EFFECT OF BUFFER COMPOSITION ON THE BACKGROUND ACTIVITIES OF CHOLINE KINASE

by

LEE SHIN YONG

Dissertation submitted in partial fulfillment of the requirements for the degree of

Bachelor of Health Sciences (Biomedicine)

May 2013

CERTIFICATE

This is to certify that the dissertation entitled "Effect of buffer composition on the background activities of choline kinase" is the bonafide record of research work done by Miss Lee Shin Yong during the period from September 2012 to May 2013 under my supervision.

ACKNOWLEDGEMENT

I would like to thank all whom directly and indirectly contribute to my thesis writing.

I would like to express my gratitude towards my supervisor, Dr Few Ling Ling for her guidance throughout my final year project. Not to forget, I would also like to thank Dr. See Too Wei Cun too. Furthermore, I would also like to thank Miss Chang Ching Ching and Mr. Sam Kuan for guiding me. They had taught me a lot and help me a lot throughout the project. Thanks for their patience in guiding me.

I would like to thank my parents for giving me their full support. Not only that, I would also like to express my gratitude towards my friends for their continuous support.

Besides that, I would like to thank all the staff at School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian for guiding me or helping me in any way throughout the year. Thanks to Desasiswa Murni for providing me a hostel to stay throughout the year which lessen my burden in travelling to and fro to the school.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOL, ABBREVIATION AND ACRONYMN

Symbol and Abbreviation	Description
%	Percentage
°C	Degree Celcius
α	Alpha
β	Beta
µg	Microgram
µL	Microlitre
ADP	Adenine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CCT	CTP: phosphocholine cytidylyltransferase
cDNA	Complementary DNA
CK	Choline kinase
CKA-2	*Caenorhabditis elegans* choline kinase
CPT	Choline phosphotransferase
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E	Enzyme
E. coli	*Escherichia coli*
ECT	CTP:phosphoethanolamine cytidylyltransferase
EDTA	Ethylenediaminetetraacetic acid
EK	Ethanolamine kinase
EPT	Ethanolamine phosphotransferase
ES	Enzyme-substrate complex
hCK	Human choline kinase
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilo dalton
K_m	Michaelis-Menten constant
LB	Luria Bertani
LDH	Lactate dehydrogenase
mA	Milliampere
mg	Milligram
mL	Millilitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
NADH	Nicotinamide adenine dinucleotide

NaOH	Sodium hydroxide
Ni-NTA	Nickel nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
P	Product
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PK-LDH assay	Pyruvate kinase-lactate dehydrogenase assay
PMSF	Phenylmethylsulfonyl fluoride
rpm	Round per minute
RNA	Ribonucleic acid
S	Substrate
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	Unit
UV	Ultraviolet
V	Volt
V_0	Initial velocity
v/v	Volume per volume
V_{max}	Maximum reaction rate
w/v	Weight per volume
x *g*	Fold gravity

ABSTRAK

Kolina kinase (hCK) manusia telah menjadi salah satu enzim popular dalam bidang penyelidikan. Enzim ini memangkinkan pemfosforilasi kolina untuk membentuk fosfokolina dengan bantuan ATP dan ion magnesium. hCK mempunyai tiga isoform iaitu CKα1, CKα2 dan CKβ yang dikodkan oleh dua gen berlainan, *Chkα* dan *Chkβ*. Pengasaian enzim gandingan piruvat kinase-laktat dehidrogenase (PK-LDH) telah digunakan untuk menentukan aktiviti enzim CKα dan CKβ. Pra-aktiviti enzim telah diperhati sebelum substrat kolina ditambah ke dalam reaksi kimia. Pra-aktiviti enzim yang dikesan melalui pengasaian enzim mungkin akan mempengaruhi ketepatan pengukuran aktiviti CK. Oleh itu, pelbagai penampan pada pH 8.5 telah digunakan untuk membuat perbandingan antara sistem penampan yang berlainan terhadap pra-aktiviti enzim untuk CKα2 dan CKβ. Tujuan penyelidikan ini dijalankan adalah untuk meminimumkan pra-aktiviti kolina kinase dengan menggunakan pelbagai sistem penampan dalam pengasaian enzim. Penampan yang digunakan dalam pengasaian enzim termasuklah penampan borat, glisina-NaOH, HEPES, Tris-asetat, Tris-maleat, dan trisin. Penentuan parameter kinetik untuk CKα2 dan CKβ diteruskan dengan penampan yang dapat mengurangkan pra-aktiviti enzim. Penampan yang dapat mengurangkan pra-aktiviti enzim termasuklah penampan glisin-NaOH, HEPES dan trisin. Hasil penentuan parameter kinetik daripada enzim CKα2 bersama dengan kolina sebagai substrate memberi V_{maks} yang paling tinggi sebesar 87.41 μmol/min/mg dengan menggunakan penampan trisin, manakala, penampan HEPES memberi V_{maks} yang paling tinggi iaitu 41.60 μmol/min/mg untuk CKβ. Untuk substrat ethanolamina, V_{maks} yang paling tinggi untuk CKα2 dan CKβ diperolehi dengan penampan trisin sebesar 37.71 dan 13.86 μmol/min/mg masing masing.

Sebagai intiha, penampan glisin-NaOH, HEPES dan trisin dapat memansuhkan pra-aktiviti CKα2 dan CKβ. Nilai aktiviti enzim ini juga bergantung kepada sistem penampan yang digunakan dalam pengasaian enzim.

ABSTRACT

Choline kinase has become one of the popular enzymes that has recently been studied extensively as it functions to catalyze the phosphorylation of choline to form phosphocholine in the presence of ATP and magnesium. Choline kinase exists in three isoforms which are CKα1, CKα2 and CKβ encoded by two separate genes, *Chkα* and *Chkβ*. Pyruvate kinase-lactate dehydrogenase (PK-LDH) coupled enzymatic assay was used to determine the activities for hCKα2 and hCKβ. Background activity was the activity observed before choline was added into the reaction. The existence of background activity in enzymatic assay of hCKα2 and hCKβ affects the accuracy of measurement for choline kinase. Thus, different buffer systems at pH 8.5 were used to compare the effect of these buffers towards the background activity of hCKα2 and hCKβ. The aim of this study was to reduce the background activity of choline kinase by using different buffer systems (pH 8.5) for enzymatic assay. The buffers tested for enzymatic assay were borate, glycine-NaOH, HEPES, Tris-acetate, Tris-maleate and tricine. Kinetic parameters for hCKα2 and hCKβ were determined with buffers that removed the background activity of the enzymes. The buffers that abolished the background activity of choline kinase were glycine-NaOH, HEPES and tricine buffers. The highest V_{max} for hCKα2 of 87.41 μmol/min/mg with choline as the substrate was obtained in tricine buffer, while HEPES buffer gave the highest V_{max} of 41.60 μmol/min/mg for hCKβ. With ethanolamine as substrate, the highest V_{max} for both hCKα2 and hCKβ were obtained in tricine buffer with the values of 37.71 and 13.86 μmol/min/mg respectively. In conclusion, glycine-NaOH, HEPES and tricine buffers were able to remove the background activities of the hCKα2 and hCKβ. The activity level of these enzymes were also dependent on the buffer system used in enzymatic assay.

CHAPTER 1 INTRODUCTION

1.1 Background of Study

This study focuses on the background activity of the choline kinase. Background activity of enzyme choline kinase is the activity that appeared in pyruvate kinase-lactate dehydrogenase (PK-LDH) assay before the substrate is added into the reaction. This background activity was observed in hCKα2 and hCKβ. However, reasons behind the appearance of background activity of choline kinase was unknown. Thus, it gives an opportunity to solve this problem of background activity.

There are several factors that could affect the measurement activity of enzyme such as pH and temperature. Since hCKα2 and hCKβ are mammalian choline kinase, the temperature of the assay was fixed at 37°C. The pH of the assay was a reference to the methods in Malito *et al.*, 2006. Screening of the optimum pH was previously carried out but it did not eliminate the background activity. Several other factors in the enzymatic assay may also affect the background activity of choline kinase. The idea of using different buffers has come up to see the effect of different buffer composition on the background activity of choline kinase.

After screening with seven different buffers, the results were evaluated and those buffers that were able to abolish the background activity of the enzyme were proceeded with determination of kinetic parameters. Hereby, the K_m and V_{max} of the different buffers were compared with Tris-HCl buffer that was originally used in the PK-LDH coupled assay.

1.2 Objectives

1.2.1 General Objective

- To reduce the background activity of the enzymatic assay of choline kinases

1.2.2 Specific Objectives

- To express and purify recombinant human choline kinases
- To compare different buffer systems (pH 8.5) for enzymatic assay of choline kinases
- To determine the enzyme kinetic parameters of hCKα2 and hCKβ with different buffer systems

1.3 Significance of Research

There is a background activity in the enzymatic assay system that disturbs the measurement of total activity of the enzyme. Thus, this study will facilitate the accurate measurement of choline kinase activities in future. Besides that, it can also be a reference.

CHAPTER 2 REVIEW OF LITERATURE

2.1 Choline Kinase

2.1.1 Structure and Function

Choline kinase is an enzyme involved in catalyzing the first step of Kennedy Pathway. It is responsible in catalyzing the phosphorylation of choline to form phosphocholine in the presence of ATP and magnesium ions (Ishidate, 1997).

The choline kinase was found by Wittenberg and Kornberg in the year 1953. In mammalian including human, choline kinase presents as three isoforms, hCKα1, hCKα2 and hCKβ encoded by two separate genes, *Chkα* and *Chkβ*. The hCKα1 (50 kDa, 439 amino acids) and hCKα2 (52 kDa protein with 459 amino acids) were found to be derived from identical gene which was *Chkα* by alternative splicing (Aoyama *et al.*, 2000; Aoyama *et al.*, 2004). The cloning of hCKβ produces a protein with 395 amino acids. In a comparison of both hCKα1 and hCKα2, hCKα2 was found to have an insertion of 18 amino acids into CKα1 sequence. hCKα1 and hCKβ are 40% identical (Gallego-Ortega *et al*, 2011). Studies found that hCKα was highly expressed in both testis and liver, while hCKβ was found in heart and liver (Aoyama *et al.*, 2004). The summary of CK isoforms of several organisms is listed in Table 2.1.

Choline kinase plays a role in cell transformation as the increased levels of choline kinase and phosphocholine has been detected in several mammalian tumours such as breast, lung, colon and prostate tumors (Aoyoma, 2004; Ramirez *et al.*, 2005). The CKα has been found to mediate human cell transformation and induce *in vivo* tumorigenesis (Ramirez *et al.*, 2005). Not only that, in a genetic study, the deletion of

Table 2.1: Summary of CK isoforms of several organisms

Origin	Source	Gene	GenBank Accession No.	Amino acids	SDS-PAGE (kDa)
Rat	(Uchida and Yamashita, 1992)	Ck-α1	D10262	435	50
	(Uchida, 1994)	CK-α2	D37885	453	52
	(Aoyama *et al.*, 1998b)	CK-β	AB006607	394	42
Mouse	(Aoyama *et al.*, 1998a)	CK-α1	AB011002	436	50
		CK-β	AB011000	394	42
Human	(Hosaka *et al.*, 1992)	Ck-α2	NM_001277.2	456	52
		Ck-α1	NM_212469	439	50
		CK-β	AB029886	395	45
S. cerevisiae	(Hosaka *et al.*, 1989)	CK1	J04454	582	66

CKα could cause embryonic lethality while loss of function of CKβ activity has caused muscular dystrophy and neonatal bone deformity (Wu *et al.*, 2009).

2.2 CDP-Kennedy Pathway

The Kennedy pathway has two branches which are CDP-ethanolamine and CDP-choline pathways (Figure 2.1). Both consist of three enzymatic steps. First, the choline kinase catalyzes the phosphorylation of choline to phosphocholine and the byproduct ADP, in the presence of ATP and Mg^{2+} (Vance, 2002; Ishidate, 1997). The second step of the pathway involves the CTP:phosphocholine cytidylyltransferase catalyzes the formation of CDP-choline from phosphocholine and CTP (Vance, 2002; Kent, 1997). Lastly, the choline phosphotransferase (CPT) catalyzes the condensation reaction of CDP-choline with diacylglycerol (DAG) (Vance, 2002; McMaster and Bell, 1997).

2.3 Choline and Ethanolamine

Choline (Figure 2.2) is important for all cells for structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signalling and lipid-cholesterol transport and metabolism (Zeisel and Blusztajn, 1994; Zeisel, 2006b). The ethanolamine is also involved in the Kennedy Pathway in synthesizing phosphatidylethanolamine which is the second most abundant mammalian phospholipid membrane Thus, ethanolamine also play a role in structural integrity of cell. Besides that, it is also important in cellular processes such as membrane fusion (Deeba *et al.*, 2005), cell cycle (Emoto *et al.*, 1996), autophagy (Ichimura *et al.*, 2000), and apoptosis (Emoto *et al.*, 1997). The structure of ethanolamine was shown in Figure 2.3.

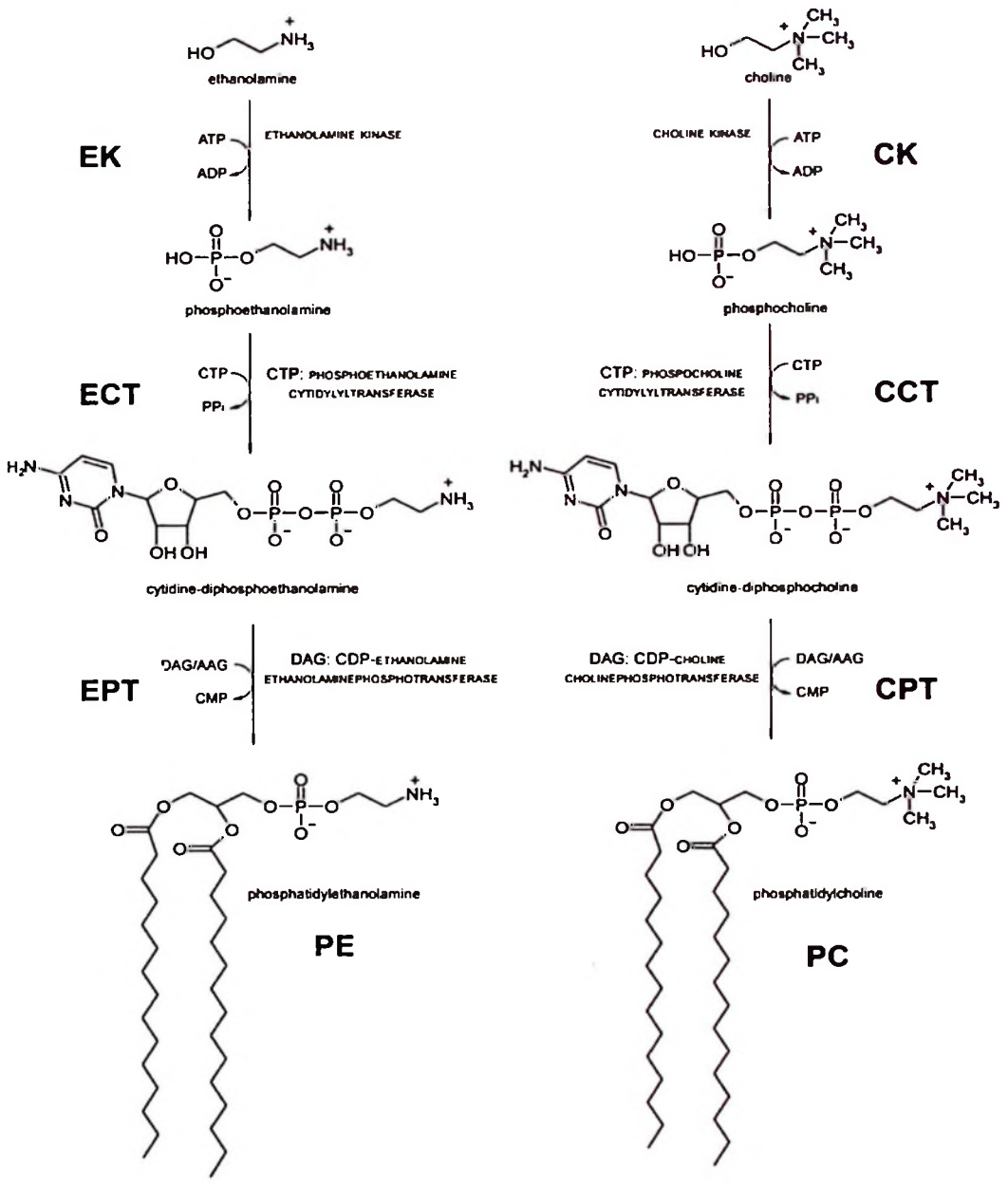


Figure 2.1 : Kennedy Pathway

Abbreviation :

EK - Ethanolamine Kinase
ECT- ethanolamine-phosphate cytidylyltransferase
EPT- ethanolamine phosphotransferase
CK- Choline Kinase
CCT-choline-phosphate cytidylyltransferase
CPT- choline phosphotransferase
PC - Phosphocholinase

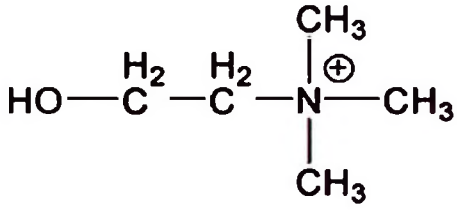


Figure 2.2 : Structure of Choline

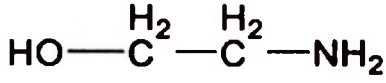


Figure 2.3 : Structure of Ethanolamine

2.4 The Michaelis-Menten Equation

The Michaelis-Menten equation (Eq. 2.1) was introduced by Michaelis and Menten but further developed by Briggs and Haldane (Michaelis and Menton, 1913; Briggs and Haldane, 1925). This equation is characterized by two constants which are the Michaelis- Menten constant, K_m and the indirectly obtained catalytic constant, k_{cat}.

2.4.1 Kinetic Parameters of Michaelis-Menten Equation

Kinetic description of an enzyme activity is needed to understand the function of enzyme. The Michaelis constant, K_m and the maximal rate, V_{max} are the usual kinetic parameters that are being used to describe the enzyme activity. These kinetic parameters can be derived from rates of catalysis measured at a variety of substrate concentrations if an enzyme works according to this formula (Eq. 2.1) :

$$V_o = V_{max} \frac{[S]}{[S]+ K_m} \quad \text{.....(Eq. 2.1)}$$

The derivation of K_m and V_{max} are derived with curve-fitting programs on a computer. The K_m value for an enzyme is dependent on the particular substrate and also the environment factors such as pH, temperature and ionic strength. The K_m value is defined as the concentration of the substrate which half of the active sites are filled. The K_m value also reflect the concentration of substrate required for the significant catalysis to occur. Besides that, K_m is related to this equation :

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} E + P$$

K_m is also the dissociation constant of the ES complex if k_2 is much smaller than k_{-1}. In this condition, K_m measures the strength of the ES complex where a high K_m indicates a weak binding, while a low K_m indicates strong binding. When k_{-1} is greater than k_2, the K_m indicates the affinity of ES complex only.

V_{max} shows the turnover number of an enzyme that represents the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. It is equal to the kinetic constant k_2 which is also known as k_{cat}. The turnover number of an enzyme is determined if the concentration of active sites $[E]_T$ is known in this equation (Berg *et al.*, 2002) :

$$V_{max} = k_2 [E]_T$$

2.5 Measurement of Enzyme Activity

In order to study the reaction mechanism of purified enzymes from individual organisms or tissues, enzymatic assay has been used to determine the enzyme activity and constants in Michaelis-Menten equations. Enzyme activity is being determined through the measurement of amount of product formed or substrate consumed under known conditions of temperature, pH and substrate concentration. There are a few methods being used to measure enzyme activity such as spectrophotometric method and enzyme immunoassays.

2.5.1 Quantification Techniques

Quantifications techniques have been used to determine the changes in concentration of substrates or products of enzymatic reactions. The commonly used principles includes UV-visible spectrophotometry (Bergmeyer, 1987), fluorimetry (Gomes *et al.*, 2006; Greenberg, 1962), and luminometry (Ching, 1982; Cole *et al.*, 1967; Fan and Wood, 2007).

Spectrophotometric methods are commonly used due to the fact that many reactions involve directly or indirectly the oxidized and reduced forms of NADH that

absorbed specifically at 340 nm. This spectrophotometric property of NADH can be useful in monitoring enzyme reactions as coupling agent in secondary reaction with the product (Matsumoto and Ukeda, 2005). Fluorometric measurement involved the usage of fluorogenic substances that are commercially available. Luminometric method which involves luciferase and luciferin as substrates is commonly used to measure ATP, ADP and AMP (Cole *et al*, 1967).

Radioactivity method is being used when conventional methods cannot achieve specificity or sensitivity in detecting the enzyme activity. However, throughput of such methods are usually low and care in handling waste is needed as it may affect environment health and safety. Besides that, mass spectrometry can also be used in determining enzyme activities (Gries, 2007).

2.5.2 Coupling Reactions

Some of the enzyme activities could not be measured directly. One or more coupled reactions are being used to convert a product of the enzyme reaction to another quantifiable product. One of the example is the pyruvate kinase-lactate dehydrogenase (PK-LDH) assay. If velocity of the coupling system equals the velocity of the reaction of interest, thus, the coupled assay is valid. Efficient coupling happens when steady-state concentrations of the product of the primary reaction are smaller than the corresponding K_m (Storer and Cornishb, 1974).

2.5.3 Measurement of Choline Kinase Activity

Radioactive assay is one of the most common enzyme assay being used to measure the activity of choline kinase. Besides that, the choline kinase activity can also

be determined by using the periodide precipitation method of Appleton *et al.*, 1953 where it measure the residual of choline. Not only that, pyruvate kinase-lactate dehydrogenase assay can also be used to measure the activity of choline kinase (Malito *et al.* 2006).

2.6 Factors Affecting on Enzyme Activity

The factors that affect the enzyme activity includes pH, temperature and concentration of the substrate. The pH affects the environment of the reaction in the assay. Slight changes in pH could result in enzyme denaturation and lead to loss of catalytic activity. Most of the enzymes only work in a narrow pH range. Thus, it is important to optimize the pH of assay as at optimum pH, enzyme exhibits maximum activity. Buffers help to maintain optimum pH for an enzyme.

Besides that, temperature also affects the activity of an enzyme. At high temperatures, the molecules move faster and collide even more frequently. Thus, the substrate molecules and enzymes collide more frequently. When the temperature increases over a certain threshold, the increased in energy will cause disruptions of the tertiary structure of enzyme. Thus, optimization of temperature is needed for an enzyme to exhibit its maximum activity. Human enzymes such as choline kinase usually have optimum temperature at 37°C as human body temperature.

When the substrate concentration increases while the enzyme concentration is at its constant, at certain point, the enzyme reaches its maximum extent of activity. When the enzyme is working at its maximum extent, there is no more active site to bind to the incoming substrate molecules. The rate of an enzyme accepting substrate molecules and

releasing product molecules at substrate saturation is decided by its turnover number. Turnover number of an enzyme is defined as the number of substrate molecules transformed per minute by one molecule of enzyme under optimum conditions of temperature, pH and saturation. (Stoker, 2010).

2.7 Buffers

Buffers are compounds that undergo reversible protonation and, thus aid in maintaining the pH of a solution (Thiel *et al*, 1998). It is also important in biological reactions that are often sensitive to small changes in pH. Buffer system with different pH are often used to optimize pH in assays.

Tris-HCl buffer is one of the commonly used buffer in assays. It is inexpensive and readily available in highly purified form. Tris buffer is widely used as a component of a buffer solution such as Tris-acetate-EDTA (TAE) buffer. Tris is a nucleophile and has a lone pair of electrons on its sole nitrogen atom. However, it also has its disadvantages where it displaces electron transport and phosphorylation. Besides that, Tris-borate-EDTA (TBE) buffer and Tris-acetate-EDTA (TAE) buffer form complexes with DNA. (Stellwagen *et al.*, 1997; Stellwagen *et al.*, 2000, Stellwagen *et al.*, 2002).

Glycine buffer, on the other hand, has a physiological range of pH 8.2 to 10.1. Tris and glycine have amine group as their buffering chain side at alkaline pH. The amine group of Tris is in NH_2 form while amine group of glycine is in the NH_3^+ form (Hethey *et al.*, 2002).

HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) is one of the ideal biological buffer suggested by Good and Izawa (1968). It has a physiological range between pH 6.8-8.2. Besides that, its pK_a is not strongly affected by ionic strength, temperature or even buffer concentration. It does not form complexes with metal or interfere with most chemical reactions. Apart from the good points, HEPES is found to inhibit acetylcholine-induced currents in Helix neurons (Witte *et al.*, 1985) and competitively inhibit Na-independent binding of γ-aminobutyric acid (GABA) to its receptor (Tunnicliff and Smith, 1981).

Good's buffers refer to the group of buffers described in the research of Dr. Norman Good *et al.* in 1966. These buffers are selected because of their characteristics that are useful in biology and biochemistry research. Good and his coworkers (1966) uses nine criteria to evaluate the new hydrogen ion buffers which includes tricine buffer. The nine criteria includes high solubility in water, minimal salt effects and temperature effects, stability in solution and absence of absorption bands in the visible and ultraviolet regions of the spectrum. Tris(hydroxymethyl)methylglycine which is also known as tricine is one of the Good buffer. The physiological range for this tricine buffer is between pH 7.2 to pH 8.5. Tricine buffer is considered as a weaker base in comparison to glycine buffer. The good criteria of tricine buffer includes its solubility up to 0.8 M at 0°C, stable in solution, could be purified easily and the compound exists in zwitterionic form.

The physical properties and ionization reaction of buffers is summarized in Table 2.2.

Table 2.2 : Physical Properties of Buffer

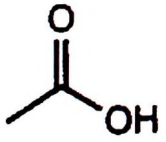
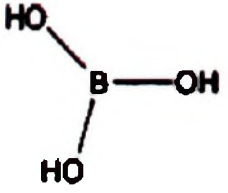
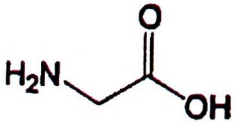
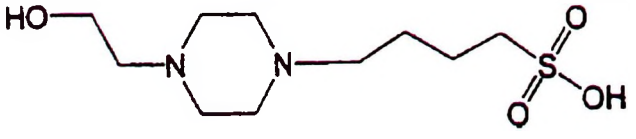
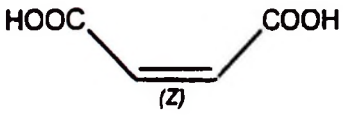
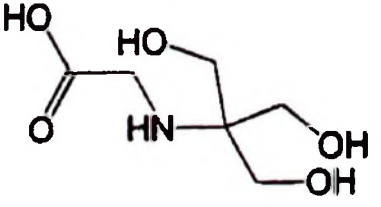
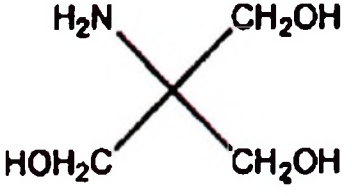
Name	Structure	Ionization Reaction	pK
Acetate		$CH_3COOH=H^+ + CH_3COO^-$	4.756
Borate		$H_3BO_3=H^+ + H_2BO_3^-$	9.237
Glycine		$H_2L^+=H^+ + HL^\pm$ (1) $HL^\pm=H^+ + L^-$ (2) where $HL=C_2H_5NO_2$	9.780 (for reaction 2)
HEPES		$HL^\pm=H^+ + L^-$, where $HL=C_{10}H_{22}N_2O_4S$	7.564

Table 2.2 : Continued

Name	Structure	Ionization Reaction	pK
Maleate		$HL^- = H^+ + L^{2-}$ (2) where $H_2L = C_4H_4O_4$	6.27
Tricine		$HL^{\pm} = H^+ + L^-$ (2) where $HL = C_6H_{13}NO_5$	8.135
Tris		$HL^+ = H^+ + L$, where $L = C_4H_{11}NO_3$	8.072

Data from Goldberg *et al.* (2002)

CHAPTER 3 MATERIALS AND METHODS

3.1 Workflow of the Study

The chart for workflow of the study is shown in Figure 3.1

3.2 Materials

3.2.1 Chemicals

The chemicals used in this study are listed in Table 3.1.

3.2.2 Molecular Biology Reagents

The molecular biology reagents used in this study are listed in Table 3.2.

3.2.3 Consumable Items

The consumable items used in this study are listed in Table 3.3.

3.2.4 Apparatus and Instruments

All apparatus and instruments used throughout this study are listed in Table 3.4.

3.2.5 Computer Softwares

The computer softwares used in this study are listed in Table 3.5.

3.2.6 Microorganisms

The lists of microorganisms used during the study are listed in Table 3.6.

3.2.7 Plasmids and Oligonucleotides

Plasmid vectors and oligonucleotides used in this study are listed in Table 3.7

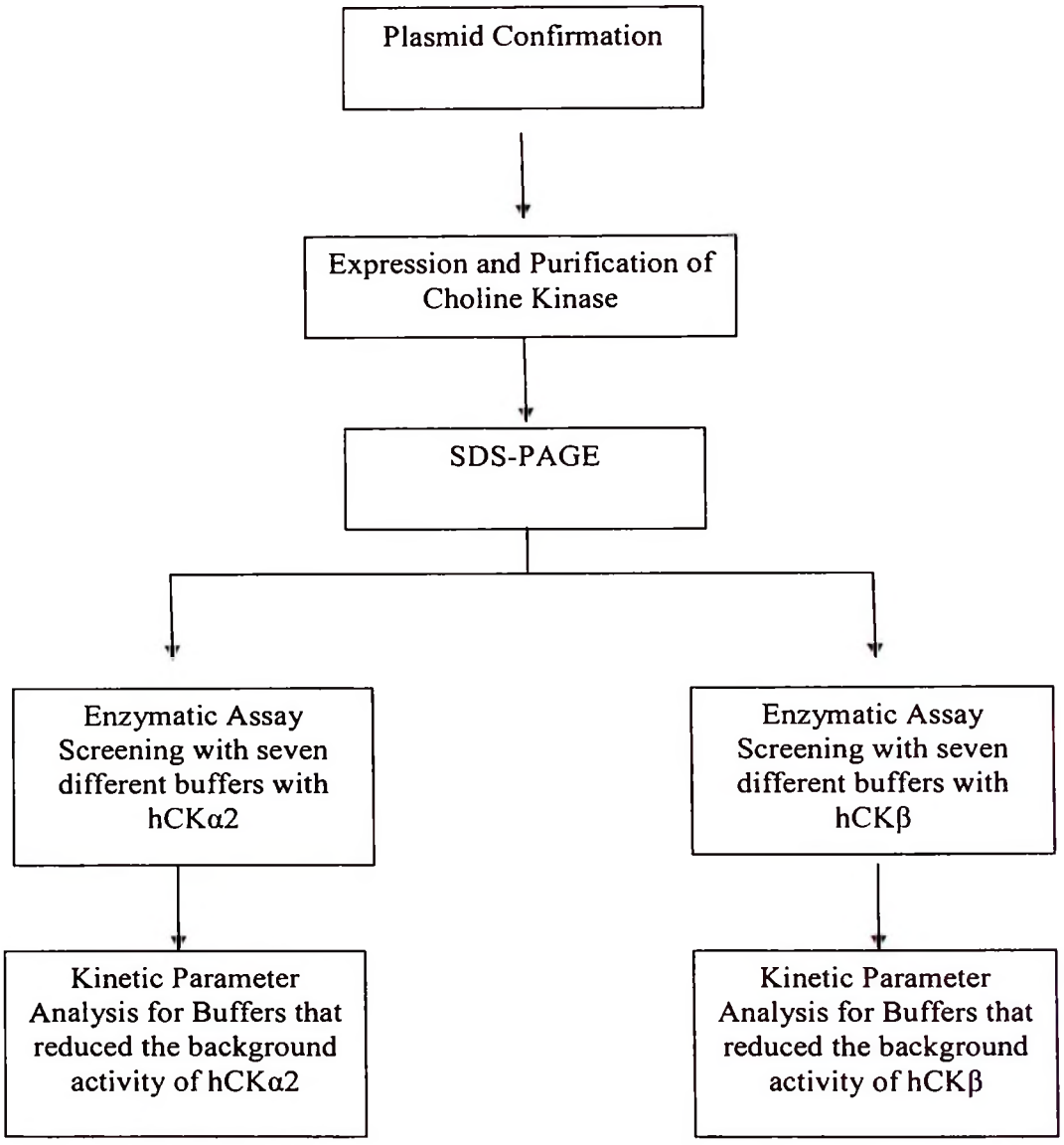


Figure 3.1 : Workflow of the Study

Table 3.1 : List of Chemical Reagents

Name	Manufacturer	Location of Origin
30% Acrylamide/Bisacrylamide Solution	Bio-Rad	California, USA
Acetic acid (glacial) 100%	Merck	Darmstadt, Germany
Ammonium persulfate (APS)	Bio-Rad	California, USA
Ampicillin sodium salt	Amresco	Ohio, USA
ATP disodium trihydrate	Amresco	Ohio, USA
B-mercaptoethanol	Amresco	Ohio, USA
Bio-Rad Protein Assay Reagent	Bio-Rad	California, USA
Boric Acid	Sigma-Aldrich	Missouri, USA
Bromophenol blue	Amresco	Ohio, USA
Calcium chloride	Sigma-Aldrich	Missouri, USA
Choline chloride	Sigma-Aldrich	Missouri, USA
Coomassie brilliant blue R-250	Amresco	Ohio, USA
Ethanolamine	Sigma-Aldrich	Missouri, USA
Ethidium bromide	Amresco	Ohio,USA
Ethylenediaminetetraacetic acid (EDTA) disodium salt	Asia Pacific Specialty Chemicals	New South Wales, Australia
Glycerol	Amresco	Ohio, USA
Glycine	Merck	Darmstadt, Germany
HEPES	Merck	Darmstadt, Germany
Hydrochloric acid fuming 37%	Merck	Darmstadt, Germany

Table 3.1 : Continued

Name	Manufacturer	Location of Origin
Imidazole	Merck	Darmstadt, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Fermentas	USA
Kanamycin Sulphate	Amresco	Ohio, USA
L-arabinose	Merck	Darmstadt, Germany
LB broth, Miller	Merck	Darmstadt, Germany
Magnesium chloride	Merck	Darmstadt, Germany
Maleic Acid	Merck	Darmstadt, Germany
Methanol	Merck	Darmstadt, Germany
Reduced Nicotinamide adenine dinucleotide (NADH) disodium salt trihydrate	Amresco	Ohio, USA
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich	Missouri, USA
Phospho(enol)pyruvic acid monopotassium salt (PEP)	Sigma-Aldrich	Missouri, USA
Potassium chloride	Merck	Darmstadt, Germany
Protease Inhibitor Cocktail, EDTA free	Roche	Mannheim, Germany
SeaKem LE Agarose	Cambrex	Iowa, USA
Sodium chloride	Merck	Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Amresco	Ohio, USA
Tetramethylethylenediamine (TEMED)	Vivantis	Selangor, Malaysia

Table 3.1 Continue

Name	Manufacturer	Location of Origine
Tris-Hcl	Amresco	Ohio, USA
Tricine	Amresco	Ohio,USA
Triton X-100	Amresco	Ohio,USA

Table 3.2 : List of Molecular Biology Reagents.

Name	Manufacturer	Location of Origin
6x DNA Loading Dye	Fermentas	Ontario, Canada
*Bam*H1	New England Biolabs	Massachusetts, USA
GeneRuler DNA Ladder Mix	Fermentas	Ontario, Canada
*Nde*I	New England Biolabs	Massachusetts, USA
Ni-NTA agarose	QIAGEN	Hilden, Germany
PageRuler Unstained Protein Ladder	Fermentas	Ontario, Canada
Pyruvate kinase(PK) from rabbit muscle	Sigma	Missouri, USA
L-Lactate dehydrogenase (LDH) from rabbit muscle	Sigma	Missouri, USA
Taq DNA polymerase	Fermentas	Ontario, Canada

Table 3.3 : List of Consumable Items.

Name	Manufacturer	Location of Origin
0.5 mL PCR tube	Axygen	California, USA
1.5mL microcentrifuge tube	Axygen	California, USA
1.6mL cuvette	Greiner Bio-One	Frickenhausen, Germany
15mL & 50mL centrifuge tube	TPP	Trasadingen, Switzerland
Glasswares	DURAN	Mainz, Germany
Pipette tips	Axygen	California, USA

Table 3.4 : List of Apparatus and Instruments

Name	Manufacturer	Location of Origin
Adhesive matting incubator shaker	Infors HT	Bottmingen, Switzerland
Agarose gel electrophoresis tank	Owl	USA
Autoclave	Hirayama	Saitama, Japan
Balance	Mettler Toledo	Greifensee, Switzerland
Bioimaging system	UVP	California, USA
Centrifuge	Hettich	Tuttlingen, Germany
Deep Freezer (-80°C)	Ilshin	Seoul, Korea
Freezer (-20°C)	Fiocchetti	Luzzara, Italy
Ice maker	Scotsman	Edinburgh, UK
Incubator	Binder	Tuttlinger, Germany
pH meter	Hanna Instruments	Rhode Island, USA
Micropipette	Gilson	Wisconsin, USA
Power pack	Bio-Rad	California, USA
Refrigerator	Hitachi	Tokyo, Japan
Rotator	Becton Dickinson	New Jersey, USA
UV-visible spectrophotometer	Varian	California, USA
Vortex mixer	Erla	Selangor, Malaysia

Table 3.5 : List of Computer Softwares.

Name	Application	Developer	Web Page
Adobe Acrobat 8 Professional	For creating, editing and viewing file in Portable Document Format (PDF)	Adobe System Inc.	http://www.adobe.com/Acrobat
Graphpad Prism 5	Curve fitting and scientific graphing program	GraphPad Software, Inc.	http://www.graphpad.com/prism/prism.htm
Microsoft Office 2007	For word processing, spreadsheets, presentations and graphics	Microsoft Corp.	http://office.microsoft.com/