Original Article

MOLECULAR METABOLISM

The human digestive tract has proteases capable of gluten hydrolysis

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ABSTRACT

Objective: To identify, purify, and characterize the proteins responsible for glutenase activity in the feces of healthy subjects and patients with celiac disease (CD).

Methods: Sixteen subjects were included in this study; 8 were healthy with no known food intolerances, and 8 were treated CD patients on a gluten-free diet. Fecal samples were homogenized, and precipitated proteins were purified by chromatography. Glutenase activity was evaluated by bioassays, zymography, and high-performance liquid chromatography with immunogenic 33-mer, 19-mer, and 13-mer gliadin peptides.

Results: The gastrointestinal elastase 3B (CEL3B), elastase 2A (CEL2A), and carboxypeptidase A1 (CBPA1) enzymes degraded human gluten. These proteins fully hydrolyzed 13-mer and 19-mer gliadin peptides that trigger immune-mediated enteropathy in individuals genetically predisposed to CD and partially digested a 33-mer. Feces from patients with CD showed more glutenase activity than feces from individuals without CD (171−466% higher). Peptidase activity against the gliadin peptides also increased in patients with CD.

Conclusion: The digestive tracts of patients with CD and healthy subjects have enzymatic machinery needed for gluten degradation. Patients with CD showed more gluten hydrolysis than did healthy individuals, although, in both cases, a fraction of 33-mer peptide remained intact. Gliadin peptides derived from gastrointestinal digestion, especially the 33-mer, can potentially be used by commensal microbiota from both CD-positive and CD-negative individuals, and differences in bacterial hydrolysis can modify its immunogenic capacity.

Keywords Gluten; Gliadin; Celiac disease; Glutenase fecal activity; Gliadinase activity

1. INTRODUCTION

Celiac disease (CD) is a chronic, small intestinal, immune-mediated enteropathy triggered by the exposure to gluten proteins in genetically predisposed individuals [1−3]. The pathogenesis of CD involves genetic and environmental factors. Susceptibility to CD is strongly associated with the human leukocyte antigen (HLA) genes of the major histocompatibility complex, and approximately 90% of patients express alleles coding for the HLA-DQ2 (HLA-DQ2.5 and HLA-DQ2.2) and HLA-DQ8 haplotypes [1,4,5]. The diet is the fundamental factor contributing to the development of CD. The gliadin prolamin (from gluten) and related prolamins (from wheat, barley, and rye) are resistant to complete digestion by human digestive enzymes due to their high glutamine and proline contents. Their digestion results in the production of large peptides (10 to ≥30 amino acids) that cross the small intestinal barrier, some of which (such as 13-, 19-, or 33-mer), are capable of triggering inflammatory processes associated with CD [6−8].

Nevertheless, although 30% of the general population carries a genetic predisposition for CD, only approximately 3% will develop this disease. Moreover, additional environmental cofactors may be required [9], including intestinal pathogens that can enhance gluten immunogenicity and toxicity, e.g., rotavirus infections [10]; an altered gut-microbiota composition [11,12]; and some immune-modulatory drugs, e.g., IFN-alpha [13]. As such, intestinal dysbiosis has been associated with patients with CD [9,14], and it has been purposed that gliadin-metabolizing bacteria may represent one of the missing environmental links in the development of CD [15,16].

Previous findings have revealed that human feces show glutenase activity in both healthy individuals [17] and CD patients [9] and that significant differences in fecal glutenase activity may occur between these two groups [9]. These results suggest that human feces are

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Abbreviations: CD, celiac disease; HLA-DQ, human leukocyte antigen; LPLC, low-performance liquid chromatography; NCD, non-celiac-disease; CEL3B, gastrointestinal elastase 3B; CEL2A, gastrointestinal elastase 2A; CBPA1, human carboxypeptidase A1; ATIs, α-Amylase/trypsin inhibitors

Received April 7, 2017 • Revision received May 9, 2017 • Accepted May 15, 2017 • Available online xxx

http://dx.doi.org/10.1016/j.molmet.2017.05.008

potentially a good sample source for evaluating and identifying the proteases involved in gluten metabolism.
Accordingly, in this work, we identified and characterized the proteins responsible for fecal glutenase activity, and we compared the proteolytic profiles involved in degrading gluten peptides between healthy and individuals with CD.

2. MATERIALS AND METHODS

2.1. Fecal sampling

Sixteen subjects were included in this study; 8 were healthy with no known food intolerances (mean age 41; range 25–67), and 8 were CD patients on a gluten-free diet (mean age 42; range 27–57). The healthy subjects were symptom-free volunteers for whom CD was ruled out based on normal serum tissue Transglutaminase Antibody (tTGA) levels and an HLA-DQ phenotype that was not DQ2 or DQ8. Treated CD patients were diagnosed on the basis of positive tTGA and duodenal mucosa biopsy with villous atrophy (Marsh III in all of the cases). They complied with a strict gluten-free diet for at least 2 years, showed negative serology markers, and displayed complete recovery during the initial villous atrophy (Marsh 0 or Marsh I in the biopsy control). No participants in this study were treated with antibiotics in the month before the provided samples. The study complied with the Declaration of Helsinki guidelines, and all procedures involving human subjects were approved by the local ethics committee of our hospital. Written informed consent was obtained from all subjects. Fresh stools from both subject groups were collected and immediately stored at −80 °C. Previously, we showed that glutenase activity (evaluated by bioassay) in stool samples was not modified by freezing at −80 °C. Fecal samples were homogenized and processed immediately.

2.2. Purification of different fecal glutenase activities

All procedures were performed at 4 °C unless otherwise indicated.
Step 1: Sample preparation — Fecal samples were homogenized by mechanical stirring (90 min) in 10 mM Tris–HCl, pH 7.5 (1:5 w/v) and centrifuged (3,100 $\times g$, 30 min). The supernatant was re-centrifuged at 30,000 $\times g$ for 15 min and filtered through a 0.44-μm nitrocellulose filter.
Step 2: Ammonium sulfate fractionation — Each filtered supernatant obtained from step 1 was precipitated with ammonium sulfate. The fraction precipitating between 35 and 65% ammonium sulfate (containing glutenase activity) was collected by centrifugation at 30,000 $\times g$, 15 min. The pellet was dissolved in 10 mM Tris–HCl (pH 7.5). Ammonium sulfate was eliminated by passing through a Sephadex G-25 (PD-10) column (GE Healthcare Live Sources, San Diego, CA) equilibrated with Tris-ClH (pH 7.5) according with manufacture indications.
Step3: Ion-exchange low-performance liquid chromatography (LPLC) — Samples from step 2 were injected into an LPLC system (BioLogic LP Systems, BioRad) equipped with a cation-exchange column (BioRad Macro-Prep High Q, 1,000 Å, 50 μm), equilibrated with 10 mM Tris–HCl buffer, pH 7.5. Next, the column was washed with the same buffer and eluted with a linear 0-to-0.3-M KCl gradient. Fractions (1 mL) were collected at a flow rate of 1 mL/min and assayed in MCG-1 plates to detect glutenase activity (see below). The non-retained proteins from a cation-exchange column were applied to an anion exchange column (BioRad Macro-Prep High S, 1,000 Å, 50 μm) equilibrated with 10 mM Tris–HCl (pH 7.5) or 10 mM MOPS (pH 6.5). The column was washed with either buffer and proteins were eluted using a linear KCl gradient (0–0.3 M). One-milliliter fractions were collected.

2.3. Evaluation of fecal glutenase activity in bioassays

Fecal glutenase activities were measured as described [17]. Briefly, fecal samples were spread on agar plates (MCG-1) containing gluten (1.5%), 20 g/L glucose, 0.05 g/L $CaCl_2$, 0.07 g/L $ZnSO_4$, 0.05 g/L L-cysteine, 0.1% Tween 80, 60 mM phosphate buffer (pH 6.5), and 1.5 g/L agar, and incubated at 37 °C for 24 h. The plates were evaluated by measuring the diameter of the halo formed. Trypsin, at different concentrations, was used to generate a standard curve. Fecal glutenase activity (FGA) was expressed as trypsin-activity equivalents/g feces [17].

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (12%) was run as described by Laemmli [18], with modifications described by Helmerhorst and Wei [19]. Protein molecular weights were estimated using standard protein markers (97–14.4 kDa; GE Healthcare, Amersham LMW, UK). Gels were stained with Coomassie Brilliant Blue R-250.

2.5. Evaluation of gliadin activity by zymography

Gliadin degradation was assessed with 12% SDS-PAGE zymogram gels containing wheat gliadin (0.6 mg/mL; Sigma, St. Louis, MO), without β-mercaptoethanol. The protein samples were diluted (1/20) in 10 mM Tris–HCl (pH 7.5), and electrophoresis was performed at 100 V at 4 °C. Protein renaturing in the gels was achieved by washing twice for 30 min at room temperature in renaturing buffer containing 2% (v/v) Triton-X-100, 0.1 M NaCl, and 0.05 M Tris–HCl (pH 7.8). Subsequently, the Triton-X-100 was removed by washing (3 × 20 min) in developing buffer (0.05 M Tris–HCl, pH 7.8). After overnight in developing buffer at 37 °C, gels were stained for 30 min with 0.1% (w/v) Coomassie Brilliant Blue R-250.

2.6. Densitometric evaluation of the electrophoretic results

SDS-PAGE and zymogram gels were digitalized using a densitometer (Bio-Rad GS800) and Quantity One 1-D analysis software (Bio-Rad Laboratories Inc., Hercules, CA). Densitometry analyses were performed using lane-based background subtraction, followed by measuring the peak areas, and optical density (OD) values were used for statistical analysis. Each sample was analyzed on duplicate gels. Proteins were represented by comparing their relative mobilities with those of molecular weight standards.

2.7. Determining 33-mer, 19-mer, and 13-mer hydrolysis

Peptides were synthetized by Proteogenix SAS (Schiltigheim, France). The 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), 19-mer (LGQQQPFPPQQPYPQPQPF), and 13-mer (LGQQQPFPPQQPY) peptides had purities of 95%, 97%, and 96%, respectively. Since the HPLC chromatograms of the commercial 19-mer and 13-mer peptides showed 2 peaks, purity was verified by the Laboratorio de Técnicas Instrumentales (Universidad de León, Spain), confirming that the appearance 2 peaks were caused by the assay pH and not by impurities (Figures 2, 5 and 7).
We assayed 33-mer, 19-mer, and 13-mer hydrolysis as described previously [20], with modifications. Reaction mixtures (40-μL) contained 3.4 μL (0.8 mg/mL) of desalted protein extract (purification step 2, described above) or purified proteins (above); 4.7 μL of 33-, 19-, or 13-mer peptide (60 μM); and 31.9 μL of phosphate-buffered saline (pH 7.3). Reactions were incubated at 37 °C for 60 min and stopped by boiling at 100 °C for 10 min. Each reaction was filtered using a 0.22-μm Cellulose Acetate Spin-X Centrifuge Tube Filter (Thermofisher), and 10-μL aliquots were subjected to reverse-phase HPLC using a C-18