SCREENING OF MICROBIAL INHIBITORS AGAINST GLYCOGEN SYNTHASE KINASE-3 IN EUKARYOTIC SIGNAL TRANSDUCTION SYSTEM OBTAINED FROM ACTINOMYCETES USING A YEAST BASED SCREENING SYSTEM

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THIS DISSERTATION IS SUBMITTED TO FULFILL THE PARTIAL REQUIREMENT TO OBTAIN THE DEGREE OF BACHELOR OF SCIENCE WITH HONOURS

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DECLARATION

I hereby declare that the work in this project is of my own except for the quotations and summaries from the references which have been fully acknowledged.

21 February 2005

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ABSTRACT

Screening Glycogen Synthase Kinase-3 (GSK-3) inhibitors is an essential task as GSK-3 inhibitors could serve as drugs to treat diabetes, Alzheimer's disease and other diseases. In this project, a yeast-based screening system comprises of two mutant yeasts, H10075 and H10079 was used to search for GSK-3 inhibitors. A total of 133 (listed in appendix C) actinomycetes crude extracts which contain secondary metabolites were firstly screened using the mutant yeast H10075 to find out whether they have the potential to inhibit mammalian GSK-3β. The mammalian GSK-3β gene was inserted into a plasmid and the plasmid was transformed into a mutant yeast *Saccharomyces cerevisiae*. This was done by Professor Tomoko Andoh of Hiroshima University in Japan. All these 133 extracts screened were made from 133 actinomycetes strains isolated from soil samples collected at Danum Valley Conservation Area, Sabah. Of all these 133 extracts screened, only 3 (H11526, H11668 and H11686) were found to be able to inhibit the mammalian GSK-3β in the mutant yeast. Another 6 extracts (H11520, H11888, H11900, H11612, H11635 and H11911) were found to be toxic against the mutant yeast and the rest have no activity against the mutant yeast. Presence or absence of inhibition zones around paper discs loaded with extracts is a mean to detect the presence or absence of GSK-3 inhibitors in this screening system which is based on temperature sensitivity of the yeast. An extract which has the potential to inhibit mammalian GSK-3β will exhibit inhibition zone around paper disc on the screening plate incubated at 37 °C but not at 25 °C after 72 hours of incubation. An extract which is toxic to the yeast will exhibit inhibition zones on paper discs on both plates. An extract which shows no activity against the yeast will not show any inhibition zones at all on both plates. The 3 extracts of actinomycetes strains H11526, H11668 and H11686 were then screened by using the H10079 mutant yeast based on the same principles above. It was found that these 3 extracts are able to inhibit MCK1, the gene in yeast which is homologous to mammalian GSK-3. It was suggested that these 3 extracts to be further screened by using a screening system based on galactose sensitivity in yeast.

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LIST OF UNITS

°C	Degree Celcius
mg	Milligram
g	Gram
µl	Micro liter
ml	Milliliter
l	Liter
µm	Micrometer
mm	Millimeter
cm	Centimeter
m	Meter
km^2	Kilometer square
psi	Pounds per square inch
rpm	Rounds per minute
M	Molarity (mol dm^{-3})

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CDKs	Cyclin dependent kinases
GBP	GSK-3 Binding Protein
GC	Guanine and Cytosine
GDP	Guanosine Diphosphate
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine Triphosphate
IRS	Insulin Receptor Substrate
NIDDM	Non-Insulin Dependent Diabetes Mellitus
PDK1	Phosphoinositide-dependent protein kinase-1
PIP_3	Phosphatidyl inositol (3,4,5) trisphosphate
PKB	Protein Kinase B
PTB	phosphotyrosine binding
Rog	Revertant of gsk-3
RTK	Receptor protein tyrosine-kinase
Ser	Serine
Ser/Thr	Serine / Threonine
SH2	Src homology 2
Thr	Threonine
Tyr	Tyrosine

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CHAPTER 1

INTRODUCTION

Signal transduction systems are important for cells to respond to stimulation from the environment outside the cells. In eukaryotic organisms, signal transduction is actually a mean of communication between cells, as well as for the cells to respond to stimulations. In signal transduction, signals will be transduced from the environment outside the cell into cells. Signaling molecule which contains the information to be sent to the target proteins will binds to the specific receptor. The receptor will change its conformation, which triggers and activates a relay protein molecule. This relay molecule will activates a protein kinase by phosphorylation. Protein kinases are enzymes that transfer phosphate groups from the ATP molecules to another protein. A phosphorylated protein kinase becomes active and it activates another protein kinase by phosphorylation. This series of protein kinase phosphorylation forms a protein kinase cascade. At the end of the cascade, the last activated protein kinase will phosphorylates the target protein. The target protein will becomes active and carries out its function to triggers cellular response.

Glycogen synthase kinase-3 (GSK-3) is a protein serine threonine kinase that phosphorylates the enzyme glycogen synthase (Doble and Woodgett, 2003). Glycogen synthase is the rate limiting enzyme in glycogen synthesis (Dent *et al.*, 1989). Phosphorylated glycogen synthase become inactive, thus the process of glycogen synthesis is halted. Therefore GSK-3 play a key role in glycogen synthesis, and glycogen synthesis is related to the stimulation of insulin. If GSK-3 is inhibited, the enzyme glycogen synthase is still active and the production of glycogen can takes place, and maintains the blood sugar level. If glycogen synthesis cannot takes place as a result of inactivation by GSK-3, the blood sugar level will increase; leading to the disease such as diabetes mellitus, therefore GSK-3 inhibitors are important to be used as drugs to treat diseases.

The gram positive bacteria Actinomycetes, especially species from the genus *Streptomyces*, can produce various types of useful secondary metabolites during their growth. Certain secondary metabolites are very useful to humans because these secondary metabolites may have the potential of inhibiting certain protein kinases, and can be used as drugs such as antibiotics.

1.1 Objectives Of Research

One of the objectives of this project is to collect soil samples from the tropical rain forest in Danum Valley, Sabah. Next, actinomycetes will be isolated out and purified from the soil samples. These actinomycetes strains are then used to produce secondary metabolites. These secondary metabolites will be tested by using the screening method to find out whether they have the potential in inhibiting the protein kinase Glycogen synthase kinase-3, GSK-3.

1.2 Background Of The Research Site

The tropical rain forest in Danum Valley Conservation Area, Sabah, is chosen as the site to collect soil samples to be used in this project. Danum Valley Conservation Area is located at the south east of Sabah. The area spread across 438 km^2 of land and is an undisturbed lowland rain forest. In this conservation area it has a large variety of plants and lowland fauna, which includes the Sumatran rhino, banteng (tembadau), Asian elephant, clouded leopard, orang utan and proboscis monkey. The area is stocked with trees of the Dipterocarpaceae family. About 90 percent of the Conservation Area is classified as lowland dipterocarp forest with the remaining being low canopy, sub-montane forest. The soils under leaf litter of identified trees will be collected in this area.

CHAPTER 2

LITERATURE REVIEW

2.1 Actinomycetes

Actinomycetes are bacteria which are gram positive. Actinomycetes are GC rich in DNA base composition, with more Guanine and Cytosine bases. Actinomycetes form filaments and spores in their growth (Madigan *et al.,* 2003). Roughly observed, the external morphology of actinomycetes look similar to those of filamentous fungi, but the filaments of actinomycetes are made up of prokaryotic cells and the diameters are smaller than those of fungi. Certain actinomycetes may look more similar like molds, by producing external asexual spores for reproduction. Actinomycetes are soil microbes and usually found in soils that are alkalytic or neutral in pH, rich in humus and good in aeration. Therefore, formation of branches in growth is an advantage for actinomycetes, because this enables actinomycetes to move across gaps between soil particles to move to a new nutritional site (Tortora *et al.*, 2002). Actinomycetes strains used in this project were isolated from soil samples. There are many genuses of Actinomycetes, some of them are *Streptomyces, Frankia, Actinomyces, Mycobacterium, Norcardia, Rhodococcus* and others.

2.1.1 *Streptomyces*

Streptomyces are the best studied actinomycetes. About 500 species of *Streptomyces* are found. *Streptomyces* contain high level of GC in their DNA base composition, which is from 69 percent to 73 percent. Asexual reproductive spores of *Streptomyces* are called conidia and were found at the tips of the aerial filaments. Each conidium can germinates and forms a single colony if placed with a suitable substrate (Tortora *et al.*, 2002). In the filament of *Streptomyces*, the cytoplasm of each cell is continuous with that of the adjacent cells. This is known as the coendocytic feature (Nester *et al.*, 1998). Filaments of *Streptomyces* are about 0.5 μm to 1.0 μm in diameter.

Streptomyces are also bacteria which were mostly isolated from soil. *Streptomyces* require soils which have alkalytic or neutral pH, and low water potential for growth. *Streptomyces* only need nitrogen sources and very rarely need any growth factors to live. But *Streptomyces* can utilize various types of compounds as carbon sources, such as alcohols, organic acids, amino acids, and certain aromatic compounds. *Streptomyces* are aerobic microorganisms and produce extracellular enzymes to digest protein, polysaccharides and many other organic substances in soils. *Streptomyces* can produce gaseous compounds known as geosmins which gives odor to the soil. Geosmins are made up of carbons, hydrogens and oxygens. One of the geosmins regularly found is trans-1,10-dimethyl-trans-9-decalol (Madigan *et al.*, 2003).

Streptomyces are valuable because *Streptomyces* are able to synthesize many commercial used antibiotics. More than 500 types of antibiotics were found to be produced by *Streptomyces*. Certain *Streptomyces* species may produce more than one type of antibiotic. In certain cases, several types of antibiotics synthesized by a species of *Streptomyces* may have no relations or resemblances chemically. One species of *Streptomyces* is immune to the antibiotics synthesized by it, but it becomes sensitive against antibiotics synthesized by another species of *Streptomyces* (Madigan *et al.*, 2003).

2.2 Secondary Metabolites

In cells, there are certain synthetic processes that do not play any roles in the metabolic pathway of an organism. The products of these processes are known as secondary metabolites. Secondary metabolites are not required in both growth and reproduction.

In the growth cycle of a microorganism, secondary metabolites will only start to be synthesized when a large portion of the microorganism has completed the rapid logarithmic growth phase, known as trophophase and has entered the stationary phase in the cell cycle. Idiophase refers to the period during which most of the secondary metabolites are produced. This happens during the stationary phase, which most cells are not multiplying (Tortora *et al.*, 2002).

Production of secondary metabolites is very dependent on the growth conditions, especially the composition of the growth medium. Secondary metabolites usually synthesized in groups which have chemically related structure, but they are totally different. Generally synthesis of secondary metabolites requires many enzymes and involved many complicated steps. For example, 72 enzymatic steps are involved in the synthesis of the antibiotic tetracycline (Madigan *et al.,* 2003).

The starting material for the synthesis of secondary metabolites is usually comes from the major biosynthetic pathways. Therefore the production of secondary metabolites can be considered as a microbial conversion of primary metabolites (Tortora *et al.*, 2002). Primary metabolites such as amino acids, lipids and polysaccharides are among the primary metabolites used in secondary metabolites synthesis (Nester *et al.,* 1998). Many secondary metabolites which are complex in structures are actually originated from precursor molecules which have similar structures (Madigan *et al.,* 2003).

In industrial processes, the products synthesized by microorganisms are secondary metabolites. Secondary metabolites can be obtained in an amount far higher than required in industrial processes (Madigan *et al.,* 2003). Secondary metabolites may have medicinal values, such as antibiotics, antitumor agents or to be used as enzyme inhibitors. Production of secondary metabolites in actinomycetes may be used as defense mechanism; this is because secondary metabolites produced by one type of bacteria can be toxic to other types of bacteria around them, and defect the growth of

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