EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF TRIOSE PHOSPHATE ISOMERASE (TIM) FROM PSYCHROPHILIC BACTERIUM

by

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CERTIFICATE

This is to certify that the dissertation entitled

"Expression and Biochemical Characterization of Triose Phosphate Isomerase (TIM) from a Psychrophilic Bacterium"

is the bonafide record of research work done by

Ms Lee Sze Yea

during the period from July 2009 to October 2009 under my supervision.

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LIST OF SYMBOLS

°C	Degree celcius
%	Percent
α	Alpha
β	Beta
π	Phi
Δ	Delta
μ	Micro

LIST OF ABBREVIATION

AA	Amino acid
APS	Ammonium persulfate
BSA	Bovine serum albumin
dH_2O	Distilled water
GRAVY	Grand average of hydropathy
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria Bertani
M	Molar
mA	Miliampere
min	Minute
mM	Milimolar
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
nm	Nanometer
OD	Optical density
pI	Isoelectric point
rpm	revolution per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
TIM	Triose phosphate isomerase
U	Unit
USA	United Stated America
UV-Vis	Ultraviolet-Visible
v/v	volume/volume
w/v	weight/volume

ABSTRACT

Psychrophiles are organisms that grow rapidly below 20°C. In order to overcome the inherent challenges in cold, cold-active enzymes with high catalytic efficiency at low temperature and heat-labile properties were evolved as one of their adaptive strategies. In this study, triose phosphate isomerase (TIM) of psychrophilic bacterium π9, which was isolated from sea ice of Antarctic at Casey station, was overexpressed in *Escherichia coli* BL21 (DE3) host under IPTG induction and purified to homogeneity for subsequent biochemical characterization. TIM is a dimeric enzyme that consists of two identical subunits, each containing about 250 residues. It catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate in glycolysis. π9 TIM activities at temperatures range from 20 to 45°C were studied. The optimum temperature for π9 TIM activity was found to be in the range of 35 to 40°C. While, thermostability study showed π9 TIM was quite thermostable. It remained stable at 40°C after 2 hours incubation and was gradually inactivated at 50°C. These suggest π9 TIM might not possess psychrophilic features. Other than that, comparative protein sequence analysis that was performed on TIM sequences from psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria revealed an amino acid property groups preference in psychrophilic and mesophilic TIM as compared to thermophilic and hyperthermophilic TIM. The deeper understanding of strategies evolved by TIM enzymes that adapted to varied environments provides contributive information for further studies on those valuable cold-adapted enzymes.

ABSTRAK

Psikrofil adalah organisma yang tumbuh pesat di bawah suhu 20°C. Untuk mengatasi cabaran semula jadi dalam kesejukan, enzim "*cold-active*" dengan kecekapan pemangkinan yang tinggi pada suhu yang rendah dan sifat labil terhadap kepanasan telah berkembang sebagai salah satu strategi adaptif mereka. Dalam penyelidikan ini, "*triose phosphate isomerase*" (TIM) dari Antarctic psikrofilik bakteria π9 yang diasingkan dari laut ais di Stesen Casey telah dihasilkan dalam *Escherichia coli* BL21 (DE3) di bawah rangsangan IPTG dan ditulenkan untuk kajian biokimia yang kemudian. TIM adalah enzim dimer yang terdiri daripada dua subunit identiti yang mempunyai 250 "*residue*" masing-masing. Ia pemangkin penukaran antara dihydroxyaceton fosfat dan D-glycealdehyde-3-fosfat pada glikolisis. Aktiviti π9 TIM pada suhu 20 hingga 45°C telah dikaji. Suhu optimum untuk aktiviti π9 TIM didapati pada suhu 35 hinggan 40°C. Sementara itu, kajian thermostabiliti menunjukkan π9 TIM agak thermostabil. Ia kekal stabil pada suhu 40°C selepas eraman yang selama 2 jam dan ia di-nyahaktifkan secara perlahan pada suhu 50°C. Ini menunjukkan kemungkinan π9 TIM tidak mempunyai sifat psikrofilik. Selain itu, analisis perbezaan rangkaian protein yang dilakukan ke atas rangkaian TIM daripada psikrofilik, mesofilik, thermofilik dan hyperthermofilik menemui keutamaan kumpulan amino acid dalam TIM psikrofilik dan mesofilik berbanding dengan TIM thermofilik dan hyperthermofilik. Pengetahuan tentang strategi yang dikembangkan oleh enzim TIM yang dapat menyesuaikan diri dengan persekitaran yang berbeza harus didalami bagi menyediakan maklumat yang menyumbang kepada kajian lanjutan ke atas enzim "*cold-adapted*".

CHAPTER 1

INTRODUCTION

Earth biosphere is the part of the earth, which includes atmosphere, hydrosphere and lithosphere, in which living organism are found, and with which they interact to form the global ecosystem (Park, 2001). Originally, the concept was applied just to the earth surface where obviously occupied by plants and animals. Therefore, biosphere was thought as a very thin layer around the earth. However, the actual thickness of the biosphere on earth became hard to measure as more organisms live at unexplored extreme environment were discovered. These organisms, known as extremophiles, are organisms that not only survive but actually require the specific extreme environmental condition that beyond the normal acceptable range, which are too harsh for normal life to exist, for their survival and growth (Satyanarayana *et al.*, 2005).

Extremophiles are classified according to its environmental niche difference. Psychrophiles, one type of extremophiles, are organisms that grow rapidly at about 15°C or lower, having a maximal temperature for growth at about 20°C, and a minimal temperature for growth at 0°C or below (Morita, 1975). They are found at permanently cold terrestrial environment as well as at aquatic niche, snow, glacier, sea ice, and other cold ecosystems, which in facts occupied more than three-quarters of the earth surface. Cold environments restrict growth of organisms. Hence, the ability of these organisms to survive and proliferate at low temperatures indicates a vast array of cold adaptations of them, which enables their colonization in these extreme environments.

Low temperatures slow down biochemical reaction rates catalyzed by enzymes, thus, it strongly inhibits the life at cold environments. To counteract the negative effect of cold on the activity of an enzyme, some enzymes of psychrophiles have evolved sufficient activities and efficiencies to support the growth of cold-tolerant organisms at low temperatures. Those enzymes are known as psychrophilic enzymes or cold-active enzymes, which are enzymes that have high catalytic efficiency at low temperatures, and are inactivated at moderate temperature (D'Amico *et al.*, 2006, Gerday *et al.*, 2000).

In recent years, increased attention has been focused on psychrophilic enzymes. These enzymes are suggested to display high catalytic activities at low temperatures by having improved flexibility at active site and more rigidity at other protein regions that are not involved in the catalysis, as compared to their mesophilic counterparts. Due to these attractive properties, psychrophilic enzymes offer considerable potential for fundamental research and biotechnological application. Their application in the detergent and food industries, and for the production of fine chemicals are significant (Gerday *et al.*, 2000). Industrial application of cold-active enzymes has greatly increased because of their active catalytic activity at extreme conditions. Besides that, they have energy saving advantage and consecutive economic benefits originate from their specific properties (Hoyoux *et al.*, 2004).

Triose phosphate isomerase (TIM) is a dimeric enzyme formed by two identical subunits each consisting of about 250 residues. It is a central enzyme in the glycolytic pathway. Beginning from glucose, glycolytic pathway is catalyzed by the sequential

action of ten enzymes, in which, TIM enzyme catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate (Figure 1).

Glycolysis is one of the most universal metabolic pathways found in all living organisms. It is the initial process of many carbohydrate catabolism pathways, which include the most usual glucose metabolism and also metabolism of fructose, galactose, and other carbohydrates. It mainly serves two principal functions, which are generating energy and providing intermediates for other metabolic pathways. Glycolysis provides majority of the organism's energy requirement. Hence, TIM is an important enzyme in the energy-harvesting reaction which sustains the life of organism.

TIM enzyme has a tertiary α/β barrel structure. Each TIM monomer structure has eight α-helices alternating with eight β-strands along the polypeptide chain. The βα units fold up in a regular way, such that the β-strands are hydrogen-bonded to each other and form an interior, solvent-excluded, eight-stranded β-barrel, which surrounded by the eight α-helices on the outside (Alvarez *et al.*, 1998, Maes *et al.*, 1999).

In this research, TIM enzyme of bacterium π9, an Antarctic psychrophile that was isolated from sea ice of Antarctic at Casey Station, was purified and characterized. The gene from the psychrophilic bacterium has been isolated and cloned into a pET14b plasmid. Overexpression and purification of TIM were carried out. Enzymatic assays were performed to analyze its biochemical characteristics. This study served to gather information on the enzyme produced by a psychrophilic bacterium. The understanding of

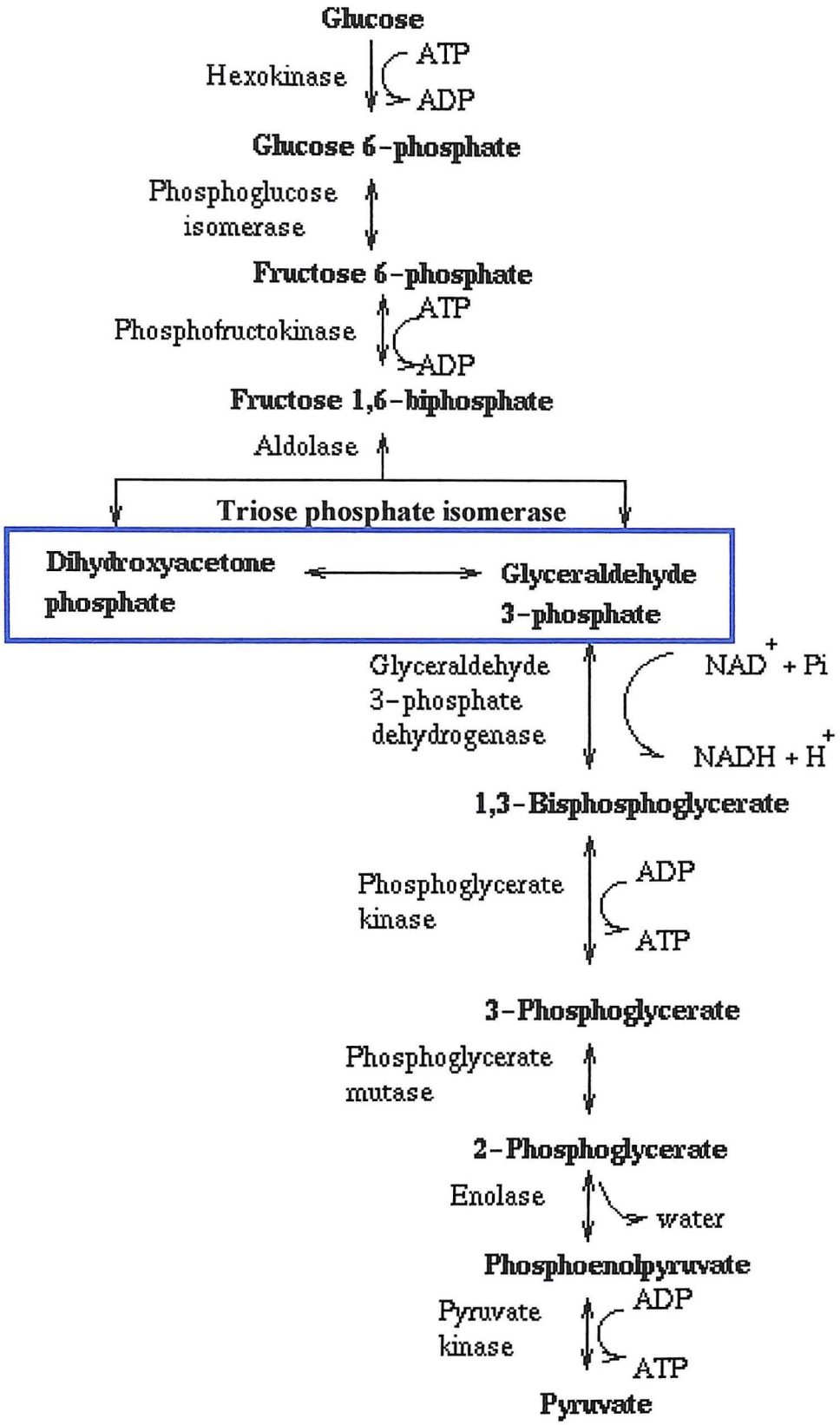

Figure 1: Glycolytic pathway. Reaction catalyzed by TIM is shown in blue box.

the properties of enzyme from psychrophilic bacterium is commonly used to gain further insights of the adaptive strategies of cold-active enzymes. By having a deeper understanding of their evolved strategies in cold-adaptation, their application in industrial and biotechnological uses can be enhanced.

CHAPTER 2

OBJECTIVES OF RESEARCH

The main objectives of this study include:

1. To overexpress TIM enzyme of bacterium π9 as His-tag fusion protein in *Escherichia coli* BL21 (DE 3).

2. To purify TIM enzyme of bacterium π9 to homogeneity.

3. To determine the optimum temperature for π9 TIM activity.

4. To study the thermal stability of π9 TIM at different temperatures.

CHAPTER 3

LITERATURE REVIEW

3.1 Life in the cold

Life under low temperatures was reported as early as at 1887 by Forster, who isolated microorganisms from fish that could grow well at 0°C (Zecchinon *et al.*, 2001). Since then, numerous organisms particularly prokaryote but also eukaryote, have been found successfully colonized permanently cold environments.

Temperature limits the growth of organism. Living at cold environments requires organisms to overcome the barriers, which include: reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; protein cold-denaturation; inappropriate protein folding; and intracellular ice formation, that inherent to low temperatures (D'Amico *et al.*, 2006). Hence, thrives of psychrophilic organisms at cold environments somehow imply the evolution of these organisms at the level of their membranes, constitutive proteins and enzymes, which enables them to adapt to low temperatures.

In order to sustain the growth in these extreme conditions, psychrophiles have been found to develop various adaptive strategies from the molecular level to that of the whole organism. Those adaptations include: the regulation of membrane fluidity; the synthesis of specialized molecules known as cold-shock proteins, cryoprotectors and antifreeze molecules; the regulation of ion channels permeability; microtubules

polymerization, seasonal dormancy, and importantly, the modification of enzyme kinetics (Georlette *et al.*, 2004).

3.2 Characteristics of cold-active enzymes

One of the main challenge to survive at low temperature is the exponentially decrease in the rate of biochemical reactions with any decrease in temperature. Therefore, to make the organism compatible with life in cold, enzymes that capable of catalyze the biochemical reactions occurring within an organism are an essential target for the cold adaptation. Synthesis of cold-active enzymes was found to be one of the strategies developed by psychrophilic organisms. Those psychrophilic enzymes are the main physiological adaptation at the enzyme level (Feller and Gerday, 2003). They have higher catalytic activity at low temperatures, and thus, enable psychrophiles to maintain appropriate rate for enzyme-catalyzed reactions that are involved in essential biochemical processes.

Temperature profiles of enzymes from psychrophilic and their mesophilic counterparts were first determined by Morita (Morita, 1975). To date, extensive studies on the thermodependence of psychrophilic enzymes activities had revealed that these cold-active enzymes were adapted to have their higher catalytic activities at temperatures lower than their mesophilic and thermophilic counterparts (Brenchley, 1996). Study of the lactate dehydrogenase from *Vibrio marinus* by Mitchell and coworkers (1985) showed that psychrophilic lactate dehydrogenase had an optimum activity at 10 to 15°C, which was different from that described for their mesophilic counterparts. In another

work, researchers reported that alkaline metalloprotease from psychrophilic *Pseudomonas sp.* was three times more active at 20°C than its mesophilic counterpart (Chessa *et al.*, 2000). The shift of psychrophilic enzyme activity to a lower temperature range renders psychrophiles to cope with the reduction of chemical reaction rate induced by low temperatures.

Besides that, comparison of temperature dependence activity of psychrophilic enzymes with their mesophilic homologues have showed that their high efficacy at low temperatures often up to an order of magnitude higher than those observed for their mesophilic homologues (Feller and Gerday, 2003, Georlette *et al.*, 2004). This interesting phenomenon was shown in a study of cellulase CelG from Antarctic bacterium *Pseudoalteromonas haloplanktis* (Garsoux *et al.*, 2004). The study revealed that psychrophilic cellulase is at least 15 times more active at 4°C than its mesophilic counterparts.

Another main property of cold-active enzymes which have been characterized is their heat lability. This feature was commonly observed in studies, for example, in the study of isocitrate lyase from *Colwellia maris*. The psychrophilic isocitrate lyase has been shown to rapidly inactivate at the temperatures above 30°C (Watanabe *et al.*, 2001). In another study of psychrophilic alanine racemase, the enzyme showed heat sensitivity. The enzyme activity was lost quickly after incubation over 35°C for 1 hour, while mesophilic and thermophilic enzymes showed stability up to 55°C and 75°C respectively, under the same conditions (Okubo *et al.*, 1999).

The higher catalytic activity at low temperatures and low stability of cold-adapted enzymes as compared to their mesophilic and thermophilic counterparts were proposed as a result of increased structural flexibility (Lonhienne *et al.*, 2000). This hypothesis was verified as studies have proved that cold-adapted enzymes have higher flexibility in the catalytic important areas of the structure as compared to their mesophilic homologues (Georlette *et al.*, 2004, Olufsen *et al.*, 2005). Apart from that, the occurrence of structural flexibility and rigidity responsible for high efficacy of cold-active enzymes is also supported by analysis of the activation parameters (Lonhienne *et al.*, 2000).

3.3 Kinetic adaptation

Cold-adapted enzymes were characterized as enzymes with enhanced catalytic efficiency k_{cat}/K_m. The analysis of the differences between activation parameters of psychrophilic enzymes with those of their mesophilic counterparts indicates that the high k_{cat} of cold-active enzymes at low temperatures is due to a significant decrease of the activation enthalpy $\Delta H^{\#}$ (D'Amico *et al.*, 2002). The low activation enthalpy results in less temperature dependence of psychrophilic enzyme activity than the mesophilic enzymes, and it was considered as the main kinetic adaptive character to low temperatures.

The decrease in activation enthalpy is achieved structurally by a decrease in the number of enthalpy-driven interactions that have to be broken during catalysis. As these interactions are contributing to the conformation of the active site, reduced number of