ISOLATION AND CHARACTERISATION OF MITOCHONDRIAL PROTEIN FROM *Orthosiphon stamenius* IN NORMAL AND COPPER STRESS CONDITIONS

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Specially dedicated to
my beloved husband, ibu, ayah,
daughters, son, family members
and friends

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ABSTRACT

Orthosiphon stamenius is a species of medicinal herb that can be found in tropical area. Traditionally, the leaves of this plant have been used as diuretics, treatment of rheumatism, kidney and bladder inflammation, as well as abdominal pain and gout. This herbaceous plant requires in-depth research not only for medical field, but also to understand their properties during normal and stress environment. The purpose of this study is initially to search for the most suitable technique for mitochondrial isolation from *Orthosiphon stamenius* leaves. Three protocols for isolation of mitochondria were performed and the mitochondrial enzyme activities were determined. The highest activity of Cytochrome c Oxidase (1.423 U/g) and the highest mitochondrial protein concentration (2.656 mg/ml) was obtained from mitochondrial isolation using a method from Millar *et al*. (2007). This protocol was further used for mitochondrial isolation from *Orthosiphon stamenius* leaves treated with different concentrations of Copper (20 ppm, 60 ppm, 140 ppm). The activity of Cytochrome c Oxidase (4.217 U/g, 4.429 U/g, 4.887 U/g) and the concentration of mitochondrial protein (2.604 mg/ml, 2.623 mg/ml, 4.887 mg/ml) showed significant increase in 20 ppm, 60 ppm and 140 ppm Copper treatment, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been conducted to check the mitochondrial protein profiles in normal and Copper treated plants. There was no obvious difference obtained in the protein profiles from normal and treated plants. There were six bands clearly visible on the gel for untreated and treated sample. Even though the protein profiles were similar, the increasing activity of Cytochrome c Oxidase and concentration of mitochondrial protein in Copper treated plants showed that plants do response under Copper stress. This study showed interconnected responses of mitochondrial activity and mitochondrial protein when exposed to different level of Copper.

ABSTRAK

Orthosiphon stamenius adalah tumbuhan herba yang biasanya digunakan dalam bidang perubatan tradisional. Ianya boleh didapati di kawasan tropika. Secara tradisi, daun tumbuhan ini boleh digunakan untuk merawat penyakit sendi, buah pinggang, keradangan pundi kencing, sakit perut dan gout. Kajian yang terpeinci mengenai tumbuhan ini diperlukan memandangkan bukan sahaja kerana kepentingannya dalam bidang perubatan malah pemahaman tentang tindakbalas tumbuhan ini dalam keadaan stress dan normal juga perlu diberi perhatian. Tujuan kajian ini adalah untuk memilih satu teknik terbaik untuk pengasingan mitokondria dari daun *Orthosiphon stamenius*. Tiga teknik untuk pengasingan mitokondria telah dijalankan ke atas daun *Orthosiphon stamenius*. Aktiviti tertinggi Cytochrome c Oxidase (1.423 U/g) dan kepekatan protein mitokondria tertinggi (2.656 mg/ml) telah diperolehi dari pengasingan mitokondria dari Millar *et al*. (2007). Teknik ini telah dijalankan ke atas daun *Orthosiphon stamenius* yang ditambah dengan kepekatan kuprum yang berbeza (20 ppm, 60 ppm, 140 ppm). Aktiviti Cytochrome c Oxidase (4.217 U/g, 4.429 U/g, 4.887 U/g) dan kepekatan protein mitokondria (2.604 mg/ml, 2.623 mg/ml, 4.887 mg/ml) menunjukkan peningkatkan jumlah pada kepekatan kuprum 20 ppm, 60 ppm dan 140 ppm. Kajian ini juga mengunakan natrium sulfat dodecyl polyacrylamide gel elektroforesis (SDS-PAGE) untuk menganalisa profil protein mitokondria dalam keadaan normal dan keadaan ditambah kuprum. Profil protein yang diperolehi adalah sama. Terdapat enam jalur yang terhasil dalam gel bagi sampel yang normal dan sampel yang ditambah kuprum. Walaupun profil protein adalah sama, peningkatan aktiviti Cytochrome c Oxidase dan kepekatan protein mitokondria adalah saling berkaitan dengan peningkatan kepekatan kuprum. Kajian ini menunjukkan terdapat tindak balas mitokondria dan protein mitokondria apabila terdedah kepada beberapa tahap kepekatan kuprum.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP	-	Adenosine Tri Phosphate
BN	-	Blue Native
BSA	-	Bovine serum albumin
Cu	-	Copper
$CuSO.5H_2O$	-	Copper (II) sulphate pentahydrate
DNA	-	Deoxyribonucleic Acid
DTT	-	Dithiotheitol
EGTA	-	ethylene glycol-bis-(2-aminoethylether)-n, n'tetraacetic acid
et al.,	-	and Others
Fe	-	Ferum
g	-	Gram
GDC	-	Glycine Decarboxylase Complex
H_2O	-	Water
HCl	-	Hydrogen Chloride
HSP	-	Heat Shock Protein
kDa	-	kilo Dalton
M	-	Molar
MCO	-	Metal catalysed oxidation
mg	-	Milligram
min	-	Minute
mL	-	Millilitre
mM	-	Millimolar
MOPS-KOH	-	3-(N-morpholino) propanesulfonic acid-Potassium hydroxide
MPP	-	Mitochondrial Processing Peptidase
nm	-	nanometre

OPGDC	-	2-Oxoglutarete Dehydrogenase Complex
PDC	-	Pyruvate Dehydrogenase Complax
PVP 40	-	Polyvinylpyrrolidone
QCR	-	Cytochrome c Reductase
RNA	-	Ribonucleic Acid
ROS	-	Reactive oxygen species
SDH	-	Succinate Dehydrogenase
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel
SPSS	-	Statistical Package for the Social Sciences
STDEV	-	Standard deviation
TEMED	-	*N,N,N',N'*-tetramethylethane-1,2-diamine
TES	-	n-tris (hydroxymethyl) methyl-2-aminoethane-sulphonic acid
TOM	-	Translocate of the Outer Mitochondrial Membrane
v	-	Volume
w	-	Weight
Δ	-	Delta
ε	-	Epsilon
°C	-	Degree Celsius
µg	-	Microgram
µL	-	Microlitre
µM	-	Micromolar

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Mitochondria are important organelles involved in Adenosine Tri Phosphate (ATP) production and many other functions in living organisms. They are found in the cytoplasm and occupy about 20% of the cell's volume (Douce, 1985). The number of mitochondria in a cell depends upon the metabolic requirements of that cell, and may range from a single large mitochondrion to thousands of the organelles (Mader and Windelspecht, 2010). According to Douce (1985), the most common shape for plant mitochondria is that of a rod with hemispherical ends about 2µm in length and 0.5µm in diameter. Mitochondria are often called the powerhouses of the cell because they produce most of the ATP utilized by the cell (Mader and Windelspecht, 2010).

Mitochondrion is a double membrane-surrounded organelle, in which cellular respiration takes place. The enzymes of the mitochondrial respiratory chain are located in the inner mitochondrial membrane and synthesize most of the cell's energy equivalents to ATP. Each mitochondrion contains a circular DNA molecule coding for ribosomal and mitochondrial transfer RNA molecules and for several mitochondrial proteins. The inner membrane is folded to cristae comprising the mitochondrial matrix space (Schwab, 2012). They contain enzymes for pyruvate

oxidation, the citric acid cycle, the β-oxidation of fatty acids, and oxidative phosphorylation, as well as the electron transport chain. The matrix of mitochondria is highly concentrated mixture of enzymes. Various enzymes and proteins present in the matrix of mitochondria, which helps in processing and break down carbohydrates and other nutrient molecules obtained from food (Mader and Windelspecht, 2010).

To prepare a crude mitochondrial fraction by differential centrifugations is not easy because some degree of structural damage has occurred during the isolation procedure (Moore *et al*., 1993). That is why the isolation of plant mitochondria has always been complicated primarily by biochemical changes that occur during plant cell development and across plant species (Mackenzie, 1994). These techniques, while maintaining mitochondria morphological structure and their functional characteristics have required methods that avoid osmotic rupture of membranes that protect organelles from harmful products released from other cellular compartments (Millar *et al*., 2007), such as hydrolytic enzymes, phenolic compounds, alkaloids and terpenes (Neuberger, 1985). Most of the problems involved in the isolation of fully functional mitochondria occur during the homogenization phase because of the high shearing forces required to rupture the plant cell wall (Moore *et al*., 1993). Thus the effects of homogenization on mitochondrial structure and function range from undesirable to totally destructive. The ability to isolate functional mitochondria from plant tissues is a key technique in the study of proteome and metabolic function of the plant mitochondrion (Sweetlove *et al*., 2007).

Recent advances in proteomic approaches led to the identification of large sets of mitochondrial proteins (Hajek *et al*., 2004). According to Eubel (2007) one of purpose for mitochondrial isolation is to identify their proteomes and changes in these proteomes during development and environmental stress treatments. One of the threatening stresses is heavy metal. Transition metal ions are essential in biochemical function by being incorporated into or associating with proteins for living cell to functions effectively. For an example in plant mitochondria, key functions of metal cofactors include metabolism, electron transport, ATP synthesis and the detoxification of reactive oxygen species (ROS), however, metal ions can also be

highly toxic to cells and cells organelle functions (Tan *et al*., 2010). The high levels metals become toxic for the cell due to displacement of essential elements for enzymatic functions, interference with functional sites in protein, or enhanced ROS production (Garcia *et al*., 2014).

The presence of free metal cations, redox active or inactive such as Copper may significantly contribute to the initiation of oxidative stress. Metal-catalysed oxidation (MCO) of protein is mechanisms for metal-linked damage which involved the oxidation of susceptible amino acids (Tan *et al*., 2010) MCO of protein can be highly specific event when the site of protein oxidation can be defined on the protein surface that binds to metal ions and where proteins are more susceptible to damage if they bind metal ions (Stadman, 1990; Tan *et al*., 2010). According to Gupta *et al*. (2012), mitochondrial enzymes often require metals as cofactors such Ferum (Fe) and Copper during electron transfer. However, higher concentrations of these essential metals induce ROS production. The presence of free metals ions in plant mitochondria could be crucial in the initiation of oxidative stress, resulting in oxidative damage to respiratory and other mitochondrial proteins.

1.2 Problem Statement

Mitochondria are important organelles that involved in ATP production and many other functions in organisms, including plants. Currently, development of techniques for the isolation of plant mitochondria lack far behind the progress made in the upstream processes such as protein and metabolite detection. As a common herbal plant, there are growing numbers of studies related to *Orthosiphon stamenius* in Malaysia. However, there is no attempt to develop a technique for mitochondrial isolation and analyse the protein profiles from this species yet.

1.3 Objectives

(i) To deduce the best method for mitochondrial isolation from *Orthosiphon stamenius.*

(ii) To determine the activity of Cytochrome c Oxidase from isolated mitochondria.

(iii) To evaluate mitochondrial protein obtained from *Orthosiphon stamenius* under normal and metal treatment conditions.

1.4 Scope of Study

In this research, *Orthosiphon stamenius* plants about three months old were obtained from local supplier. Then, leaves were harvested to obtain and isolate the mitochondria. The crude of mitochondria was isolated using by three different centrifugation methods. First centrifugation method used by Wilson and Chourey (1984). Second and third method used the modification of a protocol by Millar *et al.* (2007), where the mannitol was replaced by sucrose. Total mitochondrial protein quantification for each protocol was determined using Bradford method. The activity of Cytochrome c Oxidase for each samples from each isolation protocol were determined and protein profiles were analysed by Sodium Dodecyl Sulfate Polyacrylamide Gel-Electrophoresis (SDS-PAGE).

Another sample of *Orthosiphon stamenius* about two months old were also obtained from local supplier and was treated with three different concentration of Copper ($CuSO.5H_2O$). Then, the leaves were harvested after one month. The crude of mitochondria was isolated using the best method. Total mitochondrial protein quantification was determined using Bradford method. The activity of Cytochrome c

Oxidase weas determined and protein profiles were analysed by Sodium Dodecyl Sulfate Polyacrylamide Gel-Electrophoresis (SDS-PAGE).

1.5 Significance of Study

There are several techniques to isolate mitochondria from plants. Studies relating to such methods are imperative and these suitable techniques will determine the potential application their respective industries. By establishing a method for mitochondrial isolation from *Orthosiphon stamenius,* it will enhance our knowledge and open new possibilities for its application in biotechnology. Information on specific mitochondrial protein from the leaves under normal and metal treatment condition is important for further upstream studies related to mitochondrial functions in plants. Furthermore, this will help us to understand better on *Orthosiphon stamenius* response under metal stress.

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