IDENTIFICATION OF AMINO ACID RESPONSIBLE FOR THE BINDING OF MANGANESE ION IN *ENTAMOEBA HISTOLYTICA* CHOLINE KINASE

by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

3D	Three-dimensional
Ala/A	Alanine
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CAEP	Ceramide aminoethyl phosphonate
CDP-Cho	Cytidine diphosphate-choline
CDP-Etn	Cytidine diphosphate-ethanolamine
CMP	Cytidine monophosphate
CT	Choline-phosphate cytidyltransferase
CTP	Cytidine triphosphate
DG	Diacylglycerol
EDTA	Ethylenediaminetetraacetic acid
EhCK	*Entamoeba histolytica* choline kinase
EK	Ethanolamine kinase
ELISA	Enzyme-linked immunosorbent assay
ET	Cytidine triphosphate:phosphoethanolamine cytidylyltransferase
Fe-SOD	Iron superoxide dismutase
Gly/G	Glycine
hCK	Human choline kinase
hCKα2	Choline kinase alpha isoform a
hCKβ	Choline kinase beta
hEK	Human ethanolamine kinase

hEK1	Human ethanolamine kinase isoform a
hEK2α	Ethanolamine kinase 2 isoform a
G	Gram
Gal/GalNac	Galactose/N-acetylgalactosamine
GST	Gluthathione S-transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
L	Liter
LB	Luria-Bertani
Mg	Milligram
µg	Microgram
mL	Milliliter
µL	Microliter
M	Molar
Mg	Magnesium
mM	Millimolar
µM	Micromolar
Mn	Manganese
mRNA	Messanger RNA
N	Nano
NCBI	National Center for Biotechnology Information
°C	Degree celcius
OD	Optical density
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PC	Phosphatidylcholine
PCho	Phosphocholine

PCR	Polymerase chain reaction
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEMT	Phosphatidylethanolamine N-methyltransferase
Psi	Pounds per square inch
RNAP	RNA polymerase
RE	Restriction enzyme
Rpm	Revolutions per min
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	*Thermus aquaticus*
TEMED	Tetramethylethylenediamine
T_m	Melting temperature
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume to volume
w/v	Weight to volume
x g	Fold gravity
Vmax	Maximum velocity

PENGENALPASTIAN ASID AMINO KOLINA KINASE *E. HISTOLYTICA* YANG BERINTERAKSI DENGAN ION MANGAN

ABSTRAK

Amebiasis merupakan penyakit akibat jangkitan *Entamoeba histolytica* yang menyebabkan kadar kematian dan kemorbidan yang tinggi di negara-negara yang mundur. Membran *E. histolytica* memainkan peranan yang penting kepada *E. histolytica* termasuk pengawalan bahan yang memasuki sel dan jangkitan hos. Fosfatidilkolina merupakan salah satu fosfolipid yang terbanyak pada membran *E. histolytica*. Proses sintesis fosfatidikolina bermula dengan pemfosforilan kolina oleh kolina kinase. Penggunaan ion magnesium (Mg^{2+}) oleh kolina kinase daripada organism lain dalam proses pemfosforilan telah diketahui umum. Namun begitu, kajian terdahulu telah menunjukkan bahawa kolina kinase *E. histolytica* lebih cenderung menggunakan ion mangan (Mn^{2+}) berbanding dengan Mg^{2+} dalam proses sintesis. Oleh itu, kajian ini telah dijalankan dengan tujuan untuk mengenalpasti asid amino yang berinteraksi dengan Mn^{2+}. Perbandingan jujukan amino asid kolina kinase dan ethanolamina kinase yang terpilih telah dibuat dan Glisina-45 telah dipilih sebagai calon asid amino dalam kajian ini. Mutasi telah dibuat untuk menggantikan Gly-45 kepada alanina (A). EhCK-G45A telah diklonkan ke dalam vektor pGEX-RB dan induksi penghasilan dan penulenan protein telah dibuat. Namun begitu, tiada protein yang dihasilkan oleh EhCK-G45A. Selain daripada itu, model struktur EhCK-G45A telah dibuat. Melalui analisis model struktur yang terhasil, Ala-45 didapati terletak di kawasan simpulan protein EhCK. Kesimpulannya, kajian ini telah mengenalpasti asid

amino yang disyaki berinteraksi dengan Mn^{2+} dan juga menghasilkan model struktur EhCK-G45A yang berguna untuk penyelidikan perencatan EhCK pada masa hadapan.

IDENTIFICATION OF AMINO ACIDS RESPONSIBLE FOR THE BINDING OF MANGANESE ION IN *ENTAMOEBA HISTOLYTICA* CHOLINE KINASE

ABSTRACT

Amoebiasis is an infection by *Entamoeba histolytica* that causes serious mortality and morbidity cases in developing countries. *E. histolytica* plasma membrane plays vital roles in survival, ranging from controlling substance movement across the cell to invasiveness of *E. histolytica*. Phosphatidylcholine (PC) is one of the predominatant phospholipid of the plasma membrane in *E. histolytica*. PC synthesis begins with the phosphorylation of choline by choline kinase (CK). It is widely accepted that CK from other organisms utilizes magnesium ion (Mg^{2+}) as cofactor for phosphorylation. Interestingly previous study showed unusual preference in manganese ion (Mn^{2+}) by *Entamoeba histolytica* choline kinase (EhCK). Hence, this study aims to identify potential amino acid residue responsible for Mn^{2+} preference. Protein sequence alignment of selected CK and ethanolamine kinase (EK) was done. Glycine-45 residue was selected as a potential amino acid responsible for Mn^{2+} preference. Gly-45 was replaced by alanine using PCR site directed mutagenesis. Mutant EhCK-G45A was cloned into pGEX-RB vector, expressed and purified. No protein expression by mutant EhCK-G45A was observed. In addition, structural modeling of mutant EhCK-G45A was done. From the model generated Ala-45 resides in the loop region of the model. In conclusion, the study predicted the amino acid that favors Mn^{2+} binding and also generated EhCK mutant was done to lay the groundwork for future study on EhCK inhibition.

CHAPTER 1

INTRODUCTION

1.1 Entamoeba histolytica and Amoebiasis

1.1.1 *Entamoeba histolytica* and Epidemiology of Amoebiasis

Entamoeba histolytica is an intestinal parasite from Genus *Entamoeba*. Amoebiasis is the term for amoebic infection. *E. histolytica* lives in human host and causes both intestinal amoebiasis and extraintestinal amoebiasis. *E. histolytica* causes serious morbidity and mortality cases in developing countries such as Nigeria and certain region in Philipines (Ajero *et al.*, 2008; Perez Jr, 2006), as invasive amoebiasis causes 40,000 to 100,000 death annually worldwide (Hung *et al.*, 2008).

1.1.2 Morphology

E. histolytica can exist in two forms, depending on their stage of life cycle. When exist as a trophozoite, it is irregular in shape and about 20-30 μm in length. It has two layers of cytoplasm, namely ectoplasm and endoplasm. Trophozoites are motile and in active feeding stage. Meanwhile in cyst form, *E. histolytica* appears to be in spherical form, where the number of nuclei varies (uninucleate, binucleate and quadrinucleate). A chitin layer surrounds the cyst to act as a barrier to protect the cyst against harsh outer environment. Chromidial bars are only present in cyst form. *E. histolytica* in cyst form are non-motile.

1.1.3 *E. histolytica* Life cyle

The life cycle of *E. histolytica* is shown in Figure 1.1. Along its life cycle, *E. histolytica* changes its form between trophozoite and cyst. Trophozoite is the active

form of *E. histolytica*. Trophozoite grows and feeds on bacterial flora and multiply via binary fission in large intestine. During encystation, trophozoite encysts in colon lumen and excreted along with feces. First, precyst grows into uninucleated cyst. The nucleus undergoes multiplication to form binucleated cyst and ultimately become quadrinucleated cyst. Some trophozoites may also be excreted out through host feces but they are unable to last long outside of human body. The cyst is surrounded by chitin wall, thus allowing the survival of *E. histolytica* against harsh conditions outside of the host for few weeks. The chitin wall also allows it to withstand acidic condition in the stomach before reaching the small intestine. *E. histolytica* does not require any intermediate host.

When the cysts of *E. histolytica* were ingested, the cysts will be carried to the intestine. Excystation occurs in terminal ileum to form trophozoite. During excystation, each nucleus of the metacyst divides by binary fission and eventually the cytoplasm of metacyst becomes separated to produce 8 metacystic trophozoites. These metacystic trophozoites will eventually grow into mature trophozoites during transition from small intestine to large intestine.

1.1.4 Mode of Transmission

Invasive amoebiasis cases are mostly found in developing countries due to numerous factors that favour the transmission of *E. histolytica*. A study in an urban slum in Dhaka, Bangladesh that spanned for a year showed that 39% of children in that area were infected by *E. histolytica* (Haque *et al.*, 2001). *E. histolytica* transmits through oral-fecal route. Poor sanitation level in developing countries encourages transmission

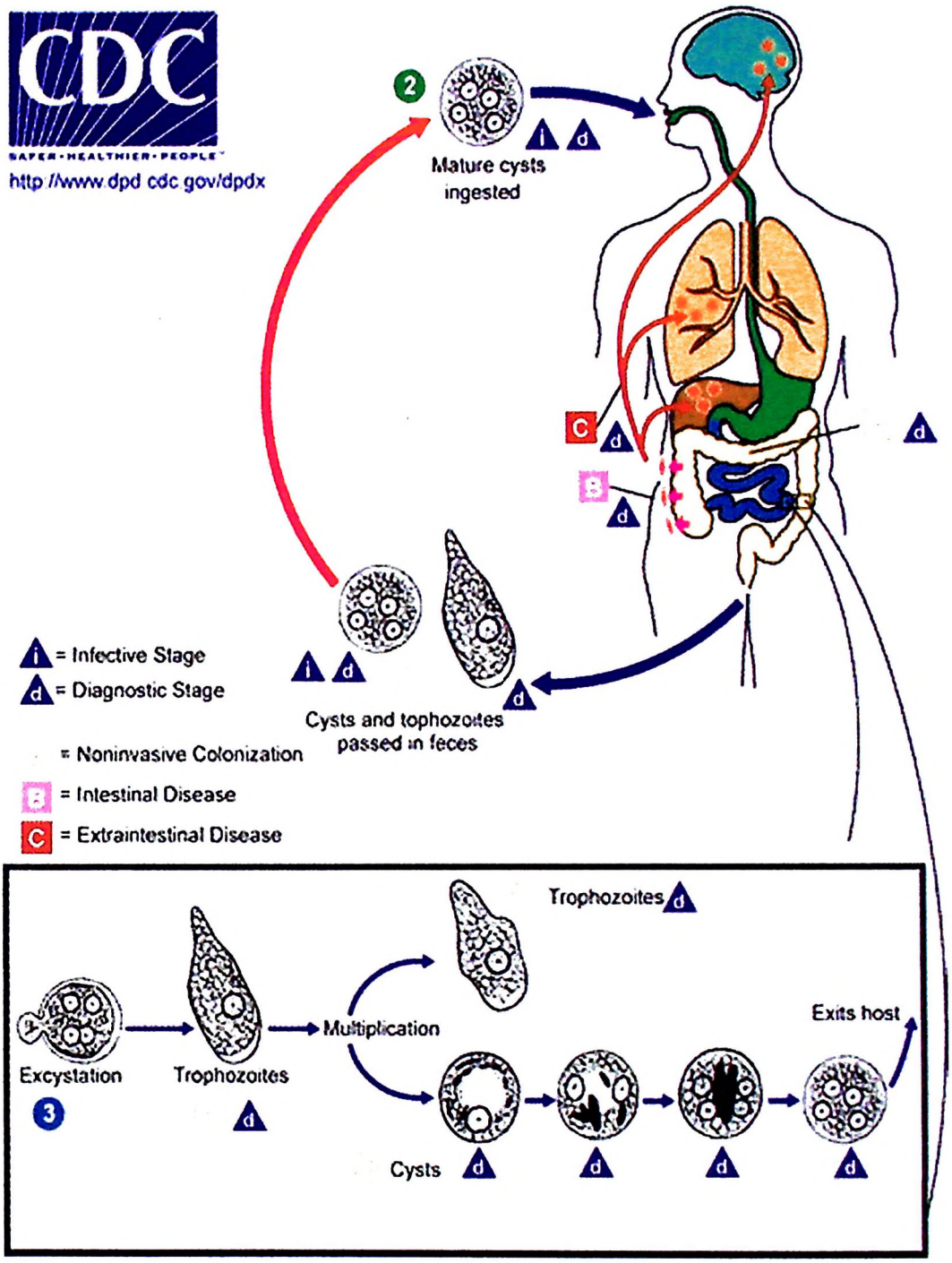

Figure 1.1 *E. histolytica* life cycle. Figure is adapted from *Amebiasis* (2013)

of *E. histolytica* cysts. Improper sewage disposal creates chances for *E. histolytica* cysts to be ingested as the sewage potentially contaminates drinking water and food. On the contrary, amoebiasis is rare in developed countries with good sanitation system. Amoebiasis may also occur to travellers that travel to endemic regions, where they bear the risk of taking contaminated food or drinks.

Amoebiasis can also be transmitted through anal-oral contact. Thus homosexual men bear higher risk of getting amoebiasis. A study done in 1979 showed 31.7% out of 126 homosexual men in New York were infected by *E. histolytica* infection (Kean *et al.*, 1979). The paper also stated that the water supply in highly infected area was clean, thus eliminating the potential of cause due to intake of contaminated water supply. Another study shows that the male homosexual population in Japan shows high seropositivity for antibodies to *E. histolytica* (Nozaki *et al.*, 1989; Takeuchi and Reue, 2009). As a summary, any activity that allows possible ingestion of *E. histolytica* cyst bears the possibility of getting amoebiasis.

1.1.5 Amoebiasis Infection

There are at least 8 amoebas such as *E. dispar*, *E. moshkovskii*, *E. coli* and *E. hartmanni* that thrive in human intestinal lumen and act as commensal organisms except *E. histolytica*, which is both pathogenic and invasive (Tanyuksel and Petri, 2003). *E. histolytica* causes amoebiasis, however this only accounts for around 10-20% of *E. histolytica* infection as most infections remain asymptomic. Amoebiasis can be categorized into intestinal amoebiasis and extraintestinal amoebiasis. Minor infection such as luminal amoebiasis causes symptoms such as flatulence, loose stools, stomach ache and cramp.

E. histolytica produces cytolytic amoebapore. Amoebapore is a protein capable of forming ion channels in lipid membrane. The formation of pore in cell membrane breaks the ionic balance between cell and outer environment, thus depolarize the target cells (Lynch *et al.*, 1982; Rosenberg *et al.*, 1989; Young *et al.*, 1982). Amoebapore acts by binding to anionic phospholipid of eukaryotic cells.

Amoebic colitis is one of the implications of intestinal amoebiasis. Colitis is the inflammation of the large intestine. Amoebic colitis shows multiple discrete ulcers thicken by mucosa, diffusely inflamed and oedematous mucosa and also necrosis and perforation of intestinal wall (Stanley, 2003). The infection occurs when *E. histolytica* attaches to epithelial cells in colon by galactose/N-acetylgalactosamine specific lectin. This is supported by a finding that showed mammalian cell that lack N-terminal galactose or N-acetylgalactosamine residues are protected against *E. histolytica* attachment (Stanley, 2003). The disease onsets gradually where multiple mucoid stools and profuse diarrhea might be seen. The stool is haem positive even without apparent blood seen in the stool (Adams and MacLeod, 1977). Other signs such as fever, weight loss and anorexia may be present.

Extraintestinal amoebiasis is the clinical manifestation of *E. histolytica* outside of the digestive tract. This occurs when *E. histolytica* spreads outside the patient's gut. The lipophosphoglycan/ proteophosphoglycan glycosyl phosphatidylinositol-anchored molecules that covered the trophozoites act as physical barriers to complement molecules in the blood stream. The presence of an inhibitor region in Gal/GalNAc lectin may also cross react with C5b-9 attack complex in red blood cells, thus protecting *E . histolytica* trophozoite from lysis (Braga *et al.*, 2001). Besides that, the cysteine

proteinase of *E. histolytica* further protects itself from human immune defense system by cleaving and inactivating C3a, C5a, IgA and IgG (Kelsall and Ravdin, 1993; Reed *et al.*, 1988). Liver abcess development is the most common extraintestinal amoebiasis manifestation. When *E. histolytica* enters the portal venous system after breaching colonic mucosa, it will colonize the liver and causes abcess. The abcess are filled with liquefied cells, cellular debris and dead hepatocytes, confined by a rim of connective tissues, inflammatory cells and amoebic trophozoites (Reed *et al.*, 1988). Symptoms that surface are fever, right upper quadrant pain, substantial hepatic tenderness and cough, while jaundice may also present (Stanley Jr., 2003).

1.1.6 Diagnosis

E. histolytica diagnosis is relied upon microscopic examination. Fresh stool samples are required for microscopic examination as trophozoites cannot survive in outer environment for long. Microscopic examination can be done by saline wet mount technique to seek for motile trophozoites, iodine-stained wet mount or by using trichrome staining method. Iodine stain aids detection by staining the nuclei. However, microscopic examination has many drawbacks. One of the drawbacks of this method is the morphological similarity between *E. histolytica* and non-pathogenic *E. dispar*. The morphological similarity between them causes confusion and leads to many wrong diagnosis. Hence one should be aware that some *E. histolytica* past infections in older data may be confused with *E. dispar* infections (Tanyuksel and Petri, 2003). This is because 90% of Entamoeba species cases reported worldwide were caused by *E. dispar* while pathogenic *E. histolytica* infection only takes up 10% of the total infection (Braga *et al.*, 2001). The World Health Organization had recommended that treatment should only be done on *E. histolytica* infection after distinguishing it from *E. dispar* infection,

where no treatment will be given for *E. dispar* infection (WHO/PAHO/UNESCO, 1997). One of the ways to differentiate *E. histolytica* from *E. dispar* is through observation for erythrophagocytosis. However, this identification method is unreliable as red blood cell ingestion may not appear in some chronic amoebic infection cases (Markell et al., 1999). Besides that in some cases *E. dispar* had been observed to contain red blood cell (Haque et al., 1995). Microscopic observation result may be adversely affected by factors such as delayed sample delivery or insufficint well-trained microscopist.

Stool antigen test is another alternative test to look for the presence of *E. histolytica* antigen by using enzyme-linked immunoassay (ELISA). ELISA test utilizes monoclonal antibody to detect *E. histolytica* antigen. A study conducted to evaluate ELISA detection method showed that ELISA detection method has higher sensitivity than standard diagnostic method (Merino *et al.*, 1990). The study also showed that *E. histolytica* antigen can be used for detection regardless of the condition of the parasite.

Serological test can also be used for *E. histolytica* detection. The presence of *E. histolytica* causes the production of anti-*E. histolytica* antibody. Detection of amoebiasis via serological method is useful in developed country as amoebiasis infection is rare. On the downside this method is not practical to be practiced in high endemic area due to inability to distinguish current infections from past infections. Indirect hemagglutination assay (IHA) is one of the examples of assay for anti-*E. histolytica* antibody detection. It involves in binding of *E. histolytica* antibodies in serum to *E. histolytica* antigen sensitized red cells. The addition of anti-human antibody causes the agglutination of red cells.

Molecular diagnosis is another alternative available for *E. histolytica* detection. PCR is used to amplify *E. histolytica* specific gene. Molecular diagnosis holds great advantage for being highly specific and sensitive. However this method is not practical in developing country as it is time consuming, expensive and highly technical.

1.1.7 Treatment

Antiamoebic drugs can be classified into luminal, tissue and mixed amoebicides (James *et al.*, 2009). Drugs with nitroimidazole derivatives such as metronidazole, tinidazole and ornidazole are highly effective against anaerobic organisms. Metabolic pathway in anaerobic organism reduces the nitro group of metronidazole. The reduction of nitro group produces reduced cytotoxic intermediate that destabilizes DNA (Müller, 1983). Luminal agent such as paromomycin and iodoquinol are used to eradicate amoebic colonization (Powell *et al.*, 1969). Unlike other microbacteria that acquired drug resistance, nitroimidazole derivatives remain as an effective drug for treating amoebiasis (Stanley, 2003). However a study conducted 1999 showed resistance development in *E. histolytica in vitro* towards metronidazole via episomal transfection of antioxidant enzymes such as Fe-SOD and flavin peroxidase (Wassmann *et al.*, 1999). Hence it would be wise to discover an alternative method against *E. histolytica*.

1.2 Phospholipid Metabolism in *E. histolytica*

1.2.1 Plasma membrane in *E. histolytica*

E. histolytica is relatively rich in lipid where phospholipid allocates 60-70% of it (Sawyer *et al.*, 1967). Phospholipids are basic building blocks of cell membrane. Phospholipids consist of a polar head group that is connected to a non-polar fatty acid tail via glycerol linkage. A study done on *E. histolytica* showed that

phosphatidylcholine (PC) makes up most of the phospholipid composition of the whole cell, followed by ceramide aminoethyl phosphonate (CAEP) and dieonic phosphatidylethanolamine (PE2) (Aley *et al.*, 1980). However, CAEP is the most common phospholipid found at the plasma membrane, following by PE2 and PC. Meanwhile other types of phospholipid just make up a small percentage of the total phospholipid content of the cell. These show that majority of the phospholipid in the cell constituted of PC, CAEP and PE2.

Plasma membrane is an important component for *E. histolytica* as the membrane involves in the movement of substances across the cell. Furthermore, invasiveness of *E. histolytica* depends on a heterodimeric glycoprotein on the plasma membrane named galactose-inhibitable lectin, which not only allows the adherence to host cell to conduct contact dependent killing, but also acts as complement resistance (Petri and Mann, 1993). Plasma membrane composition of *E. histolytica* also plays a vital role in protecting itself from its own pore-forming toxin. Since amoebapore binds to anionic phospholipids, the lack of anionic phospholipids on the outer membrane leaflet in *E. histolytica* compare to other eukaryotic cells protects itself from its own toxin. Besides that, cholesterol on *E. histolytica* plasma membrane further protects itself from own amoebapore (Andrä *et al.*, 2004). These show the importance of plasma membrane component, especially phospholipids to *E. histolytica*.

1.2.2 Phosphatidylcholine Synthesis

PC is a class of phospholipid which incorporates choline as a headgroup. PC plays an important role as building block of plasma membrane, as it is the third most abundant phospholipid type on plasma membrane.

Choline (trimethyl-beta-hydroxyethylammonium) is a quaternary ammonium compound which can be found in plants and animals (Zeisel, 1981). Choline is an essential dietary nutrient used as a component in phospholipid. Kennedy pathway is a cascades of reaction for phospholipid synthesis. CDP- ethanolamine pathway and CDP-choline pathway are branches of Kennedy pathway (Gibellini and Smith, 2010). Phosphatidylcholine synthesis was shown in Figure 1.2. CDP-choline pathway starts from choline phosphorylation by choline kinase (Haque *et al.*, 2001) to produce phosphocholine (PCho). Phosphocholine is an intermediate in phosphatidylcholine (PC) synthesis. After that a cytidylyl group from cytidine triphosphate (CTP) will be transferred to PCho to form cytidine diphosphate-choline (CDP-Cho). This reaction was catalysed by choline-phosphate cytidyltransferase (CT). CTP is a pyramidine nucleoside triphosphate which acts as energy coupler just like adenosine triphosphate (ATP), despite involved in limited metabolic reactions. In the last step of the reaction cascade, PC was synthesized by the transfer of CDP-Cho to diacylglycerol (DG). This reaction is catalysed by CDP-Cho: 1, 2-diacylglycerol cholinephosphotransferase (CPT). The by-product of the reaction is cytidine monophosphate (CMP) and a proton.

DG is a glyceride which made up of 2 fatty acid chain that is covalently bonded to a glycerol molecule via ester linkage. DG synthesis started with phosphorylation of glycerol by glycerol kinase to form glycerol-3-phosphate (G-3-P). Next, glycerol-3-phosphate O-acyltransferase acted as catalyst to transfer acyl group to G-3-P to form 1-acyl-G-3-P. Phosphatidic acid (PA) was formed from 1-acyl-G-3-P. This reaction is catalysed by 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT). Ultimately phosphatidic acid phosphatase (PAP) dephosphorylates PA into DG. PAP can be divided into PAP type 1 (PAP1) and also PAP type 2 (PAP2). Both PAP1 and PAP2

are present in mammal, where PAP1 is responsible for phospholipid biosynthesis while PAP2 was not suspected to be involved in intracellular phospholipid biosynthesis (Gomez-Muñoz *et al.*, 1992; Takeuchi and Reue, 2009; Vance and Vance, 2008). However, PAP2 was believed to be involved in phospholipid biosynthesis in *E. histolytica* due to lack of PAP1 (Lykidis *et al.*, 2001).

1.2.3 Phosphatidylethanolamine

Phosphatidylethanolamine (PE) is another important component that makes up *E. histolytica* plasma membrane (Aley *et al.*, 1980). PE is structurally similar to PC, with the exception of the head group where ethanolamine acts as the head group for PE. However, the distribution between PE and PC in plasma membrane differs from each other, where PE is more commonly found at outer leaflet of the membrane (Vance and Vance, 2008). Similar to choline, ethanolamine cannot be synthesized *de novo* (Zeisel *et al.*, 1991). PE synthesis was carried out via CDP-Ethanolamine pathway, which is one of the branches of Kennedy pathway along with CDP-Choline pathway (Gibellini and Smith, 2010). The synthesis pathway for EhEK was shown in Figure 1.2.

Similar to PC synthesis, PE synthesis starts with phosphorylation of ethanolamine by ethanolamine kinase (EK). Phosphate group from ATP was transferred to ethanolamine with the presence of Mg^{2+} as cofactor (Weinhold and Rethy, 1974). Hence, both CK and EK require Mg^{2+} as cofactor for phosphorylation. Next, cytidine triphosphate:phosphoethanolamine cytidylyltransferase (ET) catalyzed the conversion of phosphoethanolamine (PEtn) into cytidine diphosphate-ethanolamine (CDP-Etn). Finally PE was synthesized when CDP-Etn was transferred to DG. Interestingly, PE

can be converted into PC through catalyzation by phosphatidylethanolamine N-methyltransferase (PEMT).

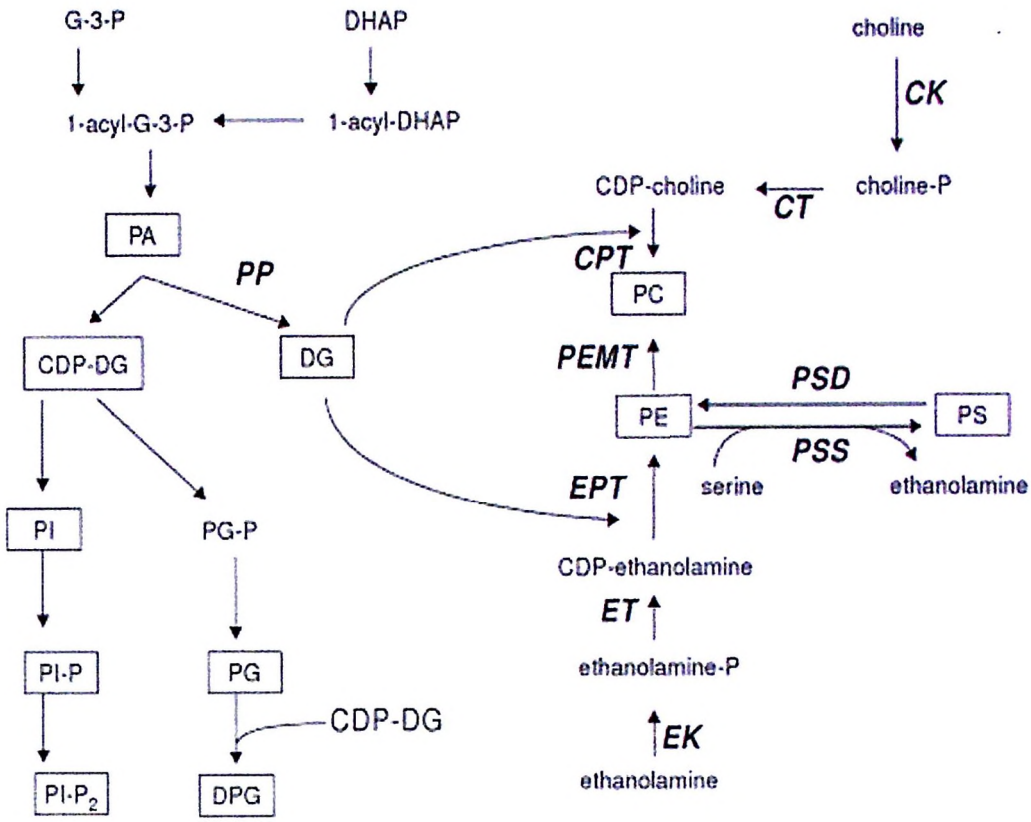

Figure 1.2 Phospholipid biosynthesis pathways in animal cells. Part of the pathway shows biosynthesis of diacylglycerol (DG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The abbreviations are: G-3-P, glycerol-3-phosphate; PA, phosphatidic acid; DG, diacylglycerol; CK, choline kinase; CT, choline-phosphate cytidyltransferase; CDP-Cho, cytidine diphosphate-choline; CPT, CDP-Cho: 1, 2-diacylglycerol cholinephosphotransferase; PC, phosphatidylcholine. EK, ethanolamine kinase; ET, cytidine triphosphate:phosphoethanolamine cytidylyltransferase; PEtn, phosphoethanolamine, CDP-Etn, cytidine diphosphate-ethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase. Figure is adapted from Vance and Vance (2008).

1.2.4 Choline Kinase

CK is the first enzyme involves in PC synthesis pathway. CK transfers a phosphate group from ATP to choline to produce PCho. This is done with the aid of Mg^{2+} as cofactor for enzyme catalysis. CK is one of the well studied enzymes and it is ubiquitous in eukaryotes (Yamashita and Hosaka, 1997). CK enzyme was indicated to be existed in few isoforms (Kozo *et al.*, 1985; Porter and Kent, 1990; Uchida and Yamashita, 1990). There are 2 CK genes in mammals, namely *ckα* and *ckβ* which responsible in encoding CKα1, CKα2 and CKβ (Aoyama *et al.*, 2002).

Most of the choline kinases are also involved in phosphatidylethanolamine synthesis by catalyzing phosphorylation of ethanolamine to phosphoethanolamine. However the *Km* values for choline phosphorylation are relatively lower than in ethanolamine phosphorylation (Peisach *et al.*, 2003). Choline kinase alignment shown in Figure 1.3 shows several highly conserved regions shared among CKs and EKs among different species (Peisach *et al.*, 2003). Brenner`s motif is one of the commonly found motifs among choline kinases and ethanolamine kinases of different species. Brenner motif was also been identified in many different enzymes such as aminoglycoside phosphotransferases (Fong *et al.*, 2010). Another conserved motif is choline kinase motif where it is believed to be involved in choline or ethanolamine binding. There are 3 highly conserved region which is proposed to be involve in ATP binding (Peisach *et al.*, 2003). Figure 1.4 showed the interaction between ATP along with Mg^{2+} and ATP binding site. The presence of same conserved motifs between CKs and EKs showed connectivity between these two different kinases.

Interestingly, over-expression of CK and PC increases the risk of cancer development in human, and CK inhibition was shown to inhibit tumour growth *in vitro* and *in vivo* study (Ramirez *et al.*, 2002). An increased in CK activation was shown in ras-transfomed cell (Hernandez-Alcoceba *et al.*, 1997). The study also showed cell proliferation could be suppressed through CK inhibition, thus making CK a potential target for antiproliferative and anticancer drug design target.

As for lower eukaryotes, CK was studied for drug designation against infective eukaryotes. *Plasmodium falciparum* is a malaria-causing parasite. Malaria is one of the most significant parasitic diseases in human as it causes high morbidity and mortality rate worldwide. Proliferation of *P. falciparum* within erythrocyte was associated with mass synthesis of PC and PE (Alberge *et al.*, 2010). That study also shows that antimalarial drug called bis-thiazolium compound T3. The drug works by impairing phosphatidylcholine synthesis in *P. falciparum* by acting as competitive inhibitor. This shows potential way to treat *E. histolytica* infection via plasma membrane impairation.

1.2.5 *E. histolytica* Choline Kinase

E. histolytica choline kinase (EhCK) can be divided into EhCK1 and EhCK2. Most of the CK also possess EK activity (Gee and Kent, 2003; Gibellini *et al.*, 2009). *E. histolytica* choline kinase (EhCK) is responsible for phosphocholine and phosphoethanolamine synthesis in *E. histolytica*. *EhCK* gene can be divided into *EhCK1* and *EhCK2*, where *EhCK1* located at position 109,594 to 110,670 and EhCK2 located at 247,582 to 248757 within positive strand of *E histolytica* genome (Chang, 2012). The length of *EhCK1* spans 1077 bp while *EhCK2* spans 1176 bp, which is the same as respective mRNA length. This indicates that there are no introns on both genes

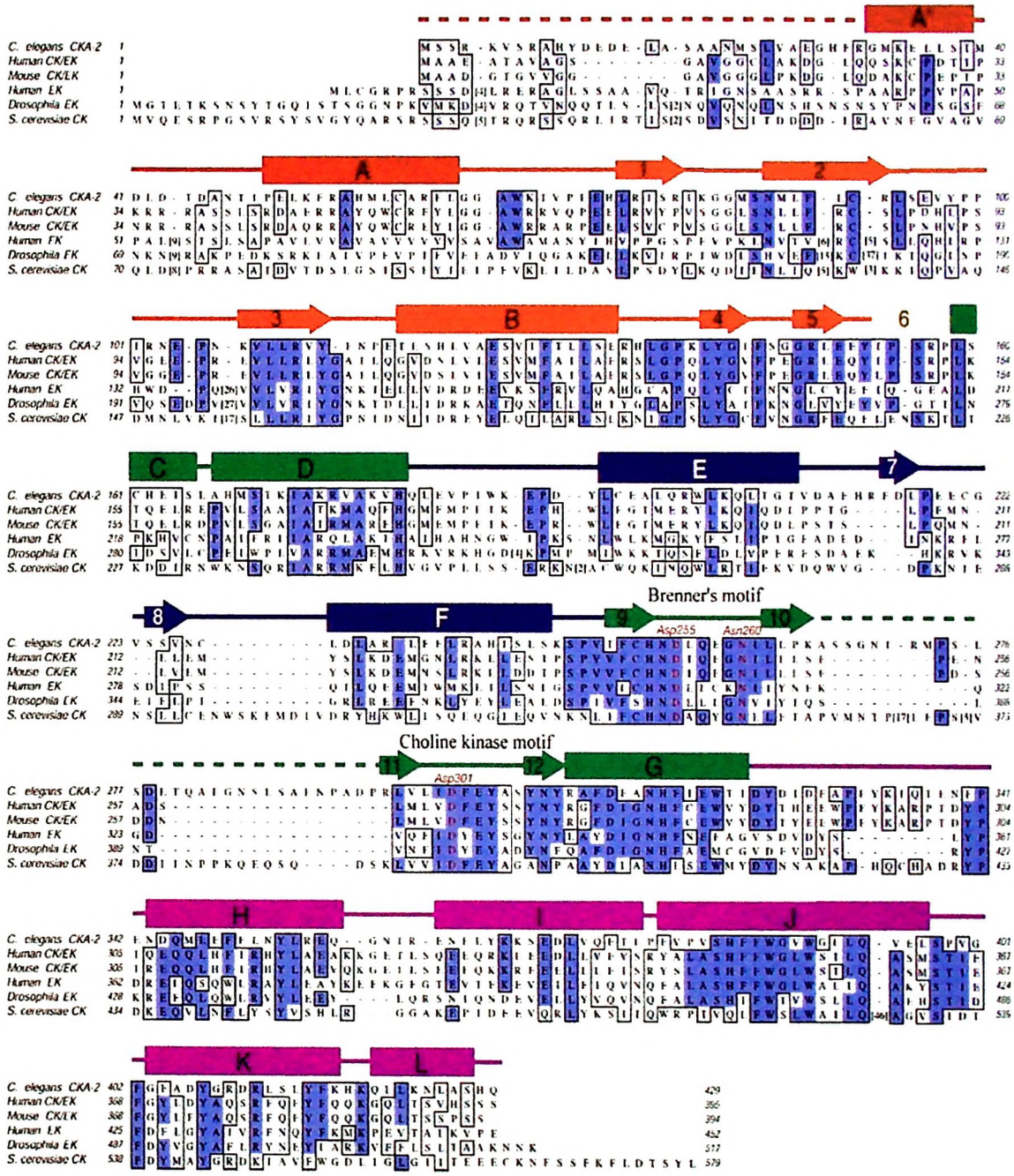

Figure 1.3 Sequence alignment of choline/ethanolamine kinases. The sequences of *C. elegans* choline kinase CKA-2. human choline/ethanolamine kinase (CK/EK), mouse choline/ ethanolamine kinase, human ethanolamine kinase (Thompson *et al.*, 1994), *Drosophila* ethanolamine kinase and S. cerevisiae choline kinase alignment by using ClustalW. Choline kinase motif and Brenner's motif are labeled in the alignment. Proposed ATP binding site are indicated with red letters (Peisach *et al.*, 2003).

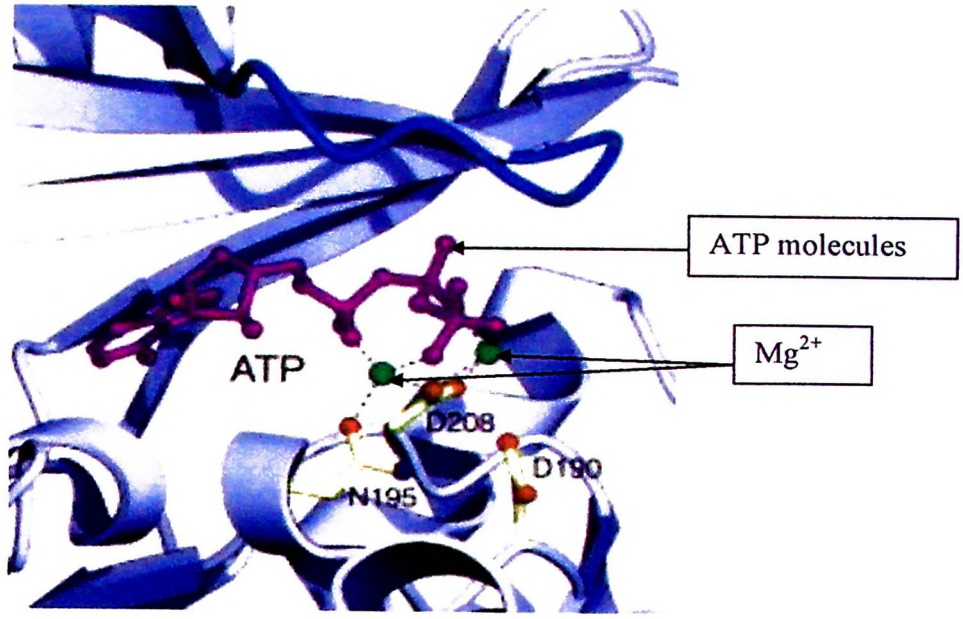

Figure 1.4 The proposed ATP binding site in *C. elegans* choline kinase (CKA-2). ATP molecules are represented by balls and sticks. Mg^{2+} are represented by spheres (Peisach *et al.*, 2003).

(Chang, 2012). According to Chang (2012), no ethanolamine phosphorylation activity was detected by EhCK1 with 5 mM or 10 mM ethanolamine while no choline phosphorylation activity was detected by EhCK2. This may indicate that EhCK1 are choline-specific kinase while EhCK2 is ethanolamine-specific kinase. Hence in this study, EhCK1 will be referred as EhCK while EhCK2 will be referred as EhEK.

Though CK is widely accepted to use Mg^{2+} as cofactor (Ishidate, 1989) , study by Chang (2012) showed a drastic increment in EhCK activity when Mn^{2+} was used as cofactor instead. The finding is tabulated in Table 1.1. Although some CK such as hCKα2 and hCKβ showed CK activity in using Mn^{2+} as cofactor as shown in Table 1.2, the activity (U/mg) is minimal compared to using Mg^{2+} as cofactor (Chang, 2012).

1.2.6 Magnesium

Magnesium is an alkaline earth metal with the symbol Mg and atomic number 12. Due to its electron configuration, magnesium can only be found in combination with other elements, where it appears to be in +2 oxidation state. Magnesium can be found in fruits and vegetables (Davies, 2014). Magnesium is one of the most abundant trace elements in the body. Most of the magnesium is distributed between skeletal system and within body cells. Magnesium intake is always inversely proportional to calcium intake in the body. Mg also involved in acting as antioxidant against mitochondria free radical damage (Barbagallo *et al.*, 2015). Mg ion deficiency affects mitochondria energy production pathway in generating ATP as well in mechanisms for synaptic plasticity and neuronal properties (Barbagallo *et al.*, 2015).

Table 1.1 Comparison of EhCK kinetic parameters for different metal ions. Fold changes (in brackets) are relative to Mg^{2+} dataset. Values are means ± S.D. of triplicate (Chang, 2012).

Metal ion	**V_{max} (U/mg)**	**$K_{0.5}$ (Lykidis *et al.*)**	**V_{max}/ $K_{0.5}$**	**Hill Coefficient**	**Minumum Detectable Activity (Lykidis *et al.*)**
Mg^{2+}	3.5 ± 0.1 (1)	13.9 ± 0.2	0.3 (1)	3.9 ± 0.1	6
Mn^{2+}	149.1 ± 2.5 (42.6)	9.5 ± 0.1	15.7 (52.3)	9.7 ± 1.1	3

oxidation form of Mn in manganese protein is +2 oxidation form (Yamamoto *et al.*, 2003). Manganese is an essential trace element needed by the body. In general manganese intake per day ranges from 2.0 to 5.0 mg for adults, while 1.0 to 3.0 mg per day is needed for children (Tran *et al.*, 2002). Mn is taken into the body via gastrointestinal tract and respiratory tract (Aschner and Aschner, 1991). Mn plays role as cofactor to many enzymes that catalyze vital functions in our body. For instance, enzyme such as phosphoenolpyruvate carboxykinase which involves in gluconeogenesis requires Mn as cofactor (Miller *et al.*, 1968). Though Mn is vital for human body function, excess Mn in central nervous system (CNS) induces neurological disorders (Aschner and Dorman, 2006).

1.2.7 Ion Preference as Cofactor in Phosphorylation by CK and EK

Generally, phosphorylation of choline and ethanolamine requires Mg^{2+} to act as cofactor (Aoyama *et al.*, 2004; Weinhold and Rethy, 1974). However according to the study by Chang (2012) on CK and EK activity of human choline kinases (hCKs) and human ethanolamine kinases (hEKs) in presence of Mg^{2+} or Mn^{2+} which is tabulated in Table 1.2, choline kinase alpha isoform a (hCKα2) and choline kinase beta (hCKβ) have greater preference for Mg^{2+} while human ethanolamine kinase isoform A (hEK1) and ethanolamine kinase 2 isoform 1 (hEK2α) prefer Mn^{2+} as cofactor instead. All hCK and hEK except hEK2α shows certain degree of both CK activity and EK activity.

1.3 Problem and Rationale of the Study

PC is one of the major phospholipid that can be found in the phospholipid bilayer membrane in eukaryotes. Hence, disruption of biosynthesis of PC could severely deform the membrane, ultimately leading to cell death. Inhibition of CK activity has

Table 1.2 CK and EK activity of hCKs and hEKs in the presence of Mg^{2+} or Mn^{2+}. Values are means ± S.D. of triplicate (Chang, 2012).

	Activity (U/mg)		Fold change
	Mg2+	Mn2+	
Detection of CK activity			
His-hCKα2	44.94 ± 4.8	3.5 ± 0.005	12.8
His-hCKβ	29.6 ± 1.6	3.8 ± 0.2	7.8
His-Δ89N-hEK1	0.438 ± 0.009	2.2 ± 0.1	5.0
hEK2α	ND	ND	-
Detection of EK activity			
His-hCKα2	15.7 ± 3.4	ND	-
His-hCKβ	ND	0.23 ± 0.10	-
His-89N-hEK1	8.9 ± 0.7	23.5 ± 1.8	2.6
hEK2α	0.490 ± 0.007	0.9 ± 0.3	1.8

been shown to have anticancer effect and also as an anti-malarial strategy. The understanding on EhCK cofactor preference paves groundwork for drug development in inhibiting EhCK activity, which would lead to distruption of PC synthesis in *E. histolytica*.

According to Chang (2012), EhCK activity was studied with different ions to determine the divalent metal ion cofactor needed by EhCK. Interestingly, EhCK activity in 12 mM Mn^{2+} was approximately way higher than equimolar of Mg^{2+}. The findings also showed significant higher Hill coefficient in different Mn^{2+} concentration compared to Mg^{2+}. These data showed greater preference of Mn^{2+} compared to Mg^{2+} by EhCK. This finding opposed the common perception where Mg^{2+} is the most favourable divalent metal ion cofactor for CK (Aoyama *et al.*, 2004).

The sequences that showed preference to Mn^{2+} are EhCK, EhEK, hEK1 and hEK2α, while hCKα2 and hCKβ showed preference towards Mg^{2+} (Chang, 2012). hCKα2, hCKβ and hEK1 showed both CK and EK activity to certain degree. Besides that, the selected EKs for this study showed preference in Mn^{2+} instead of Mg^{2+} while CKs selected except EhCK prefers Mg^{2+} as cofactor (Chang, 2012). Similarity of few conserved motifs by CKs and EKs such as Brenner's motif and choline/ethanolamine binding site as shown in Figure 1.3 shows some connection between CKs and EKs. These factors allow comparison between CKs and EKs with different ion preference to identify amino acid residue responsible for unusual Mn^{2+} preference in EhCK.

1.4 Aim of the Study

The previous study showed unusual preference of Mn^{2+} over Mg^{2+} as cofactor in EhCK. Therefore this study aimed to identify the amino acid residues responsible for Mn^{2+} preference in EhCK.

To achieve the general objective, the first specific objective of the study was to predict the amino acid residues responsible for Mn^{2+} preference in EhCK. This was done by comparing amino acid residues from selected CK and EK sequences that either prefers Mg^{2+} or Mn^{2+} as cofactor based on Chang (2012). Comparison was done *in silico*. The amino acid suspected to be important for Mn^{2+} preference was subjected for PCR site-directed mutagenesis (SDM). The plasmid with mutated *EhCK* was transformed into *E. coli* followed by protein expression and purification. In addition to that, structural modeling of mutated EhCK will be done to identify the location of mutated amino acid.

1.5 Outputs and Benefits

The study allows the comparison of amino acid sequences of selected CK and EK among eukaryotes. This study also allows the identification of possible amino acid responsible for the unusually Mn^{2+} preference in EhCK compared to other CK enzymes.

This study provides valuable information to serve as groundwork for future study in developing EhCK inhibitor. The study that discovered resistance towards metronidazole by *E. histolytica in vitro* does raise some concern about the emergence of development of nitroimidazole-resistance *E. histolytica* strain in future (Wassmann *et al.*, 1999). Hence, this study helps in the future study on developing drug design against *E. histolytica* infection through membrane disruption.

CHAPTER 2

MATERIALS AND METHODS

An overview of this study is illustrated in Figure 2.1.

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study were of biotechnology, molecular biology or analytical grade. The chemical reagents are listed in Table 2.1.

2.1.2 Molecular biology reagents

The molecular biology reagents used in this study are listed in Table 2.2.

2.1.3 Kits

QIAamp DNA Mini Kit and QIAquick Gel Extraction Kit were purchased from QIAGEN (Hilden, Germany).

2.1.4 Consumable Items

The consumable items used in this study are listed in Table 2.3.

2.1.5 Apparatus and Instruments

The apparatus and instruments used in this study are listed in Table 2.4.

2.1.6 Computer Softwares

The computer softwares used in this study are listed in Table 2.5.