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Short communication

Method development for analysis of proteins extracted from the leaves of *Orthosiphon aristatus*

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ABSTRACT

Orthosiphon aristatus is a traditionally used medicinal plant. In order to study the proteome of the plant, we have developed a simple plant protein extraction method by direct extraction of protein using a modified 2D-gel compatible tris–sucrose buffer followed by a double TCA–acetone precipitation. This method omitted the use of toxic phenol which is widely used in the studies of plants proteins. Moreover, it shortens the lengthy extraction procedure of phenol extraction and back-extraction method and therefore reduced the extraction time (by 2 h) while increased in protein yields (by 50%). Comparison of the 2D-gel images of the two extracts revealed that >60 extra protein spots were detected in the extract of our current method. The method was applied on the leaves of *O. aristatus* collected from six geographical areas in Malaysia. The correlation coefficient of each replicate gels from the six areas ranged from 0.70 to 0.90 indicating good reproducibility of the method.

1. Introduction

Orthosiphon aristatus is a herb used traditionally as medicine. The therapeutic effects of *O. aristatus* were ascribed to its polyphenols content [1]. We believed that knowing the proteome of the plant will lead to better understanding of its medicinal value. To date, there is no protein composition data available for the plant. Extraction of plant proteins remains to be the greatest challenge in studying plant proteomics [2]. Plant cell wall comprises of a complex mixture of polysaccharide, cellulose, hemicellulose and pectin that are difficult to disrupt [3]. A few common plant protein extraction methods are based on phenol extraction [4] and combination of TCA/acetone precipitation and phenol extraction [5]. Phenol extraction on plant proteins has been a widely used method [6,7] due to its ability to extract proteins that are free from polysaccharides, lipids and phenolic compounds [8] that may interfere with proteins separation method. However, phenol is a toxic compound, a major drawback to the phenol extraction method. Furthermore, the multiple steps in phenol extraction and ammonium acetate precipitation [5] are time consuming and also lead to protein loss.

Many different types of extraction methods have been used to investigate the proteome of plants, yet given the numerous herbal plants new to this field, it is important to develop fresh protein extraction method as it is a critical step leading to good two-dimensional gel electrophoresis [9]. The objective of this study was to develop a simple protein extraction method for the leaves of *O. aristatus*.

2. Experimental

2.1. Material and reagents

Electrophoresis grade chemicals: trizma base (tris), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), potassium chloride (KCl), sucrose, thiourea, urea, *beta*-mercaptoethanol, phenol, Protease Inhibitor Cocktail, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Carrier Ampholytes, DL-dithiothreitol (DTT), iodoacetamide, trichloroacetic acid (TCA), ammonium bicarbonate (NH_4HCO_3) and calcium chloride ($CaCl_2$) from Sigma. Ready Strip Immobilized pH Gradient (IPG) Strips 11 cm pH 4–7 and glycerol from Bio-Rad. Analytical grade acetone and hydrochloric acid (HCl) were obtained from Qrec. High-performance liquid chromatography (HPLC) grade acetonitrile (ACN) was from JT Baker.

2.2. Plant samples

Leaves of *O. aristatus from the family of Lamiaceae* were freshly harvested from the herbal garden of Universiti Sains Malaysia, Selangor, Kedah, Penang, Johor and Sarawak. Voucher number (11210) of the specimen was deposited at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia. The leaves

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were washed with tap water to remove any traces of dirt and rinsed with distilled water before stored at −80 °C.

2.3. Sample preparations

2.3.1. Phenol extractions of protein

Protein extraction was carried out according to the phenol extraction method described by Faurobert et al. [8]. One gram of leaves was subjected to the extraction procedure.

2.3.2. Protein extraction with tris–sucrose extraction buffer

One gram of fresh plant leaves were frozen and ground in liquid nitrogen, and suspended in 1.5 mL tris–sucrose extraction buffer [300 mM tris, 25.5 mM EDTA, 40 mM KCl, 180 mM sucrose, 2% (v/v) beta-mercaptoethanol and 1% (v/v) Protease Inhibitor Cocktail, adjusted to pH 8.0 with HCl]. The mixture was vortexed, incubated on ice for 10 min and centrifuged (14,000 rpm, 4 °C, 15 min). The supernatant (1 mL) was recovered and subjected to protein precipitation by TCA/acetone precipitation procedure of Méchin et al. [10]. The supernatant was added with 9 mL precipitation solution [10% TCA in acetone, 20 mM DTT] and incubated at −20 °C for 1.5 h. After centrifugation (14,000 rpm, 4 °C, 15 min), the pellet was recovered and rinsed with 5 mL rinsing solution [cold acetone, 20 mM DTT], suspended in 5.4 mL precipitation solution [10% TCA in acetone, 20 mM DTT], incubated at −20 °C for 1.5 h and followed by centrifugation. The pellet was again rinsed with 3 mL rinsing solution [cold acetone, 20 mM DTT] twice. The pellet was air-dried at room temperature and then resolubilized in 0.2 mL TLB [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (v/v) carrier ampholytes, 50 mM DTT]. The mixture was left for 1 h at room temperature and centrifuged (14,000 rpm, 20 °C for 10 min). The supernatant was recovered and stored in aliquots of 100 μL at −80 °C.

2.4. Protein assay

The protein concentration was determined by the *RC–DC* Protein Assay (BioRad). The standard assay protocol given by the manufacturer was applied.

2.5. Protein separation

2.5.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The plant proteins (45 μg for each lane) were separated on 12.5% gel by using SDS-PAGE method of Laemmli [11]. The running time was 45 min at 200 V constant voltages. Gel-electrophoresis was carried out on a vertical electrophoresis cell (PROTEAN II XL cell; BioRad). The gel was stained with Commassie Blue dye.

2.5.2. Two-dimensional (2-D) gel electrophoresis

Plant protein (500 μg) was separated by 2D-gel electrophoresis according to Weiss and Gorg [12]. The protein was loaded to IPG strip by passive dehydration for 3 h and focused at a maximum voltage of 8000 V/h for a total of 40,000 V. The second dimension of separation was carried out on 12.5% polyacrylamide gel of 1 mm thickness and size 16 cm × 16 cm using vertical electrophoresis cell. The running time was 4 h at 200 V constant voltages. The gel was stained with Commassie Blue dye. The gel images were captured using VersaDoc Imaging System and analyzed using PDQuest software (BioRad).

2.6. In-gel digestion

Protein spots in 2D-gel were excised and subjected to in-gel digestion according to the method described by Gam and Aishah [13]. In short, the gel pieces were washed, the Coomassie Blue stain was removed by dehydration and hydration steps using acetonitrile and NH_4HCO_3 buffer, respectively. Finally the protein in the gel was digested using trypsin and the resulted peptides was eluted from the gel, blow-dried using oxygen free nitrogen and were kept at −20 °C.

2.7. Protein identification

2.7.1. Mass spectrometry analysis

The peptides were reconstituted in 30 μL of 0.1% formic acid in DI water/ACN at a ratio of 85:15 (v/v), 15 μL of the sample was injected and pre-concentrated at the enrichment column (Zorbax SB C18, 35 mm × 0.5 mm, particle size 5 μm) by using a binary pump at a flow rate of 0.1 mL/min. The mobile phase used was 0.1% formic acid in DI water/ACN at a ratio 97:3 (v/v). The concentrated peptides were then transferred to a reverse phase column (Zorbax 300SB C18, 150 mm × 0.3 mm, particle size 5 μm) using a gradient mode from 5% to 95% B in 65 min at a flow rate of 4 μL/min pumped by a capillary pump, where mobile phases A and B were DI water containing 0.1% formic acid and ACN containing 0.1% formic acid, respectively. The effluent was interfaced with an ion trap mass spectrometer. A data dependent scan mode comprised of two scan events, which were full scan MS and data dependent MS/MS scan was programmed for MS analysis. Two most intense ions from the each MS scan that exceeded the threshold of 5000 counts were subjected to data dependent MS/MS scan. The MS parameters were as follow: dry gas flow rate of 6.00 L/min, the nebulizer pressure of 15.0 psi and dry gas temperature of 300 °C and the parameters for MS/MS scan were; default collision energy (voltage) of 0.95 V, charge state of 2 and isolation width of 2 *m/z*.

2.7.2. Mascot protein identification

The MS/MS data obtained was subjected to Mascot Protein Search Database (MSDB) search engine (www.matrixscience.com) for protein identification under the *Viridiplantae* (green plants) taxonomy. Trypsin was selected as enzyme used for digestion and carboxymethyl (C) selected for fixed modification. The peptide mass tolerance and fragment mass (MS/MS) tolerance allowed were ±2 and ±0.8 Da, respectively. 2 primary tools used for researching the functions and characteristics of identified proteins were SwissProt (www.expasy.org) and NCBI (www.ncbi.nlm.nih.gov).

2.8. Applications

The method developed was applied on a study of protein profiles of the leaves of *O. aristatus* obtained from six different geographical locations, which was carried out in duplicate for each location. The reproducibility of the 2-D gel images was analyzed using PDQuest software.

3. Results and discussion

Extraction of plants' proteins involved breaking of plant cell wall by physical grinding and followed by extraction of proteins using suitable buffers. The commonly used plant protein extraction methods included extraction of proteins using a mixture of phenol saturated solution and tris–sucrose buffer [4] or pre-extraction of proteins using phenol saturated solution followed by back-extraction using tris–sucrose buffer and finally subjected to ammonium acetate precipitation [4,5] or a single TCA/acetone precipitation [5]. Pre-extraction step with phenol is meant to avoid the extraction of polysaccharide, lipids and other non-protein components, nevertheless, the subsequent protein precipitation step by using ammonium acetate or single TCA/acetone precipitation

is still required to produce good quality of protein extract for 2D-gel separation. The main drawbacks of the method are the use of toxic phenol and the back-extraction steps that are not only time consuming but also causes lost of proteins.

In our present study, we have simplified this extraction method by omitting the pre-extraction of protein using phenol and therefore the multiple back-extraction steps was not needed and therefore shorten the extraction time by two hours. Our extraction method involved direct extraction of proteins using a suitable buffer. This direct extraction of plant protein resulted in the co-extraction of other non-protein components, which interfered with the subsequent gel electrophoresis separation. We found that these non-protein components in the protein extract can be effectively removed by using a double TCA/acetone precipitation method, where the purified proteins can be subjected to 2D-gel electrophoresis. A single TCA/acetone precipitation as reported by Wang et al. [5] was found only effective when coupled with tris–HCl buffered phenol pre-extraction. Fig. 1 shows the outcome of protein extracts after a double and a single TCA/acetone precipitation, where the latter appeared as smearing and streaking protein bands.

A previously reported tris–sucrose buffer [8] that was coupled with tris–HCl buffered phenol pre-extraction cannot be used singularly to extract plant proteins as it was not 2-D gel compatible even after coupled with a double TCA/acetone precipitation. We have modified the composition of tris–sucrose buffer by reducing the concentrations of all the reagents, where sucrose was reduced from 700 mM to 180 mM; tris from 500 mM to 300 mM; KCl from 100 mM to 40 mM; EDTA from 50 mM to 25.5 mM. This new composition of tris–sucrose buffer not only making it a more cost effective buffer, it can also produce protein extracts that was compatible to 2-D gel separation when coupled with a double TCA/acetone precipitation, which was not possible with the former buffer. Moreover, the quality of the proteins extracted was equal if not better than the widely used method [8]. As shown in Fig. 1, after optimization of buffer composition, the protein bands appeared to be sharper and the number of protein extracted was also increased.

The images of phenol extract (Fig. 2, panel A) and optimized tris–sucrose extract (Fig. 2, panel B) of *O. aristatus* produced relatively similar profile although the amount of extract loaded from the phenol extract was double than that of tris–sucrose extract. The recovery of protein in tris–sucrose extract was >50% higher than that of phenol extract (2.116 vs. 1.181 mg protein/g leaves) when the extractions were carried out on the similar amount of leaf tissue by weight. Despite the relatively similar image, the number of protein spots in tris–sucrose extract was higher (>60 spots) than the phenol extract. The LC/MS/MS analysis on the protein spots indicated that most of the proteins extracted by the two buffers were belonged to identical proteins. Nevertheless, there was greater number of proteins identified in tris–sucrose extract than the phe-

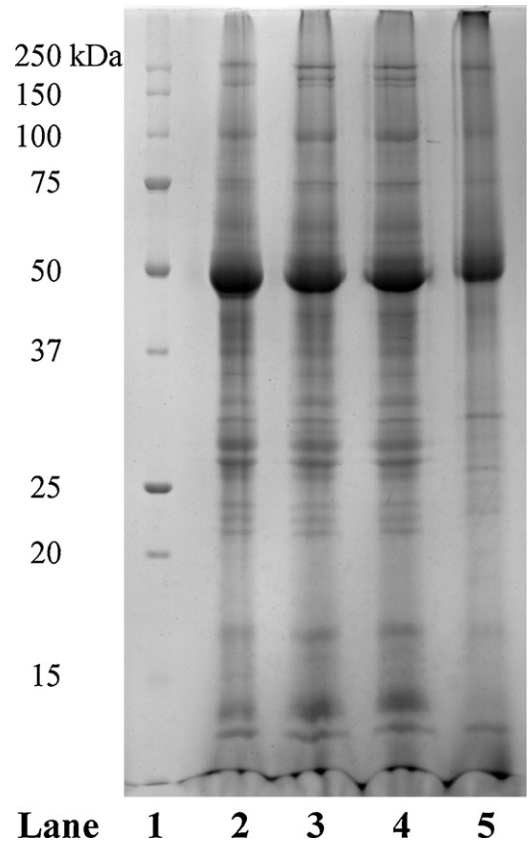

Fig. 1. Protein profiles in SDS-PAGE using different extraction buffers. Lane 1 are protein standards; Lane 2 shows protein profile of protein extracted with tris–sucrose extraction coupled with single TCA/acetone precipitation; Lanes 3 and 4 show protein extracted with tris–sucrose extraction coupled with double TCA/acetone precipitation of tris–sucrose buffers before optimization (3) and after optimization (4), respectively; Lane 5 shows protein profile of tris–HCl buffered phenol pre-extraction buffer.

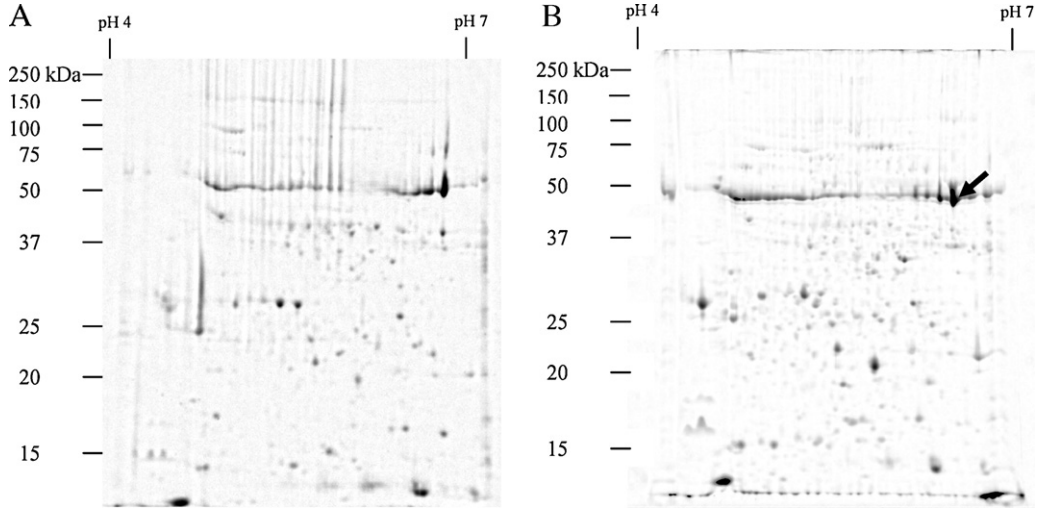

Fig. 2. 2-D gel images for tris–HCl buffered phenol pre-extraction buffer and tris–sucrose extraction proteins. Panel A shows the protein profile of *Orthosiphon aristatus* using phenol extraction method. Panel B shows the protein profile using the direct tris–sucrose extraction buffer. The arrows show the landmarks used in the respective gels.

Table 1
Lists of proteins identified.

Protein name	SwissProt accession no.	Score	Sequence coverage (%)	Gravy	Polarity	Functions
Tris–sucrose extract proteins						
Transketolase	Q43848	240	16	−0.206	Hydrophilic	Involves in carbohydrate biosynthesis
Ribulose bisphosphate carboxylase	P00869	196	16	−0.182	Hydrophilic	Photosynthetic enzyme responsible for the CO_2 fixation during photosynthesis process
Phosphoglycerate kinase	P12782	143	3	0.172	Hydrophobic	Functions in the Calvin cycle by catalyzing the conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate
ATP synthase beta subunit	Q9MRM0	132	17	−0.054	Hydrophobic	Synthesizes adenosine triphosphate (ATP) from adenosine diphosphate (ADP) in the presence of a proton gradient across the cell membrane
Oxygen-evolving enhancer protein 2	Q00434	114	7	−0.208	Hydrophilic	Involves in photosystem II; interacts with the cytoplasmic kinase domain of wall-associated receptor kinase and acts as a substrate for wall-associated receptor kinase
Triosephosphate isomerase	Q9M4S8	97	12	−0.125	Hydrophilic	Involves in carbohydrate biosynthesis
2-Cys peroxiredoxin BAS1	Q96291	87	9	−0.112	Hydrophilic	An antioxidant enzyme involved in the detoxification of H_2O_2 and alkyl hydroperoxides, reducing H_2O_2 and alkyl hydroperoxides by the use of hydrogen provided by thioredoxin, thioredoxin reductase and NADPH
Ferredoxin-NADP reductase	P41344	78	7	−0.34	Hydrophilic	Catalyzes the final step in the oxygenic photosynthetic electron transport
Carbonic anhydrase	P17067	64	7	−0.121	Hydrophilic	Catalyzes the reversible hydration of carbon dioxide
Sedoheptulose-1,7-bisphosphatase	P46285	62	6	−0.088	Hydrophilic	Involves in carbohydrate biosynthesis
Nucleoside diphosphate kinase 2 (NDPK2)	Q852S5	60	18	−0.124	Hydrophilic	Multiple functions enzymes: participates in protection against reactive oxygen species stress, auxin signaling and stimulation of GTPase activities for small G proteins in plants
Putative blue light receptor	P93025	53	3	−0.560	Hydrophobic	Acts as a blue-light photoreceptor controlling photo-induced movements of plants such as leaf positioning, leaf flattening and stomatal opening
NAD(P)H-quinone oxidoreductase subunit 5	Q0G9H2	42	2	0.520	Hydrophobic	NADH provides electrons from NAD(P)H plastoquinone
Invertase	P40912	37	10	−0.498	Hydrophilic	Involves in the hydrolysis of terminal non-reducing D-fructofuranoside residues in beta-D-fructofuranosides
GDSL esterase/lipase At1g29670	Q9C7N4	33	3	−0.155	Hydrophilic	A lipolytic enzyme
Anaphase promoting complex subunit 4	Q91W96	31	1	−0.210	Hydrophilic	Part of the component of the anaphase promoting complex involves in protein modification
Cytokinin-O-glucosyltransferas 2	Q9SK82	28	1	−0.074	Hydrophilic	Involves in the O-glucosylation of trans-zeatin and dihydrozeatin
Phenol extract proteins						
Plastocyanin A	P00299	63	7	0.232	Hydrophobic	A type of chloroplastic protein participating in electrons transfer between P700 and the cytochrome b6-f complex in photosystem I
Flavonol synthase	Q41452	34	2	−0.434	Hydrophilic	Catalyzes the formation of flavonols from dihydroflavonols

nol extract, where only two unique proteins were identified from the latter (Table 1).

The good protein quality produced by the developed extraction method was revealed in the quality of 2-D gel images and the LC/MS/MS data. The result of analysis of 12 gel images of leave extracts from 6 different geographical areas (Samples 1–6) (Table 2) showed that the 2-D gel images were reproducible for each replicate group, producing an average correlation coefficient

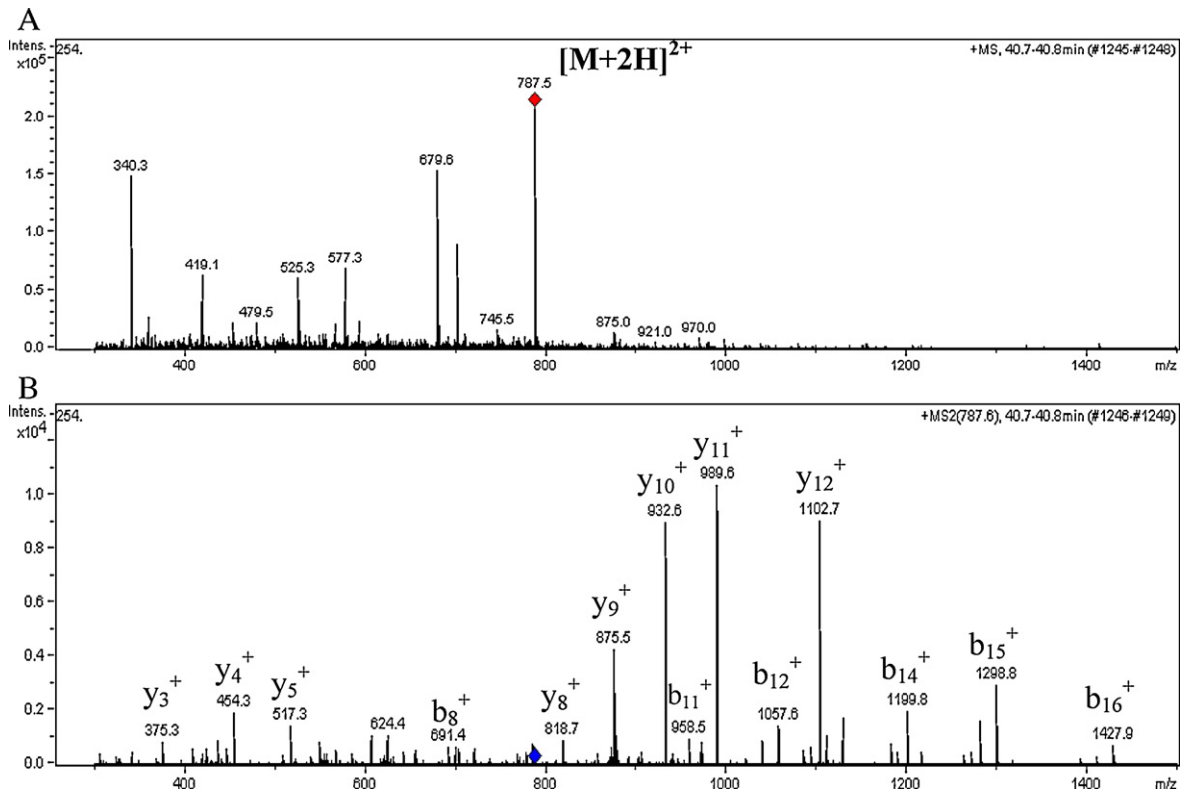

Fig. 3. Mass spectrometry analysis of one of the identified protein, phosphoglycerate kinase. Panel A: MS scan showing a doubly charged peptide ion with *m*/*z* 787.5 as precursor ion that exceeded the threshold set for data dependent scan; Panel B: MS/MS scan showing series of y and b product ions that revealed the amino acid sequence of the peptide.

Table 2
Analyses of *Orthosiphon aristatus* leaves sample collected from six different geographical areas tested in replicate.

Gel name	Protein yield (mg/g)	Average number of resolved spots	Average correlation coefficient, $R^2 \pm$ standard deviation
Sample 1	5.078	292	0.90 ± 0.14
Sample 2	3.070	256	0.85 ± 0.21
Sample 3	3.240	249	0.94 ± 0.08
Sample 4	2.116	176	0.89 ± 0.15
Sample 5	4.129	173	0.83 ± 0.24
Sample 6	2.495	180	0.87 ± 0.19

(R^2) ranging between 0.70 and 0.90, where correlation coefficient of 1.00 indicating two perfectly similar images (PDQuest and The Discovery Series). In LC/MS/MS, due to the high protein quality, the signal of the peptide ions was not suppressed by the non-protein components, which resulted in generation of good MS data for protein identification (Fig. 3).

Table 1 shows the list of proteins detected in this study, tris–sucrose buffer extracted both hydrophilic and hydrophobic proteins as indicated by proteins' Grand Average of Hydropathy (GRAVY) score [14]. The functions of the proteins were reported in Table 1.

4. Conclusion

We have developed a plant extraction method by omitting the use of phenol and therefore eliminating the multiple steps after phenol extraction. We also modified the tris–sucrose buffer composition, although the concentrations of all the reagents were concomitantly reduced, the quality of protein extracts was equal if not better than the former. The extraction procedure was coupled with TCA/acetone precipitation method to purify and concentrate proteins which resulted in good reproducibility of 2D-gel images. The newly developed method was cost effective, shorter extraction time and produced good quality of plant extract that enable detection of greater number of protein spots in 2D-gel than phenol extraction method. Therefore, we believe that we provide an alternative method for studying plant proteomics.

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