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Research Article

Protein Profiling of *Orthosiphon stamineus* Embryogenic Callus using Liquid Chromatography Coupled with Tandem-Mass Spectrometry (LC-MS/MS)

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ABSTRACT

Orthosiphon stamineus is one of the most prominent medicinal herbs widely grown in Southeast Asia. Propagation of *O. stamineus* using tissue culture technique helps to rapidly produce samples for research purposes, increase the production of secondary metabolites and is considered as one of the strategies in plant genetic improvement. Despite vast information on *O. stamineus* pharmacological properties, the protein profile of this species is currently underexplored. In the present study, proteins expressed in embryogenic callus developed from *O. stamineus* leaves in Murashige and Skoog medium supplemented with 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were identified via Liquid chromatography- tandem mass spectrometry (LC-MS/MS). The presence of embryogenic callus was confirmed with microscope observation and Evan Blue assay. Using this proteomic approach, we identified peptides that represent 22 proteins localized to different cell compartments and involved in various biological and metabolic functions in plants. This finding is useful to improve our understanding of protein functions, thus their future applications could be further explored.

Keywords: Embryogenic, Callus, Herb, Orthosiphon stamineus, Proteins

Introduction

Orthosiphon stamineus or commonly known as “Misai Kucing” in Malaysia is a medicinal herb rich in bioactive compounds that possess valuable pharmacological properties. *O. stamineus* leaf extracts can be used to treat diseases such as hypertension, gout, fever, diuretic problems and many more [1]. To date, extensive studies have been conducted and many literatures are available on anti-oxidant, anti-bacterial, anti-inflammatory and anti-angiogenic properties of *Orthosiphon* [2, 3, 4]. However, the detailed study on the protein profile that drives these aforementioned properties is still lacking. To date, protein profiling of purple variety *O. stamineus* leaves have been conducted using a proteomic approach [5, 6].

O. stamineus is generally propagated using stem cutting. However, *in vitro* cell culture technique also available to be used for mass propagation of *O. stamineus* clones derived from any parts of the plant. *In vitro* studies of *Orthosiphon* have been well progressing for several years [7, 8, 9]. Cell culture system such as direct and indirect somatic embryogenesis in response to wounding or hormones [10] is the most common plant regeneration method for genetic improvement and large-scale synthetic seed production [11]. The unorganized cell masses like callus responding to their environs including various biotic and abiotic stresses [12]. Viability checks on the embryogenesis of the callus is very important to determine whether it holds a complete genetic data. Staining method using Evans blue dyes is one of the techniques available to distinguish between cells that

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possessed embryogenic potential or not by determining the percentage of embryogenic callus.

Studies on protein functions in *O. stamineus* are important to understand how plants develop and produce valuable bioactive compounds. Using a proteomic approach, a list of proteins available in each cell type, tissue or organelles could be determined [13]. These proteins are directly responsible for the majority of biological processes in living cells [14]. On top of that, changes in protein expression facilitate understanding of the cellular metabolic event in plants [15]. Biochemical and pharmacological properties of a protein are not only determined by its amino acids sequence but also largely influenced by a pallet of modification that proteins undergo co or post-translationally [16]. In this study, we aimed to identify the protein profiles from white variety *O. stamineus* callus. Information on the total protein profiling of embryogenic *O. stamineus* callus can be used as a platform to provide useful insights on the roles of proteins expressed during callus development which will be useful to understand their functions and applications.

Material and Methods

Plant materials

O. stamineus plants were purchased from a local nursery in Skudai, Johor, Malaysia. Plants were grown in a glasshouse at Universiti Teknologi Malaysia with daily temperature of 26ºC to 33ºC. The relative humidity is above 80%. Plants were watered twice daily early in the morning and late in the evening.

Surface sterilisation and callus development

Matured leaf explants from *O. stamineus* plants were selected and smeared in commercial liquid soap. The leaf explants were then washed under running tap water for 30 minutes and placed in sterilized jars. After that, 70 % (v/v) ethanol were applied for one minute. 20 % of bleach solution was also applied for 30 minutes with an addition of a few drops of Tween 20. This is followed by three times rinse with sterile distilled water. Using a sterilized cutter blade, plant leaves were cut to form 1 cm × 1 cm square shape each. Five pieces of the cut leaves were cultured into each Petri dish containing agar MS medium plus NAA 1 mg/Land 2,4-D 1 mg/L for callus induction. Callus of *O. stamineus* was established until maturation by day-24.

Determination of embryogenic callus

Callus viability test was conducted as described in Fernandez and Menendez, 2006 [17]. Approximately 100 µl of 1% (w/v) of Evans blue solution was applied directly onto one mg sample of six-week-old callus culture. The callus was incubated at room temperature for 10 minutes. The callus was then rinsed with distilled water to remove the remaining dye. The percentage of viable callus cells was observed under the hemocytometer and viewed under the Nikon Eclipse E200 microscope.

Total protein extraction of Orthosiphon stamineus cell suspensions

Calli (100 mg) was powdered in a pre-chilled mortar and pestle. Powdered calli was homogenized with 0.23 mL of extraction buffer containing 50 mM Tris-HCl pH8, 5% (v/v) glycerol, 0.1 mM EGTA, 0.2 KCl, 1.5% (v/v) polyvinylpyrrolidone, 1% β-mercaptoethanol. The proteins precipitated by addition of 1/10 vol. 100% trichloroacetic acid (TCA) 4°C for overnight. Extracted protein was collected by centrifugation at 10000 rpm for 20 min, 4°C and resuspended to sample buffer consisting 60mM Tris-HCl (pH6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 14.4mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue and stored in -80° C freezer prior to usage.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis SDS-PAGE

SDS-PAGE was conducted based on Laemmli as described in Bollag *et al*., 1996 [18]. The stacking gel solution (125 mM Trishydrochloride (Tris-HCl) pH 6.8, 0.1 % (w/v) SDS, 5 % (w/v) acrylamide (acrylamide: bis-acrylamide ratio of 37.5: 1), 0.07 % (w/v) ammonium persulphate and 0.03 % (v/v) N,N,N',N'-tetrame-thylethane-1,2-diamine (TEMED) was overlaid on the separating gel solution (373 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 12 % (w/v) acrylamide, 0.04 % (w/v) ammonium persulphate and 0.05 % (v/v) TEMED). Samples were mixed with 5 × sample buffer (60 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 25 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue, 14.4mM 2-mercaptoethanol). Samples were then heated for 5 min at 90ºC, prior to centrifugation at 10,000 × g for 5 min at 25ºC. The same amount of protein samples was loaded on the gel and separated at 150 V until the bromophenol blue reached the bottom of the gel. For staining, the gel was incubated in

Coomassie blue R-250 (0.1 % (w/v), 10 % (v/v) acetic acid, 45 % (v/v) methanol) for 16 h and transferred to destaining solution (10 % (v/v) methanol, 10 % (v/v) acetic acid). Then, the gel was de-stained until the protein bands are visible. Protein samples were subjected to trypsin digestion prior to mass spectrometry analysis.

Mass spectrometry analysis

Peptides were analyzed by electrospray ionization mass spectrometry using the Agilent 1260 Infinity HPLC system (Agilent) coupled to an Agilent 6540 mass spectrometer (Agilent) using the method provided. Tryptic peptides were loaded onto a C18 column 300 SB, 5 µm (Agilent) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science, London, UK) at http://www.matrix-science.com with MSPnr100 database against *Arabidopsis thaliana* genome sequence.

Results and Discussions

Embryogenic callus production from Orthosiphon stamineus

From our results, 91.3% of the callus exhibited a light blue to brownish colour after being exposed to Evan Blue solution, while only 8.69% of the total callus exhibited a dark blue colour. This indicates that the callus produced are composed of embryogenic cells. This is consistent with previous findings from Rashid *et al*., 2012 [19]. *In vitro* propagation study conducted using *O. aristatus* explants showed that optimum concentration of plant medium is required for embryogenic callus induction [20]. Due to totipotency, embryogenic cells will normally retain all genetic information, able to form somatic embryo and produce whole new plant faster with organized growth [21]. The callus structure was examined under a microscope. The embryogenic callus that formed organized cells are depicted in Figure 1A, while unorganized cells of non-embryogenic callus are shown in Figure 1B. Figure 1C serves as control.

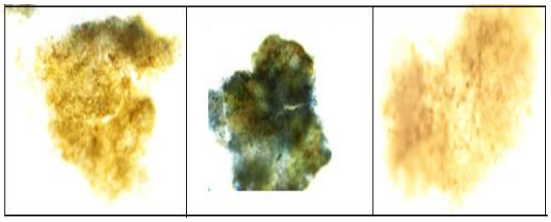

Figure 1. Callus stained with Evans-Blue solutions. (A) Light blue color or colorless on embryogenic callus at a 10 × magnification. (B) Non-embryogenic cells react strongly to Evans-Blue dyes at a 10 × magnification (C) Callus immersion in 70 (v/v) % alcohols (control).

Protein Identification from Orthosiphon stamineus Embryogenic Callus

Proteins were extracted from 6 weeks-old embryogenic callus cultured on MS media supplemented with 1.0 mg/L 2, 4-D and 1.0 mg/L NAA. Prominent protein bands separated using one dimensional (1D) gel electrophoresis were excised and subjected to trypsin digestion. The analysis using liquid chromatography coupled with tandem-MS (LC-MS/MS) identified peptides that represent a total of 22 proteins, approximately in the range of 9 kDa to 56 kDa (Table 1). These proteins were categorized according to nine main biological functions based on Gene Ontology, a database for the description of gene products

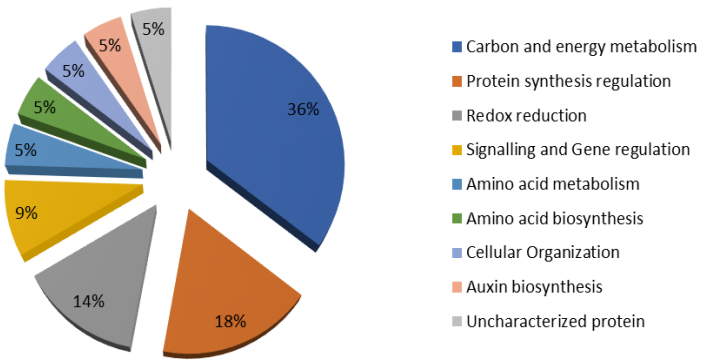

Figure 2. Categories of proteins based on GO annotation Biological Process

(http://www.uniprot.org/help/gene_ontology) (Figure 2).

The biggest portion of the identified proteins involved in carbon and energy metabolism (36%). Based on the database, these proteins were localized either to the cell cytoplasm or plasma membrane (Table 1). The energy storage that functions together with other machineries are important to regulate embryo development in somatic embryogenesis [22]. In callus culture, carbohydrates supplied in the medium were metabolized and used not only as the main source of carbon and energy but also to maintain the osmotic potential of the cells [23]. This indicates the significance roles of these proteins for cell growth and survival. Based on the Gene Ontology term associations of biological processes, proteins in this group were also associated with defense response to bacterium, oxidation-reduction and oxidative stress.

Four proteins, including elongation factor and

Table 1. Identified Proteins and Gene Ontology for the proteins from *Orthosiphon stamineus* callus. Accession number was obtained from NCBI based on search against *Arabidopsis* database. MW = Molecular Weight; pI = Isoelectric Point; GO = Gene Ontology; B= Biological Processes; C = Cellular Component; M = Molecular Function.

Accession Number	Protein pI	MW (Da)	Identified Protein Name	GO
Carbon and energy metabolism (36%)				
NP_001077530	6.67	55810.3	Glyceraldehyde 3-phosphate dehydrogenase	M: glyceraldehyde-3-phosphate dehydrogenase (NADP+) (non-phosphorylating) activity C: Cytoplasm
NP_172801	6.67	36890	Glyceraldehyde-3-phosphate dehydrogenase GAPC2, cytosolic	B: Glycolysis M: glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity C: Cytoplasm
NP_187062	6.62	36891	Glyceraldehyde-3-phosphate dehydrogenase GAPC1, cytosolic	B: Glycolysis M: NAD and NADP binding C: Cytoplasm
NP_199147	7.00	35639	cytosolic malate dehydrogenase	B: malate metabolic process M: malate dehydrogenase activity C: Cytoplasm
NP_190861	6.05	38516	Fructose-bisphosphate aldolase	B: glycolytic process/ Glucose catabolic process M: fructose-bisphosphate aldolase activity C: Cytoplasm
NP_001078156	9.49	33113	Malate dehydrogenase	B: malate metabolic process M: L-malate dehydrogenase activity C: Cytoplasm
NP_178073	4.82	23260	Phosphoglycerate kinase	B: glycolytic process M: ATP binding C: Plasma Membrane
NP_001154216	4.62	35816	14-3-3-like protein GF14 chi	B: regulation of metabolic process C: Plasma Membrane
Protein synthesis regulation (18%)				

Continue

Accession Number	Protein pI	MW (Da)	Identified Protein Name	GO
NP_193102	5.79	35393	Pinoresinol reductase 2	B: lignan biosynthetic process M: pinoresinol reductase activity (oxidoreductase)
NP_001077627	4.62	28758	Elongation factor 1B beta	B: Protein Biosynthesis M: Elongation factor C: Cell Membrane
NP_001031846	10.00	27690	40S ribosomal protein S4	B: Translation M: rRNA binding C: Ribosome
NP_001190274	10.85	22083	40S ribosomal protein S6-2	B: Translation M: structural constituent of ribosome C: Ribosome
Redox reduction (14%)				
NP_177837	5.83	41152	Alcohol dehydrogenase class-P	B: response to osmatic stress M: zinc ion binding C: Cytoplasm
NP_174553	5.37	33593	At1g32760	B: cell redox homeostasis M: electron carrier activity
NP_181965	6.06	28003	At2g44370	B: oxidation-reduction process M: protein-disulfide reductase activity C: Nucleus
Signalling and Gene regulation (9%)				
NP_190687	10.54	27980	60S ribosomal protein L8-2	B: Translation M: rRNA binding C: Ribosome
NP_566174	4.74	29806	14-3-3-like protein GF14 nu	M: protein domain specific binding C: Plasma Membrane
Amino acid metabolism (5%)				
BAH57232	8.08	32089	Adenosylhomocysteinase 1	B: one-carbon metabolic process M: adenosylhomocysteinase activity C: Plasma Membrane
Amino acid biosynthesis (5%)				
Q9LJA0	7.714	19625	Putative inactive cysteine synthase 2	B: lignin biosynthetic process M: pinoresinol reductase activity
Cellular Organization (5%)				
NP_565347	4.66	9349	14-3-3 family protein	B: protein coding M: Amino acid binding C: Plasma membrane
Auxin biosynthesis (5%)				

Continue

Accession Number	Protein pI	MW (Da)	Identified Protein Name	GO
NP_565176	4.71	29144	14-3-3-like protein GF14 omega	B: brassinosteroid mediated signaling pathway C: Golgi apparatus *Continue*
Uncharacterized protein (5%)				
NP_191606	8.79	62744	Putative uncharacterized protein T8B10	B: Biological process C: Plasma membrane

40S ribosomal proteins, represent 18% of the total proteins identified in embryogenic *O. stamineus* callus are responsible on regulation of protein synthesis, the second largest group based on gene ontology categories. Besides that, 14% of the identified proteins were categorized under redox reduction, an important event in plant cells especially in homeostasis and osmotic regulation. Pullman *et al.*, (2015) demonstrated that the alteration of medium redox potential can increase the initiation of embryogenic tissue and promote somatic embryo germination [24].

A smaller portion of proteins, 60S ribosomal protein L8-2 and 14-3-3-like protein GF14 nu occupying 9 % of the total proteins were grouped in signalling and gene regulation function. Ribosomal proteins play important roles in protein synthesis and involve in controlling cell growth and development [25].

Other group of proteins; amino acid metabolism, amino acid biosynthesis, cellular optimization, auxin biosynthesis and uncharacterized proteins represent 5 % of each group, respectively. A putative inactive cysteine synthase 2 categorized under amino acid biosynthesis involved in lignin biosynthesis were identified in *O. stamineus* embryogenic callus. Lignin is a complex phenolic polymer and the various lignifications studies have been investigated in plant cell cultures [26]. 14-3-3-like protein GF14 omega under the category of auxin biosynthesis associated in brassinosteroid mediated signalling pathway. Brassinosteroid are naturally produced plant hormones that regulates many developmental processes from seed germination to flowering and senescence [27], also important during somatic embryogenesis [28]. The dynamics of protein synthesis in callus were previously investigated in perennial soybean, Glycine wightii (Wight & Arn.) Verdc, a family of Leguminosae [29]. Embryogenic callus culture showed various metabolic as well as physiological properties which could be narrated with differential expression of proteins compared to non-embryogenic callus [22, 30].

Conclusion

The objective of this research has been achieved by the identification of peptides that represent proteins from *O. stamineus* embryogenic callus using liquid chromatography coupled with tandem-mass spectrometry. The identified proteins in *O. stamineus* callus suggest that embryogenic status is related to the ability of callus to grow by maintaining basic mechanisms controlling the process in cells, besides responding to general stress from its surroundings. Several major protein networks are crucial not only for intact plants, but also in developing somatic tissues. The proteins described can be good candidates for embryogenic cells marker and may be used for the improvement of somatic embryo production and enlighten the possibility of improving somatic embryogenesis in this species by manipulating *in vitro* growth.

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