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Biochemical modifications of gliadins induced by microbial transglutaminase on wheat flour

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ABSTRACT

Background: Celiac disease (CD) is an immune-mediated disorder caused by the ingestion of wheat gluten. A life-long, gluten-free diet is required to normalize the intestinal mucosa. We previously found that transamidation by microbial transglutaminase (mTGase) suppressed the gliadin-specific immune response in intestinal T-cell lines from CD patients and in models of gluten sensitivity.
Methods: SDS-PAGE, Western blot, ELISA, tissue transglutaminase (tTGase) assay and nano-HPLC–ESI-MS/MS experiments were used to analyze prolamins isolated from treated wheat flour.
Results: Gliadin and glutenin yields decreased to 7.6 ± 0.5% and 7.5 ± 0.3%, respectively, after a two-step transamidation reaction that produced a water-soluble protein fraction (spf). SDS-PAGE, Western blot and ELISA analyses confirmed the loss of immune cross-reactivity with anti-native gliadin antibodies in residual transamidated gliadins (K-gliadins) and spf as well as the occurrence of neo-epitopes. Nano-HPLC–ESI-MS/MS experiments identified some native and transamidated forms of celiacogenic peptides including p31–49 and confirmed that mTGase had similar stereo-specificity of tTGase. Those peptides resulted to be 100% and 57% modified in spf and K-gliadins, respectively. In particular, following transamidation p31–49 lost its ability to increase tTGase activity in Caco-2 cells. Finally, bread manufactured with transamidated flour had only minor changes in baking characteristics.
Conclusions: The two-step transamidation reaction modified the analyzed gliadin peptides, which are known to trigger CD, without influencing main technological properties.
General significance: Our data shed further light on a detoxification strategy alternative to the gluten free diet and may have important implications for the management of CD patients.

1. Introduction

Coeliac disease (CD) is an immune-mediated enteropathy of the small intestine developed in genetically susceptible individuals and caused by the ingestion of wheat gluten and related prolamins present in barley and rye [1]. Currently, a lifelong, gluten-free diet (GFD) is required to alleviate symptoms of CD and to normalize the antibodies in the intestinal mucosa [2]. However, dietary compliance is poor, necessitating the development of new approaches to treat CD. Furthermore, gluten plays a key role in establishing the unique rheological properties and baking quality of wheat, which are only partially restored in GF products. Moreover, to improve palatability, many GF products are manufactured with purified wheat starch, which invariably contains residual gluten.

Gluten is essentially composed of two protein fractions, gliadins (alcohol-soluble) and glutenins (alcohol-insoluble) characterized by high levels of glutamine (30–35%) and proline (10–15%) residues that are involved in CD pathogenesis. In the small intestine of CD patients, specific glutamine residues are converted to glutamic acid by tissue transglutaminase (tTGase); this post-translational modification plays a major role in intestinal T cell activation [3]. Proline residues are resistant to intestinal proteases, ensuring the survival of immunostimulatory epitopes to digestion [4]. Interestingly, gliadin could be cleaved by bacterial prolyl endopeptidases (PEPs) into short peptides that lost their immunological activity [5]. Accordingly, PEPs have been evaluated as a technological tool for gluten detoxification. In particular, a 60-day diet of baked goods made from PEP-hydrolyzed wheat flour was found to be not toxic to CD patients [6].

We previously reported a novel approach to reduce gluten immunogenicity by transamidation using food-grade microbial transglutaminase (mTGase), a transamidase of the endo-γ-glutamine:ε-lysine transferase type [7]. Unlike tTGase, mTGase is a calcium-independent, low molecular weight protein, which has several advantages for food industrial

Abbreviations: CD, Celiac disease; tTGase, tissue transglutaminase; mTGase, microbial transglutaminase; GFD, gluten-free diet; spf, water-soluble protein fraction; K-gliadins, insoluble transamidated gliadin; PEPs, prolyl endopeptidases; K-C_2H_5, lysine ethyl ester; nano-HPLC–ESI-MS/MS, tandem mass spectrometry coupled with nano-reverse phase liquid chromatography

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applications [8]. This enzyme is commercially available as a dough improver that adds stability and elasticity to dough. Importantly, the covalent attachment of amino acids by enzymatic procedures is also a generally accepted means of improving the nutritional quality and functional properties of food proteins. More specifically, a previous study showed that the presence of isopeptide linkages in gliadins did not impair their digestibility [9], indicating that this treatment was safe. The final catabolic step in gluten transamidation occurs largely in kidneys, where ε-(γ-glutamyl)-lysine provides a substrate for γ-glutamylamine cyclotransferase [10]. Interestingly, mTGase was shown to exhibit a similar site specificity as tTGase on synthetic peptides, but lacked deamidase activity [11]. Most importantly, we found that the transamidation of gliadin following the treatment of wheat flour with mTGase and lysine methyl ester (K-CH_3) caused a dramatic down-regulation in IFNγ production *in vitro* in the intestinal T cells of CD patients [11]. Very recently, we demonstrated that wheat flour, following transamidation using a new "two-step" procedure, was selectively associated with positive changes in the phenotype of the antigen-specific immune response in models of gluten sensitivity [12].

In the present study, the biochemical features of gluten following the two-step transamidation reaction of wheat flour were examined. This enzymatic treatment was associated with a progressive production of new forms of gliadins with higher molecular weights that became soluble in water. Structural analyses of known celiacogenic epitopes of gliadin by tandem mass spectrometry coupled with nano-reverse phase liquid chromatography experiments (nano-HPLC–ESI-MS/MS) highlighted that isopeptide bonds involved glutamine residues already reported as tTGase deamidation sites [4,13–15], thus confirming that transamidation reactions performed on wheat flour could successfully prevent the formation of immunodominant gluten peptides. Furthermore, we evidenced for the first time the ability of mTGase to inhibit the activity of the celiacogenic p31–49 in Caco-2 cells.

2. Materials and methods

2.1. Transamidation reaction of wheat flour

A commercial preparation of bread wheat flour was used. A total of 100 g of flour was suspended in 8 volumes of 0.4 M NaCl and stirred for 10 min to extract albumin/globulins. The flour suspension was then centrifuged at 1000 *g* for 10 min, and the supernatant was discarded. The recovered pellet was exhaustively washed with water to eliminate any residual soluble protein, suspended in 1 volume of water containing 20 mM pharmaceutical grade lysine ethyl ester (K-C_2H_5; NutraBio.com, Middlesex, NJ, USA) and 8 U/g flour mTGase (ACTIVA®WM, specific activity: 81–135 U/g, Ajinomoto Foods Hamburg, Germany), and incubated for 2 h at 30 °C. The suspension was then centrifuged at 3000 *g* for 10 min, and the soluble protein fraction (spf) was recovered in the supernatant. In the two-step process, the pellet was suspended again in 1 volume of water containing a final concentration of 20 mM K-C_2H_5 and fresh mTGase (8 U/g flour) and incubated for 3 h at 30 °C. For the control, the sample was prepared using the same protocol in the absence of mTGase.

2.2. Peptide synthesis and transamidation reaction

Peptide p31–49 was synthesized by GeneCust Europe Laboratoire de Biotechnologie du Luxembourg S.A (Dudelange, Luxembourg). Purity (>95%) was assessed by the manufacturer by means of RP-HPLC and mass spectrometric analyses. Transamidation reaction of the peptide was carried out in 25 mM ammonium bicarbonate (pH 8.3) containing 20 mM K-C_2H_5 and 0.015 U/10 μg peptide mTGase at 37 °C for 30 min.

2.3. Purification of protein fractions

The gliadin and glutenin fractions were extracted from the protein pellet using a modified Osborne procedure [16]. Protein content was assessed by Bradford analysis [17] and protein pattern was qualitatively analyzed by 8–16% SDS-PAGE and Coomassie R-250 blue staining.

2.4. Western blotting of gliadins

Protein aliquots (50 μg) were fractionated by 12% SDS-PAGE, blotted onto ImmobilonTM PVDF membranes (Millipore, Billerica, MA, USA) and probed with in-house produced mouse anti-sera toward native gliadins (1:10,000 dilution), transamidated gliadins (K-gliadins; 1:10,000 dilution) or spf (1:10,000 dilution). After washing, the membranes were incubated with peroxidase-conjugated antibodies against mouse IgG (Dako SpA, Milano, Italy, 1:4000 dilution). Finally, immunodetection was performed using Hyperfilm and ECL reagents (Amersham-GE Healthcare Europe GmbH, Glattbrugg, Switzerland). In some experiments anti-K-gliadin antiserum (1 mL, 1:1000 dilution) was pre-adsorbed with native gliadins (10 mg) for 2 h at 37 °C.

2.5. Chymotryptic digestion of gliadins

Enzymatic digestion was performed in 50 mM Tris–HCl (pH 8.5), at 37 °C for 4 h using an enzyme to substrate ratio of 1:50 w/w.

2.6. Nano-HPLC–ESI-MS/MS experiments for peptide structural analyses

Nano-HPLC–ESI-MS/MS experiments were carried out on a Q-TOF Micro instrument equipped with a nano-electrospray source (Z spray) and a capillary liquid chromatography system (CapLC, Waters, Milford, MA, USA). Samples (1 μg of chymotryptic digest) were loaded, concentrated and desalted on a pre-column Symmetry300 C18 Trap Column, 0.18 × 23 mm, 5 μm (Waters) at 20 μL/min flow rate. Peptides were separated on a Atlantis dC18 3 μm 75 μm × 150 mm nano-column, (Waters) using 2% Acetonitrile in 0.1% Formic acid as eluent A and 98% Acetonitrile in 0.1% Formic acid as eluent B and a linear gradient from 2 to 60% B over 58 min and from 60 to 95% B over 5 min. Flow rate was split from 5 μL/min to approximately 220 nL/min. TOF analyzer was calibrated in MS/MS mode using Glu1-Fibrinopeptide B (doubly charged ion at m/z 785.84, 1 pmol/μL (Waters) in the m/z range 50–1500). MS mode acquisition was performed over the m/z range 350–1500.

Collision induced dissociation experiments (MS/MS) were carried out on multiply charged molecular ions to generate peptide fragments using argon as collision gas. The instrument was operated in the MS survey scan mode (Data Dependent Acquisition — DDA). MS to MS/MS switch criteria were as follows: threshold 20 counts/s, charge states from +2 to +5. MS/MS spectra were acquired in the m/z range 70–1500. Experiments were carried out in duplicate.

Mass spectrometric data obtained from the analysis of native gliadin samples were used for database searches against the NCBInr database by means of the proteomic tool ProteinProspector Batch-tag Web (http://prospector.ucsf.edu), in order to identify peptides of interest containing immunogenic gliadin epitopes already reported in literature [4,13–15]. Parameters for all searches were as follows: no taxonomic category, no enzyme, neither of fixed and variable modifications, error window on experimental peptide mass values 0.2 Da and on MS/MS fragment ion mass values 0.1 Da.

Transamidated forms of the previously selected peptides of interest were identified in nano-HPLC–ESI-MS profiles of K-gliadins and spf chymotryptic digests and modification sites were defined by manual interpretation of the corresponding MS/MS spectra.

Semi-quantitative analyses on the native and modified chymotryptic peptides of K-gliadins and spf were carried out by means of tools of MassLynx software 4.1 (Waters) to measure the integrated peak area from the extracted ion chromatograms of the doubly charged ions generated by native and modified forms of selected peptides.

Matrix Assisted Laser Desorption Ionization–Time of Flight-Mass Spectrometry (MALDI–TOF-MS) and nano-HPLC–ESI-MS/MS experiments were performed to define the extent of modification