

Prolyl Endopeptidase Mediated Destruction of T Cell Epitopes in Whole Gluten – Chemical and Immunological Characterization

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D) Abbreviations:

LC-MS – Liquid chromatography – mass spectrometry

RP-HPLC – reverse phase – high performance liquid chromatography

PTCEC- pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase

E.) Gastrointestinal, Hepatic, Pulmonary, and Renal

ABSTRACT

Celiac Sprue is a widely prevalent immune disease of the small intestine induced by dietary gluten intake in genetically susceptible individuals. It has been suggested that prolyl endopeptidases (PEPs) may be useful catalysts for gluten detoxification. We have investigated this hypothesis using food-grade gluten as the target antigen, and a combination of mass spectrometry and patient-derived T cells as quantitative assay systems. Spectrometric characterization of physiologically proteolyzed gluten revealed a number of 10 to 50 residue peptides containing known T cell epitopes involved in Celiac Sprue pathogenesis. Several of these peptides were multivalent, suggesting they may be potent triggers of the inflammatory response to gluten in celiac patients. Treatment of proteolyzed gluten with recombinant bacterial prolyl endopeptidase (PEP) decreased the number of potentially immunostimulatory peptides. Substantially reduced immunogenicity was also quantified in 12 out of 14 intestinal polyclonal T cell lines from celiac patients. Kinetic investigations using eight T cell clones showed rapid destruction of α -gliadin epitopes, but less complete processing of γ -gliadin epitopes. Given the difficulty associated with a strict lifelong gluten-exclusion diet, the ability of a single enzyme to greatly reduce the antigenic burden of grocery store gluten reinforces the case for developing oral peptidase therapy against Celiac Sprue.

INTRODUCTION

Celiac Sprue, also known as Celiac Disease, is a multifactorial disorder that affects children and adults with high prevalence (1:100-1:300) (Maki et al., 2003). The disease is characterized by an inflammatory response to ingested wheat gluten (and the similar proteins in rye and barley) which leads to destruction of the intestinal villi and impairs ability to absorb nutrients (Farrell and Kelly, 2002; Green and Jabri, 2003; Sollid, 2002). The disease may present in early childhood with malabsorption, diarrhea and failure to thrive, but often manifests later in life with predominant extraintestinal symptoms like fatigue, osteoporosis, anemia, psychiatric syndromes and neurological conditions (Farrell and Kelly, 2002; Green and Jabri, 2003). Among complex immune disorders, Celiac Sprue is unique in that critical factors that contribute to its etiology have been identified. Gluten proteins are obligate environmental triggers, whereas human leukocyte antigen (HLA) genes (DQ2 and DQ8) are a primary genetic factor (Sollid, 2002).

Several studies have mapped epitopes in gluten that stimulate intestinal CD4⁺ T cells from Celiac Sprue patients but not control subjects. These T cells are invariably restricted HLA-DQ2 or -DQ8 (Sollid, 2002). They focus on Gln and Pro rich peptides that are deamidated by tissue transglutaminase (TG2) (Molberg et al., 1998; van de Wal et al., 1998; Arentz-Hansen et al., 2000; Arentz-Hansen et al., 2002; Vader et al., 2002), an enzyme that is also the target of disease specific autoantibodies (Dieterich et al., 1997). T cell epitopes have been identified in both the major classes of gluten proteins, the monomeric gliadins and the polymeric glutenins, but it is clear that a hierarchy exists between different epitopes. In particular, the α -gliadin epitopes are recognized by T cells from almost all patients, whereas responses to the γ -gliadin and glutenin epitopes are less frequent in intestinal T cell lines derived from patients (Arentz-Hansen et al., 2000; Arentz-Hansen et al., 2002; Vader et al., 2002; Sjostrom et al., 1998; Shan et al., 2002; Vader et al., 2003). T cell epitopes are unevenly distributed in the sequences of gliadin proteins; they cluster in Pro- and Gln-rich regions (Arentz-

MATERIALS AND METHODS

Materials-All enzymes were purchased from *Sigma* (St. Louis) except for Pepsin, which was obtained from *American Laboratories* (Omaha) and Trypsin+Chymotrypsin, which was purchased from *Enzyme Development Co.* (New York). The specific activities of all enzymes were tested using standard chromogenic assays prior to use, and were found to be: Pepsin: 4000 U/mg; Trypsin: 2000 USP/mg; Chymotrypsin: 800 USP/mg; Elastase: 5 U/mg; Carboxypeptidase A: 30 U/mg. Wheat gluten flour was from *Bob's Red Mill* (Milwaukie). All other reagents were food or reagent grade. Substrates for the chromogenic assays were purchased from *Sigma* except for Z-Glu-Pro-p-nitroanilide, which was purchased from *Bachem* (Basel).

Enzyme activity assays: Activity assay protocols for pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, PEP, as well as dipeptidyl peptidase IV (DPP IV) and aminopeptidase N (APN) from the rat brush border membrane (BBM) are described in detail in the Supplementary Materials.

Isolation of rat brush border membrane — *Sprague-Dawley* rats were anesthetized with ketamine and 25 cm of its jejunum was removed; the rat was then killed by exposure to CO₂ and subsequent cervical dislocation. The jejunum was flushed gently with 0.9% NaCl, 1mM dithiothreitol (DTT) and cut longitudinally, and the mucosa was scraped off with glass microscopic slides. The recovered tissue was homogenized in a *Dounce* homogenizer in 5 mM ethylene diamine tetraacetic acid (EDTA) and 5 mM histidine-imidazole, pH 7.4, and centrifuged at 55,000×g for 20 min at 4°C. 15 ml of 0.25 M sorbitol, 12.5 mM NaCl, 0.5 mM EDTA and 5 mM histidine-imidazole, pH 7.4 was added to the pellet, which was then homogenized again, diluted to 25 ml and centrifuged at 1,400×g for 10 min at 4°C. 5 ml of 50 mM mannitol in 2 mM Tris-HCl, pH 7.1, was added to the pellet, and the solid was homogenized in a *Polytron* homogenizer at setting 3 for three 30 sec periods. The homogenate was then diluted to 10 ml and CaCl₂ was added to a final concentration of 10 mM. The suspension was

stirred for 30 min at 4°C and subsequently centrifuged at 2,000×g for 10 min at 4°C. The supernatant was collected and centrifuged for 20 min at 20,000×g at 4°C. The resulting pellet was re-suspended in PBS, pH 7.1. Activity of the preparation was confirmed by analysis of APN and DPP IV activities to be 10-15 fold purified over the initial tissue homogenate.

Gluten treatment with gastric and pancreatic enzymes— Wheat gluten flour (3.0 g) was added to 100 ml of water with addition of HCl to achieve a stable suspension at pH 2.0. Pepsin (Pepsin NF powder, 1:10000, 60 mg) was mixed in to the gluten suspension and the gluten-pepsin mixture was shaken constantly at 37°C for 2 h. After the pepsin treatment had been completed, 350 mg of Na₂HPO₄ were added and the pH adjusted to 7.9 by addition of 0.1 M NaOH. A mixture of trypsin/chymotrypsin (38 mg) was added and the mixture was shaken again at 37°C for 2 h and then heated to >95°C for 10 min. After cooling to room temperature, 74 µL of the mixture was treated with elastase (20 µL, 1 mg/ml) and the mixture was diluted to 1.7 mg/ml using PBS buffer, pH = 7.5. The mixture was maintained at 37°C. After 2 h the digested gluten suspension was heated to 95°C for 10 minutes and cooled to room temperature. Carboxypeptidase A (20 µL) was added and incubated at 37°C for 2 h, and subsequently heat deactivated at 95°C for 15 min.

Prolyl endopeptidase (PEP) and Brush Border Membrane (BBM) treatment—Recombinant PEP (Sp. Act ~40 U/mg), prepared as described elsewhere (Shan et al., 2004), was added at a ratio of 200 mU/mg of the PTC-digested gluten substrate, and incubated at 37°C for 15 min to 1 h and then heat de-activated, depending on the sample. Subsequently BBM (65 mU (DPP IV activity)/mg substrate) was added and the mixture was incubated for 1 h and heat deactivated at 95°C for 15 min. Control samples contained PEP and BBM buffer solutions without the enzymes.

Reverse Phase-HPLC- Samples were chromatographically separated on a 4.6 x 150 mm reverse phase C-18 protein & peptide column (Vydac, Hesperia) using Rainin Dynamax SD-200 pumps (1 ml/min), a Varian 340 UV detector set at 215 nm and a Varian Prostar 430 autosampler. Solvent A was H₂O with 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile with 0.1% TFA. Samples were centrifuged for 10 min at 13,400× g, prior to injection.

HPLC-MS- Separations were performed on a Surveyor HPLC system (ThermoFinnigan, San Jose). Solvent A was H₂O with 0.1% formic acid and 0.025% TFA; solvent B was acetonitrile with 0.085% formic acid and 0.022% TFA. Samples were chromatographed on a Vydac 2.0 x 150 mm reverse phase C18 column at a flow rate of 200 uL/min, using a gradient of 5-35% B over 30 min. The column was connected directly to the LCQ quadrupole ion trap mass spectrometer equipped with an electrospray ion source operating in positive ion mode. The sheath gas was set to 60 (arbitrary units), spray voltage to 4.5 kV, and capillary temperature to 200° C. The system acquires full MS, zoom scan, and MS/MS spectra in an automatic data dependent mode.

Data was processed with the *Sequest* Browser software. Detected ions and fragments were searched against all sequences in the *Entrez Protein* database of the National Center for BioInformatics (NCBI) that responded to the search term “*Triticum*” (>2000 protein sequences). Sequences with a cross correlation value below 1.5 were rejected; the remaining sequences were searched for epitope sequences listed in Table S1 (Supplementary Materials).

T cell assays- The generation of polyclonal gluten-specific T cell lines derived from *ex vivo* gluten challenged small intestinal biopsy specimens of adult Celiac Sprue patients have been described elsewhere (Arentz-Hansen et al., 2002). For this study 14 different intestinal T cell lines derived from 13 DQ2+ celiac patients were selected based on two criteria: (i) effective recognition of TG2-treated

digests of gluten, and (ii) significant responses to at least two different gluten epitopes (Table S2, Supplementary Materials). The generation of intestinal T cell clones and the epitope specificity of these clones, except TCC 387.3, has been described elsewhere (Arentz-Hansen et al., 2000). The TCC 387.3 recognizes a TG2-dependent epitope, defined as γ -VI that is contained within residues 62-72 (PQQPFPQQPQQ) of recombinant γ -5 gliadin (Ø. Molberg, unpublished). The freeze-dried, proteolyzed gluten samples were dissolved in PBS with 2 mM CaCl₂ and treated with 150 µg/ml human recombinant TG2 (Piper et al., 2002) or, in some experiments 200 µg/ml guinea pig transglutaminase (Sigma), for 2 hours at 37 °C. The TG2-treated samples were incubated overnight in triplicates on U-bottomed 96 well plates with 75 000 cells per well of DQ2 homozygous, irradiated (75 Gy) EBV-transformed B-lymphoblastoid cell lines in a volume of 100 µl 15% pooled, inactivated human serum and RPMI1640. After the incubation 50 µl of freshly thawed T cells (1 x 10⁶ cells / ml) were added to each well. The proliferation in the wells was evaluated by ³H thymidine incorporation from 48-72 hours after the addition of the T cells.

RESULTS

Proteolysis of Gluten and Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis

Wheat gluten was proteolyzed with pepsin, followed by pancreatic proteases (trypsin, chymotrypsin, elastase and carboxypeptidase A), followed by PEP (or vehicle), and eventually BBM (or vehicle). The overall proteolytic protocols are as detailed in the Materials and Methods section, and summarized Figure 1. Samples 1 to 7 were prepared as described in Table 1. Sample 1, the reference PTCEC-gluten sample, was not exposed to BBM or PEP. Samples 2 and 3 were exposed to PEP for increasing durations, without subsequent exposure to BBM. Sample 4 was exposed to BBM but not

PEP. Samples 5 and 6 were exposed to both PEP (for increasing durations) and BBM. Sample 7, which was quenched immediately after adding PEP and BBM, was included as a control to verify the lack of effect of either PEP or BBM components on mass spectrometric or T cell assays.

The gluten samples 1-7 were initially analyzed using RP-HPLC (Figure 2). Due to the complexity of the mixtures analyzed, individual peaks could not be readily identified; most of the observed peaks (including the major peaks in the 3-13 min range) are likely to represent mixtures of peptides. As expected, the HPLC traces of the samples 1 (blue) and 7 (not shown) showed no qualitative difference. Both samples revealed a complex mixture of peaks in the 20-30 min range. Most known immunogenic peptides, such as PQQQLPYPQQQLP (which elutes at ca. 20 min) and the 33-mer LQLQPFPPQQQLPYPQQQLPYPQQQLPYPQQQPF (which elutes at ca. 30 min), elute within this range under similar conditions. Treatment of samples with PEP only (e.g. sample 3) resulted in attenuation of the 20-30 min envelope, especially the late-eluting peptide peaks (>23 min) which included several long, multivalent peptides (vide infra, see also Tables S3-S5 in Supplementary Information). Thus PEP treatment of PTCEC gluten appeared to result in a substantial reduction in the median peptide length. Treatment with BBM and PEP (e.g. sample 6) led to almost complete decomposition of all peaks eluting after 15 min, with a concomitantly strong increase of peaks eluting at 3, 7 and 13 min. This is consistent with the known ability of BBM exopeptidases to efficiently recognize the short peptidic products generated by PEP action (Hausch et al., 2002).

Liquid Chromatography coupled tandem Mass Spectrometry (LC-MS/MS)

Overall analysis. The number of distinct peptides with lengths between 4 and 55 AA in each gluten sample varied between 133 (sample 6) to 314 (sample 7) (Figure S1, Supplementary Materials). Overall, 1549 sequences with a cross-correlation > 1.5 were identified, including duplicates. When the cross-correlation, which serves as a measure for the reliability of an identified sequence, was lowered

to 1.0, the number of distinct peptides increased from 314 to 444 in sample 7 (treated only with PTCEC). If the cross-correlation was increased to 2.0, the number decreased from 314 to 162 for the same sample.

Length distribution of identified sequences $x_C=1.5$. The average length of the 1549 identified sequences identified in samples 1 through 7 was 20.3 residues. No clear trend could be deduced from the data; the minimum average length was 17.3 residues (sample 2), whereas the maximum average length was 26.0 residues (sample 7).

Abundance of epitope-containing peptides. The amino acid sequences of the proteolytic fragments identified in samples 1-7 were screened for core DQ2-binding epitopes (9 residues) corresponding to the majority of (DQ2 restricted) T cell stimulatory gluten peptides identified to date (see Table S1, Supplementary Materials). In total, 68 distinct epitope containing peptides were identified (Table 2 and Tables S3-S5, Supplementary Materials). Except for the γ -VI epitope, nearly all of the epitope containing peptides were present in non-PEP treated samples. The percentage of the epitope-bearing peptides in each sample is shown in Figure S1 (Supplementary Materials). As can be seen, there is a pronounced reduction in the prevalence of epitope-bearing peptides in samples treated with PEP (samples 2, 3, 5 and 6). Notably, BBM treatment without PEP of the PTCEC-gluten led to only a modest reduction in epitope-containing peptides (see Table 2 and Figure S1, Supplementary Materials).

Origins and sequences of epitope-containing peptides. The origins of the 68 epitope-containing peptides in samples 1-7 are summarized in Table 2, and their individual sequences are detailed in Tables S3-S5 (Supplementary Materials). Notably, although a majority of gluten proteins in the Entrez database include γ -gliadin and glutenin sequences rather than those of α - or ω -gliadins, α -gliadin peptides comprise 32% of all identified epitope-bearing peptides.

α -gliadin epitopes. Whereas each of the three known α -gliadin epitopes (α -I, α -II and α -III) were found in untreated and BBM treated gluten (samples 1 and 4; sample 7 revealed α -I and α -II, but not

α -III, epitopes), PEP treatment (samples 2, 3, 5 and 6) led to the elimination of all α -gliadin epitope-bearing peptides (Table 2). All α -gliadin epitopes were found to occur in two closely related families of gluten peptides (Table S3, Supplementary Materials; entries 1-2 and entries 4-6); the first of these families is closely related to the previously identified 33-mer α G-33 (Shan et al., 2002).

γ -gliadin epitopes. Although the γ -VI epitope was identified in all samples except in sample 6, there were fewer γ -VI epitope-bearing peptides in the PEP-treated samples (Table 2 and Table S4, Supplementary Materials). Thus, this epitope appears to be somewhat resistant to cleavage by the *F. meningosepticum* PEP. The 9-mer core region of this epitope was identified in at least nine different gluten peptides. This is likely a major factor contributing to the many hits observed for this epitope. The γ -II epitope was found in sample 1 and 7 and Glia- γ 2 was found in sample 1 (Table 2 and Table S4, Supplementary Materials).

Other epitopes. Epitope Glt-17 was found in samples 1, 3 and 7, and occurs in the context of multiple peptides (Table 2 and Table S5, Supplementary Materials).

T cell assays – Polyclonal T cell lines

T cell assays were performed on 14 polyclonal cell lines derived from small intestinal biopsies of Celiac Sprue patients as well as 8 intestinal T cell clones that recognize distinct epitopes. These gluten responsive, HLA-DQ2 restricted T cells are a hallmark of Celiac Sprue: notably, they are not found in the intestinal mucosa of control individuals, regardless of their HLA background (Molberg et al., 1997). As was expected from previous experience (7,8), all T cell lines displayed vigorous gluten dose-dependent increases in ^3H -thymidine incorporation, reflecting T cell replication in the presence of TG2-treated gluten (sample 1) and DQ2+ antigen presenting cells (Figures 3 and 4). All samples were pre-treated with TG2, and tested at four different dilutions. From previous studies we determined that 250 μg / ml TG2-treated gluten is sufficient to obtain near maximal T cell proliferation; therefore,

this ratio was thus chosen as the highest test concentration for all samples. Figure 3 shows the responses of 14 polyclonal T cell lines to 250 µg / ml of alternatively prepared gluten samples. BBM treatment alone can attenuate the T cell response somewhat (sample 4 versus sample 1), as illustrated by the T cell lines TCL 421.1.4 and 451.1.1. However, this effect is modest compared to the dramatic decline in T cell response seen after 15 min, and especially 60 min of PEP treatment (samples 2 and 3). Notably, in many cases the combined effects of BBM and PEP appear to be highly complementary. Figure 4 shows the dose-dependence of responses to samples 1-6 in six of the T cell lines. A similar picture was obtained with the remaining eight T cell lines.

To quantify the effects of PEP and BBM treatment of gluten on its ability to induce proliferation of different T cell lines, the proliferative responses to samples 3 (PEP alone), 4 (BBM alone) and 6 (PEP+BBM) were calculated as a percentage of proliferation observed with sample 1 (no PEP or BBM) (Table S6, Supplementary Materials). Based on this analysis, PEP treatment alone completely detoxifies gluten from the viewpoint of a majority of the celiac lesion derived T cell lines (8 out of 14); the same PEP-treated gluten can be considered partially detoxified as assessed by the remaining T cell lines. No case was identified where the antigenicity of gluten was unaffected by PEP treatment. The complementary effect of BBM is also apparent, since 12 out of 14 T cell lines indicated a complete destruction of T cell epitopes when treated with PEP and BBM in sequence; the remaining two T cell lines showed some residual activity of the PEP and BBM treated gluten. In contrast, BBM treatment alone led to complete abrogation of T cell response for only one line (TCL 451.1.1) and partial abrogation of T cell responses in only six lines. The responses of the remaining lines were unaltered by BBM treatment of gluten. Together these results make a compelling case for the potential benefit of PEP in the digestive system of the Celiac Sprue patient.

T cell assays – T cell clones

Since individual T cell clones recognize distinct epitopes from gluten, they provide an excellent assay system to quantify the abundance of that epitope in a complex but physiologically relevant material such as proteolyzed gluten. Eight celiac lesion derived T cell clones were characterized using the same assay set-up as in the case of polyclonal T cell lines (Figure 5A). The kinetics of destruction of gluten epitopes were also monitored by exposing gluten to PEP for varying time periods while keeping the BBM exposure constant (Figure 5B). A pattern emerges from this data set. The α -gliadin epitopes are highly susceptible to PEP-mediated destruction, the α -II and α -III more so than the α -I. PEP-treated gluten retains some activity for the α -I specific intestinal T cell clone 387E9 even after 60 min PEP treatment, whereas the peptides recognized by the α -II specific T cell clone 430.1.135 and the α -III specific 370E3.19 are almost completely destroyed at the earliest time point (Figures 5A and 5B). This is consistent with the notion that the immunodominant α -G 33 peptide is destroyed by PEP (Shan et al., 2002).

In contrast, destruction of some γ -gliadin epitopes is considerably less efficient by the *Flavobacterium meningosepticum* PEP. Indeed, BBM treatment alone has a dramatic effect on the γ -I and IV epitopes (TCC 423.1.3.8 and TCC 430.1.112), but only a partial effect on the γ -II, γ -III and γ -VI epitopes (TCC 430.1.41, 430.1.134 and 387.3) (Figure 5B). Whereas the γ -III epitope is rapidly eliminated by this PEP after only 10 min, gluten fragments containing the γ -II and γ -VI epitopes persist for longer durations.

DISCUSSION

Strict adherence to a gluten exclusion diet is currently the only treatment for Celiac Sprue patients. Compliance is difficult but critical, because untreated disease causes morbidity and is associated with increased mortality (Farrell and Kelly, 2002; Green and Jabri, 2003). Alternative therapeutic options

that could ameliorate the burden of dietary restriction are thus in demand. Based upon studies with a recombinant gliadin protein, we recently proposed that a prolyl endopeptidase (PEP) could be an effective catalyst for destruction of proline-rich T cell epitopes (Shan et al., 2002). Here we have taken a significant step forward through the detailed chemical and biological analysis of the effects of PEP on whole gluten as found in a typical grocery store. Gluten was proteolyzed by pepsin and the pancreatic proteases under physiological conditions to yield a complex mixture of thousands of peptides of varying lengths. Our chemical analyses involved high-resolution chromatographic and mass spectroscopic procedures, whereas our biological analyses involved the use of DQ2-restricted, gluten-responsive T cells from 14 Celiac Sprue patients. In addition to lending further support to the proposal that immunogenic fragments of gluten are also proteolytically resistant, our results demonstrate that a suitable PEP, or a mixture of PEPs with complementary substrate specificities, may be an effective way to render gluten harmless for many Celiac Sprue patients.

LC-MS/MS of whole gluten proteolyzed under various conditions demonstrated that a large fraction of epitope-containing peptides were between 21 and 30 residues in length (31 out of 68, 46%), although a few peptides (10 out of 68, 15%) were longer than 30 residues, and 27 out of 68 (40%) were between 11 and 20 residues in length. In contrast, the distribution of non-antigenic peptides was biased toward shorter lengths: 1-10 residues, 38%; 11-20 residues, 27%; 21-30 residues, 22% and >30 residues, 14%. There may be an overall bias in the length assessment, as reliable detection of long peptide sequences using ion trap MS/MS detection is limited due to incomplete fragmentation of longer peptides. The problem is exacerbated for peptides rich in Pro and Gln residues, as they inhibit complete fragmentation. Finally, our LC-MS/MS based assessment of the residual immunotoxicity of individual gluten samples may be affected by other biasing factors. For example, the multiple hits for the γ -VI epitope are likely due to the large number of parent sequences harboring this epitope in the *Entrez Protein* database; they may not necessarily imply an exceptionally high abundance of this

sequence. Also, quantification of peptide abundance by MS requires the use of internal standards (Gygi, 1999; Turecek, 2002), which are difficult to generate for complex, uncharacterized and variable materials such as grocery store gluten. Notwithstanding these limitations, we have employed LC-MS/MS to demonstrate that PEP treatment and, to a lesser extent, BBM treatment, results in a substantial reduction of abundance of immunogenic peptides in gluten that has been pre-treated with pepsin and pancreatic enzymes.

T cells derived from small intestinal biopsy samples of Celiac Sprue patients are exquisitely sensitive and accurate monitors of the inflammatory potential of a processed gluten sample. In the absence of an animal model for Celiac Sprue, such T cell proliferation assays are the best approximation for quantifying the toxic effects of gluten in Celiac Sprue patients. Although intact small intestinal biopsies from celiac patients have been used to demonstrate gluten toxicity *ex vivo* (Maiuri et al., 2003), their scarcity and sample-to-sample variability makes them unsuitable for quantitative pharmacological evaluation of experimental therapeutic agents. Both monoclonal and polyclonal T cell samples have been utilized in this study to evaluate the scope of PEP therapy for Celiac Sprue. In many cases PEP alone is enough to achieve complete detoxification of gluten pre-digested by pepsin and the pancreatic enzymes without the need for exposure to BBM, whereas in some cases, although PEP treatment results in partial processing of gluten, the action of BBM peptidases is necessary to achieve complete detoxification. These findings reinforce the heterogeneity of disease-associated T cells among Celiac Sprue patients, which in turn may forebode the heterogeneity of patient response to a selected PEP such as the *Flavobacterium meningosepticum* enzyme. For example, to the extent that the observed T cell responses in Figs. 3 and 4 accurately reflect *in vivo* conditions, one might predict (Table S6, Supplementary Materials) that patients 421, 422, 432, 437, 446, 451 and 491 would respond well to PEP treatment. Additionally, patients 461, 482, 488, 494 and 496 may also derive some benefit from PEP treatment, although complete benefit would require optimal small intestinal

mucosal structure to yield active BBM peptidases. In contrast, patient 502 is unlikely to stay in remission when challenged with gluten even when treated with an otherwise effective PEP. From the known reactivity pattern of this T cell line (see Table S2, Supplementary Materials), one might have predicted that it should be sensitive to PEP. The unexpected finding brings up the possibility that some of the T cells within the line respond to an unidentified gluten peptide that is only infrequently recognized by T cells, but is resistant to treatment with PEP and BBM. Ultimate verification of such hypotheses will require controlled in vivo and ex vivo clinical studies with PEP in Celiac Sprue patients.

In addition to polyclonal T cell lines, monoclonal T cells are also valuable reagents for evaluating the efficacy of PEP (Fig. 5). In particular, since their epitope specificity has been precisely characterized, they serve as excellent reporters of the persistence of individual epitopes in a complex gluten mixture that has been treated with a given PEP. For example, from analysis of 8 different T cell clones, it can be concluded that, whereas the *F. meningosepticum* enzyme has high specificity for α -gliadin epitopes, it has lower specificity for γ -gliadin epitopes. Epitopes γ -II (TG2-treated IQPQQPAQL) and γ -VI (TG2-treated PQQPFPQQPQQ) recognized by the intestinal T cell clones 430.1.41 and 387.3, respectively, seem to be stable against treatment with both PEP and BBM. It should be noted however that these two epitopes, despite their resistance to the PEP and BBM treatment, are not frequently recognized by intestinal T cell lines (Table S2, Supplementary Materials; and Shan et al., 2002). This could be related to low levels of proteins containing these epitopes in ingested gluten, inefficient presentation by DQ2+ antigen presenting cells in the celiac lesions and/or lack of T cells with appropriate T cell receptors. Encouragingly, parallel studies have revealed that PEPs from different sources have different substrate specificities, both with regard to cleavage sites and substrate chain lengths (Shan et al., 2004). Thus, there remains considerable potential for combining PEPs with

complementary substrate specificities, or for screening and/or engineering PEPs with optimized ability to render gluten non-stimulatory to intestinal T cells in Celiac Sprue patients.

In summary, this study has substantially strengthened our earlier proposal that enzymatic detoxification of gluten has the potential to be an effective method for treating Celiac Sprue. It should be noted that, although the pivotal role of gluten responsive CD4+ T cells in Celiac Sprue pathogenesis is well established, alternative mechanisms for the initiation of the celiac lesion in the small intestine have been proposed (Maiuri et al., 2003; Tuckova et al., 2002). The precise structural basis for gluten toxicity via these mechanisms remains to be elucidated; consequently, the ability of PEP to cleave the corresponding peptides in grocery store gluten cannot be systematically evaluated at present. However, in those cases where specific gluten peptides have been proposed as pathogenic triggers the intestinal brush border membrane peptidases can efficiently cleave these peptides either alone or in conjunction with PEP (Piper et al., 2004). Ultimate verification of the therapeutic utility of PEP will of course require patient trials, which might initially consist of exposing Celiac volunteers to PEP-treated and untreated gluten.

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FOOTNOTES

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LEGENDS TO FIGURES

Figure 1. Overall strategy for simulating enzymatic detoxification of gluten. In a step designed to mimic the action of the stomach on dietary gluten, commercial gluten was first treated with pepsin under acidic conditions. The reaction mixture was neutralized and treated with trypsin, chymotrypsin, elastase and carboxypeptidase to simulate the effect of pancreatic secretions on dietary gluten emptied into the duodenum from the stomach. The resulting material, which is known to be toxic for Celiac Sprue patients, was optionally treated with PEP and/or BBM. Rat BBM preparations contain membrane bound exopeptidases; their in vitro activity mimics the intact digestive surface in the small intestine. PEP, Prolyl Endopeptidase; BBM; Rat brush border membrane enzymes.

Figure 2. UV₂₁₅ traces of PEP and BBM treated PTCEC gluten. Samples 1, 3 and 6 are as described in Table 1. The inset shows an expanded view of the chromatogram in the 20-30 min region, where most immunogenic gluten peptides elute.

Figure 3. Response of fourteen T cell lines challenged with 250 µg/ml gluten. The conditions under which individual gluten samples were treated are summarized in the table below. Sample 1 data for TCL 461.4 was lost, and has therefore not been plotted.

Figure 4. Response of six polyclonal T cell lines to varying concentrations of individual TG2-treated PTCEC-gluten samples.

Figure 5. Response of T cell clones to gluten. A: Titration with increasing concentrations of gluten. B: Kinetics of gluten destruction upon treatment with PEP (all samples treated with BBM for 60 min).

Table 1: Treatment scheme of pepsin/trypsin/chymotrypsin/elastase/carboxypeptidase A (PTCEC) treated whole gluten.

Sample	1	2	3	4	5	6	7
Duration PEP treatment (min)	no PEP	15	60	no PEP	15	60	0
Duration BBM treatment (min)	no BBM	no BBM	no BBM	60	60	60	0

Table 2: Number of distinct peptides in the individual samples that harbor the 9-mer core regions of T cell epitopes (Table 1). The sequences of these peptides are given in Supplemental Table 1.

Epitope	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample
α -I	2	-	-	3	-	-	1
α -II	2	-	-	1	-	-	1
α -III	2	-	-	1	-	-	-
Glia- α 20	-	-	-	1	-	-	-
γ -I	-	-	-	-	-	-	-
γ -II	2	-	-	-	-	-	2
γ -III	-	-	-	-	-	-	-
γ -IV	-	-	-	-	-	-	-
γ -VI	12	3	4	7	3	-	6
Glia- γ 2	2	-	-	-	-	-	-
Glu-5 (Var 1)	-	-	-	-	-	-	-
Glu-5 (Var 2)	-	-	-	-	-	-	-
Glt-17 (Var 1)	4	-	2	-	-	-	7
Glt-17 (Var 2)	-	-	-	-	-	-	-
Total	26	3	6	13	3	-	17

Figure 1

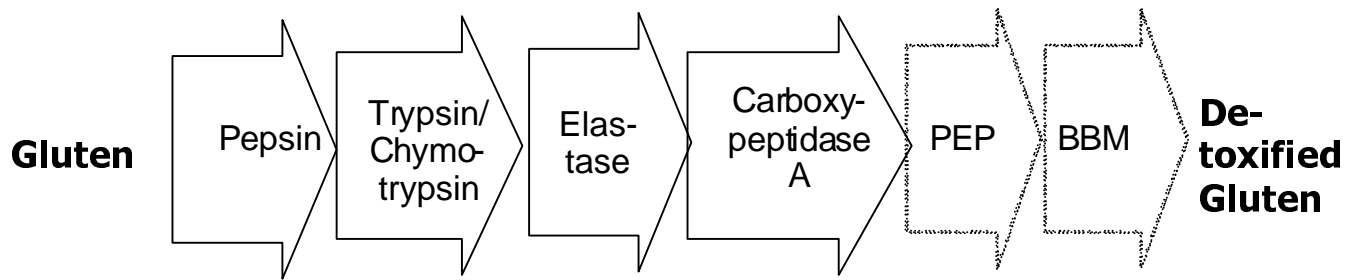


Figure 2

Sample 1
Sample 3
Sample 6

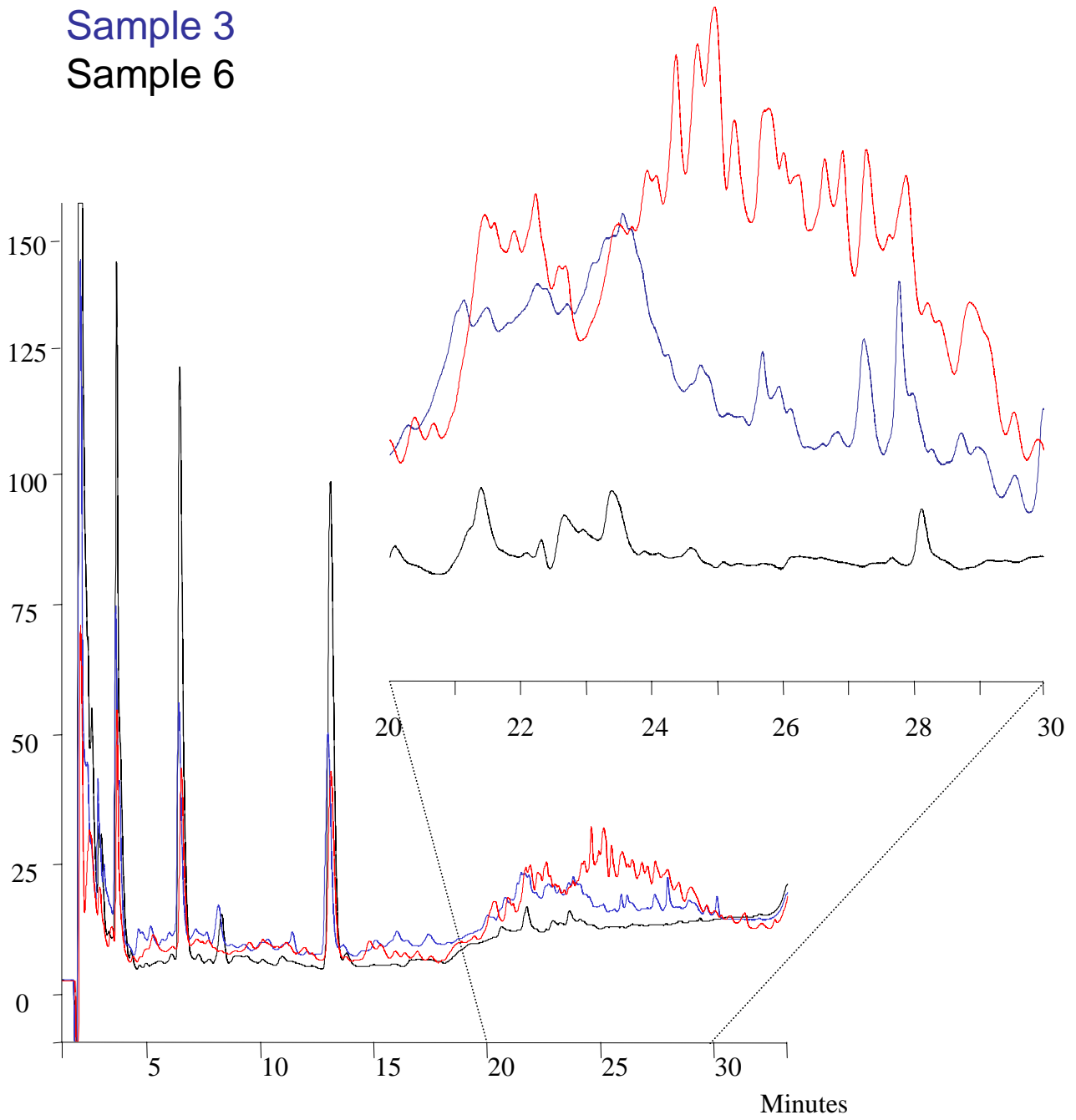
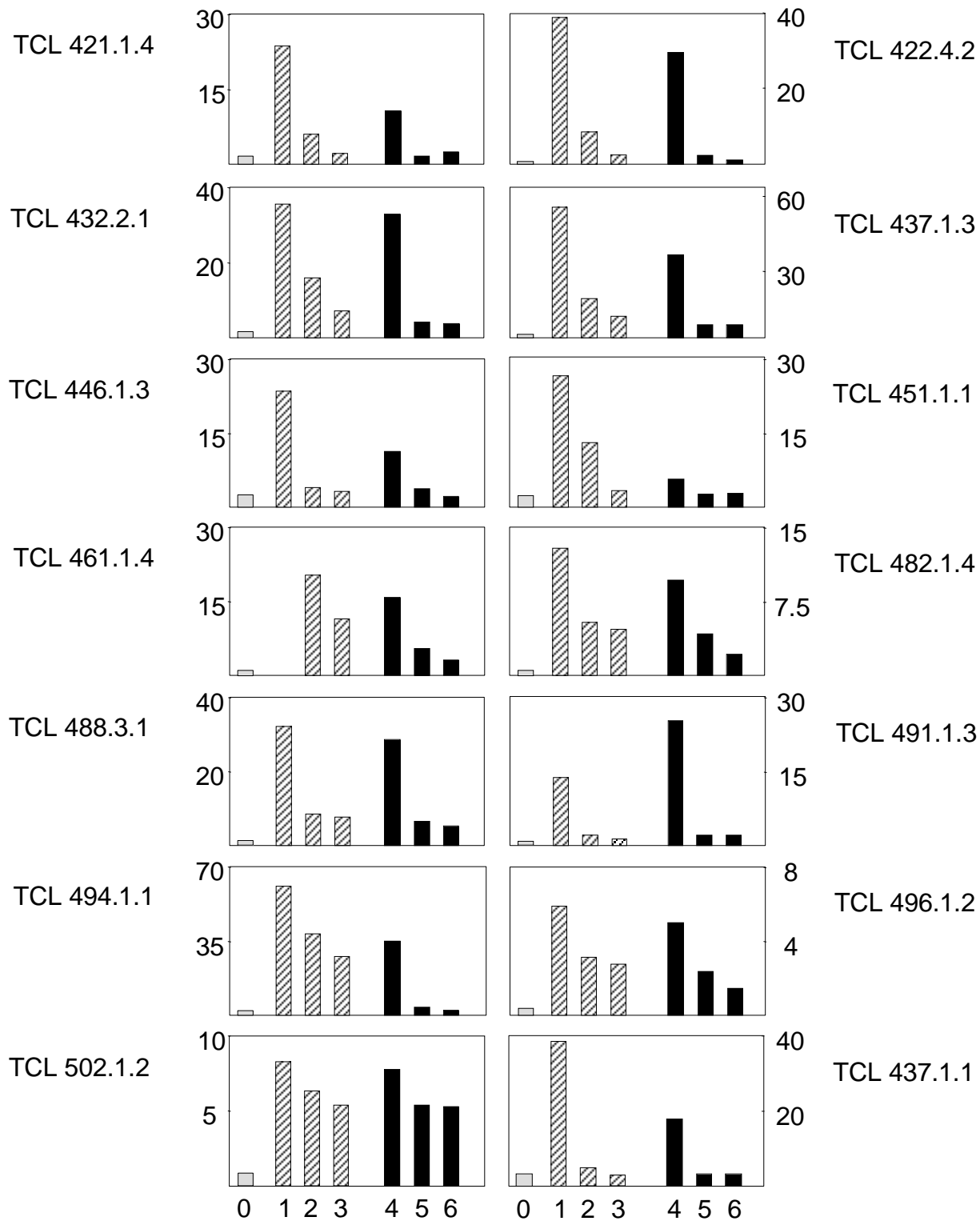


Figure 3



	0	1	2	3	4	5	6
Gluten	-	+	+	+	+	+	+
TG2	-	+	+	+	+	+	+
PEP	-	-	15'	60'	-	15'	60'
BBM	-	-	-	-	60'	60'	60'

Figure 4

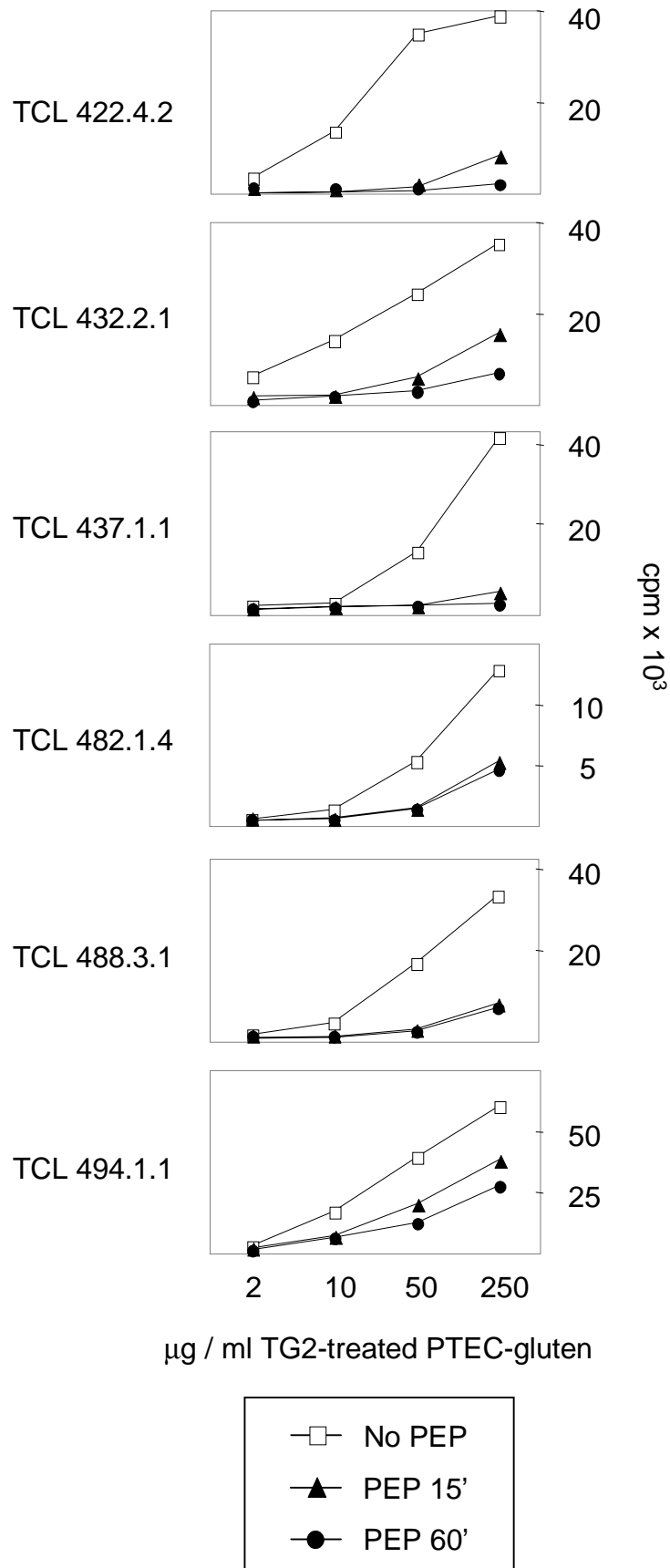


Figure 5

