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The Study of Food Allergy in Patients and Experimental Model

Ph.D. Thesis

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This dissertation describes my original work except where acknowledgement is made in the text. It is not substantially the same as any work that has been, or is being submitted to any other university for any degree, diploma or any other qualification.

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RNDr. Jaroslav Goliáš

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Abstract

Food allergy belongs among the most frequent disorders and its incidence is continuously rising over the last two decades in the developed world. Although the methods used in the diagnostics of food allergies are high sensitive, they have low specificity, which is affected by a purity of used extracts. Therefore, it is important to develop new proteomic procedures for isolation of food allergens in the pure and the biologically active forms, thereby improving the diagnostics of food allergies. Another approach for studying allergies is using an experimental model, which can help us to clarify the mechanisms of allergic response and the acquired findings employ in prophylaxis or allergy treatment.

In the first part, we have developed a new proteomic procedure for isolation of wheat allergens in the purified form. By this procedure, using Rotofor, HPLC and electrophoretic methods, we identified 27 potential wheat allergens, from which 7 were newly identified: endogenous α-amylase/subtilisin inhibitor, trypsin/α-amylase inhibitor CMX1/CMX3, TLP, XIP-1, β-glucosidase, class II chitinase, and 26 kDa endochitinase. Further, we showed that isolated allergens (α-amylase 0.19, LTP, TLP, and wheatwin) retained their biological activity and were capable to activate basophils (BAT).

In the second part, we isolated and identified rice allergens. For identification, we used raw and boiled forms of rice, which is the most common form for rice consumption. We identified 22 potential rice allergens, from which 6 were newly identified: glutelin C precursor, granule-bound starch synthase 1 protein, disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and protein encoded by locus Os02g0453600. Moreover, for patients with food allergy (mainly wheat allergy), who were strongly positive in immunoblots and in BAT, we recommend to perform additional skin prick tests (SPT) with the boiled rice homogenate including both water-soluble and water-insoluble rice allergens.

In the third part, we introduced a mouse model of food allergy in which we showed that even small irreversible changes in the structure of ovalbumin after thermal processing and enzymatic digestion led to the formation of new epitopes shifting the immune system to Th1 response and reducing the allergic reaction. Furthermore, our preliminary experiments have shown that germ-free mice were not capable to develop the food allergy and even the colonization of germ-free mice by probiotic bacterium *Lactobacillus plantarum* was not sufficient to induce the food-allergy symptoms.

Abstrakt

Potravinová alergie patří mezi nejčastější alergická onemocnění, jejíž incidence má ve vyspělém světě v posledních dvou desetiletích stoupající tendenci. Metody používané v diagnostice potravinových alergií jsou sice vysoce senzitivní, ale mají nízkou specificitu, která je ovlivněna čistotou používaných extraktů. Proto je důležité vyvíjet nové proteomické postupy pro izolaci potravinových alergenů v čisté a biologicky aktivní formě, čímž můžeme zlepšit diagnostiku potravinových alergií. Jiný přístup pro studium alergií je využití experimentálního modelu, který nám může pomoci objasnit mechanizmy alergické odpovědi a získané poznatky pak uplatnit v profylaxi nebo léčbě alergií.

V první části jsme vyvinuli nový proteomický postup pro izolaci pšeničných alergenů v čisté formě. Tímto postupem, využívajícím Rotofor, HPLC a elektroforetické metody, jsme identifikovali 27 potenciálních pšeničných alergenů, z kterých 7 bylo nově identifikováno: endogenní inhibitor α-amylázy, inhibitor α-amylázy CMX1/CMX3, TLP, XIP-1, β-glukosidáza, chitináza II třídy a 26 kDa endochináza. Dále jsme ukázali, že námi izolované alergeny (α-amyláza 0.19, LTP, TLP a wheatwin) si zachovávají svoji biologickou aktivitu a byly schopné aktivovat bazofily (BAT).

V druhé části jsme izolovali a identifikovali potenciální rýžové alergeny. Při identifikaci alergenů jsme použili syrovou a vařenou formu rýže, což je nejběžnější forma její konzumace. Identifikovali jsme 22 potenciálních rýžových alergenů, z nichž 6 bylo nově identifikováno: glutelin C prekurzor, v granulích vázaná škrobová syntáza 1, disulfid izomeráza 1-1, hypotetický protein Os_13867, prekurzor kyselé fosfatázy 1 a protein kódovný na lokusu Os02g0453600. Navíc pro pacienty s potravinovou alergií (hlavně pšeničnou alergií), kteří jsou silně pozitivní na imunoblotech a v testech aktivace basofilů (BAT), doporučujeme provést doplňující kožní testy (SPT) s homogenátem vařené rýže, který obsahuje jak ve vodě rozpustné tak ve vodě nerozpustné rýžové alergeny.

Ve třetí části jsme zavedli myší model potravinové alergie. Ukázali jsme, že i malé nevratné změny ve struktuře ovalbuminu po tepelném zpracování a po enzymatickém štěpení vedou ke tvorbě nových epitopů, posouvajících imunitní systém směrem k prozánětlivé Th1 odpovědi, čímž dochází k redukci alergické reakce. Mimoto, naše předběžné experimenty ukázaly, že bezmikrobní myši nejsou schopny rozvinout potravinovou alergii, a dokonce že kolonizace bezmikrobních myší probiotickou bakterií *Lactobacillus plantarum* není dostatečná pro vyvolání příznaků potravinové alergie.

Contents

Abbreviations

1-DE	one-dimensional electrophoresis
2-DE	two-dimensional electrophoresis
AD	atopic dermatitis
ACD	allergic contact dermatitis
ALP	alkaline phosphatase
APC	antigen-presenting cell
APT	atopy patch test
AR	allergic rhinitis
BALB/c	laboratory-bred strain of the “house mouse”
BAT	basophil activation test
b-OVA	boiled-ovalbumin
CD	cluster of differentiation
CFU	colony-forming units
CM	chloroform/methanol mixtures proteins
CTL	cytotoxic $CD8^+$ T cells
CTLA-4	cytotoxic T-lymphocyte antigen 4; a receptor for the chemokine fractalkine
CV	conventional
CX3CR1	a high-affinity inhibitory receptor on T cell
DBPCFC	double-blind, placebo-controlled food challenge
DC	dendritic cell
DTH	delayed type hypersensitivity
Fas	a member of the TNF receptor family with death domain
FasL	trimeric Fas ligand
Fc	antibody fragment crystallizable
FcεRI	high affinity receptor for IgE antibodies
Foxp3	forkhead box P3; transcription factor
FPIES	food protein-induced enterocolitis syndrome
GALT	gut-associated lymphoid tissue
GF	germ-free
GIT	gastrointestinal tract
HMW	high molecular weight

h-OVA	heated-ovalbumin
HPLC	high-performance liquid chromatography
IDO	indoleamine-2,3-dioxygenase
IEF	isoelectric focusing
IFN-γ	interferon-γ
Ig	immunoglobulin
i.g.	intragastric
IL	interleukin
ILF	isolated lymphoid follicle
i.p.	intraperitoneal
kDa	kilo Dalton
LAP	latency-associated peptide
LMW	low molecular weight
LP	lamina propria
Lp	*Lactobacillus plantarum*
LTP	lipid transfer protein
M cell	microfold cell
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MAMP	mucosal-associated molecular pattern
MCPT-1	mass cell protease-1
MLN	mesenteric lymph node
MRS	bacterial growth medium (agar) so-named by its inventors: de Man, Ragosa and Sharpe
OIT	oral immunotherapy
OVA	ovalbumin
PAC	perennial allergic conjunctivitis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pH	potential of hydrogen
PPs	Peyer’s patches
PPT	prick to prick test
PRR	pattern recognition receptor
RA	retinoic acid
SAC	seasonal allergic conjunctivitis

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPT	skin prick test
Th	helper T cell
Th3	a subset of regulatory T cell
TGF-β	transforming growth factor-β
TLP	thaumatin-like protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
Tr1	a subset of regulatory T cell
Treg	a subset of regulatory T cell
iTreg	induced regulatory T cell
nTreg	natural regulatory T cell
WDEIA	wheat-dependent exercise induced anaphylaxis

1. Introduction

1.1. Mucosal immune system

The interface of organism and outside world is selectively permeable for small molecules (*e.g.*, nutrients) and at the same time it must constitute the barrier against environmental threats (*e.g.*, pathogens). This natural barrier is formed by mucosal and skin surfaces covered with special single epithelial cell layers. The intestinal mucosal surface covers the largest area of the body (approximately 300 m^2), and the mucosal immune system situated under this surface is the biggest reservoir of immune cells in the body, protects the body from invading pathogens from the intestinal lumen and keeps the commensal microbiota compartmentalized. The epithelial cells secrete a number factors that contribute to barrier function, including mucins, antimicrobial peptides (*e.g.*, defensins, lysozymes), and trefoil factors secreted by goblet cells and Paneth cells, respectively. Below the epithelial layer is mucosa densely populated by resident immune cells, such as $CD4^+$ and $CD8^+$ T cells, regulatory T (Treg) cells, antibody-secreting B cells, dendritic cells (DCs), macrophages, and eosinophils (Tlaskalová-Hogenová et al., 2002; Chehade and Mayer, 2005).

The important role in the mucosal immune system plays the commensal microbiota in the intestine. Each human adult harbors approximately 10^{14} bacteria in the gastrointestinal tract (GIT), which is about 10 times more than the number of all cells making up the human body (Hart et al., 2002; Penders et al., 2007). There are more than 1000 different species (including bacteria, archaea, fungi, and protozoans) divided into different strains, highlighting the enormous complexity of this environment (Maynard et al., 2012; Smythies L.E. and Smythies J.R., 2014). Only the minority of the species (300–500) present in the intestine is aerobic and can currently be cultured *in vitro* (Borchers et al., 2009). The microbiota differs depending on their location in the GIT; the concentration of bacteria ranges from 10^3 CFU/ml in the stomach, in which the number of ingested bacteria is reduced by contact with the gastric acid, to 10^{11}–10^{12} CFU/ml in the colon (Hart et al. 2002; Ley et al., 2006).

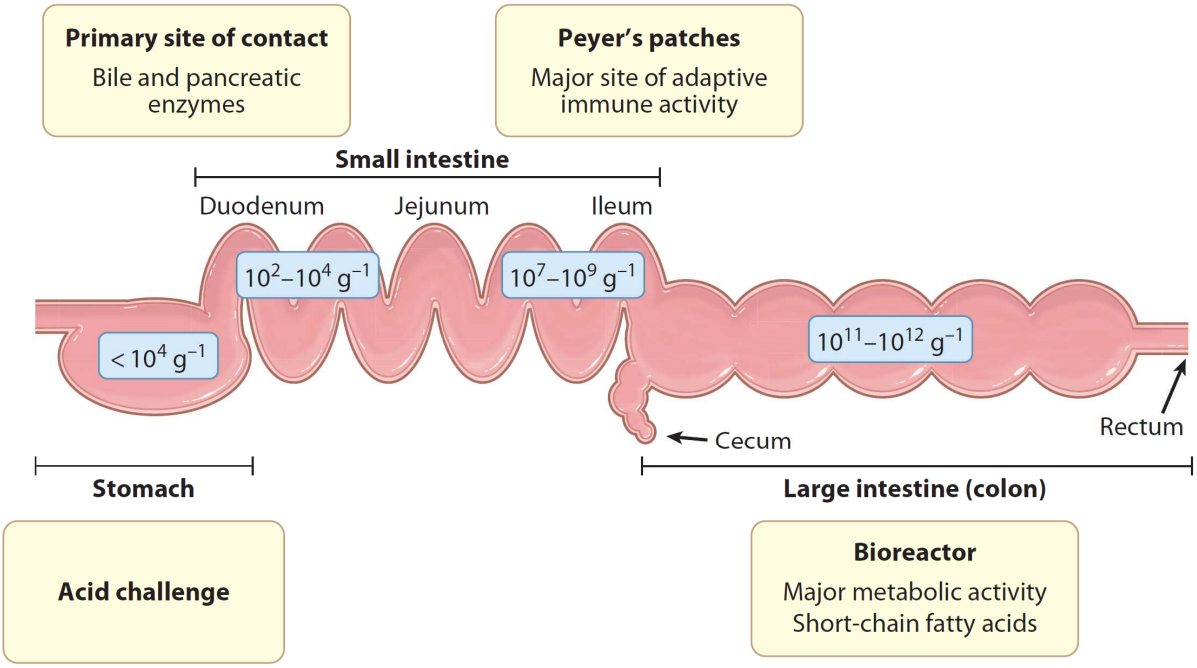

Figure 1. Schematic representation of the human intestine indicating its different regions and the overall sizes of the residing bacterial population. The population of endogenous and probiotic bacteria are increasing from the stomach to the colon. The low amount of these bacteria in the stomach and in the duodenum is caused by acid stress and by bile and pancreatic enzyme stress, respectively. In the jejunum, the population of bacteria is increasing and the most abundant are probiotic bacteria (*e.g.*, *Lactobacilli*, *Bifidobacteria*). Lower in the small intestine (ileum), the population of probiotic bacteria is gradually replaced by endogenous bacteria, and in the colon, the endogenous bacteria are only present (Kleerebezem and Vaughan, 2009).

The human GIT, at birth, is sterile and the immune system is naïve. Soon after birth, the GIT is colonized by a numerous types of microorganisms. Normally, this intestinal colonization of newborns is dominated by transmission of bacteria from the maternal vaginal flora, which is less diverse than that of the lower intestinal tract (Dominguez-Bello et al., 2010). The bacterial species received from the mother in this manner are very important, because infants born by Caesarean section, who are initially colonized by bacterial species of the skin, are much more predispose to development of allergies and asthma later in their life (Bager et al., 2008). Another important source of bacteria colonizing the infant intestine is mother's milk, which contains up to 10^9 microbes/L in a healthy mother (Castellazzi et al., 2013).

The infant microbiota varies erratically until about 1 year of age when is stabilized and resembles that of adults. During this period, the microbiota is required for the maturation of the neonatal immune system and for the development of immune tolerance, not only towards

themselves, but also towards other antigens such as food components. The healthy human intestinal microbiota is mainly composed by *Bacteroides*, *Clostridia*, *Enterobacteria*, *Bifidobacteria* and *Lactobacilli*. These bacterial species, as dietary supplements (probiotics), can be used for improving the intestinal homeostasis or for the recolonization of the intestine by commensal bacteria after infectious diseases. The probiotics are defined as live microorganisms that when are administered in adequate amounts confer a health benefit on the host (Kleerebezem and Vaughan, 2009). The probiotics and other dietary factors, such as prebiotics (compounds specifically promote the growth of beneficial bacteria), antioxidants, polyunsaturated fatty acids, folate and vitamins, have also positive effects on the function of the immune system (Castellazzi et al., 2013).

Dysbiosis and unbalanced shifts in the composition of the microbiota may contribute to development of various gastrointestinal diseases such as inflammatory bowel diseases (Conte et al., 2006; Sokol et al., 2006), irritable bowel syndrome (Malinen et al., 2005), necrotizing enterocolitis (Terrin et al., 2014), and food allergy (Macdonald and Manteleone, 2005).

1.2. Oral tolerance

The mucosal immune system must maintain tolerance to the commensal microbiota, foods and self-antigens while initiating the defensive response to pathogens to prevent uncontrolled infection of the host. This oral tolerance can be divided into inductive and effector sites. The inductive site represents the gut-associated lymphoid tissue (GALT), which including Peyer's patches (PPs) and isolated lymphoid follicles (ILFs), and gut-draining mesenteric lymph nodes (MLNs). Peyer's patches and MLNs develop prenatally, but ILFs develop postnatally; however, each of these lymphoid tissues requires additional signals from endogenous antigens for their complete development. Lamina propria (LP) and gut epithelium constitute the main effector sites, harboring large populations of T cells and antibody-secreting plasma B cells (Brandtzaeg et al., 2008). The endogenous antigens (*e.g.*, intestinal bacteria, nutrients) are processed by the GALT system and utilized for the suppression mechanisms to maintain the host unresponsive to them (Faria and Weiner, 2005; Strobel and Mowat 2006).

The effector site of oral tolerance includes not only anergy or apoptosis of antigen-specific T cells in the intestine, but also the active suppression through the induction of

antigen-specific regulatory T cells (Dubois et al., 2005; Tsuji, 2006). The primary factor, that determines which form of peripheral tolerance develops after oral administration of antigen, is the dose of the antigen fed. The high doses of orally administered antigen result in anergy or deletion of antigen-specific T cells. On the other hand, the low doses of antigen result in the generation of antigen-specific Treg cells following the presentation of antigen by gut associated antigen-presenting cells (APCs).

Regulatory T cells appear to come in various forms – Tr1, Th3, induced Treg (iTreg), and natural Treg (nTreg) cells. Interleukin-10-producing Tr1 cells develop from naïve $CD4^+$ T cell into unique $CD4^+CD25^-Foxp3^-$ cells with suppressive function attributed to the secretion of IL-10 and TGF-β. Both these cytokines have effective suppressive properties that inhibit the cytokine production by activated T cells, the expression of costimulatory molecules on APCs, and the antibody production. The Th3 cells are TGF-β-producing antigen-specific $CD4^+$ T cells and are considered to be a part of the latency-associated peptide (LAP^+) T cell population that exert potent immunosuppressive properties via TGF-β and form effective anti-inflammatory cell populations for the maintenance of immune homeostasis. Because TGF-β induces expression Foxp3, the Th3 cells can affect the T cell development of neighboring cells into the so-called induced Treg cells (Carrier et al., 2007). Furthermore, in the intestine, antigens are captured, processed and presented by $CD103^+$ subpopulation of DCs which subsequently migrate to MLNs and induce gut-homing iTreg cells by a mechanism dependent on TGF-β, retinoic acid (RA) and indoleamine-2,3-dioxygenase (IDO) (Coombes et al., 2007). These iTreg cells expand in LP by IL-10-expressing $CX3CR1^+$ macrophages and can then suppress immune responses, including allergic sensitization. The last Treg cell population is nTreg cells which develop in the thymus as $CD4^+CD25^+$ cells and express high levels of Foxp3, and their suppression mediators include inhibitory molecules such as CTLA-4 receptor and immunomodulatory cytokines IL-10, TGF-β, or IL-35. All these regulatory T cells are involved in the development of oral tolerance (McHugh and Shevach, 2002; Sakaguchi, 2004; Tsuji and Kosaka, 2008; Pabst and Mowat, 2012).

The development and induction of oral tolerance can be also affected by the commensal microbiota. This effect of the oral tolerance induction was observed for example in mice grown under germ-free conditions which do not have developed normal tolerance (Sudo et al., 1997). Moreover, the normal intestinal microflora affects the development of both the intestinal lymphatic tissue and the whole immune system, as demonstrated by comparing germ-free animal models and conventionally or specific pathogen free animal models (Hooper et al., 2001).

The induction of oral tolerance, mainly in a susceptible individual, may not be always dependent on antigen digestion, insomuch as its induction might be bypassed by the presentation of allergens through alternative route, such as through the respiratory tract or even the skin. Pollen-food allergy is an appropriate example, in which oral tolerance is bypassed, because sensitization occurs through the respiratory route (Fernández-Rivas et al., 2006).

1.3. Hygiene Hypothesis

Hygiene hypothesis postulates that the decreasing incidence of infectious diseases is the primary factor behind the increasing incidence of allergic diseases in the industrialized countries. The hygiene hypothesis was first proposed by Strachan, who observed an inverse correlation between hay fever and the number of elder siblings. According this observation the child, who is growing among siblings, has overcome the common child infections and had lesser probability to develop the allergy (Strachan, 1989).

The western lifestyle is generally understood as a main factor in the increasing predisposition towards allergic diseases due to improved socioeconomic and hygienic conditions. These habits in the lifestyle can reduce infections due to decontamination of the water sources, pasteurization and sterilization food products (*e.g.*, milk). Moreover, vaccination against common childhood infectious diseases and the wide use of antibiotics lead to the eradication of ordinary infections and the emergence of allergic and autoimmune diseases (Weiss, 2002).

Several epidemiological studies found less frequent incidence of allergic diseases among people living in less developed regions. One of these studies reported the results from Germany shortly after unification; prevalence of asthma and other atopic diseases was significantly higher among children from West Germany than those from East Germany, regardless of their similar genetic base (von Mutius et al., 1994). As changes in the genetic factors are negligible in these populations, the changes in the environment of the developed countries, as a result of modernization, may lead to the rise in prevalence of allergic diseases (Wills-Karp et al., 2001).

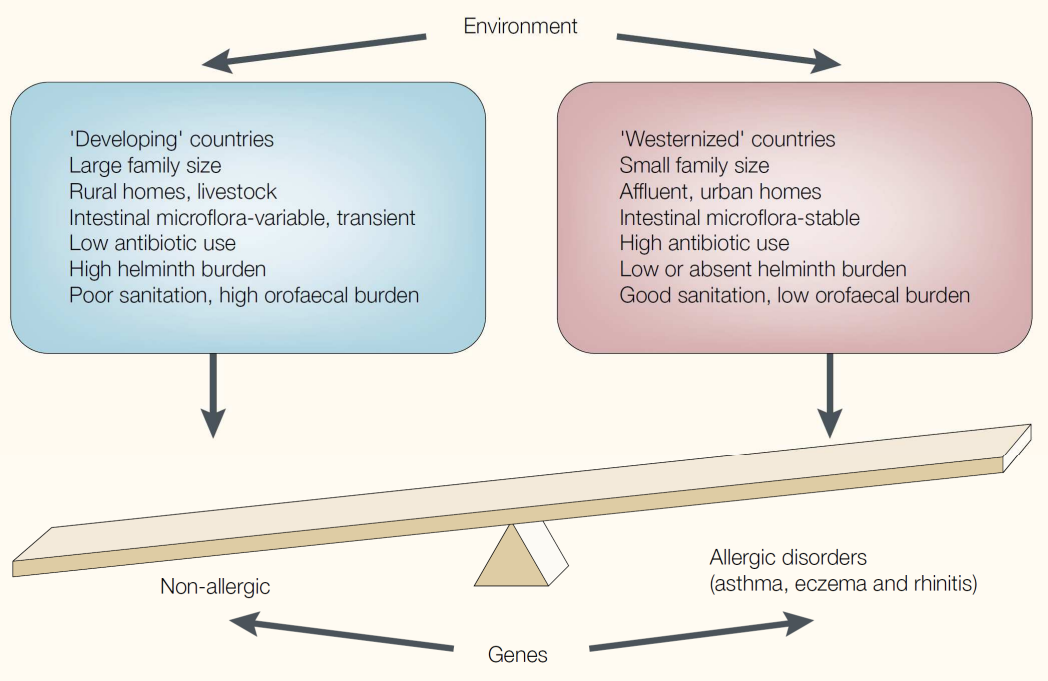

Figure 2. Environment and genetic factors in allergy prevalence. Although both genetic and environmental factors influence the etiology of asthma and allergy, changes in the genetic make-up of stable populations does not occur in this time frame. The recent rise in the prevalence of asthma and allergies is therefore thought to be primarily due to changes that have taken place in the environment in developed countries as a result of modernization (Wills-Karp et al., 2001).

The immunologic explanation of the hygiene hypothesis is based on the balance between Th1 and Th2 immune response. The Th1 and Th2 cells are antagonistic and their differential activation is important in the development of immune-mediated diseases. Allergic diseases arise as a result of the systemic imbalance characterized by the predominance of Th2 cells, whereas infectious diseases and many autoimmune diseases are directed by a predominance of Th1 cells. This Th1/Th2 balance was the first major candidate mechanism for explaining the protective influence of infectious agents from immunological disorders. Subpopulation of Th1 cells produces inflammatory cytokines, such as IL-2, IFN-γ, and TNF-α, that are important in the cell-mediated immunity including autoimmune disorders. In contrast, subpopulation of Th2 cells, producing IL-4, IL-5, and IL-13, contribute to the IgE production and the allergic response (Okada et al., 2010).

The hygiene hypotesis can be explained by so-called antigenic competition. The development of strong immune response against antigens from infectious agents could inhibit the response to weak antigens such as allergens or autoantigens. Moreover, highly specific T cell receptors, DCs and other cells interact with potential infectious agents by pattern

recognition receptors (PRRs). Natural ligands of these receptors constitute pathogen-associated molecular patterns (PAMPs) that are typical for potentially pathogenic structures on bacteria, viruses and fungi. Within PRRs, the Toll-like receptor (TLR) family represents the best characterized class which is crucial for the initialization of inflammatory and immune defense responses. Allergens themselves do not represent or carry typical PAMPs, but common bacteria carry these ligands which are named as mucosal-associated molecular patterns (MAMPs). Uptake and presentation of such MAMPs leads to the activation of Th1 immune response through TLRs and prevent the Th2-driven allergic immune response. Hence, the TLRs together with commensal microbiota have a very important role in the regulation of allergies (Rakoff-Nahoum et al., 2004; Garn and Renz, 2007).

1.4. Hypersensitivity reactions

Before proceeding to a direct focus on food allergic diseases, it is useful to divide harmful immune responses, or hypersensitive reaction, initially outlined by Coombs and Gell. The hypersensitivity reactions are usually divided into four classes: (a) type I, or IgE-mediated disorders; (b) type II, or antibody-mediated disorders; (c) type III, or complement-mediated immune disorders; and (d) type IV, or delayed-type hypersensitivity (Gell and Coombs, 1963; Descotes and Choquet-Kastylevsky, 2001; Rajan, 2003).

1.4.1. Type I hypersensitivity, immediate IgE-mediated allergic reaction

Type I hypersensitivity, known as allergy, is an immediate IgE-mediated reaction to various antigens or allergens. The type I hypersensitivity is usually divided into two phases – the sensitization and the allergic reaction. During the sensitization phase, allergen specific IgE antibodies bind to the high affinity IgE receptors (FcεRI) situated on the plasma membrane of mast cells leading to their activation. During the allergic reaction, the subsequent exposure to the same allergen cross-links the surface-bound IgE antibodies and activates intracellular signals from the cytoplasmic portion of the FcεRI receptors (De Angelis et al., 2010).

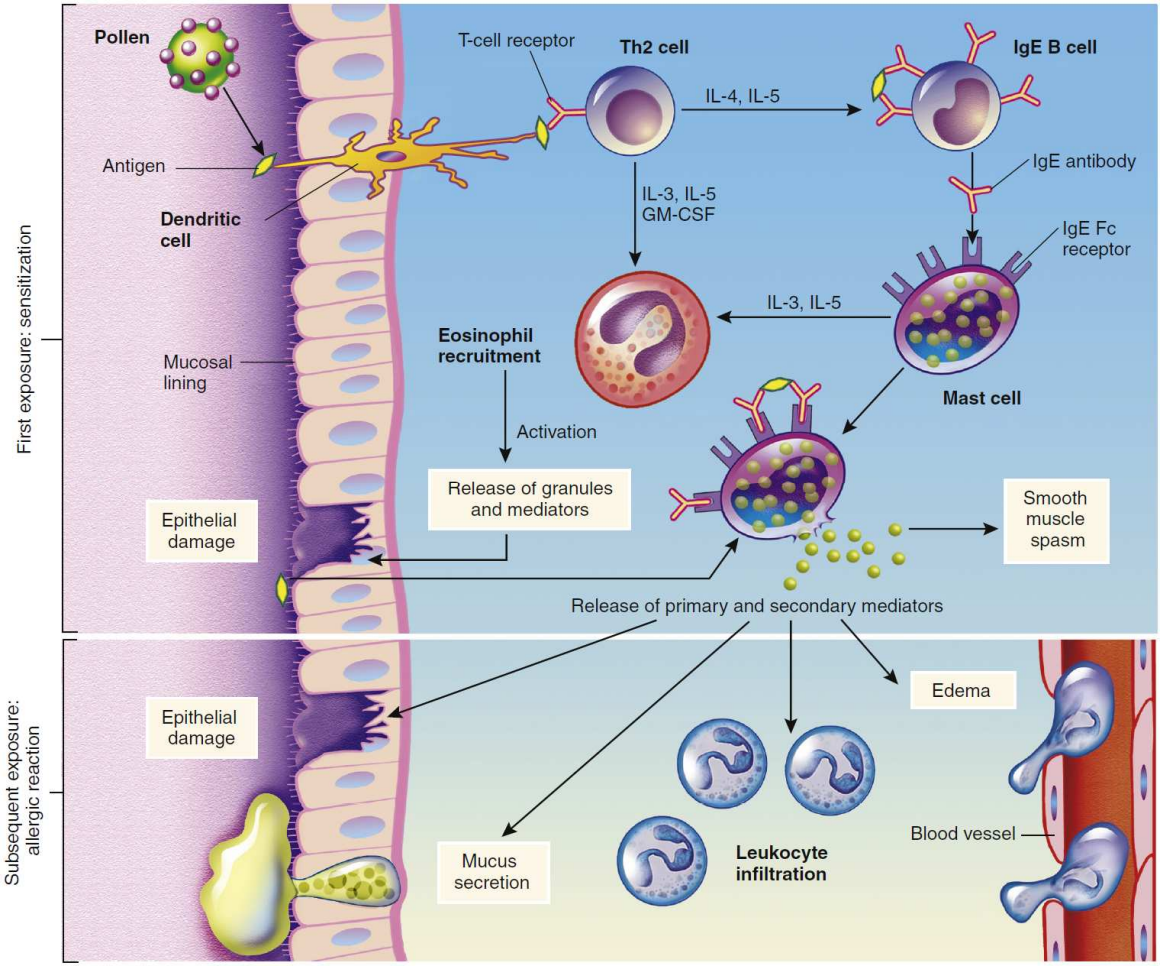

Figure 3. Type I IgE-mediated allergic reaction. Th2 cells are activated by antigen-presenting dendritic cells to produce cytokines, including IL-3, IL-4, IL-5, and GM-CSF. These cytokines attract and promote the survival of eosinophils and induce B cells to class-switch to IgE-producing plasma cells. The IgE coats the surface of the mast cells by binding with IgE-specific Fc receptor and activates signaling from the cytoplasmic portion of Fc receptors. These signals initiate two parallel and independent processes: mast cell degranulation of preformed mediators (*e.g.*, histamine) and the production of newly formed mediators such as arachidonic metabolites (leukotrienes, prostaglandins). Many local type I hypersensitivity reaction have two phases, the initial phase (vasodilation, smooth muscle spasm, etc.) occurring within 5 to 30 minutes, and late phase occurring 2 to 8 hours later and leading to more intense infiltration of tissues with granulocytes and lymphocytes and tissue destruction (McCance et al., 2014).

The own allergic reaction has two well-defined phases – the initial and late phase. The initial phase is characterized by vasodilation, vascular leakage, and, depending on the location, smooth muscle spasm or granular secretions. This phase usually becomes evident within 5 to 30 minutes after exposure to the allergen and is caused by mast cell degranulation and releasing preformed mediators (*e.g.*, histamine, eosinphil-chemotactic factor of anaphylaxis). The late phase occurs 2 to 8 hours later without additional exposure to the

antigen which is caused delayed production of newly formed mediators such as arachidonic metabolites (*e.g.*, leukotrienes, prostaglandins). This phase leads to infiltration tissue with granulocytes (eosinophils, neutrophils, basophils) and lymphocytes and tissue destruction in the form of mucosal epithelial cell damage.

The IgE antibodies, the main antibodies involved in the allergic reaction, are normally present in very low numbers in the circulation, with the normal concentration of 50-200 ng/ml in non-allergic individuals (Gould et al., 2003). The half-life of IgE in the serum is 2.5 days and much of the IgE is sequestered in tissues due to binding on Fc receptors on mast cells or eosinophils. Despite of much lower half-life of IgE in the circulation, the half-life of IgE antibody captured on high-affinity FcεRI receptor is approximately 14 day and it seems that the IgE-FcεRI interaction has a potent stabilizing effect on the IgE half-life (Hellman, 2007).

1.4.2. Type IV hypersensitivity, cell-mediated reaction

Type IV hypersensitivity reaction, also known as a delayed-type hypersensitivity (DTH), involves cell-mediated (T-cell-mediated mechanism) rather than antibody-mediated immune response. The typical DTH reaction develops within 24 to 48 hours. Due to the heterogeneity of T-cell function in this hypersensitivity reaction, DTH reactions may be divided into two groups – Th1-mediated or cytotoxic-mediated reaction. The Th1-mediated DTH reaction is maintained by activation of macrophages and the mechanism of this reaction is similar to the immune response against a variety of microorganisms, including intracellular pathogens such as *Mycobacterium tuberculosis* and viruses, as well as extracellular agents such as fungi, protozoa, and parasites. The cytotoxic-mediated reaction is maintained by activation cytotoxic lymphocytes (CTLs), which attack and destroy cellular targets directly inducing of apoptosis through Fas–FasL interactions or perforin-granzyme mediated apoptosis. Typical examples of these reactions are contact dermatitis (allergic reaction to latex, poison ivy and metals such as nickel), chronic asthma, chronic allergic rhinitis, insulin dependent (type 1) diabetes mellitus, rheumatoid arthritis, Hashimoto´s thyroiditis, graft rejection, etc. (Rajan, 2003; Khan et al., 2010; McCance et al., 2014).

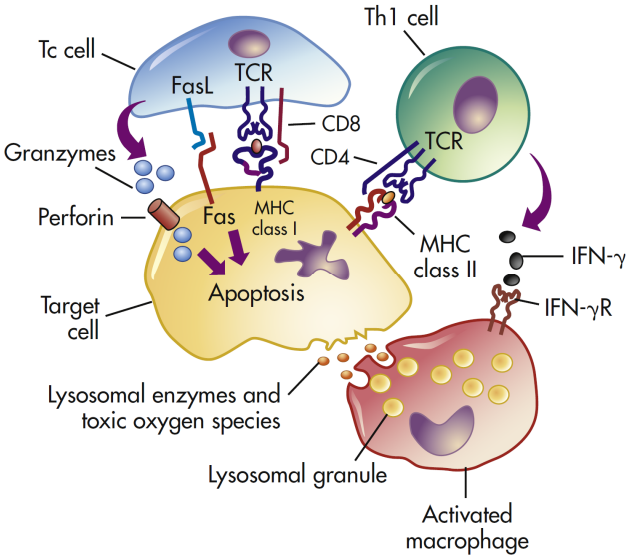

Figure 4. Type IV hypersensitivity reaction. Antigens from target cells stimulate T cells to differentiate into cytotoxic (Tc) lymphocytes with direct cytotoxic activity, and Th1 cells involved in delayed hypersensitivity. The Th1 cells produce cytokine IFN-γ that activate macrophages which can attach to targets and release enzymes and reactive oxygen species that are responsible for most of the tissue destruction (McCance et al., 2014).

1.5. Allergy

The immune system, in some circumstances, reacts against innocuous "environmental" stimuli such as pollen, foods, drugs, or other environmental components. This inadequate reaction of the immune system is generally known as an allergic reaction and this state is called atopy. The atopic individuals have higher total levels of IgE antibodies in the circulation and are more susceptible to allergic diseases. The IgE-mediated allergic reaction is also defined as an immediate hypersensitivity or anaphylactic reaction, because occurs very soon after the contact of atopic individuals with an allergen (Sampson, 2003; Johansson et al., 2004).

1.5.1. Allergens

Allergens, by definition, are environmental proteins, largely derived from complex living organisms such as plant, fungi, insects, and other mammals. The allergen has the ability to elicit powerful Th2 responses, culminating in the IgE antibody production (Wills-Karp,

1999). Allergens are predominantly small proteins with molecular mass within 10 to 70 kDa, and their most typical representatives are pollen, house dust mites, animal dander, foods, and chemicals and drugs, such as penicillin (Cochrane et al., 2009; Kanagawa et al., 2009). Most of allergens belong to the relatively low number of different protein families according to intrinsic features such as similar amino acid sequences and/or three-dimensional folding. For instance, plant food allergens belong to only a few protein families (*e.g.*, prolamin superfamily, cupin superfamily, thaumatin-like proteins (TLPs), Bet v 1 family, etc.), which conserved structure and biological activities play the important role in determining or promoting the allergenic properties. In addition, the members of the same protein family from diverse species may share the same antigenic epitopes that are recognized by the same IgE antibody, resulting to the cross-reactivity. For instance, birch pollen allergen Bet v 1 has homologous sequences in various foods such as allergen Mal d 1 in apple, Cor a 1 in carrot or Pru av 1 in cherry (Breiteneder and Mills, 2005a; Breiteneder and Mills, 2005b; Jahn-Schmid et al., 2005).

1.5.2. Clinical symptoms of allergy

The clinical manifestation of allergic diseases is depended on the main portal of entry for the allergen. For instance, pollen and other airborne allergens usually cause respiratory allergies, but food proteins most often cause food allergies. The exposure to the allergen may be through inhalation, ingestion, injection, or skin contact. The manifestation of the allergic diseases includes conjunctivitis, rhinitis, asthma, urticaria or hives, atopic and contact dermatitis, food allergy symptoms, and anaphylaxis. Type I reactions may occur as a local or an atopic reaction that is merely annoying (*e.g.*, seasonal rhinitis) or severe (*e.g.*, asthma), or as a systemic and potentially life-threatening anaphylactic reaction (Johansson et al., 2001; Johansson et al., 2004).

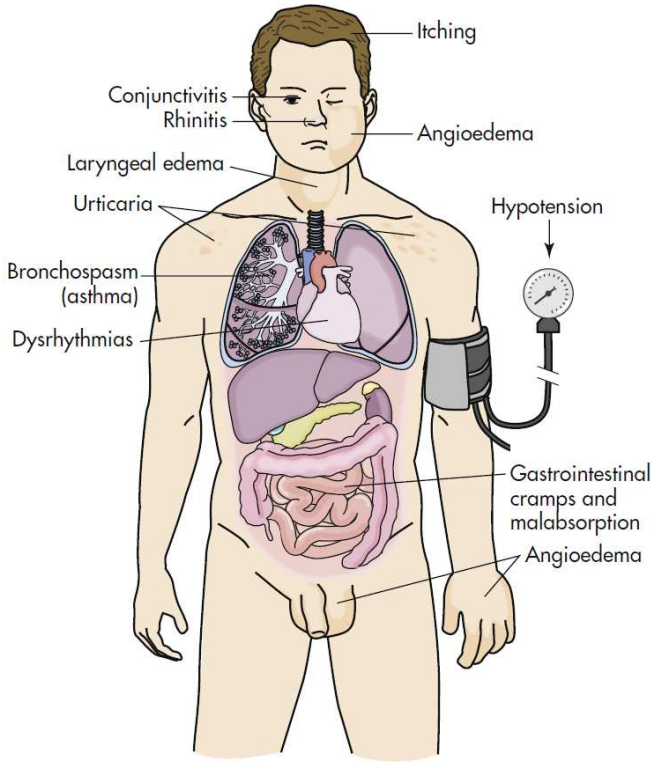

Figure 5. Symptoms of the allergic reaction. The manifestation of allergy include itching, angioedema (swelling caused by exudation), edema of the larynx, urticaria (hives), bronchospasm (constriction of airways in the lungs), hypotension (low blood pressure), dysrhithmias (irregular heartbeat) due to the anaphylactic shock, and gastrointestinal cramping (McCance et al., 2014).

Ocular allergy or conjunctivitis represents one of the most common allergic reactions in clinical practice, and numerous factors contribute on its manifestation, including genetics, air pollution in urban areas, pollens, pets, etc. (Leonardi et al., 2007). The most common forms of conjunctivitis are seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC). Signs and symptoms of both disorders are the same (itching, redness, swelling of the conjunctiva), but the difference is in the specific allergen to which the patient is allergic; SAC is usually caused by airborne pollens and symptoms are seasonal (*e.g.*, the spring), but PAC may occur throughout the year with exposure to perennial allergens (Friedlaender, 2011; La Rosa et al., 2013).

Allergic rhinitis (AR) or hay fever is another representative of the allergic reaction to outdoor allergens (*e.g.*, grass and tree pollens). The prevalence of AR is within 10% to 20%. The symptoms are characterized by itching, sneezing, increased secretion or blockage, and severe form of AR has a negative effect on the quality of patient's life (Johansson et al., 2004; Greiner et al., 2011). Allergic rhinitis occurs frequently with asthma and there is a widespread

assumption that AR in children often predicts the development of asthma (Burgess et al., 2007; Dretzke et al., 2013).

Asthma is heterogeneous disease, usually characterized by airway inflammation, bronchial hyperresponsiveness, and structural changes in the bronchial walls (airway remodeling) (Wenzel, 2013). The clinical symptoms of allergic asthma are recurrent episodes of wheeze, shortness of breath, chest tightness, and cough. The asthmatic response is provoked by allergy in 80% of all asthmatic cases. The clinical manifestation of asthma often appears during childhood and the world prevalence of asthma is approximately 10% (Holgate, 2008; Madore and Laprise, 2010).

Urticaria, also known as hives, and angioedema are the most common allergic reactions manifested on the skin. The manifestation is characterized by the appearance of fleeting wheal, which is caused by activated mast cells in the superficial skin in urticaria or activated mast cells deeper in dermis in angioedema. Urticaria and angioedema is typical for food allergies and the reaction to food can occur immediately or within several hours after consumption. Urticaria may also develop after the direct contact with the allergen (*e.g.*, latex allergy) or in individuals with dog's saliva allergy (Johansson et al., 2004; Spickett, 2014).

Atopic dermatitis (AD) is often the first manifestation of allergic disease. Most patients with AD are suffering from another atopic disorder, such as AR, asthma, or food allergy. Atopic dermatitis affects up to 25% of children and 1% to 3% of adults (Novak and Simon, 2011; Flohr and Mann, 2014). Pruritus, scratching, chronic relapsing, or eczematous lesions are major hallmarks of AD. Children with AD have higher risk of developing food allergy. The prevalence of food allergy in this group of patients is within 20% to 80% (Oehling et al., 1998; Werfel and Breuer, 2004).

A close contact with low molecular weight chemicals or irritants may provoke another local allergic reaction on the skin. This reaction is predominantly mediated by Th1 cells and is named as allergic contact dermatitis (ACD). Typical allergens causing ACD include nickel, chromium ions, fragrances, preservatives, etc. (de Waard-van der Spek et al., 2013). The curious type of ACD is protein contact dermatitis caused by absorption of proteins through damaged skin (Rocha et al., 2010; Barata and Conde-Salazar, 2013).

Anaphylaxis is a serious, life-threatening, and generalized hypersensitivity reaction leading to the rapid release of mediators from tissue mast cells and peripheral basophils into the bloodstream (*e.g.*, insect venom, drugs). The release of mediators leads to loss of blood pressure due to vasodilatation, airway constriction and may lead to the anaphylactic shock and death (Boden and Burks, 2011).

The allergic reaction can occur after the consumption of any allergens present in food. These food components cause releasing of mediators from activated mast cells associated in gastrointestinal mucosa and lead to the manifestation of various symptoms that are rapid in the onset and may be presented as itchy flushing of the skin or urticaria, abdominal pain and vomiting, occasionally blood in the stool, and mild or severe bronchospasm, respiratory distress, and hypotension (Sicherer, 2002; Wang and Sampson, 2011; Burks et al., 2012). In some cases, the food allergy may also lead to the fatal anaphylactic reaction. This situation is primarily reported in the allergic reactions to peanuts and tree nuts, and can occur in teenagers or young adults with the combination of asthma and food allergy (Bock et al., 2007).

1.6. Food allergy

The adverse food hypersensitivity includes a broad spectrum of disorders divided into the immune mediated and non-immune mediated reactions. Among the immune mediated reactions belong food allergy and celiac disease. According to immunopathologic mechanisms, food allergies are further divided and classified into three groups: (a) IgE-mediated allergic reaction, in which IgE antibodies are produced; (b) cell-mediated allergic reaction, in which cells of the immune system are important (*e.g.*, eosinophiles) located mainly in the GIT; and (c) mixture of IgE-mediated and cell-mediated allergic reaction (Cianferoni and Spergel, 2009; Burks et al., 2012). The non-IgE-mediated immune reactions include celiac disease, eosinophilic esophagitis, eosinophilic gastroenteritis, and food-protein-induced enterocolitis syndrome (FPIES) (Liacouras et al., 2011; Leonard and Nowak-Węgrzyn, 2012; Katz and Goldberg, 2014; Wechsler et al., 2014).

The second group, the non-immune mediated reactions, represents primarily the food intolerance. The food intolerance could be caused either by food properties such as toxicity (*e.g.*, fish toxin), metabolic insufficiency (*e.g.*, lactose intolerance), pharmacologic intolerance (*e.g.*, caffeine, tyramine in rotten cheese), or by other undefined intolerance (*e.g.*, sulfites, food additives) (Mills and Breiteneder, 2005; Ramesh, 2008; Cianferoni and Spergel, 2009; Boyce et al., 2010). These facts point out that only 50% of adults, who reported an adverse reaction after food consumption, were confirmed as a food-allergic patient, whereas the rest of them suffered from one type of the food intolerance (Seitz et al., 2008).

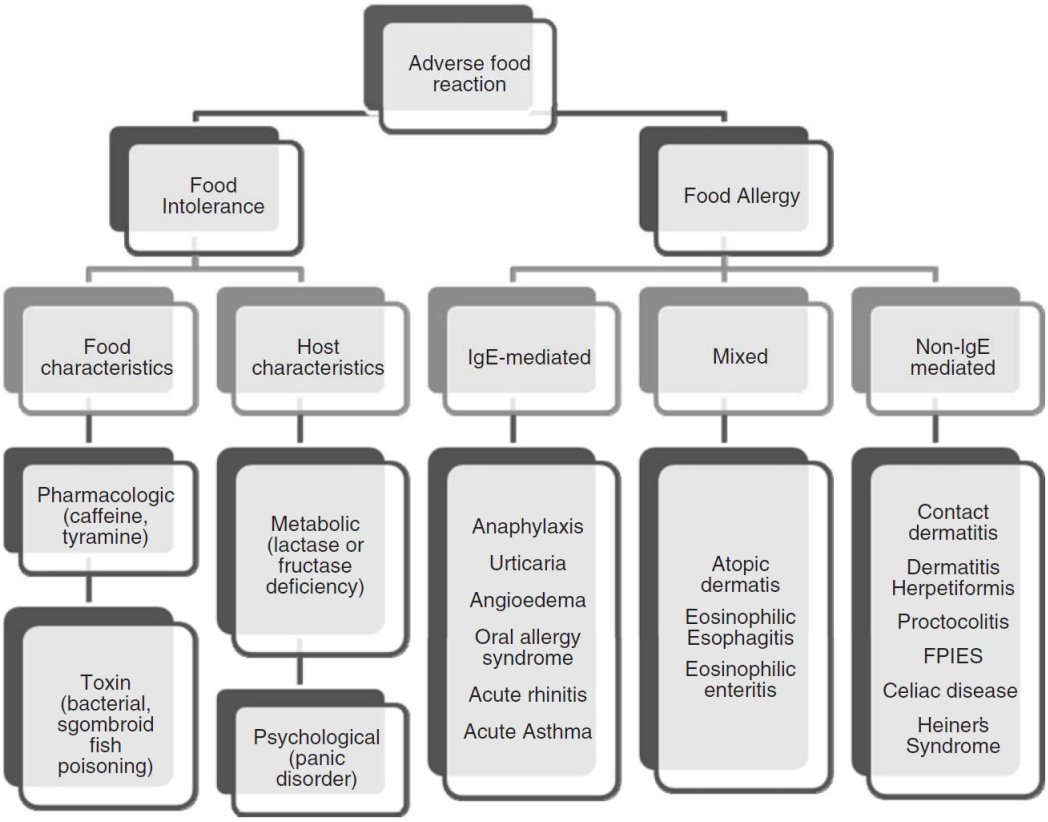

Figure 6. Classification of adverse reactions to foods (Cianferoni and Spergel, 2009).

The classification of adverse reactions to food from Figure 6 was revised and updated in guidelines developed by the coordinating committee of National Institute of Allergy and Infectious Diseases (Figure 7) (Boyce et al., 2010).

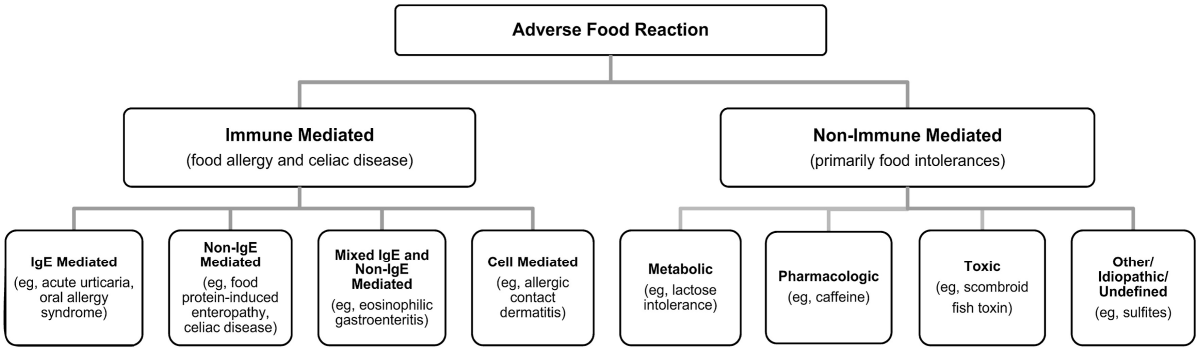

Figure 7. Revised and updated adverse food reactions (Boyce et al., 2010).

However, food allergy is the most common IgE-mediated adverse reaction to food, appearing mainly in early childhood and currently affecting up to 8% of children and 3% to 4% of adults in the developed and industrialized countries. In addition, the incidence of food allergies is continuously rising over the last two decades (Björkstén, 2001; Wang and Sampson, 2012). The prevalence of food allergies may be influenced by various factors, such as genetic predispositions or environmental factors (Prescott and Allen, 2011).

Food allergens are most often proteins, which can be of plant or animal origin, and differ in their structure and molecular weight (Mills and Breiteneder, 2005; Wills-Karp et al., 2010). Major food allergens share a number of common conserved features which play a crucial role in their allergenicity; therefore atopic patients may usually react with allergens from various foods (Cochrane et al., 2009; Kanagawa et al., 2009). Although food allergy could be triggered by virtually any food, some foods indicate predominate prevalence of food allergies (Rona et al., 2007). The food allergens responsible for most allergic reactions include milk, eggs, peanuts, tree nuts, shellfish (*e.g.*, crustaceans and mollusks), fish, wheat, and soy. These food allergens together are responsible for almost 90% of all food allergies, mainly in children (Inomata, 2009; Sicherer and Sampson, 2010). Most commonly foods causing allergies in adults are peanuts, shellfish, and fish. However, in adults, up to 60% of all food allergies are due to cross reactions between food and inhalative allergens, even if pollen-associated fruit, vegetable, and spice allergies may occur during childhood (Werfel, 2008).

Most children may ‘outgrow’ their allergy to some foods, such as milk, eggs, wheat, and soy during their first decade of life. Unfortunately, other allergies to foods such as peanut, tree nuts, fish, and shellfish are often retained for the whole life (Wood, 2003; Wang and Sampson, 2011). Children with non-IgE-mediated cow’s milk allergy retain their sensitivity and about half of them develop allergy to other foods (Høst et al., 2002; Saarinen et al., 2005). Interestingly, the children with egg allergy may develop egg tolerance in later age, but the children with high specific IgE antibodies to egg allergen (especially those $\geq$ 50 kU/L) are unlikely to develop egg tolerance (Savage et al., 2007). Higher prevalence of food allergies in childhood may also be influenced by reduced gastric acidity in young children (Hyman et al., 1985; Untersmayr and Jensen-Jarolim, 2006). Furthermore, the increased permeability of mucosal surfaces shortly after birth can lead to the easy sensitization of infants with food allergens (Bresson et al., 1984).

1.6.1. Mechanism of food allergy

The first contact with a food allergen results in the sensitization of an allergic sensitive individual. The initial sensitization to the food allergen occurs either via the intestinal mucosa or through the skin exposure. The food allergen can be transported from the luminal side of the intestine through damaged epithelial cell layer or *via* active uptake and transport by enterocytes known as M cells (transcytosis). The M cells are specialized for active transporting the intestinal contents (*e.g.*, macromolecules and small particles) from the lumen into the organized lymphoid follicles, such as Peyer's patches (PPs). Another way of allergen transporting through the epithelial barrier is arranged by DCs which are able to continuously sample food allergens by phagocytosis, macropinocytosis or by extending processes between epithelial cells without disturbing their integrity (Niess and Reinecker, 2006; De Angelis et al., 2010).

After the transfer of food allergens, antigen-presenting cells, such as DCs, process of food allergens into the short peptides for the presentation to lymphocytes in PPs and MLNs. The peptides presented on APCs stimulate the differentiation of Th2 lymphocytes to the production of pro-allergenic cytokines such as IL-4, IL-5, IL-9, and IL-13. These cytokines activate mast cells and basophils, stimulate differentiation of B cells into the IgE-producing plasma cells, and recruit and activate eosinophils. The IgE antibodies produced by plasma cells consequently bind to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils (Vickery et al., 2011; Gill, 2012; Salazar and Ghaemmaghami, 2013).

Repeated exposure to the same food leads to binding of allergen to IgE antibodies linked to the FcεRI receptors mast cells and basophiles. Binding the food allergen on IgE antibodies leads to cross-linking of Fc receptors on mast cells and the immediate release of their pro-inflammatory mediators, such as histamine and heparin in the first phase, and arachidonic metabolites (leukotriens, prostaglandins, and tromboxanes) in the late phase of allergic reaction (van Wijk and Knippels, 2007; Salazar and Ghaemmaghami, 2013). Moreover, activated mast cells produce enzyme mast cell protease-1 (MCPT-1), which can support their further activation and degranulation (Vaali et al., 2006).

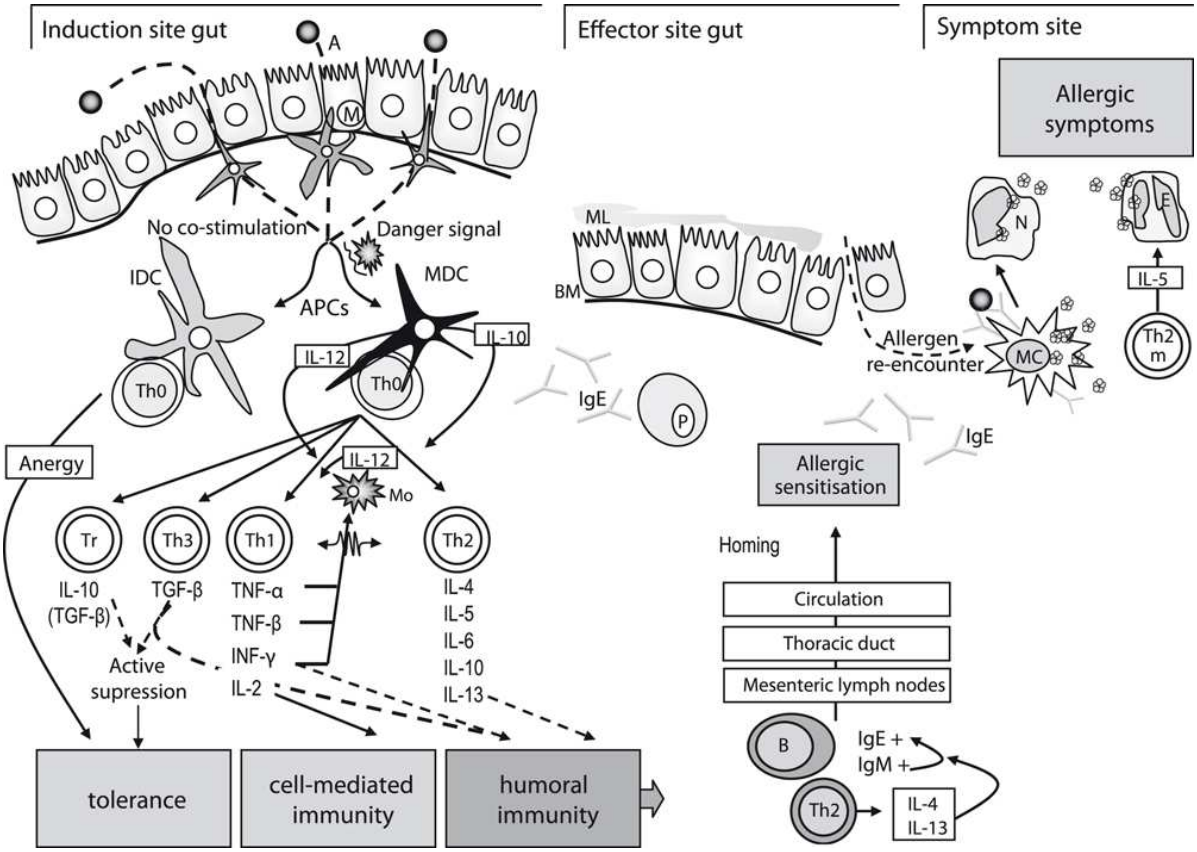

Figure 8. Schematic depiction of the mechanism of food allergy. A: allergen, APCs: antigen presenting cells, B: B cell, BM: basement membrane, E: eosinophil, IDC: immature dendritic cell, IL: interlukin, m: memory cell, M: M cell, MC: mast cell, MDC: matured dendritic cell, ML: mucous layer, Mo: macrophage, N: neutrophil, P: plasma cell, TGF: transforming growth factor, Th: T-helper cell, TNF: tumor necrosis factor, Tr: T-regulatory cell (De Angelis et al., 2010).

1.6.2. Diagnosis of food allergies

Obtaining an accurate patient history is crucial in diagnosis of food allergy. The patient clinical history can help to understand the relationship between clinical symptoms and food that patient consumed. The dietary diary can be very useful for obtaining this information. When the patient's clinical history is known, the elimination diet is the first step used for diagnosis, because the successful elimination of potential allergenic food can confirm that the correct allergen was identified. However, elimination diet alone is insufficient for diagnosing the food allergy. Therefore, two types of diagnostic procedures are useful for diagnosing IgE-mediated allergic disorder – *in vitro* and *in vivo* testing.

In vitro tests are preferred over *in vivo* test, especially for infants and young children. The most frequent test is the measurement of specific IgE antibodies. Serum allergen-specific

IgE antibodies can be detected by using immunoassays (*e.g.*, ImmunoCAP, Immunlite), which provide reliable and reproducible measurements. Traditionally, most tests for specific IgE use extracts derived from the whole food. The results obtained from such allergen-specific tests can be inaccurate. Therefore, the development of recombinant or purified allergen-specific IgE tests against the individual major allergenic components in food (e.g., Ara h 2 in peanut) might improved the diagnosis of allergic disease (Burks et al., 2012). Second most often used *in vitro* method for diagnosing of food allergy is basophil activation test (BAT). The activation test is now accepted as an additional diagnostic tool using flow cytometry to measure activated basophils that express two activation markers – CD63 and CD203c (Sturm et al., 2009).

The 'gold standard' for *in vivo* diagnosis of potential food allergens is double-blind, placebo-controlled food challenge (DBPCFC) (Shaker and Woodmansee, 2009). This test is a highly valuable diagnostic tool, but in sensitive patients with food allergy, severe or even fatal reactions can occur; therefore DBPCFC are generally considered unsuitable for patients with severe immediate hypersensitivity reactions. The other useful *in vivo* diagnostic tool is skin prick test (SPT) which is the standard method for the detection of food allergy (Mehl et al., 2012). The skin prick test is quick and simple to perform and the measured wheal size correlates with the likelihood of food allergy (Knight et al., 2006; Imai et al., 2014). The risk of the systemic allergic reaction in SPT is low, but it must not be undervalued in patients with suspected severe food allergy. The SPT is highly sensitivity method, but it has a low specificity (Sampson and Ho, 1997; Eigenmann and Sampson, 1998). Another useful method for diagnosis of food allergies is prick to prick test (PPT) which is very similar to SPT. The PPT with raw food allergen is a special type of this test, in which a needle is firstly inserted into the food and then into the patient's skin. This type of PPT has higher sensitivity than using commercial extracts, but can be used only for good defined foods, such fruits or vegetables, which must be fresh, never frozen, because thawing can destroy allergenic structures (Heinzerling et al., 2013). Others tests used for diagnosis of food allergy include atopy patch test (APT), and scratch (scarification) and rub test. The APT is used for diagnosis of DTH (*e.g.*, metals), less often for diagnosis of IgE-mediated food allergy in patients with atopic dermatitis (*e.g.*, cow's milk and hen's egg) or for non-IgE-mediated disorders such as eosinophilc esophagitis, and FPIES (Fogg et al., 2006; Heine et al., 2006). The APT uses small pads soaked with the suspected allergen which are applied to the intact skin. The tested food allergen (fresh or from powders) is applied to the skin for 48 hour and read at 72 hours following application (Boyce et al., 2010). Compared to SPT, the APT is more specific, but

less sensitive (Niggemann, 2002; Spergel et al., 2007). Scratch and rub tests are less relevant in food allergy diagnosis. The former test traumatizes the skin, potentially leading to false-positive results, and the latter is compromised by lacking clinical reproducibility.

1.6.3. Therapy and prevention of food allergy

At the moment, there is no 'cure' for food allergy; therefore the primary therapy is to avoid the food containing the suspicious allergen. Education about avoidance includes careful attention to label reading, care in obtaining foods from restaurants, and avoidance of cross-contact of foods with an allergen during the meal preparation. Special care is required for patients who respond to allergens with severe anaphylactic reactions even in negligible amounts. Such allergens include milk, eggs, fish, shellfish, peanuts, tree nuts, sesame, and celery (Sicherer and Sampson, 2010).

One exception to the recommendations for allergen avoidance, in some cases, is heat-sensitive allergens. These include tree-pollen associated foods such as stone fruit, pip fruit, vegetable, such as apple, cherry and celery. The responsible allergen is generally unstable and is destroyed by thermal processing. It was demonstrated that children, who had an allergic reaction to unheated cow milk, tolerated milk in baked products (Nowak-Węgrzyn et al., 2008). In contrast to these so-called pollen-related allergens, the roasting increases the allergenicity of peanuts. For example, protein extracts of thermally treated peanuts bind IgE antibodies from patients' sera at up to 90-fold higher levels than extracts obtained from the corresponding non-treated peanuts (Maleki et al., 2000).

A standardized oral immunotherapy (OIT) is usually used for treatment of various food allergies. The aim of this therapeutic procedure is to receive significant decreasing food-specific IgE antibodies and increasing specific IgG_4 antibodies. This treatment involves administering the allergenic food daily in increasing doses. The majority of food allergic patients (83%) which completed the OIT protocol could subsequently tolerate the food to which they were previously allergic (Patriarca et al., 2003; Patriarca et al., 2007).

Further and future perspective therapeutic approaches comprise engineered recombinant food proteins and anti-IgE antibody therapy (Skripak and Sampson, 2008). Anti-IgE antibody therapy can be used for highly sensitive patients. The main aim of this treatment

is to decrease the amount of IgE antibodies which are capable to bind the allergen and subsequently trigger the anaphylactic reaction (Leung et al., 2003).

A very interesting way to decrease the risk of food allergy is prevention or prophylaxis. An example of very good prophylaxis is a maternal avoidance of potential food allergens during pregnancy or lactation. The ingested food allergens can pass in immunologically intact form into the mother's breast milk and induce reactions in the infant (Isolauri et al., 1999; Vadas et al., 2001). Similarly, the avoidance of solid foods for the first 4 to 6 months of infant's life can decrease the risk of developing the food allergy (Sicherer and Sampson, 2010). Breastfeeding and the delayed introduction of solid foods are associated with the reduction in atopic diseases. Mainly, exclusive breastfeeding is recommended for the first 6 months of life. However, there is inconclusive evidence that either early introduction solid foods before 4 months of age increases or delayed introduction after 6 months of age reduces the risk for subsequent food allergic sensitization, especially for children with atopic risk factors (Poole et al., 2006; Nwaru et al., 2013; Luccioli et al., 2014).

1.6.4. Wheat allergy

Wheat (*Triticum aestivum*) belongs together with rice and maize among the most consumed crops around the world. The wheat products are mainly consumed in Europe and America. In this part of world, the consummation of wheat products leads to higher incidence of wheat food allergy (Shewry, 2009). The prevalence of food allergy to wheat in children is about 0.5% and in adults is less than 1% (Zuidmeer et al., 2008).

Apart from a classical example of wheat food allergy which is primarily dependent on the exposure to allergen via the GIT, there are two other well-characterized types of wheat allergy, occupational asthma (also known as baker's asthma) and wheat-dependent exercised induced anaphylaxis (WDEIA). Baker's asthma is allergic response to the inhalation of wheat and cereal flours and dust. Most patients with backer's asthma do not present wheat food allergy (Tatham and Shewry, 2008). The WDEIA is an allergic reaction induced by the ingestion of food containing wheat following physical exercise within 2 to 4 hours, but neither the food not the exercise alone do not trigger this type of allergy; the combination of both is required. Patients with WDEIA display a wide range of clinical symptoms from local or generalized urticaria to dyspnea, hypotension, collapse and shock (Inomata, 2009). There are

occasions when more than two triggers, such as exercise and cold stimulation, are needed to provoke the symptoms in WDEIA (Benhamou et al., 2007). A skin contact with wheat components, included in moisturizing body cream, may lead to IgE-mediated contact urticaria (Varjonen et al., 2000).

The wheat grains are composed from three major components – starch, proteins, and fibre (cell wall polysaccharides). The proteins constitute 10% to 15% of the dry weight. Wheat proteins may be divided into four fractions, which are extracted sequentially in water (albumins), dilute saline (globulins), alcohol/water mixtures (prolamins) and dilute acid (glutenins). This basic classification is widely used by cereal chemists, but it is now more usual to classified wheat proteins based on their functions and properties. Approximately 80% of the total grain protein mass is the major storage protein fraction. This mass, containing the gliadin and glutenin fractions, is termed gluten. The gluten proteins are crucial for the processing properties of wheat flour, conferring the cohesiveness and viscoelasticity that allows dough to be processed into bread and other foods. The gliadins are monomeric proteins classified into three groups on the basis of their electrophoretic mobility at low pH – α/β-gliadins (fast), γ-gliadins (intermediate), and ω-gliadins (slow). The ω-gliadins are further divided into ω1-, ω2- and ω5-gliadin components also according to their electrophoretic mobility. The ω5-gliadin is a major allergen involved in WDEIA (Snégaroff et al., 2006; Mameri et al., 2012). Moreover, gliadins are generally known as being responsible for celiac disease (Ciccocioppo et al., 2005; Briani et al., 2008). The glutenins are polymers of individual proteins linked by interchain disulphide bonds classified into high molecular weight (HMW) glutenins and low molecular weight (LMW) glutenins after separation by sodium dodecylsulphate polyacrylamide gel electrophoresis. The HMW glutenins belong among the main allergens involved in WDEIA (Morita et al., 2009; Takahashi et al., 2012).

The rest 20% of the total wheat grain protein mass comprises over 1000 various proteins, many of which correspond to ‘house keeping’ proteins (present throughout the plant), but several groups of proteins are specific for grains such as α-amylase inhibitor protein family, non-specific lipid transfer protein (LTPs), peroxidases, and puroindolines. Many of these proteins protect grain against fungal pathogens and invertebrate pests (Tatham and Shewry, 2008).

Alpha-amylase inhibitors constitute a large protein family and belong to the salt soluble fraction of wheat (de Gregorio et al., 2009). Alpha-amylase inhibitors play an important role in wheat food allergy (James et al., 1997; Armentia et al., 2002), and in baker’s asthma are major allergens (Palosuo, 2003; Constantin et al., 2008). Wheat contains two types

of α-amylase inhibitor. One of them is a bifunctional protein (20.5 kDa) capable of inhibiting endogenous wheat α-amylase and the protease subtilisin. The second type forming the major group of α-amylase inhibitors called chloroform methanol proteins (known also as 'CM' proteins) such as CM1, CM2, CM3, CM7, CM16, and CM17. They exist in a mixture of monomeric (0.28), dimeric (0.19 and 0.53) and tetrameric (CM proteins) forms based on at least 11 subunits with molecular weight ranging from about 12 to 16 kDa. These all forms inhibit mammalian and insect enzymes but not the endogenous α-amylase of wheat, implying protective role (Amano et al., 1998; Weichel et al., 2006).

Lipid transfer proteins (~ 9 kDa) are ubiquitous lipid binding proteins of the plant kingdom (Douliez et al., 2001), and are of great technological importance, because they are involved in the beer foam formation (Sørensen et al., 1993). The LTPs differ in their structure stability; for example, wheat LTP was not resistant to thermal or chemical processing, but maize LTP is, on the contrary, highly resistant to cooking (Pastorello et al., 2003). These proteins belong among the most important panallergens due to their wide-spread presence in the plant kingdom and sensitization occurs via both ingestion and inhalation causing wheat food allergy and backer's asthma (Pastorello et al., 2007; Tordesillas et al., 2009). Another wheat allergen with molecular weight 36 kDa was identified as a seed-specific peroxidase and it is associated mainly with patients who have Backer's asthma. This allergen may play a role in cross-reaction, because IgE-binding glycoproteins with similar size were detected also in barley (Sánchez-Monge et al., 1997).

1.6.5. Rice allergy

Rice (*Oryza sativa*) is the predominant staple food in Asia and Latin America, and after maize, it is the grain with the second highest worldwide production. In contrast to its wide consumption, rice allergy seems to be exceedingly rare, and only few cases of IgE-mediated allergic reaction are documented. The most often symptoms are rhinoconjunctivitis, bronchial asthma or generalized urticaria after inhalation of rice flour or steam of cooked rice (Fiocchi et al., 2003; González-Mendiola et al., 2003; Orhan and Sekerel, 2003; Nambu et al., 2006). There are only few reports on immediate IgE-mediated allergic reactions after ingestion of rice leading to rhinoconjunctivitis or gastrointestinal symptoms (Kumar et al., 2007; Monzón

et al., 2008). Rice, for its low allergenicity, is usually considered to be a hypoallergenic food and is frequently recommended as a diet alternative for patients with food allergies.

Rice proteins account for 10% of composition of rice grain by weight, and are 80% acid- and alkali-soluble glutelins, 10% salt-soluble globulins, 5% water-soluble albumins, and 5% alcohol-soluble prolamins (Cagampang et al., 1966; Satoh et al., 2011). The allergenicity of rice is partly dependent on globulin and albumin protein fractions, such as 14-16 kDa albumin and α-globulin (Shibasaki et al., 1979; Ito et al., 2005). Among major allergens characterized in these fractions were a 16 kDa and a 33 kDa protein. The 16 kDa allergen is homologous to α-amylase/trypsin-inhibitor family proteins; the 33 kDa allergen was identified as a novel type of plant glyoxalase I (Urisu et al., 1991; Usui et al., 2001). Further rice allergens are a 14 kDa LTP (Ory s 14) and the whole spectrum of rice proteins with molecular weight 50 kDa to 60 kDa. These allergens lead to various clinical symptoms, such as AD, urticaria, and angioedema (Asero et al., 2007; Kumar et al., 2007). Other four potential rice allergens with molecular weight 49 kDa to 98 kDa were identified, but only 56-kDa glycoprotein was partially independent on cross-reactivity, thereby this protein seems to be a genuine rice food allergen (Trcka et al., 2012).

1.6.6. Hen egg's allergy

Together with milk, hen's egg allergy is one of the most common food allergies in infants and young children. It usually presents around 1 year of age, reflecting the typical age of introduction of eggs into the child's diet (Benhamou et al., 2010). The prevalence of egg allergy in children from 1 to 3 years varies between 1% and 2% (Eggesbø et al., 2001). The prevalence confirmed by a challenge with raw egg is about 9%, but 80% of these patients tolerate baked egg (Osborne et al., 2011).

Although the hen's egg is composed from egg white and yolk, a large number of egg allergens are in egg white (Hildebrandt et al., 2008). Egg white contains about 40 different proteins, but four main egg white proteins are responsible for the majority of the allergic reaction to hen's egg: ovomucoid (Gal d 1), ovalbumin (OVA, Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4). The protein α-livetin (Gal d 5) is the major allergen in the egg yolk (Jacobsen et al., 2008). Ovalbumin (42 kDa) forms 54% of the whole egg white and therefore is the most important egg's allergen. However, OVA is sensitive to thermal

denaturation, with the resultant decrease its allergenicity (Urisu et al., 1997; Benedé et al., 2014). Ovomucoid (28 kDa, 11% of the total egg white protein) is heat resistant and remains soluble after extensive heating, and due to these facts ovomucoid is considered to be the dominant allergen in the egg white (Lemon-Mulé et al., 2008). Ovotransferrin (77.7 kDa, 12% of the total egg white protein) is present in a large number of isoforms due to different levels of Fe saturation of this protein (Leduc et al., 1999). Despite the lower content in the egg white, ovotransferrin belong among the important egg allergen. Lysozyme (14.3 kDa, 3.4% of the total egg white protein) is the least abundant allergens in the egg white (Pérez-Calderón et al., 2007).

1.6.7. Processing of allergens

There are many ways we may process foods leading to their better edibility or their preservation. Processing procedures divide on thermal or non-thermal. Thermal processing can be accomplished by dry heating (*e.g.*, oven and oil roasting) or by cooking (*e.g.*, boiling, steaming). Non-thermal processing includes germination, fermentation, soaking, etc. The food processing for the preservation of foods include smoking, salting, pickling with low pH agents such as vinegar or acidic fruit juices, and the fermentation using microorganisms such as yeast and lactic bacteria. The food processing leads to improvement of food qualities such as flavor, taste, or color (Sathe et al., 2005; Sathe and Sharma, 2009).

The food processing induces chemical and physical modifications causing various changes in the secondary and tertiary structure of proteins. For example, the most frequently used processing of raw ingredients into finished foods is cooking and baking. This procedure may result in modifications including protein denaturation or aggregation, hydrolysis of peptide bonds, induction of various non-covalent and covalent modifications (Koppelman et al., 1999; Maleki, 2004). The modifications arise also by the interaction with other food components such as sugars, carbohydrates, lipids, additives, and preservatives. One of the most important covalent modifications introduced into food proteins by thermal processing is non-enzymatic glycation as a result of the Maillard reaction between proteins and sugars. This modification involves the reaction of free amino groups, generally lysine residues, on proteins with reducing sugars such as glucose and lactose (Davis et al., 2001; Clare Mills and Mackie, 2008). The Maillard reaction may lead to increased binding food allergens to IgE antibodies,

such as peanut allergens (Ara h 1 and Ara h 2) in peanut-allergic patients (Maleki et al., 2000; Beyer et al., 2001; Gruber et al., 2005). However, in some cases, severe heating causes the significant decrease of food allergenicity (*e.g.*, apple allergen Mal d 3), due to the protective effect of the Maillard reaction on thermostability of LTPs (Sancho et al., 2005). On the other side, there is evidence that glycated OVA may decrease the allergic immune response in a BALB/c mouse model of egg allergy (Rupa et al., 2014).

As mentioned above, the structure and protein modifications may improve the stability of allergens or can lead to production new allergens. Cooking can reduce the allergenicity of certain food allergens in pollen-related fresh fruit and vegetable foods and their products (Brenna et al., 2000; Eigenmann, 2000; Fiocchi et al., 2004). The thermal processing reduces also the allergenicity of egg; children with egg allergy were tolerant of heated egg white food challenge (Urisu et al., 1997), and tolerated egg baked into a cake (Des Roches et al., 2006). Similarly, one adult patient who experienced anaphylaxis to raw egg was tolerant to heated egg (Eigenmann, 2000). Baking also leads to decreasing the allergenicity of hazelnuts (Hansen et al., 2003).

1.7. Animal models of food allergy

In the last decades, there has been a substantial increase in the prevalence of allergic diseases. Hence, there is a basic necessity to investigate the mechanisms of allergic diseases and to trace novel treatment approaches (Meyer-Martin et al., 2014). The allergic disorders were explored using various species such as rats, swine, guinea pig and dog (Knippels and Penninks, 2003; Schmied et al., 2013; Abramo et al., 2014; Smit et al., 2014). Very suitable animal models for studying allergies, especially food allergy, are mouse models (Oyoshi et al., 2014).

The mouse model have been developed for almost all allergic diseases, such as asthma, allergic rhinitis, food allergy, AD, and allergic conjunctivitis (Takeda and Gelfand, 2009). The most suitable mouse for studying the food allergy in the conventional conditions is the BALB/c mouse strain (Mine and Yang, 2007). The mouse, as a model animal, has a short life-cycle and has relatively uncomplicated and quick reproduction. Moreover, mice are easy to handle and to gain of genetically modified transgenic or knock-out strains (Shapiro, 2008).

Multiple methods are used to trigger food allergy in the mouse model. The main differences consist of the food antigen used and the strategy to sensitize the animal prior to oral challenge. The sensitization of mice may be performed by oral, intraperitoneal, and intradermal sensitization (Corazza and Kaufmann, 2012). Depending on the route of exposure, dose of allergen and the presence of suitable adjuvant, the immune response can result in either sensitization or tolerance induction (Mine and Yang, 2007; Repa et al., 2008; Perrier et al., 2010). In mouse models of food allergy, the oral administration of the allergen usually results in oral tolerance induction; however, the co-administration with strong adjuvant such as Cholera toxin or with anti-acid drugs (increasing gastric pH) could be used for the successful allergic sensitization (Lee et al., 2001; Diesner et al., 2008; Brunner et al., 2009; Pali-Schöll et al., 2010). Another reliable and effective approach to overcome the oral tolerance induction is the sensitization of mice by intraperitoneal (*i.p.*) administration of allergen with aluminium hydroxide (alum) as adjuvant following by repeated intragastric (*i.g.*) challenges. This experimental model mimics a mild form of human allergy with the IgE-mediated mast cell degranulation leading to the increased small-intestine permeability and resulting to the manifestation of allergy symptoms, such as diarrhea (Dearman and Kimber, 2007; Brandt et al., 2009; Herouet-Guicheney et al., 2009).

In the food allergy models, the histological examination of small intestine reveals changes of epithelium, *e.g.* alteration in number of goblet cells and mucine production, and the damage of tips of villi, as well as changes of lamina propria, *e.g.* increased cell infiltration and activation (Li et al., 1999; Saldanha et al., 2004; van der Ventel et al., 2011). Moreover, the mucosa of the small intestine is an actively metabolizing, rapidly proliferating, and absorptive epithelium with the crucial nutritional and homeostatic function. The activity of brush border enzymes is very sensitive marker of the intestinal cell differentiation and the postnatal development, reflecting both dietary changes and microbial colonization (Simon et al., 1979; Kozáková et al., 2001; Hudcovic et al., 2009). Partial and subtotal atrophy of the villous was shown to correlate with the activity and expression of alkaline phosphatase (Lallès, 2010). This enzyme may be also involved in host defense against pathological stress-induced damage during inflammation and infectious disease (Harada et al., 2003).

An invaluable tool for studying food allergies is germ-free (GF) or gnotobiotic (colonized with at least one known bacterium) animal models. The GF animal (*e.g.*, mouse) is maintained free from demonstrable microbial associates such as bacteria, viruses, fungi, and parasites throughout its life. The GF animals exhibit extensive distinctive characteristics in immune functions compared to conventional mice; they have fewer and smaller lymphatic-

system and lymphatic-tissue components such as PPs, MLNs, mucin producing goblet cells and antibody production (Macpherson and Harris, 2004; Round and Mazmanian, 2009). The GF mice secrete decreased levels of IgA antibodies which play an important role in the intestinal mucosal immunity. Moreover, the intestinal commensal bacteria, normally present in the intestine of conventional mice, are an important factor in induction of oral tolerance and in the development of allergy. As a consequence, GF mice, which lack indigenous bacteria in their GIT, provide valuable tools for analyzing the direct modulation of the immune system by selected probiotic bacteria (Tsuda et al., 2010; Yi and Li, 2012). The best way for studying these immunomodulation effects is to use a mouse model in which mice are mono-colonized with a single probiotic bacterium strain. The probiotic bacteria can be obtained from the feces of healthy conventional mice. The most frequently used bacteria in gnotobiotic mouse models are lactic acid bacterial strains – *Lactobacillus sp.* and *Bifidobacteria sp.*, which are non-invasive and non-pathogenic Gram-positive bacteria (Kim et al., 2008; Borchers et al., 2009; Yi and Li, 2012,).

2. Importance and aims of the thesis

The food allergy has increasing prevalence in the industrialized world. It is very important to characterized and identify these food allergens in the purified form for their use in diagnostics and treatment of allergic disease. Another research approach is to develop a suitable animal model for studying food allergies which can help us to understand the mechanisms of food allergy development and to show the route how to treat allergic diseases.

In the thesis we focused on:

- Development of a new technical approach for isolation and purification of proteins from natural sources, such as wheat flour.

- Isolation, characterization, and identification of the most important water/salt-soluble wheat allergens.

- Characterization and identification of potential water/salt-soluble and water/salt-insoluble rice allergens from raw and boiled rice.

- Confirming the opinion that rice is a suitable hypoallergenic diet for patients with food allergy.

- Development of a mouse model for studying the mechanisms of immune responses to food allergen and for verification of hygiene hypothesis.

- The effect of changes in the secondary structure of hen egg's ovalbumin on its allergenic potential in the mouse model of food allergy.

- Studying the effect of germ-free conditions and colonization of germ-free mice by probiotic bacterium *Lactobacillus plantarum* on the immune response in the mouse model of food allergy.

3. Results and discussion

3.1. Publication I

Šotkovský, P.; Sklenář, J.; Halada, P.; Cinová, J.; Šetinová, I.; Kainarová, A.; **Goliáš, J.**; Pavlásková, K.; Honzová, S.; Tučková, L. A new approach to the isolation and characterization of wheat flour allergens. ***Clin. Exp. Allergy*** **2011, *41*, 1031–1043**.

Wheat is an important part of human diet but its consumption may often lead to IgE-mediated food allergy. Therefore, it is necessary to isolate wheat allergens in pure and native (biologically active) form in amounts sufficient for studying their structural and modification changes, and for improving the accuracy of clinical diagnostic tests (Inomata, 2009). The isolated wheat proteins can be characterized by IgE antibodies of allergic patients, and then, IgE recognized wheat proteins can be further identified by proteomic techniques, such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

We developed a new three-step procedure for isolation of wheat proteins in the high purity, consisting of ultrafiltration, native (liquid-phase) preparative isoelectric focusing (IEF), and the high-performance liquid chromatography (HPLC). In the first step, salt-soluble wheat proteins with molecular mass within 5 to 120 kDa were extracted from the flour and then separated using Amicon 100k, 30k, and 10k filter devices. In this manner, we obtained two fractions – ‘fraction 1’ containing the wheat proteins with molecular mass from 30 to 100 kDa, and ‘fraction 2’ containing proteins with molecular mass below 30 kDa. These individual fractions were further separated in the Rotofor cell equipment utilizing liquid-phase IEF for fractionating the proteins according to their mobility in the pH gradient ranging from 3.0 to 9.4. The separated wheat proteins were further purified by HPLC and the resulting fractions were characterized by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting using patients’ sera. The IgE-binding proteins were identified by MALDI-TOF mass spectrometry.

Using this approach and protein database searches, we succeeded in indentifying 27 wheat proteins. The most abundant wheat allergens among identified proteins were α-amylase

protein family, such as previously described α-amylase inhibitors 0.19 (Tri a 28.0101), 0.28 (Tri a aAI), and 0.53 (Tri a 28); α-amylase/trypsin inhibitors CM1, CM2 (Tri a 29), CM3 (Tri a 30), and CM16 (Tri a CM16), which belongs to the most important wheat allergens responsible for baker's asthma and food allergy to wheat (Pastorello et al., 2007; Šotkovský et al., 2008). The other identified wheat allergens include previously characterized allergens from serpin protein family, β-amylase, CM17, non-specific LTP, wheatwin-1 and wheatwin-2 (Palacin et al., 2007; Šotkovský et al., 2008; Sander et al., 2011). Out of the 27 identified proteins, the following 7 are new potential wheat allergens: endogenous α-amylase/subtilisin inhibitor, trypsin/α-amylase inhibitor CMX1/CMX3 protein, thaumatin-like protein (TLP), xylanase inhibitor protein-1 (XIP-1), β-glucosidase 1, class II chitinase, and 26 kDa endochinase. The reactivity of patients' sera with α-amylase inhibitors is not unambiguous. For instance, although the endogenous α-amylase/subtilisin inhibitor has not been previously reported as an allergen (Tatham and Shewry, 2008), we observed the reactivity of this purified protein with IgE antibodies from 19 out of 22 patients (85%), albeit with medium intensity. The TLP is other newly isolated IgE-binding protein associated with food allergy to wheat, which was previously described and associated with fruit allergy, such as kiwi, apple, cherries, and grapes (Breiteneder, 2004; Pastorello et al., 2003; Gavrović-Jankulović et al., 2002). Moreover, the TLP and other new IgE-binding wheat protein XIP-1 were previously identified in patients with baker's asthma, but not in connection with food allergy (Lehto et al., 2010).

Further we characterized the intensity and frequency with which IgE antibodies bind the individual wheat proteins using immunoblots and sera from allergic patients, disease and health controls. All allergic patients (100%) showed positive IgE reaction with α-amylase inhibitor 0.28, α-amylase/trypsin inhibitor CM16, α-amylase/trypsin inhibitor CM2, TLP, and tritin. More than 90% of sera reacted with proteins CM17, α-amylase inhibitor 0.53, 26 kDa endochitinase, XIP-1, and class II chitinase. The frequency of IgE recognition of other proteins was ranging from 54% to 86%.

In the last step, we tested the biological activity of purified wheat allergens using BAT. The blood samples form patients and controls were exposed to four isolated wheat proteins – α-amylase inhibitor 0.19, LTP, TLP, and wheatwin. The positive cell activation was detected within the range 70% to 80%. Finally, we compared the number of patients positive for these wheat proteins either in IgE immunoblots or in BAT. For all allergens tested in immunoblots, the proportions of positive cases ranged from 60% to 100%, whereas this proportion was lower in BAT, but the range was more narrow (70% to 80%).

In summary, we developed a new three-step procedure enabling us to isolate and purify wheat allergens in their native forms and in amounts sufficient for further structural and functional studies. We identified 27 IgE-binding wheat proteins, including 7 new potential wheat allergens. Moreover, we showed for the first time that the purified allergens can activate basophils, documenting their retained biological activity. Better knowledge about the allergenicity of native proteins could help us to prepare and select appropriate vectors for the recombinant allergens preparation, improving diagnostic test and subsequent therapy.

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ORIGINAL ARTICLE **Allergens**

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

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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
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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 $1 \rightarrow 801$). 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

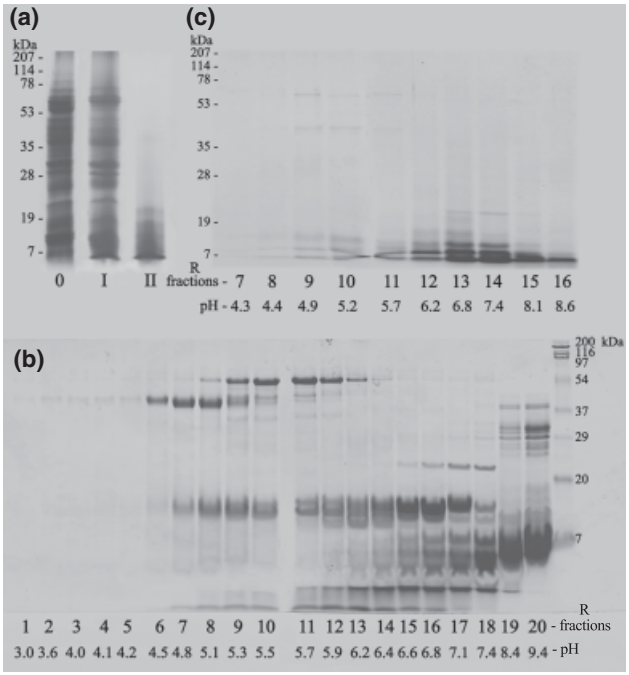

Fig. 1. SDS-PAGE analysis of wheat flour PBS extracts (a) and Rotofor® fractions (b and c). (a) Spectrum of wheat flour proteins extracted with PBS (0) and purified using Amicon 100k and 30k filter devices (fraction I). Fraction I was concentrated using a 10k filter device (fraction II). (b and c) Proteins 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

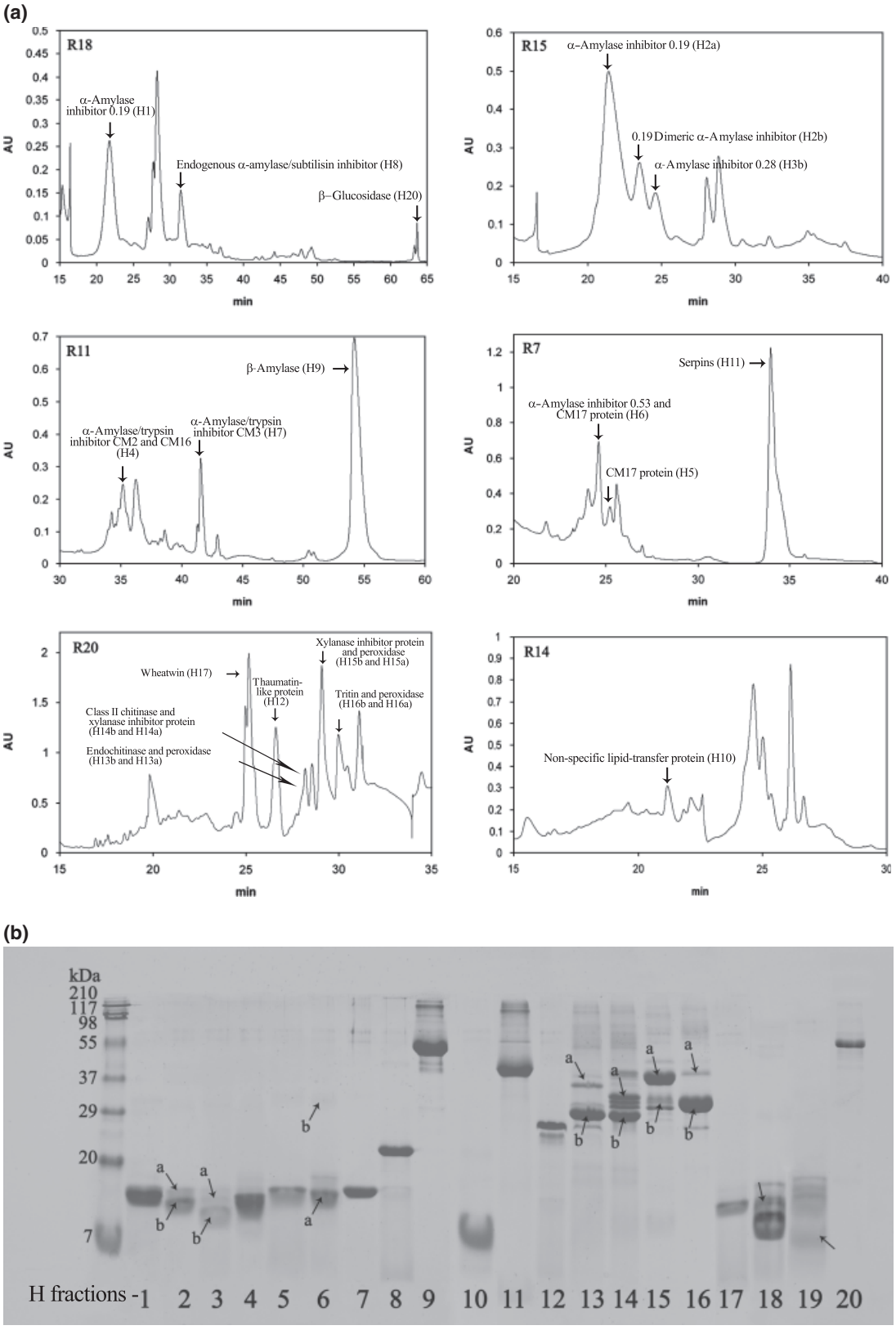

Fig. 2. (a) Examples of the chromatographic profiles of Rotofor® fractions R18, R15, R11, R7 and R20 (shown in Fig. 1b) and fraction R14 (shown in Fig. 1c) obtained from reversed-phase HPLC. The HPLC fraction numbers (H1–H20) and the peaks with arrows indicate purified and identified wheat proteins corresponding 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.

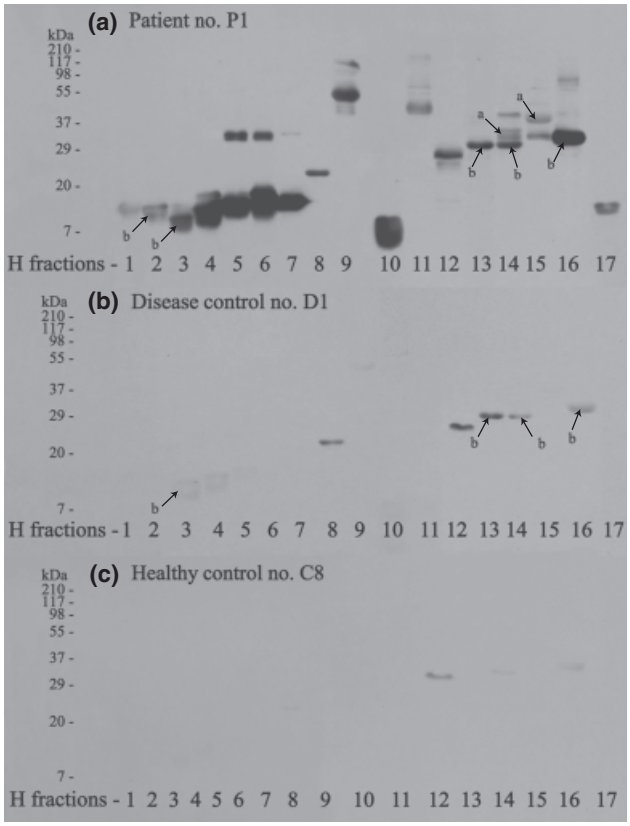

Fig. 3. An example of IgE immunoblotting of purified wheat proteins with the sera of (a) one patient, (b) a disease control and (c) a healthy control. The HPLC fraction numbers indicate the bands corresponding to the allergens identified in Table 2.

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 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).

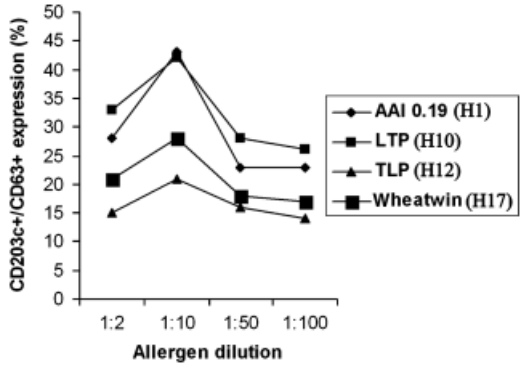

Fig. 4. CD203c/CD63 expression of basophils from seven patients in a dose–response curve after incubation with allergens: AAI 0.19 (H1), LTP (H10), TLP (H12) and wheatwin (H17) (dilution: 1 : 2, 1 : 10, 1 : 50 and 1 : 100).

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.

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3.2. Publication II

Goliáš, J.; Humlová, Z.; Halada, P.; Hábová, V.; Janatková, I.; Tučková, L. Identification of rice proteins recognized by the IgE antibodies of patients with food allergies. ***J. Agric. Food Chem.*** **2013, *61*, 8851–8860**.

Rice, as well as wheat, belongs among the most consumed foodstuff worldwide. Rice food is commonly considered to be hypoallergenic and is frequently recommended as a diet alternative for patients with food allergy, especially to wheat. However, several rice proteins recognized by IgE antibodies from patients with food allergies were previously described (Wüthrich et al., 2002; Monzón et al., 2008). In the view of the fact that all previously described rice allergens were water/salt-soluble and rice is most often consumed after thermal processing, the aim of our study was to isolate and characterize potential rice allergens from water-soluble (extracted into PBS) and water-insoluble (extracted into SDS) fractions of raw or boiled rice, and to compare their IgE reactivity with wheat allergens.

In the first step, we characterized the intensity and frequency of reactivity with which specific IgE antibodies bind PBS- and SDS-extracted proteins of raw and boiled wheat and rice using immunoblots and patients' sera from food-allergic patients, pollen-allergic patients as positive controls, and health controls. The individual IgE reactivity to PBS-extracted wheat proteins was markedly reduced by boiling, which is in the correlation with the previously described results, where the wheat-derived foodstuffs had a lower allergenicity than raw flour (de Gregorio et al., 2009). Surprisingly, the individual IgE reactivity to SDS-extarcted wheat proteins was affected by boiling only slightly, pointing out, in all probability, their higher heat resistance. The boiling of rice reduced IgE reactivity to PBS-extracted proteins, but the IgE reactivity to SDS-extracted proteins was increased and the frequency of their recognition was also high (up to 80%). These findings indicate that the thermal processing may either reduce or increase IgE reactivity of patients' sera to food allergens. It could be explain by forming complexes among proteins and fats, sugars, and other food components, and also by protein modifications, such as the Maillard reaction (Schmitt et al., 2010; Wagner et al., 2011). Furthermore, the thermal processing may also uncover so-called cryptic epitopes, which can influence the allergenicity of proteins (Beyer et al., 2001; Paschke, 2009).

In the next step, the IgE-mediated cell response (BAT) to individual allergens was compared using commercial and prepared PBS extracts from wheat and rice. The level of cell activation was comparable for commercial extracts and prepared PBS extracts of both wheat and rice. However, after boiling, the level of cell activation was dramatically decreased to values close to the negative controls. This finding can be explain by absence of SDS-extracted rice components in PBS extracts which retained their IgE-binding capacity also after boiling, as showed in the immunoblots. Therefore, we further tested the patients with high levels of specific IgE antibodies to wheat, with positivity to rice in BAT, and with high IgE reactivity to boiled SDS-extarcted rice proteins in immunoblots by SPT using the commercial wheat and rice extracts, and the boiled rice homogenate in PBS solution. Surprisingly, we observed that the allergic response of these patients in SPT was higher in comparison with their lower positivity in BAT, probably due to the presence of both soluble and insoluble rice proteins in the used homogenate. We assume that the boiled rice homogenate is beneficial for additional testing in SPT, especially for those patients who have increased specific IgE antibodies to wheat and who have positive IgE reaction to rice proteins in immunoblots and BAT, before the prospective rice recommendation as a suitable hypoallergenic diet.

In the last part of this study, we focused on the identification of rice allergens. The rice proteins were separated either by one-dimensional electrophoresis (1-DE) or two-dimensional electrophoresis (2-DE). The IgE-binding proteins were detected using the pooled sera from three patients selected according to their IgE reactivity in immunoblots and their positivity in BAT and SPT. The 1-DE-separated proteins recognized by IgE antibodies with the highest frequency were identified by MALDI-TOF mass spectrometry. In the SDS extract, we identified three seed allergenic proteins (RA5, RAG1, RAG2) belonging to the α-amylase/trypsin inhibitor family, from which RAG2 was previously characterized as an IgE-binding protein in the raw rice extract (Adachi et al., 1993). Further identified rice proteins corresponded to the large protein family of glutelins with subfamilies GluA and GluB, belonging to the major seed storage proteins (Katsube-Tanaka et al., 2010). Glutelin C precursor and granule-bound starch synthase 1 chlorplastic/amyloplastic protein were identified as new potential rice allergens. Simultaneously with us, the latter rice protein was identified also as a potential rice allergen by Krishnan's group (Krishnan and Chen, 2013). The other identified IgE-binding protein, 19 kDa globulin, belonging to α-globulins, was identified previously in salt extracts (Satoh et al., 2011). Unfortunately, the others proteins were identified in mixture, therefore we used 2-DE to separate potential rice allergens much more effectively. By this way, we isolated 16 spots from SDS extract and succeeded to

identify 9 IgE-binding proteins, from which 4 were identified as new potential rice allergens – disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and protein encoded by locus Os02g0453600.

Taking together, we demonstrated that patients with food allergy, mainly to wheat, have often increased IgE reactivity to rice proteins. More than 80% of these patients reacted to SDS-extracted boiled rice proteins in immunoblots and 26% from them were positive in BAT. Hence, before rice recommendation as a suitable hypoallergenic diet, we can suggest additional testing by SPT with the boiled rice homogenate, including both soluble and insoluble rice proteins, because the standard diagnostic tests, such as BAT, do not cover all allergens capable of binding IgE antibodies, and therefore they may not be sufficient for the detecting the potential rice allergenicity. Finally, we succeeded to identify 6 new potential rice allergens that retain their IgE-binding capacity after boiling. Two identified rice proteins, 19 kDa globulin (Ory s 19kD) and RAG2 (Ory s aA_TI), were recently included to Allergome – the international database of allergens.

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Identification of Rice Proteins Recognized by the IgE Antibodies of Patients with Food Allergies

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ABSTRACT: Similarity among food allergens is a great problem affecting the specificity of diagnosis and treatment of allergic patients. We have observed that 80% of patients with food (including wheat) and pollen allergies have increased IgE antibodies against rice proteins. By immunoblotting, we documented that boiling decreased solubility and IgE reactivity of PBS-extracted rice and wheat proteins, yet in SDS extracts this reactivity was only slightly changed. The sera of patients highly positive on the IgE immunoblot and positive in basophil activation and skin prick test with boiled rice components were used for characterizing the IgE-binding proteins separated by 1D or 2D electrophoresis. Using mass spectrometry, we identified 22 rice SDS soluble proteins. Six of them were new thermostable potential rice allergens: glutelin C precursor, granule-bound starch synthase 1 protein, disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and a protein encoded by locus Os02g0453600. All of the identified rice proteins differed from known wheat allergens, except proteins belonging to the α-amylase/trypsin inhibitor family. Furthermore, we would suggest that in patients with high IgE reactivity to wheat and rice components, the IgE immunoblot and skin prick test with boiled rice proteins could be beneficial before diet recommendation.

KEYWORDS: *food allergy, thermal processing, potential rice allergens, basophil activation test, skin prick test*

INTRODUCTION

Food allergy belongs among the world's most widespread disorders, appearing mainly in early childhood and affecting up to 8% of children and 3−4% of adults in Western countries. In addition, its incidence has continuously risen over the past two decades.[1−3] Wheat (*Triticum aestivum*) and rice (*Oryza sativa*) are important parts of the human diet worldwide. Wheat, however, belongs to the six food allergens, including milk, egg, wheat, peanut, soy, and fish, that account for 90% of food hypersensitivity reactions.[3,4] Wheat proteins could be involved in the three routes of sensitization: inhalation, contact, and ingestion. Inhalation of wheat components may cause baker's asthma,[5] whereas contact with skin may trigger IgE-mediated contact urticaria,[6] and ingestion may cause atopic dermatitis, wheat-dependent exercise-induced anaphylaxis, or gastrointestinal symptoms.[7,8] Diagnostic tests including the estimation of specific IgE antibodies are highly sensitive, but less specific. This low specificity may be due to the insufficient purity of currently used protein extracts instead of isolated allergens but also perhaps because of cross-reactivity among similar proteins in various foods.[9]

In contrast, rice is commonly considered to be hypoallergenic and is frequently recommended as a diet alternative for patients with food allergies. However, several studies describe immediate hypersensitivity reactions to rice. It has been documented that the inhalation of rice flour or vapors from boiling rice may cause bronchial asthma,[10,11] that contact of skin with raw rice may lead to contact urticaria,[12,13] and that ingestion may cause urticaria, bronchial asthma, rhinoconjunctivitis, and anaphylaxis.[14−17]

Rice allergens have already been described, but only two, Ory s 1 (β-expansin) and Ory s 12 (profilin A) (both isolated from rice pollen), were well characterized and have been included in the official allergen database of the IUIS.[18] Analysis of grains from boiled rice revealed in a water/salt-soluble extract the α-amylase/trypsin inhibitor family of proteins of about 14−16 kDa, cross-reacted with other cereal grains, which are recognized by IgE antibodies from the majority of patients with a rice allergy.[19,20] The other rice allergens were identified as a 26 kDa major seed storage protein α-globulin and a 33 kDa plant glyoxalase I.[21,22] Lipid transfer protein (LTP, Ory s 14) was also described as a rice allergen, which cross-reacts with peach/apple LTP.[23] Recently, Satoh et al. have characterized novel IgE-binding rice components such as 52 and 63 kDa globulin-like proteins,[24] which are homologous to cupin superfamily allergens belonging to seed storage proteins. Trcka et al. described a 56 kDa glycoprotein responsible for anaphylaxis after the consumption of food containing boiled rice.[17] All of the rice allergenic components described so far have been water/salt-soluble proteins, but we could not find any information about water/salt-insoluble rice allergens.

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Table 1. Demographic, Clinical, Serological, and Therapy Characterization of Patients and Disease Controls[a]

				RAST value (kIU/L)				
patient	sex	age	clinical symptoms	wheat	rice	specific IgE for allergens	restricted diet	therapy
1	F	7	FA, AD	0.75	0.75	eg, mi, so, ha, gl	yes	AH
2	F	28	AR, AB, FA	2.22	0.59	gcp, we, bp, mo, dg, ct	yes	AH
3	M	34	AR, AB	0.65	0.49	gcp, mo, mt, ca, ap	no	AH, ICS
4	M	10	FA, anaphylaxis	0.88	0.64	gcp, mo, ps, ki, nu, ap, gl	no	AH
5	M	3	AD, FA	4.78	1.06	gl, mi, eg, cf, nu	no milk	AH, local CS
6	M	15	AD, FA	1.66	0.86	gr, ha, so, mo, mt, dg, ct	no	AH, Tacrolimus, local CS
7	M	11	AD, FA	1.13	0.68	gr, eg, so, ha, mo, mt, dg, ct	yes	AH, Tacrolimus, local CS
8	M	65	AR, AB	0.71	0.44	gcp, bp, wo, am, po, nu	no	AH, ICS
9	M	32	AR, AB	1.18	0.93	gcp, bp, am, wo, nu, to, ce	no	AH, ICS
10	M	40	AD, AR	1.33	0.62	gcp	no	AH, ICS
11	F	62	FA, AR, AB, CoD	5.27	3.62	ry, gl, gcp	yes	AH, β-mimetics
12	M	51	AC, FA, AB	6.46	0.98	gr, nu, gl, ki, or, la, mt, ct	yes	AH, ICS
13	F	44	AD, FA, AR	5.70	8.37	ry, gcp, nu, ca, po, ce, mt	no	
14	M	62	FA, AB	5.11	3.70	bp, gcp, gl	yes	AH, ICS
15	M	37	AB	0.63	0.67	bp, po, ce, to, ki	no	AH
16	F	39	FA, AB	2.23	0.92	gl, he	yes	AH
17	F	62	FA, AR, AB	2.15	1.33	ry, bp, gr, so, ce, ma, mi, nu, ca, ap, po, to	yes	Nalcrom, Ketotifen, AH, ICS
18	F	51	FA, AB	2.29	1.31	gl, nu, ap, gp	yes	AH, Nalcrom, ALTs
19	F	22	FA, AR, AB	1.43	0.94	gcp	yes	AH, ICS
20	F	56	U, AR, AB	3.83	neg	bp	no	AH, ALTs
21	M	30	AD, AB	0.86	0.22	nu, mt, mo, dg, ct	no	AH, ICS, local CS
22	M	8	FA	1.55	0.25	eg, mi, ry, so, ba	yes	AH
23	F	5	FA, AD	3.60	neg	eg, ha, gl	yes	AH, Tacrolimus, Emolentia
24	F	4	FA, AD	0.47	neg	eg, mi, de	yes	AH, local CS
25	F	58	FA, AB	1.89	neg	ry	yes	AH, β-mimetics
26	M	4	FA, AD	1.12	neg	eg, mi, ha	yes	AH, local CS
27	M	44	FA, AB	1.49	0.19	ry	yes	AH, ICS
28	F	49	FA, AB, WA	30.6	neg	gl, wv	yes	AH, Alutard, ICS
29	F	3	AD, FA	0.73	neg	eg, gl, so, ha, dg	yes	AH
30	M	3	AD, FA	2.85	neg	eg, gl, dg	yes	AH, Tacrolimus, local CS
31	M	2	AD, FA	5.27	0.10	eg, mi, gl, ha, so	no milk	Neocate
32	M	2	AD, FA	0.40	neg	ry, nu, pe, eg, po, dg	yes	AH, Neocate, Nalcrom, ALTs
33	F	2	AD, FA	0.84	neg	eg, mi	yes	AH
34	M	2	AD, FA	10.9	0.10	mi, eg, so, nu, ki, ba, dg	yes	AH, local CS
35	F	24	AD, FA, AB	12.1	0.10	mi, eg, gl, so, ha, dg	yes	AH, IKS, local CS
36	M	23	AD, AR, acne	neg	neg	bp, gr, rp, ct	no	local ATB for acne
37	M	15	AR, AB, CD	neg	0.61	bp, rp, wv, be, fe, mt, dg, hr	no	AH, ICS, local CS
38	M	21	AR	neg	neg	bp, gr, rp, ct	no	AH, NCS
39	M	38	AR	neg	neg	bp, gr, we, rp, wo, fe, mt, mo, ct	no	AH, Cromoglycate
40	F	48	AR	0.22	0.19	bp, gr, wo, wv	no	AH, NCS
41	M	20	AR, AB	0.11	neg.	bp, gr, wo, mt	no	AH
42	F	31	AR	neg	0.13	bp, gr	no	AH
43	M	61	AR	neg	neg	gr, da, dn	no	AH, NCS
44	F	68	AR	neg	neg	bp, wo, gr	no	AH
45	F	65	AR, AB	neg	neg	gr, bp	no	AH, NCS, ICS
46	M	47	AR	neg	neg	gr, bp	no	AH, NCS, ICS
47	F	30	AR	neg	neg	gr, bp	no	AH, NCS
48	F	39	AR	neg	neg	gr, bp	no	AH, NCS, ICS
49	F	27	AR	neg	neg	gr, bp, wo	no	AH
50	F	25	AR	neg	neg	gr, bp, mt	no	AH

[a]Abbreviations: F, female; M, male; AB, asthma; AD, atopic dermatitis; AR, allergic rhinitis; CD, contact dermatitis; CoD, celiac disease; FA, food allergy; U, urticaria; WA, wasp allergy; neg, negative reaction; am, *Ambrosia* spp.; ap, apple; ba, banana; be, bee venom; bp, birch pollen; ca, carrot; ce, celery; cf, cod fish; ct, cat; da, daisy; de, *Dermatophagoides* spp.; dg, dog; dn, dandelion; eg, egg; fe, feather; gcp, grass and cereal pollen; gl, gluten; gp, green pepper; gr, grass pollen; ha, hazelnut; he, herbal; hr, horse; ki, kiwi; la, latex; ma, maize; mi, milk; mo, mold; mt, mites; nu, nuts; or, orange; pe, peanut; po, potato; ps, poppy seed; rp, rye pollen; ry, rye flour; so, soybean; to, tomato; we, weeds; wv, wasp venom; wo, wormwood; AH, antihistamines; ALTs, antileukotriens; ICS, inhalation corticosteroids; CS, corticosteroids; NCS, nasal corticosteroids.

The aim of our study was to characterize and compare potential rice allergens, soluble in phosphate-buffered saline (PBS) and sodium dodecyl sulfate (SDS) buffers extracted from raw or boiled rice using proteomic techniques. IgE-

binding proteins from boiled rice were detected by immunoblotting using sera from patients with food (mainly wheat allergy), food and pollen, or only pollen allergies (disease controls) and from healthy donors (controls). The extracted rice proteins were separated by one-dimensional electrophoresis (1-DE) and two-dimensional electrophoresis (2-DE), and the most frequently recognized IgE-binding proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Moreover, the reactivity to rice proteins was also tested by basophil activation test (BAT) and skin prick test (SPT), and the clinical significance of all tests is discussed herein.

MATERIALS AND METHODS

Chemicals. The chemicals used for preparing the PBS and SDS extracts for 1-DE and 2-DE and for the visualization of separated proteins were sourced from Serva (Heidelberg, Germany), Sigma (St. Louis, MO, USA), and Lach-Ner (Neratovice, Czech Republic); protein concentrations in extracts were determined by using a Bicinchoninic Acid Protein Assay Kit from Pierce (Rockford, IL, USA). The other chemicals used for 2-DE were DeStreak and Pharmalyte (pH 3−10 or pH 8−10.5) from Amersham Bioscience (Uppsala, Sweden) and Immobiline DryStrips (18 cm) with a nonlinear pH 3−10 gradient from GE Healthcare (Uppsala, Sweden). For blocking of nitrocellulose membranes (from Serva) was used Tween 20 from Duchefa (Haarlem, The Netherlands) and powdered defatted milk from Promil (Nový Bydžov, Czech Republic). The peroxidase-labeled sheep anti-human IgE antibody was from the Binding Side (Birmingham, UK). Development solution, SuperSignal West Femto Maximum Sensitivity Substrate kit, was from Pierce. For clinical tests were used commercial allergen extracts from Alyostal-Stallergenes (Antony, France).

Characterization of Patients. We characterized IgE reactivity to wheat and rice proteins in the cohort of 50 patients (24 females, 26 males; mean age = 30.8 years; age range, 2−68 years) with food and/or pollen allergies. Thirty-five patients with food allergy (group I), all with a positive reaction to wheat allergens, were further divided into two subgroups according to their IgE reactivity to rice proteins (rice positive, denoted Ia, patients 1−19; and negative or low positive, denoted Ib, patients 20−35). Fifteen patients positive for pollen allergens, but negative for wheat or other food allergens, were used as a disease control (group II, patients 36−50). Sera of 10 healthy donors were used as a healthy control (group III). The clinical symptoms, allergen specificity, and treatment of allergic patients are summarized in Table 1. All sera were stored at −20 °C until use. Written informed consent was obtained from all patients and healthy donors before sampling, and the study was approved by the Ethics Committee of the General Teaching Hospital in Prague (Czech Republic).

Immunoglobulins and Specific IgE. Total IgA and IgG were measured by nephelometry[25] using a Dade-Behring BNII nephelometer (Dade-Behring, Marburg, Germany). The reagents used included OSAR15 N Antiserum to human IgA and OSAS15 N Antiserum to human IgG. The standard laboratory referential ranges are 6.90−14.0 g/L for IgG and 0.70−3.70 g/L for IgA immunoglobulins.

Total and specific IgE antibodies were measured by a chemiluminescent enzyme immunoassay method using an IMMULITE 2000 system (Siemens, Erlangen, Germany). The standard laboratory referential range for total IgE antibodies is 0−100 IU/mL. Allergen specific IgE antibody concentrations above 0.35 kU/L were considered positive. The detection limit was 0.1 kU/L, and functional sensitivity was 0.2 kU/L.

Eosinophilic Cationic Protein. The level of eosinophilic cationic protein (ECP) was measured by a chemiluminescent enzyme immunoassay method using an IMMULITE 2000 system (Siemens). A value within the range of 0−24 ng/mL was considered normal.

Preparation of Extracts from Wheat Flour and Rice Grains. The extracts from wheat flour (Sulamit cultivar) or commercially available long-grain rice (*Oryza sativa* L.) were prepared from raw and boiled forms. Wheat flour and rice grains were boiled for 10 min and dried at 37 °C; the boiled wheat and raw and boiled rice were milled to a fine powder. Proteins from 3 g of wheat or rice were extracted into 10 mL of PBS containing 0.9% NaCl, 0.02% NaH_2PO_4, and 0.05% Na_2HPO_4, pH 7.2; agitated at 37 °C for 2 h; and centrifuged at 3000*g* for 20 min at 20 °C. The PBS-soluble proteins were collected, divided into aliquots, and stored at −20 °C until use. The remaining pellets were resuspended in 10 mL of SDS/2-mercaptoethanol containing buffer, containing 1% SDS, 10% glycerin, 5% 2-mercaptoethanol, and 50 mM TRIS; agitated at 37 °C for 1 h; and centrifuged at 3000*g* for 20 min at 20 °C. The SDS-soluble proteins were collected, divided into aliquots, and stored at −20 °C until use. Protein concentrations in extracts were determined using a Bicinchoninic Acid Protein Assay Kit (Pierce) and slightly modified Bradford method.[26] Final concentration of all samples was adjusted to 2 mg/mL by precipitation with acetone.

Basophil Activation Test. Flow cytometric basophil activation tests (BAT) were performed after the stimulation of blood cells from patients with commercial wheat and rice allergens (Alyostal-Stallergenes), PBS extracts of wheat (Sulamit cultivar), and PBS extracts of raw and boiled rice. Activation of basophiles was measured in whole blood in heparin using the commercially available BasoFlow kit (Exbio, Prague, Czech Republic) following the manufacturer's instruction. The activated basophils expressing CD63 and CD203c were measured by FC500 flow cytometry (Beckman-Coulter, Miami, FL, USA). Samples containing >15% basophils, expressing CD63 (CD203c+, CD63+), were considered positive.

Prick Tests. Skin prick tests were performed with commercial allergens (Alyostal-Stallergenes) and with boiled rice homogenate in PBS. The following food allergens were used for testing: wheat (catalog no. 105), rice (catalog no. 160), boiled rice homogenate in PBS (concentration = 100 μg/mL), negative and positive (histamine) controls. The size of a weal 3 mm or more in diameter was considered as a positive reaction to the tested allergen.

SDS-PAGE and Immunoblotting. Extracted proteins (in a final concentration of 2 mg/mL) were separated by SDS-PAGE as described by Laemmli[27] using a 5−20% polyacrylamide gradient gel under reducing conditions (with 5% 2-mercaptoethanol) and electrophoresis run on a Mini Protean 3 cell (Bio-Rad, Hercules, CA, USA). The separated proteins were stained with Coomassie Brilliant Blue R-250 (CBB) or electrotransferred using a Mini Tran-Blot cell (Bio-Rad) onto nitrocellulose membranes (NC) for 1 h at room temperature. The membranes were blocked with TBS containing 0.1% Tween 20 and 1% milk (TTBS; 0.9% NaCl, 100 mM TRIS, 0.1% Tween 20, and 1% powdered defatted milk), and NC strips were incubated with patient sera, disease, and healthy controls, diluted 1:40 in the blocking buffer (TTBS containing 0.1% milk) overnight at 4 °C. After a washing with TTBS, the strips were incubated with peroxidase-labeled sheep anti-human IgE antibodies diluted 1:5000 with TTBS containing 1% milk for 1 h at room temperature. Development was carried out using a SuperSignal West Femto Maximum Sensitivity Substrate kit, and the exposure times onto the films (Kodak, Rochester, NY, USA) were 30 s, 1 min, and 5 min to characterize and compare IgE reactivity of the individual patient.

SDS-PAGE using longer polyacrylamide gels (18 cm) with a gradient from 5 to 20% was applied for the identification of potential rice allergens, and electrophoresis was run on a Protean II xi cell (Bio-Rad) at a constant current of 5 mA/gel for 1 h, and then 40 mA/gel for 4 h at a temperature of 20 °C. The separated proteins were stained with CBB or by slightly modified silver staining.[28,29] Development and exposure times were the same as described above.

2D Electrophoresis and Immunoblotting. Wheat and rice proteins were precipitated overnight in 20% TCA in acetone containing 0.2% DTT at −20 °C, centrifuged at 3000*g* for 15 min, resuspended in acetone containing 0.2% DTT, centrifuged at 3000*g* for 15 min, and dried. The precipitated samples were dissolved in a rehydration buffer containing 6 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris-base, 0.1% w/v bromophenol blue, 1.2% v/v DeStreak, 1% v/v Pharmalyte (pH 3−10), and 0.5% v/v Pharmalyte

Table 2. Total and Specific IgE, IgA, and IgG Antibodies and ECP (SEM)[a]

			total			specific IgE (IU/mL)	
group	*n*	ECP (ng/mL)	IgE (IU/mL)	IgA (g/L)	IgG (g/L)	wheat	rice
I	35	31.54 ± 8.20	1550.4 ± 600.8	1.37 ± 0.17	8.97 ± 0.70	3.57 ± 0.92	0.86 ± 0.26
Ia	19	26.72 ± 4.54	1528.8 ± 491.6	1.72 ± 0.21	10.81 ± 0.55	2.45 ± 0.45	1.52 ± 0.44
Ib	16	45.18 ± 29.96	1576.0 ± 1202.2	0.96 ± 0.24	6.76 ± 1.18	4.91 ± 1.93	0.08 ± 0.03
II	15	34.32 ± 6.79	272.1 ± 58.1	2.1 ± 0.23	11.49 ± 0.55	0.02 ± 0.02	0.06 ± 0.04
III	10	37.85 ± 10.43	40.0 ± 11.4	1.26 ± 0.17	9.79 ± 0.67	neg	neg

[a]Abbreviations: *n*, number of patients in the tested group; neg, concentration of specific IgE antibodies was under detection limit.

(pH 8−10.5). Eighteen centimeter Immobiline DryStrips with a nonlinear pH 3−10 gradient were used, and the strips were swollen in a total volume of 350 μL with the rehydration buffer containing 200 or 500 μg of protein samples.

Separation in the first dimension (isoelectric focusing) was performed with a Multiphor II unit (Amersham Bioscience). 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 3500 V for 18 h; at constant conditions of 6 mA, 15 W, and 20 °C. Proteins on strips were separated in the second dimension on a 12% SDS-PAGE using a Protean II xi cell (Bio-Rad) at a constant current of 5 mA/gel for 1 h and then 40 mA/gel for 4 h, at a temperature of 20 °C.[30]

Proteins were visualized by modified silver staining[28,29] or electrotransferred with TE77XP Semidry Blotters (Hoefer, Holliston, MA, USA) onto NC membranes, and their IgE-binding proteins were visualized with pooled patient serum (patients 71, 113, and 134) or the serum of disease or healthy controls, as described above (1-DE and immunoblotting).

MALDI-TOF MS and Protein Identification. CBB-stained protein bands or spots were excised from the gel, washed, and digested with trypsin as described previously.[31] Mass spectra were acquired on an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with LIFT technology for MS/MS analysis. The mass spectra were searched against SwissProt 2012_06 or NCBInr 20120623 database subsets of rice proteins, using an in-house MASCOT search engine. Proteins with a MOWSE score over the threshold of 48 (SwissProt) or 64 (NCBInr), calculated for the utilized settings, were considered as identified. If the score was only slightly higher than the threshold value or the sequence coverage was too low, the identity of the protein candidate was confirmed by MS/MS analysis.

RESULTS AND DISCUSSION

Reactivity of Patient IgE Antibodies with Wheat and Rice Extracts. Because rice is frequently recommended as an alternative diet for patients with food allergies and due to the relatively frequent occurrence of wheat allergy, we focused on testing IgE reactivity to rice in patients having food allergies, which included wheat (group I, $n = 35$), with pollen allergy (group II, disease controls, $n = 15$), and healthy controls for comparison (group III, $n = 10$). As shown in Table 1, patients 1−19 had IgE antibodies specific to rice (subgroup Ia), whereas others (20−35) had very low or no IgE reactivity to rice (subgroup Ib). In the disease controls suffering only from a pollen allergy (group II), IgE reactivity to rice was very low, if at all existent. As shown in Table 2, the mean level of total IgE was increased in patients with food allergies (groups I, Ia, and Ib) and in patients with a pollen allergy (group II), as compared with healthy donors, whereas the mean levels of total IgA, IgG, and ECP were very similar in all three groups.

To characterize IgE reactivity to wheat and rice allergens in more detail, we employed immunoblotting techniques. As both wheat and rice are most often consumed after thermal processing, we prepared PBS and SDS extracts of raw and boiled wheat and rice. The heterogeneity of the extracted proteins was characterized by SDS-PAGE (Figure 1). The

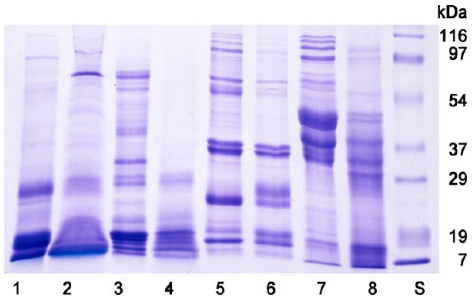

Figure 1. SDS-PAGE protein profiles of PBS and SDS extracts from raw and boiled wheat flour or rice grains. Lanes: 1, PBS extract of raw rice; 2, PBS extract of boiled rice; 3, PBS extract of raw wheat; 4, PBS extract of boiled wheat; 5, SDS extract of raw rice; 6, SDS extract of boiled rice; 7, SDS extract of raw wheat; 8, SDS extract of boiled wheat; S, molecular weight standard. Ten micrograms of protein was loaded per well, and the gel was stained with CBB.

spectra of PBS-extracted rice and wheat proteins were decreased after boiling, and in the case of the rice extract, high molecular weight components above 80 kDa newly appeared. The increased temperature slightly affected the profile of SDS-extracted rice proteins and partially changed the heterogeneity of the SDS-extracted wheat proteins. In general, the effect of boiling can alter the solubility of proteins by changing the protein structure and triggering the formation of protein aggregates.[32−34]

The specific IgE-binding to proteins in raw or boiled wheat flour was demonstrated by immunoblot using sera of the patient group and controls, as described above. An illustrative example of IgE reactivity of five patients to wheat proteins is documented in Figure 2. The individual IgE reactivity with raw wheat components in PBS extract, ranging from 7 to 80 kDa (Figure 2A), was markedly reduced by boiling (Figure 2B). Only the low molecular weight IgE-binding proteins were detected, which corresponds to the lower extraction of high molecular weight wheat proteins in PBS (Figure 1, lane 4). The similar effect of thermal processing (such as baking) on the IgE binding to Tris-HCl-extracted wheat proteins has also been described previously.[35] Our data support the finding that the low molecular weight wheat components of about 14 kDa belonging to the α-amylase/trypsin inhibitor family were identified as major wheat allergens.[4,36] Interestingly, the heterogeneity of IgE reactivity with SDS-extracted proteins from raw and boiled wheat was affected only slightly, which may be explained by the higher thermal stability of these proteins.

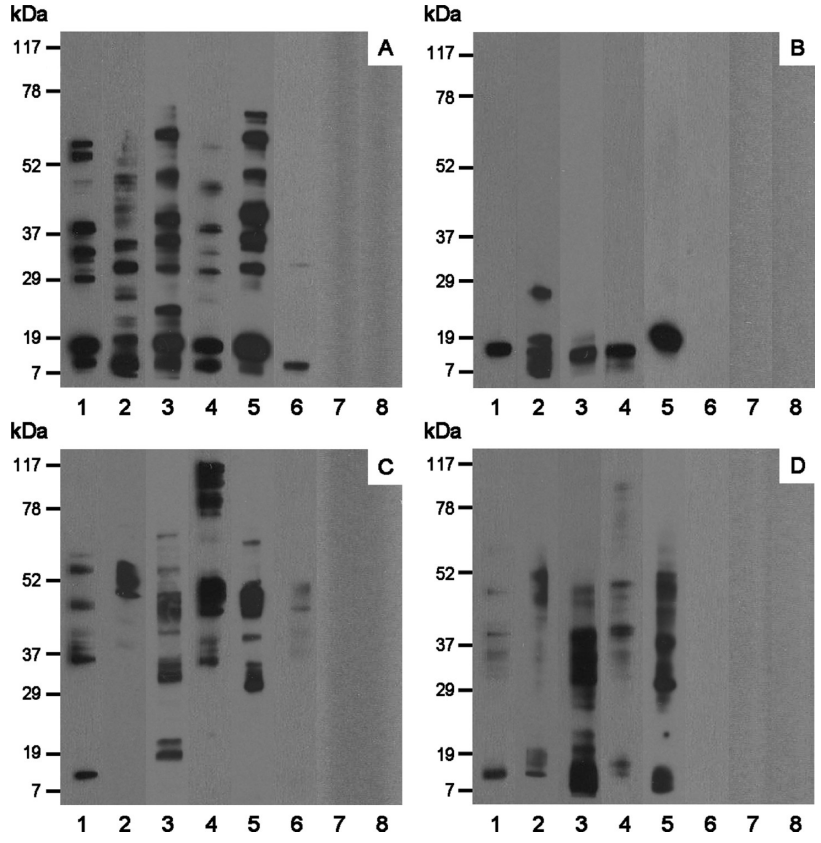

Figure 2. IgE immunoblotting results for wheat protein components in PBS or SDS extracts, from raw and boiled forms of wheat: (A) PBS extract of raw wheat; (B) PBS extract of boiled wheat; (C) SDS extract of raw wheat; (D) SDS extract of boiled wheat. The figure provides an example of the IgE reactivity of five patients with food allergies (lanes 1−5), a disease control with a pollen allergy (lanes 6), a healthy donor (lanes 7), and the secondary anti-human IgE antibody (lanes 8).

We further characterized the heterogeneity of IgE-binding components in PBS and SDS extracts of raw or boiled rice using 19 patients' sera (group Ia) with IgE antibodies specific to rice (Table 1). An illustrative example of IgE reactivity of five patients to rice components on an immunoblot is shown in Figure 3. We detected the whole spectrum of IgE-binding proteins from 9 to 117 kDa in a PBS extract from raw rice (Figure 3A). By evaluation of individual reactivity to separated proteins, we estimated that rice proteins of about 14, 29, 37, 52, and 60−65 kDa are recognized by IgE antibodies, with the highest frequency about 60−70%; proteins of about 19, 24, 33, 45, 78, 98, and 117 kDa were detected with a lower frequency from 30 to 45%. The thermal processing of PBS-extracted rice proteins changed the number of IgE binding PBS-extracted proteins (Figure 3B), as well as the frequency of recognition (from 10 to 30%). The main IgE-binding proteins in the SDS extract from raw rice were of about 37 and 60−65 kDa and were detected with frequencies of 53 and 32%, respectively. Surprisingly, in the SDS extract the number of IgE-binding proteins increased after boiling (Figure 3D), and the frequency of recognition of these proteins with molecular weights of about 14, 19, 24, 29, 37, and 60−65 kDa varied from 35 to 80%.

We can conclude that thermal processing had a different effect on the solubility of wheat and rice PBS- or SDS-extracted proteins and either reduced or increased IgE-binding capacity and the heterogeneity of individual proteins. In general, food proteins exposed to heat can form complexes with fats, sugars, and other food components that could affect the extractability of proteins. One of the most important modifications of food proteins is a nonenzymatic glycation, Maillard reaction.[37,38] All of these processes may uncover so-called cryptic allergenic epitopes or even introduce new structural modifications (including the formation of new antigenic epitopes) and markedly affect the allergenicity of these food proteins.

Reactivity of Patients to Wheat and Rice Extracts in BAT and SPT. To compare IgE-mediated cell response, we used commercial and PBS extracts from wheat and rice. We found a comparable level of basophil activation in the group Ia patients (n = 10) with commercial and PBS wheat extracts (mean values of 49.0 ± 9.5 and 49.9 ± 8.8%, respectively). The similarly small differences in BAT values were obtained using commercial and PBS rice extracts (mean values of 20.8 ± 9.9 and 25.9 ± 9.7%, respectively). However after boiling rice, BAT activation with a PBS extract was decreased to values close to the negative control. The activation of basophils was negative for the disease controls, employing all tested extracts (n = 4).

Patients with high specific IgE serum antibodies and positive in BAT to raw wheat and rice (patients 14 and 17−19) were further tested by the skin prick test (SPT) using wheat and rice extracts. The SPT was performed with commercial extracts of wheat and rice and with a boiled rice homogenate in PBS (100 μg/mL) containing both PBS- and SDS-soluble rice proteins. The data, summarized in Table 3, indicated that three of four patients tested clearly reacted to the boiled rice homogenate. These patients (14, 17, and 18; characterized in Table 1) were IgE positive for more allergens and were on a wheat-free diet.

The lower positivity in BAT compared to SPT could be caused by using different rice proteins in the tests. In BAT, only

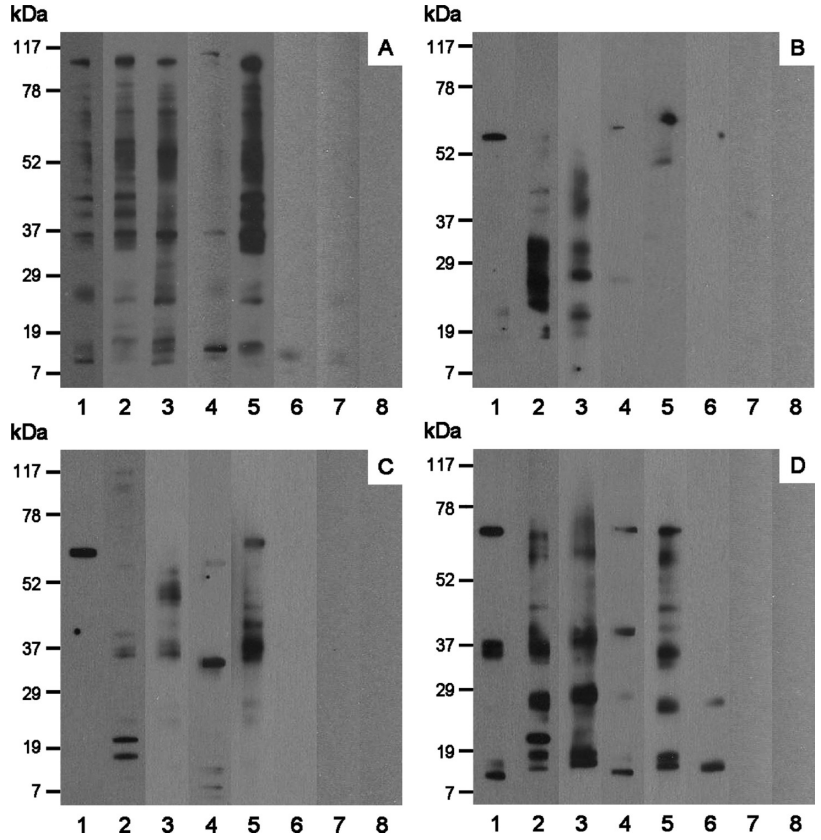

Figure 3. IgE immunoblotting results for rice protein components in PBS and SDS extracts, from raw and boiled forms of rice: (A) PBS extract of raw rice; (B) PBS extract of boiled rice; (C) SDS extract of raw rice; (D) SDS extract of boiled rice. The figure provides an example of the IgE reactivity of five patients with food allergies (lanes 1−5), a disease control with a pollen allergy (lanes 6), a healthy donor (lanes 7), and the secondary anti-human IgE antibody (lanes 8).

Table 3. Skin Prick Test

	diameter of weal on skin (mm)				
patient	wheat[a]	rice[a]	boiled rice homogenate	negative control	positive control[b]
14	5	5	3	0	8/25
17	5	3	3	0	10
18	3	3	3	0	7/25
19	5	0	2	0	7/20

[a]Commercial extract. [b]Assessments of both the weal (swelling and edema) and erythema (flame, redness) reactions were recorded. Patient 17 had only weal reaction.

PBS-soluble proteins from boiled rice could be used, whereas in SPT the whole rice homogenate (containing both PBS- and SDS-soluble proteins present in real foodstuffs) was applied. We showed that rice proteins soluble in SDS maintained IgE-binding capacity, also after boiling, as detected by immunoblotting. These proteins cannot be tested in the BAT assay (using PBS extracts) but could be involved in the SPT reactivity of IgE highly positive patients.

Although rice is often recommended as a diet alternative to wheat-allergic patients, our data suggest that additional testing of a boiled rice homogenate in SPT could be beneficial, especially for patients with high IgE antibodies specific to rice (measured, e.g., by an IMMULITE 2000 system and/or immunoblot), prior to the prescription of a suitable diet.

Characterization and Identification of IgE-Binding Rice Proteins. We identified potential IgE-binding rice proteins in PBS- and SDS-extracted boiled rice. The proteins were separated by either 1-DE or 2-DE, and the IgE-binding proteins were detected using the pooled sera from three selected patients (14, 17, and 18 with IgE antibodies specific to rice and positive reaction to rice in BAT and SPT). The 1-DE-separated proteins recognized by patient IgE antibodies with the highest frequency were identified by MALDI-TOF MS (Figure 4). Rice proteins (one or two) reacting with the pooled sera of healthy donors or with secondary antihuman IgE antibodies were not included in the MS analysis (data not shown). We identified IgE-binding proteins in the SDS (Figure 4, lane 1) and in the PBS extracts (Figure 4, lane 2). A list of all of the identified proteins can be found in Table 4. In the SDS extract, bands 1 and 2 were a mixture of two or three rice seed proteins (RA5, RAG2, and RAG1) belonging to the α-amylase/trypsin inhibitor family. One of them, RAG2, was previously identified in a raw rice PBS extract as an IgE-binding protein.[39] Bands 5 and 8 corresponded to the large family of glutelins, major seed storage proteins encoded as a multigene family. Six major glutelin genes or subunits have been identified in the Asian japonica rice subspecies. These genes have been classified into two subfamilies, GluA and GluB, according to their relatedness in nucleotide sequences.[40] Bands 3 (SDS extract) and 10 (PBS extract) corresponded to a 19 kDa globulin belonging to the α-globulins, previously isolated and identified in a salt extract from rice by Satoh et al.[24] Two separated proteins, glutelin C precursor (band 4) and granule-bound starch synthase 1 chloroplastic/amyloplastic protein (band 9), were identified as new potential rice allergens. Simultaneously

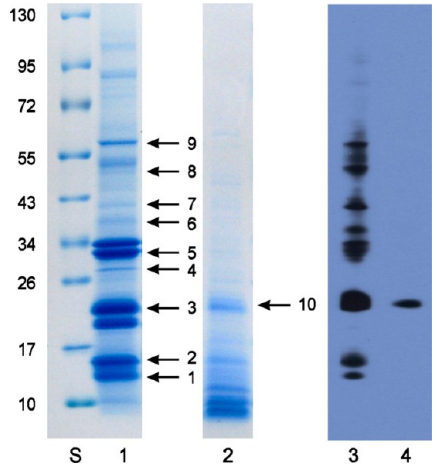

Figure 4. Proteins separated under reducing conditions on a large 1-DE gel from SDS (lane 1) and PBS (lane 2) boiled rice extracts. The gel was stained by CBB, and bands selected for identification were marked by arrows. The bands were selected on the basis of the IgE reactivity of pooled patient sera with SDS (lane 3) and PBS (lane 4) boiled rice extracts on immunoblot; lane S is molecular weight standard. The numbering of the selected protein bands refers to Table 4.

with us, this rice protein was identified as a potential rice allergen by Krishnan and Chen.[41] Band 7 was also a mixture of proteins: aspartate aminotransferase and two rice proteins encoded by gene loci Os08g0545200 and Os01g0905800. In summary, from the 1-DE gel we identified two separated unique IgE-binding rice proteins as new potential rice allergens: glutelin C precursor and granule-bound starch synthase 1 chloroplastic/amyloplastic protein; others were identified in a combined mixture.

Therefore, we utilized 2-DE to further separate proteins far more effectively. We analyzed 16 spots by MS (numbered 101−116, Figure 5A,B) and identified 9 IgE-binding proteins (Table 5). Spot 101 corresponded to disulfide isomerase-like 1-1 protein. Spots 102 and 103 were identified as a hypothetical protein OsI_13867, and spot 108 was identified as a putative acid phosphatase precursor 1. Spots 109 and 110 corresponded to a glutelin type-A 1, a large seed storage protein also identified in the 1-DE gel. Spot 111 was a mixture of two proteins, a glutelin type-A 1 and a rice protein encoded by the gene locus Os02g0453600. A rice protein encoded by the same gene locus was identified in spot 112 together with a glutelin type-B 1. Herein, we could only speculate about the IgE-binding capacity of the protein encoded by locus Os02gO453600, because both spots 111 and 112 were mixed with the already known IgE-binding proteins, glutelin type-A 1 or glutelin type-B 1, respectively.[40] Glutelin type-B 1 was also identified in the separated spot 113. Spots 114, 115, and 116 corresponded to a 19 kDa globulin, the seed allergenic protein RAG2, and a protein encoded by the gene locus

Table 4. MALDI-TOF MS Identification of Potential Rice Allergenic Proteins from SDS-PAGE Experiments

band[a]	extract	protein (potential allergen) name	accession no.	MW (kDa)	peptides matched	sequence coverage (%)	MS/MS confirmation
1	SDS	seed allergenic protein RA5	RA05_ORYSJ	17	6	41	no
		seed allergenic protein RAG2	RAG2_ORYSJ	18	6	34	no
2	SDS	seed allergenic protein RA5	RA05_ORYSJ	17	11	53	ELGAPDVGHPMSEVFR CEAISHMLGGIYR
		seed allergenic protein RAG2	RAG2_ORYSJ	18	9	65	ELGATDVGHPMAEVFPGCR
		seed allergenic protein RAG1	RAG1_ORYSJ	18	5	49	QLAAVDDGWCR GAASAADEQVWQDCCR
3	SDS	19 kDa globulin	GL19_ORYSJ	21	12	22	no
4	SDS	glutelin C precursor[b]	37993736	55	13	21	no
5	SDS	glutelin type-A 1[b]	GLUA1_ORYSJ	56	10	26	LQAFEPIR GLLLPHYTNGASLVYIIQGR
		glutelin type-A 3[b]	GLUA3_ORYSJ	56	5	13	GLLLPHYSNGATLVYVIQGR
		glutelin type-B 1[b]	GLUB1_ORYSJ	56	4	7	no
7	SDS	Os08g0545200	115477633	39	13	40	LPPVGPYDVR FGFSQEDVEEAFEVSAR
		aspartate aminotransferase	AATC_ORYSJ	44	9	34	LIFGADSPAIQENR
		Os01g0905800	297598143	39	8	23	IGPNEPSQLSIDLNAQGLAR
8	SDS	glutelin type-A 1	GLUA1_ORYSJ	56	8	26	VEHGLSLLQPYASLQEQEQGQVQSR
		glutelin type-B 1	GLUB1_ORYSJ	56	8	17	YTNIPGVVYIIQGR
		glutelin type-B 4	GLUB4_ORYSJ	57	6	19	no
9	SDS	granule-bound starch synthase 1 chloroplastic/amyloplastic protein	SSG1_ORYSI	66	15	30	no
10	PBS	19 kDa globulin	GL19_ORYSJ	21	6	20	FQPMFR

[a]Band 6 was not identified by mass spectrometry. [b]Identified as an N-terminal fragment of the intact protein.

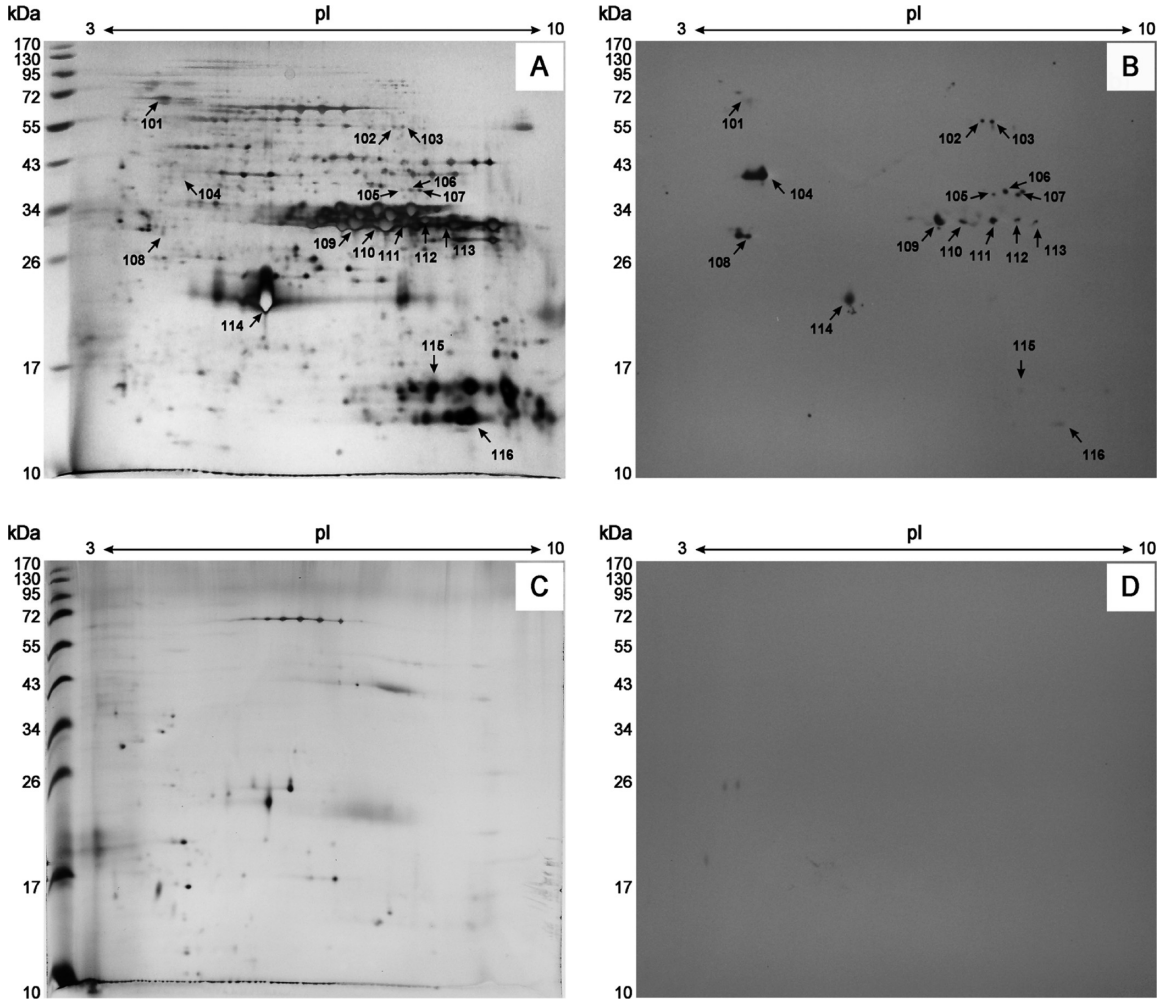

Figure 5. Spectra of proteins in the boiled form of the rice SDS and PBS extracts separated by 2-DE: (A) proteins from the SDS extract visualized by silver staining; (B) proteins recognized by the IgE antibodies of patients with food allergy; (C) proteins from the PBS extract visualized by silver staining; (D) proteins recognized by the IgE antibodies of patients with food allergy. The spots selected for identification are numbered. The numbering refers to Table 5.

Table 5. MALDI-TOF MS Identification of Potential Rice Allergenic Proteins in the SDS Extract from 2-DE Experiments

spot[a]	protein (potential allergen) name	accession no.	MW (kDa)	p*I*	peptides matched	sequence coverage (%)	MS/MS confirmation
101	protein disulfide isomerase -like1-1	PDI11_ORYSJ	57	5.0	11	31	SDYDFGHTLHANHLPR
102	hypothetical protein OsI_13867	218193892	52	7.0	7	20	no
103	hypothetical protein OsI_13867	218193892	52	7.0	4	10	FPDEQVVGAAVGGYR
108	putative acid phosphatase precursor 1	53792717	28	4.9	5	26	LYNELQGLGIHIILLTGR
109	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	10	12	QFQCTGVSVVR LQAFEPIR
110	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	9	13	GLLLPHYTNGASLVYIIQGR
111	Os02g0453600	115445979	57	9.0	5	11	EFFLAGKPR
	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	4	8	LQAFEPIR
112	Os02g0453600	115445979	57	9.0	8	14	QFSFGGSPLQSPR
	glutelin type-B 1	GLUB1_ORYSJ	57	9.3	3	7	no
113	glutelin type-B 1	GLUB1_ORYSJ	57	9.3	7	13	QLFNPSTNPWHSPR
114	19 kDa globulin	GL19_ORYSJ	21	7.5	6	43	QGYYGEGSSEEGYYGEQQQQPGMTR
115	seed allergenic protein RAG2	RAG2_ORYSJ	18	8.1	8	51	QLAAVDDSWCR CQPGMGYPMYSLPR
116	Os07g0216700	115471187	16	7.5	10	68	AGYGGYGDVGEYCR ELAAVPMQCR

[a]Spots 104, 105, 106, and 107 were not identified by MS.

Os07g0216700, respectively. We also tried to characterize rice proteins from the PBS extract (Figure 5C), but no or very low IgE reactivity was detected (Figure 5D). We can suppose that the loss of IgE binding to PBS-extracted proteins, including the

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19 kDa globulin identified from 1-DE, may be caused by the damage of IgE epitopes during the treatment of the sample for 2-DE (higher salt concentration and detergent). In summary, from the 2-DE gel we identified nine rice proteins, of which five were already known and four have newly been described as potential rice allergens: disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and protein encoded by locus Os02g0453600. Interestingly, although plant food allergens belong to a rather limited number of proteins, the already known IgE-binding rice proteins and those newly identified by us differ widely from the known IgE-binding wheat proteins (except proteins belonging to the α-amylase/trypsin inhibitor family).[31]

In conclusion, we have demonstrated that patients with food (mainly wheat) and pollen allergies often have increased levels of IgE antibodies specific to rice. More than 80% of these patients reacted to SDS-extracted boiled rice proteins, and those with the highest IgE-binding capacity were positive in a SPT. On the basis of these data, we could suggest that the patients with high IgE antibodies specific to rice, and positive on immunoblot, should be further tested by a SPT with boiled rice components before rice is recommended as a suitable hypoallergenic diet alternative. Using proteomic techniques we analyzed SDS-extracted rice proteins and identified six new potential rice allergens (from 1-DE and 2-DE) that also retain IgE-binding capacity after thermal processing, potentially representing important allergens in rice-containing foodstuffs. However, further experiments are necessary to confirm these proteins as newly defined rice allergens.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; BAT, basophil activation test; CBB, Coomassie Brilliant Blue; ECP, eosinophilic cationic protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NC, nitrocellulose; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate buffer; SDS-PAGE, sodium dodecyl sulfate solution−polyacrylamide gel electrophoresis; SPT, skin prick test; TBS, Tris-buffered saline

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3.3. Publication III

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

The majority of food allergens are consumed after thermal processing leading to the alteration of their molecular structure, as well as their allergenicity. Among such allergens belongs hen's egg white. Forming approximately 60% of total egg white protein, ovalbumin (OVA) is the most abundant protein of the egg white (Huntington and Stein, 2001). Egg allergens, such as OVA, are processed at different temperature (*e.g.*, baked, scrambled or soft/hard boiled eggs) and these processing conditions can have an important impact on their secondary structure, their susceptibility to enzymatic digestion in the GIT and their allergenicity. Partial decrease of IgE binding after OVA thermal processing suggested that both linear and conformational epitopes participate in the OVA-IgE specific interactions (Lemon-Mulé et al., 2008; Martos et al., 2011; Nowak-Węgrzyn and Sampson, 2011). As the impact of heating on the changes in the secondary structure of OVA and on its ability to induce the clinical symptoms of food allergy has not been previously studied, we tried to develop a suitable mouse model for studying this effect on the immune system in detail.

We showed that heating OVA causes irreversible changes in its secondary structure, although heating to 70°C (h-OVA) has only minor effect on the secondary structure of OVA compared to heating to 90°C (b-OVA). However, these minimal changes were accompanied by different susceptibility of OVA to pepsin digestion. Both h-OVA and b-OVA were much more resistant to pepsin digestion than native form of OVA. We used these findings in our mouse model of food allergy.

During the experiment, we assessed allergic diarrhea, the main clinical symptom of food allergy, which was induced in both native OVA and h-OVA stimulated BALB/c mice, but the disease symptoms appeared much earlier and with higher frequency in native OVA fed mice. As the small intestine has a great plasticity in the response to various dietary changes, which may be reflected in activation of enterocyte brush-border enzymes, we further

analyzed these enzymes involved in intestinal homeostasis. We showed for the first time, that one of the enzymes, alkaline phosphatase (ALP), could be very promising marker in food allergy, because its specific activity was significantly increased in native OVA fed mice compared to h-OVA fed and control mice. This result is in accordance with recent findings in which the ALP plays a crucial role in the regeneration of intestinal enterocytes (Lynes and Widmaier, 2011; Lallès, 2010). Furthermore, h-OVA induced lower activity of serum MCPT-1 enzyme, which is very good marker of activated mast cells, and stimulated higher secretion of IFN-γ supporting Th1 immune response (Vaali et al., 2006).

For testing the effect of thermal processing on OVA allergenicity, we measured the serum levels of OVA-specific antibodies (IgA, IgE, IgG1, and IgG2a) against either native OVA or h-OVA in the course the experiment. We detected higher levels of specific IgE antibodies against native OVA and lower levels of specific IgG2a antibodies compared to h-OVA. Higher levels of IgG2a antibodies in h-OVA fed mice could probably lead to allergen competition and thus may block the allergy development (Johansen et al., 2005; Lemon-Mulé et al., 2008).

Furthermore, heating of OVA led to decreasing in the levels of proallergenic cytokines (IL-4, IL-5, IL-13), and stimulated higher secretion of IFN-γ supporting Th1 immune response. Higher production of blocking IgG2a antibodies and secreting of IFN-γ in h-OVA fed mice are in accordance with their lower production of specific IgE antibodies and later onset of allergic symptoms, such as diarrhea. These results confirm the tendency to shift the immune system from Th2 towards Th1 response.

To characterize the effect of heating and enzymatic digestion on T cell subpopulations, especially on Treg cell differentiation, we cultured *in vitro* splenocytes isolated from naïve (non-stimulated) BALB/c mice either with native OVA, h-OVA or b-OVA as well as with their enzymatic digests. Interestingly, we detected that 20-minute peptic digests of both heated forms of OVA induced differentiation of Treg cells, but this ability decreased again after 40-minute digestion.

In summary, we showed that even mild changes in the secondary structure of OVA after thermal processing has far-reaching consequences concerning its antigen properties. After digestion of h-OVA, fragments with different immunogenic properties are formed leading to the shift from Th2 towards Th1 immune response compared to native OVA. Nevertheless, h-OVA still has an ability to induce the allergy symptoms, but this ability is less pronounced and needs longer time to develop.

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

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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.

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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 (4×10^4 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-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader (λ_{ex}:360 nm/λ_{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 (6×10^5/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 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 (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

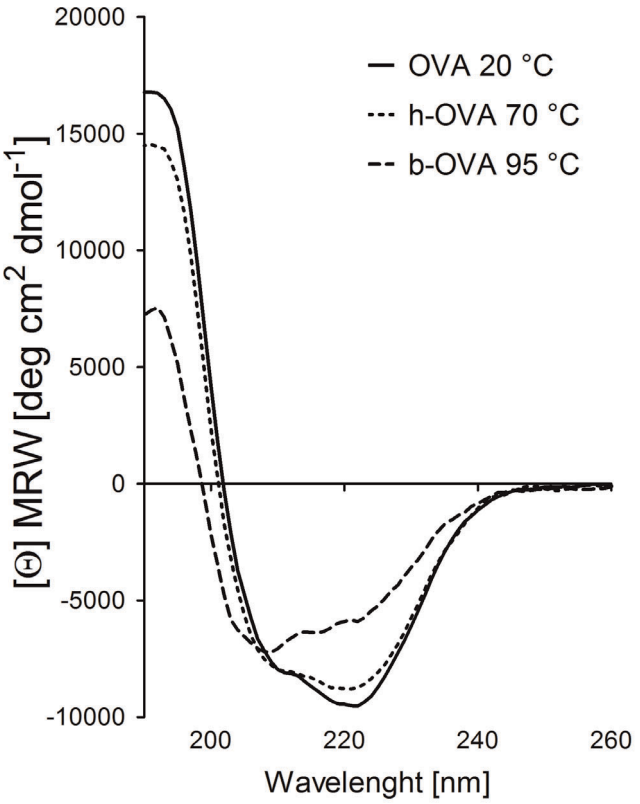

Figure 1. Circular dichroism spectra of native and heated-OVA. Circular dichroism spectra showed only minor irreversible structural changes of hen egg ovalbumin-OVA heated for 10 minutes at 70°C (h-OVA, dotted line) as compared to OVA heated at 95°C (b-OVA, dashed line). Spectra were taken after renaturation 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

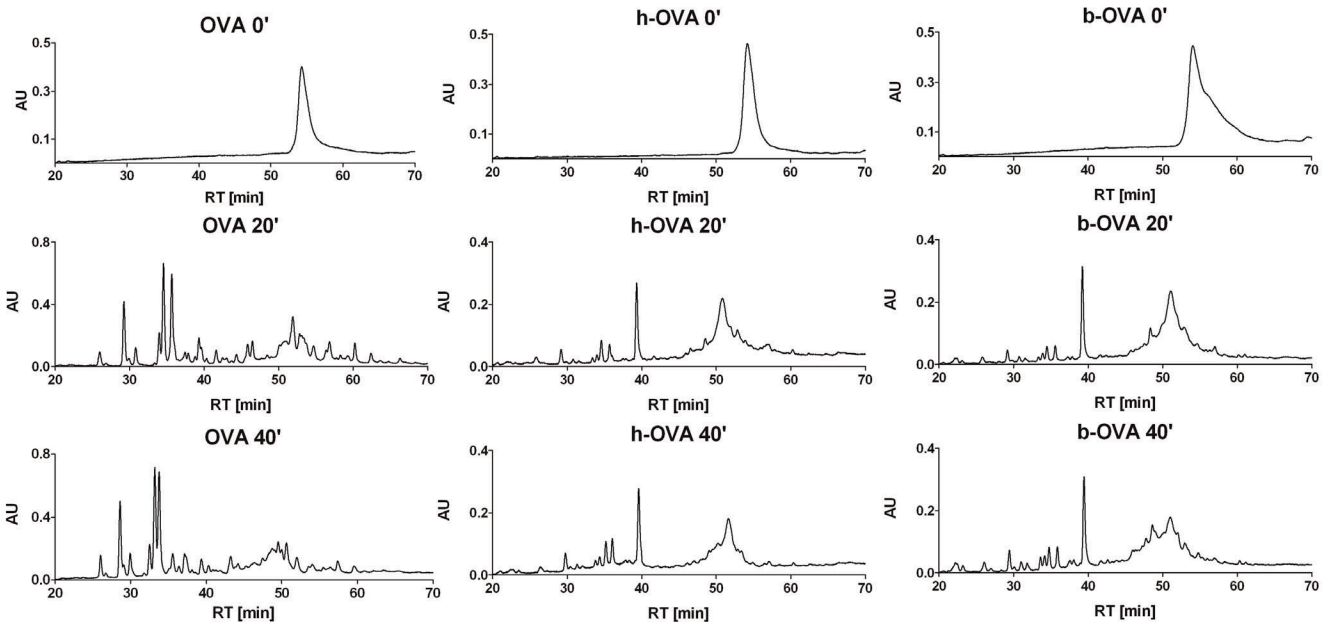

Figure 2. RP-HPLC separation profile of native-OVA and heated-OVA peptic digests. RP-HPLC separation profile monitored at 280 nm corresponds to OVA and OVA heated at 70°C (h)-OVA or boiled at 95°C (b)-OVA undigested (0′) and after 20 (20′) and 40 minutes (40′) of digestion by pepsin. RT – retention time.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

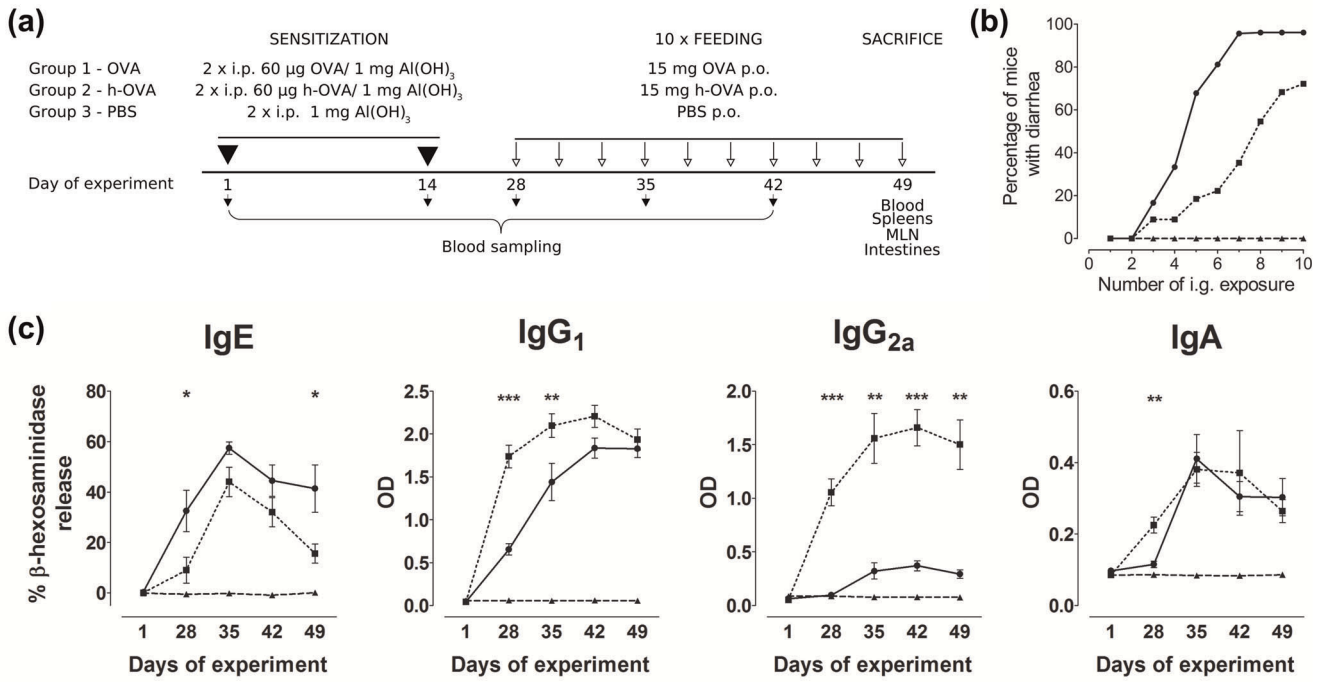

Figure 3. Impact of heating on OVA-induced allergic response. Experimental design (a). Mice were sensitized twice intraperitoneally (*i.p.*) with OVA/Al(OH)$_3$, heated OVA (h-OVA)/Al(OH)$_3$ or PBS/Al(OH)$_3$ alone and subsequently challenged with ten doses of OVA, h-OVA or PBS by intragastric tubing (*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).

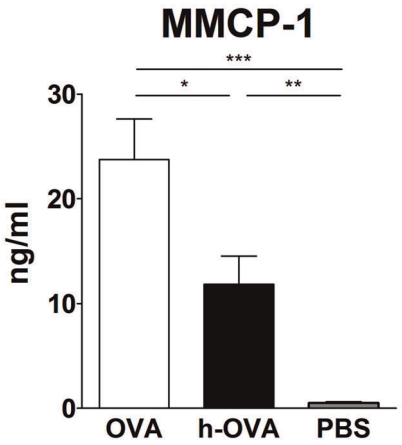

Figure 4. Decreased mast cell protease induction by heated-OVA. Heated OVA (h-OVA, black bar) induced significantly lower amounts of mast cell protease (MMCP-1), the marker of mast cell activation, compared to mice fed with native OVA (white bar). Data are represented as mean ± SEM (n = 10 mice/group), representative data from one out of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001.
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-α 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-γ, 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-γ production by splenocytes of mice challenged with heated fish extract. Our findings suggest that heating of OVA induces

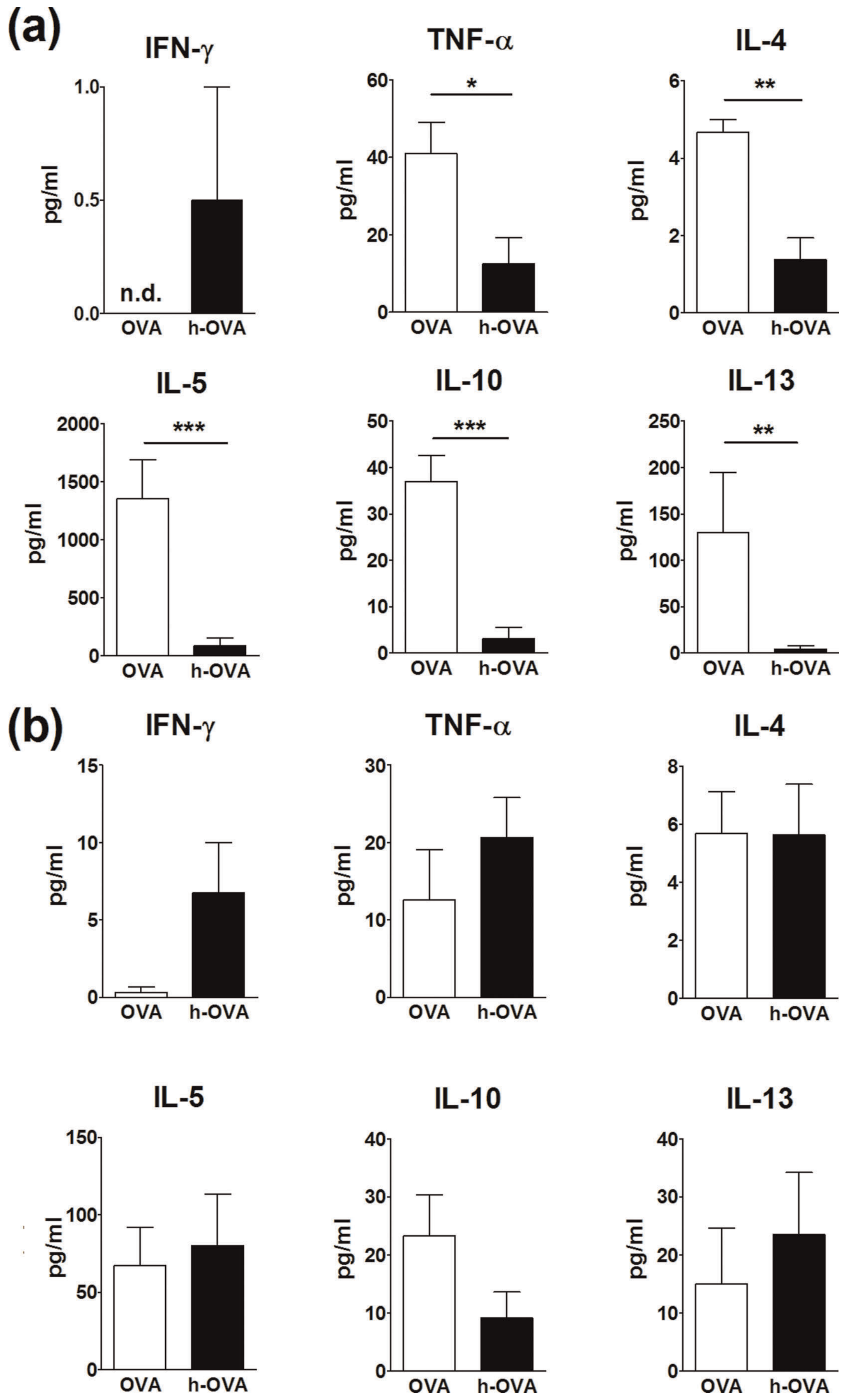
(a)
IFN-γ
TNF-α
IL-4
pg/ml
1.0
0.5
0.0
n.d.
60
40
20
0
*
6
4
2
**
OVA
h-OVA
IL-5
IL-10
IL-13
2000
1500
1000
500
***
50
30
10
250
200
150
100
(b)
15
5
8
7

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

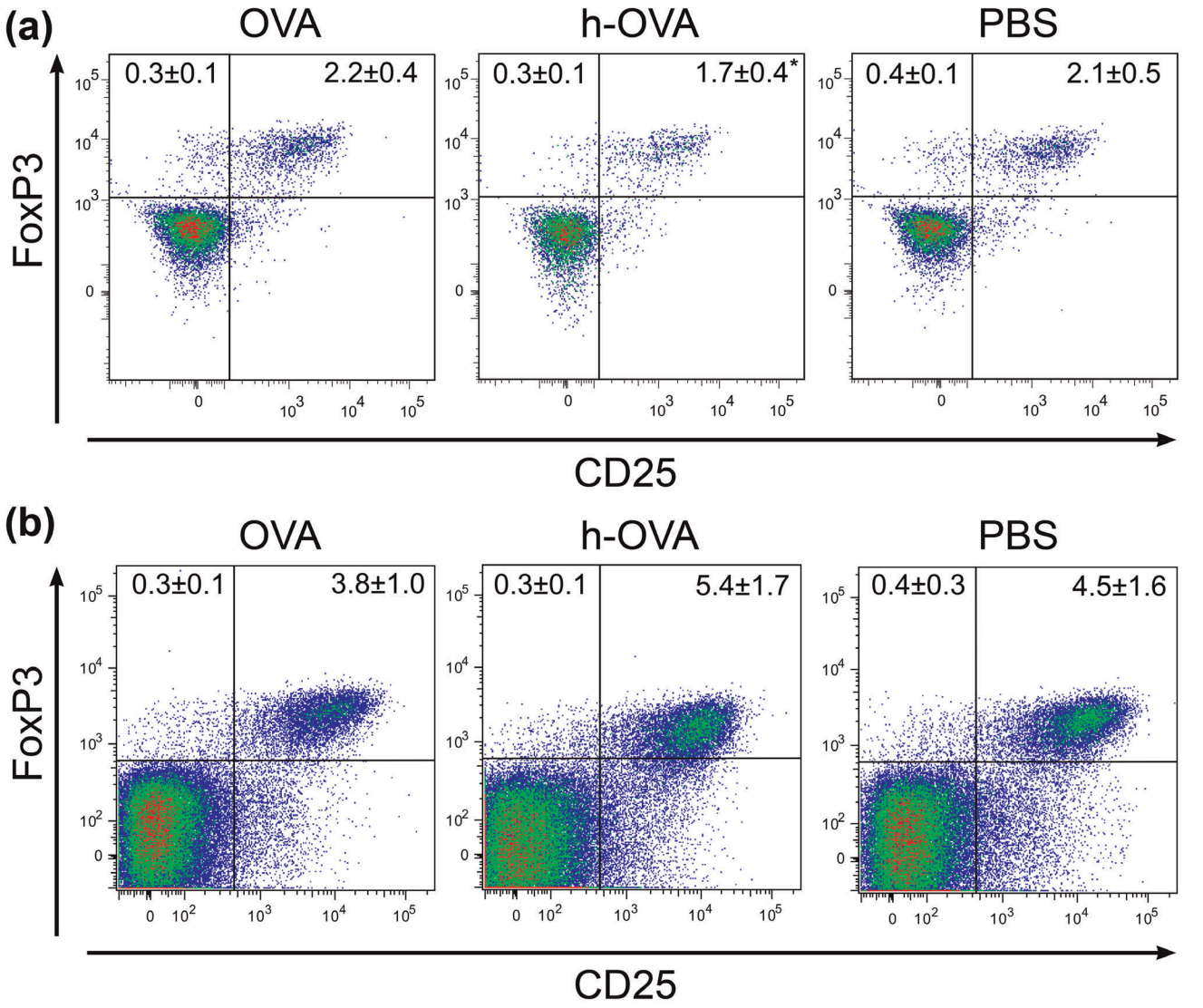

Figure 6. Numbers of Tregs in splenocytes and mesenteric lymph nodes of OVA treated mice. Typical plots depicting numbers of Tregs in mouse splenocytes (a) and mesenteric lymph node (b) in gated CD3+CD4+CD8– T helper cells after feeding with OVA, h-OVA or PBS, respectively. Numbers in upper quadrants 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

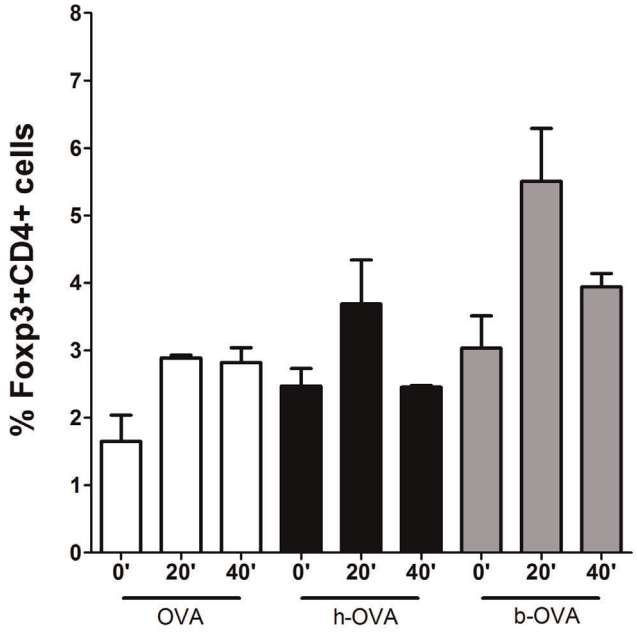

Figure 7. Number of Tregs in spleen cell suspensions co-cultured *in vitro* with OVA digests. The percentage of Tregs in cell suspension isolated from spleens of non-stimulated (naïve) BALB/c mice cultured *in vitro* for 48 hours with undigested (0′) and after 20 (20′) and 40 minutes (40′) peptic digest 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)

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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.

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Supporting Information

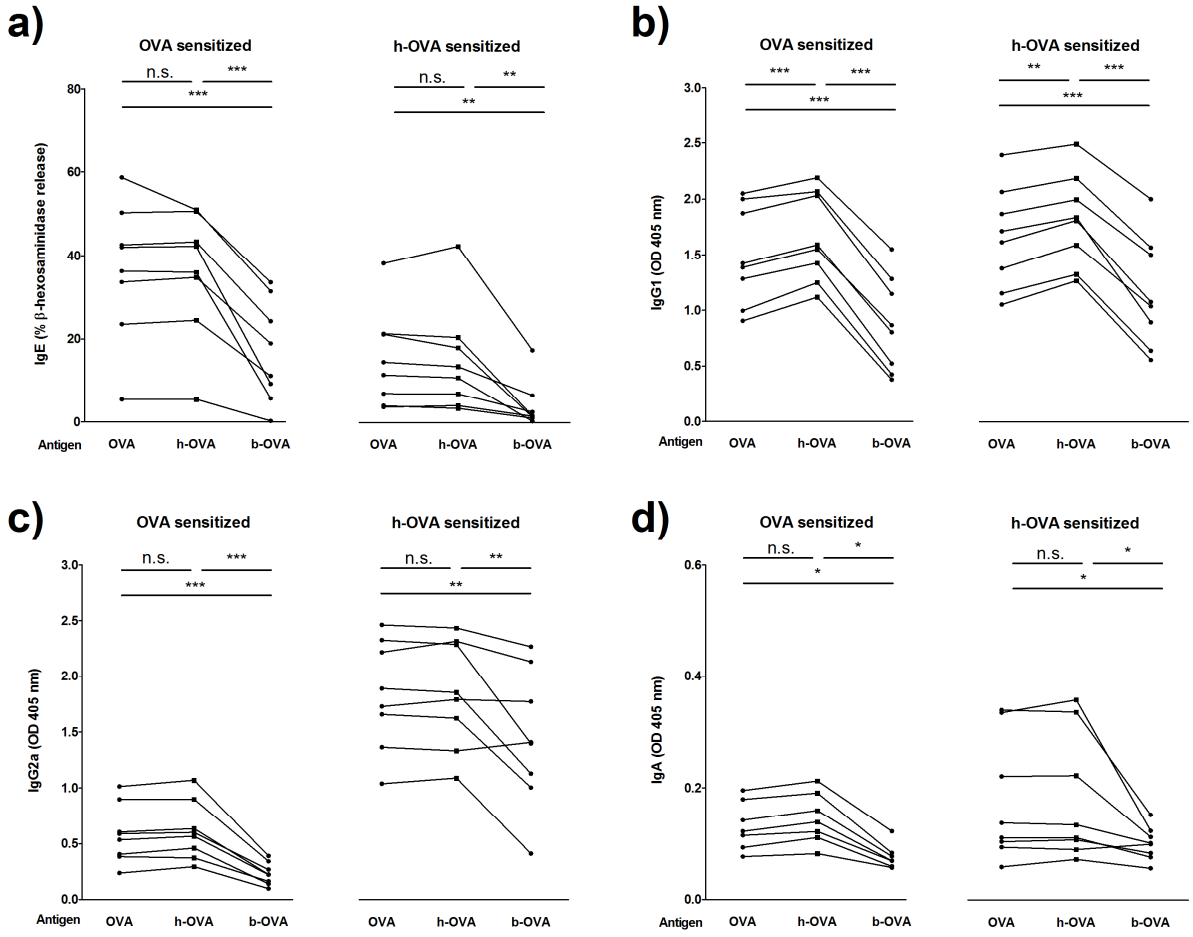

Figure S1. Cross-reactivity of anti-OVA specific antibodies. At the end of the experiment we determined the levels of OVA-specific antibodies in OVA and heated (h)-OVA treated mice against OVA, h-OVA (70°C) and boiled (b)-OVA (95°C). The levels were retained for IgE, IgG2a and IgA (a, c, d) when we used 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.

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3.4. Unpublished results

The colonization of the intestine by probiotic bacteria may modulate the induction of oral tolerance and allergy development. Therefore, GF or gnotobiotic (model animal colonized minimally by one good defined microorganism) mouse model is an invaluable tool for studying the host-microbiota interactions and can be used for evaluation of the effect of selected bacterial species on the development of mucosal and systemic immunity. The aim of our study was to analyze the impact of probiotic bacterium *Lactobacillus plantarum* in monocolonized mice on the food allergy development.

Eight weeks old female mice (BALB/c) were divided into six groups – conventionally reared mice (CV), GF mice, GF mice colonized with *Lactobacillus plantarum* (Lp), and three control groups for each condition. The germ-free female mice were colonized by intragastric administration with the single dose (2×10^8 CFU) of freshly grown bacterium *Lactobacillus plantarum* in 200 μl of sterile PBS and mated 20 days later. The success and stability of colonization was checked by plating of feces on MRS agar and CFU were counted after 48-hours cultivation at 37°C.

Thereafter, all three groups of mice were twice sensitized intraperitonealy (i.p.) in two week intervals (day 1 and 14 of experiment) by OVA with alum adjuvant. Fourteen days after the second sensitizing dose, the mice were challenged 8-times (at 2–3 day intervals) by intragastric (i.g.) dosages of 15 mg of OVA (Figure 9).

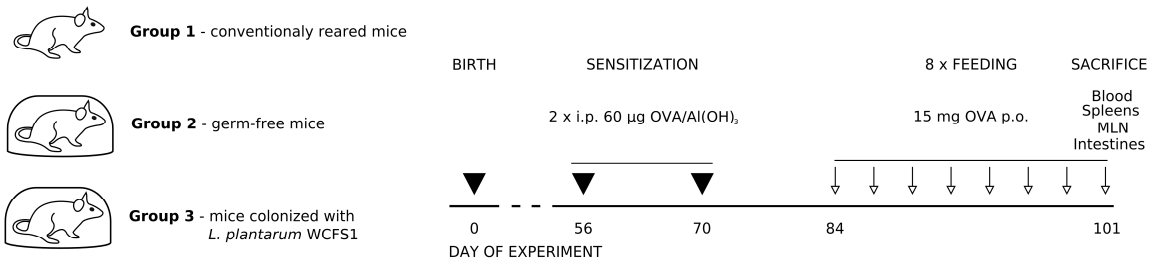

Figure 9. Experimental design.

During the experiment, we evaluated the onset of allergic symptoms, such as diarrhea, changes in temperature and weight of mice. The allergic diarrhea appeared in all CV mice (100%) already after the sixth i.g. dosage of OVA, whereas both GF and Lp mice have no diarrhea throughout the whole feeding (Figure 10A). The temperature was decreased in CV

mice as a consequence of the allergy onset, whereas in GF and Lp mice it was slightly increased (Figure 10B).

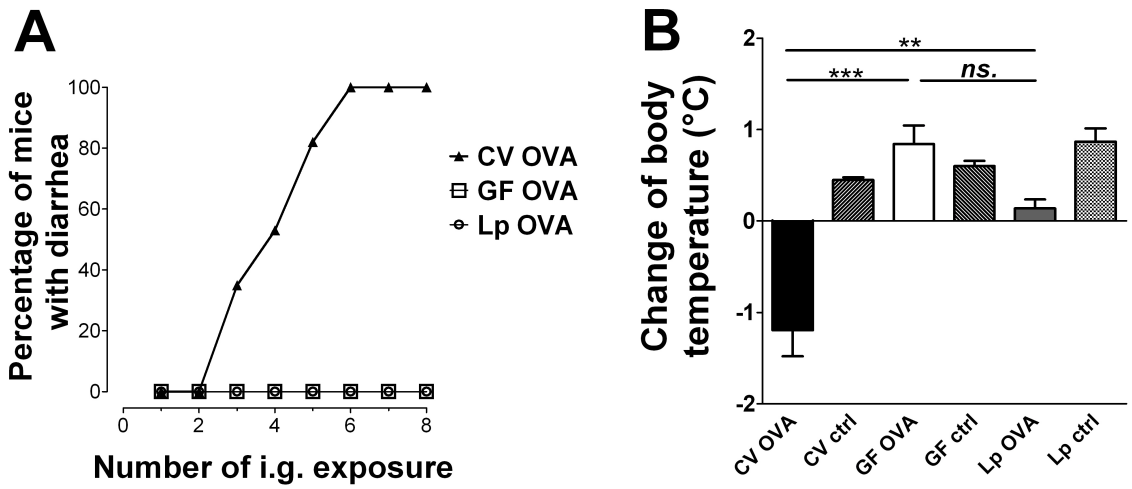

Figure 10. Onset of diarrhea (A) and change of body temperature (B) in mice during the experiment; CV OVA – conventional mice fed ovalbumin, CV ctrl – conventional mice fed PBS, GF OVA – germ-free mice fed ovalbumin, GF ctrl – germ-free mice fed PBS, Lp OVA – mice colonized by *Lactobacillus plantarum* fed ovalbumin, Lp ctrl – mice colonized by *Lactobacilus plantarum* fed PBS, i.g. – intragastric dosage, ns. – non-significant.

The level of MCPT-1 enzyme in mouse sera was significantly higher in CV mice compared to GF and Lp mice (Figure 11A). The significantly lower level of MCPT-1 was measured also in jejuna of both GF and Lp mice (Figure 11B). These results indicate that the early colonization of the intestine by commensal bacteria is necessary for the proper development and function of mast cells in the intestinal mucosa.

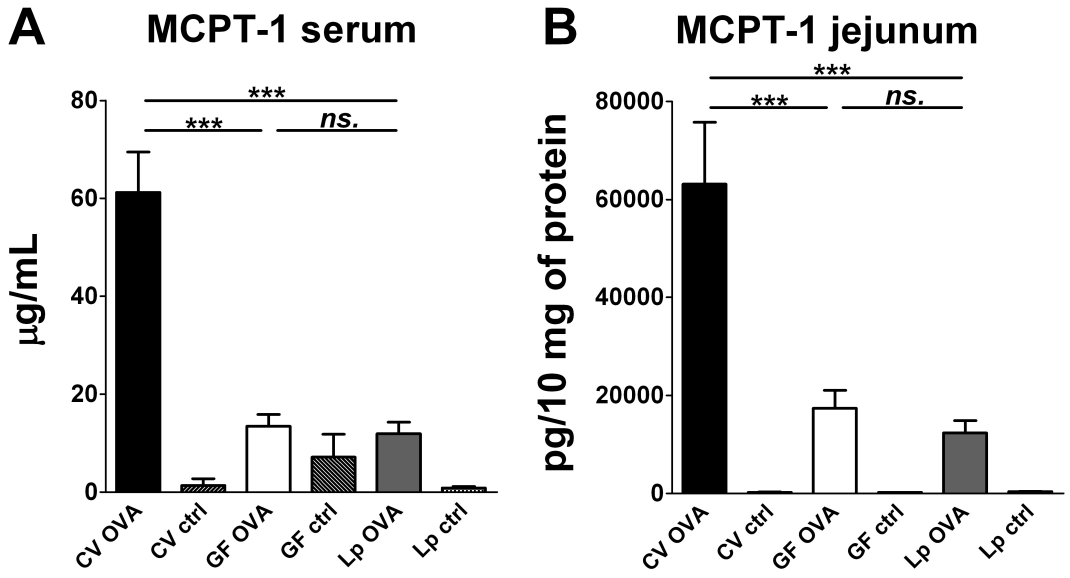

Figure 11. Levels of MCPT-1 enzyme in mouse sera (A) and in mouse jejuna (B); CV OVA – conventional mice fed ovalbumin, CV ctrl – conventional mice fed PBS, GF OVA – germ-free mice fed ovalbumin, GF ctrl – germ-free mice fed PBS, Lp OVA – mice colonized by *Lactobacillus plantarum* fed ovalbumin, Lp ctrl – mice colonized by *Lactobacilus plantarum* fed PBS, ns. – non-significant.

Further, we evaluated the differences in the serum levels of specific IgA, IgE, IgG1, and IgG2a antibodies (Figure 12). The level of specific IgE antibodies was significantly lower in Lp mice compared to CV and GF mice. Likewise, the level of specific IgG1 (proallergenic) antibodies was also decreased. On the other hand, the level of specific IgG2a antibodies, shifting the immune system towards Th1 response was distinctly decreased in both GF and Lp mouse groups. Interestingly, the level of specific IgA antibodies was decreased in GF mice compared to CV and Lp mice. This findings indicates that for the production of IgA antibodies in the intestinal mucosa is crucial the stimulation of the immune system by bacteria.

In summary, we showed that GF and Lp mice affected the development of food allergy. We showed that GF mice were not capable to develop the food allergy at all, probably due to their immature immune system, and the colonization by *Lactobacillus plantarum* was not sufficient to induce the allergic symptoms, such as diarrhea, during the experiment. However, the colonization of mice by this probiotic bacterium led to the suppression of Th2 immune response by decreasing the IgG1 and IgE antibody production.

The cell subpopulations, cytokine production, total IgA and IgE antibodies production in jejunum, and histology are under investigation.

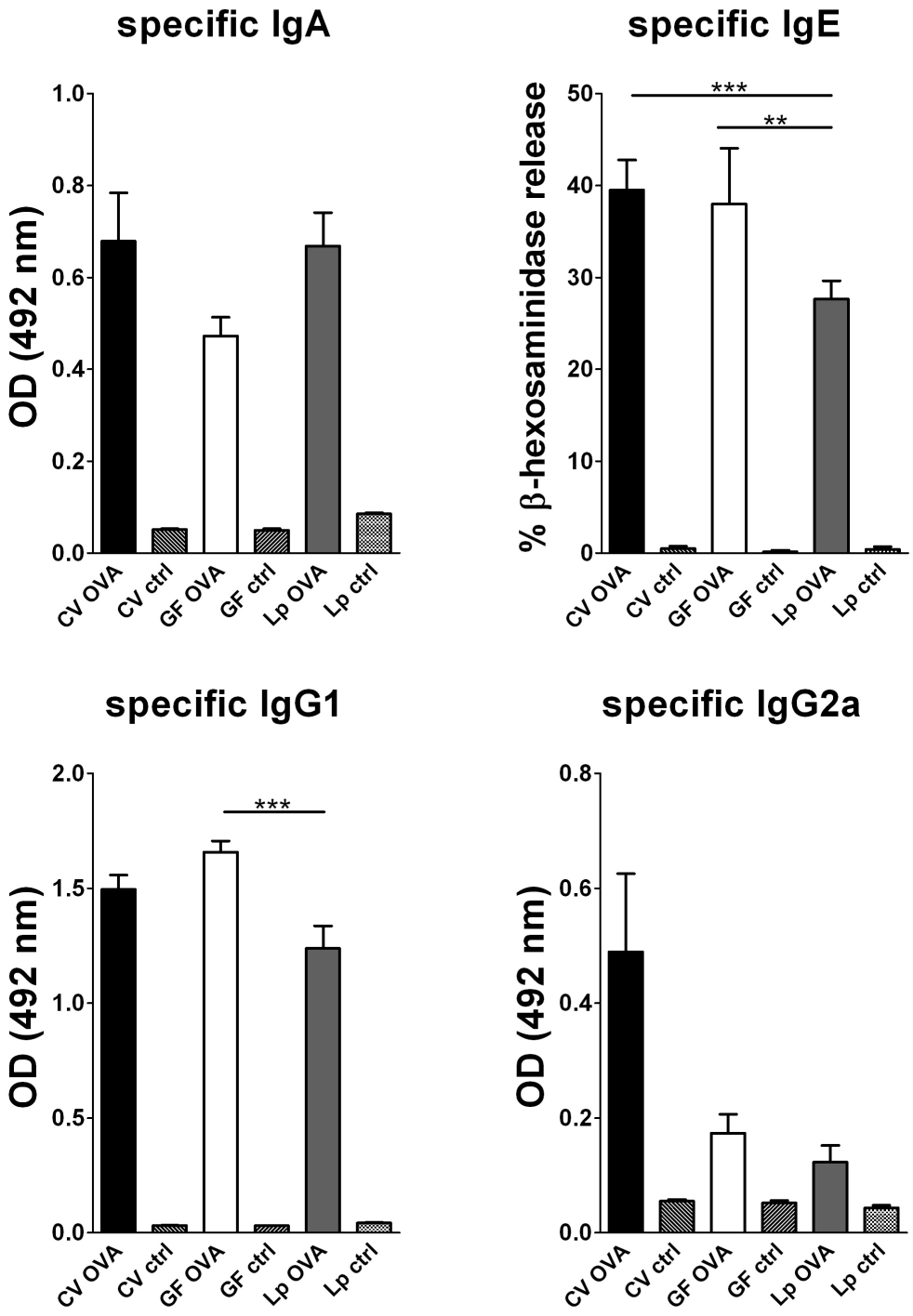

Figure 12. Levels of specific antibodies in mice sera; CV OVA – conventional mice fed ovalbumin, CV ctrl – conventional mice fed PBS, GF OVA – germ-free mice fed ovalbumin, GF ctrl – germ-free mice fed PBS, Lp OVA – mice colonized by *Lactobacillus plantarum* fed ovalbumin, Lp ctrl – mice colonized by *Lactobacilus plantarum* fed PBS, ns. – non-significant, OD – optical density.

4. Conclusions

- We succeeded to develop the new three-step procedure for isolation and purification of proteins from natural sources, such as wheat flour. This protocol, including ultrafiltration, preparative isoelectric focusing using Rotofor, and the subsequent purification by HPLC, enabled us to isolate and to purify wheat proteins in native form and amount sufficient for further structural and functional studies. Moreover, the isolated proteins retained their biological activity.

- Using this new approach and patients' sera, we characterized IgE-binding wheat proteins and identified 27 allergens, from which 7 were identified as new potential wheat allergens: endogenous α-amylase/subtilisin inhibitor, trypsin/α-amylase inhibitor CMX1/CMX3 protein, thaumatin-like protein (TLP), xylanase inhibitor protein-1 (XIP-1), β-glucosidase 1, class II chitinase, and 26 kDa endochinase.

- Further, we aimed to characterize and identify potential rice allergens in water/salt-insoluble (SDS extract) fraction, which maintained their IgE-binding capacity after boiling. We succeeded to identify 22 rice proteins, from which 6 proteins were identified as new potential rice allergens: glutelin C precursor, granule-bound starch synthase 1 protein, disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and protein encoded by locus Os02g0453600.

- We showed that patients with food allergy (mainly to wheat allergens) have increased levels of specific IgE antibodies to rice components. Moreover, up to 80% of these patients had increased IgE reactivity to SDS-extracted boiled rice proteins in immunoblots and 26% of them were also positive in BAT. Therefore, for these patients, we can suggest additional testing by SPT using the boiled rice homogenate, containing both water-soluble and water-insoluble rice proteins, before rice recommendation as a suitable hypoallergenic diet.

- We succeeded to develop the suitable mouse model for studying food allergies. Our model is a convenient tool for studying the mechanisms of food allergy and verification of hygiene hypothesis, using animals reared under conventional or germ-free condition. This model can be also used for studying the immunomodulating properties of probiotic bacteria on the immune system in the gnotobiotic mouse model.

- We described that heating of OVA to 70°C led to mild irreversible changes in its secondary structure, which also affected its enzymatic digestibility. The introduction of these findings to the mouse model of food allergy, we showed that even mild irreversible changes in the OVA structure led to the decreased manifestation of clinical symptoms (diarrhea), lower production IgE antibodies, proallergenic cytokine secretion, and activation of mast cells. Moreover, heating of OVA led to higher production of IgG2a antibodies shifting the immune system to Th1 response. Nevertheless, the h-OVA still had the ability to induce the food-allergy symptoms, but these were less pronounced and needed longer time to develop.

- Finally, we showed that germ-free and *Lactobacillus plantarum* colonized mice affected the allergic sensitization and the onset of food allergy. We showed that germ-free mice were not capable to develop the food allergy at all, probably due to their immature immune system, and the colonization by *Lactobacillus plantarum* was not sufficient to induce the allergic symptoms, such as diarrhea, during the experiment.

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