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Enzymatic and Chemical Modifications of Food Allergens

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1. Introduction

Food allergy is defined as an abnormal immunological reaction to food proteins, which causes an adverse clinical reaction. Today 4–6% of children and 1–3% of adults are affected with food allergy in Europe (Mills et al., 2007), while in the USA 6–8% of children and 4% of adults are affected (Gupta et al., 2008). The incidence of food allergy has been increasing over the years, and to date, no effective treatment of food allergies is available. Therapies involving drugs (antihistamines, decongestants, or steroids) only combat the symptoms of an allergic reaction and do not prevent allergenic reactions due to future exposures to the allergen. Specific immunotherapy (SIT) is the only prophylactic desensitizing therapy for allergy (Bousquet et al., 1998; Durham et al., 1999). However, for food allergies immunotherapy safety-profile is extremely poor because allergic reactions are associated with a significant risk of anaphylactic side-effects (Nelson et al., 1997; Skripak et al., 2008), even with fatal outcome. Therefore, allergen modification resulting in almost complete reduction of IgE-binding, while retaining its immunogenicity, could improve formulations for immunotherapy of food allergies.

The best way to prevent unintended exposure to a food allergen is the strict avoidance of foodstuffs containing allergens. Such avoidance may not always be possible, and in certain instances impossible. Furthermore, complete elimination of foodstuffs causing allergy also has negative impact on healthy diet as about 90 % of allergens are present in food of high nutrition value (milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soybeans) (Thompson et al., 2006). Therefore production of hypoallergenic food become increasingly indispensable part of food industry with growing demand for methods aimed to reduce allergenic potential of food products. There is increasing number of publications describing successful attempts to reduce allergenicity of food allergens. A number of studies of food processing on allergen reactivity are largely focused on thermal treatments. Although thermal treatments can be used on many products to alter food allergen reactivity, thermal processing has also been known to modify organoleptic properties, including flavor, color, and nutrient content (Holdsworth, 1985). Therefore, novel non-thermal food processing techniques are being explored in order to create hypoallergenic products (Shriver & Yang, 2011). Variable success of methods for allergenicity reduction, due to limited knowledge of allergen and epitope structures and the factors governing their stability, imposes need for an empirical approach (Soler-Rivas & Wichers, 2001). Chemical or enzymatic modification of allergen molecule may alter its structure or physically obstruct binding of IgE antibodies to

conformational or linear epitopes. The objective of this review is to thoroughly explore chemical and enzymatic processing techniques utilized to modify food allergens, highlighting the efficacy of these methods in their ability to alter food allergen reactivity. As there are comprehensive reviews on food allergen treatment with digestive enzymes and the effects of fermentation on food reactivity, this review is focused on chemical and enzymatic modification of food allergen in the narrow sense.

2. Methods for assessing allergenicity of food products

The efficacy of chemical and enzymatic methods used to alter food allergen reactivity must be verified by analyzing the treated allergen's (or treated allergenic food) ability to trigger an immune response. As the reactivity of an allergen is often described by its ability to bind IgE antibodies, reduced IgE activity may indicate a modification or removal of food allergen(s). A variety of rapid assay methods are in use as analytical tools in research of molecular structure, integrity and biological activity of food allergens and their epitopes. Allergen reactivity can be determined by *in vitro*, *ex vivo* and *in vivo* testing. *In vitro* tests are often inexpensive, quick and without a threat to human or animal subjects. On the other hand *in vivo* assays provide a more accurate representation of the research. *Ex vivo* tests are advantageous because they are measuring allergenic response on effector cell level, using human subject blood without exposing them to risk. Animal models may also be used, though these models are not always analogous to the human. However, animal models may be a useful tool for predicting sensitizing potential of proteins introduced into diet by genetic manipulation or modified allergens which may carry higher allergenic risk.

In vivo tests. In vivo detection includes skin prick tests (SPT) and oral food challenges (OFC). SPT is simple and inexpensive, but the wheal sizes can vary by allergen and subject (Sampson, 1999), subjective results can differ between evaluators (Poulsen, 2001) and patients with atopic dermatitis may develop false-positive wheals (Fleischer et al., 2010). OFC is considered the "gold standard" in diagnosing food allergy as it can provide more accurate information regarding food allergy. However, these tests are complex, expensive and time consuming. As subject may experience severe adverse reactions patients who are susceptible to anaphylaxis should not be included in this type of study (Fleischer et al., 2010). However, final proof of improved safety of potentially less allergenic foods must be provided through human test studies, via double-blind placebo-controlled food challenge (DBPCFC)-tests.

Animal models. Rodent animal models are the most often exploited animal models of food allergy. Though the dosage of the allergen, route of exposure and duration of the build-in phase of allergy may differ depending on the allergen used and animal strain, many food allergy models have been described in the literature providing generation of IgE in the serum upon immunization and/or anaphylaxis reaction upon challenge with allergen. Animal models are useful in elucidation of mechanisms involved in reduction of allergenicity, especially on B cell and T cell level (Hattori et al., 2000; Kobayashi et al., 2001; Kobayashi et al., 2003).

Ex vivo tests. Ex vivo tests include histamine release or up regulation of surface molecules CD63 or CD203c on basophile granulocytes, known as the basophile activation test, BAT. By measuring specific IgE we only measure one interaction, between IgE and an allergen, whilst an allergic response requires two simultaneous interactions of allergen with IgE on the same effector cell. This is simulated in the *ex vivo* tests based on basophile granulocytes.

In vitro tests. *In vitro* studies for determining allergen reactivity include the measurement of serum IgE using radio-allergosorbent tests (RAST), enzyme-allergosorbent tests (EAST), enzyme linked immunosorbent assay (ELISA), ImmunoCAP assays (Phadia, Uppsala, Sweden); and immunoblotting. By RAST, EAST and ELISA (competitive inhibition and indirect) multiple samples can be tested at once. However, due to differences with solid phase and sample preparation among analysts standardization is the main problem. Furthermore, IgG antibodies can compete with IgE antibodies for similar epitopes. Although expensive, an ImmunoCAP tests have increased sensitivity compared to RAST, EAST and ELISA with minimized nonspecific binding by non-IgE binding antibodies. Immunoblotting includes Western blot and dot blot allergen analysis. In Western blotting proteins are most often tested in their linear conformation so that conformational epitopes may not be represented and new IgE binding epitopes, in native conformation hidden within the protein, may be uncovered. Immunoblotting is frequently used since protein bands can be individually analyzed to determine the changes in a specific allergen. In dot blot conformational epitopes may be preserved due to non-denaturing conditions, but in the case of protein mixture immunogenicity of the entire sample is analyzed.

Digestibility assays for food protein allergenicity assessment. The observation that many food allergens exhibit proteolytic stability led to allergy assessment strategy that use of digestion stability as a criterion for protein allergenicity assessment (Becker, 1997; Besler et al., 2001; Taylor & Lehrer, 1996). Although the relationship between the stability of proteins in simulated gastric fluid (SGF) and allergenicity has been inconsistent among studies, many recent reports proved increased incidence of food allergies in relation to impaired gastric digestion in human and animal studies. (Untersmayr & Jensen-Jarolim, 2008). Thomas et al. (Thomas et al., 2008) demonstrated that common protocol for evaluating the *in vitro* digestibility of proteins is reproducible and yields consistent results when performed using the same proteins at different laboratories. However, digestibility assays of pure proteins may not be always relevant in assessing allergenicity potential as they do not account for the effects of food matrices (Ofori-Anti et al., 2008). Therefore, *in vitro* gastrointestinal digestion protocols should be preferably combined with immunological assays in order to elucidate the role of large digestion-resistant fragments and the influence of the food matrix (Moreno, 2007). For a comprehensive review on digestibility of food allergens refer to Cirkovic Velickovic et al. (Cirkovic Velickovic et al., 2009). Anyway, comparison of unmodified and modified food allergen in digestibility studies can give valuable information about allergic potential of modified food protein and efficiency of modifying method used. As enzymatic and chemical methods change allergen structure digestibility of modified allergen can be increased or decreased thus directly influencing allergen availability to gut immune system.

3. Chemical modifications

The aim of chemical modifications of proteins used for nutritional purposes, in addition to reduction of allergenic properties, is obtaining of proteins with required techno-functional properties (solubility, emulsification, foaming, gelling, etc.) and preserved nutritional value. Chemical modifications of food proteins can lead to a change in the charge and hydrophobicity of proteins, which in turn can diminish or eliminate allergenicity of food allergens. However, regardless the application of non-toxic reagents, chemical modifications are not so often applied in the food industry regarding complex procedures of removing remaining chemical agents.

3.1 Covalent modifications

Acylation. Acylation of allergens by treatment with anhydrides, such as acetic or succinic, blocks positively charged amino groups on the protein molecule and the remaining free carboxyl groups of aspartic and glutamic acid residues make the net charge of the modified protein more negative. Szymkiewicz & Jędrychowski (Szymkiewicz & Jedrychowski, 2009) modified pea proteins with acetic or succinic anhydride. Immunoreactivity of albumins and legumin, as estimated by ELISA with rabbit polyclonal antibodies, was reduced by 91-99% and 78-97% after succinylation and acetylation, respectively, while immunoreactivity of vicilin fraction was reduced down to 12% and 17%, respectively. In their other study (Szymkiewicz & Jędrychowski, 2008) the authors combined enzymatic hydrolysis by Alcalase with chemical modification by acylation of pea proteins. The enzyme hydrolysis of acylation-modified pea proteins caused significant reduction in the immunoreactivity of pea proteins, especially vicilin, whose epitopes were the most resistant to the modifications, both chemical (Szymkiewicz & Jedrychowski, 2009) and enzymatic one (Szymkiewicz & Jędrychowski, 2005), applied separately. The application of Alcalase lowered the immunoreactivity of vicilin to 2-2.5%, while immunoreactivity of legumin and albumin decreased by nearly 100%. Thus, combining various methods of protein modification is more efficient and can be a promising approach for preparing products with reduced allergenicity. However, in ELISA tests with individual sera of patients allergic to leguminous seeds, IgE binding of pea proteins were reduced only by 40-75%, indicating that considerable decrease in the antigenicity of proteins not always guarantees their lower allergenicity and also shows differences in individual patients.

Carbamoylation. Mistrello et al. (Mistrello et al., 1996) chemically modified ovalbumin by reaction with potassium cyanate (KCNO), which transforms the ϵ -amino group of the lysine of proteinaceous allergens into ureido groups. KCNO-modified (carbamylated) allergens have low allergenic potency, as demonstrated *in vitro* (RAST inhibition) and *in vivo* (passive cutaneous anaphylaxis). When used to immunize rabbits, carbamylated allergens still induce IgG antibodies able to cross-react with native allergens (immunoblotting experiments). Although potentially useful for immunotherapy formulations preparation this method is not suitable for hypoallergenic food preparation.

Nitration. In study of Untersmayr et al. (Untersmayr et al., 2010) BALB/c mice were immunized intragastrically by feeding untreated ovalbumin (OA) and nitrated ovalbumin (nOA) with or without acid-suppression. While oral immunizations of nOA under anti-acid treatment did not result in IgG and IgE formation, intraperitoneal immunization induced high levels of OA specific IgE, which were significantly increased in the group that received nOA by injection. Furthermore, nOA triggered significantly enhanced mediator release of effector cells of sensitized allergic animals. In gastric digestion experiments nOA was degraded within few minutes, whereas OA remained stable up to 120 min. Additionally, one tyrosine residue being very efficiently nitrated is part of an ovalbumin epitope recognized exclusively after oral sensitization. These data indicated that despite the enhanced triggering capacity in existing allergy, nitration of OVA may be associated with a reduced *de novo* sensitizing capability via the oral route due to enhanced protein digestibility and/or changes in antibody epitopes. Although the authors considered effects of endogenously nitrated allergen, these results imply that nitration of food allergen as method have no potential for reducing its allergenic potential.

Conjugation with synthetic copolymer. Conjugates of ovalbumin (OA) and the copolymer of N-vinyl pyrrolidone and maleic anhydride (VMA) modified with epsilon-aminocaproic

acid (Acp) were prepared by Babakhin et al. (Babakhin et al., 1995). Of all conjugates injected intraperitoneally into mice only the conjugate containing 20%OA (OA-Acp-VMA) did induce anti-OA IgG antibodies without significant quantities of anti-OA IgE. In passive cutaneous anaphylaxis, RAST inhibition and leukocyte histamine release, OA-Acp-VMA have shown significant reduction of allergenicity and stimulated activation of the OA-specific T-cell comparable to that of unconjugated OA. During experimental allergen-specific hyposensitization with OA-Acp-VMA, suppression of anti-OA IgE response and elevation of anti-OA IgG responses were noted. By using the carrier Acp-VMA to reduce allergenicity there is selective blockage of B-cell epitopes of allergen without affecting T-cell epitopes, thereby preserving immunogenicity, which enable creation of preparations for allergen-specific immunotherapy.

Polymerization by glutaraldehyde. By treatment with glutaraldehyde aldehyde groups of glutaraldehyde react with the amino groups of protein resulting in cross-linked allergen proteins with altered immunological characteristics (Patterson et al., 1979). Recently the ability of glutaraldehyde-treated allergens to stimulate T-cells has been disputed (Wurtzen et al., 2007). XiYang et al. (Yang et al., 1993) demonstrated that whereas *in vivo* administration of ovalbumin (OA) induces cytokine synthesis that is neither Th1 nor Th2 dominated, administration of glutaraldehyde polymerized, high relative molecular weight OA (OA-POL) leads to 20-fold increase in the ratio of interferon γ (IFN- γ)/IL-4 and IFN- γ /IL-10 synthesis observed after short-term, antigen-mediated restimulation directly *ex vivo*. In contrast, concurrent *in vivo* administration of anti-IFN- γ /mAb and OA or OA-POL results in marked increases in IL-4 and IL-10, and decreased IFN- γ production, reflecting a polarization of the response towards a Th2-like pattern of cytokine synthesis. This is approach that allows selective activation of strongly Th1-dominated immune responses to protein antigens and it may be useful in clinical settings where the ability to actively select specific patterns of cytokine gene expression would be advantageous. Koppelman et al. (Koppelman et al., 2010) demonstrated that modification of peanut conglutin with glutaraldehyde (GA) does not result nor in a change of secondary structure nether in substantial decrease of IgE binding (only 2-3 fold). Reduction/alkylation treatment (RA) change secondary structures, whereas RA treatment followed by GA modification (RA-GA) results in a tertiary structure that differs from that of conglutin treated only with RA, due to modification of Cys and Lys residues. As demonstrated by IgE-ELISA and IgE blot treatment with RA-GA decreases IgE-binding up to a hundred fold and also induce a strong T cell response in T cell proliferation tests. These data demonstrate that all three modifications lead to a reduction in IgE binding, with the strongest reduction observed after both reduction/alkylation and glutaraldehyde treatment.

3.1.1 Covalent hydrophilisation - conjugation with polysaccharides

The shielding of epitopes by materials having low-antigenicity and immunogenicity may be efficient way of reducing allergenicity of the protein, especially with the use of a high molecular weight modifier to achieve effective shielding of epitopes.

Carboxymethyl dextran. Hattori et al. (Hattori et al., 2000) prepared BLG-carboxymethyl dextran conjugates (BLG-CMD), by using a water-soluble carbodiimide. The anti-BLG antibody response was markedly reduced after immunization with the BLG-CMD conjugates in mice. Linear epitope profiles of the BLG-CMD conjugates were similar to those of BLG, while the antibody response for each epitope was dramatically reduced. Reduction of immunogenicity of BLG depend on CMD content indicating that masking of epitopes by

CMD is responsible for the decreased immunogenicity of the BLG conjugates due to effective shielding by CMD. Similar results were obtained by Kobayashi et al. (Kobayashi et al., 2001) who prepared BLG-CMD with different molar ratios. Results of both studies show that conjugation with CMD of higher molecular weight is effective in reducing the immunogenicity of BLG by masking of B cell epitopes by CMD. In their further study Kobayashi et al. (Kobayashi et al., 2003), investigated changes in the T cell response to BLG after conjugation with CMDs. When lymph node cells from mice immunized with BLG or the conjugates were stimulated with BLG, T cells from the conjugate-immunized mice showed a lower proliferative response comparing to BLG-immunized mice (Fig.1). T cell epitope profiles of the conjugates were similar to those of BLG, whereas the proliferative response to each epitope was reduced, indicating that the lower *in vivo* T cell response with the conjugates was not due to induction of conjugate-specific T cells, but due to a decrease in the number of BLG-specific T cells. In addition, conjugation with CMD enhanced the resistance of BLG to cathepsin B and cathepsin D, suggesting that conjugation with CMD inhibited the degradation of BLG by proteases in antigen presenting cells (APC) and led to suppression of the generation of antigenic peptides including T cell epitopes from BLG. Therefore, the authors considered that the suppressive effect on the generation of T cell epitopes reduced the antigen presentation of the conjugates and this reduction led to a decrease in the number of BLG-specific T cells *in vivo*. As a result, the decreased help to B cells by T cells would have reduced the antibody response to BLG leading to conclusion that suppression of the generation of T cell epitopes by conjugation with CMD is important to the mechanism for the reduced immunogenicity of BLG.

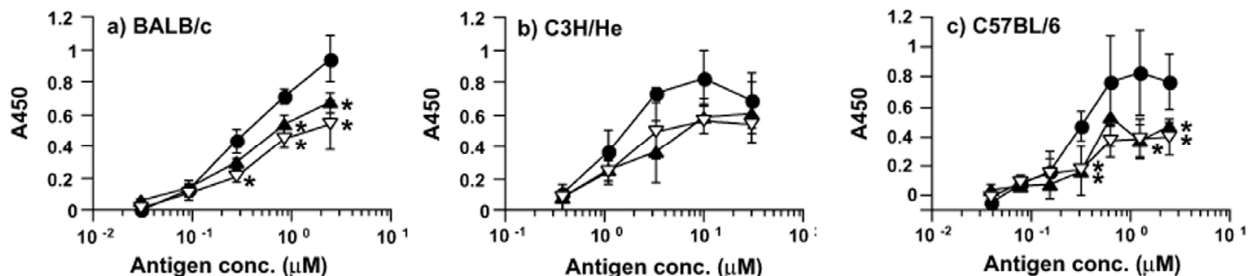


Fig. 1. Proliferative response to BLG of lymph node cells from mice immunized with BLG (circles) or the BLG-CMD conjugates (triangles) and stimulated with BLG at various concentrations. The magnitude of the *in vivo* T cell response was evaluated as the *ex vivo* proliferative response by BrdU ELISA. Each value is expressed as the mean absorbance at 450 nm and standard deviation of triplicate cultures after subtracting the background values (stimulated with PBS). Significant differences ($p < 0.05$) between BLG and each conjugate were determined by Student's t-test and are indicated by asterisks. Reprinted with permission from (Kobayashi et al., 2003). Copyright (2003) American Chemical Society.

Acidic polysaccharides. Hattori et al. (Hattori et al., 2004) conjugated BLG with the acidic oligosaccharides, alginic acid oligosaccharide (ALGO) and phosphoryl oligosaccharides (Pos), by the Maillard reaction. Fluorescence studies indicated surface of each conjugate was covered with a saccharide chain. The anti-BLG antibody response was markedly reduced after immunization with both conjugates in mice. Linear epitope profiles of the conjugates were found to be similar to those of BLG, whereas the antibody response to each epitope

was dramatically reduced. In particular, effective reduction of the antibody response was observed in the vicinity of the carbohydrate-binding sites. Obtained conjugates are edible, have higher thermal stability and improved emulsifying properties than those of native BLG, thus being very useful for food application. Yoshida et al. (Yoshida et al., 2005) demonstrate that the T cell response was reduced when mice were immunized with BLG-ALGO conjugates and that novel epitopes were not generated by conjugation. The authors clarified that the BLG-ALGO conjugate modulated the immune response to Th1 dominance and considered that this property of the BLG-ALGO conjugate would be effective for preventing food allergy as well as by its reduced immunogenicity. Therefore, conjugation with acidic oligosaccharides could be applied to various food allergens to achieve reduced allergenicity with multiple improvements in their properties.

Galactomannan. Babiker et al. (Babiker et al., 1998) prepared soy protein-galactomannan conjugate by the Maillard reaction. Conjugation removed the allergenicity of the 34 kDa protein which is frequently recognized by the IgE antibody in the sera of soybean-sensitive patients as a major allergen. Monitoring of polyclonal antibody titers by an indirect enzyme-linked immunosorbent assay and immunoblotting of rabbit sera, monoclonal antibody, and human allergic sera showed that soy protein-galactomannan conjugation was more effective in reducing the allergenicity of the soy protein than transglutaminase treatments and/or chymotrypsin. Additionally, heat stability, solubility and emulsifying properties were greatly improved by conjugation with galactomannan.

Chitosan. Aoki et al. (Aoki et al., 2006) conjugated BLG with chitosan (CHS) by means of a water-soluble carbodiimide to reduce its immunogenicity. The antigenicity of the BLG-CHS conjugates was similar to that of BLG in C3H/He mice, while immunogenicity of BLG was reduced by conjugation. The linear epitope profiles of the conjugates were found to be similar to those of BLG, while the antibody response to each epitope was dramatically reduced. The researchers suggested masking of B cell epitopes as one of the mechanisms in reduction of immunogenicity.

Dextran-glycylglycine and amylose-glycylglycine. Nodake et al. (Nodake et al., 2010) conjugated BLG with the N-hydroxysuccinimide ester of the dextran-glycylglycine adduct (DG-ONSu) to reduce the immunogenicity of BLG. Conjugation with DG-ONSu greatly decreased the reactivity of BLG with anti-BLG antibodies and suppressed their production *in vivo* due to its shielding action for epitope(s) on the protein's molecular surface. Beside, DG-BLG was resistant to proteolytic enzymes. In the other study (Nodake et al., 2011) these authors demonstrated that conjugation BLG with the N-hydroxysuccinimide ester of the amylose-glycylglycine adduct (AG-ONSu) also greatly decreased the reactivity of BLG. The authors proposed usage of DG-ONSu and AG-ONSu to suppress the hypersensitivity mediated by IgE antibodies in milk allergy.

3.1.2 Covalent lipophilization

Stearic acid. In the study of Akita and Nakai (Akita & Nakai, 1990) BLG was chemically modified by covalent attachment of different levels of stearic acid. A decreased *in vitro* digestibility was observed with extent of stearic acid incorporation (lipophilization). At low lipophilization increased ability to elicit IgE antibodies, determined by heterologous passive cutaneous anaphylaxis, was observed. Medium level of lipophilization decreased this ability, while high lipophilization almost destroyed the ability to elicit IgE antibodies. The researchers also found that low- and medium-lipophilization increased while high lipophilization decreased the IgG binding ability, measured by ELISA.

3.2 Noncovalent modifications

Components of food matrix can interact noncovalently with food allergens resulting in insoluble complexes thus lowering the level of soluble allergens and reducing their allergenic properties. Also they can reduce digestibility of food allergens and consequently their allergenicity by hindering access of digestive enzymes. Phenolic compounds and phytic acid are known to form soluble and insoluble complexes with proteins. For instance, it was shown that multivalent hydrophobic interactions cause compaction of cow's milk caseins with the polyphenol epigallocatechin in way that individual casein molecules “wrap around” polyphenol (Jobstl et al., 2006).

Phytic acid. Chung et al. (Chung & Champagne, 2007) treated peanut extract with phytic acid and demonstrated that phytic acid formed complexes with the major peanut allergens (Ara h 1 and Ara h 2), reducing their solubility in acidic and neutral conditions. Also, 6-fold reduction in IgE binding of the extract was observed after treatment with phytic acid, observed by competitive inhibition ELISA using a pooled serum from peanut-allergic individuals. Similar result obtained with peanut butter slurry led to the suggestion that phytic acid treatment might reduce the allergenicity of peanut-based products due to reducing their solubility (Fig 2). In another study by the same group of authors, a facilitated IgE binding in vitro was observed with peanut allergens and phytic acid. Apparently, phytic acid was able to stabilize allergen-antibody interactions (Chung & Champagne, 2006) However, usage of phytic acid might be limited considering its anti-nutritive properties due to ability to chelate iron.

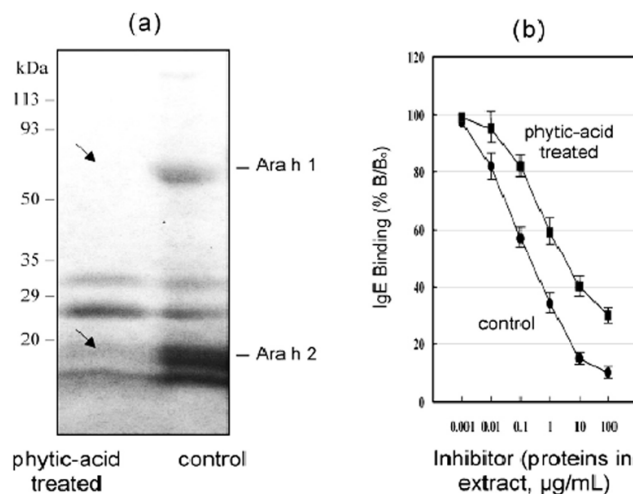


Fig. 2. a) SDS-PAGE and b) competitive inhibition ELISA of natural peanut butter slurry treated and centrifugated with and without phytic acid. Values are means \pm SD (n=3). Values of the treated samples at 0.01–100 $\mu\text{g}/\text{mL}$ are significantly different from those of the control ($P < 0.05$, n=3). Reprinted with permission from (Chung & Champagne, 2007). Copyright (2003) American Chemical Society.

Phenolic compounds: Adding of phenolics such as caffeic, chlorogenic and ferulic acids to peanut extracts, liquid peanut butter and peanut butter slurries, precipitated most of the major peanut allergens, Ara h 1 and Ara h 2, and complexation was irreversible (Chung & Champagne, 2008; Chung & Champagne, 2009). Of the three phenolics, caffeic acid formed the most precipitates with peanut extracts. IgE binding was reduced approximately 10- to 16-fold as determined by inhibition ELISA (Fig. 3). Assuming that the insoluble complexes are not absorbed by the body the researchers concluded that reducing IgE binding by

phenolics is feasible and have a great potential in development of less allergenic liquid peanut-based products.

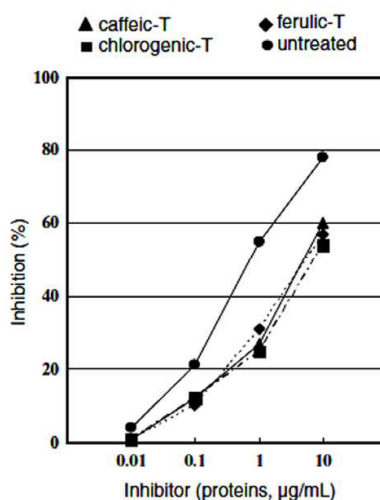


Fig. 3. IgE binding of phenolic-treated and untreated peanut extracts in competitive inhibition ELISA using pooled human plasma from peanut-allergic individuals. Values (on semi-log scale) are mean of three determinations. Values between treatments (caffeic, chlorogenic, and ferulic) are not significantly different from each other but from the untreated ($P < 0.05$). Reprinted from (Chung & Champagne, 2009), Copyright (2009), with permission from Elsevier.

Polysaccharides. Mouecoucou et al. (Mouecoucou et al., 2004) examined the influence of polysaccharides, i.e., gum arabic, low methylated pectin (LMP) and xylan, on the *in vitro* hydrolysis of peanut protein isolate (PPI) and the *in vitro* allergenicity of the digestion products. PPI was hydrolyzed *in vitro* by pepsin, followed by a trypsin/ chymotrypsin (T/C) mixture in dialysis bags. Hydrolysis by all of the digestive enzymes showed retention of some proteins in the dialysis bags in the presence of gum arabic and xylan. The retentates were recognized by IgG and IgE, but IgE binding of retentate containing xylan was reduced. The immunoreactivity of hydrolysis products in dialysates was considerably reduced by polysaccharides. Polovic et al. (Polovic et al., 2007) demonstrated that addition of apple fruit pectin (1.5% and 3%) to the purified kiwi allergen Act c 2 was able to protect it from pepsin digestion *in vitro*. *In vivo* experiments on healthy non-atopic volunteers have shown that 1 h after ingestion of kiwi fruit in gastric content intact Act c 2 was still present. In their further work Polovic et al. (Polovic et al., 2010) discovered that after *in vivo* digestion of Act c 2 with pure pectin in rats both gastric acidity, as well as specific and total pepsin activity declined and thus protected 23% of the ingested allergen from digestion for 90 minutes. These results show that although presence of polysaccharides can be effective in masking of IgE epitopes, thus reducing allergenicity, it also reduces allergen digestibility, enabling higher dosages of the allergen to reach the immune system.

Oxidized lipids. In study of Doke et al. (Doke et al., 1989) though oxidized soybean oil did not show any allergenicity, the IgE titer of sera from soybean-sensitive patients (in ELISA) was greatly increased when oxidized soybean oil was incubated with soybean 2S-globulin. The IgE titer became higher when greater amounts of oxidized soybean oil were used, but little difference was noted when soybean 2S-globulin was replaced by other food proteins. A

similar tendency was noted when soybean oil was replaced by other vegetable oils or fatty acids. The authors speculated that proteins interacted with oxidized lipid are allergenic to soybean-sensitive patients probably due to creation of new epitopes.

3.3 Modifications by oxidation/reduction

Oxidation. Heavy metal ions (Cu^{2+} , Fe^{2+} , etc.) with hydrogen peroxide can cross-link proteins through oxidation of their tyrosine residues and forming dityrosine and isodityrosine, as well as by oxidation of sulfhydryles resulting in disulfides. In study of Chung et al. (Chung, 2005) extracts from raw and roasted peanuts were treated with $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ and only roasted peanuts were affected by $\text{Cu}^{2+}/\text{H}_2\text{O}_2$. In this case cross-links were formed and levels of Ara h 1 and Ara h 2 were reduced as shown in competitive inhibition ELISA with pooled serum of peanut-allergic individuals. IgE binding, overall, was lower despite some binding of IgE to cross-links leading to conclusion that $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ reduced peanut allergenicity by inducing the decrease and cross-linking of peanut allergens.

Reduction. The proteins are allergenically active and less digestible in the oxidized (S-S) state. When reduced (SH state), they lose their allergenicity and/or become more digestible. Allergen reduction can be performed by using a reducing agent such as 2-mercaptoethanol, dithiothreitol, cysteine, glutathione, etc., or by using proteins glutaredoxin or thioredoxin. In study of Buchanan et al. (Buchanan et al., 1997) thioredoxin mitigated the allergenicity of whey flour proteins, gliadins and glutenins, as determined by skin tests with a canine model for food allergy, but gave less consistent results with albumins and globulins. In the study of de Val et al. (del Val et al., 1999) after reduction of one or both of its disulfide bonds by thioredoxin, BLG became strikingly sensitive to pepsin in simulated gastric fluid and lost its allergenicity as determined by skin test responses and gastrointestinal symptoms in inbred colony of high IgE-producing dogs sensitized to milk. Koppelman et al. (Koppelman et al., 2007) modified 2S albumins by reduction by thioredoxin, resulting in breakage of disulfide bonds, followed by alkylation for prevention of reformation of disulfide bonds. Oral administration of native 2S albumin resulted in the development of Th-1 mediated IgG1- and Th-2 mediated IgG2a and IgE responses in the rat, as determined by ELISA. Oral exposure to RA-2S albumin did not result in the development of specific IgE against RA-2S, but IgG1 and IgG2a antibodies against RA-2S albumin were formed in a lower level compared to native 2S albumin. Dosing of the animals with the low dose RA-2S albumin (0.1 mg protein/rat/day) did not result in an antibody response at all in the rats whereas the same dose of native 2S albumin induced specific IgG1, IgG2a and IgE responses, again indicating a lesser immunogenicity. Taken together, these data show that reduction of the disulphide bonds of 2S albumin results in loss of allergenicity and an increased sensitivity to digestion. All these results provide evidence that thioredoxin can be applied to enhance digestibility and lower allergenicity of food proteins. However, thioredoxins represent a novel family of cross-reactive allergens involved in the pathogenesis of atopic eczema and asthma. Also, cross-reactivity to human thioredoxin can contribute to the exacerbation of severe atopic diseases by involvement of IgE-mediated autoreactivity (Glaser et al., 2008). Considering these facts usage of thioredoxin in food allergen modification might be limited.

4. Enzymatic modifications

Beside proteolytic enzymes, enzymes able to cross-link proteins have shown to be promising tools for reduction of allergenicity of food proteins. Enzymatic cross-linking of proteins, by

transglutaminases, peroxisases and phenol oxidases (such as tyrosinases and laccases), is currently exploited in the food processing industry (Buchert et al., 2007).

4.1 Transglutaminases

Transglutaminases (TG) catalyze formation of a covalent bond between a primary amines (including ϵ -amino group of lysine residues) and the γ -carboxamid group of protein-bound glutamine leading to protein cross-linking.

Villas-Boas et al. (Villas-Boas et al., 2010) polymerized heat treated BLG and TG (BLG-TG) and untreated BLG in the presence of cysteine and TG (BLG-Cys-TG). BALB/c mice sensitized with BLG-Cys-TG showed lower levels of IgG1 and IgE than those immunized with native BLG or BLG-TG, suggesting that polymerization in the presence of Cys modified or hid epitopes, reducing the potential antigenicity of BLG. Clare et al. (Clare et al., 2007) cross-linked peanut flour dispersions with transglutaminase in the presence and absence of the dithiothreitol. Transglutaminase treatment did not diminish IgE binding responses in ELISA implying only that transglutaminase cross-linking do not enhance potential for allergic responses. In their further study Clare et al. (Clare et al., 2008) cross-linked light roasted peanut flour (PF) with transglutaminase with casein (CN) as co-substrate. In immunoblotting, in some patient sera IgE binding to TGase-treated PF-CN fractions appeared less compared to equivalent polymeric PF dispersions lacking supplemental CN and non-cross-linked PF-CN samples. The researchers assumed that covalent modification masked IgE peanut protein binding epitopes, at least to some degree, on an individual patient basis.

In study of Wroblewska et al. (Wroblewska et al., 2008) whey protein concentrate (WPC) was modified by two enzymes: proteinase Alcalase and transglutaminase. The new products were characterized by 2D electrophoresis, immunoblotting, and ELISA methods. The WPC hydrolysate obtained with Alcalase contained proteins and peptides showing strong immunoreactive properties, as revealed by immunoblotting with α -la and β -lg polyclonal rabbit antibodies. However, the immunoblot analysis demonstrated that WPC showed a stronger reactivity towards IgE of allergic patients then WPC treated with transglutaminase. ELISA assay with human sera showed that two-step modification with Alcalase followed by TG significantly reduced the immunoreactive properties of whey proteins. Patients with wheat-dependent, exercise-induced anaphylaxis (WDEIA) experience recurrent anaphylactic reactions when exercising after ingestion of wheat products.

Palosuo et al. (Palosuo et al., 2003) digested purified ω -5 gliadin, major allergen in WDEIA, with pepsin or with pepsin/trypsin and treated with tissue transglutaminase (tTG). The IgE-binding ability of ω -5 gliadin was retained after pepsin and pepsin-trypsin digestion, as shown in IgE ELISA test. tTG treatment of the whole peptic digest resulted in cross-linked aggregates which bound IgE antibodies in immunoblotting more intensely than untreated, pepsin-digested, or pepsin-trypsin-digested ω -5 gliadin. In the 20 WDEIA patients the mean skin prick test wheal elicited by tTG treated peptic fraction was 77% larger than that elicited by the untreated peptic fraction and 56% larger than that elicited by intact ω -5 gliadin. These results suggest that activation of tTG during exercise in the intestinal mucosa of patients with WDEIA could lead to the formation of large allergen complexes capable of eliciting anaphylactic reactions. Leszczynska et al. (Leszczynska et al., 2006) modified wheat flour by the treatment with transglutaminase and demonstrated, in indirect non-competitive ELISA with human sera, reduction of glutenin immunoreactivity to below 30%.

Watanabe et al. (Watanabe, 2004) have made hypoallergenic flour by flour treatment with actinase, collagenase, and transglutaminase and that TG is the least effective of tested enzymes in reducing flour allergenicity. Soy protein-galactomannan conjugation was more effective in reducing the allergenicity of the soy protein than transglutaminase treatments (Babiker et al., 1998) (Fig. 4). In the study of Monogioudi et al. (Monogioudi et al., 2011) β -casein was cross-linked by transglutaminase and demonstrated that enzymatically cross-linked β -casein was stable under acidic conditions and was more resistant to pepsin digestion when compared to non cross-linked β -casein. In the study of Stanic et al. (Stanic et al., 2010) TG-treated casein showed no mitigated IgE binding reactivity compared with the untreated CN in BAT.

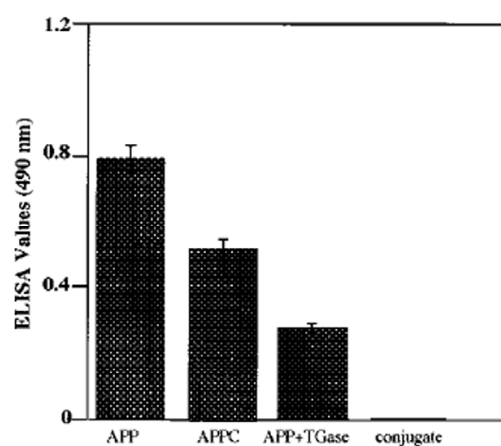


Fig. 4. Determination of antibody titers by indirect ELISA of acid precipitated soy protein (APP), chymotrypsin digest (APPC), transglutaminase treated APP and galactomannan conjugate dry heated for 7 days. Error bars indicate the standard deviations (n=6). Reprinted with permission from (Babiker et al., 1998). Copyright (1998) American Chemical Society.

As a microbial TG is included in many food technological processes, safety of the TG itself, as well as safety of the deamidated/cross-linked proteins generated by this enzyme should be checked (Malandain, 2005). In their study, Pedersen et al. (Pedersen et al., 2004) investigated the allergenicity of TG from *Streptoverticillium mobaraensis* by evaluation of amino acid sequence similarity to known allergens, pepsin resistance, and detection of protein binding to specific serum immunoglobulin E (IgE) (RAST) evaluated as recommended by 2001 FAO/WHO Decision Tree, recommended for evaluation of proteins from genetically modified organisms (GMOs). All tests demonstrated that there is no safety concerns with regard to the allergenic potential of tested TG.

4.2 Peroxidases

Peroxidases (POD) are a heme-containing enzymes catalyzing the oxidation of a variety of organic compounds by hydrogen peroxide or hydroperoxides. Acting on phenolic compounds POD generate o-quinones, which further react with other phenolics, amino, or sulfhydryl compounds in proteins to form cross-linked products.

In their study Chung et al. (Chung et al., 2004) have treated protein extracts from raw and roasted defatted peanut meals with POD in the presence of hydrogen. While POD treatment had no effect on raw peanuts, a significant cross-linking and decrease in the levels of the

major allergens, Ara h 1 and Ara h 2, in roasted peanuts (Fig.7) was observed in immunoblots and IgE ELISA. The authors suppose that POD induced the cross-linking of mainly Ara h 1 and Ara h 2 from roasted peanuts and that, due to POD treatment, IgE binding was reduced.

Garcia et al. (Garcia et al., 2007) investigated effect of peroxidase and antioxidant diethyldithiocarbamic acid (DIECA) on IgE-binding by Mal d 1, the major apple allergen. In competitive ELISA IgE-binding by Mal d 1 decreased by adding peroxidase with more pronounced effect in presence of catechin. DIECA protected the IgE-binding by the allergen, protection being less strong in the presence of exogenous peroxidase (Fig 5).

Weangsripanaval et al. (Weangsripanaval et al., 2003) purified and characterized new allergenic protein from the tomato identified as suberization-associated anionic peroxidase 1. Furthermore, SanchezMonge et al. (SanchezMonge et al., 1997) purified and characterized allergenic protein from wheat flour identified as seed-specific peroxidase. These facts imply that peroxidases themselves can be allergens and safety for use them must be assessed.

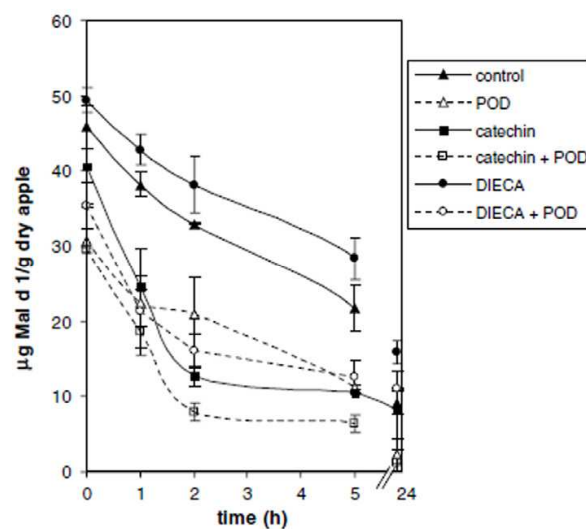


Fig. 5. Influence of the treatments performed on apple peel samples on Mal d 1 concentration determined by competitive ELISA using a pool of sera. The treatments consisted of: additional peroxidase (POD), additional substrate catechin, additional catechin combined with POD, additional diethyldithiocarbamic acid (DIECA) and additional DIECA combined with POD. The incubation times were 0, 1, 2, 5, and 24 h at room temperature. Reprinted from (Garcia et al., 2007), Copyright (2007), with permission from Elsevier.

4.3 Phenol oxidases

4.3.1 Polyphenoloxidases (tyrosinases)

Polyphenol oxidases (PPO) or tyrosinases are bifunctional enzymes catalyzing o-hydroxylation of monophenols (including protein-bound tyrosine residues) to o-diphenols and subsequent oxidation of o-diphenols to o-quinones (Lerch, 1983). Reactive o-quinones can further undergo non-enzymatic polymerization or can react with amino acid residues in proteins.

In the study of Gruber et al. (Gruber et al., 2004) incubation of recombinant Pru av 1, major cherry allergen, with phenol compounds in the presence of tyrosinase led to decrease in IgE-binding activity of the protein as revealed by EAST and inhibition assays. Caffeic acid and

epicatechin showed to be the most efficient in decreasing of rPru av 1 IgE-binding activity, followed by catechin and gallic acid, while quercetin and rutin were the least efficient. However, PPO without the addition of a phenolic compound did not display a reduction in IgE binding. The researchers speculated that, reactive intermediates formed during enzymatic polyphenol oxidation are responsible for modifying nucleophilic amino acid side chains of proteins, thus inducing an irreversible change in the tertiary structure of the protein and resulting in a loss of conformational epitopes of the allergen. In study of Chung et al. (Chung et al., 2005) peanut extracts were treated with and without PPO, PPO/caffeic and caffeic acid. All treatments resulted in cross-linking and decreased the levels and IgE binding (competitive inhibition ELISA) of two peanut major allergens, Ara h 1 and Ara h 2, with PPO/caffeic as the most effective (Fig.6).

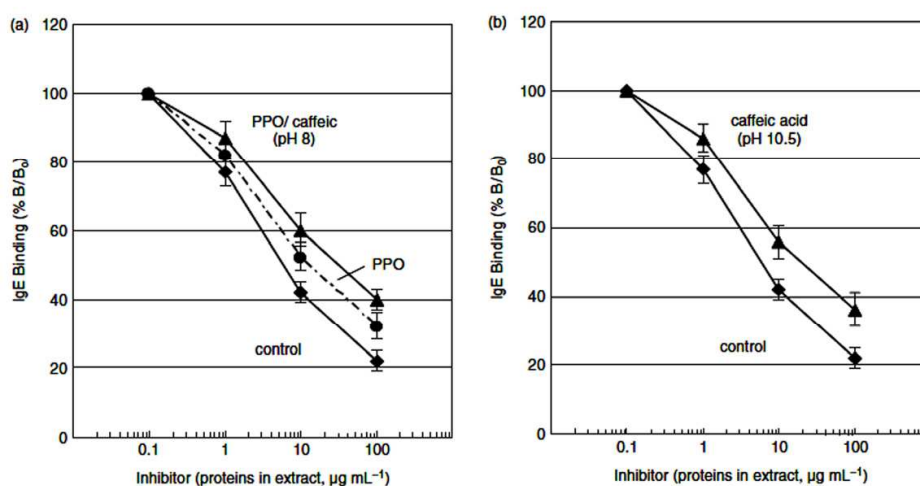


Fig. 6. Inhibition of IgE antibodies in a competitive inhibition ELISA by (a) PPO- and PPO/caffeic-treated extracts, pH 8, and (b) caffeic-treated extract, pH 10.5. Extracts, diluted at the concentration indicated, were each mixed with a pooled serum from peanut allergic individuals, and then added to an allergen-coated microtiter plate. Values are means \pm SD ($n = 3$). Values of the treated samples at 1–100 $\mu\text{g mL}^{-1}$ are significantly different from those of the control ($P < 0.05$, $n = 3$). Reprinted from (Chung et al., 2005), Copyright (2005), with permission from John Wiley and Sons.

In their further study Chung et al. (Chung & Champagne, 2008) treated peanut butter slurries with phenolic compounds/PPO and obtained reduction in IgE-binding, despite the formation of soluble allergen complexes or cross-links, for which authors assume that are less allergenic. Novotna et al. (Novotna et al., 2011) investigated effects of celery juice by oxidation by utilising its natural polyphenol oxidase (PPO) content on the reduction of the content of the Api g1, the main celery allergen. Oxidation failed to eliminate the allergenicity of pure celery juice, but oxidation in apple-celery juices reduced the allergenicity of the mixture. However, the basophil activation test showed no reduction in the allergic response to the oxidised juice mixture. Skin testing showed that the prolonged oxidation of juice mixture showed significantly lower reaction, while apple juice stabilised with ascorbic acid did not have effect. Due to the contradictory results in different tests, the method cannot be declared successful or safe, even for mixtures of apple-celery juices. In competitive ELISA Garcia et al. (Garcia et al., 2007) demonstrated decreased IgE binding of Mal d 1 after enrichment of apple extract with PPO, with the strongest effect in presence of

catechin. Antioxidant DIECA protected the IgE-binding by the allergen, protection being less strong in the presence of exogenous PPO. Schmitz et al. (Schmitz-Eiberger & Matthes, 2011) evaluated relationship between Mal d 1, the main apple allergen, content and PPO, total phenol content and antioxidative capacity in different apple varieties. Whereas higher PPO activities and polyphenols contents result in less extractable Mal d 1, higher antioxidative activity can inhibit the interaction between oxidised phenols and Mal d 1, resulting in higher allergenicity (extractable Mal d 1). In the study of Monogioudi et al. (Monogioudi et al., 2011) β -casein was cross-linked by tyrosinase and demonstrated that enzymatically cross-linked β -casein was stable under acidic conditions and was more resistant to pepsin digestion when compared to non cross-linked β -casein. In the study of Stanic et al. (Stanic et al., 2010) tyrosinase treated casein showed mitigated IgE binding reactivity, compared with the untreated CN, only in the presence of caffeic acid as mediator in BAT.

4.3.2 Laccases

Laccases catalyse oxidation of various phenolic compounds with one electron mechanism generating free radical species. Reactive free radicals can further undergo non-enzymatic polymerization or can react with high redox potential substrate targets, such as amino acid residues in proteins (Canfora et al., 2008).

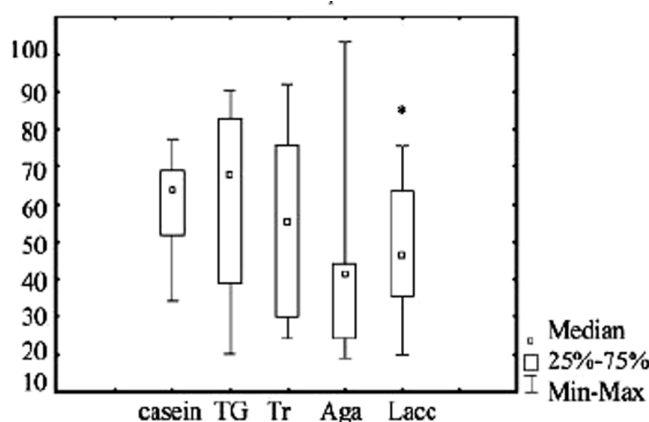


Fig. 7. Basophil activation to casein and crosslinked caseins in seven patients with CMA. $p < 0.05$ was statistically significant difference in a Wilcoxon-matched pairs test. Reprinted from (Stanic et al., 2009), Copyright (2009), with permission from John Wiley and Sons.

Tantoush et al. (Tantoush et al., 2011) cross-linked BLG by laccase in the presence of an sour cherry phenolics. In a BAT assay, the allergenicity of the cross-linked protein was shown to decrease in all nine cow's milk-allergic patients, while digestibility of the remaining monomeric BLG in simulated conditions of the gastrointestinal tract increased. In the further study of Tantoush et al. (Tantoush et al., 2011) cross-linking BLG by laccase in the presence of apple phenolics (APE) rendered the protein insoluble in the reaction mixture consisting of cross-linked BLG, with a fraction of the BLG remaining monomeric. Enzymatic processing of BLG decrease the bi-phasal pepsin-pancreatin digestibility of the monomeric and cross-linked protein, thus decreasing its nutritional value. Stanic et al. (Stanic et al., 2010) crosslinked β -casein (CN) by laccase and caffeic acid and demonstrated that crosslinking was not very efficient, leaving mostly monomeric CN modified by caffeic acid. Regardless to that ability of crosslinked CNs to activate basophils was significantly reduced in seven

patients and reduced inhibition potential is possibly due to hindering of epitopes by monomer modification. Also, digestion of crosslinked CN by pepsin was hampered.

As enzyme preparations used in food technology are food grade, but often not of the highest purity, they can contain contaminating enzyme activities. These so-called “side activities” even if present only in trace quantities, can have unpredictable influence on functional properties, nutritional quality and safety of food implying that effects of contaminating enzymes in used enzyme preparations should be carefully monitored. Stanic et al. (Stanic et al., 2009) found out that in the presence of high purity commercial laccase and tyrosinase preparations, both variants of BLG (A and B) underwent removal of a peptide from the N-terminus. The truncated forms were more susceptible to digestion by pepsin and thus with lower allergenic potential.

5. Conclusion

In development of new methods for food allergen modification there are two main applications. First one, and the most frequent, is creation of hypoallergenic food and food additives, and the second one is preparation of formulations for safe immunotherapy of food allergies. In addition to the main aim, reduction of allergenicity, there are several other requirements that must be satisfied for certain application. For both applications safety is obligatory, implicating usage of minimal toxic and nonallergenic agents, including the main and auxiliary one. Preferred reagents used for allergen modification are those acceptable for the production of foodstuffs or pharmaceuticals. In that sense, recent researches are more focused on usage of enzymes as modification agents, as well as compounds naturally occurring in food. Also, all products of modification reaction, not only modified allergen, must be safe or successfully eliminated/degraded in further processing and expenses of method have to be reasonable. For food industry desirable physico-chemical properties of modified proteins are important as they determine technological properties such as solubility, pH stability, gelling, foaming, emulsifying properties etc., as well as nutritional value and impact on taste and flavor. Allergen modifications, although efficient in reducing allergenicity, do not bare potential for application in food industry if mentioned properties are significantly disturbed, as for food industry the most important is consumer's acceptance of certain food (texture, taste, stability, natural origin, etc.). Typical example are extensive protein hydrolysates, although highly hypoallergenic, having bitter taste, which is difficult to mask, and high osmolarity. For immunotherapy preparations, in addition to reduced/eliminated allergenicity and preserved immunogenicity, immunomodulatory properties are highly desirable for increasing immunotherapy efficiency. Also, physico-chemical properties of modified proteins define their stability, such as susceptibility for proteolysis and heat denaturation.

There is numerous studies focusing on modification of food protein as new properties enable design of more different food products and exploitation of cheap high nutritional food sources. Many of methods for reducing food protein allergenicity emerged from these studies, as evidenced by in parallel investigation of techno-functional properties and allergenicity testing in some publications. Reducing of allergenic properties by chemical or enzymatic methods was demonstrated on full extract, raw or pretreated, or on isolated allergens from peanut, wheat, whey, soy, pea and cherry. The most frequently modified food allergens are BLG and ovalbumin. BLG was interesting due to its high nutritional value, important functional properties and its availability as by-product cheese industry.

Ovalbumin was the most frequently used as a model allergen in studies using animal models.

As reducing of allergenicity can be achieved by removal of epitopes, destruction of epitopes, and by their masking, knowledge of the structural aspects of allergenicity and of the factors that determine epitope integrity will lead to novel and more specific approaches to decrease allergenicity. By treatment with chemical or enzyme agent only few amino acid residues of allergen react, such as residues of lysine, arginine, cysteine, tyrosine. Therefore, epitope containing these residues are expected to be changed/masked in significant extent and knowing the epitope structure can help in prediction which method would be the most successful. In addition to B cell epitope changes, changes in T cell epitopes can significantly contribute reduction of allergic response as well. T cells provide help to B cells, by direct interaction and secretion of cytokines, and promote their proliferation and differentiation into antibody producing cells (Kaminogava, 1996). Therefore, during allergen modification formation of bonds non-hydrolyzable by processing machinery in APC, can change T cell epitope profiles of allergens and have influence on direction of immune response, potentiating allergy or tolerance.

It seems that the most effective approach for allergen modification is combination of two or more methods acting by different mechanisms, such as combination of acylation and proteolysis (Szymkiewicz & Jedrychowski, 2008), reduction, alkylation and cross-linking (Koppelman et al., 2010), cross-linking and reduction (Villas-Boas et al., 2010), proteolysis and cross-linking (Wroblewska et al., 2008). Covalent modification of allergens by edible high molecular mass polysaccharides is effective and acceptable way for designing hypoallergenic food formulations offering also improved techno-functional properties. However, low immunogenicity of modifications limits this method for creation of effective immunotherapy preparations.

Of all applied cross-linking enzymes it seems that transglutaminase is the least efficient in reducing protein allergenicity, and only when it is combined with allergen reduction or in the presence of co-substrate or with proteolysis. Although for the efficiency of peroxidase there are only sparse data, food allergen cross-linking with phenol oxidases (tyrosinases, laccases) with assistance of small phenolic compounds have great potential. It looks like that protein cross-linking itself is not enough for significant masking IgE epitopes and that phenolic mediators contribute by modification of the nucleophilic surface residues in the protein and the hindering of the epitopes by their covalent attachment in a highly aggregated product, as well as to monomer allergen. Also, it seems that cross-linked allergens are more resistant to digestion than their monomeric form. It is well known that proteins resistant to both gastric and intestinal digestion are not allergenic. The data from animal models show that both gastric and intestinal-resistant allergens do not carry strong food-allergy risk or induce oral tolerance, simply because both food-allergy and oral-tolerance are MHC class II-dependent processes and require antigen presentation to the immune system i.e. readily available peptides in intestinal fluids (Bowman & Selgrade, 2008).

For the most methods mentioned in this review exhaustive studies on human subjects, including DBPCFC tests, are needed in order to design food with label hypoallergenic. As substantial part of the population reacts to very low amounts of allergens, residual unmodified allergen that remain after modification can still bind IgE and elicit symptoms in highly sensitive patients (Wensing et al., 2002). Also some modifications may be clinically relevant for some allergic individuals but not for others. The method for allergen

modification applied to food source, such as wheat, peanut, soy flour or WPI, may change different allergens to a different extent. Therefore, methods enabling a reduction of allergenicity of all proteins should be used or different types of modification should be applied simultaneously.

In the future further and more fundamental researches in this multidisciplinary field is necessary in order to develop novel hypoallergenic foods and food additives on one hand, and to obtain suitable preparations enabling safe and efficient food allergen-specific immunotherapy in clinical practice on the other hand.

6. Acknowledgment

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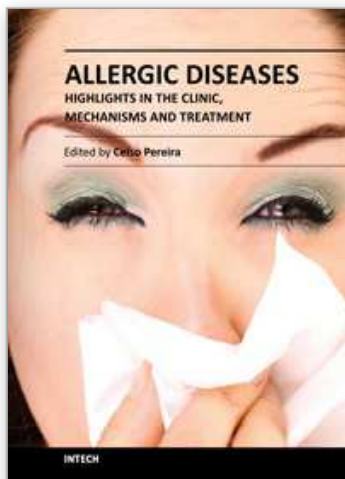
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