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Proteome changes of *Pteridium aquilinum* during postharvest storage

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Abstract

Postharvest quality deterioration directly limited the shelf life and edibility of the stem of *P. aquilinum*. Proteome changes in response to quality worsening of the vegetable were investigated using 2-DE and differential proteins were identified by MALDI-TOF/TOF. The results demonstrated seventeen proteins were identified and these proteins were classified into five functional groups based on the protein functions and relevant literatures. Among them, six proteins were related to carbohydrate metabolism, three proteins were connected with to energy and amino acid metabolism, three proteins were involved in cell structure formation, three proteins were associated with stress response and defense and two proteins were engaged in protein synthesis. The biological mechanisms of quality deterioration was shows as follow: (1) The decline of antioxidant capacity would be responsible for accumulation of ROS, and excessive ROS leads to cell oxidation and senescence (2) *P. aquilinum* senescence begins and protein content declines when the vegetable is harvested and stored. (3) Cell structure integrity could be destroyed during postharvest storage.

Keywords: Proteomics; *P. aquilinum*; Quality deterioration; Postharvest storage.
Abbreviation: *P. aquilinum_Pteridium aquilinum*; MALDI_matrix assisted laser desorption ionization; TOF_time of flight; 2-DE_two dimensional gel electrophoresis; ROS_reactive oxygen species; TCA_the tricarboxylic acid; MDHAR_monodehydroascorbate reductase; Hsp_Heat shock proteins; MDHA_monodehydroascorbate; ADF_actin-depolymerizing factor 10-like

Introduction

The ferns are the sporous plants with fascicular vascular cambia, and about 12000 species are recorded around the world (Xu et al., 2009). Studies conducted in several countries showed that the plant not only invaded crop fields and pastures, but also produced toxic effects (carcinogens and cyanogenic glycosides) that seriously affected human and animal health (da Costa RM et al., 2012). As one of important fresh vegetables, the use of its rhizomes and fronds as food appeared to be prevalent due to its special flavor and taste in recent years in China. However, postharvest *P. aquilinum* has a short shelf life without appropriate treatments resulting from quality deterioration including tissue hardening, taste bastardizing and discoloration during storage (Huang et al., 2013). The postharvest *P. aquilinum* was believed to be under genetic control and usually interacted with environmental stimuli such as heat, dark, water loss and microorganism attack and mechanical injury which sped up quality worsening. Their nutritional value and visual appeal rapidly decreased, making them unmarketable within three to five days (Huang et al., 2013). Therefore, the research on retarding quality worsening was the core problem for *Pteridium* production. Recent researches on *P. aquilinum* mainly focused on bracken distributions (Holland and Aplin, 2013), fresh-keeping by modified atmosphere (Guo et al., 2010), active substances extraction (Kardong et al., 2013; Wang and Wu, 2013) and pathogenetic chemicals (Fletcher et al., 2011) at home and abroad. Recently, proteomics technology has been applied to investigate molecular mechanisms of various stress responses in plants such as *Arabidopsis thaliana* (Mukherjee et al., 2010), maize (Li et al., 2011), rice (Zhang et al., 2012), fresh-cut *Zizania latifolia* (Luo et al., 2012) and Lotus tuber (Jiang et al., 2012). To our knowledge, studies on biological mechanisms of the vegetable on quality worsening from proteomic angles have not yet been performed up to now. Thus, we used 2-DE technology followed by MALDI-TOF/TOF to identify the differential proteins which may partially explain the molecular mechanisms on quality deterioration. The study may provide useful molecular information for taking effective measures to prolong marketability of a vegetable product in the future.

Result

The functional classification of differential proteins

The results showed that seventeen proteins were identified by MALDI-TOF/TOF and these proteins were involved into five metabolic pathways, including carbohydrate metabolism (6, 35.3%), protein synthesis (2, 11.9%), energy and amino acid metabolism (3, 17.6%), cell structure (3, 17.6%), and stress response and defense (3, 17.6%) based on biological functions of differential proteins and relevant literatures.

The subcellular localization of differential proteins

According to WOLF PSORT database (http://wolfpsort. org/), nine proteins were located to two sub-organelles (Table 1),

including mitochondrial (6, 35.3%) and chloroplast (3, 17.6%), while other eight proteins were cytoplasmic proteins (8, 47.1%).

The comparison on experimental Mr/pI and theoretical Mr/pI of differential proteins

As shown in Table 1, the experimental Mr/pI of the identified proteins was in reasonable agreement with the theoretical values of them (Table 1). However, differences between the experimental and theoretical values of Mr/pI for few proteins were noticeable (Table 1). The theoretical Mr of the elongation factor (spot 1) was markedly higher than the experimental value (Table 1). In addition, the theoretical pI (9.39) of prohibitin (spot 4) was far beyond the pH range of IPG strips we used. The discrepancy of Mr/pI might be due to the presence of different isoform, the lack of genomic information for the organisms, the expressions in different organisms and post-translational modification of the same gene expression.

The determination of H_2O_2 and protein content

The protein content has been on the decline during postharvest storage. The H_2O_2 content was increasing in the former six days storage, while went down on the ninth day storage. The results showed in Fig 5 and Fig 6.

Discussions

Proteomic changes in *P. aquilinum* are associated with the regulation of different metabolic pathways when the vegetable was harvested and subsequently stored for a few days. However, little is known on the molecular mechanisms of the quality deterioration. V-ATPase is a key player in several aspects of cellular function, including acidification of intracellular organelles and regulation of extra-cellular pH. The protein is indispensable for plant growth under normal conditions due to its role in energizing secondary transport, maintenance of solute homeostasis and facilitating vesicle fusion (Dietz et al., 2001). The expression of V-ATPase was reported to be involved in adapting plants under different stresses such as salinity (Podda et al., 2013), drought, acid stress, anoxia and cold (Dietz et al., 2001). However，the abundance of V-ATPase was significantly down-regulated in CK3, CK6 and CK9 compared to CK0. Postharvest *P. aquilinum* may lack of the regulatory mechanisms to cope with adverse condition when it encountered multiple stresses during storage. Proteomic studies showed enzymes involved glycolysis in the chloroplast were down-regulated when flag leaves senesces (Zhang et al., 2010). In this experiment, three enzymes of glycolysis, including triosphosphate isomerase (spot 3), phosphoglucomutase family protein isoform 1 (spot 17) and phosphoglycerate mutase (spot 15) in abundance were all down-regulated. It seemed that *P. aquilinum* senescence begins when the vegetable was harvested and subsequently stored. Furthermore, three proteins were associated with TCA cycle including isocitrate dehydrogenase (spot 7), malate dehydrogenase (spot 10) and ATP-Citrate synthase alpha chain protein 2-like (spot 12). The authors considered the down-regulation of these proteins in abundance due to the influence of upstream glycolysis.

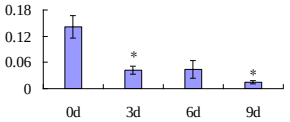
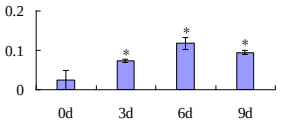
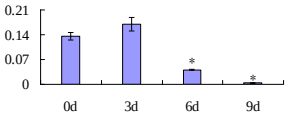
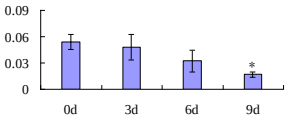
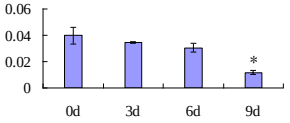
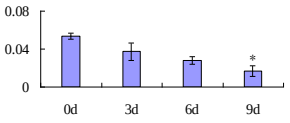
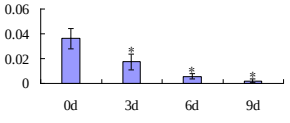
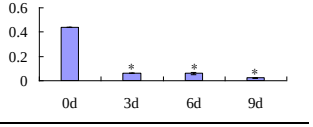
Three proteins were related to cell structure. Actin 2 (spot 14), an indispensable constituent of the cytoskeleton, played vital functions in cell division, intracellular transport, cell growth, cell to cell communication and organelle positioning. ADF (spot 1) was one of eukaryotic actins regulatory proteins, which accelerated the turnover rate of actins by depolymerizing actins monomers from the pointed ends and severing the filaments (Luo et al., 2012; Zhang et al., 2011). Actins monomers were dissociated and cytoskeleton integrity could be destroyed due to the up-regulation of the protein in abundance during storage. Prohibitins (spot 4) played a crucial role in mitochondrial biogenesis and protection against various stresses and senescence in plant cells (Ahn et al., 2006). Plants are generally subjected to biotic and abiotic stresses during their lifetime and correspondingly evolved a wide range of defense mechanisms to protect themselves. After harvest, *P. aquilinum* will suffer from the main stresses such as microorganism attack, water loss, lack of nutrients supply and mechanical damage. Water loss from plant cells causes a burst of oxidative stress, generating excessive ROS, which results in protein denaturation and lipid peroxidation. Five proteins which are connected with ROS metabolism, protein synthesis and protein fate. MDHAR (spot 3) could deacidize MDHA to ascorbic acid and have a positive effect in eliminating ROS and maintaining reduced power, however ROS production may be accumulated due to downregulation of MDHAR. Structural damage, funciton loss and programmed cell death in plant cell may be caused by excessive ROS production (Liang et al. 2014). Glutamate dehydrogenase (spot 6, GDH) plays an important role in providing carbon skeletons for effective functioning of TCA cycle. Lightfoot reported transgenic plants expressing GDH gene are improved in tolerance to, water deficit and pathogen infections (Lightfoot et al., 2007; Pageau et al., 2006). The authors speculated that the up-regulation of GDH in abundance may be responsible for microorganism infection or other stresses during storage. Heat shock proteins function as protein and membrane stabilizers, are elicited by biotic and abiotic stresses (Qin et al., 2009) and also expressed during fruit ripening (Prinsi et al., 2011) and vegetable's quality deterioration (Jiang et al., 2012). In general, principal Hsps are expressed in large quantities because they participate in facilitating the normal situation of the organism or ameliorate the problems of unsuitable folding and aggregateion against biotic and abiotic stresses (Mohamed and Whaibi, 2011; Shi et al., 2014). To our surprise, variation trend of Hsp 101 in abundance was just the opposite of Hsp17.5. It is difficult for the authors to explain the difference. Elongation factor (spot 8 &11) was highly conserved among different species and may be involved in protein synthesis and aging (Riis et al., 1990). As shown in Table 1, the two proteins in abundance were steadily decreased, and the above results indicated protein synthesis could be retarded and the protein content may decrease during storage. The content of H_2O_2 and protein content were examined (Fig 5, Fig 6) , and the results validated above deduction. As discussed above, *P. aquilinum* inevitably encountered the cell structure disassembly and cell senescence after harvesting. ROS production increased and protein content decreased when the vegetable was harvested and stored. These results may jointly lead to quality deterioration of *P. aquilinum* during postharvest storage.

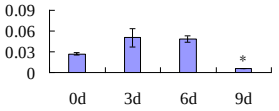
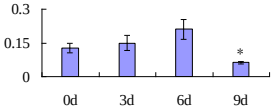
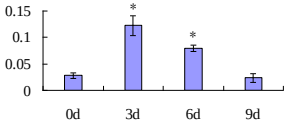
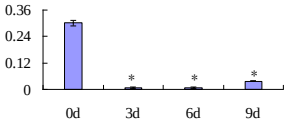
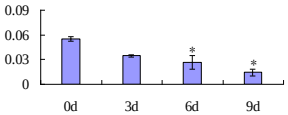
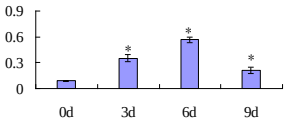
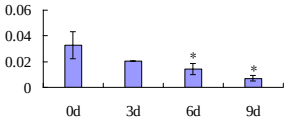
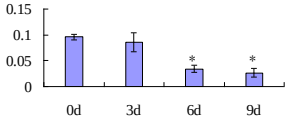
Materials and Methods

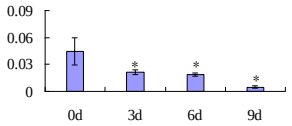
Plant material

P. aquilinum was harvested at vegetative stage from the "SanTai hill", situated in ChiZhou city, Anhui province, China and immediately transported to experimental laboratory. The individuals of the *P. aquilinum* (CK0) were selected for uniform size (20 cm in length) and color and the absence of visual defects. The stems of the vegetable were

Table 1. Identification of 17 differential proteins from *P. aquilinum* during postharvest storage.

[a]Spot no	[b]Protein name	[c]Accession no/species	[d]Sequence coverage (%)	[e]Matched peptide sequence	[f]Theor/exp Mr(kDa)/pI	[g]Protein subcellular localization	[h]Score/ thershold	[i]Spot % volume variations (p<0.05)
[j]Energy and amino acid metabolism								
16	V-ATPase subunit A	gi|137460 *Daucus carota*	11	YSNSDTVVYVGCGER EASIYTGITIAEYFR LAEMPADSGYPAYLAAR LEGDSATIQVYEETAGLMVNDPVLR	69.1/70.1 5.29/5.61	cytoplasm	509/50	
6	glutamate dehydrogenase	gi|4150965 *Asparagus officinalis*	8	FHGYSPAIVTGKPVDLGGSLGR	44.5/44.9 6.52/6.93	mitochondrial	168/50	
5	methionine synthase protein	gi|18483235 *Sorghum bicolor*	6	YGAGIGPGVYDIHSPR IQEELDIDVLVHGEPER	84.1/90.2 5.93/7.67	mitochondrial	371/48	
[j]Carbohydrate metabolism								
3	cytosolic triosephosphate isomerase	gi|310768790 *Pteris vittata*	20	FFVGGNWK VIACIGETLEQR ESNEFVADKVAYALSQGLK	27.3/27.3 5.04/6.11	chloroplast	316/49	
17	phosphoglucomutase family protein isoform 1	gi|508709149 *Theobroma cacao*	6	FLFEDGSR QHWATYGR ATGAFILTASHNPGGPHEDFGIK	63.5/70.9 5.40/6.88	chloroplast	235/49	
15	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1	gi|18391066 *Arabidopsis thaliana*	11	ALEYEDFDKFDR AVGPIVDGDAVVTFNFR	60.8/64.7 5.32/6.04	chloroplast	386/49	
12	PREDICTED: ATP-Citrate synthase alpha chain protein 2-like	gi|357155751 *Brachypodium distachyon*	5	WGNVEFPLPFGR	47.1/49.4 5.55/6.11	mitochondrial	107/49	
7	isocitrate dehydrogenase	gi|19171610 *Cucuis sativus*	16	HAFGDQYR TIEAEAAHGTVTR NILNGTVFREPILCK GGETSTNSIASIFAWSR	46.4/47.4 6.00/6.93	mitochondrial	386/49	

10	malate dehydrogenase, cytoplasmic	gi|11133373 *Medicago sativa*	9	VLVTGAAGQIGYALVPMIAR	35.9/41.3 6.39/6.48	mitochondrial	129/49	
[j]Cell structure								
14	Actin 2	gi|3746938 *Anemia phyllitidis*	25	GYSFTTTAER SEYDESGPSIVHR IWHHTFYNELR SYELPDGQVITIGAER VAPEEHPVLLTEAPLNPK TTGIVLDSGDGVTHTVPIYEGYALPHAILR	41.8/44.2 5.31/5.55	cytoplasm	574/49	
1	PREDICTED: actin-depolymerizing factor 10-like (ADF)	gi|460406249 *Solanum-lycopersicum*	8	IFFVAWSPETAR	49.0/14.6 6.57/5.72	cytoplasm	64/49	
4	prohibitin	gi|384245685 *Coccomyxa subellipsoidea*	8	EFNILVDDIAITHLSFGTEFTK AKEFNILVDDIAITHLSFGTEFTK	31.0/32.1 9.39/7.27	mitochondrial	177/49	
[j]Stress response and defense								
13	monodehydroascorbate reductase	gi|113472842 *Rheum australe*	8	AYLFPEGAAR LPGFHTCVGSGGER	47.4/45.6 5.63/5.95	cytoplasm	180/49	
2	heat shock protein 17.5 (sHsp 17.5)	gi|186694325 *Malus domestica*	22	IDQVKAAMENGVLTVTVPK	17.5/13.4 6.20/6.42	cytoplasm	188/49	
9	101kDa heat shock protein (Hsp101)	gi|11561808 *Triticum aestivum*	10	RPYSVILFDEVEK ALAEQLFDDENLLVR VILFIDEIHLVLGAGR FQQVYVAEPSVADTISILR	101.3/115.0 5.84/6.70	cytoplasm	402/49	
[j]Protein synthesis								
11	elongation factor 1-gamma	gi|351722398 *Glycine max*	4	VPVLETPDGPIFESNAIAR	47.9/55.5 6.30/6.05	cytoplasm	150/50	

8	elongation factor, putative	gi|255565836 *Ricinus communis*	10	ILSEEFGWDKDLAK AYLPVIESFGFSGTLR GHVFEELQRPGTPLYNIK STLTDSLVAAAGIIAQEVAGDVR	95.0/115.8 5.87/6.65	cytoplasm	410/50	

a Numbering corresponds to the 2-DE gel in Fig 1
b Names of the protein obtained via the MASCOT software (www.matrixscience.com) from the NCBInr database
c Accession numbers and species from the NCBInr database
d Percent sequence coverage
e Matched peptide sequence
f Theoretical molecular mass/experimental molecular mass(Mr) and theoretical isoelectric point and experimental isoelectric point (pI)
g The protein sub-cellular location of seventeen differently proteins according to PSORT (http://wolfpsort.org).
h MOWSE score probability (protein score) for the entire protein and for ions complemented by the percentage of the confidence index
i X-axis denotes storage time, and Y-axis denotes protein abundance (normalized volume of spots), values are expressed as the mean of three replications±standard deviation. The values which are statistically changed (more than 2-fold and $p<0.05$, according to Student *t*-test) in comparison with the CK 0 are marked by asterisks (*)

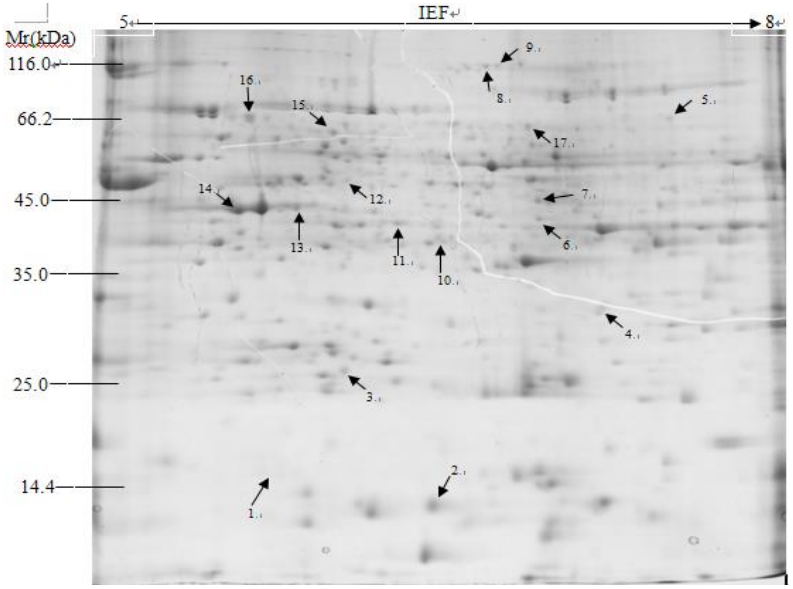


Fig 1. Identification of seventeen protein spots was performed by 2-DE and MALDI-TOF-TOF analysis. The numbers with arrows indicated the differential expressed protein spots

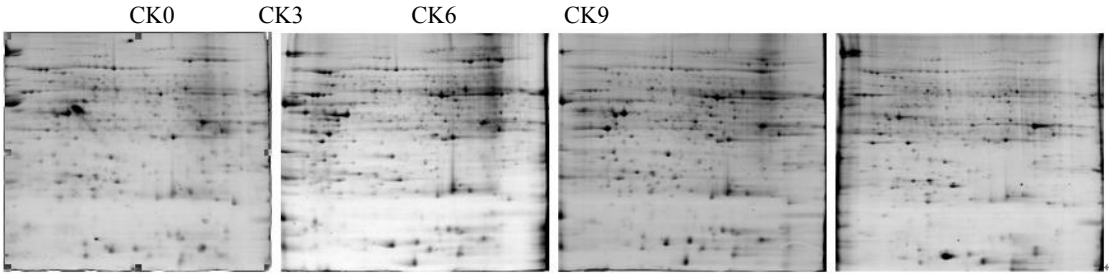


Fig 2. Representative 2-DE maps of *P. aquilinum* at different storage time

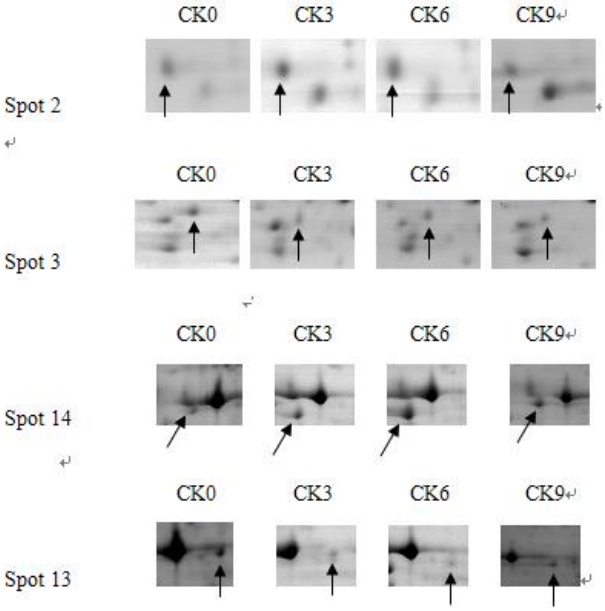


Fig 3. Magnified views of some differential proteins marked in Fig. 1. Arrows and numbers indicated the differential spots at different storage time.

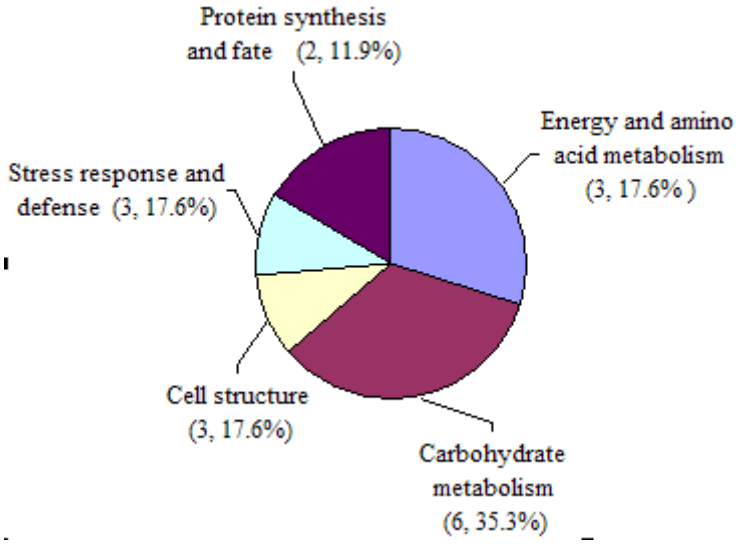


Fig 4. The categorization of metabolism pathways of identified proteins according to their putative functions. The percentage and the number of protein of each group were indicated.

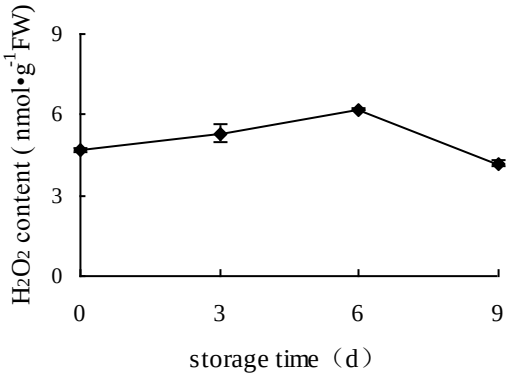


Fig 5. The change of H_2O_2 content during postharvest storage Storage.

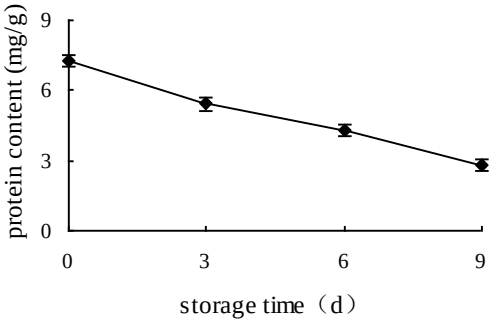


Fig 6. The change of protein content during postharvest.

packaged into 12 polyethylene plastic bags (each bag containing approximately 250 g). Twelve bags were randomly divided into four segments, one segment was taken for immediate use, and other segments were stored at 14± 1℃ with RH 80-90% for duration of 3, 6, 9 d (annotated in CK3, CK6, CK9), respectively. Samples were taken at corresponding storage time and then frozen in liquid nitrogen followed by storing at -20 ℃ prior to protein extraction. Three biological replicates were performed for each storage time point.

Protein extraction and quantification

Protein was extracted using the method of Wang (Wang, 2006). The proteins were dissolved overnight at 4℃ in lysis buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, and 0.5% (v/v) pH3-10 IPG buffer), and then centrifuged at 10000×g for 30 min at 4 ℃. The supernatants were collected and protein content was determined according to Bradford method (Bradford, 1976).

2-DE and staining

Protein extracts (1.2mg) were subjected to 17cm pH 5-8 IPG strips (Bio-Rad). The iso-electric focusing was performed on a PROTEAN IEF system (Bio-Rad) at 50v for 13h, at 100v, 200v, 500v and 1000v for 1h, respectively and at 4000v, 8000v in linear for 2h, respectively and then run at 8000v to until 70 kVh at 20℃. After isoelectric focusing, the strips were equilibrated by gentle stirring for 15 min with 2% dithiothreitol in equilibration buffer (50mM Tris-HCl, pH8.8, 6M urea, 20% glycerol, and 2% SDS). A second equilibration was carried out for 15 min in the same equilibration buffer containing 2.5% iodoacetamide. Following equilibration, the strips were run in 12% polyacrylamide gels using the Ettan DALT Twelve vertical set (GE Healthcare) for 1 h at 1 W/gel, followed by 15w/gel until the bromophenol blue reached the gel bottom. After SDS-PAGE, gels were stained with 0.12% colloidal Coomassie brilliant blue G250 overnight.

Image analysis and data

The gels were scanned at 300 dpi with a Versdoc 3000 scanner (Bio-Rad) and image analysis was performed using PDQuest analysis software. The protein spots were checked manually and added or deleted if necessary. Protein spots were considered valid according to their presence in at least three spots of 2-DE gels for each sample. Protein spot intensities were normalized as a percentage of the total volume in all the spots present in the whole gel. Protein spots with at least 2-fold increase/decrease and statistically significant (Student's *t*-test, $p<0.05$) of were considered as differential proteins.

Protein identification

For MALDI-TOF/TOF analysis, protein spots showing changes statistically significant ($p<0.05$) and a 2-fold threshold were excised from the gels and digested by addition of trypsin (Promega, USA). The resulting peptides were analyzed by a 4800 MALDI-TOF/TOF proteomic analyzer (Applied Biosystems, USA). Proteins were identified by searching based on the NCBInr (non-redundant) database of plants using the MASCOT search engine v3.5 (Matrix Science Ltd, London). Only the best matches with higher score were accepted when the result showed more than one eligible result.

The determination of H_2O_2 content

Two gramme of bracken stem was homogenized with 6 mL of chilled 100% acetone and then centrifuged at 10000×g for 20 min at 4°C. The supernatant was collected immediately for H_2O_2 analysis according to the method of Patterson (Patterson et al., 1984). H_2O_2 content was expressed as μmol /gFW.

Conclusions

Proteome analysis had provided extensive information for understanding the biological mechanisms of quality deterioration after harvesting: (1) The decline of antioxidant capacity would be responsible for accumulation of ROS, and excessive ROS leads to cell oxidation and senescence (2) *P. aquilinum* senescence begins and protein content declines when the vegetable is harvested and stored for a few days (3) Cell structure integrity could be destroyed during postharvest storage. For better understanding the above results, molecular biological analysis and other sub-cellular proteomic studies should be performed in the future.

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