Alimentary tract and pancreas

Breakdown of gliadin peptides by intestinal brush borders from coeliac patients

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SUMMARY The 'missing peptidase' hypothesis to explain the aetiology of coeliac disease has never been satisfactorily resolved and recent reports suggest that coeliac brush borders may have depressed levels of specific peptidase enzymes. It has been inferred from these studies that the subsequent brush border digestion of gliadin peptides may therefore be defective. In this present study a sensitive fluorometric assay was used to measure the hydrolysis of a peptic-tryptic digest of gliadin by both normal and coeliac brush borders. The coeliac brush borders were as efficient as the normals in hydrolysing gliadin peptides and showed no depression of any specific peptidase activity.

The aetiology of coeliac disease is not known. In recent years support for an immune based theory has emerged but there is no unifying concept of the pathogenesis¹ and some reports would mitigate against an immune defect being the primary cause of the syndrome.² Unquestionably there are changes in some immunological parameters but many of these may be secondary responses to the gluten induced damage of the intestinal mucosa – for example see reference 3.

The 'missing peptidase' hypothesis emerged in the 1950s when Frazer⁴ observed that gliadin could be detoxified by treatment with an extract of pig intestinal mucosa. This led to the postulation that the coeliac intestinal mucosa was lacking a peptidase required for the normal digestion of gliadin peptides. Since then this theory has never been satisfactorily resolved and earlier studies of both the enzymology⁵⁻⁸ and the digestive capacity⁹⁻¹⁰ of the coeliac mucosa have produced conflicting results.

Two more recent reports¹¹⁻¹² have suggested that there were specific peptidase deficiencies in the coeliac mucosa in remission, but a later third report¹³ showed no significant differences in the levels of two peptidases when coeliac biopsies were compared with controls. All these *in vitro* studies have involved the use of whole homogenates of biopsies, where peptidases from a number of sub-cellular loci will be present, and the specific enzymes

measured using synthetic substrates.

It is now recognised that it is the brush border which is the major site of mucosal protein digestion and is known to contain numerous peptidase enzymes.¹⁴⁻¹⁷ Given also that the brush border represents the interface between the intracellular milieu and the toxic gliadin peptides then this membrane becomes the most logical site for any enzyme defect. Supporting such an idea is the fact that other brush border membrane enzyme defects are well documented, including enterokinase deficiency¹⁸ and sucrase-isomaltase deficiency.¹⁹

This present study represents the first detailed study of the enzymology and digestive capacity toward gliadin of the isolated coeliac brush border membrane. The ability to study the coeliac brush border hydrolysis of gliadin peptides has been made possible by the development of a sensitive micro-assay²⁰ designed specifically to measure this digestion.

Methods

BIOPSIES

Jejunal biopsies were obtained using a steerable biopsy capsule (Meditech) under fluoroscopic screening. The subjects for the control group were patients being biopsies for routine diagnostic purposes. The coeliac group were patients in remission, who had been on a gluten free diet for at least a year, and who were being checked for the maintenance of their response to the diet. Biopsies

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Received for publication 18 November 1983

were divided for routine histology, and for biochemical studies were wrapped in parafilm and immediately frozen on solid CO₂. Biopsies were stored at -20°C and analysed within three weeks. Biopsies were coded so that the analysis was carried out blind and only those which subsequently showed normal histology have been included in the data.

BRUSH BORDER MEMBRANE PREPARATION

Brush border membranes were purified from the frozen intestinal biopsies using a modified and scaled-down method of Schmitz *et al.*²¹ Each biopsy was homogenised with a pestle homogeniser in 8.0 ml 50 mM mannitol, 2 mM tris-HCl buffer pH 7.1, and the homogenate was made 10 mM with respect to CaCl₂ by addition of solid. After slow stirring for 30 minutes, the homogenate (H₁) was centrifuged at 2000 *g* for 20 minutes. The pellet (P₁) was resuspended in a small volume of buffer and the supernatant (S₁) poured off and centrifuged at 25 000 *g* for 30 minutes. The resultant supernatant (S₂) was poured off and the brush border pellet (P₂) was washed twice and resuspended in 250 µl of buffer.

BIOCHEMICAL ASSAYS

Alkaline phosphatase (EC 3.1.3.1), Zn⁺⁺ resistant α-glucosidase (EC 3.2.1.20), N-acetyl-β-glucosaminidase (EC 3.2.1.30), aminopeptidase N (EC 3.4.11.20), aminopeptidase A (EC 3.4.11.7), diaminopeptidase IV (EC 3.4.14.-), and γ-glutamyl transpeptidase (EC 2.3.2.2) were assayed using scaled down methods of those described by Sterchi and Woodley.²² Lactic dehydrogenase was measured fluorimetrically by the method of Lowry *et al.*²³ Peptidyl dipeptidase (EC 3.4.15.1) was assayed using a scaled down method of Cheung and Cushman.²⁴ Carboxypeptidase P (EC 3.4.12.-) was assayed using the incubation conditions of Auricchio *et al.*²⁵ with N-carbobenzyloxypolyalalanine as substrate, the released alanine being detected fluorimetrically.²⁶ All enzyme activities are expressed as µmol substrate hydrolysed per minute at 37°C. Protein estimation was by the method of Lowry *et al.*²⁷ using bovine serum albumin standards.

GLIADIN PEPTIDES

A highly purified α-gliadin preparation of proven toxicity was a kind gift of Dr W Th J M Hekkens, Leiden, Netherlands. A peptic-tryptic digest (PT-gliadin) was prepared as described previously.²⁰ This digest contained 38% glutamine and glutamic acid.

BREAKDOWN OF GLIADIN PEPTIDES

The brush border breakdown of PT-gliadin was

measured using a modification of the previously published enzyme linked assay,²⁰ in which free released glutamine and glutamic acid were measured fluorimetrically. Two parameters of breakdown were measured which differed only in the incubation conditions used: (i) initial rate of breakdown: 40 µl of brush border fraction (P₂) and 40 µl of a PT-gliadin solution (0.9 mg/ml in 0.1M Tris HCl buffer pH 7.5) were incubated on a roller incubator for 60 minutes at 37°C. (ii) Extent of breakdown: 40 µl of brush border fraction (P₂) and 40 µl of a PT-gliadin solution (0.15 mg/ml in 0.1M Tris HCl buffer pH 7.5) were incubated overnight on a roller incubator at 37°C.

In both cases appropriate blanks were carried out and the released glutamine and glutamic acid was measured on 40 µl of the deproteinised and neutralised incubation mixture as described previously²⁰ using appropriate volumes of reagents.

EXPRESSION AND COMPARISON OF BREAKDOWN

(i) Initial rate of breakdown of gliadin peptides was expressed as the release of µmol of glutamine and glutamic acid per minute at 37°C. In order for comparisons to be made the initial rate of breakdown was correlated to both the amount of protein used (specific activity - U/mg protein) and also to the activity of the brush border marker enzyme Zn⁺⁺ resistant α-glucosidase. This latter correlation enables the digestive capacity of the membrane to be expressed as a function of its non-peptidase activity, and avoids changes in specific activity which may represent alterations in protein content rather than enzyme concentrations.

(ii) Extent of breakdown was expressed in term of % of total breakdown. Total (100%) breakdown was measured using conventional acid hydrolysis as described previously.²⁰

Results

BRUSH BORDER MEMBRANE PREPARATION

The results of fractionation of 25 biopsies from non-coeliac patients are shown in Table 1. These biopsies are a different set from those used as controls in the coeliac study. The brush border fraction (P₂) showed a 12.1±1.4 enrichment of the brush border marker enzyme Zn⁺⁺ resistant α-glucosidase. The contamination by lysosomal enzymes was negligible, only 0.7±0.1% of the activity of the marker enzyme N-acetyl-β-glucosaminidase being detected in the brush border fraction (P₂). Lactic dehydrogenase activity (not shown) was barely detectable and was less than 0.5% of the total, showing that the brush borders

Table 1 Distribution and specific activity of marker enzymes and gliadin hydrolysing activity in subcellular fractions prepared from 25 intestinal biopsies

Fraction	Protein distribution (%)	α -Glucosidase		Activity against PT-gliadin		N-acetyl- β -glucosaminidase	
		Distribution (%)	Sp activity (U/mg protein $\times 10^3$)	Distribution (%)	Sp activity (U/mg protein $\times 10^4$)	Distribution (%)	Sp activity (U/mg protein $\times 10^3$)
Homogenate			3.34 $\pm$ 0.57		0.93 $\pm$ 0.05		3.6 $\pm$ 0.5
P ₁	41.6 $\pm$ 2.9	32.7 $\pm$ 2.7	2.68 $\pm$ 0.46	29.9 $\pm$ 3.1	0.78 $\pm$ 0.09	9.2 $\pm$ 1.3	0.78 $\pm$ 0.1
P ₂ (brush border)	2.4 $\pm$ 0.4	23.8 $\pm$ 2.2	40.5 $\pm$ 8.5	15.9 $\pm$ 3.7	5.96 $\pm$ 1.46	0.7 $\pm$ 0.1	1.45 $\pm$ 0.45
SN	42.9 $\pm$ 3.1	21.3 $\pm$ 2.8	1.54 $\pm$ 0.38	11.1 $\pm$ 1.4	0.27 $\pm$ 0.03	74.5 $\pm$ 2.9	7.4 $\pm$ 0.97
Total recovery (%)	91.1 $\pm$ 2.4	82.2 $\pm$ 2.6		54.4 $\pm$ 2.7		87.5 $\pm$ 3.3	
Brush border enrichment			12.1 $\pm$ 1.4		6.4 $\pm$ 0.1		0.5 $\pm$ 0.06

Values refer to means of 25 biopsies $\pm$ SE.

Units for activity against gliadin are μ mol of glutamine and glutamic acid released per min, and for the other enzymes are μ mol of substrate hydrolysed per min, all at 37°C.

were not contaminated with cytosol. Initial rate activity against PT-gliadin was measured in 14 of these preparations and was enriched in the brush border fraction (6.4 $\pm$ 0.1) although not to the same extent as the brush border marker enzyme. Also the total recovery of this activity was low (54.4 $\pm$ 2.7) despite good recoveries of both marker enzyme activities and protein. Both these results can be explained by the fact that the activity against PT-gliadin in the original homogenate (H₁) is not because of one enzyme but the combination of various enzymes from different subcellular loci and this combination of activities (particularly by the brush border and cytosol enzymes) is lost when the cell is fractionated. It should be noted that the homogenate will also contain lysosomal enzymes which may be contributing to breakdown. The amount of protein in the brush border fraction is small (<3%) but represents uncontaminated brush border membranes.

COELIAC AND NON-COELIAC BRUSH BORDER ACTIVITIES

Brush border fractions (P₂) were prepared from coeliac and non-coeliac biopsies. These fractions were assayed for the following brush border enzymes: Zn⁺⁺ resistant α -glucosidase, alkaline phosphatase, aminopeptidase N (ApN), aminopeptidase A (ApA), diaminopeptidase IV (DAP IV), γ -glutamyl transpeptidase (γ GT), peptidyl dipeptidase (Pdp), and carboxypeptidase P (CpP). Both the initial rate and extent of breakdown of PT-gliadin were measured on these fractions.

The Figure shows the initial rate activity correlated to the Zn⁺⁺ resistant α -glucosidase activity while Table 2 shows all the other activities expressed as specific activities (U/mg protein).

Discussion

The 'missing peptidase' hypothesis to explain coeliac disease postulates that the coeliac mucosa is deficient in an enzyme normally involved in digesting gliadin peptides. The digestion of gliadin peptides within the small intestinal mucosa is an extremely important stage in the overall digestion of the gliadin protein. This is because, unlike other dietary proteins, the preceding stages of digestion are relatively ineffective. Indeed, peptides as large as mol wt 12 000 have been reported after exhaustive *in vitro* digestion by pepsin and trypsin²⁸ and it is these relatively large peptides which are

Figure Initial rate activity against PT-gliadin correlated to Zn⁺⁺ resistant α -glucosidase activity; (●) coeliac and (○) non-coeliac controls. Correlation ($r=0.85$) line is of non-coeliac controls.

Table 2 Specific activities of enzymes in purified brush borders from coeliac patients in remission and non-coeliac controls

	Non-peptidase		Peptidases						Activity against PT gliadin	
	α -Glucosidase	Alkaline phosphatase	DAP IV	γ GT	CpP	ApA	ApN	Pdp	Initial rate	Extent (%)
Non-coeliac controls										
Mean	65.0	218.0	13.9	1.95	1.58	3.95	80.7	60.8	6.32	100.9
Standard deviation	32.9	159.0	3.4	1.27	0.75	2.39	35.8	37.6	3.19	7.5
n	20	18	11	11	11	11	11	11	20	17
Coeliac										
Mean	61.4	253.0	14.9	2.63	1.83	2.88	142.0	37.0	6.19	97.3
Standard deviation	52.6	112.0	5.6	1.98	0.61	1.39	39.4	22.8	1.67	7.9
n	10	10	10	10	10	10	10	10	10	10
p value	>0.1	>0.1	<0.1	>0.1	>0.1	>0.1	<0.005	>0.05	>0.1	>0.1

Specific activity for activity against gliadin peptides is U/mg protein $\times 10^4$, where units (U) are μ mol of glutamine and glutamic acid released per min at 37°C. For all other enzymes, specific activity is U/mg protein $\times 10^3$, where units (U) are μ mol of substrate hydrolysed per min at 37°C.

DAP IV = diaminopeptidase IV, γ GT = γ -glutamyl transpeptidase, CpP = carboxypeptidase P, ApA = aminopeptidase A, ApN = aminopeptidase N, Pdp = peptidyl dipeptidase.

toxic to coeliac patients.^{28, 29} The digestion of these peptides within the intestinal mucosa is achieved by the enzymes of the brush border and cytosol although as the cytosol enzymes are unable to cleave peptides containing more than two amino acid residues¹⁴ the digestion of the large toxic gliadin peptides has to be achieved by the brush border enzymes. This brush border digestion becomes more important when also considering the observation that glutamine (the predominant amino acid in gliadin) containing peptides are poor substrates for cytosol peptidases.³⁰ Although the digestion of gliadin peptides within the human intestinal mucosa has yet to be studied in detail, studies on the hog intestinal mucosa³¹ have confirmed the brush border as the major site of this digestion. Subsequently it was shown that a purified rat brush border membrane fraction could completely hydrolyse a peptic-tryptic digest of gliadin.²⁰

In the light of these observations the search for a 'missing peptidase' within the coeliac mucosa switched from the enzymes of the cytosol to those of the brush border,^{11, 12} although all previous studies have used whole biopsy homogenates and not purified brush borders. Such studies have produced conflicting results, which might be partly explained by the existence of non-brush border peptidase activity in the whole homogenate. An impaired coeliac mucosal digestion of gliadin peptides was reported by Pittman and Pollitt¹⁰ and by Cornell^{6, 7} whereas Douglas and Booth,⁹ using similar methods, showed no such impairment. The study of individual brush border peptidases has also produced conflicting results. These enzymes have been assayed on whole biopsy homogenates using

synthetic substrates thought to be hydrolysed exclusively by particular brush border peptidases. Using this approach Sjostrom *et al*¹² reported low concentrations of aminopeptidase N and diaminopeptidase IV in coeliac patients in remission. Andria *et al*¹¹ could not confirm these findings but instead claimed that the activity of aminopeptidase A remained low in coeliac patients after all other enzymes had returned to normal. Most recently, Andersen *et al*¹³ showed that levels of aminopeptidase N and α -glutamyl transpeptidase in homogenates of biopsies from treated coeliacs were the same as normal controls.

These discrepancies may be because of the fact that the proteolytic activity of a whole biopsy homogenate does not accurately reflect that of the brush border membrane, as non-brush border peptidases are involved in the observed *in vitro* activity, a situation which does not occur *in vivo*. It should also be noted that it was not known which of the brush border peptidases were actually involved in the degradation of gliadin.

The proteolytic activity of the brush border membrane can only be studied using purified uncontaminated brush border membranes.

In this study, a modified and scaled down method of Schmitz *et al*²¹ was used to purify brush border membranes from intestinal biopsies. Only about 20% of the original membranes were recovered in the brush border fraction (P₂) as measured by the recoveries of the marker enzymes but an 11 fold purification was achieved, and the membranes were free of lysosomal contamination (a possible major source of non-brush border peptidases) and cytosolic contamination as represented by lactic

dehydrogenase activity. The ability to study the brush border digestion of PT-gliadin was made possible by the development of a sensitive assay²⁰ designed specifically to measure gliadin peptide breakdown by measuring the released glutamine and glutamic acid. Using this assay, both initial rate of breakdown of gliadin peptides and the extent to which they could be broken down were measured. Of the six brush border peptidases measured particular attention was given to the enzymes aminopeptidase N and peptidyl dipeptidase. This is because these two enzymes have been shown to be responsible for most of the observed brush border hydrolysis of PT-gliadin (unpublished data).

There was no significant difference ($p>0.1$) in the activities of the two non-peptidase brush border marker enzymes Zn^{++} resistant α -glucosidase and alkaline phosphatase between the coeliac and control groups. This was an important observation as any reduced peptidase activity could not be attributed to poor mucosal recovery. A slow mucosal recovery was thought to be responsible for the previously observed reduction in α -glucosidase activity.³²

Of the six peptidases measured there was no significant difference in the activities of: diamino-peptidase IV, γ -glutamyl transpeptidase, aminopeptidase A, and carboxypeptidase P (all $P>0.1$) and peptidyl dipeptidase ($p>0.05$) while the coeliac activity of aminopeptidase N was significantly higher ($p<0.005$) than the non-coeliacs. Ultimately though there was no significant difference ($p>0.1$) in the ability to digest PT-gliadin, both in the initial rate and also in the extent of breakdown. Indeed all brush border fractions studied, coeliac and non-coeliac, could completely (93.1–112.3%) hydrolyse PT-gliadin under our assay conditions. Even when correlated to Zn^{++} resistant α -glucosidase activity (Figure) the initial rate of breakdown by coeliac brush border was indistinguishable from the non-coeliacs. Any sample showing impaired gliadin breakdown would fall substantially below the correlation line, and the Figure shows this not to be the case.

Thus it has been shown by this study that the coeliac brush border membrane does not lack the ability to digest gliadin peptides. This observation would appear to refute the 'missing peptidase' hypothesis proposed to explain the aetiology of coeliac disease.

This work was supported by funds from the West Midlands Regional Health Authority, Birmingham, UK. The authors wish to gratefully acknowledge the support of the Stoke Coeliac Society, and the help of Sister Ravenscroft and her staff.

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