Chapter 21

Immunotoxic Gluten Fraction Detection: Applications in Food Safety

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Abstract

The only currently available therapy for celiac patients is a life-long strict gluten-free diet, however, it generates numerous social and economic repercussions. Various studies have suggested that failure to comply with the diet is frequent in celiac patients. For this reason, and because of the currently recognized importance of nutrition in the management of CD, the development of new strategies for monitoring the gluten-free diet is essential. The toxicity of cereals such as oats is questioned. Studies have shown that oat's immunogenicity depends on interindividual sensitivity and the cultivar used. The incorporation of harmless oat varieties in food products may improve the nutritional quality of the gluten-free diet. Additionally in the search for a less-toxic barley, it has been demonstrated that cultivated varieties contain lower levels of immunogenic gluten than the wild ones. This fact is important in breeding programs of cultivated species and in the preparation of certain foods and beverages derived from toxic cereals.

1. Introduction

Currently, the only existing treatment for patients with celiac disease (CD) is to follow a strict life-long gluten-free diet (GFD) by excluding toxic dietary wheat proteins (gliadin and glutenin), and their counterparts in barley (hordeins), rye (secalins), and oats (avenins), as well as in hybrids of these grains (such as kamut and triticale) and derivatives thereof (starch, flour, etc.)[1].

In most celiac patients, strict compliance with a GFD leads, in a few months, to the rapid and complete recovery of the normal architecture and function of the small intestinal mucosa, as well as to symptom remission and normalization of serological tests[2]. However, maintaining a GFD is not easy, not only due to the high cost involved, but there are also situations that favor involuntary gluten intake, such as its presence in a high proportion of manufactured products. Approximately, more than half of the commercial food contains gluten from wheat, barley, rye or oats, including those in which it only acts as a thickening agent or binder. The risk posed by these foodstuffs for celiac patients makes it convenient to carry out a rigorous gluten content control.

In European legislation, the acceptable gluten amount in food which seeks to be labeled "gluten-free" is of 20 parts per million (ppm or mg/kg). Another category has also been provided, food with "very low gluten content" which is used for products made with wheat, rye, barley, oats or their crossbred varieties, but which have been specially treated to eliminate gluten. Food labelled as "very low gluten content" may not exceed 100 ppm (REGULATION (CD) Number 41/2009 concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten, http://bit.ly/RdEqVI). Therefore, control of gluten-free products requires the use of quantitative methods with highly specificity and sensibility. The use of inadequate control methods exposes celiac patients to important health problems. This also leads to severe economic losses and legal problems associated with questionable identification of gluten-free products. At industrial level, rigorous control of the raw materials used and the final marketed product must be excercised.

To certify suitable food, no product is exempt from analysis. Inadvertent contamination and adulteration seriously compromise the health and quality of life these patients. The industrial use of wheat flour and/or derived components (starch, gluten) used to increase water retention capacity, improve texture, preserve structure and quality attributes, leads to the presence of toxic proteins. Furthermore, during the production process, foods are subjected to heat treatments and other processes able to modify their gluten content. This product modification is a problem in order to quantify the gluten immunotoxic fractions.

Due to the complexity of the system being analyzed, the only way to provide a safe diet for celiac patients is the use of highly sensitive and specific tests. The techniques for gluten analysis are mass spectrometry, immunological methods based on monoclonal antibodies (MAbs) or PCR techniques.

Mass spectrometry is based on the determination of the characteristic mass spectra of different gluten fractions. Furthermore, through these techniques the peptides contained in different types of food can be characterized[3]. They require complex instrumentation and equipment calibration, expensive equipment, extensive facilities and a complex process of developing spectral profile libraries.

The most frequently used method in food analysis are MAbs produced specifically against gluten. These antibodies recognize gluten repetitive regions[4,5] or have been designed from toxic regions in the gluten protein sequences[6-9]. Some of these antibodies have been incorporated into various ELISAs to be used in food gluten content analysis[8-10]. These methods are the most convenient and widely used as they unite simplicity, sensitivity and economy, in addition to being able to directly detect proteins toxic to celiac patients.

Another option, used primarily as a complement to the above mentioned ones, is based on PCR techniques using primers that encode prolamine repetitive sequences[11,12]. Unlike ELISA, PCR is an indirect technique for detecting gluten protein which does not quantify the presence of these proteins, but that of the DNA which encodes them.

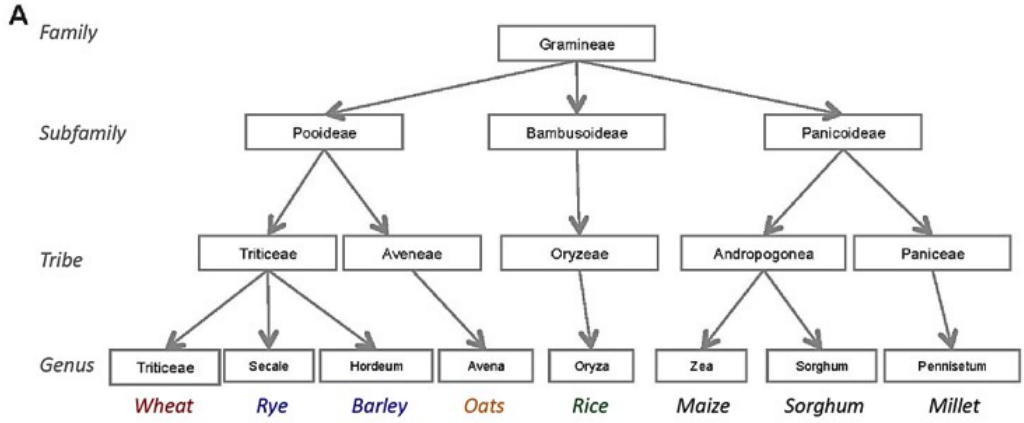
2. Suitability of Oats in the GFD

The introduction of oats in the GFD has been a topic subject to debate in recent years[13,14]. Adherence to a strict GFD may sometimes be difficult due to the narrow range of permitted ingredients and any dietary restrictions, such as oat consumption, can be a relief for celiac patients. Nutritionally wise, oatmeal is an important source of protein, fat, vitamins, minerals and fibers, and therefore, could be beneficial for people with CD. In addition, the palatability of oats and their wide availability may contribute to greater acceptance in a diet free of wheat, barley and rye.

Oats differs from other cereals in their prolamin content, which is of 10-20% of the total protein, in contrast to wheat prolamins, which can be between 40-50%. Furthermore, various cereal prolamins differ in molecular size and amino acid content. In avenin, the proline and glutamine proportion (amino acids rich in toxic regions) is lower than in other toxic cereals (Figure 1).

Janatuinen *et al*.[17] lconducted the first controlled study on the toxicity of oats in CD. Since then, several studies have evaluated the safety of oat consumption for celiac patients. Some researchers claim that celiac patients tolerate oats with no sign of intestinal inflammation[14,17,18], in fact, many countries allow the use of oats in "gluten-free" food, for example Gluten-Free Oats®. On the other hand, there are studies that confirm the toxicity of certain types of oats for celiac patients and the impossibility of regular oats consumption. Arentz-Hansen *et al*.[15] described the intestinal damage suffered by some patients after consuming oats and a GFD. In these patients an immune response against avenins may be triggered similar to that produced by gluten from wheat, rye or barley. A study led by Dr. Knut Lundin[19] with 19 celiac patients who were consuming 50 grams of oats/day for 12 weeks showed that one of the celiac patients proved to be oat sensitive. This suggests the need to distinguish groups of celiac patients according to their sensitivity to cereals, and to identify the immunogenicity source in avenin peptides.

A



B

Basic prolamin characteristics	Wheat	Oats	Rice
Number of genes	>100	8-25	34 (21 transcribed)
Size	20-40 kDa	19-31 kDa	10-16 kDa
aas prevalence	Gln (35%) Pro (25%)	Gln (30%) Pro (10%)	Gln (22%) Pro (<other cereals)
Prolamins based on total proteins in grain	40-50%	10-20%	25%

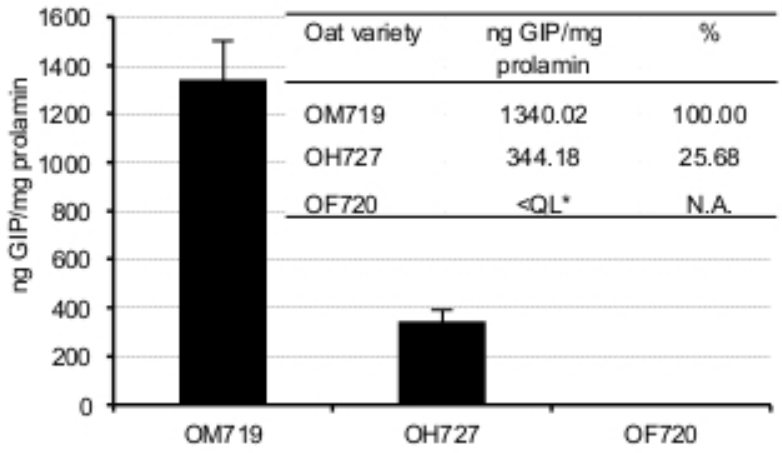
Figure 1. Taxonomic and molecular relationship of oats to other food cereals in the context of CD. A. Taxonomy of oats in the grass family in relation to cereals toxic for celiac patients, such as wheat, barley and rye, and non-toxic cereals such as rice, maize, sorghum and millet. B. Molecular characteristics of the prolamins from wheat, oats and rice. Modified according to Kagnoff.[16]

Silano *et al.*[20,21] conducted a series of *in vitro* tests with different varieties of oats and found that all varieties tested were toxic to celiac patients, with differences in the levels of toxicity. Therefore, it is critical to qualitatively and/or quantitatively determine the immunotoxic potential of oats due to the clinical implications for celiac patients.

2.1. Diversity in the Potential Immunogenicity of Different Oat Varieties

The differences in the type of oats used, the oat purity and the study design did not allow a clear answer on whether or not oats are safe for all celiac patients. Besides, "pure" (uncontaminated) oats are considered gluten-free according to CD regulation No. 41/ 2009. However, a study by our research group explains the apparent contradictions found in previous research related to the safety of oats for celiac patients[21]. We demonstrated that oat immunogenicity varies depending on the cultivar used.

Nine oat varieties from various Australian and Spanish commercial sources were used. The purity of the oat material was carefully controlled and shown to be free of contamination. The analysis of DNA amplification products confirmed that the oat samples were not contaminated with wheat, barley, rye or mixtures of these grains. The toxicity of the different oat varieties was evaluated by MAb G12 immunoassay, an antibody obtained from one of the most toxic peptides described for CD, the α-2 gliadin 33-mer peptide. Three varieties of oats were distinguished based on their MAb G12 reactivity: a group with high reactivity, a group which showed an intermediate reactivity and another without detectable reaction (Figure 2). The potential immunotoxicity of three oat types was evaluated by cell proliferation and interferon-γ release (IFN-γ), using peripheral blood T lymphocytes from celiac patients. Thus, it was demonstrated that mAb G12 reactivity against the storage proteins of different oat varieties correlated with immunological studies of samples from celiac patients[21].



Oat variety	ng GIP/mg prolamin	%
OM719	1340.02	100.00
OH727	344.18	25.68
OF720	<QL*	N.A.

Figure 2. GIP concentration in different oat varieties. The GIP concentration is determined by competitive ELISA using G12-HRP. OM719, OH727, OF720: oat varieties. (GIP: Gluten Immunogenic Peptides)
%: Percentage of GIP in each variety in relation to the more reactive, OM719.
**GIP concentration below the assay's quantification limit (5.4 ng/mL).*
N.A.: Not applicable. Modified according to Comino et al.[21]

In comparison with wheat gliadins, the avenins have been little studied, and the number of full avenin genes present at the moment in the databases is limited and from few genotypes, so that the variability of avenin genes in oats is not well represented. It has recent been known that, like wheat, oat grains have both monomeric and polymeric avenins[22]. A direct correlation between the immunogenicity of the different varieties of oats and the presence of the specific peptides with a higher/lower potential immunotoxicity has been found, that could explain why certain varieties of oats are toxic for celiac patients and other not[22,23].

The addition of some oat varieties to gluten-free food could not only improve the patient's nutritional status but it may also provide some benefits in the treatment of some diseases

related to cholesterol, diabetes or intestinal transit problems. These studies provides new insights into the dilemma of oats in CD and suggests practical methods for selecting those varieties tolerable by celiac patients.

Given the importance of the source of oats used, this topic should be taken into account in food safety regulations, in the labeling of gluten-free products that may contain oats, as well as in the design of clinical trials on the effect of oats in celiac patients.

3. Natural Immunotoxicity Variation in Cultivated and Wild Barley Varieties

Compliance with GFD present difficulties due to inadvertent ingestions or voluntary transgressions. Consequently, different strategies have been proposed to develop new therapies for CD[24-27]. A possible alternative is based on the identification of new cereal varieties with low toxicity profiles, which could contribute to improving the quality and variety of foods destined to the celiac community. In the case of oats, immunological studies revealed that certain varieties had no toxicity for celiac patient[21]. Different studies have investigated the possible immunogenicity of wheat varieties by means of antibodies to immunogenic wheat peptides and T cell reactivity from celiac individuals[6]. It is unknown whether all barley varieties are equally toxic to celiac patients. In this sense, our research group has studied the toxicity of different barley lines, investigating *Hordeum vulgare*, a cultivable barley variety, and *Hordeum chilense*, a wild barley variety, used for the development of new cultivable cereals.

Barley is an important cereal crop, mainly used for food, obtaining malt, making beer and distilled spirits. In recent years, the use of barley has been increased, largely due to its high nutritional value. Barley seeds provide complex carbohydrates (mainly starch), minerals, vitamins, and fiber, which provide benefits in helping reduce blood cholesterol. In addition, its high fiber and other components have a satiating effect, which can positively affect weight control as well as improved intestinal transit[28,29].

In our study we first compared the differences in toxicity levels between different varieties of barley[30]. Rigorous control of sample purity both by visual examination as well as by PCR techniques was executed, afterwards, the hordein banding pattern was analyzed by MALDI-TOF MS. Our results showed there was a greater number of hordein bands for wild varieties. These mass spectrum differences may be related both to the seed's functional properties as well as the toxicity in connection with CD. The results obtained by G12 immunological techniques showed large differences between the *H. vulgare* and *H. chilense* lines, the wild barley lines being more immunogenic. Also, differences in immunotoxic potential were found between varieties of a same barley species (Figure 3). The stimulatory capacity of these barley varieties was evaluated by peripheral blood cell proliferation and IFN-γ release and from the intestinal mucosa of active celiac patients. All barley varieties were able to stimulate IFN-γ secretion, at both in peripheral blood and in the intestinal mucosa. However, one of the wild varieties was the one that showed stronger activity in relation to the pathogenesis of CD.

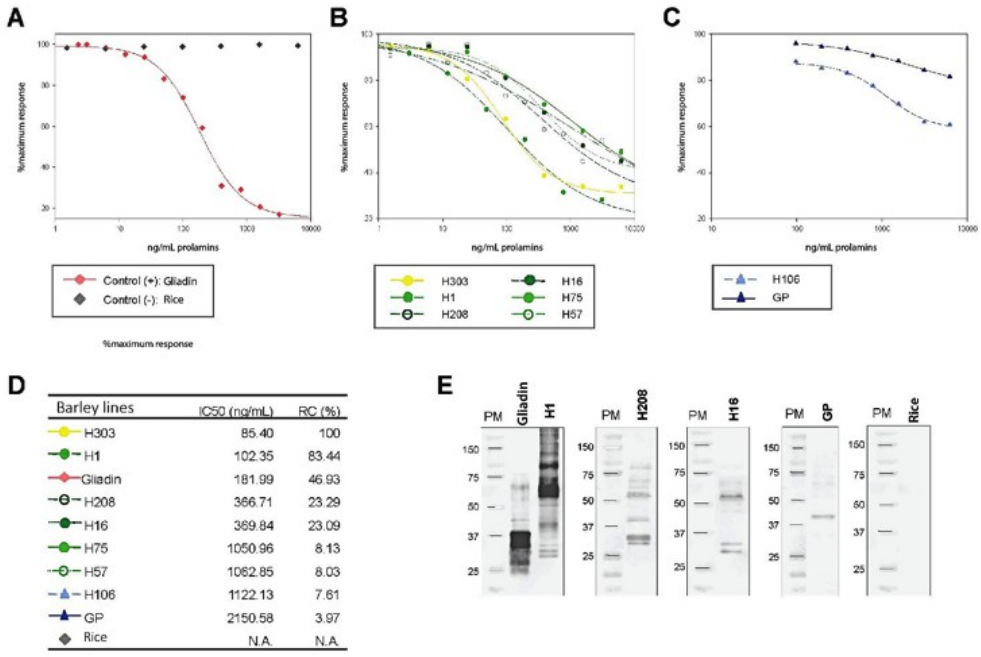


Figure 3. Relative affinity of anti-gliadin 33-mer G12 mAb against different barley lines.
(A, B, C and D) G12 competitive ELISA to determine the relative antibody affinity to the various barley lines. Gliadin and rice were used as positive and negative controls, respectively.
(E) G12 Western blot prolamins of different barley lines. The membranes were revealed with mAb G12. MW, molecular weight (kDa).
IC50: antigen concentration of which a 50% reduction of the maximum signal is obtained.
CR: Cross-reactivity. Modified according to Comino et al.[28]

A correlation between the type of barley used and immunotoxicity for celiac patients has established. It has been shown that cultivated barley varieties exhibit lower levels of toxic gluten than wild ones. These findings could help develop new lines with low gluten levels, which may be intended for the manufacture of food and beverages with gluten amounts below the threshold allowed for celiac patients[31]. Thus, for example, during the brewing process the initial quantity of toxic peptides can be lowered a thousand times in the different extraction and fermentation processes[32]. Barley varieties with reduced immunotoxicity[30] could be included in genetic breeding aimed at developing varieties that could serve as raw material for the production of toxic peptide-free beers.

The incorporation of wild germplasm in breeding programs is a common practice to increase the genetic base of cultivated species. However, care must be exercised not to increase the toxicity of cultivated varieties, as in the case of barley, because, according to the results obtained by Comino et al.[30], wild varieties may contain higher levels of toxic gluten than cultivated varieties.

4. Conclusions

The GFD is currently the only treatment for celiac patients, therefore, the characterization and quantification of the toxic gluten fraction in food and raw materials for the celiac patients is essential. There is a wide variability in the immunotoxic potential of different cereal varieties. It has been demonstrated that there is no strict correlation between gluten content and immunotoxic potential, due to the fact that some gluten epitopes may be less immunogenic than others.

Immunogenicity of oats varies depending on the cultivar used, there being varieties which could be safe for celiac patients and be enrich the GFD. Likewise, it has been shown that cultivated barley varieties, although there are differences between them, exhibit lower levels of toxic gluten compared to wild ones. This fact is important for breeding programs of cultivated species and for the preparation of certain foods and/or beverages derived from toxic cereals.

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