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Structural Analysis and Caco-2 Cell Permeability of the Celiac-Toxic A-Gliadin Peptide 31−55

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Supporting Information

ABSTRACT: Celiac disease is a chronic enteropathy caused by the ingestion of wheat gliadin and other cereal prolamines. The synthetic peptides 31−43 (P31−43) and 31−49 (P31−49) from A-gliadin are considered to be model peptides for studying innate immunity in celiac disease. Our previous study demonstrated that P31−43 and P31−49 are encrypted within peptide 31−55 (P31−55), which is naturally released from gastropancreatic digestion and is not susceptible to hydrolysis by brush border membrane enzymes. Here, we analyzed the permeability of P31−55 through the epithelial cell layer of confluent Caco-2 cells using high-performance liquid chromatography, mass spectrometry, and fluorescence-activated cell sorting. Twenty-three percent of the P31−55 added to the apical chamber was transported to the basolateral chamber after 4 h of incubation without being degraded by hydrolysis. Treatment of Caco-2 cells with whole gliadin digests extracted from a common wheat cultivar increased the epithelial P31−55 translocation by approximately 35%. Moreover, we observed an atypical chromatographic profile consisting of a double peak. Chromatography using different column temperatures and circular dichroism highlighted the presence of more conformational structures around the amide bond of the two adjacent prolines 38 and 39. These findings confirm that P31−55 is gastrointestinally resistant and is permeable across a Caco-2 monolayer. Moreover, we hypothesize that the various conformations of P31−55 may play a role in the activation of innate immunity.

KEYWORDS: *gliadin, celiac disease, Caco-2 cell, gastropancreatic digestion, brush border membrane enzymes, innate immunity*

INTRODUCTION

Gluten is considered to be the environmental factor triggering celiac disease (CD) in genetically susceptible patients.[1] The term gluten mainly refers to wheat storage proteins. It is an extremely heterogeneous mixture of proteins, which are classified as gliadins and glutenins. Gliadins are monomeric proteins that are subdivided into α/β-, γ-, and ω-fractions according to their electrophoretic profile. Glutenins are present as polymeric complexes that are comprised of two subunit types, the high molecular weight and the low molecular weight subunits.[2] Rye and barley cereal also contain related gluten proteins (secalin and hordein, respectively), which are toxic and immunogenic in CD patients.[3]

A common feature of gluten proteins is the presence of a high level of proline and glutamine residues, which renders gluten highly resistant to proteolytic degradation in the gastrointestinal tract.[4,5] Consequently, the released peptides can cross the gut epithelial barrier and reach the lamina propria of the intestinal mucosa, triggering two immunological pathways: the adaptive and the innate immune responses.[6]

The adaptive immune response has been intensely studied through the identification of specific immunogenic peptide sequences, which are presented by HLA-DQ8 or HLA-DQ2 to gluten-sensitive CD4+ T-cells.[6] The binding of gluten peptides to HLA molecules occurs with enhanced affinity when a specific glutamine residue is deamidated to glutamic acid by the enzyme tissue transglutaminase.[7,8] Following gluten recognition, prevalent proinflammatory cytokines (mainly γ-interferon) are released, leading to profound tissue remodeling.[1] At least 50 T-cell stimulatory gliadin and glutenin epitopes in their native or deamidated forms have been identified,[6] but a 33-mer peptide released from α2-gliadin through gastropancreatic enzyme digestion is currently considered the predominant immunogenic peptide because its sequence includes six overlapping epitopes.[9] This 33-mer peptide is highly resistant to further digestion by intestinal brush border membrane (BBM) enzyme. Hence, it has been suggested that the 33-mer could reach the underlying lamina propria and thus play a central role in the pathogenic cascade of CD.[9,10]

In concert with adaptive immunity, gliadin peptides elicit an innate immune response in professional antigen-presenting cells (monocytes, macrophages, and dendritic cells), which predominantly activates intraepithelial lymphocytes but also activates intestinal epithelial cells.[1] Contrary to the adaptive response, the innate immune response has only begun to receive attention in recent years. Almost all research regarding the involvement of innate immunity in CD has been performed using the model P31−43 (LGQQQPFPPQQPY) from A-gliadin.[1,6,11−14] Different from immunogenic peptides (i.e., the

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33-mer), whose release has been demonstrated in vitro and in vivo,[9,14] P31−43 was discovered in early experiments that were conducted on a panel of synthetic peptides containing the sequence of A-gliadin.[15,16] P31−43 is considered a toxic peptide, as it induces mucosal damage when added to duodenal mucosal biopsies and when administered in vivo to the proximal and distal intestine.[1,6] In particular, P31−43 up-regulates the expression of interleukin (IL)-15, cyclooxygenase-2 (COX-2), and the cell activation markers CD25 and CD83 on LP macrophages, monocytes, and dendritic cells without stimulating $CD4^+$ T-cells.[17] More recently, it was demonstrated that P31−43 affects the actin cytoskeleton and cell proliferation, both of which depend on the activation of the epidermal growth factor receptor (EGFR), in several cell types and in organ cultures of celiac mucosa. In this system, P31−43 interferes with EGFR decay and prolongs EGFR activation.[11] Although few other peptides have been identified,[6] P31−43 currently represents a unique model peptide for the study of the innate immune system in CD. To date, the mechanisms underlying the biological properties of the gliadin peptides and the metabolic pathways involved in the activation of innate immunity in CD are unclear. Furthermore, no receptor has been found, and the mechanism by which it enters the cell is unknown.

We previously demonstrated that P31−43 (or P31−49 as well) is not released during the digestion of proteins from food intake but is encompassed within a longer peptide, P31−55 (LGQQQPFPPQQPYPQPQPFPSQQPY), which is resistant to gastropancreatic and to subsequent BBM enzymes even after prolonged exposure. This finding was demonstrated using recombinant A-gliadin and the synthetic peptide as well as with whole gliadin extracted from a common wheat cultivar.[18]

Here, we studied the transepithelial transport of P31−55 across Caco-2 cell monolayers, used as model of the gut epithelium, and measured its permeability and deliverability. The peptide remained intact through the cell monolayer, and no hydrolytic processing was observed. The structure and conformational dynamics of P31−55 were investigated in detail by reverse phase high-performance liquid chromatography (RP-HPLC) and circular dichroism.

MATERIALS AND METHODS

Preparation of Synthetic Peptides. Peptides were produced by solid-phase Fmoc synthesis by means of a Pioneer Peptide Synthesis System 9050 instrument (PE-Biosystems, Framingham, MA). Subsequent N-terminal labeling of P31−55 with the fluorescein-5-isothiocyanate (FITC) (Sigma Aldrich, Milan, Italy) was done according to the manufacturer's protocol. Peptides were purified by HPLC, and their identity was assessed by mass spectrometry (MS) analysis.

Preparation of the Pepsin-Trypsin-Chymotrypsin-Carboxypeptidase-Elastase (PTCCE) Hydrolysis of Gliadins. Gliadins were extracted from an Italian common wheat cultivar and hydrolyzed according Mamone et al.[18] Briefly, 1 mg of gliadins was dissolved in 0.01 M chloride acid and incubated with pepsin (1:100, enzyme:substrate) for 30 min. Then, the pH of the sample was increased to 7.5 with phosphate buffer and incubated with a mixture of trypsin (1:100), chymotrypsin (1:100), elastase (1:100), and carboxypeptidase A (1:500) for 1 h at 37 °C. Finally, the sample was immersed in a boiling water bath for 5 min and stored at −20 °C.

Cell Culture. The human colon carcinoma cell line Caco-2 was obtained from the ATCC (Philadelphia, PA). Cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum, 1% nonessential amino acids, 2 mM L-glutamine, and penicillin/streptomycin. Caco-2 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells used in this study were at passage 20−30. Upon reaching 80% confluence, cells in the logarithmic phase were subcultured weekly at a split ratio of 1:3 by trypsinization. The medium was changed every other day. The cell viability was tested using the vital dye neutral red according to the manufacturer's instructions (Sigma).

Transepithelial Transport Studies. For transport studies, cells were seeded at 450000 cells/cm^2 in cell culture inserts with PET (polyethylene terephthalate) membranes (0.4 μm pore size, 23.1 mm diameter, 4.2 cm^2 growth surface area; BD Falcon, Italy). Cell attachment was improved by preliminarily covering the filter membrane with bovine collagen type I (Gibco, Invitrogen, Italy) following the supplier's instructions. The monolayer became confluent after 4 days, and the cells were allowed to differentiate for at least another 20 days before performing transepithelial transport experiments. The integrity of the cell layer was evaluated by transepithelial electrical resistance (TEER) measurements using an epithelial volt-ohm meter (Millicell ERS-2, Millipore, Italy). TEER values were expressed as ohms per square centimeter. Only Caco-2 monolayers showing TEERs higher than 700 Ω/cm^2 were used for the experiments. Following TEER measurement, Caco-2 cell monolayers were gently rinsed twice with PBS. Volumes of 2 and 3 mL of transport medium (Hank's balanced salt solution supplemented with 25 mM glucose and 10 mM HEPES, TM) were added to the apical (AP) and basolateral (BL) compartments, respectively. The inserts were incubated at 37 °C for 330 min, and samples from the AP and BL compartments were collected at the beginning and end of the incubation period for HPLC and liquid chromatography (LC)-ESI/MS analyses. At the end of the experiments, the TEER was remeasured. In the same set of cells, the integrity of the layer was evaluated with the lucifer yellow (LY) permeability assay.[19] Fluorescence leakage was determined for LY using 485 nm excitation and 530 nm emission on a fluorescence plate reader (Biorad, Milan, Italy). Transport buffer (HBSS with Ca^{2+}, Mg^{2+}, and 10 mM HEPES, pH 7.4) was added to the BL compartment. LY was diluted in transport buffer and added to the AP compartment at a final concentration of 100 μM. The AP to BL permeability coefficients (P_c) were calculated according to the following equation: $P_c = [V/(A \times C_i)] \times [C_f/T]$, where V is the volume of the basal chamber (mL), A is the area of the membrane insert (cm^2), C_i is the initial concentration of the fluorochrome, C_f is the final concentration of the fluorochrome, and T is the assay time (seconds). Moreover, cell differentiation was evaluated by alkaline phosphatase activity assayed according to the method described by Garen and Levinthal,[20] with *p*-nitrophenolphosphate as the substrate.

Flow Cytometric Analysis. Caco-2 cells were incubated in the presence or absence of labeled fluorescent peptide. Following incubation, Caco-2 cells were harvested with trypsin at various time points (1−3 h), washed twice with PBS, and then resuspended in PBS. Caco-2 cells were analyzed by FACS using a FACSCalibur Flow Cytometer (BD Biosciences, Italy) equipped with CellQuest software (BD Biosciences). At least 50000 cells were collected.

RP-HPLC Analysis. RP-HPLC of the synthetic peptide was performed using a reverse-phase column (2.0 mm i.d. × 250 mm, C18, 5 μm; Phenomenex, Hesperia, CA) with a flow rate of 0.2 mL/min on an Agilent 1100 modular system with an integrated diode array detector (Palo Alto, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) (v/v) in water; solvent B was 0.1% TFA in acetonitrile. A 60 min gradient of 5−60% buffer B was used. The chromatographic separation of the peptide was performed at ambient (25 °C), low (∼4 °C), and high (50 °C) temperatures, using a thermostatic column holder or chilling the column in an ice-cold bath.

LC-Tandem Mass Spectrometry (MS/MS). Synthetic and PTCEE samples recovered from both the AP and the BL compartments of the Caco-2 monolayers were separated by μ-HPLC using an Integral 100Q HPLC system (PerSeptiveBiosystems, Framingham, MA). The flow rate was split from 200 to 5 μL/min using a flow splitter. The eluents used were as follows: (A) 5% ACN in 0.08% FA and 0.01% TFA and (B) 95% ACN in 0.08% FA and 0.01% TFA. Peptides were separated on a capillary column (C_{18}PepMap, 15

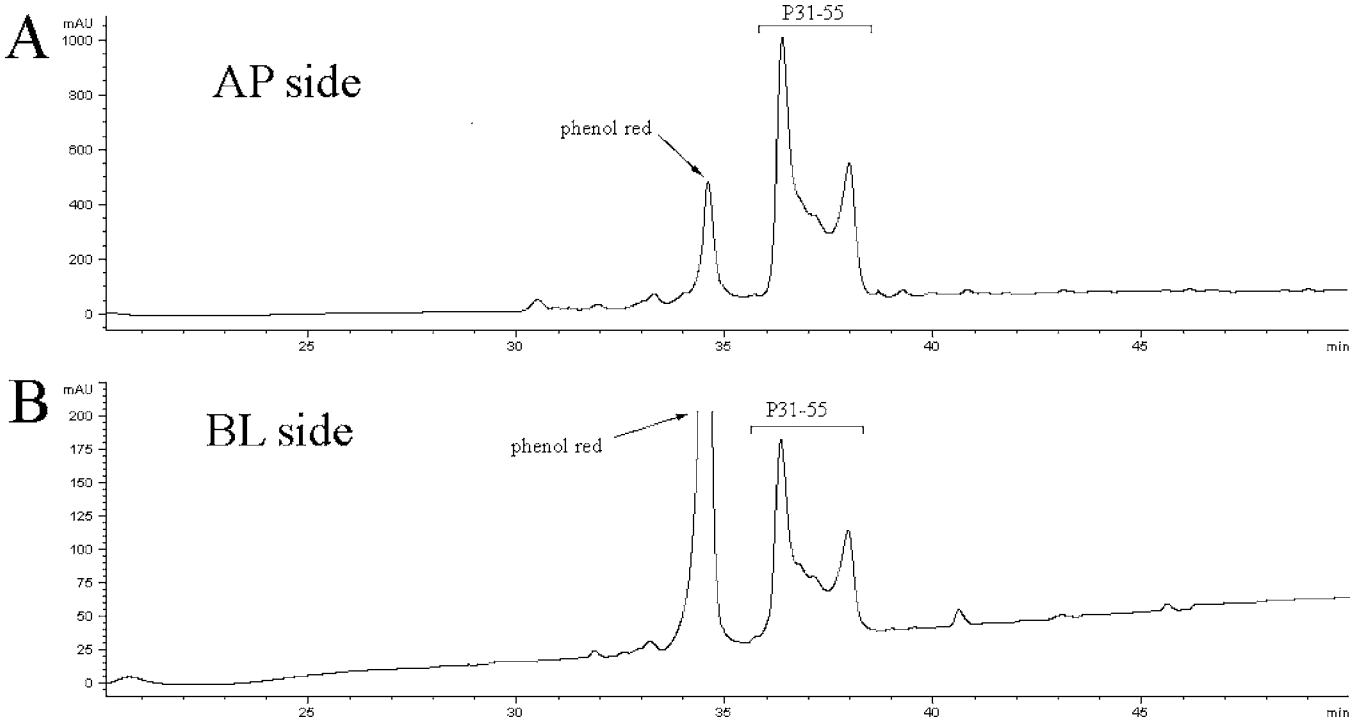

Figure 1. HPLC-UV analysis of P31−55 recovered from AP (A) and BL (B) compartments after 4 h of incubation time.

cm length, 300 μm i.d., 300 Å; LC Packings) using a linear gradient of 5−40% B over 60 or 90 min. For MS/MS analysis, a Q-Star Pulsar mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray ion source was used. Experiments were performed in IDA mode. Precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: ions greater than 400.0 m/z, charge state of 2−4, intensity exceeds 15 counts, former target ions excluded for 60 s, and ion tolerance of 50.0 mmu. Ions were fragmented by collision-induced decay (CID) using nitrogen as the collision gas.

Circular Dichroism Spectropolarimetry. Circular dichroism was performed on homogeneous samples of P31−55 at a concentration of 0.2 mg/mL in 2 mM Tris/HCl, pH 7.4. We used a J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Neslab RTE-110 temperature-controlled liquid system (Neslab Instruments, Portsmouth, NH).

Far-UV circular dichroism spectra were recorded in a 1 mm path length cell from 250 to 190 nm with a step size of 0.1 nm, a bandwidth of 1.0 nm, and an averaging time of 4 s. For all spectra, an average of five scans was obtained. Circular dichroism spectra of the appropriate buffers were recorded and subtracted from the peptide spectra. Before undergoing circular dichroism analyses, all samples were kept at the experimental temperature for 5 min.

Statistical Analysis. Each experiment was performed at least three times. Student's t tests were used to analyze the statistical significance of the results. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Caco-2 Cells Experiment. Previous studies have suggested that the gastropancreatic hydrolysis of recombinant A-gliadin and whole gliadin digests from wheat results in the release of P31−55. Further treatment with BBM hydrolase from human gut does not affect the peptide sequence.[18] To verify the permeability of P31−55, confluent Caco-2 cells were grown on transwell filter systems. Despite their colonic origin, the human Caco-2 cell line is considered a useful model of the intestinal epithelium for predicting absorption by jejunal enterocytes.[21,22] Caco-2 monolayers have previously been used to study the effects of gliadin on metabolism, oxidative balance, apoptosis, and permeability.[11,23−26] This study was conducted using synthetic P31−55 and PTCCE, which contains the natural form of P31−55 that is released following in vitro digestion of whole gliadin.[18] The samples were applied to the AP compartment of Caco-2 monolayers and collected from the BL compartment after a 4 h incubation. The integrity of the mucosal aspect of the monolayers was verified before and after the experiment by observing the cells under phase contrast microscopy. The TEER remained almost unmodified during the experiment, at approximately 700 Ω/cm^2, indicating that the membrane was undamaged and that tight junctions were well formed. The incubation of the cells with synthetic P31−55 did not change the TEER value, while as expected, the addition of PTCCE induced a drastic decrease to 250 Ω /cm^2. This finding is in accordance with a study by Sander and co-workers who proposed an increase in permeability upon treatment with whole gliadin digests treatment.[26]

The permeability of synthetic P31−55 was verified by HPLC-UV analysis of samples collected from the BL compartment of the Caco-2 monolayer. No additional peaks were observed other than those from the intact P31−55 loaded into the AP compartment, indicating that P31−55 was not hydrolyzed by BBM peptidases during trans-epithelial translocation (Figure 1A,B). As noted in our previous study,[18] the chromatogram of P31−55 showed an atypical profile that consisted of a double peak. The MS/MS spectra of the two peaks gave fragmentation patterns that were ascribed to P31−55 (Figure S1 in the Supporting Information), ruling out the presence of impurities or aggregation. These data suggest that the two resolved forms might be conformational isomers.

Natural P31−55 contained in the PTCEEE sample was also analyzed by LC-MS/MS (Figure 2). The total ion current (TIC) chromatogram of samples collected from both compartments showed a high complexity of the peptide mixture,

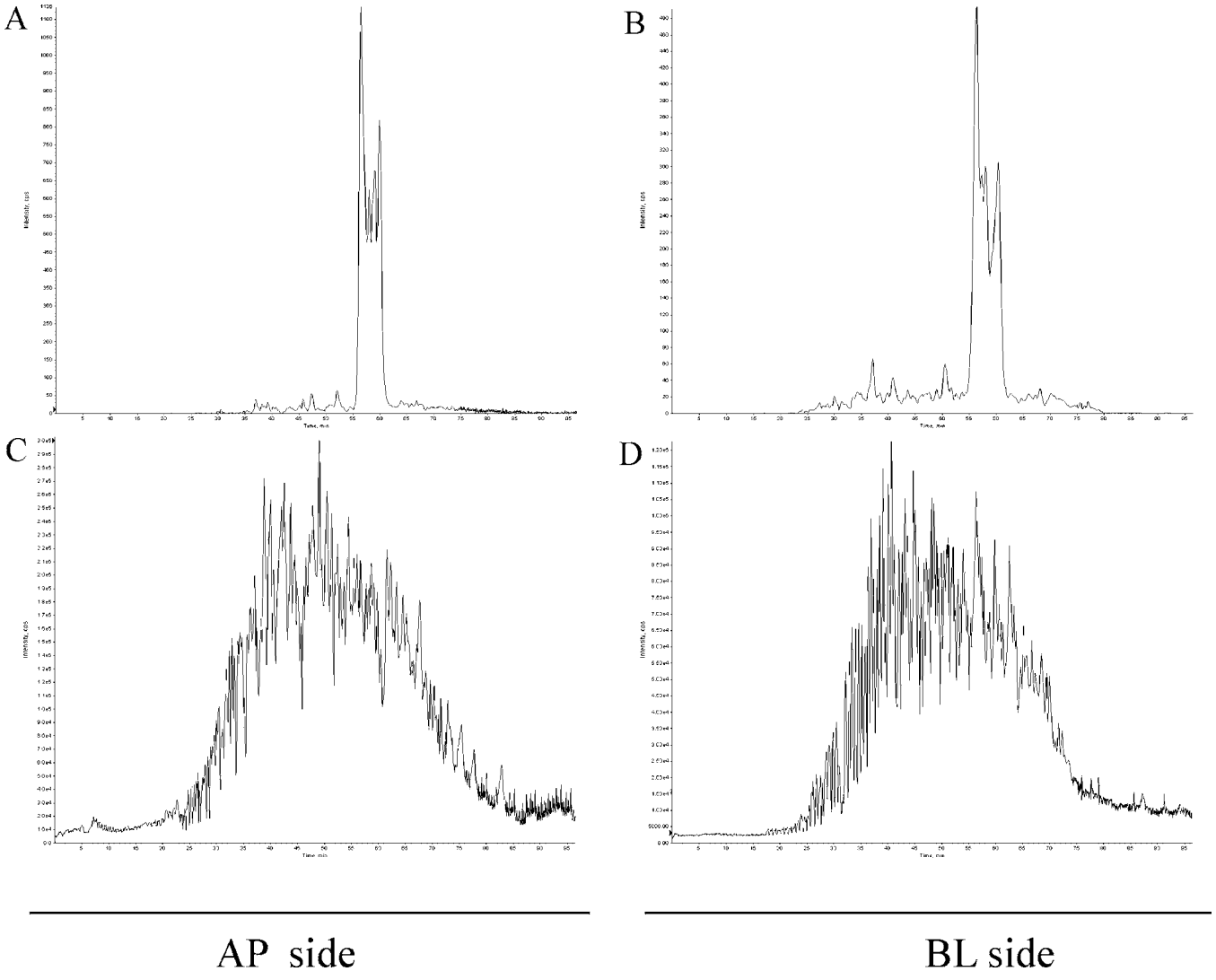

Figure 2. LC-MS/MS analysis of PTCEE samples recovered from AP (C) and BL (D) compartments after 4 h of incubation time. P31−55 was identified by ion extraction of doubly $[(M + 2H^+)^{2+} = 1461.7]$ and triply charged $[(M + 3H^+)^{3+} = 974.8]$ in AP (A) and BL (B) compartments.

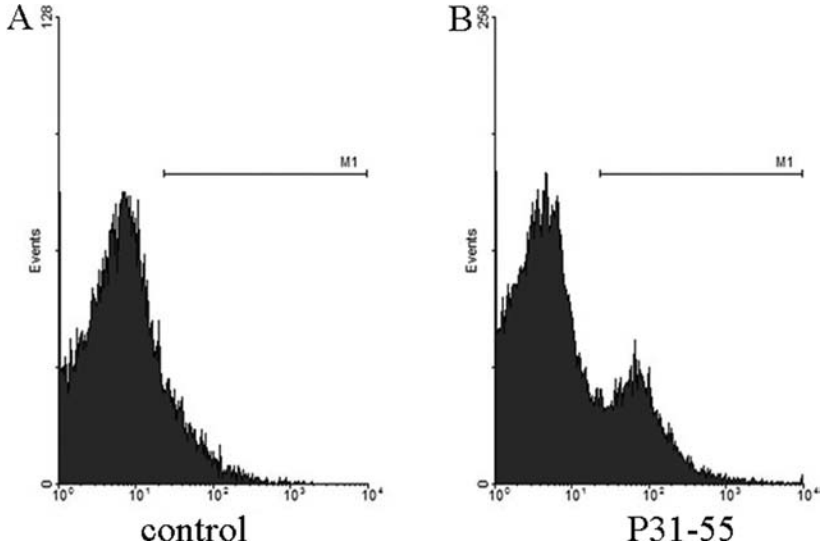

Figure 3. FACS analysis of Caco-2 cells incubated with FITC-tagged P31−55. After 60 min of incubation time, more than 15% of the Caco-2 cell population resulted in FITC-positive in comparison to control.

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because of the high molecular heterogeneity of wheat containing hundreds of gliadin species (Figure 2C,D). P31−55 was identified in PTCEE sample by software extraction of the doubly $[(M + 2H^+)^{2+} = 1461.7]$ and triply charged $[(M + 3H^+)^{3+} = 974.8]$ ions (Figure 2C,D). These signals occurred at the expected retention time (Rt) and emerged under the characteristic double peak (panels A and B). Comparison of the AP and BL samples in the PTCEE chromatogram showed that a high number of epitopes were permeable in addition to P31−55.

Translocation of 25-mer Fragments. Simulated intestinal uptake was analyzed by incubating Caco-2 cells in the presence or absence (control) of FITC-tagged P31−55. FITC-positive cells were counted by FACS analysis (Figure 3). The distribution of the Caco-2 cells appeared very similar with respect to granulation and size as compared to the total cell population according to the SSC/FSC dot blot. After 1 h of incubation, 15% of the Caco-2 cell population was FITC-positive in comparison to the control. A swift uptake was detected by flow cytometry, which confirmed that P31−55 could be efficiently internalized into cells. Microscopy analysis was used to obtain merged phase contrast and fluorescence images and confirmed the intracellular internalization of the FITC-tagged peptide (Figure S2 in the Supporting Information).

HPLC-UV Analysis of P31−55. The LC-MS analysis of both synthetic and natural P31−55 showed two partially resolved peaks (Figure 1). We assumed that this atypical behavior was most likely a consequence of isomerization of two conformers. At room temperature (25 °C), the peptide eluted at an approximately 60:40 molar ratio based on peak area (Figure 4A). Each HPLC-UV fraction that was collected and reinjected into the column regenerated these two peaks, which eluted in positions corresponding to the original fractions (not showed). For different peptides, it has been demonstrated that two adjacent prolines may cause the presence of slow interconverting cis−trans isomers with respect to the amide bond in solution.[27−30] P31−55 includes nine proline residues, two of which are contiguous in positions 38 and 39. The partial rotational hindrance around the Pro−Pro bond may explain the slow mutual conformational interconversion that causes peak splitting during HPLC analysis. Because the rate of proline isomerization is a function of temperature, the HPLC analysis was repeated but with sample that was previously incubated at 4 °C and a column that was chilled in ice (Figure 4 B). The low temperature induced a decrease in the rate of the interconversion, and two resolved peaks were observed. On the contrary, elution at high temperatures up to 50 °C led to an acceleration of the conformational rotation, resulting in coalescence of the two conformers that produced a sharp unique peak (Figure 4C). A chromatographic temperature gradient assay highlighted how the structures of the conformers were sufficiently different to cause them to interact differently with the hydrophobic adsorbing surface of the C18 column, resulting in different Rt values.

Additional evidence of the influence of the configuration of the Pro38−Pro39 residues on the isomerization of P31−55 was obtained by analyzing two synthetic analogues of P31−55 in which Ala was substituted for either Pro38 or Pro39. Mutation of each residue in the synthetic peptide resulted in a unique single peak, which was independent of column temperature (Figure 3S in the Supporting Information).

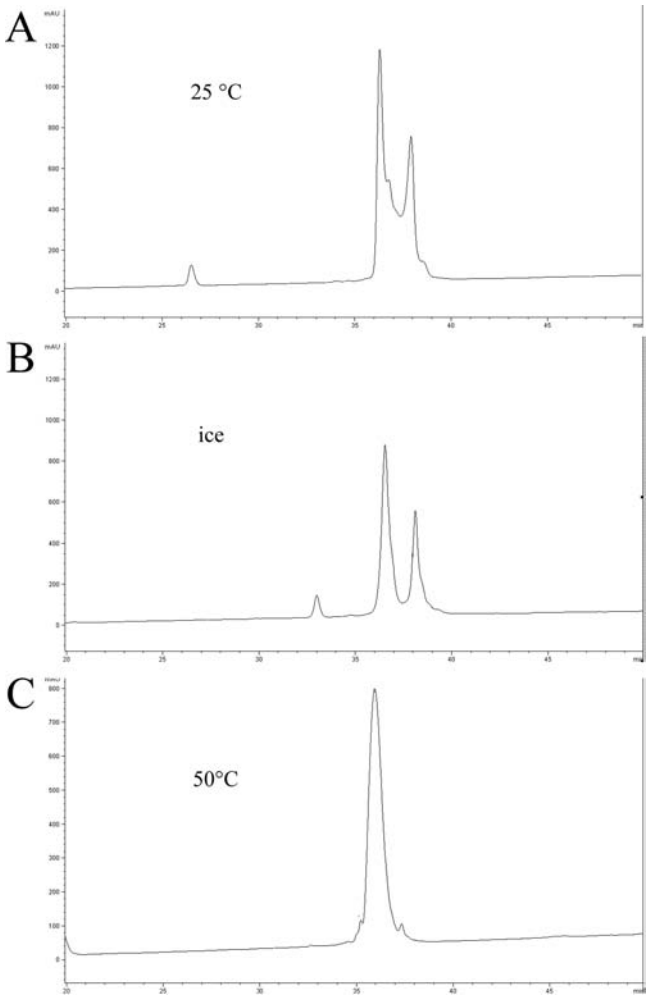
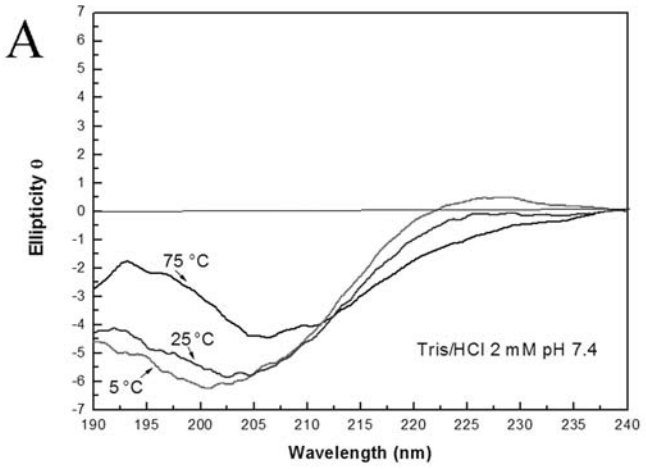


Figure 4. Effect of column temperature on the peak shape of P31−55. At room temperature (25 °C), peptide eluted atypically in double peak (A). At 50 °C (C), P31−45 eluted in a single peak, while when analyzed at low temperature (column chilled in ice), peptides eluted in two well-resolved peaks (B).

The influence of temperature on retention time and peak shape was also investigated using truncated forms of P31−55: P31−43 and P44−55. The latter peptide gave a single resolved peak independent of column temperature (not showed), while the former showed a similar behavior to P31−55 (Figure S4 in the Supporting Information); even at room temperature, P31−43 was less resolved, indicating a more rapid isomerization of the two conformers.

Circular Dichroism of P31−55. The global secondary structure of P31−55 was studied by CD at temperatures between 5 and 75 °C (Figure 5A). At 25 °C, the peptide showed a prevalently unordered structure, which was characterized by a broad negative extremum centered at 205 nm and a weak shoulder at 225−230 nm. By increasing the temperature up to 75 °C, the spectrum changed markedly. An increase in ellipticity in the 212−235 nm region was observed when the temperature was raised from 5 to 75 °C (Figure 5 and Figure S5 in the Supporting Information). The spectra exhibited an isodichroic point at approximately 212 nm, which indicated constant ellipticity as a function of temperature and suggests that at least two conformers were present and existed in equilibrium. The secondary structure content, as evaluated by the method of Yang et al. [31] at 5, 25, and 75 °C, is



B

Temperature	α-helix	β-sheet	turn	random
5 °C	0.0 %	50.8 %	10.5 %	38.8 %
25 °C	0.0 %	54.8 %	8.2 %	37.0 %
75 °C	5.4 %	49.1%	10.9 %	34.7 %

Figure 5. (A) Far-UV circular dichroism spectra of P31−55 at 5, 25, and 75 °C. (B) Evaluation of secondary structure content at 5, 25, and 75 °C.

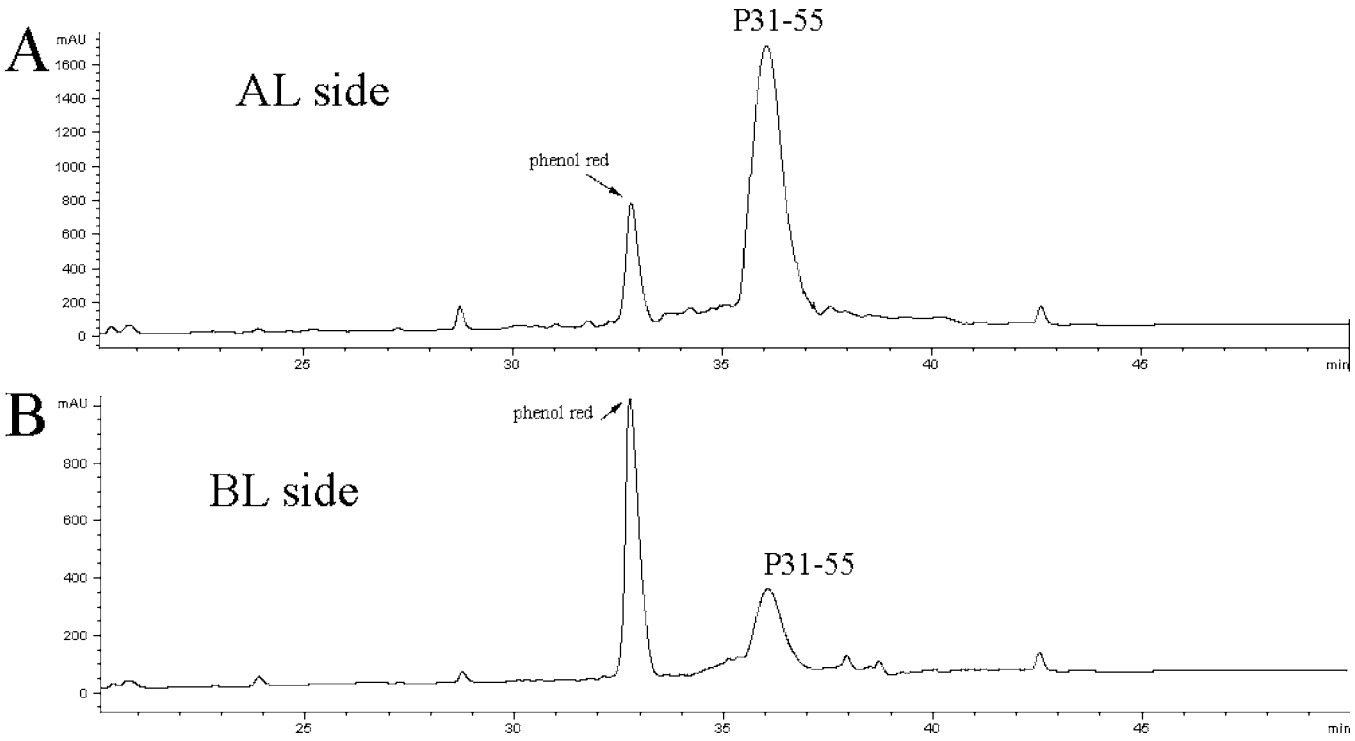


Figure 6. HPLC-UV analysis of P31−55 peptide from the AP and BL compartments after 4 h of incubation and analyzed by HPLC-UV. Chromatography was performed at 50 °C. Comparing the UV peaks, 22% of P31−55 was estimated to have crossed the Caco-2 epithelial barrier.

summarized in Figure 5B. The obtained value shows a variation in secondary structure content with increasing temperature. In particular, at 75 °C, an increase in the α-helix content was observed with respect to that at 5 °C.

Quantitative Recovery of P31−55 through the Caco-2 Monolayer. Synthetic P31−55 peptide was recovered from the AP and BL compartments after 4 h of incubation and analyzed by HPLC-UV (Figure 6). Chromatography was performed at 50 °C to allow P31−55 to elute in a unique resolved peak (Figure 4C). By estimating the area ratio under the UV peaks from AP and BL samples, 22% of the synthetic P31−55 was estimated to have crossed the Caco-2 epithelial barrier. Analogous results were achieved by analyzing the AP and BL samples by LC-MS (not shown).

The relative quantitative recovery of natural P31−55 from PTCEE was determined by comparing the ion intensity of triply charged $[(M + 3\ H^+)^{3+} = 974.8]$ (Figure S6 in the Supporting Information). A higher percentage of P31−55 was able to cross the epithelium (35%) because of the alteration of the TEER in the monolayer. This result indicates that because

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of a probable synergistic effect, the total amount of P31−55 and that of all of the other toxic or immunogenic peptides may reach levels well above that of the single peptide, when complex mixtures of gliadins-derived peptides are applied to Caco-2 monolayers.

DISCUSSION

Over the years, various studies have aimed to identify gliadin peptides that reach unhydrolyzed the lamina propria where they can exert an immunogenic or toxic effect in CD patients. Traditionally, these studies have attempted to identify immunogenic peptides that stimulate specific T-cell lines and clones derived from the jejunal mucosa or peripheral blood of celiac patients. On the contrary, only a few toxic peptides are known to be able to induce mucosal damage when added in culture to duodenal mucosal biopsies or when administered in vivo to the proximal or distal intestine.[6] Both classes of peptides are greatly resistant to proteases, which allows them to reach and cross the intestinal epithelium intact. A digestion model that mimics in vivo gastric−pancreatic intestinal processes has been applied to gliadin proteins.[9,18] The human Caco-2 cell line has been used as a model of the gut epithelium to predict the physiological behavior and the uptake of some gliadin peptides. Transport studies across Caco-2 monolayer represent one of the most widely accepted models of in vitro permeation of drugs and peptides as they well correlate, at least under a qualitative standpoint, with intestinal absorption in vivo.[32] The epithelial translocation of a canonical immunogenic peptide 33-mer by transcytosis was observed in Caco-2 cells.[23] Similarly, the permeability of toxic p31−43 and p31−49 across Caco-2 cell layer was also demonstrated.[33,34] Barone et al. showed that toxic P31−43 enters Caco-2 cells, interacts with the endocytic compartment, and interferes with the endocytic pathway by delaying the maturation of early endosomes into late endosomes.[12]

Our previous study proved that canonical toxic P31−43 and P31−49 are not released by enzymatic hydrolysis but are encrypted within P31−55, which is resistant to in vitro gastric and pancreatic digestion and is not susceptible to hydrolysis by BBM enzymes.[18] The toxicity of P31−55 was established in vitro in organ culture of treated biopsies.[15,16]

In the current experiment, we demonstrated that P31−55 crossed the intestinal epithelium simulated by a Caco-2 monolayer without being degraded by hydrolysis. Although the TEER measured was high enough to ensure paracellular tightness, ∼22% of the synthetic P31−55 added to the AP compartment was transported to the BL compartment after 4 h of incubation (Figure 6). FACS (Figure 3) and microscopy (Figure S2 in the Supporting Information) analysis revealed an active uptake of P31−55, suggesting that the translocation of this peptide involves the transcellular rather than the paracellular pathway. However, deeper investigation of the transport mechanism with selective inhibitors is required to confirm this hypothesis.

Unlike synthetic P31−55, PTCCE rapidly decreases the permeability of Caco-2 cells.[26] The effect of whole gliadin digests on permeability was demonstrated in previous studies. Specific gliadin peptides induce modifications in the actin cytoskeleton and junctional proteins, which in turn alter the barrier function of the monolayer.[26,35] This mechanism is regulated by zonulin, a protein that is released from Caco-2 cells upon gliadin treatment.[36] The alteration of the TEER causes an increase in the translocation of natural P31−55 up to 35% (Figure S6 in the Supporting Information). At the same time, chromatographic profiles of PTCEE samples from the AP and BL compartments showed that a large number of peptides were permeable (Figure 2C,D), and their characterization deserves further peptidomic investigation. Preliminary MW exploration by MS revealed that the peptides from the BL side were all smaller than 4000 Da (data not shown), indicating that the permeability linearly decreased with the peptide size, in agreement with previous determinations.[37] The natural release of P31−55 during the gastropancreatic digestion of whole gliadin and its transport across Caco-2 monolayers further strengthens the result that P31−55 is digestion-resistant throughout the gastrointestinal tract.[18] This achievement represents a turning point in the study of CD because the studies conducted to date on innate immunity in CD have all been performed using peptides (i.e., P31−43 or P31−49) whose actual release in vivo has never been established. Matysiak-Budnik et al. demonstrated that AP to BL transport and processing of P31−49 are severely altered in active CD, leading to the release of intact peptides on the basal side, whereas the same peptide is almost entirely degraded during its intestinal transport in control individuals and treated CD patients.[38,39] In our opinion, these experiments should also be revised using the model P31−55 because the size and shape of the peptide affects the action of the BBM enzymes in addition to intestinal translocation. To this purpose, it has to be considered that the 33-mer peptide (LQLQPFPQPQLPYPQ-PQLPYPQPQPF) is highly resistant to gastrointestinal digestion, but its shortened form (P56−68, LQLQPFPQP-QLPY), which corresponds to the first 13 residues, is rapidly degraded by BBM.[18,38]

Our experiments also revealed that P31−55 exhibits some unique biochemical characteristics. Circular dichroism spectra showed a markedly changed of global secondary structure of P31−55 increasing temperature from 5 to 75 °C (Figure 5 and Figure S5 in the Supporting Information). An anomalous chromatographic behavior of P31−55 was also detected by eluting peptides under different column temperatures (Figure 4). At room temperature, the peptide was present in two partially resolved peaks in equilibrium that coalesced into a single peak at elevated column temperatures. According to previous studies, this peak splitting is actually due to slow cis−trans isomerization of the imido peptide bond between two adjacent prolines. On-column cis−trans isomerization of the peptide bond at Pro−Pro is strongly visible by HPLC, as the rate of isomerization is typically too slow to permit the separation of the isomers[27,28] In this study, we demonstrated decisive evidence of the slow Pro38−Pro39 isomerization of P31−55 in that the selective substitution of each proline with an alanine residue resulted in the elution of the peptide as a single peak, which was independent of column temperature (Figure S3 in the Supporting Information). A similar behavior was also observed for P31−43 (Figure S4 in the Supporting Information), while its complementary sequence P44−55 was unaffected (not showed). Further NMR experiments are required to elucidate the cis/trans isomer for each proline residue.

The existence of isomerization between Pro38−Pro39 may have some relevant biological significance and could play an important role in the mechanism of toxicity in celiac subjects. This hypothesis is supported by an interesting study that demonstrated that modification of P38 and P39 abrogates peptide pathogenicity, as evaluated by morphometric analysis

on duodenal biopsy specimens of patients with CD.[40] We can hypothesize that P31−55 assumes different conformations of similar energy and that the hypothetical receptorial cavity is flexible. Indeed, a number of reports indicate that proline cis−trans isomerization plays the role of a molecular switch in receptor binding activation.[41,42] Representative examples to address the question of the bioactive conformation applying this concept are studies of various biological processes such as protein folding/refolding,[43] the immune response,[44] and opioid receptor recognition.[45] The major limitation of all of these considerations is that the receptor(s) activating innate immunity in CD are far from being fully revealed. Barone et al.[12] found that P31−49 is similar to amino acids 719−731 of human hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is a key protein in endocytic maturation.[46] In particular, the motif PPQQPY of P31−55 is common to human Hrs. This finding is interesting because Hrs is localized both in the cytosol and in endocytic vesicles and is involved in the transport of EGFR, PDGFR and other receptor-containing early endosomes and lysosomes.[47]

In summary, our study demonstrates that P31−55 is resistant to degradation throughout the gastrointestinal tract and is permeable across Caco-2 monolayers, providing fundamentally new insights into the naturally formed toxic peptides released from in vivo digestion. The identification of a conformers of P31−55 uncovers a new structural hypothesis on the activation of innate immunity. Future identification of receptor(s) will not only aid in understanding the molecular basis of CD but will also be useful in the design of selective inhibitors of P31−55.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

LC-MS analysis of P31−55 (Figure S1), uptake analysis performed by incubation of Caco-2 cells with the 31−55 FITC-tagged peptide (Figure S2), HPLC-UV analysis at room temperature of two modified forms of P31−55 (Figure S3), effect of column temperature on the peak shape of P31−43 (Figure S4), temperature dependence of CD spectra of the P31−55 (Figure S5), and extracted ion triply charged $[(M + 3H^+)^{3+}$ = 974.8 of P31−55 from LC-MS analysis of PTCCE-gliadin (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AP, apical; BL, basolateral; BBM, brush border membrane; CD, celiac disease; EGFR, epidermal growth factor receptor; FITC, fluorescein-5-isothiocyanate; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PET, polyethylene terephthalate; PTCCE, pepsin-trypsin-chymotrypsin-carboxypeptidase-elastase; TEER, transepithelial electrical resistance; TIC, total ion current

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