REGULAR ARTICLE

Characterization of wheat gliadin proteins by combined two-dimensional gel electrophoresis and tandem mass spectrometry

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A proteomics-based approach was used for characterizing wheat gliadins from an Italian common wheat (*Triticum aestivum*) cultivar. A two-dimensional gel electrophoresis (2-DE) map of roughly 40 spots was obtained by submitting the 70% alcohol-soluble crude protein extract to isoelectric focusing on immobilized pH gradient strips across two pH gradient ranges, *i.e.*, 3–10 or pH 6–11, and to sodium dodecyl sulfate-polyacrylamide electrophoresis in the second dimension. The chymotryptic digest of each spot was characterized by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and nano electrospray ionization-tandem mass spectrometry (MS/MS) analysis, providing a "peptide map" for each digest. The measured masses were subsequently sought in databases for sequences. For accurate identification of the parent protein, it was necessary to determine *de novo* sequences by MS/MS experiments on the peptides. By partial mass fingerprinting, we identified protein molecules such as α/β-, γ-, ω-gliadin, and high molecular weight-glutenin. The single spots along the 2-DE map were discriminated on the basis of their amino acid sequence traits. α-Gliadin, the most represented wheat protein in databases, was highly conserved as the relative *N*-terminal sequence of the components from the 2-DE map contained only a few silent amino acid substitutions. The other closely related gliadins were identified by sequencing internal peptide chains. The results gave insight into the complex nature of gliadin heterogeneity. This approach has provided us with sound reference data for differentiating gliadins amongst wheat varieties.

Keywords:
Gliadin / Peptide mass fingerprinting / Tandem mass spectrometry / Wheat

1 Introduction

Gliadins, the alcohol-soluble fraction of glutens, constitute the major protein fraction of wheat endosperm. They belong to the proline and glutamine-rich prolamin family, and are subdivided into α, β, γ, and ω fractions [1, 2]. In recent years, research on gliadin fractions has been channeled into two main directions aimed at: (i) detecting mature proteins expressed by the multigene gliadin family [3, 4], and (ii) identifying specific gliadin sequences within prolamin-derived peptides responsible for triggering celiac disease [5, 6]. While several cDNA sequences are available in databases [7], the reverse is not true for mature gliadins [8], whose structural analysis is hindered by the high number of wheat phenotypes, each containing at least 25 closely-related gliadin forms [9]. Efficiency in fractionating gluten proteins by HPLC has been accomplished through

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Abbreviation: HMW, high molecular weight

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gel IEF in the second dimension [8]. Although some HPLC peaks migrated as single bands, others were still cross-contaminated by coeluting proteins [10]. The molecular mass of gliadins varies from 30 to 40 kDa, and up to ~80 kDa for ω-gliadins [11]. Using MS as an alternative tool has been given a more important role in the characterization of high molecular mass proteins, even when analyzing complex mixtures. We have seen MALDI-TOF MS in studies that have characterized wheat α-gliadins and high molecular weight (HMW) glutenin subunits [12–14]. In addition, it has been applied to the direct analysis of bread and durum wheat gliadins, and glutenin subunits [15]. MALDI-TOF MS is not merely a fast and specific tool for protein analysis, but it can also give high mass-resolution with accuracy up to 0.1% SD. Molecular weight determination can also be obtained by ESI-MS coupled with HPLC. In this way, more than 50 protein species with different molecular masses were found in the gliadin and glutenin fraction [16].

A proteomics-based approach serves as a powerful tool for gene expression analysis, as is implied by the term "proteome" referring to the full complement of proteins in a genome or tissue [17, 18]. A proteomic study involves the extraction, solubilization, and separation of proteins using high-resolution 2-DE combined with micro-analytical techniques and bioinformatics, to characterize and identify proteins [19]. Hence, efficient clarification of changes in protein composition in a range of biological systems is more likely achieved.

The aim of the present study was to determine the gliadin species for rationalizing the molecular basis of either intravarietal heterogeneity or intervarietal polymorphism. Our analytical strategy involved the probing of one or more sequence stretches to obtain a straightforward clarification of the classes of gliadin, and afterwards, of gliadin heterogeneity. Additionally, the substantial information obtained can further broaden our knowledge of the gene expression.

2 Materials and methods

2.1 Materials

Urea, guanidine/HCl, and 1-propanol, CBB R250, agarose, ACN, TFA were supplied by Bio-Rad (Milan, Italy). NaCl and $KHNaPO_4$ were provided by Carlo Erba (Milan, Italy). SDS, ammonium bicarbonate, CHAPS, DTT, and EDTA CHCA were obtained from Sigma (St. Louis, MO, USA). Tris-HCl and glycine were supplied by ICN Biomedicals (Costa Mesa, CA, USA). Bromophenol blue was provided by ESA Electrophoresis (Chelmsford, MA. USA). Iodoacetamide was obtained from Fluka (Milan, Italy). Glycerol was provided by J.T. Baker. Wheat flour was largely obtained from an Italian winter variety (cv. Risciola).

2.2 Sample preparation and 2-DE

Separation of gliadin proteins was carried out as previously described [20]. Pre-extraction of albumins and globulins from nondefatted flour (100 mg) was achieved by stepwise addition (three times) of 1 mL of 0.4 mol/L NaCl, 0.067 mol/L $HKNaPO_4$, pH 4.6. Subsequently, gliadins were extracted with 0.5 mL of 70% v/v ethanol (three times). Each extraction step started with vortexing for 2 min at room temperature and magnetic stirring for 10 min. The suspensions were centrifuged for 15 min (albumins, globulins) and 20 min (gliadins), respectively, at 7000 rpm using a refrigerated centrifuge. The supernatants of gliadins were combined and lyophilized.

IEF was performed using ready-to-use Immobiline DryStrips, linear pH gradient 3–10 and 6–11, length 18 cm (Amersham Biosciences, Uppsala, Sweden), and in-gel sample rehydration method [21]. IEF was run on a Multiphor II electrophoresis unit (Amersham Biosciences) at 5°C constant temperature; it was run for 1 h at 1000 V, 1 mA, 5 W, followed by 16 h at 3500 V,1 mA, 5 W. After IEF, the IPG gel strips were incubated at room temperature for 15 min in 6 M urea, 30% w/v glycerol, 2% w/v SDS, 2% DTT, 50 mM Tris-HCl, pH 8.6. A second equilibration step was carried out for 7 min in the same solution with the exception that DTT was replaced by 2.5% iodoacetamide plus a trace of bromophenol blue as tracking dye. The strips were sealed at the top of the 1.0 mm vertical second dimension gel (Ettan Dalt six electrophoresis unit, power supply EPS 3501XL; Amersham Biosciences) with 0.5% agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. SDS-PAGE was carried out on linear 12%. The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, and running conditions were 400 mA constant, 5 W/gel for 30 min, then 15 W/gel for 5 h until the bromophenol blue reached the bottom of the gel. Molecular weight markers were broad ranged (Bio-Rad). Gels were stained with CBB R-250 for several hours followed by partial destaining with 30% methanol and 10% acetic acid in deionized water.

2.3 Protein reduction and alkylation

The protein spots were cut from the gel using a sterile scalpel and destained overnight with a solution of 25 mM ammonium bicarbonate and 50% ACN. Then the gel pieces were washed in distilled water for 20 min. The distilled water was removed and the gel was dehydrated with ACN for 10 min. After the ACN was removed, the gel piece was dried in a SpeedVac for 5 min. The gel piece was then incubated at 56°C for 60 min in 50 mM, containing 10 mM DTT. The DTT solution was allowed to cool and then the gel pieces were incubated at room temperature in the dark 50 mM ammonium bicarbonate pH 8.0, containing 20 mM iodoacetamide for 45 min. The iodoacetamide was removed and the gel pieces washed for 10 min. The gel pieces were then dehydrated with ACN and dried in the SpeedVac.

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2.4 Enzymatic digestion

The reduced and alkylated gel pieces were covered with 50 mM ammonium bicarbonate, pH 8.0, containing trypsin or chymotrypsin (both 12.5 ng/mL) and left on ice for 45 min. Excess enzyme was removed and 20 mL of ammonium bicarbonate was added to stop the gel from drying out. The gel pieces were then incubated overnight at 37°C. Peptides were extracted from the gel piece having 50% ACN, 5% formic acid by three extraction, with intermitant sonication. The peptide extracts were combined and dried in a SpeedVac.

2.5 MS analysis

MS experiments were carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO instrument equipped with an N_2 laser (337 nm 3 ns pulse width). Each spectrum was taken by the following procedure: 1 μL aliquot tryptic or chymotryptic digest solution from each extracted spot was loaded on a stainless steel plate together with 1 μL of CHCA matrix (10 mg in 1 mL aqueous 50% ACN). Mass spectrum acquisition was performed in both positive linear and reflectron mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with mass peptide standards (Sigma).

MS/MS data for the extracted peptides were obtained using a Q-STAR mass spectrometer (Applied Biosystems) equipped with nanospray interface (Protana, Odense, Denmark). Dried samples were resuspended in 0.1% TFA, desalted using ZipTip™ C18 microcolumns (Millipore) and sprayed from gold-coated "medium length" borosilicate capillaries (Protana). The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by the quadrupole mass filter (MS1) and then induced to fragment by collision. The collision energy was 20–40 eV, depending on the size of the peptide. The CID was processed using Analyst 5 software (Applied Biosystems). The deconvolution MS/MS was manually interpreted with the help of the Analyst 5 software. For the interpretation of MS spectra of protein digests MS-Pattern (http://prospector.ucsf.edu/ucsfhtml4.0/mspattern.htm) and PeptIdent (http://www.expasy.ch/tools/peptident.html) programs were used.

3 Results and discussion

Owing to the high complexity of wheat proteins [1, 2], we chose to use the procedure described by Osborne to fractionate wheat proteins selectively [20], whereby three raw protein fractions were obtained: albumins/globulins, gliadins, and glutenins. For the 2-D analysis, the 70% ethanol-soluble gliadin fraction was used for separation on IPG strips across pH range 3–10, giving the protein map shown in Fig. 1 with most of the spots migrated in the alkaline side of the gel. To improve the resolution amongst these components, we opted for an IEF separation across pH 6–11 range (Fig. 2). Ultimately, both maps were utilized for semi-preparative use. The protein spots on the gliadin map shown in Fig. 1 are labeled from "a-m", and those in Fig. 2 are numbered from 1 to 28.

The excised spots were digested with trypsin for protein identification, as described in Section 2, and a few high molecular mass tryptic peptides missing in databases were obtained due to the low level of lysine and arginine residues in parent proteins [22, 23]. Chymotrypsin, having a broader specificity than trypsin, provided an efficient gliadin spot digestion with a greater number of medium-sized peptides, better suited for a mass spectrometric identification. MALDI-TOF MS analysis on the chymotryptic digest gave a complex

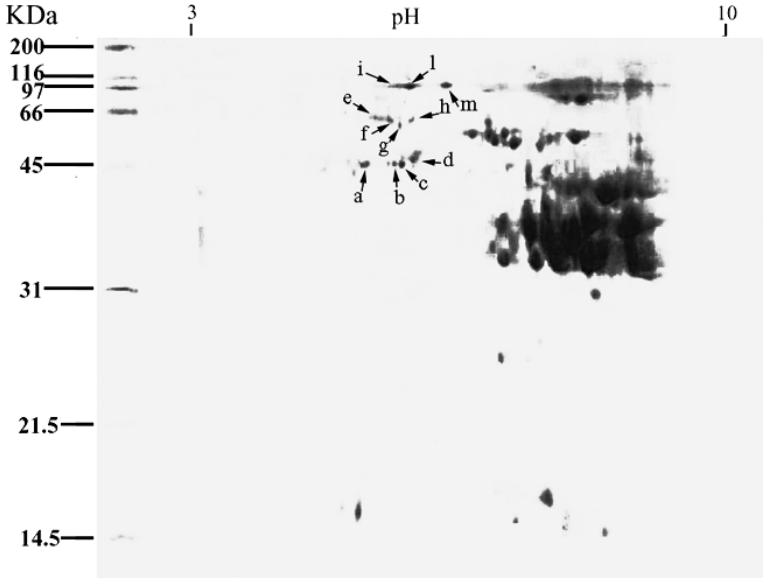

Figure 1. 2-DE of gliadin extracted from wheat. Proteins were separated in the first dimension by IEF using a pH range 3–10 linear 18 cm IPG strip and in the second dimension on linear 12% SDS-PAGE. Proteins were stained using CBB R-250. Only the well resolved spots were labelled a→m and excised from the gel for protein characterization as reported in Table 1.

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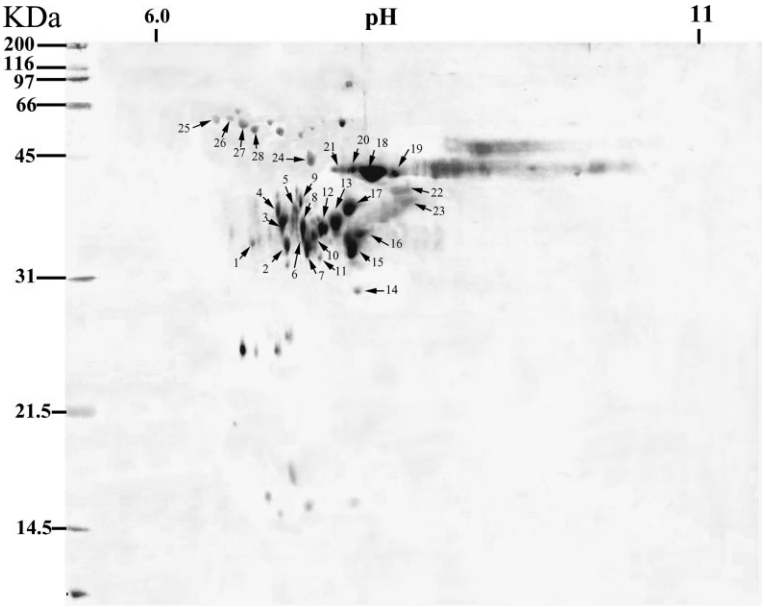

Figure 2. 2-DE of gliadin extracted from wheat. Proteins were separated in the first dimension by IEF using a pH range 6–11 linear 18 cm IPG strip and in the second dimension on linear 12% SDS-PAGE. Proteins were stained using CBB R-250. Only the well resolved spots were numbered 1→28 and excised from the gel for protein characterization as reported in Table 1.

peptide profile, which was difficult to rationalize due to the high sequence variability amongst the components of the gliadin fraction. In order to overcome this drawback, we performed nanoESI-MS/MS analysis of the chymotryptic peptides in each spot (Figs. 1, 2). The results are grouped for each gliadin subfraction and reported in the following sections.

3.1 α-gliadin

The MALDI-TOF MS spectrum of the chymotryptic digest from protein spot 15 in Fig. 2 is shown as example in Fig. 3. A similar spectrum was given by spots 1–14 and 22–24 differing by the p*I* and molecular mass (M_r) coordinates. The spectra shared several mass signals, marked by an asterisk in Fig. 3, for which each corresponding molecular mass is reported in Table 1.

Database search *via* molecular mass of the peptides did not give any parent protein identification. With nanoESI-MS/MS analysis for identification of some derived chymotryptic peptides, we were able to determine α-gliadin in spots 1–15 and 22–24. Figure 4 shows the spectrum of the double-charged ion at *m/z* 784.93 (1567.84 Da), which matched α-gliadin sequence 56–68 (Swiss-Prot, accession number Q9ZP09). In the same manner, we found that spots 1–15 and 22–24 shared the following peptides 1–20 (2296.19 Da), 1–24 (2733.46 Da), 75–87 (1625.79 Da), 1–30 (3491.82 Da), as shown in Fig. 5 (Swiss-Prot accession number Q9ZP09), further indicating that the *N*-terminal region of α-gliadin sequence is well conserved [24]. Through MS/MS analysis we established the *N*-terminal sequence portion of the 268 amino acid residues long α-gliadin, present in spots 1–15 and 22–24. Another common structural feature of these spots was the presence of the PQLPQFLQPQLPYPQPQL PYPQPQPF (3085.61 Da) peptide, which was not found in the database. This sequence stretch however, possibly corresponds to that of α-gliadin 64–89 (NCBI, accession number AJ133612.1) with a single amino acid substitution, $I^{70} \rightarrow P^{70}$. Identifying α-gliadin as a heterogeneous protein migrated in a wide area with different p*I* and/or M_r is in agreement with Shewry *et al.* [24], indicating that α-gliadin contains a well conserved *N*-terminal region, and has highly repeated sequences, which include PQQP and PQQPY as common elements. This evidence, together with other quite common

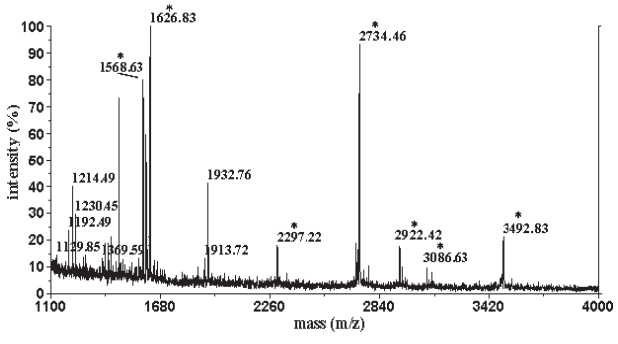

Figure 3. MALDI-TOF MS relative to spot 15, excised from 2-DE gel show in Fig. 2. Asterisk indicates mass signals common to spots 1→15 and 22→24.

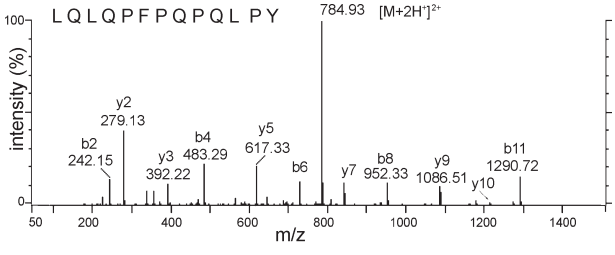

Figure 4. nanoESI-MS/MS spectra of double ion charged 784.93 (1567.84 Da) of spot 15 digest with chymotrypsin. The peptide sequence is reported.

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Table 1. Identification by nanoESI-MS/MS of chymotryptic gliadin peptides generated from hydrolysis of spots in Fig. 1 and 2. Boxed residues indicate substituted or deleted amino acid residues along the peptide chain found under the indicated accession number in the Swiss-Prot or NCBI(*) data bank archive (lower line)

Spot	Molecular Mass	Identyfied Sequence by nESI-MS/MS	SWISS-Prot, NCBI* accession number
1–15, 22–24	3491.82	V R V P V P Q L Q P Q N P S Q Q Q P Q E Q V P L V Q Q Q Q F	α-gliadin (1–30) Q9ZP09
	2733.46	V R V P V P Q L Q P Q N P S Q Q Q P Q E Q V P L	α-gliadin (1–24) Q9ZP09
	2921.41	L G Q Q Q P F P P Q Q P Y P Q P Q P F P S Q Q P Y	α-gliadin (31–55) Q9ZP09
	1567.84	L Q L Q P F P Q P Q L P Y	α-gliadin (56–68) Q9ZP09
	1625.79	R P Q Q P Y P Q P Q P Q Y	α-gliadin (75–87) Q9ZP09
	2296.19	V R V P V P Q L Q P Q N P S Q Q Q P Q E	α-gliadin (1–20) Q9ZP09
	3085.61	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-gliadin (64–89) AJ133612.1*
18–21	1396.75	V P P E C [V/S] I [I/M] R A P F	γ-gliadin (287–298) P08453
	1384.72	V P P E C S I [I/M] R A P F	γ-gliadin (287–298) P08453
	2958.45	[P/F] [Q/P] Q P Q [A/Q] [P/Q] [F/L] P Q P Q Q P Q Q S F P Q Q Q R P F	γ-gliadin (137–153) P08453
	516.27	L P C K	γ-gliadin (163–16) P08453
	871.52	F L L Q V P R	γ-gliadin
25–28, e–m	1190.59	K E L Q S P Q Q S F	ω-gliadin (9–18) AAGI7702*
	1403.72	P [Q/I] P Q Q P Q Q P F P L	ω-gliadin (204–215) AAGI7702*
	3212.56	P Q Q P F P Q Q P Q Q P Q Q [P Q Q N L S E Q P Q Q L F]	ω-gliadin (63–76) AAGI7702*
	1534.71	X X Q Q L T P P F P A P F	ω-gliadin
	1590.76	L Q S E P Q P Q Q P Q Q P S	ω-gliadin
a–d	1403.64	[F/G] Q Q P E Q [F/G] Q Q P [/G] Q	HMW-glutenin (221–231) P10387
	1275.58	[F/G] Q Q P E Q [F/G] Q Q P	HMW-glutenin (220–231) P10387
	774.36	Q [F/G] Q Q P [Q/G]	HMW-glutenin (151–156); (226–231); (522–527) P10387

events such as amino acid substitution, insertion, or deletion in the gliadin sequence, could justify the different coordinates of gliadins species along the 2-DE gel map. However, we did not investigate further elements which could differentiate the protein spots since the defining of the entire protein sequence went beyond the scope of this study.

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α-gliadin, (Swiss-Prot, accession no. Q9ZP09)

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    1          11         21         31         41         51
    |          |          |          |          |          |
  1 VRVPVPQLQP QNPSQQQPQE QVPLVQQQQF LGQQQPFPPQ QPYPQPQPFP SQQPYLQLQP  60

 61 FPQPQLPYSQ PQPFRPQQPY PQPQPQYSQP QQPISQQQQQ QQQQQQQQQQ QQQQQILQQI 120

121 LQQQLIPCMD VVLQQHNIAH GRSQVLQQST YQLLQELCCQ HLWQIPEQSQ CQAIHKVVHA 180

181 IILHQQQKQQ QQPSSQVSFQ QPLQQYPLGQ GSFRPSQQNP QAQGSVQPQQ LPQFEEIRNL 240

241 ALQTLPAMCN VYIPPYCTIT PFGIFGTN
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Figure 5. Amino acid sequence of α-gliadin sequences (NCBI, accession number Q9ZP09). Continued lines mark the peptides identified by nanoESI-MS/MS.

3.2 γ-gliadin

The chymotryptic digest of spots 18–21 shared the same MALDI-TOF MS signals listed in Table 1. We performed nanoESI-MS/MS analysis on the chymotryptic digest to get some structural information of the parent proteins. The V^{287}PPECVIIRAPF^{298} peptide sequence established by nanoESI-MS/MS matches the γ-gliadin fragment 287–298, V^{287}PPECSIMRAPF^{298} (Swiss-Prot database, accession number P08453), but differs by two silent amino acid substitutions $V^{292} \rightarrow S^{292}$ and $I^{294} \rightarrow Met^{294}$. Similarly, the peptide at 1385.66 Da corresponded to the γ-gliadin 287–298 sequence with a single substitution $I^{294} \rightarrow M^{294}$. The presence of a peptide with 1396.75 Da containing a further amino acid residue substitution, *i.e.*, $V^{292} \rightarrow S^{292}$ indicated the presence of at least two silent comigrating γ-gliadin variants. The mass signal at 2958.45 Da corresponded to the PQQPQAPFPQPQ QPQQSF PQQQRPF peptide different from the γ-gliadin 137–153 found in the database (Swiss-Prot accession number P08453) for five amino acid substitutions: $P^{137} \rightarrow F^{137}$, $Q^{138} \rightarrow P^{138}$, $A^{142} \rightarrow Q^{142}$, $P^{143} \rightarrow Q^{143}$, and $F^{144} \rightarrow L^{144}$ (Table 1). The peptides at 871.52 Da (sequence FLLQVPR, no match in databases) and 516.27 Da (sequence LPCK, γ-gliadin 163–167, Swiss-Prot accession number P08453) both occurred in spots 18–21. In conclusion, γ-gliadin components were concentrated in spots 18–21, although the relatively similar M_r proteins presented a different p*I*.

3.3 ω-gliadin

The chymotryptic digest of spots 25–28 and e-m (Fig. 1) shared the same mass signals reported in Table 1. Amongst the peptides of protein databases, only that at 1190.59 Da matched the ω-gliadin 9–18 peptide (*N*CBI, accession number AAG17702) indicating a well-conserved *N*-terminal region of ω-gliadin. The peptide at 1403.64 Da with the amino acid sequence PQPQQPQQPFPL corresponded to ω-gliadin 204–215 (AAG17702), however with the single substitution $Q^{205} \rightarrow L^{205}$. Only the first 14 amino acid residues of peptide PQQPFPQQPQQPQQNLSEQPQQLF (3212.56 Da) was identified as ω-gliadin 63–76 (AAG17702, database NCBI).

Though the entire peptide LQSEPQPQQPQQPS (1590.77 Da) was not found in protein databases, the partial sequence, PQPQQPQQP, is known as a repeated typical motif of many ω-gliadins. Moreover, the peptide at 1534.71 Da found in all the ω-gliadin spots corresponding to the sequence XXQQLTPPFPAPF (X indicates an unidentified residue) was missing in protein databases. In conclusion, the above indicated spots belonged to the ω-gliadin family.

3.4 HMW glutenins

Three peptides at 1403.64, 1275.58, and 774.36 Da identified as FQQPEQFQQPQ, FQQPEQFQQP, and QFQQPQ, respectively, were common to the protein in spots a–d (Fig. 1). These sequences only partly correspond to those from protein databases. As shown in Table 1, the main difference between the sequences of these peptides and the counterpart found in databases is the substitution F → G. The occurrence of glutenin in the 70% ethyl alcohol-soluble fraction is not surprising, as a chemical procedure fractionation could lend itself to a cross contamination of gliadins by glutenins as previously reported [26, 27]. Intensely lower spots a–d, detected in the middle region of the 2-D map on Fig. 1, identified by coordinates M_r 43–46 kDa and p*I* 5–6 range belonged to the HMW glutenins.

4 Concluding remarks

The main difficulty in gliadin structural analysis lies in high protein heterogeneity owed to numerous duplications, and subsequent divergences of the gliadin multigene family encoding a polymorphic set of polypeptides [25–28]. Southern blot analysis of wheat DNA has suggested that the number of genes encoding the α/β-type gliadin is extremely high (greater than 100 copies/haploid genome) [28]. In the light of this, conventional electrophoretic and chromatographic techniques alone, routinely used in gliadin analysis, must be considered inadequate when facing the problem of rationalizing the complexity. As regards molecular-based investigations of protein diversity, a proteomics-based analytical approach allows us to obtain more accurate information on gliadin identity. Though preliminary, this approach can further supply genome analysis with supplementary and complementary data since it comprises the study of functional rather than informational molecules. It is expected that proteomics combined with transcriptome analysis will become a very effective approach for controlling the genetic improvement of wheat.

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The work here describes the complexity of the gliadin fraction in terms of molecular species derived from a high resolution analysis of PMF. The results reported suggest that the coupling of 2-DE to MS/MS can successfully map any gliadin or glutenin subunit, hence representing the most precise method currently available for direct testing of the gene-derived sequence.

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