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Analysis of Wheat Gluten Proteins by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) was used to analyze the protein composition in several common and durum wheat varieties. Mass spectra were obtained directly from crude and partially purified wheat gliadin and reduced glutenin subunit fractions. Mass spectra of the gliadins and the low molecular weight (LMW) glutenin subunits show a complex pattern of proteins in the 30–40 kDa range. The observed gliadin patterns may be suitable for differentiation between wheat varieties, but the complexity of the mass spectra precludes the use of MALDI/MS as a stand-alone technique for the identification of most individual gliadin components. The mass spectra of the high molecular weight (HMW) glutenin subunits are much simpler and the complete HMW subunit profile can be determined directly from a single mass spectrum. This may prove particularly useful in wheat breeding programs for rapid identification of lines containing subunits associated with superior quality. The correspondence between previously identified HMW subunits and the mass spectral peaks was established with MALDI measurements of HPLC-separated subunits. Delayed extraction proved effective in improving the mass resolution for the monomeric gliadins and LMW glutenin subunit fractions, with masses less than 40 kDa. However, it gave little improvement for the HMW glutenin subunits which have masses of ~80 kDa.

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INTRODUCTION

The physical dough properties and processing characteristics of wheat flours are determined primarily by the quantitative and qualitative properties of the gluten protein complex. Both major protein groups in this complex, the monomeric gliadins and the polymeric inter-chain disulfide-bonded glutenins, are associated with quality differences among wheat varieties.[1–4] The gliadins are a heterogeneous group consisting of more than 100 proteins[5] divided into four subgroups (α, β, γ, ω) based upon electrophoretic mobility.[6] Most have molecular masses in the 30–40 kDa range, although some ω-gliadins have a molecular mass near 80 kDa.[7] The polymeric glutenin proteins, with molecular masses ranging from less than 300 kDa into the millions,[4,7–10] are composed of two groups of subunits. The low molecular weight (LMW) glutenin subunits are similar in size and structure to the γ-gliadins (30–40 kDa). The high molecular weight (HMW) glutenin subunits range in molecular mass from ~65–90 kDa.[7] There is a substantially larger variety of LMW subunits compared with the HMW subunits and they account for most of the mass of the glutenin proteins.[4,7]

At present, characterization of the protein composition in wheat and other grains is done almost exclusively by a combination of gel electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC). These techniques have been used to establish strong positive relationships between dough strength and/or baking quality and the size distribution of the glutenin polymers.[11] This size distribution appears to be determined by the genetically controlled composition of both LMW and HMW subunits,[4,8,9,12,13] although the relative amount of the larger polymers appears to correlate most strongly with the distribution of HMW subunits.[8,9] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC methods are used routinely in many breeding programs for the selection of specific HMW subunits associated with superior quality.[4] Varietal quality can also be assessed by similar analysis of the gliadins,[14,15] but this method is not currently widespread in breeding programs. On the other hand the wide diversity in the gliadin patterns is very useful for varietal identification.[16,17] In Canada and other exporting countries, these procedures are used extensively to confirm the classification of commercial samples.[18]

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Mass spectrometry has recently emerged as an alternative method to characterize high molecular mass molecules and molecular composition with the development of new ionization methods. In particular, matrix-assisted laser desorption/ionization (MALDI) in combination with time-of-flight mass spectrometry (TOF-MS) has made it possible to study complex mixtures of large, thermally labile, non-volatile biopolymers such as peptides, proteins and oligonucleotides with molecular masses up to several hundred kilodaltons.[19–21] The technique typically requires less than 1 pmol of sample and is relatively tolerant of impurities compared with other MS techniques, allowing analyses of crude or partially purified protein or peptide mixtures.[21,22] Compared with the separation methods described above, MALDI is much more accurate and much faster, requiring only a few minutes per sample to perform the measurement. The high throughput is particularly attractive for the possibility of rapid varietal identification in the field.

MALDI has recently been used to assess the molecular mass of purified wheat α-gliadins[23] and HMW glutenin subunits after separation by HPLC[24] and for the direct analysis of unfractionated prolamins from various grains, including the gliadins from wheat.[25] This latter study showed that the observed pattern of the prolamins is characteristic of the grain.

Here we report the use of MALDI for assessing the composition and mass distribution of crude and partially purified wheat gliadin and reduced HMW and LMW glutenin subunit fractions from common and durum wheat varieties.[26] MALDI was also used to determine the molecular masses of several HPLC-purified HMW glutenin subunits to establish a correspondence between proteins observed in the mass spectra of the crude mixtures and proteins already identified and coded by HPLC and electrophoresis. The results are discussed in relation to potential applications involving wheat gluten protein component identification for quality assessment in breeding programs, for molecular mass characterization and for varietal identification.

EXPERIMENTAL

Pure samples of the following wheat varieties were obtained from stocks maintained at the Grain Research Laboratory in Winnipeg: Katepwa, Teal, Columbus and Pasqua varieties from the Canada Western Red Spring common (hexaploid) commercial class; Kyle, Plenty and Arcola varieties from the Canada Western Amber Durum (tetraploid) commercial class; Biggar and AC Tober varieties from the Canada Prairie Spring commercial class; and Glenlea and Wildcat varieties from the the Extra Strong Red Spring commercial class. Each sample was confirmed for varietal purity by electrophoresis.[27] All chemicals were of reagent grade unless stated otherwise. HPLC-grade acetonitrile, dithiothreitol (DTT) and ethanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and trifluoroacetic acid (TFA) from Sigma (St Louis, MO, USA). HPLC-grade deionized water, prepared with a Milli-Q plus-TOC water purification system (Millipore, Bedford, MA, USA), was employed to prepare all solutions.

Protein extraction

Wheat samples (25 g) were ground in a UDY cyclone sample mill equipped with a 1 mm sieve. Gliadins were extracted from ground grain (1.0 g) into 70% (v/v) ethanol (6 ml) at room temperature for 60 min. Extracts were then centrifuged at 20000*g* for 10 min. Glutenins were extracted sequentially from ground grain (1.0 g) into 50% (v/v) propan-1-ol containing 1% (w/v) DTT as described previously[28] with some modification (albumins and globulins were not pre-extracted with 0.5% (w/v) salt). HMW glutenins were purified by precipitation as described previously[28] and the remaining LMW glutenin supernatant fraction was retained. All fractions were filtered through Gelman 0.45 μm syringe filters prior to HPLC analysis and aliquots of each extract (150 μl) were dried using a Juoan Vacuum Concentrator prior to MALDI analysis.

HPLC

A Waters (Milford, MA, USA) HPLC system with Waters Millenium 2010 software was used to fractionate HMW glutenin subunits for some measurements. Analyses were performed with a Zorbax 300SB-C8 column (300 Å pore size, 5 μm particle size, 15 cm × 4.6 mm i.d.; Chromatographic Specialties, Brockville, ON, Canada). The column temperature was 50 °C and the eluent was monitored at 210 nm. Three aliquots (3 × 5 μl) were injected on to the column and the proteins were resolved with an aqueous (0.1% TFA) linear gradient extending from 24–48% acetonitrile at a flow rate of 1 ml min^{-1} for 120 min.[29] Individual HMW glutenin subunits were collected and dried as above.

MALDI

The dried gliadin samples were reconstituted in 300 μl of 0.1% trifluoroacetic acid in water. The dried LMW and HMW glutenin samples were reconstituted in 150 μl of acetonitrile mixed with 150 μl of 0.1% trifluoroacetic acid in water. All reconstituted stock protein solutions were then kept in a water-bath at 60 °C for 30 min with intermittent vortexing to dissolve the protein completely. The resulting stock solutions had concentrations varying from 30–600 μmol l^{-1}. A saturated solution of the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in aqueous 0.1% TFA–acetonitrile (2:1, v/v) was first applied to the sample probe, dried and then crushed.[30] The stock solution was then mixed (1:10, v/v) with the matrix solution and a few microliters were applied to the crushed spot on the sample probe. When a visible film began to form on the probe surface (about 10 s), the spot was rinsed with cold deionized water for 10 s and then allowed to dry thoroughly prior to analysis.[30]

The above washing procedure significantly improved the signal-to-noise ratio in the mass spectra, particularly

for the LMW and HMW glutenin subunit fractions. This may be a result of the removal of DTT, which was used as a reducing agent during the preparation of the subunits. Spectra of alkylated (with vinylpyridine) and non-alkylated reduced glutenin subunits were comparable, which confirmed that alkylation was not required to prevent re-oxidation (results not shown).

Positive ion mass spectra were obtained on a MALDI/TOF mass spectrometer built in-house (Manitoba TOF II)[31] and run in the linear mode. A nitrogen laser (VSL 337 ND, Laser Science, Cambridge, MA, USA) was used to illuminate the target with a pulse frequency of 2–3 Hz. For some of the gliadin and LMW glutenin subunits, a two-grid delayed extraction system[32] was employed using a 25 kV d.c. accelerating potential on the probe and first grid with a pulse of 3 kV applied to the probe 1.2 μs after the laser pulse. The 3 kV pulse was supplied by a high-voltage switch (Behlke, Frankfurt, Germany). For the HMW glutenin subunits, d.c. extraction was employed with target and first grid potentials of 30 and 18 kV, respectively. Following desorption and acceleration, ions drift through a field-free region of 1.2 m in which the nominal pressure was 3×10^{-7} Torr (1 Torr = 133.3 Pa) measured with an ion gauge. The ions were detected with microchannel plates and the signal was recorded with a transient recorder (LeCroy TR8828D) from which the spectra were summed by an Atari (TT030) computer. There were ~150–200 shots accumulated per spectrum. The gliadin and LMW glutenin subunit spectra were calibrated externally with carbonic anhydrase (29 021 Da[33]) whereas the HMW glutenin subunit spectra were calibrated externally with human transferrin (79 549 Da[33]). Better mass accuracy would be possible with internal calibration but the emphasis here is more on the overall pattern and the feasibility of the technique for rapid and routine analysis.

RESULTS AND DISCUSSION

Delayed extraction

The use of delayed extraction in MALDI/TOF-MS to give improved resolution has mainly been emphasized for peptides and small proteins up to about 10 kDa,[32,34,35] although some examples up to 30 kDa have been presented.[34,36] Above ~20 kDa, the improvement compared with d.c. extraction drops rapidly and above 30 kDa little or no improvement has been reported. Recently, Bahr *et al.*[37] reported that with judicious choice of matrix and pulsing conditions to minimize metastable fragmentation, delayed extraction can give enhanced resolution up to about 60 kDa. In our measurements, using sinapinic acid as the matrix, we observed a significant improvement in the mass resolution using delayed extraction compared with d.c. extraction for proteins up to ~40 kDa. The enhanced resolution is particularly beneficial for the complex mixtures of gliadins (compare Figs 1 and 2) and reduced LMW glutenin subunit fractions. No significant improvement for the HMW glutenin subunits was observed.

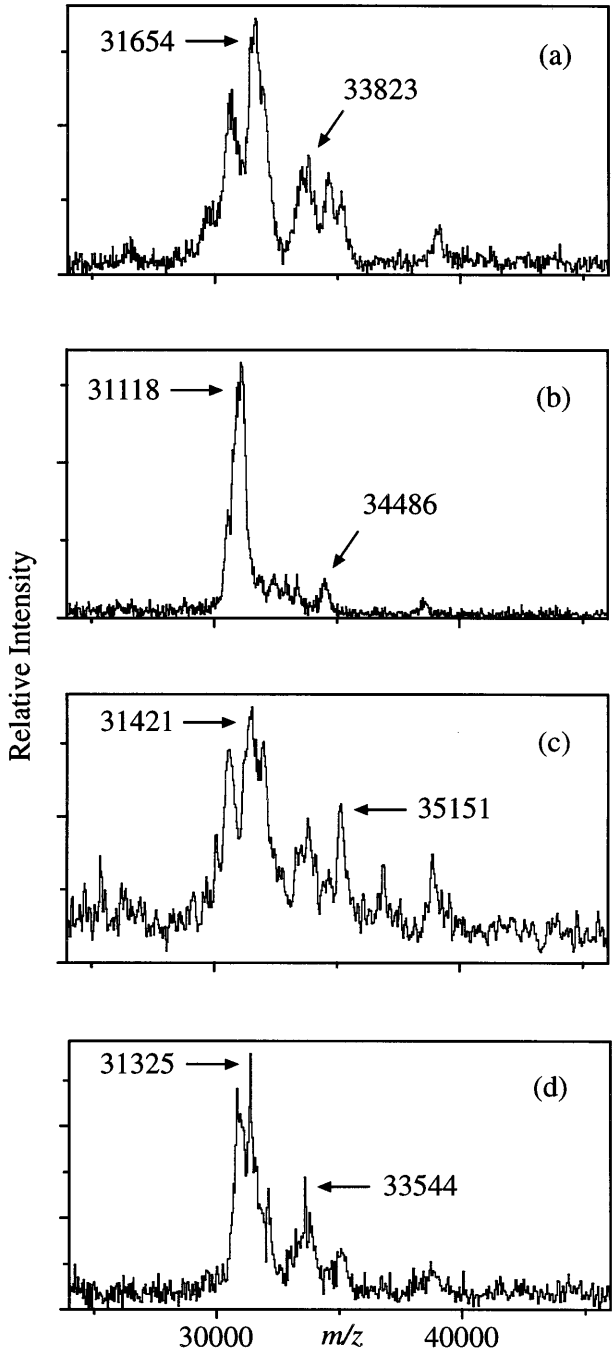

Figure 1. D.c.-extraction MALDI mass spectra of gliadin fractions from four commercial wheat classes: (a) Katepwa variety from the Hard Red Spring class; (b) Kyle variety from the Amber Durum class; (c) Biggar variety from the Canada Prairie Spring class; (d) Glenlea variety from the Extra Strong Red Spring class. Selected peaks are labeled in daltons.

Gliadins

Mass spectra were obtained for gliadin fractions from ten different wheat varieties in four commercial classes, using d.c. MALDI. Each sample was run several times to determine the reproducibility. Although the absolute signal strength is variable from sample to sample and from shot to shot, the measured masses and the observed intensity patterns were consistent for different samples of the same fraction. Four representative spectra are shown in Fig. 1, one from each of the four

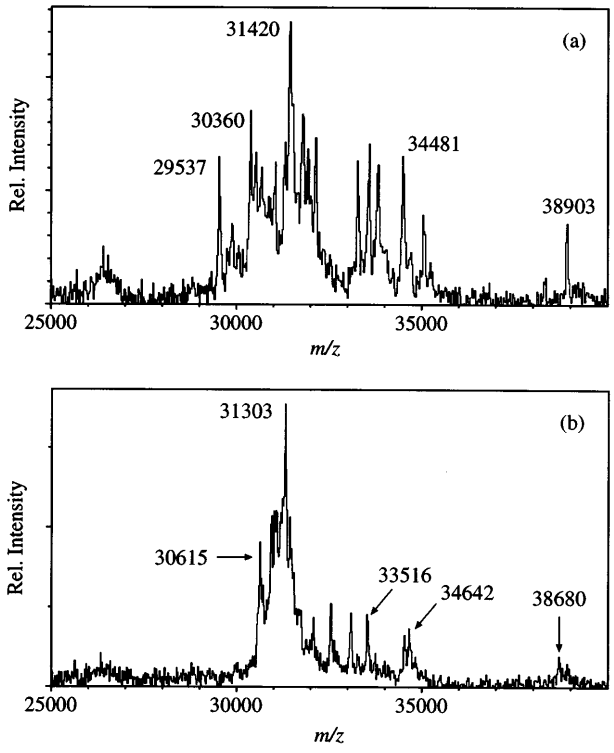

Figure 2. Delayed-extraction MALDI mass spectra of gliadins from the (a) Katepwa and (b) Kyle wheat varieties with selected peaks labeled in daltons.

commercial classes. Although the spectra consist of many unresolved peaks, the different wheat varieties showed distinct mass spectral fingerprints indicating the potential of automated MALDI for rapid varietal identification. At present, electrophoretic and HPLC patterns of gliadin proteins are used extensively in variety identification of commercial samples,[16–18] but this is much more time consuming than MALDI. Further experiments are in progress to evaluate a larger sample set and to use delayed extraction to improve discrimination.

Delayed extraction mass spectra were obtained for two of the gliadin fractions, Katepwa and Kyle. The increased number of resolved peaks in the spectra shown in Fig. 2 illustrates the advantage of delayed extraction. Even so, only about 20 protein masses can be determined from the spectra, compared with more than 100 gliadin proteins indicated by two-dimensional electrophoresis.[5] The many different gliadin proteins have not been characterized in detail so their molecular masses are not accurately known, but the broad width and irregular shape of the observed gliadin peaks (compared with spectra of mass standards in this range) strongly suggest that most of the components are simply unresolved. The majority of components for the wheat variety Katepwa exhibit masses ranging from about 29.5–32.5 kDa whereas a smaller number of components fall in a second region between 33.5 and 35.7 kDa. The spectrum of the durum wheat variety Kyle shows less complexity than Katepwa. Presumably the tetraploid durum wheat, which is missing the D chromosome, has a smaller number of gliadin proteins than the hexaploid common wheat. Even so, the distribution of durum wheat components is similar to that of common wheat components with most of the masses ranging from about 30.5–32.2 kDa and a smaller number being present between 32.5 and 35 kDa. No major peaks are visible for either variety above 39 kDa indicating that most, if not all, of the gliadins are within the normal mass range normally attributed to the majority of gliadin proteins.[7]

Although the observed patterns show some potential to differentiate between varieties, the complexity of the gliadin mass spectra precludes the use of MALDI/MS, even with delayed extraction, as a stand-alone technique for identification of most *individual* gliadin components unless much higher mass resolution can be obtained. Characteristically, other common one-dimensional fractionation techniques such as electrophoresis[38] and HPLC[39] also are unable to resolve all gliadin components. Currently, two-dimensional electrophoresis offers the best indication of the gliadin composition.[5] Similar separation may be possible with MALDI used in combination with this technique or another technique such as HPLC or capillary electrophoresis (CE). Such a combination might also provide a means of obtaining more accurate molecular masses.[24,40]

Low molecular mass glutenin subunits

The LMW glutenin subunits of Katepwa and Kyle were analyzed by delayed-extraction MALDI. Like the spectra of the gliadins, the spectra of the LMW subunits, shown in Fig. 3, reflect a complex mass spectral pattern, consistent with a large number of subunits.[4,5] Three major regions with masses from ~30.5–33, 37.5–39 and 42–43 kDa are present, with no peaks evident above 43 kDa. The first region is similar in mass to the

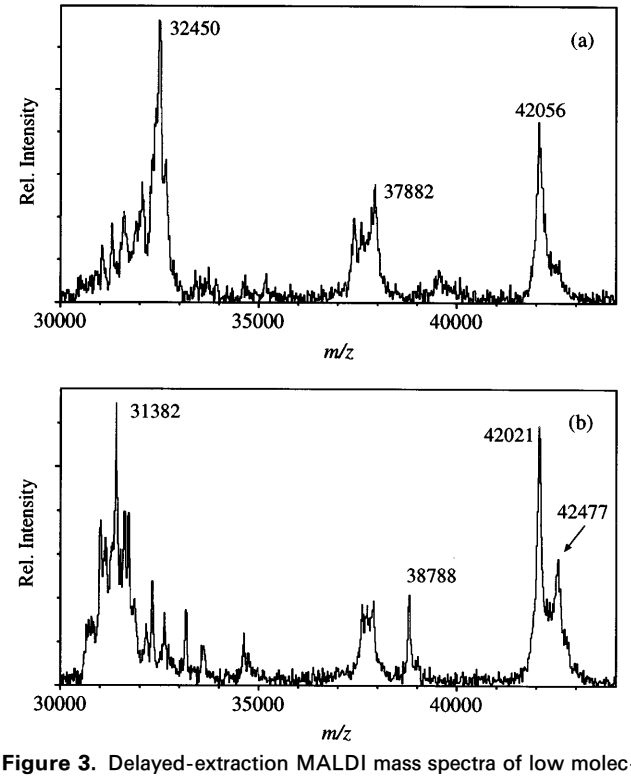

Figure 3. Delayed-extraction MALDI mass spectra of low molecular weight glutenins from the (a) Katepwa and (b) Kyle wheat varieties with selected peaks labeled in daltons.

major gliadin range. This is probably a reflection of their similar evolutionary relationship.[4] The other LMW glutenin subunit regions appear to be distinct from those obtained for the gliadins. These results are consistent with SDS-PAGE, which shows extensive overlap between gliadins and LMW glutenin subunits in addition to lower mobility LMW glutenin subunit regions distinct from the gliadins.[28,38] In view of the increasing interest in LMW glutenin subunits associated with their potential important relationship to wheat protein quality,[41,42] a two-dimensional approach using HPLC followed by MALDI may prove useful for the further characterization of individual components.

High molecular mass glutenin subunits

Mass spectra of the HMW glutenin subunits for Katepwa and Kyle are shown in Fig. 4. In contrast to the mass spectra of gliadins and LMW subunits, the mass spectra of HMW subunits are fairly simple. Five distinct, well separated peaks are observed in the spectrum for Katepwa and four distinct peaks are evident in the spectrum for Kyle. The relatively simple spectra are consistent with the small number of these subunits previously identified in both wheat varieties.[12,38] The results indicate the feasibility of using MALDI to obtain a rapid and complete profile of HMW glutenin subunits without prior separation by HPLC. Automated sample analysis using this technique may prove particularly useful in wheat breeding programs where rapid isolation of lines containing subunits associated with superior quality is a major objective.[4] Hickman *et al.*[24] reported difficulty in obtaining molecular ion signals of the unseparated HMW subunits and the peaks obtained from the separated proteins were broader those in Fig. 4, but they did not use a washing procedure prior to MALDI analysis and they used a different matrix (α-cyano-4-hydroxycinnamic acid).

Most HMW glutenin subunits have been identified by both SDS-PAGE and HPLC and have been classified based upon electrophoretic mobility, but a size-fractionation technique such as SDS-PAGE does not provide accurate mass values.[43] To establish a correspondence between the classified subunits and the observed molecular ion peaks in the mass spectra, HPLC was used to separate the glutenin subunit fraction into constituent components (described previously[40]) before analysis by MALDI. This step would not be necessary to obtain the HMW profile of a wheat sample once the known subunits have been characterized individually by mass spectrometry; measurements are in progress to determine the molecular mass of the known HMW subunits in Canadian wheat varieties.[44]

Five subunits have previously been identified in Katepwa with the designations 2*, 5, 7*, 9 and 10,[45] and there is a one-to-one correspondence between them and the mass spectral peaks; each HPLC component gave a single mass peak. The measured masses are listed in Table 1 along with predicted masses, if available, from the corresponding gene sequences.[46] The difference between the prediction and the measured mass is about 200 Da or less in all cases, which is consistent

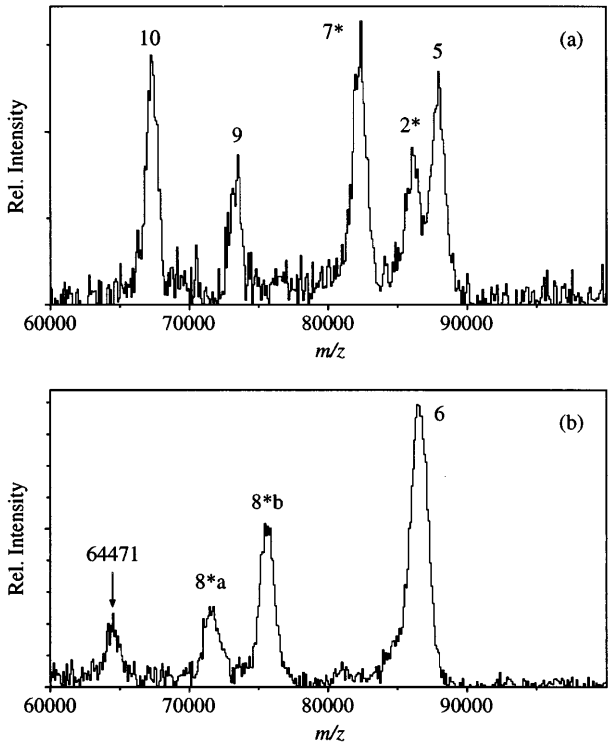

Figure 4. MALDI mass spectra of high molecular weight glutenins from the (a) Katepwa and (b) Kyle wheat varieties. The peaks are labeled with the subunit number; corresponding masses are given in Table 1.

with an estimated measurement accuracy of a few tenths of a per cent for broad peaks in this mass range. The correspondence is remarkably good and it is tempting to attribute some significance to the systematic mass deficit in the measurements and assume a precision better than 0.1%, but it should be mentioned that there are discrepancies in the literature of 500–1000 Da for the predicted masses from gene sequences of subunits 5,[47] 9[48] and 10,[47] and that previous MALDI results for these three subunits differ from the present results by 1000 Da or more.[24] Measurements of the mass spectra of enzymatic digests of the proteins may help to resolve the differences.

The situation is less straightforward for the Kyle sample. Four mass spectral peaks were detected in the HMW glutenin fraction of Kyle [Fig. 4(b)] whereas only two main peaks were resolved by HPLC, as shown

Table 1. Measured masses of high molecular weight protein subunits from the Katepwa and Kyle wheat varieties and predicted molecular masses from available gene sequences[46]

	MALDI		Gene sequence[46]		
Subunit	Cultivar	Mass (Da)	Cultivar	Mass (Da)	Difference
2*	Katepwa	86 202	Cheyenne	86 309	−107
	Katepwa	87 936	Cheyenne	88 137	−207
6	Kyle	86 539			
7*	Katepwa	82 279			
8a*	Kyle	71 520			
8b*	Kyle	75 624			
9	Katepwa	73 308	Cheyenne	73 518	−210
10	Katepwa	67 280	Cheyenne	67 495	−215

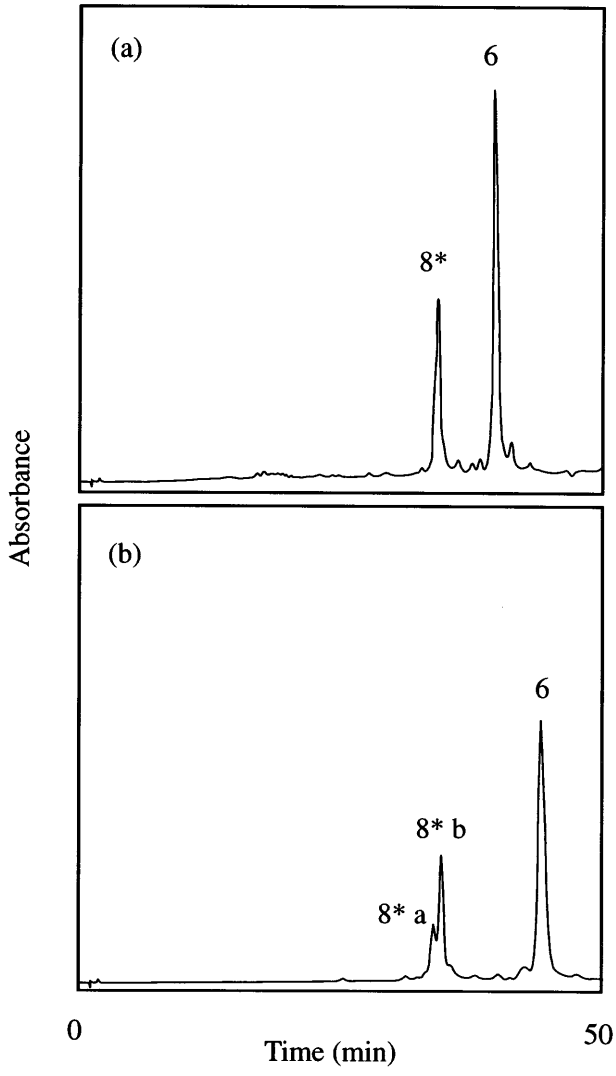

Figure 5. HPLC traces of high molecular mass glutenins from the Kyle wheat variety, showing subunits (a) 6 and 8* and (b) 6, 8*a and 8*b.

in Fig. 5(a). As established by SDS-PAGE,[38,45] the HPLC peaks correspond in position to those of subunits 6 and 8*. To determine the identity of the mass spectral peaks, the two HPLC peaks were collected and then analyzed separately by MALDI. Comparison of the mass spectra showed that the highest molecular mass peak at 86 539 Da corresponds to the HMW glutenin subunit 6 (Table 1). The other HPLC fraction, corresponding in elution time to subunit 8*, gave the two mass spectral peaks with masses of 71 520 and 75 624 Da (Table 1). This result is consistent with recent studies by Marchylo and Nightingale (unpublished), which show that the HPLC 8* peak in Kyle can be resolved into two closely spaced peaks when a modified higher resolution HPLC procedure is employed. The HPLC spectrum is shown in Fig. 5(b); the two peaks and the corresponding mass peaks in Fig. 4(b), are designated 8a* and 8b*. The remaining lower mass peak at 64 471 Da [Fig. 4(b)] was not present in any HPLC fractions eluting in the HMW glutenin subunit region. This peak may represent an ω-gliadin contaminant present in the HMW glutenin extract since these proteins are difficult to extract completely into the gliadin fraction.[28]

CONCLUSIONS

MALDI mass spectra can be obtained directly from crude and partially purified wheat gliadin and reduced glutenin subunit fractions from common and durum wheat varieties without prior separation by HPLC. The spectra of the gliadins and the LMW glutenin subunits show some promise for variety identification but reliable identification of individual components will probably require a second spectroscopic dimension such as HPLC or CE. The spectra of the HMW glutenin subunits are much simpler and the complete HMW subunit profile can be determined directly from a single mass spectrum. This may prove particularly useful in wheat breeding programs for rapid identification of lines containing subunits associated with superior quality.

Acknowledgements

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