SCREENING AND CHARACTERIZATION OF MICROBIAL INHIBITORS AGAINST EUKARYOTIC PROTEIN PHOSPHATASES (PP1 and PP2A)

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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ABSTRAK

SCREENING AND CHARACTERIZATION OF MICROBIAL INHIBITORS AGAINST EUKARYOTIC PROTEIN PHOSPHATASES (PP1 and PP2A)

Sampel tanah diperoleh dari tanah hutan Sabah di Ulu Padas, Lower Segama, Melalap, Maliau Basin, Tabin, Kpg Limau-limauwan dan kawasan di sekitar Kota Kinabalu. Sejumlah 283 actinomycetes dan 44 mikrofungi telah berjaya diasingkan dan ditulenkan. Unsur asing ini telah dikaji dan disaring dengan menggunakan sistem penyaringan perencat PP1. Dua jenis yis telah digunakan sebagai sasaran penyaringan terhadap PP1; yis jenis liar yang membawa *GLC7* dan yis mutan yang membawa alel glc7 (*glc7-10*) yang sensitif terhadap suhu yang tinggi. Mutan menyebabkan kitaran sel terencat dan keutuhan dinding sel terjejas pada 37°C tetapi diselamat dengan penambahan 1M sorbitol. Perencat berpotensi yang bertindak ke atas Glc7p jenis liar akan menunjukkan ciri yang sama seperti strain mutan. Ekstrak kasar aceton H7520, H9318 dan H9978 menunjukkan perencatan positif terhadap sistem penyaringan perencat PP1. Ujian perencat yang melibatkan aktiviti enzim telah dijalankan oleh Prof Michael J.R. Stark, dengan esktrak kasar aseton H9318 dan H9978 yang telah dibeku-kering menunjukkan aktiviti perencatan terhadap PP1 dan PP2A, sementara esktrak H7520 hanya merencat PP2A dan bukannya PP1. Tahap kesensitifan bagi perencat berpotensi yang telah disaring terhadap yis *S. cerevisiae*, PP1 atau PP2A diuji dalam ujian kekurangan haploid. Strain ASY25 (*PPH21/pph21::LEU2 pph22::URA3/PPH22*) dan ASY27 (*glc7::LEU2/GLC7*) adalah sensitif terhadap ekstrak kasar aceton H9318 dan H9978 yang telah dibeku-kering berbanding dengan strain AYS927 (*PPH21/PPH21 PPH22/PPH22 GLC7/GLC7*). Strain ASY25 adalah lebih sensitif terhadap ekstrak kasar aceton H7520 yang telah dibeku-kering berbanding dengan strain AYS927 dan ASY27. Pengesktrakan jenis cecair-cecair telah dijalankan dan lapisan ekstrak etil asetat (H7520, H9318 dan H9978) menunjukkan aktiviti perencatan. Hanya lapisan etil acetat H9318 diuji dengan RP-HPLC. Dua kompaun aktif, S1 dan S2 telah dikesan dan diasingkan keluar. S1 dan S2 bagi H9318 menunjukkan aktiviti perencatan terhadap sistem penyaringan perencat PP1. Tahap kesensitifan bagi S1 dan S2 yang telah disaring terhadap yis PP1 atau PP2A diuji dalam ujian kekurangan haploid. Strain ASY27 menunjukkan kesensitifan yang lebih tinggi terhadap S2 berbanding dengan strain diploid yang lain. S1, strain ASY25 adalah lebih sensitif daripada strain AYS927 dan ASY27. S1 dan S2 merencat kedua-dua PP1 dan PP2A dalam ujian perencat yang melibatkan aktiviti enzim. Dalam kitaran sel *S. cerevisiae*, S1 menunjukkan kesan yang sama seperti mutan pph22 (binuklear di dalam sel induk) dan mengumpul pelbagai nuklei. S2 menunjukkan kesan yang sama seperti *glc7-10* (mempamerkan morfologi pertunasan secara sisi) dan juga perbelahan nukleus. Dengan bantuan daripada Dr Linda Morris dan Ling-say Wong, S1 dan S2 dikenalpasti dengan menggunakan MS. Sebahagian struktur yang dicadangkan ialah tripeptida, S1 ialah satu komplex kuprum-tripeptida, GHC (Glycine-Histidine-Cystein) dan bergabung dengan ion kuprum, sementara S2 mempunyai 2 tripeptida yang tidak bergabung dengan ion kuprum, GHC dan GHK (Glycine-Histidine-Lysine).

ABSTRACT

SCREENING AND CHARACTERIZATION OF MICROBIAL INHIBITORS AGAINST EUKARYOTIC PROTEIN PHOSPHATASES (PP1 and PP2A)

Soil samples were collected from Sabah forests soils in Ulu Padas, Lower Segama, Melalap, Maliau Basin, Tabin, Kpg Limau-limauwan and area around Kota Kinabalu. A total of 283 actinomycetes included and 44 microfungi were successfully isolated and purified. These isolates were studied and screened against PP1 inhibitor screening system. Two types of yeast were used as the screening targets against protein phosphatase 1; wild-type yeast which carried GLC7 and mutant yeast which carried a high temperature sensitive glc7 allele (glc7-10). The mutant causes cell cycle arrest and cell wall integrity defect at 37°C but was rescued by the addition of 1M sorbitol. A potential inhibitor that acted on the wild-type Glc7p will show similar characteristic as the mutant strain. Crude acetone extracts of H7520, H9318 and H9978 demonstrated positive inhibitions on PP1 inhibitor screening system. Enzymatic inhibitor assays were performed by Prof Michael J.R. Stark, where freeze-dried crude acetone extracts of H9318 and H9978 showed inhibitory activities on PP1 and PP2A while extract H7520 inhibited on PP2A but not PP1. The degree of sensitivity of the potential inhibitors screened against yeast S. cerevisiae, PP1 or PP2A were examined in the haploid-insufficiency test. Strain ASY25 (PPH21/pph21::LEU2 pph22::URA3/PPH22) and ASY27 (glc7::LEU2/GLC7) were both sensitive to freeze-dried crude acetone extract of H9318 and H9978 compare to strain AYS927 (PPH21/PPH21 PPH22/PPH22 GLC7/GLC7). Strain ASY25 was more sensitive to freeze-dried crude acetone extract H7520 compare to strain AYS927 and ASY27. Liquid-liquid extractions were performed and ethyl acetate layer extracts (H7520, H9318 and H9978) exhibited inhibitory activities. Only ethyl acetate layer of H9318 was performed in RP-HPLC. 2 active compounds, S1 and S2 were detected and were fractions out. Fractions S1 and S2 of H9318 showed inhibitory activity on PP1 inhibitor screening system. The degree of sensitivity of the S1 and S2 fractions screened against yeast PP1 or PP2A were examined in the haploid-insufficiency test. Strain ASY27 showed higher sensitivity to fraction S2 compared to other diploid strains. For fraction S1, strain ASY25 was more sensitive than strains AYS927 and ASY27. S1 and S2 inhibited both PP1 and PP2A on enzymatic inhibitor assay. In yeast (S. cerevisiae) cell cycle, fraction S1 showed similar effect as pph22 mutant (binucleate in mother cell) and accumulate multiple nuclei. Fraction S2 showed similar effect as glc7-10 (displays aberrant bud morphology) and also nucleus fragmentation. With the help from Dr Linda Morris and Ling-say Wong, the active fractions of S1 and S2 were identified using MS. The proposed partial structures of these 2 fractions were tripeptides, S1 was a tripeptide-copper complex, GHC (Glycine-Histidine-Cystein) and incorporate with copper ion while S2 had 2 tripeptides and both without any incorporate ion, GHC and GHK (Glycine-Histidine-Lysine).

LIST OF ABREVIATIONS

Abs	Absorbance
DO	Dissolved oxygen
GLC7	The gene that encodes PP1 in yeast, *Saccharomyces cerevisiae*
glc7-10	The mutated gene of *GLC7* which shows temperature sensitivity in which was due to the replacement of the amino acid phenylalanine with leucine at the position 135 on the catalytic subunit of *GLC7* protein
Glc7p	The protein that is encoded by *GLC7*
HV agar	Humic acid agar
MAPK	Mitogen activated protein kinase
min	Minutes
mm	Millimeter
MP media	Mannitol peptone glucose media
PDA	Potato dextrose agar
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPH21	One of the gene encoded PP2A in yeast, *Saccharomyces cerevisiae*
PPH22	One of the gene encoded PP2A in yeast, *Saccharomyces cerevisiae*
YPD	Yeast extract peptone agar

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KEYWORDS

Protein phosphatase 1, protein phosphatase 2A, secondary metabolites, inhibitor, *Penicillium*, *Streptomyces*

CHAPTER 1

INTRODUCTION

1.1 Research Background

Microorganisms are associated with the lives of humans, animals and plants. Selman A. Waksman, who received the Nobel Prize in 1952 for the discovery and the use of the antibiotic streptomycin, said the following (Batzing, 2002):

> *There is no field of human endeavor, whether it be in industry or in the preparation of food or in connection with the problems of shelter or clothing or in the conservation of human and animal health and the combating of disease, where the microbe does not play an important role and often the dominant role.*

Study of microorganisms for the secondary metabolites production has been intensively pursued for many decades. Actinomyetes and fungi are the well known microorganisms which have capability to produce variety of secondary metabolites. Some of secondary metabolites from actinomycetes and fungi can be used as antibiotic. For example, penicillin, a well known antibiotic found in 1929 by Sir Alexander Fleming from *Penicillium notatum* has been used widely in the pharmaceutical field. Streptomycin an antibiotic produced by *Streptomyces griseus*, is used to against the infection that is caused by *Mycobacterium tuberculosis*.

Since then, research on secondary metabolites production from actinomycetes and fungi, has been generated for treatment of various diseases. For example, rapamycin an antifungal agent purified from *Streptomyces hygroscopicus* has immunosuppressive effects where rapamycin inhibit both host rejection following

organ transplantation and the restenosis of coronary arteries after angioplasty besides as TOR inhibitor (Inoki *et al*, 2005). Even tautomycetin produced by *Streptomyces griseochromogenes* (Kobayashi *et al*, 1988) has been found as a specific inhibitor of Protein serine/threonine phosphatase 1 (Mitsuhashi *et al*, 2001) where PP1 is a positive regulator of ras-1 *in vivo* in MAPK signaling pathway (Mitsuhashi *et al*, 2003). Tautomycetin also discover as a novel immunosuppressant in transplantation (Han *et al*, 2003; Chae *et al*, 2004). A fungus strain, *Trichoderma harzianum* produce an antibiotic, harzianic acid that shows antimicrobial activity against *Pasteurella piscicida* (Sawa *et al*, 1994) and later found to be specific inhibitor of Protein serine/threonine phosphatase 2A (Kawada *et al*, 2004) where PP2A serve as a regulatory functions at G_2/M transition and the exit from mitosis (Janssens & Goris, 2001). Cytostatin produced by *Streptomyces* sp (Masuda *et al*, 1995) is a novel inhibitor of PP2A and can inhibit B16 melanoma pulmonary metastasis by the expansion and activation of NK (natural killer) cells (Kawada *et al*, 2003). Fostriecin produced by *Streptomyces pulveraceus* and is the most selective small molecule inhibitor of serine/threonine phosphatases yet discovered. Fostriecin is an antibiotic that displays 40000-fold selectivity for PP2A over PP1 (McCluskey, Sim & Sakoff, 2002).

Signal transduction pathway plays an important role in signaling cell growth, differentiation, development, metabolism and apoptosis. Disruption or over-stimulation can lead to diseases like cancer, neurological disease, diabetes and immune disorder. It is sensible to understand the signaling pathway and search for novel secondary metabolites from microorganisms that can inhibit the signal transduction. For many years protein phosphatases were seen as playing a very passive role as the providers of a blanket level of dephosphorylation to oppose the protein kinases, while the protein kinases were perceived to take the active role in the process. However, it is becoming increasingly clear that protein phosphatase activity

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