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ARTICLE

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High Molecular Weight Glutenin Subunits in Some Durum Wheat Cultivars Investigated by Means of Mass Spectrometric Techniques

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ABSTRACT: The primary structures of high molecular weight glutenin subunits (HMW-GS) of 5 *Triticum durum* Desf. cultivars (Simeto, Svevo, Duilio, Bronte, and Sant'Agata), largely cultivated in the south of Italy, and of 13 populations of the old spring Sicilian durum wheat landrace Timilia (*Triticum durum* Desf.) (accession nos. 1, 2, 3, 4, 7, 8, 9, 13, 14, 15, SG1, SG2, and SG3) were investigated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and reversed-phase high performance liquid chromatography/nanoelectrospray ionization mass spectrometry (RP-HPLC/nESI-MS/MS). M_r of the intact proteins determined by MALDI mass spectrometry showed that all the 13 populations of Timilia contained the same two HMW-GS with 75.2 kDa and 86.4 kDa, whereas the other durum wheat cultivars showed the presence of the expected HMW-GS 1By8 and 1Bx7 at 75.1 kDa and 83.1 kDa, respectively. By MALDI mass spectrometry of the tryptic digestion peptides of the isolated HMW-GS of Timilia, the 1Bx and 1By subunits were identified as the NCBInr Acc. No AAQ93629, and AAQ93633, respectively. Sequence verification for HMW-GS 1Bx and 1By both in Simeto and Timilia was obtained by MALDI mass mapping and HPLC/nESI-MSMS of the tryptic peptides. The Bx subunit of Timila presents a sequence similarity of 96% with respect to Simeto, with differences in the insertion of 3 peptides of 5, 9, and 15 amino acids, for a total insertion of 29 amino acids and 25 amino acid substitutions. These differences in the amino acidic sequence account for the determined Δm of 3294 Da between the M_r of the 1Bx subunits in Timilia and Simeto. Sequence alignment between the two By subunits shows 10 amino acid substitutions and is consistent with the Δm of 148 Da found in the MALDI mass spectra of the intact subunits.

KEYWORDS: gluten proteins, mass spectrometry, sequence determination, Timilia, *Triticum durum*

INTRODUCTION

Prolamins are a group of cereal seed storage proteins characterized by their solubility in aqueous alcohol. These protein fractions are currently identified according to gel electrophoretic mobility or through 2D coordinates.[1] In wheat, the gliadins (α/β, γ, and ω)[2] are monomeric proteins with molecular masses in the 30−40 kDa range, although some ω-gliadins can reach 80 kDa. They can contain either no disulfide bonds or only intramolecular disulfide bonds. The glutenin fraction is composed of polymeric aggregates[3] in which constituent polypeptides, subdivided into high molecular weight (HMW, 65−90 kDa) and low molecular weight (LMW, 20−40 kDa) glutenin subunits (GS), are linked by intra and interchain disulfide bonds, the latter responsible for the polymeric nature of gluten.

In common wheat (*Triticum aestivum*), the high molecular weight gluten subunit (HMW-GS) genes (Glu-1) are located on the long arms of homologous chromosomes 1A, 1B, and 1D. Each locus comprises two genes linked together, encoding two different types of HMW-GSs, x- and y-types.[4] The x-type subunits generally have a slower electrophoretic mobility in SDS−PAGE and higher molecular weight (83−90 kDa) than the y-type subunits (65−74 kDa). These two types also show differences in the length of the repetitive region and in the number of cysteines residues.

Bread wheats (*Triticum asstivum*) could, in theory, contain six different HMW-GSs, but due to the silencing of some of these genes, most common bread wheat cultivars possess from three to five HMW-GSs. Thus, all hexaploid wheats contain at least the 1Bx, 1Dx, and 1Dy subunits, while some cultivars also contain a 1By subunit and a 1Ax subunit as well. As a consequence, it appears that the gene encoding the 1Ay subunit is always silent. Nevertheless, 1Ay subunits are expressed in the A-genome diploid species *T. monococcum* and *urartu*,[5] and some bread wheats with six HMW-GSs are known.[6,7] The *Triticum turgidum* ssp. *durum* groups tetraploid wheats characterized by the AABB. As for bread wheat, some of the genes may be silent, resulting in the presence of one to three HMW-GSs in the different cultivars.

The current nomenclature of HMW-GSs was developed by Payne and Lawrence in 1983.[8] It provides a chromosomal location of the genes, the subunit type (x- or y-type), and finally a number indicating the mobility of each subunit on SDS−PAGE gels (i.e., 1Bx7 and 1By9).

HMW-GSs, which represent approximately 5% of the total endosperm proteins, have been studied extensively. This interest results from the demonstration that allelic variation in the polypeptide composition of the HMW-GSs is closely correlated with the bread-making quality of wheat cultivars and their crosses.[9,10]

Nowadays, MALDI-TOF MS technology represents a powerful tool to quickly and accurately analyze glutenin compositions for breeding purposes.[11] For a number of HMW-GSs from bread

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Table 1. MALDI MS Determination of the M_r of HMW-GS Extracted from Different Cultivars[a]

cultivar	M_r 1By	M_r 1Bx
Timilia 1	75253	86299
Timilia 2	75315	86329
Timilia 3	75281	86321
Timilia 4	75277	86337
Timilia 7	75261	86329
Timilia 8	75279	86339
Timilia 9	75349	86417
Timila 13	75257	86437
Timila 14	75277	86319
Timilia 15	75273	86275
Timilia SG1	75275	86370
Timilia SG2	75229	86311
Timilia SG3	75267	86337
Simeto	75184	83106
Svevo	75178	83110
Duilio	75156	83086
Bronte	75182	83082
Sant'Agata	75197	83138

[a] The M_r values are calculated from the doubly charged ions.

wheat, whose gene derived sequence were known, verification of the sequence correctness and assessment of post-translational modifications have been obtained by direct MALDI-TOF mass spectrometry of crude extracts followed by enzymatic digestion and subsequent analysis of the proteolytic mixtures by MALDI-TOF-MS or by RP-HPLC/ESI-MS/MS.[12−18] On the contrary, few durum wheat HMW-GS gene derived sequences are known.

The aim of this work was the comparative analysis of HMW-GS extracted from the 5 most popular *Triticum durum* cultivars grown in the south of Italy (Simeto, Duilio, Svevo, Bronte, and Sant'Agata) in comparison with 13 accessions of the old Sicilian durum wheat landrace *Timilia* (*Triticum durum* Desf). The accessions of the landrace Timilia, coded with a simple figure after the name Timilia and ranging from 1 to 15 (Table 1), come from different populations collected in various Sicilian locations. Timilia SG1, Timilia SG2, and Tumminia SG3 originate from selections of pure lines of intrapopulations collected by Stazione Consorziale Sperimentale di Granicoltura per la Sicilia. The first two genotypes were selected in the 1950s, while the third one (Tumminia SG3) was selected and covered by an EU Community patent at the beginning of 2000. All of the Timilia populations and varieties mentioned above differ for some morphological and/or physiological traits. The plants of all the Timilia types analyzed in this study are very tall (above 100 cm), and all possess ears with black awns, except for Timilia 13, Timilia 15, and Timilia SG1. The Timilia ears are very long, significantly longer and less compact than those of the other varieties (Simeto, Svevo, Duilio, Bronte, and Sant'Agata) investigated here. Each Timilia population differs from the others in spike length and ear features (e.g., glumes). The seeds of all Timila types are smaller but harder in comparison to the seeds of the other varieties studied here.

Timilia wheat, largely used in the first part of the last century, represents a rich source of germplasm particularly suitable to Mediterranean conditions. It is highly resistant to drought and abiotic stresses and shows some valuable qualitative characteristics, such as a long shelf life of the derived baked products.[19,20] Nowadays *Timilia* is cultivated in some inner hilly areas of Sicily either for animal feed or for human consumption.

For the first time, we characterize HMW-GSs from Timilia by means of mass spectrometric approaches, and we compare these amino acid sequences with the corresponding ones of the five most popular *Triticum durum* cultivars in order to assess the differences at the molecular level of these subunits responsible for gluten viscoelasticity.

MATERIALS AND METHODS

Chemicals. Dithiothreitol (DTT), 4-vinylpyridine (4-VP), ammonium acetate, sodium chloride, Tris-HCl, urea, bovine cytochrome *c*, horse myoglobin, bovine β-lactoglobulin, enolase, sinapinic acid (SA), α-cyano-4 hydroxycynnamic acid (CHCA), and 2,6-dihydroxyacetophenone (2,6-DHAP) were purchased from Sigma (Milano, Italy); trifluoracetic acid (TFA) was obtained from Aldrich (Milano, Italy); and formic acid (FA) for mass spectrometry was obtained from Fluka (Milano, Italy). Promega's (Madison, WI) sequencing grade modified trypsin, a porcine trypsin modified by reductive methylation, rendering it resistant to proteolytic digestion, was used. 1-Propanol, HPLC grade H_2O, and CH_3CN were provided by Carlo Erba (Milano, Italy).

Plant Material. The *Triticum durum* Desf. cultivars (Simeto, Svevo, Duilio, Bronte, and Sant'Agata) and the populations of the old spring Sicilian durum wheat landrace Timilia (*Triticum durum* Desf.) (accession nos. 1, 2, 3, 4, 7, 8, 9, 13, 14, 15 SG1, SG2, and SG3 from the gene bank of Stazione Sperimentale di Granicoltura per la Sicilia Gene Bank) were grown on plots of 20 m^2 at the Stazione Sperimentale di Granicoltura per la Sicilia experimental farm, in the season 2005−2006. All of the genotypes were grown using the same cropping practices (sowing time, time, and dosage of fertilization and weed control). The plots were sown at the end of November 2005 using a plot seeder. At sowing, 2 kg/m^2 of diammonium phophate (18-46-0) was given, while at the beginning of rising, 0.02 kg/m^2 of urea (46% N) was given. Weeds were manually removed. The plots were harvested at the end of June 2006, and 100 g of grains of each genotype were ground using a laboratory grinder IKA-Werk under controlled temperature (4 °C). The whole meal flour of the different durum varieties were then subjected to further extractions and analyses.

Extraction and Purification of HMW-GS. The HMW-GSs fraction was isolated from flours by a modification of the Marchylo's method.[21] Protein was reduced and S-pyridylethylated, and the single subunits were isolated by RP-HPLC on a Varian 9010 high-performance liquid chromatograph (Sunnyvale, CA, USA) equipped with a Varian 9050 detector. Data were acquired by a PC using the software system Peak Simple II. Dialyzed proteins were filtered on Microspin filters (Alltech, Milan, Italy) and loaded onto a reversed-phase Vydac C4 (4.6 × 250 mm, 300 Å, 10 μm) column. Proteins were eluted, with a linear gradient of solvent B (CH_3CN + 0.05% TFA) in A (H_2O + 0.05% TFA) from 10% to 45% in 45 min at a flow rate of 1 mL/min at 50 °C. Peaks were detected by their absorption at 224 nm, collected manually, and freeze-dried.

Tryptic Digestion of the Reduced and S-Pyridylethylated Subunits. The reduced and S-pyridylethylated subunits were dissolved in 50 mM ammonium bicarbonate, pH 8.3, at a concentration of 1 μg/μL. Modified porcine trypsin, dissolved in the same buffer, was added to the proteins at a molar enzyme/substrate ratio of 1:50, and the solutions were incubated at 37 °C for 4 h. The digestion was stopped by cooling in liquid nitrogen, and the mixtures were immediately freeze-dried.

MALDI-MS. MALDI mass spectra were acquired on a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a UV nitrogen laser (337 nm). The instrument was operated in positive ion reflector-delayed extraction mode for m/z

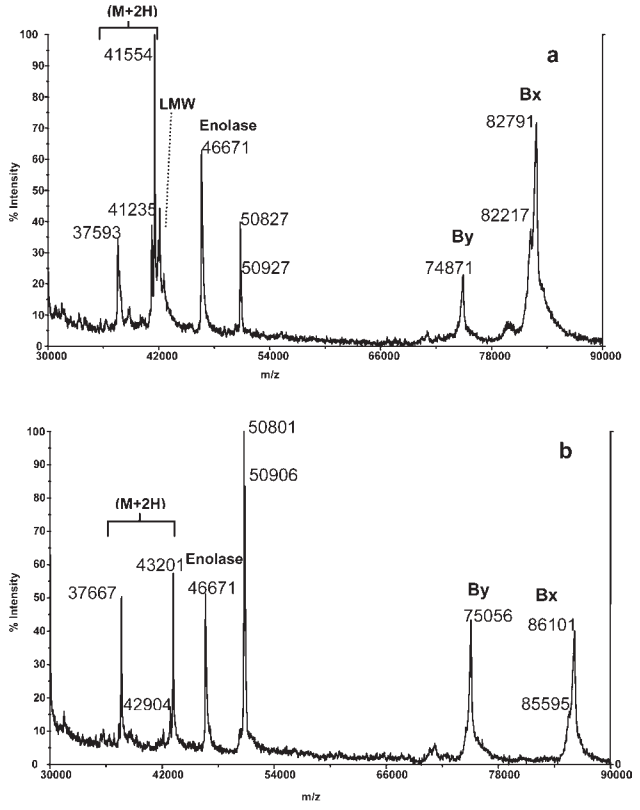

Figure 1. MALDI mass spectra of HMW subunits extracted from (a) cv. Timila 7 and (b) cv. Simeto obtained using 2,6-DHAP as the matrix. The spectra were calibrated internally with enolase (m/z 46671).

range 700−3000 and in linear mode for the high mass ranges (3000−90000). Spectra were averaged from about 150 laser shots to improve the signal-to-noise level.

Dried intact unalkylated and alkylated proteins were dissolved in $CH_3CN + H_2O$ and 0.1% TFA (50:50, v/v) at a concentration of 1 μg/μL. Sample preparation was carried out according to the dried droplet method[22] with 2,6-DHAP as the matrix. Matrix solution was prepared by dissolving 2,6-DHAP in $CH_3CN + H_2O$ and 0.1% TFA (50:50, v/v) at a concentration of 50 μg/μL. Mass assignment was made using enolase (46670 Da) as the internal standard.

The solutions containing the tryptic peptides were subjected to micropurification (desalting/concentration) with a homemade 5-mm nanocolumn packed with resin POROS R2 (Applied Biosystems, Foster City, CA, USA) in a constricted GELoader tip (Eppendorf Scientific, Westbury, NY), according to Gobom et al.[23] For analyses by MALDI-MS, the peptides were eluted with 0.6 μL of matrix solutions and deposited directly onto the MALDI target. The matrix solutions were prepared by dissolving CHCA and SA in CH_3CN + 0.1% TFA (70:30, v/v) at a concentration of 10 μg/μL.

M/Z software (Proteometrics Ltd., New York, NY, USA) was used to analyze the MALDI-TOF mass spectra, which, in the m/z range 700−3000, were externally calibrated using bovine β-lactoglobulin tryptic peptides (m/z 837.48, 2313.26, and 2707.38). Mass calibration for the other m/z ranges was made using bovine insulin (5733.6 Da), cytochrome c (12360 Da), lysozyme (14305 Da), and trypsinogen (23981 Da) as external standards.

RP-HPLC/nESI-MS/MS. Capillary RP-HPLC/nESI-MSMS was performed using an Ultimate 3000 LC system combined with an autosampler and a flow splitter 1:100 (Dionex Corporation, Sunnyvale, CA, USA) coupled online with a linear ion trap nanoelectrospray mass spectrometer (LTQ, Thermo Fischer Scientific, San Jose, CA).

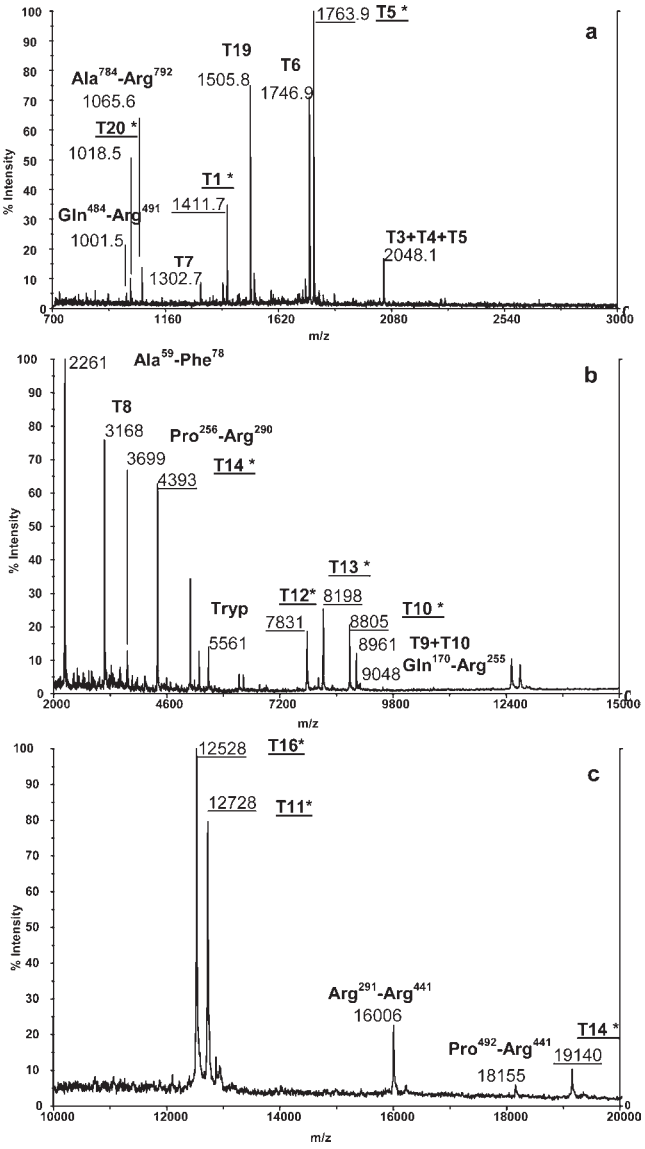

Figure 2. MALDI mass spectra of the tryptic digest of the reduced and *S*-pyridylethylated HMW subunit 1Bx from cv. Timilia in the m/z range (a) 700−3000 (reflector ion mode), (b) 2000−15000 (linear ion mode), and (c) 10000−20000 (linear ion mode). Diagnostic peptides are underlined and labeled with an asterisk.

Ionization was performed with a liquid junction using a noncoated capillary probe (30 ± 2 μm i.d.; New Objective, Woburn, MA, USA).

The peptide solution, obtained from the tryptic digestion of the isolated subunits, was diluted 1:10 with H_2O/0.1% FA; 10 μL of the sample were directly loaded onto a C18 μ-precolumn cartridge (0.3 mm × 5 mm, 100 Å, 5 μm, PepMap, Dionex) equilibrated with 0.1% FA at a flow rate of 20 μL/min for 4 min. Subsequently, peptides were applied onto a C18 capillary column (0.18 mm × 150 mm, 300 Å, 5 μm, Thermo Electron) and eluted at room temperature with a linear gradient of CH_3CN/0.1% FA/H_2O/0.1% FA from 10 to 50% in 50 min at a flow rate of 2 μL/min. Repetitive mass spectra were scanned using the following electrospray ion source parameters: capillary temperature, 220 °C; spray voltage, 1.9 kV. Peptide ions were analyzed by the data-dependent method as follows: (1) full MS scan (mass-to-charge ratio 350−2000); (2) ZoomScan (scan three major ions with higher resolution; isolation width, 2 Da); and (3) MS/MS of the three major ions

Table 2. Fragment Nomenclature, Sequence Position, and Calculated and Experimental Monoisotopic and Average Masses of Expected and Identified Tryptic Fragments of Timilia's 1Bx HMW-GS (GenBank Acc. No. AAQ93629)

		calcd MH^+[a]			RP-HPLC/n-ESI-MS/MS		
fragment	position	monoisotopic	average	MALDI-MS MH^+ measured	rt. (min)	experimental MH^+(PIM^b, charge)	sequence
T1	1−12	1411.6	1412.5	1411.7	33.9	1411.7 (706.3, 2+)	EGEASGQLQCER
T2	13−15	417.2	417.5				ELR
T3	16−16	147.1	147.2				K
T4	17−17	175.1	175.2				R
T5	18−31	1763.9	1765.0	1763.9	42.6	1763.9 (842.4, 2+)	ELEACQQVVDQQLR
T(3 + 4+5)	16-31	2048.1	2049.4	2048.1			
T6	32−47	1746.9	1748.0	1746.9	39.6	1748.0 (874.5, 2+)	DVSPGCRPITVSPGTR
T7	48−58	1302.7	1303.5	1302.7	37.5	1302.7 (651.8, 2+)	QYEQQPVVPSK
T8	59−86	3166	3168	3168	60.7	3169 (1057.0, 3+)	AGSFYPSETTPSQQLQQMIFWGIPALLR
T9	87−87	175.1	175.2				R
T10	88−169	8800	8805	8805	45.5	8805[c]	YYPSVTSSQQGSYYPGQASQQQSGQGQQPGQGQQPEQGQQDQQ PGQGQQGYYPTSPQQPGQGQQLGQGQPGYYPTSQQPGQK
T(9 + 10)	87−169	8956	8961	8961			
T11	170−290	12722	12729	12728	44.5	12730[c]	QQAGQGQQSGQGQQGYYPTSLQQSGQGQQPGQGQPGYYPTSPQ QSGQWHQPGQGQQPGQGQQSGQGQQGQQSGQGQQGQQPEQG QRPGQGQQGYYPTSPQQPGQGQQSGQGQPGYYPTSSR
Gln^{170}-Arg^{255}	170−255	9043	9048	9048			
Pro^{256}-Arg^{290}	256−290	3696	3698	3699			
T12	291−364	7828	7832	7831	46.1	7832[c]	QPGQWQQPGQGQQPGQGQQGQQPGQGQQPGQGQQGYYPTSL QQLGQGQQPGQGQPGYYPTSQQSEQGQQPGQGK
T13	365−441	8194	8199	8198			QPGQGQQGYYPTSSQQSGQGQQPGQGQPGYYPTSPQQSGQGQQ SGQGQQGYYPTSPQQSGQWQQPGQGQSGYFPTSR
T14	442−483	4390.0	4392.5	4393			QQSGQGQQLGQGQQSGQGQEGQQPGQGQQAYYPASSQQSGQR
T15	484−658	19132	19143	19140	50.3	19145[c]	QQAGQWQRPGQGQPGYYPTSPQQPGQEQQSGQTQQSGQWQLVYY PTSPQQPGQLQQPAQGQQPAQGQQSAQEQQPGQAQQSGQWQL VYYPTSPQQPGQLQQPAQGQQGYYPTSPQQSGQGQQGYYTTSLQ QSGQGQQGYYLTSPQQSGQGQQGYYPTS PQQSGQGQQPGQGQQPR
Gln^{484}-Arg^{491}	484−491	1001.5	1002.1	1001.5			
Pro^{492}-Arg^{658}	492−658	18150	18160	18155			
T16	659−774	12525	12532	12528	48.3	12532[c]	QGQQGYYPISPQQSGQGQQPGQGQQGYYPTSPQQSGQGQQPGH EQQPGQWLQPGQGQQGYYPTSSQQSGQGQQSGQGQQGYYPTSL WQPGQGQQPGQGQQGYDSPYHVSAEYQAAR
T17	775−776	260.2	260.4				LK
T18	777−779	317.2	317.4				VAK
T19	780−792	1505.8	1506.8	1505.8	43.0	1505.9 (753.4, 2+)	AQQLAAQLPAMCR
T20	793−802	1018.5	1019.1	1018.5	38.5	1018.6 (509.8, 2+)	LEGSDALSAR
T21	803−803	147.1	147.2				Q

[a] Cys: S-pyridylethylated. [b] PIM: precursor ion mass. [c] Calculated as average from all the multicharged ions.

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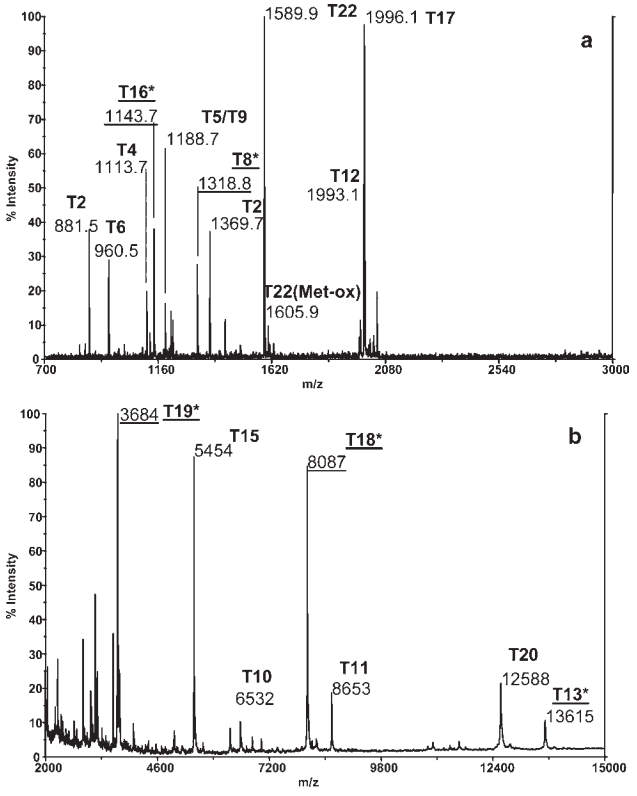

Figure 3. MALDI mass spectra of the tryptic digest of the reduced and *S*-pyridylethylated HMW subunit 1By from cv. Timilia 7 in the m/z range (a) 700−3000 (reflector ion mode) and (b) 2000−15000 (linear ion mode). Diagnostic peptides are underlined and labeled with an asterisk.

(Q 0.250, collision energy 24 au). Mass calibration was made using a standard mixture of caffeine (M_r 194.1 Da), MRFA peptide (M_r 523.6 Da), and Ultramark (M_r 1621 Da).

Bionformatic Search and Data Analysis. MALDI-TOF peptide mass data were used to perform protein identification in the Peptide Mass Fingerprint (PMF) mode by searching in a nonredundant protein sequence database (NCBInr) using the MOWSE algorithm as implemented in the Mascot search engine, version 2.2 (Matrix Science: www.matrixscience.com). The following parameters were used for database searches: taxonomy, Viridiplantae (other green plants); monoisotopic mass accuracy, 100 ppm (data acquisition performed in reflector ion-mode), average mass accuracy, and 1.2 Da (data acquisition performed linear ion-mode); cleavage specificity was set as trypsin, and 2 missed cleavages were allowed; pyridylethyl Cys (fixed), oxidation of Met (variable), transformation of N-terminal Gln, and N-terminal Glu residue in the pyroglutamic acid form (variable).

The General Protein/Mass Analysis for Windows software (http://welcome.to/gpmaw) was used for all sequence handling in MS and MSMS data analysis. The ClustalW2 tool (http://www.clustal.org/) was used for sequence alignment.

RESULTS AND DISCUSSION

In Figure 1a is reported the MALDI mass spectrum of the HMW-GS extracted from Simeto cv. This cultivar is commonly used both for pasta and bread making and its HMW-GS composition consisting of the 1Bx7 and 1 By8 subunits is well documented.[24]

The spectrum, obtained using 2,6-DHAP as a matrix and calibrated internally with enolase, shows the presence of two intense MH+ signals at m/z 74871 and 82791. These values are about 300 Da lower than the M_r of 75159 and 83122 Da calculated from the genomically deduced sequence for 1By8 (Acc. No. AAO64642) and 1Bx7 (Acc. No. AAZ23584). The MALDI mass spectrum also shows two signals at m/z 37593 and 41554 corresponding to doubly charged molecular ions of the two subunits. From the values of the doubly charged molecular ions, which are close to the enolase signal used as the internal calibrant, the M_r of 75184 and 83106 Da, respectively, can be deduced for the two HMW-GS. These values, respectively, differ by +25 and −16 Da from the M_r of 75159 and 83122 Da calculated from the genomically deduced sequence for 1By8 and 1Bx7. This result shows, as previously reported,[15−18] that measurement of the doubly charged molecular ion, which occurs in a mass region close to the molecular mass of the protein standard used, gives more reliable results in terms of accuracy than extrapolation of a single point calibration in a mass range very far from that of the protein standard.

The spectrum also shows a partially resolved signal at m/z 82217 and the corresponding doubly charged molecular ion at m/z 41235 from which a mass of 82469 Da can be calculated, corresponding to a component with a molecular mass of about 600 Da lower than that of the 1Bx7 subunit. This finding is in agreement with previous data reported for the characterization of HMW-GS type x.[15−18] Moreover, the MALDI mass spectrum, shows signals at 42 kDa due to the contamination of some LMW-GSs that occurred on HMW-GSs' pellet during the extraction procedure.

The MALDI mass spectra of the HMW-GS extracted from the other durum wheat cultivars analyzed (Svevo, Duilio, Bronte, and Sant'Agata) gave analogous results; the M_r determined for these cultivars from the m/z value of the doubly charged ions are reported in Table 1. Considering the intrinsic uncertainty of about 0.1% at this mass range, all the values are coincident, within the experimental error, with the molecular masses determined for the corresponding HMW-GS in Simeto.

To characterize the HMW-GS present in the 13 accessions of durum wheat landrace Timilia (*Triticum durum* Desf.), the proteins were extracted from the flours and analyzed by MALDI-MS. The MALDI mass spectrum of the HMW-GS fraction extracted from Timilia 7 (Figure 1b), obtained using 2,6-DHAP as a matrix and calibrated internally with enolase, shows the presence of two intense MH^+ signals at m/z 75056 and 86101. The spectrum shows also two signals at m/z 37667 and 43201 corresponding to the doubly charged molecular ions of the subunits. From these values, which are close to the enolase used as the internal calibrant, M_r of 75332 and 86400 Da can be deduced for the two HMW-GS.[17] The spectrum also shows a partially resolved signal at m/z 85595 and the corresponding doubly charged molecular ion at m/z 42904, and signals due to LMW-GS coprecipitated during the extraction procedure, as discussed above for the MALDI mass spectrum of the HMW-GS fraction extracted from Simeto.

The MALDI mass spectrum of the HMW-GS fraction extracted from Timilia 7 is representative of all the Timilia accessions investigated, as given by all of them. In fact, all the other accessions gave analogous results, and the value determined for the M_r values of the two HMW-GS present in each fraction are reported in Table 1. All the values are coincident, within experimental error, with the molecular masses determined for the corresponding HMW-GS in Timilia 7, indicating that the diverse populations contain the same two HMW-GSs.

Table 3. Fragment Nomenclature, Sequence Position, and Calculated and Experimental Monoisotopic and Average Masses of Expected and Identified Tryptic Fragments of Timilia's 1By HMW-GS (GenBank Acc. No. AAQ93633)

		calcd MH^{+a}			RP-HPLC/nESI- MS/MS		
fragment	position	monoisotopic	average	MALDI MS measured MH^+	rt. (min)	experimental MH^+(PIM^b, charge)	sequence
T1	1−6	648.3	648.6				EGEASR
T2	7−12	881.4	882.0	881.5	24.4	1.688 (441.3, 2+)	QLQCER
T3	13−23	1369.64	1370.5	1369.7	38.7	1369.8 (685.4, 2+)	ELQESSLEACR
T4	24−33	1113.6	1114.2	1113.7	36.9	1113.7 (557.3, 2+)	QVVDQQLAGR
T5	34−43	1188.6	1189.4	1188.7	46.5	1188.9 (594.9, 2+)	LPWSTGLQMR
T6	44−49	960.4	961.2	960.5	24.4	960.5 (960.5, 1+)	CCQQLR
T7	50−54	519.3	519.6				DVSAK
T8	55−65	1318.7	1319.6	1318.8	37.1	1318.9 (659.9, 2+)	CRPVAVSQVVR
T9	66−75	1188.6	1189.4	1188.7	38.8	1188.9 (594.9, 2+)	QYEQTVVPPK
T10	76−136	6529	6533	6532	58.9	6534[c]	GGSFYPGETTPLQQLQQVIFWGTSSQTVQGYYPSVSSPQQGPYYPGQ ASPQQPGQGQQPGK
T11	137−215	8650	8655	8653	45.7	8657[c]	WQELGQGQQGYYPTSLHQSGQGQQGYYPSTLQQPGQGQQTGQGQ QGYYPTSLQQPGQGQQIGQGQQGYYPTSPQHPGQR
T12	216−234	1993.0	1994.1	1993.1	37.2	1993.2 (997.1, 2+)	QQPGQGQQIGQGQQLGQGR
T13	235−361	13610	13618	13615	43.3	13622[c]	QIGQGQQSGQGQQGYYPTSPQQLGQGQQPGQWQQSGQGQQGYYP TSQQQPGQGQQGQYPASQQQPGQGQQGQYPAS QQQPGQGQQGQ YPASQQQPAQGQQGQYPASQQHPGQGQQGHYLASQQQPGR
T14	362−365	488.3	488.5				GQQR
T15	366−416	5451	5454	5454	43.8	5455[c]	HYPASLQQPGQGQQGHYTASLQQPGQGQQGHYPASLQQVGQGQQI GQLGQR
T16	417−426	1143.6	1144.2	1143.7	23.3	1143.6 (572.3, 2+)	QQLGQGQQTR
T17	427−444	1996.0	1997.1	1996.1	32.6	1996.0 (998.5, 2+)	QGQQLEQGQQPGQGQQTR
T18	445−520	8085	8089	8087	42.4	8091[c]	QGQQLEQGQQPGQGQQGYYPTSPQQSGQGQQPGQSQQPGQGQQ GYYSTSLQQPGQGQQGHYPASLQQPGQGHPGQR
T19	521−554	3682	3684	3684	42.8	3685[c]	QQPGQGQQPEQGQQPGQGQQGYYPTFPQQPGQGK
T20	555−672	12580	12588	12588	43.2	12591[c]	QLGQGQQGYYPTSPQQPGQGQQPGQGQQGHCPTSPQQTGQAQQ PGQGQQIGQVQQPGQGQQGYY- PISLQQSGQGQQSGQGQQSGQGH QLGQGQQSGQEQQGYDNPYHVNTEQQTASPK
T21	673−675	317.2	317.4				VAK
T22	676−688	1589.8	1591.0	1589.9			VQQPATQLPIMCR
T23	689−689	1065.4	1066.1		35.5	1065.6 (1065.6, 1+)	MEGGDALSASQ

[a] Cys: S-pyridylethylated. [b] PIM: precursor ion mass. [c] Calculated as the average from all the multicharged ions.

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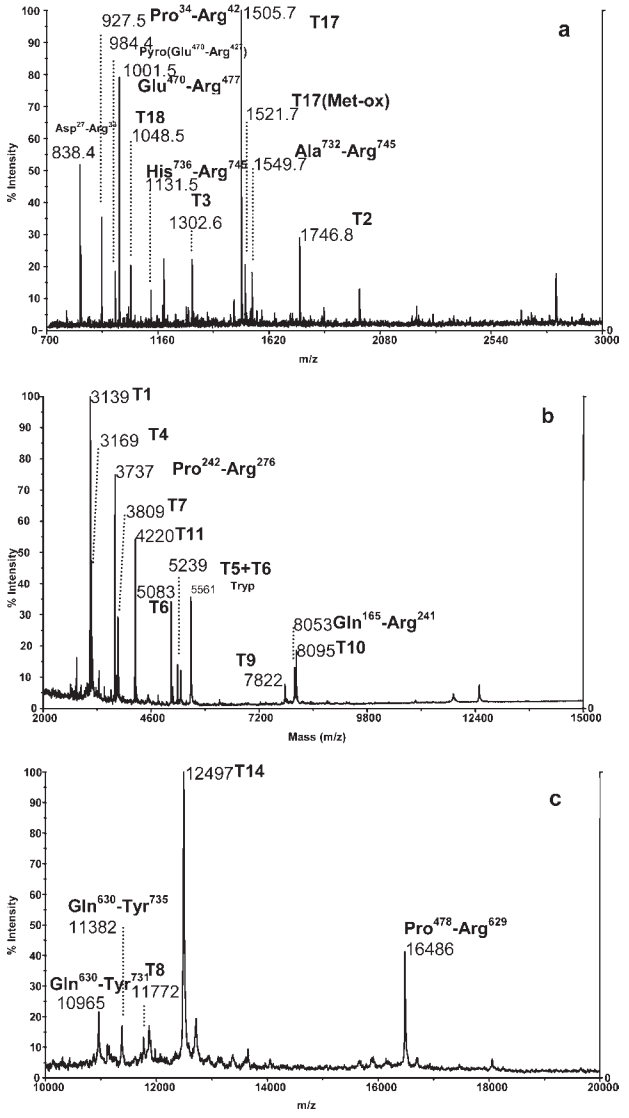

Figure 4. MALDI mass spectra of the tryptic digest of the reduced and *S*-pyridylethylated HMW subunit 1Bx from cv. Simeto in the *m*/*z* range (a) 700−3000 (reflector ion mode), (b) 2000−15000 (linear ion mode), and (c) 10000−20000 (linear ion mode).

The comparison of the HMW-GS molecular masses of the populations of Timilia and the other durum wheat cultivars (Table 1) clearly shows that the HMW-GSs contained in the Timilia accessions are different from those present in the other durum wheat cultivars. Moreover, the M_r determined by MALDI mass spectrometry for the x and y HMW-GSs in Timilia do not correspond to any of the documented M_r of HMW-GSs.

In order to determine the primary structure of the two Timilia HMW-GS and to assess their structural differences with respect to the corresponding subunits contained in Simeto and in the others durum wheat cultivars, the proteins were extracted from Timilia 7 and Simeto, as representative of the two groups, reduced, and alkylated with 4-VP and single subunits isolated by RP-HPLC-UV. The isolated HMW-GS were digested with trypsin, and the peptide mixtures were analyzed directly by MALDI-MS and RP-HPLC/ESI-MSMS.

MALDI mass spectra at low, medium, and high mass ranges were recorded for the proteolytic mixture of the HMW-GS 1Bx isolated from Timilia. The MALDI mass spectrum of the tryptic fragments in the *m*/*z* range 700−3000 is shown in Figure 2a. The spectrum shows signals at *m*/*z* 1001.5, 1018.5, 1065.6, 1302.7, 1411.7, 1505.8, 1746.9, 1763.9, and 2048.1. Figure 2b shows the MALDI mass spectrum of the tryptic mixture in the *m*/*z* range 2000−15000, in which signals at *m*/*z* 2261, 3168, 3699, 4393, 7831, 8198, 8805, 8961, and 9048 are present. Finally, the MALDI mass spectrum of the tryptic mixture in the mass range 10000−20000 (Figure 2c) shows signals at *m*/*z* 12528, 12728, 16006, 18155, and 19140. The monoisotopic and average mass values subjected to bioinformatic search by MASCOT software allowed the identification of a HMW-glutenin Bx subunit of *Triticum turgidum* (Acc. No. AAQ93629) inserted in the database as a conceptual translation of chromosome 1B.[25] By comparison of the *m*/*z* value of MALDI mass spectra signals with the *m*/*z* of the peptides originated by theoretical tryptic cleavage of the identified sequence, it was possible to match 14 theoretical peptides (T1, T5, T6, T7, T8, T10, T11, T12, T13, T14, T15, T16, T19, and T20), 4 peptides originated by aspecific cleavages at the Arg-Pro bond (Gln^{170}—Arg^{255}, *m*/*z* 9048; Pro^{256}—Arg^{290}, *m*/*z* 3699; Gln^{484}—Arg^{491}, *m*/*z* 1001.5; Pro^{492}—Arg^{658}, *m*/*z* 18155), and two peptides originated by missed cleavages (T3+T4+T5, *m*/*z* 2048.1; T9+T10, *m*/*z* 8961) (Table 2).

The data obtained allowed the identification of all the peptides originated by tryptic cleavage of the HMW-GS 1Bx of *Triticum turgidum* (Acc. No. AAQ93629) with the exception of the short peptides or single amino acid residues T2, T17, T18 and T21. The sequence is covered for 98.9%.

A similar analytical procedure was employed for the characterization of the sequence of HMW-GS 1By isolated from Timilia. The MALDI mass spectrum of the tryptic fragments in the *m*/*z* range 700−3000 is shown in Figure 3a. The spectrum shows signals at *m*/*z* 881.5, 960.5, 1113.7, 1143.7, 1188.7, 1318.8, 1369.7, 1589.9, 1605.9, 1993.1, and 1996.1. The MALDI mass spectrum of the tryptic digest in the *m*/*z* range 2000−15000 (Figure 3b) shows signals at *m*/*z* 3684, 5454, 6532, 8087, 8653, 12588, and 13615. Insertion of these *m*/*z* values into MASCOT in PMF mode resulted in the identification of a HMW-GS By of *Triticum turgidum* (Acc. No. AAQ93633), inserted in the database as a conceptual translation of chromosome 1B.[25] By comparison of the *m*/*z* value of MALDI mass spectra signals with the *m*/*z* of the peptides originated by theoretical tryptic cleavage of the identified sequence, it was possible to match all of the 23 theoretical peptides, with the exception of peptides T1, T7, T14, T21, and T23 (Table 3). Moreover, it was not possible to attribute the signal at *m*/*z* 1188.6 corresponding to the two isobaric peptides T5 and T9 (Table 3).

In order to obtain complementary data, the tryptic digest of cv. Timilia was also analyzed by RP-HPLC/ESI-MS and MS/MS. Identification of the fragments already detected by MALDI/TOF-MS was confirmed by this experiment. In addition, the tryptic fragment T23, previously undetected, was identified in the peak eluting at rt 35.5 min, which gave the expected MH^+ ion at *m*/*z* 1065.3. Unambiguous attribution of this signal to the C-terminal fragment T23 was obtained recording the MS/MS spectrum of the fragments ions of the doubly charged molecular ion at *m*/*z* 533.6, which confirmed the sequence of peptide T23. Furthermore, the TIC chromatogram showed two peaks at 38.8 and 46.5 min, which gave a MH^+ at *m*/*z* 1188.8, corresponding to fragments T10 and T5, respectively. Unequivocal attribution of the chromatographic peaks to the two isobaric fragments was achieved by recording the MS/MS spectra of the fragment ions of

Table 4. Fragment Nomenclature, Sequence Position, and Calculated and Experimental Monoisotopic and Average Masses of Expected and Identified Tryptic Fragments of Simeto's 1Bx7 HMW-GS (Acc. No. AAZ23584)

		calcd MH^+ [a]			
fragment	position	monoisotopic	average	MALDI-MS MH+ measured	sequence
T1	1−26	3137.4	3139	3139	EGEASGQLQCEHELEACQQVVDQQLR
T2	27−42	1746.9	1748.0	1746.8	DVSPGCRPITVSPGTR
Asp27-Arg33	27−33	838.4	838.9	838.4	
Pro34-Arg42	34−42	927.5	928.1	927.5	
T3	43−53	1302.7	1303.5	1302.2	QYEQQPVVPSK
T4	54−81	3166.6	3169	3169	AGSFYPSETTPSQQLQQMIFWGIPALLR
T5	82−82	175.1	175.2		R
T(5 + 6)	82−129	5236.4	5239	5239	
T6	83−129	5080.3	5083	5083	YYPSVTSSQQGSYYPGQASPQQSGQGQQPGQEQQPGQGQQDQQPGQR
T7	130−164	3806.8	3809	3809	QQGYYPTSPQQPGQGQQLGQGQPGYYPTSQQPGQK
T8	165−276	11764.4	11771	11772	QQAGQGQQSGQGQQGYYPTSPQQSGQGQQPGQGQPGYYPTSPQQSGQWQQPGQGQQP GQGQQSGQGQQGQQPGQGQRPGQGQQGYYPISPQQPGQGQQSGQGQPGYYPTSLR
Gln^{165}-Arg^{241}	165−241	8048.6	8053	8053	
Pro^{242}-Arg^{276}	242−276	3734.7	3737	3737	
T9	277−350	7817.4	7822	7822	QPGQWQQPGQGQQPGQGQQGQQPGQGQQSGQGQQGYYPTSLQQPGQGQQLGQGQ PGYYPTSQQSEQGQQPGQGK
T10	351−427	8090.6	8095	8095	QPGQGQQGYYPTSPQQSGQGQQLGQGQPGYYPTSPQQSGQGQQSGQGQQGYYPTSPQ QSGQGQQPGQGQSGYFPTSR
T11	428−467	4217.9	4220	4220	QQSGQGQQPGQGQQSGQGQQGQQPGQGQQAYYPTSSQQSR
T12	468−469	303.2	303.3		QR
T13	470−629	17459.1	17469.4		QQAGQWQRPGQGQPGYYPTSPQQPGQEQQSGQAQQSGQWQLVYYPTSPQQPGQLQQ PAQGQQPAQGQQSAQEQQPGQAQQS
Gln^{470}-Arg^{477}	470−477	1001.5	1002.1	1001.5	GQWQLVYYPTSPQQPGQLQQPAQGQQGYYPTSPQQSGQGQQGYYPTSPQQSGQGQQG YYPTSPQQSGQGQQPGQGQQPR
Pro^{478}-Arg^{629}	478−629	16476.1	16486	16486	
T14	630−745	12489.6	12497	12497	QGQQGYYPISPQQSGQGQQPGQGQQGYYPTSPQQSGQGQQPGHEQQPGQWLQPGQGQQG YYPTSSQQSGQGHQSGQGQQGYYPTSLWQPGQGQQPGQGQQGYASPYHVSAEYQAAR
Gln^{630}-Tyr^{735}	630−735	11377.1	11383	11382	
His^{736}-Arg^{745}	736−745	1131.6	1132.2	1131.5	
Gln^{630}-Tyr^{731}	630−731	10958.9	10965	10965	
Ala^{732}-Arg^{745}	732−745	1549.7	1550.7	1549.7	
T15	746−747	260.2	260.4		LK
T16	748−750	317.2	317.4		VAK
T17	751−763	1505.8	1506.8	1505.7	AQQLAAQLPAMCR
T18	764−773	1048.5	1049.3	1048.5	LEGSDALSTR
T19	774−774	147.1	147.2		Q

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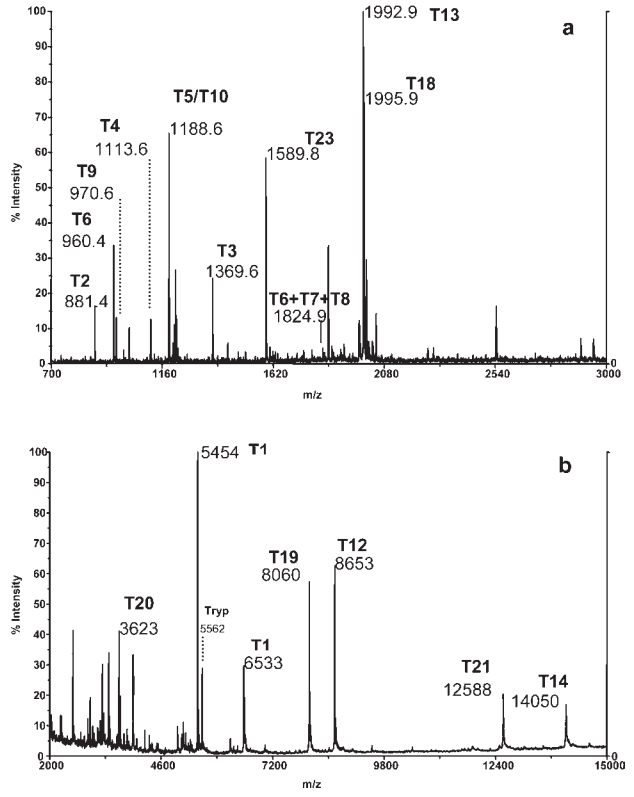

Figure 5. MALDI mass spectra of the tryptic digest of the reduced and *S*-pyridylethylated HMW subunit 1By from cv. Simeto in the m/z range (a) 700−3000 (reflector ion mode) and (b) 2000−15000 (linear ion mode).

the doubly charged ions at m/z 595.1, which confirmed the sequences of the two peptides. As a whole, the data obtained allowed the identification of all the peptides originated by tryptic cleavage of the reported gene derived sequence of HMW-GS 1By (Acc. No. AAQ93633) with the exception of the short peptides T1, T7, T14, and T21 (Table 3). The sequence was covered for 97.4%.

Using the same procedure, sequence verification was also performed for the isolated 1Bx7 and 1By8 subunits present in Simeto. MALDI mass spectra at low, medium, and high mass ranges were recorded for the proteolytic mixture of the HMW-GS 1Bx7 isolated from Simeto. The MALDI-MS spectrum of the tryptic fragments in the m/z range 700−3000 is shown in Figure 4a. The spectrum shows signals at m/z 838.4, 927.5, 984.4, 1001.5, 1048.5, 1131.5, 1302.6, 1505.7, 1521.7, 1549.7, and 1746.8. Figure 4b shows the MALDI-MS spectrum of the tryptic mixture in the m/z range 2000−15000, in which peaks at m/z 3139, 3167, 3737, 3809, 4220, 5083, 5239, 7822, 8053, and 8095 are present. Finally, the MALDI mass spectrum of the tryptic mixture in the mass range 10000−20000 (Figure 4c) shows signals at m/z 10965, 11382, 11772, 12497, and 16486. The monoisotopic and average mass values subjected to bioinformatic search by MASCOT software allowed the identification of a HMW-GS 1Bx7 subunit of *Triticum aestivum* (Acc. No. AAZ23584). By comparison of the m/z value of MALDI mass spectra signals with the m/z of the peptides originated by theoretical tryptic cleavage of the identified sequence, it was possible to match 13 theoretical peptides (T1, T2, T3, T4, T6, T7, T8, T9, T10, T11, T14, T17, and T18), 4 peptides originated by aspecific cleavages at the Arg−Pro bond (Gln^{165}−Arg^{241}, m/z 8053; Pro^{242}−Arg^{276}, m/z 3737; Gln^{478}−Arg^{477}, m/z 1001.5; Pro^{478}−Arg^{629}, m/z 16486), and 4 peptides originated by aspecific trypsin cleavages (Gln^{630}−Tyr^{735}, m/z 11382, and His^{736}−Arg^{745}, m/z 1131.6; Gln^{630}−Tyr^{630}, m/z 10965, and Ala^{732}−Arg^{745}, m/z 1549.7). The data obtained are reported in Table 4. All the peptides originated by tryptic cleavage of the HMW-GS 1Bx7 of a *Triticum aestivum* (Acc. No. AAZ23584) were identified with the exception of the short peptides or single amino acid residues T5, T15, T16, and T19. The sequence was covered for 98.96%.

A similar analytical procedure was employed for the characterization of the sequence of HMW-GS 1By8 isolated from Simeto. The MALDI-MS spectrum of the tryptic fragments in the m/z range 700−3000 is shown in Figure 5a. The spectrum shows signals at m/z 881.4, 960.4, 970.6, 1113.6, 1188.6, 1369.6, 1589.8, 1824.9, 1992.9, and 1995.9. The MALDI mass spectrum of the tryptic digest in the m/z range 2000−15000 (Figure 5b) shows signals at m/z 3624, 5454, 6533, 8060, 8653, 12588, and 14050. Insertion of these m/z values into MASCOT in PMF mode resulted in the identification of a HMW-GS 1By8 of *Triticum turgidum* subsp. *durum* (Acc. No. AAO64642). By comparison of the m/z value of MALDI mass spectra signals with the m/z of the peptides originated by theoretical tryptic cleavage of the identified sequence, it was possible to match all of the 24 theoretical peptides, with the exception of peptides T1, T16, T17, T22, and T24. Moreover, it was not possible to attribute the signal at m/z 1188.6 corresponding to the two isobaric peptides T5 and T10. In order to obtain complementary data, the tryptic digest was also analyzed by RP-HPLC/ESI-MS and MS/MS. Identification of the fragments already detected by MALDI/TOF-MS was confirmed by this experiment. In addition, the tryptic fragment T24, previously undetected, was identified. Unambiguous attribution of this signal to the C-terminal fragment T24 was obtained recording the MS/MS spectrum of the fragments ions of the doubly charged molecular ion at m/z 533.6, which confirmed the sequence of peptide T24. Furthermore, HPLC/nESI-MSMS analysis allowed one to obtain unequivocal attribution of the two isobaric fragments T5 and T10 by recording the MS/MS spectra of the fragment ions of the doubly charged ions at m/z 595.1, and it was possible to confirm the sequences of the two peptides.

The data obtained are reported in Table 5. In summary, all of the peptides originated by tryptic cleavage of the HMW-GS 1By8 of *Triticum turgidum* subsp. *durum* (Acc. No. AAO64642) were identified with the exception of the short peptides T1, T16, T17, and T22. The sequence was covered for 97%.

Sequence alignments of the verified HMW-GS Bx and By from Timilia with the HMW-GS 1Bx7 and 1By8 from Simeto are reported in Figure 6. The Timila Bx subunit presents a sequence similarity of 96% with respect to Simeto, with differences in the insertion of 3 peptides of 5, 9, and 15 amino acids, for a total insertion of 29 amino acids and 25 amino acid substitutions (Figure 6a). These differences in the amino acidic sequence clearly explains the Δm of 3294 Da between the M_r of the 1Bx subunits in Timilia and Simeto, as determined by the MALDI mass spectra of the intact subunits (Figure 1).

Sequence alignment between the two By subunits shows 10 amino acids substitutions (Figure 6b) and is consistent with the Δm of 148 Da found in the MALDI mass spectra of the intact subunits.

Amino acid insertions and substitution generate a set of diagnostic peptides for the Bx subunit (T1, T5, T10−T16, T20) and the By subunit (T8, T13, T16, T18, and T19), as compared to the Simeto subunits. These peptides are underlined and labeled with asterisks in Figures 2 and 3.

Table 5. Fragment Nomenclature, Sequence Position, and Calculated and Experimental Monoisotopic and Average Masses of Expected and Identified Tryptic Fragments of Simeto's 1By8 HMW-GS (GenBank Acc. No. AAO64642)

		MH^+ calcd[a]			RP-HPLC/nESI- MS/MS		
fragment	position	monoisotopic	average	MALDI-MS measured MH^+	rt. (min)	experimental MH^+ (PIM^b, charge)	sequence
T1	1−6	648.3	648.6				EGEASR
T2	7−12	881.4	882.0	881.4	25.9	881.5(441.2, 2+)	QLQCER
T3	13−23	1369.6	1370.5	1369.6	36.4	1369.6 (685.3, 2+)	ELQESSLEACR
T4	24−33	1113.6	1114.2	1113.6		1113.7 (557.3, 2+)	QVVDQQLAGR
T5	34−43	1188.6	1189.4	1188.6	46.3	1188.7 (594.8, 2+)	LPWSTGLQMR
T6	44−49	960.5	961.2	960.4			CCQQLR
T7	50−54	519.3	519.6				DVSAK
T8	55−56	383.2	383.5				CR
T(6 + 7+8)	44−56	1824.9	1826.2	1824.9			
T9	57−65	970.6	971.2	970.6	40.2	970.6 (485.8, 2+)	LVAVSQVVR
T10	66−75	1188.6	1189.4	1188.6	35.4	1188.7 (594.8, 2+)	QYEQTVVPPK
T11	76−136	6529.1	6533	6533	58.8	6534[c]	GGSFYPGETTPLQQLQQVIFWGTSSQTVQGYYPSVSSPQQGPYYPGQASPQQ PGQGQQPGK
T12	137−215	8648.0	8653	8653	45.9	8653[c]	WQELGQGQQGYYPTSLHQSGQGQQGYYPSSLQQPGQGQQIGQGQQGYYP TSLQQPGQGQQIGQGQQGYYPTSPQHPGQR
T13	216−234	1993.0	1994.1	1992.9	34.5	1993.1 (997.0,2+)	QQPGQGQQIGQGQQLGQGR
T14	235−365	14042.4	14050	14050	46.3	14050[c]	QIGQGQQSGQGQQGYYPTSPQQLGQGQQPGQWQQSGQGQQGYYPTSQ QQPGQGQQGQYPASQQQPGQGQQGQYPASQQQPGQGQQGQYPASQQ QPAQGQQGQYPASQQQPGQGQQGHYLASQQQPGQGQQR
T15	366−416	5450.6	5454	5454			HYPASLQQPGQGQQGHYTASLQQPGQGQQGHYPASL QQVGQGQQIGQLGQR
T16	417−421	585.3	585.6				QQPGR
T17	422−426	589.3	589.6				GQQTR
T18	427−444	1996.0	1997.1	1995.9	29.9	1996.1 (998.5, 2+)	QGQQLEQGQQPGQGQQTR
T19	445−520	8055.7	8060	806	42.6	8061[c]	QGQQLEQGQQPGQGQQGYYPTSPQQSGQGQQPGQSQQPGQGQQGYYS SSLQQPGQGLQGHYPASLQQPGQGHPGQR
T20	521−554	3621.7	3623.8	3623.8	36.3	3624.0 (1208.6, 3+)	QQPGQGQQPEQGQQPGQGQQGYYPTSPQQPGQGK
T21	555−672	12580.8	12588	12588	42.1	12588[c]	QLGQGQQGYYPTSPQQPGQGQQPGQGQQGHCPTSPQQTGQAQQP GQGQQIGQVQQPGQGQQGYYPISLQQSGQGQQSGQGQQSGQGHQL GQGQQSGQEQQGYDNPYHVNTEQQTASPK
T22	673−675	317.2	317.4				VAK
T23	676−688	1589.8	1590.9	1589.8	41.2	1589.8 (795.4, 2+)	VQQPATQLPIMCR
T24	689−699	1065.5	1066.1		35.5	1065.3 (594.8, 2+)	MEGGDALSASQ

[a] Cys: S-pyridylethylated. [b] PIM: precursor ion mass. [c] Calculated as the average from all the multicharged ions.

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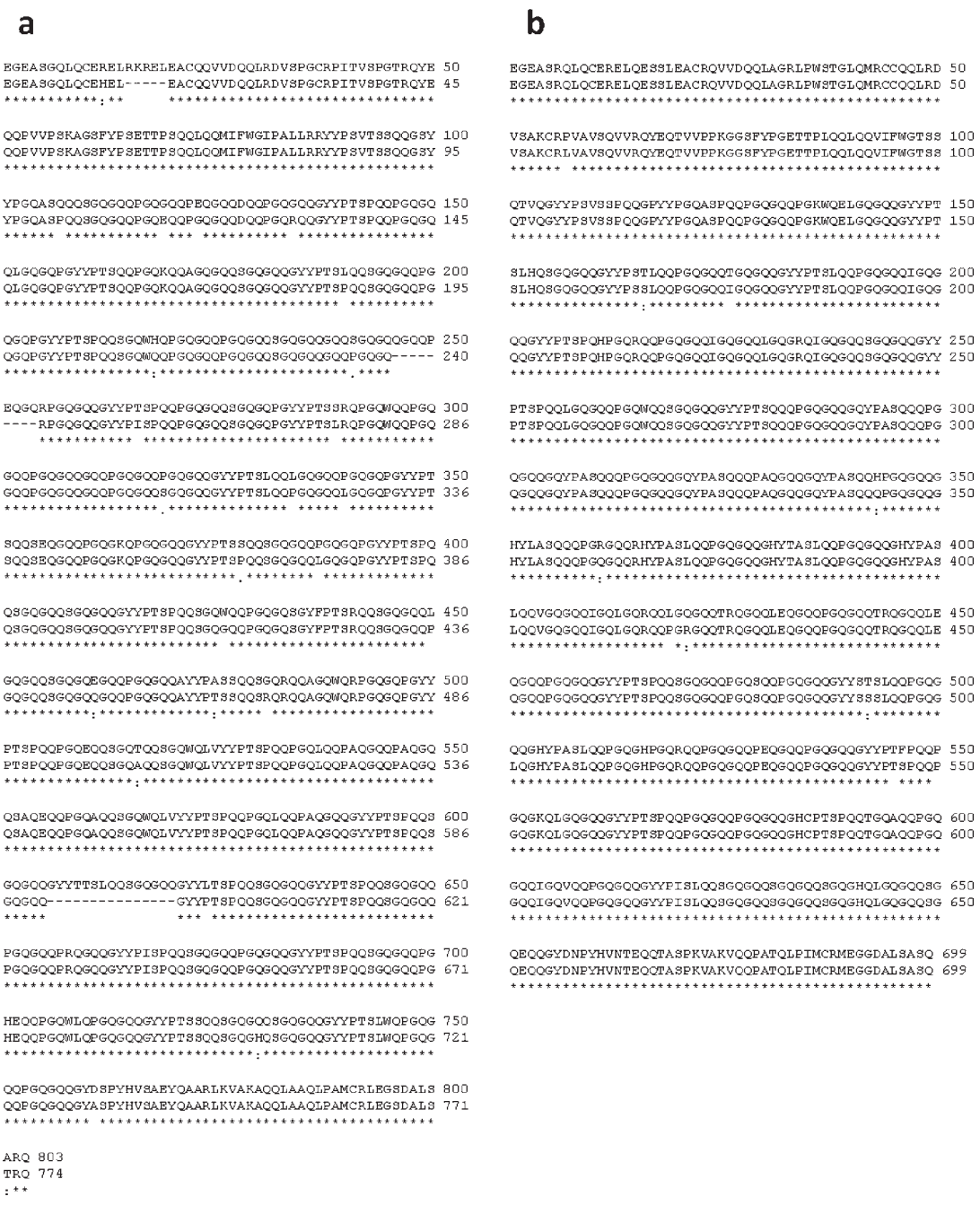

Figure 6. Comparison of the gene-derived sequences of the HMW subunits of *T. durum* cultivar Timilia (upper row) and Simeto (lower row): (a) 1Bx and (b) 1By sequence alignment.

In general, the molecular characterization of the protein components of local wheat varieties is an irreplaceable way to trace the local plant biodiversity that needs to be recognized, categorized, and preserved. An application of this study can be found in tracking commercial frauds or fakes. Semolina derived from Timilia is used to produce a traditional Sicilian bread named Pane Nero di Castelvetrano (Black Bread from Castelvetrano, Trapani),[26] whose price is higher than that for the usual bread. It can thus be subject to mixing or substitution with cheaper flours. This study highlights how Timilia's HMW-GSs could constitute protein markers, thanks to their simple isolation and characterization. The determination of HMW-GS's M_r and the presence diagnostic peptides could discriminate the presence of Timilia in a mixture with durum wheat cultivars usually employed for bread making.

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