Characterization of Wheat Gluten Proteins by HPLC and MALDI TOF Mass Spectrometry

Yuwei Qian,^a Ken Preston,^b Oleg Krokhin,^a Jean Mellish,^b and Werner Ens^a

^a Department of Physics and Astronomy, University of Manitoba, Winnipeg, Manitoba, Canada

^b Grain Research Laboratory, Canadian Grain Commission, Winnipeg, Manitoba, Canada

We have performed a detailed characterization and identification of wheat gluten proteins obtained from the Teal variety of Canadian hard red spring wheat. RP-HPLC separation of the sample into 35 fractions has reduced the spectral complexity; this was followed by MALDI mass spectrometry (MS), which showed the presence of six or fewer resolved protein components above 20 kDa in each RP-HPLC fraction, giving a total of 93 MS resolved peaks. These included 17 peaks in the ω -gliadin fractions (F1-4), 12 in the high molecular weight (HMW) glutenin subunit fractions (F5-8), 59 in the α - and β -gliadins and low molecular weight (LMW) glutenin subunit fractions (F9-31) and 5 peaks in the γ -gliadin fractions (F32-35). Peptide maps of tryptic digests of HPLC fractions were obtained from a tandem quadrupole time-of-flight mass spectrometer (MALDI QqTOF MS) and were submitted to the ProFound search engine. HMW glutenin subunits including Ax2*, Dx5, Bx7, and Dy10 (consistent with the known profile of Teal), and LMW glutenin subunits including six from group 3 type II and 1 from group 2 type I, were identified with reasonable sequence coverage from HPLC fraction 5, 7, 17, and 18. The identities of the peptides attributed to selected gluten proteins were confirmed using MS/MS with BioMultiView to match the predicted and measured partial amino acid sequences. Because of incomplete wheat DNA databases, many wheat gluten proteins could not be identified. These results suggest that the combination of RP-HPLC with MS and MS/MS techniques is a promising approach for the characterization of wheat gluten proteins. (J Am Soc Mass Spectrom 2008, 19, 1542–1550) © 2008 American Society for Mass Spectrometry

The gluten (storage) proteins in wheat are responsible for imparting the unique visco-elastic properties that allow the production of leavened bread, pasta, noodle, and related products. The two major groups of these proteins include the monomeric gliadins and the polymeric glutenins consisting of inter-chain disulphide bonding low molecular weight (LMW) and/or high molecular weight (HMW) glutenin subunits [1]. Electrophoretic and RP-HPLC patterns of gluten proteins and their gliadin and LMW glutenin subunit components are complex, showing a large number of components, while simpler patterns are evident for the HMW glutenin proteins [2]. The complexity of these patterns can be attributed to the presence of two or three sets of homologous chromosomes in durum and common wheat, respectively, and to additional polymorphism related to mutation of gluten protein genes into many allelic forms [3]. Gliadin patterns have been extensively used to provide definitive identification of all but the most closely genetically related varieties [1]. Specific peaks or patterns, particularly those of the HMW glutenin subunits, have been

associated with differences in processing performance among wheat classes and varieties, allowing their use as quality markers [4–7].

There are several difficulties associated with proteomic analysis of wheat storage proteins, including the limited databases available, the limited number of basic residues (particularly in the LMW glutenin subunits), the complexity resulting from the presence of sets of homologous proteins, and the presence of repeating motifs. In spite of this, wheat proteins have been widely studied using HPLC [1, 8], and mass spectrometry techniques are now widely applied alone or in combination with other chromatographic techniques in proteomic studies of wheat [9–12]. Previous studies have established that MALDI-TOF MS can resolve a large number of wheat gliadins and glutenin subunits [9]. MALDI-MS of purified HMW subunits yielded molecular mass determinations consistent with their cDNA derived amino acid sequences [10]. The use of MALDI-MS for mapping tryptic peptides of HMW subunits demonstrated good agreement between MS measured molecular weights and those derived from the gene sequences [11, 12]. Tryptic digests have also been analyzed using ESI mass spectrometry in MS and MS/MS modes to confirm amino acid sequences of HMW subunits [12]. In this study we demonstrate the

Address reprint requests to Dr. W. Ens, TOF Laboratory, 506 Allen Bldg, Department of Physics and Astronomy, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada. E-mail: ens@cc.umanitoba.ca

viability of detailed characterization and identification of wheat gluten proteins by RP-HPLC followed by high-resolution MALDI mass spectrometry (MS and MS/MS) of the resulting protein fractions and their trypsin peptide digests. For this study, we have selected the CDC Teal variety, which is a registered crop under Canada Western Red Spring Wheat. It is a high protein bread wheat variety that was developed by the Crop Development Centre at the University of Saskatchewan. It is one of the top five crops in Canada.

Methods

Reagents and Chemicals

All the solvents used for sample preparations and HPLC, 1-propanol (Caledon, Georgetown, ON, Canada) and acetonitrile (Fisher Scientific, Canada), were distilled or HPLC grade. The reagents for reduction and alkylation, dithiothreitol (DTT) and iodoacetamide (Sigma Chemical Co., St. Louis, MO, USA), were enzyme grade. Ammonium hydrogencarbonate was from Sigma-Aldrich Inc. (St. Louis, MO, USA). Photometric grade trifluoroacetic acid (TFA) was from Sigma-Aldrich. Sequencing grade Protease was trypsin from Roche Diagnostics Co. (Indianapolis, IN, USA); 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid from Sigma-Aldrich were used as matrices. The all-in-1 protein standard kit used for MALDI-TOF calibration was from Ciphergen Biosystems Inc. (Fremont, CA, USA). Peptides for MALDI-QqTOF calibration were obtained from American Peptide Co. (Sunnyvale, CA, USA) (enkephalin) and Sigma-Aldrich (Substance P and bee venom melittin).

Protein Extraction

Gluten proteins were extracted from a Canadian hard red spring bread wheat variety (CDC Teal). Seed was ground on an Udy cyclone mill fitted with a 1 mm sieve. Approximately 1 g of ground wheat and 6 mL of 50% 1-propanol containing 1% DTT were combined in an Oak Ridge 30 mL centrifuge tube (Nalge Co., Rochester, NY, USA) and held in a water bath at 60 °C for 0.5 h with intermittent vortexing every 10 min. The mixture

was then centrifuged for 10 min at $8000 \times g$ at 25 °C and the supernate was removed and filtered through a 0.45 μm syringe filter (Whatman, Clifton, NJ, USA) before HPLC analysis.

RP-HPLC

Extracts were analyzed on a Waters chromatography system (600S Controller, 616 Pumps and 717 Autosampler) (Waters Corporation, New Castle, DE, USA) controlled with Millennium software using a procedure similar to that of Marchylo et al. [8]. Three aliquots ($3 \times 5 \mu\text{L}$) of the filtered extract were injected into a Zorbax SB300-C18 reversed-phase column (15 cm $\times$ 4.6 mm i.d.) (Agilent Technology, Palo Alto, CA, USA) and separation was carried out at 60 °C using a linear gradient of aqueous acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min. The acetonitrile concentration was increased from 24% to 50% over 108 min and held constant thereafter. Column effluent was monitored at 210 nm. Thirty-five major peaks from the HPLC chromatogram were identified and collected individually (Figure 1). The concentrations of protein in most HPLC elute fractions were from 10 to 80 ng/ μL , measured by use of a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

MALDI-TOF of Intact Proteins

Preliminary studies showed that alkylation of the reduced protein from RP-HPLC did not interfere with the determination of mass measurements. Two hundred μL of each HPLC fraction was vacuum-concentrated in a Speed Vac (Model SC110, Savant) to almost dryness and then re-constituted in 70 μL of 50% acetonitrile containing 0.1% TFA with vortexing to dissolve the proteins. The reconstituted fractions had protein concentrations varying from 30 to 240 ng/ μL . On a metal surface sample chip (made in our lab), 1 μL of reconstituted sample was deposited onto a designated spot and dried. On the top of the dried sample, 0.5 μL of freshly prepared sinapinic acid (30 mg/mL) was added and air-dried before loading the chip onto a Ciphergen

Figure 1. Reversed-phase HPLC profile of reduced gluten extract from CDC Teal.

MALDI-TOF instrument (Model PBS-II, Ciphergen Biosystems Inc., Fremont, CA, USA).

MALDI-TOF MS in positive ion mode was optimized in the mass range of 10 to 60 kDa for α -, β -, and γ -gliadins and LMW glutenin subunit fractions. For the larger ω -gliadin and HMW glutenin subunit fractions, the optimal mass range was set from 40 to 120 kDa. External calibration was performed using the protein calibration kit (Ciphergen Biosystems Inc.) containing proteins with masses after protonation of 12,360.2 (horse cytochrome *c*), 16,951.5 (horse muscle myoglobin), 35,688.0 (rabbit GAPDH), 66,433.0 (bovine serum albumin), and 116,351.0 (*E. coli* β -galactosidase). Approximately 75 shots on each sample spot were used to obtain spectra. Delayed extraction using a pulse lag time of 1328 ns was used to enhance resolution [3].

Peptide Mass Mapping (MS) and Peptide Fragmentation (MS/MS) Using MALDI-QqTOF

One hundred μ L from each HPLC fraction was vacuum concentrated (Speed Vac) to almost dryness and then reconstituted in 5 μ L of 50% acetonitrile containing 0.1% TFA with vortexing to dissolve the proteins. Five μ L of 100 mM ammonium hydrogencarbonate was added into the solution to optimize conditions for tryptic digestion. The gluten proteins in each fraction were then reduced by adding 1.1 μ L of 100 mM DTT followed by incubation in a water bath at 60 °C for 45 min with intermittent vortexing every 10 min. The reduced proteins were alkylated by adding 11.1 μ L of 100 mM iodoacetamide and keeping the solution in the dark for 30 min with intermittent vortexing every 10 min. The reduced and alkylated proteins were incubated with 1.5 μ L of trypsin (100 ng/ μ L) at 37 °C for 20 h. Tryptic digests (1 μ L) were deposited on a metal surface sample plate, air-dried, and 160 mg/mL DHB (1 μ L) was added on top with air drying. Samples were then analyzed in positive ion mode using a MALDI QqTOF prototype instrument constructed in collaboration with MDS SCIEX [13]. The instrument was used to obtain high-resolution spectra in the single-MS and tandem MS modes. External calibration was performed using protonated ions of dalargin, Substance P, and bee venom melittin with monoisotopic masses of 726.394, 1347.736, and 2845.762, respectively. Peptide spectra were obtained using 600 to 1200 shots while MS/MS spectra were obtained using 4000 to 6000 shots. MSMS data were interpreted using BioMultiView, a commercial program from MDS-SCIEX. Monoisotopic masses from this instrument are reported for all peaks unless otherwise stated.

Results and Discussion

Analysis of Intact Proteins and Protein Subunits

The RP-HPLC profile of the reduced CDC Teal wheat gluten protein extract is shown in Figure 1. The 35

Figure 2. Mass spectra of intact proteins from selected HPLC fractions of Teal by MALDI TOF mass spectrometry: (a) fraction 5, (b) fraction 7, (c) fraction 17, and (d) fraction 18.

fractions collected, representing major peaks, can be divided into four groups based on their retention times [2, 8]: the ω -gliadins (fractions F1–F4), the HMW glutenins (F5–F9), a mixture of α - and β -gliadins and LMW glutenins (F9–F31), and the γ -gliadins (F32–F35).

Each fraction was analyzed by MALDI using linear TOF to examine intact proteins. Examples of spectra for four of the fractions are shown in Figure 2. The spectrum from each fraction resolved between 1 and 6 protein components with molecular masses over 20 kDa. The number of components observed in the mass spectra and their molecular weight range is summarized in Table 1 for each of the four groups of fractions.

Table 1. MALDI-TOF characteristics of gluten protein fractions from RP-HPLC

Region	Fractions	Number of major peaks	Molecular weight (kDa) (MALDI-TOF)
ω -gliadins	F1–F4	10 peaks	30–35
		7 peaks	42–52
HMW-glutenins	F5–F8	7 peaks	32–42
		5 peaks	67–88
α -, β -gliadins, LMW-glutenins	F9–F31	59 peaks	29–42
γ -gliadins	F32–F35	5 peaks	30–38

The ω -gliadin fractions showed molecular masses in two distinct ranges: 30–35 kDa and 42–52 kDa. These values are considerably lower than previous estimates of the ω -gliadin molecular masses based on SDS PAGE results (45–75 kDa) due to anomalous migration in SDS PAGE [14, 15]. However, more recent studies by Wieer et al. [16], based on MALDI-TOF analysis, showed a range of 36–44 kDa for ω -1,2 type gliadins and 44–55 kDa for ω -5 type gliadins, more consistent with our values, although still somewhat higher.

The m/z values from the HMW glutenin fractions also fell into two ranges, with 7 peaks ranging from about 32 to 42 kDa and 5 peaks ranging from 67 to 88 kDa. In addition to the peaks shown in Figure 2 for fractions F5 and F7, the mass spectrum of fraction F6 showed two peaks in the lower mass range, and the spectrum of F8, which eluted as a shoulder after F7, had one additional peak in the lower mass range not present in the spectrum of F7. The higher mass range is consistent with values derived from HMW glutenin gene sequences which show putative masses ranging from 67 to 74 kDa for γ -type HMW glutenin subunits and 83 to 88 kDa for α -type HMW glutenin subunits [7]. The five observed peaks are consistent with the number of HMW subunits expressed in CDC Teal as determined by genetic analysis and SDS PAGE [Lukow, O., private communication]. The origin of the peaks in the lower mass range are not yet understood. They may represent ω -gliadins that can overlap with HMW glutenins in RP-HPLC [8], and/or other wheat protein components with similar hydrophobicities such as HMW albumins, which are also extracted in 50% 1-propanol/1% DDT [10].

The third group of fractions (F9–31) consisting of α - and β -gliadins and LMW glutenin subunits gave 59 peaks ranging in molecular mass from about 29 kDa to 42 kDa. This range is also somewhat lower than the 36–44 kDa range expected as determined by SDS-PAGE [7], but consistent with MALDI-TOF MS results showing major gliadin peaks in the 30–39 kDa range and major LMW glutenin subunit peaks in the 30–43 kDa range [5].

The four γ -gliadin fractions showed five distinct peaks with masses between 30 and 38 kDa. This range is similar to that reported for α - and β -gliadins and LMW glutenin subunits [7].

Although the most effective separation of gluten protein components is still obtained by 2-D electrophoresis [2], the combination RP-HPLC and MS seems

to compare favorably with this technique. Dachkevitch et al. separated about 45 gliadin components using 2-D electrophoresis in Neepawa [14], a Canadian red spring wheat variety closely related to the CDC Teal used in this study. An advantage of the MS approach is that the measured mass is less sensitive to systematic effects than the position in a gel, and is therefore expected to be more effective as a characteristic marker for specific gluten proteins.

Protein Identifications

Each RP-HPLC fraction was subjected to tryptic digestion and then analyzed by MALDI QqTOF. The resulting peptide maps were then searched against a protein database (NCBI or SWISS-PROT) for matching proteins using the ProFound search engine [17]. Because each fraction contained multiple proteins, a tight mass tolerance of 30 ppm was necessary for peptide peak identification to reduce false positives. Fortunately, the accuracy of the QqTOF instrument is consistent with this level of accuracy. The databases currently accessible to ProFound for wheat proteins are far from complete, and as a result, most fractions did not result in a protein identification. Nevertheless, positive search responses were obtained from fractions F5, F7, F17, and F18. The MALDI QqTOF peptide maps for fraction F5, F7, F17 and F18 are shown in Figure 3.

HMW Protein Identities

Among the HMW protein subunits tentatively identified were Dy10 (P10387) from F5 and 1Ax1 (S15720), Ax2*(AAB02788), Dx5 (P10388), and Bx7 (A30843) from F7. The HMW protein subunits are subdivided into high M_r x-type and low M_r y-type subunits [3]. Two genes, encoding an x-type and y-type, are present on the 1A, 1B, and 1D chromosomes of hexaploid bread wheat [3]. All cultivars of wheat contain six HMW protein subunits, but only 3, 4, or 5 subunits are present because some of the genes are silent (1Ay gene is silent in all bread wheat varieties). The 1Ax1 gene is associated with good breadmaking quality but is not present in many cultivars [3, 18].

Sequence coverage ranged from 10% to 36% (Table 2). The relatively low sequence coverage obtained for these subunits is mainly due to the scarcity of arginine and lysine cleavage points in the large central domain of HMW glutenin subunits. This domain contains multi-

Figure 3. Tryptic peptide maps from fractions 5, 7, 17, and 18 by MALDI-QqTOF mass spectrometry.

ple copies of hexapeptide (PGQGQQ), nanopptide (GYPTSPQQ), and (in x-type subunits only) tripeptide (GQQ) motifs, and only rare arginine and lysine cleavage sites [19]. Trypsin cleavage in this region produces mainly large peptides, which are above the 5000 Da limit used by the search engine. When the observed peptide map is compared with the predicted map containing only peptides below 5000 Da, the coverage ranges from 50% for Dx5 to well over 70% for 1Ax1, Ax2*, and Dy10 (Table 2).

Additional peptides above 5000 Da were also observed but with lower mass accuracy because of the

weak signal intensity. These were compared with predicted tryptic fragments in this mass range based on average, rather than monoisotopic masses. A peak at m/z 8587 in the spectrum of fraction F5 is consistent with the tryptic peptide from residues 390 to 469 in the subunit Dy10. In the mass spectrum of the F7 fraction, peaks at m/z 7821 and m/z 8094 are consistent with tryptic peptides containing residues 298 to 371, and 372 to 448 from the subunit Bx7; a peak at m/z 8534 is consistent with residues 735 to 812 in Dx5; and a peak at m/z 8533 is consistent with a predicted tryptic fragment from both Ax2* (residues 270 to 348) and 1Ax1 (residues

Table 2. Identification of HMW glutenin subunits based on peptide patterns obtained in the MALDI-QTOF instrument and intact mass (no alkylation) measured with linear MALDI-TOF

Subunit	Code	Z ^a	HPLC fraction	Peptide coverage (%)	Maximum coverage ^b (%)	Obs'd mass (Da)	Calc. mass ^c (Da)	Δm (Da)
Dy10	P10387	2.31	F5	36	44	67401	67475	64
Ax1	Q03872	2.08	F7	26	31	87919	87679	240
Ax2*	AAB 02788	2.08	F7	31	38	86124	86317	193
Dx5	P10388	2.34	F7	10	20	87919	87191	728
Bx7	JN0690	2.34	F7	18	25	82897	82863	34

^aThe Z-score is used by ProFound as an indicator of the quality of the search result. It represents the number of standard deviations that the selected goodness-of-fit metric exceeds the mean value of the same metric for a random population. Thus, by standard statistics, for a normal distribution of random proteins, a Z-score of 1.65 represents 95% confidence that the protein is not random; Z = 2.3 represents 99% confidence. (http://cebs.niehs.nih.gov/cebs-browser/help/proteomics/profound_mascot_info.html).

^bCoverage observed if all peptides under mass 5000 Da are observed.

^cBased on the DNA sequence.

276 (y54). Only one Ax gene allele (1Ax1 or Ax2*) is present in each wheat cultivar, but according to a DNA sequence study on 1Ax1 and Ax2* [18], the two subunits have very close homology, sharing many cryptic peptides in the mass range of 500 to 5000 Da. However, two peptides were observed from Ax2* at m/z 2761 and 2842 that are not present in 1Ax1, whereas none of the observed peptides were unique to 1Ax1. This suggests that Ax2* is present, and 1Ax1 is not.

To confirm the identities of the Ax peptides, further analyses of tryptic peptides assigned to Ax2* and 1Ax1 were carried out using tandem mass spectrometry (MS/MS). BioMultView was used to extract amino acid sequence data from the spectra. The MS/MS spectrum of the 1138 Da peptide, assigned to 1Ax1 / Ax2* is shown in Figure 4. Its deduced sequence data match perfectly with the DNA derived sequence for both subunits. Both y- and b-ions are labeled for clarity, but all a-ions were also identified in the MS/MS spectrum. Results of 18 peptides assigned to Ax2* are summarized in Table 3. The position of each listed peptide corresponds to the subunit before removal of the signal peptide. The sequence data of all 18 peptides perfectly matched the DNA-translated primary sequence of the predicted Ax2* peptides identified by mass mapping.

As mentioned above, two of these peptides (2761 and 2842 Da) are not present in 1Ax1.

The predicted masses of the intact subunits identified above, based on published DNA sequences, show good agreement with the observed m/z values obtained by linear MALDI-TOF (Table 2). The peak at m/z 87,919 detected from the elution fraction F7 may be assigned to two subunits 1Ax1 and Dx5. However, the peak at m/z 86,124 is only in good agreement with the mass expected for Ax2*. Since only one Ax gene allele is expected, this result combined with the results of the peptide analysis above strongly suggests that Ax2* is present, and 1Ax1 is absent.

MS/MS analyses were also carried out on peptides assigned to the subunits Bx7 and Dx5. In these cases, weak signal intensity made complete sequence analysis impossible. However, based on peaks above m/z 500, partial sequence matches were confirmed for peptides observed at m/z 1302 (y5~y11, b4~b11), m/z 1698 (y5~y16, b5~b16), m/z 3322 (y5~13, y15, y18~y22, y24~y27, y29), and m/z 3806 (y5~y35, b5~b31, b32~b35) for Bx7. For Dx5, a full sequence match was confirmed for the peptide at m/z 1990 (y1~18) while partial matches above m/z 500 were obtained for peaks

Figure 4. MS/MS of the peptide at 1138 Da from Ax2* or 1Ax1 HMW glutenin subunit by MALDI-QTOF mass spectrometry.

Table 3. Observed amino acid sequences from MS/MS measurements using MALDI QqTOF and the corresponding association with predicted sequences in both 1Ax1 and Ax2* from the databases. The peptides are assumed to be alkylated

Obs'd mass (Da)	Amino acid sequence from MALDI QqTOF	Ax2* position	Ax1 position
826	QWLQPR	725–730	740–745
887	QGQQSGQR	349–356	355–362
982	ELQEHSLK	34–41	34–41
984	QVVDQQLR	45–52	45–52
1138	QQPGQGQQLR	195–204	201–210
1456	AQLAAQLPAMCR	792–804	807–819
1482	QGQQGQSGQGQPR	205–218	211–224
1623	DVSPECQPVGGGPVAR	53–68	53–68
1668	QQDQQSGQGQPGQR	357–371	363–377
1902	YYPTSPQQPGQEQQPR	390–405	396–411
1990	QPGYYSTSPQQLGQGQPR	372–389	378–395
2564	GQQGQSGQGQQLGQGQSGQGQPGQK	245–269	251–275
2761	QLQQPEQGQGGQGGQPEQGQGGQGGQR	406–429	×
2842	QPGYYPTSPWQPEQLQQPTQGQQR	171–194	×
2944	YYPTSSQQPGQLQQLAQGGQGGQGPQR	219–244	225–250
3209	GGSFYPGETTPPQQLQSSILWGIPALLR	80–108	80–108
3641	QQDQQSGQGQPGQRPQPGYYSTSPQQLGQGQPR	357–389	363–395
4349	QYEQVVVPPKGGSFYPGETTPPQQLQSSILWGIPALLR	69–107	69–107

at m/z 1044 (y4~y8, b4~b8), m/z 1157 (y5, y6, y8, y10, b4~b8), and m/z 1313 (y4~6, y9, y11, b4~b6, b11).

Genetic and SDS PAGE studies have identified the HMW glutenin subunits in CDC Teal as Ax2*, Bx7, Dx5, By8, and Dy10 [Lukow, O., private communication]. This is in good agreement with our results except that By8 could not be identified since no DNA sequence for this subunit has yet been published. However, a peak at mass 74.61 kDa was observed by linear MALDI TOF from the fraction F7 (Figure 2) that closely corresponds to the mass of the By8 subunit determined by capillary electrophoresis using Ferguson plot analysis [15].

LMW Protein Identities

LMW glutenin subunits were identified from database searches against peptide maps obtained from fractions F17 and F18. In F17, six group 3 type II subunits (BAB78740, BAB78741, BAB78742, BAB78743, BAB78744, and BAB78745) were tentatively identified and in F18, one group 2 type I subunit (BAB78738) was identified. Coverage with peptides below 5000 Da ranged from 22% to 26% of the sequence (Table 4). Here again, the scarcity of arginine and lysine cleavage points in the repetitive proline-rich

regions in the LMW glutenin subunits [7] results in relatively few predicted tryptic peptides below the 5000 Da limit of the search engine. With the exception of one of the subunits, all the predicted peptides in this mass range were observed; 3 of 6 were observed for BAB78738 (Table 4).

The DNA sequences for the group 3 type II LMW subunits show very strong similarities [20], so the peptide mass maps should be expected to have significant overlap. As a result, the data do not provide definitive evidence that all of the expected subunits are present. Table 5 shows the complete list of predicted peptides used to tentatively identify the subunits from fraction F17, and indicates which of the peptides is expected and observed in each case. For all six subunits, all the predicted peptides with m/z between 500 and 5000 were observed by MALDI QqTOF. However, three peptides are common to all six subunits, and two pairs (BAB78740 and BAB78745, BAB78743 and BAB78744) share identical sets of five predicted peptides with four peptides common to all four subunits. The presence of BAB78741 and BAB78742 are perhaps the most definitive, since they both have one predicted peptide not present in the others.

Table 4. Identification of LMW glutenin subunits based on peptide patterns obtained in the MALDI QqTOF instrument and intact mass measured with linear MALDI TOF

Identified subunit	Database code	HPLC	Peptide coverage (%)	Max. coverage (%)	Obs'd mass (kDa)	Pred. mass (kDa)	Trunc. mass ^a (kDa)
Group 3 type II	BAB78740	F17	22	22	42.18	42.00	40.70
Group 3 type II	BAB78741	F17	26	26	42.18	42.02	40.71
Group 3 type II	BAB78742	F17	22	22	42.18	42.00	40.69
Group 3 type II	BAB78743	F17	24	24	37.52	38.79	37.48
Group 3 type II	BAB78744	F17	25	25	37.52	37.21	35.89
Group 3 type II	BAB78745	F17	26	26	34.27	35.82	34.05
Group 2 type I	BAB78738	F18	25	37	37.84	39.45	37.50

^aTruncated mass after removal of the signal peptide.

Table 5. Ulryptic and alkylated peptides in the mass range of 500 to 5000 da, from LMW glutenin subunits

Peptides (+H)(Da)	Group 3 type II						Group 2 type I
	BAB 78740	BAB 78741	BAB 78742	BAB 78743	BAB 78744	BAB 78745	BAB 78738
637.33	—	—	—	—	—	—	×
651.35	✓	✓	✓	✓	✓	✓	—
946.50	✓	✓	—	—	—	✓	—
1109.56	—	—	✓	✓	✓	—	—
1195.64	—	—	—	—	—	—	✓
1622.81	—	✓	—	—	—	—	—
1677.85	✓	✓	✓	✓	✓	✓	—
2030.03	—	—	✓	—	—	—	—
2060.05	✓	✓	—	✓	✓	✓	—
2074.06	—	—	—	—	—	—	×
2323.98	—	—	—	—	—	—	×
2767.42	—	—	—	—	—	—	✓
3443.57	✓	✓	✓	✓	✓	✓	—
4524.30	—	—	—	—	—	—	✓

✓: predicted and observed; ×: predicted but not observed; —: not predicted.

For the tentatively identified LMW group 2 type I subunit (BAB78738), three of the six predicted peptides were observed in the MALDI QTOF peptide pattern (data not shown). A higher mass peptide was also identified at 5426.30 Da, which matches a predicted peptide at 5426.20 Da. Now, only a few DNA sequences for this group have been determined in contrast to the group 3 subunits discussed above [20].

The intact masses of the identified proteins derived from their DNA sequences give reasonable matches to the observed peaks in the linear MALDI TOF spectra (Table 4). The predicted masses are determined after the removal, if necessary, of the signal peptides in positions 1 to 4 (<http://www.cgs.dtu.dk/services/SignalP-2.0>). However, such comparisons of intact protein masses must be viewed with caution since some of the cloned LMW subunits genes used to deduce the sequence may have contained deletions of the repetitive domain [21] resulting in underestimation of the actual MW. Moreover, several of the subunits have intact molecular weights too similar to be resolved by linear MALDI TOF.

Conclusions

The results from this study show that RP-HPLC combined with MALDI mass spectrometry (MS) has the capability to effectively assess the complexity of wheat gluten proteins, and to provide reasonably accurate intact mass assignments. In combination with analysis of cryptic digests by MALDI QTOF, this approach also shows potential for the identification of specific HMW and LMW glutenin subunits, which are primary determinants of wheat processing quality. The use of sequence data from MS/MS measurements can be very valuable, and occasionally essential, to improve the confidence in protein assignments. The identification of gluten protein by this approach is currently hampered

by the lack of available DNA sequences for gluten protein, the relatively low levels of arginine and lysine in these proteins, which produces mainly large peptides, and the close similarity in sequences among groups of these subunits.

Acknowledgments

The authors acknowledge support for this work by grants from the Natural Sciences and Engineering Research Council of Canada.

References

- MacRitchie, F. Physicochemical Properties of Wheat Proteins in Relation to Functionality. *Adv. Food Nutr. Res.* **1992**, 36, 1–87.
- Bietz, J. A.; Simpson, D. G. Electrophoresis and Chromatography of Wheat Proteins: Available Methods and Procedures for Statistical Evaluation of the Data. *J. Chromatogr.* **1992**, 624, 53–80.
- Payne, P. I. Genetics of Wheat Storage Proteins and the Effect of Allelic Variation on Bread-Making Quality. *Ann. Rev. Plant Physiol.* **1987**, 38, 141–153.
- Cornish, G. B.; Skylas, D. J.; Siriamornpun, S.; Békés, F.; Larroque, O. R.; Wrigley, C. W.; Wootton, M. Grain Proteins as Markers of Genetic Traits in Wheat. *Aust. J. Agric. Res.* **2001**, 52, 1161–1171.
- Dworschak, R. G.; Ens, W.; Standing, K. G.; Preston, K. R.; Marchylo, B. A.; Nightingale, M. J.; Stevenson, S. G.; Hatcher, D. Analysis of Wheat Gluten Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *J. Mass Spectrom.* **1998**, 33, 429–435.
- Mann, M.; Hendrickson, R. C.; Pandey, A. Analysis of Proteins and Proteomes by Mass Spectrometry. *Annu. Rev. Biochem.* **2001**, 70, 437–473.
- Shewry, P. R.; Tatham, A. S. The Prolamin Storage Proteins of Cereal Seeds: Structure and Evolution. *Biochem. J.* **1990**, 267, 1–12.
- Marchylo, B. A.; Hatcher, D. W.; Kruger, J. E.; Kirkland, J. J. Reversed-Phase High-Performance Liquid Chromatographic Analysis of Wheat Proteins Using a New, Highly Stable Column. *Cereal Chem.* **1992**, 69, 371–378.
- Godovac-Zimmermann, J.; Brown, L. R. Perspectives for Mass Spectrometry and Functional Proteomics. *Mass Spectrom. Rev.* **2001**, 20, 1–57.
- Hickman, D. R.; Roepstorff, P.; Shewry, P. R.; Tatham, A. S. Molecular Weights of High Molecular Weight Subunits of Glutenin Determined by Mass Spectrometry. *J. Cereal Sci.* **1995**, 22, 99–103.
- Foti, S.; Maccarrone, G.; Saletti, R.; Roepstorff, P.; Gilbert, S.; Tatham, A. S.; Shewry, P. R. Verification of the cDNA Deduced Sequence of Glutenin Subunit 1Dx5 and an M-r 58,000 Repetitive Peptide by Matrix-Assisted Laser Desorption/Ionization Mass spectrometry (MALDI-MS). *J. Cereal Sci.* **2000**, 31, 173–183.
- Cunsolo, V.; Foti, S.; Saletti, R.; Gilbert, S.; Tatham, A.; Shewry, P. R. Structural Studies of Glutenin Subunits 1Dy10 and 1Dy12 by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry and High-

- Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 442–454.
13. Loboda, A. V.; Krutchinsky, A. N.; Bromirski, M.; Ens, W.; Standing, K. G. A. Tandem Quadrupole/Time-of-Flight Mass Spectrometer with a Matrix-Assisted Laser Desorption/Ionization Source: Design and Performance. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1047–1057.
 14. Dachkevitch, T.; Redaelli, R.; Biancardi, A. M.; Metakovsky, E. V.; Pogna, N. E. Genetics of Gliadins Coded by the Group 1 Chromosomes in the High-Quality Bread Wheat Cultivar Neepawa. *Theor. Appl. Genet.* **1993**, *86*(2/3), 389–399.
 15. Werner, W. E. Ferguson Plot Analysis of High Molecular Weight Glutenin Subunits by Capillary Electrophoresis. *Cereal Chem.* **1995**, *72*, 248–251.
 16. Seilmeier, W.; Valdez, I.; Mendex, E.; Wieser, H. Comparative Investigations of Gluten Proteins from Different Wheat Species. II. Characterization of Omega-Gliadins. *Eur. Food Res. Technol.* **2001**, *212*, 355–363.
 17. Zhang, W.; Chait, B. ProFound: An Expert System for Protein Identification Using Mass Spectrometric Peptide Mapping Information. *Anal Chem.* **2000**, *72*, 2482–2489.
 18. Halford, N. G.; Field, J. M.; Blair, H.; Urwin, P.; Moore, K.; Robert, L.; Thompson, R.; Flavell, R. B.; Tatham, A. S.; Shewry, P. R. Analysis of HMW Glutenin Subunits Encoded by Chromosome 1A of Bread Wheat (*Triticum aestivum* L.) Indicates Quantitative Effects on Grain Quality. *Theor. Appl. Genet.* **1992**, *83*, 373–378.
 19. Shewry, P. R.; Halford, N. G.; Tatham, A. S. The High Molecular Weight Subunits of Wheat Glutenin. *J. Cereal Sci.* **1992**, *15*, 105–120.
 20. Ikeda, T. M.; Nagamine, T.; Fukuoka, H.; Yano, H. Characterization of New Low Molecular-Weight Glutenin Subunit Genes in Wheat. *Theor. Appl. Genet.* **2002**, *104*, 680–687.
 21. Masci, S.; D'Ovidio, R.; Lafiandra, D.; Kasarda, D. D. Characterization of a Low-Molecular-Weight Glutenin Subunit Gene from Bread Wheat and the Corresponding Protein that Represents a Major Subunit of the Glutenin Polymer. *Plant Physiol.* **1998**, *118*, 1147–1158.