ISOLATION AND PURIFICATION OF ACTINOMYCETES FOR SCREENING OF SECONDARY METABOLITES AGAINST MAPK KINASE AND MAPK PHOSPHATASE IN EUKARYOTIC SIGNAL TRANSDUCTION PATHWAY

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March 30, 2005

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ABSTRACT

A total of 16 soil samples were collected from underneath selected and identified trees in Newbery Plot of lowland dipterocarp rainforest at Danum Valley, Lahad Datu, Sabah. The collection of soil was done around the base of dipterocarps as this area has the least tendency for cross-contamination and abundant with humus which supplies nutrients. The soil pH was measured in 1:2.5 soil-water suspension. The pH determined for the soil samples was pH 4.0 (acidic). Soil samples were first mixed, suspended in sterile dH_2O and shaken on vortex mixer. All treated samples were serially diluted up to 10^{-3} and 0.1 ml of aliquot was spread over the surface of humic acid-vitamin B agar (HV). Plates were incubated at 28ºC for growth of colonies within 7 to 14 days. Oatmeal agar (OA) was used to obtain single pure colonies. The pure cultures sporulated well on these OA media. There were 47 strains of pure actinomycetes isolated, purified and characterized by presence of aerial and substrate mycelia, extracellular pigmentation and sporulation. The colour definition for the physical and morphological characteristics was standardized according to Sissons Paint Catalogue. These 47 pure actinomycetes were grown aerobically in liquid culture for constant fermentation at 28ºC for 5 days and thereafter extracted with acetone. The 47 acetone extracts were screened for microbial inhibitors against MAPK kinase (MKK1) and MAPK phosphatase (MSG5) in budding yeast (*Saccharomyces cerevisiae*) systems. Besides of screening those 47 extracts, a total of 101 extracts owned by other coursemates were also screened. As a result, overall there would be 148 extracts that were screened, with a total of only 132 out of the 148 extracts tested against MKK1, while against MSG5 all of the 148 extracts were tested. Out of the 148 extracts tested, only 7 extracts showed activity (toxicity). All of the 7 extracts (H11610, H11612, H11635, H11640, H11813, H11428 & H11588) showed toxicity against MSG5, in which 2 extracts (H11610 & H11640) out of the 7 extracts that showed toxicity against MSG5, did not show any activity (nontoxic) against MKK1. This indicates that only 5 extracts (H11612, H11635, H11813, H11428 & H11588) out of the 7 extracts showed toxic activity against MKK1. In this research, there was no discovery of potential inhibitors against MKK1 and MSG5.

PENGASINGAN DAN PENULENAN AKTINOMISET BAGI PENSKRINAN METABOLIT SEKUNDER MENENTANG MAPK KINASE DAN MAPK PHOSPHATASE DALAM LALUAN TRANSDUKSI ISYARAT EUKARYOTIK

ABSTRAK

Sejumlah 16 sampel tanah telah dikutip dari permukaan bawah pokok-pokok terpilih yang diketahui di Plot Newbery dalam hutan hujan tanah rendah dipterokarp di Lembah Danum, Lahad Datu, Sabah. Kutipan sampel telah dibuat di sekitar kawasan dipterokarp berdekatan dengan akar kerana kemungkinan yang rendah untuk berlakunya kontaminasi silang serta kaya dengan humus yang membekalkan sumber nutrien. pH sampel tanah telah disukat dalam 1:2.5 campuran tanah-air. pH tanah yang telah ditentukan adalah pH 4.0 (asidik). Sampel tanah dicampur dengan air dan digaulkan dengan "vortex mixer". Pencairan bersiri dilakukan terhadap sampel tanah sehingga 10^{-3} dan 0.1 ml alikut telah disebarkan ke atas permukaan agar asid humik-vitamin B (HV). Piring petri telah dieram pada 28°C untuk pertumbuhan koloni dalam masa 7 sehingga 14 hari. Agar Oatmeal (OA) telah digunakan untuk mendapatkan koloni tunggal yang tulen. Kultur tulen bersporulasi dengan baik dalam media OA. Sebanyak 47 strain aktinomiset tulen telah diasingkan, ditulenkan dan dicirikan melalui kehadiran miselia aerial dan substrat, pigmentasi ekstraselular dan sporulasi. Penentuan warna bagi sifat fizikal dan morfologi telah dipiawaikan mengikut Katalog Cat Sissons. Kesemua 47 strain tersebut telah difermentasikan secara aerobik dalam kultur cecair pada suhu malar 28°C selama 5 hari, dan kemudiannya diekstrak dengan aseton. Kesemua 47 ekstrak aseton ini telah diskrin bagi perencat mikrob menentang MAPK kinase (MKK1) and MAPK phosphatase (MSG5) dalam sistem yis (*Saccharomyces cerevisiae*). Selain daripada menskrin kesemua 47 ekstrak tersebut, sejumlah 101 ekstrak kepunyaan rakan seperjuangan juga telah diskrin. Maka, jumlah keseluruhan ekstrak yang telah diskrin adalah 148 ekstrak, dengan sejumlah 132 daripada 148 ekstrak tersebut diuji menentang sistem MKK1, manakala bagi sistem MSG5 kesemua 148 ekstrak tersebut telah diuji. Daripada kesemua 148 ekstrak yang telah diuji, hanya 7 ekstrak yang menunjukkan aktiviti (ketoksikan).

Kesemua 7 ekstrak tersebut (H11610, H11612, H11635, H11640, H11813, H11428 & H11588) menunjukkan kesan ketoksikan terhadap MSG5, yang mana 2 ekstrak (H11610 & H11640) daripada 7 ekstrak tersebut yang toksik terhadap MSG5, tidak menunjukkan sebarang aktiviti (tak toksik) terhadap MKK1. Ini menunjukkan bahawa hanya 5 ekstrak (H11612, H11635, H11813, H11428 & H11588) daripada 7 ekstrak tersebut yang menunjukkan aktiviti ketoksikan. Dalam kajian ini, tiada penemuan terhadap ekstrak yang berpotensi menjadi perencat kepada MKK1 dan MSG5.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celcius
g	gram
µg	microgram
mg	miligram
L	liter
µl	microliter
ml	mililiter
m	meter
nm	nanometer
cm	centimeter
mm	milimeter
sq	square
r.p.m.	rotation per minute
v/v	volume over volume
w/v	weight over volume
No.	Number
UMS	Universiti Malaysia Sabah
DVFC	Danum Valley Field Centre
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HV	Humic acid – Vitamin B agar
OA	Oatmeal agar
HPLC	High-perfomance liquid chromatography
UV	Ultra violet
PBS	Phosphate buffer solution
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase

MAPKKK	Mitogen-activated protein kinase kinase kinase
ERK	Extracellular signal-regulated kinase
MEK	ERK-activating kinase
MEKK	MEK kinase
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MKK1	Mitogen-activated protein kinase kinase 1
MSG5	Multicopy suppressor of growth
GPCR	G protein-coupled receptor
RTK	Related tyrosine kinase
PKC	Protein kinase C
NaCl	Sodium chloride
KCl	Potassium chloride
HCl	Hydrogen chloride
$MgSO_4$	Magnesium sulphate
$FeSO_4$	Ferum sulphate
$CaCO_3$	Calcium carbonate
Na_2HPO_4	Disodium hydrogen orthophosphate anhydrous
KH_2PO_4	Potassium dihydrogen phosphate
dH_2O	Distilled water

CHAPTER 1

INTRODUCTION

Signal transduction networks enable cells to perceive specific changes (signals) in the extracellular environment, to transduce the signals through protein phosphorylation and dephosphorylation and to mount an appropriate response. Mitogen-activated protein kinase (MAPK) cascades are among the most thoroughly studied of signal transduction systems and have been shown to participate in a diverse array of cellular programs, including cell differentiation, cell movement, cell division, and cell death (Schaeffer and Weber, 1999).

Actinomycetes have been proven to be the prolific producers of bioactive secondary metabolites against signal transduction and cell cycle. Actinomycetes are the major source of diverse organic compounds with antimicrobial properties called antibiotics. Antibiotics are produced during secondary metabolism and discovered at a rate of 300 per year and 75% of those discovered are produced by actinomycetes, with *Streptomyces* species producing most of the antibiotics. Most antibiotics are useless to humans because they are extremely toxic. Many antibiotics have become useless because the target microorganisms have developed resistance to them. It is not clear why actinomycetes make antibiotics. They may give actinomycetes a competitive advantage in soil. Antibiotics production may force actinomycetes into stationary growth or signal

them to begin sporulating. Antibiotics are rarely detected in soil and it is difficult to prove that they play a role there (Coyne, 1999).

The straightforward genetics of the budding yeast *Saccharomyces cerevisiae*, and the high degree of conservation of basic cellular processes between yeast and higher organisms makes yeast an excellent tool for drug development studies, particularly in regards to anticancer and antifungal drug discovery. The budding yeast *Saccharomyces cerevisiae* offers several advantages as a biological model system for molecular studies. It is a unicellular organism with a compact genome of approximately 6000 genes and life cycle well suited to classical genetic studies. Additionally, high conservation of many cellular processes between yeast and higher organisms, especially with regards to basic cellular metabolism and cell division, makes yeast a fundamental model eukaryote. Just as budding yeast has proven an excellent model system for studying cell biology, it is also a very valuable organism for modelling drug action. Yeast is particularly relevant to anticancer drug development. Many common mutations in human cancers can be modelled in yeast (Parsons *et al.*, 2003).

The selected location of research area for soil sampling was the Danum Valley Field Centre (DVFC). Danum Valley is located 80km inland from Lahad Datu on Sabah's east coast. In the heart of the 'lost world' of Danum Valley lies a vital conservation reserve, Sabah's largest protected area of lowland rainforest. The Danum Valley Conservation Area is one of the last remaining preserves of primary lowland rainforest in Asia. As one of the premier research centre in South East Asia, it oversees

research projects, controls forest enrichment planning sites, education, training and wilderness recreation and is a meeting place for naturalists from all over the world.

For the laboratory project, the main focus was concentrated on the screening of microbial inhibitors from actinomycete strains against mitogen-activated protein kinase (MAPK) pathway by targeting the activity of MKK1 and MSG5 proteins which are involved in the yeast cell wall integrity system. In this research, MAPK kinase and MAPK phosphatase are the molecular level targeted proteins. The inhibitors for these molecular targeted proteins can act as anticancer drugs. In the search for inhibitors of MAPK kinase and MAPK phosphatase, yeast-based screening system targeting the activity of MKK1 and MSG5 proteins is an ideal system as it utilizes genetically engineered mutant yeast strains of $MKK1^{P386}$ (H10068) and $MKK1^{P386}$–MSG5 (H10069), as the molecular targets where these yeasts protein kinases are homologues to MAPK signal transduction pathway of human. So, the inhibitors for these proteins will be a good candidate of anticancer drugs.

Basically, the main scope of this research is based on the objectives as the following, (i) collection of soil samples at Danum Valley Field Centre, (ii) isolation and purification of actinomycete strains from soil samples, (iii) production of acetone extracts of secondary metabolites from actinomycetes for screening test, and (iv) screening of secondary metabolites acetone–extracted from actinomycetes by using yeast-based screening system in the search for microbial inhibitors against MAPK kinase and MAPK phosphatase.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of Research Area for Soil Sampling

In this project, the selected area for soil sampling was the Danum Valley Field Centre. Sited beside the Segama River, the Field Centre is located 81 km west of Lahad Datu and more than 20 km from any other habitation, a truly remote area. It provides facilities for research, education and wilderness recreation in one of Sabah's last strongholds of undisturbed lowland rain forest, the 438 sq km Danum Valley Conservation Area. Research efforts have revealed a tremendous variety of plants and a full range of Sabah's lowland fauna, including such rare and endangered species as the Sumatran rhino, wild cattle, banteng (tembadau), Asian elephant, clouded leopard, orang utan and proboscis monkey. Bird life is equally varied with some 290 species recorded to date in the area. The Field Centre is run by the Sabah Foundation (Yayasan Sabah) under the aegis of a Management Committee which includes the Sabah Forestry Department, the Sabah Ministry of Tourism, Environment Science and Technology, Universiti Malaysia Sabah, and seven other agencies with interests in forest research and conservation.

(Research and Development Division, Yayasan Sabah Group)

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