Effect of *Rothia mucilaginosa* enzymes on gliadin (gluten) structure, deamidation, and immunogenic epitopes relevant to celiac disease

Na Tian,1 Guoxian Wei,1 Detlef Schuppan,2,3 and Eva J. Helmerhorst1

1Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston, Massachusetts; 2Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; and 3Institute of Translational Immunology and Research Center for Immunology (FZI), University Medical Center, Johannes-Gutenberg-University, Mainz, Germany

Submitted 14 April 2014; accepted in final form 18 August 2014

Tian N, Wei G, Schuppan D, Helmerhorst EJ. Effect of *Rothia mucilaginosa* enzymes on gliadin (gluten) structure, deamidation, and immunogenic epitopes relevant to celiac disease. Am J Physiol Gastrointest Liver Physiol 307: G769–G776, 2014. First published August 21, 2014; doi:10.1152/ajpgi.00144.2014.—*Rothia mucilaginosa*, a natural microbial inhabitant of the oral cavity, cleaves gluten (gliadin) proteins at regions that are resistant to degradation by mammalian enzymes. The aim of this study was to investigate to what extent the *R. mucilaginosa* cell-associated enzymes abolish gliadin immunogenic properties. Degradation of total gliadins and highly immunogenic gliadin 33-mer or 26-mer peptides was monitored by SDS-PAGE and RP-HPLC, and fragments were sequenced by liquid chromatography and electrospray ionization tandem mass spectrometer (LC-ESI-MS/MS). Peptide deamidation by tissue transglutaminase (TG2), a critical step in rendering the fragments more immunogenic, was assessed by TG2-mediated cross-linking to monodansyl cadaverine (MDC), and by a +1-Da mass difference by LC-ESI-MS. Survival of potential immunogenic gliadin epitopes was determined by use of the R5 antibody-based ELISA. *R. mucilaginosa*-associated enzymes cleaved gliadins, 33-mer and 26-mer peptides into smaller fragments. TG2-mediated cross-linking showed a perfect inverse relationship with intact 33-mer and 26-mer peptide levels, and major degradation fragments showed a slow rate of MDC cross-linking of 6.18 ± 2.20 AU/min compared with 97.75 ± 10.72 and 84.17 ± 3.25 AU/min for the intact 33-mer and 26-mer, respectively, which was confirmed by reduced TG2-mediated deamidation of the fragments in mass spectrometry. Incubation of gliadins with *Rothia* cells reduced R5 antibody binding by 20, 82, and 97% after 30 min, 2 h, and 5 h, respectively, which was paralleled by reduced reactivity of enzyme-treated 33-mer and 26-mer peptides in the R5 competitive ELISA. Our broad complementary approach to validate gluten degrading activities qualifies *R. mucilaginosa*-associated enzymes as promising tools to neutralize T cell immunogenic properties for treatment of celiac disease.

Celiac disease (CD) is a T cell-mediated inflammatory enteropathy caused by dietary gluten. Gluten, a heterogeneous mixture of gluten- and proline-rich storage proteins from wheat, rye, and barley, is the primary environmental cause of CD. CD has become the most frequent inflammatory intestinal disease with autoimmune features, affecting roughly 1% of most populations worldwide (9). At least 50 distinct gluten peptides can be deamidated by intestinal TG2, which transforms them into peptides with an improved fit for the antigen binding groove of HLA-DQ2 or -DQ8, which is the necessary and predominant genetic predisposition of CD. The peptides presented in this context then trigger (deamidated) gluten-specific T helper 1 (Th1) T cells that elicit and maintain the mucosal destruction with villous atrophy and crypt hyperplasia, which is the histological hallmark of CD and which can prevent the effective absorption of nutrients (1, 13, 14, 26). If left untreated, people with CD can develop further complications related to malabsorption such as osteopenia or osteoporosis, anemia, steatorrhea, and weight loss, and in some cases T cell lymphoma (5). Complete gluten exclusion from the diet is effective in that inflammation recedes in the majority of patients. Yet, apart from the obvious sources, gluten is present in almost all refined foods, leading to severe dietary restrictions and in part social isolation of CD patients. Moreover, even on a strict gluten-free diet, it is often impossible to avoid traces of gluten that can cause symptoms of the disease (34). Therefore, therapies additive to the gluten-free diet are urgently needed.

The unique feature of abundant proline and glutamine residues in their primary structures makes gluten peptides highly resistant to natural human digestive enzymes (27, 28). This resistance results in intact immunogenic peptides surviving intestinal degradation. One therapeutic strategy for CD is oral enzyme therapy, which is aimed at hydrolyzing domains that resist digestion by pepsin, trypsin, and chymotrypsin (2, 6). Protease-resistant peptides of particular concern are a 33-mer from α-gliadins and a 26-mer from γ-gliadins that each contain multiple copies of highly immunogenic epitopes (27, 28). Prolyl endopeptidases cleaving gluten have been identified in various microorganisms, such as *Flavobacterium meningosepticum*, *Myxococcus xanthus*, *Sphingomonas capsulata*, and *Aspergillus niger* (12, 33). In addition, barley (*Hordeum vulgare*) produces an endopeptase, EP-B2, that cleaves preferentially after glutamine, especially with proline in P2 and P2 positions (3). Both prolyl endopeptidases and EP-B2 are currently explored therapeutically, alone and in combination, to neutralize gluten toxicity (29, 30).

Address for reprint requests and other correspondence: E. J. Helmerhorst, Dept. of Molecular and Cell Biology, Boston Univ., Henry M. Goldman School of Dental Medicine, 700 Albany St., CABR W202B, Boston, MA 02118 (e-mail: helmer@bu.edu).

http://www.ajpgi.org 0193-1857/14 Copyright © 2014 the American Physiological Society

G769
We have discovered other gluten-degrading enzymes that are associated with the human body and therefore may offer advantages over “foreign” enzymes. Thus the mixture of bacteria in human dental plaque can cleave gliadins, including immunogenic gliadin-derived 33-mer and 26-mer domains, into smaller fragments (15, 40). Twenty-two gliadin-degrading strains were identified representing seven species: Rothia mucilaginosa, Rothia aeria, Actinomyces odontolyticus, Streptococcus mitis, Streptococcus sp., Neisseria mucosa, and Capnocytophaga sputigena. Among these, the Rothia species, which are considered harmless residents of the upper gastrointestinal tract, were active in four complementary activity assays (10). Similar methods have later led to the identification of gluten-degrading species in human feces belonging to lactobacilli, staphylococci, staphylococci, bifidobacteria, and Clostridium species (4). Gluten-degrading natural microbial residents represent a promising new source of enzymes for luminal enzyme therapy since, e.g., allergic or toxic reactions to these bacteria and their products are less likely. In addition, there is a distinct opportunity that such bacteria could be actively employed as probiotics.

Although the hydrolysis of the 33-mer and 26-mer peptides by Rothia bacteria was encouraging, it remained to be established to what extent cleavage would abolish the immunogenicity of the gliadin domains. The aim of this study is to determine the degradation cascade of gliadin and gliadin-derived immunogenic peptides by R. mucilaginosa and to investigate how this degradation affects recognition by tissue transglutaminase (TG2). TG2 is a calcium-dependent enzyme that catalyzes the deamidation of glutamine (Q) residues to glutamic acid (E) residues, or, at pH values >7.3 and in the presence of a free amino donor, facilitates the formation of an isopeptide bond between a glutamyl and a lysyl group (11). The significance of TG2 in CD is evident from the high antibody titers against this enzyme in serum from patients with active CD (7, 8, 18, 25) and the increased immunogenicity of gluten peptides after TG2-mediated deamidation (36, 38). Abolishment of deamidation of the primary immunogenic gliadin peptides is highly predictive of their reduced binding to HLA-DQ2 and -DQ8, thus serving as a valid surrogate for subsequent T cell activation.

MATERIALS AND METHODS

Cultivation of Rothia mucilaginosa. Rothia mucilaginosa ATCC C25296 was grown on Brucella blood agar plates (Hardy Diagnostics, Santa Monica, CA) for 48 h at 37°C under aerobic conditions. Cells grow in light grayish smooth colonies that are sticky in nature.

Gliadins and derived peptides. Mixed gliadins were obtained from Sigma (St Louis, MO) and freshly dissolved to 5 mg/ml in 60% ethanol. Gliadin-derived 33-mer (RLQLQFPQPQPLYPQPLYPQPLYPQPLYPQPLYPQPPF), three fragments thereof designated pep1 (LPYPQPLYP), pep2 (LQLQFPQPQPLYPQPPQ), and pep3 (LQLQFPQPQPLYPQPPQ), and the gliadin-derived 26-mer (FLQPQQPPPQFQPQPQPQPQPQPQPQPQPQPPQPP) and three peptides thereof designated pep4 (PQPQPPQPPQPPQ), pep5 (QPQPPQPPQPPQ), and pep6 (PQPQPPQPPQ) were synthesized at a purity of 95% (21st Century Biochemicals, Marlboro, MA). The purity level was verified by RP-HPLC analysis using a shallow gradient as described below. All peptides were dissolved in deionized water to 10 mg/ml, aliquoted, and stored at −80°C.

Degradation of gliadins, 33-mer and 26-mer in solution. R. mucilaginosa ATCC 25296 was harvested with a sterile cotton swab from 48-h cultures on blood agar plates and suspended in saliva ion buffer containing 50 mmol/l KCl, 1.5 mmol/l potassium phosphate, 1 mmol/l CaCl₂, and 0.1 mmol/l MgCl₂, pH 7.0 to an OD₆₂₀ of 1.2. Gliadins, the 33-mer or 26-mer peptide, were added to a final concentration of 250 μg/ml. The suspensions were incubated in a 37°C water bath. At various time intervals 100-μl aliquots were removed, boiled for 5 min, and lyophilized by use of a SpeedVac (Savant, Farmingdale, NY). The gliadin mixtures were loaded onto precast 12% Bis-Tris gels (Novex, InVitrogen, Carlsbad, CA) and the 33-mer and 26-mer degradation aliquots were subjected to RP-HPLC.

RP-HPLC. RP-HPLC was carried out as reported previously (40). Briefly, the 100-μl sample aliquots were mixed with 900-μl buffer A containing 0.1% (vol/vol) trifluoroacetic acid (TFA), filtered, and analyzed by RP-HPLC using a HPLC model 715 (Gilson, Middleton, WI) and a C-18 column (TSK-GEL 5 μm, ODS-120T, 4.6×250 mm, TOSOHAs, Montgomeryville, PA). The column was equilibrated with buffer A. Peptides were eluted by using a linear gradient from 0 to 55% buffer B containing 80% (vol/vol) acetonitrile and 0.1% (vol/vol) TFA over a 75-min time interval at a flow rate of 1.0 ml/min. The eluate was monitored at 219 and 230 nm (Unipoint version 3.3, Gilson). Fractions containing the degradation fragments were collected and lyophilized by use of a Speedvac (Savant).

LC-ESI-MS/MS. The lyophilized fragments were dissolved in 25 μl of 5% acetonitrile and 0.1% formic acid. Mass spectrometry was performed by using a capillary nano-flow liquid chromatography and electrospray ionization tandem mass spectrometer (LC-ESI-MS/MS) as previously described (15). The raw MS/MS data of the peptide ions in each of the analyzed samples were searched against a limited database containing the 33-mer and the 26-mer sequences, by use of SEQUEST software (Bioworks Browser 3.3.1, Thermo-Finnigan). The cross-correlation values applied in the searches were 1.5, 2.2, and 3.5 for Z = 1, 2, and 3, respectively. The ΔCn and peptide probability values were set at >0.1 and <0.01, respectively.

Cross-linking of gliadins. 33-mer, 26-mer, and derived peptides to MDC by TG2. The TG2 used for these experiments was human recombinant TG2 (rhTG2, Zedira, Darmstadt, Germany). The preparation had a specific activity of 0.59 U/mg, where 1 U equals the formation of 1 μmol hydroxamate per minute from Z-Gln-Gly-OH and hydroxyamine (20). Cross-linking to monodansyl cadaverine (MDC; Sigma, St. Louis, MO) was determined as described (8), based on enhanced fluorescence of the dansyl group (21). Briefly, 10 μmol/l protein or peptide substrates, 30 μmol/l MDC and 20 μg/ml rhTG2 (final concentrations) were dissolved in 100 μl buffer containing 0.1 mol/l Tris-HCl, 0.15 mol/l NaCl, 5 mmol/l CaCl₂, pH 8.8. Samples were incubated at 37°C and fluorescence was measured for 1 h at λₑx 360 nm and λₑm 535 nm by using a Genios microtiter plate reader in the kinetic mode.

Detection of peptide deamidation by mass spectrometry. To examine deamidation directly, the peptides were incubated with rhTG2 as described above, except in the absence of MDC and in PBS containing 1 mmol/l CaCl₂ (pH 7.2). After 4 h incubation at 37°C, aliquots were dried and deamidation was assessed by LC-ESI-MS.

LC-ESI-MS. Deamidation of samples was determined on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo-Scientific, Tewksbury, MA). The sample aliquots were diluted 20-fold in buffer A (97.5% water, 2.5% acetonitrile, and 0.1% formic acid) and 1 μl was applied to an in-house packed Accucore column containing C₁₈ 2.6-μm resin beads (ThermoScientific). The column has an internal diameter of 100 μm and is 20–25 cm in length. Samples were applied by use of a Famos auto sampler (Dionex/I/C Packings, Vernon Hills, IL). The HPLC pump used was an Accella 600 (Thermo Scientific). Peptides were eluted applying a gradient from 0 to 65% buffer B (97.5% acetonitrile, 2.5% water, and 0.1% formic acid) over a 45-min time interval.

ELISA. Sandwich and competitive enzyme-linked immunosorbent assays (ELISAs) were carried out by employing the R5 monoclonal antibody (Ridascreen Gliadin and RIDASCREEN Gliadin com-
petitive kits, R-Biopharm, Darmstadt, Germany) (37). The R5 antibody has a high binding activity toward the potentially celiac toxic epitope QQPF. Experiments were performed according to the manufacturer’s instructions. For the sandwich ELISA 100-μl sample aliquots from the gliadin degradation experiment were centrifuged to collect the supernatant, diluted 1:1 with water and then 1:500 with dilution buffer. Aliquots of 100 μl were added to the anti-gliadin antibody-coated microtiter plate. The plates were incubated for 30 min at room temperature, washed with washing buffer, and incubated with 100 μl of peroxidase-conjugated secondary antibody for 30 min at room temperature. After washing, substrate solution containing tetramethylbenzidine was added, and after 30 min, 100 μl stop reagent was added and the absorbance was read at 450 nm.

Epitope elimination in the 33-mer and 26-mer degradation mixtures was assessed with a competitive R5 ELISA. In this assay, the incubation mixtures were diluted 1:10 with water, then 1:500 with dilution buffer, and 50 μl was added to a gliadin-coated microtiter plate, followed by the addition of 50 μl antibody-enzyme conjugate solution. Thus in this assay the experimental samples containing the 33-mer or 26-mer fragments are competing with the gliadin substrate coated onto the microtiter plate for binding to the R5 antibody. After incubation for 30 min at room temperature, wells were washed, followed by the addition of substrate solution containing tetramethylbenzidine. Stop reagent was added after 10 min and the absorbance was read at 450 nm.

Statistical analysis. SPSS 15.0 software was used for statistical analysis. A correlation between the peak height and TG2-mediated cross-linking was tested by the Pearson’s correlation test. Differences in the ELISAs between groups were determined by independent samples student’s t-test (two-tailed). Significance was set at \( P < 0.05.\)

RESULTS

Degradation of gliadins and 33-mer and 26-mer peptides by R. mucilaginosa. To test and confirm the degradation of gliadin and gliadin-derived immunogenic peptides by R. mucilaginosa, we incubated the cells with gliadins and 33-mer or 26-mer peptides. SDS-PAGE showed that the major ~37 kDa band in the gliadin preparation, representing the α/β- and γ-gliadins (39), was gradually degraded into smaller fragments in a time-dependent manner, with smaller molecular weight fragments appearing over time. The gliadins were stable when incubated with heat-inactivated cells (Fig. 1A). The 33-mer and 26-mer peptides were also degraded in a time-dependent fashion, as assessed by RP-HPLC (Fig. 1, B and C). The 26-mer and 33-mer were almost completely degraded in 30 min and 8 h, respectively, showing that the 33-mer was more resilient to proteolysis than the 26-mer peptide. Heat inactivation of the R. mucilaginosa suspension prevented gliadin peptide degradation. Overall, R. mucilaginosa has the enzymatic capacity to degrade mixed gliadins as well as 33-mer and 26-mer domains into smaller fragments.

Epitope abolishment by R. mucilaginosa: incubation mixtures poorly recognized by TG2. To study whether R. mucilaginosa-mediated degradation would affect gliadin 33-mer and 26-mer recognition and modification by TG2, cross-linking to a lysyl donor, MDC, was performed. Cross-linking is a measure to predict TG2 deamidation in vivo, which is known to provide a measure to predict TG2 deamidation in vivo, which is known...
to occur primarily in the mucosa and to significantly impact peptide immunogenicity (23, 38). The experiment was conducted at pH 8.8, favoring cross-linking over deamidation activity of TG2 (11). The rates of cross-linking of the gliadin 33-mer and 26-mer incubation mixtures to MDC is a measure for the survival of epitopes and was greatly reduced as the incubation time with _Rothia_ progressed (Supplemental Fig. S1; Supplemental Material for this article is available on the Journal website). The percent residual 33-mer and 26-mer peptide after the various incubation times with _Rothia_ (derived from Fig. 1, B and C) were plotted against the percent of the maximum MDC cross-linking rate (derived from Fig. 2, B and C). A perfect correlation was observed between both parameters, showing 50% reductions after 4.9 and 4.3 h, respectively, for the 33-mer, and 10.5 and 10.2 min, respectively, for the 26-mer (Fig. 3).

Identification of 33-mer and 26-mer degradation fragments by LC-ESI-MS/MS. The experiments above suggested that TG2 could not recognize fragments of the 33-mer and 26-mer. To confirm this, fragments of the 33-mer and 26-mer were identified and tested in the same assay. Degradation mixtures of the _Rothia_-incubated 33-mer and 26-mer were separated by RP-HPLC (Fig. 4, A and C). Peaks indicated by arrows were collected and dried, and the peptides were characterized by LC-ESI-MS/MS (Fig. 4, B and D). Results showed that frequent cleavages occurred after glutamine residues, which is consistent with the fact that _R. mucilaginosa_ rapidly cleaves the Z-YPQ-pNA substrate (40). Prominent de novo-generated peptides were chemically synthesized for further analyses. The fragments chosen to be synthesized were relatively long, covering the middle, NH₂-terminal, and COOH-terminal domains of the original 33-mer and 26-mer peptides, and were more prominent in amount as derived from the RP-HPLC peak heights. Synthesized fragments were designated pep1–pep3 (33-mer derived) and pep4–pep6 (26-mer derived), indicated in boxes in Fig. 4, B and D.

Epitope abolishment by _R. mucilaginosa_: pure fragments poorly recognized by TG2. Pure, chemically synthesized fragments pep1–pep6 derived from the 33-mer and 26-mer were tested individually in the MDC cross-linking assay. The pep1–pep6 fragments were all poor substrates for TG2, displaying an average rate of incorporation of MDC of 6.18 ± 2.20 AU/min (virtually no MDC cross-linking observed) compared with 97.75 ± 10.72 and 84.17 ± 3.25 AU/min for the 33-mer and 26-mer, respectively (Fig. 5).

Since the cross-linking to MDC is an indirect method to predict the recognition and modification of peptides by TG2, we also conducted mass spectrometry to directly observe the mass increase of +1 Da due to the conversion of a Q to an E residue. Incubations of the 33-mer, 26-mer, and pep1–pep6 with TG2 were conducted in the absence of a lysyl donor and at pH 7.2, favoring deamidation activity of the enzyme. TG2 effectively deamidated the 33-mer and 26-mer, each carrying three and five QXP sites, respectively (Supplemental Fig. S2A, 33-mer results shown). When the 33-mer was incubated with TG2, moieties with +1, +2, and +3 mass additions appeared and the nondeamidated form was no longer detected (Supplemental Fig. S2B). Pep1–pep3 were also deamidated by TG2, but, in contrast to the 33-mer, the nondeamidated forms remained clearly detectable (Supplemental Fig. S2, D, F, and H). In the case of peptide 1, most of the peptide remained in the nondeamidated form. A few additional observations of interest were made. First, even without incubation with TG2, pep2 and pep3 showed minor levels of deamidation, as evidenced from the presence of peptides with +1 Da mass additions (+0.5 m/z in the doubly charged ions) eluting just after the nondeamidated forms (Supplemental Fig. S2, E and G). Second, TG2-incubated pep2 and pep3 displayed a minor degree of deamidation at a second glutamine as evidenced by small peaks with...
+2-Da mass additions (+1.0 m/z in the doubly charged ions; Supplemental Fig. S2, F and H). Regardless of these minor species, a significant portion of TG2-incubated pep1–pep3 (and pep4–pep6; not shown) remained nondeamidated. Thus the MDC and MS approaches both indicated that deamidation of the major fragments was highly reduced, albeit not completely abolished, and suggested that a reduction in immunogenicity is achieved after incubation with *R. mucilaginosa.*

**Epitope abolishment by *R. mucilaginosa:* loss of recognition by the R5 antibody.**

Finally, to test the survival of potentially immunogenic epitopes, we used two ELISA-based assays, a sandwich assay and a competitive assay. The assays employed...
the R5 antibody, which recognizes QQPFP, a potentially celiac toxic epitope. A sandwich ELISA requires two epitopes and is suitable for analysis of larger proteins (here for gliadin analysis). For the 33-mer and 26-mer, the competitive ELISA was employed, requiring only one epitope. The binding activity of mixed gliadin to R5 decreased as the incubation time with \textit{R. mucilaginosa} increased, with a 20, 82, and 97% reduction in the 30-min, 2-h, and 5-h incubated samples, respectively (Fig. 6A).

In the competitive assay binding to R5, antibody is inversely proportional to the absorbance measured at 450 nm. The 33-mer effectively competed with gliadins coated onto the microtiter plate for binding to the R5 antibody in the \( t = 0 \) sample, and this ability disappeared gradually in the 8-h and 24-h samples, indicating abolishment of binding sites through degradation (Fig. 6B). A maximum reduction in competing ability (increase in OD450) of 65% was observed. For the 26-mer, the binding epitopes were abolished more rapidly, as evidenced by a near complete reduction in competition in the 5-h degradation mixture (Fig. 6C).

DISCUSSION

CD is induced by gluten-derived immunogenic peptides reaching the small intestine. Although some gluten peptides are highly resistant to mammalian digestive enzymes, our study indicated that oral microbes express such activity and are capable to further digest these immunogenic domains. Besides elucidating the proteolytic cascade of gliadin 33-mer and 26-mer degradation by \textit{R. mucilaginosa}, we also studied the possible influence of this hydrolysis on downstream processes relevant in CD, measured by TG2 modification and R5 antibody binding activity. After degradation of gliadin 33-mer and 26-mer peptides by \textit{R. mucilaginosa}, their modification by TG2 was reduced, and recognition by the R5 antibody was greatly diminished.

The structural requirements of gluten and TG2 modification have been well established (34). The spacing between glutamine and proline residues, the two most abundant amino acids in gluten, has a major effect on the efficiency of deamidation by TG2. In the sequences QP and QXXP, the Q is not a target for TG2, whereas in the sequences QXP, QXXF (Y, W, M, L, I, or V), and QXPX (Y, W, M, L, I, or V), the Q is a very good target for deamidation. Interestingly, besides the anticipated deamidation of QXP in pep2 and pep3, deamidation of a second glutamine in both peptides was noted. In pep2, the remaining Q residues occurred either in the QL or QP context, or at the COOH-terminus. In pep3, the second deamidated Q residue was inevitably in the QP context. The results suggest that TG2-mediated deamidation may proceed at unpreferred sites, or that some (minor) unspecific deamidation had occurred during incubation in PBS, in a TG2-independent fashion. Indeed, the non-TG2 incubated pep2 and pep3 already showed some level of deamidation.

As shown in Fig. 4, cleavage occurred after Q in the three “QLPY” repeats, which is a preferred deamidation site for TG2 and also present in multiple T cell epitopes (32). In the 26-mer, four of the potential TG2 recognition sites, “QQP,” were cleaved after the first Q. Thus \textit{R. mucilaginosa} hydrolysis specificities eliminate some predicted TG2 recognition sites of the major immunogenic domains. This is consistent with our results showing that the fragments in the degradation mixtures are not cross-linked as efficiently as the untreated proteins in TG2-mediated cross-linking and deamidation assays.

At present, the only effective treatment for CD is strict lifelong gluten-free diet (9). R5 monoclonal antibody-based analysis has been recommended by the Codex Alimentarius and the Food and Drug Administration for the detection of gluten contaminants in gluten-free foods to ensure the safety for celiac patients (19, 22, 37). As indicated the preferred binding epitope for the R5 antibody is the potentially celiac toxic epitope QQPFP. Besides this epitope, the R5 antibody also binds to structurally similar pentapeptide repeats, such as LQPFP, QLPYP, QLPTF, QQSF, EQTFP, PQPFP, QQYPFP, QQQPFP, and QVQWP (16, 24, 37). Among these epitopes, the 33-mer contains LQPFP (one repeat, strong binding) and QLPYP (three repeats, weaker binding). The 26-mer contains two copies of the strong binding epitopes QQQPFP and one weaker binding epitope QQYPFP. The different predicted affinities of the 33-mer and 26-mer for the R5 antibody is consistent with the higher competition (lower OD values) of the 26-mer in the competitive ELISA at the starting point. Given the recognition of a broad range of epitopes by R5, abolishment of all epitopes by \textit{R. mucilaginosa} enzymes would be unlikely. Nevertheless, we observed a clear reduction in binding over time to \textit{Rothia}-incubated gliadins and 26-mer peptide, consistent with the cleavage of R5 binding epitopes by \textit{R. mucilaginosa} as decoded by MS (Fig. 4). Although the
elimination of epitopes in the 33-mer was less effective than in the
26-mer, MS results indicated that R. mucilaginosa showed hydrolytic activity toward all the three major immunogenic epitopes comprised in the 33-mer. The R5 antibody binds to all peptides in the NH2-terminal part of α-type gliadin known to induce responses in patients with CD after in vivo gluten challenge (16), although there is no study to show a direct correlation of R5 antibody and immunogenicity in patients (19). Therefore, the R5 results have to be interpreted with some caution. However, the binding domains of R5 are present in multiple T cell immune-stimulatory epitopes (32), such as PQQSFEQQQQ (DQ2.5-glia-α3), PQQSFPEQQ (DQ2.5-glia-α1), PQQSFPEQPY (DQ2.5-glia-γ1), PQQSPFPWP (DQ2.5-glia-α2), PQQSPFPFPQ (DQ2.5-hor-2), EQPQFPFPFPQ (DQ8-γ1a), EQPQEPSY (DQ8-γ1b), and PQQSFPEQE (DQ8-γ1a), and these are associated with HLA types most commonly carried in patients with CD (DQ2.5, DQ8, and DQ8.5). To obtain direct evidence for the abolishment of immunogenic epitopes an experiment was conducted in which peripheral blood mononuclear cells (PBMCs) from CD patients were exposed to the Rothia-digested gliadin preparations. As a measure for T cell stimulation, the production of inflammatory cytokines was measured. Unfortunately, the negative control (Rothia supernatant without gliadins added) increased cytokine levels, possibly via TLR2 or TLR4 activation by bacterial by-products (data not shown), and thus the assay could not be used to investigate gliadin epitope elimination. However, the presented data showing reduced Tg2 recognition and reduced R5 antibody binding, are strong indications that T cell activation had been abolished by treatment with the Rothia cells. This should be validated in cell based assays with pure Rothia glucenolating enzyme preparations once they become available.

Luminal enzyme therapies are promising new avenues for the development of novel therapeutics for CD, and are actively being pursued clinically (17, 35). The current enzymes from natural resident microbes could, when further developed into medicinal agents, have a broad potential impact on a large segment of the population that is suffering from this as-yet-untreatable disease.

GRANTS
These studies were supported by NIH/NIAID grant AI087803 (E. J. Helmerhorst), AI101067 (E. J. Helmerhorst), and a grant by the German Ministry of Education and Research (BMBF 031A002) to D. Schuppan.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
N.T. and E.J.H. performed experiments; N.T., G.W., D.S., and E.J.H. analyzed data; N.T., G.W., D.S., and E.J.H. interpreted results of experiments; N.T. and E.J.H. prepared figures; N.T. and E.J.H. drafted manuscript; N.T., G.W., D.S., and E.J.H. approved final version of manuscript; G.W., D.S., and E.J.H. edited and revised manuscript; E.J.H. conception and design of research.

REFERENCES


