JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
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Engineering of Kuma030: A Gliadin Peptidase That Rapidly Degrades Immunogenic Gliadin Peptides in Gastric Conditions

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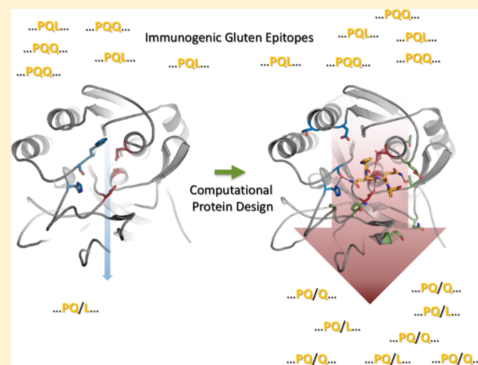
S *Supporting Information*

ABSTRACT: Celiac disease is characterized by intestinal inflammation triggered by gliadin, a component of dietary gluten. Oral administration of proteases that can rapidly degrade gliadin in the gastric compartment has been proposed as a treatment for celiac disease; however, no protease has been shown to specifically reduce the immunogenic gliadin content, in gastric conditions, to below the threshold shown to be toxic for celiac patients. Here, we used the Rosetta Molecular Modeling Suite to redesign the active site of the acid-active gliadin endopeptidase KumaMax. The resulting protease, Kuma030, specifically recognizes tripeptide sequences that are found throughout the immunogenic regions of gliadin, as well as in homologous proteins in barley and rye. Indeed, treatment of gliadin with Kuma030 eliminates the ability of gliadin to stimulate a T cell response. Kuma030 is capable of degrading >99% of the immunogenic gliadin fraction in laboratory-simulated gastric digestions within physiologically relevant time frames, to a level below the toxic threshold for celiac patients, suggesting great potential for this enzyme as an oral therapeutic for celiac disease.



INTRODUCTION

Celiac disease is a debilitating illness that afflicts approximately 0.5−1% of the global population.[1,2] The basis for celiac disease is a T_h1-mediated inflammatory immune response directed against peptides derived from gliadin, a protein component that constitutes roughly half of the total protein composition of dietary gluten. Gliadin in highly enriched in the amino acids proline (P) and glutamine (Q), which renders it recalcitrant to degradation by human digestive enzymes. PQ-rich peptide fragments derived from partial digestion of gliadin in the stomach and intestines are deamidated in the intestinal lumen by tissue transglutaminase (TG2), thereby allowing binding to HLA-DQ2 or DQ8, and stimulation of an inflammatory response in people with celiac disease.[3] Chronic inflammation resulting from continuous gluten exposure leads to damaging of the intestinal villi, which promotes malnutrition-related symptoms, and to an increased incidence of developing intestinal lymphomas,[4] among other complications.

Currently, the only treatment for celiac disease is complete elimination of gluten from the diet. The gluten-free diet is very burdensome for patients because it is difficult to strictly achieve, due to the ubiquity of gluten in modern food production.[5−8] Medical treatment that could reduce or eliminate the effects of accidental gluten exposure would significantly benefit the celiac patient population.[9] Oral enzyme therapy, in which orally administered enzymes break down the PQ-rich regions of gliadin in the gastrointestinal tract, has been proposed as a method to treat celiac disease.[10] However, the requirement for proteolytic activity by these enzymes is high, because ingested gluten must be kept at 10 mg or less to prevent intestinal damage.[11,12] Upon the accidental ingestion of 1 g of gluten (approximately the amount of gluten in 1/4 of a slice of bread), reduction to 10 mg or less would require degradation of immunogenic gluten content by 99+% at physiologically relevant time scales in order to eliminate intestinal damage. Additionally, degradation must take place in the gastric compartment, because immunogenic gliadin peptides initiate the immune response immediately upon entering the duodenum.[13] Finally, an enzyme therapeutic for celiac disease should be specific for the immunogenic fraction of gliadin, so that its activity is not markedly hindered by other proteins

Received: August 7, 2015
Published: September 15, 2015

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DOI: 10.1021/jacs.5b08325
J. Am. Chem. Soc. 2015, 137, 13106−13113

present during a meal. While several enzymes have been considered as potential oral enzyme therapeutics for celiac disease,[14,15] no enzyme has been identified that harbors all of these properties.

Previously, we reported the engineering of a novel gliadin peptidase called KumaMax (hereafter referred to as Kuma010), which demonstrates stability and functionality in postprandial gastric conditions.[16] Kuma010 specifically degrades peptides after the PQ dipeptide motif, which is found throughout the immunogenic fraction of gliadin. In this work, we describe the computational redesign of the active site of Kuma010 in order to achieve the 99% activity threshold. We then assess the potential of Kuma030 as an enzyme therapeutic for celiac disease by quantifying its ability to reduce gluten content in laboratory-simulated digestions of wheat bread and beer and to reduce the immunostimulatory potential of gliadin as determined by T cell assays.

■ RESULTS

Alteration of the Kuma010 Active Site by Computational Enzyme Design. To guide the improvement of Kuma010 by computational protein design, we solved the crystal structure of Kuma010 at 2.5 Å resolution (PDB ID 4NE7). On the basis of this structure, we utilized the Rosetta Molecular Modeling Suite[17,18] to redesign the Kuma010 active site, selecting for mutations that were predicted to increase activity against immunogenic gliadin peptides. Designed mutants were then screened for increased activity on the highly immunogenic 33mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and 26mer (FLQPQQPFPQQPQQPYPQQPQQPFPQ) gliadin peptides[19,20] (Supporting Information Table 1). These peptides harbor either the PQL or PQQ tripeptide motif, tripeptides that have been implicated in the immunogenic cores of gliadin T cell epitopes shown to be toxic for celiac patients.[21] Mutations to Kuma010 that conferred increases in activity were combined in an iterative process, and the resulting mutant enzymes were retested for increased activity (Supporting Information Table 2). In this manner, the variant Kuma030 was built. Kuma030 is 44-fold more active against peptides containing PQQ, and 11-fold more active against peptides containing PQL, than Kuma010, as assessed by k_{cat}/K_M values for these enzymes on relevant gliadin peptides (Supporting Information Figure 1).

As determined by molecular modeling using Rosetta, the putative S1′ peptide binding interface of Kuma010 consists entirely of hydrophobic residues and should therefore prefer hydrophobic residues such as leucine over polar residues such as glutamine at P1′. The S1′ binding pocket of Kuma030 includes an isoleucine to threonine mutation (I463T), which is predicted to provide a hydrogen bond with a P1′ glutamine, enabling this enzyme to accommodate both leucine and glutamine in the S1′ subsite and thereby target both PQL and PQQ tripeptides (Figure 1). Kuma030 also includes six additional mutations (K262E, E269T, S354Q, G358S, D399Q, A449Q) that are required for the enhanced catalytic efficiency on the 26mer and 33mer peptides (Supporting Information Figure 2). Two of these mutations (K262E and G358S) are substitutions of mutations previously introduced into Kuma010.[16] G358S is thought to stabilize the loop containing a histidine introduced in Kuma010 which is predicted to hydrogen bond to the P1 glutamine residue (Figure 1). The remaining mutations are predicted to stabilize the protein structure as modeled.

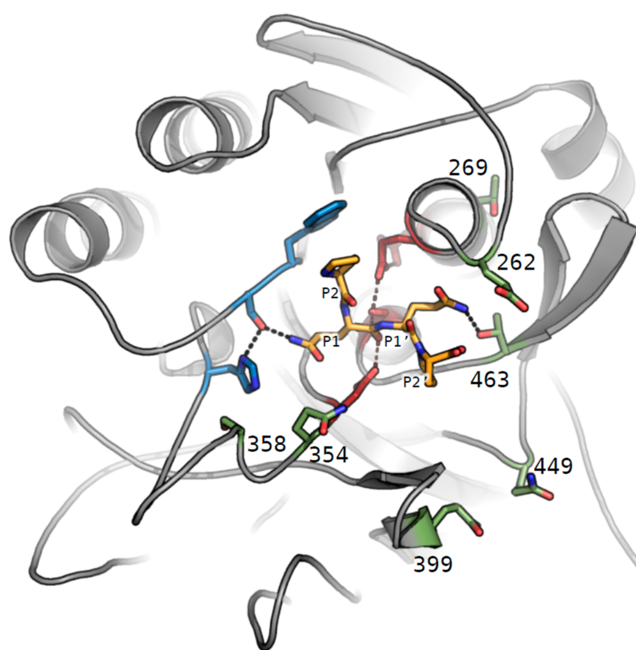

Figure 1. Amino acid substitutions in the Kuma030 endopeptidase. Model of a representative PQQP tetrapeptide in the active site of Kuma030. Enzyme backbone is shown in gray, gliadin tetrapeptide is shown in yellow. Green residues were mutated to generate Kuma030. Blue residues are thought to be important in coordinating the tetrapeptide into the active site. Image was created using PyMol.

Kuma030 Targets Immunodominant Peptides in Wheat, Rye, and Barley. While dozens of PQ-rich epitopes have been linked to celiac disease, several peptides derived from gliadin (wheat), hordein (barley), and secalin (rye) have been shown to account for the vast majority of the immune response in celiac disease and have thus been classified as immunodominant.[21] In wheat, these include the peptides W02-E07 (LQPFPQPQLPYPQPQ), W03-E07 (QPFPQPQQPFPWQP), and the 33mer peptide, which contains the W02-E07 sequence.[19,22] These peptides harbor several HLA-DQ2 or -DQ8 epitopes shown to be strongly immunogenic.[19,23] To evaluate the ability of Kuma030 to destroy these epitopes throughout gluten, purified whole gluten was incubated with Kuma030 under simulated postprandial gastric conditions (pH 4.0 at 37 °C with 0.6 mg mL^{-1} pepsin).[24] The gluten fraction remaining after degradation was quantified using ELISA assays based on either the G12 or R5 antibodies, which recognize the amino acid motifs QPQLPY and QQPFP, respectively. These motifs encompass many immunogenic gliadin epitopes, including the immunodominant peptides mentioned above,[25,26] and is therefore a reasonable assessment of the immunostimulatory potential of gluten. To compare the activity of Kuma030 to that of published glutenases, we also examined the glutenases EPB2 and SCPEP, which are currently being pioneered at a 1:1 ratio as a combination enzyme therapeutic for celiac disease. The EPB2 and SCPEP enzymes generated in this work were verified to have activities consistent with that of published values[27,28] (Supporting Information Table 3). Upon incubation with gluten, we observed a dose-dependent reduction in the QQPFP or QPQLPY load using either Kuma030, Kuma010, or a 1:1 combination of EPB2 and SCPEP (Figure 2A and Supporting Information Figure 3). At a 1:25 w:w ratio of enzyme:gluten, EPB2 and SCPEP broke down 84.8% of the gluten present, consistent with previously

DOI: 10.1021/jacs.5b08325
J. Am. Chem. Soc. 2015, 137, 13106−13113

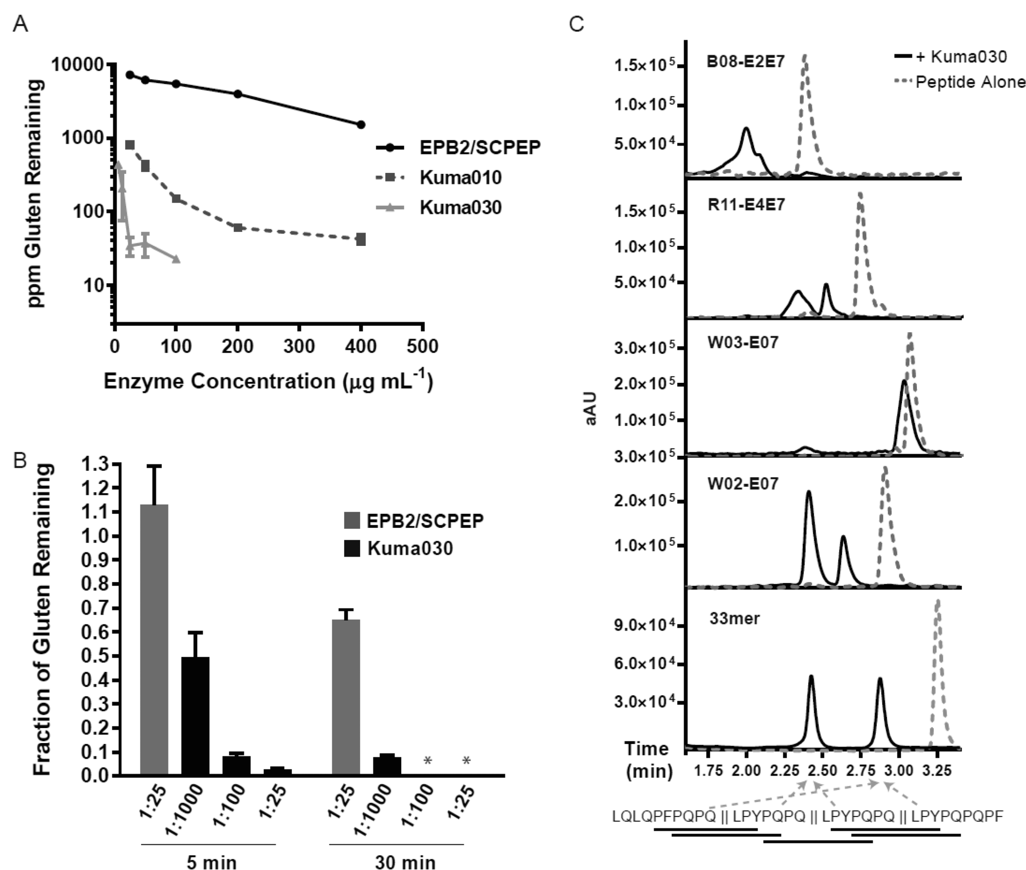

Figure 2. Kuma030 is capable of rapidly and effectively degrading the immunogenic regions of gluten in gastric conditions. (A) concentrations of either EPB2 and SCPEP (at a 1:1 ratio), Kuma010, or Kuma030 in gastric conditions, as measured by ELISA using the G12 antibody. The starting concentration of gluten was 10 mg mL^{-1} (10 000 ppm). Note that the *Y*-axis is plotted on a logarithmic scale. (B) The amount of gluten detected at 5 or 30 min after incubation with EPB2 and SCPEP or Kuma030. Shown is the ratio of enzyme:gluten. The starting concentration of gluten was 10 mg mL^{-1}. Samples were normalized to the amount of gluten remaining after incubation with pepsin alone. Asterisk indicates that greater than 99.9% of the gluten was degraded. (C) HPLC trace of full-length peptide (gray dotted lines) or breakdown products (black lines) of the immunodominant peptides from gliadin (W02-E07, W03-E07, 33mer), hordein (B08-E2E7), or secalin (R11-E4E7). The bottom of the figure shows the amino acid sequence of the 33mer peptide, position of known immunogenic epitopes (horizontal lines), location of Kuma030 cleavage site (vertical lines), and elution peaks of the resulting breakdown products (gray arrows). Immunodominant peptides displayed the following breakdown patterns: B08-E2E7: PQQPIPQ||QPQPYPQ||Q; R11-E4E5: QPFPQ||QPEQIIPQ||QP; W02-E7: LQPFPQPQ||LPYPQPQ; W03-E7: QPFPQPQ||QPFPWQP. All peptide masses and elution times were confirmed by LCMS. Note that although the undigested W03-E07 peptide eluted at approximately the same time as a W03-E07 breakdown fragment, these are separate peaks, as determined by LCMS (Supporting Information Figure 4). aAU, arbitrary absorbance units.

published reports.[14,29] Kuma030 was highly effective at eliminating these peptide epitopes throughout gluten, with a 1:400 w:w ratio of enzyme:gluten being sufficient to reduce the immunogenic gluten present by over 99.5% at 60 min, as quantified by both ELISA methods (Figure 2A and Supporting Information Figure 3), and a 1:100 w:w ratio was sufficient to decrease the detected gluten by >99.9% by 30 min (Figure 2B). Kuma030-dependent reduction in immunogenic gluten load was rapid, with >98% degradation achieved by 5 min at a w:w ratio of 1:25 (Figure 2B). HPLC-MS analysis of the Kuma030 cleavage products revealed that Kuma030 cleaved each peptide after the PQ dipeptide motif in the immunodominant epitopes from wheat (33mer, W02-E07, and W03-E07), and also those from barley B08-E2E7 (PQQPIPQQPQPYPQQ) and rye R11-E2E7 (QPFPQQPEQIIPQQP)[22] (Figure 2C, Supporting Information Figure 4). While these intact peptides are highly immunostimulatory, the peptide breakdown products are not predicted to stimulate the immune system, because Kuma030 action results in elimination of the core 9mer epitope required to trigger the immune response.[30] The ability of Kuma030 to cleave peptides containing either a PQL or a PQQ tripeptide motif, and to reduce the immunogenic gluten load as measured by both G12 and R5 antibodies, is consistent with the hypothesis that Kuma030 can bind and cleave peptides with either a leucine or a glutamine in the S1′ binding pocket.

Gliadin Treated with Kuma030 Loses Its Ability To Stimulate a T-Cell Response. The ability of Kuma030 to efficiently degrade immunogenic gliadin epitopes suggests that incubation of gliadin with Kuma030 might reduce its capacity to stimulate a T-cell-mediated immune response. T cell assays utilizing cells derived from the intestinal biopsies of celiac patients represent the gold standard for this evaluation.[31] We performed T cell assays in which cells were exposed to Kuma030-treated gliadin and the resulting T cell reaction was assessed. The gliadin-reactive intestinal CD4$^+$ T cell lines used in this study were previously generated from intestinal mucosa of celiac patients and have been shown to react to a diversity of epitopes across different gliadin families[32] (Figure 3F). Kuma030 was incubated with purified wheat gliadin at enzyme:gliadin ratios spanning 1:10−1:1000 in laboratory-

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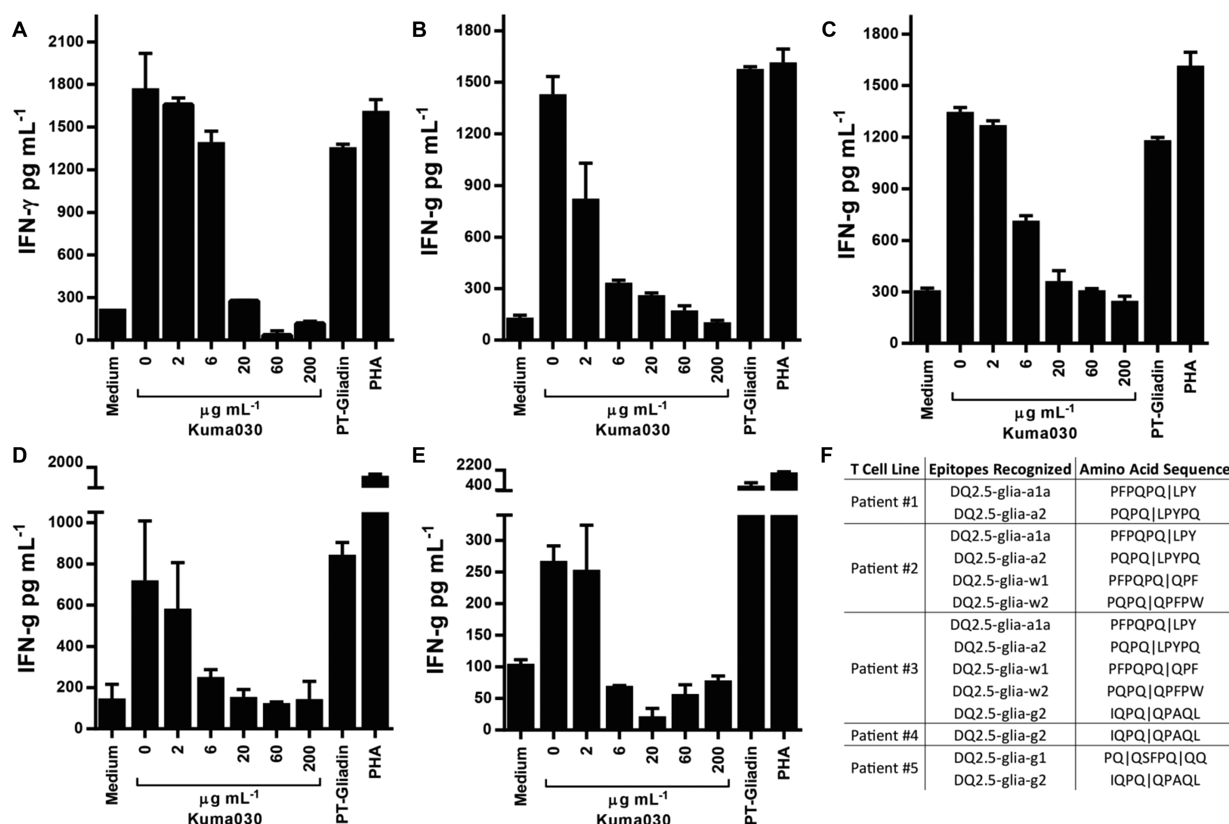

T Cell Line	Epitopes Recognized	Amino Acid Sequence
Patient #1	DQ2.5-glia-a1a	PFPQPQ|LPY
	DQ2.5-glia-a2	PQPQ|LPYPQ
Patient #2	DQ2.5-glia-a1a	PFPQPQ|LPY
	DQ2.5-glia-a2	PQPQ|LPYPQ
	DQ2.5-glia-w1	PFPQPQ|QPF
	DQ2.5-glia-w2	PQPQ|QPFPW
Patient #3	DQ2.5-glia-a1a	PFPQPQ|LPY
	DQ2.5-glia-a2	PQPQ|LPYPQ
	DQ2.5-glia-w1	PFPQPQ|QPF
	DQ2.5-glia-w2	PQPQ|QPFPW
	DQ2.5-glia-g2	IQPQ|QPAQL
Patient #4	DQ2.5-glia-g2	IQPQ|QPAQL
Patient #5	DQ2.5-glia-g1	PQ|QSFPQ|QQ
	DQ2.5-glia-g2	IQPQ|QPAQL

Figure 3. Gliadin treated with Kuma030 loses it immunostimulatory potential. (A–E) Purified gliadin was treated with Kuma030 at the specified concentration for 60 min at pH 4.0 at 37 °C in the presence of 0.6 mg mL^{-1} pepsin. After the gastric phase, the pH of the samples was increased, and samples were treated with chymotrypsin and TG2. Samples were then exposed to T cell lines from patients #1 (A), #2 (B), #3 (C), #4 (D), or #5 (E), in the presence of autologous irradiated B cell lines, and IFN-γ was measured by ELISA. Phytohemagglutenin (PHA) and a peptic-tryptic digest of gliadin (PT-gliadin) were included as positive controls. Incubation of T cell lines with antigen-presenting cells in the absence of antigens (medium) acted as a negative control. (F) T cell stimulatory epitopes recognized by the T cells used in this assay and the predicted Kuma030 cleavage sites within these epitopes.[21] Predicted Kuma030 cleavage sites are shown by a vertical line |. Cleavage sites are predicted based on Kuma030 activity on gliadin peptides as presented in Figure 2.

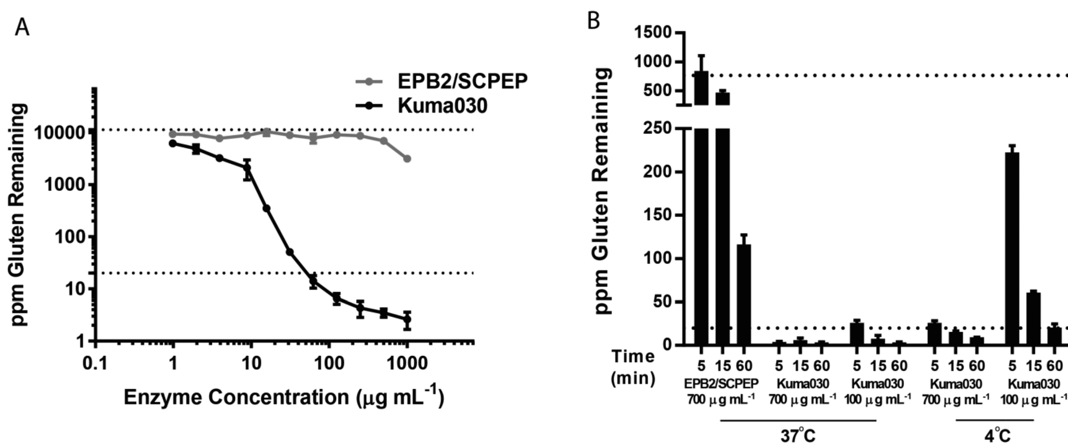

Figure 4. Kuma030 efficiently degrades gluten in complex food matrices. Gluten was detected using G12 ELISA. (A) The amount of gluten remaining in whole wheat bread after a 30 min incubation with either Kuma030 or EPB2/SCPEP at the indicated enzyme concentrations. Dotted lines represent either the initial concentration of gluten (10 000 ppm) or 20 ppm gluten. Note both axes are plotted on a logarithmic scale. (B) The amount of gluten remaining in a wheat beer after incubation with the indicated concentrations of either EPB2/SCPEP or Kuma030 at 37 or 4 °C, for 5, 15, or 60 min. Dotted lines represent either the initial concentration of gluten (764 ppm) or 20 ppm.

simulated gastric conditions for 60 min. In order to mimic transit into the intestinal compartment, the pH levels of the samples were then increased, and the samples were treated with chymotrypsin and deamidated with tTG to unmask the immunogenic epitopes. The resulting gliadin samples were presented to T cell lines, and stimulation was assessed by measuring IFN-γ production (Figure 3) and T cell proliferation (Supporting Information Figure 5). In all cell lines tested, exposure to pepsin-treated gliadin resulted in stimulation of production of IFN-γ, and treatment of gliadin with Kuma030

DOI: 10.1021/jacs.5b08325
J. Am. Chem. Soc. 2015, 137, 13106−13113

reduced this response, eliminating it at higher concentrations of Kuma030. The decreases observed in IFN-γ production were not due to Kuma030 toxicity (Supporting Information Figure 6). Importantly, Kuma030 eliminated the T cell response to gliadin in every T cell line tested regardless of T cell epitope specificity, indicating that Kuma030 is effective against all epitopes recognized by the T cell lines used in this assay. Because these epitopes span the three major gliadin families, α-, ω-, and γ-gliadin, this suggests that Kuma030 is capable of degrading immunogenic epitopes within all relevant regions of gliadin.

Kuma030 Reduces Gluten Load in Laboratory-Simulated Gastric Digestions Representing Ingestion of 4–8 g of Gluten to Below Levels Thought to Be Toxic to Celiac Patients. To evaluate the practical application of Kuma030, we tested the ability of this enzyme to break down gluten present in simulated gastric digests of whole wheat bread and wheat beer. Whole wheat bread was mashed in artificial saliva to simulate mastication. The mixture was then acidified by the addition of HCl and pepsin at a bread concentration representative of that in the stomach after ingestion of 1–2 slices of bread (assuming 4 g of gluten per slice and a stomach volume of 400–800 mL), and glutenases at various concentrations were added. The amount of gluten remaining was then quantified after 30 min of digestion, representing a lag time of food in the stomach before the commencement of ingesta release into the duodenum through the pyloric opening.[33] At the highest concentration of glutenase tested (1:10 w:w ratio), treatment with EPB2 and SCPEP resulted in 84.4% gluten degradation (Figure 4A, Supporting Information Figure 7). This is consistent with published results from a Phase I study showing that EPB2 and SCPEP eliminate 70–79% of gluten in a test meal at a 1:10 enzyme:gluten ratio in the human stomach after a 30 min incubation time.[34] At a 1:40 w/w ratio (250 μg mL^{-1} enzyme), Kuma030 achieved >99.97% degradation, reaching the limit of quantification by ELISA (3 ppm), which is well below the 20 ppm threshold for "gluten free" labeling in the U.S. (Figure 4A). A 1:320 w:w ratio (31.25 μg mL^{-1}) was sufficient to reduce the level of gluten in the bread by >99%. Finally, the gluten-degrading ability of Kuma030 was also tested directly in a wheat beer, because wheat beers demonstrate higher gluten levels than their barley-made counterparts.[35] Beer was incubated with Kuma030 at 37 or 4 °C at two enzyme concentrations. Samples were taken at various time points and the concentration of remaining gluten was quantified. We found that incubation of the beer, which demonstrated an initial gluten concentration of ~764 ppm, with Kuma030 decreased the gluten level to below 20 ppm by only 5 min (Figure 4B). Surprisingly, Kuma030 was capable of reaching this 20 ppm threshold in wheat beer even at 4 °C (Figure 4B).

DISCUSSION

Oral enzyme therapy has been considered an attractive treatment option for celiac disease since the identification of PQ-rich immunogenic gliadin epitopes that stimulate the immune response.[3] An essential characteristic of any enzyme therapeutic for celiac disease is the ability to break down immunogenic gliadin peptides in gastric conditions before these peptides can access the intestinal tract. Gluten challenge studies in celiac patients have shown that the ingested gluten load must be kept at 10 mg or less in order to prevent intestinal damage.[11,12] Indeed, the U.S. FDA currently mandates that any food labeled as "gluten free" must demonstrate less than 20 ppm gluten, because strict adherence to this standard is predicted to result in a daily ingestion of 10 mg or less. Ingestion of only 1 g of gluten would require a 99% degradation level in order to achieve this threshold; accidental ingestion of a sandwich made with gluten-containing bread (~8 g of gluten assuming 4 g of gluten per slice) would require a ~99.9% degradation level. There is therefore a clear need for glutenases that can rapidly and efficiently destroy immunogenic gliadin epitopes in gastric conditions. In a model of gastric digestion of whole wheat bread, Kuma030 degraded >99.97% of the gluten load in 30 min. While oral administration of glutenases may not be a replacement for a gluten-free diet, the ability to provide protection in the event of a large amount of gluten ingestion is important for efficacy. Thus, the fact that Kuma030 is capable of achieving this degradation level suggests its potential for use as a next-generation enzyme therapeutic for celiac disease.

T cell lines derived from celiac patients demonstrate a myriad of responses to different immunogenic epitopes. It is therefore significant that Kuma030, which demonstrates a PQ dipeptide specificity, is effective at decreasing or eliminating gliadin-mediated T cell stimulation in all cell lines tested, which represent reactivity against peptides derived from all major families of gliadin. While many immunogenic gliadin peptides have been identified, virtually all of these harbor the PQ dipeptide motif within the HLA-binding core, and cleavage after each of these motifs is predicted to destroy the HLA-binding region; thus, PQ is a reasonable proteolytic target for therapeutic glutenases. The selection of a dipeptide specificity, rather than a single amino acid specificity, may contribute to the effectiveness of Kuma030, because while this dipeptide is commonly found throughout the immunogenic regions of gluten, it is found much less frequently in the amino acid sequences of other proteins. Because dietary gluten comprises only roughly 20% of the total protein consumed in a Western diet, a dipeptide specificity may allow for the surgical removal of immunogenic epitopes of gliadin even in the presence of large amounts of other dietary protein. This factor alone may provide a significant advantage over proteases with a single amino acid specificity that indiscriminately act upon protein targets.

The ability to design novel therapeutic proteins through computational protein design holds great potential for the engineering of 21st century medicines. Kuma030 represents the first molecule originally designed by the video game Foldit[16] to move through drug development toward clinical trials. These studies demonstrate the power of protein design to incorporate many different beneficial characteristics into a single therapeutic molecule and warrant further work assessing the potential of this enzyme in vitro and in vivo.

CONCLUSIONS

In this work, we describe the engineering of the gliadin endopeptidase Kuma030. Molecular modeling and computational design allowed for the prediction of mutations on the template enzyme Kuma010 that greatly increased proteolytic activity on immunogenic epitopes relevant to individuals with celiac disease. The resulting enzyme, Kuma030, is capable of degrading all immunodominant epitopes from wheat, barley, and rye, indicating that Kuma030 may be able to degrade a broad spectrum of immunogenic targets. Indeed, treatment of wheat gliadin with Kuma030 reduced or eliminated the capacity of gliadin to stimulate T cells that are specific for a variety of

DOI: 10.1021/jacs.5b08325
J. Am. Chem. Soc. 2015, 137, 13106–13113

immunogenic epitopes. Treatment of foodstuffs with Kuma030 reduced the gliadin load by over 99% in minutes, to below the threshold level shown to be toxic to celiac patients. Future work will determine the potential of Kuma030 as a therapeutic for celiac disease using relevant in vivo models.

■ MATERIALS AND METHODS

Materials. Growth media components were obtained from MP Biomedicals. All reagents for mutagenesis were obtained from New England Biolabs (NEB) with the exception of Pfu Turbo C_x hotstart polymerase which was from Agilent Technologies. Peptides were synthesized by GenScript. Chromogenic substrates were obtained from Bachem. HPLC grade solvents were from Fisher Scientific. Unless otherwise noted, all other reagents were from Sigma-Aldrich.

Crystallization of Kuma010. Purified Kuma010 was concentrated to 9.15 mg mL^{-1} in buffer containing 5 mM DTT, 100 mM NaCl, 10 mM Tris-HCl pH 7.5, and 0.02% NaN_3. The protein was crystallized using the hanging drop vapor evaporation method, at 277 K. The best crystals were obtained at the following condition: 15% PEG 8000, 0.1 M MES pH 6.0, 0.2 M zinc acetate. Diffraction data set to 2.5 Å was collected at the National Synchrotron Light Source, with beamline X4C, and the data were processed with HKL-2000 (HKL Research, Inc.). The structure was determined by molecular replacement using Phaser,[36] with the wild type structure (PDB code 1SN7) as initial search model. The refinement was carried out using Phenix,[37] and model adjusting was done in Coot.[38] The crystal structure was quite close to the original design model for Kuma010, with a C_α RMSD of 0.676 Å.

Computational Enzyme Design. The enzyme design module[17] of the computational protein design software Rosetta was used to suggest mutations that stabilize the PQLP-Kuma010 and PQQP-Kuma010 complexes under the assumption that a higher affinity binding would lead to enhanced catalytic properties. Both the PQLP- and the PQQP-bound models were relaxed in Rosetta using coordinate and catalytic constraints,[39] and then the sequences of the proteins were optimized for peptide−protein interaction energy using fixed-backbone Monte Carlo sequence design.[17] Designable residues were restricted to those within 10 Å of the peptide and were not allowed to mutate to cysteine or proline. Ten to twenty design trajectories were run through design protocols. Mutations were selected for experimental characterization based on occurrence in the designs and in a multiple sequence alignment of Kumamolisin (the Kuma010 template protease), Rosetta, and FoldIt[40] scores, and manual inspection.

Production and Purification of Kuma010 Variants and SCPEP. All mutations to the *kuma010* gene were incorporated using the site-directed mutagenesis method PFunkel.[41] Protein was expressed from pET29B plasmids harboring Kuma010 enzyme variants or SCPEP by *Escherichia coli* BL21(DE3). Enzymes were purified by immobilized-metal affinity chromatography (IMAC) using Ni-NTA Superflow resin (Qiagen) and dialyzed in storage buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM β-mercaptoethanol, 10% (v/v) glycerol). Aliquots were stored at −80 °C until use.

EPB2 Expression and Purification. Expression and purification of recombinant EPB2 proenzyme was performed according to published methods.[27,42] Briefly, EPB2 was expressed from cultures of *E. coli* BL21(DE3). Cultures were centrifuged, and pellets were resuspended in disruption buffer (200 mM sodium phosphate, pH 7.0, 200 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 30% (v/v) glycerol), lysed by sonication, and clarified by centrifugation. Inclusion bodies containing EPB2 were washed twice with water and then dissolved in EPB2 solubilization buffer (50 mM Tris-HCl, pH 8.0, 7 M urea, 2 mM β-mercaptoethanol). The solution was clarified by centrifugation, and the enzyme was purified by IMAC. Fractions containing proEP-B2 were pooled, diluted with refolding buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM β-mercaptoethanol, 15% (v/v) glycerol), and stirred overnight. Refolded pro-EPB2 was filtered and concentrated to 15 mg mL^{-1}. Aliquots were stored at −80 °C until use.

KumaMax Kinetic Characterization. Kinetic parameters were assessed using gliadin-derived substrates Glia_α1 (PFPQPQLPY) and Glia_ω1 (PFPQPQQPF). Enzymes were incubated at 37 °C for 10 min and diluted to 100 nM with reaction buffer (100 mM NaOAc, pH 4.0) containing either Glia_α1 or Glia_ω1 at 0−18.4 mM. Degradation was monitored by periodic sample removal and quenching, followed by quantification of the PFPQPQ breakdown product by HPLC/ESI-MS. The k_{cat}/K_M value was calculated using the Michaelis−Menten equation in Graphpad Prism.

SCPEP and EPB2 Kinetic Characterization. Kinetic parameters of SCPEP and EPB2 were measured on chromogenic substrates Suc-Ala-Pro-pNA (SCPEP) and Z-Phe-Arg-pNA (EPB2) following previously published protocols.[27,28] pNA release was spectrophotometrically monitored. Buffers were as follows: 100 mM $NaHCO_3$, pH 6.0, 150 mM NaCl (SCPEP), or 100 mM NaOAc pH 4.0 (EPB2). The k_{cat}/K_M value was calculated using the Michaelis−Menten equation in Graphpad Prism.

Peptide Degradation Assays. Lyophilized 33mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), 26mer (FLQPQQPFQQPQQPYPQQPQQPFPQ), W03-E07 ((QPFPQPQQPFPWQP), W02-E7 (LQPFPQPQLPYPQPQ), R11-E4E7 (QPFPQQPEQIIPQQP), and B08-E2E7 (PQQPIPQQPQPYPQQ) peptides were dissolved in reaction buffer (100 mM NaOAc, pH 4.0). Enzymes were incubated at 37 °C for 10 min in reaction buffer and then incubated at 2 μg mL^{-1} with 1 mg mL^{-1} substrate (for increased activity screening) or at 500 μg mL^{-1} with 5 mg mL^{-1} substrate (for qualitative degradation assessment) at 37 °C for 60 min. Degradation of the gliadin peptides was monitored by analysis by HPLC at Ab_{215}, or by LC/ESI-MS on a TSQ Quantum Access (Thermo Fisher Scientific).

Degradation of Purified Gluten. Purified wheat gluten was suspended in 70% ethanol at 200 mg mL^{-1}. Enzymes were diluted in reaction buffer (100 mM NaOAc, pH 4.0) and incubated at 37 °C for 10 min. Suspended gluten was rapidly diluted 10-fold with reaction buffer and aliquoted into reaction tubes. Pepsin at 0.6 mg mL^{-1} and enzymes were added, and samples were incubated at 37 °C. The final concentration of gluten in the reaction mixture was 10 mg mL^{-1}. Gluten degradation was halted at ≥95 °C for 10 min, and the gluten level was quantified by competitive ELISA using either the R5 Ridascreen (R-Biopharm) or the G12 GlutenTox kit (Biomedal).

Gluten Degradation in Whole Wheat Bread. Bread slurry was prepared by mashing whole wheat bread (Oroweat: approximately 4 g of protein per slice) with a spatula in artificial saliva (20 mM $NaHCO_3$, 25 mM KCl, 2 mM $CaCl_2$, 0.3 mM $MgCl_2$, pH 7.0), followed by acidification with HCl and blending for 2 min in a Waring blender, at which the pH of the bread mixtures was approximately pH 4.5. Degradation was initiated by the addition of pepsin at 0.6 mg mL^{-1} and either Kuma030 or a 1:1 mixture of EP-B2 and SC PEP. The final gluten concentration in the bread mixture was 10 mg mL^{-1} and was verified by ELISA. Samples were incubated at 37 °C for 30 min with periodic agitation. At the end of the degradation period, samples were incubated at ≥95 °C to halt enzyme activity. Gluten levels were quantified by the G12 and R5 ELISA methods described above.

Gluten Degradation in Wheat Beer. Wheat beer (Chainbreaker White IPA, Deschutes Brewery) was aliquoted on ice. Either Kuma030 or a 1:1 mixture of EPB2 and SCPEP was added to the beer and incubated at 37 or 4 °C for 60 min. Samples were incubated at ≥95 °C to halt enzyme activity. The gluten level for each sample was measured by the G12 ELISA method described above.

T Cell Immunogenicity Assays. Gliadin-specific intestinal T cell lines (iTCLs) were previously generated from duodenal biopsies of 5 HLA-DQ2 celiac disease patients.[32] Briefly, mucosal cells were stimulated for two to three cycles with irradiated autologous peripheral blood mononuclear cells (PBMCs) and deamidated peptic-tryptic digest of gliadin extracted from the hexaploid wheat variety Sagittario (PT-Gliadin). Stable TCLs were kept in culture by repeated stimulation with heterologous irradiated PBMCs and PHA, and IL-2 as a growth factor. The peptide specificity of the iTCLs was evaluated by assaying their reactivity toward a panel of immunogenic gluten

DOI: 10.1021/jacs.5b08325
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peptides[32] (and unpublished data), and revealed an oligoclonal pattern of peptide recognition.

The immune response of iTCLs to gliadin treated with Kuma030 at different concentrations was assayed by detecting IFN-γ production and cell proliferation.[32] T cells (3×10^4) were coincubated with irradiated autologous EBV-transformed, B-lymphoblastoid cell lines (B-LCLs, 1×10^5), in 200 μL of complete medium (X-Vivo plus 5% human serum, Lonza-BioWhittaker, Verviers, Belgium) in U-bottom 96-well plates. Gliadin samples, digested with Kuma030 at the specified concentration, were then added at 50 $\mu g\ mL^{-1}$. Cells incubated in absence of antigens acted as negative control while phytohemagglutinin (PHA, 1 $\mu g\ mL^{-1}$; Boehringer; Mannheim, Germany) and PT-gliadin (50 $\mu g\ mL^{-1}$) were included as positive controls. After a 48 h incubation, cell supernatants (50 μL) were collected for IFN-γ determination and T cells were pulsed overnight with [^{3}H]-thymidine (0.5 μCi per well; GE Healthcare; Buc Cedex, France) and therefore harvested for the evaluation of cell proliferation. Each antigen preparation was assayed in duplicate and in at least two independent experiments.

IFN-γ was measured by a classic sandwich ELISA using purified and biotin-conjugated anti-IFN-γ Abs purchased from Mabtech (Nacka Strand, Sweden). The sensitivity of the assay was 32 pg mL^{-1}.

To evaluate possible toxic effects of enzyme-treated gliadins, PBMCs from healthy donors (2×10^5) were stimulated with PHA (1 $\mu g\ mL^{-1}$), or PHA + samples treated with Kuma030 (50 $\mu g\ mL^{-1}$), in 200 μL of complete medium in U-bottom 96-well plates. IFN-γ production and cell proliferation were assayed as described for TCLs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08325.

> Lists of mutations on the Kuma010 background and their effects on activity; experimentally determined kinetic parameters for selected enzymes; data supporting conclusions in the main text derived from gluten and gliadin peptide degradation assays; T cell proliferation and toxicity data (PDF)

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Notes

The authors declare the following competing financial interest(s): I.S.P., J.S., and D.B. are founders in a company, PvP Biologics, that intends to license the technology described herein. C.W., I.S.P., J.S., D.B., and C.T. are inventors on a patent that describes the technology presented herein.

■ ACKNOWLEDGMENTS

We thank Jayaraman Seetharaman for data collection during X-ray crystallography. This work was supported by the Life Sciences Discovery Fund of Washington State, the Washington State Research Foundation, the University of Washington, and private philanthropy.

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DOI: 10.1021/jacs.5b08325
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