IDENTIFICATION OF PROTEIN FROM *EURYCOMA LONGIFOLIA* ROOT EXTRACT

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
Universiti Teknologi Malaysia

AUGUST 2014

Specially dedicated to my beloved umi, abah, siblings, supervisor and caring friend

Thank you for your support

"Jika mahu cemerlang, mesti sanggup menempuh semua halangan. Halangan terbesar ialah MINDA kita sendiri, yang hanya sukakan sesuatu yang selesa dan mudah."

ACKNOWLEDGEMENT

By the Name of Allah, the Most Merciful, the Most Beneficent

First and foremost, I am thankful to Allah s.w.t, as I have completed this study fulfilling my master degree successfully. In preparing this thesis, I was helped and assisted by many inspiring people. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation and gratitude to my supervisor, Dr. Chua Lee Suan and Professor Dr Mohamad Roji bin Sarmidi for their encouragement, patient guidance and criticism throughout this project. It is my honour to work under their supervision and all of their efforts are greatly appreciated.

Acknowledgment and appreciation also goes to all my laboratory friends and staff at Institute of Bioproduct Development in Universiti Teknologi Malaysia for their help and guidance which enabled me to complete my work.

Appreciation also especially goes to my beloved ‘umi’, ‘abah’ and my siblings for their precious love, time and countless support that was an extraordinary help towards the success of this project.

Last but not least, I would like to specially thank my beloved friends especially Nurul ‘Amirah who always besides me to guide and support me. Million thanks to all.

ABSTRACT

This study was carried out to identify plant protein from the root samples of *Eurycoma longifolia* harvested from Pahang and Perak, Malaysia. Protein usually presents in small quantity which is only 0.001% in plant, therefore it is critical to determine the extraction method for high yield and good quality of protein for the subsequent process of protein identification spectrometrically. Four extraction methods, namely water, Triton X-100 (non-ionic detergent), phenol-SDS precipitation and TCA-acetone precipitation were investigated for plant protein extraction. The yield of protein extracted from the plant samples was determined using Bradford assay. Both water extracts (water and Triton X-100) methods contained slightly higher protein content (0.2-0.53 mg protein/ml crude protein) than the extracts of precipitation (phenol and TCA) methods (0.12-0.29 mg protein/ml crude protein). The water extraction method also produced the highest resolution of 15% polyacrylamide gel with six and five protein bands for *E. longifolia* Pahang and Perak, respectively. However, the number of protein bands decreased from five to three for the extraction method of Triton X-100, phenol-SDS and TCA-acetone, respectively. After trypsin digestion of the protein bands, the presence of protein was analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The mass spectra matched with the databases showed that the addition of Triton X-100 could assist the extraction of mitochondrial protein, namely superoxide dismutase. Proteins that involved in energy metabolism such as phosphoenolpyruvate carboxykinase and plant protein inhibitor were also detected in the phenol-SDS buffer extraction because SDS acted as ionic detergent for cell lysis in this method. In line with previous studies, TCA-acetone did not exhibit clear gel image and subsequently less peptides were detected spectrometrically. In the present study, the addition of detergent (Triton X-100 and SDS) could enhance plant protein extraction from *E. longifolia* root from 46.7 to 72.7% w/w, but the use of TCA did not improve protein precipitation effectively.

ABSTRAK

Kajian ini dijalankan untuk mengenal pasti protein tumbuhan daripada sampel akar *Eurycoma longifolia* yang dituai dari Pahang dan Perak, Malaysia. Protein biasanya dalam kuantiti yang sedikit dalam tumbuhan, maka adalah penting untuk menentukan kaedah pengekstrakan bagi mendapatkan kandungan protein yang tinggi dan berkualiti supaya proses pengenalpastian protein dapat dilakukan secara spektrometrik. Empat kaedah pengekstrakan, iaitu air, Triton X-100 (detergen bukan ionik), pemendakan phenol-SDS dan pemendakan TCA-aseton telah dikaji untuk mengekstrak protein tumbuhan. Hasil protein daripada sampel tumbuhan telah ditentukan dengan menggunakan ujian Bradford. Hasilnya didapati pengekstrakan menggunakan air dapat mengekstrak hasil protein yang lebih tinggi (0.2-0.53 mg protein/ml protein mentah) daripada kedua-dua kaedah pemendakan (0.12-0.29 mg protein/ml protein mentah). Kaedah pengekstrakan air juga menghasilkan 15% poliakrilamida gel beresolusi tinggi dengan 6 dan 5 jalur protein bagi *E. longifolia* Pahang dan Perak masing-masing. Walau bagaimanapun, bilangan jalur protein menurun daripada 5 hingga 3 jalur bagi kaedah pengekstrakan Triton X-100, phenol-SDS dan TCA-aseton masing-masing. Selepas dicerna oleh trypsin, kehadiran protein dianalisis dengan menggunakan kaedah kromatografi cecair diintegrasikan dengan jisim spektrometri sejajar (LC-MS/MS). Spektra jisim dipadankan dengan pangkalan data dan didapati bahawa penambahan Triton X-100 membantu pengekstrakan protein mitokondria iaitu superosida dismutase. Protein terlibat dalam metabolisma tenaga seperti fosfenolpiruvate karboxykinase dan pemangkin protein dikesan dalam kaedah pemendakan phenol-SDS kerana SDS bertindak sebagai detergen ionik yang boleh memecahkan sel tumbuhan. Sebagaimana kajian sebelumnya, TCA-aseton tidak menunjukkan imej gel jelas dan kurang peptida dapat dikesan oleh spektrometer. Dalam kajian ini, penambahan detergen (Triton X-100 dan SDS) boleh meningkatkan proses pengekstrakan protein daripada tumbuhan sebanyak 46.7 hingga 72.7% w/w, tetapi penggunaan TCA tidak dapat memberikan hasil pemendakan protein secara berkesan.

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LIST OF ABBREVIATIONS

µl	-	Microliter
2DE	-	Two-Dimensional Electrophoresis
ACN	-	acetonitrile
AIDS	-	acquired immunodeficiency syndrome
APS	-	ammonium persulfate
ATP	-	Adenosine trisphosphate
BCA	-	Bicinchoninic acid assay
BLAST	-	Basic Local Alignment Search Tool
BPB	-	bromophenol blue
BSA	-	bovine serum albumin
CBB	-	Coomassie Brilliant Blue
cm	-	centimeter
DNA	-	Deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	enzyme-linked immunosorbent assay
EPF	-	Epidermal Patterning Factor
FDA	-	US Food and Drug Administration
FRIM	-	Forest Research Institute Malaysia
FTIR	-	Fourier transform infrared spectroscopy
GC–MS	-	Gas chromatography–mass spectrometry
HCl	-	hydrogen chloride

HIV	-	human immunodeficiency virus
HPLC	-	High-Performance Liquid Chromatography
IAA	-	iodoacetamide
KCl	-	potassium chloride
kD	-	kilo Dalton
LC-MS/MS	-	Liquid Chromatography Coupled with Tandem Mass
MALDI-TOF	-	Matrix-assisted laser desorption/ionization - time-of-flight
mg	-	Miligram
ml	-	Mililiter
mM	-	Mili Molar
Mn	-	Manganese
MS	-	Mass Spectrometry
NCBI	-	National Center for Biotechnology Information
nm	-	nanometer
PCA	-	Principal component analysis
PMF	-	Peptide Mass Fingerprinting
pmol	-	picomoles
PMSF	-	phenylmethylsulfonyl fluoride
RALF	-	Rapid Alkalinization Factor; 49 amino acid residues
RIA	-	Radioimmunoassay
rpm	-	round per minute
SDS-PAGE	-	Sulphate Polyacrylamide Gel Electrophoresis
SELDI-MS	-	Surface Enhance Laser Desorption Ionization Mass Spectrometer
SPME	-	Solid Phase Microextraction
SRM	-	selected reaction monitoring
TCA	-	Trichloroacetic acid
TDIF	-	Tracheary Element Differentiation Inhibitory Factor
TEMED	-	tetramethylethylenediamine
TMV	-	Tobacco Mosaic Virus
TPD1	-	Tapetum Determinant 1
UV-Vis	-	Ultraviolet-visible

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CHAPTER 1

RESEARCH BACKGROUND

1.1 Introduction

Eurycoma longifolia or commonly known as Tongkat Ali in Malaysia is a tropical herbal plant found in several parts of South East Asia countries. It is also known as Payung Ali, Penawar Pahit, Setunjang Bumi, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Tongkat Ali Hitam, Pokok Jelas and Jelaih. There are four common species of Tongkat Ali, namely *Eurycoma longifolia*, *Eurycoma apiculata*, *Polyathiabullata* and *Goniothalamus* sp. (Aziz *et al.*, 2003). Among these species, *Eurycoma longifolia* is commonly used in traditional medication for various diseases. Traditionally, *E. longifolia* is used for its aphrodisiac, anti-pyretic and anti-malarial effects as well as a general tonic (Kuo *et al*., 2003). A decoction of its long woody tap root is taken orally. The benefits of the roots of *E. longifolia* include restoring energy and vitality, enhancing blood flow and functioning as herbal ingredient for women after child birth (Ismail *et al.*, 1999). This plant bears fruit after two and a half years of cultivation, while the root is generally taken to be processed after 4 years of cultivation (Athimulam *et al*., 2006).

To our knowledge, plant protein from *E. longifolia* is seldom studied by researchers. It is believed that besides small metabolites, plant protein from the plant could be the bioactive constituent that contributed to the reported pharmacological properties (Chua *et al*., 2011). The detection of bioactive peptide (4.3 kD) which had been reported to have aphrodisiac activity from *E. longifolia* (Asiah *et al*., 2007) has encouraged more intensive research on protein or peptide in the plant. In the following year, another two plant proteins from *E. longifolia* with the molecular weight of 7.5 and 6.0 kD had been identified by Farouk *et al*. (2008). They reported the proteins showed antibacterial activity against human pathogenic bacteria. Antimicrobial peptides are ribosomally synthesised as natural antibiotics by almost all living organisms including bacteria, animals and plants. The plant still contains many proteins to be discovered, particularly for pharmacological applications.

1.2 Problem Statement

The hard cell walls of the plant have been reported to be the limitation factor for plant protein extraction and separation (Granier, 1988). Therefore, plant protein identification is a challenge since plant protein usually presents in small quantity. Plant proteins need to be extracted efficiently without degradation to ensure maximal yield of protein extraction. This study investigated the method of protein extraction that can give the highest number of proteins and the highest protein content. Usually, detergent is used to break the cell wall for proteins extraction. However, the type of detergent chosen must contribute to the lowest protein loss during extraction.

Recent technical advancement in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS) have made the effort of protein separation and identification to be faster and higher in accuracy and sensitivity. There are limited studies on proteins from *E. longifolia* from gel electrophoresis to LC-MS/MS technique. This is mainly due to the minute amount of plant protein. Fortunately, this technique only requires small amount of sample for analysis. Somehow, extra precaution in sampling handling such as protein alkylation and reduction must take into consideration before mass spectrometric analysis.

There are still many types of proteins need to be explored, mainly for pharmacological application. For instance, phytohormones which are used in intercellular responses for plant growth and development might be useful for drug development. Phytohormones involve in plant defense mechanism in response to wound signal transduction by pests (Lindsey *et al.*, 2002). Plant protein could also be used as a carrier such as plant non-specific lipid-transfer proteins in drug delivery (Cheng *et al.*, 2004). However, the number of plant protein in the database is lesser than protein from bacteria and animals (Kim *et al.* and Park *et al*., 2002). Only 0.001% of the total 961,019 number of total protein sequence belongs to plant protein (National Center for Biotechnology Information (NCBI) database, assessed on 26th April 2014).

1.3 Objective

The objective of this study was to identify plant proteins from *Eurycoma longifolia* (Tongkat Ali) root extracts collected from Pahang and Perak in Malaysia.

1.4 Scope

In order to achieve the objective of this study, the scopes were:

1. To investigate the protein extraction methods for *E. longifolia* roots using four different extraction methods, namely water, Triton X-100 (non-ionic detergent), phenol-SDS buffer and TCA-acetone precipitation methods.

2. To fingerprint plant proteins extracted from *E. longifolia* roots using one dimensional gel electrophoresis and mass spectrometric approaches.

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