Pepsin Digest of Gliadin Forms Spontaneously Amyloid-Like Nanostructures Influencing the Expression of Selected Pro-Inflammatory, Chemoattractant, and Apoptotic Genes in Caco-2 Cells: Implications for Gluten-Related Disorders

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Scope: Proteolysis-resistant gliadin peptides are intensely investigated in biomedical research relates to celiac disease and gluten-related disorders. Herein, the first integrated supramolecular investigation of pepsin-digested gliadin peptides (p-gliadin) is presented in combination with its functional behavior in the Caco-2 cell line.

Methods and Results: First, gliadins are degraded by pepsin at pH 3, and the physicochemical properties of p-gliadin are compared with gliadin. An integrated approach using interfacial, spectroscopic, and microscopic techniques reveals that the p-gliadin forms spontaneously soluble large supramolecular structures, mainly oligomers and fibrils, capable of binding amyloid-sensitive dyes. The self-assembly of p-gliadin starts at a concentration of 0.40 µg mL⁻¹. Second, the stimulation of Caco-2 cells with the p-gliadin supramolecular system is performed, and the mRNA expression levels of a panel of genes are tested. The experiments show that p-gliadin composed of supramolecular structures triggers significant mRNA up-regulation (p < 0.05) of pro-apoptotic biomarkers (ratio Bcl2/Bak-1), chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8), and the chemokine receptor CXCR3.

Conclusions: This work demonstrates that p-gliadin is interfacial active, forming spontaneously amyloid-type structures that trigger genes in the Caco-2 cell line involved in recruiting specialized immune cells.

1. Introduction

Gluten-related disorders, a group of conditions induced by gluten, have an increasing global prevalence of 5%.⁵ A recent systematic review and meta-analysis demonstrated that Celiac Disease (CeD), which is at the center of gluten-related disorders, has a global prevalence of 1.4% based on serological tests and a prevalence of 0.7% biopsy-confirmed CeD.⁶ Gluten-related disorders are triggered by proteins present in wheat, rye, barley, and some oats. In wheat gluten, gliadin proteins make 50% of the total gluten composition, and they are the most studied proteins connected with these diseases.⁷ At the molecular level, gliadins are classified into three groups α, γ, and α, which differ in their electrophoretic mobility. The α, γ, and α gliadins have a high percentage of prolines (P), 16%, 15%, 27%, and glutamines (Q), 36%, 35%, 37%, respectively.⁸ Moreover, the Q residues in gliadin primary sequences confer a non-ionic amphiphilic nature and gliadin
supramolecular structures were reported in water or water–ethanol mixtures. Moreover, gliadin nanoparticles performed by the anti-solvent method have foaming properties with interfacial activity. On the other hand, at the stomach physiological pH 3.0, gliadins behave as a self-organized solution composed of stable 30 nm micellar type-nanostructures. By changing the pH to 7.0, the micelles convert to solid-type aggregates remaining dispersed in solution. Similar behavior was observed for caseins, the main protein of milk that produces cow milk allergy.

Large proteolysis-resistant gliadin peptides elicit an immunological and toxic response in predisposed individuals. In vitro, gliadin, proteolytic resistant peptides are obtained after treatment with pepsin or followed by trypsin and extensively investigated in cellular models. Manai et al. showed that pepsin-trypsin gliadin digest is composed of large aggregates that interfere in the autophagy process. The structures were visualized by confocal microscopy, pointing out the relevance of the aggregation process in connection to the disease. Nevertheless, the physicochemical behavior of the gliadin digest remains elusive. Gliadin impaired proteolysis, and its toxicity seem to be related to its unusual primary structure. However, CeD patients present a differential gliadin degradation compared to healthy ones, producing more toxic fragments after complete gliadin digestion. Therefore, avoiding specific sequences from degradation may exert a protective effect in particular individuals. Prandi et al. have shown that different peptide fragments were obtained depending on degradation conditions. Van Buiten et al. have also demonstrated that green tea extract enriched on polyphenols forms a complex with gliadin. This complex is less degraded by digestive proteases, reducing the gliadin effect on cell permeability and interleukin release. Although the reason for such differential proteolysis remains elusive, these results suggested that the environmental conditions modulate the degradation process, which could produce different levels of the toxic peptides. Each of these peptides elicits a different immune response; however, together in the digested gliadin, they might cooperate in the activation of immune cells. In this regard, a physicochemical evaluation of the digestion process is needed to understand better the role of gliadin digested peptides in balancing tolerance and disease.

In general, the complicated relationship between food properties, behavior during digestion, and their contribution to human nutrition and health are not well understood. To tackle this complexity, we envisaged as a first stage to investigate the pepsin gliadin digest (p-gliadin) for the first time using a physicochemical approach. Next, we screened in the Caco-2 cell line the capability of the characterized p-gliadin system to activate a panel of selected pro-inflammatory, chemoattractant, and apoptotic genes observed in CeD patients. The Caco-2 cell line is a validated in vitro cellular model to test the effect of gliadins and their peptides, which are considered the triggers of CeD unanimously. As proof of concept about the pathogenicity of p-gliadin in this cell model, we selected a panel of known upregulated genes observed in CeD patients. Enterocytes are a tightly epithelial monolayer that establishes continuous crosstalk with intestinal antigens and immune cells, inducing their activation and migration by releasing inflammatory mediators.

Our findings suggest that p-gliadin forms spontaneously nanostructures with amyloid-like characteristics, triggering a set of selected pro-inflammatory and apoptotic genes in Caco-2 cells, providing for the first time understanding of the toxicity of the whole p-gliadin system at the supramolecular level. Therefore, these findings might open avenues to the rational design of nutritional or therapeutic strategies for CeD and probably the other gluten-related disorders.

2. Results and Discussion

2.1. The p-gliadin Digest Spontaneously Forms Supramolecular Structures

After enzymatic proteolysis, gliadins are not fully degraded to amino acids or short peptides as occurs in the case of model proteins like bovine serum albumin (BSA) or α-Hemoglobin (Hb), among others. To investigate the theoretical role of pepsin, trypsin, and chymotrypsin in the degradation of α- γ- and α-ω-gliadins in comparison with BSA and α-Hb, we employed the bioinformatics tool Expasy PeptideCutter (see supplementary material).

The analysis has shown that pepsin, which is the main enzyme present in the gastric fluid, has theoretically the highest degradation capability once compared to trypsin and chymotrypsin together. Pepsin was the most relevant enzyme, representing more than 50% of all the proteins. Furthermore, when comparing the theoretical digestibility of gliadin to the other proteins, it has been confirmed that gliadins have less than half of the pepsin cleavage sites. Considering that pepsin is the most relevant protease and relevant in gastric digestion, we envisaged to evaluate the physicochemical characteristics of the gliadin peptides obtained by pepsin digestion, p-gliadin, in comparison to gliadin and to screen the inflammatory role of p-gliadin in the enterocyte model Caco-2 cell line.

The micellar gliadin solution at pH 3.0 was treated with pepsin for 2 h at 37 °C, which was sufficient to degrade gliadins into p-gliadin, as shown in the SDS-PAGE (Figure 1B). Since all the gliadin bands disappeared during proteolysis, this suggests that the presence of micellar gliadin does not interfere with pepsin activity (Figure 1B). Furthermore, a UV–vis spectra analysis before and after treatment with pepsin showed an increment of the aggregation index (AI) from approximately 4–12% without detecting any precipitation. This result suggests that p-gliadin is a stable self-organized solution as well as gliadin (Figure 1C).

To get a further understanding of both solutions’ colloidal properties, we performed a surface tension analysis. Previous reports have shown that gliadins reduce the surface tension to 45 mN m⁻¹ in water at acidic pH or PBS, suggesting that these proteins are interfacial active. Under our experimental aqueous conditions, gliadin reduced the water surface tension to 50 mN m⁻¹, and a critical aggregation concentration (C.A.C.) of 0.39 µg mL⁻¹ was obtained by linear fitting (Figure 1D). This behavior is characteristic of water–air interface active proteins where a decrease of the surface tension is observed until concentration and conformation equilibrium is achieved in the surface.

Similar behavior was observed in other protein aggregates like human serum albumin nanofibrils and the Aβ amyloid
peptide.[27] P-gliadin induced an overall decrease of the surface tension to 55 mN m⁻¹ with two inflection points. The first C.A.C. was calculated at 0.40 µg mL⁻¹ and the second at 1.25 µg mL⁻¹, showing p-gliadin self-organized system complexity. A comparable two-stage decrease in surface tension was observed in the surface-active Sap-Pc protein from a marine strain of *Penicillium chrysogenum*.[28] Altogether these results indicate for the first time, at least to our knowledge, that the p-gliadin is composed of self-associated peptides that are active at the water/air interface. These C.A.C values are much below than the 1 mg mL⁻¹ usually employed in biomedical research, which implies that large p-gliadin supramolecular structures should be present in vitro studies.

Previously, DLS experiments of gliadin have shown that the mean hydrodynamic radius of the aggregates was 30 ± 15 nm, as shown in Figure 2A. Here, the evaluation of p-gliadin showed that it is composed of two types of aggregates, one with a hydrodynamic radius around 100 ± 40 nm and another larger of 500 nm ± 200 nm (Figure 2A). This distribution is indicative of at least two main species in solution, showing that the p-gliadin forms larger nanostructures than gliadin, which are in agreement with the higher aggregation index obtained. In the DLS distribution, larger particles dominate due to r⁶ dependence of the Rayleigh scattering intensity, with r being the aggregate radius, implicating that the smaller particles are the majority in the distribution of p-gliadin but do not significantly contribute to the scattering signal.

Next, we took advantage of tryptophan (Trp) intrinsic fluorescence because it is sensitive to changes in its microenvironment’s polarity. Trp fluorescence was used to evaluate folding modifications due to oligomerization in different protein systems, and accurate data is obtained by the second derivative of the spectra.[29] In this case, Trp fluorescence emission spectra of gliadin and p-gliadin were taken and compared in Figure 2B. As reported previously, Trp emission of gliadin nanostructures is broad; detecting two minima, one at 338 nm, which is characteristic of a more buried Trps in the protein core and the second at 347 nm, indicating that Trps are exposed to the solvent.[8] In p-gliadin, we observed only a broad minimum at 347 nm, indicating that Trps residues in the p-gliadin are more exposed to solvent than in the gliadin protein (Figure 2B).

Considering these results, we investigated the formation of p-gliadin aggregates in situ by following gliadin proteolysis by pepsin at 37 °C, using two independent techniques such as
SLS experiments and Trp fluorescence (Figure 2C) in a time-dependent manner. While SLS could be used to inquire about the formation of aggregates in situ over time, Trp fluorescence contributes to following the kinetics of the degradation considering the redshift of the signal at around 335 nm. SLS showed an increment of the size of the aggregates after the addition of pepsin. The data were fitted to an exponential curve, and a half-lifetime of 13 ± 0.8 min was obtained. The decay of the fluorescence intensity at 335 nm was followed, confirming that Trp residues are less buried after proteolysis. The data were fitted to an exponential decay curve, leading to a half-lifetime of 22.5 ± 0.2 min (Figure 2C). The observed half-time differences suggest that the aggregation process could start before tryptophan surrounding environment change.

Interestingly, degradation and aggregation reached equilibrium after 40 min. Finally, the degradation over time was assessed by SDS-PAGE (Figure 2D). During the first 30 min, a band between 10 and 15 kDa was detected, indicating the formation of peptides; meanwhile, a band around 33 kDa remained. After 60 min, the high molecular weight protein bands decrease their intensity until their disappearance at the end of the proteolysis (2 h). Together all the above-mentioned experiments indicate that only peptides are present in p-gliadin at the end of pepsin digestion, and these fragments self-aggregate into large assemblies.

The fact that p-gliadin forms large aggregates in solution reinforces the relevance of previous findings on synthetic gliadin fragments that are proteolytic resistant and self-assembled into soluble aggregates immunodominant 33-mer and the toxic p31-43 peptides.[30a-c,31]

2.2. The p-gliadin System Has a Preponderantly Disordered Conformation with PPII as the Dominant Secondary Structure in Equilibrium with β-Structures

For identifying secondary structure differences between gliadin and p-gliadin, far-UV circular dichroism spectra (CD) were performed. Due to the concomitant presence of different species in the mixture, only qualitative information about the secondary structure at a constant concentration was obtained. As previously reported, the circular dichroism spectrum of gliadin is compatible with a structure mainly composed of random and alpha-helix presenting a negative peak at around 207 nm, an negative shoulder at 220 nm, and a positive band at 190 nm.[8,32] Here, we investigated the role of the temperature in the gliadin folding. Only a small hypochromatic displacement of the negative band from −6.2 to −5.5 ΔE, and a small shift of the minimum around 207 nm was observed when the temperature increases from 5 to 37 °C (Figure 3A). Lower temperature led to the formation of a gel (not shown).

On the other hand, p-gliadin spectra at −10 °C showed a negative band at around 202 nm together with the appearance of a small band at 223 nm; these two bands are characteristic of a polyproline II (PPII) conformation.[33] Similar PPII type CD
Figure 3. Temperature-dependent circular dichroism spectra show that p-gliadin has a predominantly disordered conformation with PPII as the dominant secondary structure. A) Gliadin solution and B) p-gliadin solution. The legends indicate the corresponding temperature of each spectrum.

spectra were found in caseins, proteins with a high P and Q residue content like gliadins. When the temperature was increased up to 37 °C, the negative band minimum was red-shifted to 205 nm, and a hypochromic displacement of the band from -5.5 to -4.8 ΔE was observed (Figure 3B). In both gliadin and p-gliadin, the occurrence of more than one isodichroic point confirms that many conformations are present, which is expected considering the complex mixture (Figure 3A and B). When analyzing and comparing gliadin and p-gliadin secondary structure content at 37 °C by BeStSel web server, it was observed that p-gliadin has a higher content of parallel and anti-parallel beta-sheets and turns and less alpha-helix structure than gliadin (Table S1, Supporting Information). In the case of 33-mer and p31-43 gliadin synthetic fragments, a predominant PPII structure was reported. In the particular case of 33-mer peptide, folding to a parallel beta structure has been observed increasing peptide concentration. More than one isodichroic point was detected when 33-mer gliadin peptide oligomeric species were cross-linked by forming di-tyrosine bonds. To summarizing, p-gliadin has a predominantly disordered conformation with PPII as the dominant secondary structure in equilibrium with β-structures.

2.3. The p-gliadin Structures Have a Compact Quaternary Structure Able to Bind Amyloid Sensitive Dyes

In general, fluorescent molecular probes are key tools to evaluate biomolecular interactions and the presence of oligomers and fibrils. We showed that gliadin micelle type aggregates bind the Nile Red (NR) fluorophore in our previous work. NR changes its emission, sensing its microenvironment’s polarity, leading to a blueshift of the maximum wavelength emission after binding to a hydrophobic pocket. BODIPY dye has shown to be sensitive to oligomers and fibrils of the β-amyloid peptide, meanwhile, Thioflavin T (ThT) is a standard probe used to follow the aggregation of different amyloid systems. Here, we employed NR, triazole-based BODIPY dye, and ThT because they are complementary probes that do not have significant fluorescence emission in water solutions, but their fluorescence intensity increases after binding (Figure 4). Gliadin micelles bind to NR, showing a maximum fluorescence emission at 640 nm; meanwhile, the binding to p-gliadin led to a strong blueshift to 625 nm. This blueshift of 15 nm is associated with the location of NR in an apolar environment buried from the solvent, confirming that p-gliadin is a self-organized system in solution (Figure 4A). Next, we tested if gliadin and p-gliadin aggregates can bind to the amyloid-sensitive dyes BODIPY and ThT (Figure 4B and C). Both dyes bind to gliadin and p-gliadin with slight opposite effects. These results are in agreement with the CD spectra analysis by BeStSel and confirm that p-gliadin aggregates show an amyloid-like character. This is a distinctive characteristic of p-gliadin, in comparison with 33-mer gliadin peptide aggregates that did not bind to these amyloid sensitive dyes.

2.4. The p-gliadin Solution is an Organized System Composed of Spheres and Fibrillar Structures

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) experiments of the p-gliadin were performed to visualize the nanostructures detected by UV-Visible, DLS, and extrinsic fluorescence methods. We observed fibrils of a transversal diameter of 129 ± 62 nm and spheres 98 ± 26 nm (Figure 5A and B). Also, some linear arrangements of spheres are observed, showing their tendency to be self-organized. Importantly, the detected fibrils can be responsible for the binding to amyloid-sensitive probe ThT observed in the fluorescence experiments, as well as the soluble species of 500 nm detected by DLS (Figure 2A). The transformation from spheres to the fibrillar structure has been observed for Phe-Phe derivates, the D-Leu-Phe-Phe, and the 33-mer gliadin peptide. By SEM, it was observed that p-gliadin interaction with the surface generated a three-dimensional arrangement (Figure 5C). Generally, when the solvent evaporates, the precipitation of soluble species continuing with protein deposition and aggregation may occur, resulting in complex self-assemble architectures as the ones observed in SEM. In general, kinetic and thermodynamic analyses are...
required to clarify the complex mechanism of fibril formation, which exceed this initial report of p-gliadin aggregation.\footnote{42} In the meantime, based on our findings, we provide a first insight considering the morphology of the detected species in comparison with other better-studied systems.\footnote{30b,30c,43}

We hypothesized that the proximity and reduction of the degrees of freedom on the glass surface facilitate the organization of oligomers into fibrils. Further lateral association between fibrils could build up the detected planar structures, which “sheet”-like structures are reminiscent of plaques. The simultaneous detection of planar and spherical structures, in the absence of any intermediate particles, implies that the fibrils act as a second nucleation site for the particles, thus limiting the interaction between particles and favoring the formation of the fibrils, which lateral organization led to the formation of the planar structures observed (Figure 5C). The occurrence of different morphological structures observed in TEM and SEM, suggests that this is not a classical nucleation process and that the intrinsic characteristics of the p-gliadin molecules would favor the formation of such morphologies. In comparison with 33-mer, the lateral association of the fibrils in the p-gliadin seems to be a dominant effect. For p-gliadin, there is a higher abundance of fibrils and higher coverage of the surface than the 33-mer-gliadin peptide.\footnote{30b-d} The higher abundance of p-gliadin fibrils than the 33-mer gliadin peptide is also confirmed by binding the amyloid fluorescence probes, ThT and BODIPY.

2.5. The Self-organized Solution of p-gliadin Digest Induces Pro-Inflammatory, Chemoattractant and Apoptotic Responses in the Caco-2 Cell Line

One yet unresolved issue in gluten-related disorders is whether a specific trigger exists that could induce peaks of gliadin peptides translocation and, thereby, potentially induce active disease periods.\footnote{44} In this regard, we hypothesized that the proteolysis-resistant gliadin superstructures could form a biofilm in the epithelial barrier, which may alter its homeostasis. The formation of gliadin peptide superstructures could be potentially responsible for tissue inflammatory responses, like those observed in CeD.\footnote{45} Before the T-cell promoted immune response to gliadin observed in CeD, the first interaction of gliadin digest with the host are intestinal epithelial cells.\footnote{20d} Herein, we explored, as a proof of concept, the effect of the characterized p-gliadin system on an enterocyte model by measuring the mRNA expression levels of a panel of some selected genes involved in inflammation and apoptosis in a cellular model. The Caco-2 cell line was selected as an enterocyte model, and p-gliadin obtained as described above was used as a stimulus for 20 h,\footnote{17b,46} and the comparison with unstimulated cells are presented in Figure 6.

First, cell viability was assessed using a lactate dehydrogenase (LDH) assay showing no significant cellular death/damage when cells were treated with 0.064 mg mL$^{-1}$ of p-gliadin (Figure 6A) as previously reported.\footnote{47}

Second, we evaluated the presence of pro-apoptotic signals by testing mRNA gene expression levels of Bak-1 and Bcl-2. Meanwhile, Bak-1 is known to induce a pro-apoptotic response, Bcl-2 acts as an anti-apoptotic factor. Together, they play a crucial role in regulating and modulating mitochondrial depolarization and

Figure 4. Fluorescence analysis of gliadin and p-gliadin binding to Nile Red, BODIPY and Thioflavin T probes. Gliadin (○) and p-gliadin samples (⬤) were treated with (A) NR, (B) BODIPY, and (C) ThT, excitation. (See Materials and Methods section)
The increased expression levels of chemokine and pro-apoptotic genes that were tested in this work reveal that the self-organized p-gliadin is a pathogenic stimulus to Caco-2 epithelial cells. The up-regulated mRNA levels of CCL3, CCL4, and CCL5, were observed in untreated celiac disease patients. These chemokines and their common receptor, CCR5, and the CXCR3 and its ligands (CXCL9, -10, -11), are likely involved in the attraction and migration of macrophages, neutrophils, and gluten-specific CD4+T cells into the gut lesions. We also confirmed the upregulation of CXCR3/CXCL8 pathway which are also observed in CeD biopsies, pointing out that the self-organized p-gliadin may play a pathological role in vivo. Interestingly, the p-gliadin treated monolayers showed a decrease in CCL2 mRNA expression. These results may be a cell-specific feature as other groups have observed an increment of CCL2 expression in monocytes from CeD patients.

3. Conclusions

We demonstrated that p-gliadin spontaneously self-assembles during gliadin proteolysis by pepsin at pH 3.0. The self-assembly process occurs at a low concentration, such as 0.40 µg mL⁻¹, detected by surface tension measurements. The p-gliadin aggregates were characterized by DLS experiments, UV-Visible spectroscopy, and fluorescence experiments which demonstrated that large p-gliadin structures are in solution. Notably, gliadin and the p-gliadin structures can bind to two amyloid-sensitive dyes, showing a potential dysfunctional folding. Although the p-gliadin showed a predominantly disordered conformation, a PPII character was detected at low temperature, accompanied by a higher beta sheet structure than in gliadin. The electron microscopy analysis showed that oligomers and fibrils mainly compose p-gliadin system. In this regard, it is possible to conclude that p-gliadin is structurally a complex system that self-organizes into amyloid-like nanostructures in solution as determined by spectroscopic and microscopic techniques.
Figure 6. P-gliadin induces pro-apoptotic signals and affects chemokines mRNA and CXCR3 expression in Caco-2 cell model. Caco-2 cells were incubated 20 hours with p-gliadin. Both LDH activity and mRNA gene expression of genes involved in cell activation were determined. Graph A) LDH activity results (in milli-units mL$^{-1}$). Graphs B-I depict differences in gene expression levels of stimulated cells compared to gene expression levels of unstimulated cells as fold change (with unstimulated cell expression being normalized to 1). Graph B & C) Bak1 and Bcl-2, Graph D) CCL2, Graph E) CCL3, Graph F) CCL4, Graph G) CCL5, Graph H) CXCL8 and Graph I) CXCR3. The statistical analysis was performed by Mann–Whitney U test, $p < 0.0005$. Results ($n = 6$ wells per condition) are expressed in mean ± SEM.
When the model epithelial cells Caco-2 were exposed to the self-organized p-gliadin solution, the up-regulation of a set of selected pro-inflammatory, chemotactant, and apoptotic mRNA were detected. Besides, we demonstrate that p-gliadin up-regulates the expression of the receptor CXCR3 associated with increased intestinal permeability and CXC18, a marker of inflammation. Stimulation of the cells with 33-mer supramolecular structures did not show any effect, obtaining comparable results to the unstimulated. We are aware that the upregulation of the selected genes is neither specific nor unique for CeD; nevertheless, they prove that the p-gliadin amyloid-like aggregates, and not the 33-mer aggregates, have a pro-inflammatory, chemotactant, and apoptotic role in this in vitro model pointing out the necessity to further evaluate their role in biomedical research.

Considering the complexity of the digestion process and the possible occurrence of gliadin peptide aggregates in the gastrointestinal tract, changes in its self-assembly process may affect its interaction with the enterocyte barrier. Thus, its inflammatory role could be modulated. In this sense, nutritional or pharmacological interventions towards the inhibition or modulation of the formation of gliadin amyloid-type nanostructures could be a potential novel therapeutic strategy in connection with CeD and possible to the other less understood gluten-related disorders. Our efforts are directed towards this goal.

4. Experimental Section

Preparation of Gliadin and p-gliadin Digest Solutions: A dispersion of 1.0 mg mL\(^{-1}\) wheat gliadin (Sigma Aldrich) was prepared in filtered 0.001 M HCl, 10 × 10\(^{-3}\) M NaCl, pH 3.0. MilliQ water. The gliadin dispersion was homogenized at room temperature and stabilized at 10 °C for 12 h, followed by centrifugation at 15000 rpm. The supernatant was collected, and gliadin concentration was determined by Bradford colorimetric assay at 0.16–0.18 mg mL\(^{-1}\).

Pepsin digestion was carried out by adding pepsin from pig gastric mucosa (1:10000 NF, Anedra) dissolved in 0.001 M HCl, 10 × 10\(^{-3}\) M NaCl, pH 3.0, immediately before use to the stabilized gliadin solution at 37 °C for 20 min in a ratio 1:3. The enzymatic reaction was stopped after 2 h by keeping the sample at 95 °C for 5 min. Electrophoretic analysis of the samples was done as described in the supplementary material.

UV-Visible Spectroscopy: UV-vis spectra of gliadin and p-gliadin were acquired at room temperature in VWR 3100PC spectrophotometer. The aggregation index was calculated as described in the supplementary material.

Tensiometry: The critical aggregation concentration of gliadin and p-gliadin was determined using the Wilhelmy plate. Measurements were done at 22 °C with a DCAT 21 tensiometer from Dataphysics (Filderstadt, Germany) using a platinum-iridium-plate. After reaching buffer stability, the critical aggregation concentration of gliadin and p-gliadin were recorded on a Jasco J-810 CD spectrometer using a Peltier system as a temperature controller. Five scans were acquired for each sample with a scanning speed of 50 nm min\(^{-1}\) and 0.2 nm quartz cuvettes. Gliadin was analyzed at 5, 25, and 37 °C. In the case of p-gliadin, spectra at −10, 5, 25, and 37 °C were analyzed. Controls of pepsin alone at the working concentration were measured under the same conditions and subtracted from the spectra, and smooth noise reduction was applied as a binomial method.

Steady-State Fluorescence Spectroscopy: Tryptophan intrinsic fluorescence of gliadin and p-gliadin was done by exciting the samples at 295 nm, and the emission collected in the 300–450 nm range at 25 °C. The second derivative analysis determined the accurate \(\lambda_{\text{em}}\) max emission. The kinetic assay of gliadin degradation followed by tryptophan fluorescence and the ability of gliadin and p-gliadin to bind to Nile Red, Thioflavin T, and the triazole-containing BODIPY probes are detailed described in the supplementary material.

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM): TEM was performed depositing 20 µL of the sample in a SiO\(_2\) grid fed by the conventional freeze-fixation and freeze-drying method. The visualization was carried out with a CM 100 microscope from Philips at 100 kV acceleration voltage with a high-contrast lens. For SEM, 20 µL of the solution was deposited on a coverslip and left to dry. The specimen was coated with Au(0) and observed using a JSM-35 CF with a secondary electrons’ detector.

Cell Culture: Enterocytes from the Caco-2 cell line (ATCC, UK) were cultured in 75 cm\(^2\) flasks (Falcon, Corning Incorporated, USA) in minimal essential medium supplemented with 10% fetal bovine serum, 2 × 10\(^{-3}\) M l-glutamine, 1% non-essential amino acids (Gibco, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA) at 37 °C in a 5% CO\(_2\) incubator. Cells were passed at 80% confluency. For experiments, Caco-2 enterocytes (passages 7–18) were seeded in 6-wells (9.5 × 10\(^5\) wells) or 12-wells plates (3.8 × 10\(^5\) wells) (Costar, Corning Incorporated) and allowed to grow confluently till 12 days (SI). Stimulation experiments were performed by incubation with medium alone (negative control) or p-gliadin digest at a final concentration of 0.064 mg mL\(^{-1}\) for 20 h.[17b,46] Previously, 33-mer gliadin peptide was physicochemically characterized, showing the formation of fibrils at concentrations above 0.78 mg mL\(^{-1}\);[30] therefore, this system was employed as second control at a final concentration of 0.98 mg mL\(^{-1}\) (250 × 10\(^{-6}\) M). Supernatants were collected and stored at −80 °C until RNA isolation. The cell integrity was measured by measuring the lactate dehydrogenase (LDH) activity (SI).

Gene Expression Measurement: RNA was extracted from Caco-2 monolayers with RNeasy kit (Qiagen, USA). Complementary DNA (cDNA) was synthesized from 1 µg RNA per sample using the ReverTaid First Strand cDNA Synthesis kit (Thermo Fisher Scientific).

Messenger RNA (mRNA) expression of an array of primers was quantified by RT-PCR using SYBRgreen fluorescence (Applied Biosystems, Foster City, CA). Specific primers for CXC18 were: 5′-CCACCCAGACCTCATAACG-3′ (forward), 5′-GATGGTTCTTCCTGGTGTTGTT-3′ (reverse), for CCL2: 5′-TCTGGTCCTGCTCTACATC-3′ (forward), 5′-GCCGATTCCATTGATCTGGC-3′ (reverse), for CCL3: 5′-TCCGTACATCCTGAAATGCT-3′ (forward), 5′-GGCAAGTCGTACGAGAAGTCT-3′ (reverse), for CCL4: 5′-CCCACTGACGCCTTTATC-3′ (forward), 5′-GCCGGAGAGTACTTCC-3′ (reverse), for CCL5: 5′-CTCAGTTGGACACCCAGGTAATCC-3′ (forward), 5′-CTGCGGTGACAAAGACGACTG-3′ (reverse), for CXCR3: 5′-TGTCACCCACAGGATATC-3′ (forward), 5′-GGCAAGTAGGCTCTTCC-3′ (reverse), for CXCL1: 5′-CCATGGCCCTCCTGTTGAG-3′ (forward), 5′-TGGGTGACAAAGACGACTG-3′ (reverse), for Bak-1: 5′-CCACGACCTGTTCCTAC-3′ (forward), 5′-GCTGTAGCCAGGTGTTTCC-3′ (reverse), and for house-keeping gene, 18S: 5′-CATACGGCCTGCTGACTCT-3′ (forward), 5′-TCCCTTCTGACCACGCCACCC-3′ (reverse). These primers were purchased from Eurofins Genomics (Ebersberg, Germany).

cDNA was run on a LightCycler 480 System (Roche Molecular Diagnostics, Pleasanton, CA) with the following protocol: one cycle of 3 min at 95 °C, 45 cycles of 10 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C.
Relative gene expression was calculated using the comparative ΔCt method with 18S as a housekeeping gene. Relative expression of genes was calculated as $2^{-\Delta\Delta Ct}$. The fold (± SEM) change in gene expression after stimulation was relative to medium (normalized to 1). Differences between the two groups were compared using the Mann–Whitney U test. For the significance of differences among the three groups, the Kruskal–Wallis test and post Dunn’s multiple comparison tests were used. Values of $p < 0.05$ were considered significant.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
M.G.H., F.N., P.W.D., M.G., Y.H., I.E., K.M.L, V.I.D. designed and performed the experiments. M.G.H., F.N., M.G., P.W.D., N.T., Y.H., T.H., K.M.L, V.I.D. contributed to data analysis. M.G.H. and V.I.D. drafted the manuscript. M.G.H., N.T., F.N., T.H., A.H., K.M.L., and V.I.D. discussed the overall research. V.I.D. conceived the idea, obtained the funding to develop the study, and supervised it. All authors reviewed and approved the final version of the manuscript.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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