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Proteomic Profiling of *Stemona* Alkaloids Production Response to Chitosan Elicitor

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Abstract: The study purposed to investigate the protein expression of *Stemona* alkaloids biosynthesis response to chitosan elicitor by 2D gel electrophoresis. The total proteins extraction of *Stemona* roots were performed for comparison with the control and chitosan treatments. It was found that 15 out of 150 protein spots exhibited different expression between control and chitosan culture treatment. The identified 15 protein spots were subjected to amino acid sequencing and two proteins appeared interesting for examining *Stemona* alkaloids biosynthesis. After treated with chitosan, glutathione S-transferase became down-regulated while heat shock protein up-regulated in relation to the control treatment. These proteins may play roles in alkaloids biosynthesis via plant defense metabolism from the presumptions that chitosan might weaken the detoxifying function of glutathione S-transferase, then, heat shock protein is probably produced to signal for tissue protection mechanism. Thus, *Stemona* alkaloids may be responsive to this stress.

Key words: *Stemona* sp., protein expression, chitosan, glutathione S-transferase, heat shock protein

INTRODUCTION

Proteomics has already been used for many different applications in plant sciences. It is a tool for the identification of proteins involved in cellular processes, including the enzymes, transport and regulatory proteins. The applications are related to the analysis of various functional proteome such as post-translational modifications, protein-protein interactions, activities and structures. Proteomic researches have been endeavored at the levels of cell or tissue of plants including chloroplast, mitochondria, nucleus, plasma membrane and cell wall (Park, 2004). Many natural products contain antibiotic, anticancer and antifungal properties. Proteomic approach has been a powerful tool to enhance discovery of their biosynthetic pathways. Plant cell cultures under controlled conditions are a good system for comparative proteome and metabolites studies, including the study of proteins of biosynthetic pathways leading to secondary metabolites. Proteomic analysis of Korean ginseng (*Panax ginseng* C.A. Meyer) by Nam *et al.* (2005) regarding secondary metabolism reveal that ginsenosides, the main components of ginseng, have pharmacological effects and the biosynthetic pathway of ginsenosides was studied by proteomics. The proteins identification found post-translational modifications proteins and the enzymes involved in ginsenosides biosynthesis.

Stemona spp. are a source valuable alkaloids production. The *Stemona* alkaloids have been proven to have insect toxicity properties (Tang *et al*., 2008) and pharmaceutical activities (Brem *et al*., 2004; Lin *et al*., 2008). Sastraruji *et al*. (2005) reported the isolation of 1', 2'-didehydrostemofoline and Baird *et al*. (2009) found that this alkaloid has the inhibitory activity against acetylcholinesterase. These findings indicated their being a potential therapeutic agent in the treatment of the initial symptoms of Alzheimer's disease. Moreover, stemofoline, other *Stemona* alkaloids that was obtained from *Stemona* sp., has been shown to have the inhibitory activity against acetylcholinesterase as well (Sastraruji *et al*., 2010). In addition, stemofoline could also increase the anticancer drugs sensitivity as previously described by Chanmahasathien *et al*. (2010). The knowledge on alkaloids biosynthetic pathway or the regulatory mechanisms controlling alkaloid biosynthesis is still appears inadequate. However, the systems biology approaches associated with genomics, transcriptomics or proteomics ultimately lead to discovery of new pathways, the modeling of metabolic networks and predictive metabolic engineering (Goossens and Rischer, 2007). In order to find novel proteins involved in the alkaloid biosynthesis, a proteomic approach was undertaken. The changes in protein expression could be correlated with the accumulation of secondary metabolites.

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MATERIALS AND METHODS

Plant material: Shoot tips and auxiliary buds were surface sterilized and cultured on MS (Murashige and Skoog, 1962) agar medium supplemented with 3 mg L^{-1} benzyladenine for multiple shoot induction. For root induction, half-MS medium supplemented with 2 mg L^{-1} indolebutyric acid was the appropriate medium (Chaichana *et al*., 2011).The cultures were transferred in a growth room at 25±2°C under a daily photo period of 16 h. The *Stemona* alkaloids production by feeding elicitors was earlier examined. It was found that chitosan elicitor resulted in the highest production of *Stemona* alkaloids, 1', 2'-didehydrostemofoline and stemofoline, over than any elicitors (Chaichana *et al*., 2012; Chaichana and Dheeranupattana, 2012). Therefore, chitosan culture treatment was selected for proteomic analysis compared with control culture treatment in protein profiling expression.

Protein extraction of *Stemona* sp. root: The study on total protein expression of *Stemona* sp. was performed to establish the relationship between protein expression and *Stemona* alkaloids biosynthesis. Direct IEF buffer extraction method was considered the appropriate method. IEF buffer is composed of 8 M urea, 20 mM DTT and 4% CHAPS as previously described by Sheoran *et al.* (2009). The *Stemona* roots were ground and added with IEF buffer. Then, the solution was centrifuged at 10,000 g, 4°C for 40 min and then supernatant was collected. The solution was cleaned with 2D clean up kit to wash the interfering substances. Then, the total protein concentrations were measured by Bradford method and analyzed with 2D gel electrophoresis.

Protein expression by two-dimensional gel electrophoresis: The first-dimensional electrophoresis was carried out on an Isoelectric Focusing (IEF) system with immobilized pH gradient (IPG) strip. In the first step, 13 cm, pH 3-10 IPG strip was tested and the proteins were mostly expressed in pH range of 4-7. Therefore, the IPG strip 13 cm, pH 4-7 was subsequently used. The protein sample was mixed with IPG buffer and rehydration solution to be loaded to strip holder. Then, IPG strip was analyzed by IPG phore II machine. After process was finished the first dimension, the IPG strips were further subjected to a second-dimensional SDS gel electrophoresis. The protein samples were taken in triplicate. After that, the gels were strained by Coomassie Brilliant Blue G250. Finally, the different expression of protein spots between with and without elicitor-induction was analyzed by Image Master Plantinum TM v. 2005 software. The difference among proteins spots were further identified by LC MS/MS method.

Research location: This research was studied at Plant Tissue Culture Laboratory, Department of Biology and Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand for 1 year (September 2011-August 2012).

RESULTS AND DISCUSSION

The proteome patterns of control and chitosan culture treatment were shown in Fig. 1(a) and (b), respectively. All of the gels were matched with reference gel and the different spots were investigated. The difference protein spot was refined the selection from Max value with≥1.2-fold of up or down regulation. The report from selection established the 15 different protein spots. Three protein spots located up or down regulation between control and chitosan treatments. The expression of different protein spots were shown in Table 1. A total of 18 protein spots were chosen and analyzed for protein identification by LC MS/MS method. After LC MS/MS analysis, the amino acid sequences were analyzed into MASCOT database to identify protein matching. The proteins were searched from all entry organisms because the *Stemona* protein database is not available. There are many proteins which are matched from all of protein spots such as glutathione-S-transferase, heat shock protein, mitochondrial processing peptidase subunit, gluconate utilization system Gnt-I transcriptional repressor, glucan-binding proteins, hemocyamin, hypothetical protein, ras GTPase-activating-like protein, kduD2 gene product and replication protein C as shown in Table 1. The interested proteins that may involve in plant secondary metabolism are glutathione S-transferase and heat shock protein. Both proteins are known to relate to plant defense metabolism and thus, may be pertinent relate in *Stemona* alkaloid biosynthesis mechanism via chitosan stress response. There exist the experimental evidences that glutathione S-transferase and heat shock protein play role in plant secondary pathway.

Glutathione S-transferase (GSTs) is a enzyme that finds in many plant species. The functions of this enzyme include detoxifying oxidative-stress metabolites and response to biotic and abiotic stress involve in plant defense metabolism. The glutathione S-transferase gene expression of maize (*Zea mays* L.) due to hydrogen peroxide was investigated. Hydrogen peroxide (H_2O_2) can cause oxidative stress and damage to the cells. The experiments revealed that H_2O_2 induced glutathione S-transferase gene expression to higher levels

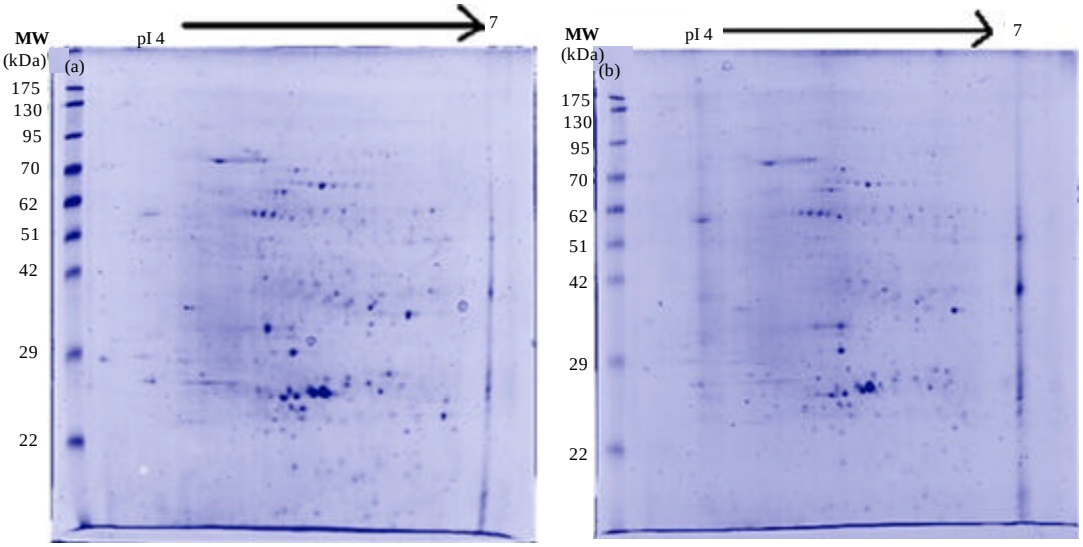

Fig. 1(a-b): 2D gel spot (a) Pattern of control and (b) Chitosan culture treatment

Table 1: The protein spots identification from 2D gel electrophoresis

Spot No.	Experiment group	Protein identification
1	Control	Hemocyanin
	Chitosan (down-regulation)	
2	Control	Glucan-binding proteins
3	Control	Hemocyanin
4	Control	Ras GTPase-activating-like protein
	Chitosan (up-regulation)	
5	Control	Hemocyanin
6	Control	Hypothetical protein BC1G_02232
7	Control	Heat shock protein
	Chitosan (up-regulation)	
8	Control	Hypothetical protein CHLNCDRAFT_134899
9	Control	Predicted protein (Phosphoglycerate mutase, EC 5.4.2.4.)
10	Control	Mitochondrial processing peptidase subunit
11	Control	Hypothetical protein AZC_3383
12	Control	kduD2 gene product
13	Control	Gluconate utilization system Gnt-I transcriptional repressor
14	Control	Glutathione S-transferase
15	Control	Replication protein C

(Polidoros and Scandalios, 1999). Moreover, the glutathione S-transferase activity of maize by herbicides attack was studied. It was found that the GST activity was increased toward herbicides and the highest activity expressed in the roots more than shoot (Dixon *et al*., 1997). However, the GSTs was normally increased when the plant were under stress to detoxification system but some of GST isozymes, such as wheat GST25, was present at the high level in control plant and then did not accumulate further after inoculation with pathogen (Mauch and Dudler, 1993).

Heat shock proteins (HSPs) are found in eukaryotes, including plants. The important functions of HSPs are clearly in protection against high-temperature stress. There are several classes of HSPs with high and low molecular weights that are heat induced to occur in a majority of cell types in a wide range of organisms (Vierling, 1991). However, heat shock proteins may play distinct roles in various stress responses. It was found that HSPs were stimulated by several of stress conditions and signals such as osmotic stress, electric shock, pathogen attack, light, chemical and plant hormones. Almost all stresses induce the production of a group of proteins called heat-shock proteins or stress-induced proteins (Cheong *et al*., 2002; Al-Whaibi, 2011). In previous report, mechanisms of plant adaptation to thermal stress by HSPs were associated with the metabolite level (Kaplan *et al*., 2004). In addition, there is proteomic profiling of opium poppy (*Papaver somniferum*) cell cultures response to elicitor treated. Opium poppy is a source of bioactive benzylisoquinoline alkaloids. Proteome analysis of elicitor-treated opium poppy cell cultures by 2D gel electrophoresis revealed that heat shock proteins were the one group of protein that found on the spot analysis coordination with plant defense responses (Zulak *et al*., 2009).

The relationship between proteins, GST and HSP and *Stemona* alkaloids response to chitosan elicitor was discussed. Chitosan probably increased the alkaloids production by plant defense metabolism via the expression of GST and HSP. GST plays an important role

in protecting tissue from oxidative damage and oxidative stress. From 2D gel electrophoresis, GST was found in control treatment but it was down-regulated in chitosan treatment gel. It may be assumed that chitosan culture treatment decreased the enzyme activity. To prevent tissue destroyed, the response, may be heat shock protein, was produced and sending the signal for tissue protection. So, in chitosan treatment was found the heat shock protein up-regulate than control treatment. The secondary metabolite in plant was produced to protect plant from invasive by tissue damage or herbicide. The chitosan treatment with 25 mg L^{-1} was the most appropriate concentration for *Stemona* alkaloid production, the treatments with higher concentration were affected on *Stemona* tissue plantlets and also decreased *Stemona* alkaloid production (Chaichana *et al*., 2012). Chitosan may elicit *Stemona* alkaloids via down-regulation of the GST function and up-regulation of heat shock protein for tissue damage protection. In the earlier study, proteomic analysis of differentially expressed proteins in elicitor-treated *Arabidopsis thaliana* cell cultures was performed. The study was aimed to identify changes in protein abundance regulating the plant defense response. It was found that GSTs and HSPs were elicitor responsive proteins required for signaling of the defense response to detoxification and localizes cell death (Chivasa *et al*., 2006). In addition, many studies supported that GST and HSP functioned responding to signaling stress such as heavy metal stress (Maksymiec, 2007) and cold stress (Cui *et al*., 2005). However, further experiment should be pursued to confirm the up or down regulation of these proteins by western blot or real time PCR.

CONCLUSION

The highest *Stemona* alkaloids production (25 mg L^{-1} chitosan) was select for proteomic profiling compare with control treatment. After the protein analyze with 2D gel electrophoresis, 15 out of 150 protein spots were found the difference exoression between control and chitosan treatment. The interested proteins that may involve in plant secondary metabolism were glutathione S-transferase and heat shock protein. Glutathione S-transferase down-regulated whereas heat shock protein up-regulated from control treatment. These proteins may play role in *Stemona* alkaloids biosynthesis via plant defense metabolism. Chitosan may cause decreasing detoxifying function of glutathione S-transferase. Then heat shock protein may be produced to signal for tissue protection and *Stemona* alkaloids may response for this stress.

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