Research article

Immunoreactivity of chemically cross-linked gluten and hydrolysates of wheat flour

Iwona Majak, Joanna Leszczyńska, Agata Łącka*

Institute of General Food Chemistry, Technical University of Lodz, 90-924 Łódź, Poland

*agata.lacka@p.lodz.pl

Abstract: *The immunoreactivity of gluten and wheat flour proteins cross-linked with chosen chemical reagents was investigated. Native proteins and flour hydrolysates subject to enzymatic proteolysis with collagenase and subtilisin were studied. Determination of immunoreactivity was performed with noncompetitive ELISA method with coeliac patients' sera. The lowest immunoreactivity values were obtained during cross-linking of wheat flour hydrolyzates with polyethyleneimine, below 5% of the values for non-modified flour.*

Keywords: *wheat; gluten; cross-linking; ELISA*

Introduction

Cereals are the base of human diet all over the world. However, consumption of wheat or products enriched with wheat proteins can manifest with different undesirable reactions of the organism. Among the most prevalent symptoms of food allergies are: urticaria, atopic dermatitis (AD) and wheat-dependent exercise-induced anaphylaxia (WDEIA) [1]. Apart from food allergies, disorders related with food intolerance of gluten, known as coeliac disease (CD), can occur. Consumption of gluten causes small intestine disorders resulting in destruction of epithelium and inflammatory response. The strongest response is evoked by peptides obtained as a result of digestion of one of the gluten fractions of wheat proteins [2].

One of the methods applied to decrease the immunoreactivity of proteins is the formation of covalent, intra- and intermolecular, bonds between them, so called cross-linking. It allows for covering of the molecular epitopes structure, which previously were presented on the surface of the allergogenic protein and therefore, the depletion of their recognition of antibodies. It is an interesting method, because the created bonds can not be hydrolysed in human intestinal tract by the digestive enzymes. What is more, the cross-linked proteins may favour an enhancement of organoleptic or technological properties of the initial product.

The aim of this work was to find an appropriate chemical reagent for covalent cross-linking of gluten proteins and wheat hydrolyzates.

Experimental

Reagents

Gluten (Sigma) and wheat flour type 500 from "Kruszynek" mill, containing 18.8% of gluten, 0.51% ash and 14.9% moisture content were chemically modified. To 1g of gluten or wheat flour 2ml of distilled water and 2% or 10% in regard to substrate (gluten or flour) mass of chemical reagent were added. The modifications were performed at the temperature of 37°C on a shaker with an incubation time of 4 hours. After the termination of the reaction all samples were centrifuged and washed twice with distilled water and centrifuged again. Supernatant was used for further analysis.

As cross-linking reagents were used: diethanolamine (D-8885), glutaraldehyde (G-7526), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (E-6383), poliethylenimine (P-3143), 4-vinylpyridine (V-3877), polyethyleneglycol PEG 3350 (P-3640) and PEG 1450 (P-5402), D-sorbitol (S-1876), formaldehyde (F-1635), polyvinyl alcohol (P-8136, all of the above from Sigma) glycerol (analytically pure) (POCh, Poland).

Enzymatic modifications

Enzymatic modifications were carried out accordingly to chemical modifications. Two proteolytic enzymes were used: collagenase from *Clostridium histolyticum* (EC 3.4.21.3) and subtilisin from *Bacillus licheniformis* (EC 3.4.21.62). Both enzymes were chosen basing on previous research [3]. The substrate (gluten or flour) was incubated with enzyme for 18 hours at 37°C on a shaker, the weight ratio of enzyme to substrate was 1:100. The enzymatic reaction was terminated by 5 min heating in boiling temperature.

Immunoreactivity analysis

To estimate the immunoreactivity of gliadin fraction isolated from modified flour or gluten the indirect enzyme-linked immunosorbent assay (ELISA) technique was used, in which two human sera containing antigliadin antibodies and monoclonal antibodies against human antiglobuline IgG conjugated with an alkaline phosphatase were used [4]. Antibodies from both sera did not recognize proteins of albumin and globulin fractions contained in the wheat flour (data not shown).

Proteins extracts were isolated according to the Osborne's procedure [5].

Microtitre plates EB 92029330 (Labsystem, Helsinki, Finland) were coated overnight at 4°C with 100 µl of the antigen solution (100 times diluted extracts) in 0.1 M carbonate buffer (pH 9.6), containing about 1.5 mg of protein. Plates were washed and free binding sites were blocked by incubation of the plates with a 3% solution of low fat milk in phosphate buffer (pH 7.2), containing 0.1% Tween-20 for 2 h. This was followed by removal of buffer solution, rinsing the plates five times and further incubation with human sera containing antigliadin antibodies diluted with phosphate buffer for 1 h at room temperature. The plates

were washed again and 100 µl of 1000-fold diluted solution of anti-IgG antibodies conjugated with an alkaline phosphatase from mouse, clone GG-5 (Sigma, A 2064) was added. After incubation of the plates for 1 h and rinsing with phosphate buffer the bound phosphatase activity was determined by reaction with p-NPP with Multiscan MC reader at 405 nm. A 0.1% milk solution in phosphate buffer (pH 7.2), containing 0.05% Tween-20 was used for all washings and dilutions of antibodies.

The residual immunoreactivity of probes was estimated in ratio to untreated wheat flour or gluten.

Results

The cross-linking reaction was carried out for all of the cross-linking reagents (table 1). Both, the purified gluten proteins and the analysed commercial flour underwent the cross-linking. What is more, different amounts of cross-linking reagent per substrate were used (2% or 10%). The expected cumulative effect of an increased dose of the cross-linking reagent was not achieved in all samples.

Table 1. The residual immunoreactivity of gluten and flour subjected to the action of cross-linking reagents in reference to the immunoreactivity of the non-modified samples

Lp	Cross-linking reagent	Residual immunoreactivity [%]			
		Gluten		Wheat flour	
		The amount of cross-linking agent (w/w)			
		2 %	10 %	2 %	10 %
1	4-vinylpyridine	73,3±4,2	81,6±4,1	96,5±1,9	92,1±4,7
2	diethanolamine	62,8±3,1	58,1±3,9	48,4±1,8	27,2±3,2
3	EDC	66,8±4,1	55,9±3,7	76,7±2,4	28,4±2,1
4	formaldehyde	56,9±2,7	50,3±2,7	67,8±3,3	65,2±2,8
5	glutaraldehyde	87,1±5,0	69,6±2,5	56,9±3,5	39,5±2,9
6	glycerol	91,8±3,3	89,9±1,9	90,8±4,4	82,6±3,1
7	PEG 1450	81,0±4,3	68,9±1,7	94,4±2,4	91,2±3,4
8	PEG 3350	88,8±4,7	93,1±2,6	95,0±3,7	92,1±3,6
9	poliethylenimine	16,3±2,2	2,7±1,1	49,2±2,8	40,9±2,9
10	polyvinyl alcohol	65,7±2,7	69,7±2,2	68,7±2,9	59,9±2,9
11	sorbitol	80,7±3,6	76,7±2,3	77,1±3,2	76,9±2,8

Among the applied chemical compounds polyethyleneimine turned out most favourable. Product modified with this reagent was characterized by the lowest immunoreactivity (below 20%). Diethanolamine also proved useful for decreasing of gluten immunoreactivity, causing its significant depletion. In the case of this cross-linking reagent, presence of other protein fractions in wheat flour enhanced the desired effect - the decrease of wheat flour immunoreactivity was greater. An analogous phenomenon was observed for sorbitol and glutaraldehyde. What is more, a significant decrease of immunoreactivity of the

analysed proteins (from 70 to 30%) in reference to unmodified sample was obtained with 1-ethyl-3(3-dimethylamino-propyl)carbodiimide (EDC).

In the second part of the experiment a reaction of plasteinisation was conducted, that is an enzymatic hydrolysis of gluten proteins to polypeptides with a subsequent cross-linking reaction with chosen cross-linking reagents. All of proteins contained in wheat flour were subject to plasteinisation due to the possibility of reaggregation of polypeptides, and derived from albumin and globulin hydrolysis. The hydrolysis was conducted paralelly with two proteases: subtilisine and collagenase, chosen on the basis of previous research on decreasing of wheat gliadins immunoreactivity through enzymatic modifications [3]. To the subsequent cross-linking only those reagents were chosen, which were characterized by the greatest decrease of gluten IR (dithyleneamine, EDC, polyethyleneimine) or those approved as food additives (glycerol, sorbitol, PEG 1450, PEG 3350). The obtained results are presented in table 2.

Table 2. The residual immunoreactivity of wheat flour subjected to the action of protease (collagenase, subtilisin) and cross-linking reagents (10%) in relation to native sample immunoreactivity

Cross-linking reagent	Residual immunoreactivity [%]	
	collagenase	subtilisin
diethanolamine	33,8±1,9	3,5±1,6
EDC	19,1±1,8	6,1±1,8
glycerol	31,7±2,1	39,2±2,0
PEG 1450	41,6±2,3	78,7±2,3
PEG 3350	40,7±2,4	61,1±2,4
poliethylenimine	4,6±2,3	3,3±1,5
sorbitol	37,4±2,6	42,3±2,4
none	29,3±1,9	70,3±2,6

The combination of enzymatic hydrolysis and chemical cross-linking allowed for significant decreasing of wheat flour gliadins' immunoreactivity down to about 3%, when subtilisin was used as the hydrolysing enzyme. The application of collagenase as the hydrolysing enzyme also proved effective (the remaining immunoreactivity of 4,6%), but only when the cross-linking reagent was polyethylenimine. In other cases the remaining immunoreactivity of the gliadins modified with cross-linking as well as plasteinisation was of the same order of magnitude. Surprising results were obtained for two reagents: PEG 1450 and PEG 3350, for which the flour samples exhibited lower immunoreactivity after hydrolysis than after the subsequent cross-linking reaction.

Discussion

Cross-linking as a method of decreasing of allergenic proteins immunoreactivity is relatively little investigated, although it gives a possibility of blocking contact of the antibodies present in the organism with epitopes of immunogenic polypeptides contained in food, especially ingredients of those food products that cannot be entirely eliminated from diet due to their common application in production technology of a wide variety of foods. One of the main components presenting such character is wheat gluten, which is responsible for both, food allergies as well as immunological reactions connected with its intolerance (coeliac disease).

Up to this time, researchers put the main emphasis on the enzymatic, and therefore strictly oriented, cross-linking of gluten proteins. Cross-linking of prolamines is a beneficial phenomenon in technology of wheat flour processing, enhancing the physico-chemical parameters of the obtained bread, regardless of the initial half-products quality [6, 7, 8]. A number of enzymes allowing for cross-linking of gluten proteins has been known, however, an impact of cross-linking on the immunoreactivity of protein was investigated only for one of them – transglutaminase (TG).

Gluten proteins cross-linked with TG express much lower immunoreactivity towards IgE class antibodies (coming from allergy sufferers) [9], however similar or even higher towards IgG [10] and IgA [11] class antibodies (both classes from coeliac patients) than the native protein, owing to which, gluten modified with this enzyme should not be intended for people with gluten intolerance [12]. A significant problem is, therefore, finding of another gluten proteins cross-linking method suitable for both, allergy sufferers and patients with coeliac disease.

The change of cross-linked with chemical reagents proteins' immunoreactivity was analysed mainly for cross-linking with polyethyleneglycols (PEG). PEGylation of proteins caused depletion of immunological response of antibodies to cow's milk proteins: α-lactoalbumin and β-lactoglobulin. The proteins expressed residual immunoreactivity below 0,1% of the initial milk immunoreactivity in *in vitro* tests [13].

Cross-linking reagents used in this work, forming covalent bonds between protein molecules, were chosen basing on the analysis of literature reports concerning cross-linking of proteins, both for food products production as well as biodegradable plastics obtained from protein materials, used as packing materials. So far, only four out of 11 analysed cross-linking reagents have been legally admitted as food additive (glycerol, sorbitol, PEG 1450 and PEG 3350).

An addition of polyols and amines to gluten proteins was considered as plastifier in biodegradable protein films production for packing materials. Among the large group of investigated compounds the best mechanical properties of the films were obtained for low molecular weight amines: diethanolamine and triethanolamine, and glycerol [14]. In that research an addition of plastifiers exceeds 20% in reference to the gluten proteins. In our research we applied lower

amounts of chemical reagents, because protein cross-linking can take place at low concentrations of cross-linking reagent, e.g. gluten proteins cross-linked with glutaraldehyde lost bands below 250 kDa on SDS electroforegrams already at concentration of 0,05% [15], however the cross-linking required longer reaction time.

Too high temperatures of protein cross-linking (above 50°C) have a significant influence on the time of reaction, however, in such conditions a simultaneous reaction of polymerisation of the cross-linking reagent alone can proceed [15], and most of all, an independent of the reagent used intermolecular regrouping of disulphide bonds between glutenin residues [16] or gliadins integration to glutenins polymers [17]. Therefore, the cross-linking reactions were carried out below 40°C, in neutral environment, because at such conditions, at minimum gluten proteins solubility in water, the cross-linking reagent is in contact only with aminoacids on the surface of proteins, which in turn favours creation of intermolecular bonds [18], without formation of intramolecular bonds in glutenins' subunits.

The differences between the immunoreactivity of gluten and flour subject to cross-linking can be explained with competitiveness of other proteins present in flour (albumins, globulins) as substrates for cross-linking. Only glutaraldehyde, diethanolamine and sorbitol caused a stronger depletion of protein immunoreactivity in flour than in purified gluten. It points to the lack of substrate competition of the remaining fractions present in flour or attachment of those proteins to gluten in regions recognized by antibodies.

Low values of the remaining gluten immunoreactivity after cross-linking with EDC at the applied quantities of cross-linking reagent (0.13 i 0.64 mmol EDC/g gluten) are surprising, because research on gluten cross-linking a molar ratio 4:1 of EDC to carboxyl groups in proteins was recognized as optimal [18], while at ratios below 0.1:1 no significant changes pointing to cross-linking were observed [19]. Since 1g of gluten contains about 0.4 mmol of free carboxylic groups, then the applied in this work amounts of EDC are far from optimal for cross-linking (the ratio EDC : COOH was 0.325:1 and 1.6:1), however, even in this quantity EDC has a significant impact on gluten immunoreactivity.

The next step of the research was based on plasteinisation, that is hydrolysis of native wheat flour protein with two chosen non-digestive enzymes (subtilisin, collagenase) and a following reaction of chemical cross-linking. Differences in enzymatic hydrolysates of wheat flour immunoreactivity were connected with substrate diversity of the enzymes used. Collagenase is an enzyme hydrolysing peptide bond on the side of carboxylic residue neighbouring proline residue. It is significant, because majority of epitopes recognized by anti-gluten antibodies contain proline residues, which hamper hydrolysis by digestive enzymes. Subtilisin is a nonspecific enzyme, which destructive action towards gluten was confirmed in an earlier study [3, 20]. Both enzymes proved to be a suitable choice for protein hydrolysis before cross-linking, allowing for binding of inert

polypeptide fragments from hydrolysis of other wheat protein fractions to immunogenic polypeptides. Persistence of such bonds during chemical treatment and digestion requires separate research. The only inconvenience of the process turned out to be the fact, that the mass distributions of the plasteinised proteins was not coordinated with the obtained immunoreactivity values (data not shown).

The above research is purely cognitive, however, there are two reasons allowing to hope, that flour modified with chemically induced cross-links will not be toxic. The first one arises from the dose of the reagent; an application of such a small concentration should not leave reaction residues. Secondly, the chosen chemical compounds are applied for production of gluten films used as an easily biodegradable packing material, not polluting the environment. These are not circumstances sufficient to conclude about their usefulness or uselessness for food purposes, however, a research on the toxicity of the chosen cross-linking reagents and emerging cross-linking reaction products is required before they are admitted for consumption.

References

1. Battais F, Courcoux P, Popineau Y, Kanny G, Moneret-Vautrin DA, Denery-Papini S. Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. J Cereal Sci **2005**, 42:109-117.
2. Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C. Structural basis for gluten intolerance in celiac sprue. Science **2002**, 297:2275-2279.
3. Leszczyńska J, Łącka A, Pytasz U, Lukamowicz J, Szemraj J, Lewiński A. Influence of proteolysis on gliadin immunoreactivity. Pol J Food Nutr. Sci **2002**, 11(SI2):145-148.
4. Leszczyńska J, Łącka A, Szemraj J, Lukamowicz J, Żegota H. The influence of gamma irradiation on the immunoreactivity of gliadin and wheat flour. Eur Food Res Technol **2003**, 217:143-147.
5. Osborne TB. The proteins of the wheat kernel. Carnegie Institute, Washington DC, Publ. No. 84, p.1907.
6. Larre C, Denery-Papini S, Popineau Y, Deshayes G, Desserme C, Lefebvre J. Biochemical analysis and rheological properties of gluten modified by transglutaminase. Cereal Chem **2000**, 77:121-127.
7. Bonet A, Caballero PA, Gomez M, Rosell CM. Microbial transglutaminase as a tool to restore the functionality of gluten from insect-damaged wheat. Cereal Chem **2005**, 82:425-430.
8. Caballero PA, Bonet A, Rosell CM, Gomez M. Effect of microbial transglutaminase on the rheological and thermal properties of insect damaged wheat flour. J Cereal Sci **2005**, 42:93-100.
9. Watanabe M, Ikezawa Z, Arai S. Fabrication and quality evaluation of hypoallergenic wheat products. Biosci Biotechnol Biochem **1994**, 58:388-390.
10. Leszczyńska J, Łącka A, Bryszewska M, The use of transglutaminase in the reduction of immunoreactivity of wheat flour. Food Agric Immunol **2006**, 17:105-113.
11. Berti C, Roncoroni L, Falini ML, Caramanico R, Dolfini E, Bardella MT, Elli L, Terrani C, Forlani F. Celiac-related properties of chemically and enzymatically modified gluten proteins. J Agric Food Chem **2007**, 55:2482-2488.

12. Gerrard JA, Sutton KH. Addition of transglutaminase to cereal products may generate the epitope responsible for coeliac disease. Trends Food Sci Technol **2005**, 16:510-512.
13. Wróblewska B, Jędrychowski L. Effect of conjugation of cow milk whey protein with polyethylene glycol on changes in their immunoreactive and allergic properties. Food Agric Immunol **2002**, 14:155-162.
14. Irissin-Mangata J, Bauduin G, Boutevin B, Gontard N. New plasticizers for wheat gluten films. Europ Polymer J **2001**, 37:1533-1541.
15. Reddy N, Tan Y, Li Y, Yang Y. Effect of glutaraldehyde crosslinking conditions on the strength and water stability of wheat gluten fibers. Macromol Mater Eng **2008**, 293:614-620.
16. Lagrain B, Goderis B, Brijs K, Delcour JA. Molecular basis of processing wheat gluten toward biobased materials. Biomacromolecules **2010**, 11:533-541.
17. Lagrain B, Thewissen BG, Brijs K, Delcour JA. Mechanism of gliadin-glutenin cross-linking during hydrothermal treatment. Food Chem **2008**, 107:753-760.
18. Tropini V, Lens JP, Mulder WJ, Silvestre F. Cross-linking of wheat gluten using a water-soluble carbodiimide. Cereal Chem **2000**, 77:333-338.
19. Tropini V, Lens JP, Mulder WJ, Silvestre F. Wheat gluten films cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. Ind. Crop Prod **2004**, 20:281-289.
20. Leszczyńska J, Bryszewska M, Łącka A, Wolska K, Żegota H. Immunoreactivity reduction of wheat flour proteins modified by the treatment with subtilisin, Pol J Food Nutr Sci **2008**, 58:339-344.