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Journal of Chromatography B, 855 (2007) 236–241
JOURNAL OF CHROMATOGRAPHY B
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Identification of a peptide from α-gliadin resistant to digestive enzymes: Implications for celiac disease

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Received 26 February 2007; accepted 7 May 2007
Available online 18 May 2007

Abstract

Current knowledge indicates that both innate and adaptive immune responses are involved in Celiac disease (CD) driven by different gliadin peptides. By studying a representative recombinant α-gliadin form, a further 25-mer peptide resistant to gastric, pancreatic, and human intestinal brush-border membrane enzymes was detected. This peptide latter encompasses the sequence 31–43 known to elicit the innate immune response in CD. The resistance of 25-mer, as well as that of the already described 33-mer related to the CD adaptive immune response, was confirmed on a standard flour wheat sample representative of the most widespread European varieties.

Keywords: Celiac disease; Gliadin; Brush-border membrane enzymes; Prolamin Working Group standard; Mass spectrometry

1. Introduction

CD is a common enteropathy induced by ingestion of wheat gluten proteins and related prolamins from oat, rye and barley in genetically susceptible individuals. The genetic predisposition is fundamental for the onset of the disease considering that 95% of celiac subjects express the HLA-DQ2 heterodimer and most of the remaining patients the HLA-DQ8 molecule [1]. The main toxic components of gluten belong to a family of closely related proline- and glutamine-rich proteins called gliadin. It is an extremely heterogeneous mixture of proteins that contains at least 50 components which can be assigned to three major groups (i.e. α/β-, γ- and ω-gliadins) [2], each group consisting of a mixture of different proteins [3,4]. The high percentage of proline residues makes gliadin resistant to gastric–pancreatic and intestinal digestive proteases, so that long gliadin fragments can reach high concentration levels in the gut epithelium [5].

The role of adaptive immunity in CD is very well defined. To date, a very high number of gluten peptides, deriving from α- and γ-gliadins, and recently from glutenins, have been reported to stimulate $CD4^+$ T lymphocytes isolated from the small intestinal mucosa of CD patients [6–8]. Importantly, most of these peptides became very potent T-cell stimulators only after deamidation by tissue transglutaminase, an enzyme present in the intestinal mucosa [9]. Although at least 50 T-cell stimulatory gluten epitopes in native or deamidated form have been identified, the 33-mer peptide is considered the most immunogenic including six overlapping epitopes [8]. The 33-mer peptide, released from recombinant α2-gliadin (Swiss-Prot accession number: Q9M4L6) through gastric–pancreatic enzyme digestion, is highly resistant to further digestion by intestinal brush-border enzymes (BBM) [8]. Hence, it has been suggested that the 33-mer could reach the underlying lamina propria (LP) and, following deamidation, play a central role in the pathogenic cascade of CD.

More recently, attention has been directed to the possible involvement of innate immune mechanisms in CD [10,11]. In particular, Maiuri et al. [11] showed that the synthetic

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doi:10.1016/j.jchromb.2007.05.009

peptide α-gliadin 31–43 (LGQQQPFPPQQPY) is able to up-regulate the expressions of interleukin (IL)-15, of the enzyme cyclooxygenase-2 (COX-2) and of the cell activation markers CD25 and CD83 on LP macrophages, monocytes, and dentritic cells, without stimulating $CD4^+$ T-cells. The sequence 31–43 is comprised in an α-gliadin (accession number: Q9ZP09), previously expressed in *E. coli* [12]. This sequence is very similar to that of A-gliadin from which a large amount of data on CD has been presented in the last years [13].

In the present study, we have characterised the peptides generated from the hydrolysis of Q9ZP09 α-gliadin resistant to digestive enzymes. Structural data have shown that only α-(31–55) peptide was stable to human hydrolases and BBM enzyme. Wheat gliadin behaved similarly, releasing both α-(31–55) and 33-mer peptides.

2. Experimental

2.1. Chemicals and reagents

Pepsin, trypsin, chymotrypsin and elastase, carboxypeptidase A, elestase, dithiothreitol (DTT), iodoacetamide, α-cyano-4-hydroxycinnamic acid, and sinapinic acid, were purchased from Sigma (St. Louis, Missouri, USA). All of the other reagents and solvents were of the highest purity available from Carlo Erba. Whole gliadine was a gift from Prolamin Working Group (PWG) [14].

2.2. Expression of recombinant α-gliadin

A recombinant alpha gliadin (Q9ZP09) was purified from *E. coli* essentially as described in Senger et al. [12]. Briefly, bacteria were grown until the OD at 590 nm reached 0.6; then gliadin expression was induced by adding 0.4 mM isopropyl β-D-thiogalactoside (Sigma) to the culture medium, followed by further culturing for 18 h. Harvested bacteria were washed once in 20 mM Tris/HCl pH 8.0, resuspended in 70% ethanol and incubated at 60 °C for 2 h. After removal of the bacteria cell debris by centrifugation, proteins were precipitated overnight by adding two volumes of 1.5 M NaCl. The precipitate was collected by centrifugation at 14,000 × *g* for 30 min, solubilised in 0.1 M Tris/HCl, pH 8.5 containing 2 M urea and subjected to purification as reported [12]. Protein samples were dried in a Speed-Vac centrifuge (Savant), lyophilized twice and stored at −20 °C.

2.3. Preparation of human BBM

The methods as described by Shirazi-Beechey et al. [15] were used with little modification. Frozen surgical specimens of intestinal jejunum (1 cm) were thawed in ice-cold 50 mM mannitol, 2 mM Tris/HCl pH 7.1. Cells were removed from the underlying connective tissue by using a Vibromixer (model E-1, Alpha Laval, UK) at max speed 2 × 1 min. The cell suspension was homogenised at maximum speed with an Ultraturrax T 25 (IKA, Works, Inc., USA). 500 mM $MgCl_2$ stock solution was then added to a final concentration of 10 mM. The suspension was stirred 20 min at 0 °C and then centrifuged at 3000 × *g* 15 min at 4 °C to eliminate cell debris, basolateral membranes, nuclei, and mitochondria. The supernatant was centrifuged at 30,000 × *g* 30 min at 4 °C. The pellet was resuspended in 300 mM mannitol, 0.1 mM $MgSO_4$, and 2 mM Tris pH 7.4 to give a protein concentration of 10–30 mg/mL and made homogeneous by passing several times through a 27-gauge needle; the brush-border vesicles were used immediately or stored in aliquots at −80 °C. Shortly before use, the vesicles were thawed on ice and washed with the buffer required for the planned experiments. The purification degree of BBM preparation was determined by measuring sucrase specific activity, as reported below. Sucrase activity was measured using a procedure based on that of Dahlqvist [16].

2.4. Preparation of synthetic peptides

The peptide 31–55 from α-gliadin (Q9ZP09) and the 33-mer peptides from α2-gliadin (Q9M4L6) were produced by solid-phase Fmoc synthesis by means of a Pioneer Peptide Synthesis System 9050 instrument (PE-Biosystems, Framingham, MA, USA). Peptides were purified by high-performance liquid chromatography (HPLC) and their identity was assessed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS)

2.5. Gastric–pancreatic and BBM digestion

Recombinant α-gliadin (100 mg), PWG gliadin (0.5 mg) and 25-mer synthetic peptide (10 μg) were each dissolved in 5% formic acid and incubated at 37 °C with pepsin (1:100 protease to protein, w/w ratio) for 30 min. Before pancreatic digestion, the samples were evaporated and washed twice with deionised water. Trypsin (1:100, w/w), chymotrypsin (1:100, w/w), elastase (1:500, w/w) and carboxypeptidase (1:100, w/w) were added in phosphate buffer (pH 7.0) and the mixtures were incubated at 37 °C for 1 h. The reaction was stopped by heating for 5 min and samples were freeze dried.

The samples from gastric–pancreatic digestion were dissolved in phosphate buffer pH 7.2 and incubated after BBM supplementation (650 mU/mg) for 1 h. The 25-mer was also incubated with BBM for 2 and 4.

2.6. HPLC analysis

Liquid chromatography was performed using a 2.0 mm i.d. × 250 mm, C18, 5 mm reverse-phase column (Phenomenex, Torrance, CA, USA) with a flow rate of 0.2 mL/min on a HP1100 modular system (Agilent, Palo Alto, CA, USA). Solvent A was 0.03% trifluoroacetic acid (TFA) (v/v) in water; solvent B was 0.02% TFA (v/v) in acetonitrile. The column was equilibrated at 5% solvent B. Separation of the peptides was effected with a gradient of 5–70% solvent B over 90 min. The column effluent was monitored by UV detection (220 nm) and each peak was manually collected.

For HPLC with positive ionisation mass spectrometry (LC–ESI/MS) the effluent from the column 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 1800 to 300 amu 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 a reference compound.

2.7. MALDI mass spectrometric analyses

MALDI MS experiments were carried out on a PerSeptive Biosystems Voyager DE-PRO instrument equipped with an N_2 laser (337 nm 3 s pulse width). Each spectrum was taken by the following procedure: 0.5 μL of HPLC peaks were loaded on a stainless steel plate together with 0.5 μL of α-cyano-4-hydroxycynnamic acid matrix (5 mg in 0.5 mL aqueous 50% acetonitrile) and dried under ambient conditions. Mass spectrum acquisition was performed in positive ion reflectron mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with mass peptide standards (Sigma).

MALDI MS/MS spectra were acquired on an Applied Biosystems 4700 TOF/TOF Proteomics analyzer, equipped with delayed extraction and a 200-Hz repetition rate UV laser (355 nm). Typically, 600 shots/spectrum were accumulated in the MS mode and 1000 shots/spectrum in the MS/MS mode. An external standard was used for mass calibration.

3. Results

3.1. Digestion of recombinant α-gliadin

Within the α-gliadin family, the Q9ZP09 has the most conserved sequence [12] including both the peptides 31–43 (LGQQQPFPPQQPY), and 56–68 (LQLQPFPQPQLPY). The former was recently recognised to elicit an innate immune response in CD [11], while the latter is a DQ2-restricted peptide [17]. In contrast, the Q9ZP09 gliadin does not contain the multivalent 33-mer peptide (LQLQPF-PQPQLPYPQPQLPYPQPQLPYPQPQPF) [8]. In order to identify the digestive enzyme-resistant bonds, hydrolysis was carried out with gastric (pepsin) and pancreatic proteases, i.e. carboxypeptidase A, chymotrypsin, elastase and trypsin. These enzymes were chosen on the basis of the major roles during the digestion process [18]. As result of the combined action of hydrolytic enzymes on Q9ZP09, gliadin 30 peaks appeared along the HPLC profile shown in Fig. 1A. Peaks singularly collected were analysed by MALDI MS/MS and the identified peptides shown in Table 1. The 25-mer LGQQQPFP-PQQPYPQPQPFPSQQPY (MH^+ 2922.41, residues 31–55) was identified in fractions 21 and 22. No shorter forms of this 25-mer peptide occurred in gliadin hydrolyzate indicating that this peptide could resist digestion in the stomach and upper small intestine. The 25-mer peptide harbours the 31–43 sequence of noteworthy interest for the innate immune response in CD [11].

Peptide 56–68, containing the immunodominant epitope PFPQPQLPY [17], was slowly digested by proteolytic enzymes because three shortened forms, i.e. 57–67, 57–68, and 56–67

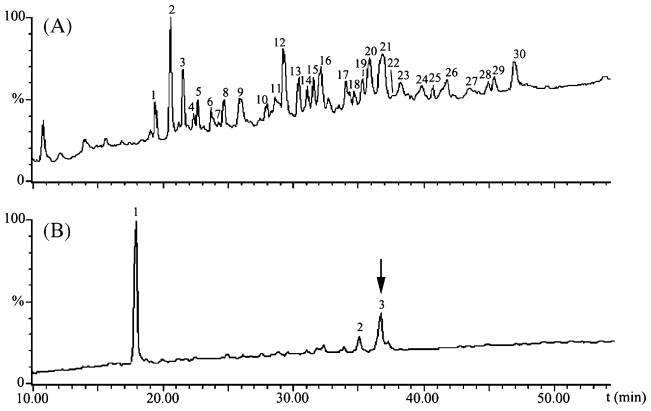

Fig. 1. HPLC analysis of α-gliadin digests with gastric and pancreatic enzymes (panel A) and then with BBM enzymes (panel B). Numbered peaks were collected and identified by MALDI MS/MS (Table 1). The peak arrowed and marked with 3 corresponds to the undigested 31–55.

Table 1
Identification by MALDI MS/MS of the gliadin peptides occurring in the gastric–pancreatic enzyme digests (Fig. 1)

HPLC peak[a]	Measured mass (MH^+)[b]	Peptide[c]
1	1007.60	181–188
2	2237.09	239–257
3	2226.05	143–160
4	2216.11	214–233
5	1371.74	131–142
6	1486.78	130–142
7	2692.31	209–233
8	2450.20	212–233
9	1078.63	180–188
10	2148.05	69–86
11	1055.50	217–226
12	2838.37	119–142
13	1752.80	163–176
14	1215.69	179–188
15	3321.63	204–233
16	3787.88	200–233
17	3345.79	1–29
18	2635.39	2–24
19	2479.29	3–24[d]
20	4022.0	198–233
21	2922.41	31–55[d]
22	2922.41	31–55[d]
	2734.42	1–24
23	4121.01	197–233
24	3493.83	1–30
25	2080.00	197–214
26	2687.40	172–194
27	1292.70	57–67
28	1455.79	57–68
29	1405.77	56–67
30	1568.84	56–68

[a] Number refers to the peak on the chromatogram shown in Fig. 1A.
[b] Peptide molecular weight measured by MALDI MS.
[c] Numbers indicate the amino acid residues at the extremity of each peptide refers to α-gliadin Q9ZP09.
[d] Peptides found also after BBM supplementation (Fig. 1B).

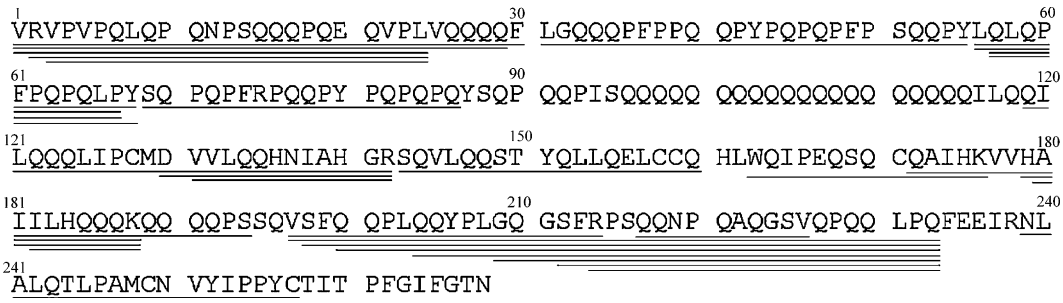

Fig. 2. Amino acid sequence of α-gliadin incubated with gastric–pancreatic enzyme. The lines mark peptides identified by MALDI MS/MS.

were identified in the hydrolyzate together with the parent peptide.

Careful analysis of Table 1 and Fig. 2 revealed that the protein regions 1–30, 119–142, 179–188, and 197–233 were also affected by digestion due to the separate action of endopeptidases. Differently from these ones, 69–86 143–160, 163–176, 239–257 resisted digestion by gastric and pancreatic enzymes.

Such proteomic approaches allowed us to map 87% of the gliadin sequence (Fig. 2); the remaining 13% escaped MALDI identification presumably due to a more thorough digestion of the primary peptides into oligopeptides and free amino acids. Nevertheless, the high protein coverage strongly suggests an overall high resistance of gliadin peptides to the concerted action of endo- and exo-peptidases.

3.2. BBM hydrolysis

Because the essential pre-requisite for promoting toxicity is the digestive-resistance of gastric and pancreatic peptides on the surface of the small intestine, incubation of recombinant gliadin peptides with BBM enzymes was investigated by HPLC. Peptides are expected to rapidly breakdown into free amino acids, dipeptides or tripeptides. The resulting 1 h-incubation gastric/pancreatic digests was analysed in Fig. 1B. Comparison of the latter with Fig. 1A shows that the majority of peaks actually disappeared after incubation with BBM preparation, while two peaks still resisted in the digests. MS analysis showed that peak 1 was constituted of low-molecular mass oligopeptides (<500 Da). By contrast, peak 3 contained the undigested 25-mer peptide 31–55. The latter actually resists the strong proteolytic action of BBM enzymes making it a possible candidate to cross the gastric-intestinal barrier. The N-terminal region of gliadin formed the resistant peptide 3–24 (peak 2, Fig. 1B) found, however, at lower levels than the 25-mer (Table 1). In contrast, the immunogenic peptides 56–68, 56–67 and 57–68 were hydrolysed by BBM enzymes into lower molecular mass peptides.

3.3. Resistance of 25-mer synthetic peptide to BBM enzymes action

In order to determine whether the 25-mer peptide resisted further hydrolysis, the synthetic 25-mer peptide mimicking the amino acid sequence of recombinant gliadin was assayed for the resistance to gastro-intestinal enzymes. The HPLC analysis produced a double-shaped peak (Fig. 3D) similar to that observed for the recombinant α-gliadin hydrolyzate (Fig. 1A, peaks 21 and 22). Each HPLC peak manually collected and individually injected into the LC–ESI/MS gave the same two peaks in the same ratio of area (data not shown). This result, also confirmed by MALDI MS analysis, suggests that the two resolved forms of the 31–55 peptide can be conformational isomers, in agreement with previously reported data for other proline-containing peptides [19].

The hydrolysis of the synthetic 25-mer peptide by gastric and pancreatic enzymes was followed by LC–ESI/MS (Fig. 3A–C). Even after a 4-h incubation with BBM enzymes, the 25-mer was substantially unchanged.

3.4. Identification of 25- and 33-mer in standard gliadin

Peptides resistant to digestive enzymes were then searched in authentic wheat gliadin. As PWG constitutes the only available reference gliadin material derived from a mix of 28 European wheat cultivars [14], it was submitted to the hydrolytic procedure as recombinant α-gliadin. The total ion current (TIC) profile of the LC–ESI/MS analysis (Fig. 4A) was more complex than that obtained from the recombinant α-gliadin sample. This is consistent with the higher molecular heterogeneity of wheat gluten containing hundreds of gliadin species compared to the single chain of recombinant α-gliadin.

In the PWG sample we performed the search of peptides expected to be released as a result of peptide breakdown by

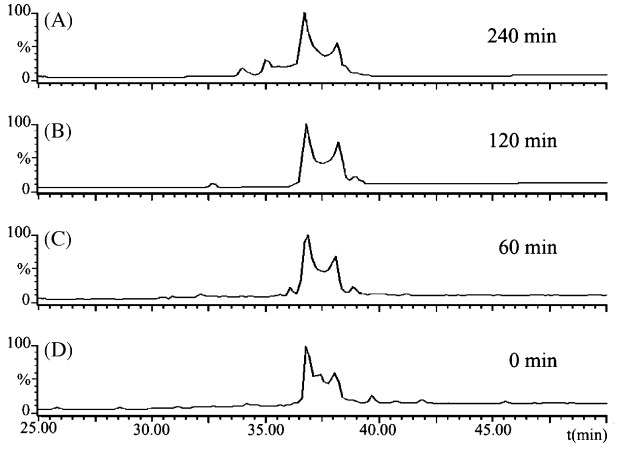

Fig. 3. HPLC analysis of the 25-mer synthetic peptide incubated with BBM at different times: 0 h (panel D), 1 h (panel C), 2 h (panel B) and 4 h (panel A).

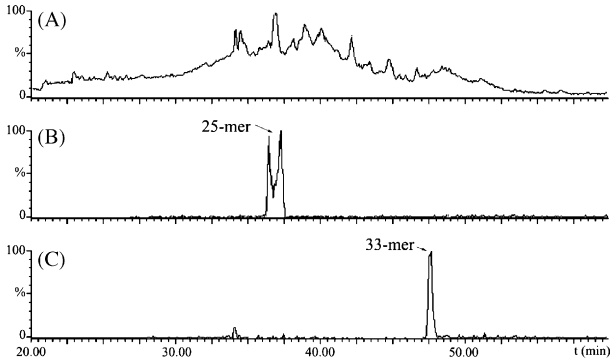

Fig. 4. LC–ESI/MS chromatogram of PWG gliadin digests with gastric–pancreatic and BBM enzymes. Panel A, TIC; panels B and C, ion extraction of 25- and 33-mer multiply charged ions, respectively.

gastric–pancreatic and BBM enzymes. By software extraction, the doubly $[(M+2H^+)^{2+} = 1461.7]$ and triply charged $[(M+3H^+)^{3+} = 974.8]$ states of the 25-mer peptide were identified. This signal occurred at the expected retention time and emerged under the characteristic double-shaped peak (Fig. 4B).

The 33-mer peptide from Q9M4L6 gliadin was localised along the HPLC pattern by comparison with the retention time of the synthetic standard peptide (not shown). Like the 25-mer, the intact 33-mer peptide in the standard gliadin digests was identified by extracting its triply and quadruply charged ions at $(M+3H^+)^{3+} = 1304.8$ and $(M+4H^+)^{4+} = 978.9$ (Fig. 4C).

4. Discussion

The present work first considered the digestion of recombinant α-gliadin Q9ZP09, chosen as reference sample. This was justified by the assumption that this gliadin was one of the most representative within the α-gliadin family, because of the high homology with other known gliadins [12]. The *in vitro* digests of the recombinant protein produced a number of peptides (Fig. 1A), but supplementation with BBM greatly simplified the digests up to the dominant 25-mer peptide 31–55 (Fig. 1B). Incubation of the synthetic 25-mer with BBM enzymes for longer reaction times confirmed its high resistance to proteolytic enzymes (Fig. 2). The 25-mer peptide is of particular interest as it comprises the 31–43 sequence, which was found to be a useful probe for stimulating the innate immune response [11]. In particular, *in vitro* 24-h stimulation of organ cultures from intestinal biopsies of CD patients with peptide 31–43 induces COX-2 increase, and promotes CD25 expression on monocytes and macrophages, and CD83 on dentritic cells in the LP compartment. It is intriguing that all these events are inhibited by antibodies neutralizing IL-15, a promoting factor of intraepithelial lymphocytes (IEL) expansion. Innate immunity mechanisms precede activation of the adaptive $CD4^+$ T-cells-mediated immune response in CD [11]. In particular, the increased expression in epithelial cells from CD patients of MICA, a MHC class I-like molecule that interacts with the NKG2D receptor of IEL, and of IL-15 [20], highlights a specific role of innate immunity in the disease.

An immunoactive epitope for the adaptive T-cell mediated immune response, also comprised within gliadin Q9ZP09, encompasses the sequence 56–68 [17] partly digested by gastric/pancreatic enzymes (Fig. 1A) and completed after BBM supplementation (Fig. 1B). This finding indicates that gliadin Q9ZP09 undergoes *in vitro* extensive hydrolysis causing loss of a known HLA-DQ2-restricted epitope while the peptide sequence stimulating the innate response in CD was unaffected. Further studies are needed to better define the residual toxicity of Q9ZP09 gliadin digests for celiac patients.

Other studies carried out on α-gliadin Q9M4L6 allowed detection of the 33-mer peptide 57–89 involved in the $CD4^+$-mediated immune response to gluten of celiac patients [8]. This peptide first recognised as resistant to digestion is nowadays generally acknowledged as the main modulator of the adaptive immune response in CD.

Likely, the recombinant gliadin did not produce the multitude of harmful gluten peptides reaching the intestine of celiac patients. In commercial flour samples gliadins are more heterogeneous than the recombinant protein. Therefore, the analytical approach extended to the PWG gliadin wheat sample allowed us to identify both the 25- and 33-mer peptide (Fig. 4). This indicates that Q9ZP09- and Q9M4L6-like proteins are co-present in the gliadin PWG wheat sample. This finding strongly suggests that both the peptides are capable of reaching the intestinal lumen and triggering two distinct immunological pathways. The 25-mer is thought to have a rapid effect on enterocytes and/or other immune cells involved in the innate immunity [10,11] whereas that 33-residue long peptide activates the subsequent adaptive immune response by inducing gliadin-specific $CD4^+$ T-cells in the LP [8]. The complex HPLC pattern of the PWG sample digests was indicative of a broadly heterogeneous mixture harbouring, in addition to the 25- and 33-mer, a still undetermined number of epitopes deserving further structural investigation.

Acknowledgements

This work was supported by "Rete di Spettrometria di Massa" (Contract Fondo Europeo di Sviluppo Regionale 94.05.09.103, ARINCO 94.IT.16.028). The authors are grateful to Dr. Annarita Formisano and Dr. Chiara Stella Sacco for technical assistance. G.M. gratefully acknowledges the Italian National Research Council (CNR) for the "Short-Term Mobility Program 2005" grant.

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