Journal of Biomolecular Techniques, Volume 13, Issue, 3, September, 2002

Identification of Wheat Endosperm Proteins by MALDI Mass Spectrometry and LC-MS/MS

William H. Vensel*[1], Leslie Harden[2], Charlene K. Tanaka[1], William J. Hurkman[1], and William F. Haddon[2] , Crop Improvement and Utilization Research Unit[1] and Produce Safety and Microbiology Research Unit[2]. Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710

Running Title:

Wheat Proteins by MALDI and LC-MS/MS

*To whom correspondence may be addressed:
William H. Vensel
Crop Improvement and Utilization Research Unit
Western Regional Research Center,
800 Buchanan Street, Albany, CA 94710

Vensel@pw.usda.gov
510-559-5674 phone
510-559-5777 FAX

Abstract

The commercial value of the wheat crop is a function of the quality and amount of the storage protein and starch present in the grain, which in turn are influenced by environmental conditions during grain-fill. To understand how environment modifies the size and composition of wheat grains, we set out to identify the key metabolic and regulatory proteins in developing grain. We present results of initial studies aimed at establishing instrument conditions that will allow us to identify cytoplasmic proteins present in wheat endosperm. Proteins were isolated, separated by 2-D gel electrophoresis and stained with Coomassie blue to visualize and quantify changes in protein expression. Mass spectrometry was used to identify protein spots in 2-D gels by means of "peptide mass maps" of in-gel enzymatically digested protein spots. Because only about 30% of the proteins could be identified by “peptide mass mapping” we developed nano-flow LC-MS/MS techniques that allowed us to identify about 80% of the salt soluble proteins in wheat endosperm.

Keywords: doubly-charged ion, endosperm, MALDI, LC-ESI-MS/MS, wheat

Introduction

The discovery that it was possible to generate gas-phase protein ion beams of the principle storage protein of maize kernels[1] followed by subsequent developments in matrix-assisted laser desorption ionization[2] (MALDI) and electrospray ionization[3] (ESI) mass spectrometry resulted in routine peptide and protein identification by these processes. Commonly employed techniques that are outgrowths of this discovery include "peptide mass mapping" by mass spectrometry and the generation of sequence specific fragments from individual peptides by tandem mass spectrometry. We are using mass spectrometry to identify the hundreds of proteins that are involved in the complex program of wheat grain development. We fractionated wheat endosperm into two protein fractions[4], gliadins and glutenins (storage proteins), and the albumin and globulin fraction (salt soluble proteins). We focused on the salt-soluble protein fraction, because it contains most of the enzymes and factors that are important in cellular metabolism. In this paper, we report on the development of methods and instrument conditions that we found necessary to identify the proteins in this fraction.

Materials and Methods

Growing Conditions: Wheat (*Triticum aestivum* L. cv. Butte 86) plants were grown in a climate-controlled greenhouse that had an average daily maximum daytime temperature of 24^{o}C and nighttime temperature of 17^{o}C. Water and fertilizer were supplied by drip irrigation. Grain was harvested from developing heads at 10 and 36 days post-anthesis (dpa). Endosperm was collected, frozen in liquid nitrogen, and stored at –80^{o}C.

2-D Gel Electrophoresis: Endosperm was ground in liquid nitrogen with a mortar and pestle and proteins were extracted with a high salt buffer. Proteins were precipitated, solubilized in urea, separated by 2-D gel electrophoresis, and stained with Coomassie blue G-250. Methods were similar to those described previously[5] with the following modifications: a 3–10% ampholyte mixture was used and the second dimension gel was a NuPAGE 4-12% acrylamide, Bis-Tris gel (Invitrogen, Carlsbad, CA).

Protein Spot Digestion: Protein spots were excised from 2-D gels with razor-shortened plastic pipette tips and the gel plugs dislodged with an intact pipette tip into microcentrifuge tubes for storage or into DigestPro (INTAVIS Bioanalytical Instruments AG, Bergish Gladbach, Germany) reaction tubes for processing. The DigestPro was used to automatically carry out alkylation, in-gel tryptic digestion and elution into collection tubes using programs and conditions supplied by the manufacturer. The eluent was reduced in volume to about 20 to 30 :L by use of a Speed Vac SC100 (Thermo Savant, Holbrook, NY). Initially the elution buffers contained 0.01% SDS, but the SDS was later omitted because of the need to remove it prior to electrospray.

MALDI Mass Spectrometry: Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was used to obtain mass fingerprints of the peptide fragments from proteins separated by 2-D gels. Approximately 0.5 μL of the eluent from the DigestPro was spotted onto an already-prepared MALDI matrix spot, ~0.4 μL of a mixture of 20 mg/mlalpha-cyano-4-hydoxy-trans-cinnamic acid and 5 mg/ml of nitrocellulose dissolved in acetone-propanol (1:1) as described by Schevchenko et al[6] . After spotting, the sample was allowed to dry for 30 min at room temperature and the matrix spot washed 3 times with 5 μL of 0.1% TFA. Mass spectra were obtained using a Reflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) that was operated using delayed extraction in the reflectron mode. Porcine trypsin fragments were used as internal calibrants.

ESI-Ion-trap Mass Spectrometry: Electrospray ionization mass spectrometry was carried out using a Finnigan LCQ Classic Ion-trap Mass Spectrometer (Finnigan MAT, San Jose, CA) that was running version 1.1 of the Xcalibur software package and was equipped with a New Objective nano-spray interface (New Objective Inc., Woburn, MA). Operation of the LCQ was carried out *via* the Instrument Method files in the Sequence Setup window of the Finnigan Xcalibur software. The temperature of the heated desolvation capillary was set to 160^{o}C. The number of microscans was set to 3 and the injection time was set to 50 ms *via* the TunePlus window. The instrument was operated in the "triple play" mode with the LCQ set to acquire a full MS scan between 400 and 1400 m/z followed by a zoom scan of the most intense ion from the preceding scan followed by MS/MS fragmentation. Collision energy for collision-induced dissociation was set to 35% and the activation time to 30 msec. Dynamic exclusion was set for a repeat count of three and a repeat duration of 0.5 min with a 10 min exclusion duration window.

Nano flow HPLC: An LC Packings nano flow LC system (Dionex, Sunnyvale, CA) with autosampler, column switching device, loading pump, nano-flow solvent delivery system, and UV detector was used with the LCQ. An anion exchange cartridge was placed in line after the injection loop to remove SDS from samples. Peptide digests (typically 20 μL in volume) from the autosampler were loaded into a 50 μL loop and then pumped (40 μL/min.) by means of a sample-loading pump through the anion exchange trap cartridge through the 10-port injection valve and onto the C-18 trap cartridge. Five minutes after the start of sample loading the injection valve was switched to place the C-18 trap cartridge in line with the nano-flow solvent delivery system (400 nL/min.), thus enabling the trapped peptides to be eluted onto the C-18 nano-bore separation column. The SDS trap cartridge continued to be eluted by solvent from the loading pump. The SDS trap cartridge was then stripped of SDS with 90% acetonitrile/0.1 N HCL for

five minutes before washing with the starting trap-column-eluent (0.1% TFA and 2% acetonitrile). The gradient conditions were as described in Fig. 1.

Two different C-18 separation column configurations were tested. In one configuration, an LC Packings C-18 column (75 microns by 15 cm) was plumbed through the UV detector in front of the electrospray. In the other configuration, a New Objective C-18 PicoFrit (75 uM x 10 cm) column with integral spray tip was used. When the LC Packings column was used, the electrospray voltage was applied to the column effluent after the UV detector, just before the effluent left the emitter tip and passed through the New Objective Proximally Coated Tip Module. When the New Objective PicoFrit column was used, the electrospray voltage was applied at the head of the column by means of a titanium union (Valco Instruments Co., Houston, Texas) located inside the New Objective Distally Coated Tip Module. Column flow rate was measured by collecting (into a 1 μL calibrated capillary) the droplet that appeared during a timed interval on the emitter tip.

Maximizing the Number of Doubly Charged Ions: The LCQ was "tuned" from the TunePlus window of the Xcalibur software using the doubly charged ion of Bombesin (200 fm/μL). After tuning, approximately 95% percent of the signal from the infused sample appeared as the doubly charged ion. The optimum temperature for the heated desolvation capillary was determined by chromatographing samples of a tryptic digest of pyridylethylated bovine serum albumin (BSA) while operating the instrument in the “triple play mode”. The spectra were analyzed and the number of doubly charged ions determined at heated desolvation capillary temperatures ranging from 150 to 200^{o}C. The maximum number of doubly charged ions occurred at 160^{o}C and this setting was used for all subsequent work.

Spot Identification: Spectra were transferred to a PC and identification was carried out using a suite of programs from Genomic Solutions (Madison, WI). The Knexus automation client was used for peptide mass mapping and the Sonar package for MS/MS data.

Results

Consistent and reliable identifications are the goal of any analytical technique. We found that the most important factor in obtaining reproducible peptide identifications by LC-MS/MS was the ability to sustain a stable electrospray. Initially we utilized the UV detector to monitor peptide elution patterns. However, we encountered a number of cases in which the UV spectra were good while data obtained from the mass spectrometer were of poor quality. The cause for this appeared to be the erosion of the conductive coating from the electrospray tip. As a result we switched to a column that had an uncoated emitter tip even though this did not allow UV monitoring. Another important factor in determining reproducibility was correct adjustment of the column flow rate.

Use of the trap cartridges to scrub contaminants and/or sample from a relatively large (20 μL) volume greatly reduces the time required to load samples on to the nano-bore columns that typically operate at 200 to 400 nL /minute. Tests of the efficiency of trapping by the C-18 trap cartridges and the effect of the anion exchange (SDS trap) cartridge on peptide recovery were carried out by means of the UV detector while using the LC Packings column. To estimate the amount of sample that was retained by the trap cartridges, we compared direct 1 μL loop injections onto the analytical column with a 1μL injection onto the trap cartridge followed by elution onto the analytical column. The signal response was lower by a factor of three when sample was injected first onto the trap cartridge instead of directly onto the analytical column. Similar peak intensities were observed whether the anion exchange column was present or absent from the flow path. The anion exchange cartridge was extremely effective; in its absence standards and unknown proteins that had been treated with the SDS could not be identified. However severe tailing accompanied by inability to identify any peptides occurred in some cases suggesting that not all SDS was removed (data not shown). Base peak chromatograms for BSA and a SDS treated unknown protein (Fig. 1) illustrate that good chromatography and successful identification can be obtained from SDS treated digests. Sequence specific fragments from 10 individual peptides identified the unknown as triosephosphate isomerase. The anion exchange and C-18 trap cartridges appeared to have a useful life expectancy of approximately 100 injections.

The 2-D gels of salt-soluble endosperm proteins present at 10 and 36 dpa revealed that the protein population changed dramatically during grain development. Compared to the protein pattern at 10 dpa, a number of new proteins were present at 36 dpa. These proteins were particularly noticeable, because, in general, they had lower molecular weights and higher isoelectric points. (Fig. 2).

We set out to identify the most abundant 250 protein spots in the 2-D gels. Initial experiments using MALDI-TOF mass spectrometry allowed us to identify only about 30% of the gel spots analyzed. Using MALDI-TOF mass spectrometry we obtained 125 identifications, 67 of which represented unique proteins. We stored samples at –80°C until we developed the capability to analyze them by ESI-MS/MS. Thus far we have carried out approximately 100 analyses using the combination of LC-ESI-MS/MS, and have been able to positively identify 80% of the samples. Samples stored in solution at -80°C for as long as two years have given positive results regardless of prior exposure to SDS. So far we have identified 106 different proteins by a combination of peptide mass mapping and MS/MS fragmentation. These proteins can be classified in 10 functional classes (Table 1). Most are involved in protein synthesis and turnover, carbohydrate transport and metabolism, and defense against predators and pathogens. It is worth noting that none of the proteins identified are storage proteins, indicating the efficacy of the fractionation process.

Conclusions

2-D gel electrophoresis was used to analyze the accumulation patterns of salt soluble wheat endosperm proteins during early and late stages of grain development. As would be expected, the 2-D gel patterns were significantly different at 10 and 36 dpa. Mass spectrometry was used to identify the proteins in the 2-D gels by means of "peptide mass maps" of the in-gel enzymatically digested protein spots. As would be expected, the majority function in protein synthesis and starch metabolism. We found that we could identify approximately 30% of the protein samples by MALDI mass mapping, and approximately 80% by LC-ESI-MS/MS. Based on these results we plan to continue using MALDI-TOF in the first stage of sample screening and reserve LC-ESI-MS/MS for samples that do not provide positive identifications by peptide mass mapping. Identification of many more proteins and careful analysis of changes in protein accumulation profiles over a number of time points will result in a better understanding of the timing of metabolic events during grain development and allow us to determine the effect of environment on these events.

References

1. Clegg GA, and Dole M. Molecular Beams of Macro Ions. III. Zein and polyvinylpyrrolidone. Biopolymers 1971;10: 821-826.

2. James P, Quadroni M, Carafoli E, Gonnet G. Protein identification by mass profile fingerprinting. Biochem. Biophys. Res. Commun. 1993;195: 58-64.

3. Wilm M, Mann M. Analytical properties of the nanoelectrospray ion source. Anal. Chem. 1996;68: 1-8.

4. MacRitchie F, Kasarda DD, Kuzmicky DD. Characterization of wheat protein fractions differing in contributions of breadmaking quality. Cereal Chem.1991;68:122-130.

5. Hurkman WJ, Tao HP. Tanaka CK. Germin-like polypeptides increase in Barley roots during salt stress. Plant Physiol. 1991;97: 366-374.

6. Schevchenko A, Wilm M, Vorm O, and Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 1996;68:850-858.

Mention of a particular product or instrument is for purposes of information only and is not meant to imply endorsement by the US Department of Agriculture over others that may be suitable.

Figure legends

Fig. 1. LCQ base peak chromatograms (Filter: m=400-1400) from the separation of 200fM of BSA (A) and of an unknown sample exposed to SDS during tryptic digestion (B). Nano flow HPLC conditions are as described in the Methods section. Solvent A was 0.05% formic acid and 5% acetonitrile. Solvent B was 0.05% formic acid and 80% acetonitrile. The column was equilibrated with 95% solvent A, and at five minutes after injection a gradient to 50% of solvent B in 25 minutes was started. The column was stripped for five minutes at 90% of solvent B and returned to the starting conditions in one minute.

Fig. 2. Digital 2-D gel images of salt-soluble proteins from wheat endosperm. Grain was harvested at 10 (A) and 36 DPA (B). MW, Molecular weight standards $x10^{-3}$.

Table 1

Function	
Amino acid transport and metabolism	8
Carbohydrate transport and metabolism	14
Cell Structure	12
Defense	19
Energy	7
Metabolism	7
Protein Destination and Storage	25
Protein Synthesis	10
Signal Transduction	3
Unknown	1

Functional Categories are based on the descriptions posted from the laboratory of Dr. Robert Goldberg at: http://www.mcdb.ucla.edu/Research/Goldberg/