Susceptibility to transglutaminase of gliadin peptides predicted by a mass spectrometry-based assay

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Abstract A peptidomics approach was developed to identify transglutaminase-susceptible Q residues within a pepsin–trypsin gliadin digest. Based on tagging with a monodansylcadaverine fluorescent probe, six α/β-, γ-gliadin, and low molecular weight glutenin peptides were identified by nanospray tandem mass spectrometry. In functioning as an acyl acceptor, tissue transglutaminase was able to form complexes with the glutamine-rich gliadin peptides, whereas by lowering pH, the peptides were deamidated by transglutaminase at the same Q residues, which were previously transamidated. The main common feature shared by the peptides was the consensus sequence Q-X-P. Our findings offer relevant information for the understanding of how dietary peptides interact with the host organism in celiac disease.

Key words: Transglutaminase; Celiac disease; Gliadin; Mass spectrometry; Peptidomics

1. Introduction

Celiac disease (CD) is an enteropathy of the small intestine induced by dietary exposure to gluten proteins and other prolamins [1]. As a result of gluten ingestion CD patients develop the anti-gliadin antibody and anti-tissue transglutaminase (tTG) autoantibodies [2]. tTG is a calcium-dependent enzyme capable of deamidating peptide-bound glutamine (Q) residues to glutamic acid (E) and cross-linking Q residues with ε-amino groups of peptide-bound Lys (K) [2,3]. The Q-X-P consensus sequence (X represents any amino acid residue) is mostly preferred by tTG [3].

Gluten proteins and derived peptides are also good substrates for tTG-catalyzed cross-linking given the high frequency of Q residues, which account for about 40% [4,5]. In addition to forming gliadin–gliadin complexes, tTG can be incorporated into high molecular weight complexes with gliadins [6], giving rise to novel antigenic epitopes [2]. Furthermore, it has been demonstrated that tTG can selectively deamidate gluten peptide, which results in strongly enhanced T cell-stimulatory activity [6–9].

In a slightly acidic environment, as in chronic inflammation, tTG-induced gliadin peptide cross-linking (transamidation) decreases, whereas deamidation increases [3]. Both post-translational modifications, transamidation and deamidation, play a key role in the pathogenesis of CD by generating a variety of haptens which, in addition to others, are responsible for an autoimmune response [2,3,6–9].

The majority of these studies have been carried out mainly on recombinant gliadin and synthetic peptides [6–9]. Gluten has been so infrequently used in such studies because the complexity of the chemistry of the cereal proteins has made it extremely difficult to find their toxic components. Previous studies have demonstrated that gliadin and glutenin are each composed of at least 50 closely related species [10].

Peptidomics has become an efficient tool for the rapid identification of peptides in complex mixtures, and could therefore facilitate identification of tTG-mediated modifications of peptides. In the present study, we developed a peptidomics-based procedure to individuate selectively, amongst the complex gliadin fraction of a wheat variety, the main gliadin peptides that are susceptible to tTG.

2. Materials and methods

2.1. Materials

When not specified, reagents were from C. Erba (Milan, Italy). Guinea pig tTG, monodansylcadaverine (MDC), pepsin (porcine stomach mucosa), goat anti-rabbit IgG alkaline phosphatase, and trypsin TPCK were from Sigma Chemical Co. (St. Louis, MO, USA). Guinea pig tTG (Sigma) was purified according to Leblanc et al. [11]. Wheat flour was obtained from a 25 kg grain lot of Italian winter variety (cv. Strampelli). Synthetic peptides and polyclonal antibody were from PRIMM (Milan, Italy).

2.2. Peptic–tryptic (PT) gliadin digestion and treatment with tTG

One milligram of gliadin purified according to Wieser et al. [12] was dissolved in 1 ml of 5% formic acid and incubated in a 37°C water bath with pepsin (1:100 enzyme:substrate ratio) for 2 h. Before trypsin digestion, the sample was evaporated and washed twice with deionized water. Trypsin was added at an enzyme/substrate ratio of 1:100 in 1 ml of 100 mM ammonium bicarbonate (pH 8) and the mixture was incubated for 4 h at 37°C. The reaction was stopped by heating for 5 min and the sample was evaporated and washed twice with deionized water. 1 mg of PT was incubated with tTG (1:50 enzyme:substrate ratio) at 37°C for 0, 2, or 4 h in 5 mM Tris–HCl buffer, pH 7.6, containing 2 mM MDC, 5 mM $CaCl_2$, 10 mM NaCl, and 10 mM dithiothreitol (final volume 1 ml). PT and synthetic pep-

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Abbreviations: tTG, tissue transglutaminase; CD, celiac disease; PT digest, pepsin–trypsin digest; MDC, monodansylcadaverine; LC/MS, liquid chromatography-electrospray mass spectrometry; nES-MS/MS, nano-ES tandem mass spectrometry

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tide deamidation were carried out with tTG (1:1 enzyme:substrate ratio) in the buffer used for transamidation without MDC brought to pH 6.8. The tTG-catalyzed reaction was stopped by adding 8 μl 0.4 M EDTA.

2.3. High performance liquid chromatography (HPLC) and liquid chromatography/electrospray mass spectrometry (LC/MS)

Liquid chromatography was performed using a 2.1 mm i.d.×250 mm, C18, 5 μm (Vydac, Hesperia, CA, USA) reverse-phase column with a flow rate of 0.2 ml/min on a HP1100 modular system (Hewlett-Packard, Palo Alto, CA, USA). Solvent A was 0.1% trifluoroacetic acid (TFA) (v/v) in water; solvent B was 0.1% TFA in acetonitrile. The column was equilibrated at 5% solvent. Separation of the peptides was effected with a gradient of 5–70% solvent B over 90 min. The column effluent was monitored both by UV detector (absorbance at 220 nm) and by fluorimetric detector (λ_{exc} 338 nm, λ_{em} 500 nm).

The effluent from the detector was injected on-line into a Platform (Micromass, UK) mass spectrometer equipped with a standard electrospray source via a 75 μm i.d. fused silica capillary. The mass spectra were scanned from 2000 to 400 atomic mass units at a scan cycle of 5 s/scan. The source temperature was held at 180°C and the cone voltage at 40 V. Mass scale calibration was obtained using myoglobin as reference compound.

2.4. Identification of MDC-tagged and deamidated peptide by nano-ES tandem mass spectrometry (nES-MS/MS)

Each HPLC fluorescent top peak was manually collected and analyzed by nES-MS/MS to identify peptides. A Q-STAR mass spectrometer (PE Sciex, Toronto, ON, Canada) equipped with a nanospray interface (Protana, Odense, Denmark) was used. The capillary voltage used was 800 V. Doubly and triply charged precursor ion isotopic clusters were selected by the quadrupole mass filter (MS1) and then induced to fragment by collision with collision energy varied between 20 and 40 V, depending on the mass and charge state of the peptides. The deconvoluted MS/MS spectra were manually interpreted with the aid of the Analyst software (PE Sciex).

The determined amino sequence was used as input to search the corresponding peptides in the NCBI database. The search was carried out using the PROTEIN PROSPECTOR/MS-PATTERN software (http://prospector.ucsf.edu).

2.5. Immunoblotting

One hundred microgram of both 19-mer synthetic peptide (LGQQQPFPPQQPYPQPQPF) and purified guinea pig tTG dissolved in a 1 ml pH 7.6 buffer was incubated for 4 h and at 37°C. The reaction was stopped by freeze-drying and the sample analyzed on standard 12.5% Tris-glycine sodium dodecyl sulfate (SDS) gel electrophoresis. 20 μg incubated protein was applied per lane and proteins were transferred to a nitrocellulose membrane sheet by a routine semi-dry blotting technique. Subsequently, the membrane was incubated overnight at 4°C in a pH 7.5 TBS buffer containing 10% horse serum as blocking solution followed by a 60 min incubation with anti-synthetic peptide antibody preparation, 1:1000 (v/v) diluted. After three washes with 10% horse serum the membrane was further incubated with an anti-rabbit goat IgG-alkaline phosphatase conjugate containing 0.1% Tween 20 (Bio-Rad, Hercules, CA, USA). To visualize adducts, an incubation with Western blue-stabilized substrate for alkaline phosphatase (Immuno-Star; Bio-Rad) was carried out.

3. Results

In order to detect gliadin peptides derived from gastric and pancreatic digestion possibly modified by tTG, we developed an analytical approach capable of selective probing of susceptible Q sites within these peptides. The whole gliadin fraction, isolated from a winter wheat flour variety according to the procedure described by Wieser et al. [12], was therefore digested with pepsin and trypsin. LC/MS of the PT digest showed the presence of an extraordinary number of peaks (Fig. 1A). Running the molecular mass value through data bank archives did not give us identification of the formed peptides. To compensate, we developed a basic strategy using

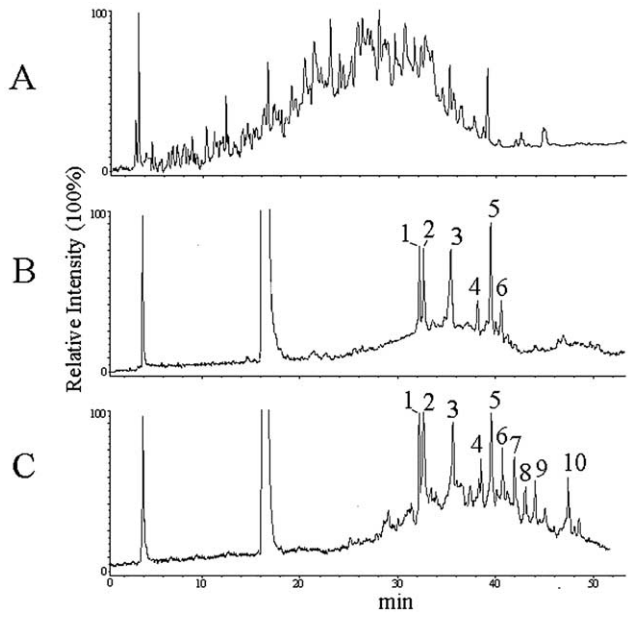

Fig. 1. HPLC analysis of the PT digest of the gliadin fraction isolated from a common wheat variety. Detection of peptides by (A) LC/MS, (B,C) fluorescence after PT 2 h and 4 h incubation, with tTG, MDC and calcium ions, in a pH 7.6 buffer. Numbered peaks were manually collected and each directly analyzed by nES-MS/MS analysis.

MDC as chemical label to identify the tTG-susceptible peptides in the complex PT digest. Fluorescent MDC enabled us to be selective in the labelling of Q residues in the presence of tTG by incubating the PT gliadin digest under conditions described in Section 2. The HPLC profile indicated the formation of six and 10 fluorescent peaks, after 2 and 4 h incubation time, respectively (Fig. 1B,C). Each HPLC peak top was characterized by nES-MS/MS. Peak 1 gave the spectrum in Fig. 2 corresponding to a Q-MDC-tagged peptide. The mass spectrum obtained by selecting the appropriate positively charged precursor (Fig. 2) displayed a complete '*b*-' and '*y*-ion' series. The localization within the peptide chain of the Q residue susceptible to tTG was evident in the characteristic fragmentation by the 446.22 Da loss corresponding to Q-MDC instead of 128.05 Da of the native Q residue. In this way, effective individuation of tTG-susceptible peptides was facilitated, while the interference from non-specific reagent binding peptides was minimized. In Table 1, the overall MDC-tagged peptides identified in the PT digest by tTG action are shown. The 14- (peak 1), 21- (peak 2), 14- (peak 3), 19- (peak 4), 18- (peak 5) and 25-mer (peak 6) peptides bound each MDC residue at sites Q^9, Q^{12}, Q^{10}, Q^4, Q^9 and Q^{16} in the respective chains, while other Q endoresidues were unaffected.

After 4 h incubation, tTG further modified Q residues of peptides 1, 3, 4, and 6, giving rise to the respective two-MDC derivatives (Table 1).

With this amino acid sequence information, we searched for a counterpart in data bank archives. All the identified sequence peptides differed from those reported in the archives by at least one amino acid substitution or deletion. The only exception was peptide 19-mer (α/β-gliadin 31–49), which was found unaltered (peaks 4 and 9, Table 1). Sequence information on peptides reported in Table 1 made fingerprinting possible for α/β-, γ-gliadin, and low molecular weight glutenin as

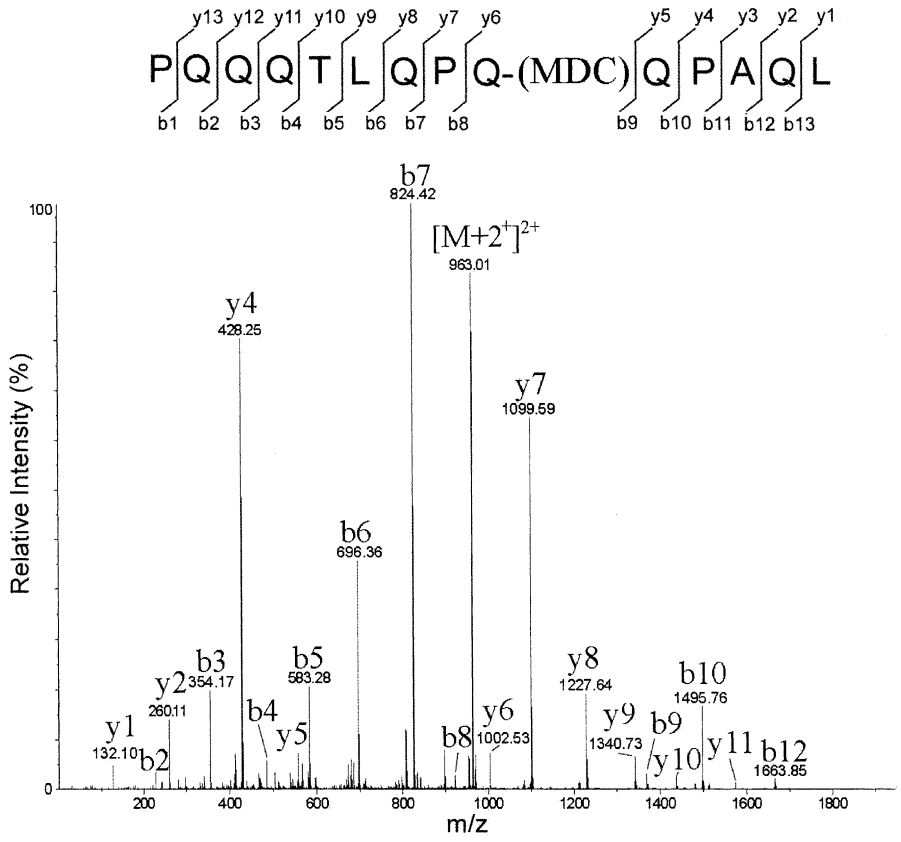

Fig. 2. nES-MS/MS analysis of the peptide isolated by HPLC (Fig. 1, peak 1) from the PT digest of gliadin incubated with tTG and MDC at pH 7.6. Q-(MDC) indicates a Q residue cross-linked with MDC.

parent protein. This means that the α/β-, and γ-gliadin and a glutenin fraction could be substrates for in vivo transamidation by tTG.

In addition, because gliadin proteins are rich in Q and poor in K residues [4,5], in the absence of MDC, tTG may act as an acyl acceptor and form complexes with gliadin peptides. Assays to detect gliadin peptide–tTG complexes were unsuccessful owing to the high heterogeneity of the reaction mixture and the very high molecular weight of the protein–peptide complexes. This heterogeneity would be resolved if, instead of a whole gliadin PT digest (mimicking in vivo digestion), single synthetic peptides amongst those identified were incubated with tTG. To this end, we developed polyclonal antibodies against the 19-mer peptide α/β-gliadin 31–49 (peaks 4 and 9, Table 1), known to be toxic for CD patients [13], as a putative substrate in forming peptide–tTG complexes. The choice of this peptide was determined also based on its high Q residue content; that is, a total of seven, with two belonging to the tTG-preferred consensus sequence Q-X-P [3]. SDS–polyacrylamide gel electrophoresis (PAGE) analysis carried out on the reaction mixture containing the gliadin peptide and tTG in the presence of calcium at pH 7.6 allowed us to detect a band of ~80 kDa recognized by the antibodies raised against the synthetic peptide (Fig. 3, lane 3). There was no reactivity in the corresponding tTG positive control (lane 2) with reference to tTG migration (lane 1). Notably, this clearly indicated that the 19-mer peptide formed relatively stable and covalent aggregates with tTG in the absence of other binding molecules.

As previously shown [3], the capacity of tTG to mediate gliadin peptides and protein cross-linking could be abolished by lowering the pH of the reaction to values around 6.8 in the absence of amines. In these conditions, water may act as an acyl acceptor substrate with subsequent deamidation of protein-bound glutamine residues [3]. To test this prediction in vitro, the PT gliadin digest was incubated with tTG at pH 6.8. An HPLC profile similar to that in Fig. 1A was obtained (not shown), showing unresolved the individual peptide species that differed by either one molecular mass unit or multiple values. Therefore, to evaluate the propensity of the peptides

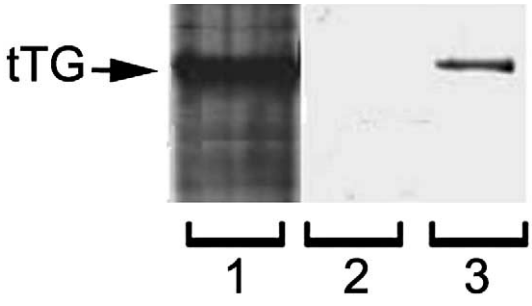

Fig. 3. SDS–PAGE separation of the 4 h incubated mixture of the synthetic 19-mer peptide (see peaks 4 and 9 in Table 1) incubated with a large tTG excess. Lane 1, reference tTG, staining with Coomassie blue. Lanes 2 and 3, staining with anti-19-mer antibodies against synthetic peptide; lane 2, reference tTG and lane 3, mixture of tTG and synthetic peptide incubated for 4 h. Control tTG showed no anti-peptide antibody binding.

Table 1
Identification by nES-MS/MS of MDC-tagged Q residue gliadin peptides isolated from the fluorescent PT digest by HPLC fractionation (Fig. 1B,C)

HPLC peak	MW measured	MDC number	Peptide identified by nES/MS-MS	Identification NCBI data bank
1	1922,00	1	P Q Q Q [T L / F P] Q P Q* Q P [A / Q] [Q L / Q]	γ–gliadin(105-118) AJ416336.1
2	2668,40	1	S H I [L / P] G [P / L] E R P S Q Q* Q P L P P Q Q T L	LMW-glutenin (19-39) AB062859.1
3	1953,01	1	[P / S] Q Q P F P S Q Q Q* Q P [L / V] I	γ–gliadin(173-186) AAA34285.1
4	2539,26	1	L G Q Q* Q P F P P Q Q P Y P Q P Q P F	α/β–gliadin(31-49) AJ133607.1
5	2467,23	1	Q P Q [/ L] P Y P Q P Q* L P Y P Q P Q P F	α/β–gliadin(71-89) AJ133612.1
6	3290,69	1	P Q L P Q F [L / P] Q P Q [/ L] P Y P Q P Q* L P Y P Q P Q P F	α/β–gliadin(64-89) AJ133612.1
7	2240,15	2	P Q* Q Q [T L / F P] Q P Q* Q P [A / Q] [Q L / Q]	γ–gliadin(105-118) AJ416336.1
8	2271,18	2	[P / S] Q* Q P F P S Q Q Q* Q P [L / V] I	γ–gliadin(173-186) AAA34285.1
9	2857,43	2	L G Q Q* Q P F P P Q* Q P Y P Q P Q P F	α/β–gliadin(31-49) AJ133607.1
10	3608,86	2	P Q* L P Q F [L / P] Q P Q [/ L] P Y P Q P Q* L P Y P Q P Q P F	α/β–gliadin(64-89) AJ133612.1

Q* indicates transamidated Q residues. Amino acid residues are indicated with a single letter nomenclature. Boxed residues indicate substituted or deleted amino acid residues along the peptide chain found under the indicated accession number in the NCBI Data Bank archive (lower line).

to deamidation, synthetic counterparts mimicking the natural gliadin components identified in Table 1 were used as tTG substrates. An example of the propensity of gliadin peptides to be deamidated is shown in Fig. 4, where the nES-MS/MS spectrum of 14-mer peptide (peaks 1 and 7, Table 1) evidenced the formation of E^2 and E^9 (Fig. 4B) from the native Q^2 and Q^9 (Fig. 4A). Similarly, all the other peptides shown in Table 1 were deamidated at the Q sites previously binding MDC, once they were incubated in conditions favoring deamidation by tTG.

4. Discussion

Identification of gliadin Q residues susceptible to cross-linking or to deamidation by tTG is a challenging task. Overlapping of peptides with similar size and hydrophobicity from

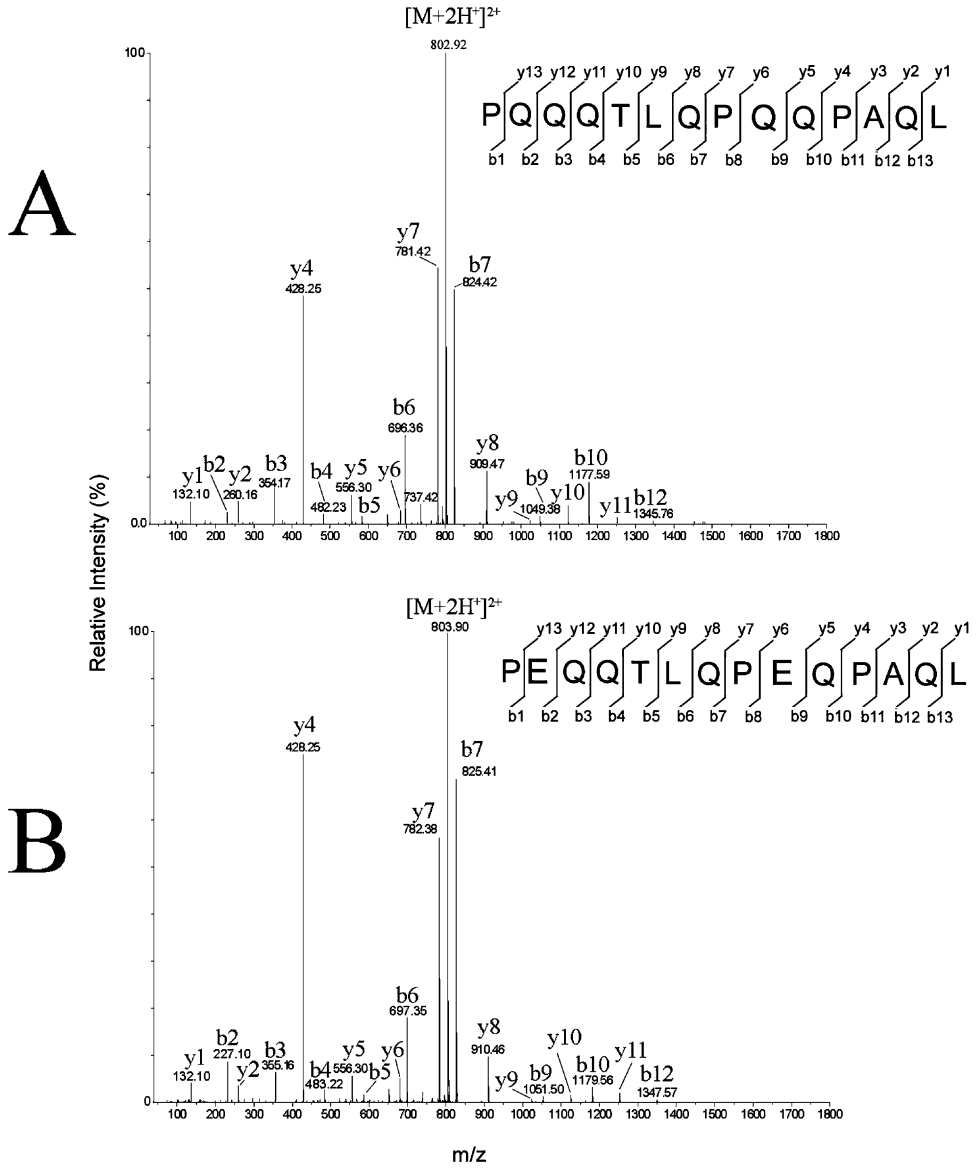

Fig. 4. nES-MS/MS analysis of the synthetic 14-mer peptide in the native (A) and deamidated form after tTG treatment (B). Peptide sequence is indicated at the top of the spectrum. The sequence of the tTG-deamidated peptide indicated native Q^2 and Q^9 were converted into E^2 and E^9.

HPLC fractionation represents one of the main problems while identification is further complicated by the analysis of a highly complex protein digest. After probing the complex mixture of the PT gliadin digest with MDC, and fractionation by HPLC, the transamidated components became fluorescent since they contain a selected consensus sequence targeted by tTG. They were sequenced in spite of the overlapping non-derivative peptides, which then did not interfere in the mass spectrum of the fluorescent peptide.

For the first time in a wheat flour, this procedure allowed us to identify, as tTG substrates in vitro, a few gliadin peptides having a specific amino acid composition amongst a myriad of other peptides. Contrary to what was expected, only a few gliadin peptides interacted with tTG even if a high content of Q residues was available along the gliadin chains [4,5]. Only one belonged to the Q-X-Q consensus sequence (peak 7, Table 1) while Q residues occupying the −2 position with respect to a P residue were preferentially modified, confirming previous data concerning tTG specificity [3].

Amongst the identified peptides, an 18 and a 25 residue long peptide (Table 1) were both internal to a 33-mer putative precursor, previously isolated from the recombinant α-gliadin digest [14] and already indicated as a possible trigger for the inflammatory response to gluten in CD patients [14]. This 33-mer peptide did not occur in the PT digest of gliadin flour. However, the 18-mer peptide (peak 5, Table 1) corresponded to a fragment of the 25-mer peptide (peaks 6 and 10) with seven truncated N-terminal residues. Compared to the peptide from recombinant gliadin [14], the natural sequence gliadin had substitution L^7 for P^7 (peaks 6 and 10, Table 1), meaning that the amino acid sequence of natural gliadin was sensitive to pepsin at the L^7-Q^8 bond, while the P^7-Q^8 peptide bond of recombinant gliadin was not. An additional peptide bond

sensitive to pepsin (or trypsin) was missing in recombinant gliadin since no homologous counterpart to the 25-mer peptide (peak 6, Table 1) was found in the PT digest. Therefore, drawing general conclusions from studies carried out on recombinant proteins should be avoided because the genetic evolution of cereals could generate new peptides with null or attenuated toxicity in CD patients.

The presence of MDC derivatives indicated that the native gliadin peptides are tTG substrates. In the absence of acyl acceptors and pH higher than 7, the immunoblotting experiment (Fig. 3) showed that gliadin peptides were perfectly capable of forming complexes with tTG, just like other structural proteins having a K site available. The conjugate constituted by one or more units of dietary peptides cross-linked to tTG, producing a net increase of antigen size, would be able to generate autoantibodies against tTG in CD patients. This reaction merely consists of a conjugation of exogenous peptide(s) to tTG as a carrier protein. In this case, a population of antibodies is produced by the living organism recognizing both the prolamin antigen and tTG itself, as this latter was modified by cross-linked gliadin peptides through an ε-(γ-glutamyl)-lysine (isopeptidyl) bond.

It can be generally concluded that in CD pathogenesis at pH above 7.0, it is possible that peptides with a high Q residue content are prevalently transamidated by tTG giving increased levels of serum antibodies specific for gluten, transglutaminase, and other antigens simultaneously. Hence, as the pH decreased from pH 7 [3], residual tTG-sensitive Q residue peptides crossing the lamina propria are deamidated and then become able to bind the HLA-DQ2/DQ8 molecule on antigen-presenting cells [6,9]. The identified gliadin peptides (Table 1), all found deamidatable by tTG, are good candidates for recognition by intestinal T cells from CD patients.

In conclusion, the outlined procedure could be used to probe peptides susceptible to tTG in any food, even in a duodenal mucosal biopsy, and to obtain their structural characterization. A positive response with the fluorescent probe would predict the presence of a specific tTG-susceptible Q residue determinant potentially toxic to a genetically predisposed subject. Therefore, a systematic screening of foods with this type of predictive test could help assure safer food for CD patients.

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