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STRUCTURE AND ALLERGENICITY OF WHEAT GLUTEN PROTEINS – A REVIEW

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In persons showing gluten intolerance, wheat storage proteins (gliadins and glutenins) cause IgE-mediated allergies and coeliac disease. Because both of these protein fractions play a significant role in the human diet, intensive research has been carried out to explore the biochemical and molecular reasons for their allergenicity, and the possibilities for eliminating or limiting it. In spite of much progress, this problem has not been solved yet. This paper reviews important information on the structure and conformation of gluten proteins in the context of their allergenicity. Three significant protein structure elements are emphasized. The first is short ("toxic") amino acid sequences that probably function as antibody-binding epitopes in immunological reactions. Other structural elements considered are beta turns, giving allergen particles a specific conformation, and disulphide (SS) bonds which stabilize that structure. Attempts to modify gluten proteins, using various enzyme systems and a thioredoxin reducing system, are also discussed. Such modifications may considerably decrease allergen immunoreactivity. Finally, an interdisciplinary strategy, using methods from genetics, plant breeding, food technology and medicine, is proposed which may aid in developing wheat having decreased allergenicity.

INTRODUCTION

Gluten proteins are an example of a substance whose favourable and usable characteristics are combined with properties that are unfavourable or even harmful to health. These proteins are classified as prolamins, composed of monomeric gliadins and polymeric glutenins. The biological function of these proteins in wheat seeds is storage, but they also have a unique technological role in breadmaking. Elastomeric gliadin and glutenin protein groups interact, forming the viscoelastic gluten matrix having properties that directly affect the quality of bakery products [Kączkowski, 1991]. Foods made of wheat seed are common in human diets, hence gliadin and glutenin are nutrients of great importance.

Unfortunately these proteins, especially gliadins, may be strong food allergens. Their specific properties cause two types of undesirable food reactions to gluten. The first include IgE-mediated allergies, such as asthma, atopic dermatitis, urticaria, angioedema, food allergy or anaphylasis, which constitutes direct mortal danger (*e.g.* food-dependent exercise-induced anaphylaxis) [Bürk *et al.*, 2001; Varjonen *et al.*, 2000; Vissers *et al*., 2001]. Secondly, these proteins may aggravate coeliac disease, a genetically-determined gluten-dependent disease in which proteins damage intestinal membranes and degenerate villi, causing serious disorders of the alimentary system [Wieser, 1996].

Unfavourable effects of gliadin in humans occur only in persons sensitized to wheat proteins, genetically-susceptible to their action. In others, these proteins cause no disease symptoms. The problem is important, however, since from 5% to more than 30% of persons in various social groups and age classes have allergies. Of this group, one-third suffer from food allergies [Crane *et al.*, 2002; Strachan *et al.*, 1997]. Statistical research shows that, in highly developed industrialized West European countries, the number of people at risk of allergy is increasing. During the last three decades, this increase has been especially large [Sly, 1999]. The magnitude of this problem may increase with time because of the irreversibility of processes accompanying cultural and technological development, which contribute to the prevalence of allergies.

Allergenicity of gluten proteins has been intensively studied, and many attempts have been made to limit it. Considering the importance of this problem, investigations of the allergenicity of wheat and other cereal proteins will likely become increasingly important. We need to understand the biochemical and molecular bases of allergenicity, as well as the interactions of the human body with food and plants from which foods are made. Successful research could limit the occurrence or effects of allergens. The research needed should be multi-disciplinary, including several fields of the life sciences, mainly medicine and food technology.

Many medical studies of food allergens have been specifically directed toward patient susceptibility. Such research evaluates the degree of disease symptoms, expressed in the amount of antibodies produced and in

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the intensity of the immunological reaction. This research has yielded important information about effects of plant allergens on the human body, and has helped us to better understand the chemical composition and action of these substances [Marsh, 1992].

Many other studies of food allergens relate to food technology and food chemistry, showing how hazardous raw materials may be biochemically or technologically modified for foods. Such research has given us information about the chemical determinants of allergenicity of various protein groups. Simulation of processes in the human alimentary system, under experimental conditions, has shown that biochemical modification may considerably lessen the hazardous effects of these substances [Maruyama *et al.*, 1998].

Further research progress concerning food allergens may be optimized by combining medical and food technology approaches. Results to date have provided a sound theoretical basis concerning plant genetics and breeding. Continued efforts in these fields could genetically alter protein loci, limiting or eliminating gluten allergenicity. Plants so modified could be used to breed new wheats used to manufacture foods for gluten-intolerant persons. Until now, the so-called "hypoallergenic foods", which cause no allergic reactions, have been produced by modifying raw materials during manufacturing. Then use of plant materials having genetically-reduced allergenicity should enable more effective control of allergies. Such an approach seems to be an effective and socially-justified method of solving the problem of intolerance to food products of plant origin.

This manuscript reviews and summarizes interrelationships between the chemical compositions and allergenicity of gluten proteins. In spite of many studies and much progress, the knowledge on how the structures of plant proteins or of their degradation products cause disease reactions in intolerant persons is still incomplete. Nevertheless, previous achievements may directly support future research which may more effectively limit or eliminate unfavourable properties of prolamins.

STRUCTURE

Most well-known plant allergens are low-molecular weight proteins (3–80 kDa) [Liebers *et al.*, 1996]. Among these are many wheat gluten proteins, including monomeric gliadins. In spite of considerable differences in structure and function, all of these proteins cause increased synthesis of immunoglobulin E (IgE) antibodies in intolerant persons.

Classification of wheat kernel proteins by Osborne. According to a classification scheme first proposed by Osborne, wheat seed endosperm contains four primary protein fractions differing in solubility. They are: (1) albumins, soluble in water; (2) globulins, soluble in dilute salt solutions; (3) gliadins (prolamins I), soluble in 70% ethanol; and (4) glutenins (prolamins II), soluble in acid solutions [Osborne, 1907]. Subsequent progress in cereal chemistry has revealed numerous protein subfractions within these classes, and has led to modern classification systems [Bietz & Wall, 1980; Kączkowski, 2002]. Nevertheless, Osborne's classification remains important today, both from a historical perspective, and because the terms he introduced are still commonly used in everyday language and publications.

Wheat's gliadin and glutenin proteins, also termed "prolamins" because of their high content of proline and glutamine, function primarily as storage proteins [Tatham *et al.*, 1990]. They differ from each other not only in primary, but also in secondary, tertiary and quaternary structures. Gliadins are monomeric polypeptides with weak hydrogen bonds. Glutenins are polymeric complexes formed by covalent intermolecular SS bonds [Kączkowski & Bernacka-Mieleszko, 1980]. Due to these differences, gliadins and glutenins are also termed prolamins I and II, respectively. Wheat's other protein fractions, the albumins and globulins, are mostly biologically-active, performing catalytic and regulating functions [Garcia-Casado *et al.*, 1994].

Physicochemical properties of gluten proteins. Important information about the physicochemical structure and inheritance of gluten proteins has come from studies using electrophoresis. For gliadins, acidic electrophoresis (A-PAGE) on polyacrylamide gels in lactate-aluminium buffer is most often used [Bushuk & Zillman, 1978]. Electrophoretic patterns among wheat cultivars and strains differ, permitting their identification [Waga, 1997]. Upon A-PAGE, gliadins separate into four groups differing in molecular weight and electric charge, designated α, β, γ and ω (Figure 1). The lowest molecular weight proteins (about 10 kDa) are in the group α, and the highest molecular weight proteins (40–100 kDa) are classified in the group ω [Woychik *et al.*, 1961]. Studies into the inheritance of storage proteins have shown that genes coding gliadin synthesis are on chromosomes *1A*, *1B*, *1D*, *6A*, *6B*, and *6D* [Payne, 1987]. Genes coding each gliadin type have specific chromosomal locations: the heaviest ω-gliadins are coded by genes on chromosome *1D*; lighter ω-gliadins by chromosomes *1A* and *1B*; γ-gliadins by chromosomes *1A*, *1B*, *1D* and *6B*; β-gliadins by chromo-

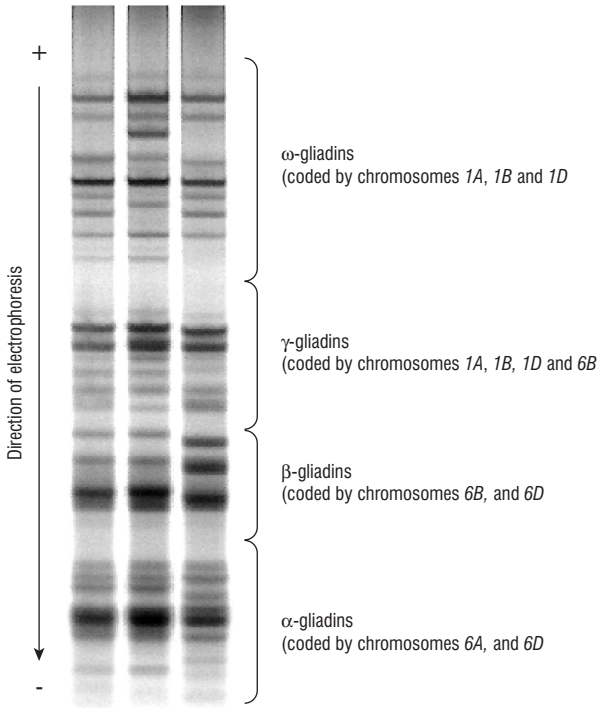

FIGURE 1. Gliadin proteins of chosen winter wheat genotypes analysed by acid polyacrylamide gel electrophoresis (A PAGE). Chromosomes coding for individual bands belonging to α, β, γ and ω fractions are indicated.

somes *6B* and *6D*; and α-gliadins by chromosomes *6A* and *6D*. This information is important not only from a genetic standpoint, but also because it relates to gliadin allergenicity, as discussed below.

While many gliadin fractions may elicit allergy symptoms, α-gliadins have long been known to be most immunoreactive [Kowlessar & Sleisenger, 1963]. A subfraction of α-gliadin, A-gliadin, is of special importance. A-gliadin can

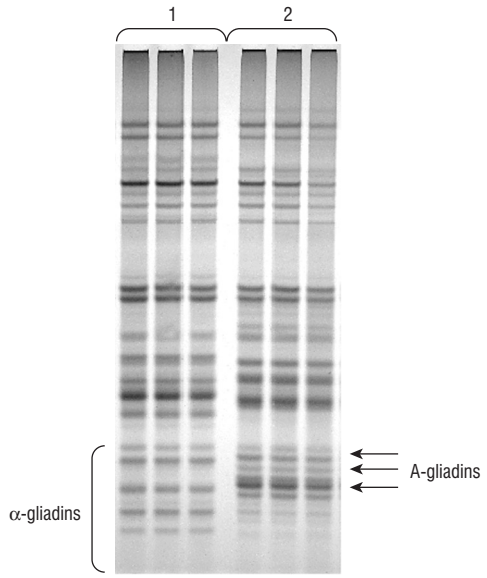

FIGURE 2. Differentation of α-gliadin zone among winter wheats. Genotype 1 does not contain while genotype 2 contains A-gliadins. Combination of electrophoretic bands typical for A-gliadins is marked by the arrows.

be isolated by ultracentrifugation [Kasarda, 1980]. Its polypeptides form aggregates, visible by electron microscopy as fibres forming a dense network. Its structure is stabilized by interchain hydrogen, ionic, and hydrophobic bonds. Genes on chromosomes *6A* and *6D* control synthesis of α-gliadins. A-gliadin is one allelic variant of α-gliadin, coded by chromosome *6A* (locus symbol *Gli A2*, designated *Gli A2-1*; "1" is the catalogue number used by this author [Waga, 2000]). Upon A-PAGE, A-gliadin shows a specific, easy to identify combination of bands (Figure 2). A-gliadin is present in more than 40% of wheat cultivars and strains in Polish working collections. Other wheats contain other α-gliadin variants [Waga & Węgrzyn, 2000].

The polymorphism of glutenin proteins is, unlike that of gliadins, best investigated using electrophoresis on polyacrylamide gel in a basic medium with the addition of sodium dodecyl sulphate (SDS) (SDS PAGE) [Laemmli, 1970]. Electrophoresis reveals two protein groups: high-molecular weight and low-molecular weight glutenin subunits (Figure 3). However the latter overlap gliadin fractions, so SDS PAGE is used mainly to identify high-molecular weight glutenin subunits. These polypeptides consist of two types of subunits: x – with higher molecular weight, and y – with lower molecular weight [Payne *et al.*, 1981]. Proteins of these groups differ considerably in immunoreactivity [Curioni *et al.*, 1991].

Regions and domains of gluten proteins. The amino acid sequences – or primary structures – of gluten proteins are

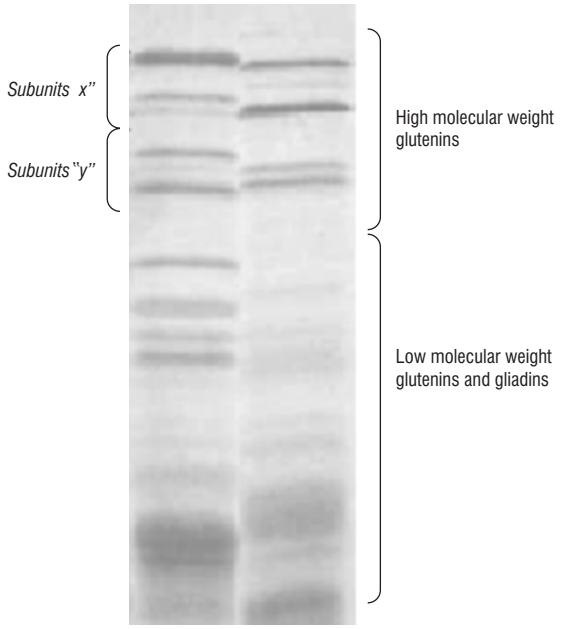

FIGURE 3. Glutenin proteins of two winter wheat genotypes analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). High molecular weight glutenins as well as subunits of "x" and "y" type are indicated.

of utmost importance in determining their characteristics. Both gliadins and glutenins have regions containing specific repeatable amino acid sequences (Figure 4), formed from short peptides, repeated many times [Tatham, 1995], but they differ in the arrangement, size, and sequence of repeated regions.

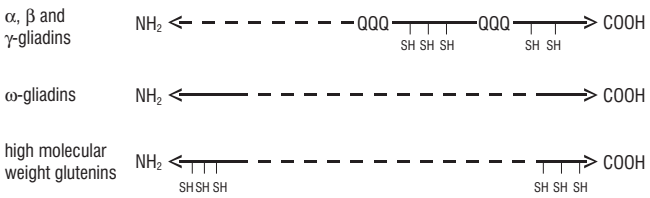

FIGURE 4. Schemes of gliadin and glutenin polypeptides. Regions of specific (continous line) and repeated (dashed line) sequences, polyglutamine regions (QQQ) and cystine residues (SH) are marked.

Alpha-gliadin proteins consist of an N-terminal region, followed by a region of repeatable sequences which is linked to a C-terminal region. The N-terminal region is very short, and the sequence repeat consists of five amino acids, not clearly separated from the C-terminal region. Thus, α-gliadins are sometimes divided into only two regions, an N-terminal region with repeatable sequences, and C-terminal region containing specific sequences. Alpha-gliadins also have a considerable number of cysteine residues in specific sequences [Shewry & Tatham, 1997]. Two domains rich in glutamine (the so-called "polyglutamine regions") are also a specific element of the primary structure of α-gliadins. One of these domains is at the border of the repeatable and specific sequences, and the other is in the middle of the specific sequences region [Anderson *et al.*, 1991]. A similar structure has been found for β and γ gliadins, and for low-molecular glutenins. For both α and β gliadins, sequences

of repeatable regions are formed of the peptides Pro-Gln--Gln-Pro-Phe-Pro and Pro-Gln-Gln-Pro-Tyr. For γ gliadins, the repeat is the peptide Pro-Gln-Gln-Pro-Phe-Pro-Gln [Sygiyama *et al.*, 1986].

A different structural model exists for the highest molecular weight gliadins. Omega-gliadins contain short, distinct C and N terminal sequences, separated by a region of repeatable sequences similar to γ gliadins. The sequence differs from that of γ gliadins, however, in having additional glutamine at the end of the peptide (Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln). In contrast to other gliadin fractions, ω-gliadins contain no cysteine. Thus, α, β and γ gliadins are defined as proteins rich in sulphur (S^+), and ω gliadins as deficient in sulphur (S^-). The formulations cited refer to sulfhydryl (SH) groups in cysteine molecules [Kączkowski, 2002].

High-molecular weight glutenin subunits contain a long central region of repeatable sequences between their N and C terminal regions, as do ω gliadins. Repeatable regions include the sequences Pro-Gly-Gln-Gly-Gln-Gln (typical for both the "x" and "y" subunits), Gly-Tyr-Tyr-Pro-Thr-Ser--Leu-Gln-Gln, Gly-Gln-Gln (only for the "x" subunits), and Gly-Tyr-Tyr-Pro-Thr-Ser-Pro-Gln-Gln (only for the "y" subunits). The terminal regions contain specific sequences and, contrary to ω gliadins, do contain cysteine molecules.

Toxic sequences. Short amino acid sequences composed mainly of proline and glutamine are important elements of the primary structure of prolamins, and are related to their allergenic properties. Allergies are related to the pentapeptide Gln-Gln-Gln-Pro-Pro, and coeliac disease to the tetrapeptides Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln. In the literature, these are referred to as "toxic peptides".

The pentapeptide Gln-Gln-Gln-Pro-Pro has the specific ability to bind to IgE antibodies in serum of patients with allergy symptoms [Watanabe *et al.*, 1995]. When gluten was digested with chymotrypsin and the resulting peptides separated by chromatography, several allergenic fractions were obtained, as revealed by ELISA using serum of persons having atopic dermatitis. The most allergenic component, a tridecapeptide, was purified and sequenced. It contained four characteristic Gln-Gln-Gln-Pro-Pro type sequences. This is actually the shortest sequence necessary for IgE binding; the N-terminal glutamine residue plus two proline residues are of prime importance, while other amino acids play a secondary role in IgE binding [Tanabe *et al.*, 1996].

Evidence for activity of short peptides in coeliac disease was presented by De Ritis *et al.* [1988], who expressed peptide activity as a function of intestinal mucosa enterocyte cell enlargement in *in vitro* cultures. When the 266-residue A-gliadin was cleaved with cyanogen bromide, three peptides (CB1, CB2 and CB3) resulted (Figure 5). CB1 and CB2 caused growth of enterocytes from persons with coeliac disease, while CB3 was inactive. CB1 was then digested with chymotrypsin, yielding three smaller fragments (Ch1.1, Ch1.2 and Ch1.3). Only Ch1.1 was active. It was again digested with chymotrypsin, yielding "toxic" peptides Ch1.1.1 and Ch1.1.2. All peptides found to be active in coeliac disease contained the sequences Gln-Gln--Gln-Pro and Pro-Ser-Gln-Gln; inactive peptides contained no such sequences. These sequences are thus of special importance for disease development [Marsh, 1992]. Most "toxic" tetrapeptides (3 of 5 identified) came from the region of repeatable sequences near A-gliadin's N-terminal region (residues 1 to 55); thus, this region is considered the most "toxic" within the protein molecule. Only two "toxic" tetrapeptides were found in the region of specific sequences, and A-gliadin's C terminal region showed no "toxicity".

The immunological activity of Gln-Gln-Gln-Pro-Pro in allergies and Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln in coeliac disease was confirmed by others [Maruyama *et al.*, 1998; Wieser, 1996]. The toxicity of these peptides was also shown by substitution of individual amino acids; such modifications caused total loss of peptide immunoreactive properties [Kocna *et al.*, 1991].

It is unclear why Gln-Gln-Gln-Pro-Pro, active in allergies, is not active in coeliac disease, even though it contains the motif Gln-Gln-Gln-Pro [Maruyama *et al.*, 1998]. It may be that these peptides cannot cause disease symptoms by themselves since they are too small to effect release of histamine from basophils, and can only function as antibody binding epitopes. It is thus hypothesized that Gln-Gln-Gln--Pro-Pro is an epitope indispensable for IgE binding, and the release of histamine results from a high concentration of tridecapeptides.

It is notable that the sequence Pro-Ser-Gln-Gln also occurs in capsule proteins of the adenovirus Ad12, which infects the alimentary tract. When a person with coeliac disease is so infected, resulting symptoms are similar to those that appear after consumption of gluten-containing products. This is further evidence that "toxic" sequences

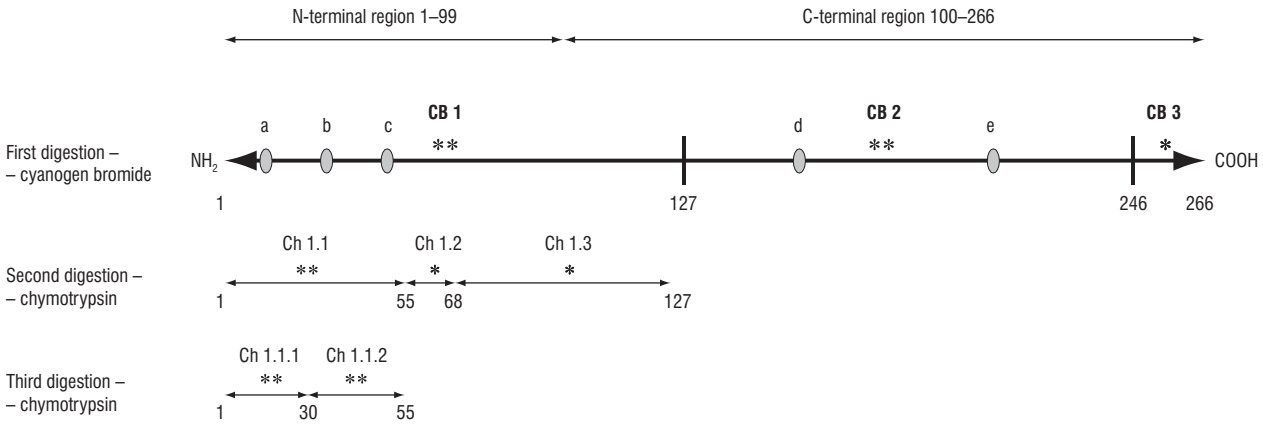

FIGURE 5. Peptides obtained after digestion of A-gliadins by cyanogen bromide (CB) and chymotrypsin (Ch) and their activity in coeliac (** high activity, * lack of activity). – Localization of "toxic" peptides: a – PSQQQP, b – QQQP, c – PSQQ, d – QQQP, e – PSQQ

cause the disease. However, antibodies against Ad12, which bind synthetic α-gliadin peptides, do not link with peptide CB1, containing two Pro-Ser-Gln-Gln sequences [Marsh, 1992]. Consideration of other differences in structures of Ad12, synthetic α-gliadin and peptide CB1 suggests that the specific "toxic" properties of Pro-Ser-Gln-Gln depend on the structure of the whole protein molecule, on combination with other amino acids, on conformation, on the degree of folding, and/or on the configuration of higher--order structures.

Beta turns. Beta-type turns, which give polypeptides a specific shape, are an important element of the secondary structure of gluten proteins, connected with their allergenic properties [Kasarda, 1994]. They result from a change in polypeptide direction caused by the presence of certain amino acids [Stryer, 2000]. For example, proline lacking a hydrogen atom at the amide group does not form hydrogen bonds, thus facilitating considerable changes in chain turn angle. Other amino acids favouring formation of β turns include glycine, asparagine and serine. The resulting polypeptide configuration often functions as a "clamp", linking two antiparallel β structures. Numerous repeat sequences also favour formation of β turns; the resulting single turns tend to overlap and form tight loops on the protein surface.

In gluten proteins, formation of such structures is much easier due to the presence of proline in the repeatable sequences: Pro-Phe-Pro in gliadins (in ω gliadins additionally Pro-Gln-Gln), and Gly-Gln-Gln in high-molecular glutenin subunits [Tatham, 1995]. Such amino acid combinations can occur in the regions of repeatable sequences, *i.e.* in the central regions of high-molecular glutenin subunits and ω gliadins, and in the N-terminal region of α, β and γ gliadins.

When analysing A-gliadin by circular dichroism, Tatham *et al.* [1990 b] found β turns in all four tetrapeptides of that type and in Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln occurring in the molecule of that peptide. In an attempt to explain the immunological activity of these structures in coeliac disease, the authors hypothesized that they form loops when overlapping on the protein surface, cause its polarization. This increases the antigenic character of the particle, facilitating its contact with both the intestinal mucosa and the antigen presenting proteins (class II surface MHC proteins), and with the receptors of auxiliary lymphocytes T [Tatham *et al.*, 1990 a]. In addition, such structures facilitate development of antibodies which cause phenomena accompanying the immunological reaction.

Disulphide bonds (SS). Another element of gluten protein structure, related to their allergenicity, is the presence of disulphide bonds (SS). They are formed by oxidation of sulfhydryl (SH) groups of cysteine molecules [Kączkowski & Bernacka-Mieleszko, 1980]. SS formation causes protein folding as two cysteine SH groups come close to each other. This process occurs in the endoplasmic reticulum and the Golgi apparatus, and is an important type of posttranslational modification of proteins [Stryer, 2000]. SS bonds are common in both plant and animal proteins, suggesting that this structure is important for proper functioning. In proteins having similar function, the location of SS bonds is conserved, confirming their evolutionary importance. They are the macroerg bonds whose energy in water is 376.6 kJ/mol and exceeds considerably the energy of ionic bonds (only 12.6 kJ/mol) and of hydrogen bonds and van der Waals forces (4.2 and 0.4 kJ/mol, respectively) [Alberts *et al.*, 1999]. To cleave SS bonds, strong reduction is required; to reconstruct them, weak oxidation is sufficient. SS bonds stiffen protein conformations, and are important in stabilizing higher-order structures. SS bonds increase proteins' resistance to thermal denaturation, proteolytic digestion, and changes in the medium reaction. SS bonds may be cleaved at >50°C, and chemical modification (such as reduction and alkylation) makes protease digestion (especially by trypsin) easier. Thus, SS bonds, due to their ability to strengthen the protein structure, are indispensable for proper functioning of various proteins, such as those which are biologically active.

In gluten proteins, SS bonds are both inter- and intra--chain. Intermolecular SS bonds occur mainly in glutenin [Shewry & Tatham, 1997]. Cysteine molecules in terminal regions of high molecular weight subunits link polypeptides in a cascade-like way, forming complex polymers. This configuration gives the gluten matrix elasticity, extensibility and shrinkability. This is a major factor explaining gluten's unique physicochemical properties, and the technological properties of bakery products made from wheat grain. Due to the great strength of SS bonds, gluten effectively resists the high pressure of CO_2 released as dough ferments, and expands. The technological parameters (such as volume, shape and texture) of wheat bakery products thus considerably exceed those of products made from other cereals. Inter-chain SS bonds are a model example of the relationship between molecular structure and final product mechanical properties [Ewart, 1985].

In contrast to glutenins, SS bonds in gliadins are primarily intra-chain. This increases amino acid chain folding [Müller & Wieser, 1997]. Their presence may relate to gliadin allergenicity, as suggested by studies on specific reduction of SS bonds to free SH groups. Reduced proteins show a considerably decreased affinity for specific antibodies, which, in consequence, can be connected with a decrease in their allergenicity [Kączkowski, 2000]. Specific reduction of intra-chain SS bonds, without breaking inter-chain bonds, may be achieved by biologically-active oxidation-reduction proteins, such as thioredoxin (TR), a low-molecular weight (12–13 kDa) protein.

In living organisms, TR occurs with thioredoxin reductase (TRR) and NADPH, forming the so-called "thioredoxin system", which plays an intermediary role in NADPH oxidation and TR reduction [Holmgren *et al.*, 1975]. TR contains, in its active centre, a disulphide bond (SS) which after reduction transfers electrons to a target protein (*e.g.* gliadin), reducing specific intra-chain SS bonds (Figure 6). Reduced target proteins contain free SH groups in place of SS bonds [Kobrehel *et al.*, 1992]. This process is a preparatory stage to proteolysis of seed storage proteins; the reverse reaction (*i.e.* formation of SS bonds) occurs during seed maturation [Gobin *et al.*, 1996, 1997].

Based on farinograph analyses, Wong *et al.* [1993] stated that specific reduction of gluten proteins improves the rheological properties of modified gluten. They suggested that SH groups released upon cleavage of intra-chain SS bonds

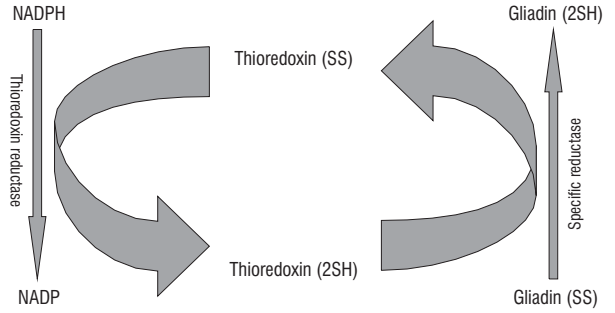

FIGURE 6. Specific reduction of gliadins' intramolecular disulphide bonds (SS) by thioredoxin system and gliadin specific reductase.

facilitate rearrangement and crosslinking of SS bonds of the gluten matrix, thus increasing its elasticity. Hence, thioredoxin decreases the amount of free gliadins and increases the molecular weight of polypeptides engaged in gluten complex formation.

One microbaking study [Waga *et al.*, 2003] showed that the quality of products made from TR-modified flour was neither improved nor decreased. However, considerably decreased affinity between modified gliadin and antigliadin antibodies was noted, proving decreased immunoreactive properties. Such a change resulting from protein modification, providing that baking properties of flour are maintained, is therefore favourable for usability of bakery products.

Glycosidic bonds. Another element of protein structure that can relate to their allergenicity is glycosidic bonds resulting from the addition of saccharides to the amino acid chain. The glycosylation process, like that of SS bond formation, takes place in the endoplasmic reticulum and is one of the ways of protein posttranslational modification [Alberts *et al.*, 1999]. Glycosylation concerns the sequences Asn-X-Ser and Asn-X-Thr, where X is any amino acid. As a result of adding a polysaccharide to asparagine, serine or threonine, an N-glycosidic linkage is formed (most frequently occurring glycoproteins) by the amide group of asparagine, or a O-glycosidic linkage is formed by the OH group of serine or threonine. The allergenicity of such structures has been illustrated by glycosylated subunits of tetrameric inhibitors of barley α-amylase [Mena *et al.*, 1991]. It is closely connected with saccharide chains added to a protein through asparagine. Carbohydrate-protein complexes have always bound strongly to IgE antibodies (from patients showing symptoms of bakers' asthma), whereas detachment of saccharides inhibited the immunological reaction. Also, when we compare glycosylated subunits of barley α-amylase, which bind IgE antibodies, with analogous rye enzymes lacking saccharides (which do not show such a property), it is evident that saccharides play an important role in binding antibodies, and hence in allergenicity of these proteins [Garcia-Casado *et al.*, 1994].

Amino acid sequences of gluten proteins contain asparagine, as well as tripeptides that can bind saccharides. In the N-terminal regions of α-gliadins (residue 12), near the sequences considered as potential allergens, Asn-Pro-Ser-Gln-Gln-Gln-Pro occurs [Shewry *et al.*, 1980]. This sequence is separated from a serine molecule by one proline molecule, giving in effect a tripeptide sequence in which asparagine can undergo glycosylation. An identical combination of three amino acids occurs in higher-molecular weight ω-gliadins.

Experimental results confirm this hypothesis. Two-dimensional electrophoresis (A-PAGE + SDS-PAGE) and immunoblotting using antibodies specific for saccharides and N-glycanes containing xylose showed that combinations with xylose occur in low-molecular glutenins, while high-molecular glutenin subunits and gliadins contain other saccharides bound to asparagine [Lauriere *et al.*, 1996]. While there is yet no empirical evidence of allergenicity of glycosidic bonds for gliadins and glutenins, the fact that they occur in gluten proteins and that they are allergenic in proteins of other cereal species suggests a relationship between the presence of glycosidic bonds in gluten proteins and allergenicity.

ALLERGENICITY

In spite of significant differences between allergy and coeliac disease, both health disorders result from improper functioning of the immunological system.

In coeliac disease, the enzyme tissue transglutaminase (tTG), occurring in various mammalian tissues and systemic fluids, is of special importance for disease development [Szaflarska-Szczepanik *et al.*, 2002]. This enzyme catalyses formation of isopeptide bonds between glutamine and lysine. Gliadin (containing much glutamine), introduced into a sensitive person, undergoes modification in which monomeric polypeptides are transformed to high-molecular weight gliadin-gliadin or gliadin-tTG complexes. They are especially effectively bound by the main histocompatibility complex proteins (of HLA DQ2 type), and are then recognized by gliadin specific T lymphocytes. These reactions initiate inflammatory processes having symptoms characteristic of coeliac disease, such as intestinal villi atrophy or small intestine mucosa damage [Szaflarska-Szczepanik *et al.*, 2001].

Only 50 years ago, gliadin was declared the main antigen in coeliac disease [Dicke *et al.*, 1952]. Modern criteria for diagnosing the disease were established by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) in 1989. Coeliac disease is now recognized when after including gluten in a diet clinical symptoms typical of that disease occur, and when in a patient's serum specific antiendomysium (EmA) or antireticulin (ARA) antibodies are identified in the class IgA, and when IgA is lacking – in the class IgG [Czerwionka-Szaflarska, 2002].

All four gliadin fractions can produce symptoms of coeliac disease in sensitive persons; α-gliadins, especially A-gliadin, are most active. A-gliadin, coming in contact with the small intestinal surface, causes characteristic changes in the epithelium. This has been confirmed by an *in vitro* study of intestine surface fragments from patients [Wieser, 1996]. Pathological changes were so severe that at first allergenicity was related only to A-gliadins. Further studies, both *in vivo* and *in vitro*, suggest that in coeliac disease all the gliadin groups can be active [Howdle *et al.*, 1984]. For example, the ability of intestinal T cells from persons with coeliac disease to recognize γ-gliadin epitopes indicates their "toxicity" [Varjonen *et al.*, 2000]. Such a possibility is also suggested by the presence of Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln tet-

rapeptides in β, γ and ω gliadins. Nevertheless, the problem is still under discussion, while the "toxicity" of A-gliadin is not questioned.

Another type of immunological activity of wheat storage proteins occurs in allergy. Sensitized persons produce excessive amounts of IgE antibodies against gluten proteins and their analogs from rye, barley, and oats. Linkage of antigens with antibodies results in release of histamine from basophils (*i.e.* cells especially rich in histamine which, as a result of the immunological reaction, come out), which, in various ways, damage organs. There is a relationship between gliadin polymorphism and the type of disease symptoms they produce; in some cases, certain groups of these proteins are stronger allergens than they are at other times.

Low-molecular weight α-gliadins, in addition to causing coeliac disease, can also cause symptoms of dermatitis [Varjonen *et al.*, 2000]. The *ca.* 14 kDa fraction, like IgA and IgG antibodies in persons with coeliac disease, can also bind IgE antibodies in patients with atopic dermatitis. Beta--gliadins, which are important allergens in those with coeliac disease showing symptoms of atopic dermatitis, have similar properties. This is proven by the ability of IgE, IgA and IgG antibodies, present in patients' serum, to bind polypeptides of molecular weight 30 to 43 kDa.

Gamma-gliadin can cause anaphylaxis symptoms. Studies using immunoblotting showed that chromatographically purified γ-gliadin forms strong bonds with antibodies of patients' serum [Palosuo *et al.,* 1999]. ELISA proved that sensitive persons' serum showed a significantly increased level of IgE against that fraction. Other studies of patients with symptoms of anaphylactic shock showed that γ-gliadins can inhibit the immunological reaction between the gluten protein complex and serum IgE [Morita *et al.*, 2001]. Thus, in the case of the anaphylactic shock, γ-gliadins are a much stronger allergen than other gluten proteins.

Considering dermal symptoms, the γ fractions (like β fractions) can be a cause of urticaria, but they do not induce atopic dermatitis. Varjonen *et al.* [1999] observed IgE antibodies against ethanol-soluble prolamins in patients with urticaria symptoms. The molecular weight of these proteins was 30–50 kDa, making it possible to classify these proteins as β and γ gliadins. Dermatological symptoms caused by food allergens can result from the ability to deposit immunological complexes in the subcutaneous layer [Vainio *et al.*, 1983]. Another cause of that phenomenon can be the nonspecific cross reaction of gliadin with reticulin protein in skin.

Omega-gliadins cause allergy symptoms in children. One chromatographically-purified ω5 fraction (about 77 kD) is an allergen responsible for breathing trouble, food allergy and atopic dermatitis. Serum of children aged about 2.5 who showed severe symptoms of these diseases contained much IgE antibody against ω5 gliadin [Palosuo *et al.*, 2001]. The same protein can also be an important allergen in anaphylactic shock in adults. An increased level of IgE, as well as of IgA and IgG antibodies, and increased release of histamine from basophils in patients accentuates the role of ω5-gliadin in this disease [Lehto *et al.*, 2003].

Although the harmful action of gliadins in allergic persons intolerant of gluten raises no serious doubts, the role of glutenins as food allergens has not been clearly explained. Some reports imply that the immunological reaction involving glutenins and anti-glutenin IgE antibodies is possible; others do not connect them to allergy symptoms. Sandiford *et al.* [1997] found IgE antibodies against high-molecular glutenin subunits in serum of persons with bakers' asthma. The immunoreactive properties of these glutenin subunits were comparable to those of α- and ω-gliadins. However, in bakers' asthma, the strongest allergens are albumins and globulins. Sandiford *et al.* [1997] accepted a hypothesis that the observed effect results from structural similarity of epitopes in the mentioned protein groups. Sutton *et al.* [1982] observed strong bonds in children with asthma and eczema between serum IgE antibodies and a glutenin fraction, which may prove the allergenic properties of glutenin polypeptides in these diseases.

Kushimoto and Aoki [1985], by digesting high-molecular glutenins with pepsin, obtained polypeptides of molecular weight 15 to 100 kDa. These peptides exhibited allergenicity in patients showing urticaria symptoms. In the authors' opinion, digestion of glutenins with pepsin and trypsin can cause increased allergenicity by releasing epitope-containing peptides directly bound to IgE antibodies.

Simonato *et al.* [2001] found that allergenicity of glutenins occurs when the gluten matrix is shaped, and as a result of processes during baking. Allergenic properties of lower molecular weight proteins then disappear, and emerge in higher molecular weight fractions.

Gluten proteins are, however, not the only allergenic components of wheat seed. Albumins and globulins also exhibit strong allergenic properties, especially in asthma and bakers' asthma [Sanchez-Monge *et al.*, 1992; Garcia-Casado *et al.*, 1994, 1996; Armentia *et al.*, 2002]. Other important allergens include lipid transfer proteins (LTP), causing urticaria and angioedema symptoms [Breiteneder & Ebner, 2000; Pastorello *et al.*, 2001].

It is noteworthy that the discussed proteins, in addition to causing alimentary and respiratory diseases (these being the main routes in which antigens are introduced), can also cause conditions such as skin diseases, cerebellar ataxia, or psychosomatic complications. This indicates that complexes of proteins with specific antibodies can go from blood to other systems, causing damage.

INFLUENCE OF BIOCHEMICAL MODIFICATIONS ON WHEAT GLUTEN PROTEIN ALLERGENICITY

Studies on limiting or eliminating allergenicity of gluten proteins by biochemical modification have been done for years. Results imply that, under laboratory conditions, gluten's unfavourable properties can largely be altered. Studies of the enzymatic digestion of prolamins enable us to perform an *in vitro* simulation of processes in the alimentary system. Such research may indicate how natural human enzymes may be used on an industrial scale to make bakery products for persons intolerant of gluten. The specificity of such modification depends on fragmentation of the amino acid chain. Proteolysis may yield fragments less allergenic than the protein substrates. However, change in hydrolysis direction can also cause a separation of complete allergenic sequences and, in effect, an increase in hydrolysate allergenicity.

Research results confirm the occurrence of both possible cases. In most studies, decreased immunoreactive properties of gluten upon digestion with pepsin, trypsin, or chymo-

trypsin was observed [Sutton *et al.*, 1982; Szabo *et al.*, 2002; Leszczyńska *et al.*, 2002; Maruyama *et al.*, 1998]. A method to produce hypoallergenic bakery products and pasta was developed, in which proteolytic digestion is a key stage in manufacturing [Watanabe *et al.*, 1994]. However, Kushimoto and Aoki [1985] found that digestion of gliadins and glutenins with pepsin formed highly allergenic 15–100 kDa peptides. They also observed that peptides formed by pepsin digestion were more allergenic than those resulting from trypsin digestion. Simonato *et al.* [2001] found that pepsin and pancreatin proteolysis, simulating digestion in the alimentary tract, unfavourably modified wheat protein allergenicity. In hydrolysates, the allergenicity of low-molecular weight proteins of the α-amylase inhibitor group disappears, and emerges in high-molecular weight fractions (*e.g.* in high--molecular glutenins).

It should be emphasized that proteolytic digestion can modify the harmful properties of gluten proteins only for typical allergies. In coeliac disease, digestion of these proteins with pepsin or trypsin did not affect their "toxicity" [Sutton *et al.*, 1982].

Another method for enzymatic modification of gluten is deamidation using transglutaminase (TG). In contrast to proteolytic enzymes, TG does not attack typical peptide bonds, but rather detaches 5-amide NH_2 groups from glutamine. TG can modify immunoreactive properties of gliadins, decreasing their ability to bind both polyclonal and monoclonal antigliadin antibodies [Leszczyńska *et al.*, 2002]. In patients intolerant of gluten, a decreased ability of enzymatically-modified gliadin to bind human serum IgE antibodies was also found. Another reaction was observed in persons with coeliac disease. As a result of deamidation, the ability of the main histocompatibility complex proteins (MHC II) to bind modified gliadin molecules increases. At the same time, the ability of auxiliary T cells, formed within the alimentary system, to recognize complexes containing an antigen increases, initiating an immunological reaction in persons with coeliac disease [Molberg *et al.*, 1998].

During chemical deamidation of gluten with acetic acid, glutamine changes to glutamic acid. This considerably decreases the ability of antigliadin antibodies from persons with coeliac disease to recognize modified gluten [Berti *et al.*, 2002].

Another modification, specific reduction of SS bonds by thioredoxin (TR), was described earlier. This type of modification is advantageous, as compared with enzymatic digestion, in that it has no negative effect on gluten's rheological properties. At the same time, it considerably decreases immunoreactivity of gliadin proteins [Waga *et al.*, 2003]. The high price of TR, however, makes this approach impractical for producing "hypoallergenic food" on an industrial scale. It would be desirable to develop a technique for producing inexpensive TR using biotechnological methods, or to find a more available natural substitute having comparable properties.

Similar results were obtained for proteins soluble in salt solutions, using buffers with or without addition of the reducing agent – dithiothreitol. As a result of structure modification, allergenicity decreased, as proven by ELISA, using antibodies from patients intolerant of gluten for analysis [Klockenbring *et al.*, 2001]. This further proved that both specific amino acid sequences and protein conformation influence allergenicity. Reduction of gluten with other reducing agents will probably give similar results.

Factors increasing allergenicity of gluten proteins should also be considered. One such factor is temperature [Simonato *et al.*, 2001; Maruyama *et al.*, 1998]. During baking, high temperature causes gluten proteins to form complexes resistant to proteolytic digestion. Thus, some hazardous gluten components could be introduced into the intestine from baked products, where they could contact mucosa and antibodies, initiating a severe immunological reaction. Heated polysaccharides can also bind amino acids *via* the Maillard reaction, favouring increased allergenicity of the complex [Hansen & Millington, 1979].

Results differ for gluten denaturated during extraction or electrophoresis. Binding of antibodies is stronger, but these results may not be practically significant, since in nature such complexes do not occur.

FINAL CONCLUSIONS AND FUTUTRE PERSPECTIVES

This review draws attention to various aspects connected with the structure and allergenicity of gluten proteins. Some of the most important aspects are here summarized.

Allergenicity of gliadins or glutenins is not a typical feature of natural proteins, but is a characteristic of their degradation products, formed by proteolysis in the alimentary system. Prior studies show that these degradation products are primarily short peptides. This suggests that genetic or biochemical modification of wheat storage proteins may enhance the health properties of wheat, a chief raw material in foods.

Also noteworthy is the variability of structures connected with allergenicity of gluten. In this paper, the role of "toxic" sequences, SS bonds, and β turns is emphasized. It can be concluded that the activity of protein allergens results both from the primary structure of epitopes ("toxic" sequences) and from protein conformation (SS bonds, and β turns). Short peptides resulting from gluten digestion probably function as epitopes. However the effectiveness of their binding with antibodies is determined by protein conformation, which is largely shaped by β turns. SS bonds probably stabilize higher-order structures and protect epitopes from digestive enzymes and extreme pH values in the alimentary tract. This hypothesis is supported by studies of specific protein reduction *via* the thrioredoxin system: unfolding protein molecules and changing their conformation facilitates access of proteolytic enzymes to digestion spots, thus damaging epitopes related to immunoreactive properties.

Various authors have related gluten protein structures to immunological activity in allergies, or to "toxicity" in coeliac disease [Kasarda, 1994; Kobrehel *et al.*, 1992; Shewry & Tatham, 1997]. These unfavourable properties are probably the result of all the above-mentioned structural elements. No single structural determinant can explain allergenicity. Both β turns and SS bonds occur in many nutritionally safe plant proteins. "Toxic" proteins show immunoreactivity only when they exist in a definite conformation [Marsh, 1992]. It can thus be concluded that combinations of gluten proteins that form a specific secondary structure, rather than individual protein structures, cause allergenicity.

Also important is the relationship between gliadin molecular weights and disease. This may make it possible to

breed wheats containing proteins with decreased immunoreactivity. By crossing cultivars varying in protein composition and selecting progeny, it may be possible to select genotypes with more favourable traits than their parents.

If individual gliadins groups cause various disease symptoms, we should differentiate allergenicity of allelic protein variants to determine the intensity with which they sensitize susceptible persons. Such studies, necessitating coordination of plant genetics, food technology, and medical approaches, have not yet been done.

This paper suggests research needed to limit the harmful properties of gluten proteins. Gluten allergenicity is a complex problem, difficult to resolve quickly. Combining plant genetics with biochemical and technological modifications, with evaluation of effects in clinical tests, may provide a solution. Nevertheless, wheat flour with modified gluten proteins may never be totally safe, since other proteins (albumins, globulins, LPT) are also allergenic. Modification or elimination of such factors should be the subject of separate studies.

It is likely, however, that genetic and biochemical modifications will enable the use of wheat seed for production of foods of high technological and nutritional value, that will be healthy and safe for all people.

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STRUKTURA I WŁAŚCIWOŚCI ALERGENNE BIAŁEK GLUTENOWYCH PSZENICY – ARTYKUŁ PRZEGLĄDOWY

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U osób z nietolerancją glutenu białka zapasowe pszenicy (gliadyny oraz gluteniny) wywołują alergie zależne od IgE oraz chorobę trzewną – celiakię. Z uwagi na znaczącą rolę, jaką obie frakcje białkowe odgrywają w żywieniu człowieka prowadzone są intensywnie badania nad określeniem biochemicznych i molekularnych podstaw ich alergeniczności oraz możliwością jej eliminacji czy chociażby tylko ograniczenia. Mimo postępu w tej dziedzinie problem nie został dotychczas rozwiązany. W artykule dokonano przeglądu ważniejszych informacji na temat struktury i konformacji przestrzennej różnych frakcji białek glutenowych w kontekście ich właściwości alergogennych. Szerzej omówiono trzy istotne elementy struktury. Pierwszy z nich to krótkie sekwencje aminokwasów (tzw. sekwencje toksyczne), które prawdopodobnie pełnią funkcję epitopów wiążących przeciwciała w reakcji immunologicznej. Kolejne elementy to skręty beta nadające cząsteczce alergenu specyficzną budowę przestrzenną oraz wiązania disulfidowe (SS) stabilizujące tę strukturę. Omówiono także próby modyfikacji biochemicznych białek glutenowych z wykorzystaniem różnych systemów enzymatycznych oraz redukcyjnego systemu tioredoksyny, w efekcie których uzyskano, niekiedy nawet znaczne, obniżenie immunoreaktywności badanych alergenów. Wreszcie zasugerowano interdyscyplinarną strategię wytworzenia form pszenicy o obniżonej alergeniczności uwzględniającą wykorzystanie metod badawczych stosowanych w genetyce, hodowli roślin, technologii żywności oraz medycynie.