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The Significance of Key Amino Acid Sequences in the Digestibility and Toxicity of Gliadin Peptides in Celiac Disease

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Abstract The importance of alternative or adjunct treatments to the gluten-free diet in celiac disease is now being recognized. This paper discusses the scientific principles behind the use of caricain for enzyme therapy. *Objective*: To review the structures of the toxic peptides in A-gliadin that relate to those found by other workers insofar as having key sequences of amino acids or motifs which relate to toxicity, especially in regard to difficulty of digestion or immunogenicity. *Methods*: Structures of synthetic A-gliadin peptides shown to be toxic in the fetal chick assay were examined before and after digestion with duodenal mucosa from patients in long remission. Synthetic peptides corresponding to the undigested residues were also assayed and the key amino acid sequences compared in order to determine if they could be related to direct toxicity and immunogenicity of the peptides. *Results*: The results showed that the smallest toxic peptides from celiac mucosal digestion were octa-peptides and that they were obtained in greater yield than similar products from normal digestion. One of those peptides corresponded to residues 12-19 of A-gliadin and contained the key motifs PSQQ and QQQP of De Ritis et al. [1], whilst the other corresponded to residues 72-79 and contained the key motif PYPQ (extending to PYPQPQ), observed by other workers, especially those who have been investigating immunological activity over the past two decades. *Conclusions*: The presence of key motifs in undigested residues from celiac mucosal digestion and the greater prevalence of these residues compared with residues from normal digestion justifies our work on enzyme therapy. These studies have also indicated that our use of caricain as an enzyme capable of digesting peptides with two different types of toxicity has a sound scientific basis.

Keywords: *celiac disease, enzyme therapy, gliadin peptides, amino acid sequences, caricain, gluten*

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1. Introduction

It is important that for the first time there appears to be either alternatives to the gluten-free diet or at least some treatments that could be called adjuncts to the gluten-free diet [2,3] for the treatment of celiac disease (CD).

CD affects an estimated 10 million people globally [4] and is triggered by glutens in wheat, rye and barley. It is one of the most common food-related lifelong disorders in the world [5].

CD is normally detected by serological tests and confirmed by duodenal biopsy [6] when the patient is on a normal gluten-containing diet. Symptoms normally found are of the gastrointestinal type such as stomach pain, bloating, diarrhea as well as lassitude and other symptoms related to malnourishment, due to damaged small intestine. Long-term complications of CD include adenocarcinoma, lymphomas and overall increased mortality [7]. At the present time, treatment of CD is based around the use of a gluten-free diet, but such a diet places a heavy burden on the individual and requires constant vigilance as gluten is a very ubiquitous material. It has been reported that, not surprisingly, a large proportion of patients report inadvertent and sometimes even deliberate ingestion of gluten [8].

In view of our extensive experience looking into the basic etiology of CD, we began our search for an enzyme which could be used to replace the defective enzyme in CD. Our first clinical trial was based upon oral administration of a pig mucosal extract. The results were encouraging as we reported that amelioration of the symptoms occurred and some signs of histological improvement were seen even when volunteers were challenged with 1 g of gluten daily [9].

We were then forced to change to a plant-derived enzyme due to emergence of animal viruses and prions capable of infecting humans. Our current clinical trials show that we are well placed, both with dermatitis herpetiformis [10] and CD [11] to provide an adjunct treatment to the gluten-free diet that will not only ameliorate symptoms but may help to provide small bowel recovery in a shorter time than without the supplement.

In order to understand the value of mucosal digestion of gliadin, the use of synthetic A-gliadin peptides and the use of other models of toxicity in our understanding of CD, the work of De Ritis et al. [1] needs to be presented again in the light of our own work.

The work of Kocna et al [12] was also very important to our studies. Working with synthetic peptides, they used the fetal chick bioassay to test the toxicity of several synthetic peptides. They found that peptide 8-19 of A-gliadin was toxic in this assay, which we confirmed. Subsequently we used this peptide for our studies of mucosal digestion [13] because we noted that it had both De Ritis motifs PSQQ and QQQP. We also studied smaller peptides within the 8-19 residues of A-gliadin. In all of these studies we relied upon the fetal chick bioassay of Mothes et al. [14] for evaluation of the toxicity of peptides coupled with the use of different synthetic peptides to give us information on the effect of structural changes [15,16].

It was important to note that the Kocna peptide 8-19, which was active in the fetal chick bioassay, was part of the toxic serine-containing peptide 5-20, found in Fraction 9 [17]. Hence in trying to pinpoint the smaller active part of the A-gliadin molecule (sometimes called an epitope) we endeavored to find a motif associated with toxicity as De Ritis et al. had done.

In all our work we focused entirely on structures in A-gliadin in order to maintain an uncluttered approach. If other programs are considered, particularly those which focus on providing immunity, if it is obvious that many other wheat proteins need to be taken into account, and for that matter, rye and barley proteins. By comparison, more complete digestion of A-gliadin proteins is easier to monitor, especially considering the opportunity it provides for making use of synthetic peptides for structural studies.

2. The Work of De Ritis et al.

De Ritis et al. [1] cleaved A-gliadin with cyanogen bromide and chymotrypsin, purified the resulting peptides and tested their toxicity by tissue culture of celiac intestinal mucosa. The sequences PSQQ and QQQP motifs were present in the toxic peptides but absent in the non-toxic peptides. The numbers refer to the particular residues in A-gliadin. The findings were:

Peptide 1 – 30 toxic, PSQQ & QQQP (present in overlapping sequences)

Peptide 31-55 toxic, QQQP and PSQQ present

Peptide 56-68 non-toxic, absence of QQQP and PSQQ

Peptide 128-246 toxic, QQQP and PSQQ present

Peptide 247-266 non-toxic, absence of QQQP and PSQQ

The motifs PSQQ and QQQP associated with toxicity of A-gliadin are located at the following residues:

Peptide 13-16	PSQQ
Peptide 15-18	QQQP
Peptide 33-36	QQQP
Peptide 50-53	PSQQ
Peptide 188-191	QQQP
Peptide 213-216	PSQQ

The A-gliadin molecule has some interesting features. There is a large molar percentage of glutamine/glutamic acid (35.3%) mostly as glutamine. There are 18 consecutive glutamine residues between 96 and 113. Proline is present at 13.5% (molar) but there is no proline from residue 94 to residue 163. The highest proline-rich areas (20 residues) are 41-60 and 61-80 each with 35% proline (molar). The toxicity of gliadin is not caused by a single fraction of α, β or γ-gliadin but is found in peptides from several parts of those molecules. For simplicity, in all subsequent work we concentrated on peptides derived from A-gliadin (like α-gliadin) as several other researchers have done. Most importantly, we have sought evidence of other amino acid motifs that could be associated with toxicity.

2.1. Relationship of De Ritis et al. Research to Our Own Work

It is obvious that PSQQ and QQQP in overlapping sequence is not sufficient for toxicity in the fetal chick bioassay since peptide 13-18 was found to be non-toxic [15].

Furthermore, even larger peptides such as 11-17 and 11-18 are non-toxic.in the fetal chick bioassay [14]. Besides size, other factors have to be taken into account. Peptide 12-19 is toxic, yet 10-19 is only slightly toxic perhaps because the latter has an N-terminal proline. It is also notable that peptide 11-18, being an octa-peptide, would be expected to have some activity, but the deletion of the C-terminal glutamine from peptide 11-19 reduces the activity considerably.

Table 1. Data showing that one or both of the two types of amino acid motifs proposed by DeRitis (PSQQ and QQQP) are present in our toxic serine – containing peptides as well as in peptides shown by other workers to be toxic in CD

Researchers	Toxic peptides (A- gliadin sequence)	Structure/key sequence
Kocna et al (1991)	8-19 (active)*	^{8}LQPQNPSQQQPQ^{19}
Cornell and Mothes (1995)	8-19 (active, confirmed)* 11-19 (very active)	^{8}LQPQNPSQQQPQ^{19} ^{11}QNPSQQQPQ^{19}
Cornell and Rivett (1995) (octapeptide residue)	12-19 (active)	^{12}NPSQQQPQ^{19}
De Ritis et al (1988)	1-30 31-55	PSQQQP QQQP, PSQQ
Giovannini et al (1997)	31-43 45-55 56-68 (non-toxic)	QQQP PSQQ (no motifs)
Mantzaris and Jewell (1991)	206-217	^{206}LGQGSFRPSQQN^{217}

*Activity refers to activity in the fetal chick bioassay of Mothes et al (1985). Not applicable to the other workers listed above.

We noted that PSQQ and QQQP were also motifs in the highly toxic peptide corresponding to A-gliadin 11-19 which explained why peptide 5-20 in Fraction 9 contributed to the toxicity of this fraction in the same assay [14]. Amino acid deletions, made in order to determine the minimum sized peptide that was still toxic showed that peptide 12-19 was still toxic but 11-18 was not toxic. The importance of peptide 12-19 is that it is one of the main peptides in the residue from celiac mucosal digestion of peptides 8-19 and 11-19 [13]. It contains both the De Ritis motifs PSQQ and QQQP. The toxic peptide of Mantzaris and Jewell [18], A-gliadin 206-217, contained only PSQQ (refer Table 1). Giovannini et al. [18] found that peptide 31-43, with the motif QQQP but without PSQQ was active in their assay (refer to Table 1).

2.2. Gliadin Peptides with Reported Toxicity to Patients with CD

A major component in Fraction 9 was shown to be a peptide near the N-terminus of A-gliadin, corresponding to residues 5-20 [17]. Hence, the toxicity of peptides of this type would be due to De Ritis motifs of PSQQ (residues 13-16) and QQQP (residues 15-18). We found that, using synthetic peptides, the most active peptide in this region was peptide 11-19, a nona-peptide with both motifs overlapping. Remembering that the peptide 13-18 (PSQQQP) was non-toxic in the fetal chick bioassay it seems that the hexa-peptide combined motif needs flanking amino acids for the motif to be associated with toxicity. The question then needs to be asked is: how big does this peptide have to be before toxicity is evident? The answer is provided by our findings of octa-peptide residues from celiac mucosal digestion of peptide 11-19, these being peptides NPSQQQPQ and QNPSQQQP containing both PSQQ and QQQP [13]. The toxic peptide 31-43 of Sturgess et al. [24] is too large to make conclusions about this issue although the work of Giovannini et al. [19] indicates that peptide 31-43 is also toxic to cells in culture, other than those from patients with CD. This peptide only has the QQQP motif, not the PSQQ motif whereas our peptide 11-19 has both. In the octa-peptide residues from celiac mucosa digestion above both motifs are present. The 33-mer peptide [20] and the Anderson et al. [21] peptide 57-73 have neither motif which suggests that they are associated more with immunogenicity than cytotoxicity.

In order to obtain evidence for a consistent pattern of a structural relationship in gliadin peptides and toxicity or immunogenicity in celiac disease it was necessary to relate the findings of our group and its collaborators with those of other researchers. In this way, points of agreement seem to suggest that there is an amino acid motif common to the work of several groups, in which tyrosine, proline and glutamine feature. The idea of this springs from the work of De Ritis et al [1] but for the first time includes tyrosine as a key amino acid, together with proline and glutamine. These types of peptides depend upon immunological reactions to bring about the mucosal damage observed in CD. They differ from peptides containing the PSQQ and QQQP motifs, which are strongly associated with cytotoxicity, but what they have in common is that they are both present in undigested residues from remission celiac digestion. Therefore in the design of any intervention for protection of coeliac patients by vaccination against gliadin peptides, those with direct toxic action must be taken into account. However, let us consider what has been learnt about gliadin toxicity by referring again to our work on Fraction 9.

2.3. Toxic Tyrosine – Containing Peptides

Another sub-fraction of Fraction 9 also showed activity in the fetal chick bioassay, but this peptide displayed rather more immunological activity than the high direct toxicity, exhibited by peptide 11-19. The sequence of this peptide was quite different from that of 11-19 and did not contain the PSQQ or QQQP motifs. Its structure was RPQQPYPQPQPQ (A-gliadin 75-86) and it appeared to be the most prevalent toxic peptide in Fraction 9 [17]. This fraction also produced the most γ-interferon in blood of celiac patients [22]. Cornell and Wills-Johnson [23] showed by computer modeling techniques that peptide 75-86 had essentially β-structure as opposed to the α-helical structure of peptide 11 – 19.

It should be noted that peptide 31-43 [19] contains the QQQP motif, whereas peptide 44-99 contains the PSQQ motif. Now, although both these peptides contain in addition, QQPY, that particular tyrosine – containing motif is not the one we are suggesting is necessary for toxicity or immunogenicity. Instead, it is PYPQPQ. QQPY and PYPQPQ however are part of both the toxic and immunogenic peptide 75-86 and the residue from celiac mucosal digestion of the toxic tyrosine -containing peptides.

The toxic peptide of Sturgess et al. (A-gliadin 31-49) [24], not only contains the De Ritis motif QQQP but also the tyrosine – containing motif PYPQPQ that one can see is common to toxic or immunogenic peptides of several workers (refer Table 2). The structure of peptide 31-49 is: 31 LGQQQPFPPQQPYPQPQPF 49.

Table 2. Proposed amino acid motifs in the tyrosine – containing gliadin peptides of other workers shown to be toxic or immunogenic in CD

Researchers	Toxic peptide (A-gliadin sequence)	Structure
Cornell and Mothes (1993)	75-86	^{75}RPQQPYPQPQPQ^{86}
Sturgess et al (1994)	31-49	^{31}LGQQQPFPPQQPYPQPQPF^{49}
Anderson et al (2000) [x]	57-73	^{57}QLQPFPQPQLPYPQPQS^{73}
Shan et al (2002)	57-89 (33-mer peptide from α_2 gliadin)	^{57}LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF^{89}
Cornell (1998)	77-84 (β peptide residue)	^{77}QQPYPQPQ^{84}
Mc Lachlan et al (2002)[xx]	Sequences common to wheat, rye and barley	PQQPYQQPYP

[x] toxicity of peptide not yet verified
[xx] both sequences present in Cornell and Sturgess peptides.

2.4. Evidence from Remission Celiac Mucosal Digestion of Synthetic Toxic Peptides of A-gliadin

Emphasis was placed on the composition of peptide residues remaining after celiac mucosal digestion of synthetic peptides. The two different types of peptides previously referred to as serine – containing and tyrosine-containing peptides were each digested with remission celiac mucosa and normal mucosa and the residues subjected to gel filtration and amino acid analysis [13,25]. The residues from celiac mucosal digestion (Mw > 400) had amino acid composition consistent with their having the molar composition NSQ4P2 and for both peptide 11-19 and peptide 8-19, the amounts of residues were much higher from celiac mucosal digestion than they were from normal mucosal digestion. On looking at the structures of the peptide 8-19, viz. 8 LQPQNPSQQQPQ 19, it is clear that the N-terminal leucine has been removed by celiac mucosal digestion but not the serine residue or the asparagine residue. In view of the ratio of 2:1 for Q: P one could speculate that the undigested residue (Mr >400) was 12 NPSQQQPQ 19, an octa-peptide found to have activity in the fetal chick bioassay [14]. It was noted that this peptide had the PSQQ and QQQP motifs of De Ritis et al. [1].

It was important to determine if the same conclusions could be drawn concerning the toxic peptide A-gliadin 75-86 mentioned previously. This peptide, together with peptide 75-86, were subjected to the celiac mucosal digestion when it was found that the undigested peptides in the Mr>400 fraction of each peptide contained glutamine, proline and tyrosine with the likelihood of it being A-gliadin 77-84 (QQPYPQPQ).

It should be noted that the arginine residue has been removed by celiac mucosal digestion, together with the adjacent proline residue. The ratio of glutamine: proline was found to be 1.27:1, which is close to expected (1:33) for peptide 77-84. This peptide has been shown to have activity in the fetal chick bioassay [16] and that it is likely to have an amino acid motif that is the reason for it being difficult to digest by remission celiac mucosa and may explain why this peptide is toxic.

2.5. The Possibility of a Tyrosine – Containing Motif Relating to Toxicity

It was necessary to consider looking for other types of motifs since neither the PSQQ nor QQQP motifs are present in the toxic peptide 75-86. Furthermore, these motifs are not present in the 33-mer peptide or the 57-73 peptide of Anderson [21]. An interesting point here is that our peptide 75-86, like the aforementioned peptides, has neither PSQQ nor QQQP. The amino acid tyrosine is the one that might be expected to be implicated, so it was important to determine if similar types of motifs existed in regard to the tyrosine containing toxic peptides of A-gliadin. There are several reasons why these tyrosine-containing toxic peptides are of interest:

1. Tyrosine is present in two of the three motifs common to the three celiac-toxic cereals – wheat, rye and barley. These motifs are PQQPY, QQPYP and QQQPFP [25].
2. Tyrosine is present in the major toxic fraction of a natural gliadin digest (Fraction 9) which, when purified was found to have the amino acid sequence corresponding to residues 75-86 of A-gliadin, this being RPQQPYPQPQPQ [16].
3. Tyrosine is present in the undigested, but still toxic, peptide residue from digestion of peptide 75-84 of A-gliadin with remission celiac intestinal mucosa, this peptide corresponding to residues 77-84 i.e. QQPYPQPQ [26]. This is expected to be the smallest peptide that retains toxicity.
4. Tyrosine is present in peptide 31-49 of A-gliadin, shown to be toxic in vivo to celiac patients [24].

The tyrosine-containing motif being sought may be from 4-6 amino acids in size and is also likely to contain proline, an amino acid often associated with the difficulty of digestion of proteins. It is notable that peptide 75-86 contains 42% proline (molar). Hence motifs of 4 amino acids that could be considered are QQPY, QPYP or PYPQ. However, all of these are present in peptide 75-86 and the toxic (in vivo) peptide 31-49 [24].

The regions of A-gliadin that have toxicity possibly due to a tyrosine motif which is expected to also contain glutamine and proline are:

Peptide 31-43 PQQPY (also has QQQP at 33-36)
Peptide 31-55 PQQPY (also has QQQP at 33-36)
Peptide 45-56 QQPY (also has PSQQ at 50-53)
Peptide 75-86 PQQPY

Tyrosine residues are at 43, 55, 68, 80, 87, 149, 204 and 250 but not all of these are close to proline.

The other amino acids in the tyrosine-containing motif are likely to be the ones which are common to the ones where toxicity has been established. For example *in vivo* toxicity has been shown with peptide 31-49 [24] which has PQQPYPQ, and *in vitro* toxicity with our 75-86 peptide which also has PQQPYPQ. Other toxic peptides cited do not have this hepta-peptide motif but we have to take into account the immuno-dominant peptide 57-73 of Anderson et al. [21] and the Shan et al. [20] 33-mer peptide 57-89, both of which have PYPQPQ. This means that perhaps the motif is the smaller PYPQP, common to all. It is important to note that the undigested residue of the peptide 75-86 is peptide 77-84, QQPYPQPQ, which contains this residue as well as one of the three amino acids sequences common to wheat, rye and barley (QQPYP). It is the smallest toxic tyrosine-containing peptide yet reported.

3. Enzyme Therapy - Structures that Need to be Targeted by Enzyme Therapy for This Treatment to be Effective

If this work and the related work of others has a sound scientific basis, it would appear that an enzyme capable of disrupting key sequences of amino acids would be likely to be effective in helping to overcome the toxicity of gluten. Such an enzyme would have to be delivered to the site where it would be most effective and one way of doing this is by the use of an enteric coating that only disintegrates when it arrives in the higher pH of the small

intestine. Here, it can be given a chance of completing the digestion of the toxic gliadin peptides so that they are no longer able to exhibit direct toxicity or initiate pathological immunological reactions.

With this in mind, we set about developing an enzyme supplement which would be able to detoxify small amounts of gluten that would otherwise continue to damage the small intestine of patients who are meant to be on a gluten free diet but inadvertently consume small amounts of gluten. Our idea of using an enzyme supplement was a logical progression because of our early findings that an enzyme deficiency was indicated [26].

The enzyme found to be most effective in detoxifying a peptic-tryptic-pancreatinic digest of gliadin was caricain, a serine hydrolase found in the plant *Carica Papaya*. We discovered that crude papaya extract was very effective in detoxifying our peptic-tryptic-pancreatinic digest of gliadin using a rat-liver lysosome assay [28]. Furthermore, we then showed that it was the enzyme caricain in this extract that was mainly responsible for the detoxification. Experiments with pure caricain confirmed this finding [29].

Other enzymes present in crude caricain extract may also play a part in detoxification of gluten [27], these are:

Chymopapain 3.4.4.11. Acts like papain and is not likely to provide specific attack that detoxifies gliadin.

Prolyl endopeptidase 3.4.21.26. Attacks prolines within peptide chain at C-terminal end

X – P – Y – Z
↑

and X – Y – P – Z etc.
↑

The particular enzyme we tested in the rat liver lysosome bioassay was not very effective [28]. However, more recently, there has been a report of highly efficient degradation of gluten by a new type of prolyl endopeptidase derived from *Aspergillus niger* [29].

Glutamine cyclotransferase (Glutamine cyclase) 2.3.2.5. N- terminal residues of glutamine are cyclized to form N-pyrolidonecarboxylyl peptides.

Other papaya proteinases are also present.

Prolidase activity is also present, whereby N-terminal proline residues are split from a peptide. Our work would suggest that these types of enzymes can also help in the initial stages of peptide digestion.

Regarding the PSQQ and QQQP motifs, attack on the proline of PSQQ at its C-terminal side would be achieved by attack by a prolidase or a proline endopeptidase. Attack on the QQQP motif would be achieved by an enzyme with prolidase activity which we think is inherent in caricain.

Detoxification as a result of enzymic attack could occur in a number of ways. They could include the disruption of the key tyrosine motif in 75-86 and likewise the key serine motif (PSQQ) in peptide 8-19 by attacking the C-terminal peptide bond of proline at position 13.

Obviously, many enzymic reactions occur with rapidly changing substrates making the complete digestion a very complex matter. Caricain is able to play a major role, particularly in attacking proline residues, which play a key role in toxicity. Peptide 75-86, believed to play an important role in damaging small intestinal tissues, contains 42% (molar) proline, as does the 33-mer peptide of Shan et al. [20]. The attack of proline at its N-terminal peptide bond is believed to be an important factor in its successful detoxification of the various A-gliadin peptides. Pure papain has not been implicated in enzyme therapy for CD [28].

3.1. The Action of Caricain on Toxic Serine Containing A-gliadin Peptides

Caricain appears to be able to disrupt the key amino acid sequences of De Ritis (PSQQ and QQQP) as well as those key tyrosine proline and glutamine – containing sequences proposed as a result of our various types of investigation. Taking these sequences found from synthetic A-gliadin peptides that are toxic to fetal chick mucosa and moreover, found in residue from celiac mucosal digestion of the toxic peptides, we propose that both types of toxic peptides i.e. serine – containing and tyrosine-containing peptides are attacked by caricain, thus rendering them harmless to the mucosa of celiac patients. Direct evidence for detoxifying serine-containing and tyrosine-containing peptides is not available, but implicated by the findings to date. For example, in order to detoxify the octa-peptide residue A-gliadin 12-19, 12 NPSQQQPQ 19, by disrupting the QQQP De Ritis motif, attack on the N-terminal side of proline residue 18 is necessary i.e. prolidase activity. The work of Mantzaris et al. [18] implied that the PSQQ motif is the important one.

An important point here is that peptides 11-17 and 11-18 are not toxic, but this could be because of their small size. This may be also the case for peptide 13-18 (PSQQQP) having both De Ritis motifs but being only a hexa-peptide, it is too small to be toxic. Peptide 11-19 had the highest activity of all the serine – containing peptides in this part of the A-gliadin molecule.

3.2. Attack by Caricain on Tyrosine – Containing Peptides

With regard to the tyrosine – containing motif, the octa-peptide residue from celiac mucosal digestion of the related synthetic peptides 75-85 and 75-86 suggests that we need to consider the active residue 77-84 of A-gliadin (QQPYPQPQ) for detoxification by enzyme therapy. As indicated previously the tyrosine containing motif is likely to be within this residue.

The toxic peptide 31-49 of A-gliadin [24] contains the QQQP motif of De Ritis and an extended tyrosine motif QQPYPQPQ which is homologous with peptide 77-84 above. It has the structure: 31 LGQQQPFPPQQPYPQP QPF 49.

It is too large to be able to define the fundamental toxic structure. It does however, contain the 77-84 structure (QQPYPQPQ) of the toxic peptide residue from celiac mucosal digestion, which has in vitro toxicity in the fetal chick bioassay. As mentioned previously, the work of other researchers has indicated that PYPQPQ (peptide 79-84) may be the residues upon which to focus. Attack of one or more of these three proline residues (79, 81, 83) by an endopeptidase could render it harmless. The same would apply to the 33-mer peptide [20] which also has PYPQPQ, like the A-gliadin 77-84 residue from celiac mucosal digestion.

If we consider the tyrosine – containing residue from celiac mucosal digestion, peptide 77-84, the points of

attack need to be as indicated below in order to remove its toxicity. We know that peptide 77-82 is non-toxic so we can presume that this bond, on the N-terminal side of proline 82, would have been cleaved by caricain as shown:

^{77} QQP – YP – Q P – Q ^{84}
↑ ↑ ↑

Cleavage at proline 79 and 81 may also occur with caricain. (Refer Figure 1). If a tetra-peptide motif is postulated as being necessary for toxicity it is likely to be PYPQ. Otherwise it could be the hexa-peptide motif PYPQPQ which is based on the commonality of the results of researchers in Table 2. The latter is one of the five sequences common to wheat, rye and barley [25]. Both these motifs are by themselves, not toxic peptides (as was the case for peptide 13-18) but depends upon the size of the peptide and perhaps also the types of flanking amino acids. An octa-peptide containing the motif appears to be the minimum size for expression of toxicity.

^{75}RPQQPYPQPQPQPQ^{86}	toxic
^{75}RPQQPYPQPQP^{86}	toxic
^{76}PQQPYPQPQP^{85}	non-toxic (N-terminal proline)
^{77}QQPYPQPQ^{84}	toxic (coeliac mucosal digestion product)
^{77}QQPYPQ^{82}	non-toxic (hexapeptide)
QQPYP	one of two sequences common to wheat, rye and barley, the other, PQQPY, is seen in peptides containing residues 76-80.

Figure 1. Structure of toxic peptide 75-86 and the toxicity of its breakdown products

Previous work showed that remission celiac mucosa does not completely digest toxic gliadin peptides and significantly large amounts of peptides of Mr greater than 400 Da remain. A typical example will the peptide 75-86 (PPQQPYPQPQPQ) [25].

An enzyme such as an aminopeptidase would attack this peptide and remove the arginine residue, thus allowing the remaining peptide to be attacked on the C-terminal side of the proline at residue 76 by the carboxypeptidase. A prolinase could then remove the newly formed N-terminal proline residue.

For peptides such as the above 76-86 and other peptides where proline is not the N-terminal amino acid, enzymes such as the prolyl endopeptidase come into play and attack proline residues on the C-terminal side.

3.3. Action of Dipeptidyl Peptidase IV (DPP.IV)

DPP IV detoxifies only peptides with proline or alanine as the penultimate amino acid. With such a complex mixture of peptides and enzymes, a number of reactions will occur in which N-terminal amino acids will be released, allowing the formation of peptides with a penultimate proline and thereby allowing reactions with DPP.IV. According to Janssen et al. [31] this enzyme has only a limited proteolytic affect.

With a peptide such as octa-peptide 12-19 the proline at position 13 can also be attacked by the enzyme DPP-IV giving NP + SQQQPQ, where the hexa-peptide product is probably too small to be immunogenic. Prolyl endopeptidase is another enzyme which could attach at the carboxyl peptide bond of P13 as well as at the P19 peptide bond.

Breakdown of the residual peptide 77-84, from digestion of toxic synthetic peptides by celiac mucosa, may not proceed any further with DPP IV, whereas it would appear to do so with caricain.

3.4. Non-celiac Gluten Sensitivity

There is increasing interest in the condition known as non-celiac gluten sensitivity (NCGS) where similar symptoms to CD are experienced, but without the duodenal villous atrophy as seen in acute CD [33]. However, serum concentrations of FABP2, a marker of intestinal epithelial damage were significantly elevated in the NCGS cohort as well as in the celiac disease group. Authors speculate that the epithelial damage may have occurred in other regions of the small intestine i.e. jejunum. It is supported by the fact that FABP2 marker is expressed primarily by the epithelial cells of the jejunum. In NCGS patients, the small intestinal damage and immune activation observed could be due to the incomplete digestion of gluten because of a partial enzyme deficiency and subsequent generation of toxic peptides. Such deficiency is not significant enough to cause the extensive damage seen in CD patients but is enough to cause a range of non-specific symptoms. NCGS patients experience onset of symptoms and activation of immune/biochemical responses on ingestion of gluten and alleviation of symptoms on withdrawal of gluten from diet, a similar pattern to CD patients. Biochemical markers would be expected to be more useful that symptoms in diagnosing the NCGS condition but the patient's own assessment of the well-being is also of paramount importance.

4. Do Opioid Receptors Mediate the Action of Gliadin?

Graf et al [34] have proposed that small peptides from gluten are exorphins because of being exogenous and having morphine like activity. They have shown that a small peptide such as α-gliadin 43-47, YPQPQ, their typical exorphin, fits with the structures of some of the tyrosine containing peptides we have proposed as motifs associated with toxicity in CD. Peptide YPQP, extending to YPQPQ, is a motif which is consistent with our work and the work of other researchers. Its relevance may be that these small peptides react with T-lymphocytes in a similar way to what happens in CD, except that the result of the peptides crossing the blood-brain barrier is a neurotoxic effect.

5. Conclusions

There is significant evidence that enzyme therapy should be able to play a vital role in addition to the gluten-free diet, in providing a safer regimen for those with CD. Properly chosen and with an enteric coating, the enzyme should compensate for the deficiency in digestion observed in the recovered small intestinal mucosa of those with CD so that harmless small peptides of gluten are produced and recovery of intestinal architecture will continue, despite traces of gluten ingested.

Studies with two different kinds of toxic peptides of gliadin have confirmed that small amino acid motifs are associated not only with the problem of digestibility by the celiac mucosa but with toxicity. In the case of the serine – containing toxic peptides, the motifs discovered by De Ritis et al, i.e. PSQQ and QQQP are also barriers to digestibility. The same principle was found to hold with the more immunogenic tyrosine containing peptides, suggesting that PYPQPQ was a barrier to digestibility by the celiac mucosa and a large part of the reason why larger peptides containing this motif are toxic.

With the aim of finding the smallest peptides of A-gliadin which are still toxic, it appears that with both types of toxic peptides i.e. directly toxic and immunogenic, the octa-peptides containing the key motifs are of the size where they still display activity in the fetal chick bioassay. Two of these octa-peptides are believed to be NPSQQQPQ and QQPYPQPQ.

The presence of key amino acid motifs, especially as they are in larger amounts from celiac mucosal digestion than from normal digestions, adds further evidence to our early investigations indicating an enzyme deficiency in CD and justifies further work on enzyme therapy. The action of caricain and other enzymes on these peptides results in small peptides without detectable toxicity and provide a sound scientific basis for enzyme therapy. Preventing the direct toxicity afforded by the toxic peptide residues present in digestates even in mucosal assays of coeliac patients in remission, should limit the entry of immunogenic peptides into the mucosa, affording a first line defence against the toxicity of gluten in coeliac patients who have an intrinsic incapacity to detoxify these gliadin residues. Nonetheless, the two approaches (reconstituting enzymatic capacity through caricain therapy) and attenuating the response to immunogenic peptides through vaccination) offer complementary and potentially synergistic therapeutic benefits

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