Coeliac disease: the abolition of gliadin toxicity by enzymes from *Aspergillus niger*

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Summary

1. Gliadin from which carbohydrate was removed by treatment with carbohydrase from *Aspergillus niger* was fed to three coeliac patients in remission.
2. Xylose absorption, mucosal morphology and brush-border enzymes were used to assess the toxicity of the carbohydrase-treated gliadin.
3. Gliadin treated with carbohydrases did not damage the intestinal mucosa of the coeliac patients.
4. The primary structure of the gliadin proteins was not altered by the enzyme treatment.

Key words: brush-border enzymes, coeliac disease, gliadin, intestine, mucosa, xylose.

Introduction

The gluten of wheat and rye was recognized to be the cause of intestinal damage in coeliac disease by Dicke (Dicke, Weijers & Van de Kamer, 1953; Dicke, 1950) and the toxicity was found to lie in gliadin, the 70% ethanol-soluble fraction (Van de Kamer & Weijers, 1955). Several investigations involving fractionation of gliadin have shown the toxicity of certain fractions to patients with coeliac disease when in remission, but have not yet identified the precise chemical nature of such fractions.

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Methods

Biochemical methods

Gliadin. Gliadin was prepared from a single variety of wheat (Triticum aestivum var. quern) according to Gehrke, Oh & Freeark (1964), and residual enzyme activity abolished by heating to 90°C for 1 min. Carbohydrate was removed from dialysed gliadin by dissolving 50 g in acetic acid (10 mmol/l) and adjusting the pH to 4.5 with dilute sodium hydroxide. A mixture of carbohydrases prepared from Aspergillus niger (Biomara Teo., Galway) was added and the solution left at 30°C overnight. When this digest was examined periodically for the appearance of amino groups with 2,4,6-trinitrobenzenesulphonic acid (Fields, 1972), proteolytic activity was found to be negligible. The gliadin was then dialysed and freeze-dried, before being baked into five 450 g loaves with gluten-free flour.

The diffusate was examined for peptides by colour development with ninhydrin and by high-voltage electrophoresis (Michl, 1951), the amount of dialysable ninhydrin-positive material being less than 0.001% of the total protein. The diffusate was also examined for carbohydrate by gas-liquid chromatography. The carbohydrate was quantitatively recovered with meso-inositol used as an internal standard (Phelan, 1974).

Peptide maps. Peptide mapping of gliadin and carbohydrase-treated gliadin was carried out by hydrolysis of approximately 2 mg of protein with pepsin at pH 2 (1% pepsin, w/w). The freeze-dried digest was dissolved in 250 µl of ammonium bicarbonate, pH 8.0 (20 mmol/l). Trypsin (1%) and chymotrypsin (1%) were then added simultaneously and the reaction was allowed to proceed for 16 h at 30°C. The entire sample was applied to Whatman 3 MM filter paper for electrophoresis at pH 3.5 for 80 min at 3000 V. The peptides were then chromatographed in the second dimension in butanol/acetic acid/water (3:1:1, by vol.) before staining with ninhydrin. The peptide maps (Fig. 1) showed no difference between the carbohydrase-treated and untreated gliadin, indicating that there was no alteration in the primary structure of the protein.

Chromatography of gliadin peptides. Untreated and enzyme-treated gliadin was chro-

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**Fig. 1.** Peptide maps of gliadin and enzyme-treated gliadin. Approximately 2 mg of protein was hydrolysed with pepsin, trypsin and chymotrypsin and applied to Whatman 3 MM filter paper for electrophoresis at pH 3.5 for 50 min at 3000 V. The peptides were chromatographed in the second dimension in butanol/acetic acid/water (3:1:1, by vol.) before staining with ninhydrin.
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Fig. 2. Chromatography of gliadin. Treated (a) or untreated (b) gliadin was applied to a column of carboxymethylcellulose in sodium acetate (50 mmol/l), pH 3.5, containing dimethylformamide (1 mol/l) and eluted with sodium chloride (Patey & Evans, 1973). Protein was monitored by absorption at 280 nm (■) and carbohydrate (▲) by the phenol/sulphuric acid test (Hirs, 1967). Peaks 1 and 2 were eluted with sodium chloride solutions (25 mmol/l and 45 mmol/l respectively); peaks 3 and 4 were eluted with salt gradients of 65–90 mmol/l and 100–500 mmol/l respectively.

Chromatography on carboxymethylcellulose (Booth & Ewart, 1969; as modified by Patey & Evans, 1973). The column was equilibrated with sodium acetate (50 mmol/l), pH 3.5, containing dimethylformamide (1 mol/l) and gliadin fractions were eluted with gradients of sodium chloride in the same buffer. Protein was detected at 280 nm and carbohydrate by the phenol/sulphuric acid test (Hirs, 1967). Chromatography of chymotryptic peptides was carried out on columns of Bio-Gel P6 (140 cm × 0.9 cm) in 1% formic acid.

Integrity of the primary structure of treated gliadin. When native gliadin was chromatographed on carboxymethylcellulose (Patey & Evans, 1973) examination of the effluent for protein and carbohydrate showed that carbohydrate was associated with the four main protein bands (Fig. 2a). When carbohydrafreated gliadin was chromatographed on the same system the results showed no alteration in the protein pattern (Fig. 2b). However, as a heat-stable cellulase was present in the enzyme preparation, which slowly cleaved the carboxymethylcellulose, the estimation of carbohydrate proved difficult. Each peak was therefore dialysed, hydrolysed with chymotrypsin and chromatographed on Bio-Gel P6. The pattern of these peptides from the γ-gliadin fraction of enzyme-treated and untreated gliadin are not appreciably altered (Fig. 3), but, more significantly, carbohydrate was completely absent from the treated gliadin. Similar results were obtained with the other peaks.
Clinical methods

Biopsies were obtained from the small intestine after a fast of at least 10 h, a Watson capsule being used in patients nos. 1, 2 and 4 and an Olympus duodenoscope GIF D2 in patient no. 3. Biopsy specimens were divided, part being immediately wrapped in Parafilm and stored at -20°C until assayed for enzyme activity. The remainder was put in 4% formalin in saline, examined under the dissecting microscope and then processed for histology, 5 μm sections being stained with Haematoxylin and Eosin. Mucosal enzymes are expressed in international units/g or mg of protein. Lactase and sucrase were assayed by the method of Dahlqvist (1968), alkaline phosphatase by the method of Kelly & Hamilton (1969) and protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Intraepithelial lymphocytes on the sides of well-orientated villi or on the luminal surface of ‘flat’ biopsies, were counted at a magnification of 400×, and are expressed per 1000 epithelial cells. The mean height of 25 epithelial cells was also measured in these sites at this magnification, with a micrometer eyepiece calibrated with a 1 mm slide.

D-Xylose absorption was measured after 0.1 mol (15 g) oral load. Venous blood samples were drawn at 0, 30, 60, 75, 90 and 120 min into fluoride-oxalate-treated tubes and stored at 4°C until assay within 36 h (Stevens, Watt, Bourke, McNicholl, Fottrell & McCarthy, 1977). Deproteinized blood was assayed for D-xylose by the method of Roe & Rice (1948). The patients’ heights and weights were compared with data of Tanner & Whitehouse (1959).
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Figs. 4-7. Histological sections of jejunal biopsies at the time of diagnosis of coeliac disease in patients no. 1 (Fig. 4), no. 2 (Fig. 5), no. 3 (Fig. 6), and no. 4 (Fig. 7).

Magnification x 270. Figs. 4-6 are sections stained with Haematoxylin and Eosin; Fig. 7 is a section stained with PAS.

(Facing p. 38)
Clinical details

Patient no. 1. Diagnosis was at age 16 years, when two younger sisters were found to have coeliac disease. At that time he was anaemic, and under the third percentile for height and weight; jejunal biopsy showed a flat mosaic pattern and severe mucosal damage on histological section (Fig. 4). At the time of the study he was 19 years old, had gained 14 kg and had grown 18 cm on a gluten-free diet for 34 months, and a jejunal biopsy, from just proximal to the ligament of Treitz, was normal (Fig. 9a).

Patient no. 2. Diagnosis was 3 years previously, at age 19 years, when he presented with diarrhoea, tetany and infantilism. A flat mucosa was seen on dissecting microscope study of a jejunal biopsy, and severe mucosal damage in histological sections (Fig. 5). On a strict gluten-free diet for 20 months he grew 21 cm and increased his weight by 32·3 kg. Before feeding with the gliadin preparation his jejunal mucosa had returned to normal (Fig. 10a).

Patients no. 1 and no. 2 are brothers from a family of 13, in which three other siblings also have coeliac disease; their parents and eight other siblings have normal intestinal biopsies. A close rapport exists between this family and the relevant hospital staff and the mother supervises adherence to the gluten-free diets.

Patient no. 3. Coeliac disease had been diagnosed in childhood and jejunal biopsy at 19 years showed a flat mosaic pattern and severe damage in histological sections (Fig. 6). She was symptomless at the time of the biopsy. At the time of the study she had been on gluten-free diet for 14 months, had gained 12 kg and the duodenal mucosa appeared normal (Fig. 11a).

Patient no. 4. This patient presented with diarrhoea, steatorrhoea and megaloblastic anaemia at the age of 48 years, when a jejunal

<p>| TABLE 1. Small-intestinal mucosal brush-border enzyme activities and intraepithelial lymphocyte counts at the time of initial diagnosis of coeliac disease |
|---------------------------------------------------------|------------|--------------|--------|------------|----------------|</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Alkaline phosphatase (i.u./mg of protein)</th>
<th>Lactase (i.u./g of protein)</th>
<th>Sucrase (i.u./g of protein)</th>
<th>Intraepithelial lymphocytes (no./1000 cells)</th>
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<tbody>
<tr>
<td>1</td>
<td>8·8</td>
<td>2·3</td>
<td>7·4</td>
<td>998</td>
</tr>
<tr>
<td>2</td>
<td>8·0</td>
<td>0·75</td>
<td>7·4</td>
<td>883</td>
</tr>
<tr>
<td>3</td>
<td>4·2</td>
<td>3·1</td>
<td>15·5</td>
<td>1024</td>
</tr>
<tr>
<td>4</td>
<td>5·0</td>
<td>2·2</td>
<td>7·4</td>
<td>524</td>
</tr>
</tbody>
</table>

<p>| TABLE 2. Feeding with treated and untreated gliadin: details of the study |
|-------------------------------|-------------------|-------------------|
| X&lt;sub&gt;1&lt;/sub&gt; and X&lt;sub&gt;2&lt;/sub&gt; = days of D-xylose absorption studies. B&lt;sub&gt;1&lt;/sub&gt; and B&lt;sub&gt;2&lt;/sub&gt; = days on which biopsies were taken. For each patient gliadin was eaten on days 3–7 inclusive. |</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gliadin preparation eaten</th>
<th>Days of study</th>
<th>Gliadin eaten (g)</th>
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<tr>
<td></td>
<td></td>
<td>1 2 3 4 5 6 7 8 9</td>
<td>Before B&lt;sub&gt;2&lt;/sub&gt; Total</td>
</tr>
<tr>
<td>1</td>
<td>Treated gliadin</td>
<td>B&lt;sub&gt;1&lt;/sub&gt; X&lt;sub&gt;1&lt;/sub&gt;</td>
<td>B&lt;sub&gt;2&lt;/sub&gt; X&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Treated gliadin</td>
<td>B&lt;sub&gt;1&lt;/sub&gt; X&lt;sub&gt;1&lt;/sub&gt;</td>
<td>B&lt;sub&gt;2&lt;/sub&gt; X&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Treated gliadin</td>
<td>X&lt;sub&gt;1&lt;/sub&gt; B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>B&lt;sub&gt;2&lt;/sub&gt; X&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Untreated gliadin</td>
<td>X&lt;sub&gt;1&lt;/sub&gt; B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>B&lt;sub&gt;2&lt;/sub&gt; X&lt;sub&gt;2&lt;/sub&gt;</td>
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</table>
biopsy showed a flat mosaic pattern and severe mucosal damage on histological sections (Fig. 7). At the time of the study she had been on a gluten-free diet for 3 months with marked clinical improvement, including a weight gain of 5 kg, disappearance of diarrhoea and anaemia, but her jejunal biopsy was still abnormal (Fig. 12a), with elevated intraepithelial lymphocytes and decreased mucosal enzymes (Table 1).

Informed consent for the study was obtained from each patient. Enzyme-treated gliadin was fed to patients nos. 1, 2 and 3 and untreated gliadin was fed to patient no. 4. In each instance 10 g of the appropriate gliadin preparation was baked under supervision in 450 g loaves of gluten-free bread; five loaves (i.e. 50 g of gliadin preparation) were given to each patient and any bread uneaten at the end of the study period was recorded. Xylose-absorption tests and intestinal biopsies were done before and after each period of feeding with specially prepared loaves (Table 2).

Results
Changes from the original blood xylose concentrations found after feeding with gliadin preparations are shown in Fig. 8, where it can be seen that only patient no. 4, fed with untreated gliadin, showed a definite impairment in xylose absorption.

![Graph showing xylose absorption](image)

**Fig. 8.** The broken line shows the 95% confidence lines of the standard error of estimate of repeat tests in the same individual. 0 (the abscissa) is the xylose absorption study (X). A positive deflection indicates an increased concentration; a negative deflection indicates a decreased concentration in the subsequent xylose absorption study (X2). Blood sample times are indicated above the results. No sample was available for patient no. 3 at 75 min.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Biopsy site</th>
<th>Before/After</th>
<th>Alkaline phosphatase (i.u./mg of protein)</th>
<th>Lactase (i.u./g of protein)</th>
<th>Sucrase (i.u./g of protein)</th>
<th>Intraepithelial lymphocytes (no./1000 cells)</th>
<th>Enterocyte height (μm)</th>
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<tr>
<td>1</td>
<td>T-2</td>
<td>Before</td>
<td>21.5</td>
<td>20.4</td>
<td>73.3</td>
<td>305</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>22.5</td>
<td>11.9</td>
<td>50.3</td>
<td>260</td>
<td>35.1</td>
</tr>
<tr>
<td>2</td>
<td>T-1</td>
<td>Before</td>
<td>22.5</td>
<td>13.2</td>
<td>75.8</td>
<td>302</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>20.8</td>
<td>5.2</td>
<td>44.6</td>
<td>234</td>
<td>38.7</td>
</tr>
<tr>
<td>3</td>
<td>D1</td>
<td>Before</td>
<td>6.1</td>
<td>1.2</td>
<td>25.5</td>
<td>321</td>
<td>30.7</td>
</tr>
<tr>
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<td>After</td>
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<td>276</td>
<td>36.1</td>
</tr>
<tr>
<td>4</td>
<td>T+1</td>
<td>Before</td>
<td>11.9</td>
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<td>42.3</td>
<td>624</td>
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<tr>
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<td>1.4</td>
<td>11.9</td>
<td>1044</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Table 3. Small-intestinal mucosal biochemical and histological variables before and after feeding studies
T indicates distance± from ligament of Treitz (in inches); D1, proximal duodenal biopsy with Olympus GIF D2.
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FIG. 9. Histological sections of mucosal biopsies before (a) and after (b) feeding treated gliadin to patient no. 1. Magnification ×400 (Haematoxylin and Eosin). The biopsy after feeding with treated gliadin does not show histological deterioration.
FIG. 10. Histological sections of mucosal biopsies before (a) and after (b) feeding treated gliadin to patient no. 2. Magnification ×400 (Haematoxylin and Eosin). The biopsy after feeding with treated gliadin does not show histological deterioration.
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Fig. 11. Histological sections of mucosal biopsies before (a) and after (b) feeding treated gliadin to patient no. 3. Magnification ×400 (Haematoxylin and Eosin). The biopsy after feeding with treated gliadin does not show histological deterioration.
Fig. 12. Histological section of mucosal biopsies before (a) and after (b) feeding untreated gliadin to patient no. 4. Magnification ×400 (Haematoxylin and Eosin). The biopsy after feeding with untreated gliadin shows histological deterioration with loss of villus height, decrease in epithelial cell height, increased intraepithelial lymphocyte counts and increased inflammatory infiltration into the lamina propria.
The histological sections from the four patients before and after feeding experiments are shown in Figs. 9(a) and 9(b), 10(a) and 10(b), 11(a) and 11(b), 12(a) and 12(b). Pairs of histological sections taken before and after the feeding experiments were examined by three of the authors, who were unaware of the identity of the initial or subsequent biopsy. General morphology including villus height, crypt depth, epithelial cell height and the presence or absence of inflammatory cells in the epithelium and lamina propria showed no change in patients nos. 1, 2 and 3, who were fed with enzyme-treated gliadin, whereas distinct changes were seen in patient no. 4 who was fed with untreated gliadin (Table 3). Epithelial cell height and intraepithelial lymphocytes revealed a marked change only in patient no. 4 (Table 3).

Mucosal brush-border enzyme activities (Table 3) showed that only in patient no. 4 was there a fall in all enzymes; patient no. 3 showed a rise in alkaline phosphatase, but this was unchanged in patients no. 1 and 2.

Patient no. 3 also showed some rise in lactase and sucrase activity, whereas patients no. 1 and no. 2 showed a decrease in lactase and sucrase activities. Patients nos. 1, 2 and 3 were symptom-free during the period of the study, whereas patient no. 4 had diarrhoea after the second xylose test.

Discussion

This study confirms our preliminary findings that the enzymic removal of carbohydrate from gliadin alters its toxicity to patients with coeliac disease. We have used several criteria for assessing the toxicity of treated and untreated gliadin, e.g. xylose absorption, mucosal histology, epithelial cell height, intraepithelial lymphocyte counts and mucosal enzymes.

As shown in our hands, depression of xylose absorption, as shown by lowering of the xylose tolerance curve, proved to be a reliable method for assessing gliadin toxicity. A depression of more than 45% in the blood xylose concentration at 1 h was found by Rolles, Anderson & McNeish (1975) when they challenged coeliac children in remission with gluten. Xylose absorption was not depressed in our patients nos. 1, 2 and 3 who were fed with carbohydrase-treated gliadin. The depression of xylose absorption in patient no. 4 was accompanied by histological deterioration, an increase in intraepithelial lymphocytes, and a fall in enzyme activity. After the enzyme-treated gliadin patients nos. 1, 2 and 3 showed none of the histological features normally associated with gliadin toxicity (Bayless, Yardley, Norton & Hendrix, 1962; Pollock, Nagle, Jeejeebhoy & Coghill, 1970; Shmerling & Shiner, 1962; Dissanayake, Truelove & Whitehead, 1974a).

Feeding toxic material to coeliac patients in remission causes a fall in mucosal brush-border enzymes. Disaccharidase activity may decrease as soon as 1 h after gliadin challenge (Bayless, Rubin, Topping, Yardley & Hendrix, 1970) and is pronounced after 24 h (Dissanayake et al., 1974a). Hekkens et al. (1974) found recovery of lactase and sucrase activity to commence within 24 h of a single intraduodenal dose of α-gliadin. Shmerling & Shiner (1970) found no fall in disaccharidase activity in one patient studied after gliadin challenge but the biopsy timings of 2 and 24 h after challenge may have been respectively too early and too late to show such a fall. Hekkens et al. (1974) showed that alkaline phosphatase activity fell by more than 65%, along with the fall in lactase and sucrase activity. Mucosal disaccharidase activities may fluctuate in coeliac patients on gluten-free diets (Dissanayake, Jerrome, Offord, Truelove & Whitehead, 1974b). We have found lactase activity to vary from 2.6% to 54% and sucrase from 6.6% to 31.6% in repeated biopsies from the same site, without any change in diet.

We have emphasized the abolition of toxicity rather than the isolation of toxic fractions from proteolytic digests, as in other studies. This approach eliminates large-scale fractionation of gliadin, which becomes more difficult as the fraction numbers increase. Its disadvantage is that, should the patient inadvertently consume even small traces of gliadin, then it cannot be stated with certainty whether or not toxicity has been completely abolished. It is noteworthy then, that patients no. 1 and no. 2 showed a fall in sucrase and lactase activities, whereas the alkaline phosphatase did not decrease, the epithelial enterocyte height did not change and the intraepithelial lymphocyte counts did not increase. Patient no. 3, although challenged with gliadin which was treated with the same enzyme as that used in patients no. 1 and no. 2, did not show a similar fall in disac-
charidase activities, which probably indicated abolition of toxicity. We believe that these results justify trials of similarly treated gliadin in newly diagnosed coeliac patients.

This study suggests that the carbohydrate bound to gliadin plays an important role in the toxicity of this substance in coeliac disease. This conflicts with previous concepts as to the nature of this toxicity, but clarifies many anomalies which have arisen since Dicke's original report (Dicke, 1950; Dicke et al., 1953). It explains why a wide range of purified proteolytic enzymes does not abolish the toxicity. It shows why the length of the peptide chain is bound to gliadin plays an important role in the nature of this toxicity, but clarifies many original report (Dicke, 1950; Dicke et al., 1953). It explains why a wide range of purified proteolytic enzymes does not abolish the toxicity. It shows why the length of the peptide chain is toxic (Van de Kamer & Weijers, 1955; Alvey et al., 1957; Frazer et al., 1958; Van Roon et al., 1960; Bronstein et al., 1966; Dissanayake et al., 1973). It has been reported that a 'deamidation' of the glutamine residues of gliadin, i.e. by boiling in HCl for 2 h, abolishes toxicity (Weijers & Van de Kamer, 1958) and crude papain possesses an enzyme which catalyses a similar reaction (Krainich & Mohn, 1959; Messer et al., 1964). When gliadin was enzymically detoxified by glycosidic enzymes, there was no deamidation of glutamine, as shown by peptide mapping, where the charge differences would be readily detected. Crude papain probably contains carbohydrate activity, which cleaves the glycosidic bonds of gliadin (unpublished studies). The hydrochloric acid procedure used by Van de Kamer also hydrolyses a wide variety of glycosidic linkages (Spiro, 1966) thus rendering gliadin non-toxic. It has been proposed that the toxicity of gliadin resulted from the cyclization of amino-terminal glutamine to form pyrrolidone carboxylic acid, and that the coeliac intestine was deficient in the enzyme which cleaves this bond (Bronstein et al., 1966; Kowlessar, Warran & Bronstein, 1970). However, Woodley (1972) found no deficiency of this enzyme in homogenates of coeliac intestinal mucosa. The suggestion that the toxicity of gliadin is due to a short chain of amino acids, including glutamine, which are peculiar to gliadin proteins, is also unlikely.

The suggestion that coeliac patients lack a specific intestinal peptidase was a natural corollary of the hypothesis that the harmful effects of gliadin were due to a peptide. Our concept is supported by reports that intestinal peptide hydrolases have broad specificity (Fottrell, Donlon, O'Cuinn & Piggott, 1975) and by the finding that coeliac patients do not lack a specific peptide hydrolase (Douglas & Booth, 1970).

We suggest that gliadin toxicity may be abolished by enzymes from A. niger, without extensive hydrolysis of the primary structure of the proteins. This study implies that the carbohydrases present in the microbial extract are responsible for detoxifying gliadin. However, until a complete characterization of the enzymes involved has been completed, the possibility remains that a side-chain substituent other than carbohydrate may account for the toxicity of gliadin.

Acknowledgments

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References

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