EXPRESSION OF RECOMBINANT HUMAN DNA TOPOISOMERASE IN *Pichia pastoris* AND ANALYSIS OF ITS ACTIVITY

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EXPRESSION OF RECOMBINANT HUMAN DNA TOPOISOMERASE IN *Pichia pastoris* AND ANALYSIS OF ITS ACTIVITY

by

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LIST OF ABBREVIATIONS

ANONA	Analysis of variance
AOX	Alcohol Oxidase
APS	Ammonium persulphate
BLASTn	Nucleotide Basic Local Alignment Search Tool
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EBL	European Bioinformatics Institute
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMBL	European Molecular Biology Laboratory
ER	Estrogen receptor
EtBr	Ethidium bromide

FBS	Fetal bovine serum
HCl	Hydrochloric acid
HIS4	Histidinol dehydrogenase
kbp	Kilo base pair
kDa	Kilo dalton
LB	Luria-Bertani
LDH	Lactate dehydrogenase
Lk	Linking number
MTT	3-(4,5 Dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide
Mut^-	Methanol utilization minus
Mut^+	Methanol utilization plus
Mut^s	Methanol utilization slow
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polimerase chain reaction
Pen/Strep	Penicillin/Streptomycin
RNA	Ribonucleic acids
SDS	Sodium dodecyl sulphate
TEMED	N, N, N' N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tw	Twist
UV	Ultraviolet

Wr	Writhe
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPDS	Yeast extract peptone dextrose with sorbitol

PENGUNGKAPAN DNA TOPOISOMERASE MANUSIA REKOMBINAN DALAM *Pichia pastoris* DAN ANALISIS AKTIVITINYA

ABSTRAK

DNA Topoisomerase manusia adalah enzim yang amat penting dalam proses-proses sel kerana ia mengaturkan topologi DNA untuk kemandirian sel. Enzim ini digunakan sebagai sasaran molekul untuk penyaringan agen anti-kanser yang berpotensi menggunakan asai kaedah gel. Disebabkan kos enzim komers ini adalah mahal, ia telah menjadi satu halangan kepada penyelidikan terutamanya yang melibatkan penyaringan agen anti-kanser secara besar-besaran. Oleh itu, kajian ini adalah bertujuan untuk mewujudkan satu kaedah bagi menghasilkan DNA Topoisomerase manusia rekombinan secara dalaman. Jujukan pengkodan DNA Topoisomerase I (Topo I) dan DNA Topoisomerase IIα (Topo IIα) telah diamplifikasi daripada templat cDNA yang dijana daripada RNA jumlah sel kanser payudara, MDA-MB-231. Jujukan pengkodan Topo I dan Topo IIα diligasi dalam vektor pengekspresan, pPICZ-α-A dan pPICZ-α-C. Seterusnya vektor rekombinan terjana ditransformasi dalam empat jenis strain, *Pichia pastoris* (X-33, GS115, SMD1168H and KM71H). Analisis PCR dijalankan bagi mengenalpasti rekombinan klon dan diikuti dengan pemilihan klon multisalinan dengan menggunakan piring agar yang mengandungi kepekatan Zeocin® yang secara meningkat. Klon multisalinan terpilih diaruh bagi pengekspresan rekombinan enzim dalam sistem kelalang goncang. Didapati Topo I telah dihasilkan paling tinggi dalam strain, GS115 berbanding dengan strain yang lain. Walau bagaimanapun, pengungkapan Topo IIα tidak dapat dikesan dalam semua strain *Pichia pastoris* dengan analisis blot Western. Enzim Topo I manusia rekombinan yang mentah dan tulen telah digunakan dalam

asai kaedah gel untuk penyaringan 4 jenis flavonoid (apigenin, krisin, kaempferol and kuersetin) dan perencat topoisomerase I (camptothecin). Hasil keputusan bagi asai kaedah gel menunjukkan bahawa apigenin, kaempferol and kuersetin adalah perencat topoisomerase I. Hanya Krisin didapati bukan perencat Topoisomerase I. Topo I manusia rekombinan mentah dan tulen mempunyai ciri-ciri aktiviti pemangkinan enzim yang sama dengan Topo I yang komers. Ini menunjukkan projek ini telah berjaya menghasilkan enzim yang boleh digunakan dalam asai kaedah gel. Penyaringan kesan perencatan pertumbuhan yang diberi oleh flavonoid dan camptothecin telah dijalankan terhadap empat jenis sel manusia (MCF-10A, MDA-MB-231, MCF-7 dan sel induk sumsum tulang) dengan menggunakan asai kesitotoksikan-kaedah sel. Didapati hasil kajian daripada asai kesitotoksikan MCF-7 dengan menggunakan kaedah sel adalah serasi dengan asai penyantaian DNA dengan menggunakan kaedah gel. Kedua-dua asai tersebut memberikan profil kesitotoksikan flavonoid yang aturan serupa bagi MCF-7. Antara semua flavonoid yang dikaji, apigenin didapati adalah paling menyakinkan dan berpotensi sebagai anti-kanser agen daripada hasil keputusan kedua-dua asai kaedah gel dan sel.

EXPRESSION OF RECOMBINANT HUMAN DNA TOPOISOMERASE IN *Pichia pastoris* AND ANALYSIS OF ITS ACTIVITY

ABSTRACT

Human DNA topoisomerases are essential enzymes in cellular processes because the enzymes regulate the topology state of DNA for cell survival. The enzymes have been used as molecule targets for the screening of potential anticancer agents using gel-based assay. As the commercial enzymes are quite costly, it becomes an obstacle to the research involving mass screening of potential anticancer agents. Therefore, this study was aimed to establish a method to produce in-house recombinant human DNA topoisomerase. The DNA topoisomerase I (Topo I) and DNA topoisomerase IIα (Topo IIα) coding regions were amplified from the cDNA of breast cancer cells, MDA-MB-231. Topo I and Topo IIα coding region were ligated into expression vector, pPICZ-α-A and pPICZ-α-C, respectively. Subsequently, the constructed recombinant vectors were transformed into four types of *Pichia pastoris* strain, X-33, GS115, SMD1168H and KM71H. PCR screening for recombinant clones was performed and followed by selection of multicopy clones using agar plates containing increasing concentrations of Zeocin®. The selected multicopy clones were then induced for enzymes expression in a shake flask system. Topo I was expressed relatively higher in GS115 than other *Pichia* strains. However, Topo IIα was not expressed in any strains of *Pichia* as assessed by Western blotting. Crude and purified recombinant human Topo I enzymes were used in the gel-based assay to screen four flavonoids (apigenin, chrysin, kaempferol and quercetin) and topoisomerase I inhibitor (camptothecin). The gel-based assay showed that apigenin, kaempferol and quercetin were topoisomerase I inhibitors. Only chrysin was

identified not a topoisomerase I inhibitor. The crude recombinant human Topo I and purified recombinant human Topo I showed catalytic enzyme properties that are similar to the commercial Topo I, indicating that human recombinant Topo I had been successfully produced and can be used for gel-based screening assay. Screening growth inhibitory effect of flavonoids and camptothecin was performed using cell-based cytotoxicity assay in four human cell lines, MCF-10A, MDA-MB-231, MCF-7 and bone marrow stem cells. The finding of cell-based cytotoxicity assay on MCF-7 is compatible to the finding using gel-based DNA relaxation assay. Both cell-based assay and gel-based assay provide the similar order of cytotoxic profile of flavonoids on MCF-7. Among the tested flavonoids, apigenin is the most promising and potential anticancer compound based on both gel-based and cell-based assays.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Globally, the incidence of cancer has increased significantly from the past years. Breast cancer the most common cause of death among women worldwide, has gradually raised major concern in public health (Jemal *et al*., 2011). According to the World Health Organisation data published in 2014 breast cancer deaths in Malaysia reached 2,535 or 1.99% of total deaths.

Over the past decades, there has been a global increase in researches conducted to develop new chemotherapy drugs. Some of these drugs offer promising anticancer proliferative properties which have ability to reduce metastatic effect and control the mortality and morbidity of these diseases. The current treatment option for cancer patients is by the use of chemotherapy drugs that selectively kill or inhibit proliferation of target cancer cells in order to minimize damage to surrounding healthy cells (Nussbaumer *et al*., 2011; Sun *et al.* 2017). However, these chemotherapy drugs always bring adverse effects such as bone marrow suppression, gastrointestinal tract lesions, hair loss, nausea and development of multidrug resistance on the cancer patients (Nussbaumer *et al*., 2011; Perez-Herrero and Fernandez-Medarde 2015). With advances in molecular biology methods, knowledge on molecular mechanisms of carcinogenesis shed new light in the identification of certain intracellular targets for chemotherapeutic and designing new effective anticancer agents. Enzyme-based anticancer agents that target DNA are reported to be the most effective chemotherapeutic agents in the treatment of human

malignancies (O'Connor and Mark 2015). These enzyme-based anticancer agents act on DNA-modifying enzymes such as DNA topoisomerases, polymerases and various transferases essential in the processes of nucleic acid transcription, replication, mitosis and repair in living cells (Dezhenkova *et al*., 2014). A potential anticancer agent can penetrate into the cell nucleus and interact directly with DNA or interrupt the function of DNA modifying enzymes. The interaction and disruption of these enzyme functions changed the structure of DNA that impairs genomic integrity. In response to the impairment of DNA repair and damage, the cell cycle is arrested and cell to cell death processes is started (Dezhenkova *et al*., 2014; O'Connor and Mark, 2015). Among these enzymes, DNA topoisomerases have been used as intracellular molecular targets for chemotherapeutic agents. DNA topoisomerases which are ubiquitous enzymes regulate the conformational changes of DNA by catalysing the concerted breakage and rejoining of DNA strands during normal cellular growth (Berger *et al*., 1996; Wang, 1996,; Topcu, 2001; Tsai *et al*., 2010). These enzymes act by introducing or removing DNA superhelical tensions, tying or untying DNA knots and catenating or decatenating circular DNA molecules (Topcu, 2001).

There are two types of DNA topoisomerases which have been isolated from prokaryotes and eukaryotes. The type I DNA topoisomerases (Topo I) act by making a transient break and rejoin in one strand of double helix DNA whereas type II DNA topoisomerases (Topo II) introduce transient double-strand DNA breaks and pass through another double strand DNA (Pommier *et al*., 1999; Champoux, 2001; Cheng *et al*., 2007; Bush *et al.* 2015). The cleaved DNA is covalently linked to the topoisomerase protein *via* the active site tyrosine hydroxyl and the covalent complex

represents an intermediate for the cleavage and religation of DNA. Anticancer drugs which are capable of stabilizing the covalent intermediate formed into topoisomerases will lead to permanent DNA strand breaks and cell death (Cheng *et al*., 2007; Tsai *et al*., 2010). Inhibition of the Topo I and II enzyme activities have proven to be a successful approach to screen anticancer agents. Agents that can inhibit enzymes are predicted to disrupt replication and proliferation of cells. Moreover, high expression level of topoisomerase in cancer cells compared to normal cells raises the use of topoisomerase as a potent target molecule for screening anticancer compounds (Cattan, 1996; Pommier *et al.* 1999; Pommier, 2006).

Topoisomerase inhibition assay kits for *in vitro* screening of topoisomerase inhibitor are commercially available in the market. To date, there are two specialized commercial sources of purified DNA topoisomerase I and II isolated from prokaryotes and eukaryotes which are supplied by TopoGen (www.topogen.com) and Inspiralis (www.inspiralis.com). However, the average cost per reaction is quite high e.g. RM 20-30 per reaction. In order to reduce the cost of screening and facilitate the mass screening of potential anticancer agents, establishing a rapid method to produce recombinant DNA topoisomerases needful to research remains warranted. It would be feasible to produce in-house DNA topoisomerases rather than commercialized enzymes as the first step to facilitate the main application of this project. Thus, enzymes produced can be beneficial to other researches utilising these enzymes for molecular biology study.

For this purpose, cloning and expression of human DNA topoisomerases were conducted to produce in house enzymes to screen topoisomerases inhibitor from the

group of flavonoids. The selected topoisomerase inhibitors and non-topoisomerase inhibitors were used to treat normal breast cells, breast cancer cells and bone marrow stem cells. The cytotoxic effects of the selected topoisomerase inhibitor and non-topoisomerase inhibitors on the cells proliferation were determined.

1.2 Supercoiling

DNA is a macromolecule which encodes genetic information for replication and transcription of an organism. It is a double helical molecule with one strand coiling around another and compactly packed into the cell nucleus. As the length of the chromosomal DNA far exceeds the size of a single human cell ($3x10^9$ base pairs corresponding to approximately 1.8 m), in order to have the chromosome well compacted into the cell nucleus (average diameter of 6 μM), the DNA is twisted upon itself to form a supercoiled DNA which is about one million times smaller than the length of the genome (Pommier *et al.*, 2010). Therefore, supercoiling is a very crucial process in the formation of the structure of double-helical DNA.

Supercoiling is defined as the twisting of double-helical molecule on itself. There are right handed coils defined by negative numbers and referred to negative supercoiling. There are also left handed coils which are defined by positive numbers and referred to positive supercoiling. Supercoiling makes the DNA under torsional stress and approximately 6% of the double helix is negatively wound (Wang, 1996; Schvartzman and Stasiak, 2004; Gilbert and Allan, 2014). The underwound double helix reserves energy to the DNA and enables genetic information to be accessible via the process of replication and transcription (McClendon and Osheroff, 2006; Gentry *et al*., 2011).

Topology is the mathematical studies in which the properties of an object remain unchanged under continuous deformations. Molecules which have a different linking number are called topological isomers. Linking number is defined as a topology state of the DNA molecule. Thus it is the number of times for the DNA molecule to wind in the right handed direction around the axis of the helix. It can be demonstrated as a mathematical equation:- linking number (Lk) equals to the sum of twist (Tw) and writhe (Wr) (Figure 1.1). Twist is defined as helical winding of DNA strands around each other. Writhe is the number of coiling of the axis of the double helix DNA molecule (Crick, 1976; Vologodskii and Cozzarelli, 1994; Koster *et al.*, 2010; Gilbert and Allan, 2014).

Although DNA is folded in a highly compact state in a cell, it can be loosen to be accessed by others factors/proteins during the cellular processes such as transcription and replication (Vos *et al.*, 2011). In the process of transcription, two strands of the DNA need to be untangled. However, in the process of replication, the helical twist of the two strands DNA need to be opened to allow RNA polymerase to move along the template for RNA elongation (Durand-Dubief *et al.*, 2011). Hence, DNA topoisomerases are the enzymes that play the roles in relieving the helical torsion stress by adding or removing the DNA supercoils through a series of catalytic functions.

1.3 DNA Topoisomerases

In 1971, the first DNA Topoisomerase that had been identified as ω protein from *Escherichia coli* was discovered by Wang (1971). All DNA topoisomerases can be

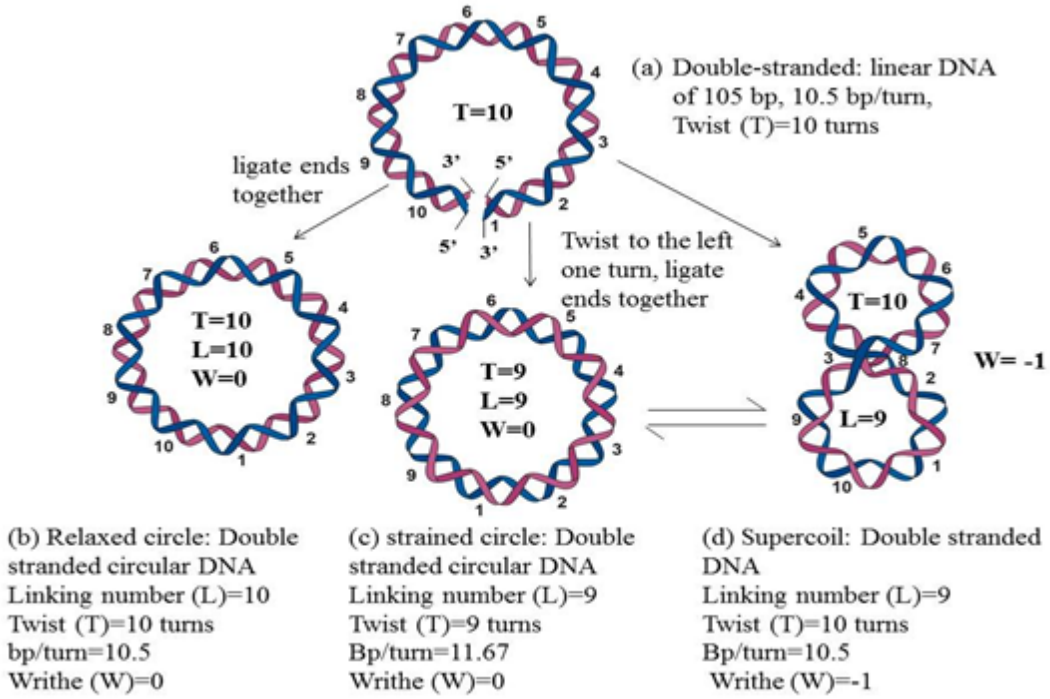


Figure 1.1 A diagram showed the different forms of supercoiling by mathematic equation of linking number (Lk) equal to the sum of twist (Tw) and writhe (Wr) twist. Adopted from Tertiary structure of DNA molecules (2005) [Online]. [Accessed 18th October 2017]. Available from World Wide Web: http://www.oregonstate.edu/instuct/bb331/lecture06/Fig4-24.gif.

divided into two classes, type I and type II depending on the capability of the enzymes to cleave one or two strands of DNA. Type I DNA topoisomerases can cleave single stranded DNA while Type II DNA topoisomerases cleave both strands of DNA. An odd Roman numeral was assigned to the name of the enzymes that fall into the type I class such as Topoisomerase I or Topoisomerase V whereas type II topoisomerase was assigned with an even Roman numeral. The type I and II enzymes are further classified as their subfamilies, A or B such as type IA or type IIA to distinguish their amino acids sequences and structures. In the type IA subfamily, the protein is linked to the 5' phosphate and the type IB is linked to the 3' phosphate as shown in Figure 1.2 (Wang, 1996; Champoux, 2001; Wang, 2002; Bush *et al.*, 2015).

Basically, topoisomerases act on the DNA by forming a transient DNA phosphodiester bond with a tyrosine residue in the active site of enzymes. The cleavage reaction of the enzyme is initiated by nucleophilic attack of OH group of the active-site tyrosine on the phosphate group of DNA to form a covalent phosphotyrosine bond between one end of the break of the DNA strand (strands) and the enzyme. The reaction will cleave the DNA strands and lead to the rotation of one of the ends of the broken strand. After the cleavage reaction, religation reaction of the broken DNA strand follows in which the hydroxyl group of the free end of the broken strand attacks on the phosphotyrosine bond of the enzyme (Berg *et al*., 2002; Pommier *et al.*, 2010). The type I topoisomerases nick in the single stranded DNA forming an intermediate covalent bond with the DNA backbone and the free strand rotates around the nicked strand to release the torsional stress (Figure 1.3). The reactions of type I topoisomerases doesn't require any energy (Stewart *et al.*, 1998).

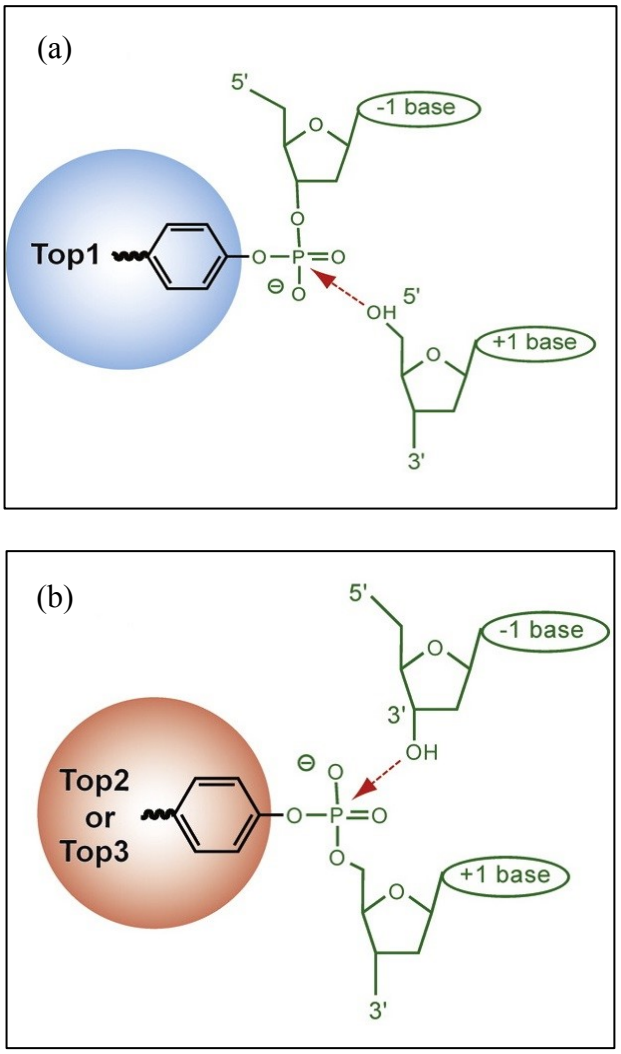


Figure 1.2 Mechanism of the topoisomerase I and II-catalyzed cleavage and ligation of the DNA. (a) The Topo I cleavable complex is formed through the 3′-phosphotyrosine covalent bond. (b) The Topo II cleavable complex is formed through the 5′-phosphotyrosine covalent bond. The arrow indicates reversibility of the cleavage and religation reactions, which is favoured under normal conditions. (Pommier *et al*., 2010).

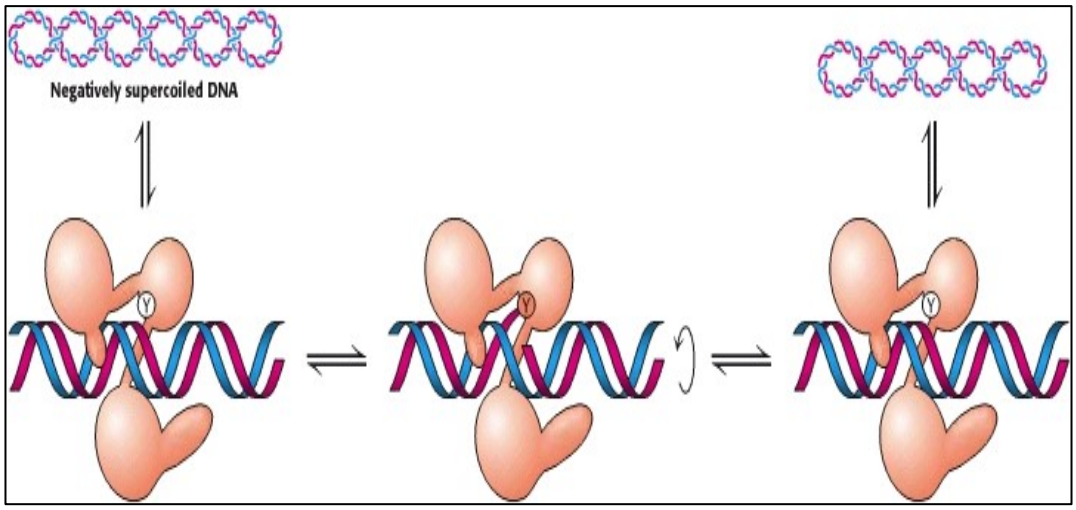


Figure 1.3 Type I topoisomerase mechanisms. Topoisomerases nicked on one DNA strand (pink) and formed a phosphodiester bond between tyrosine group of enzyme and phosphate group of the DNA. This makes the duplex DNA at the end of the nicking to rotate around and is followed by resealing of the nicking (Berg *et al*., 2002).

However, the type II topoisomerases cleave the double stranded DNA to form a covalent bond with both strands of DNA. The opposite intact double helix DNA passes through this transient break and is followed by religation of the broken strand (Figure 1.4). As a result, the enzyme relaxes the supercoiled DNA through a series of catalytic cycle (Berger *et al.*, 1996; Wang, 1998; Nitiss, 2009a).

There are six DNA topoisomerases found in human, two members derived from each of the subgroup IA (DNA topoisomerase IIIα and IIIβ), IB (DNA topoisomerase I and mitochondrial DNA topoisomerase I) and IIA (DNA topoisomerase IIα and IIβ). The enzymes function at both of the nuclear and mitochondria compartments to resolve the helical torsional stress of the supercoiled DNA during the cellular processes (Wang, 1996). Among these enzymes, DNA Topoisomerase I and DNA Topoisomerase IIα are the best studied human DNA topoisomerase in the context of their biochemical, structural and cellular function.

1.3.1 Human DNA Topoisomerase I (EC 5.99.1.2)

The Human DNA topoisomerase I (Topo I) is a monomeric enzyme and is classified as type IB subgroup of the enzymes. There are two types of IB topoisomerases in mammalian cells which are found in nuclear and mitochondrial region of the cells (Zhang *et al.*, 2001). It consists of 2298 base pairs and encodes a protein with a molecular weight about 91 kDa. Topo I comprised of four domains: the unconserved and highly charged 'amino terminal' domain (24 kDa), the conserved core domain (52 kDa), a poorly conserved and positively charged 'linker' region (3 kDa) and the highly conserved 'carboxyl-terminal' domain (10 kDa) which contained an active site tyrosine at the position 723 (Stewart *et al.*, 1996a). The 'amino terminal' domain

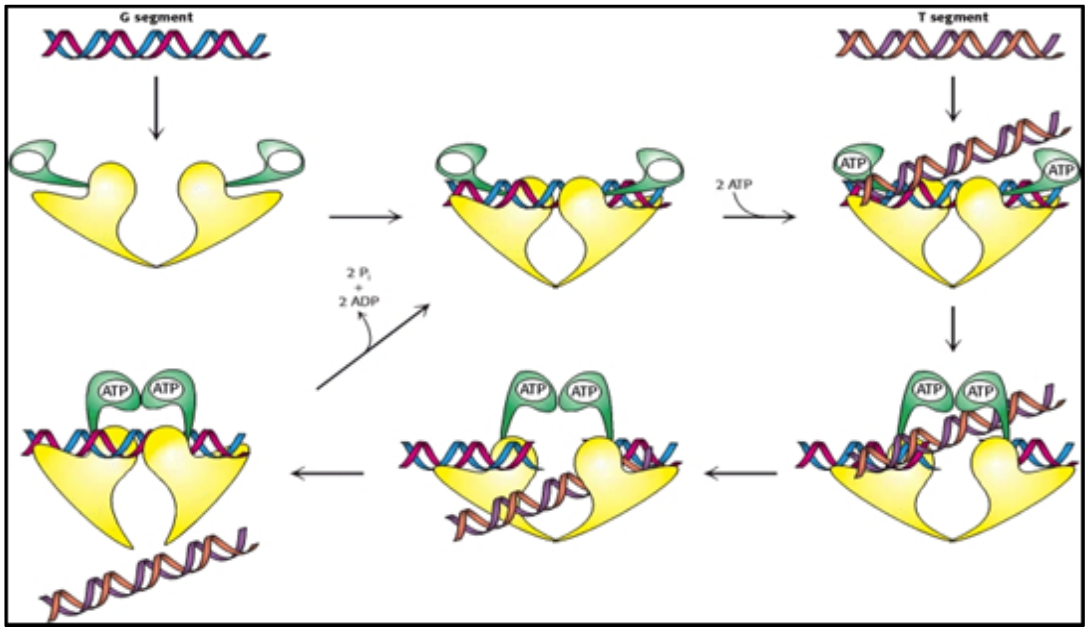


Figure 1.4 Type II topoisomerase mechanisms. Type II enzymes cleave both strands of a DNA duplex (G-segment) and pass a second intact duplex DNA (T-segment) through the transient break. Two ATPs are used for the strand passage (Berg *et al*., 2002).

is sensitive to proteolysis and is dispensable for DNA relaxation activity. It has five nuclear localization signals which are responsible for subcellular localization of the enzyme *in vivo* (Alsner *et al.*, 1992; Stewart *et al.*, 1996b). However, both of the core and the 'carboxyl-terminal' domains are required for the DNA relaxation activity *in vitro*. The domains contain five active site amino acid residues (Arg-488, Lys-532, Arg-590, His-632 and Tyr-723) conserved within the eukaryotic type IB group of the enzymes (Stewart *et al.*, 1997). The domain structure of human Topo I is depicted in Figure 1.5.

1.3.2 Human DNA Topoisomerase IIα (EC 5.99.1.3)

Human DNA topoisomerase IIα (Topo IIα) is categorised into type IIA subgroup of the enzymes and it is an ATP-dependant enzyme. Topo IIα was first cloned and sequenced in 1988 (Tsai-Pflugfelder *et al.*, 1988). It consists of 4296 base pairs and encodes 1531 amino acid residues. It is highly conserved with all the eukaryotic type II topoisomerase such as yeast.

There are three conserved domains found in the enzyme, the amino terminal domain of the enzyme contains the ATP-binding domain (1-431 amino acid residues), a central core domain which carries an active site tyrosine required for DNA cleavage (at position 805) and a 'carboxyl terminal' domain (1193-1531) characterized by clusters of charged amino acids which is required for nuclear localization and contained phosphorylation sites as shown in Figure 1.6 (Dickey and Osheroff, 2005; Nitiss, 2009a). There are three conserved motifs (EDGSA, IMTDQ and PLRGK) which are important for type II topoisomerase function (Aravind *et al.*, 1998). This enzyme can still function well and is active when trunscated at residue 1296

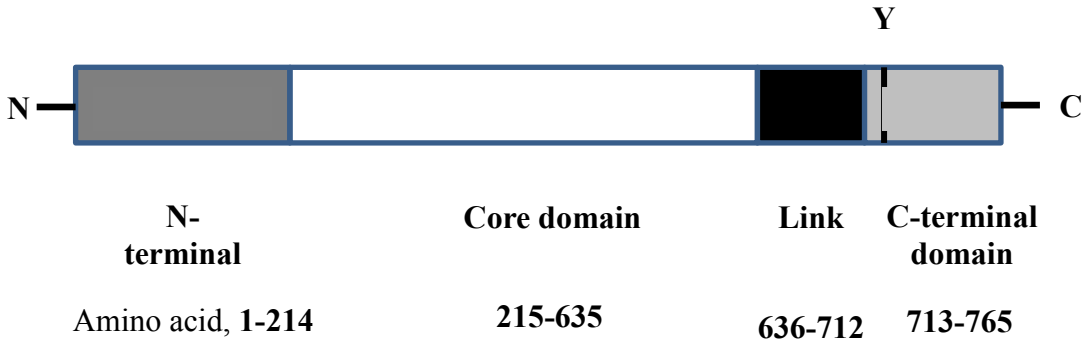


Figure 1.5 Schematic showing the domain of full length human topoisomerase I. N: Amino terminal, C: Carboxyl terminal and Y: Active site tyrosine (Stewart *et al.*, 1996a).

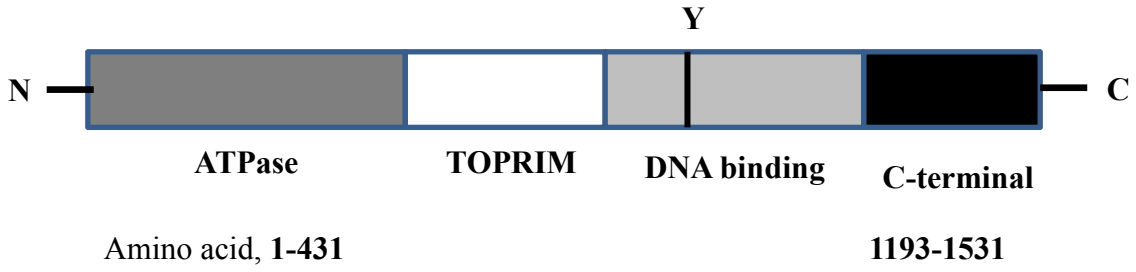


Figure 1.6 Schematic showing the domain of full length human topoisomerase II. N: Amino terminal, C: Carboxyl terminal and Y: Active site tyrosine (Gilroy and Austin, 2011).

Jensen *et al.*, 1996). Like other type II topoisomerase enzymes, human Topo IIα can introduce two transient DNA strands breaks with 4 bp stagger in a single DNA duplex (Wang, 2002).

1.4 Molecular target for primary screening of potential anticancer agent

As DNA topoisomerases are essential for many vital cellular processes such as transcription, replication and recombination, they have been used as target enzymes for chemotherapy. Agents or compounds which can inhibit the enzyme activity will interrupt the cellular processes in the cells and generate enzyme-mediated DNA damage that leads to the cell death. Both Topo I and Topo IIα are important targets for screening of anticancer drugs. Topo I is not a cell cycle-dependent enzyme which is expressed in a constant rate throughout different phases of cell growth (Sinha, 1995). This makes Topo I a useful cellular target for development of anticancer drugs. Unlike Topo I, Topo IIα is found highly expressed in proliferating cells such as cancer cells than normal cells (Holden, 2001). Overexpressing of Topo IIα in high proliferating cancer cells increase the sensitivity of the cells to drugs and resistance to drugs are found in the cells that express low level of Topo IIα (Nitiss, 2009b).

Lot of studies have been carried out to find a novel topoisomerase inhibitor using topoisomerases as molecular targets for screening of potential anticancer agents. Topoisomerase inhibitors represent a major class of anticancer agents which are widely used for chemotherapy in a broad spectrum of tumours (Holden, 2001; Nitiss, 2009b). Normally, there are two groups of topoisomerase inhibitors, known as topoisomerase poisons and topoisomerase catalytic inhibitors which are classified based on their mode of action. Topoisomerase poison acts on the enzyme-DNA

intermediate cleavage complex. It impairs the religation of the nicked DNA and induce forward rate of DNA cleavage (Froelich-Ammon and Osheroff, 1995). The broken DNA accumulates and if the DNA is not repaired, it will be lethal to the cell. In contrast to topoisomerase poison, topoisomerase inhibitor disrupts the catalytic activity of the enzyme. It does not stabilize the enzyme-DNA intermediate cleavage complex. The inhibitor normally acts on the free enzyme and prevent it carrying out its catalytic function (Bailly, 2000).

To date, topoisomerases inhibitors which target Topo I and Topo II have been developed. A list of clinically used anticancer drugs which target Topo I and Topo II is shown in Table 1.1. Camptothecin is one of the well-established and clinically approved drugs for Topo I poison but the drug can cause serious side effects to cancer patients (O'Leary and Muggia, 1998; Srivastava *et al.*, 2005). As camptothecin is not a water soluble compound, two of its improved derivative, topotecan and irinotecan have been recently approved for clinical use (O'Leary and Muggia, 1998; Takimoto *et al*., 1998). Epipodophyllotoxin etoposide (VP-16) and the anthracycline doxorubicin (Adriamycin) are categorised as Topo II poison in which they can increase the level of Topo II-DNA covalent complex. Sensitivity of the cells to the Topo II drugs also depends on the level of Topo II. However, Topo II poison can bring about secondary malignancies to cancer patients as a result of drug induced translocations of DNA (Felix, 1998). In contrast to Topoisomerase II poison, Topo II inhibitors inhibit enzyme activity through several catalytic steps in the enzyme reaction cycle but do not induce enzyme-mediated DNA damage. Agents such as novobiocin and coumermycin in the groups of coumarin drugs inhibit Topo II by blocking the ATP binding site. Topoisomerase inhibitors are found to be able to

Table 1.1: Different classes of DNA topoisomerase I and II directed drugs (Boege *et al.*, 1996; Topcu, 2001; Larsen *et al.*, 2003; Nitiss, 2009b)

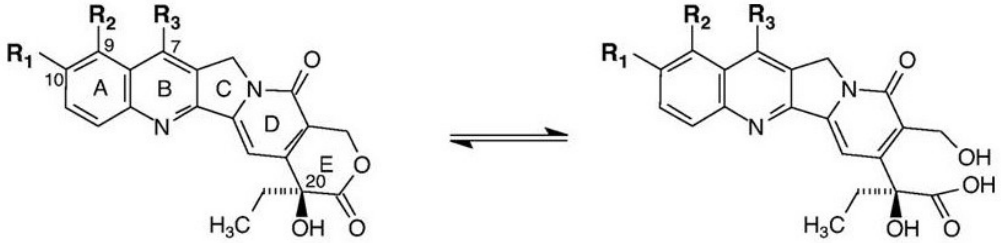
Class	**Subclass**	**Group**	**Example drug**	**Target on Topo**
Class I (Poisons)	Intercalative	acridines	M-AMSA	II
		anthracyclines	doxorubicin	II
		actinomycins	actinomycin D	I & II
		ellipticines	2-methyl-9-OH-epllipticinium acetate	II
	Non-intercalative	alkaloids	camptothecin	I
		epipodophyllotoxins	etoposide	II
		quinolones	ciprofloxacin	
Class II (Catalytic inhibitors)	Intercalative	coumarins	novobiocin	II
		anthracyclines	aclarubicin	I &II
	Non-intercalative	fostriecin analogues	fostriecin	II
		thiobarbituric acids and aniline conjugates	merbarone	II
		bisdioxopiperazine	ICRF 154, ICRF 159	II

reduce the function of the enzyme by binding into ATP binding site and trapping the enzyme as a closed clamp on DNA or docking the ATP-binding pocket and preventing nucleotide binding (Salerno *et al*., 2010).

1.4.1 Camptothecin

Camptothecin was discovered in 1950s during a search for compounds that could be used for steroid synthesis (Wall *et al.*, 1966). It was extracted from the wood, bark and fruit in the tree named *Camptotheca acuminate* (O'Leary and Muggia, 1998). The drug has been used in traditional Chinese medicine in the treatment of various diseases including tumours. Due to its limited water solubility, a water soluble salt was added for use in clinical trials (O'Leary and Muggia, 1998). Two water-soluble camptothecin analogs have been approved by the Food and Drug Administration for clinical use: topotecan, as a second-line therapy for ovarian cancer or small-cell lung cancer, and irinotecan, for the treatment of colorectal carcinoma (Bomgaars *et al*., 2001).

The lactone ring of camptothecin plays the major role for its activity and a pH dependant equilibrium existed between the lactone and carboxylate forms (Arbuck and Takimoto, 1998). The drug requires an intact α-hydroxyl lactone group in the E-ring for optimal interactions with the topoisomerase I enzyme (Hertzberg *et al.* 1987). As the E-ring is labile in aqueous solutions, it would be undergoing reversible pH-dependent hydrolysis to a relatively inactive open-ring carboxylate form (Fig. 1.7). The drug can stabilize the cleavable complex between the topoisomerase I molecule and the free 3′- phosphate of the DNA. Due to the stabilised cleavable



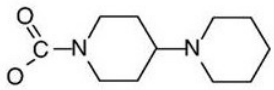
	R_1	R_2	R_3
Camptotecin	H	H	H
Topotecan	OH	$CH_2N(CH_3)_2$	H
9-Aminocamptotecin	H	NH_2	H
Irinotecin		H	CH_2CH_3
SN-38	OH	H	CH_2CH_3

Figure 1.7 Structures of camptothecin and its semisynthetic analogs in the active lactone form and the closed-ring hydroxy acid (inactive) form (Blaney *et al.*, 1993).

complex, the resulting enzyme-linked DNA breaks cannot be religated as long as drug is present (Hertzberg *et al*.,1987).

1.5 Screening of anticancer agents with DNA relaxation assay

DNA relaxation assay is a widely used *in vitro* method to determine the activity of topoisomerases. It is a gel based assay in which the DNA relaxation activity of topoisomerase reaction is based on the intensity of the substrate, a supercoiled plasmid DNA. The intensity of supercoiled plasmid DNA is visualised after electrophoretical separation of agarose gel post-stained with ethidium bromide. Supercoiled plasmid DNA represents topological constraints of DNA which is a good model to study the changes of DNA.

The structure of plasmid DNA is highly sensitive to the changes of the reaction and can be responded by different topological changes (Jiang, *et al*., 2007). Various types of supercoiled plasmid DNA can be used as substrates such as pBR 322 and pUC 18. However, high copy numbers of plasmid is more suitable for the assay. Usually, plasmids with base pairs between 2,500 and 3,500 could display a sufficient number of topoisomers and can be easily resolved on the gel. If the number of base pairs is increased to more than 4,000 bp, the number of topoisomers produced also increase. This makes the assay more difficult to manipulate and the great number of topoisomers reduces resolution on gel electrophoresis. Besides, preparation of plasmid DNA with more than 90% of supercoil form and 2–10% of plasmid DNA with open circular DNA form (nicked on one of the two strands of the DNA helix) or linear DNA form (nicked on both two strands) are crucial to give a reliable result for the relaxation assay (Nitiss *et al*., 2001; Fox, 2010).

Inhibition of DNA relaxation in Topo I by an unknown drug or compound was indicated by unrelaxed supercoiled plasmid DNA on the gel. Different forms of the plasmid DNA can be distinguished easily as they have different electrophoretic motility (Nitiss *et al*., 2001). Figure 1.8 shows the resolving of topoisomers generated from catalytic reaction of topoisomerases on agarose gel. One of the advantages of the gel based assay is that it can show a possible mechanism of the cytotoxic activity of a compound. The compound which can intercalate into plasmid DNA may cause inhibition of DNA topoisomerase and result in cell cytotoxicity. Besides, the gel based method is easily carried out in a basic laboratory set up containing water bath, agarose gel electrophoresis and UV imager. Thus, results can be obtained within a day. It has the advantage for screening large groups of compounds with anticancer property. Compared to cell based assay such as MTT and LDH assay for screening of potential anticancer agent, the gel based is more simple, economic and not time-consumed. The cell based assay needs a well-equipped cell culture laboratory, well-trained personnel in cell culture skills and more time is consumed for maintaining the cell culture.

Other methods to identify Topo I inhibitor is DNA cleavage assay. The assay is useful for comparison of the effectiveness of drugs in stabilizing the Topo I-DNA intermediate or cleavage (cleavable) complex. It can determine the action of a drug on blocking the forward cleavage or religation reactions by measuring the reversibility of the drug-induced Topo I–DNA cleavage complexes. However, the assay needs about 2 days to complete the entire protocol and much higher amounts of Topo I enzyme is required compared to the relaxation assay (Jaxel *et al.*, 1988;

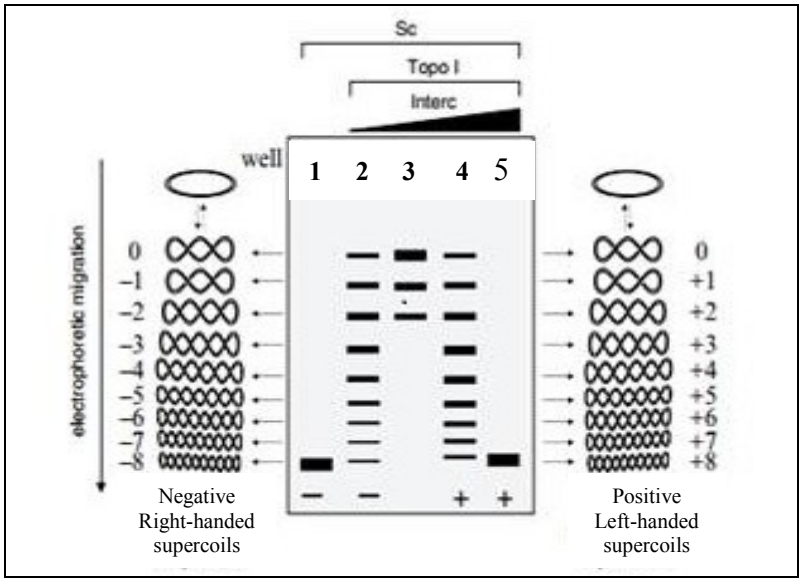


Figure 1.8 Schematic diagram of the DNA relaxation process of supercoiled plasmid DNA. Different forms of topoisomers generated by the cleavage and religation of topoisomerase I resolved on agarose gel in the absence of ethidium bromide. **Lane 1**: control for the supercoiled plasmid DNA, Sc in the form of underwound helical (negative or right handed supercoils). **Lane 2-5**: Supercoiled plasmid DNA, Sc treated with increasing concentration of intercalator (Interc) in the presence of Topo I revealed transition of the Sc from underwound helicals to fully relaxed DNA and positive supercoiled (Fox, 2010).

Dexheimer and Pommier, 2008). Compared to the cleavage assay, the DNA relaxation assay has all the steps of the Topo 1 reaction-binding, cleavage, strand transport, religation and enzyme turnover but it cannot give any particular information about the effect of drugs to the specific individual steps of Topo I reaction. Despite of the limitations of DNA relaxation assay in differentiating Topo I poisons (which trap Topo I cleavable complex), DNA intercalators (which unwind the DNA without directly affecting Topo I) and Topo I catalytic inhibitors (which inhibit Topo I catalytic activity directly but without trapping Topo I cleavable complex), the DNA relaxation assay is still very useful to be used as a primary screening method for potential anticancer drugs (Nitiss *et al*., 2001; Dexheimer and Pommier, 2008).

Similarly with Topo I, catalytic activity of Topo II can be determined by DNA relaxation assay. The difference of Topo I and Topo II in DNA relaxation catalytic activity is Topo II requires ATP and a divalent cation for the reaction (Nitiss *et al*., 2001). Besides DNA relaxation assay, decatenation assay is another option to determine the specific activity of Topo II as the enzyme has ability to catalyze the decatenation of intact double-stranded DNA (Wang, 2002; Nitiss, 2009b). Kinetoplast DNA from *Crithidia fasciculate* which forms a large network of interlocked (catenated) circles is used as a substrate in the assay (Marini *et al*., 1980). In the assay, Topo II decatenates the circles from the network and the decatenated circles are able to enter an agarose gel detected as a discrete band on the gel. This assay is specific for Topo II catalytic activity as Topo I cannot catalyze the decatenation of DNA.

1.6 Protein expression system

To date, various recombinant protein expression systems including bacteria, yeast, plant, insect and mammalian cells have been developed for heterologous protein production. Among these systems, expression of protein in bacteria is the most commonly used systems as bacterial can grow rapidly with high cell density and inexpensive substrates. The system also provides a well-characterized genetic modified host cells and a wide range of expression vectors (Terpe, 2006). One of the limitations of the system is the inability to perform post-translational of the protein such as glycosylation (Schmidt, 2004). If the heterologous protein have to be modified post-translational, the yeast expression system is one of the higher eukaryotic expression systems can be considered as the system is easy to manipulate and cheap in terms of large scale production.

However, insect and mammalian cells expression system should be chosen if the post-translational modification of the proteins will affect the biological activity of the protein as both systems are able to display posttranslational modification patterns identical to human (Schmidt, 2004). Previous studies have reported that human topoisomerase I has been expressed in bacteria (Rubin *et al*., 1994), baculovirus (Stewart *et al.*, 1996b; Stewart and Champoux, 1999) and yeast (Bjornsti *et al.*, 1989; Wasserman *et al.*, 1993). On the other hand, human topoisomerase IIα has been expressed successfully in yeast, *Saccharomyces cerevisiae* (Kingma *et al.*, 1997; Dickey and Osheroff, 2005). Expression of topoisomerases in *E. coli* has proven to be disappointing with relatively low activity, is unstable with proteolytic products and overexpression of the enzymes which was also found to be toxic to the host cells (Kikuchi and Miyaike, 1993; Nitiss *et al*., 2001). Therefore, expression of

topoisomerases is more feasible to be expressed in eukaryotic expression systems. Human Topo I has been successfully overexpressed in *Pichia* yeast expression system (Yang *et al*., 2004a). However, human Topo IIα has not been expressed in the system.

1.6.1 *Pichia* yeast expression system

Pichia pastoris is methylotrophic yeast which is easy to grow and manipulate for heterologous protein expression. It had been developed in early 1980s by Phillips Petroleum Company in cooperation with the Salk Institute Biotechnology/Industrial associates, Inc (SIBIA). To date, well-characterized expression vectors and different genetic modified *Pichia* strains has been developed for the system to facilitate production of high yield heterologous protein in *Pichia pastoris* (Cregg *et al.*, 2000). As a eukaryote, it has the ability to perform post-translational modifications of protein such as proteolytic processing, folding, disulphide bond formation and glycosylation which are found in higher eukaryotic cells (Eckart and Bussineau, 1996; Cregg *et al.*, 2000). The folding and post translational processing of vertebrate protein in yeast is very similar to the one from mammalian cells, which results in the preservation of the native receptor structure when expressed in yeast. Compared to mammalian cell system, yeasts are more economical and can produce high yield of heterologous protein (Macauley-Patrick *et al.*, 2005). However, certain complex post-translational modification such as prolyl hydroxylation and amidation, some types of phosphorylation and glycosylation cannot be carried out in yeast expression system (Cregg and Higgins, 1995).