CHAPTER 3

Cereal Proteins: Immunostimulatory and Toxic Peptides

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Storage proteins from wheat kernels are the base of a wide variety of homemade and industrial food products. Nonetheless, for a group of individuals (celiac disease (CD) patients), these proteins are toxic. Gliadins and glutenins from wheat, as well as their counterparts in barley and rye, also called prolamins, are evolutionary related, and present a high degree of homology.

Polyclonal and monoclonal antibodies raised against prolamins have been a very useful tool to characterise structural and conformational features of prolamins, and particularly, for gluten analysis based on immunochemical techniques. Complete adherence to a gluten-free diet is required to recover the normal histology of the small intestine in CD patients. To this end, the use of certified gluten-free products is mandatory.

Aqueous solvents such as 60-70% ethanol, have been used for extraction of prolamins from flours and food. This method is not selective and, therefore, results in complex mixtures of proteins which together with their low solubility in aqueous solutions, high degree of homology, and consequently crossreactivity, produce some drawbacks in gluten analysis by immunoassays.

Prolamins drive an exacerbated immune response in intestinal mucosa of CD patients. T lymphocytes are a central piece in CD pathogenesis. However, new insights in the knowledge of innate immunity point out to some gliadin peptides which can also produce structural changes in the intestine as well as inflammatory reactions.

Keywords
Gliadins, glutenins, prolamins, toxic proteins, gluten analysis, immune response.
1. Introduction

Cereal grains are one of the most important sources of protein in human nutrition. Wheat and rice comprise over 70% of the cereal grains worldwide consumed. Most of the wheat cultivars used correspond to the hexaploid (three genomes coded AABBDD) *Triticum aestivum* L. varieties which are commonly known as bread wheat. *Triticum durum*, tetraploid (genomes A and B), is primarily used for pasta production. Particularly, the massive use of wheat proteins is due to their physicochemical properties, i.e., their ability to form a particular structure called gluten. This structure is obtained from wheat flour through washing in the presence of water and elimination of some soluble components, mainly starch. As a result, an elastic and cohesive dough is obtained which is capable of retaining gas, a product of fermentation by microorganisms, usually yeast. Therefore, gluten is possibly the oldest known food and the one that is most widely distributed amongst different cultures. Due to its ability to form dough, gluten is extensively used also in the formulation of other foods and is central to the development of many products in the food industry\textsuperscript{1-3}.

Storage proteins from wheat kernels are the base of a wide variety of homemade and industrial food products. Nonetheless, for one group of individuals (celiac disease (CD) patients), these proteins are toxic. In this chapter, we will go over the structural aspects of these toxic proteins to understand their role in the pathogenesis of CD as well as the principles involved in methods for the certification of gluten-free food.

2. Classification of Cereal Proteins

Wheat, barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) are evolutionary related, and are members of the Triticeae tribe. They all contain protein groups with a high degree of homology and share some physicochemical properties. Oats, although found in the same sub-family, belong to the Aveneae tribe and present some different characteristics (Figure 1)\textsuperscript{1-3}.
Proteins from endosperm of wheat grains are complex mixtures. These proteins, which were originally classified by T.B. Osborne (1907) into four fractions according to their solubility in the following: albumins (soluble in water); globulins (soluble in saline solutions), gliadins (soluble in 60-70% ethanol) and glutenins (only soluble under stronger conditions, i.e. acids, reducing agents and detergents, urea, etc).

Gliadins and glutenins, as well as their counterparts in barley and rye, are also called prolamins. This name is due to their high content of the amino acids proline and glutamine, which along with phenylalanine explain for 60 to 80% of their amino acid content. Prolamins are synthesised and deposited in the endosperm of the grain as primary source of nitrogen for protein synthesis, which occurs later during germination. Milling process produces wheat flour, the essential primary ingredient in food manufacturing. Gliadins and glutenins, as storage proteins, comprise almost half of the protein content in wheat flour. Gliadins are found as monomers with molecular weights ranging from 30 up to 60kDa, whilst glutenins form polymers through interchain disulfide bonds with molecular weights from 80,000Da to several millions. As consequence of this crosslinking, glutenins are
poorly extracted when aqueous ethanol is used. Gliadins have been further classified into $\alpha$-, $\beta$-, $\gamma$- and $\omega$-gliadins, based on their electrophoretic mobility at acid pH (pH = 3, A-PAGE)\(^5\). The same procedure has been used to describe the homologous components of barley and rye.

### 3. Structural Characteristics and Physicochemical Properties

The primary structure of prolamins shows long regions of repeated sequences generated by insertion and duplication along the evolution, resulting in a high degree of polymorphism. The repetitive regions are formed by units of 4 to 9 aminoacid length. These units include one or more proline and glutamine, which explains for the high content of these two aminoacids in the prolamins.

Table 1 shows a prolamin classification which takes into account their composition and aminoacid sequence\(^1,3\).

| Prolamins                  | | |
|-----------------------------|--|--|
| | Gliadins (monomers) | Glutens (aggregates) |
| Wheat | $\omega$-gliadins | $\alpha$-$\beta$-gliadins | $\gamma$-gliadins | LMW glutens | HMW glutens |
| S-Poor | | | | S-Rich | |
| Barley | C hordeins | – | $\gamma$-hordeins | B hordeins | D hordeins |
| Rye | $\omega$-secalins | – | $\gamma$-secalins | LMW-secalins | HMW-secalins |

*Table 1. Classification of Wheat, Barley and Rye Prolamins.*
The amino acid sequence for $\alpha$-gliadin, a 30kDa ethanol-soluble protein, was the first to be reported. Further investigations revealed the overall structure of prolamins, consisting of typical sequences in the N-terminal end, conserved domains and repetitive regions. These characteristics are found in the homologous components of wheat, barley and rye. For example, the N-terminal regions of $\omega$-gliadins and $\omega$-secalins show a high degree of homology, and repetitive sequences account for 80% of the molecule. Two consensus sequences were found: PQQPY and PQQPFPQQ explaining for the high proline (P) and glutamine (Q) content observed in these proteins.

Sequence analysis of avenins revealed that, although some consensus sequences of repetitive units do exist, these are different from those found in wheat, barley and rye.

Based on their molecular weight, prolamins can be divided into: High Molecular Weight (HMW), Medium Molecular Weight (MMW) and Low Molecular Weight (LMW). Proteins from the HMW group include HWM-glutenins (wheat), HMW-secalins (rye) and D hordeins (barley), with molecular weights in the range of 70-90 kDa. The sequence motif QQPGQG is very frequent in the repetitive region.

The MMW group, molecular weight ranges between 50-70 kDa, includes $\omega$-gliadins, $\omega$-secalins (rye) and C-hordeins (barley). Sequences are typically formed by QPQQPFP and QQPFP repetitions. The LMW group, molecular weights ranges between 30-45 kDa, includes $\alpha$-/$\beta$-gliadins and $\gamma$-gliadins (wheat), $\gamma$-secalins (rye) and $\gamma$-hordeins (barley); these contain cysteines forming intrachain disulfide bonds. It should be pointed out that proteins homologous to $\alpha$-/$\beta$-gliadins are not found in rye and barley. The typical repetitive sequence in these proteins is QPQQPFP. In this same group, there are other proteins with interstrand disulfide bridges: LMW-GS (wheat), $\gamma$75k-secalins (rye) and B-hordeins (barley) (Figure 2 and 3A).

The secondary structure of prolamins contains $\alpha$-helix regions at the N- and C-terminal ends, and in some interspersed sequences. The repetitive regions adopt a structure called $\beta$-turn. The $\beta$-turn structural unit is composed of four residues; hydrogen bridge bonds are found between the first
carbonyl group and the amide group of the fourth residue\textsuperscript{10}. The regularity of repetitive sequences and of the $\beta$-turn structure determines the formation of a cylindrical structure with 13 residues per turn, called a $\beta$-spiral. $\beta$-turns are predominant in $\omega$-gliadins. They are also found in HMW glutenins and, to a lesser degree, in $\gamma$-gliadins. In these cases, the distribution of $\beta$-turns is irregular. In contrast, in $\alpha$-gliadins, this structure is restricted to only a few domains near the N-terminal, the ones which are more irregular and can contain interspersed sequences with $\alpha$-helix structure\textsuperscript{10}. Prolamins are compact protein structures with high physicochemical stability\textsuperscript{11}. Their rigid secondary structure is preserved, even under mild denaturalising conditions\textsuperscript{12} and only aggressive denaturing conditions, such as 4M urea, may alter their structure\textsuperscript{13}.

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**Figure 2. Outline of the structures of HMW glutenins and rich and poor sulphur gliadins.** Connecting lines 1 to 8 indicate disulfide bridges between cysteines, while SH indicates the cysteine residue positions\textsuperscript{1}. 

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4. Extraction Procedures to Obtain Prolamin Fractions

Aqueous solvents such as 60-70% ethanol, 0.01 M acetic acid or 1 M urea, among others, have been used for extraction of prolamins from flours. This method is not selective and, therefore, results in complex mixtures of proteins. Since prolamins have high tendency to aggregate in the presence of aqueous solvents, biochemical techniques to separate and purify them are not efficient. Reversed-phase high-performance liquid chromatography (RP-HPLC) is the recommended method for prolamin analysis. However, for preparative purposes only a limited amount of proteins can be purified using HPLC.

In order to obtain larger amount of these proteins, medium-pressure liquid chromatography has been used (FPLC, Fast Protein Liquid Chromatography). In this case, some enriched fractions can be obtained but they are commonly contaminated with component from other fractions. In conclusion, given their biochemical characteristics, it is not possible to obtain gliadins or other prolamins in a pure form using conventional analytic techniques. This is why, applying genetic engineering techniques, several...
gliadins have been cloned and produced in a recombinant form. These recombinant proteins have been used in functional studies\textsuperscript{16} and in the assessment of their role in the pathogenesis of the CD\textsuperscript{17,18}. Although studies carried out using individual protein used as a model, it should be take in mind that most of the processes or mechanisms to be analysed depend more on the interaction among proteins than on properties of one single component.

5. Characterisation of Prolamins by Immunochemical Methods

Polyclonal and monoclonal antibodies were raised against wheat, barley, and rye proteins were a very useful tool to characterise structural and conformational features of prolams. Though, the low solubility of prolams in aqueous solutions, the difficulty of obtaining highly purified components and the high degree of homology, and consequently crossreactivity, produce some drawbacks in this kind of studies, the information obtained by immunochemical techniques was relevant to increase the knowledge of this particular protein system. Immunochemical analysis using polyclonal antibodies obtained in rabbits immunized with $\alpha$-/$\beta$- and $\gamma$-gliadins, B-hordeins or C-hordeins, showed the immunogenicity of repetitive sequences and, in particular, that regions composed of beta-tuns mostly determine the high cross-reactivity\textsuperscript{19}. Those results revealed partial homology and the presence of similar conformational and/or lineal epitopes in $\alpha$- and $\gamma$-gliadins, B-hordeins and C-hordeins. In the same studies, $\omega$-gliadins showed much less reactivity, and no recognition of oat and rice proteins was observed.

Monoclonal antibodies were produced using different strategies for immunization and hybridoma selection. These antibodies have increased our knowledge on the structural characteristics of prolams but also they were useful for the development of quantitative assays to determine gliadin concentration in foodstuffs\textsuperscript{14,20-23}. One of these monoclonal
antibodies, called R5, has been extensively characterised and is one of the antibodies worldwide used in commercial ELISA tests for gluten control in foodstuff\textsuperscript{24-26}.

Characterisation of immunoreactivity and, in particular, the identification of the epitope recognised by a monoclonal antibody in this protein system is difficult. The complex protein system consisting of multiple antigen-antibody interactions with a broad range of affinities, and high crossreactivity makes difficult the interpretation of the immunochemical results. To identify the epitope recognised by monoclonal antibodies, synthetic peptides or phage display libraries were used. In the case of the R5 antibody, the core sequence of the epitopes was identified as QQPFP, QQQFP, LQPFP and QLPFP\textsuperscript{24}. These sequences are found in wheat, rye and barley but not in oats or rice.

In addition to the immunochemical tests, modern techniques have been developed for gluten analysis more recently. Different kind of sensors based on physical properties, electrochemical\textsuperscript{27}, and magnetic\textsuperscript{28} have been proposed to detect gluten peptides. Using a different approach, detection of DNA fragments of wheat genome by PCR has also been proposed to detect the presence of wheat components in foodstuff\textsuperscript{29}. Though, these are all powerful techniques, they could not replace the massive use of the quantitative ELISA.

6. Commonly Used Gliadins in Research of CD Pathogenesis or Gluten Analysis

To assess the role of gliadins in the pathogenic mechanisms in CD or in the development of food certification assays, the most commonly used gliadin sources have so far been: commercial gliadins, enzymatic digestion of whole gliadins and, more recently, the standard prepared by the European Working Group in Prolamin Analysis and Toxicity (PWG)\textsuperscript{30}. 
Commercial gliadins are supplied by different companies. Essentially they consist of gliadins obtained from wheat flour following conventional protocols on elimination of the albumin-globulin fraction and later extraction with aqueous ethanol. The protein fraction extracted with aqueous ethanol is then freeze dried and distributed as a lyophilized powder. It has been the most commonly used gliadin source, but it is not completely soluble. This is a relevant disadvantage when this gliadin preparation is used as standard in quantitative methods. In addition, due to the production procedure, the conformation of the proteins can be altered, and consequently their interaction with antibodies can be modified.

Gliadin fragments obtained by enzymatic digestion of commercial gliadins have often been used in characterisation of the immune response in CD patients. They are obtained, in general, through treating commercial gliadins with trypsin and pepsin, and usually called PT-gliadin. This enzymatic digestion produces a mixture of peptides of varying size. For biological assays, this preparation is used as a model of gluten-derived peptides found in the intestinal lumen after the physiological process of digestion. The disadvantage of this preparation is the high variability between batches.

The PWG gliadin was developed as part of an international multi-centre project. This preparation is an international reference material which allows the validation of quantitative tests. For the preparation, flour of 28 varieties of European wheat varieties were mixed, and the gliadin fraction was obtained following a conventional protocol for prolamin extraction. The optimization of the preparation procedure produced a high amount of gliadins. The PWG gliadin was characterized by the most wide-ranging methodology available (RP-HPLC, polyacrylamide gel electrophoresis, capillary electrophoresis, MALDI-TOF, immunoassays). Its stability and solubility were also evaluated. Thus, the PWG gliadin is a highly
stable and completely soluble reagent, which can be used as a reference material for quantitative assays in gluten analysis.\(^{30}\)

7. Prolamins and Toxicity. Induction of Innate and Adaptive Immune Response

Pioneer studies by the group of Dr. Sollid (Oslo, Sweden) at the beginning of the 1990s, demonstrated the specificity of lamina propria T lymphocytes isolated from the intestinal mucosa of untreated CD patients. Those experiments demonstrated the role of HLA alleles in CD pathogenesis.\(^{31,32}\) Following studies, using panels of T lymphocytes isolated from the intestinal mucosa allowed a deep analysis of the peptides bound to the susceptibility HLA alleles (HLA-DQ2/DQ8)\(^{33}\). Because all the information collected through these studies, the mechanism of CD pathogenesis have been defined in detail, perhaps even more than is known for other immune-mediated pathologies.

Due to their particular sequences, gluten peptides are resistant to enzymatic degradation. Consequently, partially degraded and long gluten peptides are present in the intestinal lumen. These peptides are traslocated to the lamina propria where they are uptaken and processed by dendritic cells. There, tran glutaminase 2 (TG2), a multitask enzyme, mediates deamidation of glutamine residues at selected positions of the gluten peptides.\(^{35,36}\) This modification renders peptides with higher affinity for the HLA susceptibility alleles.\(^{17,37-39}\) Taking together, the selection of peptides able to interact with the HLA susceptibility alleles and the requirement for glutamine deamidation by TG2, algorithms for prediction of toxic sequences were developed.\(^{40,41}\) Thus, the adaptive response is mainly restricted to certain gluten peptides which fulfil requirement of HLA binding and TG2 modification.\(^{42,43}\)

Though T cell reactivity seems to be heterogeneous, reactivity to \(\alpha\)-gliadin predominates to other gliadins. Immunodominant peptides, such as \(\alpha\)-gliadin p56-89\(^{44}\), induce specific immune responses in virtually all patients with celiac
disease\textsuperscript{17,45}. The major epitopes on $\alpha$- and $\gamma$-gliadins, as well as on glutenins, have been identified; many bind to HLA-DQ2 and DQ8. In most cases, TG2 deaminated peptides show a higher binding affinity and increase induction of T cell proliferation\textsuperscript{36,37,44}.

A nomenclature for relevant gluten epitopes has been proposed based on the definition of the reactivity by at least one specific T cell clone, the HLA restriction element, and the nine aminoacid core of the epitope\textsuperscript{41}. The list includes 31 epitopes recognized by CD4+ T cells, 24 HLA-DQ2 restricted (23 DQ2.5, 1 DQ2.2) and 7 HLA-DQ8 restricted (4 DQ8, 3 DQ8.5), from $\alpha$-gliadin, $\gamma$-gliadin, $\omega$-gliadin, LMW and HMW glutenins, hordeins, secalins and avenins. (Tabla 2).

It is known that gluten peptides may induce damage in cultured intestinal duodenal biopsies\textsuperscript{46}, or after being administered in vivo on the proximal or distal intestine\textsuperscript{47}. Early effects, i.e. induction of cells stress pathways and stimulation of the local innate immunity, have been described for the $\alpha$-gliadin fragments p31-49 or p31-43. Peptide 31-43 may induce the upregulation of stress inducible MHC-class I molecules MIC\textsuperscript{48}, epithelial cell death\textsuperscript{48}, and may potentiate the effect of Epidermal Growth Factor (EGF) by interference in the inactivation of its receptor\textsuperscript{50}, as well as the upregulation of mitogen-activated protein (MAP) kinase p38, CD83 and IL-15 production by mononuclear lamina propria cells\textsuperscript{51}. It has been also reported that peptide 31-43, unlike other peptides, accumulates in the intracellular lysosomes where it induces TG2 activation and degradation of Peroxisome Proliferator-Activated Receptor (PPAR) gamma, a modulator of intestinal inflammation\textsuperscript{52}. Other gliadin peptides have been involved in the expression of non-classical MHC-class II molecules HLA-E\textsuperscript{53}, and the activation of antigen presenting cells by TLR4\textsuperscript{54}, and the CXC-chemokine receptor 3 (CXCR3)\textsuperscript{55}. 
Table 2. List of relevant peptides recognized by CD4* T cells.

| EPITOPE | Current Nomenclature | Former Nomenclature | sequence* |
|---------|----------------------|---------------------|-----------|
| **DQ2.5 restricted** | | | |
| DQ2.5-glia-α1a | DQ2-α-I,α9 | PFPQP**ELPY** |
| DQ2.5-glia-α1b | DQ2-α-III | PYPQP**ELPY** |
| DQ2.5-glia-α2 | DQ2-α-II,α2 | PQPE**LPYPQ** |
| DQ2.5-glia-α3 | glia-α20 | FRPE**QPYPQ** |
| DQ2.5-glia-γ1 | DQ2-γ-I | PQQSFP**EQQ** |
| DQ2.5-glia-γ2 | DQ2-γ-II,γ30 | IQPE**EQPAQL** |
| DQ2.5-glia-γ3 | DQ2-γ-III | **QQP**EQPYPQ |
| DQ2.5-glia-γ4a | DQ2-γ-IV | SQPEQ**EFPQ** |
| DQ2.5-glia-γ4b | DQ2-γ-VIIc | PQPE**QFPQ** |
| DQ2.5-glia-γ4c | DQ2-γ-VIIa | **QQP**EQPFPQ |
| DQ2.5-glia-γ4d | DQ2-γ-VIIb | PQPE**QPCQ** |
| DQ2.5-glia-γ5 | DQ2-γ-VI | QQPFP**EQPQ** |
| DQ2.5-glia-ω1 | DQ2-ω-I | PFPQP**EQPF** |
| DQ2.5-glia-ω2 | DQ2-ω-II | PQPE**QFPWP** |
| DQ2.5-glut-L1 | glutenin-17 | FSQ**Q**ESPFP |
| DQ2.5-glut-L2 | glutenin-156 | PQPE**EQVP** |
| DQ2.5-hor-1 | Hor-α9,Ha9 | PFPQP**EQPF** |
| DQ2.5-hor-2 | Hor-α2,Ha2 | **PQP**EQPFPQ |
| DQ2.5-hor-3 | hor-I-DQ2 | **PIPEQ**PQPY |
| DQ2.5-sec-1 | Sec-α9,Sα9 | PQPFP**EQPF** |
| DQ2.5-sec-2 | Sec-α2,Sα2 | PQPEQ**FPFP** |
| DQ2.5-ave-1a | Av-α9A | **PY**EQ**EEP** |
| DQ2.5-ave-1b | Av-α9B,1490 | PQPEQ**EFP** |
| **DQ8 restricted** | | | |
| DQ8-glia-α1 | DQ8-α-I | **EGSFQ**PSQ **E** |
| DQ8-glia-γ1a | DQ8-γ-Ia | **EQPQ**QPFPQ |
| DQ8-glia-γ1b | DQ8-γ-Ib | **EQPQ**QYPYE |
| DQ8-glut-H1 | HMW-glutenin | **QGY**P**TSPQ** |

*Aminoacid sequence in one letter code. In red: Glutamate residues (E) due to TG2 desamidation are important for the affinity to DQ molecule. In blue: other Glutamine residues (Q) potential substrates for TG2**.154
However, it remains to be confirmed whether these toxic peptides are produced in the intestinal lumen by digestive enzymes, and the specific receptor for p31-43 (or related peptides) should be identified in order to understand its interaction with enterocytes and how transepithelial transport of this peptide occurs. Transcytosis experiments performed *ex vivo* suggest that transferrin receptor CD71 can mediate the translocation of IgA-gliadin complexes, though this mechanism will not be effective in patients with IgA deficiency\textsuperscript{56}. A high transepithelial transport from the apical to the basal membrane of enterocytes has been described in CD patients, mediated by an IFN$\gamma$-dependent mechanism\textsuperscript{53}. (Table 2) (Figure 3).

The current picture of CD pathogenesis involves two classes of toxic peptides: those able of generating a very fast change in the mucosa through inflammatory and innate mechanisms and others which trigger the full adaptive response. Both pathways interact and potentiate each other to sustain the chronic process of the intestinal damage\textsuperscript{42,57}.

In conclusion, studies aiming to increase our knowledge on toxic sequences derived from gliadins and glutenins, as well as from other toxic cereals have a great importance in many aspects of celiac disease. The development of analytical tools for the detection of gliadins and glutenins in food to be consumed by CD patients requires a precise immunochemical information on the reactivity of the antibodies used in quantitative techniques. Furthermore, the development of new methods requires also the identification of appropriate sequences from these proteins as target for detection by immunochemical and non immunochemical techniques. In addition, gliadin peptides can be used for the detection of specific antibodies against deamidated peptides, which are a useful tool in serology and screening strategies to detect CD patients. Besides, different gluten peptides have been reported to have a role in the pathogenesis of CD, as they are involved in both the induction of innate and adaptive immune responses. The mechanisms and sequences responsible for the induction of inflammatory reactions are still poorly understood. Some of these inflammatory pathways might also have a role in the new entity Non-Celiac Gluten Sensitivity.
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