group showed very low sequence homologies with other proteins. Independently on the degree of structural homology, each of these inhibitors was found to bind

**Table 4.** Basophil activaton test (%)

| Protein name | AAI 0.19 | LTP | TLP | Wheatwin | Wash buffer (negative control) | Stimulation with anti-IgE (positive control) |
|---|---|---|---|---|---|---|
| H fraction | 1 | 10 | 12 | 17 | | |
| Patients | | | | | | |
| P1 | 80 | 89 | ND | ND | 6 | 91 |
| P2 | 89 | 57 | 84 | 70 | 10 | 92 |
| P3 | ND | ND | 14 | 45 | 5 | 51 |
| P4 | 32 | 27 | 22 | 22 | 13 | 53 |
| P5 | 17 | 19 | ND | ND | 2 | 85 |
| P6 | 7.7 | 30 | 34 | 30 | 5 | 88 |
| P7 | ND | ND | 19 | 31 | 12 | 77 |
| P8 | ND | ND | 11 | 22 | 10 | 87 |
| P9 | ND | ND | 15 | 19 | 11 | 49 |
| P10 | 9.1 | 9.2 | 3.3 | 7.2 | 3 | 71 |
| P11 | ND | ND | 15 | 24 | 5 | 96 |
| P12 | 3.3 | 6.5 | ND | ND | 2 | 93 |
| P13 | ND | ND | 12 | 19 | 10 | 79 |
| P14 | 12 | 9.5 | ND | ND | 7 | 76 |
| P15 | 26 | 25 | 16 | 21 | 8 | 62 |
| P16 | ND | ND | 22 | 11 | 10 | 60 |
| P17 | ND | ND | 12 | 27 | 6 | 19 |
| P18 | ND | ND | 2.3 | 7.9 | 2 | 42 |
| P19 | ND | ND | 1.7 | 2.1 | 1.5 | 95 |
| P20 | 5.2 | 4.6 | ND | ND | 4 | 62 |
| P21 | ND | ND | 20 | 21 | 3 | 45 |
| P22 | ND | ND | 9.4 | 8.2 | 4 | 66 |
| Healthy controls | | | | | | |
| C1 | 6.5 | 5.9 | ND | 14 | 4.5 | 61 |
| C2 | 7.3 | 7.5 | 10 | 12 | 4.4 | 75 |
| C3 | 14 | 6.5 | 6.7 | 10 | 4.1 | 49 |
| C4 | 8.7 | 8.1 | 10 | 13 | 8 | 41 |
| C5 | 1.7 | 12 | 7.1 | 9.5 | 1.5 | 45 |
| C6 | 2.4 | 2.7 | 1.5 | 1.7 | 1 | 55 |
| C7 | ND | ND | 11 | 6.1 | 6 | 45 |
| C8 | ND | ND | 8.2 | 4.3 | 3 | 57 |
| C9 | ND | ND | 11 | 14 | 9 | 58 |
| cut-off | 9 | 9 | 11.9 | 16 | | |

AAI, α-amylase inhibitor; LTP, non-specific lipid-transfer protein; TLP, thaumatin-like protein; ND, not done.

patients' IgE Abs, but the intensities of their binding varied (Table 3).

*Activation of basophils from allergic patients with purified allergens*

The biological activity of purified allergens was tested using the basophil activation test. Blood samples were obtained from 22 patients with clinical symptoms of wheat allergy and with positive basophil activation tests to a commercial wheat extract, from 10 disease controls and from nine healthy donors. These blood samples were exposed to four isolated wheat proteins, AAI 0.19 (H1), LTP (H10), TLP (H12) and wheatwin (H17). A bell-shaped dose–response curve for all four allergens was obtained (Fig. 4) and the optimal dilution was selected (1 : 10). Positive cell activation was detected in response to LTP in eight out of 10 patients (80%), to AAI 0.19 in seven out of 10 patients (70%) and to TLP and wheatwin in 12 out of 17 patients (71%) (Table 4).

We compared the number of patients positive for allergens AAI 0.19, LTP, TLP and wheatwin in either IgE immunoblot or in the basophil activation test (analysing both IgE binding and cell activation). We have shown that for all allergens tested in IgE immunoblot, the proportion of positive cases ranged from 60% to 100%. This proportion was somewhat lower in the basophil activation test and the range was more narrow (70–80%).

## Discussion

Extensive analysis of allergenic molecules is necessary for allergy diagnosis and treatment. We developed a new, rapid procedure for isolation of wheat flour allergens in amounts allowing subsequent structural and functional analysis. We succeeded in purifying relevant IgE binding proteins from a water/salt-soluble extract of wheat flour using Amicon devices, Rotofor® and HPLC techniques.

The advantage of Rotofor® (protein separation based on pI value – preparative IEF) is its capacity to optimally fractionate milligrams to grams of total protein. However, the apparent pI of Rotofor® fractions may not exactly match the predicted pI of separated proteins; these proteins may be rather spread over two or more fractions [35]. With isolation of natural non-denatured allergen epitopes in mind, we used native (liquid-phase) preparative IEF that resulted in substantial protein enrichment and provided the basis for a successful isolation of native allergens. The purification of individual water/salt-soluble wheat proteins from their mixtures included the preparative reversed-phase HPLC. The purified proteins were subsequently analysed by SDS-PAGE, and IgE-binding proteins (selected by immunoblot) were identified by MALDI-TOF mass spectrometry. This isolation scheme yielded 27 IgE-binding wheat proteins, including the following previously described proteins: AAIs (Tri a 28; Tri a 28.0101; Tri a 29; Tri a 30; Tri a CM16 and Tri a aAI), non-specific LTP (Tri a 14), peroxidase, serpins and β-amylase (identified on 1D or 2D electrophoresis – 14, 16, 30). Some of them were purified here for the first time. In addition, we newly identified the following potential allergens: endogenous α-amylase/subtilisin inhibitor, trypsin/AAI CMX1/CMX3, TLP, XIP-1, β-glucosidase, class II chitinase and 26 kDa endochitinase as associated with food allergy. The fact that some of these proteins do not match wheat components identified so far (such as 26 kDa endochitinase, β-glucosidase) could be explained by the lack of knowledge of the complete genome. The corresponding protein/peptide sequences of wheat may be missing in the current databases.

The findings of the reactivity of patients' IgE with AAIs are not unambiguous. For instance, although the endogenous α-amylase/subtilisin inhibitor has not been reported as an allergen [25], we observed a reactivity of this purified protein with IgE Abs from 19 out of the 22 patients, albeit with only medium intensity (median 4). In previous studies, purified AATI CM3 but not CM2 or CM16 reacted with IgE from patients [17], and SPTs were positive with CM3 and CM16 but not CM2 in a patient with anaphylactic reaction to wheat flour [36]. We found that the most frequently recognized AAIs were 0.28 AAI and a mixture of CM16 and CM2, all of them reacting with IgE Abs of our patients. However, the frequency of IgE biding with other inhibitors, such as 0.19 AAI, 0.19 dimeric and CM17 was also high.

We succeeded in identifying and purifying wheat LTP, a food panallergen that is also a major allergen associated with baker's asthma [23]. We found that IgE Abs of 64% patients in our cohort reacted with our purified wheat LTP, which is a higher percentage then previously reported (41% and 28%) [16, 37]. We also purified tritin (a 29.6 kDa ribosome-inactivating protein from *T. aestivum)* previously identified from the water/salt-insoluble fraction of raw wheat [16]; its clinical relevance has not yet been clarified. This wheat component was recognized by all our patients (22/22) with high binding intensity (median 8), and also by some of our disease controls (7/10, median 2).

TLP is one of the successfully isolated new IgE-binding wheat proteins associated with food allergy. In our cohort, TLP was recognized by IgE Abs of all 22 patients and the intensity of IgE binding (median 8) was significantly higher when compared with disease controls (median 1). TLP, as well as wheatwin, are members of the family of pathogenesis-related proteins, which play a role in plant defence. TLP is an important allergen in several fruits, such as apples, cherries, kiwis and grapes [38–40], and wheat TLP has recently been reported as the allergen in baker's respiratory allergy [41]. We showed previously that wheatwin-2 is an IgE immunoreactive protein [14], and the results presented here showed that wheatwin-1

and wheatwin-2 have sequence homology of 97%. Using MALDI-TOF mass spectrometry, we observed at least two unique peptides for each wheatwin sequence (data not shown). Furthermore, we have shown for the first time that newly identified wheat components, such as TLP and wheatwin bind to IgE from patients and can activate patients' basophils to an extent comparable with that of the well-characterized allergens, AAI 0.19 (Tri a 28.0101) and LTP (Tri a 14).

In addition, we purified other new IgE-binding wheat components, such as XIP, class II chitinase and 26 kDa endochitinase, which were not previously identified as allergens in individuals with food allergy (XIP was identified in patients with baker's asthma [41]) These components reacted strongly (median 5.5–7.0) with the vast majority of patients' IgE Abs (20/22, 21/22 and 21/22, respectively) and with a lower intensity (median 0–1.5) and frequency (2/10, 5/8 and 5/9) with IgE of disease controls.

In conclusion, using preparative liquid-phase IEF and HPLC, we have developed a new procedure enabling us to purify native forms of wheat allergens in amounts sufficient for further structural and functional studies. The advantage of this approach is that it can be easily modified for isolation of various allergens (by changing pI range in Rotofor® or HPLC elution profile). We purified 27 IgE-binding wheat components, including seven new ones. We have shown for the first time that purified allergens, such as AAI 0.19, LTP, TLP and wheatwin, can activate patients' basophils, confirming that our purified proteins maintain their biological activity.

Interestingly, wheat proteins, CM17 (H5), CM17 and AAI 0.53 (H6) as well as newly identified TLP (H12), 26 kDa endochitinase (H13b), XIP (H14a) and class II chitinase (H14b), were shown to belong to those reacting with the highest frequency and intensity with IgE of patients (median 5.5–8) and with low intensity with IgE of disease controls (median 0–1). These proteins may be candidates for clinically relevant wheat allergens.

Our isolation procedure will allow preparation of native allergens for studies of their structural features (linear and conformational epitopes), including the impact of post-translational modifications (e.g. glycosylation) on allergenicity. Better knowledge of the allergenicity of native proteins could lead to a selection of an appropriate vector for recombinant allergen preparation, improving both diagnostic tests and therapy.

## Acknowledgements

This work was supported by grant GA310/07/0414 and 310/08/H077 from the Czech Science Foundation; SYNLAB.CZ (IMUMED); grant IAA500200801 from the Grant Agency of AS CR; Institutional Research Concept Grant AV0Z50200510; and grant 2B06155 and LC07017 from the Czech Ministry of Education, Youth and Sports.

## References


1 Poole JA, Barriga K, Leung DYM *et al.* Timing of initial exposure to cereal grains and the risk of wheat allergy. *Pediatrics* 2006; **117**:2175–82.

2 Ostblom E, Lilja G, Pershagen G, van Hage M, Wickman M. Phenotypes of food hypersensitivity and development of allergic diseases during the first 8 years of life. *Clin Exp Allergy* 2008; **38**:1325–32.

3 Pourpak Z, Mansouri M, Mesdaghi M, Kazemnejad A, Farhoudi A. Wheat allergy: clinical and laboratory findings. *Int Arch Allergy Immunol* 2004; **133**:168–73.

4 Varjonen E, Petman L, Mäkinen-Kiljunen S. Immediate contact allergy from hydrolyzed wheat in a cosmetic cream. *Allergy* 2000; **55**:294–6.

5 Palosuo K. Update on wheat hypersensitivity. *Curr Opin Allergy Clin Immunol* 2003; **3**:205–9.

6 Tordesillas L, Pacios LF, Palacin A *et al.* Molecular basis of allergen cross-reactivity: non-specific lipid transfer proteins from wheat flour and peach fruit as models. *Mol Immunol* 2009; **47**:534–40.

7 Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* 2001; **107**:891–6.

8 van Kampen V, Merget R, Rabstein S *et al.* Comparison of wheat and rye flour solutions for skin prick testing: a multi-centre study (Stad 1). *Clin Exp Allergy* 2009; **39**:1896–902.

9 Tokuda R, Nagao M, Hiraguchi Y *et al.* Antigen-induced expression of CD203c on basophils predicts IgE-mediated wheat allergy. *Allergol Int* 2009; **58**:193–9.

10 Constantin C, Touraev A, Heberle-Bors E, Quirce S, Huber WD, Valenta R. Detection of antigens reactive to IgE and IgA during wheat seed maturation and in different wheat cultivars. *Int Arch Allergy Immunol* 2009; **149**:181–7.

11 Werfel T, Breuer C. Role of food allergy in atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2004; **4**:379–85.

12 Brandtzaeg P. Food allergy: separating the science from the mythology. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 380–400.

13 Scibilia J, Pastorello EA, Zisa G *et al.* Wheat allergy: a double-blind, placebo-controlled study in adults. *J Allergy Clin Immunol* 2006; **117**:433–9.

14 Sotkovsky P, Hubalek M, Hernychova L *et al.* Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics* 2008; **8**: 1677–91.

15 Akagawa M, Handoyo T, Ishii T, Kumazawa S, Morita N, Suyama K. Proteomic analysis of wheat flour allergens. *J Agric Food Chem* 2007; **55**:6863–70.

16 Pastorello EA, Farioli L, Conti A *et al.* Wheat IgE-mediated food allergy in European patients: α-amylase inhibitors, lipid transfer proteins and low molecular weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int Arch Allergy Immunol* 2007; **144**:10–22.

17 Kusaba-Nakayama M, Ki M, Iwamoto M, Shibata R, Sato M, Imaizumi K. CM3, one of the wheat α-amylase inhibitor

subunits, and binding of IgE in sera from Japanese with atopic dermatitis related to wheat. *Food Chem Toxicol* 2000; **38**:179–85.

18 Kusaba-Nakayama M, Ki M, Kawada E *et al.* Intestinal absorbability of wheat allergens, subunits of wheat α-amylase inhibitor, expressed by bacteria. *Biosci Biotechnol Biochem* 2001; **65**:2448–55.

19 Weichel M, Vergoossen NJ, Bonomi S *et al.* Screening the allergenic repertoires of wheat and maize with sera from double-blind, placebo-controlled food challenge positive patients. *Allergy* 2006; **61**:128–35.

20 Jones SM, Magnolfi CF, Cooke SK, Sampson HA. Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol* 1995; **96**:341–51.

21 Sander I, Raulf-Heimsoth M, Duser M, Flagge A, Czuppon AB, Baur X. Differentiation between cosensitization and cross-reactivity in wheat flour and grass pollen-sensitized subjects. *Int Arch Allergy Immunol* 1997; **112**:378–85.

22 Pourpak Z, Mesdaghi M, Mansouri M, Kazemnejad A, Toosi SB, Farhoudi A. Which cereal is a suitable substitute for wheat in children with wheat allergy? *Pediatr Allergy Immunol* 2005; **16**: 262–6.

23 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**:1132–8.

24 Lauer I, Miguel-Moncin MS, Abel T *et al.* Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid transfer protein, Pru p 3. *Clin Exp Allergy* 2007; **37**:261–9.

25 Tatham AS, Shewry PR. Allergens in wheat and related cereals. *Clin Exp Allergy* 2008; **38**:1712–26.

26 Bohle B, Vieths S. Improving diagnostic tests for food allergy with recombinant allergens. *Methods* 2004; **32**:292–9.

27 Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005; **116**:608–13.

28 Palacin A, Varela J, Quirce S *et al.* Recombinant lipid transfer protein Tri a 14: a novel heat and proteolytic resistant tool for the diagnosis of baker's asthma. *Clin Exp Allergy* 2009; **39**:1267–76.

29 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**:7451–60.

30 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**:1229–34.

31 Bodinier M, Leroy M, Ah-Leung S *et al.* Sensitization and elicitation of an allergic reaction to wheat gliadins in mice. *J Agric Food Chem* 2009; **57**: 1219–25.

32 Mine Y, Yang M. Epitope characterization of ovalbumin in BALB/c mice using different entry routes. *Biochim Biophys Acta* 2007; **1774**:200–12.

33 Adel-Patient K, Nahori MA, Proust B *et al.* Elicitation of the allergic reaction in β-lactoglobulin-sensitized Balb/c mice: biochemical and clinical manifestations differ according to the structure of the allergen used for challenge. *Clin Exp Allergy* 2003; **33**:376–85.

34 Dearman RJ, Kimber I. Determination of protein allergenicity: studies in mice. *Toxicol Lett* 2001; **120**:181–6.

35 Gagné JP, Hunter JM, Labrecque B, Chabot B, Poirier GG. A proteomic approach to the identification of heterogeneous nuclear ribonucleoproteins as a new family of poly (ADP-ribose)-binding proteins. *Biochem J* 2003; **371**:331–40.

36 Zapatero L, Martínez MI, Alonso E *et al.* Oral wheat flour anaphylaxis related to wheat α-amylase inhibitor subunits CM3 and CM16. *Allergy* 2003; **58**:956.

37 Battais F, Courcoux P, Popineau Y *et al.* Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. *J Cereal Sci* 2005; **42**:109–17.

38 Gavrovic-Jankulovic M, Cirkovic T, Vuckovic O *et al.* Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J Allergy Clin Immunol* 2002; **110**: 805–10.

39 Breiteneder H. Thaumatin-like proteins: a new family of pollen and fruit allergens. *Allergy* 2004; **59**:479–81.

40 Pastorello EA, Farioli L, Pravettoni V *et al.* Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin. *J Allergy Clin Immunol* 2003; **111**: 350–9.

41 Lehto M, Airaksinen L, Puustinen A *et al.* Thaumatin-like protein and baker's respiratory allergy. *Ann Allergy Asthma Immunol* 2010; **104**:139–46.

## *2.3. Mouse model of food allergy*

In this study we showed that irreversible changes in secondary structure of egg allergen ovalbumin (OVA) caused by thermal processing significantly affect its digestion by gut enzymes and decrease its allergenicity in the mouse model of food allergy. Effect of increased temperature (70°C and 95°C) on OVA secondary structure was characterized by circular dichroism and by the kinetics of pepsin digestion with subsequent HPLC.

Publication:

**Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy.**
Goliáš J, Schwarzer M, Wallner M, Kverka M, Kozáková H, Šrůtková D, Klimešová K, Šotkovský P, Palová-Jelínková L, Ferreira F and Tučková L.

PLoS One. 2012; 7(5): e37156.

Egg white contains several allergens such as ovalbumin, ovomucoid, ovotransferin and lysozyme. Forming approximately 60% of the total egg white protein, OVA is by far the most abundant of them [Huntington et al., 2001]. Like the majority of food allergens OVA is consumed after thermal processing and these conditions can have a major impact on the molecular structure, susceptibility to enzymatic digestion in the gastrointestinal tract and allergenicity. However, the impact of different temperature treatment on the changes in the secondary structure of OVA and on its ability to induce clinical symptoms of food allergy hasn't been studied in detail.

BALB/c mice were sensitized intraperitoneally and challenged with repeated gavages of OVA or OVA heated to 70°C (h-OVA). Levels of allergen-specific serum antibodies were determined by ELISA (IgA and IgGs) or by β-hexosaminidase release test (IgE). Specific activities of digestive enzymes were determined in brush border membrane vesicles of jejunal enterocytes. Cytokine production and changes in regulatory T cells in mesenteric lymph nodes and spleen were assessed by ELISA and flow cytometry. Heating of OVA to 70°C caused mild irreversible changes in secondary structure compared to

boiling to 95°C (b-OVA), but both OVA treatments led to markedly different digestion kinetics and Tregs induction ability in vitro, compared to native OVA.

Although both heated and native OVA induced allergic diarrhea in BALB/c mice, the disease symptoms appeared much earlier and with a higher frequency in OVA fed mice than in those fed with h-OVA. Allergic diarrhea appeared in about 70% of mice already after the 5th i.g. dose of OVA, but only in 20% of those fed with h-OVA. After 7 i.g. doses, the disease symptoms were found in more than 90 % of OVA fed animals, but only in 35 % of those fed with h-OVA. At the end of the experiment (10 i.g. doses), the diarrhea was found in all mice fed with OVA, but only in 70% of mice fed with h-OVA.

To determine the effect of thermal processing of the allergen on the level and specificity of anti-OVA antibodies, the serum levels of IgE, IgG1, IgG2a and IgA against either OVA or h-OVA were determined in the course of the experiment. The level of IgE anti-OVA Abs was higher in response to native OVA than to h-OVA. In contrast, OVA-specific IgG2a was significantly higher after h-OVA feeding. The levels of the other two isotypes (IgG1 and IgA) were increased compared to controls but the differences corresponding to the two OVA forms were diminished towards the end of experiment. At the end of the experiment, we characterized the specificity and the degree of cross-reactivity of anti-OVA antibodies using ELISA with OVA, h-OVA or b-OVA bound as an antigen.

Increase of allergen-specific IgE is essential for mast cell activation and development of allergic diarrhea symptoms. We determined the level of MMCP-1 enzyme as the marker of mast cell activation and degranulation. In this case, the reducing effect of thermal processing was clearly demonstrated; the h-OVA induced only half the level of serum MMCP-1 compared to the native OVA.

Local and systemic cell responses to OVA and h-OVA were evaluated in all three groups of animals as in vitro cytokine production by MLN and splenocytes after exposure to corresponding allergens. The levels of TNF-α, IL-4, IL-5, IL-10 and IL-13 were higher in culture media obtained from MLN exposed to native OVA. The differences in cytokine secretion were less pronounced in the experiments with splenocytes cultures. Only the production of IFN-γ was higher after exposure to h-OVA as compared to OVA.

Further, we analysed the changes in Tregs in spleen and MLNs of OVA-, h-OVA- and PBS-treated mice at the end of the experiment. In spleen we observed a

decrease in Tregs in h-OVA treated mice, as compared to OVA- and PBS-treated mice. Only a non-significant increase was found in MLNs of h-OVA treated mice. To characterize the effect of heating and enzymatic digestion on T cell subpopulations, especially on regulatory T cell differentiation in more detail, splenocytes from naive (untreated) BALB/c mice were cultured in vitro either with OVA, h-OVA or b-OVA as well as with their peptic digests. The *in vitro* stimulation of splenocytes with undigested heated proteins led to a slight increase in proportion of CD4+Foxp3+ Treg cells compared to native form of OVA. Interestingly, 20 min peptic digests of heated forms of OVA induced increased proportion of Tregs, but this ability decreased again after 40 min of digestion. In contrast the pepsin digestion did not change the ability of native OVA to slightly increase the proportion of Tregs.

In conclusion, we showed that even a mild change in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigenic properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 to Th1-type response as compared to native OVA. Nevertheless, the h-OVA fragments still have the ability to induce allergic symptoms, but these are less pronounced and need longer time to develop.

PLoS one

# Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy

**Jaroslav Golias[1]☯, Martin Schwarzer[2]*☯, Michael Wallner[3], Miloslav Kverka[1], Hana Kozakova[2], Dagmar Srutkova[2], Klara Klimesova[1], Petr Sotkovsky[1], Lenka Palova-Jelinkova[1], Fatima Ferreira[3], Ludmila Tuckova[1]**

**1** Department of Immunology and Gnotobiology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic, **2** Department of Immunology and Gnotobiology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Novy Hradek, Czech Republic, **3** Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria

## Abstract

***Background and Aims:*** The egg protein ovalbumin (OVA) belongs to six most frequent food allergens. We investigated how thermal processing influences its ability to induce allergic symptoms and immune responses in mouse model of food allergy.

***Methodology/Principal Findings:*** Effect of increased temperature (70°C and 95°C) on OVA secondary structure was characterized by circular dichroism and by the kinetics of pepsin digestion with subsequent HPLC. BALB/c mice were sensitized intraperitoneally and challenged with repeated gavages of OVA or OVA heated to 70°C (h-OVA). Levels of allergen-specific serum antibodies were determined by ELISA (IgA and IgGs) or by β-hexosaminidase release test (IgE). Specific activities of digestive enzymes were determined in brush border membrane vesicles of jejunal enterocytes. Cytokine production and changes in regulatory T cells in mesenteric lymph nodes and spleen were assessed by ELISA and FACS. Heating of OVA to 70°C caused mild irreversible changes in secondary structure compared to boiling to 95°C (b-OVA), but both OVA treatments led to markedly different digestion kinetics and Tregs induction ability in vitro, compared to native OVA. Heating of OVA significantly decreased clinical symptoms (allergic diarrhea) and immune allergic response on the level of IgE, IL-4, IL-5, IL-13. Furthermore, h-OVA induced lower activities of serum mast cell protease-1 and enterocyte brush border membrane alkaline phosphatase as compared to native OVA. On the other hand h-OVA stimulated higher IgG2a in sera and IFN-γ secretion by splenocytes.

***Conclusions:*** Minor irreversible changes in OVA secondary structure caused by thermal processing changes both its digestion and antigenic epitopes formation, which leads to activation of different T cell subpopulations, induces shift towards Th1 response and ultimately reduces its allergenicity.

**Citation:** Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, et al. (2012) Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy. PLoS ONE 7(5): e37156. doi:10.1371/journal.pone.0037156

**Editor:** Lucienne Chatenoud, Université Paris Descartes, France

**Received** December 9, 2011; **Accepted** April 14, 2012; **Published** May 21, 2012

**Copyright:** © 2012 Golias et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by grants from the Czech Science Foundation (GA310/07/0414, 310/08/H077 and 310/08/H077), http://www.gacr.cz/; Synlab (http:www.synlab.cz), the Academy of Sciences of the Czech Republic (IAA500200801, IAA500200710 and KJB500200904), http://www.gaav.cz/; the Czech Ministry of Education, Youth and Sports (2B06155 and LC07017), http://www.msmt.cz; and by Institutional Research Concept Grant AV0Z50200510, http://www.isvav.cz. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: schwarzer@biomed.cas.cz

☯ These authors contributed equally to this work.

## Introduction

Food allergy is a serious health concern affecting 6–8% of young children and about 2–4% of adults. Allergies to eggs, milk and peanut are currently the most frequent food allergies and their prevalence, severity and persistence has been increasing during the last decades. Food allergy is considered mainly as an IgE-mediated type I hypersensitivity, characterized by an increased production of IgE antibodies and Th2 cytokines, common markers found both in human disease and in experimental models [1–4].

Depending on the route of exposure, dose of allergen and the presence of suitable adjuvant, the immune response can result in either sensitization or oral (mucosal) tolerance induction [4–6]. In mouse models of food allergy, oral administration of allergen usually results in oral tolerance induction, but its co-administration with strong mucosal adjuvant such as cholera toxin or with anti acid drugs (increasing gastric pH) could be used for allergic sensitization [7–10]. Another reliable and effective approach to overcome the oral tolerance induction is pretreatment of mice by systemic intraperitoneal (*i.p.*) administration of allergen with aluminum hydroxide (alum) as adjuvant followed by repeated

intra-gastric treatments. This experimental model mimics a mild form of human allergy with IgE–mediated mast cell degranulation causing increased small intestine permeability [2,11,12] with diarrhea as one of the symptoms of anaphylaxis. Histological examination of small intestine reveals changes of epithelium, *e.g.* alteration in number of goblet cells and mucin production and the damage of tips of villi, as well as changes of lamina propria, *e.g.* increased cell infiltration and/or activation [13–15].

The mucosa of small intestine is an actively metabolizing, rapidly proliferating, absorptive epithelium with nutritional and homeostatic functions. The activity of brush border enzymes is sensitive marker of intestinal cell differentiation and postnatal development, reflecting both dietary changes and microbial colonization [16–18]. Partial and subtotal atrophy of the villous apparatus was shown to correlate with the activity and expression of alkaline phosphatase [19]. Moreover, this enzyme may be also involved in host's defense against pathological stress-induced damage, such as during inflammation and infection [20].

Egg white contains several allergens such as ovalbumin (OVA), ovomucoid, ovotransferin and lysozyme. Forming approximately 60% of the total egg white protein, OVA is by far the most abundant of them [21]. Like the majority of food allergens OVA is consumed after thermal processing and it has been shown that after heating its molecular structure as well as allergenicity is altered [22,23]. However, it should be considered that egg allergens are processed at different temperatures (baked, scrambled or soft/hard boiled eggs or even native as whipped egg white) and these processing conditions can have a major impact on the secondary structure, susceptibility to enzymatic digestion in the gastrointestinal tract and allergenicity. Partial decrease of IgE binding after OVA thermal processing suggested that both linear and conformational epitopes participate in the OVA-IgE specific interactions [22–24]. Moreover, heating of allergens can lead to their aggregation, which reduces their absorption and transport through epithelial layer and thus decreases their allergenicity [25]. However, the impact of different temperature treatment on the changes in the secondary structure of OVA and on its ability to induce clinical symptoms of food allergy hasn't been studied in detail.

In the present study we show that heating of hen egg allergen OVA to 70°C has only minor effect on its secondary structure. However, these minor changes lead to different kinetics and occurrence of fragments after digestion. This result in activation of different T cell subpopulations and changes in both cytokine production and specific antibody formation, which leads to significant reduction of egg allergy symptoms.

## Materials and Methods

### Ethics Statement

All animal experiments were approved by the Laboratory Animal Care and Use Committee of the Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic, approval ID: 94/2006 and 244/2009.

### Animals

Two month-old female BALB/c mice (*H-2b*) (Animal facility of the Institute of Physiology ASCR, Czech Republic) were kept under standard conditions, fed by OVA-free diet and water *ad libitum*.

### Ovalbumin Preparation

For *i.p.* sensitization, OVA (Worthington, Lakewood, NJ, USA) and heated OVA (h-OVA; prepared by exposure of OVA to 70°C for 10 minutes, enabling accurate and reproducible dosing) were dissolved in phosphate-buffer saline (PBS) to a final concentration of 300 μg/ml containing 5 mg/ml of alum adjuvant (Sigma, Steinheim, Germany). For oral administration, OVA and h-OVA were dissolved in PBS to a final concentration of 100 mg/ml. For *in vitro* studies boiled OVA (b-OVA) was prepared by exposure of OVA to 95°C for 10 minutes. EndoGrade® Ovalbumin (Hyglos GmbH, Germany) with endotoxin content <1 EU/mg was used for enzymatic digestion and *in vitro* stimulation.

### Circular Dichroism

Protein secondary structure elements were determined by CD spectroscopy. Spectra were recorded in 5 mM sodium phosphate buffer (pH 7.4) with a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier single position cell holder (Jasco, Tokyo, Japan). All spectra are baseline-corrected and presented as mean residue molar ellipticity $[\Theta]_{MRW}$ at a given wavelength. Thermal denaturation of proteins was monitored from 20°C to 70°C or from 20°C to 95°C at the fixed wavelength of 222 nm with a temperature slope of 1°C/min. The melting point (Tm) was calculated from the inflection point of the resulting sigmoid curve [26].

### Enzymatic Digestion and HPLC Separation of Ovalbumin Fragments

Peptides of OVA, h-OVA or b-OVA were prepared using pepsin-agarose gel similarly as described previously [27]. Briefly, digestion of proteins was stopped after 20, 40, or 60 minutes by removing the pepsin-agarose gel by centrifugation (10 min; 1500 g) and by neutralization with 1 M NaOH to final pH 7. Digested or undigested proteins were separated using SP 250/10 NUCLEOSIL 300-7 C18 column (Macherey-Nagel, Düren, Germany) on the HPLC system Gold 125NM Solvent Module (Beckman Coulter, Miami, FL, USA). Samples were applied on columns and separated as described previously [27]. For *in vitro* stimulations, digests were dissolved in complete RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 500 μg/ml.

### Experimental Protocol

Mice were divided into the three groups according to the treatment – OVA, h-OVA and PBS (controls). Mice were sensitized *i.p.*, with a two week interval, with 60 μg of either OVA or h-OVA together with 1 mg of alum in a final volume of 200 μl PBS on day 1 and 14. Control mice received only 200 μl PBS containing 1 mg of alum. Two weeks later, the mice were challenged 10 times at 2–3 days intervals by *i.g.* gavages of 15 mg of OVA in a final volume of 150 μl PBS. Diarrhea was assessed visually by monitoring mice for 30 minutes after each *i.g.* exposure. Body weight was recorded before gavage and rectal temperature both before and 30 minutes after each *i.g.* exposure.

### Quantification of OVA-specific Antibodies and Mast Cell Protease-1

Blood samples were collected before the first *i.p.* injection, during the experiment and at sacrifice. Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA [28]. Briefly, 96-well microtiter plates were coated either with OVA, h-OVA or b-OVA (5 μg/ml). Serum samples were diluted 1/10000 for IgG1, 1/100 for IgG2a and 1/10 for IgA. Rat anti-mouse IgG1, IgG2a and IgA antibodies (Abs) (1 μg/ml Pharmingen, San Diego, CA, USA) were applied, followed by peroxidase-conjugated mouse anti-rat IgG Abs (1/1000; Jackson, Immuno Labs., West

Grove, PA, USA) for detection. Antibody levels were reported as optical density (OD). As it was shown that allergen-specific IgG interferes with allergen-specific IgE detection [29], allergen-specific IgE levels in sera were quantified by degranulation of rat basophil leukemia (RBL-2H3) cells (originally described by [30], kindly provided by prof. Ursula Wiedermann). RBL-2H3 cells were plated in 96-well tissue culture plates ($4\times10^4$ cells/per well) and passively sensitized by incubation with mouse sera in a final dilution of 1/90 for 2 hours. After washing, OVA, h-OVA or b-OVA (0.6 μg/ml) were added for 30 min at 37°C to induce degranulation. Supernatants were incubated with 4-methylum-belliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader ($\lambda_{ex}$:360 nm/$\lambda_{em}$:465 nm) Infinite M200 (Tecan Group Ltd., Grödig, Austria). Results are reported as percentage of total β-hexosaminidase release from cells after disruption with 1% Triton X-100.

Levels of serum mouse mast cell protease-1 (MMCP-1) enzyme were determined by commercial kit (eBioscience, San Diego, USA) according to manufacturer's instructions. Sacrifice sera were diluted 1/250 and the MMCP-1 levels are reported as ng/ml.

### Cell Culture and Cytokine Evaluation

Mesenteric lymph nodes (MLN) and spleens were removed at sacrifice. Single-cell suspensions were prepared in RPMI-1640 containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). Cells ($6\times10^5$/well) were cultured in a flat-bottom 96-well plate (TPP, Trasadingen, Switzerland) without any stimuli or in the presence of either OVA or h-OVA (100 μg/well) for 72 hours (37°C, 5% $CO_2$). Supernatants were collected and stored at –40°C until analyses. IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, INF-γ and TNF-α were determined by the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Panel (Millipore, Billerica, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, Hercules, USA) with sensitivities <0.3 pg/ml for IL-4, <0.8 pg/ml for IL-5, <2.1 pg/ml for IL-6, <2.6 pg/ml for IL-10, <12.4 pg/ml for IL-13, <0.7 pg/ml for IL-17, <1.1 pg/ml for IFN-γ and <3.1 pg/ml for TNF-α. Values are reported in pg/ml after subtraction of baseline levels of non-stimulated cultures. Values below assay sensitivity were considered non-detectable (n.d.). In order to measure the capacities of OVA, h-OVA and b-OVA and their peptic digests (100 μg/well) to induce Tregs, we cultivated them with naïve mouse splenocytes for 48 hours.

### Flow Cytometry Analysis

Single-cell suspensions of spleens or MLN were stained for regulatory T cells using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA, USA) with fluorochrome labeled anti-mouse monoclonal Abs: CD3e-Fluorescein isothiocyanate (eBioscience; clone 145-2C11), CD4-Qdot® 605 (Invitrogen, clone RM4-5), CD25-Alexa Fluor® 700 (eBioscience; clone PC61.5) and Foxp3-phycoerythrin (eBioscience; clone FJK-16s) according to the manufacturer's recommendation. Flow cytometric analysis was performed on LSRII (BD Biosciences, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

### Determination of Enterocyte Brush-border Enzyme Activities

Jejunum was removed, washed with cold saline and brush border membrane vesicles (BBMV) were prepared from jejunal scrapings as described by Kessler *et al.* [31]. Protein concentration in BBMV was determined by the method of Lowry *et al.* [32] using bovine serum albumin, fraction V (Serva, Heidelberg, Germany) as standard. The activity of alkaline phosphatase (EC 3.1.3.1), γ-glutamyltranspeptidase (EC 2.3.2.2), dipeptidyl peptidase IV (EC 3.4.14.5), lactase (EC 3.2.1.23/62/108) and sucrase (EC 3.2.1.48/10) were determined as described previously [33]. Enzyme activities were expressed in nkat/mg protein, 1 nkat being the amount of the enzyme that converts 1 nmol of substrate per second under the given conditions.

### Histology and Morphometry

Intestinal tissue sections were fixed immediately in 4% formalin. The fixed tissues were cut and processed using routine methods. Paraffin sections (5 μm) were deparaffinized in xylene, rehydrated through an ethanol gradient to water and stained by hematoxylin-eosin. Villus height was evaluated under the Olympus BX 40 microscope equipped with Photo camera DP 70 using program QuickPhoto Micro 23 program (Olympus, Japan). The mean height of 20–30 villi ± SEM was calculated.

### Statistical Analysis

Differences between multiple experimental groups were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and differences between two groups were evaluated using unpaired two-tailed Student's *t*-test. Data were expressed as the mean ± SEM unless otherwise stated. GraphPad Prism statistical software (version 5.03 GraphPad Software, La Jolla, CA, USA) was used for analyses.

## Results

### The Effect of Thermal Processing on OVA Secondary Structure and Enzymatic Digestion

Since eggs could be consumed after various kinds of processing, we analyzed the effect of different temperatures on the secondary structure of OVA allergen. Employing the circular dichroism technique, we found that heating to 70°C or 95°C causes irreversible changes in secondary structure of OVA allergen (Fig. 1). The structural changes induced by heating were accompanied by different susceptibility to pepsin digestion. HPLC elution profiles of pepsin-digested OVA, h-OVA or b-OVA were documented after 20 and 40 minutes (Fig. 2). The majority of native OVA was split to fragments after 20 min, while the majority of both forms of heated OVA remained undigested. However, while both h-OVA and b-OVA had similar peptide profiles after 20 or 40 min of digestion, these were both quite different from those of untreated OVA (Fig. 2). The profiles after 40 min of digestion remained almost unchanged after 60 min of digestion (data not shown).

### Experimental Allergic Diarrhea Induced by OVA and Heated-OVA

Allergic diarrhea appeared in about 70% of mice already after the $5^{th}$ *i.g.* dose of OVA, but only in 20% of those fed with h-OVA. After 7 *i.g.* doses, the disease symptoms were found in more than 90% of OVA fed animals, but only in 35% of those fed with h-OVA. At the end of the experiment (10 *i.g.* doses), the diarrhea was found in all mice fed with OVA, but only in 70% of mice fed with h-OVA (Fig. 3a, b). There were small, non-significant differences in body weight and in rectal temperature after each *i.g.* dose of either OVA or h-OVA and PBS control group (data not shown). Morphometry analysis of histological

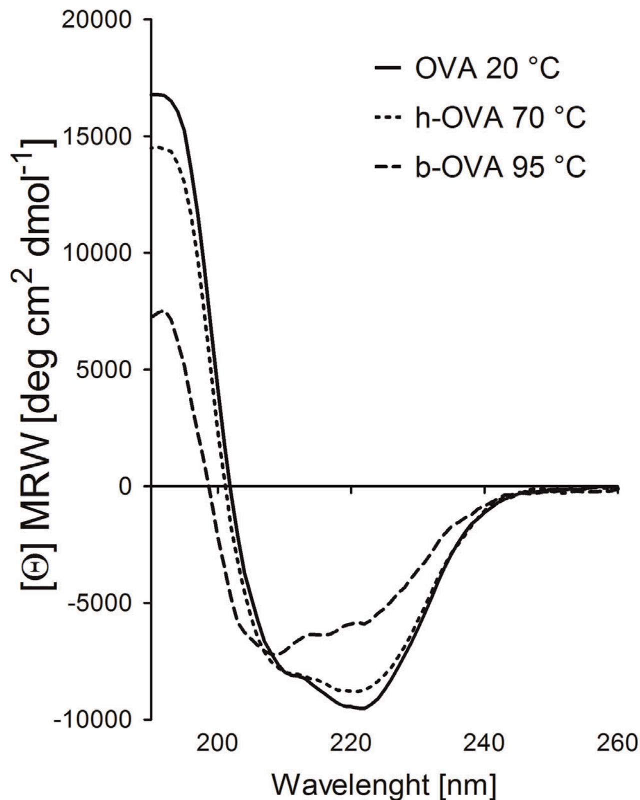



**Figure 1. Circular dichroism spectra of native and heated-OVA.** Circular dichroism spectra showed only minor irreversible structural changes of hen egg ovalbumin-OVA heated for 10 minutes at 70°C (h-OVA, dotted line) as compared to OVA heated at 95°C (b-OVA, dashed line). Spectra were taken after renaturation at 20°C, native conformation of OVA at 20°C is shown as control (solid line).
doi:10.1371/journal.pone.0037156.g001

pictures documented shortening of villi in mice treated with either form of OVA, as compared to PBS-treated controls (PBS 190.2±5.1 μm, OVA 157.7±14.0* μm, h-OVA 161.4±6.0** μm).

## OVA and h-OVA Treatment Changes Activity of Brush-border Hydrolases

The brush-border membrane hydrolases are enzymes involved in the final steps of digestion processes. We tested if these enzymes are involved in small intestine homeostasis and could be therefore considered as new markers in food allergy. We determined their activities in the jejunum of OVA-, h-OVA- and PBS-treated mice (Table 1). We found that the specific activity of alkaline phosphatase was significantly higher in mice treated with native OVA but only slightly increased in those exposed to h-OVA, as compared with PBS-treated mice. On the other hand, as compared to PBS-treated controls, both OVA and h-OVA treatments significantly decreased the specific activity of dipeptidyl peptidase IV. We did not observe any significant changes among the three experimental groups in the levels of glutamyl transpeptidase, lactase or sucrase (Table 1).

## Thermal Processing of OVA Changes the Kinetics of OVA-specific Antibody Responses and the Levels of Serum MMCP-1

To determine the effect of thermal processing of the allergen on the level and specificity of anti-OVA antibodies, the serum levels of IgE, IgG1, IgG2a and IgA against either OVA or h-OVA were determined in the course of the experiment. As shown in Fig. 3c the level of IgE anti-OVA Abs was higher in response to native OVA than to h-OVA. In contrast, OVA-specific IgG2a was significantly higher after h-OVA feeding. The levels of the other two isotypes (IgG1 and IgA) were increased compared to controls but the differences corresponding to the two OVA forms were diminished towards the end of experiment. At the end of the

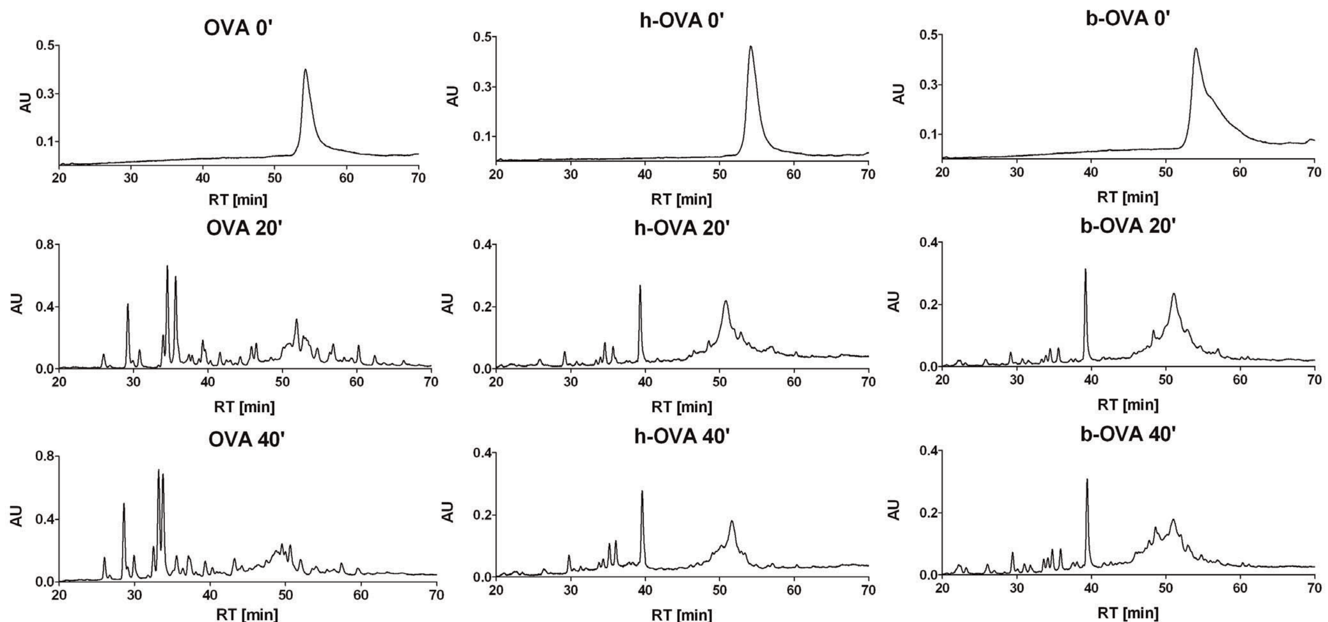



**Figure 2. RP-HPLC separation profile of native-OVA and heated-OVA peptic digests.** RP-HPLC separation profile monitored at 280 nm corresponds to OVA and OVA heated at 70°C (h)-OVA or boiled at 95°C (b)-OVA undigested (0′) and after 20 (20′) and 40 minutes (40′) of digestion by pepsin. RT – retention time.
doi:10.1371/journal.pone.0037156.g002

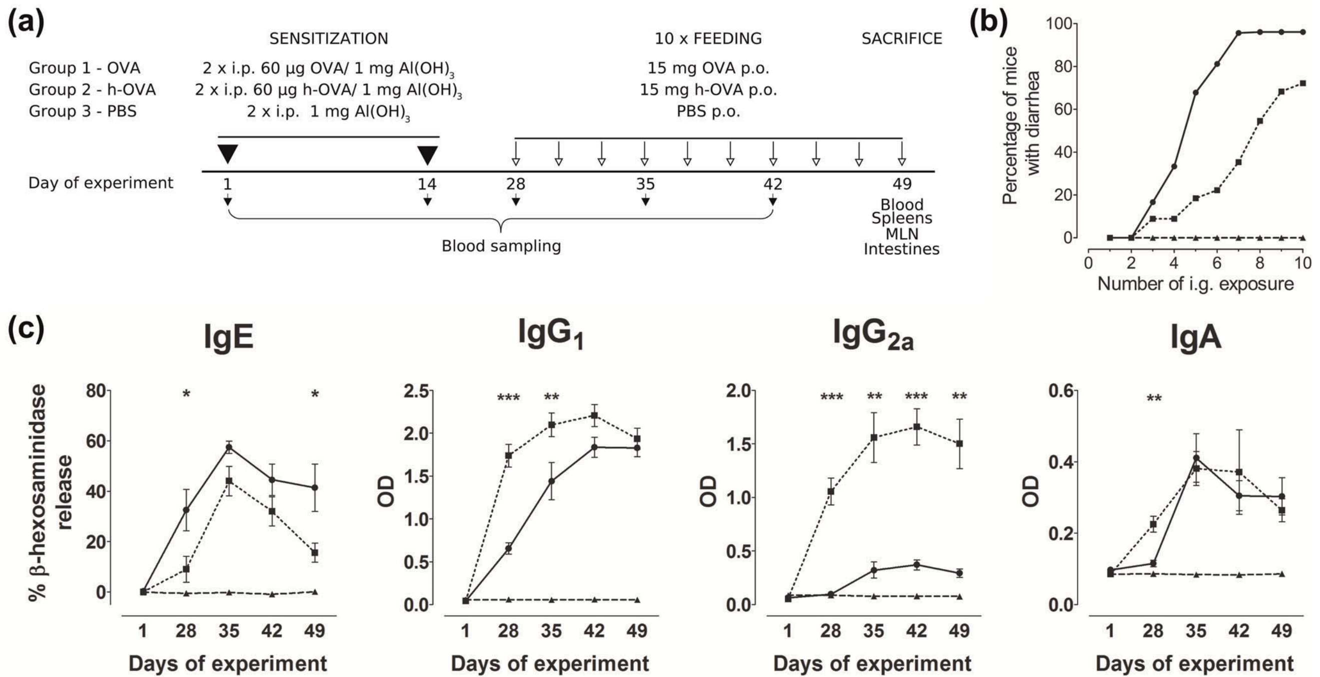



**Figure 3. Impact of heating on OVA-induced allergic response. Experimental design (a).** Mice were sensitized twice intraperitoneally (*i.p.*) with OVA/Al(OH)$_3$, heated OVA (h-OVA)/Al(OH)$_3$ or PBS/Al(OH)$_3$ alone and subsequently challenged with ten doses of OVA, h-OVA or PBS by intragastric tubing (*i.g.*). Blood samples were taken at indicated time points for antibody analysis. At the end of the experiment, spleens and mesenteric lymph nodes were taken for FACS and cytokine assays, small intestine for histology and enterocyte brush border for enzyme activity analysis. **Occurrence of allergic diarrhea** (**b**). Occurrence of allergic diarrhea in OVA (solid line) or h-OVA (dotted line) challenged mice, data pooled from three independent experiments. PBS controls are shown as dashed line. **The kinetics of specific Abs formation** (**c**). Levels of specific antibodies in sera from mice exposed to OVA (solid line), h-OVA (dotted line) or PBS (dashed line) were detected by ELISA (IgA, IgG1and IgG2a) or by β-hexosaminidase release assay (IgE). Data are represented as mean ± SEM (n = 10 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001.
doi:10.1371/journal.pone.0037156.g003

experiment, we characterized the specificity and the degree of cross-reactivity of anti-OVA antibodies using ELISA with OVA, h-OVA or b-OVA bound as an antigen (Fig. S1). The levels of OVA-specific antibodies remained unchanged, when we used h-OVA or OVA as a coating antigen, except for IgG1, which levels were significantly higher, when h-OVA instead of OVA was used. When b-OVA was used as coating antigen, the response of both OVA- and h-OVA treated mice decreased significantly in all measured isotypes.

Increase of allergen specific IgE is essential for mast cell activation and development of allergic diarrhea symptoms. We determined the level of MMCP-1 enzyme as the marker of mast cell activation and degranulation. In this case, the reducing effect of thermal processing was clearly demonstrated; the h-OVA induced only half the level of serum MMCP-1 compared to the native OVA (Fig. 4).

**Table 1.** Specific activities of enterocyte brush-border enzymes (nkat/mg protein) in jejunum of treated mice.

| Enzyme (nkat/mg protein) | OVA | h-OVA | PBS |
|---|---|---|---|
| Alkaline phosphatase | 14.26±1.09*** | 10.08±0.84[#] | 8.27±0.29 |
| GGT | 10.79±3.33 | 8.64±1.59 | 9.74±2.02 |
| DPP IV | 4.61±0.50** | 5.39±0.45* | 7.39±0.77 |
| Lactase | 9.19±0.63 | 8.37±0.59 | 9.99±1.61 |
| Sucrase | 32.16±8.04 | 36.00±4.10 | 27.30±4.24 |

GGT – gamma-glutamyltranspeptidase, DPP IV – Dipeptidyl peptidase IV. Values are expressed as the mean ± SEM.
*$P<0.05$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
**$P<0.01$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
***$P<0.001$ ovalbumin-treated group (OVA) *vs.* PBS-treated group.
[#]$P<0.05$ heated-ovalbumin-treated group (h-OVA) *vs.* ovalbumin-treated group (OVA).
doi:10.1371/journal.pone.0037156.t001

## Ex vivo Cytokine Production by MLN and Splenocytes Induced by OVA Allergens

Local and systemic cell responses to OVA and h-OVA were evaluated in all three groups of animals as *in vitro* cytokine production by MLN and splenocytes after exposure to corresponding allergens. Cytokine production from controls (PBS group) was low or not detectable and did not change after exposure to either form of OVA (data not shown). As shown in Fig. 5a, the levels of TNF-α, IL-4, IL-5, IL-10 and IL-13 were higher in culture media obtained from MLN exposed to native OVA. The differences in cytokine secretion were less pronounced in the experiments with splenocytes cultures (Fig. 5b). Only the production of IFN-γ was higher after exposure to h-OVA as compared to OVA. Levels of IL-6 and IL-17 didn't differ among the groups neither in MLN nor in spleen (data not shown).

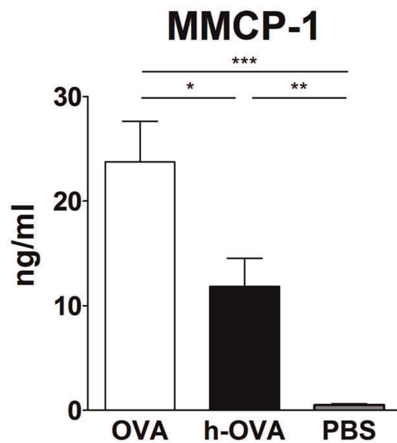



**Figure 4. Decreased mast cell protease induction by heated-OVA.** Heated OVA (h-OVA, black bar) induced significantly lower amounts of mast cell protease (MMCP-1), the marker of mast cell activation, compared to mice fed with native OVA (white bar). Data are represented as mean ± SEM (n = 10 mice/group), representative data from one out of three independent experiments. $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$.
doi:10.1371/journal.pone.0037156.g004

### Differentiation of CD4+CD25+Foxp3+ T Cells in OVA and h-OVA Fed BALB/c Mice

Since regulatory T cells (Tregs) are known to be crucial for induction of oral tolerance to protein antigens [34], we analyzed the changes in Tregs in spleen and MLNs of OVA-, h-OVA- and PBS-treated mice at the end of the experiment. In spleen we observed a decrease in Tregs in h-OVA treated mice, as compared to OVA- and PBS-treated mice (Fig. 6). Only a non-significant increase was found in MLNs of h-OVA treated mice.

### Induction of CD4+Foxp3+ T Cells by h-OVA and b-OVA in vitro is Increased After a 20-min Pepsin Digestion

To characterize the effect of heating and enzymatic digestion on T cell subpopulations, especially on regulatory T cell differentiation in more detail, splenocytes from naïve (untreated) BALB/c mice were cultured *in vitro* either with OVA, h-OVA or b-OVA as well as with their peptic digests. As shown in Fig. 7, the *in vitro* stimulation of splenocytes with undigested heated proteins led to a slight increase in proportion of CD4+Foxp3+ Treg cells compared to native form of OVA. Interestingly, 20 min peptic digests of heated forms of OVA induced increased proportion of Tregs, but this ability decreased again after 40 min of digestion. In contrast the pepsin digestion did not change the ability of OVA to slightly increase the proportion of Tregs as compared to undigested OVA.

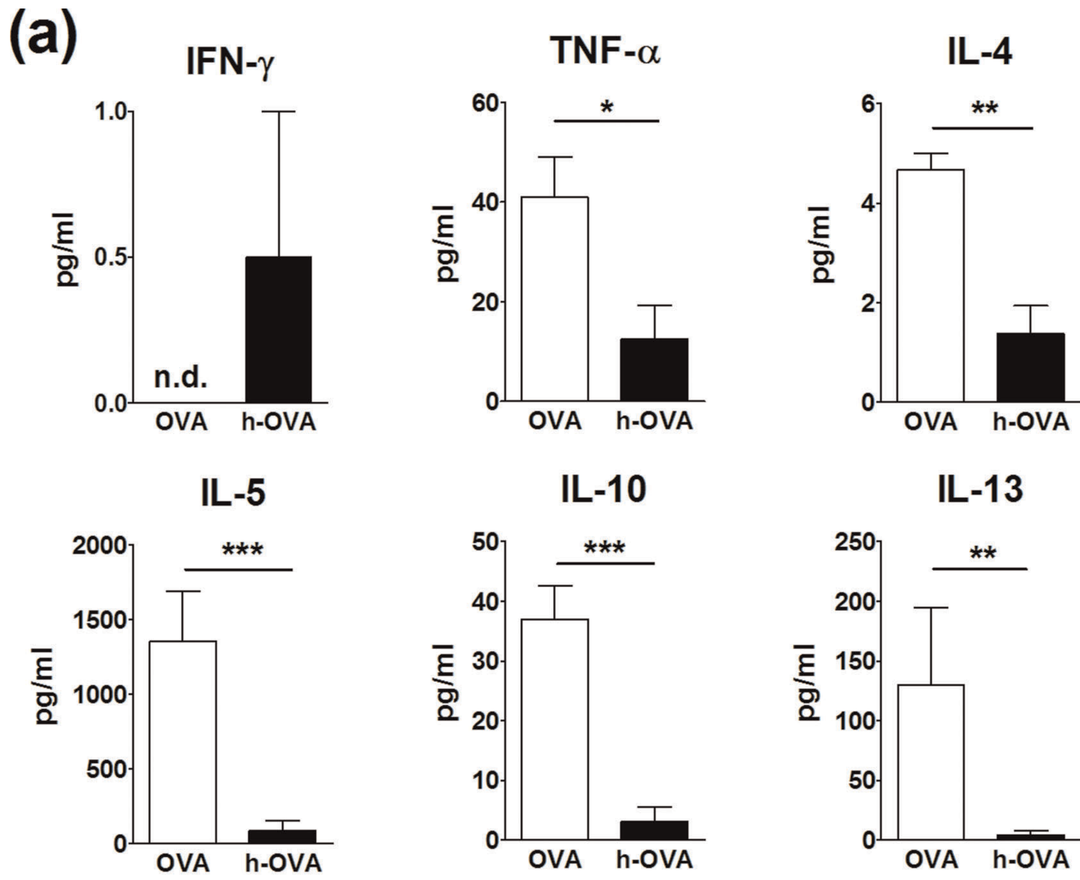
## Discussion

In this study, we showed that small irreversible changes in secondary structure of egg allergen OVA caused by thermal processing significantly affect its digestion by gut enzymes and decrease its allergenicity in the mouse model of food allergy. While both heated and native OVA induced allergic diarrhea in BALB/c mice, the disease symptoms appeared much earlier and with a higher frequency in OVA fed mice than in those fed with h-OVA. As compared to OVA-treated group, the sera of h-OVA-treated mice contained also significantly lower levels of specific IgE and MMCP-1, known markers of mast cell activation and degranulation [35]. It seems that even slight changes in the secondary structure elements have a high impact on the immunological behavior of the allergen. This could be explained by differences either in allergen absorption, which could lead to a decrease in allergen exposure, or in allergen digestion, which leads to production of peptides with different allergenicity and to a partial loss of conformational epitopes and/or exposure of new linear epitopes to immune cells.

The small intestine is noted for its plasticity in response to various dietary changes, which may be reflected in activation of enterocyte brush-border enzymes. Here we demonstrated for the first time that alkaline phosphatase (ALP) can be used as a new marker in food allergy, because its specific activity was significantly increased in OVA-treated group compared to controls. This is in line with the recent findings that ALP has a crucial role in regeneration of enterocytes and that its activity correlates with villous atrophy [19,36]. We can speculate that the increased level of ALP contributes to restoration of homeostasis in the enterocyte membranes after long-term stimulation with OVA allergens. On the contrary, dipeptidyl peptidase IV (CD26 - that cleaves L-alanine or L-proline residues in the penultimate N-terminal position) was significantly reduced in both OVA- and h-OVA-treated groups, as compared to PBS controls. Interestingly, a decrease in CD26 was found in patients with celiac disease induced by gluten, which belongs to wheat components responsible for food- or wheat-dependent exercise-induced allergy and for occupational asthma [37,38].

The changes in secondary structure by heating could influence antibody response *in vivo*. Here we report that OVA induced significantly higher levels of OVA-specific IgE and lower levels of IgG2a, as compared to h-OVA. High levels of potentially "blocking" IgG2a (mouse homolog of human IgG4) may compete for allergen [22]. The ability of heat-denatured allergens to induce Th1 associated IgG2a was also shown for other allergens, such as bee venom or birch pollen [39]. However, the effect cannot be generalized, because in a recent study by van der Ventel [15] a higher sensitizing potential was shown for cooked fish proteins. Surprisingly, when we changed the coupling allergen (h-OVA was used for OVA sensitized sera and vice versa) the binding of specific Abs was retained. Moreover, the binding was significantly higher when h-OVA antigen was used for specific IgG1 antibody determination. We assume that this is caused by heating-uncovered linear epitopes (supplementing the loss of the conformational ones), which are then presented after processing by antigen-presenting cells to T and B lymphocytes. On the other hand, when the extensively heated b-OVA was used, we observed a strong drop in the signal in all OVA-specific antibodies, which correlated with observed circular dichroism structural changes, and suggested the importance of structural epitopes in specific antibody formation.

Next, we addressed the question if the differences in OVA and h-OVA-specific antibody responses are also associated with cytokine milieu. On the local level in MLNs, we found a significantly higher production of Th2 cytokines in the OVA-treated mice, accompanied by proinflammatory TNF-$\alpha$ production after an *in vitro* exposure to OVA. Surprisingly, we determined an up-regulation of regulatory cytokine IL-10, which could be a result of a biological feedback aimed at dampening down the local inflammation, similar to chronic experimental colitis [40]. OVA-treatment did not significantly influence cytokine production in splenocytes, except for IFN-$\gamma$, which was produced predominantly by h-OVA stimulated splenocytes. The same observation was recently made by van der Ventel [15], who showed an increased IFN-$\gamma$ production by splenocytes of mice challenged with heated fish extract. Our findings suggest that heating of OVA induces


(a)
IFN-γ
TNF-α
IL-4
IL-5
IL-10
IL-13
pg/ml
n.d.
*
**
***
OVA
h-OVA

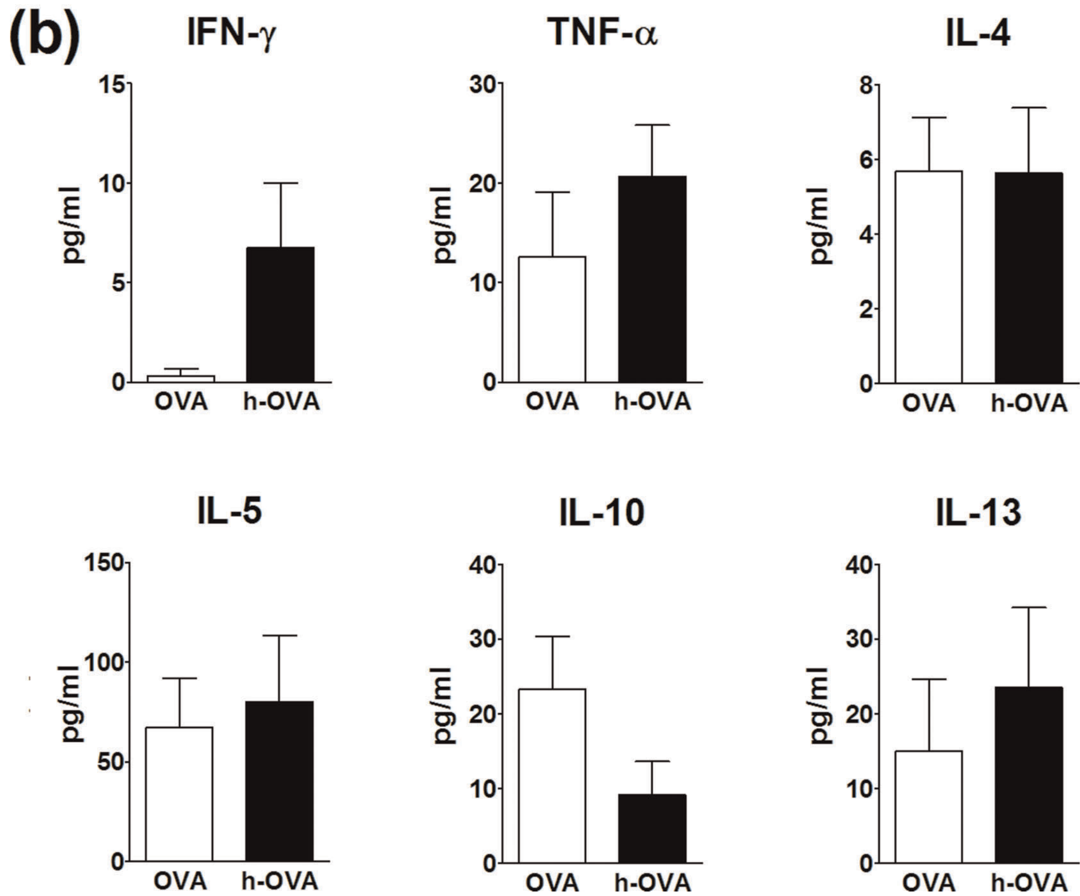

(b)
IFN-γ
TNF-α
IL-4
IL-5
IL-10
IL-13
pg/ml
OVA
h-OVA

**Figure 5. Cytokine production after *in vitro* restimulation with OVA.** The cytokine production from mesenteric lymph nodes (a) and splenocytes (b) of BALB/c mice fed with OVA (white bars) or h-OVA (black bars) and stimulated *in vitro* with appropriate allergen. Cytokine levels are expressed after subtraction of base line levels of unstimulated lymph node cells or splenocytes. Data shown are mean values ± SEM (n = 4–7 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001, n.d. = not detectable.
doi:10.1371/journal.pone.0037156.g005

changes in its digestion and processing by immune cells that lead to changes in the local cytokine environment ultimately leading to a shift from Th2- toward Th1-type response, reduction in the level of specific IgE and an increased production of blocking IgG2a antibodies [22]. These data fit well with clinical symptoms observed in allergic subjects in response to heated egg allergens [5,24].

Moreover, our results support recent data showing that thermal processing interferes with OVA stability [23]. Here, we show that h-OVA and b-OVA are initially (at 20 min) more resistant to proteolysis than native OVA. The difference in degradation kinetics could be explained by partial aggregation of heated forms of OVA, which makes the target structures less accessible for the enzyme. Nevertheless, after 40 min digestion the number of h-OVA and b-OVA fragments was even higher and their spectrum differed from those obtained from OVA. However, the spectra of h-OVA and b-OVA fragmented peptides were similar, differing only in the region corresponding to retention time of 50 min. Surprisingly, when we stimulated splenocytes from naïve mice *in vitro* we found an increase in the percentage of regulatory T cells in response to h-OVA and b-OVA. The capacity of both heated forms of OVA to induce Tregs was increased after 20 min of pepsin digestion and decreased again after 40 min digestion. The prolonged digestion had no effect on Treg inducing capacity of

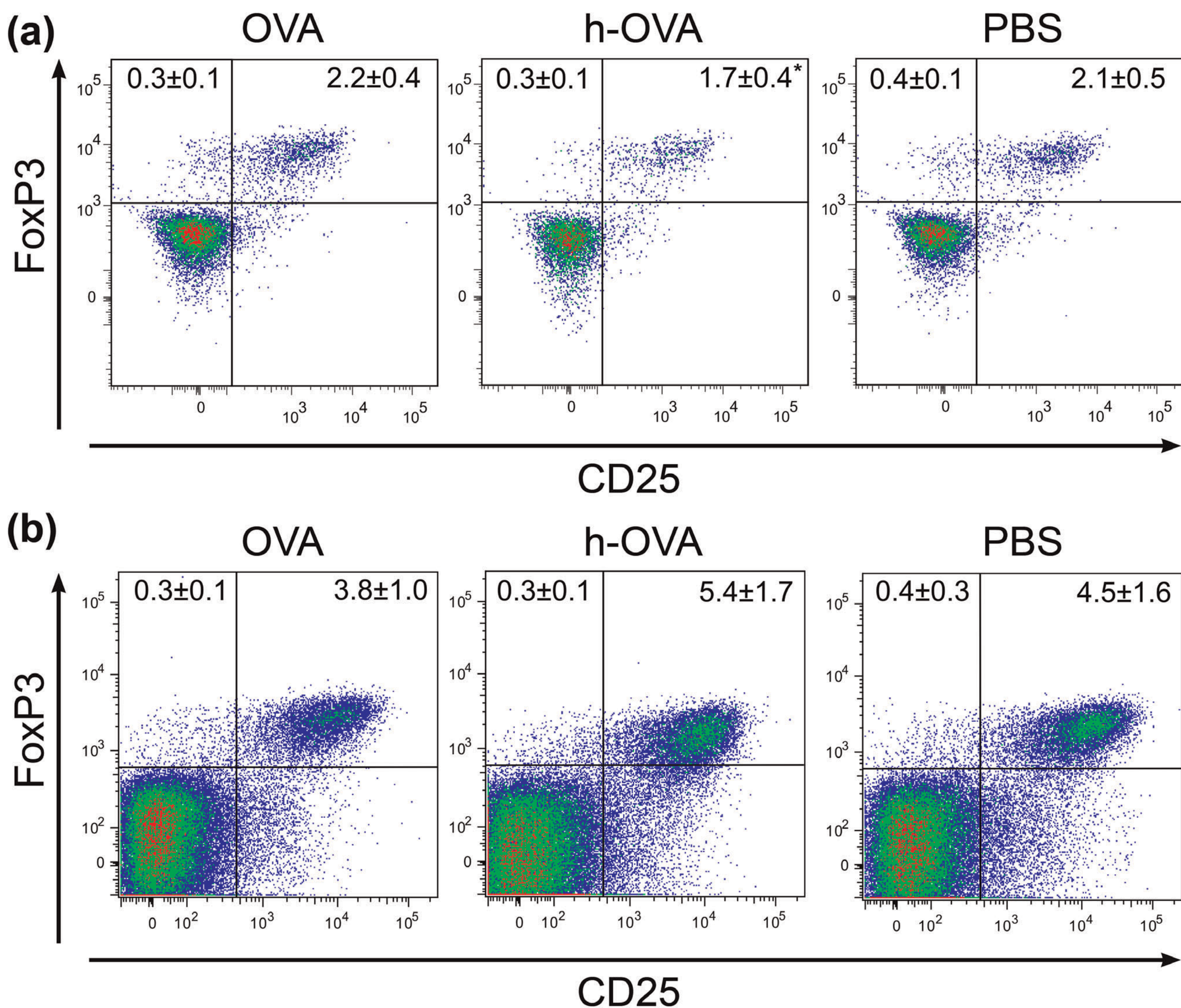



**Figure 6. Numbers of Tregs in splenocytes and mesenteric lymph nodes of OVA treated mice.** Typical plots depicting numbers of Tregs in mouse splenocytes (a) and mesenteric lymph node (b) in gated CD3+CD4+CD8– T helper cells after feeding with OVA, h-OVA or PBS, respectively. Numbers in upper quadrants shows proportions (mean ± SD) of either CD25–Foxp3+ or CD25+Foxp3+ Th cells out of all cells. Representative data from one out of three independent experiments. *P≤0.05.
doi:10.1371/journal.pone.0037156.g006

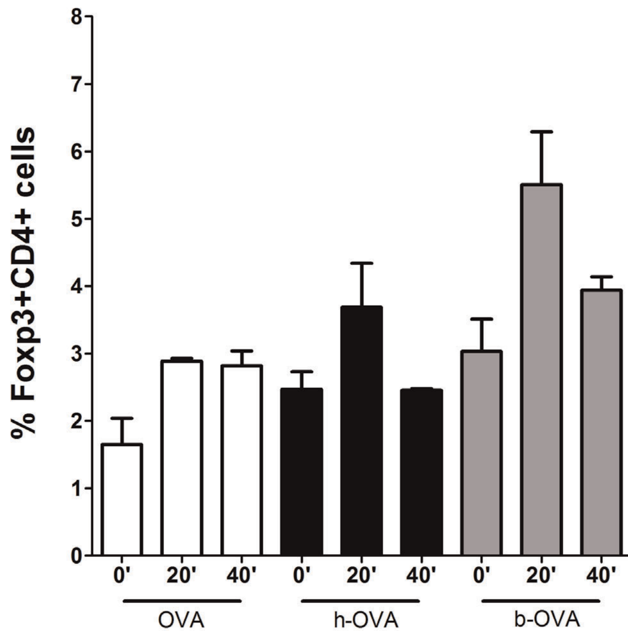



**Figure 7. Number of Tregs in spleen cell suspensions co-cultured *in vitro* with OVA digests.** The percentage of Tregs in cell suspension isolated from spleens of non-stimulated (naïve) BALB/c mice cultured *in vitro* for 48 hours with undigested (0′) and after 20 (20′) and 40 minutes (40′) peptic digest of OVA (white bars), h-OVA (black bars) or b-OVA (grey bars). The data represent the percentage of CD4+Foxp3+ cells out of all live cells as measured by FACS. Representative data from one out of three independent experiments are shown. Data are represented as mean ± SEM.
doi:10.1371/journal.pone.0037156.g007

native (heat untreated) OVA digests. These data are supported by recent evidence in experimental mouse model of suppressive effects of some OVA T cell epitope peptides on allergic immune responses via Foxp3+ T cell generation [41].

A direct continuation of the study would be the analysis of intestinal DC subsets and goblet cells [42–44] in initial steps of allergen sensitization in our model, which should contribute to understanding how the tolerance or allergic response is achieved. The analysis of the role of enzymes in brush-border membrane of epithelial cells (activated after OVA gavages) will shed light on allergen digestion and immunogenicity of fragments (esp. dipeptidases) and on regeneration of gut epithelium (ALP). Moreover, it would be of great importance to apply this model for verification of hygiene hypothesis using animals kept under conventional and/or germ-free condition and subsequently colonized with various bacterial strains.

In conclusion, we showed that even a mild change in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigenic properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 to Th1-type response as compared to native OVA. Nevertheless, the h-OVA fragments still have the ability to induce allergic symptoms, but these are less pronounced and need longer time to develop.

## Supporting Information

**Figure S1 Cross-reactivity of anti-OVA specific antibodies.** At the end of the experiment we determined the levels of OVA-specific antibodies in OVA and heated (h)-OVA treated mice against OVA, h-OVA (70°C) and boiled (b)-OVA (95°C). The levels were retained for IgE, IgG2a and IgA (a, c, d) when we used OVA as coating antigen for h-OVA-treated mice or h-OVA as coating antigen for OVA treated mice. In case of IgG1 (b) the levels were significantly higher when we used h-OVA as coating antigen for either OVA- or h-OVA- treated mice. When we used b-OVA we observed a significant drop in the signal for all measured antibodies. Representative data from one out of three experiments (n = 8). Repeated measures ANOVA with Tukey's multiple comparison test was used for analysis of differences between antibody levels of the same sample measured either against OVA, h-OVA and b-OVA antigen. n.s. non-significant, *P≤0.05, **P≤0.01, ***P≤0.001.
(TIF)

## Acknowledgments

The authors thank Jana Cinová for her help with cell cultivation, Alena Smolová for excellent technical assistance and Anna Koffer for language revision.

## Author Contributions

Conceived and designed the experiments: JG LT FF MS HK. Performed the experiments: JG MS MK DS HK MW KK PS. Analyzed the data: MS JG MK DS LT HK MW KK PS LP-J. Contributed reagents/materials/analysis tools: LT MW HK FF. Wrote the paper: LT MS JG MK MW HK PS. Obtained permission for use of animal model: LT.

## References


1. Savage JH, Matsui EC, Skripak JM, Wood RA (2007) The natural history of egg allergy. J Allergy Clin Immunol 120: 1413–1417.
2. Herouet-Guicheney C, Aldemir H, Bars R, de Barbeyrac D, Kennel P, et al. (2009) Inter-laboratory comparisons of assessment of the allergenic potential of proteins in mice. J Appl Toxicol 29: 141–148.
3. Sicherer SH, Wood RA, Stablein D, Burks AW, Liu AH, et al. (2010) Immunologic features of infants with milk or egg allergy enrolled in an observational study (Consortium of Food Allergy Research) of food allergy. J Allergy Clin Immunol 125: 1077–1083.
4. Perrier C, Thierry AC, Mercenier A, Corthésy B (2010) Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. Clin Exp Allergy 40: 153–162.
5. Mine Y, Yang M (2007) Epitope characterization of ovalbumin in BALB/c mice using different entry routes. Biochim Biophys Acta 1774: 200–212.
6. Repa A, Kozakova H, Hudcovic T, Stepankova R, Hrncir T, et al. (2008) Susceptibility to nasal and oral tolerance induction to the major birch pollen allergen Bet v 1 is not dependent on the presence of the microflora. Immunol Lett 117: 50–56.
7. Lee SY, Huang CK, Zhang TF, Schofield BH, Burks AW, et al. (2001) Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. Clin Immunol 101: 220–228.
8. Diesner SC, Knittelfelder R, Krishnamurthy D, Pali-Schöll I, Gajdzik L, et al. (2008) Dose-dependent food allergy induction against ovalbumin under acid-suppression: A murine food allergy model. Immunol Lett 121: 45–51.
9. Brunner R, Wallmann J, Szalai K, Karagiannis P, Altmeppen H, et al. (2009) Aluminium *per se* and in the anti-acid drug sucralfate promotes sensitization via the oral route. Allergy 64: 890–897.
10. Pali-Schöll I, Herzog R, Wallmann J, Szalai K, Brunner R, et al. (2010) Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization. Clin Exp Allergy 40: 1091–1098.
11. Dearman RJ, Kimber I (2007) A mouse model for food allergy using intraperitoneal sensitization. Methods 41: 91–98.
12. Brandt EB, Munitz A, Orekov T, Mingler MK, McBride M, et al. (2009) Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. J Allergy Clin Immunol 123: 53–58.
13. Li X, Schofield BH, Huang CK, Kleiner GI, Sampson HA (1999) A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 103: 206–214.

14. Saldanha JCS, Gargiulo DL, Silva SS, Carmo-Pinto FH, Andrade MC, et al. (2004) A model of chronic IgE-mediated food allergy in ovalbumin-sensitized mice. Braz J Med Biol Res 37: 809–816.
15. van der Ventel ML, Nieuwenhuizen NE, Kirstein F, Hikuam C, Jeebhay MF, et al. (2011) Differential responses to natural and recombinant allergens in a murine model of fish allergy. Mol Immunol 48: 637–646.
16. Simon PM, Kedinger M, Raul F, Grenier JF, Haffen K (1979) Developmental pattern of rat intestinal brush-border enzymic proteins along the villus-crypt axis. Biochem J 178: 407–413.
17. Kozáková H, Reháková Z, Kolínská J (2001) Bifidobacterium bifidum monoassociation of gnotobiotic mice: effect on enterocyte brush-border enzymes. Folia Microbiol 46: 573–576.
18. Hudcovic T, Kozáková H, Kolínská J, Stepánková R, Hrncír T, et al. (2009) Monocolonization with Bacteroides ovatus protects immunodeficient SCID mice from mortality in chronic intestinal inflammation caused by long-lasting dextran sodium sulfate treatment. Physiol Res 58: 101–110.
19. Lallès JP (2010) Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutr Rev 68: 323–332.
20. Harada T, Koyama I, Kasahara T, Alpers DH, Komoda T (2003) Heat shock induces intestinal-type alkaline phosphatase in rat IEC-18 cells. Am J Physiol Gastrointest Liver Physiol. 284: G255–G262.
21. Huntington JA, Stein PE (2001) Structure and properties of ovalbumin. J Chromatogr B Biomed Sci Appl. 756(1–2): 189–198.
22. Lemon-Mulé H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, et al. (2008) Immunologic changes in children with egg allergy ingesting extensively heated egg. J Allergy Clin Immunol 122: 977–983.
23. Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Węgrzyn A (2011) Mechanisms underlying differential food allergy response to heated egg. J Allergy Clin Immunol 127: 990–997.
24. Nowak-Węgrzyn A, Sampson HA (2011) Future therapies for food allergies. J Allergy Clin Immunol 127: 558–573.
25. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, et al. (2008) Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy 63: 882–890.
26. Himly M, Nony E, Chabre H, Van Overtvelt L, Neubauer A, et al. (2009) Standardization of allergen products: 1. Detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation. Allergy 64: 1038–1045.
27. Tučková L, Novotná J, Novák P, Flegelová Z, Květoň T, et al. (2002) Activation of macrophages by gliadin fragments: isolation and characterization of active peptide. J Leukoc Biol 71: 625–631.
28. Wiedermann U, Jahn-Schmid B, Bohle B, Repa A, Renz H, et al. (1999) Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant bet v 1, the major birch pollen allergen, in a murine model of type I allergy. J Allergy Clin Immunol 103: 1202–1210.
29. Lehrer SB, Reish R, Fernandes J, Gaudry P, Dai G, et al. (2004) Enhancement of murine IgE antibody detection by IgG removal. J Immunol Methods 284: 1–6.
30. Barsumian EL, Isersky C, Petrino MG, Siraganian (1981) IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. Eur J Immunol 11: 317–323.
31. Kessler M, Acuto O, Storelli C, Murer H, Müller M, et al. (1978) A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. Biochim Biophys Acta 506: 136–154.
32. Lowry OH, Rosebrough NJ, Farr Al, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.
33. Kozakova H, Kolinska J, Lojda Z, Rehakova Z, Sinkora J, et al. (2006) Effect of bacterial monoassociation on brush-border enzyme activities in ex-germ-free piglets: comparison of commensal and pathogenic *Escherichia coli* strains. Microbes Infect 8: 2629–2639.
34. Whitacre CC, Gienapp IE, Orosz CG, Bitar DM (1991) Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J Immunol 147: 2155–2163.
35. Vaali K, Puumalainen TJ, Lehto M, Wolff H, Rita H (2006) Murine model of food allergy after epicutaneous sensitization: role of mucosal mast cell protease-1. Scand J Gastroenterol 41: 1405–1413.
36. Lynes MD, Widmaier EP (2011) Involvement of CD36 and intestinal alkaline phosphatases in fatty acid transport in enterocytes, and the response to a high-fat diet. Life Sci 88: 384–391.
37. Smith MW, Phillips AD (1990) Abnormal expression of dipeptidylpeptidase IV activity in enterocytes brush-border membranes of children suffering from coeliac disease. Exp Physiol 75: 613–616.
38. Detel D, Persić M, Varljen J (2007) Serum and intestinal dipeptidyl peptidase IV (DPP IV/CD26) activity in children with celiac disease. J Pediatr Gastroenterol. Nutr 45: 65–70.
39. Johansen P, Senti G, Gómez JMM, Wüthrich B, Bot A, et al. (2005) Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. Eur J Immunol 35: 3591–3598.
40. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, et al. (2009) Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis 15: 341–352.
41. Yang M, Yang C, Mine Y (2010) Multiple T cell epitope peptides suppress allergic responses in an egg allergy mouse model by the elicitation of forkhead box transcription factor 3- and transforming growth factor-β-associated mechanisms. Clin Exp Allergy 40: 668–678.
42. Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA (1998) Expanding dendritic cells in vivo enhances the induction of oral tolerance. J Immunol 160: 5815–5825.
43. Lied GA, Vogelsang P, Berstad A, Appel S (2011) Dendritic cell populations in patients with self-reported food hypersensitivity. Int J Gener Med 4: 389–396.
44. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, et al. (2012) Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 483: 345–349.

# 3. CONCLUSIONS AND DISCUSSION

Recent research has provided clear progress in the understanding of wheat allergy, and has also revealed the problems to be resolved in the future. Population-based studies investigating trends of the prevalence of each type of wheat allergy will be important worldwide. The reasons for the increase in food allergy prevalence are not well known, but, the short period of time over which the increase occurred, suggests that environmental factors are more likely to be relevant as part of the hygiene hypothesis [Bach, 2002; Schaub et al., 2006]. Additional factor such as food preparation or exposure to medicinal skin creams containing food allergens are also of the importance [Lee et al., 2006; Nowak-Wegrzyn et al., 2006]. Moreover, the introduction of food later in the infant diet has been postulated to play a role in the increase of food allergy [Du Toit et al., 2008]. Extensive analysis of allergenic molecules is generally time-consuming, indicating the need for rapid and easy methods of allergen separation. Greater knowledge will help in the precise diagnosis and treatment of food allergies. Importantly, additional studies will be necessary to elucidate the profiles of the allergens in both the water/salt-soluble and insoluble fractions involved in each clinical type depending on the patient age, sensitization routes, symptoms, and the allergen state during exposure. The potential application of defined food allergens (natural or recombinant) will be their use in sIgE Abs testing, cell activation assays as well as in complex allergen-microarrays (biochip technology).

Proteomic analysis has proved to be a useful tool for the identification of the causative food allergens and for increasing the specificity of diagnostic tests. Using this approach we have demonstrated a large spectrum of wheat proteins recognized by IgE Abs in the sera of allergic patients. To identify IgE reactive wheat components we employed 1-DE, 2-DE separation, immunoblotting analysis, and MALDI-TOF, QTOF and ion-trap mass spectrometer. We also succeeded in purifying relevant IgE-binding proteins from a water/salt-soluble extract of wheat flour using Amicon devices, Rotofor and HPLC techniques, and we later identified these proteins by mass spectrometry. These methods allow the isolation of native allergens from wheat flour in sufficient quantities to study their function, as well as the effects of their tertiary structure, post translational modifications (*e.g.* glycosylation) on allergenicity and for testing weather they are able to

induce innate or adaptive immune response. Moreover, purified allergens could induce basophil activation.

Initially, we documented the impact of various wheat cultivars, extraction procedure, and of the technique of separation on the number of detected IgE-binding wheat components. In accordance with previously published data [Constantin et al., 2005], separation of salt-soluble wheat extracts by 1-DE under nonreducing conditions and immunoblotting revealed IgE-binding proteins of molecular mass ranging from 10 to 100 kDa. However, separation under reducing conditions revealed predominantly low-molecular mass components (ranging from 10 to 30 kDa). These changes in the IgE-binding pattern could be a consequence of conformational changes, reduction of interchain disulfide bridges and uncovering or formation of new antigenic structures (epitopes).

The 2-DE technique has been a critical tool for the separation and analysis of proteins in complex systems. Hundreds to a few thousand proteins can be separated on the basis of IEF according to the pI of the proteins in the first dimension, and their relative molecular mass in the second dimension, typically by SDS-PAGE. The problems of pH gradient instability were overcome by the introduction of immobilized pH gradient strips for IEF [Görg et al., 1988]. Wide-range pH gradients (3–12) allow the separation of highly acidic or basic form of proteins and give the information about total protein in extracts. These data are, however, influenced by protein sources used for the analysis, because of different amount of proteins extracted from various wheat cultivars. We selected cultivar Sulamit for further experiments because of its large spectrum of IgE reactive wheat components. These IgE-binding proteins were separated by 2-DE and identified by MALDI-TOF and QTOF technique. Using these methodological approaches we identified 14 IgE-binding proteins. The spectrum of identified proteins was supplemented with further 5 molecules using 1-DE separation and ion-trap mass spectrometry detection technique. These methodological approaches revealed IgE binding proteins including previously reported α-amylase inhibitors (CM16, CM17 and 0.19 dimeric) [Kitta et al., 2006; Weichel et al., 2006], profilin (of high sequence identity with birch pollen allergen) [Rihs et al., 1994], and Tri a Bd 27K protein [Yamashita et al., 2002]. Moreover, we succeeded in identifying new IgE-binding wheat proteins such as β-amylase, β-D-glucan exohydrolase, serpin, α-amylase/trypsin inhibitor CM 3, α-amylase inhibitor CIII and three protein species of 27K protein.

The SDS-PAGE followed by immunoblotting can provide information on the molecular mass of proteins that are bound by the antibodies employed for the detection. As compared with protein-based methods, DNA-based methods (*e.g.* PCR, PCR-ELISA and real-time PCR) offer both advantages and disadvantages. Although the concentration of (allergenic) proteins within an allergenic source depends on variables such as the species/variety and the growth conditions, the level of DNA is very stable [Angelis et al., 2010]. Moreover, the most of wheat allergens are commercially unavailable. Isolation and purification of natural wheat allergens is of utmost importance for subsequent structural and functional analysis. Recombinant allergens may be useful in diagnosing and/or treating allergies [Jutel et al., 2005; Jutel et al., 2012], but only a small number of wheat allergens have been cloned and produced in recombinant form [Palacin et al., 2009]. Many recombinant allergens do not have the same immunological characteristics as their natural counterparts. IgE binding to allergens often depends on the tertiary structure of the latter, because many epitopes recognized by IgE Abs are conformational. Alterations of the amino acid sequence of allergens may influence protein folding, resulting in the reduction or total prevention of IgE binding. Several recombinant allergens have been produced in bacterial expression systems as nonglycosylated proteins, although some natural α-amylase inhibitors are glycosylated [Amano et al., 1998]. Thus, highly purified natural allergens are still indispensable in determining the allergenicity of recombinant and natural forms of allergens. The detection of sIgE against natural or recombinant allergens has been especially helpful in understanding molecular mechanisms of food allergy. Protein microarrays have recently been introduced as a promising tool to measure sIgE Abs against a large number of recombinant or purified natural allergens in low amounts of patient serum. Recently, measurement of sIgE against recombinant wheat ω-5-gliadin was introduced for the diagnosis of food allergies using protein microarrays. Such component-resolved diagnosis revealed sIgE in all 10 patients with suspected WDEIA who presented with a convincing history of severe, systemic, allergic reactions after the ingestion of wheat followed by physical exercise and elevated levels of sIgE against recombinant ω-5-gliadin according to the fluorescence enzyme immunoassay. However, further large-scale studies are warranted to confirm these results [Brans et al., 2012]. Moreover, purified allergens are not only needed for analysis of their structure and function but also in animal models enabling to study immunopathogenic mechanisms of the disease. As the early step,

native proteins including β-lactoglobulin, bovine serum albumin, gliadins and ovalbumin has been studied [Dearman et al., 2001; Adel-Patient et al., 2003; Mine et al., 2007; Bodinier et al., 2009; Gourbeyre et al., 2012], but wheat water/salt-soluble allergens has not. Analysis of the ability of wheat allergens to elicit an allergic response in animal models is therefore of utmost importance.

We developed a new procedure for isolating and purifying wheat flour allergens in amounts that allowed subsequent structural and functional analyses. Wheat flour proteins were separated primarily by Rotofor and HPLC. Rotofor fractionation of these wheat proteins based on pI gradient was an efficient initial purification step and can be utilized for protein loads ranging up to tens of milligrams in contrast to 2-DE. The main step in the purification of relatively large quantities of several water/salt soluble wheat proteins was preparative reverse-phase HPLC. This technique was applicable for fractions containing both individual and mixtures of proteins. The purified proteins were subsequently separated by SDS-PAGE and identified by MALDI-TOF mass spectrometry. This purification scheme yielded 27 IgE binding proteins, including several previously described α-amylase inhibitors, non-specific lipid-transfer proteins, peroxidase, serpins and β-amylase identified by 2-DE [Pastorello et al., 2007; Sotkovsky et al., 2008], as well as several new wheat proteins, such as endogenous α-amylase/subtilisin inhibitor, wheatwin, TLP, tritin, xylanase inhibitor protein-1 (XIP), β-glucosidase, class II chitinase and 26 kDa endochitinase.

All techniques used in our studies detected members of α-amylase inhibitor family and confirmed this enzyme as one of the major wheat allergens. Although the α-amylase inhibitors are wheat allergens, the reactivity of individual members of this family with IgE has been found to differ among studies, which may reflect differences in populations and/or analytical approaches. Our findings on the reactivity of patients' IgE with α-amylase inhibitors are mutually inconsistent. When we tested the reactivity of IgE Abs of our patients with eight α-amylase inhibitors, we found that the most frequently recognized inhibitors were 0.28 and a mixture of CM16 and CM2 α-amylase inhibitors. However, the reactivity of these serum samples with other inhibitors, such as 0.19 α-amylase inhibitor, 0.19 dimeric, CM17, CM3 and endogenous α-amylase/subtilisin inhibitor was also statistically significant. Although the endogenous α-amylase/subtilisin

inhibitor has not been reported as an allergen [Tatham, 2008], we observed a reactivity of this purified protein with IgE Abs from 19 out of 22 patients. Moreover, our yield of purified natural α-amylase inhibitors 0.19 was higher than that of other purified proteins and we could study its reactivity with patients' IgE Abs [Sotkovsky et al., 2011]. This protein has been described as one of the major allergens of patients with baker's asthma as well as those with food allergy to wheat [Amano et al., 1998; Weichel et al., 2006], but it had not been isolated in its natural form in sufficient amount and purity. Recently, α-amylase inhibitors 0.19 and CM3 has been identified as strong activators of innate immune responses in monocytes, macrophages, and dendritic cells. These α-amylase inhibitors engage the TLR4–CD14 complex and lead to up-regulation of maturation markers and elicit release of proinflammatory cytokines in cells from celiac and nonceliac patients [Junker et al., 2012]. Moreover, some controversies exist regarding α-amylase inhibitors thermal resistance. Studies that confirmed them as clinically relevant food allergens merely used raw flour [James et al., 1997, Sotkovsky et al., 2008] or flour after mild heat treatment [Pastorello et al., 2007]. In contrast, when extracts from final wheat products were tested, the α-amylase inhibitors were found to be thermolabile [Pasini et al., 2001; Simonato et al., 2001; De Gregorio et a., 2009].

Despite the fact that α-amylase inhibitors were already known as allergens [Kusaba-Nakayama et al., 2001; Zapatero et al., 2003], a clinical study of quantitative estimation of IgE Abs against α-amylase inhibitors in the sera of allergic patients was missing. We developed new ELISA assay for the detection and quantification of sIgE Abs against wheat extract from the most frequently used cultivar Sulamit and/or commercially available α-amylase inhibitors type 1 and 3 as the coupling antigens. The highest levels of IgE and the highest number of positive patient's sera were detected using the water/salt soluble Sulamit extract. The mean level of IgE Abs against the α-amylase inhibitor type 1 was similar to that obtained with the whole extract and the level of IgE Abs against the α-amylase inhibitor type 3 was low in the majority of patients' sera tested. However, these commercial α-amylase inhibitors types 1 and 3 are not well defined and contained one major IgE binding protein together with small amounts of impurities. The difference between the mean value of IgE Abs in healthy donors and patients with wheat allergy was statistically significant even for this allergen, probably due to the few highly positive sera in the patients' group. Data obtained by testing disease controls (patients with pollen

allergy) using wheat extract, reflect the presence of the so-called panallergens such as lipid-transfer proteins or profilins recognized also by IgE of these patients. Interestingly, cross-reactive epitopes in pollen and food allergens have been recently demonstrated and similar epitopes may exist also in pollen and wheat allergens (including α-amylase inhibitors) [Asero et al., 2007; Pastorello et al., 2007; Weber, 2007].

We also succeeded in isolating and purifying new wheat allergens associated with food allergy, such as TLP and wheatwin. TLP isolated from wheat has been shown to be an allergen in patients with baker's respiratory allergy [Lehto et al., 2010] but not so far in patients with food allergy. In our cohort, TLP was recognized by IgE Abs of all 22 patients and wheatwin of 13 patients. The intensity of IgE binding (median 8) of TLP was significantly higher when compared with disease controls (median 1). Moreover, we identified and purified wheat LTP that is also a major allergen associated with baker's asthma [Lauer et al., 2007]. We found that IgE Abs of 64% patients in our cohort reacted with our purified wheat LTP, which is a higher percentage than previously reported (41% and 28%) [Battais, Courcoux et al., 2005; Pastorello et al., 2007]. We also purified tritin (ribosome-inactivating protein) that was recognized by all our patients (22/22) with high binding intensity (median 8), and also by some of our disease controls (7/10, median 2). In addition, we purified other new IgE-binding wheat components, such as XIP, class II chitinase and 26 kDa endochitinase, which were not previously identified as allergens in individuals with food allergy, although xylanase inhibitor protein was identified in patients with baker's asthma but not purified [Lehto et al., 2010]. These components reacted strongly (median 5.5–7.0) with the vast majority of patients' IgE Abs (20/22, 21/22 and 21/22, respectively) and with a lower intensity (median 0–1.5) and frequency (2/10, 5/8 and 5/9) with IgE of disease controls. Moreover, we have shown for the first time that purified allergens such as α-amylase inhibitor 0.19, lipid-transfer protein, TLP and wheatwin can activate patients' basophils, thus showing that our purified proteins have biological activity and that the basophil activation test is useful in diagnosing this condition [Sotkovsky et al., 2011].

In our recent study with mouse model of food allergy [Golias et al., 2012], we showed that minor irreversible changes in OVA secondary structure caused by thermal processing changes its both digestion and antigenic epitopes formation lead to activation of different T cell subpopulations, induce shift towards Th1 response and ultimately reduces

its allergenicity. It seems that even slight changes in the secondary structure elements have a high impact on the immunological behaviour of the allergen. This could be explained by differences either in allergen absorption (which could lead to a decrease in allergen exposure or in allergen digestion), by production of peptides with different allergenicity, a partial loss of conformational epitopes and/or exposure of new linear epitopes to immune cells [Lynes at al., 2011; Lallès 2010]. The changes in secondary structure by heating could influence antibody response in vivo. Here we report that OVA induced significantly higher levels of OVA-specific IgE; MMCP-1, known markers of mast cell activation and degranulation [Vaali K et al., 2006]; and lower levels of IgG2a, as compared to h-OVA. High levels of potentially "blocking" IgG2a (mouse homolog of human IgG4) may compete for allergen [Lemon-Mulé et al. 2008]. The ability of heat-denatured allergens to induce Th-1 associated IgG2a was also shown for other allergens, such as bee venom or birch pollen [Johansen et al. 2005]. However, the effect cannot be generalized, because in a recent study by van der Ventel [van der Ventel et al. 2011] a higher sensitizing potential was shown for cooked fish proteins. Surprisingly, when we changed the coupling allergen (h-OVA was used for OVA sensitized sera and vice versa) the binding of specific Abs was retained. Moreover, the binding was significantly higher when h-OVA antigen was used for specific IgG1 antibody determination. We assume that this is caused by heating-uncovered linear epitopes (supplementing the loss of the conformational ones), which are then presented after processing by antigen-presenting cells to T and B lymphocytes.

Next, we addressed the question if the differences in OVA and h-OVA-specific antibody responses are also associated with cytokine milieu. We found a significantly higher production of Th-2 cytokines in the OVA-treated mice, accompanied by proinflammatory TNF-$\alpha$ production after an in vitro exposure to OVA. Surprisingly, we determined an up-regulation of regulatory cytokine IL-10, which could be a result of a biological feedback aimed at dampening down the local inflammation, similar to chronic experimental colitis [39]. Our findings suggest that heating of OVA induces changes in its digestion and processing by immune cells that lead to changes in the local cytokine environment ultimately leading to a shift from Th2- toward Th1-type response, reduction in the level of sIgE and an increased production of blocking IgG2a antibodies [Lemon-Mulé et al. 2008]. These data fit well with clinical symptoms observed in allergic subjects in response to heated egg allergens [Nowak-Węgrzyn et al., 2011; Mine et al., 2007].

Moreover, our results support recent data showing that thermal processing interferes with OVA stability [Martos et al., 2011]. Here, we show that h-OVA and boiled OVA (b-OVA) is initially (at 20 min) more resistant to proteolysis than native OVA. The difference in degradation kinetics could be explained by partial aggregation of heated forms of OVA, which makes the target structures less accessible for the enzyme.

The study with OVA in our model is an initial step for understanding how the tolerance or allergic response is achieved. A direct continuation of the study would be the analysis of intestinal dendritic cells subsets and goblet cells in initial steps of allergen sensitization in our model, which should contribute to understanding how the tolerance or allergic response is achieved. The analysis of the role of enzymes in brush-border membrane of epithelial cells (activated after OVA gavages) will shed light on allergen digestion and immunogenicity of fragments (esp. dipeptidases) and on regeneration of gut epithelium (ALP). Moreover, it would be of great importance to apply this model for verification of hygiene hypothesis using animals kept under conventional and/ or germ-free condition and subsequently colonized with various bacterial strains. Furthermore, this model will be useful for other allergens components identified from different natural sources, for example wheat flour etc.

Nowadays, proteomics plays a vital role in many scientific disciplines, enabling discovery of disease biology and mechanisms, new drug targets, food quality evaluation and much more. Application of proteomic techniques to the study of food quality has recently revealed its power in pointing out differences in food proteomes relevant for nutrition and diseases. IgE-reactive proteome analysis of wheat components provides not only an overall map of the allergenic proteins, but also useful information for further systematic research on wheat allergens and for individual desensitising treatments and therapeutic development. Innovative analytical methods and novel applications of available techniques are required to face the food allergenicity problems in an integrated manner. Due to the tremendous diversity of the naturally occurring samples and the physicochemical properties of proteins, sample preparation knowledge has been not yet well established. However, progress in the field of allergenomics is moving fast. The research for food allergy and the implications for the clinical utility depend on a better understanding of the molecular nature of allergen components and those structures which

are important for the antibody recognition and effector cell activation. This is especially true for the design of allergen derivates to be used in immunotherapy with a reduced risk of inducing adverse reactions, such as anaphylaxis [Factor et al., 2012; Kulis et al., 2012]. Moreover, identifying whether the sensitization is primary (species specific) or a result of cross-reactivity to proteins with similar protein structures makes it easier for the clinician to judge the risk of reaction on exposure to different allergen sources. The development of allergen components in pure form has made it possible to resolve many of these problems. In terms of production techniques, they can be either produced biotechnologically in recombinant form or purified from their original sources. In other words, the main area of application for purified natural or recombinant allergen components is in the precise identification of the allergies that cause the disease [Borres et al., 2011]. Allergen extracts will be needed for the diagnosis of unusual allergies and in the cases of unusual sensitization patterns to common allergen sources, however, approaches based on well-defined individual molecules of either natural or recombinant origin are likely to supplement those based on food extracts in the future. Therefore, a library as Allergome comprising well-characterised authentic natural and recombinant allergens was formed. The protocols published in this library and the detailed physicochemical characteristics of the allergens will allow other researchers to produce batches of native allergens of similar quality. The availability of such information will improve the comparability of studies utilising purified allergens in future. The information about our contribution to identification of allergens can be found at Allergome databases. In particular, these high-quality purified food allergens will contribute to improve conventional food allergy diagnostics as well as new technologies such as protein biochips. In the near future, allergenomic approaches could be used as a standard technique for *in vitro* separation and identification of allergens from foods including novel and/or reduced allergens products.

# 4. ORIGINAL PUBLICATIONS

**Šotkovský P**, Hubálek M, Hernychová L, Novák P, Havranová M, Šetinová I, Kitanovičová A, Fuchs M, Stulík J and Tučková L. Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patiens. Proteomics 2008; 8: 1677-91.
IF= 4,505

**Šotkovský P**, Sklenář J, Halada P, Cinová J, Šetinová I, Kainarová A, Goliáš J, Havlásková K, Honzová S and Tučková L. A new approach to the isolation and characterization of wheat flour allergens. Clin Exp Allergy 2011; 41(7): 1031-43
IF= 5,030

Goliáš J, Schwarzer M, Wallner M, Kverka M, Kozáková H, Šrůtková D, Klimešová K, **Šotkovský P**, Palová-Jelínková L, Ferreira F and Tučková L**.** Minor heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. PLoS One. 2012; 7(5): e37156.
IF= 4,090

# 5. CONFERENCE ABSTRACTS

**Šotkovský P**, Fuchs M, Havranová M, Tučková L. Characterisation of wheat flour allergens. XXV. Congress of the European Academy of Allergology and Clinical Immunology, Vienna, Austria 2006 (p. 416).

**Šotkovský P**, Fuchs M, Havranová M, Tučková L. Characterisation of wheat flour allergens. $1^{st}$ Joint Meeting of European National Societies of Immunology – $16^{th}$ European Congress of Immunology, Paris, France 2006 (p. 138).

**Šotkovský P**, Stulík J, Hernychová L, Hubálek M, Fuchs M, Havranová M, Tučková L. Identifikace alergenů z pšeničné mouky. XXIII. Sjezd Českých a Slovenských Alergologů a Klinických Imunologů, Hradec Králové, Czech Republic 2006 (p. 38).

**Šotkovský P**, Hubálek M, Hernychová L, Novák P, Havranová M, Fuchs M, Stulík J, Tučková L. Identification of wheat allergens using proteomic approach. $2^{nd}$ International Symposium on Molecular Alergology, Roma, Italy 2007 (p. 41).

**Šotkovský P**, Hubálek M, Hernychová L, Novák P, Havranová M, Fuchs M, Stulík J, Tučková L. Different proteomic approaches to the identification of wheat flour allergens. XX. World Allergy Congress, Bangkok, Thailand 2007 (p. 154).

**Šotkovský P**, Hubálek M, Hernychová L, Novák P, Havranová M, Šetinová I, Stulík J, Tučková L. Identification and characterisation of main wheat allergens. $3^{rd}$ International Symposium on Molecular Allergology, Salzburg, Austria 2008 (p. 65).

**Šotkovský P**, Kitanovičová A, Hubálek M, Hernychová L, Novák P, Šetinová I, Honzová S, Fuchs M, Stulík J, Tučková L. Identification of wheat allergens and characterisation of the main factors inducing hypersensitivity reaction. XXV. Sjezd Českých a Slovenských alergologů a klinických imunologů XII. Kongres Českých a Slovenských imunologů, Prague, Czech Republic 2008, (p. 25).

**<u>Šotkovský P</u>**, Kainarová A, Šetinová I, Honzová S, Novak P, Ludmila Tučková. The impact of thermal processing and enzymatic digestion on wheat products allergens. Allergy & Asthma Symposium: Bridging Innate and Adaptive Immunity, Bruges, Belgium 2009 (p. 52).

**<u>Šotkovský P</u>**, Kainarová A, Šetinová I, Honzová S, Novak P, Ludmila Tučková. The effect of thermal processing on allergenicity of wheat products. XXVIII. EAACI Congress: Allergy and Asthma without Frontiers, 6-10 June 2009, Warsaw, Poland. Source: Allergy Eur. J. Allergy Clin. Immunol. 2009 64 SUPPL. 90 (166).

**<u>Šotkovský P</u>**, Sklenar J, Kainarová A, Šetinová I, Honzová S, Novak P, Ludmila Tučková. Hypersensitivity to wheat: resistence of main allergens to thermal and enzymatic processing.
XXI. World Allergy Congress, Buenos Aires, Argentina 2009, (no. 344)

**<u>Šotkovský P</u>**, Sklenář J, Halada P, Cinová J, Šetinová I, Kainarová A, Goliáš J, Havlásková K, Honzová S and Tučková L. Isolation and purification of wheat flour allergens. Allergy Eur. J. Allergy Clin. Immunol. 2010 65 SUPPL. 92 (734).

Kainarová A, Šetinová I, **<u>Šotkovský P</u>**, Goliáš J, Novák P, Tučková L. Allergenicity of wheat products. Allergy Eur. J. Allergy Clin. Immunol. 2010 65 SUPPL. 92 (734).

Humlová Z, Kitanovičová A, Goliáš J, Janatková I, **<u>Šotkovský P</u>**, Tučková L. Different proteins are responsible for cross-reactivity between wheat flour, rice and pollen allergens. Allergy Eur. J. Allergy Clin. Immunol. 2010 65 SUPPL. 92 (406-407).

Humlová Z., Kitanovičová-Kainarová A., Goliáš J., Janatková I., **<u>Šotkovský P.</u>** and Tučková L. Distribution of immunoglobulins, eosinophilic cationic protein and anti-nuclear antibodies in patients with allergy to wheat flour, rice and pollen allergens. Inflamm. Res. 2011 60 SUPPL. 1 (S140).

Šetinová I., **Šotkovský P.**, Sklenář J., Halada P., Cinová J., Kainarová A., Honzová S., Goliáš J. and Tučková L. Wheat flour allergens-isolation and characterisation. Allergy Eur. J. Allergy Clin. Immunol. 2011 66 SUPPL. 94 (322-323)

# 6. ORAL PRESENTATIONS

Analysis of wheat flour allergens using different proteomic approaches. EAACI Workshop in Functional Genomics and Proteomics, Aspenas, Sweden 2008.

The effect of thermal processing on allergenicity of wheat products. XXVIII. EAACI Congress: Allergy and Asthma without Frontiers, Warsaw, Poland 2009.

# 7. BIBLIOGRAPHY


Adel-Patient K, Nahori MA, Proust B, Lapa e Silva JR, Creminon C, Wal JM et al. Elicitation of the allergic reaction in β-lactoglobulin-sensitized Balb/c mice: Biochemical and clinical manifestations differ according to the structure of the allergen used for challenge. Clin Exp Allergy 2003; 33: 376–85.

Akagawa M, Handoyo T, Ishii T, Kumazawa S, Morita N, Suyama K. Proteomic analysis of wheat flour allergens. J Agric Food Chem 2007; 55: 6863–70.

Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med. 2004; 199: 1567-75.

Amano M, Ogawa H, Kojima K, Kamidaira T, Suetsugu S, Yoshihama M et al. Identification of the major allergens in wheat flour responsible for baker's asthma. Biochem J 1998; 330: 1229-34.

Angelis MD, di Cagno R, Minervini F, Rizzello CG, Gobbeti M. Two-dimensional electrophoresis and IgE-mediated food allergy. Electrophoresis 2010; 31: 2126-36.

Armentia A, Sanchez Monge R, et al. In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. Clin Exp Allergy 1993;23(5):410-5

Asero R, Mistrello G, Roncarolo D, Amato S. Detection of some safe plant-derived foods for LTP-allergic patients. Int Arch Allergy Immunol 2007; 144: 57–63.

Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002; 347: 911-20.

Balkhi MY, Trivedi AK, Geletu M, Christopeit M, Bohlander SK, Behre HM, Behre G. Proteomics of acute myeloid leukaemia: Cytogenetic risk groups differ specifically in their proteome, interactome and post-translational protein modifications. Oncogene 2006; 25: 7041-58.

Bashir ME, Louie S, Shi HN, Nagler-Anderson C. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. J Immunol 2004; 172(11): 6978-87.

Battais F, Courcoux P, Popineau Y et al. Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. J Cereal Sci 2005; 42: 109–17.

Battais F, Mothes T, Moneret-Vautrin DA, et al. Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. Allergy 2005; 60: 815–21.

Bodinier M, Leroy M, Ah-Leung S, Blanc F, Tranquet O, Denery-Papini S et al. Sensitization and elicitation of an allergic reaction to wheat gliadins in mice. J Agric Food Chem 2009; 57: 1219–1225.

Borres MP, Ebisawa M, Eigenmann PA. Use of allergen components begins a new era in pediatric allergology. Pediatr Allergy Immunol 2011; 22(5): 454-61.

Brans R, Sauer I, Czaja K, Pfützner W, Merk HF. Microarray-based detection of specific IgE against recombinant ω-5-gliadin in suspected wheat-dependent exercise-induced anaphylaxis. Eur J Dermatol. 2012; 22(3): 358-62.

Brandt EB, Munitz A, Orekov T, Mingler MK, McBride M, et al. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. J Allergy Clin Immunol 2009; 123: 53–58.

Breiteneder H. Thaumatin-like proteins: a new family of pollen and fruit allergens. Allergy 2004; 59: 479–81.

Brunner R, Wallmann J, Szalai K, Karagiannis P, Altmeppen H, et al. Aluminium per se and in the anti-acid drug sucralfate promotes sensitization via the oral route. Allergy 2009; 64: 890–897.

Bublin M, Radauer C, Wilson IBH et al. Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. FASEB J 2003; 17: 1697–9.

Buonocore V, Petrucci T, Silano V. Wheat protein inhibitors of α-amylase. Phytochemistry 1977; 16: 811-20.

Chehade M, Mayer L. Oral tolerance and its realtion to food hypersensitivities. J Allergy Clin Immunol 2005; 115: 3-12.

Cianferoni A, Spergel JM. Food allergy: review, classification and diagnosis. Allergol Int 2009; 58: 457-66.

Constantin C, Huber WD, Granditsch G, Weghofer M et al. Different profiles of wheat antigens are recognized by patients suffering from coeliac disease and IgE-mediated food allergy. Int Arch Allergy Immunol 2005; 138: 257–66.

Constantin C, Touraev A, Heberle-Bors E, Quirce S, Huber WD, Valenta R. Detection of antigens reactive to IgE and IgA during wheat seed maturation and in different wheat cultivars. Int Arch Allergy Immunol 2009; 149: 181-7.

Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? Allergy 2001; 56 Suppl 67: 56-60.

De Gregorio M, Armentia A, Díaz-Perales A, Palacín A, Dueñas-Laita A, Martín B, Salcedo G, Sánchez-Monge R. Salt-Soluble Proteins from Wheat-Derived Foodstuffs Show Lower Allergenic Potency than Those from Raw Flour. J Agric Food Chem 2009; 57: 3325-30.

De Zorzi M, Curioni A, Simonato B, Giannattasio M, Pasini G. Effect of pasta drying temperature on gastrointestinal digestibility and allergenicity of durum wheat proteins. Food Chem 2007; 104: 353–63.

Dearman RJ, Kimber I. Determination of protein allergenicity: Studies in mice. Toxicol Lett 2001; 120: 181–6.

Dearman RJ, Stone S, Caddick HT, Basketter DA, Kimber I. Evaluation of protein allergenic potential in mice: dose-response analyses. Clin Exp Allergy 2003; 33(11): 1586-94.

Dearman RJ, Kimber I. A mouse model for food allergy using intraperitoneal sensitization. Methods 2007; 41: 91–98.

Du Toit G, Katz Y, Sasieni P et al. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. J Allergy Clin Immunol 2008; 122: 984-91.

Dupont Ch. Food allergy: recent advances in pathophysiology and diagnosis. Ann Nutr Metab 2011; 59 Suppl 1: 8-18.

Eberlein-Konig B, Varga R, Mempel M, Darsow U, Behrendt H, Ring J. Comparison of basophil activation tests using CD63 or CD203c expression in patients with insect venom allergy. Allergy 2006; 61: 1084–5.

Ebo DG, Stevens WJ. IgE-mediated food allergy--extensive review of the literature. Acta Clin Belg 2001; 56: 234–247.

Factor JM, Mendelson L, Lee J, Nouman G, Lester MR. Effect of oral immunotherapy to peanut on food-specific quality of life. Ann Allergy Asthma Immunol. 2012; 109(5): 348-352.

Ferrer M, Sanz ML, Sastre J et al. Molecular diagnosis in allergology: application of the microarray technique. J Investig Allergol Clin Immunol 2009; 19: 19–24.

Finkelman FD, Wills-Karp M: Usefulness and optimization of mouse models of allergic airway disease. J Allergy Clin Immunol 2008, 121: 603-606.

Gao ZS, van de Weg WE, Schaart JG et al. Linkage map positions and allelic diversity of two Mal d 3 (non-specific lipid transfer protein) genes in the cultivated apple (Malus domestica). Theor Appl Genetics 2005; 110: 479–91.

Gavrovic-Jankulovic M, Cirkovic T, Vuckovic O, Atanaskovic-Markovic M, Petersen A, Gojgic G et al. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. J Allergy Clin Immunol 2002; 110: 805–10.

Gerrad JA. Protein-protein cross linking in food: Methods, consequences, applications. Trends Food Sci Technol 2002; 13: 391–9.

Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D, Klimesova K, Sotkovsky P, Palova-Jelinkova L, Ferreira F and Tuckova L. Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. PLoS One. 2012; 7(5): e37156.

Görg A, Postel W, Domscheit A, Günther S. Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (Hordeum vulgare): method, reproducibility and genetic aspects. Electrophoresis 1988; 9: 681-92.

Görg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004; 4: 3665-85.

Grimshaw KE, King RM, Nordlee JA, Hefle SL, Warner JO, Hourihane JO. Presentation of allergen in different food preparations affects the nature of the allergic reaction-a case series. Clin Exp Allergy 2003; 33:581-5.

Gourbeyre P, Denery-Papini S, Larré C, Gaudin JC, Brossard C, Bodinier M. Wheat gliadins modified by deamidation are more efficient than native gliadins in inducing a Th2 response in Balb/c mice experimentally sensitized to wheat allergens. Mol Nutr Food Res. 2012; 56(2): 336-44.

Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. Allergy Asthma Clin Immunol 2010; 18; 6(1):1.

Hed J. Cross-reactivity in plant food allergy – Clinical impact of Component Resolved Diagnostics (CRD). Phadia AB 2009; 6-11.

Hejgaard J. Inhibitory plant serpins with a sequence of three glutamine residues in the reactive centre. Biol Chem 2005; 386: 1319–23.

Helm RM, Furuta GT, Stanley JS, Ye J, Cockrell G, Connaughton C, Simpson P, Bannon GA, Burks AW. A neonatal swine model for peanut allergy. J Allergy Clin Immunol 2002; 109(1): 136-42.

Hudcovic T, Kozakova H, Kolınska J, Stepankova R, Hrncır T, et al. Monocolonization with Bacteroides ovatus protects immunodeficient SCID mice from mortality in chronic intestinal inflammation caused by long-lasting dextran sodium sulfate treatment. Physiol Res 2009; 58: 101–110.

Huntington JA, Stein PE. Structure and properties of ovalbumin. J Chromatogr B Biomed Sci Appl 2001; 756(1–2): 189–98.

James JM, Sixbey JP, Helm RM, Bannon GA, Burks AW. Wheat α-amylase inhibitor: A second route of allergic sensitization. J Allergy Clin Immunol 1997; 99: 239-44.

James JM, Sixbey JP, Helm RM, Bannon GA, Burks AW. Wheat α-amylase inhibitor: A second route of allergic sensitization. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 1997; 99: 239-44.

Jégou S, Douliez JP, Mollé D, Boivin P, Marion D. Purification and structural characterization of LTP1 polypeptides from beer. J Agric Food Chem 2000; 48: 5023-29.

Johansen P, Senti G, Gómez JMM, Wüthrich B, Bot A, et al. Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. Eur J Immunol 2005; 35: 3591-8.

Johansson SGO, Bieber T, Dahl R, Friedmann P, Lanier V, Lockey R, Motala C et al. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization J Allergy Clin Immunol 2004; 113: 832–36.

Jones BL, Marinac LA. Purification, identification, and partial characterization of a barley protein that inhibits green malt endoproteinases. J Am Soc Brew Chem 1997; 55: 58-64.

Junker Y, Zeissig S, Kim SJ, Barisani D, Wieser H, Leffler DA, Zevallos V, Libermann TA, Dillon S, Freitag TL, Kelly CP, Schuppan D. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. J Exp Med 2012; 17; 209(13): 2395-408.

Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. J Allergy Clin Immunol 2005; 116: 608–13.

Jutel M, Solarewicz-Madejek K, Smolinska S. Recombinant allergens: The present and the future. Hum Vaccin Immunother. 2012; 8(10).

van Kampen V, Merget R, Rabstein S, Sander I, Bruening T, Broding HC et al. Comparison of wheat and rye flour solutions for skin prick testing: a multi-centre study (Stad 1). Clin Exp Allergy 2009; 39: 1896-902.

Kimber I, Dearman RJ, Penninks AH, Knippels LM, Buchanan RB, Hammerberg B, Jackson HA, Helm RM. Assessment of protein allergenicity on the basis of immune reactivity: animal models. Environ Health Perspect. 2003 Jun; 111(8): 1125-30.

Kitta, K., Ohmishi-Kameyama, M., Moriyama, T., Ogawa, T. et al. Detection of low-molecular weight allergens resolved on two-dimensional electrophoresis with acid-urea polyacrylamide gel. Anal Biochem 2006; 351: 290–297.

Knippels LM, Penninks AH. Assessment of the allergic potential of food protein extracts and proteins on oral application using the brown Norway rat model. Environ Health Perspect 2003; 111(2): 233-8.

Kulis M, Wesley Burks A. Oral immunotherapy for food allergy: Clinical and preclinical studies. Adv Drug Deliv Rev. 2012; 22 [Epub ahead of print].

Kusaba-Nakayama M, Ki M, Iwamoto M, Shibata R, Sato M, Imaizumi K. CM3, one of the wheat α-amylase inhibitor subunits, and binding of IgE in sera from Japanese with atopic dermatitis related to wheat. Food Chem Toxicol 2000; 38: 179–85.

Kusaba-Nakayama, M., Ki, M., Kawada, E., Sato, M. et al. Intestinal absorbability of wheat allergens, subunits of wheat α-amylase inhibitor, expressed by bacteria. Biosci Biotechnol Biochem 2001; 65: 2448-55.

Lallès JP. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutr Rev 2010; 68: 323-32.

Lauer I, Miguel-Moncin MS, Abel T, Foetisch K, Hartz C, Fortunato D et al. Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid transfer protein, Pru p 3. Clin Exp Allergy 2007; 37: 261–9.

Lee LA, Burks AW. Food allergies: prevalence, molecular characterization, and treatment/prevention strategies. Annu Rev Nutr 2006; 26: 539-65.

Lehto M, Airaksinen L, Puustinen A, Tillander S, Hannula S, Nyman T et al. Thaumatin-like protein and baker's respiratory allergy. Ann Allergy Asthma Immunol 2010; 104: 139-46.

Lemon-Mulé H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, et al. Immunologic changes in children with egg allergy ingesting extensively heated egg. J Allergy Clin Immunol 2008; 122: 977-83.

Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol 2000; 106(1 Pt 1): 150-8.

Lidholm J, Ballmer-Weber BK, Mari A, Vieths S. Componentresolved diagnostics in food allergy. Curr Opin Allergy Clin Immunol 2006; 6: 234–40.

Lieberman JA, Sicherer SH. Diagnosis of food allergy: epicutaneous skin tests, in vitro tests, and oral food challenge. Curr Allergy Asthma Rep 2011; 11(1): 58-64.

Lynes MD, Widmaier EP. Involvement of CD36 and intestinal alkaline phosphatases in fatty acid transport in enterocytes, and the response to a high-fat diet. Life Sci 2011; 88: 384-91.

Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Węgrzyn A. Mechanisms underlying differential food allergy response to heated egg. J Allergy Clin Immunol 2011; 127: 990-7.

Matsuo H, Morita E, Tatham AS, et al. Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. J Biol Chem 2004; 279: 135–40.

Matsuo H, Kohno K, Niihara H, Morita E. Specific IgE determination to epitope peptides of omega-5 gliadin and high molecular weight glutenin subunit is a useful tool for diagnosis of wheat-dependent exercise-induced anaphylaxis. J Immunol 2005; 175: 8116–22.

Mesa-del-Castillo M, Martínez-Cócera C, Caballero ML, Vazquez L, Moneo I. IgE binding to pepsin-digested water soluble and insoluble wheat proteins. Allergy 2004; 59: 1229-32.

Mills EN, Jenkins JA, Alcocer MJ, Shewry PR. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. Crit Rev Food Sci Nutr 2004; 44(5): 379-407.

Mills EN, Sancho AI, Rigby NM, Jenkins JA, Mackie AR. Impact of food processing on the structural and allergenic properties of food allergens. Mol Nutr Food Res 2009; 53: 963-9.

Mine Y, Yang M. Epitope characterization of ovalbumin in BALB/c mice using different entry routes. Biochim Biophys Acta 2007; 1774: 200–12.

Mittag D, Niggemann B, Sander I, Reese I, Fiedler EM, Worm M et al. Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheatdependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility. Mol Nutr Food Res 2004; 48: 380–9.

Moneret-Vautrin DA, Sainte-Laudy J, Kanny G, Fremont S. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. Ann Allergy Asthma Immunol 1999; 82: 33–40.

Morita E, Matsuo H, Mihara S, et al. Fast omega-gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis. J Dermatol Sci 2003; 33: 99–104.

Murphy Kenneth P. Janeway´s Immunobiology. Garland Science Tylor & Francis Group 2012; 347-8, 583-5.

Neumann GM, Condron R, Thomas I, Polya GM. Purification and sequencing of a family of wheat lipid transfer protein homologues phosphorylated by plant calcium-dependent protein kinase. Biochim Biophys Acta 1994; 1209: 183-90.

Nowak-Wegrzyn A, Fiocchi A. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. Curr Opin Allergy Clin Immunol 2009; 9: 234-7.

Nowak-Węgrzyn A, Sampson HA. Future therapies for food allergies. J Allergy Clin Immunol 2011; 127: 558-73.

Ocmant A, Peignois Y, Mulier S, Hanssens L, Michils A, Schandene L. Flow cytometry for basophil activation markers: the measurement of CD203c up-regulation is as reliable as CD63 expression in the diagnosis of cat allergy. J Immunol Methods 2007; 320: 40–8.

Ostblom E, Lilja G, Pershagen G, van Hage M, Wickman M. Phenotypes of food hypersensitivity and development of allergic diseases during the first 8 years of life. Clin Exp Allergy 2008; 38: 1325-32.

Palacin A, Quirce S, Armentia A, Fernandez-Nieto M, Pacios LF, Asensio T et al. Wheat lipid transfer protein is a major allergen associated with baker's asthma. J Allergy Clin Immunol 2007; 120: 1132–8.

Palacin A, Varela J, Quirce S, del Pozo V, Tordesillas L, Barranco P et al. Recombinant lipid transfer protein Tri a 14: a novel heat and proteolytic resistant tool for the diagnosis of baker's asthma. Clin Exp Allergy 2009; 39: 1267–76.

Pali-Scholl I, Herzog R, Wallmann J, Szalai K, Brunner R, et al. Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization. Clin Exp Allergy 2010; 40: 1091–1098.

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: 912–17.

Palosuo K. Update on wheat hypersensitivity. Curr Opin Allergy Clin Immunol 2003; 3: 205–9.

Palosuo K, Varjonen E, Nurkkala J, Kalkkinen N, Harvima R, Reunala T, Alenius H. Transglutaminase-mediated cross-linking of a peptic fraction of w-5 gliadin enhances IgE reactivity in wheat-dependent, exerciseinduced anaphylaxis. J Allergy Clin Immunol 2003;111(6):1386-92

Paschke A. Aspects of food processing and its effect on allergen structure. Mol Nutr Food Res 2009; 53: 959-62.

Pasini, G., Simonato B, Giannattasio M, Peruffo AD, Curioni A. Modifications of Wheat Flour Proteins during in Vitro Digestion of Bread Dough, Crumb, and Crust: An Electrophoretic and Immunological Study. J Agric Food Chem 2001; 49: 2254-61.

Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Fortunato D, Giuffrida MG et al. Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin. J Allergy Clin Immunol 2003; 111: 350-9.

Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S et al. Wheat IgE-mediated food allergy in European patients: α-amylase inhibitors, lipid transfer proteins and low molecular weight glutenins. Aller\genic molecules recognized by double-blind, placebo-controlled food challenge. Int Arch Allergy Immunol 2007; 144: 10–22.

Perrier C, Corthésy B. Gut permeability and food allergies. Clin Exp Allergy 2010; 41: 20-8.

Perrier C, Thierry AC, Mercenier A, Corthe´sy B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. Clin Exp Allergy 2010; 40: 153–162.

Poole JA, Barriga K, Leung DYM, Hoffman M, Eisenbarth GS, Rewers M et al. Timing of initial exposure to cereal grains and the risk of wheat allergy. Pediatrics 2006; 117: 2175-82.

Ramesh S. Food allergy overview in children. Clin Rev Allergy Immunol 2008; 34: 217–30.

Repa A, Kozakova H, Hudcovic T, Stepankova R, Hrncir T, et al. Susceptibility to nasal and oral tolerance induction to the major birch pollen allergen Bet v 1 is not dependent on the presence of the microflora. Immunol Lett 2008; 117: 50–56.

Ribeiro CM, Paradiso AM, Carew MA, Shears SB, Boucher RC. Cystic fibrosis airway epithelial Ca2+ i signaling: the mechanism for the larger agonist-mediated Ca2+ i signals in human cystic fibrosis airway epithelia. J Biol Chem 2005; 280: 10202-9.

Rihs HP, Rozynek P, May-Taube K, Welticke B et al. Polymerase chain reaction based cDNA cloning of wheat profilin: A potential plant allergen. Int Arch Allergy Immunol 1994; 105: 190–4.

Salcedo G, Sanchez-Monge R, Diaz-Perales A, Garcia-Casado G, Barber D. Plant non-specific lipid transfer proteins as food and pollen allergens. Clin Exp Allergy 2004; 34(9): 1336-41.

Sampson HA. Food allergy. Part 1: immunopathogenesis and clinical disorders. J Allergy Clin Immunol 1999; 103(5 part 1):717-28.

Sampson HA: Utility of food-specific IgE concentrations in predicting symptomatic food allergy. J Allergy Clin Immunol 2001; 107: 891–6.

Sampson HA. Food allergy. J Allergy Clin Immunol 2003; 111: S540-7.

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(10):1130-7.

Sancho AI, Hoffmann-Sommergruber K, Alessandri S, Conti A, Giuffrida MG, Shewry P, Jensen BM, Skov P, Vieths S. Authentication of food allergen quality by physicochemical and immunological methods. Clin Exp Allergy. 2010; 40(7): 973-86.

Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PH, Hadjivassiliou M, Kaukinen K, Rostami K, Sanders DS, Schumann M, Ullrich R, Villalta D, Volta U, Catassi C, Fasano A. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. BMC Med 2012; 7; 10:13.

Sastre J. Molecular diagnosis in allergy. Clin Exp Allergy 2010; 40(10): 1442-60.

Schaub B, Lauener R, von Mutius E. The many faces of the hygiene hypothesis. J Allergy Clin Immunol 2006; 117: 969-77.

Schmidt-Weber CB, Blaser K. Immunological mechanisms in specific immunotherapy. Springer Semin Immunopathol. 2004; 25: 377-90.

Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. Curr Opin Allergy Clin Immunol 2006; 6: 226–33.

Sicherer SH, Sampson HA. Food allergy: recent advances in pathophysiology and treatment. Annu Rev Med 2009; 60: 261-77.

Simonato B, Pasini G, Giannattasio M, Peruffo AD, De Lazzari F, Curioni A. Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. J Agric Food Chem 2001; 49: 5668-73.

Sotkovsky P, Hubalek M, Hernychova L, Novak P, Havranova M, Setinova I et al. Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. Proteomics 2008; 8: 1677-91.

Sotkovsky P, Sklenar J, Halada P, Cinova J, Setinova I, Kainarova A, Golias J, Pavlaskova K, Honzova S, Tuckova L. A new approach to the isolation and characterization of wheat flour allergens. Clin Exp Allergy 2011; 41(7): 1031-43.

Strobel S, Mowat AM. Oral tolerance and allergic responses to food proteins. Curr Opin Allergy Clin Immunol 2006; 6: 207-13.

Takeda K and Gelfand EW. Mouse model of allergic diseases. Curr Opin in Immun 2009; 21: 660-5.

Tatham AS, Shewry PR. Allergens in wheat and related cereals. Clin Exp Allergy 2008; 38: 1712-26.

Tokuda R, Nagao M, Hiraguchi Y, Hosoki K, Matsuda T, Kuono K et al. Antigen-induced expression of CD203c on basophils predicts IgE-mediated wheat allergy. Allergol Int 2009; 58:193-9.

Tordesillas L, Pacios LF, Palacin A, Quirce S, Armentia A, Barber D et al. Molecular basis of allergen cross-reactivity: Non-specific lipid transfer proteins from wheat flour and peach fruit as models. Mol Immunol 2009; 47: 534-40.

Vaali K, Puumalainen TJ, Lehto M, Wolff H, Rita H. Murine model of food allergy after epicutaneous sensitization: role of mucosal mast cell protease-1. Scand J Gastroenterol 2006; 41: 1405-1413.

van der Ventel ML, Nieuwenhuizen NE, Kirstein F, Hikuam C, Jeebhay MF, et al. Differential responses to natural and recombinant allergens in a murine model of fish allergy. Mol Immunol 2011; 48: 637-46.

Varjonen E, Savolainen J, et al. IgE-binding components of wheat, rye, barley and oats recognized by immunoblotting analysis with sera from adult atopic dermatitis patients. Clin Exp Allergy 1994;24(5):481-9

Vickery BP, Chin S, Burks AW. Pathophysiology of food allergy. Pediatr Clin North Am 2011; 58(2): 363-76.

Wang JR, Yan YH, Wei YM, Nevo E et al. Molecularcharacterization of dimeric alpha-amylase genes in wheat and development of genome allele-specific primers for the genes located on chromosome 3BS and 3DS. J Cereal Sci 2006; 43: 360–8.

Weber RW. Cross-reactivity of pollen allergens: Impact on allergen immunotherapy. Ann Allergy Asthma Immunol 2007; 99: 203–11.

Weichel M, Vergoossen NJ, Bonomi S, Scibilia J, Ortolani C, Ballmer-Weber BK et al. Screening the allergenic repertoires of wheat and maize with sera from double-blind, placebo-controlled food challenge positive patients. Allergy 2006; 61:128–35.

Wenzel S, Holgate ST. The mouse trap: it still yields few answers in asthma. Am J Respir Crit Care Med 2006, 174: 1173-6.

Wiley PR, Tosi P, Evrard A, Lovegrove A, Jones HD, Shewry PR. Promoter analysis and immunolocalisation show that puroindoline genes are exclusively expressed in starchy endosperm cells of wheat grain. Plant Mol Biol 2007; 64: 125–36.

Yamashita H, Nanba Y, Onishi M, Kimoto M. et al. Identification of a wheat allergen, Tri a BD 36K, as a peroxidase. Biosci Biotechnol Biochem 2002; 66: 2487–90.

Zaborsky N, Brunner M, Wallner M et al. Antigen Aggregation decides the fate of the allergic immune response. J Immunol 2010; 184: 725–35.

Zapatero M, Martínez MI, Alonso E, Salcedo G et al. Oral wheat flour anaphylaxis related to wheat α-amylase inhibitor subunits CM3 and CM16. Allergy 2003; 58: 956.

Zicari AM, Indinnimeo L, De Castro G, Zappalà D, Tancredi G, Bonci E, Celani C, Duse M. Food allergy and the development of asthma symptoms. Int J Immunopathol Pharmacol. 2012; 25(3): 731-40.