Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2014 May 5; 9112: . doi:10.1117/12.2058730.

Experimental Strategy to Discover Microbes with Gluten-degrading Enzyme Activities

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Abstract

Gluten proteins contained in the cereals barley, rye and wheat cause an inflammatory disorder called celiac disease in genetically predisposed individuals. Certain immunogenic gluten domains are resistant to degradation by mammalian digestive enzymes. Enzymes with the ability to target such domains are potentially of clinical use. Of particular interest are gluten-degrading enzymes that would be naturally present in the human body, e.g. associated with resident microbial species. This manuscript describes a selective gluten agar approach and four enzyme activity assays, including a gliadin zymogram assay, designed for the selection and discovery of novel gluten-degrading microorganisms from human biological samples. Resident and harmless bacteria and/or their derived enzymes could potentially find novel applications in the treatment of celiac disease, in the form of a probiotic agent or as a dietary enzyme supplement.

Keywords

Celiac disease; gluten; enzymes; degradation; oral; saliva; proteolytic; zymography; microbial; therapy

1. Introduction

Celiac disease is the most common food disorder in the United States, affecting about 1% of the US population. It is caused by gluten proteins contained in barley, rye and wheat, and is observed in both men and women, and across all ages and races. There is a genetic predisposition for the disease in subjects carrying HLA-DQ2 and HLA-DQ8 MHC-class molecules on their antigen presenting cells. The disease is characterized by gastrointestinal discomfort and the inefficient absorption of nutrients due to duodenal inflammation and villous atrophy. The generalized GI clinical symptoms are in part to blame for the fact that many CD patients are late-or improperly diagnosed. Gluten exclusion from the diet in most cases resolves the inflammation and related clinical symptoms. Celiac patients on a gluten-free diet however have to remain aware of gluten contamination, e.g. in restaurant sauces, in improperly labeled foods, and in foods served on social events (reviewed in [1-3]).

The enzymatic digestion of gluten is being explored as a potential therapeutic avenue to neutralize the immunostimulatory properties of gluten-derived domains [4-6]. There are multiple examples where digestive disorders are being treated with dietary supplements containing the missing enzyme [7]. While celiac disease is strictly speaking not an enzyme deficiency disorder, it is noteworthy that some food intolerances are being treated with supplemental enzymes. An example is lactose intolerance which can be addressed with the enzyme lactase contained lacteeze and lactaid, two over-the-counter available dietary supplements. Gluten-degrading enzymes are expressed by various microbes (e.g. prolyl endopeptidases from *Sphingomonas capsulata* and *Aspergillus niger*), and by the barley species itself (EP-B2), and these enzymes are in clinical trials for the treatment of celiac disease [8, 9]. There is a distinct advantage if such enzymes could be isolated from microbial sources that naturally are associated with the human body [10-12]. Therapeutic application of such bacteria and their enzymes would have an expected lower incidence of allergy or other intolerances associated with the introduction of foreign substances. The present manuscript describes the experimental strategy to isolate and characterize gluten-degrading bacteria that are natural colonizers of the human gastrointestinal tract.

2. Materials and Methods

Culturing on blood agar and gluten agar

To identify novel gluten degrading bacteria colonizing the human body, various bodily fluids were screened. Our studies so far have utilized human saliva, human dental plaque and human fecal samples as microbial sources. Samples were suspended in sterile PBS containing 10 mM potassium phosphate, pH 7.2, containing 0.9% NaCl and ten-fold serial dilutions were prepared in the same buffer. Two types of nutrient agars were used for bacteria cultivation. The first is a commercial agar called Brucella agar (BA) (Hardy Diagnostics, Santa Monica, CA). The second is an in-house generated gluten agar (GA). The formulation of this agar was based in part on the composition of Fastidious Anaerobe Agar produced by LabM (Lancashire, UK), with 23 g peptone being replaced by 23 g gluten, and eliminating the addition of horse blood. Thus in the GA formulation gluten proteins are the only protein source to support bacterial growth. The composition of GA is given in Table 1. Modified gluten agar formulations containing additional proteins have been published elsewhere [11].

To prepare GA, the ingredients were first dissolved in a 100 ml volume and homogenized extensively before addition of the residual 900 ml water volume. Gluten proteins are insoluble in water and remain in suspension, and further precipitate following autoclaving. Nevertheless, most gluten precipitates will be dispersed into sufficiently small particles to pour plates. Both the Brucella agar and GA agar contain haemin and vitamin K, facilitating the growth of anaerobic microorganisms. Aliquots (50 μ l) of the diluted human biological samples in PBS were plated on BA or GA, and incubated for 24-48 h at 37°C under aerobic conditions, or in a sealed candle jar that was rendered anaerobic using GasPak pouches (Beckton-Dickinson, Franklin Lakes, MD). Plates that showed abundant growth and single separated colonies were selected for subculturing on a segmented GA plate (20 strains/plate). After incubation for 24-48 h, colonies were subcultured on BA agar, repeatedly if

necessary, until cultures were obtained which were macroscopically and microscopically pure. Lastly, the strains were subcultured on GA to reconfirm growth on this limited agar. The plating strategy is depicted in Figure 1.

Enzyme activity evaluation

While growth on GA agar is an excellent first step in selecting microbes capable of utilizing gluten for bacterial growth, the obtained strains do not necessarily produce highly active glutendegrading enzymes, or exhibit specificities of interest targeting immunogenic domains. Therefore, strains that grow well on GA agar need further evaluation in enzyme activity assays. Four assays which were developed in our laboratory are described below.

A. Hydrolysis of paranitroanilide-derivatized substrates—The substrate specificity of bacteria-associated gluten-degrading enzymes can be assessed with synthetic paranitroanilide-derivatized tripeptide substrates of the general sequence Z-XXX-pNA, where Z represents the protective group benzyloxycarbonyl and pNA stands for paranitroanilide. The tripeptides selected, YPQ, QQP, LPY, PFP and PPF, occur with high frequency in immunogenic gluten domains [13]. All substrates were custom synthesized at 21st Century Biochemicals (Marlborough, MA, USA) and dissolved in 75 to 100 % (v/v) DMSO to a final concentration of 10 mM. Bacteria were cultured on BA for 48 h at 37°C. Cells were harvested with a sterile cotton swab, suspended in 20 mM Tris-HCl solution, pH 8.0, and diluted in the same buffer to an $OD_{620}=1.2$. Aliquots of 200 μ l of the bacterial suspension were mixed with 4 μ l substrate solution, yielding a final substrate concentration of 200 μ M. Immediately thereafter, the plate was covered with MicroAmp™ optical adhesive film (Applied Biosystems, Foster City, CA) and the OD at 405 nm was measured ($t=0$ reading) using a Genios microtiter plate (Tecan, Männedorf, Switzerland). Subsequent OD_{405} readings were taken at selected time intervals where the length of the time intervals chosen depended on the level of activity associated with the bacterial species (e.g. 10 min intervals and 2 h total incubation for *R. aeria*). Release of the paranitroaniline group after hydrolysis is detected spectrophotometrically at 405 nm. At this wavelength, and under physiological salt conditions, it has an estimated specific absorption coefficient (ϵ) of 8615 $M^{-1}.cm^{-1}$ [14]. The ionic composition and pH of the incubation buffer may influence this value. From the path length of the 200 μ l sample in the microtiter plate and the absorption coefficient, the paranitroaniline concentration at the various incubation times can be calculated using the Lambert Beer equation: $E=\epsilon.c.l$, where c is the concentration (in molar) and l is the path length (in cm).

B. Degradation of gliadins in gel (gliadin zymography)—Gliadin degradation in gel was assessed using a zymogram gel containing mixed gliadins (Sigma, St. Louis, MO; catalog# G3375) as the incorporated substrate. Mixed gliadins comprise α/β , γ and ω gliadins [15, 16]. The gel was prepared according to the composition in Table 2, suitable for preparing two gels in the BioRad minigel system, with spacers of 1.5 mm thickness. It is important that the mixed gliadins are dissolved in 0.1 M acetic acid and not in 60% ethanol which is commonly used to dissolve gliadins [17]. Cells were cultured on BA for 48 h at 37°C and resuspended in 20 mM Tris-HCl, pH 8.0 and diluted in the same buffer to an OD_{620} 5.0. Aliquots of 150 μ l were centrifuged and the pelleted cells were resuspended in

2x concentrated zymogram sample buffer containing 0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS and 0.005% (w/v) Bromophenol Blue, leading to (partial) cell lysis. Electrophoresis was carried out at a constant voltage of 100 V at 4°C. Renaturing of the proteins in the gels was achieved by washing the gels for 2×30 min at room temperature in zymogram renaturing buffer (Invitrogen, Carlsbad, CA). The renaturing buffer can also be prepared as 2.5% (v/v) Triton-X100. Triton-X100 is non-ionic detergent and exchanges with the SDS present in the zymogram gels. Subsequently the Triton-X100 is removed by washing the gels for 3×20 min in zymogram developing buffer (Invitrogen). The latter can also be prepared as a solution of 20 mM Tris-HCl, pH 7.5. The pH of the renaturing and developing buffer can be adjusted according to the optimum pH of the gluten-degrading enzymes. After developing for 48 h, the gels were stained for 16 h with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid, and destained in the same solution without dye. This method is not quantitative and primarily used to screen for the presence of gliadin-degrading enzymes and to determine their approximate molecular weight.

C. Degradation of mixed gliadins in solution—A mixed gliadin stock solution of 5 mg/ml was prepared in 60% ethanol [17]. For each experiment gliadins were freshly dissolved since gliadins adhere to plastic over time, lowering the concentration in solution. Cells were suspended from plate in 20 mM Tris-HCl solution (pH 8.0) or another suitable buffer of choice, to an OD₆₂₀ of 1.2. An aliquot of the gliadin stock solution was added to the cell suspension to reach a final gliadin concentration of 250 µg/ml. The mixture was incubated in a 37°C waterbath, and at defined time intervals (e.g. t=0, 5, 15, 30, 60 and 120 min), aliquots of 100 µl were removed and heat inactivated by placing them in a boiling water bath for 10 min. The 100 µl samples were then dried using a SpeedVac (Thermo Fisher Scientific, Cambridge, MA), resuspended in 30 µl of 1x SDS sample buffer, and analyzed on NuPAGE 4-12% Bis-Tris gels (Invitrogen). Electrophoresis was carried out at a constant voltage of 120 V for 1 h. Gels were stained and destained as described above.

D. Degradation of the 33-mer and 26-mer peptides—Synthetic highly immunogenic peptides derived from α2-gliadin (LQLQPFQQLPYPQQLPYPQQLPYPQQLPYPQPF; 33-mer; [18]) or γ-gliadin (FLQPQQPFQQLPYPQQLPYPQQLPYPQQLPYPQ; 26-mer; [19]) were synthesized at a purity of 95% (21st Century Biochemicals, Marlboro, MA), and dissolved in deionized water to 10 mg/ml. The degradation assay was essentially the same as the one described above for the degradation of mixed gliadins in solution, with the exception that the 33-mer or 26 mer peptides were used as substrates. The final cell suspension density was OD₆₂₀ 1.2 and the final concentration of the peptides was 250 µg/ml. After incubation for various time intervals (e.g. t=0, 2 h and 5 h), 100 µl sample aliquots were removed and boiled for 10 min to abolish enzyme activity. After cooling, the aliquots were mixed with buffer A containing 900 µl of a 0.1% (v/v) trifluoroacetic acid (TFA). The samples, containing negligible amounts of peptides/proteins of bacterial origin, were filtered with an Acrodisc 13 mm syringe filter containing 0.2 µm HT Tuffryn membrane (Pall Corporation, Ann Arbor, MI, USA) and analyzed by RP-HPLC. The RP-HPLC used was a HPLC Model 715 (Gilson, Middleton, WI) and the column was a C-18 TSK-GEL 5 µm, ODS-120T column of 4.6 x 250 mm (Tosoh Bioscience LLC, King of Prussia, PA). The 33-mer/26-mer

and derived fragments were eluted using a linear gradient from 0% to 55 % buffer B containing 80% (v/v) acetonitrile and 0.1% (v/v) 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 and eluting fractions were collected using peak width and peak sensitivity settings of 1.2 and 5, respectively (Unipoint version 3.3 software, Gilson). The collected peaks were dried using a Speedvac (Thermo Fisher Scientific) for further structural analysis by LC-ESI-MS/MS (described elsewhere, [10, 20]).

3. Results

Figure 2 shows an example of a GA plate with 20 strains that were subcultured from BA. The presence of small gluten precipitates in the GA agar can be observed. After 24 h incubation one strain, WSA-8, grew particularly well. This strain was later speciated by 16S rDNA analysis as *R. aeria* [20]. On GA agar without gluten no bacterial growth was observed (not shown), indicating that gluten was a necessary ingredient and utilized to support bacterial growth on GA. Figure 3 summarizes the four assays used to investigate bacterial enzymatic activities towards gliadin and gliadin-derived substrates. All assays were carried out with bacterial suspensions. In A, hydrolysis is shown of pNA-derivatized tripeptide substrates by strain WSA-2B (speciated as *R. mucilaginosa*). Z-YPQ-pNA was the most rapidly cleaved substrate by this strain, followed by Z-LPY-pNA, whereas the other three substrates were not hydrolyzed. The XPQ↓ and XPY↓ cleavage specificities predict that multiple immunogenic epitopes in gliadins will be targeted, conceivably abolishing their T-cell stimulatory activities. In B, two gliadin zymograms are shown, developed at different pH. The bacterial cells applied were in lane 1, strain WSA-2B (*R. mucilaginosa*); lane 2, strain WSA-8 (*R. aeria*); lane 3, strain WSA-26 (*R. mucilaginosa*) and lane 4, *R. mucilaginosa* ATCC 25296, each 150 µl, at an OD₆₂₀ of 5.0. One gel was developed at pH 7.0 and one developed at pH 3.0, the latter representing pH values more representative of the stomach. Note that enzymatic activities associated with *R. aeria* were partly resistant to acidic conditions, which suggests that its enzymes may be active during gastric transport. In C, gliadin degradation in solution over time by strain WSA-8 (*R. aeria*) is depicted. Gliadin degradation is observed as the disappearance of the major band around 37 kDa in the preparation, representing α, β, and γ gliadins [15]. Degradation fragments stain poorly with Coomassie brilliant blue and are not easily observed. For this reason, in D, the degradation of the immunogenic 33-mer peptide over time was studied by RP-HPLC. The relevant area in the chromatograms of the t=0, 2 h and 5 h incubated samples are shown. Note that the 33-mer peptide, which is resistant to proteolysis by trypsin and chymotrypsin [18], is completely degraded by enzymes associated with this natural resident microbe.

4. Conclusions

Luminal enzyme therapy with gluten-degrading human body-associated bacteria, or their pure enzymes, is a novel therapeutic avenue in the treatment of celiac disease. It responds to the demand for a new and safe alternative or adjunctive therapy to a gluten-free diet. The selective agar plating strategy together with four complimentary enzyme assays allows for the rapid selection of strains of interest. Gluten-degrading resident bacteria hold great

promise to be developed as probiotics or enzymatic dietary supplements, not only for celiac disease but also for the treatment of non-celiac gluten sensitivity.

Acknowledgements

These studies were supported by NIH/NIAID grants AI087803 and AI101067 to EJH. The authors are indebted to Drs. Floyd Dewhirst and Detlef Schuppan for their advice and support.

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Figure 1.

Culturing strategy to enrich for gluten-degrading microorganisms from human biological samples. Aliquots of diluted suspensions of e.g. dental plaque, whole saliva or human feces were plated on gluten-limited agar (GA) or Brucella agar (BA) and subcultured on BA to obtain pure cultures as indicated. The final culture was on GA to confirm growth on gluten. Plates were incubated for 24-48 h at 37°C under aerobic or anaerobic conditions.

Figure 2.

Gluten agar plate with 20 human salivary strains subcultured from BA. One strain, designated WSA-8, showed substantial growth on GA after 24 h incubation whereas the 19 other strains did not grow. Thin arrows point to gluten particles in the agar.

Figure 3.

Four enzyme assays to evaluate gluten-degrading enzyme activities. A, tripeptide substrate hydrolysis; B, gliadin zymography; C, gliadin degradation in-gel; D, 33-mer degradation by RP-HPLC. See text for details.

Table 1

Gluten Agar Recipe

Chemical	Amount
Wheat gluten (Sigma, catalog# G5004)	23 g
Sodium chloride	5 g
Soluble starch	1 g
Agar	12 g
Sodium bicarbonate	0.4 g
Glucose	1 g
Sodium succinate	0.5 g
Sodium pyruvate	1 g
Soluble pyrophosphate	0.25 g
L-Arginine	1 g
L-Cysteine	0.5 g
Haemin	0.01 g
Vitamin K	0.001 g
Suspend in deionized water	1 liter

Table 2**Gliadin Zymogram Recipe for Two Gels**

	Separating gel (μl)	Stacking gel (μl)
Deionized water	5,352	3,000
30% Acrylamide/Bis solution 37.5:1 (6%T) *	3,200	-
30% Acrylamide/Bis solution 37.5:1 (4%T)	-	660
1.5 M Tris HCl pH 8.8 (375 mM)	4,000	-
0.5 M Tris HCl pH 6.8 (126 mM)	-	1,260
10% SDS (0.1%)	160	50
10% APS (0.05%)	80	25
10 mg/ml Gliadin in 0.1M acetic acid (2 mg/ml)	3,200	-
Undiluted TEMED (0.05%)	8	-
Undiluted TEMED (0.1%)	-	5
Total (μl)	16,000	5,000

* final concentrations are given in parentheses