VERIFICATION OF GLUTAMATE AS THE AMINO ACID RESIDUE RESPONSIBLE FOR MANGANESE ION PREFERENCE IN *ENTAMOEBA HISTOLYTICA* CHOLINE KINASE

by

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LIST OF SYMBOLS AND ABBREVIATIONS

AAG	Alkyl-acylglycerol
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Amp	Ampicillin
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CAEP	Ceramide aminoethyl phosphonate
CCT	Cytidine triphosphate:phosphocholine cytidylyltransferase
CMP	Cytidine monophosphate
CPT	Cytidine diphosphate-choline:1,2-diacylglycerol Cholinephosphotransferase
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECT	Cytidine triphosphate:phosphoethanolamine cytidylyltransferase

EDTA	Ethylenediaminetetraacetic acid
EhCK	Entamoeba histolytica choline kinase
EK	Ethanolamine kinase
EPT	Cytidine diphosphate-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
ESR	Erythrocyte sedimentation rate
GAD	Glutamate decarboxylase
Glu	Glutamate
Gln	Glutamine
GST	Gluthathione S-transferase
h	Hour
hCK	Human choline kinase
HCl	Hydrochloric acid
hEK	Human ethanolamine kinase
IHA	Indirect haemagglutination assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
$K_{0.5}$	Apparent dissociation constant
kDa	Kilo Dalton
L	Liter
LB	Luria-Bertani
LDH	Lactate dehydrogenase

Mn^{2+}	Manganese ion
Mg^{2+}	Magnesium ion
min	Minute
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National Center for Biotechnology Information
°C	Degree Celcius
OD	Optical density
ORF	Open reading frame
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PEtn	Phosphoethanolamine
PK	Pyruvate kinase
PS	Phosphatidylserine
RE	Restriction enzyme
rpm	Revolutions per min
s	Second
SDS	Sodium dodecyl sulphate

TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	Thermus aquaticus
TEMED	Tetramethylethylenediamine
Tm	Melting temperature
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	Unit
UV	Ultra violet
V	Volt
v/v	Volume to volume
w/v	Weight to volume
$\times$ g	Fold gravity
V_{max}	Maximum velocity

ABSTRAK

Entamoeba histolytica merupakan parasit protozoa yang menyebabkan jangkitan amebiasis dan merupakan penyebab masalah kesihatan awam yang utama di negara yang sedang membangun. Jangkitan amebiasis mungkin tidak bergejala, membawa gejala yang ringan ataupun yang teruk seperti sakit abdomen, cirit-birit ringan, cirit-birit berdarah ataupun kolitis teruk dengan kematian tisu dan perforasi. Membran plasma *E. histolytica* merupakan komponen yang penting untuk pengawalan bahan yang memasuki sel dan sitotosisiti berkait dengan sentuhan. Komponen yang utama (60-70%) dalam membran plasma *E. histolytica* ialah fosfolipid. Fosfatidilkolina merupakan salah satu fosfolipid yang utama pada membran *E. histolytica*. Proses sintesis fosfatidikolina bermula dengan pemfosforilan kolina oleh kolina kinase. Penggunaan ion magnesium (Mg^{2+}) sebagai kofaktor oleh kolina kinase daripada pelbagai organism dalam proses pemfosforilan telah diketahui umum. Namun begitu, kajian sebelum ini menunjukkan bahawa kolina kinase *E. histolytica* lebih cenderung menggunakan ion mangan (Mn^{2+}) berbanding dengan Mg^{2+}. Aktiviti EhCK meningkat sebanyak 24 kali ganda dengan kehadiran Mn^{2+}. Perbandingan jujukan amino asid kolina kinase dan ethanolamina kinase yang terpilih telah dibuat dan tiga amino asid termasuk Glu-100 telah dikenalpasti dan dijangka bertanggungjawab terhadap kecenderungan EhCK terhadap Mn^{2+}. Kajian ini bertujuan untuk mengenalpasti peranan Glu-100 dalam interaksi dengan Mn^{2+} ion. Mutasi telah dibuat untuk menggantikan Glu-100 dengan glutamin (Q). ORF EhCK-E100Q dan EhCK telah diklonkan ke dalam vektor pGEX-RB dan induksi penghasilan dan penulenan protein telah dibuat. Kedua-dua protein tersebut telah digunakan

untuk asai spektrofotometri gabungan piruvat kinase-laktat dehidrogenase. Kepekatan Mn^{2+} yang berlainan telah digunakan untuk menganalpasti $K_{0.5}$ EhCK dan EhCK-E100Q. $K_{0.5}$ untuk EhCK dan EhCK-E100Q didapati masing-masing ialah 10.5 mM and 9.14 mM. Sebagai kesimpulannya, kajian ini telah menunjukkan bahawa asid amino Glu-100 yang dikenalpasti bukan asid amino spesifik yang bertanggungjawab terhadap ion Mn^{2+}. Kajian yang lain boleh dilakukan pada masa hadapan untuk mengenalpasti asid amino yang berinteraksi dengan Mn^{2+}. Kajian ini berguna untuk penyelidikan perencatan EhCK pada masa hadapan.

ABSTRACT

Entamoeba histolytica is a parasitic protozoan that causes amoebiasis, a major public health problem in developing countries. Amoebiasis can be presented with no, mild, or severe symptoms such as abdominal pain, mild diarrhea, bloody diarrhea or severe colitis with tissue death and perforation. The plasma membrane of *E. histolytica* is important in its invasiveness and contact dependence cytotoxicity. The major component of its plasma membrane (60-70%) is phospholipid. Phosphatidylcholine (PC) is one of the predominant phospholipids of the plasma membrane in *E. histolytica.* PC synthesis begins with phosphorylation of choline by choline kinase (CK). It is widely accepted that the CK of many organisms prefer Mg^{2+} as their cofactor for phosphorylation. However, previous studies showed an unusual preference of *E. histolytica* choline kinase (EhCK) towards Mn^{2+} ion. EhCK activity was shown to increase 24 folds in the presence of Mn^{2+}. Based on the protein sequence alignment, three amino acid residues, including glutamate-100, were identified and predicted to be responsible for the preference of Mn^{2+} ion as a cofactor. The aim of this study was to validate the role of glutamate-100 in Mn^{2+} ion cofactor preference. Glutamate-100 was replaced with glutamine (E100Q) utilizing PCR site directed mutagenesis. Mutant EhCK-E100Q and wild type EhCK open reading frame (ORF) were respectively cloned into pGEX-RB vectors. The proteins were expressed and purified. Both of the proteins were used in the assay by employing pyruvate kinase-lactate dehydrogenase coupled spectrophotometric assay. Different Mn^{2+} concentrations were used in the assay in order to determine the $K_{0.5}$. The $K_{0.5}$ for wild type EhCK and EhCK-E100Q were 10.5 mM and 9.14 mM, respectively. In conclusion, this study showed that the predicted amino acid glutamate-100 was not the specific amino acid residue

that was responsible for the protein preference using Mn^{2+} as its cofactor. Further studies need to be carried out on other amino acid residues to identify the correct amino acid that actually plays the role in the Mn^{2+} preference. This study lays the groundwork for future study on EhCK inhibition.

CHAPTER 1

INTRODUCTION

1.1 *Entamoeba histolytica*

1.1.1 *E. histolytica* Epidemiology

Amoebiasis is caused by the parasite *E. histolytica* and it is the second leading cause of death from parasitic disease worldwide (Stanley, 2003). Approximately 50 million cases of invasive *E. histolytica* disease occured worldwide each year, resulting in as many as 100,000 deaths (Ximénez *et al.*, 2009). Most amoebic infections occur in Central and South America, Africa, and Asia (Petri and Singh, 1999).

In the United States, the overall prevalence of amoebiasis is approximately 4%. The prevalence of amoebiasis increases in immunocompromised persons, male homosexuals and persons living in communal settings. There are several studies to evaluate the association of amoebiasis with AIDS (Bowley *et al.*, 2006). However, the AIDS pandemic impact on the prevalence of invasive amoebiasis remains controversial. Some reports stated that invasive amoebiasis does not increase in patients with HIV infection (Moran *et al.*, 2005); however, others reported that amebic liver abscess is an emerging parasite infection in individuals with HIV infection living in disease-endemic areas, as well as in non–disease-endemic areas (Hung *et al.*, 2008).

Pathogenic *E. histolytica* is morphologically identical with the non-pathogenic *E. dispar* (Gonin and Trudel, 2003) but they are different biochemically and genetically (Hamzah *et al.*, 2006). Asymptomatic *E. dispar* infection is 10 times more common than *E. histolytica*

infection, only 10% of *E. histolytica* infections cause invasive disease. Thus, only 1% of persons with *Entamoeba* infection develop symptomatic amoebiasis with stool microscopy findings.

The ability of *E. histolytica* trophozoites to invade the intestinal mucosa caused clinical manifectations such as amoebic colitis and amoebic liver abscess (Stanley, 2001).

1.1.2 *E. histolytica* Structure

E. histolytica is a pseudopod-forming non-flagellated protozoan parasite existed in two forms which are invasive trophozoites and infective cyst form depending on their life cycle (Petri *et al.*, 1999). *E. histolytica* cysts are round in shape, usually 10-15 µm in diameter. They are surrounded by a refractile wall (Stanley, 2003). The trophozoites of *E. histolytica* are 10-20 µm. A picture of *E. histolytica* trophozoites was shown in Figure 1.1.

1.1.3 *E. histolytica* Life cycle and Mode of transmission

The cysts and trophozoites of *E. histolytica* are passed in feces. The ingestion of mature cycts in fecally-contaminated food or water is the main reason of *E. histolytica* infection. When the ingested cyst reaches small intestine, excystation occurs and trophozoites are released, the trophozoites then migrate to the large intestine. In the large intestine, trophozoites reproduce through binary fission to produce cyst. Both cyst and trophozoites are able to pass in the feces. Cysts can survive in external environment because of protection conferred by their walls and they are responsible for transmission. Trophozoites are mainly confined in the intestinal lumen of asymptomatic carriers. The invasion of trophozoites in intestinal mucosa causing pathologic manifestations in patients (CDC, 2013). The life cycle of *E. histolytica* was illustrated in Figure 1.2.

E. histolytica can be transmitted to human through fecally contaminated food and water. Accidental consumption of *E. histolytica* cyst from contaminated surface or fingers also causes the transmission of *E. histolytica* (CDC, 2013) .

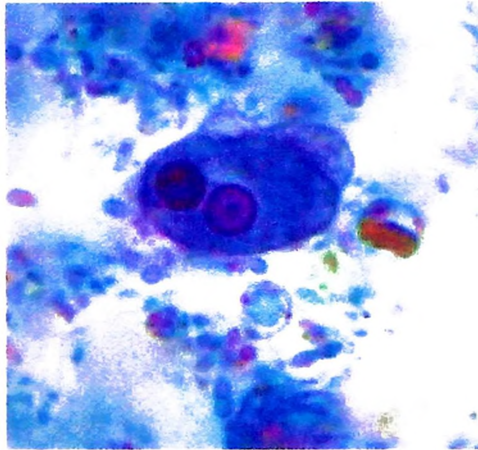

Figure 1.1 *E. histolytica* trophozoites.

Trichome stained *E. histolytica* trophozoites with ingested erythrocytes. The ingested erythrocytes appeared as dark inclusions. The parasite above showed nuclei that have the typical small, centrally located karyosome, and thin, uniform peripheral chromatin (CDC, 2013).

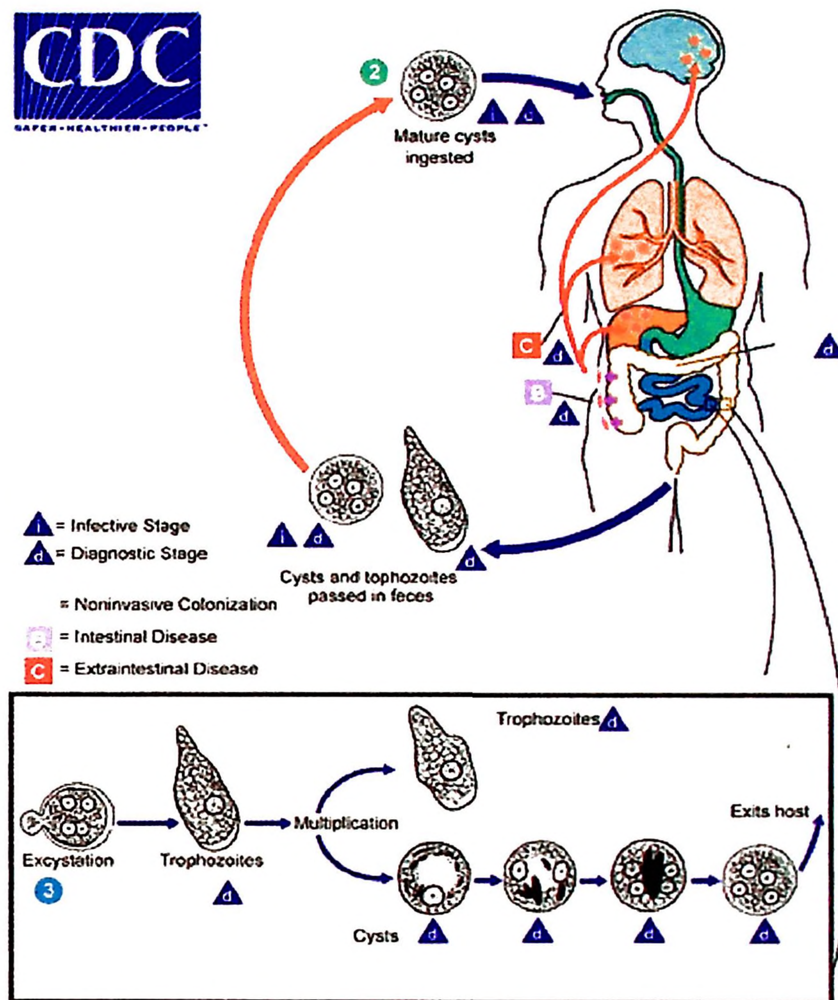

Figure 1.2 Life cycle of *E. histolytica* (CDC, 2013).

1.2 Amoebiasis

1.2.1 Clinical Manifestations of Amoebiasis

The major clinical manifestations of *E. histolytica* are amoebic colitis and amoebic liver abscesses (Stanley, 2001). Amoebic colitis is characterized by ulceration and inflammation of the colon. Amoebiasis infection caused by *E. histolytica* leads to severe gut inflammation. This is the reason why amoebic colitis is always confused with inflammatory bowel disease (Tucker *et al.*, 1975). Amoebic liver abscess is the most common extraintestinal manifestation of *E. histolytica* infection. It has circumscribed regions of dead hepatocytes, liquefied cells and cellular debris surrounded by a rim of connective tissue, with a few inflammatory cells and amoebic trophozoites (Reed *et al.*, 1988).

The spreading of *E. histolytica* trophozoites through the submucosal layers of the intestine caused the formation of ulcers and the presence of neutrophils and other inflammatory cells in the lamina propria and submucosal layers (Prathap and Gilman, 1970). Besides, there are also other clinical manifestations caused by *E. histolytica* infection, such as pleuropulmonary amoebiasis, cerebral amoebiasis, amoebic pericarditis, amoebic peritonitis, genitourinary amoebiasis, amoeboma and amoebic appendicitis (Dhawan, 2015).

1.2.2 Diagnosis of Amoebiasis

The World Health Organization has recommended that intestinal infection can be diagnosed with an *E. histolytica*–specific test (WHO, 1997). Immunologic and parasitology techniques in diagnosing amoebiasis have been established. Clinical findings from immunologic studies that are able to be obtained from blood test include elevated erythrocyte sedimentation rate (ESR), leukocytosis without eosinophilia, elevated transaminase level, elevated alkaline phosphatase level, mild elevated bilirubin level as well as reduced albumin level. These

clinical findings are shown in 80% of the patients infected with amoebiasis. However, the diagnosis cannot be 100% confirmed. Thus, other laboratory studies are also employed in the diagnosis of amoebic infections that include microscopy, culture, serologic testing and polymerase chain reaction (PCR) assay (Dhawan, 2015). However, the detections of amoebiasis through culture and PCR are mostly for research purpose, they are yet to be approved for clinical diagnostic use (Haque *et al.*, 1998).

E. histolytica stool antigen detection test is the only available specific test for pathogenic amoeba *E. histolytica* (Haque *et al.*, 1998). The detection of serum antibodies to amoeba is an important adjunct to antigen detection. In case of amoebic liver abscess, most patients do not have detectable parasites in the stool, thus, the presence of antibodies to amoebae is very useful for diagnosis (Petri and Singh, 1999). Tests for antibodies to amoebae are >90% sensitive for amoebic liver abscess and 70% sensitive for amoebic colitis. If antigen detection is negative, colonoscopy may be helpful in diagnosis of amoebic colitis.

Serological test of *E. histolytica* is useful in the developed countries. *E. histolytica* antibody detection such as indirect hemagglutination assay (IHA) is useful for amoebiasis detection. This assay involves the binding of *E. histolytica* antibodies that are present in serum to *E. histolytica* antigen sensitized red cells. However, this method is not able to distinguish between current infection and past infection which makes it a non-practical method to be practiced in high endemic area.

Microscopic examination from fresh stool smear for trophozoites contained ingested red blood cell is most commonly done (Freedman *et al.*, 2006). Microscopic examination can be done by saline wet mount technique to observe for motile trophozoites, iodine-stained wet

mount or by using trichrome staining method. However, sole microscopic examination is not able to differentiate between *E. histolytica* and *E. dispar.*

1.2.3 Treatment of Amoebiasis

Amoebiasis treatment includes pharmacologic therapy, surgical intervention, and preventive measures. Drugs used for treating amoebiasis are metronidazole, nitroimidazole, chloroquine and broad-spectrum antibiotics for bacterial superinfection (Dhawan, 2015). Surgical intervention might be needed for certain conditions which involve perforated amoebic colitis, massive gastrointestinal bleeding and toxic megacolon (Athié-Gutiérrez *et al.*, 2010). Amoebiasis can be prevented by taking good care of basic hygiene, water treatment and preventing fecally contaminated food and water through improved sanitation.

1.3 Phospholipid Metabolism in *E. histolytica*

1.3.1 Plasma Membrane of *E. histolytica*

Plasma membrane of *E. histolytica* was first isolated after stabilization by crosslinking the surface glycoproteins of intact cells with concanavalin A (Aley *et al.*, 1980). Studies on the plasma membrane showed that *E. histolytica* trophozoites are relatively rich in lipid where 60-70% of its phospholipid is composed of lipid (Sawyer *et al.*, 1967). The lipid components of the membrane, the cholesterol to phospholipid molar ratio in the plasma membrane is 0.89 (Aley *et al.*, 1980). Phospholipid composition of the plasma membrane of *E. histolytica* differed substantially from the composition of whole cells and internal vesicles (Sawyer *et al.*, 1967). Plasma membrane aids in the movement of *E. histolytica.* The cell surface molecules on *E. histolytica* also play an important role in determining the invasiveness of *E. histolytica* and the contact dependence of *in vitro* trophozoite cytotoxicity (Trissl *et al.*, 1977).

The great membrane forming capability of the amoebae is due to its high phospholipid to protein ratio. The presence of heterodimeric glycoprotein on the plasma membrane allows *E. histolytica* to adhere to host cell to perform contact-dependent killing and also as complement resistance (Petri and Mann, 1993). All of these features make *E. histolytica* to be invasive to human, particularly its plasma membrane that acts as a protection which protecting itself from its own pore-forming toxin.

1.3.2 Kennedy Pathway

The *de novo* biosynthesis of phosphatidulethanolamine (PE) and phosphatidylcholine (PC) were discovered by Kennedy and Weiss in 1956 using rat liver enzyme. The PE and PC branches of this pathway are based on the formation of high-energy intermediates cytidine diphosphate-ethanolamine (CDP-ethanolamine) for PE synthesis and cytidine-diphosphocholine (CDP-choline) for PC synthesis (Gibellini and Smith, 2010). Kennedy pathway is divided into two branches known as CDP-ethanolamine and CDP-choline pathway, respectively. The reaction is catalyzed by ethanolaminephosphotransferase (EPT) or cholinephosphotransferase (CPT). CMP is produced as its by-product (Gibellini and Smith, 2010) as visualized in Figure 1.3.

The products of Kennedy pathway, PE and PC play critical roles in human parasites as they are the essential structural components of parasite membranes. Studies had shown that the *de novo* pathway for the biosynthesis of PC is very important for *Plasmodium falciparum* development and survival. The inhibition of PC biosynthesis by selective inhibitors of CK ultimately leads to the parasite's growth inhibition as well as infection prevention in animal model (Alberge *et al.*, 2010).

1.3.2.1 Phosphatidylethanolamine (PE)

Two PE species (PE_1 and PE_2) were identified through the separation of *E. histolytica* phospholipids by two-dimensional thin-layer chromatography (Aley *et al.*, 1980). PE is the major component of membrane phospholipid in most bacteria followed by phosphatidylglycerol and cardiolipin, it is synthesized via phosphatidylserine (PS) decarboxylation (Dowhan, 1997). In bacteria, PE is a macromolecule precursor such as lipopolysaccharides (LPS) and periplasmic membrane derived oligosaccharide (Raetz *et al.*, 2007). In eukaryotes PE is the donor for the phosphoethanolamine capping of the glycosylphosphatidylinositol anchor that is required for protein attachment on cell surface (Menon *et al.*, 1993).

1.3.2.2 Phosphatidylcholine (PC)

In *E. histolytica*, the major phospholipid is PC. Besides, other components such as phosphatidic acid, phosphatidylinositol, phosphatidylserine and two species of phosphatidylethanolamine were also present (Espinosa-Cantellano and Martínez-Palomo, 1991). Sphingomyelin and ceramide aminoethyl phosphonate (CAEP) are present as minor component in *E. histolytica* plasma membrane (Cerbón and Flores, 1981). PC levels were substantially lower in the plasma membrane while CAEP, PE and PS were higher (Aley *et al.*, 1980).

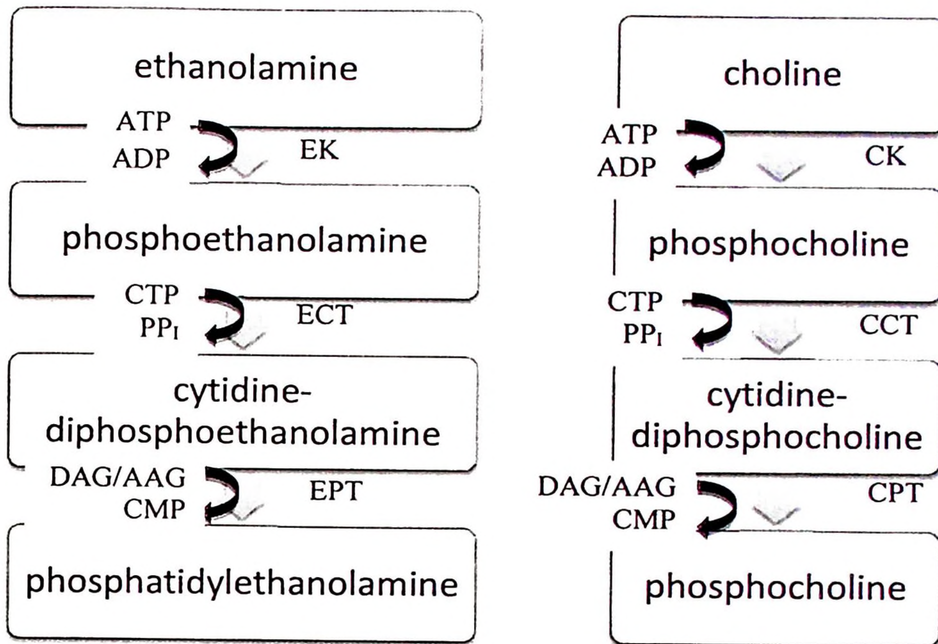

Figure 1.3 The two branches of the Kennedy pathway.

The CDP-ethanolamine and the CDP-choline pathways (Gibellini and Smith, 2010). Enzymes: AAG, alkyl-acylglycerol; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CK, choline kinase; CCT, phosphocholine cytidylyltransferase; CMP, cytidine monophosphate; CPT, cholinephosphotransferase; CTP, cytidine triphosphate; DAG, diacylglycerol; EK, ethanolamine kinase; ECT, phosphoethanolamine cytidylyltransferase; EPT, ethanolaminephosphotransferase.

1.3.2.3 Choline Kinase (CK)

CK was first described as a cytosolic enzyme that was able to phosphorylate both choline and ethanolamine (Shields *et al.*, 2003) in the initial step of Kennedy Pathway. Usually, CK utilizes both choline and ethanolamine as substrates although choline is much preferable, EKs are generally specific for ethanolamine (Gibellini and Smith, 2010). Overexpression of EK in mammalian cells does not alter the steady-state levels of PE but it leads to the accumulation of glycerol-phosphoethanolamine (Lykidis *et al.*, 2001). In contrast, changes in CK activity influence the rate of PC synthesis.

1.4 *Entamoeba histolytica* Choline Kinase (EhCK)

E. histolytica choline kinase (EhCK) and ethanolamine kinase (EhEK) are responsible for the phosphocholine and phosphoethanolamine synthesis in *E. histolytica.* The gene of *EhCK* is located at position 109,594 to 110,670 and *EhEK* is located at 247,582 to 248,757 within the positive strand of *E. histolytica* genome (Chang, 2012). The length of *EhCK* is 1077 bp while *EhEK* is 1176 bp. The gene lengths of *EhCK* and *EhEK* are the same as their respective mRNA length which indicates that there are no introns on both genes (Chang, 2012). No ethanolamine phosphorylation activity was detected in *EhCK* with 5 mM or 10 mM ethanolamine while no choline phosphorylation activity was detected in *EhEK* (Chang, 2012). This indicates that *EhCK* is a choline-specific kinase while *EhEK* is an ethanolamine-specific kinase.

1.4.1 Ion preference in EhCK

Mammalian CK is widely known to use Mg^{2+} as cofactor (Aoyama *et al.*, 2004). However, according to study by Chang (2012) when Mn^{2+} was used as the cofactor, a drastic increment

in EhCK activity was shown. The V_{max} of EhCK was shown to increase 42.6 folds from 3.5±0.1 U/mg to 149.1±2.5 U/mg when the Mn^{2+} was used to replace the Mg^{2+}.

Study by Chang (2012) on CKs and Ethanolamine Kinase (EK) showed that CKs normally prefer Mg^{2+} while EKs prefer Mn^{2+} as cofactor. The unusual preference of Mn^{2+} over Mg^{2+} in EhCK was an exception. So as a continuous study, several amino acid residues that are predicted to be responsible for Mn^{2+} preference in EhCK have been identified and Glutamate (Glu-100) is one of them (Hoi, 2015).

In this study, glutamate (Glu-100) will be replaced with glutamine (Gln-100) amino acid residue by using PCR site directed mutagenesis method. Enzymatic assay will be done to determine the ion preference of this mutant protein and wild type EhCK protein. This is to verify that Glu-100 is the amino acid residue that is responsible for Mn^{2+} preference.

1.5 PCR Site Directed Mutagenesis

PCR site directed mutagenesis is a method in molecular biology which utilizes PCR to create a targeted change in a double stranded plasmid DNA. There are variety of ways to make DNA alterations such as insertions, substitutions and deletions. Site directed mutagenesis is used in such studies to study the changes in protein activity as a result of DNA manipulation. Site directed mutagenesis is also used to introduce or remove restriction endonuclease sites or tags and to select or screen for mutations at the DNA, RNA or protein levels that have a desired property. PCR site directed mutagenesis by using primer extension method was first described by Ho et al., (1989) and involves incorporating mutagenic primers in independent or nested PCRs to ultimately combine the first round of PCR products to produce a final PCR product (Ho *et al.*, 1989). The reaction uses flanking primers on either end of the target

sequence, plus two internal primers that contain the mismatched or inserted bases and hybridize to the region where the mutation will occur. How the reaction happens was illustrated in Figure 1.4. The final product will contain the mutated sequence and to confirm the success in introducing the mutation, the product will be sent for DNA sequencing.

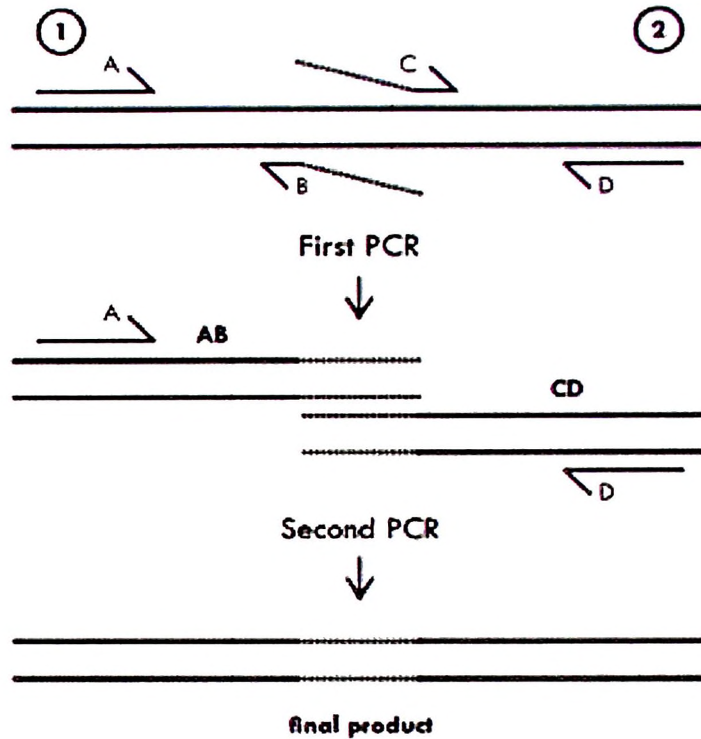

Figure 1.4 PCR Site Directed Mutagenesis.

The first round of PCR utilizes four primers A, B, C and D which create the two PCR fragments AB and CD. The two PCR products AB and CD are mixed together for a second round of PCR. Due to the complementary ends in between the two PCR fragments from the first round of PCR, the two fragments will hybridize in the second PCR reaction which will form the final PCR product (Ho *et al.*, 1989).

1.6 Problem and Rationale of the Study

E. histolytica is associated with high morbidity and mortality and it is a major health threat throughout the world especially in developing countries. Asymptomatic individuals account for almost 90% of the infections (CDC, 2013). Indiscriminately treating asymptomatic individuals may lead to drug resistance. Failed treatment with metronidazole has been reported recently (Hanna, 2000). The differences in drug sensitivity in *E. histolytica* isolates shown in recent studies indicate that there might be a small percentage of amoebae which are either resistant or may eventually become resistant due to the indiscriminate usage of anti-amoebic agents (Burchard and Mirelman, 1988). Besides, clinical resistance to metronidazole and laboratory induced metronidazole resistance in *E. histolytica* have been reported (Bansal *et al.*, 2006). Thus, the development of new intervention in treating *E. histolytica* infection is in need to overcome the problem of drug resistance in current treatment.

E. histolytica plasma membrane contains 60-70% of phospholipid and EhCK is one of the important enzymes that involve in the synthesis of these phospholipids. Biochemical characterization of EhCK, such as study on its cofactor preference, would lead to the potential designing of specific inhibitors for EhCK. Inhibition of PC synthesis by these EhCK inhibitors will ultimately inhibit the plasma membrane formation of *E. histolytica*. The disruption of plasma membrane formation of *E. histolytica* might be a new intervention for treatment of the infection.

1.7 Aim of the Study

Through previous study, the unusual ion preference of EhCK towards manganese ion and the amino acid residue (Glu-100) which is predicted to be responsible for manganese ion preference of EhCK have been identified. The main objective of this study is to validate the role of amino acid residue (Glu-100) in EhCK Mn^{2+} preference.

The specific objectives of this study are:

i. to perform PCR site directed mutagenesis of the predicted amino acid that is responsible for Mn^{2+} preference on the wild type EhCK ORF
ii. to clone the mutated EhCK ORF into pGEX-RB plasmid
iii. to express and purify the mutated and wild type EhCK proteins
iv. to determine the $K_{0.5}$ of EhCK with different Mn^{2+} concentration

1.8 Expected Outcome of the Study

This study is expected to produce a mutated EhCK ORF and a mutated EhCK protein. The study on this mutated EhCK protein will provide information on the divalent metal ion preference in EhCK which will be able to serve as a groundwork for future study in developing EhCK inhibitors. The developing of alternative treatment for *E. histolytica* becomes critical when recent studies revealed metronidazole resistance in *E. histolytica in vitro*. In conclusion, this study will be the stepping stone for future study in the development of novel treatment method for *E. histolytica* infection through membrane disruption of the microorganisms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Table 2.1 shows a list of all the chemical reagents that were used in this study, together with their respective manufacturers and country of origin.

2.1.2 Apparatus and Instruments

Table 2.2 shows a list of all general apparatus and instruments that were used in this study.

2.1.3 Kits used in this study

Table 2.3 shows a list of kits that were used in this study.

2.1.4 Consumables

Table 2.4 shows a list of consumables that were used in this study together with their respective manufacturers and their country of origin.

2.1.5 Oligonucleotides

All the oligonucleotides used in this study were purchased from 1st Base Sdn. Bhd. (Selangor Malaysia). Stock solution of 100 pmole of oligonucleotides was prepared with TE buffer. Working solution of 10 pmole was prepared by diluting the stock solution with TE buffer. The oligonucleotides were delivered in lyophilized form. All oligonucleotides that were used in this study are listed in Table 2.5.

Table 2.1 List of Chemical Reagents

Name	Manufacturer	Country of origin
Absolute ethanol	Merck	Darmstadt, Germany
Acetic acid (glacial) 100%	Merck	Darmstadt, Germany
30% Acrylamide/ Bisacrylamide solution	Bio-Rad	California, USA
Ammonium persulfate (APS)	Bio-Rad	California, USA
Ampicillin sodium salt	Amresco	Ohio, USA
ATP disodium trihydrate	Amresco	Ohio, USA
Bacteriological agar	Oxoid	Cambridge, UK
B-mercaptoethanol	Amresco	Ohio, USA
Bovine serum albumin	New England Biolabs	California, USA
Bio-Rad protein assay reagent	Bio-Rad	California, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Missouri, USA
Ethylenediaminetetraacetic acid (EDTA) disodium salt	Asia Pacific Specialty Chemicals	New South Wales, Australia
Ethidium bromide	Amresco	Ohio, USA
Glycerol	Amresco	Ohio, USA
Glycine	Merck	Darmstadt, Germany
LB Broth, Miller	Merck	Darmstadt, Germany
Magnesium chloride hexahydrate	Merck	Darmstadt, Germany
Manganese (II) chloride tetrahydrate	Merck	Darmstadt, Germany
Methanol	Merck	Darmstadt, Germany
Phospho(enol)pyruvic acid monopotassium salt (PEP)	Sigma-Aldrich	Missouri, USA
Polyethylene glycol (PEG) 8000	Merck	Darmstadt, Germany
Protease inhibitor cocktail, EDTA free	Roche	Mannheim, Germany
Sodium dodecyl sulfate (SDS)	Amresco	Ohio, USA
Sodium chloride	Merck	Darmstadt, Germany

Table 2.1 Continued		
Tetramethylethylenediamine (TEMED)	Vivantis	Selangor, Malaysia
Thrombin	MP Biomedicals	Ohio, USA
Tris•Base	Amresco	Ohio, USA
6× DNA loading dye	Fermentas	Ontario, Canada
Amylose resin	New England Biolabs	Massachusetts, USA
*Bam*H1	New England Biolabs	Massachusetts, USA
*Nde*1	New England Biolabs	Massachusetts, USA
GeneRuler™ DNA 1kb ladder	Fermentas	Ontario, Canada
GST•Bind Resin	Fermentas	Ontario, Canada
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
KOD Hot Start DNA Polymerase	Novagen	Madison Wisconsin, USA
Dream*Taq* DNA Polymerase	Fermentas	Ontario, Canada
T4 DNA ligase	New England Biolabs	Massachusetts, USA