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Peptic-tryptic digests of gliadin: contaminating trypsin but not pepsin interferes with gastrointestinal protein binding characteristics

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Abstract

For many years, peptic-tryptic digests of gliadin, known as Frazer's fraction III, have been used in investigations of gliadin effects. Potential contamination by the proteases pepsin and trypsin, however, was not considered. To investigate the influence of contaminating proteases on binding of gliadin peptides to rat small intestinal brush border membranes we compared binding characteristics of different gliadin digests. Binding of biotinylated probes was studied in dot blots and Western blots with an enhanced chemiluminescence system. In gliadin peptide preparations only contaminating trypsin, but not pepsin, was detectable by specific antisera. Digestion with insoluble proteases attached to cross-linked beaded agarose yielded gliadin peptides free of contaminating pepsin and trypsin. These peptides bound 30% less to brush border membranes. Using these peptides, there was no trypsin-typical binding pattern to low molecular mass membrane proteins in contrast to peptide preparations which contained contaminating trypsin. In conclusion, contaminating trypsin might alter gliadin peptide binding characteristics by direct binding to brush border membranes and by interfering with interactions between gliadin peptides and brush border membranes.

Keywords: Brush border membranes; Coeliac disease; Gliadin peptic-tryptic digests; Frazer's fraction III

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1. Introduction

Gliadin fractions prepared by peptic-tryptic digestion according to Frazer et al. [1] have been widely used for studying pathogenesis of coeliac disease. Changes in mucosal architecture were shown for biopsies after in vitro incubation [2–4] as well as in vivo for small intestinal mucosa after oral challenge [5,6]. Frazer's fraction III was considered to be suitable for identifying 'toxic' sequences responsible for induction of mucosal damage. Proteases used for digestion were inactivated by heat treatment [1,2,7,8] or by addition of protease inhibitors [9]. Up to now there is no report about interference of proteases with biochemical and immunological effects of peptic-tryptic gliadin digests.

The aim of this study was to clarify whether remaining proteases, or their fragments, alter binding characteristics to small intestinal mucosa of gliadin peptides obtained by peptic-tryptic digestion.

2. Materials and methods

2.1. Biochemicals

Biotin-labelled lectins from *Ulex europaeus* (UEA, L-8262) and *Triticum vulgaris* (WGA, L-5142) were purchased from Sigma, Deisenhofen, Germany. Biotinylated lectins from *Galanthus nivalis* (GNA, 1143000), *Sambucus nigra* bark (SNA, 1124617) and *Arachis hypogaea* (PNA, 1172859) were from Boehringer, Mannheim, Germany. Bovine serum albumin (BSA, A-4378), α-casein (CAS, C-7891), β-lactoglobulin (BLG, L-0130), α-lactalbumin (ALA, L-6010), trypsin agarose beads (T-1763), pepsin agarose beads (P-3286) and Tween 20 were from Sigma. Bovine trypsin (24579) and porcine pepsin (7192) used for immunoblotting were obtained from Merck, Darmstadt, Germany. Bovine trypsin (37257) and porcine pepsin (31820) used for immunization were from Serva, Heidelberg, Germany. Biotin-conjugated goat antiserum to rabbit IgG and peroxidase-conjugated streptavidin were from Dianova, Hamburg, Germany. All other chemicals were from Merck, Darmstadt, Germany.

2.2. Preparation of antisera to trypsin and pepsin

Pepsin and trypsin (1 mg/ml) were incubated for 1 h at 70°C in phosphate-buffered saline pH 7.4 (antigen preparation I). The inactivated enzyme solution (1 ml) was mixed with 0.25 ml of complete or incomplete Freund's adjuvants (antigen preparations II or III). Rabbits were injected intracutaneously four times every 2 weeks in the following sequence with 1 ml

of II, III, I, and III containing either pepsin or trypsin. After 10 weeks the animals were bled for 15 ml and the serum was used for immunoblotting.

2.3. Preparation of gliadin peptides

Peptic-tryptic digests of gliadin of the wheat variety Kolibri (PT-GLI I, previously termed 'B3' [10] or 'G3' [11]), of bovine serum albumin (PT-BSA) and of casein (PT-CAS) were obtained from Dr. H. Wieser, German Research Institute of Food Chemistry, Garching, Germany. Gliadin (GLI) was isolated from the wheat variety Kanzler and tryptic digests of this gliadin (T-GLI) were prepared according to Osman et al. [12,13].

For isolation of gliadin peptides free of contaminating proteases (PT-GLI II), insoluble enzymes attached to cross-linked beaded agarose were used. Briefly, gliadin (variety Kanzler) was dissolved in distilled water, adjusted to pH 2 and incubated for 2 h at 37°C with pepsin agarose beads. The digestion was stopped by low speed centrifugation. After filtration (0.22 μm, Millipore) the supernatant was adjusted to pH 7.4 and incubated for 2 h at 37°C with trypsin agarose beads. Thereafter gliadin peptides were separated by centrifugation, filtered and freeze-dried.

2.4. Biotinylation of proteins and peptides

PT-GLI I, cow's milk proteins, trypsin and pepsin were dissolved in 0.1 mol/l $NaHCO_3$. Biotinamidocaproate-*N*-hydroxysuccinimide ester (Sigma), dissolved in dimethylsulfoxide, was added in a final concentration of 1 mg per 10 mg protein. The reaction was allowed to proceed for 4 h at room temperature. Afterwards the biotinylated proteins were dialyzed (benzoylated cellulose dialysis sacks, Sigma) overnight at 4°C against phosphate–buffered saline with several buffer changes. In case of gliadin and gliadin peptides T-GLI and PT-GLI II, distilled water and biotinamidocaproate-*N*-hydroxysulfosuccinimide ester (Sigma) were used. Approximately 70% of initial peptides and 85% of initial protein could be recovered after biotinylation.

2.5. Small intestinal brush border membrane vesicles

Adult female Sprague–Dawley rats (WIGA-Charles River Laboratories, Sulzfeld, Germany) weighing between 200 and 250 g were fasted overnight prior to the study. Newborn rats were allowed to suckle freely and were sacrificed after a 30 min fast at 24 h of age. Brush border membrane (BBM) vesicles were prepared by Ca^{2+}-precipitation [14]. Small intestines of 20–60 newborn animals and mucosa scrapings of 2 adult rats were pooled for preparation. Disaccharidase activities (lactase in newborns and sucrase in

adults) were determined according to Dahlqvist [15]; protein concentration was measured according to Lowry et al. [16].

2.6. Immunoblotting

Food peptide preparations were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) [17] under non-reducing conditions and transferred by Western blot [18] to nitrocellulose sheets. Nitrocellulose membranes were blocked with 2% (v/v) Tween 20 [19]. Phosphate-buffered saline containing 0.05% (v/v) Tween 20 was used in the washing steps. Rabbit antisera to pepsin or trypsin were incubated overnight at 4°C. Detection of bound immunoglobulins was performed using biotinylated goat antiserum to rabbit IgG followed by peroxidase-conjugated streptavidin and enhanced chemiluminescence Western blotting detection reagents (Amersham, Braunschweig, Germany) [20].

2.7. Binding assay

In dot blots, intact brush border membranes were applied to nitrocellulose and dried [21]. In Western blots, separated membrane proteins were transferred to nitrocellulose. After blocking biotinylated samples were incubated overnight at 4°C. Bound proteins were detected using peroxidase-conjugated streptavidin and enhanced chemiluminescence. Chemiluminescence signals were documented using Hyperfilm (Amersham) or photographic paper (Tetenal, Norderstedt, Germany). Quantitation was performed by analysis with the videodensitometer system Bioprofil (Fröbel, Wasserburg, Germany). Results were expressed in relative densitometric units per μg brush border membrane protein. Calculation of densitometric units was based on biotinylated standard proteins (Bio-Rad, München, Germany), which were directly applied to nitrocellulose in each experiment and were set as 100% optical density. Student's *t*-test was used to assess statistical significance of differences.

3. Results

3.1. Preparation and biotinylation of gliadin peptides

Digestion of gliadin with insoluble proteases resulted in a peptide pattern comparable with conventional peptic-tryptic digests, whereas trypsin alone produced only partial disintegration of gliadins (Fig. 1A). Biotinylation of gliadin peptides did not change peptide composition of the preparations and was very efficient: all peptides were labelled (Fig. 1B). No significant change in molecular mass was observed by comparing unmarked with biotinylated gliadin peptides in silver stained gels.

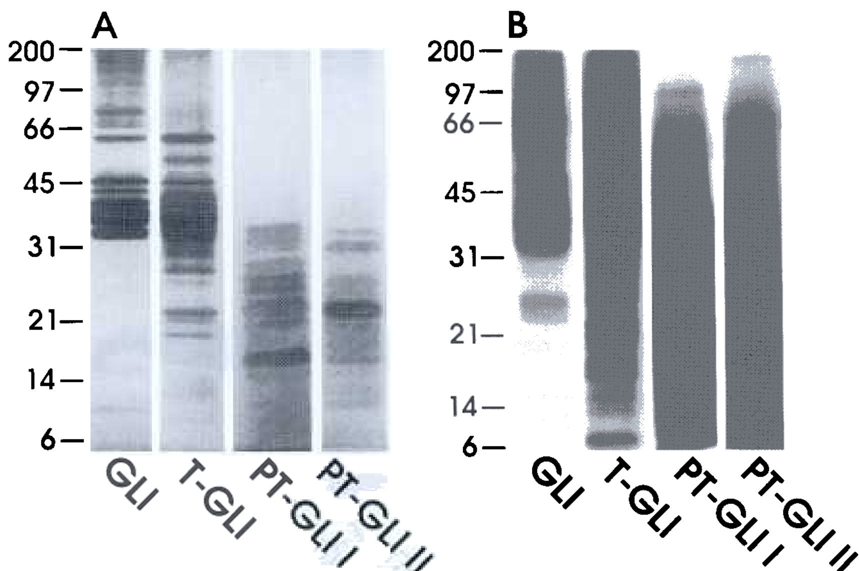

Fig. 1. Biotinylation of different gliadin peptide preparations: all peptide components are effectively marked; 3 μg GLI, 5 μg T-GLI, 10 μg PT-GLI I and 10 μg PT-GLI II were subjected to gel electrophoresis and silver stained (A) or blotted to nitrocellulose for detection of biotin-labelled components by peroxidase-conjugated streptavidin and enhanced chemiluminescence (B). Positions of molecular mass markers (kDa) are indicated.

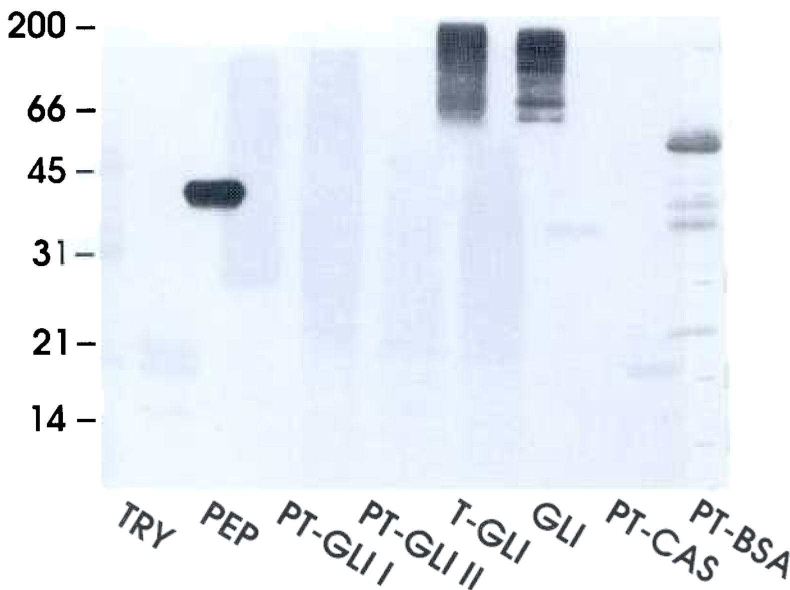

Fig. 2. Absence of pepsin from peptic-tryptic digests. Blots containing 0.3 μg pepsin as positive control and 0.3 μg trypsin, 10 μg PT-GLI I, 10 μg PT-GLI II, 10 μg T-GLI, 5 μg GLI, 10 μg PT-CAS and 10 μg PT-BSA as probes were incubated with rabbit antiserum against pepsin (1:10 000) overnight at 4°C. Bound rabbit antibodies were detected by biotinylated goat antiserum to rabbit IgG (1:10 000) followed by peroxidase-conjugated streptavidin (50 ng/ml).

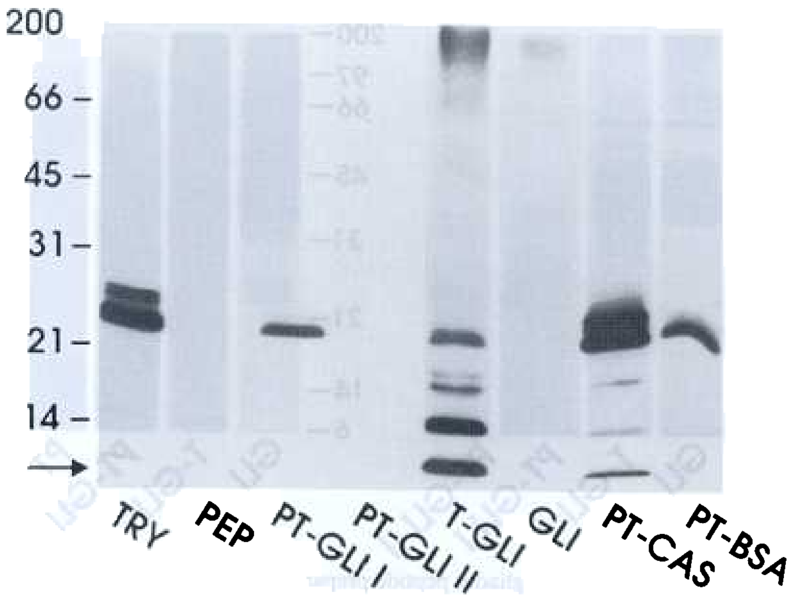

Fig. 3. Detection of contaminating trypsin in peptic-tryptic peptide preparations by specific antiserum (1:10 000). For further information see legend to Fig. 2. The arrow marks the running front of the gel.

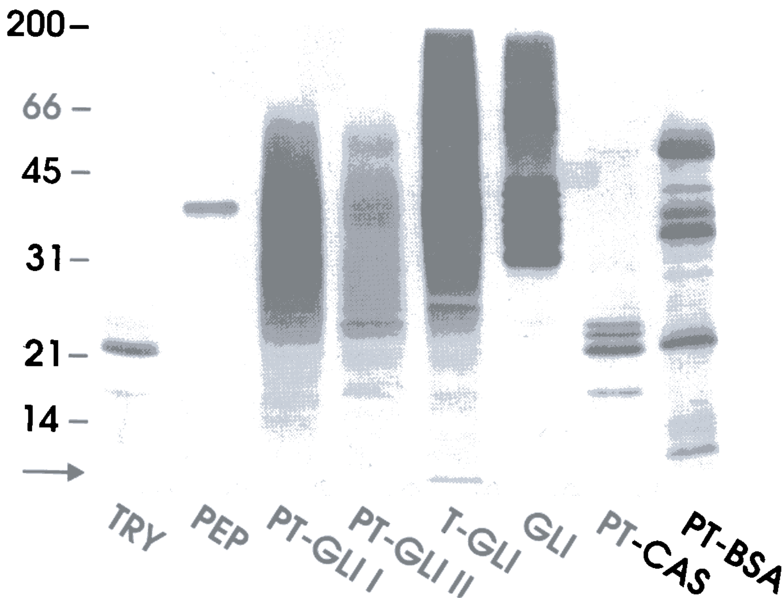

Fig. 4. Effectiveness of electrophoretic transfer of peptic-tryptic digests and gliadin to nitrocellulose was checked by protein gold staining. For further details see legend to Fig. 2. The arrow marks the running front of the gel.

3.2. Demonstration of contaminating proteases in gliadin peptide preparations

For detection of small amounts of trypsin and pepsin, we used rabbit antisera in combination with a sensitive chemiluminescence detection system. With rabbit antiserum against pepsin, no pepsin contamination of peptide preparations was seen (Fig. 2). Incubation of pepsin antiserum overnight resulted in unspecific binding to BSA and gliadin peptides as well as to intact gliadin. Binding of rabbit antiserum to BSA peptides was not seen with shorter incubation times (data not shown). Using rabbit antiserum against trypsin, bovine trypsin was detected as a 22 kDa main band by Western blot (Fig. 3). Peptide fractions PT-GLI I, T-GLI, PT-BSA and PT-CAS contained detectable amounts of trypsin. In addition, T-GLI and PT-CAS contained trypsin fragments of low molecular mass. Gliadin peptides prepared with insoluble proteases were not contaminated with trypsin. A slight unspecific binding of trypsin antiserum to gliadin components was observed. Electrophoretic transfer of peptides and proteins in a range of 10–200 kDa to nitrocellulose was complete (Fig. 4).

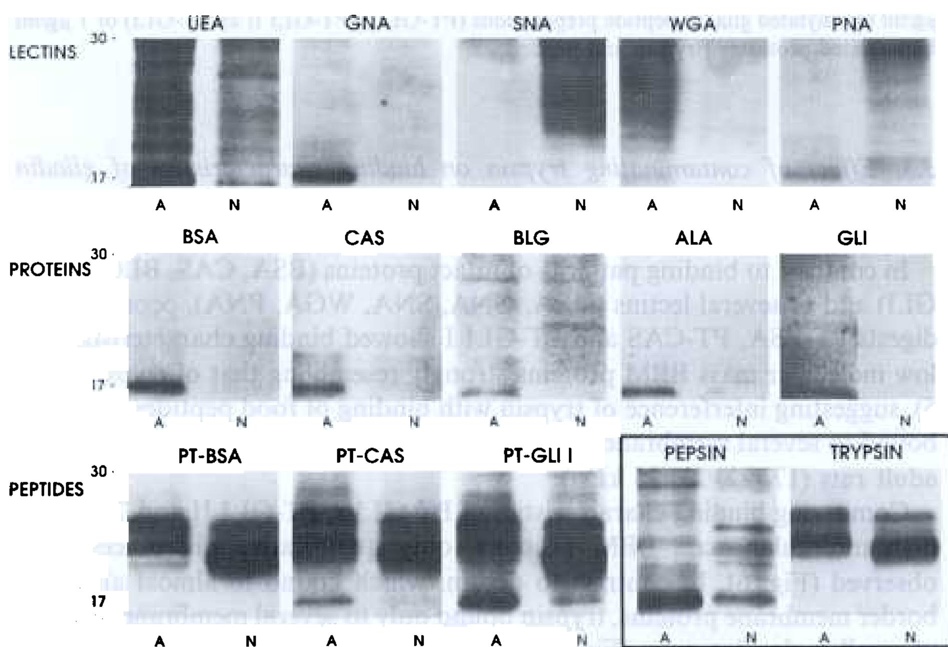

Fig. 5. Binding patterns of peptic-tryptic digests to low molecular mass brush border membrane proteins correspond with binding characteristics of trypsin in contrast to intact food proteins and lectins. Western blots were performed with 8 μg BBM proteins of adult (A) or newborn (N) rats. As biotinylated probes, 2 μg/ml lectins, 20–50 μg/ml cow's milk proteins and peptides, 5 μg/ml gliadin, 6 μg/ml PT-GLI I, 1 μg/ml pepsin and trypsin were used.

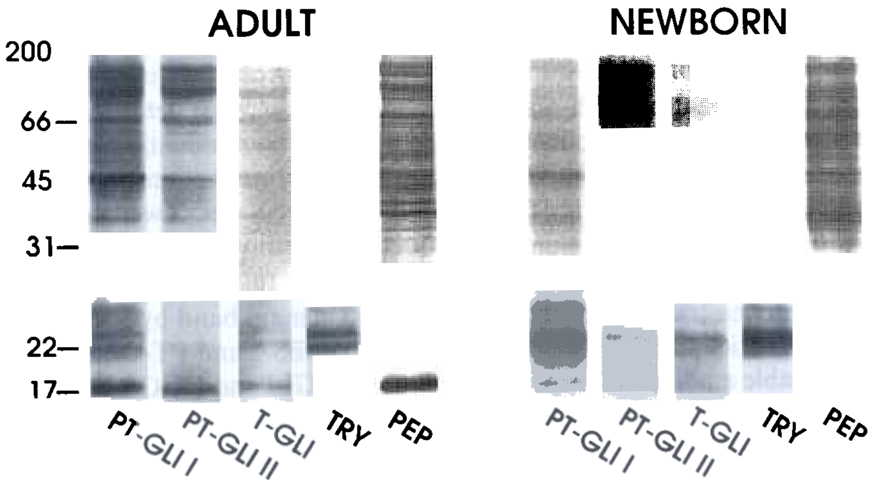

Fig. 6. Different gliadin peptide preparations show similar binding patterns to high molecular mass brush border membrane proteins, but differ in binding intensities. Western blots of 8 μg BBM proteins of adult (left panel) and newborn (right panel) rats were incubated with 10 μg/ml biotinylated gliadin peptide preparations (PT-GLI I, PT-GLI II and T-GLI) or 1 μg/ml biotinylated proteases (trypsin and pepsin).

3.3. *Effect of contaminating trypsin on binding characteristics of gliadin peptides*

In contrast to binding patterns of intact proteins (BSA, CAS, BLG, ALA, GLI) and of several lectins (UEA, GNA, SNA, WGA, PNA), peptic-tryptic digests PT-BSA, PT-CAS and PT-GLI I showed binding characteristics for low molecular mass BBM proteins strongly resembling that of trypsin (Fig. 5), suggesting interference of trypsin with binding of food peptides. Peptides bound to several membrane proteins of newborn (17, 22, 24, 25 kDa) and of adult rats (17, 22, 23, 25 kDa).

Comparing binding characteristics of PT-GLI I, PT-GLI II and T-GLI to high molecular mass BBM proteins, only quantitative differences were observed (Fig. 6). In contrast to pepsin, which bound to almost all brush border membrane proteins, trypsin bound only to several membrane proteins of small molecular mass (Fig. 6). Therefore, we conclude that quantitative differences of binding seen with high molecular mass brush border membrane proteins was not due to trypsin contamination of gliadin peptides. Undigested gliadin did not bind to BBM proteins (data not shown).

Studying interactions of gliadin peptides with intact brush border membranes by dot blots, however, approximately 30% less PT-GLI II bound to BBM in comparison with PT-GLI I (Fig. 7). Although contaminating trypsin

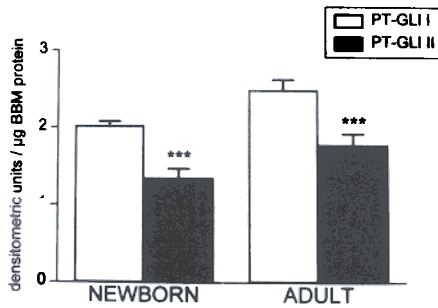

Fig. 7. Contaminating trypsin contributes significantly to overall binding of gliadin peptides PT-GLI I to brush border membranes in contrast to PT-GLI II, which is free of contaminating protease; 5 µg/ml biotinylated gliadin peptides were used in dot blots ($n = 10$). ***$P < 0.001$.

constituted only a minimal part of peptide preparations, it contributed significantly to overall binding.

Obviously, binding characteristics of PT-gliadin and other digested fractions were partly due to binding of trypsin and not exclusively to the respective food protein used.

4. Discussion

Our data show that small amounts of trypsin remained in gliadin peptide preparations T-GLI and PT-GLI I, produced by tryptic or peptic-tryptic digestion. This contaminating trypsin was also biotinylated by our standard technique. It altered gliadin peptide binding characteristics by interacting specifically with low molecular mass membrane proteins: differences seen between binding of intact food proteins (cow's milk proteins and gliadin) and of respective peptide preparations (PT-CAS, PT-BSA, PT-GLI I) to low molecular mass BBM proteins were mainly due to trypsin contamination.

Although we demonstrated trypsin contamination, using rabbit antiserum and by specific binding of trypsin inhibitor (data not shown) in all the peptic-tryptic digests tested, we were not able to measure any pepsin contamination. We assume that pepsin is degraded during further tryptic digestion.

By a modification of Frazer's method, using insoluble proteases, we were able to prepare trypsin-free gliadin peptides (PT-GLI II). In contrast to protease-contaminated gliadin peptides, this peptide preparation showed no trypsin binding pattern for small molecular mass BBM proteins. Moreover, decreased binding of PT-GLI II to intact brush border membranes was observed in dot blots.

Tryptic and peptic-tryptic digests of gliadin showed similar binding patterns concerning high molecular mass membrane proteins, but differed

significantly in binding intensities. Small amounts of contaminating pepsin, not detectable by antiserum, might contribute to binding intensities of PT-GLI I in contrast to PT-GLI II and T-GLI. In addition, differences of the wheat varieties Kolibri and Kanzler should be considered, although peptic-tryptic digests of these varieties showed similar peptide profiles in SDS–PAGE.

Gliadin peptides bound to high molecular mass membrane proteins, which were shown to be glycosylated and to interact also with cow's milk proteins [22]. Binding patterns of gliadin peptides showed no similarity to binding characteristics of lectins. In contrast to former studies using miniature ultracentrifugation [23], we found more gliadin peptide binding to adult BBM than to newborn membranes, using the dot blot system. The changed test system probably is the reason for the differences observed.

Our results demonstrate clearly that heat inactivation of proteases in gliadin peptide preparations is not sufficient to abolish adverse protease effects, especially in studies of gliadin peptide binding.

Protease contaminations may act in different ways to alter gliadin peptide binding characteristics. Firstly, measurement of bound labelled components does not discriminate between gliadin peptides and contaminating proteases. Second, contaminating proteases might possibly affect in vitro gliadin peptide binding to glycoproteins [9], to small intestinal brush border membranes [23], to isolated enterocytes [8] and to jejunal biopsy sections [24]. They could also interfere with interactions between enterocytic glycoproteins and gliadin peptides (Frazer's fraction III) bound covalently to Sepharose beads [25]. Third, contaminating proteases might interact directly with other components of different test systems. In this respect, several studies on small intestinal biopsies, indicating changes in enterocyte height [3], uptake of gliadin peptides [26] and increase in activated lamina propria T cells [4] after incubation with gliadin digests, did not describe any investigation of potential protease effects. Including such experiments, Lundin et al. [27] reported that T cells isolated after in vitro challenge of biopsies with Frazer's fraction III exhibited only gluten specificity and no pepsin or trypsin specificity.

Auricchio et al. [2] demonstrated counteraction of spermidine against PT-gliadin-induced agglutination of K 562 cells and inhibition of fetal rat intestinal development, but they did not prove absence of proteases in their peptide preparation. Increased activity of fraction 9 (peptic-tryptic-pancreatinic gliadin digest) in assays using fetal chick intestine and rat liver lysosomes might be influenced by remaining proteases [28]. Differences between undigested gliadin and gliadin peptides reported for complement activation via the alternative pathway [7] and for synergistic action on γ-interferon induced major histocompatibility complex expression by HT-29 cells [13] might partly be caused by protease contamination.

In our study the proteases pepsin and trypsin bound differently to rat

small intestinal brush border membranes. Pepsin bound to almost all membrane proteins and strongly to intact BBM. Trypsin, however, bound weakly to BBM and showed a specific binding pattern to three membrane proteins of newborn (22, 24, 25 kDa) and adult rats (22, 23, 25 kDa). This binding pattern was not seen with cow's milk proteins, lectins, or gliadin. A selective interaction of trypsin with components of brush border membranes might facilitate further uptake followed by transport of undegraded trypsin across the small intestinal mucosal barrier [29].

In order to prevent indirect or direct interference of contaminating proteases with small intestinal BBM or with different test systems for investigation of gliadin effects, the use of single peptides or of peptide fractions prepared by insoluble proteases is suggested. Frazer's fraction III or comparable peptic-tryptic gliadin digests for use in any system should be checked for potential contaminations. In view of these data, former studies using Frazer's fraction III or their variations should be reconsidered carefully.

Acknowledgements

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