Appl Biochem Biotechnol (2011) 165:1577–1586
DOI 10.1007/s12010-011-9377-x

Proteome Analysis of Abundant Proteins Extracted from the Leaf of *Gynura procumbens* (Lour.) Merr

Chaw-Sen Hew · Lay-Harn Gam

Received: 22 April 2011 / Accepted: 5 September 2011 /
Published online: 22 September 2011

Abstract *Gynura procumbens* (Lour.) Merr. is a traditionally used medicinal plant to decrease cholesterol level, reduce high blood pressure, control diabetics, and for treatment of cancer. In our present study, a proteomic approach was applied to study the proteome of the plant that had never analyzed before. We have identified 92 abundantly expressed proteins from the leaves of *G. procumbens* (Lour.) Merr. Amongst the identified proteins was miraculin, a taste-masking agent with high commercial value. Miraculin made up ~0.1% of the total protein extracted; the finding of miraculin gave a great commercial value to *G. procumbens* (Lour.) Merr. as miraculin's natural source is limited while the production of recombinant miraculin faced challenges of not being able to exhibit the taste-masking effect as in the natural miraculin. We believe the discovery of miraculin in *G. procumbens* (Lour.) Merr., provides commercial feasibility of miraculin in view of the availability of *G. procumbens* (Lour.) Merr. that grow wildly and easily in tropical climate.

Keywords *Gynura procumbens* (Lour.) Merr · Proteomics · Leaf extract · Protein profiling · Miraculin

Introduction

Gynura procumbens (Lour.) Merr. (Family Asteraceae) is a widely used medicinal plant in South East Asia to treat kidney discomfort, inflammation, rheumatism fewer, and viral ailments [1]. In addition, it has been used traditionally to decrease cholesterol level, reduce high blood pressure, control diabetics and for the treatment of cancer [2]. The studies on its small molecules content revealed that *G. procumbens* (Lour.) Merr. possess anti-cancer activity [3], antioxidant property [4], anti-hyperglycemic, and anti-hyperlipidemic activities [5] and anti-inflammatory activity [6].

Gynura procumbens (Lour.) Merr. is a plant of ~10–25 cm in length, the leaves are succulent, elliptic, glossy with purplish hairs and arranged alternately on hairy purple

C.-S. Hew · L.-H. Gam (✉)
School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
e-mail: layharn@usm.my

stem [1]. In Malaysia, the leaves of *G. procumbens* (Lour.) Merr. are consumed as raw vegetable.

G. procumbens (Lour.) Merr. is a medicinal plant that had been used for decades, nevertheless, there is no study conducted to analyze the protein profile of the plant, which may reveal the potential useful proteins from the leaves of *G. procumbens* (Lour.) Merr. Therefore, our aim in this study was to analyze the abundant proteins from the leaf extract of *G. procumbens* (Lour.) Merr.

Materials and Methods

Plant Materials

Leaves of *G. procumbens* (Lour.) Merr. were collected from Kepala Batas, Penang. Voucher number (11209) of the specimen was deposited at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia. All the leaves were kept fresh and transported to our laboratory within 3 days. The leaves were washed twice with tap water followed by rinsing with distilled water. The leaves were then ground into fine powder form in liquid nitrogen using mortar and pestle. Ground leaf powder was stored at −80 °C until used.

Protein Extraction

Ten grams of ground leaves sample was added with 20 mL of cold acetone and 2 mL of trichloroacetic acid (TCA), the mixture was vortex thoroughly and kept at −20 °C for 90 min, it was then centrifuged at 16 000 g at 4 °C for 20 min. The pellet was collected and washed twice with 20 mL of cold acetone and air-dried at room temperature. It was then added with 3 mL of thiourea lysis buffer (TLB) [8 M urea, 2 M thiourea, 4% (*w*/*v*) 3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate (CHAPS), 0.4% (*v*/*v*) carrier ampholytes, 50 mM dithiothreitol (DTT)], mixed thoroughly by vortexing and followed by incubation for 5 min. The mixture was centrifuged at 16,000×*g*, 18 °C for 20 min and the supernatant was recovered, added with 4 volumes of 10% (*v*/*v*) TCA/cold acetone containing 20 mM DTT, incubated for 90 min at −20 °C and centrifuged at 16,000×*g*, 4 °C for 15 min. The pellet was washed twice with 0.5 mL cold acetone containing 20 mM DTT and centrifuged at 16,000×*g*, 4 °C for 15 min. Subsequently, the pellet was air-dried, reconstituted in TLB buffer and vortex until it was fully dissolved. The extract was then subjected to protein assay by using Bio-Rad RC/DC™ Protein Assay Kit (Bio-Rad Laboratories).

Two-Dimensional Electrophoresis

The extract containing 500 μg of protein in 185 μL of TLB buffer was subjected to two-Dimensional (2D)-gel electrophoresis. The protein was loaded to immobilized pH gradient (IPG) strip (11 cm, pH 4–7) by using passive rehydration method for at least 12 h. The rehydrated strips were transferred to the PROTEAN IEF System (Bio-Rad) and focused at 20 °C using voltage gradient of: 1 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, 1 h gradient from 1,000 to 8,000 V, 5 h at 8,000 V. After focusing, strips were subjected to two equilibration steps by using 2 mL of equilibration buffer [6 M urea, 0.375 M tris-hydrochloride acid (HCl), pH 8.8, 2% (*w*/*v*) sodium dodecyl sulfate (SDS), 20% (*v*/*v*) glycerol] containing 2% (*w*/*v*) DTT for 15 min for the first step and subsequently in 2 mL of equilibration buffer containing 2.5% (*w*/*v*) iodoacetamide for 15 min for the second step. Strips were then

subjected to 12% SDS-polyacrylamide gels (PAGE) electrophoresis separation at a constant voltage of 200 V throughout the run. The gel was stained with Coomassie Blue stain and the gel image was captured using VersaDoc Imaging system (Bio-Rad). Gel images were analyzed with PDQuest software (Bio-Rad).

In-gel Digestion

In-gel digestion was performed following the method by Gam and Aishah [7]. In short, the protein spot was excised from the gel and the Coomassie Blue dye was removed by three cycles of dehydration and hydration steps using acetonitrile (ACN) and 100 mM ammonium bicarbonate, respectively. The protein in the gel was then subjected to in situ reduction, alkylation and finally digestion using trypsin overnight. The peptides were eluted from the gel and blow dried using nitrogen gas.

Mass Spectrometry Analysis

Tryptic-digested peptides were reconstituted in 30 μL of 85:15 ddH_2O/ACN containing 0.1% (*v*/*v*) formic acid. It was then subjected to LC/MS/MS analysis using ion-trap mass spectrometer (Agilent Technologies). Ten microliters of the samples were injected into a 2D liquid chromatography separation device consisting of an enrichment column (Zorbax SB C18, 35×0.5 mm, particle size 5 μm) and a reverse phase column (Zorbax 300SB C18, 150×0.3 mm, particle size 5 μm) connected by a valves switching device. The enrichment column was connected to a binary pump pumping at isocratic flow rate of 0.1 mL/min, mobile phase used was 0.1% (*v*/*v*) formic acid in 97:3 ddH_2O/ACN. The reverse phase column was connected to a capillary pump pumping at a gradient mode from 5% B to 95% B in 65 min with a flow rate of 4 μL/min. Mobile phase A was 0.1% (*v*/*v*) formic acid in ddH_2O and mobile phase B was 0.1% (*v*/*v*) formic acid in ACN. The eluent of enrichment column was subjected to further chromatographic separation using reversed phase column, while the eluent of reversed phase column was subjected to electrospray ionization in mass spectrometer detector. Two scans analysis consisting of full-scan MS and data- dependent MS/MS scan was programmed for the mass spectrometer analyzer. The two most intense ions from the full-scan MS will be subjected to MS/MS scan. The MS parameters were dry gas flow rate of 6.00 L/min, the nebulizer pressure of 15.0 psi, and dry gas temperature of 300 °C; the parameters for MS/MS scan were: default collision energy (voltage) of 0.95 V, charge state of 2, minimum threshold of 5,000 counts, and isolation width of 2*m*/*z*.

Mascot Protein Identification

The MS/MS data was subjected to The National Center for Biotechnology Information (NCBI) Protein Search Database (MSDB) through search engine (www.matrixscience.com) under viridiplantae (green plants) taxonomy. Trypsin was selected as the enzyme and fixed modification was carboxymethyl (C). The peptide mass tolerance was set at ±2 Da, the fragment mass (MS/MS) tolerance was set at ±0.8 Da.

Results

The leaves of *G. procumbens* (Lour.) Merr were generally heavy due to its high water content. The average protein yield for the leaves extract was 0.442 mg protein/g of fresh

leaves. Figure 1 shows the 2D gel image of the mature leaves extract of *G. procumbens* (Lour.) Merr., the gel was stained with Coomassie Blue dye, therefore the protein spots detected were proteins in microgram quantities. A total of 292 protein spots were detected

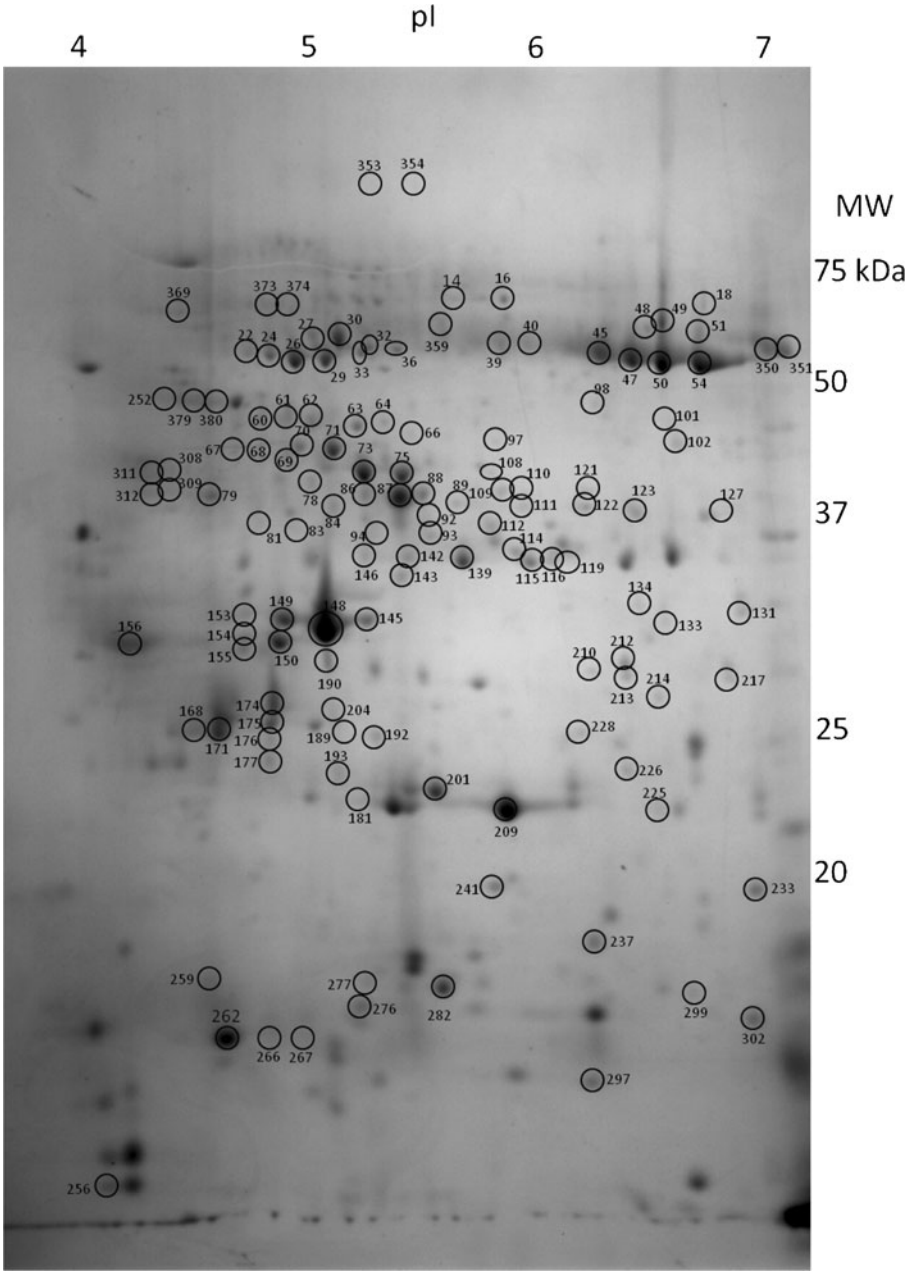

Fig. 1 2-D gel image of the leaves extract of *G. procumbens* (Lour.) Merr. The *x* and *y* axis are isoelectric point (*pI*) and molecular weight (*MW*) markers, respectively. *Spots in circles* were those with successful protein identifications

and all the spots were excised for in-gel digestion prior to subsequent LCMS/MS analyses. More than half of the protein spots were presented with no significant protein hits. Out of 123 spots (Fig. 1) that were successfully analyzed, we have identified 92 unique proteins from the plant extract. The list of the proteins identified is shown in Table 1. Most of the proteins belonged to photosynthesis and energy metabolism proteins. Other proteins were in the categories of growth and cell structure, plant defense, signal transduction, nucleic acid synthesis, nitrogen metabolism, protein destination and storage, secondary metabolism and transport. Amongst the proteins, miraculin, a plant defense protein was of very high commercial value, it has been used as low-calorie sweetener for diabetics and dieters. Based on the intensity of the protein spots detected in the gel, the ratio of miraculin protein spot intensity to the total intensity of all protein spots in the gel (n=2) was 0.001 and when considering the total amount of protein loaded onto the gel (500 μg). The quantity of miraculin detected was approximately 0.5 μg, which was 0.1% of the total protein extracted. This quantity of protein was within the detection limit of Coomassie Blue dye that is in the range of 0.5–200 μg [8]. Other interesting proteins identified were caleosin, peroxidase, catalase, glutathione transferase, and harpin protein.

Discussion

The common problem encountered in plant proteomics is the difficulty in obtaining good plant extract for subsequent electrophoresis separation. The interferences in plant extract such as high content of secondary metabolites, phenolics, polysaccharides and low protein content [9–11] can cause inefficient protein separation. In addition, the leaves of *G. procumbens* (Lour.) Merr. are succulent, which poses an additional problem to successful protein extraction. In this study, we have adapted TCA–acetone prewash method proposed by Wang et al. [11], which resulted in successful protein extraction from the succulent leaves of *G. procumbens* (Lour.) Merr. The 2D gel profile of *G. procumbens* (Lour.) Merr. leaves extract was presented as distinct and sharp protein spots, indicating the removal of the interferences from the leaves extract upon TCA–acetone prewash.

In this study, due to limitation of ion-trap mass spectrometer sensitivity for protein analysis, we focused only on the abundant proteins which can be stained with Coomassie Blue dye. Initial separation of leaves extract using IPG strip range from pH 3 to 10 revealed that majority of protein spots were found between pH 4 to 7. Thus, the subsequent isoelectric focusing separation of the leaves extract was carried out on pH 4–7. Most of the proteins migrated within the range of MW 15–100 kDa, which is similar to those reported in peanut [10], pea [12], *Arabidopsis thaliana* [13] and ginseng [14].

Only 42% of the protein spots were successfully identified with protein identities. Besides the list of proteins reported in Table 1, a total of 35 proteins were belonged to hypothetical and unknown protein groups. One of the reasons for low protein identification was the limited plant protein database [10]. Post translational modification of proteins is the main explanation for multiple electrophoretic migrations of proteins in 2D-gel observed in the leaves extract *of G. procumbens* (Lour.) Merr (Fig. 2), the similar phenomenon was also reported in peanut leaf [10] and *A. thaliana* [13]. In contrary, some of the spots were found to contain more than one type of proteins.

In our previous report on protein analysis on leaves extract of *G. procumbens* (Lour.) Merr by using SDS-PAGE separation method [15], we have identified peroxidase, a protein with existing commercial value. However, due to poor resolution of SDS-PAGE, only a few proteins were identified. In this present study, 2D-gel electrophoresis separation has

Table 1 List of identified proteins

Spots	Identified protein	Accession no.	Score	MW (kDa)	p*I*	Sequence coverage (%)
	Plant defense/repair					
87	ATS1 (*Arabidopsis thaliana* SEED GENE 1)	gi|15236846	51	28.1	5.81	3
192	ATGSTU20 (glutathione *S*-transferase TAU 20)	gi|15218311	53	25.1	5.63	5
190, 156	Harpin binding protein 1	gi|38679311	75	31.3	9.61	4
181	Miraculin homologue	gi|5689162	54	15.9	4.47	14
350, 131	Putative peroxidase	gi|56311333	66	14.2	7.07	8
351, 350	Catalase	gi|39103352	78	57.0	6.58	15
	Energy and metabolism					
168	PSI type III chlorophyll *a*/*b* binding protein	gi|159138839	51	13.9	4.51	13
171, 121	Chlorophyll *a*/*b* binding protein	gi|398599	75	28.7	5.68	17
168	Chlorophyll *a*/*b* binding protein 215	gi|115797	54	28.9	5.48	11
201	Chloroplast chlorophyll *a*/*b* binding protein 8	gi|149392115	60	20.9	5.81	9
175, 174	Chlorophyll *a*/*b* binding protein precursor	gi|6716783	62	28.5	5.29	11
209, 225	Oxygen-evolving enhancer protein 2	gi|11134156	98	28.3	8.61	7
193, 171, 175,176	Light-harvesting complex	gi|302816085	92	30.5	9.32	6
148, 153, 150	Oxygen-evolving enhancer protein 1	gi|11134054	258	35.4	5.89	21
83	PS II stability/assembly factor HCF136	gi|255559812	82	43.4	7.11	7
139, 115	Ferredoxin-NADP reductase	gi|61969078	91	35.4	7.71	12
177	Thylakoid lumen 18.3 kDa protein	gi|18405061	51	31.1	8.80	2
204, 189	Type I (26 kD) CP29 polypeptide	gi|19184	79	30.6	5.73	9
237	Photosynthetic electron transfer-like protein	gi|89475526	52	19.9	5.76	14
50, 47, 36, 39, 40, 45, 49, 54, 359, 18, 350, 373, 51, 374, 48, 94, 127	RuBisCo large subunit	gi|156634942	164	53.2	6.00	13
71, 70	RuBisCo activase	gi|158726716	212	48.8	6.10	18
122, 121, 127	Glyceraldehyde-3-phosphate dehydrogenase	gi|237648946	149	21.0	7.92	24
62, 61, 66, 60, 379	Phosphoglycerate kinase	gi|186491325	121	42.7	5.39	18
311, 121, 81, 94, 312, 84, 92, 112	Fructose-bisphosphate aldolase	gi|62732954	72	40.0	6.85	8
88	Aldolase	gi|2407279	55	39.6	8.90	2
228	Ribulose-phosphate 3-epimerase	gi|2833386	74	30.6	8.23	10
79, 312	Sedoheptulose-1,7-bisphosphatase	gi|159467635	71	42.4	8.59	3
110	Malate dehydrogenase	gi|27462764	79	35.9	6.01	10
	Transport					
14, 67, 68	Phosphate translocator-related	gi|15228248	66	39.1	9.99	3
30, 27	ATP synthase CF1 alpha subunit	gi|126723802	243	55.6	5.09	15
29, 22, 24, 26, 33, 16,	ATP synthase beta subunit	gi|66276267	263	51.0	5.20	16
89	ATP synthase gamma chain	gi|231610	76	41.7	8.16	6
115	Citrate binding protein	gi|24745940	52	25.2	9.25	11
	Growth and division					
63, 61	Actin	gi|113217	103	42.3	5.64	11

Table 1 (continued)

Spots	Identified protein	Accession no.	Score	MW (kDa)	p*I*	Sequence coverage (%)
93, 94, 109	Auxin-induced protein	gi|1184121	73	34.2	5.35	3
73	Alpha-L-fucosidase 2 precursor	gi|255552566	52	41.3	8.02	2
75	F-box family protein	gi|297839561	52	43.1	9.08	4
380	Putative kinesin-like protein	gi|238908346	53	117.8	6.29	1
214	PAG1; endopeptidase/peptidase/ threonine-type endopeptidase	gi|15225839	163	27.7	5.93	14
	Signal transduction					
252, 61	Putative blue light receptor	gi|20797092	57	82.0	8.67	1
210	Protein kinase family protein	gi|223452424	61	68.9	6.33	2
	Protein synthesis					
64	Elongation factor Tu	gi|1169494	82	52.6	6.33	8
133	Ribosomal protein L4	gi|2791998	61	30.5	8.92	4
259	Small ribosomal protein 4	gi|37992507	61	21.7	10.00	6
256, 354	RNA polymerase II second largest subunit	gi|270304976	58	27.0	9.67	5
122	mRNA-binding protein CSP41 precursor	gi|1532135	56	45.1	6.11	3
134	Maturase K	gi|28200503	56	19.8	9.75	7
	Nucleic acid synthesis					
155, 154	PUR5; ATP binding/ phosphoribosylformylglycinamidine cyclo-ligase	gi|15233161	55	41.7	5.31	2
	Nitrogen metabolism					
73	Glutamine synthetase	gi|1169931	129	39.6	5.24	9
297	Nucleoside diphosphate kinase B	gi|1346675	64	16.2	6.42	22
	Protein destination and storage					
111	Heat-shock protein 60-3A	gi|18400195	56	60.8	5.85	2
241, 233	Heat-shock protein	gi|255558876	104	17.5	5.34	14
	Secondary metabolism					
226, 93	Quinone-oxidoreductase	gi|90811717	101	21.8	6.97	13
146, 143	Cysteine synthase	gi|126508778	139	34.6	5.53	16
146	3-Mercaptopyruvate sulfurtransferase	gi|6686778	54	42.0	6.08	2
146	Similar to gb|AF109156 thiosulfate sulfurtransferase (TST) from *Datisca glomerata* and contains PF| 00581 Rhodanese-like domain. ESTs gb|F19994 and gb|F19993 come from this gene	gi|4966366	51	36.1	5.27	3
	Unclassified					
282	Hydin-like protein	gi|302783012	55	532.9	9.54	0

Spot no. refer to the protein spot excised from 2D gel (Fig. 1). Accession numbers according to NCBI database under *Viridiplantae*. Mascot score obtained for each protein was greater than 50, where score 50 indicates identity or extensive homology at a significance level ($p<0.05$). Sequences coverage refers to the percentage of amino acids sequence of the protein that was detected by LCMS/MS for the identified protein

allowed greater number of protein to be identified, amongst these proteins was Caleosin. Caleosin is also known as *A. thaliana* Seed Gene 1 (ATS1). Caleosin has an activity of peroxygenase, which is used in the production of hydroxy fatty acid derivatives with anti-fungal activity [16]. We have also identified a few abundant proteins from the leaves extract of *G. procumbens* (Lour.) Merr with high commercial value. Miraculin was a protein

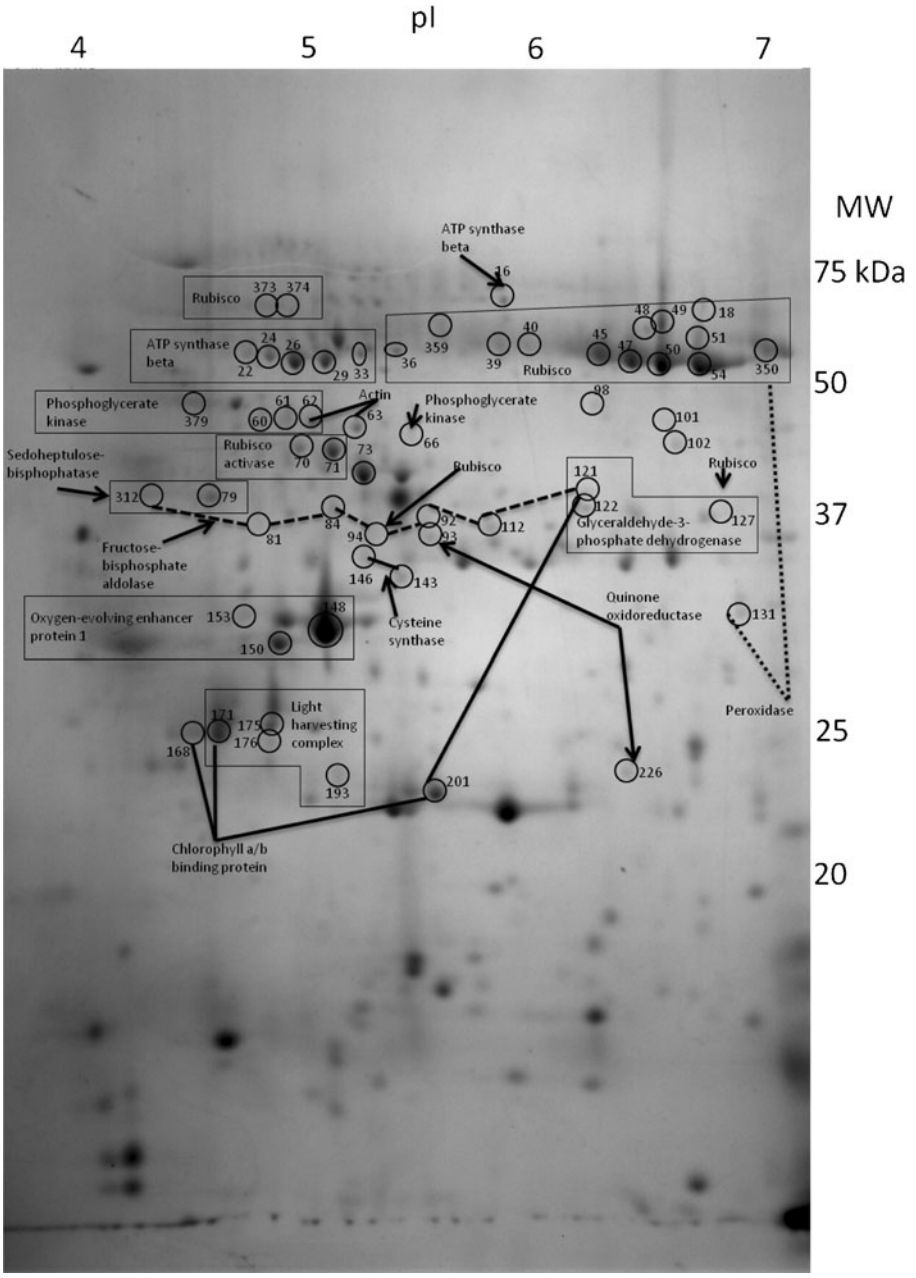

Fig. 2 2D gel image of the leaves of *G. procumbens* (Lour.) Merr. The *circled spots* in the same box indicated similar type of protein with multiple migrations. *Circled spots* connected by *dotted line* and *continuous line* were also proteins of the similar types

detected in the leaf extract of *G. procumbens* (Lour.) Merr. The name miraculin derived from its miraculous property, the protein itself is not sweet; however, it can modify the sour taste into sweet taste at 3,000 times higher than that of sucrose on a per weight basis [17].

Miraculin exhibits its taste-masking property at a very low concentration and therefore, it had been used commercially as a low-calorie sweetener for diabetics and dieters [18] in the United State and also in Japan. The natural source of miraculin is red berries (*Richardella dulcifica*). Nevertheless, due to its great demands, attempts have been made for mass production of miraculin in foreign hosts [18, 19]; however, the recombinant source of miraculin did not exhibit the taste-masking effect as in it natural source [19]. Due to this reason, miraculin is not commercially feasible. The finding of miraculin in *G. procumbens* (Lour.) Merr may lead to commercial feasibility of miraculin, this is because the plant grows wildly and easily in the tropical climate, which enable mass production of the proteins under a proper agriculture program.

Other interesting proteins identified from *G. procumbens* (Lour.) Merr were peroxidase, catalase, glutathione transferase and harpin protein. Harpin binding protein 1 was marketed as bio-activator to counteract the undesirable effects of pesticides. Harpin protein activates plant pathway that control several growth mechanisms, such as jasmonic acid pathway for plant defense [20]. On the other hand, peroxidase, catalase and glutathione transferase are proteins involve in metabolism of hydrogen peroxides (H_2O_2), an indicator to environmental stress [21]. One of the transport proteins identified was phosphate translocator, an enzyme involves in shikimate pathway [22] for synthesis of secondary products of commercial interest [23].

Conclusion

We have analyzed the abundant proteins from the leaf of *G. procumbens* (Lour.) Merr. Miraculin, a high commercial value protein was one of the proteins identified, the detection of this protein in *G. procumbens* (Lour.) Merr. opens a new dimension of research on the plant, as it was always being referred to as medicinal plant, we have showed in this study that the industrial value of the plant may be equal, if not greater than its well-known medicinal value.

Acknowledgments This work was supported by RU grant from Universiti Sains Malaysia (Project number: 1001/PFARMASI/815034). We thank the National Institute of Pharmaceutical and Nutraceutical, the Ministry of Science, Technology and Innovation, Malaysia for the use of the mass spectrometry instrument to analyze proteins, the Institute for Graduate Studies for providing scholarship to Hew Chaw-Sen.

References

1. Wiart, W. (2006). *Medicinal plants of the Asia-Pacific: drugs for the future?* Singapore: World Scientific.
2. Perry, L. M. (1980). *Medicinal plants of east and southeast Asia*. Cambridge: MIT Press.
3. Jenie, R. I., & Meiyanto, E. (2007). *Majalah Farmasi Indonesia, 18*, 81–87.
4. Puangpronpitag, D., Chaichanadee, S., Naowaratwattana, W., Sittiwet, C., Thammasarn, K., Luerang, A., et al. (2010). *Asian Journal of Plant Science, 9*, 146–151.
5. Zhang, X. F., & Tan, B. K. (2000). *Singapore Medical Journal, 41*, 9–13.
6. Iskander, M. N., Song, Y., Coupar, I. M., & Jiratchariyakul, W. (2002). *Plant Food for Human Nutrition, 57*, 233–244.
7. Gam, L. H., & Aishah, L. (2002). *Journal Biosains, 13*, 27–34.
8. López, J. L. (2007). *Journal of Chromatogr B, 849*, 190–202.
9. Isaacson, T., Damasceno, C. M. B., Saravanan, R. S., He, Y., Catala, C., Saladie, M., et al. (2006). *Nature Protocols, 1*, 769–774.
10. Katam, R., Basha, S. M., Suravajhala, P., & Pechan, T. (2010). *Journal of Proteome Research, 9*, 2236–2254.

11. Wang, W., Vignani, R., Scali, M., & Cresti, M. (2006). *Electrophoresis, 27*, 2782–2786.
12. Schiltz, S., Gallardo, K., Huart, M., Negroni, L., Sommerer, N., & Burstin, J. (2004). *Plant Physiology, 135*, 2241–2260.
13. Giavalisco, P., Nordhoff, E., Kreitler, T., Klöppel, K.-D., Lehrach, H., Klose, J., et al. (2005). *Proteomics, 5*, 1902–1913.
14. Nam, M. H., Heo, E. J., Kim, J. Y., Kim, S. I., Kwon, K.-H., Seo, J. B., et al. (2003). *Proteomics, 3*, 2351–2367.
15. Hew, C.-S., & Gam, L.-H. (2010). *Biotechnol & Biotech Eq, 24*(4), 2132–2136.
16. Partridge, M., & Murphy, D. J. (2009). *Plant Physiology and Biochemistry, 47*, 796–806.
17. Gibbs, B. F., Alli, I., & Mulligan, C. (1996). *Nutrition Research, 16*, 1619–1630.
18. Sun, H.-J., Kataoka, H., Yano, M., & Ezura, H. (2007). *Plant Biotech Journal, 5*, 768–777.
19. Bachchu, M. A. A., Jin, S.-B., Park, J.-W., Boo, K.-H., Sun, H.-J., Kim, Y.-W., et al. (2011). *J. Korean Soc Appl Biol Chem, 54*, 24–29.
20. Akbudak, N., Tezcan, H., Akbudak, B., & Seniz, V. (2006). *Scientia Horticulturae, 109*, 107–112.
21. Mhamdi, A., Hager, J., Chaouch, S., Queval, G., Han, Y., Taconnat, L., et al. (2010). *Plant Physiology, 153*, 1144–1160.
22. Streatfield, S. J., Weber, A., Kinsman, E. A., Hausler, R. E., Li, J., Post-Beittenmiller, D., et al. (1999). *The Plant Cell, 11*, 1609–1622.
23. Herrmann, K. M., & Weaver, L. M. (1999). *Annu Rev of Plant Physiol and Plant Mol Biol, 50*, 473–503.