THE IDENTIFICATION OF HIGH ABUNDANT PROTEINS IN THE LEAVES OF GYNURA PROCUMBENS

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ABSTRACT
The leaves of Gynura procumbens have been traditionally used for their medicinal properties. The study of the content of the small molecule compounds of the plants has proven that the leaves contained anti-hyperglycaemic, anti-hyperlipidaemic and anti-inflammatory activities. By using proteomic approach, we have identified few abundant proteins from the leaves of G. procumbens. Among these proteins, peroxidase was found to be the most abundant extracted protein. In view of the high commercial demand for the natural source of peroxidase, we foreseen the added value of G. procumbens in cosmetic and skin care industry besides its existing medicinal values. Other extracted proteins included two pathogenesis-related proteins.

Introduction
Plants are natural resources that serve as valuable commodity and many of them are traditionally used for treatment of diseases. However, the usage for treatment has been scientifically proven for only few of these plants. As scientific technologies advance, many of the plants with medicinal properties are analyzed and are found to contain bioactive components for healing of various types of disease. For example: pacilitaxel, which was detected from the bark of the Pacific Yew, Taxus brevifolia, is a bioactive component of an anticancer medicine (4); zingerone, which was isolated from Zingiber officinale, was used in an antidiarrheal drug (5); and more examples of this kind are reported (4, 5, 9, 20). In this study, we investigated Gynura procumbens, which was traditionally used to decrease the cholesterol level, to reduce high blood pressure, to control diabetics, and for the treatment of cancer. Extract of the leaves of G. procumbens was shown to exhibit anti-hyperglycaemic and anti-hyperlipidaemic activities (23) and anti-inflammatory activities (12).

Proteomics is a branch of the biotechnology that studies protein components in organisms. To date, there is no report on the protein analysis of the leaves of G. procumbens. In this study, proteins from the leaves of G. Procumbens were extracted, a challenging task, considering that the leaves of the plant are succulent. Furthermore, besides the low protein content of the leaves, the content of secondary metabolites, phenolics, polysaccharides were high and these components interfere with protein separation and analysis, a common problem encountered in plant proteomics studies (11, 21). These interference caused streaking, smearing and thus reduced the numbers of proteins that can be detected after separation by gel electrophoresis (21). In order to obtain a high quality of protein profiling from the leaves of G. procumbens, trichloroacetic acid (TCA)-acetone wash was applied prior to extracting the leaves’ proteins with extraction buffer (21).

Materials and Methods
Plant materials
Leaves of G. procumbens were collected from Indonesia. All the leaves were kept fresh and transported to our laboratory within 3 days. Leaves were washed twice with tap water, rinsed with distilled water and ground to a fine powder form in liquid nitrogen using mortar and pestle. Ground leave powder was stored at -80°C until used.

Protein extraction
Ten grams of ground leaves sample was transferred to a 50 mL centrifuge tube. Twenty millilitres of cold acetone and 2 mL of TCA were added into the centrifuge tube and vortexed thoroughly. The mixture was kept at -20°C for 90 min and then centrifuged at 16 000 g at 4°C for 20 min. The supernatant was discarded and the pellet was washed twice with 20 mL of cold acetone. The pellet was air-dried at room temperature. Three millilitres of TLB buffer (8M urea, 2M thiourea, 4% (w/v) CHAPS, 0.4% (v/v) carrier ampholytes, 50 mM dithiothreitol (DTT)) was added to the dried-pellet, vortexed and incubated for 5 min. The TLB extract was centrifuged at 16 000 g, 18°C for 20 min. The supernatant was mixed with 4 volumes of 10% (v/v) TCA/cold acetone containing 20 mM DTT and incubated for 90 min at -20°C. The mixture was 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 centrifuge at 16 000 g, 4°C for 15 min. The pellet was air-dried, reconstituted in TLB buffer and further processed.
buffer and vortexed until the pellet was fully dissolved. The extracted protein was subjected to protein assay and separation.

**Protein assay**

Protein concentration was determined using Bio-Rad RC/DC Protein Assay Kit (Bio-Rad Laboratories) and the analysis was carried out according to the guideline given by the manufacturer.

**SDS-PAGE**

The proteins separation was carried on SDS-PAGE utilizing a 10% gel. Protein extracted from the leaves was added with sample buffer (0.5 M Tris-HCl (pH6.8), 10% (v/v) glycerol, 0.02% (w/v) SDS and 0.1% (w/v) Bromophenol Blue). Electrophoresis process was run at a constant voltage of 200V. The gel was stained with Coomassie Blue for 4 h, destained and the gel image was captured using VersaDoc Imaging system (Bio-Rad Laboratories) and protein bands were analyzed using Quantity One software (Bio-Rad Laboratories).

**In-gel digestion**

In-gel digestion was performed following the method by Gam and Aishah (7). Electrophoretically separated protein bands were excised from SDS-PAGE. The gel pieces were washed for 10 min in 100 mM ammonium bicarbonate and then 5 min with acetonitrile (ACN). This step was repeated twice. Gel pieces were then dried in a speed-centrifuge under vacuum. The dried gel pieces were added with 10 mM DTT in 100 mM ammonium bicarbonate (sufficient to cover the gels) and incubated for 1 hour at 56°C. Excessive solution was removed and then gel pieces were covered with 55 mM iodoacetic acid in 100 mM ammonium bicarbonate in dark for 45 min at room temperature. The washing and dehydration steps were repeated as discussed above. The gel pieces were re-swollen in the presence of 15 ng/µL of trypsin in digestion buffer (50 mM ammonium bicarbonate, 5 mM CaCl₂) and incubated at 37°C overnight. The supernatant of the trypic digest was collected and the remaining peptides were extracted 3 times in 5% (v/v) formic acid in 30:70 of ddH₂O:ACN for 20 min each. Supernatants were pooled and blow dried using nitrogen.

**Mass spectrometry analysis**

Tryptic-digested samples were mixed with 30 µL of 0.1% (v/v) formic acid in 85:15 ddH₂O:ACN and analyzed using Agilent 1100 series LCMS/MS. The samples were injected into enrichment column (Zorbax SB C18, 35 x 0.5 mm, particle size 5 µm) with 0.1% (v/v) formic acid in 97:3 of ddH₂O:ACN for 6 min at flow rate of 0.1 mL/min. The peptides were then separated using a reverse phase column (Zorbax 300SB C18, 150 x 0.3 mm, particle size 5µm) with 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 ddH₂O and mobile phase B was 0.1% (v/v) formic acid in ACN. Two scans analysis was used: first scan was a full scan MS and the second scan was data dependent MS/MS scan which isolated two most intense ions from the first 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, minimum threshold of 5000 counts, and isolation width of 2 m/z.

**Mascot protein identification**

The MS/MS data was used to identify proteins identity through Mascot Protein Search Database (MSDB) search engine (www.matrixscience.com) under the *viridiplantae* (green plants) taxonomy. Trypsin was selected as 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. The proteins’ functions and their characteristics were obtained from SwissProt (www.expasy.org) and NCBI (www.ncbi.nlm.nih.gov).

**Results and Discussion**

The images of SDS-PAGE protein separation for proteins extracted from the leaves of *G. Procumbens* (Fig. 1) show the use (a) of direct TLB buffer extraction and (b) TCA-acetone wash prior to TLB buffer extraction. Each of the lanes was loaded with similar amount of proteins. It is clear that when TCA-acetone wash was applied prior to TLB buffer extraction, the protein bands appeared distinct and better shaped compared to those without the TCA-acetone wash.

![Fig. 1](image_url)

(a) direct TLB extraction (50 µg/lane); (b) TCA-acetone wash prior extracting with TLB buffer (50 µg/lane). The numbers on the right show the protein bands excised for in-gel digestion. M: molecular-mass markers (sized indicated in kDa)
The mass spectrometry analysis of peroxidase, a peptide with molecular mass of 873.8 m/z was detected as the most abundance ion in the scan. This peptide ion was subjected to MS/MS scan, where through collision induced dissociated (CID), the peptide fragment forms a profile of fragment ions that can be used to predict the amino acid sequence of the peptide and thereby indicate the identity of the protein. All the MS/MS data obtained in the mass spectrometry analysis were used for Mascot protein database search according to Mowse scoring algorithm (Matrix Science, London, UK) under taxonomy of Viridiplantae (Green Plants). A total of 247880 sequences were used to identify the matching amino acid sequence in the protein database for measurement of protein identity.

The Mascot search result for peroxidase where the full amino acid sequence of the protein is obtained is shown at Fig. 3. Using this approach, we have identified all the proteins reported in this study. The list of proteins identified in this study is shown at Table 1. Mascot score obtained for each protein was greater than 42, where score >42 indicates identity or extensive homology at a significance level (p>0.05). Sequence coverage refers to the percentage of amino acids sequence of the protein that was detected when analyzed by LCMS/MS for the identification of the protein. GRAVY value refers to the property of the proteins, where greater values indicate more hydrophobic protein and more negative values indicate hydrophilic property of the protein. The band number refers to the numbered protein bands as shown in Fig. 1.

The leaves of G. procumbens have been traditionally used for its medicinal properties, although there were studies supporting the medicinal properties of G. procumbens, these studies focused mainly on the small molecule compounds extracted from the plant. To date, there is no report on the macromolecule content of the plant, which may be valuable for the medicinal or industrial use of G. Procumbens.

This proteomic study on the leaves of G. procumbens included extraction and separation of the proteins from the mixture and analysis for their identification. Because high levels of secondary metabolites, phenolics and polysaccharide are present in the protein extracts from the plants, separation and analysis of the proteins is troubled as these compounds interfere with the gel electrophoresis separation and also may suppress the ion signal of mass spectrometry. A few extraction methods had been suggested to be useful in order that this problem is overcome. These include phenol extraction (11), TCA-acetone wash prior to extraction (21), hot SDS extraction (24), acetone wash prior to extraction (10).
separation and cause the smearing of the gel image. Thus, the protein extraction is a crucial step in plant protein analysis. By using TLB buffer for protein extraction, two types of proteins were extracted i.e. proteins with hydrophilic and hydrophobic properties. The GRAVY values obtained for the proteins indicated that the proteins were consisted of both hydrophilic and hydrophobic properties although most of the identified proteins were hydrophilic proteins. Coelution of proteins that produce a similar band in SDS-PAGE is common and in this study we have identified the presence of more than one protein in a single band. However, this resolution limitation of SDS-PAGE was compensated with the high specificity of mass spectrometry analysis. This allows that multiple proteins which produce a single band to be identified. The discrepancy in molecular weight of proteins based on their migration in the SDS-PAGE gel and their reported theoretical mass can be explained by post translational modification of the proteins i.e. glycosylation, phosphorylation and etc.

Peroxidase was identified as the abundant protein in the leaves of *G. procumbens*, where it was detected in multiple bands in SDS-PAGE. Peroxidase is involved in the removal of hydrogen peroxide. In plants, the peroxidase family carries multiple functions, such as oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls, auxin catabolism, defensive responses to wounding and defense against pathogen or insect attack (22). In term of industrial use, plant peroxidase is a common skin care ingredient that is used to remove hydrogen peroxide from cells. Hydrogen peroxide is able to penetrate cell membranes and react with ferrous ions to form highly dangerous hydroxyl radicals that cause toxic effects to the cell (2). Furthermore, hydrogen peroxide is a weak oxidizing agent that can inhibit glycolysis. The use of natural biomaterials in cosmetic products is preferable to the synthetic molecules as the latter have shown to cause various side effects in cosmetic products (2). The unusually high abundance of peroxidase content in the leaves of *G. procumbens* can be further explored as a source of the natural enzyme for the cosmetic industry. Apart from this, plant peroxidase is also used commercially as a biobleach in the paper recycling process (14) and decolorization of textile dyes (19).

Another valuable protein identified in the leaves of *G. procumbens* was osmotin like protein i and thaumatin like protein i (TL). They both belong to pathogenesis-related protein 5 (PR-5) with known antifungal activity (8, 16, 18, 25). Osmotin like protein I was reported to possess dual functions i.e. in osmotic stress and plant pathogen defence (25). TL protein was isolated from kiwi, mugwort and apple and was reported to cause allergic reactions in human besides its

**TABLE 1**

<table>
<thead>
<tr>
<th>Group based on biology process</th>
<th>Band</th>
<th>Mascot Protein Hits (SwissProt accession)</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Theoretical Molecular mass (kDa)</th>
<th>pI</th>
<th>Sequence Coverage (%)</th>
<th>GRAVY</th>
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<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>9</td>
<td>Q9M502 HELAN (P45739)</td>
<td>Catalase 4 (EC 1.11.1.6)</td>
<td>44</td>
<td>57.2</td>
<td>6.62</td>
<td>3</td>
<td>-0.590</td>
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<tr>
<td></td>
<td>13</td>
<td>Q5Q0L6 LINUS (Q96520)</td>
<td>Peroxidase 12</td>
<td>54</td>
<td>14.2</td>
<td>7.07</td>
<td>8</td>
<td>-0.019</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Q87B2 LINUS (Q96520)</td>
<td>Peroxidase 12</td>
<td>98</td>
<td>38.6</td>
<td>8.07</td>
<td>8</td>
<td>-0.019</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Q5J5A4 ORYSA (Q96520)</td>
<td>Peroxidase 12</td>
<td>42</td>
<td>40.1</td>
<td>7.56</td>
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<td></td>
<td>15</td>
<td>AAM61588 (Q9LVL2)</td>
<td>Peroxidase 67</td>
<td>68</td>
<td>35.1</td>
<td>9.83</td>
<td>6</td>
<td>-0.134</td>
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<tr>
<td>Photosynthesis</td>
<td>1</td>
<td>CDNTCC (P12469)</td>
<td>Chlorophyll a/b-binding protein C (cab-C)</td>
<td>46</td>
<td>28.5</td>
<td>5.69</td>
<td>2</td>
<td>-0.015</td>
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<tr>
<td></td>
<td>21</td>
<td>S02065 (P10649)</td>
<td>Chlorophyll a/b-binding protein type 1</td>
<td>52</td>
<td>28.5</td>
<td>5.31</td>
<td>8</td>
<td>-0.003</td>
</tr>
<tr>
<td>Glycolate pathway and photorepiration</td>
<td>16</td>
<td>Q6YT3 ORYSA (Q9LRR9)</td>
<td>Probable peroxisomal (S)-2-hydroxy-acid oxidase 2</td>
<td>63</td>
<td>40.3</td>
<td>8.50</td>
<td>7</td>
<td>-0.071</td>
</tr>
<tr>
<td>Calvin cycle, carbon dioxide fixation, photosynthesis</td>
<td>10,11,12</td>
<td>Q9TJY5 9GENT (P48694)</td>
<td>Ribulose 1,5-bisphosphate carboxylase</td>
<td>60</td>
<td>52.1</td>
<td>6.22</td>
<td>7</td>
<td>-0.295</td>
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<tr>
<td>Plasma membrane ATP synthesis coupled proton transport</td>
<td>10</td>
<td>Q3ZU70 9ROS1 (Q09G39)</td>
<td>ATP synthase beta subunit</td>
<td>50</td>
<td>50.8</td>
<td>5.17</td>
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<td>-0.030</td>
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<td>Translation</td>
<td>20</td>
<td>Q8S9G2 ORYSA (P41129)</td>
<td>60S ribosomal protein L13-2</td>
<td>58</td>
<td>24.1</td>
<td>10.91</td>
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<tr>
<td>Responds to salt stress</td>
<td>23</td>
<td>Q2HPG3 GOSH1 (P50706)</td>
<td>Osmotin-like protein I</td>
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<td>7.85</td>
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<tr>
<td>Plant defense</td>
<td>24</td>
<td>Q8LSM9 HELAN (P81370)</td>
<td>Pathogenesis-related protein 5-1/Thaumatin-like protein (Allergen Act d 2)</td>
<td>43</td>
<td>24.9</td>
<td>6.51</td>
<td>17</td>
<td>-0.186</td>
</tr>
</tbody>
</table>

Pl - Isoelectric point; GRAVY - Grand average of hydrophobicity
antifungal activity (1, 8, 15). The presence of TL protein in the leaves G. procumbens indicates that it may not be suitable as a food in a raw state because there is a possibility of an allergic reaction. However, raw eating of the leaves of G. procumbens is a common practice for the people in the Asian region.

Other abundant proteins isolated from the leaves of G. procumbens belong to the light-harvesting chlorophyll a/b-binding protein family. Chlorophyll a/b-binding protein type I precursor and chlorophyll a/b-binding protein type I are involved in light receptor captures and deliver light energy to photosystems (6). Ribulose 1,5-bisphosphate carboxylase catalyzes two reactions, carboxylation of D-ribulose 1,5 bisphosphate and oxidative fragmentation of the pentose substrate in the photorespiration process (17). Probable peroxisomal (S)-2-hydroxy-acid oxidase 2 plays an important role in photorespiration; it catalyzes the oxidation of glycolate to glyoxylate and oxidation of glyoxylate to oxalate (13). ATP synthase beta is used to provide energy to the plant and 60S ribosomal protein L13-2 is involved in translation of RNA to protein.

**Conclusions**

In this study, we have identified an abundant protein, peroxidase in the leaves of G. Procumbens. The high commercial value of peroxidase renders G. procumbens to be a useful natural resource for industrial use, besides its established value in the medicinal property.

**Acknowledgments**

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**REFERENCES**