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Changes of Protein Profile in Fresh-Cut Lotus Tuber before and after Browning

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ABSTRACT: Browning is a critical problem, which often limits the shelf life and marketability in fresh-cut lotus tuber. Proteome level changes in response to the browning metabolism were investigated using two-dimensional electrophoresis (2-DE) and MALDI-TOF-TOF. A total of 34 functional protein spots were identified by comparing 2-DE protein patterns of fresh-cut lotus tuber before and after browning. These 34 identified proteins could be classified into 7 functional groups based on the NCBI database, that is, material and energy metabolism (35%), stress response (20%), respiration metabolism (12%), cell structure (12%), signal transduction (6%), gene expression regulation (6%), and unclassified proteins (9%). The group with the greatest difference in protein expression was related to material metabolism and regulation, reactive oxygen species metabolism, and respiratory control. The distinct proteins included universal stress protein (USP), superoxide dismutase (SOD), peroxidase (POD), ferritin, and ATPase.

KEYWORDS: *lotus tuber, fresh-cut, browning, protein profile, 2-DE, MALDI-TOF-TOF*

INTRODUCTION

Lotus tuber (*Nelumbo nucifern*) is a multipurpose aquatic economic crop in China, which has been recognized as a medicinal-food plant.[1] As its tissue is crisp and tender with high water content, the lotus tuber is easy to slit and suitable for fresh-cut use. Currently there is a great demand and market for fresh-cut products in the People's Republic of China. The lotus tuber has a smooth white rind and white flesh that easily brown during storage and processing. The color change from pure white to brown negatively influences the products' sensory characteristics. There exists a subvital status in which the respiratory and behavioral physiology of fresh lotus tuber stops immediately after harvest.[2] Fresh-cut lotus tubers start browning due to mechanical damage, and browning leads to the changing of product color and flavor, resulting in the loss of nutrients and reduction in commercial value.[3] This has been the main limitation in the export and domestic sale of lotus tuber fresh-cut products.

The pathways prevalent in browning of fresh-cut products are of two types, that is, enzymatic and nonenzymatic. Enzymatic browning results from oxidation of polyphenols to quinines catalyzed by browning enzymes, such as polyphenol oxidase (PPO), peroxidase (POD), *o*-diphenol oxidase, catechol oxidase, and subsequent further reaction and polymerization of the quinines. The occurrence of enzymatic browning can limit the shelf life of fresh-cut vegetables. Nonenzymatic browning is a chemical process that produces a brown color in foods without the activity of enzymes, and the main form of nonenzymatic browning is Maillard reaction. The Maillard reaction is a chemical reaction between carbonyl and free amino groups, that is, reducing sugars and amino acids, which produces melanoidin pigments in a wide variety of foods. There are three hypotheses considered to be responsible for the mechanism of enzymatic browning, namely, phenol−phenolic enzyme regional distribution,[4] free radical damage,[5] and protective enzyme system.[6] Therefore, these browning enzymes, such as PPO and POD, are the important factors and necessary conditions for browning.[7] Because all enzymes are proteins, research on protein changes is particularly essential. Vegetables could be susceptible to browning as a result of changing gene expression and metabolism.[8] In browning mechanism research, it is an important factor to determine protein expression before and after browning. Protein expression is a reflection of the actual state of the vegetable cell and structure and is the key link of gene expression and ultimate metabolism.

Protein is one kind of executor of physiological functions in an organism and is a direct reflection of life phenomenon. Studies on protein structure and function may clarify mechanisms in physiological or pathological conditions directly.[9] Different proteins have their own specific activity patterns and do not read directly from gene information. Types and numbers of proteins in different cells vary and change not only continuously but also systematically.[10] Even in the same cell, protein components are constantly changing at different times or under different conditions. On the other hand, protein information from a gene is incomplete and information such as its activity, formation of protein structure, modification, transport position, and interaction cannot be found.[11]

Plant proteomics, although still at an early stage of development, is an important part of proteomics.[12] Plant physiological proteomics has important significance in understanding the injury mechanism of abiotic stress, the adaptation

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mechanism to abiotic environment, the interaction mechanism, and the regulation of plant hormones.[13] After experiencing adverse environmental signals, the plant regulates expressions of intracellular resistance-related proteins by signal transduction and then adjusts its physiological state to adapt to the environment. Reactive oxygen species (ROS) generation is often the first detectable response to stress in plants; for example, browning could lead to ROS production, causing detrimental effects to cellular functions.[14] Plant cells regulate ROS levels through scavenging mechanisms with antioxidant defense proteins, such as catalyzers superoxide dismutase (SOD) and POD. These proteins realize several functions as key players in the modulation of ROS levels and ROS-mediated stress signaling in grape and other plants.[15] Recent results from studies demonstrated that some induced proteins, a peroxiredoxin enzyme (Prx1) and ascorbate peroxidases (APX) from grape, might be correlated with increased tolerance to ROS.[16]

Research on proteins or genes associated with stress resistance has an important role in understanding resistance mechanisms and raising resistance performance. Being a form of mechanical damage, fresh-cut treatment also adversely affects the lotus tuber. Progress, mainly on the characteristics of browning enzymes, how to control their activities, and inhibitor utilization, has been made in previous studies on lotus tuber browning.[17,18] However, research on changes of protein types, quantity, and structures during browning are lacking. In this study, we have investigated the identification and functional analysis of differently expressed proteins in lotus tuber before and after browning to elucidate the molecular mechanism of the biological process, especially browning, at proteomic level. This study provides a theoretical basis for better browning control of fresh-cut lotus tuber in practice.

MATERIALS AND METHODS

Plant Materials. Lotus tubers cv. 3735 were purchased from markets in Nanjing, Jiangsu Province, China. Sturdy tubers of moderate maturity, with fine appearance and without injury, were dipped in running water and gently scrubbed with a soft cloth. After cleaning, the selected lotus tubers were peeled and cut into slices of 0.3−0.5 cm thickness and free from nodes. The tuber slices were divided into two groups. One group was immediately frozen in liquid nitrogen and stored at −20 °C for future use, whereas the other group was packaged in loosely sealed high-density polypropylene (HDPE; thickness = 15 μm; size = 25 cm × 38 cm) plastic bags and then stored at 20 °C until completely brown. Samples were also frozen in liquid nitrogen and stored at −20 °C for future use.

Sample Preparation. Extraction of total proteins from the lotus tuber samples was performed according to two different methods namely, phenol extraction (Phe)[19] and trichloroacetic acid/acetone precipitation (TCA/acetone)[20] with minor modifications. Frozen tuber samples (2.0 g) were ground to a fine homogeneous powder in liquid nitrogen and extracted with 5 mL of extraction solution (60 mM Tris, 1.05 M sucrose, 10 mM EGTA, 1% Triton X-100, 1 mM DTT). Protein was precipitated from the extraction buffer with chilled acetone and collected by centrifugation for 30 min at 5000g after standing for 2 h. The supernatant was collected for the next extraction by different solutions.

The supernatant was collected with the same volume of ice-cold, pH 8.0, Tris-saturated phenol by using the Phe method. The mixture was centrifuged for 30 min at 5000*g* after standing for 2 h. The upper phenolic phase was precipitated overnight with 5 volumes of chilled acetone at −20 °C. Precipitated proteins were centrifuged for 30 min at 5000*g* again and then rinsed twice with ice-cold methanol, followed by rinsing with chilled acetone twice. The pellets were air-dried at room temperature and stored at −20 °C for future use.

The supernatant was collected with 5 volumes of ice-cold TCA/acetone (10% TCA, 20 mM DTT) according to the TCA method. The mixture was centrifuged for 30 min at 5000*g* after standing for 2 h. The precipitated proteins were rinsed and treated as for the above Phe method after the supernatant has been discarded.

Dried protein powders were finally solubilized in 350 μL of rehydration buffer containing 7 M urea, 1% DTT, 2 M thiourea, 4% CHAPS, and 0.5% IPG buffer, pH 4−7. The samples were centrifuged at 5000*g* for 10 min. The supernatants were collected for the determination of protein content and application to two-dimensional electrophoresis (2-DE). Total protein concentration was determined according to the method of Bradford.[21] The absorbance was measured at 595 nm and protein concentration calculated using bovine serum albumin (BSA) as a standard.

2-DE. About 800, 1000, or 1500 μg of total protein was resuspended in rehydration buffer to a final volume of 350 μL. The mixture was pipetted in a 17 cm ceramic strip holder in which an immobilized linear pH gradient (IPG) strip, pH 4−7, 17 cm, was placed gel side down. The strips were overlaid with mineral oil. Strips were rehydrated overnight at 20 °C. Focusing was performed for a total of 60 kVh at 20 °C. The voltage was set at 100, 200, 500, and 1000 V for 1 h; at 4000 and 8000 V for 2 h; and at 8000 V until final volt-hours (60 kVh) was reached with a current limit of 50 mA/strip in an Ettan IPGphor. After isoelectric focusing (IEF), the strips were equilibrated by gentle stirring for 15 min in an equilibration buffer of 1.5 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, and 4% SDS with added 2% DTT. A second equilibration step was carried out for 15 min in equilibration buffer containing 2.5% iodoacetamide (IAA). For the second dimension, the strips were positioned on top of a 12% polyacrylamide SDS gel and sealed with 0.5% agarose. Electrophoresis was performed at 15 °C using the Protean II XL Cell system at 0.5 W/gel for 1−2 h, followed by 6 h at 15 W. Gels were stained with 0.12% Coomassie brilliant blue (CBB) G-250 overnight and destained with distilled water.[22] At least three biological replicates were performed for each treatment.

Gel Scanning and Image Acquisition. All of the gel images were digitized with a densitometer Power Look 2100XL at 300 dpi resolution and analyzed with PDQuest software (version 8.0). All gels were scanned immediately after CBB-250 destaining to minimize any possibility of fading. The percentage volumes were used to designate the significant differentially expressed spots (at least 2-fold increase/decrease and statistically significant as calculated by one-way ANOVA, $P < 0.05$). Triplicate gels were used for each sample, and the SD was calculated. Only those with reproducible and significant changes were considered to be differentially expressed proteins.

Protein In-Gel Digestion. Spots showing changes statistically significant (at $P < 0.05$) and above a 2-fold threshold were automatically excised and digested with modified porcine trypsin with minor variations.[23] Gel plugs were destained by incubation (twice for 20 min) with 50 mM ammonium bicarbonate in 50% ACN, then subjected to dehydration with pure ACN, and finally dried in the oven at 37 °C. Trypsin solution of 5−10 μL, at a final concentration of 12.5 ng/μL in 50 mM ammonium bicarbonate, was added to the dry gel pieces, and the digestion proceeded at 37 °C overnight. Peptides were extracted from gel plugs by adding 60 μL of 0.1% TFA (30 min of incubation), and all extracts were pooled.

MS Analysis and Protein Identification. Samples were analyzed in a 4700 Proteomics Analyzer MALDI-TOF-TOF mass spectrometer (Applied Biosystems, USA), in the m/z range 700−3500, with an accelerating voltage of 20 kV, in reflectron mode and with a delayed extraction set to 120 ns. Spectra were internally calibrated with peptides from trypsin autolysis. The three most abundant ions were then subjected to MS/MS analysis, providing information that could be used to determine the peptide sequence. A combined search (MS plus MS/MS) was performed using GPS Explorer software v 3.5 (Applied Biosystems) over NCBI databases using the MASCOT search. Amino acid sequence data acquired from tandem mass spectrometry were compared to previously sequenced proteins in the National Center for Biotechnology Information − Basic Local Alignment SearchTool (BLAST) database.[24]

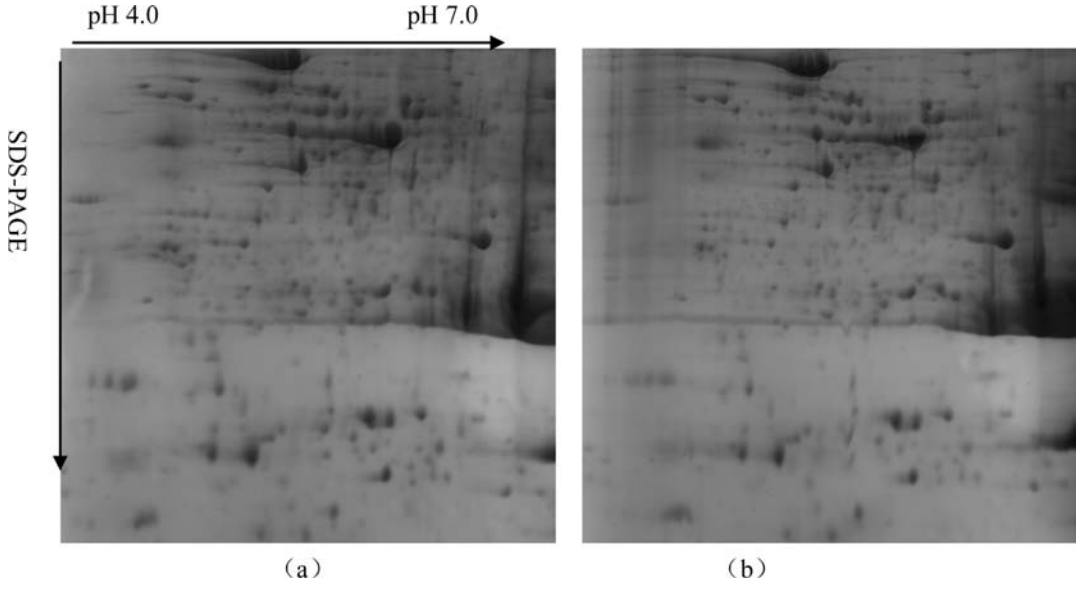

Figure 1. Representative spot maps of fresh-cut lotus tuber. Map a represents tubers before browning and map b tubers after browning. 2-DE was performed using phenol extraction method, 1500 μg of protein, linear 17 cm IPG strips (pH 4–7) and 12% SDS-PAGE gels for second-dimension electrophoresis. Gels were stained with CCB G250.

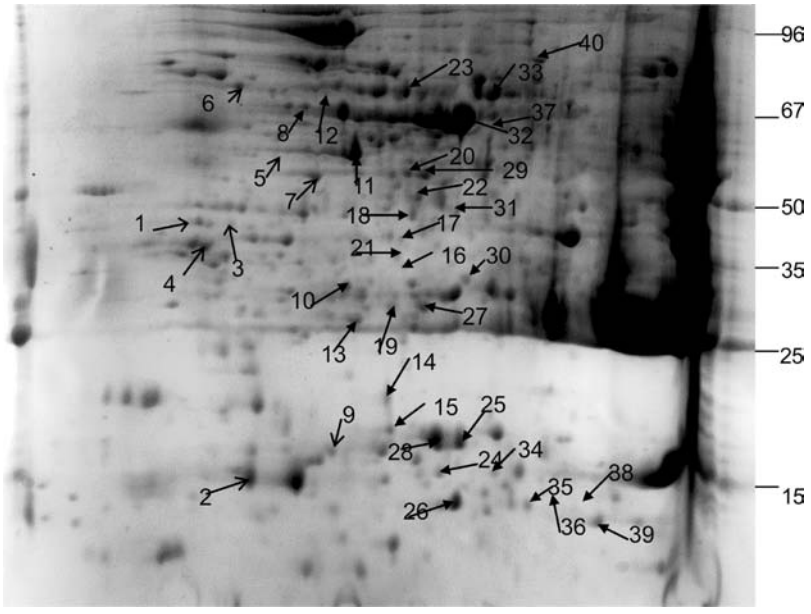

Figure 2. Differential proteins identified from lotus tuber before browning. Identification of 40 protein spots from lotus tuber before browning was performed by 2-DE and MALDI-TOF-TOF analysis. The numbers with arrows indicate the differentially expressed and identified protein spots.

RESULTS AND DISCUSSION

Establishment of Total Protein Extraction Method. Protein extraction is a key step in 2-DE;[25] the extraction method affects the electrophoretic quality, and its quality directly affects its resolution and reproducibility. TCA/acetone and phenol extractions are common methods for protein extraction.[12,26] The former is simple and quick nd is effective to precipitate protein with acetone; it can remove some interfering substances to purify protein.[27] By this extraction procedure, many sugars and other substances are precipitated with protein, which can seriously affect the quality of electrophoresis. Whereas phenol extraction is complex and time-consuming, its selective dissolution of different phases can effectively remove organic impurities in the sample, resulting in a good separation of protein.[28]

In this study, protein yields were 261 ± 11 μg/g FW (phenol) and 142 ± 7 μg/g FW (TCA/acetone) with the same sample volume. This means the loss of protein from TCA/acetone extraction was higher. All other conditions being equal, the average number of protein spots of phenol extraction was more than 400 with a clear gel surface; only about 120 protein spots were found for TCA/acetone extraction, with some spots appearing. This may be because the lotus tuber tissues are rich in secondary metabolites, such as polysaccharides, phenols, and quinines. They can all increase protein gravity and change the protein isoelectric point by binding with or being wrapped around proteins.[29] Moreover, their weak dissolvability in

Table 1. Identification of 34 Proteins in Lotus Root before and after Browning

spot[a]	protein name[b]	accession no.[c]	theor M_r (kDa)/pI[d]	obsd M_r (kDa)/pI	matched peptide sequences (m/z)[e]	matched peptides[f]	coverage rate (%)	protein score[g]	fold[h]
Stress Response[i]									
L1*	universal stress protein 1 (USP) [*Picea sitchensis*]	gil116779221 (NCBInr)	23.2/4.99	29.8/4.81	LCLEVER (918.51) LGLSAVIMGSR (1119.65) IAIAVDLSDESAFAVR (1648.92) KIAIAVDLSDESAFAVK (1777.03) LGSVSDYCVHHCVCPVVVR (2342.20)	5	26	341	
L2	pathogenesis-related protein (PR) [*Vitis vinifera*]	gil288557890 (NCBInr)	17.9/5.38	14.1/4.99	ALILDSHNLCPK (1380.76)	1	7	88	3.62
L6	heat shock protein 60 (HSP60) [*Vitis vinifera*]	gil147819511 (NCBInr)	61.4/5.20	64.1/4.96	NVYLDEFGSPK (1204.59) GYISPQFVTNPEK(1479.71) ALVAPASLIAHNAGVEGEVVVEK (2273.19)	3	8	275	5.25
L13	10 kd chaperoned (Cpn10) [*Oryza sativa* japonica group]	gil115479353 (NCBInr)	25.5/5.97	24.7/5.58	DLKPLNDR (970.66)	1	3	45	2.25
L15*	universal stress protein 2 (USP) [*Populus trichocarpa*]	gil224108456 (NCBInr)	17.8/5.56	16.5/5.75	EAICEAVEK (1048.63)	1	5	42	
L24	17.5 kDa heat shock protein (sHSP) [*Nelumbo nucifera*]	gil118452817 (NCBInr)	17.6/5.94	14.4/6.03	AMASTPADVK (1006.57) VQVEDGNVLLISGER (1628.00) EYPNSYAFIVDMPGLK (1844.04)	3	26	169	4.07
L28	17.7 kDa heat shock protein (sHSP) [*Pennisetum glaucum*]	gil1122317 (NCBInr)	17.9/5.82	16.1/6.02	FRLPENAK (974.57) IDWKETPEAHVFK (1599.87)	2	13	112	0.49
Gene Expression Regulation									
L3*	RNA recognition motif (RRM) [*Micromonas pusilla* CCMP1545]	gil303275622 (NCBInr)	32.9/9.51	37.5/4.95	M[i]KIVSNQMK (1094.61)	1	2	43	
L25	eukaryotic translation Initiation factor 5A (eIF5A) [*Populus trichocarpa*]	gil224143955 (NCBInr)	17.7/5.60	15.9/6.15	TYPQQAGTIR (1134.65) TYPQQAGTIRK (1262.75) CHFVGIDIFNGK (1406.76) DDLRLPTDENLLTQIK (1884.11) DLVVTVMSSMGEEQICALK (2126.15) KLEDIVPSSHNCDVPHVNR (2216.21)	6	38	289	6.97
Cell Signaling									
L4	14-3-3 protein [*Populus trichocarpa*]	gil224066959 (NCBInr)	29.1/4.94	34.7/4.87	MKGDYYR (932.43) MKGDYYR (948.42) LDVELTVEER (1202.62) LAEQAERYDEMVEAMNK (2042.95) LAEQAERYDEMVEAMNK (2058.94)	5	13	187	2.92
L5**	DNA-directed RNA polymerase (DDRP) [*Arabidopsis thaliana*]	Z33867 (EST)	14.2/4.99	17.2/5.19	IAYVVENAR (1034.56) LQLDSSFSPVRR (1404.75)	2	16	99	
Nucleotide Metabolism									
L7	adenosine kinase (AK) [*Vitis vinifera*]	gil225449016 (NCBInr)	38.3/5.31	44.6/5.36	RPENWALVEK (1241.61) SLIANLSAANCYK (1424.65) VHGWETDNVEEIALK (1739.78) YNVEYIAGGATQNSIR (1755.79) ALPYMDFVFGNETEAR (1875.79)	6	26	608	2.13

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Table 1. continued

spot[a]	protein name[b]	accession no.[c]	theor M_r (kDa)/pI[d]	obsd M_r (kDa)/pI	matched peptide sequences (m/z)[e]	matched peptides[f]	coverage rate (%)	protein score[g]	fold[h]
Nucleotide Metabolism									
					ITVITQGADPVVVAEDGKVK (2039.05)				
L39	nucleoside diphosphate kinase (NDK) [*Camellia sinensis*]	gil330318624 (NCBInr)	16.4/6.52	12.5/6.84	IIGATNPSDSAPGTIR (1569.87) KIIGATNPSDSAPGTIR (1697.97)	2	11	227	2.09
Amino Acid Metabolism									
L18	cysteine synthase [*Glycine max*]	gil126508780 (NCBInr)	34.2/6.26	39.4/5.88	YLSSVLFESVR (1299.83) LIVVIFPSFGER (1376.93)	2	7	151	3.47
L29	glutamate-ammonia ligase [*Arabidopsis thaliana*]	gil99698 (NCBInr)	40.9/5.40	44.8/5.94	EHIAAYGEGNER (1345.70) HKEHIAAYGEGNER (1610.90) HETADINTFSWGVANR (1818.00)	3	8	310	2.57
L33	3-phosphoglycerate dehydrogenase [*Populus trichocarpa*]	gil224137644 (NCBInr)	63.9/6.37	61.7/6.29	GLGMHVIAHDPYAPADR(1819.84) GLGMHVIAHDPYAPADR (1835.84) GLIEPISSVFVNLVNADFTAK (2234.15)	2	6	98	0.4
Phenol Metabolism									
L20*	naringenin 3-dioxygenase [*Prunus avium*]	gil326366179 (NCBInr)	40.5/5.66	45.2/5.86	EIVTYFSYPIR (1387.86) NADHQAVVNSSR (1498.85)	2	6	152	
L12**	cytochrome P450 [*Hordeum vulgare* subsp. *vulgare*]	gil326487171 (NCBInr)	58.4/6.76	59.5/6.45	SQLLMAGPR (972.66)	1	1	44	
Lipid Metabolism									
L22	esterase [*Vitis vinifera*]	gil297736351 (NCBInr)	44.1/5.73	41.4/5.89	FFDPEFYR (1120.59) IENHYFVNK (1163.66) RFFDPEFYR (1276.70) LNSDDLEIQYAAAR (1578.89)	4	11	280	2.43
Respiratory Regulation									
L8	vacuolar H^+-ATPase subunit B (H^+-ATPase) [*Zostera marina*]	gil118721470 (NCBInr)	54.5/5.18	57.5/5.30	YQEIVNIR (1034.53) KFVTQGAYDTR (1285.59) YTTVQFTGEVLK (1385.66) QIYPPINVLPSLSR (1596.83) IALTTAEYLAYECGK (1702.74) VTLFLNLANDPTIER (1715.85) GYPGYMYTDLATIYER (1912.78) GYPGYMYTDLATIYER (1928.77) IPLFSAAGLPHNEIAAQICR (2178.04) AVVGEEALSSEDLLYLEFLDKFER (2772.26)	10	27	657	2.45
L10	6-phosphogluconolactonase [*Vitis vinifera*]	gil302143957 (NCBInr)	28.0/5.66	29.3/5.53	LAYDGFLSK (1013.62) DSPKPPPER (1022.62)	2	7	120	2.25
L23	pyruvate decarboxylase (PDC) [*Vitis vinifera*]	gil10732644 (NCBInr)	63.5/6.09	62.6/5.83	ELLEWGSR (989.56) VSAABSRPPNPQ (1237.70) CNTTAYENYHR (1428.69)	3	5	170	2.65
L32	enolase [*Ricinus communis*]	gil1169534 (NCBInr)	48.1/5.56	55.8/6.12	AGWGVMASHR (1071.49) AGWGVMASHR (1087.49) AAVPSGASTGIYEALELR (1804.91)	6	21	312	0.28

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Table 1. continued

spot[a]	protein name[b]	accession no.[c]	theor M_r (kDa)/pI[d]	obsd M_r (kDa)/pI	matched peptide sequences (m/z)[e]	matched peptides[f]	coverage rate (%)	protein score[g]	fold[h]
Respiratory Regulation									
					LAMQEFMILPVGASSFK (1900.92)				
					SGETEDTFIADLAVGLSTGQIK (2252.09)				
					YGQDATNVGDEGGFAPNIQENKEGLELLK (3106.46)				
Reactive Oxygen Species Metabolism									
L9	thioredoxin-dependent peroxidase (TPx) [*Nelumbo nucifera*]	gil125620178 (NCBInr)	17.6/5.35	15.4/5.45	HBPGFIEK (926.46)	5	25	352	2.41
					RFALLVDDLK (1189.64)				
					FALLVDDLKVK (1260.67)				
					HVPGFIEKAEELK (1496.70)				
					GVDEILLISVNDPFVMK (1904.86)				
L19	ferritin [pea, seed]	gil259470 (NCBInr)	23.6/5.56	26.1/5.76	USETVAQKR (1078.69)	3	5	130	2.65
					KISEYVAQLR (1206.80)				
					ISEYVAQLRR (1234.81)				
L27	ferritin [*Phaseolus vulgaris*]	gil21027 (NCBInr)	28.4/5.64	26.2/5.95	ISEYVAQLR (1078.62)	2	3	136	2.42
					KESEYVAQLR (1206.72)				
L26	copper−zinc superoxide dismutase (Cu/Zn-SOD) [*Nelumbo nucifera*]	gil58616005 (NCBInr)	15.6/5.51	12.7/6.14	QIPLTGPYSIIGR (1414.86)	3	33	263	2.79
					EGVSGTIYFTEEEDGSTK (1948.95)				
					AVVVHADPDDLGKGGHELSK (2044.13)				
Cell Structure									
L11	actin (ACT3) [*Arabidopsis thaliana*]	gil11276980 (NCBInr)	47.3/5.19	47.1/5.57	GYTFSTTAER (1132.68)	1	2	64	0.44
L14	actin depolymerizing factor 11 (ADF11) [*Arabidopsis thaliana*]	gil15223471 (NCBInr)	16.5/5.54	18.6/5.73	IFFIAWSPDTSR (1439.86)	1	8	66	7.42
L31	Bms1 [*Populus trichocarpa*]	gil224133372 (NCBInr)	136.1/8.53	39.8/6.12	AVVMEPDER (1061.55)	2	0	45	2.93
					AVVMEPDERK(1189.64)				
L40	tetratricopeptide repeat domain [*Populus trichocarpa*]	gil224071575 (NCBInr)	65.8/6.17	82.5/6.52	DYEPAIETFQK (1340.77)	2	3	96	2.95
					DLEQQEYFDPK (1411.70)				
Unclassified									
L16	hypothetical protein [*Vitis vinifera*]	gil225440390 (NCBInr)	27.7/5.85	29.7/5.86	ALFSQISTRF (1169.77)	1	3	58	0.36
L17	Os11g0116550 [*Oryza sativa* japonica group]	gil297727911 (NCBInr)	31.6/5.50	36.9/5.87	SQCINHGR (971.57)	1	2	46	0.44
L21	hypothetical protein VOLCADRAFT_90329 [*Volvox carterif. nagariensis*]	gil302836820 (NCBInr)	101.8/9.33	32.87/5.85	ALDALLRAANR (1183.77)	1	1	44	0.25

[a]Spot number corresponds to the 2-DE gel in Figure 2. *, only in lotus root before browning. **, only in lotus root after browning. [b]Names and species of proteins obtained via the MASCOT software from the NCBInr/EST database. [c]Accession number from the NCBInr/EST database. [d]Experimental molecular mass (M_r) and isoelectric point (pI) estimated in comparison to a 2D gel with marker proteins; theoretical molecular mass (M_r) and isoelectric point (pI) of the homologous protein calculated with a tool available at NCBInr/EST database. [e]The sequences of all identified peptides with the corresponding m/z ratio in parentheses. [f]The total number of identified peptides. [g]MOWSE score probability (protein score) for the entire protein and for ions complemented by the percentage of the confidence index (CI). [h]Fold = average normal quantity of lotus root before browning/after browning. [i]Functional classification. [j]M, + oxidation (M).

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ampholyte,[30] resulting in most of the protein aggregates being in the macromolecule tip at the gel surface or forming a large number of stripes, then strongly interferes with protein extraction and 2-DE analysis. The phenol extraction can well remove these interfering substances and obtain a pure protein sample and, hence, is more suitable for 2-DE proteome analysis in fresh-cut lotus tuber during browning. Effective protein spot numbers were 101, 251, and 371 on the maps with 800, 1000, and 1500 μg protein loading amounts, respectively. With 1500 μg protein loading amount, some low-abundance protein spots were clearly observed. Most protein spots were almost round in shape, with a clear boundary and no spread, without tailing, and well distributed. Therefore, suitable 2-DE conditions for lotus tuber were selected as phenol extraction, 1500 μg sample volume, pH 4−7, 17 cm IPG, and 12% gel concentration.

Protein Identification of Lotus Tuber before and after Browning. A series of physiological and biochemical changes (e.g., changes in respiration rate, lipid peroxidation, secondary metabolites, callus formation, and nutrient losses) take place when fresh-cut vegetables get brown,[31] and the proteome information is related to the biological process. In this work, protein extracts were obtained from lotus tubers before and after browning. After 2-DE separation and protein staining, the proteome profiles were visible (Figure 1) and showed clear differences. Total protein spots of the two samples were 337 ± 3 (before browning) and 328 ± 4 (after browning) respectively, containing 40 differentially expressed protein spots significant differentially expressed at >2-fold (Figure 2). Of the 40 spots expressed, 5 were observed in lotus tubers only before browning and 5 only after browning. In addition, 22 spots were obviously higher in the tubers before browning, whereas 8 spots were clearly abundant after browning. The protein spots, which showed a >2-fold difference in expression values, present in all extract replicates were considered. The expressed 40 spots were characterized primarily by MALDI-TOF-TOF. Of the 40 spots, only L35 and L37 could not be identified; the remaining 38 spots (95% of the processed proteins) were confidently identified. The 38 protein spots were annotated by BLAST searches against the NCBInr database, including 34 functional proteins (Table 1) and four storage proteins (L30, L34, L36, and L38).

For most of the identified proteins, experimental M_r and pI were in reasonable agreement with the theoretical values of the matched proteins (Table 1). However, some identified proteins had noticeable differences between the observed and theoretical values of M_r and pI. For RNA recognition motif (RRM, L3), the theoretical pI (9.51) is beyond the pH range of the strips we used. In addition, for hypothetical proteins VOLCADRAFT_90329 (L21) and Bms1 (L31), not onlywere the theoretical pI values (9.33 and 8.53, respectively) beyond the pH range of the strips we used, but also their theoretical M_r values are markedly higher than the experimental values. The phenomenon about protein profiles has been reported in *Arabidopsis* tubers[32] and citrus flesh[33] and could be due to various reasons, such as post-translational modification, variable splicing, protein degradation, human modification during the process of protein extraction, and electrophoresis.

The 34 identified proteins were classified into seven functional classes, and the functional categories are shown in Figure 3. According to protein homologies, the literature, and the distributions, the identified proteins contained (1) material and energy metabolism (35%) including lipid, phenol, nucleotide, amino acid, and ROS metabolism; (2) stress response (20%); (3) respiratory regulation (12%); (4) cell structure (12%); (5) gene expression (6%); (6) cell signaling (6%); and (7) unclassified (9%).

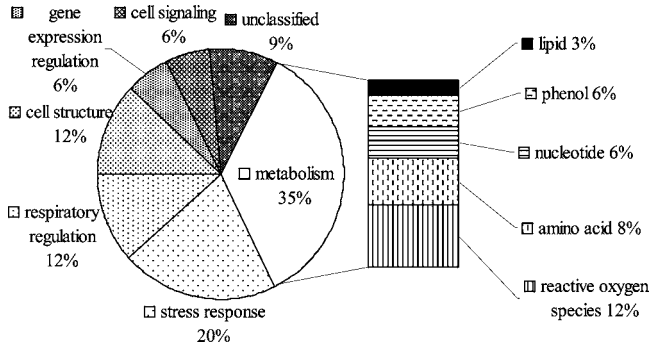

Figure 3. Functional groups of differentially expressed proteins identified from fresh-cut lotus tuber before and after browning included 34 protein spots. This classification is based on a BLAST search and their homologies and the literature.

ROS Metabolism. High levels of ROS might disrupt the normal cell functions and were considered a signal induced by an active cell against stress conditions and a means of pathogen defense. ROS damage to plants produced directly or indirectly lipid peroxidation. The cells are normally protected against ROS by the operation of intricate antioxidative systems comprising enzymatic (SOD, CAT, POD, and enzymes of the Halliwell−Asada pathway) and nonenzymatic (ascorbate, glutathione, and phenolic compounds) systems. Thioredoxin-dependent peroxidase (TPx, L9), copper−zinc superoxide dismutase (Cu/Zn-SOD, L26), and ferritin were found in this study. SOD is the first defense line in the ROS scavenging system and serves as an antioxidant.[34] SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide[35] and inhibits DNA structure injury. Cu/Zn-SOD, commonly known as SOD1, is an enzyme that has been proved to scavenge ROS directly and is mainly used to remove $O_2^{\bullet-}$.[36] After browning occurred in lotus tubers, Cu/Zn-SOD expression decreased, indicating ROS scavenging activity weakened during browning. The enzymatic processes basically involve dismutation of $O_2^{\bullet-}$ by SOD that generates another partially reduced oxygen species, H_2O_2.[37] Normally, POD and CAT take care of the cellular H_2O_2.[38] POD catalyzes the oxidation of phenol and amine compounds, on the one hand, and scavenges H_2O_2, toxic phenol, and amines, on the other.[39] So there are two POD mechanisms: one has a protective effect when plants face adversity or aging, and the other causes damage at the end of adversity and aging.[38] POD was specifically expressed in TPx in lotus tuber. As a member of the peroxides (POD), TPx can clear the toxic product H_2O_2 produced by ROS.[40,41] The down-regulation of TPx after browning indicates its role may be a stress response for browning damage in cells, and it was inhibited when cell structures were damaged. After cutting of lotus tubers, ROS balance was damaged. ROS content increased, and browning ensued. The expression of ROS clearance enzymes, such as TPx and Cu/Zn-SOD, decreased, suggesting the free radical scavenging ability of lotus tuber gradually weakened along with the occurrence of browning.

Some metals, such as iron and copper, can catalyze ROS generation, causing gene mutations and DNA damage.[37] It is considered to be an essential metal catalysis for ROS generation and reaction. Metal storage and transport proteins, such as

ferritin and transferrin, may provide additional redox active metal.[42] Iron reacts with ROS through the Fenton reaction and plays a protective role in cell damage.[43−45] As the main sources of iron in ROS metabolism, ferritin (L19 and L27) expressions decreased after browning, indicating the cell damage induced by ROS increased further during the release of iron in ferritin. When lotus tubers completely turned brown, cell metabolism disorder occurred, and ferritin expression decreased. In other words, cells produced stress-responsive proteins to resist damage due to harmful substances. When tissue proteins could not express normally or if expression was decreased, the cell structure was destroyed.

Other Substrate Metabolism. Proteins related to nucleotide metabolism were of two kinds: adenosine kinase (AK, L7), which is associated with the balance of the extra- and intracellular ATP levels;[46,47] and nucleoside diphosphate kinase (NDK, L39), which catalyzes the exchange of phosphate groups between GTP and ATP.[48] Both of them were down-expressed after browning. Their down-regulations indicated that substance metabolism was out of control after browning and the abilities of energy synthesis and utilization declined, accompanied by disorder of ATP energy metabolism. There were three kinds proteins relating to amino acid metabolism: cysteine synthase (L18) used for regulation of cysteine[49] and glutamate-ammonia ligase (L29) used for regulation of glutamine[50] expression reduced by 3.47 and 2.57, respectively, after browning. Expressions of some proteins (e.g., Cys), which had antibrowning ability decreased, resulted in promotion of the browning reaction. Involved in serine metabolism,[51] 3-phosphoglycerate dehydrogenase (L33) was expressed only after browning, an indication that browning may affect the production of serine. Related to lipid metabolism, esterase (L22) had a down-regulation of 2.43-fold after browning, a hint that esterase may be related to plant stress tolerance. Cytochrome P450 (CYP450, L12) related to secondary metabolism expressed only after browning, indicating that browning was beneficial for secondary metabolite production. Naringenin 3-dioxygenase (L20) was expressed only before browning, revealing that phenol metabolic balance was broken and activities of relevant enzymes decreased until they disappeared.

Plant Stress Response. There were seven stress-responsive proteins. Universal stress protein (USP) encompasses an ancient and conserved group of proteins the expression of which is enhanced when the cell is exposed to stress agents.[52] USP includes heat shock proteins (HSPs), low-temperature-induced proteins, pathogenesis-related protein (PR), and other stress-responsive proteins.[53] HSPs are a class of functionally related proteins the expressions of which are increased when cells are exposed to elevated temperatures or other stresses.[54] HSPs are acidic proteins (pI = 5.0−6.5)[55] including HSP110, HSP90, HSP70, HSP60, and sHSP (15−30 kDa) on the basis of their approximate molecular weights in kilodaltons.[56] HSPs play a protective and tolerant role by the proper fold, shift, maintenance, and degradation of protein through chaperones, antioxidant, signal transduction, cell stress protection, and so on. The higher expression level of HSPs is the self-protective reaction of the host, which can clear abnormal or denatured proteins induced by stress in cells, activate the expressions of other cell genes, inhibit apoptosis caused by ATP breakage, and enhance the bearing ability to many stressors.[57] Expression of HSP is the most prominent reaction at the molecular level, and the main product is sHSP.[58] Enhancement of sHSP expression can raise the plant's capacity to resist stress. There were two sHSPs, 17.5 kDa sHSP (L24) and 17.7 kDa sHSP (L28), in this study. Expression of L24 decreased after browning, indicating that HSP was highly expressed to maintain cell homeostasis when cutting occurred. Increase of L28 expression was also observed when there was extensive damage. HSP60 (L6) is considered to be a typically mitochondrial chaperone[59] and plays a role in assisting polypeptides to reach a native conformation, and it interacts with chaperonid10 (Cpn10, L13).[60] Just as the other kinds of stress protein, PR protects the plant from pathogen infection.[53] The expressions of HSP60 (L6) and chaperonid10 (Cpn10, L13) decreased by >50% after browning. Their down-regulations suggest the immune system in lotus tuber was weak. USP (L1, L5) was expressed only before browning, meaning that the plant protection activity was limited when browning was intensive. The expression of PR (L2) decreased by 72.4%, and this also indicated that browning reduces the tissue's resistance to disease. All of the above results suggest that the expression of stress proteins was complex and most of them portrayed a tendency to decrease, an indication that after browning of fresh-cut lotus tubers, environmental stress factors causing browning weakened.

Respiration Metabolism. There were four kinds of proteins related to respiration metabolism. H^+-ATP synthase (H^+-ATPase, L8) is an important functional protein that creates energy for the cell to use during the synthesis of ATP.[61,62] Its change has a direct or indirect relationship with plant resistance. After browning occurred, its down-regulation showed a weakening of the electron transport and energy transfer, and energy efficiency decreased. 6-Phosphogluconolactonase (L10) is an enzyme in the pentose phosphate pathway;[63] it also down-expressed after browning, meaning the pentose phosphorylation pathway weakened. Pyruvate decarboxylase (PDC, L23) is a homotetrameric enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide in the cytoplasm.[64] PDC's down-regulation after browning meant that acetaldehyde accumulated and cell damage increased. Enolase (L32), known as phosphopyruvate hydratase, is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the ninth and penultimate step of glycolysis.[65,66] Its up-regulation after browning indicated glycolysis increased the role played by browning on respiration was not consistent. These results suggest that the pathway and direction of respiration changed a lot after browning due to cell damage and catabolism.

Cell Structure. There are four proteins related to the composition of cell structure. Actin (L11) is a ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton.[67] It plays an irreplaceable role as a link between proteins controlling cell regulations and their new function.[68] Its up-regulation indicated that the incremental expression favors the maintenance of cell integrity during browning. However, actin depolymerizing factor (ADF11, L14) is important for regulating actin dynamics and plays the reverse role with actin by actin depolymerization.[69] ADF11 down-regulation and actin up-regulation after browning showed different effects in browning on cell structure. Bms1 (L31) is an essential, evolutionarily conserved nucleolar protein.[70] Down-regulation of Bms1 (L31) indicated ribosome synthesis was blocked. Tetratricopeptide repeat domain (L40) is involved in a variety of functions including protein−protein interactions, but common features in the interaction partners have not been

defined.[71] The down-regulation of triangle tetrapeptide repeat domain also suggests that browning had damaged the cell structure.

Gene Expression Regulation and Signal Transduction. RNA recognition motif (RRM, L3) and eukaryotic translation initiation factor 5A (eIF5A, L25) were related to gene expression regulation. RRM (L3), also known as RBD (RNA binding domain) or RNP (ribonucleoprotein domain), is a highly abundant domain in eukaryotes found in proteins involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export, and RNA stability.[72,73] Browning showed some influence on RRM's expression. It is expressed only before browning, indicating that fresh-cut injury led to RRM expression, although deepening of browning and cell damage inhibit its expression. On the basis of protein synthesis, eIF5A (L25) produces response and resistance to environmental stress by promoting the transfer of some specific mRNA and expression of related specific genes.[74,75] Decreased expression of eIF5A showed that proteins' (such as POD, SOD, and Cys) synthesis abilities weaken rapidly, which causes decrease of abilities to control browning.

14-3-3 protein (L4) and DDRP (L5) were related to signal transduction. A striking feature of the 14-3-3 proteins is their ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors.[76] 14-3-3 protein is an important regulatory factor in plants. It can directly regulate signal transduction through the combined capacity of target phosphorylation proteins.[77] It also plays important roles in low-temperature stress and mechanical injury.[78] Up-regulation of 14-3-3 protein after browning proved that signal transduction was enhanced. DDRP is the main undertaker of mRNA synthesis.[79] Its expression only after browning showed that browning did favor DDRP expression in fresh-cut lotus tuber.

Unclassified Proteins. Among the three unknown proteins, L16 and L21 subjected to mass spectrometry were identified as hypothetical proteins, whereas L17 was accessed with an unknown function. It was therefore difficult to characterize their responses to browning in fresh-cut lotus tuber. Their down-regulations after browning showed they might play roles directly and/or indirectly in response to browning. Among the three unclassified proteins, L16 and L17 were accessed from the NCBI database and identified through BLAST matching, but their functions were still unclear. L12 was not BLAST matched.

In conclusion, our results showed that the most important proteins differentially expressed and related to browning in fresh-cut lotus tuber were SOD, POD, ferritin, USP, ATPase, and actin, with their functions mainly concentrated on substances and energy metabolism, stress response, cell respiration, and maintenance of cell structure. The conclusions with respect to browning metabolism at proteome level were as follows: (1) Browning can induce the expression of USP against stress response decrease. (2) Down-regulations of Cu/Zn-SOD and TPx diminished the scavenging capacity of ROS to increased ROS contents. (3) The increase of ROS aggravated glycolysis, weakened the pentose pathway, and then changed the pathways and directions of cell metabolism regulations. (4) Browning can regulate the expressions of adenylate kinase and nucleotide kinase, enzymes related to protein synthesis such as cysteine synthase and glutamate-ammonia ligase to strengthen substance metabolism. Down-regulation of ATPase led to reduction of energy efficiency. (5) Browning can induce the expression of actin, Bms1, and tetratricopeptide repeat domain to make it difficult for the cell to maintain its normal structure, which leads to severe cell damage. The study has put forward interpretable data and provided new perspectives for browning metabolism in fresh-cut lotus tuber. However, further investigations should be performed to assess the specific roles and functional correlation of these proteins.

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