Proteome Analysis of Fruit Development in Physic Nut (*Jatropha curcas* L.)

การวิเคราะห์โปรตีโอมระหว่างพัฒนาการของผลสบู่ดำ (*Jatropha curcas* L.)

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ABSTRACT

Jatropha curcas L. seeds contain high quantity of oil and protein but utilization of oil and seed kernel are restricted because of many toxic compounds especially phorbol esters (PEs). The morphology of physic nut fruit including pulp and seed changed significantly during developmental stages. In this study, the proteomics analysis approach was then used to study the differentially expressed proteins in pulp and seed kernel during developmental stages. The 1,316 proteins were identified in both tissues and divided into seven major groups according to their functions. They were involved in transcription and translation 5.32%, cell cycle and cell division 0.60%, fatty acid biosynthetic 0.53%, photosynthesis 0.30%, embryo development 0.15%, isoprenoid biosynthetic 0.15%, and unknown proteins 4.33%, respectively.

บทคัดย่อ

เมล็ดสบู่ดำ (*Jatropha curcas* Linnaeus) ประกอบด้วยโปรตีนและน้ำมันอยู่สูง แต่การนำน้ำมันและเนื้อในเมล็ดมาใช้ประโยชน์ยังมีข้อจำกัดเนื่องจากมีสารพิษอยู่หลายชนิด เช่น สารฟอร์บอลเอสเทอร์ ซึ่งเป็นสารพิษหลักที่พบในสบู่ดำ ลักษณะทางสัณฐานวิทยาของเนื้อผลและเมล็ดสบู่ดำระหว่างพัฒนาการของผลมีการเปลี่ยนแปลงอย่างเห็นได้ชัดเจน จากการศึกษาการแสดงออกของโปรตีนจากระยะพัฒนาการของผลสบู่ดำในทั้ง 2 เนื้อเยื่อ คือ เนื้อผลและเนื้อในเมล็ดพบว่ามีโปรตีนจำนวน 1316 ชนิด ที่แสดงออกในทั้ง 2 เนื้อเยื่อ จากการระบุหน้าที่ของโปรตีนทั้งหมดและจัดกลุ่มตามหน้าที่สามารถแบ่งโปรตีนออกเป็น 7 กลุ่ม หลักๆ โดยพบโปรตีนที่เกี่ยวข้องกับการถอดรหัสและการแปลรหัส 5.32% การแบ่งเซลล์และวัฏจักรของเซลล์ 0.60% การชีวสังเคราะห์กรดไขมัน 0.53% การสังเคราะห์แสง 0.30% พัฒนาการของเอมบริโอ 0.15% การชีวสังเคราะห์ไอโซพรีนอยด์ 0.15% และโปรตีนที่ไม่ทราบชนิด 4.33% ตามลำดับ

Key Words: *Jatropha curcas* Linnaeus, Phorbol esters, Proteomics

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Introduction

The developmental stages of *J. curcas* fruit differ in morphology and function. The seed is major organ for reproduction. So, it accumulates several compounds necessary for seed germination (Lui et al., 2009). *J. curcas* or physic nut (Euphorbiaceae) is originated from South America. It is can be found widespread in subtropical and tropical regions of Latin America, Africa and Asia countries (Siang et al., 2012). *J. curcas* has survived in several climates, areas and any type of soil. It is a multipurpose plant that all parts such as bark, leaves, seeds and latex can be used (Singh et al., 2010). The seed contained 54-58 % of oil and the seed kernel has high crude protein (22-28%). The seed kernel after solvent extraction has higher protein content than soybean meal. The oil was used for biodiesel feedstock product and the seed kernel (after oil extraction) was used for livestock feed after detoxification (Deveppa et al., 2010; Yunping et al., 2012). Although the oil and the seed kernel are consist of high quantity of oil and protein, however, it utilize was restricted because of toxins such as curcin, trypsin inhibitors, lectins, phorbol esters (PEs) (Deveppa et al., 2010). PEs are the main toxic substance in *J. curcas*. The amount of PEs differ by species with additional factors including climate and soil in each area. In general, PEs are known to promote tumor growth and directly poisonous to men and animals when received the toxins. Booranasrisak et al. (2013) reported that 22 proteins involved in fatty acid biosynthesis were identified during seed development in *J. curcas* by using proteomics. The expression pattern of 4 transcripts encoding for acetyl CoA carboxylase, phosphoenolpyruvate carboxylase, mercaptopyruvate sulfurtransferase and 4-coumarate: coenzyme A ligase similar to fatty acids level. Yang et al. (2009) reported that 50 proteins were identified in germinated and post-germinated *J. curcas* seed. They played role in several pathway such as β-oxidation, glyoxylate cycle, glycolysis, citric acid cycle, gluconeogenesis and pentose phosphate pathway. Accordingly, Liu et al. (2011) studied the differential proteome of endosperm and embryo in mature seed of *J. curcas*. The proteomics analysis demonstrated that 28 identified proteins were found in both tissues and involved in oil mobilization, signal transduction, transcription and cell cycle.

In this study, shotgun proteomics approach was used to analyze proteins expression profile in developmental *J. curcas* fruits. The obtained result will be used to understand the terpenoid biosynthesis pathway and related PEs biosynthesis. The changes of proteins in fruits would be highly beneficial in *J. curcas* breeding species to reduce PEs.

Objective of the study

To analyze proteome of the *J. curcas* fruits during development stage.

Materials and methods

Plant material

The five developmental stages of *J. curcas* fruits: premature fruits (P), mature green fruits (M), mature ripe fruits (yellow) (R), senescent fruits (black) (S) and dried fruits (D) were collected from Plant Genetic Conservation Center, Nakhonratchasima province, Thailand. After collection the fruits were kept at -80°C until used.

Total protein extraction

The pulps and the seeds kernel from each development stage were ground into a fine powder in liquid nitrogen with a precooled mortar and pestle.

About 250 mg of each ground sample was dissolved / suspended in 1 ml of 0.1% (w/v) sodium dodecyl sulfate (SDS). Each protein mixture was then precipitated with 4 volumes of cold acetone overnight at -20°C followed by centrifugation (10,000 x g, 15 min at 4°C). After removal of the supernatant, the protein pellet was resuspended in 0.5% (w/v) SDS and the protein concentration of each sample was determined by the Lowry method using bovine serum albumin (BSA) as a protein standard.

SDS-PAGE separation of proteins

Each 10 µg protein sample, and the protein standard marker (Low Molecular Weight SDS Marker Kit, GE Healthcare Bio-Sciences AB, Sweden), were mixed with loading buffer [0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2 M dithiotreitol (DTT) and 0.02% (w/v) bromophenol blue] and heated at 95°C for 10 min before loading onto and resolved through a 9 cm long 15% (w/v) acrylamide resolving SDS-PAGE. Gel electrophoresis was carried out at 30 V for the stacking gel and 50 V for the separating gel (8 cm wide × 1 mm thick gels) using an electrophoresis power supply (Electrophoresis Power Supply, GE Healthcare Bio-Sciences AB, Sweden). After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250.

In gel digestion

The protein bands from SDS-PAGE were excised into small gel pieces (gel plugs) according to the molecular mass range. The gel plugs were dehydrated with 100% acetronitrile for 3 times (5 min each) and dried at room temperature. The protein were with 10 mM dithiothreitol in 10 mM ammonium bicarbonate solution for 1 hour at room temperature followed by alkylated with 100 mM Idoacetamide in 10 mM ammonium bicarbonate solution at room temperature in the dark for 1 hour. After that, dehydrated with 100% acetronitrile was performed. The gel plugs were digested with trypsin solution (10 ng trypsin in 50% acetronitrile/10 mM ammonium bicarbonate) at room temperature overnight. Peptides were extracted and pooled in a new microtube. After drying at 40°C, peptides were kept at -80°C until prior to LC-MS/MS analysis.

LC-MS/MS Identification of proteins

The peptides were analyzed by LC-MS/MS (HCT ultra PTM Discovery System, Bruker Daltonik). The condition for analysis was according to the method described by Booranasrisak et al. (2013). After analysis, the MS/MS spectra were analyzed with DeCyder MS 2.0 differential analysis software (GE Healthcare). The differentially expressed peptides were identified with Mascot software (Matrix Science, London, UK) using Viridiplantae (Green plants), carbamidomethyl (C), oxidation (M), peptide charge 1+, 2+ and 3+ and instrument ESI-TRAP. Function of all protein were analyzed with Uniprot (http://www.uniprot.org/) and GoCat (http://www.eagl.unige.ch/GoCat).

Results

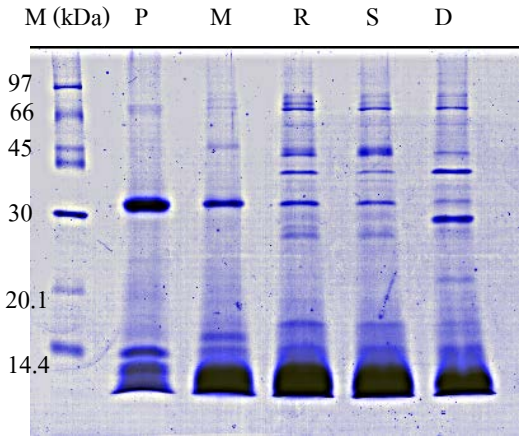
Protein profile analyses

After Coomassie Brilliant Blue G-250 staining. Many protein bands in range of 20.1 kDa to 45 kDa were detected. The proteins bands of seed kernel are higher than pulp. The results are shown in Fig. 1. After shotgun proteomic analysis, 1316 proteins were identified in seed kernel and pulp tissues. However, protein expression profile of each tissue was different. They can classified into seven groups according to function: transcription and translation proteins 5.32%, cell cycle and cell division proteins 0.60%, fatty acid

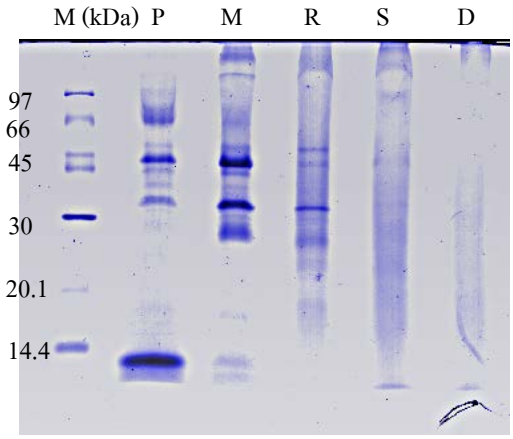
biosynthetic proteins 0.53%, photosynthesis proteins 0.30%, embryo development proteins 0.15%, isoprenoid biosynthetic proteins 0.15%, and unknown proteins 4.33%. The results are shown in Fig. 2.

Furthermore, each development stage of *J. curcas* fruits showed the protein's expression level related to isoprenoid biosynthesis. They were geranyl diphosphate synthase and diphosphomevalonate decarboxylase.

In pulp tissue, the expression level of diphosphomevalonate decarboxylase was highest in stage P, decreased in stage M to S and then increased again in stage D. The expression level of geranyl diphosphate synthase increased in stage P to R, decreased in stage S and then increased again in stage D. In kernel tissue, the expression level of diphosphomevalonate decarboxylase increased in stage P to R and then decreased in stage S to D. The expression level of geranyl diphosphate synthase was high in stage P, decreased in stage M, increased in stage R to S and then decreased again in stage D. The results are shown in Fig. 3.



(A)



(B)

Fig 1 The 12.5% SDS-PAGE of proteins isolated from *J. curcas* fruit during developmental stages. (A) Seed kernel tissues (B) Pulp tissues

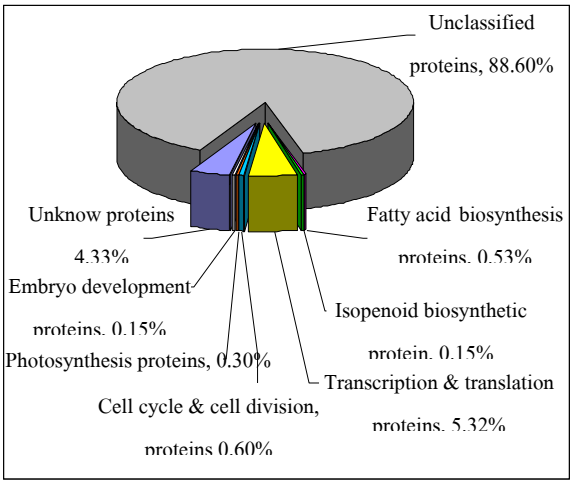
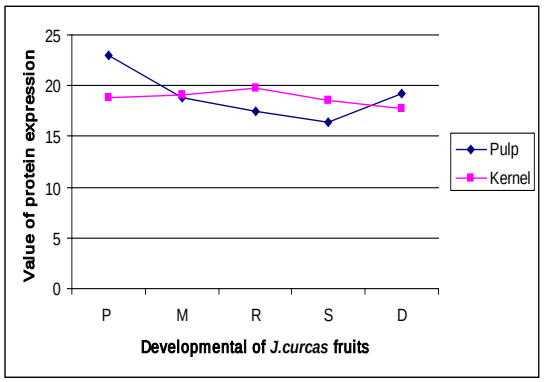
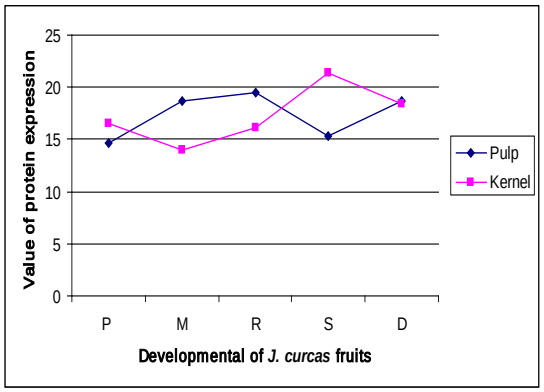


Fig 2 Functional classification percentages of total 1,316 identified proteins in seed kernel and pulp tissues



(A)



(B)

Fig 3 The proteins expression profile related to isoprenoid biosynthetic during developmental stages. (A) diphosphomevalonate decarboxylase (B) geranyl diphosphate synthase

Discussion

The data proteomics showed that 1316 proteins were found in developmental stages in tissues, pulp and seed kernel. And some proteins can find in specific tissue.

J. curcas is a multipurpose plant but hard to utilize because of the PEs. Isoprenoid biosynthesis pathway is an important part of PEs biosynthesis. In this study, we focused in the expression level of protein relate to isoprenoid biosynthesis. So, the change of proteins expression level of both enzymes (diphosphomevalonate decarboxylase and geranyl diphosphate synthase) during developmental stages would expect relates to produce and accumulate isoprenoid compound such as PEs.

Conclusions

1316 proteins were found the expression profile level in all developmental stages of *J. curcas* seed kernel and pulp tissues. They were divided into seven major groups according to their functions. For example the proteins with the functions of fatty acid biosynthesis and oil mobilization: acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase (Booranasrisak et al., 2013; Yunping et al., 2012), cell cycle and cell division: cyclin-dependent kinase inhibitor 3-like, photosynthesis: photosystem I assembly protein Ycf3, embryo development: late embryogenesis abundant protein, transcription and translation: transcription factor, putative and ribosome recycling factor. In addition, the mainly proteins with the functions of isopenoid biosynthesis: geranyl diphosphate synthase and diphosphomevalonate decarboxylase. This result would benefit to further understand PEs biosynthesis.

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References

Booranasrisak, T., Phaonakrop, N., Jaresitthikunchai, J., Virunanon1, C., Roytrakul, S., and Chulalaksananukul, W. 2013. Proteomic evaluation of free fatty acid biosynthesis in *Jatropha curcas* L. (physic nut) kernel development. Afr. J. Biotechnol. 12: 3132-3142.

Deveppa, RK., Makkar, HPS., and Becker, K. 2010. *Jatropha* toxicity: a review. J. Toxicol. Environ. Health B Crit. Rev. 13: 476–507.

Lui, H., Liu, YJ., Yang, MF., and Shen, SH. 2009. A comparative analysis of embryo and endosperm proteome from seeds of *Jatropha curcas*. J. Integr. Plant Biol. 1-8.

Liu, H., Yang, Z., Yang, M., and Shen, S. 2011. The differential proteome of endosperm and embryo from mature seed of *Jatropha curcas*. Plant Sci. 181: 660-666.

Siang, CT., Soong, TS., and Yien, ATS. 2012. Plant regeneration studies of *Jatropha curcas* using induced embryogenic callus from cotyledon explants. Afr. J. Biotechnol. 11: 8022-8031.

Singh, P., Singh, S., Mishra, S.P., and Bhatia, S.K. 2010. Molecular characterization of genetic diversity in *Jatropha curcas* L. genes, Genom Genomics. 4: 1-8.

Yang, MF., Liu, YJ., Liu, Y., Chen, H., Chen, F., and Shen, SH. 2009. Proteomic analysis of oil mobilization in seed germination and postgermination development of *Jatropha curcas*. J. Proteome Res. 8: 1441–1451.

Yunping, B., Ha, BTN., Eunice, Y., Chueng, LL., and Yan, H. 2012. Light induced degradation of phorbol esters. Ecotoxicol. Environ. Saf. 84: 268–273.