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The behavior of hypersensitivity-causing proteins during food processing

1. Summary

Since the components causing hypersensitivity reactions (allergies, celiac disease) are usually proteins, therefore, learning about their potential changes is important from the food safety point of view. If the proteins undergo various structural modifications during processing, their determination in foods could be problematic. Nevertheless, the fact that these altered proteins cannot be detected using analytical methods does not necessarily mean that they cannot cause adverse reactions in the human body. Answering the questions that arise in connection with this topic requires the cooperation of patients, clinicians and analysts as well.

Foods intended for final consumption undergo several processing steps while going from raw material to final product. Each process that alters the structure of proteins is also expected to have an effect on their binding to antibodies. Food processing procedures cause a number of physical, chemical and biochemical changes that can affect the allergenic properties of a protein. Depending on the properties of the protein, the type, length and intensity of the processing operation, or the matrix, the allergenic effect of a protein can be increased, decreased, or left unchanged by processing.

ELISA tests, which are currently used in routine methods, employ various antibodies, so the epitopes targeted in the immune responses can also be different. The various epitopes can undergo different modifications during food processing, therefore, their affinity to the antibody can also change, which can affect the results provided by the method. This phenomenon calls attention to the fact that the accuracy of commercially available methods is questionable, and so both the improvement and harmonization of immunoanalytical methods is necessary.

2. Introduction

Hypersensitivity reactions to foods present a severe food safety problem. For the majority of non-toxic reactions, eight food ingredients are responsible: gluten, raw materials made from crustaceans, eggs, fish, peanuts, soy, milk and products made of nuts. In addition to these, six other components that cause hypersensitivity reactions (celery, mustard, sesame seeds, lupine, mollusks, sulfur dioxide) also have to be indicated on food packaging in the EU **[1]**. With the exception of sulfur dioxide, disorders are caused by the protein components of the given food. It can be said about each food ingredient that they have very diverse protein compositions, and they contain a number of proteins in which the hypersensitivity reaction triggering special amino acid sequences, the so-called epitopes can be found.

Proteins that trigger the reactions usually enter the body with foods which can be matrices with different compositions and these undergo many and various processing steps of different nature. As a result of these effects, the proteins might denature, which is due to intra- or intermolecular interactions in most cases, and during which changes in composition and

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structure can occur. All of these can have an effect on the structure and behavior of the protein, and also on the epitopes that trigger the reaction. However, there is no consensus in the current literature on the topic. The number of proteins triggering the reaction, the epitope sequences of the individual proteins, as well as the behavior of the proteins are all subjects to discussion.

To ensure the safety of the consumers concerned, it is essential to develop and apply reliable analytical methods. Changes of proteins might have an effect on the efficiency of the sample preparation – including the extraction – prior to the analytical determination, and they can also influence the accuracy and the precision of the results. For this reason, a better understanding of proteins and studying their behavior is absolutely necessary.

3. Proteins causing hypersensitivity reactions and their changes

The reactivity of proteins can be influenced by several factors, the most important of which is the primary structure. The type and ratio of reactive groups are obviously determined by the amino acid composition and sequence. Hydrophobic amino acids are responsible for the conformation of the protein, for hydration, and they also affect solubility and gel-forming properties. Charged amino acids are capable of creating electrostatic interactions, and they also affect the water-binding capacity of proteins. The formation of interactions is also affected by the size, shape and charge distribution of the protein. Reactions can take place under the conditions applied during food processing (temperature, pH, presence of enzymes, etc.) **[2], [3]**.

The most critical and most frequently used technological step is heat treatment with varying intensity (time, temperature, dynamics). Heat treatment at 55-70 °C results the loss of the secondary structure, disulfide bonds break at 70-80 °C, new intra- and intermolecular interactions are formed at 80-90 °C, and at 90-100 °C protein aggregates are formed **[4]**. As a result of heat treatment, intramolecular interactions within a given protein molecule or intermolecular ones between proteins may form (**Figure 1**), by covalent or non-covalent bonds.

The most common protein-protein interaction is the creation of intra- or intermolecular disulfide bridges, which are formed between two cysteine moieties through the oxidative coupling of the free thiol groups. The bonds between the sulfur atoms contribute greatly to the heat stability of proteins. Heat treatment under alkaline conditions can lead to the racemization of amino acids, dehydroproteins are formed this way, and they are very reactive, and can be coupled to the thiol group of cysteine or ε-amino group of lysine. Tyrosine amino acids, under certain conditions, are capable of forming dityrosine bonds. In addition, polyphenol oxidase can also be an indirect source of protein interactions between cysteine, tyrosine or lysine, and the reactive benzoquinone that comes from the oxidation of phenols. As the combined effect of heat treatment and the transglutaminase enzyme, a glutamyl-lysine bond can be formed between the γ-carboxamide group of glutamine and the ε-amino group of lysine, which is a type of isopeptide bonds. Furthermore, in low carbohydrate-content foods, other isopeptide bonds can also form as a result of heat treatment between the ε-amino group of lysine and the amide groups of asparagine and glutamine **[2], [5]**.

The most common participants of protein-non-protein interactions are carbohydrates and lipids, however, interactions can also form with other macro- and microcomponents of the food matrix. The Maillard reaction is a reaction of an amine of the protein and a carbonyl group, where the latter can come from a reducing sugar or a fat decomposition product. In addition, due to the reactions of local dipoles of polysaccharides and charged ions of the proteins, weak complexes may form. A number of interactions are possible between proteins and lipids: electrostatic interactions may form between the positively charged group of a phospholipid and the negatively charged group of a protein, or the negatively charged phosphate group of a phospholipid and the positively charged group of a protein; the formation of covalent interactions is typical between oxidized lipids and proteins, and fatty acids can also form hydrogen and hydrophobic bonds with proteins **[2], [6]**.

4. Epitopes that trigger the reactions

The binding of the proteins that trigger hypersensitivity reactions to the antibodies of the immune system occurs through the epitopes. Epitopes are short peptide fragments of the protein, which are recognized by the antibody. It can be said about almost all of the proteins that trigger hypersensitivity reactions that they contain several epitopes. However, in the case of a certain individual, not all of them trigger the immune response, or not to the same extent. Epitopes that are most easily recognized by the immune system, or the ones that trigger the most intense response, are called immunodominant epitopes. In terms of epitope structure, there exist linear epitopes (with a length of 12 to 18 amino acids), in which case the reactive section is determined by the primary structure of the protein. At the same time, we can talk about conformational epitopes as well, which are formed as a result of the secondary and tertiary structures of the protein. Due to the spatial structure of proteins, the location of an epitope can be accessible to antibodies, but other epitopes can be hidden **[7]**.

Naturally, changes that occur in proteins due to processing can influence epitope structure and their availability as well. Linear epitopes are more likely to change in the hydrolyzed state, while conformational

epitopes are much more sensitive to changes that occur during processing. As a result of denaturation, epitopes may be lost, but hidden structures may also become available, and changes in the primary structure of the protein may result in the formation of new epitopes (**Figure 2**) **[7], [8]**.

The current available scientific literature regarding to the identification, behavior and allergenic activity of certain proteins and their epitopes is incomplete and, in many cases, contradictory. In the following sections, characteristic changes of certain food proteins (milk proteins, egg proteins, soy proteins, gliadin), responsible for frequently occurring disorders are reviewed.

4.1. Allergy-triggering milk proteins and their stability

In the case of milk, α-lactalbumin (ALA), β-lactoglobulin (BLG), bovine serum albumin (BSA), lactoferrin, immunoglobulins and caseins participate in triggering the hypersensitivity reaction, however, most studies demonstrate that the main allergens are caseins, BLG and ALA **[9]**.

Due to heat treatment, the solubility of milk proteins decreases, as a result of aggregate formation. Whey proteins have a globular structure, they are sensitive to heat, they denature and then disintegrate into their peptides, followed by the formation of aggregates. β-Lactoglobulin and α-lactalbumin are capable of forming covalent bonds with κ-casein. Aggregate formation is limited in the case of serum albumin, however, it is glycolized due to heat, which results in a conformation change. The thermal stability of caseins is significantly higher – α_S and β-caseins being the most stable – , however, protease enzymes can break them down quickly. The presence of caseins inhibits the aggregation of other proteins **[10], [11]**.

Cooking significantly reduces, or even completely eliminates the allergenic activity of β-lactoglobulin and serum albumin, and it also decreases the IgE-binding ability of ALA and caseins. However, it was proven by several studies that caseins do not lose their antigenicity due to heat. Whey proteins are capable of conjugation with carbohydrates, which decreases the allergenicity of α-lactalbumin and β-lactoglobulin **[10]**.

4.2. Egg protein allergens and their characteristic changes

Proteins that trigger egg allergy can be found both in the egg white (ovomucoid, ovalbumin, ovotransferrin, lysozyme, ovomucin) and the egg yolk (lipovitellins, phosvitin, α-livetin, apovitellenin I, apovitellenin VI), but the main allergens are egg white components **[12]**.

Ovalbumin (OVA) is a thermally not very stable protein, it denatures due to heat, and then aggregates are formed. Aggregates are held together by disulfide bonds, dimeric, oligomeric and polymeric forms all occur, with very little monomeric OVA remaining. Ovalbumin can form complexes with cereal proteins, and can also conjugate with carbohydrates – with reducing sugars through the Maillard reaction. The effect of the latter is controversial, according to some studies, the denaturation temperature of ovalbumin is increased by conjugation, while according to others it is decreased. In contrast, ovomucoid (OM) is a heat resistant protein, stabilized by sulfur-sulfur bonds. It is not prone to coagulation, and is resistant to denaturing agents, but it can form intermolecular bonds as a result of heat, for example, with the serum albumin proteins of milk in case of complex matrices. Ovotransferrin is very thermolabile, changes in the secondary structure occur already at 80 °C, and dimeric aggregates are formed. However, this protein that plays a role in iron transport, forms thermally stable complexes with the metal ions transported. Its solubility is irreversibly reduced with increasing heat treatment intensity and time **[13], [14]**.

There is a lot of uncertainty regarding to the change in the allergenicity of egg proteins due to heat treatment, detailed scientific research of the topic is still to be performed. It was determined about ovalbumin and ovomucoid that they form complexes with polysaccharides, held together by non-covalent forces. In this complex state, their IgE-binding capacity is increased, compared to the native proteins. At the same time, in case of ovomucoid, a decreased allergenicity can be observed in the combined presence of eggs and wheat, and this is attributed to the complex of OM and wheat proteins, stabilized by intermolecular disulfide bridges **[14]**.

4.3. Allergenic soy proteins and their changes

Very different opinions are formulated in the literature discussing the allergenic proteins of soy. Overall, the number of soy proteins identified as allergenic is estimated to be between 16 and 33, however, the most important allergenic fractions – glycinin, β-conglycinin, profilin, P34, Kunitz trypsin inhibitor (KTI) – are well known **[15]**.

The denaturation temperature of soy proteins is strongly dependent on pH and ionic strength. During heat treatment, glycinin aggregates in several stages: first, it decomposes into its subunits, and then sulfur-sulfur bonds holding together acidic and basic peptides break. Following this, soluble aggregates are formed, however, acidic polypeptides remain unchanged. Different subunits of β-conglycinin possess different heat tolerance, with the α' and β subunits being more stable. Heat causes the secondary structure to change, soluble aggregates form, which are held together by non-covalent binding forces. In addition, glycinin and β-conglycinin are also capable of forming aggregates with each other. Profilin, P34 and KTI are thermally labile proteins. As a result of heat

treatment, the secondary structure of profilin changes, aggregates form, but the stability increases with decreasing pH. The P34 protein is linked to 7S globulins via sulfur bridges. The Kunitz trypsin inhibitor is already irreversibly denatured at 90 °C, and then it forms aggregates with disulfide bridges and/or non-covalent bonds **[16], [17]**.

There is limited information available about the effect of heat transfer on the immune activity. However, it can be said that glycolysis by the Maillard reaction reduces the antigenicity of soy proteins **[18]**.

4.4. Wheat proteins triggering hypersensitivity reactions and their stability

In addition to classic allergy, wheat proteins can also trigger so-called wheat-dependent exercise-induced anaphylaxis, which occurs when the consumption of a food containing wheat proteins is followed by vigorous exercise. Furthermore, certain wheat proteins contain sequences that trigger celiac disease. Celiac disease is a genetics-based autoimmune enteropathy that develops upon provocation, and it is accompanied by destruction of the villi of the small intestine, crypt hyperplasia and lymphocytic infiltration **[19], [20]**.

Proteins that belong to the α-amylase inhibitor family have been identified as wheat allergens, as well as the LMW glutenin subunit with a QQQPP sequence, α- and β-gliadins, lipid transfer proteins, profilin proteins, and certain proteins of the albumin/globulin fraction. For the development of celiac disease, mainly gliadin and glutenin proteins are responsible, and a sequence of α2-gliadin, consisting of 33 amino acids (57-89), has been identified as the immunodominant epitope. However, this disorder can be triggered by several other protein epitopes as well **[5], [20]**.

With a few exceptions, albumin and globulin proteins of wheat are thermally labile, their secondary structure is altered due to heat treatment. In contrast, storage proteins and α-amylase are heat stable. The solubility of α-, β- and γ-gliadins decreases with increasing temperature, because they are capable of forming disulfide bonds with each other and with glutenin proteins through their cysteine amino acids, and this results in aggregates of different sizes. Glutenins can also participate in other intermolecular interactions (e.g., isopeptide bonds, β-elimination). Furthermore, it can be said that the number of isopeptide bonds increases with increasing heat treatment length **[21], [22]**.

As a result of baking, the IgE-binding activity of α-amylase disappears, while that of prolamins does not. Most of the allergenic epitopes remain stable during the baking of bread, and it was even demonstrated that some proteins become more resistant to pepsin digestion due to heat treatment **[5]**.

5. The effect of chemical and structural changes in proteins on immunoanalytical results

Of the number of analytical methods available for the detection and quantification of food allergens, the most often used are immunoanalytical methods (ELISA: Enzyme-linked Immunosorbent Assay, LFD: Lateral Flow Devices), because of their high degree of specificity and sensitivity. The structure of proteins can be influenced by reactions with matrix components and chemical changes due to food processing, and so these can also affect the results of analytical methods **[23]**.

In our research, the effects of processing operations on the analytical results were investigated in case of milk, egg, soy and wheat proteins. For the experiments, model matrices (powder mixture, dough and baked cookies) of known allergen content, but of partly different composition were developed, and then we tested how the analytical results were influenced by the individual steps of processing. Different or partly different sample preparation methodologies are used by the currently commercially available ELISA tests, and the target protein can also be different, therefore, the antibody employed is also different. For this reason, we sought to answer the question whether the analytical results was also affected by the application of different kits. So ELISA measurements were performed using the kits of two manufacturers for all four components tested: Ridascreen Fast Milk, Fast Egg, Fast Soya and Gliadin kits from R-Biopharm and AgraQuant Casein, Egg White, Soy and Gluten tests from Romerlabs were used in our experiments. (Since it was not our goal to grade the manufacturers, tests of the individual manufacturers are referred to by the letters A and B.)

In general, it can be said about the effects of processing operations on ELISA results that, compared to the powder mixture, only a slight decrease could be observed in the case of raw doughs, but a great decrease in the measurable concentration could be observed after heat treatment (**Figure 3**). The observed differences were significant in almost all cases.

The extent of concentration decrease which could be measured in the raw dough can be explained in most cases by the diluting effect of the added water and margarine, but in some cases (for example, when measuring the soy-containing model product using kit B) a stronger decrease was experienced. It could also be observed that, for model products containing milk, eggs and soy, the allergenic protein content that could be measured in the baked product was less than 60%, and in several cases it was below the detection limit of the given ELISA test.

Simultaneous application of two ELISA kits allowed for the examination of the influence of the selected method on the analytical results, in addition to that

of the processing operations. It could be observed in case of all four measured components that the results afforded by the tests of manufacturer "B" were higher than the results of the "A" kits, and the differences were significant in most cases. At the same time, when analyzing the deviation from the theoretical concentration, it can be stated that the recovery percentages of tests „A" are closer to 100%, but still differ from it significantly. However, a tendency for the direction of the deviation cannot be established for the tests of either manufacturer.

Effects of food processing operations on ELISA results can be experienced practically everywhere, but the extent of the effects differ as a function of the thermal stability of the protein sources tested. Naturally, in the case of processing operations, heat treatment has a greater effect, and it influences mainly the accuracy of the measurement (recovery percentage) significantly and decisively. The phenomenon is explained mainly – partly with the exception of gliadin – not (only) by protein-protein interactions, but reactions between protein and non-protein components, basically changing the solubilities of target proteins. Therefore, mainly the reactions and the formed products have to be identified and, following this, a solution has to be found for the efficient protein extraction from heat-treated sample matrices. Unfortunately, it must be noted that, using current sample preparation steps, certain commercially available ELISA kits can provide analytical results in the case of heat-treated products only with significant errors. It could be observed for all components analyzed that different results were obtained using different ELISA kits. When testing the same sample, large differences were already observed in the recovery values of model products containing native proteins, however, their precisions were satisfactory. There could be significant differences in the sample preparation protocols, in the target proteins and in the analytical performance of the ELISA tests of different manufacturers as well. Based on the statistical evaluation of the factors influencing measurement accuracy it can be stated that in case of all four tested components the used ELISA kit and the processing stage have the greatest influence on the measurable result.

These results draw attention to the significance of the matrix effect, to the importance of clarifying the phenomena behind it, and, by using them, to the necessity for further development and harmonization of immunoanalytical methods.

6. Conclusions

Effects of the processing operations on the analytical results can be identified in the case of all hypersensitivity-triggering components analyzed. In our work, changes in accuracy and precision as performance characteristics were studied. In case of the examined protein sources using the same matrices different extent of changes could be identified but the negative impact of the applied technological steps could be detected in all target protein group. Among the investigated technological steps, heat treatment has the greatest impact on the accuracy of measurements. The background of this phenonmenon can be explained not (only) by protein-protein intercations but also by reactions between protein and non-protein components which supposedly modify the solubility of target proteins. Therefore, mainly the occuring reactions and the formed products have to be identified and thereafter a solution should be found for the protein extraction from heat-treated sample matrices. The reducing agents and detegents currently applied for sample preparation do not seem to be satisfactory. Partially exempt from this are thermally less sensitive gluten proteins, where the formation of sulfur bridges is a major accompanying phenomenon of heat treatment. The application of currently available ELISA tests can lead to partly different and erroneous analytical results which has to be taken into consideration both in regulation and in allergen management. The identification of target proteins, epitopes, other matrix components and the physicochemical reactions which affect the analytical methods and based on this knowledge the improvement of the methodology can lead to the solutions.

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8. References

[1] EU Regulation No 1169/2011 of the European Parliament and of the Council. Official Journal of the EU, 304:18-63.

[2] Gerrard J. A. (2002): Protein-protein crosslinking in food: methods, consequences, appli-cations. Trends in Food Science & Technology, 13: 391-399.

[3] Breitender H., Mills E.n.c. (2005): Molecular properties of food allergens. Journal of Allergy and Clinical Immunology, 115(1): 14-23.

[4] Thomas K., Herouet-Guicheney C., Ladics G., Bannon G., Cockburn A., Crevel R., Fitzpatrick J., Mills C., Privalle L., Vieths S. (2007): Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. Food and Chemical Toxicology, 45: 1116-1122.

[5] Tatham A.S., Shewry P.R. (2008): Allergens in wheat and related cereals. Clinical and Experimental Allergy, 38: 1712-1726.

[6] Mccann T. H., Small D. M., Batey I. L., Wrigley C. W., Day L. (2009): Protein–lipid interactions in gluten elucidated using acetic-acid fractionation. Food Chemistry, 115: 105-112.

[7] Abbas A. K., Lichtman A. H., Pillai S. (2005): Cellular and Molecular Immunology. Elsevier Saunders, Philadelphia, USA. ISBN: 978-1-4377-1528-6.

[8] Davis P. J., Smales C. M., James D. C. (2001): How can thermal processing modify the antigenicity of proteins? Allergy, 67: 56-60.

[9] Diaz-Amigo C. (2010): Towards a Comprehensive Validation of ELISA Kits for Food Allergens: Case 1—Milk. Food Analytical Methods, 3: 351-356.

[10] Monaci L., Brohée M., Tregoat V., Van Hengel A. (2011): Influence of baking time and matrix effects on the detection of milk allergens in cookie model food system by ELISA. Food Chemistry, 127: 669-675.

[11] Pesic M. B., Barac M. B., Stanojevic S. P., Ristic N. M., Macej O. D., Vrvic M. M. (2012): Heat-induced casein-whey protein interactions at natural pH of milk: A comparison between caprine and bovine milk. Small Ruminant Research, 108: 77-86.

[12] Caubet J. C., Bencharitiwong R., Moshier E., Godbold J. H., Sampson H. A., Nowak-Wegrzyn A. (2012): Significance of ovomucoid- and ovalbumin-specific IgE/IgG4 ratios in egg allergy. Journal of Allergy and Clinical Immunology, 129(3): 739-747.

[13] Croguennec T., Renault A., Beaufils S., Dubois J., Pezennec S. (2007): Interfacial properties of heat-treated ovalbumin. Journal of Colloid and Interface Science, 315: 627–636.

[14] Azarnia S., Boye J. I., Mongeon V., Sabik H. (2013): Detection of ovalbumin in eggwhite, whole egg and incurred pasta using LC–ESI-MS/MS and ELISA. Food Re-search International, 52: 526-534.

[15] Cucu T., De Meulenaer B., Devreese B. (2011): MALDI based identification of soybean protein markers – Possible analytical targets for allergen detection in processed foods. Peptides, 33(2): 187-196.

[16] Nishinari K., Fang Y., Guo S., Philips G. O. (2014): Soy proteins: A review on composition, aggregation and emulsification. Food Hydrocolloids, 39: 301-318.

[17] Chen Y., Xu Z., Zhang C., Kong X., Hua Y. (2014): Heat-induced inactivation mechanisms of Kunitz trypsin inhibitor and Bowman-Birk inhibitor in soymilk processing. Food Chemistry, 154: 108-116.

[18] Van De Lageemat J., Silvan J.m., Moreno F. J., Olano A., Del Castillo M. D. (2007): In vitro glycation and antigenicity of soy proteins. Food Research International, 40: 153-160.

[19] Battais F., Courcoux P., Popineau Y., Kanny G., Moneret-Vautrin D. A., Denery-Papini S. (2005): Food allergy to wheat: differences in immunoglobulin E-binding pro-teins as a function of age or symptoms. Journal of Cereal Science, 42: 109-117.

[20] Tollefsen S., Arentz-Hansen H., Fleckenstein B., Molberg O., Ráki M., Kwok W. W., Jung G., Lundin K. E. A., Sollid L. M. (2014): HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. The Journal of Clinical Investigation, 116(8): 2226-2236.

[21] Singh H. (2005): A study of changes in wheat protein during bread baking using SE-HPLC. Food Chemistry, 90: 247-250.

[22] Rasheed F., Newson W. R., Plivelic T. S., Kuktaite R., Hedenqvist M. S., Gallstedt M., Johansson E. (2014): Structural architecture and solubility of native and modified gliadin and glutenin proteins: non-crystalline molecular and atomic organization. RSC Advances, 4: 2051-2060.

[23] Kerbach S., Alldrick A. J., Crevel R. W. R., Dömötör L., Dunngalvin A., Mills E. N. C., Pfaff S., Poms R. E., Popping B., Tömöközi S. (2009): Managing food aller-gens in the food supply chain - viewed from different stakeholder perspectives. Quality Assurance and Safety of Crops & Foods, 50-60.