The toxicity of wheat prolamins

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Introduction

Since the work of Weijers, van de Kamer and Dicke [1], we know that the prolamins from different cereals are toxic to the intestinal wall of patients with coeliac disease (CD). Early experiments of Frazer *et al.* [2] had shown that this toxicity remains after digestion of gluten by peptic-tryptic and pancreatic enzymes. This means that the toxicity is localised in one or more than one peptide in the digested mixture. Much work has been done since that time to elucidate the amino acid sequence of that part of the gliadin molecule that damages the intestinal wall of a gluten-sensitive patient.

Cereal grains

Cereals have been cultivated by man for about 10000 years, probably starting in southwestern Asia, from the mediterranian coast to the plains of the Euphrate and Tigris. In that region wild ancesters of our wheat are still found.

Wheat, rye and barley belong to one of the tribes of the gramineae. Oats and rice are two other members of the same family. Millet, sorghum and maize belong to an other family of the gramineae. This relationship is reflected in the toxicity of the different cereals.

The proteins from the different species have a number of physical characteristics in common. They can be divided in water soluble and water insoluble proteins. The main fraction is the insoluble part, i.e. the gluten. This gluten again is divided into glutenins and prolamins. Only the prolamins from wheat, rye, barley and oats are proven to be toxic to patients with CD. The protein and prolamin content of the cereal grains are given in Table I [3].

Table I: Protein and prolamin content of the cereal grains.

Cereal	Prolamin	Protein in %	Prolamin in %
wheat	gliadin	10-15	4.0-7.5
rye	secalin	9-14	3.0-7.0
barley	hordein	10-14	3.5-7.0
oats	avenin	8-14	0.8-2.1
maize	zein	7-13	3.5-7.0

Wheat prolamins

Wheat is the most cultivated plant in the world. The world production amounted in 1990 to about six hundred million metric tons [4] .Wheat and products made from wheat therefore have a considerable impact on human nutrition. The number of cultivars from wheat is about 20000. They all differ in technologic applicabilities, yield, resistance to disease or to climate; but also in protein content and composition.

Since wheat production exceeds direct need in developed countries and wheat starch is produced for applications in food and non-food industry, the gluten has found its way to many other products. It is used as an inexpensive and technologic favorable protein source in soups, cake mixes, stabilisers, glues, chewing gum and, partly hydrolysed, as foam stabiliser and many other applications.

Most of the protein of wheat is in the endosperm, the part that is milled to wheat flour. Glu-

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ten constitutes 90% of the endosperm proteins. It also contains 5-10% of lipids. Gluten consists roughly of equal parts of gliadin and glutenin. Gluten is obtained by washing a dough of wheat flour in water. When dried at low temperature, in order to avoid denaturation, the dry product "vital gluten" is stable (apart from possible rancidity of the lipids) and is used in the baking industry to improve the baking quality of bread.

Gluten can be separated into its two fractions by extraction with 70% ethanol. The ethanol soluble fraction is the gliadin; the insoluble fraction glutenin. In the same manner alcohol-soluble protein fractions from other cereals can be prepared. All these fractions are referred to as prolamins.

Gliadin has a relatively low molecular weight (16 000-40 000) and consists of a number of more or less related proteins. When iso-electric focussing is combined with electrophoresis, about 50 different proteins can be distinguished. On the basis of their electrophoretic mobility the gliadins are divided into four groups; α-, β-, γ- and ω-gliadins. Later analysis has demonstrated that ß-gliadin is a subgroup of α- and γ-gliadin and therefore the indication α-type and γ-type is used. The relative amounts of the different fractions is given in Table II.

Table II: Relative amounts of the gliadin subfractions as determined by HPLC.

Fraction	Relative amount in %	SD
α-type	47.9	2.9
γ-type	34.4	3.6
ω-type	17.7	3.6

The gliadin proteins are heterogenous in different regions of the molecule and consequently differ in physical and chemical properties. About 35% of the gliadin molecule is in the α-helix form, whereas 35% are ß-turns [5]. The latter are concentrated in the N-terminal and C-terminal more apolar parts of the gliadin. The remaining part has a random structure. These forms have consequences for the immunogenecity of the different regions in the molecule. Especially ß-turns are immunogenic.

The secondary, tertiary and quarternary structures of the gliadins are altered by denaturation. Heat denaturation is the commonly used process in households and industry for the preparation of food from wheat flour. The degree of denaturation depends again on the primary structure of the molecule. This is seen in the difference of denaturation between the ω-gliadin and the other gliadins; ω-gliadin with its high content of proline is much less sensitive to denaturation than the other gliadins.

Amino acid sequence

Since Kasarda *et al.* [6] have determined the amino acid sequence of α-gliadin, a number of other structural analyses could be made. They divided the gliadin molecule into six domains: individual N-terminal and C-terminal sequences, two very similar prolin-poor sequences, one glutamine-rich sequence and a typical individual repeating sequence. About 30% of the roughly 300 amino acids they found is glutamine and another 16% is proline. When we consider the general structure we see that most of the proline is in the first and last part of the molecule. As could be expected in these parts we find also the ß-turns, being related to the proline in the chain.

This analysis is of importance for the understanding of the process of antibody formation as these antibodies are primarily formed against the easily recognised structures of the ß-turns. The recognition of these epitopes in the different gliadins and other prolamin molecules by an antiserum against gliadin demonstrates the close relationship between the prolamins from wheat, rye, barley and oats. As they crossreact with antiserum against one of these prolamins, they must share at least some epitopes [7]. This will have consequenses for the immunologic analysis of the prolamins. It remains to be seen whether these epitopes are also related to the toxicity of the gliadin for CD patients.

One could ask if there are other groups bound to the amino acid backbone that could relate to its toxicity. Wheat germ agglutinin [8] and purothionin represent possible candidates. Purothionin is bound to lipids and can be extracted by the normal lipid solvents like hexane. Toxicity remains however in the defatted flour and therefore can be

excluded. Antibodies against wheat germ agglutinin have been demonstrated in the serum of coeliac patients with an enzyme-linked immunosorbent assay (ELISA) method, and it has been claimed that these agglutinins could be the cause of the intestinal damage [9]. This finding has not been replicated by other research groups [10]. The claim that glycosidic groups bound to the proteins are the toxic principle [11] has not been confirmed by others; no glycosides have been demonstrated in purified toxic preparations.

The toxic mechanism

When gliadin reaches the intestinal wall, peptic digestion has then already taken place. Further digestion by pancreatic enzymes does not destroy the toxicity of gliadin although this digestion is never complete. Some protein is still intact and can be transported through the intestinal lining. Experimental work has demonstrated that some changes in the transported molecules take place in mice [12]. In normal circumstances we develop a tolerance for proteins in our food. Whether the enzyme system of coeliac patients has a missing component or whether there is a failure of the normal tolerance is uncertain. Cornell [13] found that a homogenate of biopsies from normal subjects digest gliadin to the level that it does not damage rat liver lysosomes, whereas a digest from a homogenate of biopsies from coeliac patients causes such a lysosomal damage. He could isolate a peptide from the coeliac incubation mixture that was not found in the normal controls. A transglutaminase – an enzyme that can use gliadin as a substrate – was also found to be lacking in the brushborder of coeliac patients, even when they had a normalised intestinal architecture on a gluten free diet [14].

The way of presentation of gliadin fragments or their handling during transport can be the first step in the reaction to gliadin in the enterocytic lining the intestinal wall. Passage of insufficiently digested peptides or break of tolerance then brings the immune system into action. Apart from the formation of antibodies, it is the cellular immune system in particular that reacts to the peptides that are presented to them [15]. This immune reaction in turn is an important factor in the structural and functional changes of the intestinal cell lining. More protein or peptide is able to pass through the brush border layer of the enterocytes, leading to mucosal atrophy in coeliac patients.

Kagnoff *et al.* [16] suggested that infection with adenovirus 12 with an amino acid sequence identical to the 206-217 sequence of A-gliadin, preceeded the toxic effects of gliadin. However Carter *et al.* [17] could not find evidence of persistent virus DNA, even with a method that has a sensitivity of one copy of virus genome per cell. Figure 1 summarises the possible events in the toxic action of gliadin.

Gliadin toxicity is not a quantitative problem. From the Dutch food consumption survey 1987-1988 [18] the normal consumption of gliadin for the different age groups in the Netherlands can be calculated. For children from 2-5 years this amount is 200-250 mg/kg/day; for adults it is about 100 mg/kg/day. Tests with much higher quantities of gluten did not result in villus atrophy in normal individuals, so the effect is not due to differences in sensitivity between normal and CD patients.

Table III: Evaluation of toxicity of prolamin derivatives in coeliac patients.

In vitro	*in vivo*
biopsy culture	*in man*
lysosomal damage	challenge
lymphocyte migration	instillation
K-cell agglutination	rectal instillation
mannane interaction	*in animal*
foetal rat intestine	Irish setter dog?
foetal chicken intestine	cynomolgus monkey?

Measuring toxicity

The only 100% reliable proof of toxicity is the administration of the peptide – isolated or synthesised – to a patient in remission and verifying the damage by taking biopsies. This procedure poses ethical problems: it needs the cooperation of patients; and could require considerable quantities of substance to be tested in each patient. The problem with all tests for toxicity is that to make a quantitative study, all the prolamins, fractions or

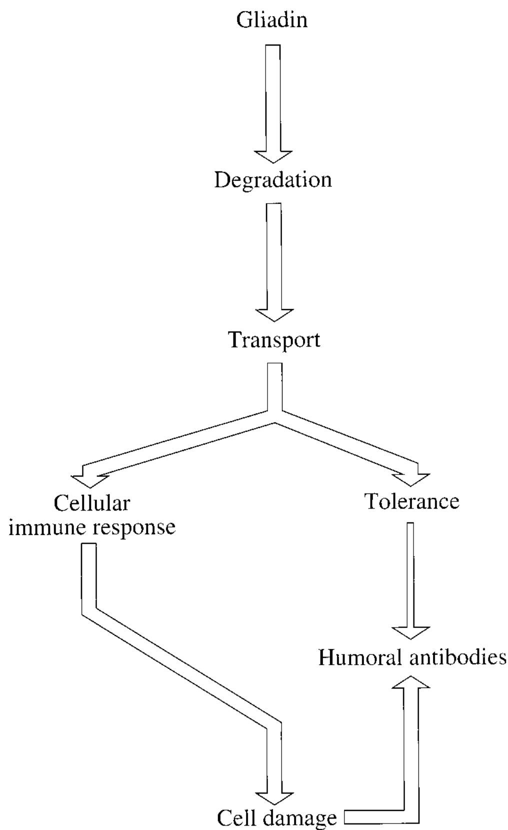

Fig. 1. Summary of the events that can play a role in the toxic reaction of gliadin. Both degradation and transport can alter the presentation of toxic material. The low level of humoral antibodies formed under normal tolerance are greatly increased when toxic material damages through cellular response the intestinal defence system.

peptides should be tested in random order in the same group of patients under strictly constant conditions. Therefore a number of other methods have been developed that at least give an indication of whether or not a peptide is potentially toxic (Table III).

With these methods, the culture of biopsies from patients comes very close to the clinical challenge of instillation of gluten directly into the duodenum [19] or the rectum [20], measuring the effect of a preparation on the intestinal wall by biopsy within 24 hours. From the other *in vitro* methods, the culture of foetal rat intestine [21] or chicken [22] are the most elegant and promising. Two animal species [23, 24] are reputed to be sensitive to gluten but are not yet used routinely in testing potentially toxic substances.

The chase for the toxic factor

From the start of the search for the toxic priciple a great deal of work has been focussed on identifying a fragment of minimum length that causes the toxic reaction in coeliac patients. The general idea was that by defining such a fragment it would be possible to define the enzyme or immune defect and perhaps to find a wheat species in which this sequence was missing.

Since the classical experiments of Dicke, Weijers and van de Kamer, we know that gliadin is the toxic fraction in wheat. They also tested glutenin and could not demonstrate its toxicity [25]. There is however no confirmation of these experiments published in literature and according to Kasarda there are structural elements in glutenin that potentially are toxic to patients with CD [26].

From the gliadin fractions, the α-, β- and γ-gliadin are proven to be toxic. There is some doubt about the toxicity of ω-gliadin. The toxicity is in any case less than that of the other gliadins [27]. There is however no systematic study available to establish these relative effects. This is also the case with the prolamins from rye, barley and oats. The toxicity of oats is definitely less than that of the other prolamins.

Most of the efforts were directed to the separation, breakdown and identification of the gliadins, the prolamins from wheat. The first attempt to identify a pure protein was made by Hekkens *et al.* [19]. Since then, together with the sequence work of Kasarda a more systematic approach to the fractionation problem has been followed. The origin and sequence of the most important gliadin fragments reviewed are summarised in Figure 2.

Cleavage of gliadin into three fragments revealed that the C- and N-terminal parts were toxic. As seen above, they contain most of the β-turns, the regions that are especially immunogenic, so it

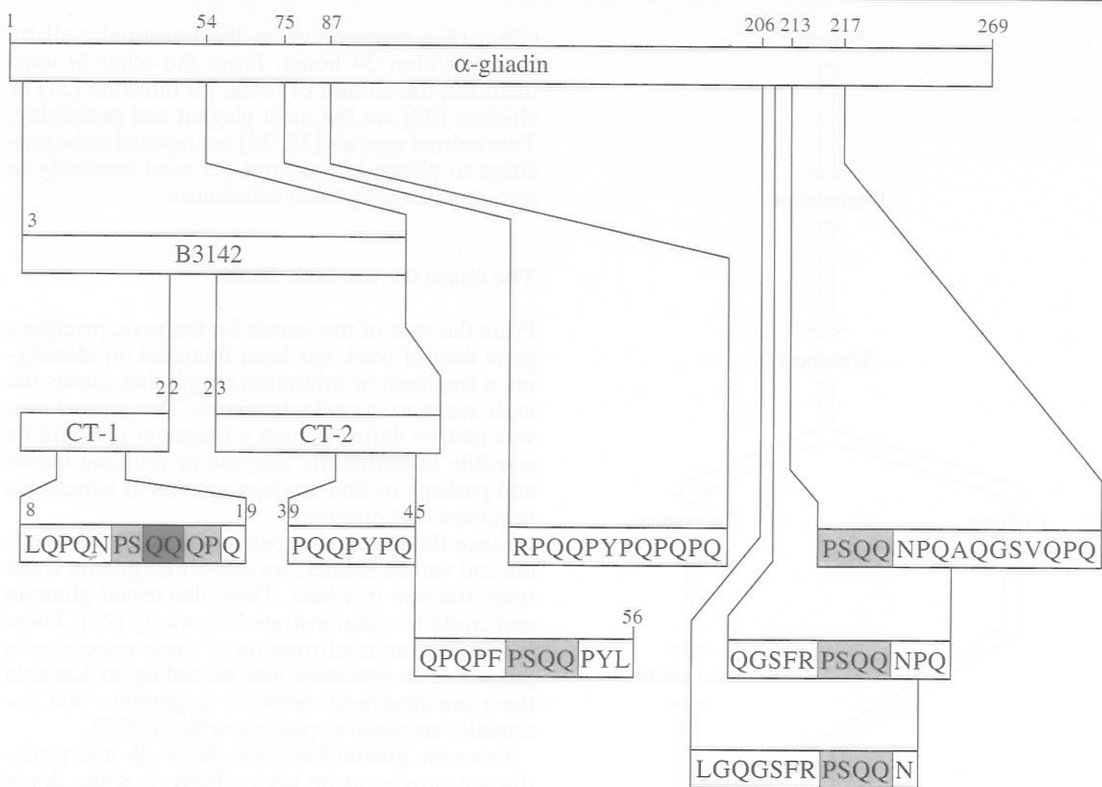

Fig. 2. Fragments of α-gliadin that have been tested by several authors and reviewed in this article. Fragment 3-54, CT-1, CT-2 and 39-45 by Wieser et al. *[28]; 8-19, 45-56 and 206 -220 by Kocna* et al. *[32]; 75-86 and 213-227 by Cornell [29] and 206-217 by Mantzaris and Jewell [33]. Amino acids in one-letter code: A=alanine; R=arginine; F=phenylalanine; G=glycine; L=leucine; N=asparagine; P=proline; Q=glutamine; S=serine and Y=tyrosine. The two tetrapeptides that are candidates for the toxic principle are hatched in the different peptides.*

is logical to look for a relation between immunogenicity and toxicity. Of special interest are of course the peptides that are shared by gliadin and the other toxic cereals: rye, barley and oats. Common pentapeptides were found in gliadin and hordein (prolamin from barley), but no longer peptides, nor were they shared by the other toxic prolamins, avenin (prolamin from oat) and secalin (prolamin from rye). Tetrapeptides are more common and Kasarda proposed the peptides:

proline-serine-glutamine-glutamine (in one-letter code PSQQ)
and glutamine-glutamine-glutamine-proline (code QQQP)

as possible candidates for the toxic effects.

Wieser *et al.* [28] isolated the first 54 amino acids from a digest and proved that that part was toxic *in vivo* and *in vitro*. It contained both sequences that were proposed by Kasarda. The peptide isolated by Cornell [29] that was not digested by mucosal preparations from patients with CD, however, did not contain this sequence. In a later study Cornell [30] isolated the peptides corresponding respectively to the sequences 75-86 and 213-227 of Kasarda's α-gliadin. Both fractions were active in the foetal chicken assay. The last one also contained the sequence PSQQ. A shorter peptide than that similar to 75-86, was not active.

Further evidence for the sequence QQQP being toxic comes from studies of Auricchio [31] using

the organ culture of foetal rat intestine and untreated coeliac mucosa. In both systems the gliadin peptide 31-55 is toxic; the toxicity is still present in the 31-43 peptide (containing QQQP), but not in the 44-55 fragment containing the other putative toxic sequence PSQQ.

Kocna *et al.* [32] synthetisized a number of peptides from the N-terminal and C-terminal part of gliadin, being the two parts that were found toxic in foetal rat intestine culture and tried to prove their effect on foetal chicken intestine. They found a pronounced effect of only one of these peptides and a lesser effect in two others. All these peptides contain the sequence PSQQ, the most active peptide also has the sequence QQQP. The tetrapeptides as such did not have a toxic effect.

Mantzaris and Jewell [33] synthesised a peptide corresponding to the sequence 206-217 of A-gliadin. This peptide corresponds with a sequence in the E1B-protein from adenovirus 12. They tested this peptide by instillation in the duodenum of two coeliac patients and in two controls with irritable bowel syndrome. In the coeliac patients the disaccharidase activities decreased within 24 hours, infiltration of the lamina propria with mononuclear cells was seen after 2 hours and a rise in IgA-containing cells was seen in one patient. No changes were seen in the controls.

This very important step in the elucidation of the toxic fragment is certainly not the last. It is however a turning point in the hunt for the relation between peptide structure and effect on the intestinal mucosa. It is the first clear indication of an effect of synthetic peptides in coeliac patients. It opens the way to a more systematic approach to the definition of the toxic sequences in coeliac disease. It proves that it is the peptide sequence that determined the effect.

The "gluten-free" diet

The Codex Alimentarius standard defines a diet as "gluten-free" when *the total nitrogen content of the gluten containing cereal grains used in the product does not exceed 0.05 g/100 g of these grains on a dry matter basis.*

There are however a number of possibilities to contaminate products that are in their normal form gluten-free. The long and tortuous route from field to customer provides a number of opportunities for contamination. A limit based on the nitrogen content of a product therefore does not exclude the presence of gliadin in the product.

When sensitive immunochemical methods were developed the gliadin content could be measured. From the determinations of Skerrit and Hill [34] by a monoclonal ELISA method and by our own measurements with a polyclonal antibody, it has been shown that in wheat starches, meeting the Codex Standard on nitrogen content, an amount of 20-30 mg/100 g of gliadin is still present. Apparently this amount is low enough to be tolerated by the great majority of coeliac patients when normal quantities of these products are eaten.

Apart from the concentration of gluten in a product we also need to know what quantity of that product a patient can tolerate. There is only one publication of Ejderhamn *et al.* [35] which addresses this issue: they followed 11 children aged between 13 and 17 years over 10 years. Calculating the daily gliadin intake by a three day-record method they found an average amount of 8 mg/ day. When they calculated the intake after determination of the gliadin content in the ingredients by an ELISA-method, they found an average intake of 1.4 mg/day. No intestinal damage could be found in these patients.

This quantitative aspect of intake per day has not been taken into consideration in the Codex Standard. Although a few coeliac patients may be highly sensitive to small amounts of gluten, a zero level of gliadin in cereal based gluten free starches is neither possible nor necessary. We therefore propose to set the level in the revised Codex Standard to an amount of 10 mg gliadin/100 g of product on a dry matter basis. It is expected that this level will not cause damage to the majority of patients who have lived without problems under the previous nitrogen based Standard with an even higher maximum level of gliadin.

There is need for further research in order to develop immunological techniques for the detection of gliadin in multi-ingredient cooked food. At the same time efforts should be made by the legislator to ensure that: 1) the addition of gluten as an ingredient to food should be mentioned on the label; 2) gluten-free food should be labeled to

indicate the origin of the starch used. Particular attention should be paid to prevent the addition of gluten to infant formulas, as there is evidence that this could change the natural history and the time of presentation of the disease.

Gliadin analysis

The analytical method for the determination of gliadin has its problems. As long as the structure of the toxic peptide is not known, the best method is to determine the total gliadin amount in a product by a method that is specific and has a sensitivity that meets the Codex Standard. This means that the lower limit of the method should be under 10 mg/100 g; or under 100 ppm. A number of methods have been published to date (Table IV).

Table IV: Analytical methods available for the determination of gliadin in food.

Method	Reagent used	Detection limit	Evaluation	Reference
Elisa	polyval.	10 ng/ml	no	[36]
Elisa	polyval.	3 ng/ml	no	[37]
Elisa	polyval.	3 ng/ml	no	[38]
Elisa	polyval.	5 ng/ml	no	[39]
Elisa	polyval.	5 ng/ml	no	[40]
Elisa	monoclonal anti-B3144	15 ng/ml	no	[41]
Electroblot	polyval.		no	[42]
DNA-probe	polymerase chain react.	500 ng as wheat	no	[43]
Ouchterlony	polyval.	50 ppm	no	[44]
Elisa	monoclonal anti ω	16 ppm	ringtest	[34]
HPLC	column		no	[45]

We developed an ELISA-technique based on a polyvalent antiserum and ring-tested samples in the lowest part of the scale, making it not an easy but at least a realistic method in relation to the expected values in gluten-free food. When we compared our values with values from a method developed by Skerrit and Hill [34] we saw that in most cases the values agreed. The main problem in both methods is the variability of the results in different laboratories.

There are some other problems that are not yet solved by the laboratory methods in current use. They relate the stability of antibodies, cross reactivity, their avidity and subclass differences; the interference of carbohydrates and lectins, the reactivity of cooked products and of partly digested gluten.

Conclusion

More than one hundred years after Samuel Gee's statement: "If the disease can be cured it must be by diet" and nearly fifty years after Dicke's, Weijers' and van de Kamer's proof that it was the gliadin in wheat that caused the disease, we still do not know the exact structure of the toxic factor; nor do we know the amount of gliadin that is necessary to elicit the damage to the intestinal wall nor the differences in sensitivity between individual patients. When we accept that even a gluten-free diet contains some gliadin, then the conclusion must be that the intestine can handle at least small amounts of prolamins.

The length of the peptide must be between 8 and 12 amino acids to reveal its toxic effect although the toxic sequence is perhaps not longer than four amino acids. From the results obtained with synthetic peptides, we know that it is the amino acid sequence that causes the toxicity and not any glyco-, lipo- or other side chain in the peptide. A limit of 10 mg gliadin per 100 g product on a dry matter basis is tolerated in the diet of the majority of coeliac patients.

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