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PhD thesis

IMMUNE-ANALYTICAL DETECTION OF THE MAJOR CEREAL ALLERGENS

Krisztina Takács

Central Food Research Institute
Unit of Biology

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Name: PhD School of Food Science

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Head: Prof. Péter Fodor
Corvinus University of Budapest

Supervisor: Dr. Éva Gelencsér
Food Safety Department
Unit of Biology
Central Food Research Institute

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Prof. Péter Fodor
Head of School

Dr. Éva Gelencsér
Supervisor

1. INTRODUCTION

Cereals have a significant role in the human nutrition from a long while. Beside their energy content they are essential sources of necessary nutrients such as carbohydrate-, vitamins-, minerals-, dietary fibres- and proteins.

Among cereals, wheat is considered as one of the most important crop, which is grown on the widest area. Despite of its favourable nutritional effects, wheat is one of the most prevalent allergen sources for patients suffering from celiac disease and wheat allergy. In the background of the two different sorts of diseases, there are different immunological processes, in which different allergens play different role in their elicitation. According to our recent knowledge the strong allergen components, which can cause most often wheat allergy are the alcohol-soluble gliadins (α és ω5), the water-soluble α-amylase inhibitors (CM3, 0.53) and the non-specific lipid transfer proteins. These components can cause a wide range of symptoms. The sensitization can occur either via the respiratory organs or via the gastrointestinal tract. In the case of celiac disease, the toxic allergenic factors are the gliadins and the prolamins having similar immunological structure to that of gliadins (e.g. rye secalin, barley hordein, oat avenin), which can cause alterations mainly in the tract villus. The only therapy of both diseases is the avoidance of the symptom-causing allergens and intolerance factors, namely to exclusively consume only allergen-free foodstuffs.

Since many of the wheat proteins with allergenic properties have been revealed the research on their chemical, immunological, clinical and molecular based examinations became reasonable. As a matter of fact it is not an easy task as the clinical symptoms occurring in the patients and the heterogeneity of the symptom-causing wheat allergens make difficult, to a considerable extent, their examination.

The recognition of allergens and determination of their activity, epitops and mechanism of actions necessarily requires method developments to detect the presence and to determine the concentration of the allergens in foods.

Different methods are available for the risk managers to examine the gluten-free nature of foods, for instances gliadin-specific polyclonal, ω-gliadin-specific monoclonal and gliadin epitop (R5) specific monoclonal antibody-based ELISA methods and other fast methods as well. According to the Codex regulations, the threshold limit of the detection (as related to gluten) should be as minimally as 10 ppm. A question may arise about suitability of the methods used for the achievement of this goal taking into consideration that specificity, sensibility and protocol of all the applicable methods are different. In order to solve the problems relating to the aforementioned question, it is necessary to deeply deal with the reliability and reproducibility of the method to be used for the detection of gluten.

2. OBJECTIVES

- Comparative examination of the significant food allergens in the respect of celiac disease and wheat allergy on the basis of the domestic cereal and pseudo-cereal cultivars.
- Investigation of the applicability of the immune-analytical methods with special respect to gluten detection in raw and processed food models.
- Development of transglutaminase enzyme-treated high quality pasta models with reduced gluten content (wheat based) and free from gluten ones (yellow pea based) to be used in gluten-free diets.

3. MATERIALS AND METHODS

3.1. Cereals, pseudo-cereals and model samples

- Domestic cereal (5-5 species of wheat, rye, barley, triticale, oat, corn, rice) and pseudo-cereal (3 species of amaranth, 5 species of buckwheat) cultivars were provided by the Cereal Research Non-Profit Company and Klorofill Limited.
- The raw ingredients of gluten-free bread (rice flour, corn starch, potato flake, egg powder, yeast, finished bread powder), gluten containg ingredients (wheat flour, rye flour), control bread samples (white bread, rye bread, gluten-free bread) and ten pieces of bread models prepared during processing of gluten-free bread and contaminated on purpose with wheat/rye flour at three contamination points (kneading dish, baking form, drying place) were provided by Dunakenyér Bread-making and Trade Company.
- Models of *T. aestivum* és *T. durum* wheat flour and yellow pea flour-based dry pastas prepared with the use of transglutaminase enzyme at different concentrations (0-200 TG/kg flour) were obtained from the University of Szeged, Faculty of Engineering, Institute of Food Engineering.
- Individual anonym and clinically certified sera from celiac and wheat allergic humans were originated from the Gastro-entherology II Serum Bank of the Heim Pál Hospital.

3.2. Methods applied for the preparation of protein fractions and models

- Extractions with Tris-HCl buffer (pH 8.5) and 70% ethanol were performed for the preparation of water/salt- and alcohol-soluble fractions from domestically cultivated cereals and pseudo-cereals.

- In case of wheat samples, DEAE-cellulose anion-exchange chromatographic method was developed and applied to prepare protein fractions rich in α-amylase inhibitors.
- In case of transglutaminase–treated pastas, extractions with 0.5M NaCl (pH 6.8), 68% ethanol solutions and borate buffer containing β-mercapto-ethanol were carried out to prepare water/salt- and alcohol-soluble and then acid/alkaline-soluble fractions.
- Combination of SDS-PAGE with immune blotting was used for the determination of allergenic proteins. In the immune blot tests, antigen-specific polyclonal antibody produced in rabbit, celiac (IgA) and wheat-sensitive (IgE) human sera were used.
- For the detection of gluten, different antibody-based (polyclonal, monoclonal ώ-gliadin, monoclonal R5) methods were applied.
- To identify allergenic proteins liquid chromatography coupled with mass spectroscopy (LC-MS/MS) and NCRInr Plant database were used.
- The determination of resistance of the allergenic proteins towards pepsin was carried out using PVDF membrane-blotted proteins.
- Wheat/rye flour contamination was monitored at selected critical points during processing of gluten-free bread (kneading dish, baking form, drying place).
- Transglutaminase enzyme was used for the preparation of wheat - and yellow pea flour-based dry pasta models.

4. RESULTS

- IgE and IgA reactive proteins were detected with the use of sera from wheat sensitive and celiac patients respectively in domestically cultivated wheat varieties.
- Presence of proteins having cross-reactivity with wheat proteins was detected by immune blotting using wheat allergic and celiac human sera in domestically cultivated cereals and pseudo-cereals (triticale, rye, barely, oat, corn, rice and amaranth, buckwheat).
- Stability of the allergenic proteins was confirmed by a newly developed immune blotting method to investigate the resistance of IgE-reactive proteins towards pepsin.
- The most frequently recognized major cereal allergens, by wheat-sensitive sera, were identified by LC-MS/MS after SDS-PAGE separation. The identification was achieved by using the NCBInr Plant database.
- In model studies (models of cereals, pseudo-cereals, breads and dry pastas) the applicability of ELISAs using antibodies with different specificities it was found that the gliadin specific polyclonal antibody-based ELISA was appropriate for the quantitative measurement of

gliadin over 200 ppm, while ELISA methods based on monoclonal antibodies specific for ω-gliadin and R5-gliadin epitop were suitable only for the determination of traces (<200 ppm) of the contaminating gliadin. Detection sensitivity of ELISAs decreased as a function of technological treatments (enzyme and heat treatments). Because the aforementioned methods are suitable for the detection of gliadin, they can be used only for the indirect determination of gluten content.

- By developing the dry pasta models it was found that the transglutaminase enzyme treatment did not decrease significantly the amount of proteins recognized with gliadin-specific antibodies in the wheat based pastas, while cross-reactivity was not identified in the yellow pea flour based pastas which can be a good source to be inserted in gluten-free diets.

5. NEW SCIENTIFIC FINDINGS

- By DEAE-cellulose anion-exchange chromatographic method selective and reproducible procedure was developed for the concentration of α-amylase inhibitor (as the major, well-known cereal allergens) separated from water- and salt-soluble fraction of wheat proteins.
- Most frequently recognized IgE-reactive proteins towards the majority of wheat allergic human sera were identified by using LC-MS/MS technique with the application of NCBInr Plant database in concentrated fractions of α-amylase inhibitors of domestically cultivated wheat varieties. Among allergenic proteins identified by several peptides covering the protein structure in high extend, xilanase inhibitor precursor, xilanase inhibitor, α-amylase inhibitor (monomer, dimer, CM 17 protein precursor), serpin, tritin, GSP1 protein, 5a2 protein, LTP1 precursor, β-amylase, manganese- superoxide dismutase, peroxidase, and corn ubiquitin-like wheat protein were found. The proteins which were identified with only one peptide were: peroxidase, PR4, 15 kDa protein, WSCI proteinase inhibitor, avenin-type precursor, serpin, and one *Arabidopsis thaliana* EDA 18-like wheat protein. It was proven that α-amylase inhibitors, which are reported in the literature as the most important allergens and serpins, which are found in the most recent researches as new allergens, could be primarily detected and identified as allergens in domestically cultivated wheat varieties.
- Foremost, IgA-reactive proteins, by the use of celiac sera, were detected among water- and salt-soluble proteins of wheat, corn, amaranth, and buckwheat. Identification of such proteins has special importance in the methodology of gluten-free food control and diagnostic examination of celiac disease because, the safety to choose food ingredients that can be used without risk in gluten-free diets can be highly improved

- Also, foremost, was carried out an in-vitro investigation of pepsin digestion the membrane-blotted wheat proteins after SDS-PAGE separation (No similar method has been published in the literature yet). With such studies it was confirmed that allergens are, in fact, resistant towards pepsin digestion. It was also evident that the pepsin-resistant and strongly reactive proteins towards human sera were mostly serpins (45 kDa) and α-amylase inhibitors (16 kDa).
- Polyclonal antibody-based competitive indirect ELISA was developed for the quantitative determination of gliadin and immunologically gliadin-cross-reactive proteins from wheat and triticale. The detection limit of the method was 10 ppm and the measurement range was between 10 and 100 ppm. The detection sensitivity was found to decrease in the heat-treated food matrices.
- With different antibody-based methods, it was proved that in wheat flour-based dry pasta models treated with transglutaminase enzyme the immune reactive gliadin content cannot be masked to a level lower than the tolerated limit of 100 ppm, therefore, such products cannot be used in diet with reduced gluten content.
- In case of yellow pea pasta model, a novel product with improved quality was developed that can be inserted in gluten-free diet by using transglutaminase enzyme treatment. The gluten-free nature of the product was assured by checking the gliadin cross-reactivity against polyclonal gliadin-specific antibodies produced in rabbit.

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