RECOMBINANT EXPRESSION OF β-GLUCOSIDASE FROM THAI ROSEWOOD (*Dalbergia cochinchinensis* Pierre) IN YEAST

Pornphimon Metheenukul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry

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การแสดงออกของเอนไซม์บีตา-กลูโคซิเดสจากพะยูงไทย (*Dalbergia cochinchinensis* Pierre) ในยีสต์

นางสาวพรพิมล เมธีนุกูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ได้ทำการโคลนยีนเอนไซม์บีตา-กลูโคซิเดสจากพะยูงไทย (*Dalbergia cochinchinensis* Pierre) ลงใน *Escherichia coli*, ยีสต์ *Saccharomyces cereviseae* และ ยีสต์ *Pichia pastoris* จากนั้นได้ศึกษาสมบัติของเอนไซม์บีตา-กลูโคซิเดสที่ได้จากสิ่งมีชีวิตข้างต้น พบว่าเอนไซม์บีตา-กลูโคซิเดสจากพะยูงไทยที่แสดงออกในยีสต์ *P.pastoris* มีแอกติวิตีของเอนไซม์บีตา-กลูโคซิเดสและเอนไซม์บีตา-ฟิวโคซิเดสสูงที่สุด จากนั้นได้แยกเอนไซม์จากอาหารเลี้ยงโดยใช้วิธี Immobilized Metal Affinity Chromatography (IMAC) พบว่าไม่สามารถทำให้เอนไซม์บีตา-กลูโคซิเดสจากพะยูงไทยในอาหารเลี้ยงเชื้อที่มีการเติมส่วน polyhistidine tag ให้กับด้าน C-terminus หรือ N-terminus หรือเติมให้กับทั้งสองด้านให้บริสุทธิ์ได้โดยวิธี IMAC ในขณะที่ใช้วิธี IMAC ทำให้เอนไซม์บีตา-กลูโคซิเดสจากพะยูงไทยที่ด้าน N-terminus มีฮิสติดีน 8 ตำแหน่ง และการตัดที่ปลายด้าน N-terminus ที่วาลีน ตำแหน่งที่ 14 บริสุทธิ์ได้ นอกจากนั้นการศึกษาสมบัติของเอนไซม์บีตา-กลูโคซิเดสที่แสดงออกในยีสต์ *P.pastoris* ได้ผลว่าค่า K_m, k_{cat}/K_m สำหรับ *p*NP-β-D-glucoside และ *p*NP-β-D-fucoside, อุณหภูมิ และค่าพีเอชที่เหมาะสมมีค่าใกล้เคียงกับเอนไซม์บีตา-กลูโคซิเดสจากเมล็ดพะยูงไทย

สาขาวิชาชีวเคมี
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ลายมือชื่ออาจารย์ที่ปรึกษา James R McCarthy
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

PORNPHIMON METHEENUKUL : RECOMBINANT EXPRESSION OF β-GLUCOSIDASE FROM THAI ROSEWOOD (*Dalbergia cochinchinensis* Pierre) IN YEAST. THESIS ADVISOR : ASST. PROF. JAMES R. KETUDAT-CAIRNS, Ph.D. 208 PP. ISBN 974-533-475-8

β-GLUCOSIDASE AND THAI ROSEWOOD

The cDNA for β-glucosidase from Thai rosewood, *Dalbergia cochinchinensis* Pierre, was produced and cloned into *Escherichia coli*, *Saccharomyces cereviseae* and *Pichia pastoris* recombinant expression systems, and the β-glucosidase was expressed. Recombinant expression of Thai rosewood β-glucosidase in *P.pastoris* gave the highest levels of both β-glucosidase and β-fucosidase activities. The enzyme was purified from culture medium by Immobilized Metal Affinity Chromatography (IMAC). The recombinant proteins produced with C-terminal His-tag, N-terminal His-tag or both N-terminal and C-terminal His-tags of the full mature protein could not be purified by IMAC. However, the recombinant protein with an N-terminal 8 x histidine tag and its N-terminus truncated at valine residue 14 could be purified from culture medium by IMAC. It was found that the recombinant Thai rosewood β-glucosidase expressed in *P.pastoris* had K_m and k_{cat}/K_m values for *p*NP-β-D-glucoside and *p*NP-β-D-fucoside, temperature and pH optima similar to those of native enzyme purified from Thai rosewood seeds.

School of Biochemistry

Academic Year 2005

Student's Signature Pornphimon Metheenukul

Advisor's Signature James R Ketudat Cairns

Co-advisor's Signature

Co-advisor's Signature

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Pornphimon Metheenukul

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LIST OF ABBREVIATIONS

A	Absorbance
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine Serum Albumin
°C	Degrees Celsius
cDNA	Complementary deoxynucleic acid
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	dATP, dCTP, dGTP and dTTP
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene diamine tetraacetic acid
g	Gravitational acceleration
(m, n) g	(milli, nano) Gram
h	Hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
min	Minute

LIST OF ABBREVIATIONS (Continued)

(m, μ, n) M	(milli, micro, nano) Molar
(m, μ) L	(milli, micro) Liter
(μ, n, pmol) mol	(micro, nano, pico) Mole
mRNA	Messenger ribonucleic acid
Mr	Molecular weight
4MUGlc	4-Methylumbellriferyl-β-D-glucoside
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
p-NP	*p*-Nitrophenol
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
s	Second
TEMED	Tetramethylenediamine
UV	Ultraviolet
U	Unit, μmol/min
v/v	Volume/volume

CHAPTER I

INTRODUCTION

1.1 Overview of β-glucosidases

β-glucosidases or β-glucoside glucohydrolases, EC 3.2.1.21, are a group of glycosidase enzymes (EC 3.2) which catalyze the hydrolysis of alkyl- and aryl- β-glucosides, as well as diglucosides and oligosaccharides, to release glucose and aglycones (Reese, 1977). These enzymes are found in many kinds of organisms, including fungi, bacteria, animals and plants, indicating their general importance to life. Therefore, β-glucosidase structures, catalytic properties, and biological functions are of interest. β-glucosidases have been studied by many researchers because of their important roles and medical, biotechnological, agricultural and industrial applications.

Most β-glucosidases share some features, such as being absolutely specific for β-glycosidic linkages, having acidic pH optima (pH 5-6), and subunit molecular weights of 55 to 66 kD (Esen A, 1993). The enzymes from different sources show broad specificities for both the aglycone and glycone part of their substrates, and this property is an advantage for industrial applications.

In microorganisms, β-glucosidases have an important role in biomass conversion (the hydrolysis of cellulose and cellobiose). This process has important applications in industry, such as the production of alcohol based fuels (Bothast and

Saha, 1997) and sugars (Woodward and Wiseman, 1982). In addition, these enzymes have been used in degradation of polysaccharide wastes, development of novel carbohydrate foods, and release of aromatic compounds from flavorless glucoside precursors in wine and fruit juice processing (Riou *et al*., 1998).

Mammals contain two β-glucosidases, lysosomal glucocerebrosidase and cytosolic β-glucosidase. Glucocerebrosidase catalyzes the degradation of the glycosphingolipid glucocerebroside to glucose and ceramide (Beutler, 1992). It has an acidic pH optimum. Deficiency of this enzyme in the lysosome causes Gaucher disease. Such deficiency is caused by mutations in the gene encoding the enzyme that result in altered β-glucosidase forms which lack catalytic activity or are not properly targeted to the lysozyme. When catalytic activity of the enzyme is lacking, the accumulation of the glucosylceramide in the lysosome of reticuloendothelial cells occurs (Beutler, 1992). Many studies have been conducted to investigate the structure-function relationships and mechanism of catalysis of glucocerebrosidase in order to understand the molecular pathology of Gaucher disease, and to use the enzyme or gene therapy for treatment of this disease (Berrin *et al*., 2002). In contrast, cytosolic β-glucosidase, which belongs to the glycosyl hydrolase family 1, has very broad substrate specificity, is most active at neutral pH and is not glycosylated (Daniels *et al*., 1981). Berrin *et al*. (2002) showed human cytosolic β-glucosidase hydrolyses a number of xenobiotic glycosides and suggested a role in xenobiotic metabolism.

In plants, β-glucosidases have been implicated in several important functions, including defense mechanisms, phytohormone regulation, release of glucose from cell wall oligosaccharides, and lignification. In defense against pathogens and herbivores,

β-glucosidases act by releasing toxic defense compounds, such as coumarins, saponins, thiocyanates, hydrogen cyanide, hydroxamic acids and terpenes (Bell, 1981; Conn, 1993; Niemeyer, 1988; Nisius, 1988; Poulton, 1990; Sahi *et al*., 1990), and by releasing inducing factors, such as volatiles that attract parasitoids of herbivores to attack them, from their inactive and nonvolatile glycosides (Mattiacci *et al*., 1995). β-glucosidases regulate the biological activity of plant phytohormones, such as cytokinins, gibberellins, auxins and abscisic acids by releasing active forms from inactive hormone glucoside conjugates (Gaskin and MacMillian, 1975; Ganguly *et al*., 1974; Brzobohaty *et al.*, 1994; Millborrow, 1970). Leah *et al*. (1995) and Hrmova *et al*. (1996, 1998) found that barley β-glucosidase (BGQ 60) hydrolyzed cellobiose, laminaribiose and other short-chain polysaccharides produced by cellulases and β-glucanases. β-glucosidases are also critical for activation of lignin precursors, release of plant volatiles from their glycoside storage forms, and metabolism of many other important natural products (Esen, 1993; Geerlings *et al.*, 2000).

Over the past few decades, substrate specificity of β-glucosidases has been the subject of extensive research. There are various natural substrates of β-glucosidases, such as steroid β-glucosides, the β-glucosyl ceramides of mammals, the cyanogenic β-glucosides of plant secondary metabolism and oligosaccharide products released from digestion of the cellulose of plant cell-walls (Clarke *et al*., 1993). In fact, β-glucosidases from different sources have similar specificity for glycone (glucose) and some non-physiological aglycone moieties, such as *p*-nitrophenol (Esen, 1993). However, in plants, the aglycone specificity of β-glucosidases has been well established by numerous studies (e.g. Babcock and Esen, 1994; Hösel and Conn, 1982;

Hughes and Dunn, 1982). The aglycone moieties, which are important for the specificity of plant β-glucosidases, include plant hormones, hydroxamic acids, flavonols, cyanoglucosides and mandelonitrils (Smitt and Van Staden, 1978; Campos *et al*., 1993; Cuevas *et al*., 1992; Niemayer, 1988; Conn, 1993). Since, as mentioned above, these aglycone moieties are implicated in a number of functions in plant metabolism, it should be emphasized that the aglycone specificity is important in determining the functions of β-glucosidases in plants (Hösel and Conn, 1982).

1.2 Plant β-glucosidase purification and characterization

β-glucosidases have been isolated and characterized at the biochemical and DNA levels in many species of plants, both from dicots and monocots. Most β-glucosidases isolated from dicots so far, including *Trifolium repens* (Kakes, 1985), *Brassica napus* (Hoglund *et al*., 1992) and black cherry (Poulton and Li, 1994) are localized in the cell wall or vacuole, while the enzymes isolated from *Polygonum tinctorium* are localized in the plastid (Minami *et al*., 1997). In addition, most dicot β-glucosidases have been shown to be glycosylated (Esen, 1993). Initially, most β-glucosidases isolated from monocots, including sorghum (Thayer and Conn, 1981), oat (Nisius, 1988) and maize (Esen, 1992) were localized in the plastid. Later, several monocot β-glucosidases including the endosperm specific β-glucosidase from barley and two β-glucosidases found in germinating rice were found to have signal peptides for endoplasmic reticulum (ER) targeting (Leah *et al*., 1995; Opassiri *et al*., 2003) and another was found to localize to rice cell walls (Akiyama *et al*., 1998). Complete cDNA and deduced amino acid sequences of plant β-glucosidases have been

determined from at least 14 different species, such as *Arabidopsis thaliana*, white clover, cabbage, *Brassica napus*, sweet cherry, black cherry, sweet almond, cassava, costus, maize, sorghum, barley, oat, rice and Thai rosewood. Comparison of amino acid sequences of these β-glucosidases has shown that their similarity ranged from 35-90% depending, in some cases, on evolutionary distances among taxa. For instance, the deduced amino acid sequence of maize β-glucosidase showed 70% identity with dhurrinase from sorghum (Cicek and Esen, 1998), 55% identity with oat, and 47% identity with *Prunus serotina,* respectively. However, the similarity between the sequences of plant β-glucosidases cannot be used to infer the specific functions of enzymes, because only small sequence differences affect substrate specificity of the enzymes (Esen, 1993).

1.3 Overview of Glycosides

A glycoside is a compound formed between a furanose or pyranose sugar and one or more nonsugar (aglycone) compounds through a glycosidic linkage (Noggle and Fritz, 1976). The glycoside structure is an ether-like compound containing a group or a molecule with a nucleophilic atom which is usually an oxygen atom. The linkage between the glycone and the aglycone through an oxygen atom yields O-glycosides, i. e., C-O-C. Less commonly, the nuclephilic atom is carbon, giving rise to the C-glycosides in which the sugar-aglycone linkage is C-C. When the nucleophilic atom is nitrogen or sulphur, N-glycosides and S-glycosides are obtained, giving rise to C-N-C or C-S-C, respectively. Whereas the monosaccharide-aglycone linkage of oxygen, nitrogen and sulphur is susceptible to acid, alkali or enzyme-catalyzed hydrolysis, the C-C linkage of the C-glycoside is not (Datta, 1994).

The sugar component of glycosides is usually D-glucose, although D- and L-galactose, D-mannose, D-fucose, and L-rhamnose have also been found. Some pentoses, for example, D- and L-arabinose, and D-ribose, have also been detected. In some glycosides the glycone parts are present as disaccharides or, in some case, as trisaccharides (Dake, 1960).

1.4 Catalytic mechanism of β-glucosidases

β-glycosidases and other glycosidases can be divided into two classes, inverting and retaining glycosidases, by whether the anomeric configuration of the released monosaccharide or oligosaccharide is inverted or retained compared to the substrate. The mechanisms of both enzymes differ in that inverting glycosidases catalyze via a direct displacement of the aglycone (leaving group) by water (which acts as a nucleophile), whereas retaining glycosidases catalyze through a glycosyl-enzyme intermediate in a double displacement mechanism (McCarter and Withers, 1994).

The inverting glycosidases use a direct displacement mechanism in which the two carboxylic acids at the active site are suitably positioned, such that one provides general base catalytic assistance to the attack of water on the anomeric carbon, while the other provides general acid catalytic assistance to cleavage of the glycosidic bond. The reaction proceeds through an oxocarbenium ion like transition state (Esen, 1993).

Catalysis by retaining glycosidases proceeds via a two step double displacement mechanism involving the formation and hydrolysis of a covalent glycosyl enzyme intermediate, both steps again proceeding through oxocarbenium ion-like transition states (Esen, 1993). Two active site carboxylic acids are also

involved in this mechanism, but have somewhat different roles. One functions as the nucleophile, attacking at the sugar anomeric center to form the glycosyl-enzyme species, the other carboxyl group acts as an acid/ base catalyst, protonating the glycosidic oxygen in the first step (general acid catalysis) and deprotonating the water in the second step (general base catalysis) (Esen, 1993).

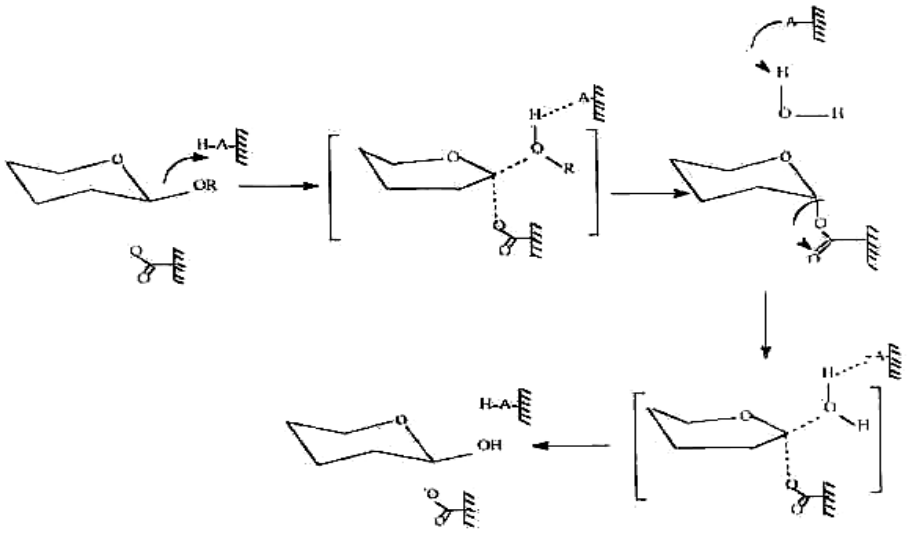


Figure 1.1 Generally accepted endocyclic pathway of the double placement mechanism proposed for retaining β-glucosidases (Esen, 1993)

In spite of the difference, there are several similarities between the two mechanisms. Both types of enzymes employ the same principle of catalytic mechanism, general acid-base catalysis. They both have a pair of conserved carboxylic amino acids, which may be aspartate (Asp) or glutamate (Glu) (McCarter and Withers, 1994). In inverting glycosidases, one residue acts as a general acid and the other as a general base. In retaining glycosidases, one residue functions both as a general acid and general base, while the other residue functions both as a nucleophile

and a leaving group. The cause of distinct mechanisms in both enzymes is the distance between the two carboxylic groups. In retaining glycosidases, they are close enough to form a glycosyl-enzyme intermediate. However, in the inverting glycosidases, the carboxylates have greater distance separation, allowing the insertion of a water molecule for direct attack (McCarter and Withers, 1994).

β-glucosidases fall into groups called glycosyl hydrolase (GH) families related by sequence, with most plant β-glucosidases characterized so far falling in GH family 1 (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996). Several structures have been solved for GH family 1 enzymes, which are $(\beta/\alpha)_8$ barrels with the catalytic acid-base and catalytic nucleophile, used for hydrolysis with retention of anomeric configuration, on β-strands 4 and 7, respectively (Barrett *et al.*, 1995; Jenkins *et al.*, 1995; Henrissat *et al.*, 1995; Lesk *et al.*, 1989; Sanz-Aparicio *et al.*, 1998). The substrate binding site lies in a funnel-shaped pocket on the C-terminal end of the β-barrel, and residues found in this region dictate the substrate-specificity of the enzyme (Barrett *et al.*, 1995; Burmeister *et al.*, 1997; Czjzek *et al.*, 2000, 2001). This specificity may be quite broad or narrow, both in terms of aglycone and glycone moieties, but β-D-glucose and β-D-fucose are generally the most active glycones (Esen, 1993).

This structural organization is also found in F-xylanases, β-galactosidases (families 2 and 35), bacterial chitinases (family 18), and β-glucanases (family 17) (Henrissat *et al.*, 1995; Perrakis *et al.*, 1994; Jenkin *et al.*, 1995), which have similar structures and also fall in the Clan A or β4,7 superfamily. However, the catalytic region in both bacterial chitinase and barley glucanases (1-3,1-4-β-glucanase

isoenzyme EII and 1-3-β-glucanase isoenzyme GII) is different. Their active sites are in the form of grooves or channels located across the C-terminus of the barrel of β-glucanases (family 17) (Varghese *et al*., 1994; Perrakis *et al*., 1994).

Three-dimensional structures of several of the glycosyl-enzyme complexes, along with those of Michaelis complexes, have been determined through x-ray crystallographic analysis, revealing the identities of important amino acid residues involved in catalysis (Davies and Henrissat, 1995; McCarter and Withers, 1994). They reveal the involvement of the carbonyl oxygen of the catalytic nucleophile in strong hydrogen bonding to the sugar 2-hydroxyl for the β-retainers or in interactions with the ring oxygen for α-retainers. The glucose ring in the -1 (cleavage) site in the intermediates formed on several cellulases and a β-glucosidase adopts a normal 4C1 chair conformation (Figure 1.1). By contrast, the xylose ring at this site in xylanase is substantially distorted into a 2,5β boat conformation (McCarter and Withers, 1994).

Withers *et al*. (1990), found that Glu 358 from *Agrobacterium feacalis* β-glucosidase (Abg) acted as the nucleophile by inactivating the enzyme with [1-^{3}H]-2′-4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside, cleaving the inactivated enzyme into peptides using pepsin, separating the peptides by reverse phase HPLC and sequencing the labeled peptide by Edman degradation. The labeled amino acid was glutamate in the peptide sequence Y-I-T-E-N-G, which matched a motif highly conserved in all family 1 glycosyl hydrolases. Trimbur *et al*. (1992) also used inhibitor studies and sequence alignments of related enzyme to target a region around the active site nucleophile in β-glucosidase from *A. faecalis* for mutagenesis. Multiple sequence alignment of family members shows Glu358 is absolutely

conserved in all catalytically competent members of the group, and the 5 residues around Glu358 (YITENG) are also highly conserved. A combination of site-directed and *in vitro* enzymatic mutagenesis was carried out on the β-glucosidase to probe the structure of the active site region. Forty-three point mutations were generated at 22 different residues in the region surrounding the active site nucleophile, Glu358. Only five positions were identified which affected enzyme activity, indicating that only a few key residues are important to enzyme activity for hydrolysis of *p*-nitrophenyl-β-D-glucoside. Thus, the enzyme can tolerate a number of a single residue changes and still function. The importance of Glu358 to enzymatic function was confirmed and other residues important to enzyme structure or function were identified (Asp374, Arg377, Tyr380, Asn359, and Gly360).

Several β-glucosidases have also been found to have a carboxylate residue as the acid catalyst involved in stabilizing the positive charge of the intermediate (Keresztessy *et al*., 1994 and Rojas *et al*., 1995). Keresztessy *et al*. (1994) kinetically characterized linamarase from cassava in mixed substrate systems and with the transition-state analogue glucono(1-5)lactone and a series of 1-thio substrate analogues using either linamarin or *p*-nitrophenyl β-D-glucopyranoside as the substrate. The modification indicated that one molecule of water-soluble carbodiimide or Woodward's reagent K was required to bind for inactivation. The linamarase was protected against inactivation by the competitive inhibitors, *p*-nitrophenyl β-D-glucopyranoside, β-D-glucopyranosylamine, and glucono(1-5)lactone. Of the three carboxylate groups modified by Woodward's reagent K, essentially one was protected by *p*-nitrophenyl β-D-glucopyranoside. The V_{max} of the modified linamarase decreased to 30% and K_m remained unchanged. Treating linamarase

modified with 0.5 M hydroxylamine at pH 7.0, could regenerated 92% of the original enzyme activity. The presence of the competitive inhibitor β-D-glucopyranosylamine protected the enzyme against inactivation, preventing the modification of one histidine residue.

To identify the amino acid residues which play the role of the acid/base catalyst, the kinetics of the mutants which replace the conserved glutamic and aspartic residues with sterically conservative and non-ionizable amino acids such as asparagine and glutamine, or alanine have been observed (Trimbur *et al*., 1992). The activity of such mutants should be reduced, especially the hydrolysis rate of substrates with poor leaving groups which require protonic assistance. With retaining glycosidases, poor leaving group substrates should be hydrolyzed very slowly, at least 10^5–fold slower than wild type, whereas hydrolysis of very good substrates should be less affected. Also, comparison of the pH dependence between the mutant and wild type enzyme can demonstrate the acid/ base mutant.

Keresztessy *et al*. (2001) identified essential amino acid residues in the active site of cyanogenic β-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz) on the basis of the white clover linamarase structure. They proved that the E413G mutant had no hydrolytic activity against either linamarin or *p*NP-glucoside, indicating an essential function for E413 as the catalytic nucleophile, which is located in the I/VTENG motif. Also, when Q339, a residue which is near E413 in the cassava β-glucosidase, was mutated to glutamate (E) to test if it has any influence on the pH-activity profile, the mutant enzyme's activities toward of linamarin and *p*NP-glucoside were 300 times lower than wild type. An explanation was derived from molecular modeling, which predicts that E339 forms a salt bridge with R106, so the

mutation would disrupt the essential salt bridge between R106 and E413 and an H-bond between Q339 and E198. In addition to Q339, A201 in the cassava enzyme was mutated to valine (V) to probe the extent of such a water sheltering effect. They found that the Michalelis constant (K_m) for the hydrolysis of linamarin was five times higher than the wild type, whereas the K_m for the hydrolysis of *p*NP-glucoside remained unchanged. The effect of the Ala→Val mutation suggested that the steric hindrance introduced by V201 may selectively inhibit docking of the tetrahedral acetone cyanohydrin moiety of linamarin, but not the planar *p*-nitrophenyl aglycone of *p*NP-glucoside.

1.5 Substrate specificity of plant glycosyl hydrolases

The fundamental factors determining substrate specificity of enzymes are conformational and chemical complementary between the substrate and its binding site on the enzyme (Hrmova and Fincher, 2001a). Hrmova and Fincher (2001a) compared the protein structures of plant glycosyl hydrolases and explained how their structures can be related to the substrate specificities. Although the enzymes in a family have similar structures and the same stereospecificity, hence the same mechanism of glycoside hydrolysis, they may have different specificities and modes of the action (Warren RAJ., 2000). The difference of substrate specificity between endohydrolases, exohydrolases, and β-glucosidases is derived from the shape of their binding sites (Hrmova and Fincher, 2001a). Endohydrolases usually have substrate binding grooves or depressions that extend across their surface, and the catalytic amino acid residues are located in the substrate-binding cleft. As a result, the endohydrolase can essentially bind anywhere along the polymeric substrate and

hydrolyze internal linkages. In contrast, an exohydrolase has a substrate binding site in the shape of a dead-end tunnel, slot, or funnel shape. Substrate specificity depends on how the substrates can fit into the substrate-binding pockets to contact the catalytic amino acid residues at the bottom of the funnel (Hrmova and Fincher, 2001a, 2001b). The tight specificity of some β-glucosidases may be due to their much longer binding sites than less specific exohydrolases. Barley β-glucosidase has a substrate binding site in a shape of shallow coin slot in which only two glucosyl residues of the substrate can fit. Therefore, most β-glucan substrates can penetrate to the bottom of the slot, while the majority of the polysaccharide substrate remains outside the enzyme. This tolerance of a wide range of substrate shapes is reflected in the broad substrate specificity of this enzyme (Hrmova and Fincher, 2001a).

Although the mechanism of catalysis and the roles of the two catalytic glutamates have been studied, the determination of the precise substrate specificity, including the site and mechanism of aglycone binding still remain to be understood.

The crystal structure of maize β-glucosidase 1 (Czjzek *et al*., 2000, 2001) was solved and the amino acids within the active site that are involved in glycone recognition and binding identified (Q38, H142, E191, E406, E464, and W465). The majr mechanism of aglycone recognition and binding appears to be aromatic stacking π-interactions between aromatic aglycones and the above-mentioned amino acids. Several other amino acids (e.g., P377, D261, M263, A467, and Y473) were also identified to be potentially involved in substrate (Czjzek *et al*., 2000).

The crystal structure of ZMGlu1 and a model of its complex with *p*-nitrophenyl β-D-thioglucoside (*p*NPTGlc) were studied by Czjzek *et al*. (2001). This

structural data allowed definition of the active site of the maize β-glucosidase, and a model of the ZMGlu1-*p*NPTGlc complex was generated. The structural analysis of ZMGlu1 provides a working hypothesis as to how substrate (i.e. aglycone) specificity is achieved within the active site of a family 1 β-glucosidase. The possible interaction of the inhibitor with Trp-378 shows that this residue is an important component of the aglycone-binding pocket. Site-directed mutagenesis later provided more information about the structure-function relationships and the questions being asked.

Czjzek *et al*. (2000) investigated the mechanism and the site of substrate (i.e., aglycone) recognition and specificity in maize β-glucosidase by x-ray crystallography using crystals of a catalytically inactive maize β-glucosidase containing its substrate DIMBOAGlc, the free aglycone DIMBOA, and a competitive inhibitor, dhurrin. The structural data from these complexes allowed visualization of an intact substrate, free aglycone, or a competitive inhibitor in the slot-like active site of the β-glucosidase. These data show that the aglycone moiety of the substrate is sandwiched between W378 on one side and F198, F205, and F466 on the other. Thus, specific conformations of these four hydrophobic amino acids and the shape of the aglycone-binding site they form largely determine aglycone recognition and substrate specificity in Glu1.

F269 in cassava linamarase is equivalent to V254 of the clover linamarase, which is located in a competent position for a contact with the substrate aglycone, and is replaced by asparagine (N) in the disaccharide-cleaving bacterial β-glucosidase. The result of a mutation between the two was that F269N had no detectable cellobiase activity; however, and it was still active against linamarin and *p*NP-glucoside (Keresztessy *et al*., 2001). However, the K_m of linamarin hydrolysis was increased 16

fold by the mutation while the K_m for *p*NP-glucoside hydrolysis was increased only 2.5 fold. In contrast, the catalytic constant (k_{cat}) measured with the linamarin substrate was decreased only 1.9 times, whereas the k_{cat} for *p*NP-glucoside is 1.5 times higher in the F269N mutation. So, replacing F269 with a polar residue confered a significant effect on the K_m of linamarin cleavage without a similar change in the k_{cat}, which can only be attributed to selective alterations in the rate of binding/ dissociation of this substrate to and/or from the enzyme, since not much affect was seen on the K_m for *p*NP-glucoside hydrolysis. This result established that F269 in the wild type enzyme has a more important function in binding the natural substrate, linamarin, than *p*NP-glucoside.

1.6 Transglycosylation activity of the β-glucosidase

Like other glycosidase enzymes, some β-glucosidases may catalyze reverse hydrolysis and transglycosylation, leading to synthesis of oligosaccharides, and alkyl glucosides (Makropoulou *et al*., 1998; Fischer *et al*., 1996; Svasti *et al*., 2003). Glycosidases and glycosyl transferases are responsible for the transfer of glycosyl moieties from a donor sugar to an acceptor. For hydrolysis (glycosidase activity), the acceptor is water, the result being hydrolysis. For transferase activity, the acceptor is typically an alcohol from another sugar, but it could be from a lipid, an aryl moiety, or a range of other components of glycoconjugates. Transfer can also occur to nitrogen or sulfur nucleophiles, for example nucleotides and plant thioglycosides (Ly and Withers, 1999).

Transglycosylation involves the use of a reactive glycosyl donor to generate a steady state concentration of the glycosyl-enzyme intermediate, which can be

intercepted with a second sugar or other alcohol acceptor molecule rather than water (Withers, 2001). The transglycosylation mechanism was described by van Rantwijk *et al*. (1999). Transglycosylation, according to reaction (1), is based on monopolization of the catalyst by a reactive glycosyl donor.

$$\text{Glycosyl-OR}_1 + \text{R}_2\text{OH} \rightarrow \text{Glycosyl-OR}_2 + \text{R}_1\text{OH} \qquad (1)$$

In transglycosylation, the nucleophiles which act as glycosyl-acceptors are considered as substrates also. The specificity for these nucleophiles is defined as "acceptor specificity", which seems to be broad, accepting various structures of nucleophiles. However many glycosidases have been reported to be able to glycosylate only the aliphatic hydroxyl group of the compounds, whereas phenolic hydroxyl groups could not be glycosylated (Dintinger *et al*., 1994). Thiol groups can also be glycosylated also. Transglycosylation, which is kinetically controlled, is much faster than reverse hydrolysis, which is thermodynamically controlled, and reactions generally take a few hours rather than days.

Because the reaction is controlled kinetically, it becomes possible to overshoot the equilibrium conversion of reactant into product (van Rantwijk et al., 1999). As the reactant is consumed the concentration of the product will peak when its rates of synthesis and dealkylation become equal, at which kinetic control is lost and the reaction should be stopped before thermodynamic control takes over and the product undergoes enzymatic hydrolysis (Figure 2.1).

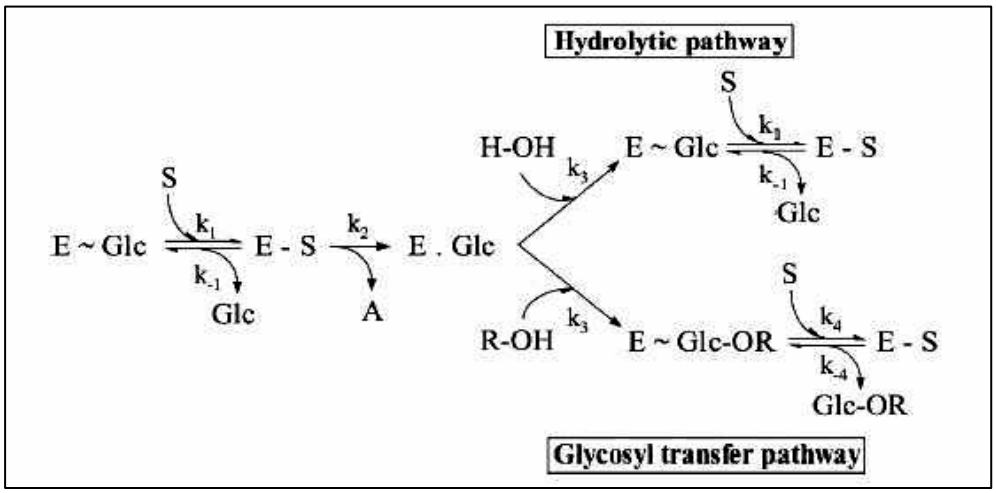
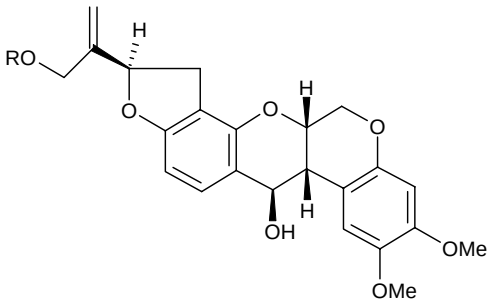


Figure 1.2 Kinetics of hydrolysis or glycosyltransfer reactions catalysed by a plant family 1 β-glucosidase (Hrmova *et al*., 2002). After the enzyme containing a noncova,ently bound glucose (Glc) product in the active site (E ~Glc) binds the first molecule of substrate (S), the Michaelis complex (E – S) is formed (k_1) and the Glc product of the previous reaction is released from the active site. In the second step, the glycosidic bonded is cleaved (k_2), and the aglycone part of the substrate becomes attached covalently to the enzyme to produce a metastable covalent glycosyl-enzyme intermediate (E .Glc). At the same time, the aglycone part of the substrate (A) is released. In the third step, the covalent glycosyl enzyme intermediate is subjected (k_3) to cleavage by a water molecule (H-OH), and a noncovalent E ~Glc product complex is formed, which is ready to interact (k_1) with the second substrate molecule (S) generate the next Michaelis complex (E - S), and again, the Glc molecule (Glc) is released from the active site. Alternatively, in the third step, the covalent glycosyl-enzyme intermediate (E .Glc) can be cleaved by an activated substrate (R-OH), leading to glycosyl transfer (E ~Glc-OR), which remains noncovalently bound to the enzyme and is released (k_4) when a second substrate molecule approaches the active site and from the next Michaelis complex (E - S) (Hrmova *et al*., 2002).

1.7 β-Glucosidase and its natural substrate from Thai Rosewood Seeds

The Thai rosewood β-glucosidase was discovered before its natural substrate, 12-dihydroamorphigenin-8'-β-D-glucoside or dalcochinin glucoside, by screening of glycosidase enzymes in Thai plant seeds for potential use in oligosaccharide synthesis (Svasti *et al*., 1999). Thai rosewood seeds show very high levels of both β-glucosidase and β-fucosidase activities (Surarit *et al*., 1995). It was found that β-D-glucosidase and β-D-fucosidase from the seeds of *Dalbegia cochinchinensis* were co-purified, since both activities were found in the same peak in both isoelectric focusing and in the gel filtration, which indicated it has pI of 5.5 and molecular weight of 330,000 daltons respectively (Srisomsap *et al*., 1996). The purified enzyme showed a single band at the same position for both activity and protein in non-denaturing gels, and also gave one band with a molecular weight of 66,000 dalton on SDS-PAGE. These results indicated that both β-glucosidase and β-fucosidase activities were likely to be found in the same enzyme. Kinetic studies on purified enzyme showed that both enzyme activities are located at the same active site.

The enzyme hydrolyses commercially available natural glycosides poorly, with hydrolysis rates lower than 5% of its *p*NP-β-D-glucoside hydrolysis rate. Therefore, investigation of the enzyme's natural substrate was carried out (Svasti *et al*., 1999). The natural substrate was found in Thai Rosewood seeds at high levels of about 3.5% (w/w). It was 12-dihydroamorphigenin-8'-β-D-glucoside (Figure 1.3), with an aglycone structure being similar to rotenone, and it was given the trivial name "dalcochinin".



dalcochinin-8'-*O*-beta-D-glucoside

Figure 1.3 The Thai rosewood β-glucosidase's substrate named Dalcochinin-8'-β-D-glucoside (Svasti *et al*., 1999)

1.7.1 Physical and kinetic properties of the Thai rosewood β-glucosidase

The purified β-glucosidase from Thai rosewood seeds was shown to have native molecular weight (M_r) of 330 kD by gel filtration on Sephacryl S-200, and subunit M_r of 66 kD on SDS-PAGE, so the native enzyme was believed to consist of 4 subunits (Srisomsap *et al*., 1996). The optimum pH of the enzyme for hydrolysis of both *p*NP-β-glucoside and *p*NP-β-fucoside was pH 5.0, while its pI was 5.6. The enzyme was able to hydrolyse several *p*NP-glycosides; but showed poor hydrolysis of commercially available natural glucosides and glucose disaccharides (Table 1). There was little or no hydrolysis of cyanogenic glucosides; although amygdalin was hydrolysed, HPLC analysis showed that products were glucose and prunasin, due to cleavage of the β(1-6) linkage in the gentiobiose moiety of amygdalin, rather than a cleavage that would result in release of cyanide.

Table 1.1 Hydrolysis of some synthetic and natural glycosides by Thai Rosewood β-glucosidase (Srisomsap *et al*., 1996)

Substrate *p*-nitrophenol glycoside [a]	Relative activity (%)	Substrate Naturally occurring substrate [b]	Relative activity (%)
*p*NP-β-D-glucoside	100	Sophorose [β(1-2)]	0.39
*p*NP-β-D-fucoside	124	Laminaribiose [β(1-3)]	0.34
*p*NP-β-D-galactoside	8.95	Cellobiose [β(1-4)]	0.06
*p*NP-β-D-mannoside	0.26	Gentiobiose[β1-6]	0.29
*p*NP-β-D-xyloside	3.91	Linamarin	<0.05
*p*NP-β-L-fucoside	0.05	Prunasin	<0.10
*p*NP-α-D-glucoside	0.18	Amygdalin	4.55
*p*NP-α-D-galactoside	0.03	Salicin	3.75
*p*NP-α-D-mannoside	0.36	Phloridzin	<0.05
*p*NP-α-L-ararabinoside	4.89	Arbutin	1.15
*p*NP-α-L-fucoside	0.08	Sinigrin	0.86
*p*NP-β-D-maltoside	0.21	Laminarin	<0.05
*p*NP-β-D-thioglucoside	0.02		
*p*NP-β-D-thiofucoside	0.03		
Methyl-β-D-glucoside	0.18		
Phenyl-β-D-glucoside	5.00		

Reactions employed 5 mM glycosides in 0.1 M sodium acetate pH 5.0 at 30°C

[a] *p*-nitrophenol release in 10 min was measured; [b] glucose release (glucose oxidase kit) in 30 min was measured.

The kinetics studies of Thai Rosewood enzyme (Srisomsap *et al*., 1996) were also performed to determine the K_m and k_{cat} values of the enzyme for various *p*NP-glycosides (Table 2). The enzyme shows both higher K_m value and k_{cat} value for of *p*NP-β-D-glucoside than *p*NP-β-D-fucoside. Moreover, the k_{cat}/ K_m ratio of the enzyme is highest for *p*NP-β-D-fucoside, indicating that this substrate is the most efficiently hydrolyzed. Since the *p*NP-β-D-fucoside has a hydrogen atom at position C-5, while the fucoside has a methyl group at C-5, and the other hexosides have a $-CH_2OH$ at C-5, it is possible that the presence of a hydrophilic substituent a C-5 detracts from binding efficiency (Srisomsap *et al*., 1996).

Table 1.2 Michaelis-Menten parameters for hydrolysis of some *p*NP-glycosides (Srisomsap *et al*., 1996)

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
*p*NP-β-D-glucoside	5.37±0.09	307±4.6	57,300
*p*NP-β- D-fucoside	0.54±0.04	151±3.0	283,100
*p*NP-β- D-galactoside	14.58±0.71	44±0.8	3,000
*p*NP-α-L-arabinoside	1.00±0.03	6.8±0.04	6,900
*p*NP-β-D-xyloside	2.45±0.14	1.8±0.04	730

The effects of various potentially inhibitory compounds on the Thai rosewood enzyme were tested (Srisomsap *et al*., 1996). The strongest inhibition was observed with mercuric compounds, at the level of 10^{-7} M for $HgCl_2$ and the level of 10^{-5} to 10^{-6} M for *p*-chloromercuribenzoate (*p*-CMB). Mercuric compounds usually inhibit enzymes by reaction with sulfhydryl groups, but with this enzyme, another sulfhydryl reagent, iodoacetate, caused no inhibition. Therefore, it is possible that the very

strong inhibition by mercuric compounds results from chelation of catalytically active acidic amino acids in the enzyme.

It was investigated whether the β-glucosidase and β-fucosidase activities are found in the same active site in Thai rosewood enzyme (Surarit *et al*., 1997). Conduritol B-epoxide (CBE), a racemic mixture of 1-L-1,2-anhydro-myo-inositol, inhibited both β-glucosidase and β-fucosidase activities to similar extents, with a pseudo-first-order rate constant (k_{obs}) of inactivation of 5.56 x 10^{-3} sec^{-1}, and binding stoichiometry of 0.9 mol per subunit. Partially inactivated enzyme showed similar kinetics with *p*NP-β-glucoside and *p*NP-β-fucoside as substrates, and Tris at 300 mM protected both β-glucosidase and β-fucosidase activities from inactivation by 6 mM CBE. Since the CBE inactivates enzymes by binding to a COO^- group at the active site, at least one acidic amino acid may be involved in the catalytic action of the enzyme. *p*NP-β-D-fucoside showed competitive inhibition of *p*NP-β-D-glucoside hydrolysis with an apparent K_I of 0.42 mM. Moreover, hydrolysis of mixtures of *p*NP-β-D-glucoside and *p*NP-β-D-fucoside at fractional ratios ranging from 0 to 1 showed Lineweaver-Burk plots intermediate between the two extremes, and the apparent K_m curve predicted the existence of a single common active site for the hydrolysis of two substrates.

Ketudat-Cairns *et al*. (2000) determined the cDNA sequence of Thai rosewood β-glucosidase and its derived amino acid sequence was compared to various β-glucosidases. The sequence of the full length cDNA of the Thai rosewood β-glucosidase transcript was composed of 1,955 bases, which included an open reading frame (ORF) that translated to a 547 amino acid polypeptide, including a signal

peptide of 23 residues and a 524 amino acid mature protein. Its amino acid composition is similar to that of purified enzyme from Thai rosewood seeds and it contained several peptides derived from this protein, indicating the clone encoded the same β-glucosidase enzyme originally found in dried seeds. The amino acid sequence of the Thai rosewood β-glucosidase precursor showed the closest identity to β-glucosidase from sweet cherry (*Prunus serotina*) (56.7%) and cyanogenic β-glucosidase (linamarase) from white clover (*Trifolium repens*) (56.3%). Thai rosewood β-glucosidase was classified into glycosyl hydrolase family-1 based on homology with other enzymes of this family. Sequence alignment identified the conserved catalytic nucleophile residue on the enzyme as Glu419 (Glu396 of the mature protein) and the catalytic acid/ base residue as Glu205 (Glu182 of the mature protein), as shown in Figure 1.4.

```
Thai rosewood  -23:MLAMTSKAILLLGLLALVSTSASIDFAKEVRETITEVPPFNRSCFPSDFIFGTASSSYQY 37
Dnbglu1        -23:MHAMTFKAILLLGLLALVSTSASIAFAKEVRATITEVPPFNRNSFPSDFIFGTAASSYQY 37
1CBG             1:-------------------------F-KPLPISFDDFSDLNRSCFAPGFVFGTASSAFQY 34

Thai rosewood   38:EG---E-GRVPSIWDNFTHQYPEKIADRSNGDVAVDQFHRYKKDIAIMKDMNLDAYRMSI 93
Dnbglu1         38:EG---E-GRVPSIWDNFTHQYPEKIADGSNGDVAVDQFHHYKEDVAIMKYMNLDAYRLSI 93
1CBG            35:EGAAFEDGKGPSIWDTFTHKYPEKIKDRTNGDVAIDEYHRYKEDIGIMKDMNLDAYRFSI 94

Thai rosewood   94:SWPRILPTGRVSGGINQTGVDYYNRLINESLANGITPFVTIFHWDLPQALEDEYGGFLNH 153
Dnbglu1         94:SWPRILPTGRASGGINSTGVDYYNRLINELLANDITPFVTIFHWDLPQALEDEYGGFLNH 153
1CBG            95:SWPRVLPKGKLSGGVNREGINYYNNLINEVLANGMQPYVTLFHWDVPQALEDEYRGFLGR 154

Thai rosewood  154:SVVNDFQDYADLCFQLFGDRVKHWITLNEPSIFTANGYAYGMFAPGRCSPSYNPTCTGGD 213
Dnbglu1        154:TIVNDFRDYADLCFNLFGDRVKHWITVNEPSIFTMNGYAYGIFAPGRCSSSYNPTCTGGD 213
1CBG           155:NIVDDFRDYAELCFKEFGDRVKHWITLNEPWGVSMNAYAYGTFAPGRCSDWLKLNCTGGD 214

Thai rosewood  214:AGTETYLVAHNLILSHAATVQVYKRKYQEHQKGTIGISLHVVWVIPLSNSTSDQNATQRY 273
Dnbglu1        214:AGTEPDLVAHNLILSHAATVQVYKKKYQEHQNGIIGISLQIIWAVPLSNSTSDQKAAQRY 273
1CBG           215:SGREPYLAAHYQLLAHAAAARLYKTKYQASQNGIIGITLVSHWFEPASKEKADVDAAKRG 274

Thai rosewood  274:LDFTCGWFMDPLTAGRYPDSMQYLVGDRLPKFTTDQAKLVKGSFDFIGLNYYTTNYATKS 333
Dnbglu1        274:LDFTGGWFLDPLTAGQYPESMQYLVGDRLPKFTTDEAKLVKGSFDFVGINYYTSSYLTSS 333
1CBG           275:LDFMLGWFMHPLTKGRYPESMRYLVRKRLPKFSTEESKELTGSFDFLGLNYYSSYYAAKA 334

Thai rosewood  334:DASTCCPPSYLTDPQVTLLQQRNGVFIGPVTPSGWMCIYPKGLRDLLLYFKEKYNNPLVY 393
Dnbglu1        334:DASTCCPPSYLTDSQVTFSSQHNGVFIGPVTPSGWMCIYPKGLRDLLLYIKEKYNNPLVY 393
1CBG           335:PRIPNARPAIQTDSLINATFEHNGKPLGPMAASSWLCIYPQGIRKLLLYVKNHYNNPVIY 394

Thai rosewood  394:ITENGIDEKNDASLSLEESLIDTYRIDSYYRHLFYVRYAIRSGANVKGFFAWSLLDNFEW 453
Dnbglu1        394:ITENGMDELDDPSQSLEESLIDTYKIDSYYRHLFYVRSAIGSGANVKGFFAWSLLDNFEW 453
1CBG           395:ITENGRNEFNDPTLSLQESLLDTPRIDYYYRHLYYVLTAIGDGVNVKGYFAWSLFDNMEW 454

Thai rosewood  454:AEGYTSRFGLYFVNY-TTLNRYPKLSATWFKYFLARDQESAKLEILAPKARWSLSTMIKE 512
Dnbglu1        454:NEGFTSRFGLNFVNY-TTLTRYHKLSATWFKYFLARDQEIAKLDITAPKARWSSSTMIKE 512
1CBG           455:DSGYTVRFGLVFVDFKNNLKRHPKLSAHWFKSFLKK----------------------- 490

Thai rosewood  513:EKTKPKRGIEGF                                               524
Dnbglu1        513:EKRKPKWAIQAF                                               524
1CBG           491:------------                                               490
```

Figure 1.4 Protein sequence alignment of β-glucosidase from *Dalbergia cochinchinensis* (Thai rosewood, AAF04007), *Dalbergia nigrescens*; Dnbglu1 (Chaunkhayan, 2004), and cyanogenic β-glucosidase from white clover, 1CBG (Barrett, 1995). The catalytic acid/base and nucleophile consensus sequences are underlined with the corresponding glutamates in bold. The Thai rosewood β-glucosidase residues R488, which corresponds with the last residue of 1CBG Lys490, and R519, which was mutated to Trp to remove a protease site, are in bold.

1.7.2 Identification of the natural substrate of the Thai Rosewood β-glucosidase

The natural substrate of the enzyme was extracted from Thai rosewood seed with absolute ethanol, purified by silica gel and preparative reverse phase C_{18} TLC (Svasti *et al*., 1999). Its structure was identified by ^{1}H-NMR, ^{13}C-NMR, COSY (H-H correlation) and mass spectrometry, and shown to be 12-dihydroamorphigenin-8'-β-D-glucoside (named dalcochinin glucoside). The molecular weights of dalcochinin and its glucoside were 412 and 574, respectively, as determined by mass spectrometry (Svasti *et al*., 1999). The hydrolytic activity of the Thai rosewood β-glucosidase towards dalcochinin glucoside was determined, and compared to other β-glucosidases and natural substrates (Table 4). The K_m of the Thai rosewood enzyme for dalcochinin glucoside was intermediate between that for *p*NP-β-D-glucoside and *p*NP-β-D-fucoside (Table 2). The V_{max} value for dalcochinin glucoside (452 nkat/mL) was slightly higher than for *p*NP-β-D-glucoside (423 nkat/mL).

The content of dalcochinin glucoside in the seed was estimated to be around 3.5% (0.35 g per 10 g seed), based on the losses during purification. Neither dalcochinin nor its glycoside could be detected in other plants tissues including leaf, stem petiole and shoot top. The high levels of both the enzyme and its natural substrate only in the seed suggest that they have an important role in the seed and may act as defense against herbivores or insects, since the structure of dalcochinin is similar to rotenone, which is a well-known insecticide (Svasti *et al*., 1999).

Table 1.3 Hydrolytic activity of β-glucosidases towards natural substrates (Champattanachai, 1998)

	Relative activity (%)			
Substrate (2mM)	Thai Rosewood seed β-glucosidase	Mustard seed thio β-glucosidase	Cassava Linamarase	Almond β-glucosidase
*p*NP-β-D-glucoside	100.0	100.0	100.0	100.0
Gentiobiose	0.2	69.8	<0.1	0.5
Phloridzin	<0.1	72.4	<0.1	<0.1
Prunasin	<0.1	46.9	0.2	158.1
Sinigrin	<0.1	16,211.0	<0.1	<0.1
Linamarin	<0.1	47.1	53.1	<0.1
Amygdalin	2.0	55.6	<0.1	124.8
Salicin	1.5	58.2	0.5	11.6
Dalcochinin	184.2	75.0	0.1	0.1

1.7.3 Transglycosylation by Thai Rosewood β-glucosidase

Srisomsap *et al*. (1999) found that β-glucosidase from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) catalyzes the synthesis of disaccharides and trisaccharides at 40-70% (w/w) glucose. Gentiobiose (β1-6-diglucose) was the major disaccharide. The reaction was optimal at pH 5.0 and 60-65°C. When the reaction was performed in mixtures of glucose and fucose, a novel product was found, which was purified and characterized by NMR spectroscopy to be D-fucosyl (β1-6)-D-glucose.

Lirdprapamongkol and Svasti (2000) found that β-glucosidase from Thai rosewood (*Dalbergia cochinchinensis* Pierre) catalyses transglucosylation of alcohols to synthesize alkyl glucosides. Good yields were obtained with primary alcohols, often approaching 90% in terms of alkyl glucoside, but secondary alcohols gave

poorer yields, and tertiary alcohols did not react. The enzyme showed higher transglucosylation yields than almond β-glucosidase with all alcohols tested.

Svasti *et al*. (2003) compared the ability of β-glucosidases from cassava, Thai rosewood, and almond to synthesize alkyl glucosides by transglucosylating alkyl alcohols of chain length C1 to C8. Cassava linamarase shows greater ability to transfer glucose from *p*-nitrophenyl-β-glucoside to secondary alcohol acceptors than other β-glucosidases, and is unique in being able to synthesize C4, C5, and C6 tertiary alkyl β-glucosides with high yields of 94%, 82%, and 56%, respectively. Yields of alkyl glucosides could be optimized by selecting appropriate enzyme concentrations and incubation times. Cassava linamarase required *p*NP-glycosides as donors and could not use mono- or di-saccharides as sugar donors in alkyl glucoside synthesis.

1.8 Recombinant expression of β-glucosidases

Human tissues such as liver, small intestine, spleen and kidney contain a cytosolic β-glucosidase (CBG) that hydrolyses various β-D-glycosides, but whose physiological function is not known. Berrin *et al*. (2000) described the first heterologous expression of human CBG, a system that facilitated a detailed assessment of the enzyme specificity towards dietary glycosides. A full-length CBG cDNA (*cbg-1*) was cloned from a human liver cDNA library and expressed in the methylotrophic yeast *Pichia pastoris* at a yield of approximately 10 $mg \cdot L^{-1}$ secreted in the media. The recombinant CBG (reCBG) was purified from the supernatant using a single chromatography step and was shown to be similar to the native enzyme isolated from human liver in terms of physical properties and specific activity towards 4-

nitrophenyl-β-D-glucoside. Furthermore, the reCBG displayed a broad specificity with respect to the glycone moiety of various aryl-glycosides (β-D-fucosides, α-L-arabinosides, β-D-glucosides, β-D-galactosides, β-L-xylosides, β-D-arabinosides), similar to the native enzyme. The human enzyme has significant activity towards many common dietary xenobiotics including glycosides of phytoestrogens, flavonoids, simple phenolics and cyanogens with higher apparent affinities (K_m) and specificities (k_{cat} /K_m) for dietary xenobiotics than for other aryl-glycosides. These data indicate that human CBG hydrolyses a broad range of dietary glucosides and may play a critical role in xenobiotic metabolism (Berrin *et al*., 2002).

Minami *et al*. (1999) isolated cDNA from leaves of the indigo plant (*Polygonum tinctorium*), which contain a β-glucosidase with a high activity for the substrate indican. The indigo plant β-glucosidase cDNA was expressed in *E. coli* and the cell extract was confirmed to have β-glucosidase activity.

Keresztessy *et al.* (1996) cloned linamarase from cassava into the glutathione S-transferase (GST) gene fusion protein expression vector pGEX-2T to produced the construct named pEXCAS and expressed in *E.coli*. Bacterial chaperonin GroEL was found to be tightly associated with the fusion protein linamarase and co-purificed. The K_m, V_{max} and V_{max}/ K_m for the hydrolysis of linamarin and *p*NP-Glc by the recombinant protein were identical with those of native linamarase.

Keresztessy *et al*. (2001) cloned mature cyanogenic β-glucosidase (linamarase) into the vector pYX243 modified to contain the SUC2 yeast secretion signal sequence and expressed it in *Saccharomyces cerevisiae*. The recombinant protein was active and characterized. The K_m and pK_a were similar to linamarase from cassava.

Opassiri *et al*. (2003) isolated cDNA for 2 isozymes, *bglu*1 and *bglu*2, from rice (*Oryza sativa* L.) and expressed their proteins in *E.coli*. A recombinant thioredoxin fusion protein produced from the bglu1 cDNA in redox-deficient *E.coli* (BGlu1) hydrolyzed *p*-nitrophenol β-D-glucoside, *p*-nitrophenol β-D-fucoside, and other *p*-nitrophenol-β-D-glycosides, and was strongly inhibited by glucono-1,5-lactone. It also hydrolyzed some natural glucosides, including the rice-derived pyridoxine-5'-O-β-D-glucoside, and hydrolyzed and transglycosylated short β-(1→ 3) and β-(1→ 4) linked gluco-oligosaccharides. The protein from *bglu*2 resulted in mainly insoluble and inactive protein. From these studies, possible functions suggested for BGlu1 include hydrolysis and recycling of oligosaccharides generated from rapid cell wall expansion during seed germination and flower expansion, and release of the coenzyme pyridoxine from its glucose-conjugated storage form.

Previously, the Thai rosewood protein was expressed as a prepro-alpha-mating factor fusion in *P. pastoris*, and the activity of the secreted enzyme was characterized. The recombinant enzyme and the enzyme purified from seeds had the same K_m values for *p*NP-glucoside and *p*NP-fucoside, had the same ratio of V_{max} for these substrates, and similarly hydrolyzed the natural substrate, dalcochinin-8'-beta-glucoside (Ketudat Cairns *et al*., 2000).

Having a robust system for recombinant expression allows engineering of enzymes by site-directed mutagenesis and other methods. Site-directed mutagenesis is being used extensively in almost every subdiscipline of biochemistry to explore the structure and function relationship in proteins and nucleic acids. This technique allows changes to be made to any amino acid residues in a recombinant protein. β-glucosidase can be also engineered by altering catalytic domain or by moving

modules (Warren, 2000). The potential changes to a catalytic domain include changing the thermal stability, changing the pH optimum, increasing the catalytic activity, altering the substrate specificity, changing the stereo-specificity, and, for a retaining enzyme, increasing or decreasing the transglycosylating activity. Modules can be interchanged to give new combinations of catalytic domains and substrate binding domains, and substrate binding domains can be fused to virtually any protein to serve as affinity tags. Therefore, it is of interest to develop a more robust system for expression and purification of for Thai rosewood β-glucosidase in order to allow such engineering of the enzyme in the future.

1.9 Objectives

The objectives of this thesis include:

1. To develop a reliable recombinant expression system for Thai rosewood β-glucosidase enzyme in yeast that allows rapid purification of the enzyme from recombinant yeast.
2. To purify the protein produced in this system and characterized its catalytic properties in comparison to the enzyme purified from seed.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide, bis-*N*,*N*′-acrylamide, ammonium persulfate, *N*,*N*′, *N*′′,*N*′′′-tetramethylethylenediamine (TEMED), lysozyme, sodium dedocyl sulfate (SDS), Triton X-100 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Ammonium hydroxide, bromophenol blue, chloroform, copper sulfate, disodium ethylenediamine tetraacetate (EDTA), ethanol, glacial acetic acid, glycerol, glycine, hydrochloric acid, methanol, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), potassium chloride, potassium hydroxide, sodium acetate, sodium bicarbonate, sodium chloride, sodium hydroxide, sodium dihydrogen phosphate, and sodium hydrogen phosphate were purchased from Carlo ERBA (Rodano, Milano, Italy). Calcium chloride, chloramphenicol, Coomassie brilliant blue R-250, imidazole, diethyl pyrocarbonate, ethidium bromide, magnesium chloride, phenyl methylsulfonyl fluoride (PMSF), and sodium citrate were purchased from Fluka (Steinheim, Swizerland). Bacto tryptone, yeast extract, and Bacto agar were purchased from DIFCO (Grayson, IL, USA). Ampicillin was purchased from Merck (Damstadt, Germany). Platinum *Pfx* DNA polymerase, pPICZαB, and Zeocin were purchased from Invitrogen (Carlsbad, CA, USA). *Taq* DNA polymerase, *Pfu* DNA polymerase, agarose (molecular grade), deoxyribonucleotides (dATP, dCTP, dTTP, dGTP), and X-

gal, were purchased from Promega (Madison, WI, USA). Restriction endonucleases *Nco*I, *Xho*I, *Eco*RI, *Pst*I, *Xba*I, *Eco*RV, and *Sna*BI were purchased from New England Biolabs (Beverly, MA, USA). Other chemicals and molecular reagents used but not listed here were purchased from a variety of suppliers.

2.1.2 PCR primers

Oligonucleotide primers were ordered from Geneset Proligo (Singapore), the BioServices Unit (BSU) of the National Science and Technology Development Agency (Thailand) and ESPEC Oligo Service Corp. (Japan).

2.2 General methods

2.2.1 DNA analysis by agarose gel electrophoresis

The amplified PCR products were analyzed by 1% agarose gel electrophoresis in 1 X TAE buffer (0.04 M Tris-HCl pH 8.0, 0.04 M acetic acid, 0.001 M EDTA pH 8.0), as described by Sambrook *et al*. (1989). The agarose gel (molecular grade) was prepared using a Pharmacia gel electrophoresis apparatus GNA-100 (Pharmacia Biotech, San Francisco, CA). DNA samples were prepared by mixing 5:1 with 6 X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) and applied to the gel wells. Electrophoresis was performed at a constant 80 V for 45-50 min. After electrophoresis, the gel was stained in 0.1 μg/mL ethidium bromide solution for 5-10 min, and was destained with distilled water for 30 min. The DNA bands in the gel were visualized by UV light transillumination with a Fluor-S™ MultiImager (Bio-RAD Laboratories, Hercules, CA). The sizes of DNA fragments were estimated by comparing with the 2-log DNA or 1 kb marker ladders

(New England Biolabs), or 100 bp and 1 kb EZ load DNA marker (Bio-RAD).

2.2.2 Purification of PCR products from agarose gels

The DNA bands in agarose gels were extracted with the QIAQuick gel purification kit (QIAGEN Corp., Hilden, Germany) by the vendor's recommended protocol. The PCR products from 100 μL PCR reaction mixtures were separated on 0.7% TAE agarose gels. The DNA bands were seen by ethidium bromide staining. The DNA bands were excised and placed in 1.5 mL microtubes. Then, 3 volumes of QG buffer were added to 1 volume of gel (by weight) and the tube was incubated at 50^{o}C with shaking for 10 min or until the gel was completely dissolved. The samples were applied to the QIAQuick columns, which were centrifuged for 1 min at 12,000 rpm, and the flow-through was discarded. Seven hundred fifty microliters of PE buffer was added to the columns and they were centrifuged at 12,000 rpm for 1 min. The flow-through was discarded and the columns were centrifuged for an additional 1 min. Each column was placed into a new 1.5 mL microcentrifuge tube, 40 μL of distilled water was added, and the column was incubated for 1 min and centrifuged at 12,000 rpm for 1 min. The eluted DNA products were combined and kept at -30^{o}C.

2.2.3 Quantitation of DNA

Five microliters of DNA solution was mixed with 995 μL distilled water. The absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) was measured and the A_{260}/A_{280} ratio was calculated with a Lamda Bio20 UV/VIS Spectrometer (Perkin Elmer, Shelton CT). The DNA content was calculated using the A_{260} value of double strand DNA with the following equation:

$$\mu g/mL \text{ of DNA} = (A_{260} \text{ x dilution factor x } 50\ \mu g/mL)$$

$$= (A_{260} \text{ x } 500 \text{ x } 50\ \mu g/mL)$$

One A_{260} unit equals 50 μg of double strand DNA/mL (Sambrook *et al*., 1989).

2.2.4 Vector preparation for ligation reactions with DNA fragments

The plasmid vector, eg. pBlueScript II SK(+) (2.996 kb) (Stratagene, La Jolla, CA) was isolated from the culture of DH5α cells with a QIA miniprep kit, as described in method 2.2.14. The plasmids were digested with the restriction enzyme *Eco*RV (Promega, Madison, WI) or *Sma* I (GIBCO, BRL, Invitrogen Corp.) to obtain blunt-ended vectors. The digestion reaction mix for the restriction enzyme *Eco*RV included 10 μg pBlueScript II SK(+) plasmid, 2 μL 10 X Buffer D, 0.2 μL 100 X BSA (10 mg/mL), 10 units of *Eco*RV and sterile distilled water to bring the volume up to 20 μL. The digestion reaction mix for the restriction enzyme *Sma*I included 10 μg pBlueScript II SK(+) plasmid, 2 μL 10 X React® 4 buffer, 0.2 μL 100 X BSA, 10 units of *Sma*I, and sterile distilled water to bring the volume up to 20 μL. The reaction mix was incubated at 37°C for 3-4 h. To remove the uncut plasmid, the digested reaction products were separated on a 0.7% agarose gel and purified with a QIAQuick gel extraction kit, as described in method 2.2.2.

2.2.5 Ligation of DNA fragments into vectors

Purified blunt-end PCR products were ligated into pBlueScript II SK(+) vector with T4 DNA ligase (GIBCO-BRL, Invitrogen Corp., Carlsbad, CA) according to the supplier's directions. The amount of vector and purified PCR product (ng) used

for the ligation was calculated according to the following equation:

$$\text{Amount of PCR product (ng)} = (\text{amount of vector, ng}) \times (\text{size of PCR product, kb}) \times \frac{(\text{molar ratio of PCR product: vector})}{\text{size of vector (kb)}}$$

For good ligation efficiency, the amount of vector is about 60-100 ng, and the molar ratio between PCR product insert and vector is 3:1 to 5:1 for blunt-end ligation. Just prior to use, the 5 X DNA ligase reaction buffer was thawed at room temperature and mixed vigorously to dissolve any precipitated material. The reaction mix consisted of 4 µl 5 X ligase reaction buffer, 60-100 ng purified pBlueScript II SK(+) vector digested with *Eco*RV or *Sma*I, 60-120 ng purified PCR product, 1 unit T4 DNA ligase, and sterile distilled water to bring the volume up to 20 µL. The reaction mix was incubated at 14°C for 16-24 h. This reaction mix was transformed directly into the cloning host strain. It is noted that T4 DNA ligase is unstable on ice for long periods, so it was returned to -20°C within 5-10 min after taking it out.

For sticky-end ligation, the purified PCR products were adenylated by *Taq* polymerase before ligated with pGEMT-easy vector by using T4 DNA ligase (Promega, Madison, WI) according to the supplier's directions. Four microliter of PCR product was mixed with 1 µL 1 mM dATP , 0.5 µL 25 mM $MgCl_2$, 1 µL 10X *Taq* polymerase buffer and 1 µL of *Taq* polymerase (Promega), then sterile distilled water was added up to 10 µL. The reaction tube was incubated at 70°C, 20 min. The DNA was precipitated with 1 µl 3 M sodium acetate, pH 4.8, and 25 µL absolute ethanol at -10°C, 30 min. The reaction tube was centrifuged at 12,000 rpm, 20 min, then the supernatant was aspirated away. The precipitated DNA was mixed with 200

μL 70% ethanol and centrifuged at 12,000 rpm, 2 min, and the supernatant was aspirated away. The precipitated DNA was dried and used for the ligation reaction.

Dry purified *Pst*I and *Xba*I products were ligated into *Pst*I and *Xba*I digested pPICZαB vector with T4 DNA ligase (GIBCO-BRL, Invitrogen Corp.) according to the supplier's directions.

2.2.6 Competent cell preparation

E.coli strain DH5α, a cloning host strain, was used to prepare competent cells by the $CaCl_2$ method, as described by Sambrook *et al*. (1989). A single colony of DH5α *E.coli* was inoculated into 5 mL of LB broth (Appendix C), and grown at 37°C overnight (12-14 h) with 160 rpm shaking. The overnight culture was used as inoculum for preparation of competent cells. One milliliter of inoculum culture (1%) was added into a 500 mL flask containing 100 mL of LB broth and grown at 37°C with 200 rpm shaking until the optical density (OD) at 600 nm was about 0.3-0.4 (about 3 h). The cell culture was transferred into a pre-chilled polypropylene tube, chilled on ice for 10 min, and pelleted by centrifugation at 3000 x g at 4°C, 10 min. The cell pellets were resuspended gently and completely with 20 mL of ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 10 mM PIPES pH 7.0, 15% glycerol) (Appendix C). The resupended cells were pelleted by centrifugation as described above. The cell pellets were resuspended gently and completely again with 20 mL of ice-cold $CaCl_2$ solution and chilled on ice for 30 min. The pellets were centrifuged as above, resuspended in 2 mL ice-cold $CaCl_2$ solution containing 7% dimethyl sulfoxide (DMSO), and aliquoted into microcentrifuge tubes (100 μL/tube). These competent cells were immediately kept at -70°C in a freezer until transformation.

2.2.7 Transformation and selection

Transformation of ligation reactions or plasmid into *E.coli* was done as described by Sambrook *et al.* (1989). A 50 μL aliquot of frozen competent cells was thawed on ice for 5 min. The plasmid (10-100 ng) or 5 μL ligation reaction mix was added to thawed competent cells, mixed gently by swirling the tube and incubated on ice for 30 min. The cells were transformed by heat shock at 42°C for 90 s and immediately chilled on ice for 2 min. The transformed cells were grown in 450 μL of SOC medium or LB broth (Appendix C) by shaking at 200 rpm for 45 min. For blue-white colony selection of recombinant pBlueScript II SK(+) or pGEMT-easy vector, 200 μL of cell culture was spread on an LB plate containing 100 μg/mL ampicillin which was pre-spread with 25 μL of 50 mg/mL X-Gal and 50 μL of 50.1 M IPTG (Appendix C). For antibiotics resistant selection of transformant clones, 200 μL of cell culture was spread on an LB plate containing the appropriate antibiotic(s) for each vector and host system. After spreading, the plate was sat-upright at room temperature for 15-20 min to let the spread cells and medium absorb into the agar, then the plate was inverted and incubated at 37°C overnight (16-18 h). Among the recombinant clones identified by blue-white color screening, the white colonies generally contained inserts while the blue colonies had no inserts (self-ligated pBlueScript II SK(+) or pGEMT-easy).

2.2.8 Plasmid isolation by boiling miniprep

Preparation of recombinant plasmid for preliminary analysis was done using the boiling miniprep method (Sambrook *et al.*, 1989). Normally, the size of recombinant plasmid is larger than non-recombinant plasmid. Single colonies were

picked with sterile toothpicks, restreaked on LB plates containing appropriate antibiotics, and grown at 37°C overnight. The fresh overnight colonies were used to inoculate 5 mL LB broth containing the appropriate antibiotic(s) and grown overnight with 200 rpm shaking at 37°C. The cell cultures were pelleted in microtubes by centrifugation at 3,000 x g for 3 min. The cell pellets were resuspended in 350 μL STET buffer (Appendix C) and mixed with 20 μL freshly prepared 10 mg/mL lysozyme. The suspensions were boiled at 100°C for 40 s to break the cells. The broken cells were pelleted by centrifugation at 12,000 x g for 10 min at room temperature, and the cell pellets were removed by sterile toothpick and discarded. To precipitate the plasmids in the supernatant, 400 μL isopropanol was added and the tubes were incubated at room temperature for 5 min, then centrifuged at 12,000 x g for 5 min at 4°C to pellet the plasmids. The pellets were washed with 500 μL 75% ethyl alcohol and dried for 15-20 min. The pellets was resuspended in 50 μL 10 mM Tris-HCl, pH 8.0, containing 0.1 mg/mL RNase A, and incubated at 37°C for 30 min to degrade the RNA. The electrophoretic mobility of circular purified plasmids were compared to plasmid without insert by analysis on 0.7% agarose gels. The plasmids containing insert were stored at -30°C.

2.2.9 Plasmid isolation by alkali lysis miniprep

Preparation of recombinant plasmid for preliminary analysis was done using the alkali lysis miniprep method (Sambrook *et al*., 1989). Single colonies were picked with sterile toothpicks, restreaked on LB plates containing appropriate antibiotics, and grown at 37°C overnight. The fresh overnight colonies were used to

inoculate 3 mL LB broth containing the appropriate antibiotic and grown overnight with 200 rpm shaking at 37°C. The cell cultures were pelleted in microcentrifuge tubes by centrifugation at 3,000 x g for 3 min. The cell pellets were resuspended in 100 μL resuspension buffer (0.2 M Tris, 0.025 M EDTA, 0.1 M glucose pH 8.0), mixed with 200 μL 1% SDS/ 0.2 M NaOH and briefly inverted. The suspensions were incubated on ice for 3 min. After that, the suspensions were mixed with 150 μL 3 M CH_3COOK, pH 4.8. The broken cells were precipitated by centrifugation at 12,000 x g for 5 min at 4°C, and the supernatants were transferred into new microcentrifuge tubes with 400 μL phenol: chloroform (1:1), then centrifuged at 12,000 x g for 5 min at room temperature. The supernatants were transferred into new microcentrifuge tubes with 900 μL absolute ethanol and then centrifuged at 12,000 x g for 10 min at 4°C. The pellets were resuspended in 100 μL TE (10 mM Tris-HCl, pH 8.0) containing 0.2 mg/mL RNase A and incubated at 37°C for 15 min. Then, the plasmids were precipitated with 70 μL PEG6000/ NaCl at 4°C for 1 h. The pellets were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were removed. The pellets were washed with 500 μL 70% ethyl alcohol and dried for 15-20 min. The pellets were dissolved with 20 μL sterile distilled water.

2.2.10 Restriction enzyme digestion for plasmid analysis

To determine the size of DNA inserts in the pBlueScript II SK(+) plasmid, restriction endonucleases whose sites are present in the region flanking the *Eco*RV site (*Pst*I and *Sac*II or *Pst*I and *Xba*I site of insert) were used to cut the recombinant plasmids. To determine the size of DNA inserts in the pGEMT-easy or

pPICZαB plasmid, restriction endonucleases whose sites are present in the flanking region of *Pst* I and *Sac*II or *Pst*I and *Xba*I at the cloning site 5′ and 3′ ends, were used to cut the recombinant plasmid. The reaction mix was prepared including 3-5 μg plasmids, 2 μL 10 X Buffer 2, 5 units of each restriction enzyme and sterile distilled water to 20 μL. The reaction was incubated at 37°C overnight. 0.7% agarose gel electrophoresis was carried out to determine the DNA insert size, as described previously in method 2.2.1.

2.2.11 QIAGEN plasmid miniprep

The QIA prep spin miniprep plasmid extraction kit (QIAGEN) was used to purify recombinant plasmid for sequencing according to the supplier's directions. A recombinant colony was inoculated into 5 mL LB broth with appropriate antibiotics, and the cells were grown by shaking at 200 rpm (12-16 h) at 37°C. The cultured cells were pelleted by centrifugation at 3,000 x g for 3 min at room temperature. The cell pellets were resuspended completely in 250 μL P1 buffer containing RNase A. Two hundred fifty microliters of P2 buffer was added to the resuspended cells, and mixed by inverting the tube gently (to avoid genomic DNA shearing) 4-6 times until the solution became viscous and slightly clear. Three hundred fifty microliters of N3 buffer was added and mixed immediately (to avoid localized precipitation) by inverting the tube gently 4-6 times. The tube was centrifuged at 13,000 x g for 10 min to compact the white pellet. The supernatant was applied to a QIA prep column by pipetting, centrifuged at 13,000 x g for 1 min, and the flow-through was discarded. The column was washed by applying 0.75 mL PE buffer and centrifuged at 13,000 x g for 1 min. The flow-through was discarded, and

the column was centrifuged for an additional 1 min. The column was placed in a new 1.5 mL microtube and 25-50 μL of distilled water or 10 mM Tris-HCl (pH 8.5) was added to the center of column. The column was allowed to stand for 1 min, and centrifuged at 13,000 x g for 1 min to elute the plasmid DNA. The DNA was stored at -30°C.

2.2.12 DNA Sequencing

Purified DNA samples were sequenced in cycle-sequencing using the ABI PRISM dye-labeled terminator kit (Big Dye) (PE Applied Biosystems, Foster City, CA, USA) with the recommended protocol. The reaction mix was composed of 4 μL Terminator Ready Reaction Mix, 2 μL purified plasmid DNA (100 μg/μL), 1 μL 3.2 pmol/μL primer and 3 μL distilled water. Amplification was done using the Gene Amp PCR system 9700 (PE Applied Biosystems). The program was conducted by rapidly increasing the temperature to 96°C, followed by 25 cycles of 96°C for 20 s, 50°C for 10 s and 72°C for 4 min. To the amplified products were purified by ethanol / sodium acetate precipitation, as described in the kit manual. The amplified products were mixed with 2 μL of 3 M sodium acetate, pH 4.6, and 50 μL of 95% ethanol, and the reaction was left at room temperature for 15 min to precipitate the extension products. After that, the reaction tubes were spun down at 12,000 rpm for 20 min, and the supernatant was carefully aspirated with a pipette and discarded. The pellet was washed with 250 μL of 70% ethanol, the supernatant was carefully aspirated with a pipette and discarded and the pellet was dried. The purified DNA pellet was dissolved in 20 μL TSR solution, heated at 100°C for 2 min, quickly cooled on ice for 3 min. The samples were again centrifuged to collect them in the bottoms of the tubes

and sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) or other automated sequencer.

2.2.13 Analysis of DNA sequences

The DNA sequence data obtained from the DNA sequencing software of the automated sequencer was interpreted and converted to single letter code in text file format by the Chromas 1.56 program (Technelysium Pty. Ltd, Tewantin, Qld, Australia). The DNA sequence data was also corrected by manual inspection of the chromatogram. The DNA sequence data was analyzed using the computer analysis programs in the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/). The sequences were confirmed to correspond to Thai rosewood β-glucosidases by a local alignment search of the Genbank database by the BLAST program at the National Center for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Comparison of the DNA fragment sequences was done with the ClustalX implementation of ClustalW (Jeanmougin *et al*., 1998; Thompson *et al*., 1994) or BLAST2 at NCBI.

2.2.14 Protein determination

Protein concentration was determined by the Bio-RAD Protein Assay kit (Bio-RAD) using bovine serum albumin (BSA) as the standard (2-10 μg). Appropriate dilutions of protein samples were prepared in 0.2 mL and mixed with 0.8 mL Bio-RAD Protein Assay Solution. The reaction was allowed to proceed for 10 min and the absorbance at 595 nm was measured using a Jenway 6405 UV/VIS Spectrophotometer (Essex, UK).

2.2.15 Analysis of protein purity and subunit molecular weight by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). The stock solution of polyacrylamide consisted of 29% (w/v) acrylamide and 1% (w/v) of *N, N'*-methylene-bis-acrylamide. The separating gel consisted of 10% polyacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, and 0.05% TEMED. The separating gel consisted of 5% polyacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% ammonium persulphate, and 0.05% TEMED. Four parts of protein solutions were mixed with 1 part of 5 X sample buffer (2.5 M Tris, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 20% β-mercaptoethanol). The suspension was boiled for 5 min, 8-12 μL aliquots were loaded onto a 10% SDS polyacrylamide gel (SDS PAGE) in a Mini-protein 3 cell (Bio-RAD), and electrophoresed in 1 X Tris-glycine/ SDS buffer, pH 8.3 (Appendix C) at a constant 150 V for 1 h from the cathodic (-) to the anodic (+) end. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 (Appendix C) for 30 min and destained with destaining solution (Appendix C) for 1 h or until the protein bands appeared clearly. The sizes of protein bands were estimated by comparing with Bio-RAD protein markers (Bio-RAD): phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and bovine α-lactalbumin (14.0 kDa).

2.2.16 Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) without SDS. Native gels were prepared

from a stock solution of polyacrylamide consisting of 30% (w/v) acrylamide and 1% (w/v) of N,N'-methylene-bis-acrylamide. The separating gel contained 7% acrylamide in 0.375 M Tris-HCl buffer, pH 8.8, while the stacking gel contained 3% acrylamide in 0.065 M Tris-HCl buffer, pH 6.8. The gel was chemically polymerized by addition of TEMED to 0.05% and ammonium persulfate to 0.5%. The samples were mixed 1:4 with 5x loading dye sample buffer containing 0.5 M Tris-HCl buffer, pH 6.8, 50% (v/v) glycerol and 0.025% bromophenol blue. Electrophoresis was run at a constant voltage of 120 V for 1 h 30 min in 1X Tris-glycine buffer, pH 8.3. The gel was washed with 1% Triton X-100 (v/v) 3 times, 15 min/time, and then washed and equilibrated in 0.1 M sodium acetate buffer, pH 5.0, for 10 min. Then, the gel was stained separately for β-glucosidase activities using 1 mM 4-methylumbelliferyl-β-glucopyranoside, for 5 min in the dark at room temperature, and fluorogenic bands of activity were detected with a Fluor-S™ MultiImager (Bio-RAD) with UV transillumination.

2.3 Recombinant Thai rosewood β-glucosidase protein purification by IMAC

The recombinant Thai rosewood β-glucosidase enzyme which included a histidine tag was purified by using an immobilized metal affinity column (IMAC) to specifically bind the protein from the cell extract or media.

2.3.1 Purification of recombinant protein by HiTrap chelating HP column (immobilized nickel)

The recombinant protein was purified from the cell lysate or expression medium with a HiTrap chelating HP column from Amersham Phamacia Biotech for

histidine-tagged protein separation. All solutions and samples used with the HiTrap chelating HP column were filtered through a 0.45 μm filter. The HiTrap column was equilibrated with 10 mL (10 volumes) equilibration buffer (20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl). Five milliliters of the soluble extract of bacteria expressing Thai rosewood β-glucosidase enzyme from pET-32a(+) or *Pichia* expression medium was loaded on to a 1 mL HiTrap chelating HP column. The column was washed with 10 mL (10 volumes) of equilibration buffer. The recombinant protein was eluted sequentially with 1 mL (1 volume) of elution buffer 1 (100 mM imidazole in equilibration buffer), 2 mL (2 volumes) of elution buffer 2 (150 mM imidazole in equilibration buffer), and 2 mL (2 volumes) of elution buffer 3 (200 mM imidazole in equilibration buffer). Elution fractions were collected for each 1 mL and analysed for protein and β-glucosidase and β-fucosidase activities. The fractions which had β-glucosidase/ β-fucosidase activity were pooled and concentrated 10 fold and exchanged with sodium phosphate buffer pH 7.4 in a 30 kDa cutoff centrifugal ultrafiltration membrane (YM-30, Amicon).

2.3.2 Purification of recombinant protein by BD Talon™ (immobilized cobalt) metal affinity resin

Pichia cultured medium was isolated from *P.pastoris* cells by centrifugation. Before separation, the end cap was firmly placed on the 20-mL plastic column, the top cap was removed, 20 mL culture was added and the top cap was replaced. The *Pichia* cultured medium was mixed with 1 mL BD Talon™ metal affinity resin on a carousel shaker for 30 min at room temperature. After that, the top cap and the end cap were removed, the column placed back into the receiving tube,

and the medium drained by gravity flow. Then, the column was removed from the receiving tube and the end cap replaced. The column was filled with 20 mL 1X Equilibration/Wash Buffer (50 mM sodium phosphate 150 mM NaCl, pH 7.0), and put into a fresh receiving tube, the top cap was replaced and the resin was resuspended by inverting the column. The end cap was removed and the column put back into the receiving tube, allowing the wash to drain by gravity flow. The end cap was replaced and the column was put into a fresh receiving tube, then 20 mL Wash-2 Buffer (50 mM sodium phosphate 150 mM NaCl buffer pH 7.0, 20 mM imidazole) was added. The top cap was replaced and the resin resuspended by inverting the column. The end cap was removed and the column placed back into the receiving tube. The buffer was drained by gravity flow. The end cap was replaced, then 2.0 mL Elution Buffer was added, the 20-mL column placed into a fresh receiving tube, and the resin was resuspended by inverting the column for 2 min. For an additional 10-15% of purified protein, the elution was repeated with an additional 2.0 mL Elution Buffer. Then, the end cap was removed and the column placed back into the receiving tube. The elution fraction was allowed to drain by gravity flow. All the fractions were assayed for β-glucosidase and β-fucosidase activities. The elution fractions were collected and desalted by centricon (MW cutoff 10 kD).

2.4 Amplification of Thai rosewood β-glucosidase cDNAs

The Thai rosewood β-glucosidase cDNA in pGEM3Z.10 and pPIC9K no. 16 were obtained from Mr. Voraratt Champattanachai. The primers, in Table 2.1, were designed for appropriate construction into the desired pPICZαB or pET-32a(+) plasmid.

The PCR reaction mix included 2 μL 10 X *Pfu* DNA polymerase buffer, 4.8 μL 2.5 mM dNTP mix, 1 μL 10 μM forward primer, 1 μL 10 μM reverse primer, 0.4 μL *Pfu* DNA polymerase (Promega, Madison, WI) and sterile distilled water to a final volume of 20 μL. The Thai rosewood β-glucosidase cDNA amplification was done with an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, 57-60°C for 60 s, and 72°C for 2 min, and a final extension of 5 min at 72°C after the last cycle. The annealing temperature of each primer pair was determined by Mastercycler® gradient (Eppendorf, Hamburg, Germany). The PCR product was analyzed by 0.7% agarose gel electrophoresis, as described in method 2.2.1, and purified with the QIA quick gel extraction kit. The purified PCR product was ligated into pET-32a(+) or pPICZαB plasmid.

Table 2.1 Primers used for amplification of the Thai rosewood β-glucosidase cDNA

Primer name	Primer sequence
forpyg	5′-CATGCCATGGTTGACTTTGCAAAAGAAGTCCGTG-3′
rev2	5′-TAGCTCGAGCAATCAAAAGCCTTCAATGCCCCAC-3′
DalYEXf1	5′-CTTTAAGCTTATGCTTGCAATGACATC-3′
PleBamPreproAFor	5′-GGGATCCATGTTTCCTTCAATTTTTAC-3′
PMcterm1	5′-AAGATCTAGATCAAAAGCCTTCAATGCCTCTC-3′
PMCtermH2	5′-AAGATCTAGATCAATGATGATGATGATGATGAAAGCCTTCAATGCCCCTC-3′
DcbgSacIIRev	5′-TCCCCGCGGCAATCAAAAGCCTTCAATGCCCCAC-3′
DncVPPFpst1f	5′-CATTCCTGCAGTTCCTCCATTCAATCGAAG-3′
DcR488StopXbar	5′-CCCCTAGACTTAACGTGCCAGAAAATACTTGAAC-3′

Table 2.2 Primers used for sequencing the Thai rosewood β-glucosidase cDNA

Primer name	Primer sequence
T7	5′-TAATACGACTCACTATAGGG-3′
T3	5′-AATTAACCCTCACTAAAGGG-3′
5′-AOX	5′-GACTGGTTCCAATTGACAAGC-3′
3′-AOX	5′-GCAAATGGCATTCTGACATCC-3′

2.5 Production of antibodies to Thai rosewood β-glucosidase

In order to aid in detection of Thai rosewood β-glucosidase during expression, antibodies against the Thai rosewood β-glucosidase enzyme were produced. The antigen was prepared from N-glycosidase F (PNGase F) digested Thai rosewood β-glucosidase seed protein in dried SDS-polyacrylamide gel. Twenty microgram of Thai rosewood β-glucosidase seed protein was digested with 20 units PNGase F at 37°C for 1 h. Then, the deglycosylated Thai rosewood β-glucosidase seed protein was subjected in to 10% SDS-PAGE, as described in section 2.2.14. The gel was stained with coomassie blue for 10 min, destained, and soaked in 2% glutaraldehyde for 1 h. The gel was washed with distilled water until the smell of glutaraldehyde disappeared, then dried. These dried SDS-polyacrylamide gel was mixed with complete Freund's adjuvant for the first injection, two 2 boosters injections were made at 3 week intervals by subcutaneous injection with dried SDS-polyacrylamide gel mixed with incomplete Freund's adjuvant. The rabbits' serum was collected once before the first injection (pre-immune bleed) and 10 days after each antigen injection. The rabbit's serum titer was tested by indirected ELISA.

2.5.1 Determination of titer from rabbit's serum by indirect ELISA

Thai rosewood β-glucosidase purified from seed was dissolved in phosphate buffer saline (PBS), pH 7.4, to 5 mg/mL. One hundred microliters were added to each well of a 96-well plate, while 100 µL/ well of 100 mg/mL BSA was added to negative control wells. The plate was then wrapped and kept at 4°C overnight. After that, the coated plate was washed 3 times with PBS, 200 µL/ well of 4% skim milk powder in PBS was added and left for 1 h at room temperature. Then, the blocked plate was washed 3 times with PBS, and 100 µL/well of diluted rabbit anti-Thai rosewood β-glucosidase serum in 4% skim milk powder in PBS with 0.05% tween 20 was added. The plate was incubated at room temperature for 2 h, followed by washing 3 times with PBS. The plate was then reacted with a 1:4000 dilution of HRP-conjugated goat anti-rabbit antibody in PBS at room temperature for 1 h, followed by washing 3 times with PBS. The antigen-antibody reaction was visualized by adding 100 µL/well of 3, 3′, 5, 5′-tetramethylbenzidine (0.1 mg TMB in 100 µL DMSO with 9.9 µL of 0.1 sodium citrate, pH 6.0, and 5 µL 30% H_2O_2), incubating at room temperature in the dark for 45 min and stopping the reaction with 50 µL/well of 12% H_2SO_4, before measuring absorbance at 450 nm.

2.5.2 Immunoblot detection with rabbit anti-Thai rosewood β-glucosidase serum

Protein samples were mixed with one-third volume of the 5 X SDS-gel sample buffer and heated at 100°C for 5 min. The protein samples were loaded onto a 10% SDS-PAGE gel and electrophoresed as described in section 2.2.14. After

electrophoresis, the gel was soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) (Towbin *et al*. (1979)), and the proteins were electrophoretically transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) on a TE 22 Mighty Small™ Transphor Tank Transfer Unit (Amersham Pharmacia Biotech) (400 mA, 2 h). After transfer, the membrane was stained with FAST-Green BB dye (0.1% (w/v) Fast green, 20% (v/v) methanol, 5% (v/v) glacial acetic acid) and destained with destaining solution (20% (v/v) methanol, 5% (v/v) glacial acetic acid) to ensure good separation and transfer before proceeding to immunological detection.

The blotted nitrocellulose membrane was blocked in 5% skim milk in Tris buffered saline with Tween (TBST: 150 mM NaCl, 5 mM Tris-HCl, pH 8.0, with 0.5% Tween 20) at 4°C, overnight. Then, the blot was probed with a 1:1000 dilution of rabbit anti- Thai rosewood β-glucosidase serum in 5% skim milk in Tris buffered saline (TBS) at room temperature for 2 h, followed by washing 2 times in TBST 10 min. The blot was then reacted with a 1: 5000 dilution of HRP-conjugated goat anti-rabbit in TBS at room temperature for 1 h, followed by washing 2 times in TBST for 10 min each. The antigen-antibody reaction was visualized using ECL™ western blotting reagents (Amersham Biosciences, UK) at a 1:1 ratio between detection reagent 1 and detection reagent 2 for 1 min, and the blot was exposed to x-ray film.

2.6 Recombinant protein expression in *Escherichia coli*

The procedure for production of Thai rosewood β-glucosidase in *E.coli* is summarized in Figure 2.1.

2.6.1 Amplification of the cDNAs to insert in expression vectors

A cDNA insert with *Nco*I and *Xho*I sites was produced by PCR with the

forpyg and rev2 primers (table 2.1) using Platinum *Pfx* DNA polymerase and pPICZαB-Thrombin-PYG no. 5 as template. The Thai Rosewood β-glucosidase PCR products were cloned into a pBluescript II SK(+) plasmid as cloning vector to aid in restriction digests. pPICZαB-Thrombin-PYG (R519W) no. 5 was constructed from *Pst*I and *Sac*II digested product from pBluescript II SK(+)-PYG (R519W) and pPICZαB-thrombin. The PCR reaction mix included 0.5 μL pPICZαB-Thrombin-PYG(R519W) no. 5, 2.5 μL of 10 X *Pfx* amplification buffer, 2.5 μL of 10 X PCR enhancer solution, 3 μL of 2.5 mM dNTP, 0.5 μL of 50 mM $MgSO_4$, 0.5 μL of 10 μM forpyg primer, 0.5 μL 10 μM rev2 primer, 0.75 unit of Platinum *Pfx* DNA polymerase (Invitrogen), and sterile distilled water to give a final volume of 25 μL. The PCR was performed by incubating at 94°C for 2 min as initial denaturation step, followed by 30 cycles of 94°C for 1 min, and 68°C for 4 min.

2.6.2 Construction of recombinant plasmids

The PCR products were cloned into pGEMT-easy as described in method 2.2.5, and transformed into DH5α *E.coli*. The resultant recombinant plasmids were purified and digested with *Nco*I and *Xho*I restriction enzymes, as described in methods 2.2.7-2.2.10. The gel-purified inserts for Thai rosewood β-glucosidase were cloned into the *Nco*I and *Xho*I sites of pET-32a(+), which has an N-terminal Trx tag, internal and C-terminal His-tags, and internal S tag, as described in method 2.2.5. The ligation reaction mix was transformed into DH5α *E.coli* and spread on LB agar plates containing 100 μg/mL ampicillin. Transformation of DH5α *E.coli* with the ligation reaction mix of recombinant plasmid helped to multiply the number of recombinant

plasmids, and these plasmids can be kept permanently (1-4 years) in these host cells.

2.6.3 Identification and isolation of recombinant expression plasmids

The recombinant colonies were grown in LB broth with 100 μg/mL ampicillin, the plasmids isolated by alkaline lysis and checked by restriction enzyme digestion, as described in methods 2.2.9 and 2.2.10.

2.6.4 Transformation of recombinant plasmid into expression host cells

The recombinant pET-32a(+)-PYG was transformed into BL21(DE3) and AD494(DE3) *E.coli* (Novagen). The competent cells of these host strains were prepared as described in Appendix C. Plasmids (10-100 ng) were used to transform a 50 μL aliquot of the frozen competent cells, as described in method 2.2.10, and spread on LB agar plates containing 100 μg/mL ampicillin. The recombinant expression plasmids in the clones were isolated by the alkaline lysis method in section 2.2.9.

Table 2.3 pET system host strain characteristics

Strain	Genotype	Description application	Antibiotic Resistance
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	General purpose expression host for T7 promoter plasmid	None
AD494(DE3)	K-12 Δ *ara–leu7697* Δ *lacX74* Δ *phoAPvuII phoR* Δ *malF3* F'[*lac*+(*lacIq*)*pro*] *trxB*::kan(DE3)	*trxB*– expression host; allows disulfide bond formation in *E.coli* cytoplasm	Kanamycin (15 μg/mL)

1. The appropriate antibiotic to select for the target plasmid must also be added

2.6.5 Protein expression in *Escherichia coli*

For expression, the selected clones were grown overnight in LB broth containing 100 μg/mL ampicillin. The fresh inoculum cultures were then diluted to a ratio 1:100 with LB broth containing 100 μg/mL ampicillin. The cultures were grown in a shaking incubator at 37°C. After the optical density at a wavelength of 600 nm of the cultures reached 0.5-0.6, IPTG was added to a final concentration of 0.3 mM and the cultures were shaken at 200 rpm at 20°C for an additional 10 h. Induced cultures were transferred to 50 mL centrifuge tubes and chilled on ice for 10 min and then harvested by centrifugation at 3000 x g in a Sorvall® Biofuge Stratos centrifuge (Dupont, Germany) at 4°C for 10 min, and the cell pellets were kept at -80°C for 30 min or longer until they were used for protein extraction.

To optimize protein expression, the optimal IPTG concentration, temperature, time and medium type for growing cells during induction of expression from the cells were determined. During induction of protein expression, IPTG concentrations were varied between 0.1, 0.2, and 0.3 mM. The induction temperatures tested were 20° and 25°C with times of induction of 5 and 10 h.

To determine the protein expression level, total protein profiles of host cells containing control-empty plasmids and recombinant plasmids were analyzed. For this, the cells were pelleted from 1 mL liquid culture in a microcentrifuge tube and the pellets resuspended in 20 μL of 2X SDS sample buffer (125 mM Tris pH 6.8, 15% glycerol, 1% SDS, 5.6% β-mercaptoethanol, 0.05% bromophenol blue). The suspension was boiled for 5 min and 8-12 μL aliquots were loaded onto 10% SDS polyacrylamide gels (SDS-PAGE) and separated as described in method 2.2.14.

2.6.6 Extraction of recombinant protein from induced cells

Bacterial culture pellets were thawed, suspended in 5 mL per gram freshly prepared extraction buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) to prevent recombinant protein degradation. The cell suspensions were sonicated with an Ultrasonic Processor GE 100 probe sonicator (Treadlitei, Woodstock, MA) 5 times at 50 W output for 10 s, with 1 min cooling in between. The pellets were extracted twice with extraction buffer to increase the amount of soluble protein. The insoluble protein fraction was removed by centrifugation at 12,000 x g at 4°C for 10 min and 10 μL of supernatant was subjected to electrophoresis by SDS-PAGE.

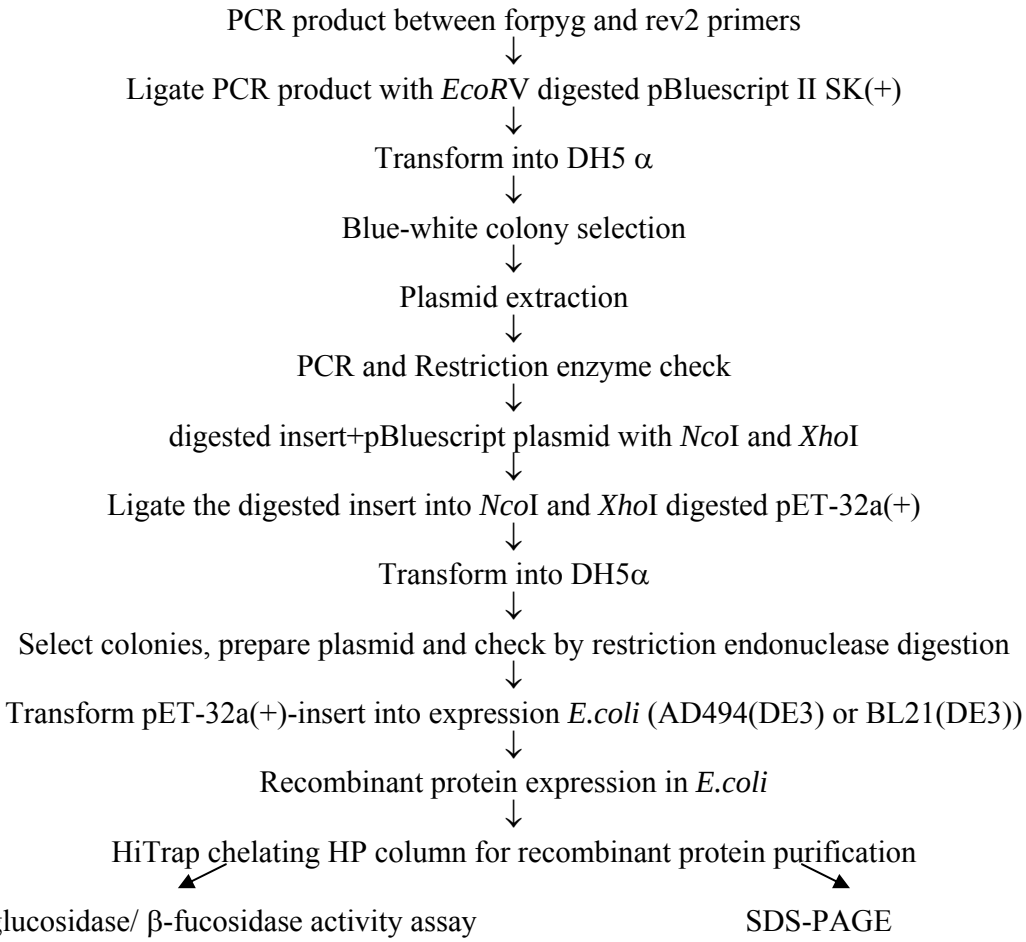


Figure 2.1 Flow-chart of recombinant Thai rosewood β-glucosidase expression in *E.coli*

2.7 Recombinant protein expression in *Saccharomyces cerevisiae*

The methods for recombinant expression of Thai rosewood β-glucosidase in *S.cerevisiae* are outlined in Figure 2.2.

2.7.1 Expression of Thai rosewood β-glucosidase enzyme in *S.cerevisiae*

Cloned Thai rosewood β-glucosidase cDNA was used as template for PCR cloning to amplify a Thai rosewood β-glucosidase cDNA fragment containing *Hind*III and *Xba*I sites for cloning into the pYES2 expression vector. The primers used were DalYEXf1 primer, which contains a with *Hind*III site and the start of Thai rosewood β-glucosidase's signal sequence, and PMctermI primer, which contains an *Xba*I site, or, PMCtermII primer which contains a 6 x histidine tag before the stop codon and *Xba*I site. They were used to PCR amplify the corresponding cDNA using *Pfu* polymerase and pPIC9K Thai rosewood β-glucosidase cDNA no. 16 as template. This PCR product was cloned into a pBluescript II SK(+) plasmid as a cloning vector to aid in digests of the restriction sites included in the primers. Then, the pBluescript II SK(+)-PYG plasmids were cut with *Hind*III and *Xba*I restriction enzymes and inserted in pYES2 to produce constructs named pYES2-BgluI and pYES2-BgluII. pYES2 is a yeast episomal plasmid. pYES2 has a GAL1 promoter, which can be activated by galactose, and contains both *E.coli* and *S.cerevisiae* origins of replication so it can replicate in both *E.coli* and *S.cerevisiae*. This plasmid can be selected by uracil deficientcy (URA3) as a metabolic selective marker in yeast. This constructed yeast plasmids were cloned in *S.cerevisiae* URA3-deficient strain BJ 5462 (*MATα ura3 trp1 pep4::His3prb1Δ can1GAL*) by lithium acetate transformation.

Thai rosewood β-glucosidase cDNA which were cloned into the

S.cerevisiae vector for expression as a fusion with the *S.cerevisiae* α-factor prepro sequence was produced by amplification with the PleBamPreproAFor (*BamH*I primer with a *S.cerevisiae* prepro-α signal sequence) and PMctermI primer or PMCtermH2 primer by PCR using *Pfu* polymerase and pPIC9K Thai rosewood β-glucosidase cDNA no. 16 as template. The cDNA PCR product with and without the C-terminal His-tag and *Bam*HI and *Xba*I sites was ligated into pBluescript II SK(+) which had been digested with *Eco*RV, and transformed into DH5α to clone it. The constructs with the signal sequence were pBluescript-PreproαI and pBluescript-PreproαII. The plasmids from transformants were extracted by boiling preparation, then checked with *Bam*HI and *Xba*I digestion. The correct plasmids were digested with *BamH*I and *Xba*I, the insert was gel purified and then ligated into *BamH*I and *Xba*I digested pYES2, and the ligation reaction was transformed into DH5α *E.coli*. Colonies were screened by plasmid extraction with the boiling preparation method, then checked with *Bam*HI and *Xba*I digestion. The correct clones were named pYES2-BgluI, pYES2-BgluII, pYES2-PreproαI and pYES2-PreproαII. The correct plasmids were transformed into BJ5462 yeast cells and plated on minimal media plate with leucine and tyrosine (MM+LT) to select yeast cells which contained the constructed pYES2 plasmid. The transformed colonies were subjected to the expression process described in sections 2.7.2 and 2.7.3.

2.7.2 *S.cerevisiae* transformation by lithium acetate

The *S.cerevisiae* BJ5462 yeast was pre-cultured in 3 mL YPD at 30°C, overnight. 500 μL of pre-culture was inoculated into 50 mL YPD and cultured until

$OD_{600} \geq 0.4$. Yeast cells were collected by centrifugation at 3000 rpm, 5 min and medium was removed. The yeast cells were resuspended with 1 mL sterile deionized water and centrifuged at 3000 rpm, 5 min. The supernatant was removed. The yeast cells were resuspended with 1 mL of solution I (100 mM Lithium acetate in TE, pH 8.0), transferred to a microcentrifuge tube and centrifuged at 3000 rpm, 5 min. The supernatant was removed. The yeast cells were resuspended with 1 mL of solution I, and 50 µL aliquoted in each microcentrifuge tube. One microgram of the constructed pYES2 recombinant plasmids in 10 µL and 10 µL 1x TE as negative control were mixed to 50 µL yeast competent cells. Five microliters of 10 mg/mL salmon sperm DNA carrier, boiled for 10 min before use, was mixed and 300 µL of solution II (100 mM lithium acetate, 40% (w/v) polyethylene glycol 4000 in TE, pH 8.0) into the transformation reaction. The transformation reactions were incubated at 30°C, 30 min with shaking 200 rpm. After that, transformation reactions were incubated at 42°C, 25 min and centrifuged at 10,000 rpm, 10 sec and the supernatant was discarded. Twenty microliters sterile distilled water was added to resuspend. The transformed yeast cells were plated on MM+LT plates and then, plates were incubated at 30°C, until colonies appear.

2.7.3 Protein expression in *S.cerevisiae*

The transformed yeast was inoculated in uracil drop-out medium (0.67% YNB without amino acids, 0.01% L-Leucine, 0.01% L-Tryptophan, 0.005% L-Proline, 0.005% L-Histidine) with 1% raffinose and 2% glucose, cultured at 30°C, overnight. After that, the culture yeast was changed to uracil drop-out medium with 1% raffinose and 2%galactose cultured at 30°C, 5 days. The recombinant yeast cells and medium

were collected daily. Yeast cells were mixed with 25 mM sodium phosphate buffer saline, pH 7.4, then frozen. The cell lysates were subjected to SDS-PAGE, as described in section 2.2.14. The culture medium and cell lysates were assayed for β-glucosidase and β-fucosidase activity.

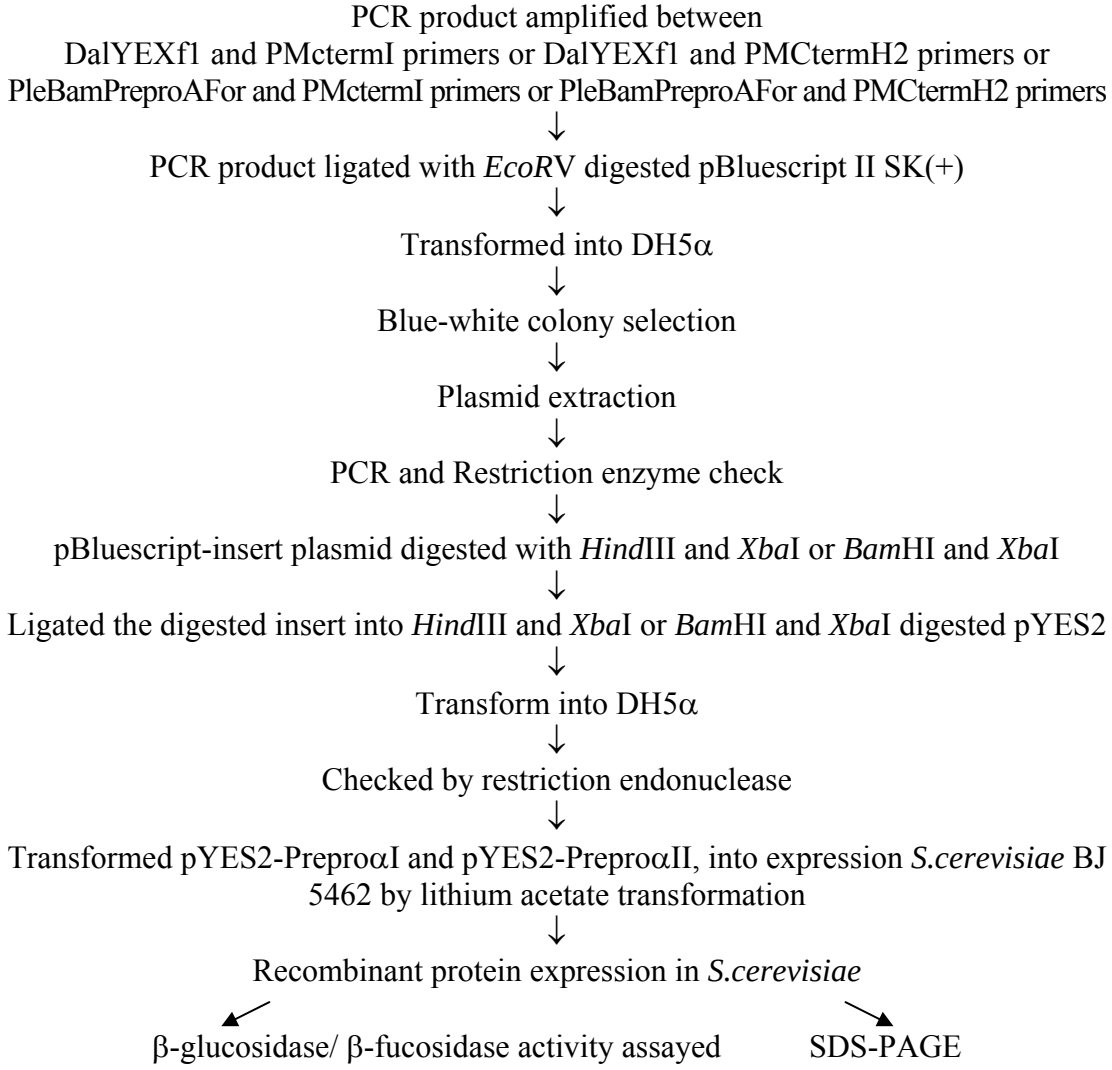


Figure 2.2 Flow chart of recombinant Thai rosewood β-glucosidase expression in *S. cerevisiae*

2.7.4 Yeast cell lysis

S.cerevisiae cell was lysed by Y-PER® yeast protein extraction reagent (Pierce, Rockford, IL, USA). Yeast cell pellet was colleted by centrifugation at 3000 rpm, 10 min. 0.1 g of yeast cell pellet was resuspended with 50 μL Y-PER® yeast protein extraction reagent until the mixture was homogeneous. The mixture was agitated at room temperature, 20 min. The mixture was centrifuged at 12,000 rpm, 10 min to cell debris separation. The supernatant was aspirated and subjected to protein determination and β-glucosidase activity assay.

2.8 Recombinant protein expression in *Pichia pastoris*

2.8.1 Thai rosewood β-glucosidase cDNA construction in pPICZαB plasmid

2.8.1.1 The pPICZα-Thrombin-PYG (R519W) construction (C-terminal 6 x histidine tag)

The pPICZαB-thrombin vector was selected to express and secrete recombinant proteins in *Pichia pastoris*. Proteins were expressed as fusions to an N-terminal peptide encoding the *S.cerevisiae* α-factor secretion signal and some also included a C-terminal peptide containing the *myc* epitope for detection and a polyhistidine tag for purification on metal chelating resin. In addition, a thrombin site was introduced before the *myc* epitope, to remove the C-terminal tag from the target protein after purification, as may be useful for crystallization of the protein (Mariena Ketudat-Cairns, personal communication).

pPICZαB-Thrombin-PYG (R519W) no. 5 was used as template for PCR to amplify Thai rosewood β-glucosidase cDNA containing *Pst*I and

*Xba*I or *Sac*II sites for cloning into pPICZαB vectors. The amplification was done with *Pfu* polymerase with and DncVPPFpst1F and DalSacIIRev primers. These PCR products were appended with a 3′A by *Taq* polymerase as described in section 2.2.5. Then, these 3′A-overhang PCR products were cloned into pGEMT-easy as described in section 2.2.6. After that, recombinant pGEMT-easy plasmids were transformed into DH5α completent cells, plated on LB with 100 μg/mL ampicillin plates with X-Gal and incubated at 37°C overnight. The white colonies were picked and inoculated into LB with 100 μg/mL ampicillin broth and incubated at 37°C overnight. The plasmid DNA was extracted from bacterial cultures by the alkaline lysis method (section 2.2.9). The extracted plasmid was checked by restriction endonuclease digestion. The correct recombinant plasmid was digested with *Pst*I and *Sac*II or *Xba*I restriction enzymes and ligated into the corresponding sites in pPICZαBNH8. The recombinant pPICZαBNH8 plasmids were transformed into DH5α competent cells, plated on LB with 25 μg/mL zeocin and incubated at 37°C overnight. The colonies were picked and inoculated into LB broth with 25 μg/mL zeocin and incubated at 37°C overnight. The extracted plasmids were checked by *Pst*I and *Sac*II or *Xba*I restriction endonuclease digestion. The correct recombinant plasmids were linearized with *Sac*I restriction enzyme, were transformed into *P.pastoris* GS115 by electroporation. The transformed yeast was plated on YPD with 100 μg/mL zeocin plate and incubated in the dark at 30°C until transformed colonies appear. These transformed colonies were picked and restreaked on YPD with 100 μg/mL zeocin plate and incubated in dark at 30°C overnight. All of transformed colonies were checked for recombinant Thai rosewood β-glucosidase expression upon methanol

induction, as described below.

2.8.1.2 The pPICZαBNH-PCR (R519W) construct with N-terminal 6 x histidine tag

PICZαBNH-PYG(R524W) was constructed from 5'AOX and DcbgSacIIRev primer with platinum *pfx* polymerase and pPICZαB-Thrombin-PYG(R519W) clone no.5 as template. The PCR condition was 95°C, 1 min, 55°C, 1 min, and 72°C, 2 min for 30 cycles. The PCR product was constructed into *EcoR*V digested pBluescript SK(+) and transformed into DH5α competent cell. The transformed colonies were selected by blue-white colony screening. The recombinant plasmids were extracted by alkaline lysis and digested check using *Pst*I and *Sac*II. The corrected plasmid was digested with *Pst*I and *Sac*II and ligated with *Pst*I and *Sac*II digested pPICZαBNH, named pPICZαBNH-PCR (R519W). The pPICZαBNH-PYG(R519W) plasmid was linearized by *Sac* I and alcohol precipitate before transform into GS115 *P.pastoris* competent cell then plated on YPD with 100 μg/mL zeocin and incubated in dark at 30°C overnight. The transformed colonies were transformed and expressed as described in sections 2.8.2 and 2.8.3.

2.8.1.3 Thai rosewood β-glucosidase from the pPICZαBNH-PYG (R519W) construct with N-and C-terminus 6 x histidine tag

pPICZαBNH-PYG(R519W) was constructed from digestion of pPICZαB-Thrombin-PYG(R519W) clone no. 5 with *Pst*I and *Xba*I. The insert was digested with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαBNH,

named pPICZαBNH-PYG (R519W). The transformed colonies were transformed and expressed as described in sections 2.8.2 and 2.8.3.

2.8.1.4 pPICZαB- Truncated R488 Thai rosewood β-glucosidase construction

The Thai rosewood β-glucosidase was truncated at arginine 488 to reduce C-terminal protease susceptible sequence. pPICZαB-Truncated R488 was constructed from DalYEXf1 and DcR511StopXbar primer with *pfu* polymerase and pPICZαB-Thrombin-PYG(R519W) clone no. 5 as template. The PCR condition was 95°C, 1 min, 55°C, 1 min, and 72°C, 2 min for 30 cycles. The PCR product was constructed into *Sma*I digested pBluescript SK(+) and transformed into DH5α competent cell. The transformed colonies were selected by blue-white colony screening. The recombinant plasmids were extracted by alkaline lysis and digested check using *Pst*I and *Xba*I. The corrected plasmid was digested with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαBNH, named pPICZαBNH- truncated R488. The transformed colonies were transformed and expressed as described in sections 2.8.2 and 2.8.3.

2.8.1.5 The pPICZαBNH-Truncated N construction (truncated N terminus with N-terminal 8 x Histidine tag)

pPICZαBNH8-Truncated N was constructed from DncVPPFpst1f and PMctermI primer with *pfu* polymerase and pPICZαB-Thrombin-PYG(R519W)clone no.5 as template. The Thai rosewood β-glucosidase was

truncated at valine 14 to reduce N-terminal protease susceptible sequence. The PCR condition was 95°C, 1 min, 65°C, 1 min, and 72°C, 2 min for 35 cycles. The PCR product was add adenine and constructed into pGEMT-easy plasmid and transformed into DH5α competent cell. The transformed colonies were selected by blue-white colony screening. The recombinant plasmids were extracted by alkaline lysis and digested check using *Pst*I and *Xba*I. The corrected plasmid was digested with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαBNH8, named pPICZαBNH8-Truncated N. The transformed colonies were transformed and expressed as described in sections 2.8.2 and 2.8.3.

2.8.2 Preparation of *Pichia* for electroporation

A single colony of *Pichia pastoris* strain GS115 was inoculated in 50 mL YPD (1% yeast extract, 2% peptone, 2% glucose) in a 250 mL Erlermeyer flask, which was then incubated at 30^{o}C overnight with shaking at 250 rpm, until the OD_{600} of the culture reached 1.3-1.5. The cells were collected by centrifugation at 1,500 x g for 5 min at 4^{o}C. Then, the pellet was resuspended with 50 mL of ice-cold (0^{o}C), sterile water and centrifuged again at 1,500 x g for 5 min at 4^{o}C. The pellet was again resuspended with 25 mL ice-cold (0^{o}C), sterile water, and centrifuged, as described above, then resuspended in 2 mL of ice-cold (0^{o}C) 1 M sorbitol. The cell pellet was collected by centrifugation as before, then resuspended in 1 mL of ice-cold (0^{o}C) 1 M sorbitol. The cells were kept on ice and used on the same day.

2.8.3 Transformation of *Pichia pastoris* by electroporation

Eighty microliters of the cells from method 2.4.14 were mixed with 5-10 μg of linearized pPICZαB plasmid and transferred to an ice-cold (0^oC) 0.2 cm electroporation cuvette. The cells in the cuvette were incubated on ice for 5 min. The cells were pulsed using a GenePulser electroporator (BioRad) which generated a pulse length of approximately 5-10 ms with 1,500 V charging voltage, 25 μF capacitance and 200 Ω resistance. Immediately after that, 1 mL of ice-cold (0^oC) 1 M sorbitol was added to the cuvette and the pulsed cells were transferred to a sterile 1.5 mL tube, and incubated at 30^oC with shaking for 1 h. The cells were collected by centrifugation at 5,000 rpm for 1 min, the pellet was resuspended with 0.2 mL YPD media and the cells spread on YPD agar plates containing 100 μg/mL zeocin. The plates were incubated at 30^oC for 2-3 days until colonies formed. Single colonies were streaked on fresh YPD plates containing 100 μg/mL zeocin.

2.8.4 Expression of Thai rosewood β-glucosidase enzyme in *Pichia pastoris*

Screening of expression was started by inoculation of each transformed colony into 10 mL of BMGY and incubating the culture at 30°C overnight. The cultured yeast was collected by centrifugation and transferred to 2 mL of BMMY with 1% methanol with a total culture OD_{600} = 20. Then, 0.5 mL of culture media was collected each day for 3 days. The enzyme activity and OD_{600} were determined from collected media. The transformed clones which gave the highest activity were selected for recombinant Thai rosewood β-glucosidase expression.

The expression levels were determined at 20°C and 30°C with 0%, 0.5% and 1% casamino acids, which act as a protease inhibitor. The optimal amount of methanol for *Pichia* expression was determined at 30°C with 0.5% and 1% methanol. The expression condition: 30°C, 1% casamino acid and 1% methanol in BMMY, which gave highest activity, was selected for recombinant Thai rosewood β-glucosidase expression.

2.9 Biochemical characterization

2.9.1 β-glucosidase assays and kinetics study

β-glucosidase activity on various substrates was assayed by determining either the *p*-nitrophenol liberated from the *p*NP derivatives of monosaccharides, as previously described (Babcock and Esen, 1994). All solutions, except those specified otherwise, were prepared in 100 mM NaOAc buffer, pH 5.0. In this assay, 900 μL of various dilutions of substrates were placed into the test tubes. Next, 100 μL of appropriately diluted enzyme were added to the test tubes to start the reaction. The assays were done at 30°C for 10 min for *p*-nitrophenol β-D-glucoside or *p*-nitrophenol β-D-fucoside. The reaction was stopped by adding 2 mL of 2 M Na_2CO_3 prepared in water. The absorbance of the *p*-nitrophenol liberated was measured at 405 nm, as compared to a p-nitrophenol standard curve prepared in the same buffers.

One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 μmoL of *p*-nitrophenol per min.

Kinetic parameters, K_m and V_{max} (at pH 5.0, at 30°C), of purified recombinant Thai rosewood β-glucosidase were calculated according to the method

of Lineweaver and Burk and nonlinear regression of the Michelis-Menten equation using the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). For each substrate, 3 replicates each of 5-7 substrate concentrations were used. Protein assays were performed colorimetrically by the Bio-RAD Protein Assay (Bio-RAD) using BSA as a standard, as described in method 2.2.14.

2.9.2 pH and temperature profiles of activity and stability

To determine the pH optimum for enzyme activity, recombinant Thai rosewood β-glucosidase was prepared in different buffers ranging from pH 3 to 10 (0.1 mM sodium phosphate-citrate buffer, pH 3, 4, 4.5, 5, 5.5, 6, 7, 8, 9, and 10). The activity at a given pH was measured by mixing the enzyme solution with a 1 mM final concentration of *p*-nitrophenol β-D-glucoside or *p*-nitrophenol β-D-fucoside in the same pH buffer. The reactions were allowed to proceed for 10 min at 30°C and the amount of product released was determined as described in method 2.7.1.

The temperature optimum was determined by incubating the recombinant Thai rosewood β-glucosidase with 1 mM *p*NPG in 0.1 mM NaOAc pH 5.0 over a temperature range of 20 to 70°C at 10° increments for 10 min. The amount of product released was determined as described in method 2.5.1.

Thermostability of the enzyme was measured by incubating the enzyme in 50 mM NaOAc buffer pH 5.0 over a temperature range of 20° to 70°C at 10° intervals. At 10 min increments from 0 to 60 min, samples incubated at each temperature were placed on ice. All the samples were assayed for activity with 1 mM *p*NPG in 0.1 mM NaOAc pH 5.0 at 30°C for 10 min.

CHAPTER III

RESULTS

3.1 Production of antibodies to Thai rosewood β-glucosidase

In order to aid in detection of Thai rosewood β-glucosidase during expression, antibodies against the enzyme were produced. The antigen was prepared from N-glycosidase F (PNGase F) digested seed Thai rosewood β-glucosidase protein purified by SDS-polyacrylamide gel, as seen in Figure 3.1. The dried SDS-polyacrylamide Thai rosewood β-glucosidase gel band was used as antigen to immunize rabbits. Rabbit serum was collected before and after the antigen injection. The rabbits' serum titer was tested by indirect ELISA.

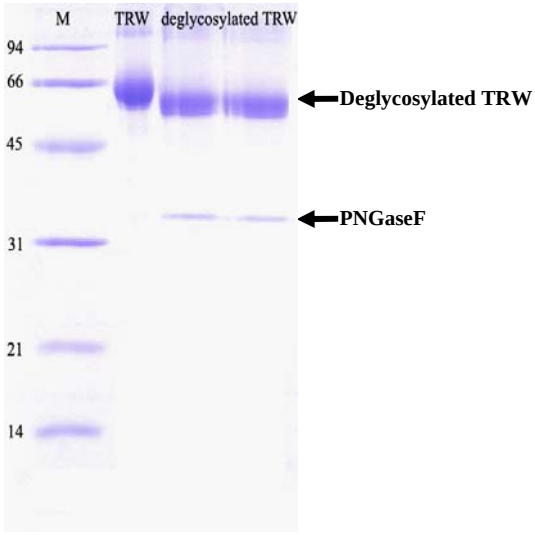


Figure 3.1 10% SDS-PAGE of Thai rosewood β-glucosidase protein from seed, before and after deglycosylation with PNGase F. Twenty microgram of Thai rosewood β-glucosidase seed protein was digested with 20 units PNGase F at 37°C for 1 h. Then, the deglycosylated Thai rosewood β-glucosidase seed protein was subjected in to 10% SDS-PAGE and coomassie blue staining. Marker (M), Thai rosewood β-glucosidase protein from seed (TRW) and deglycosylated Thai rosewood β-glucosidase protein from seed (deglycosylated TRW) were loaded and stained with coomassie brilliant blue.

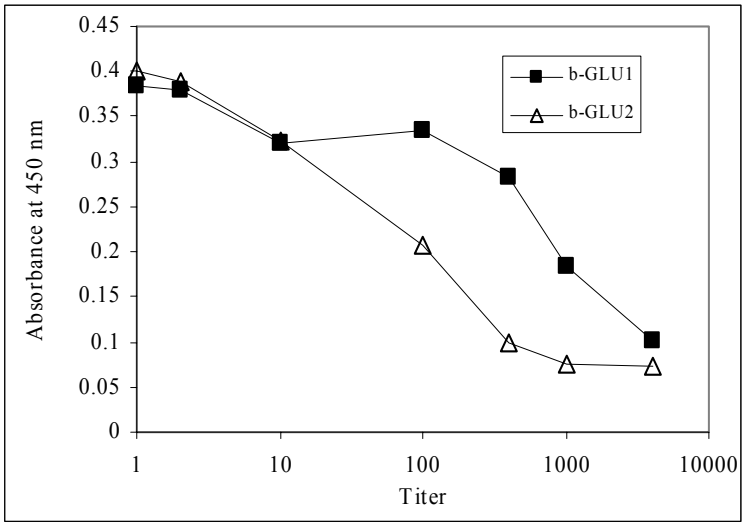


Figure 3.2 Titer determination of polyclonal antibody anti-Thai rosewood β-glucosidase from rabbit no. 1 (b-Glu1) and rabbit no. 2 (b-Glu2) by indirect ELISA. Thai rosewood β-glucosidase purified from seed was coated on plate. Thai rosewood β-glucosidase was reacted with diluted rabbit anti-Thai rosewood β-glucosidase serum and then reacted with a 1:4000 dilution of HRP-conjugated goat anti-rabbit antibody. The antigen-antibody reaction was visualized by adding 100 μL/well of 3, 3′, 5, 5′-tetramethylbenzidine incubating at room temperature in the dark for 45 min and stopping the reaction with 12% H_2SO_4, before measuring absorbance at 450 nm.

From the titer determination depicted in Figure 3.2, anti-Thai rosewood β-glucosidase polyclonal antibody from rabbit no. 1 (b-Glu1) was more suitable, compared with rabbit no. 2 (b-Glu2). Because, at the same titer anti-Thai rosewood β-glucosidase polyclonal antibody from rabbit no. 1 (b-Glu1) was higher absorbance, compared with polyclonal antibody from rabbit no. 2 (b-Glu2). Therefore, the anti-

Thai rosewood β-glucosidase polyclonal antibody from rabbit no. 1 (b-Glu1) was used at a titer of 1000, followed with goat anti-rabbit IgG monoclonal tagged with horseradish peroxidase at a dilution of 1:5000 and detected by ECL™ reagents for detection of the protein on immunoblots.

The polyclonal antibody from rabbit no. 1 (b-Glu1) was used to determine the sensitivity against Thai rosewood β-glucosidase from seed. The Thai rosewood β-glucosidase from seed was varied from 0.1 to 1.5 μg, loaded on 10% SDS-PAGE gel and then blotted on nitrocellulose before immuno detection with polyclonal anti-Thai rosewood β-glucosidase antibody from rabbit no. 1 (b-Glu1) at a dilution of 1:2000. The immunoblot result showed that polyclonal antibody can detect 0.8 μg of Thai rosewood β-glucosidase from seed, as shown in Figure 3.3.

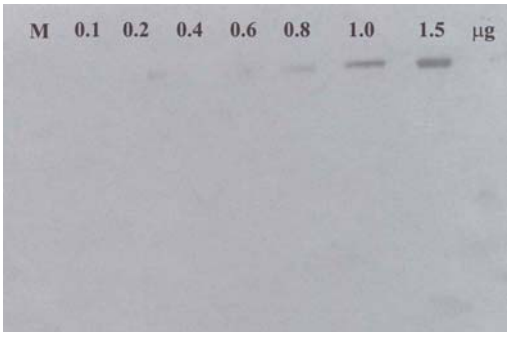


Figure 3.3 Immunoblot of polyclonal antibody anti-Thai rosewood β-glucosidase to determine the detection limit for the amount of Thai rosewood β-glucosidase from seed. Marker (M) and various concentration from 0.1 to 1.5 μg of Thai rosewood β-glucosidase from seed were blotted into nitrocellulose membrane. The serum from rabbit no. 1 (b-Glu1) was used at a dilution of 1:2000.

3.2 Expression of Thai rosewood β-glucosidase enzyme in *Escherichia coli*

PCR was done with forpyg and rev2 primers using pPICZαB-thrombin-PYG (R519W) as template to produce the appropriate cDNA for *E.coli* expression. The PCR product with *Nco*I and *Xho*I sites had the expected size of 1668 bp shown in Figure 3.4.

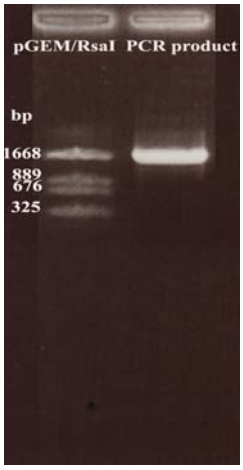


Figure 3.4 0.7% agarose gel of PCR product of forpyg and rev2 primers for *E.coli* expression using pET-32a(+). pGEM/*Rsa*I was loaded as marker size, 1668, 889, 676, and 325 bp.

After gel purification, PCR product was ligated with *Sma*I digested pBluescript II SK(+) and transformed into DH5α. The transformed colonies were picked and plasmid extracted. The pBluescript II SK(+)-PYG plasmid was checked by *Nco*I and *Xho*I digestion. The correct pBluescript II SK(+)-PYG plasmid was digested by *Nco*I and *Xho*I. *Nco*I and *Xho*I digested insert and *Nco*I and *Xho*I

digested pET-32a(+) were ligated and cloned into BL21(DE3) and AD494(DE3) *E.coli*.

The β-glucosidase and β-fucosidase activities from expression of Thai rosewood β-glucosidase from pET32-a(+)-PYG clones no. 2 and 3 in BL21(DE3) were determined at 20°C, with 0.1, 0.2, and 0.3 mM IPTG for 5 h, and 10 h. to find the optimal conditions for expression, as shown in Figure 3.5.

For pET-32a(+)-PYG clone no. 2 in BL21(DE3), the expression condition which give highest activity was 20°C, 10 h with 0.1 mM IPTG for β-glucosidase and 10 h with 0.3 mM IPTG for β-fucosidase. For pET-32a(+)-PYG, clone no. 3 in BL21(DE3), the expression condition which give highest activity is 20°C, 10 h with 0.1 mM IPTG.

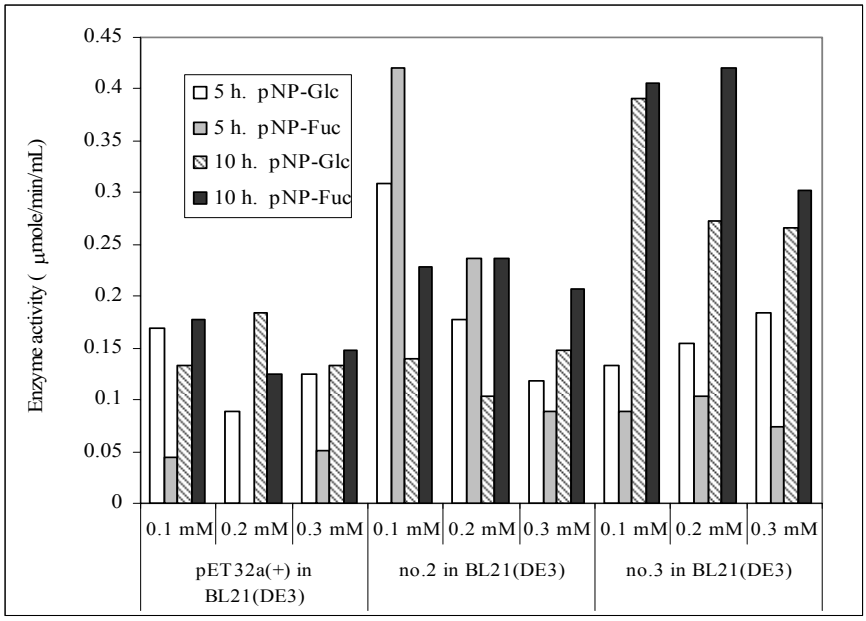


Figure 3.5 Recombinant Thai rosewood β-glucosidase expression in *E.coli* BL21(DE3). Expression from pET32-a(+) (control) and pET32-a(+)-PYG, clones no. 2 and 3, in BL21(DE3) at 20°C, with 0.1, 0.2, and 0.3 mM IPTG for 5 h and 10 h, and was evaluated by β-glucosidase and β-fucosidase assays.

The pET-32a(+)-PYG, clone no. 2 in BL21(DE3) was selected for expression of Thai rosewood β-glucosidase and cell lysate was purified by IMAC on a HiTrap immobilized nickel column. The results are shown in Figures 3.6 and 3.7. The all fractions from the HiTrap immobilized nickel column purification were assayed for β-glucosidase, β-fucosidase activity and protein. Most protein and the β-glucosidase and β-fucosidase activities were found in the flow-through fraction. All fractions were also subjected to SDS-PAGE. Most cell lysate proteins were also found in the flow-through fraction which corresponded to the protein concentration in the purification fractions. In elution fractions, E2 and E3 prominent bands at 66 and 22 kD were found.

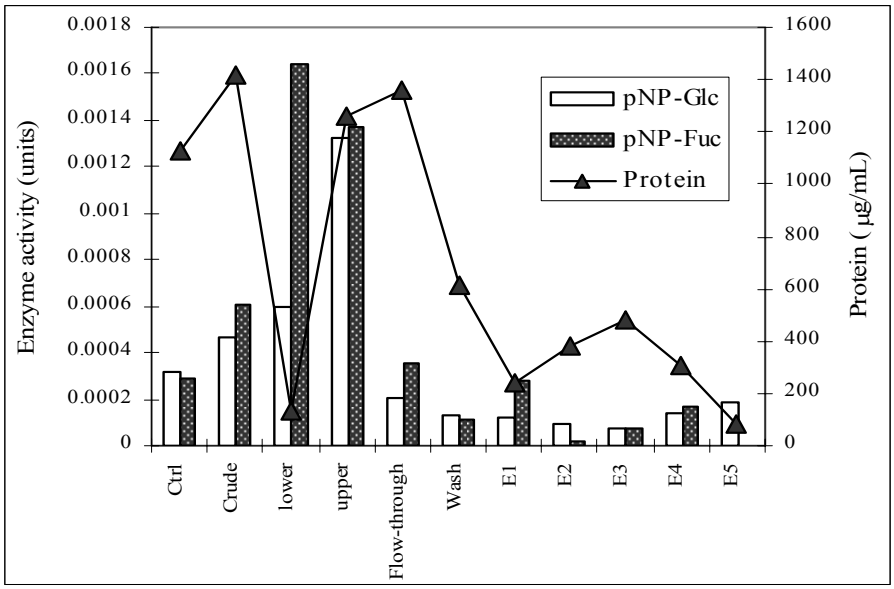


Figure 3.6 Protein and enzyme activity assay of pET-32a(+)-PYG in BL21(DE3) clone no. 2 cell lysates, flow-through, wash, and elution fractions from HiTrap immobilized nickel column purification. pET-32a(+)-PYG in BL21(DE3) clone no. 2 cell lysates (crude) was concentrated by centrifugal filtration and concentrated fraction (upper) and flow-through (lower) fractions were tested. pET-32a(+) in BL21(DE3) cell lysates (control) was used as expression control. The concentrated cell lysate (upper) was subjected to HiTrap column and then the elution fractions (E1-E5) were collected. All samples were tested with protein concentration by Bradford. The β-glucosidase and β-fucosidase activities at absorbance of 405 nm were tested in all samples.

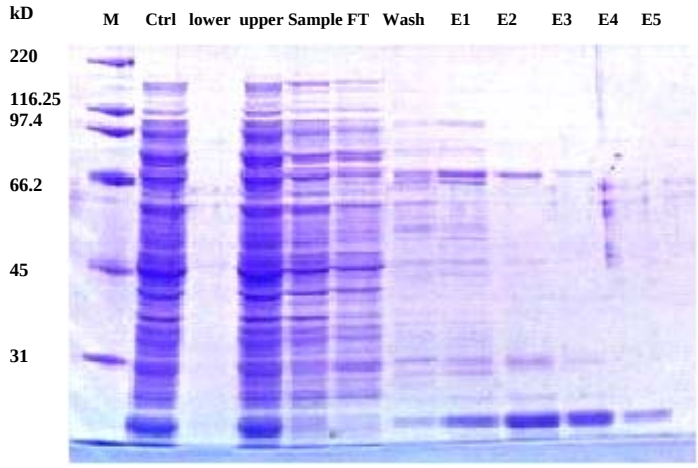


Figure 3.7 Ten percent SDS-PAGE showing recombinant Thai rosewood β-glucosidase expression purification fractions from pET-32a(+)-PYG in BL21(DE3) *E.coli*. The pET-32a(+)-PYG in BL21(DE3) *E.coli* cell lysate was concentrated by centrifugal filtration and compared with a control lysate of *E.coli* containing empty pET-32a(+) plasmid (ctrl). The upper part or concentrated lysate, as sample, was subjected to IMAC purification on a HiTrap immobilized nickel column. All fractions, upper and lower fractions from centrifugal filtration, and HiTrap flow-through (FT), wash and elution fractions (E1-E5), were loaded on the gel.

The expression of β-glucosidase and β-fucosidase from pET32-a(+)-PYG, clones no. 2 and 3, in AD494(DE3) was determined at 20°C, with 0.1, 0.2, and 0.3 mM IPTG for 5 h and 10 h to find the optimal conditions for expression, as shown in Figure 3.8.

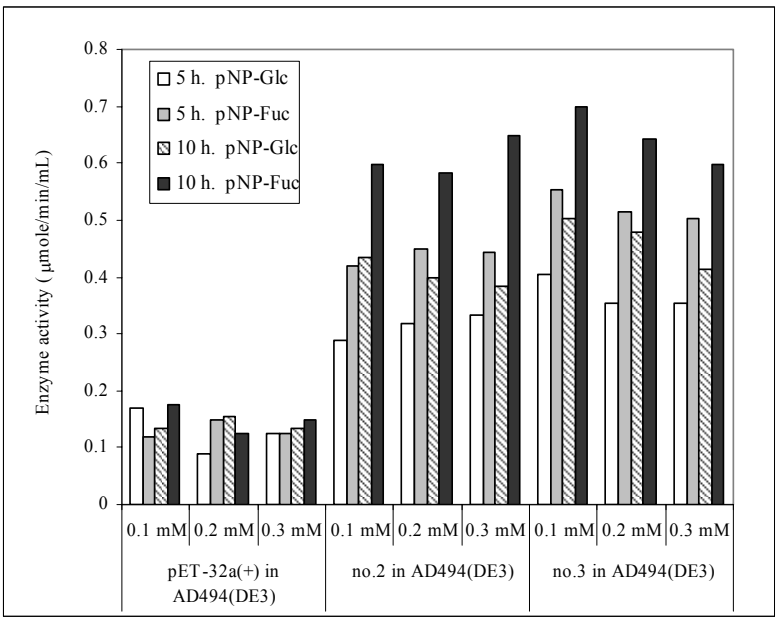


Figure 3.8 Enzyme activities in cell lysates from recombinant Thai rosewood β-glucosidase expression from pET-32a(+) control and pET-32a(+)-PYG, clones no. 2 and 3 were induced in AD494(DE3) at 20°C, with 0.1, 0.2, and 0.3 mM IPTG for 5 h and 10 h, and evaluated by β-glucosidase and β-fucosidase assays done at 30°C, 45 min with 1mM *p*NP-glucoside and 1 mM *p*NP-fucoside.

For pET-32a(+)-PYG, clone no. 3 in AD494(DE3), the expression condition which gave highest β-glucosidase and β-fucosidase activities was 20°C, 10 h with 0.1 mM IPTG. The pET-32a(+)-PYG, clone no. 3 in AD494(DE3) was selected to expression Thai rosewood β-glucosidase and protein were purified from the cell lysate with a HiTrap immobilized nickel column.

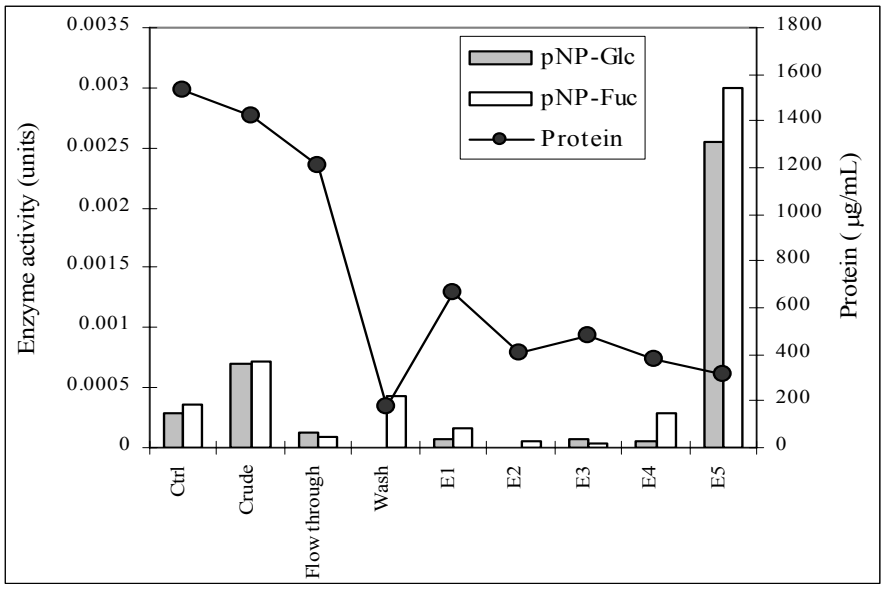


Figure 3.9 Fractions include cell lysate, flow-through, wash, and elution fractions (E1-E5) from the HiTrap immobilized nickel column. All samples were tested with protein concentration by Bradford. The β-glucosidase and β-fucosidase activities at absorbance of 405 nm were tested in all samples.

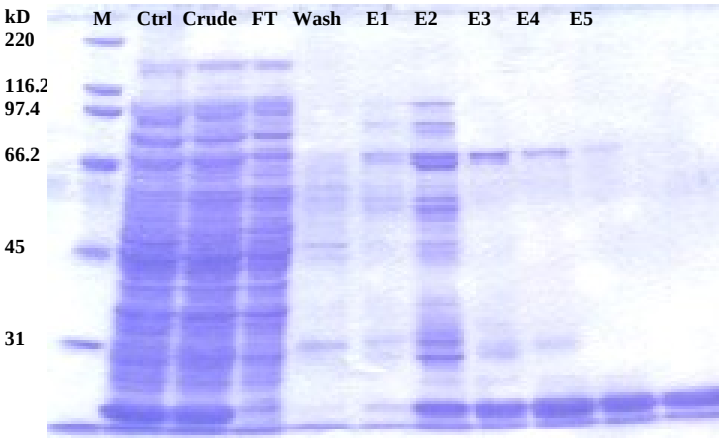


Figure 3.10 10% SDS-PAGE of recombinant Thai rosewood β-glucosidase expression from pET-32a(+)-PYG in AD494(DE3) *E.coli* and IMAC purification. The cell lysate from pET-32a(+) in AD494(DE3) as control (ctrl), pET-32a(+)-PYG clone no. 3 in AD494(DE3) as crude, flow-through (FT), wash and elution fraction (E1-E5) were loaded. The proteins were stained with coomassie brilliant blue.

Thai rosewood β-glucosidase was expressed from pET-32a(+) at 20°C in BL21(DE3) *E.coli* for 5 h with 0.1 mM IPTG and AD494(DE3) *E.coli* for 10 h with 0.1 mM IPTG. The levels of recombinant protein β-glucosidase and β-fucosidase activities were quite low (Figures 3.6 and 3.9). However, this recombinant Thai rosewood β-glucosidase bound with immobilized nickel resin in a HiTrap column. β-glucosidase and β-fucosidase activities and protein were determined in all fractions. Ten microliters of all fractions were subjected to SDS-PAGE and the protein bands were stained with coomassie blue. The protein bands from BL21(DE3) and AD494(DE3) *E.coli* bacterial cell lysates of the control and sample were similar, as

shown in Figures 3.7 and 3.10. Most of the protein from the bacterial cell lysate was in the flow-through and elution fractions, in which the concentration of imidazole was increased. The Thai rosewood β-glucosidase expression in AD494(DE3) *E.coli* eluted fractions E3 and E4, had 2 major bands corresponding to MW of 66 and 22, as shown in Figure 3.10. These two bands were confirmed by N-terminal sequencing. The N-terminal sequence of both bands corresponded to thioredoxin protein, which was the N-terminal tag fused to Thai rosewood β-glucosidase in this construct.

3.3 Expression of Thai rosewood β-glucosidase enzyme in *Saccharomyces cerevisiae*

3.3.1 Expression of Thai rosewood β-glucosidase from pYES2-BgluI and pYES2-BgluII constructs

The expression vector construction and expression of Thai rosewood β-glucosidase from pYES2 in *S.cerevisiae* are shown in Figure 3.11. The DalYEXf1 and PMctermI primers or DalYEXf1 and PMctermH2 primers were used to produce PCR products. The DalYEXf1 primer was designed with Thai rosewood β-glucosidase's signal sequence. The PCR product between the DalYEXf1 and PMctermI primers was composed of signal sequence and Thai rosewood β-glucosidase cDNA. The PCR product between DalYEXf1 and PMctermH2 primers was composed of signal sequence, Thai rosewood β-glucosidase cDNA and 6xhistidine tag was added at the C-terminus from PMctermH2. Both PCR products were ligated with *EcoR*V digested pBluescript II SK(+) and transformed. The correct plasmids were selected and digested by *Hind*III and *Xba*I, as shown in Figure 3.11.

The *Hind*III and *Xba*I insert was ligated with *Hind*III and *Xba*I digested pYES2.

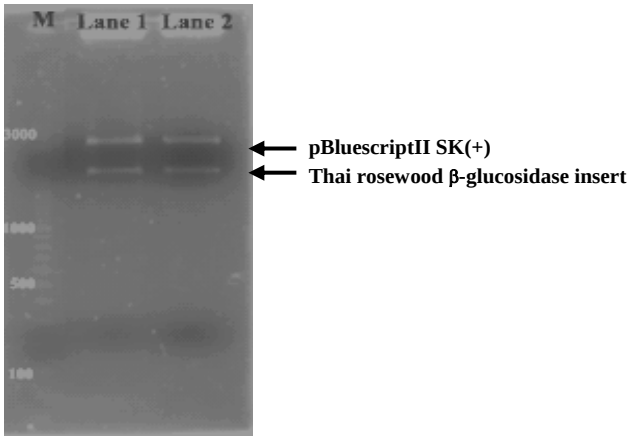


Figure 3.11 Electrophoresis of cDNA for yeast expression digested from pBluescript II SK(+) on a 0.7% agarose gel.

M: EZ load 100 bp PCR Molecular Ruler

Lane 1: *Hind*III and *Xba*I digested pBluescript II SK(+)-BgluI

Lane 2: *Hind*III and *Xba*I digested pBluescript II SK(+)-BgluII

The pYES2-BgluI and pYES2-BgluII constructs are shown in Figure 3.12. The pYES2-BgluI and pYES2-BgluII plasmids were selected and checked by restriction digest, then the correct plasmids were transformed into *S.cerevisiae* BJ5462. The transformed *S.cerevisiae* BJ5462 were selected and tested for protein expression.

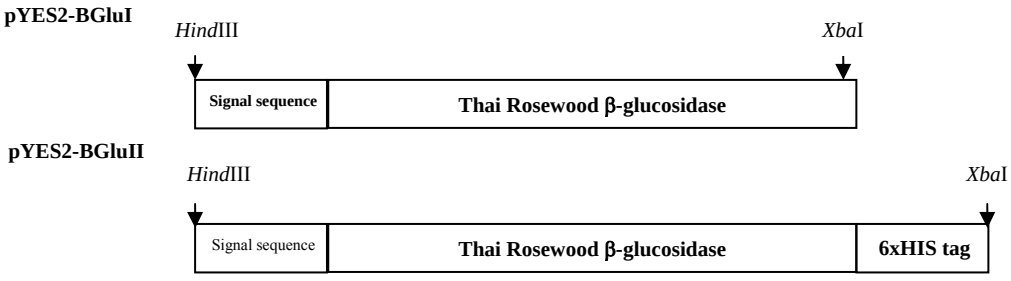


Figure 3.12 pYES2-BgluI and pYES2-BgluII constructs for expression of Thai rosewood β-glucosidase in *S.cerevisiae* BJ5462 with its own signal sequence.

The GAL1 promoter of pYES2 plasmid was induced by galactose at 30°C, 5 days to produce the proteins. The yeast cells were collected and lysed each day. The β-glucosidase and β-fucosidase activities were tested, as shown in Figure 3.13.

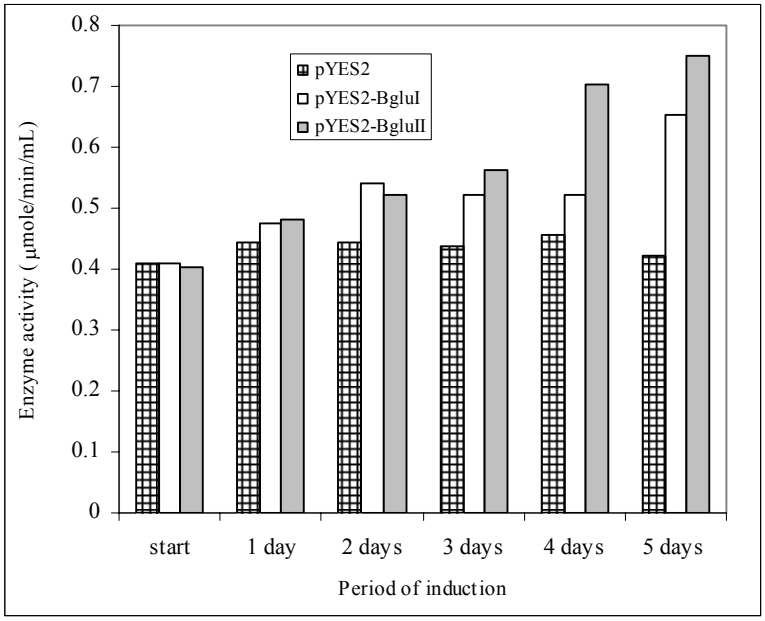


Figure 3.13 β-glucosidase activity in yeast cell lysates from recombinant Thai rosewood β-glucosidase expression with pYES2-BgluI and pYES2-BgluII plasmids in *S.cerevisiae* BJ5462. pYES2 is the empty vector control. The yeast cell was collected and lysed from start or 0 day, 1 day, 2 days, 3 days, 4 days, and 5 days, after galactose induction. β-glucosidase activity was assayed with 1 mM *p*NP-glucoside at 30°C for 30 min.

The recombinant Thai rosewood β-glucosidase expression level was highest at 5 days, the activity was 0.25 µmole *p*NP/min/mL of culture for pYES2 plasmid, 0.23 µmole *p*NP/min/mL of culture for pYES2-BgluI plasmid and 0.33

μmole *p*NP/min/mL of culture for pYES2-BgluII plasmid expressed in *S.cerevisiae* BJ5462. The total activity was 0.5 μmole *p*NP/mL of culture for pYES2 plasmid, 0.46 μmole *p*NP/min of culture for pYES2-BgluI plasmid and 0.66 μmole *p*NP/min of culture for pYES2-BgluII plasmid expressed in *S.cerevisiae* BJ5462. The transformed cells were broken after expression in order to measure the activity, which was somewhat labor intensive and prone to release proteases, so a secreted system seemed desirable.

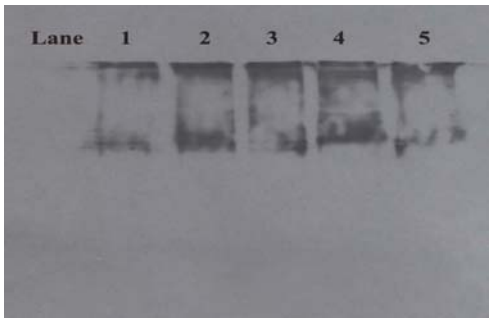


Figure 3.14 Immunoblot of cell lysate of *S.cerevisiae* BJ5462 transformed with pYES2-BgluII plasmid with polyclonal anti-Thai rosewood β-glucosidase at 1:1000 dilution. Lane 1 –Lane 5 were from 1-5 days of culture. Lane 1 was lysate from pYES2 plasmid in *S.cerevisiae* BJ5462, Lanes 2-5 were lysate from pYES2 BgluII plasmid in *S.cerevisiae* BJ5462 clone no. 1-4.

As shown in Figure 3.14, the polyclonal anti-Thai rosewood β-glucosidase reacted with many of the proteins that blotted onto the nitrocellulose, so it was not useful for detecting the recombinant protein.

3.3.2 Expression of Thai rosewood β-glucosidase from pYES2-PreproαI and pYES2-PreproαII secretion constructs

Production of secreted recombinant protein might be convenient for protein purification. Therefore, the prepro-α sequence from *S.cerevisiae* α-factor (a secreted hormone) was added to the mature Thai rosewood β-glucosidase with the PleBamPreproAfor primer. The PleBamPreproAfor and PMctermI primers or PleBamPreproAfor and PMctermH2 primers were used in PCR to produce cDNA products for expression with yeast α-factor prepropeptide for secretion. The product between PleBamPreproAfor and PMctermI primers encoded prepro-α factor and mature Thai rosewood β-glucosidase cDNA. The product between PleBamPreproAfor and PMctermH2 primers encoded the prepro-α factor and mature Thai rosewood β-glucosidase cDNA with a C-terminal 6 x histidine tag. The correct-size PCR products were produced, as shown in Figure 3.15. Both PCR products were ligated with *EcoR*V digested pBluescript II SK(+) and transformed in DHα *E.coli*. The correct plasmid was selected by digestion with *BamH*I and *Xba*I. The *BamH*I and *Xba*I insert was ligated with *BamH*I and *Xba*I digested pYES2 to produce the construct named pYES2-PreproαI and pYES2-PreproαII, as shown in Figure 3.16.

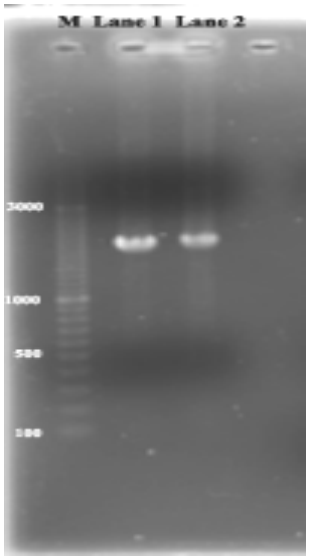


Figure 3.15 Electrophoresis of PreproI and PreproII Thai rosewood β-glucosidase cDNA PCR products 0.7% agarose gel.

M: EZ load 100 bp PCR Molecular Ruler

Lane 1: Prepro I PCR product

Lane 2: Prepro II PCR product

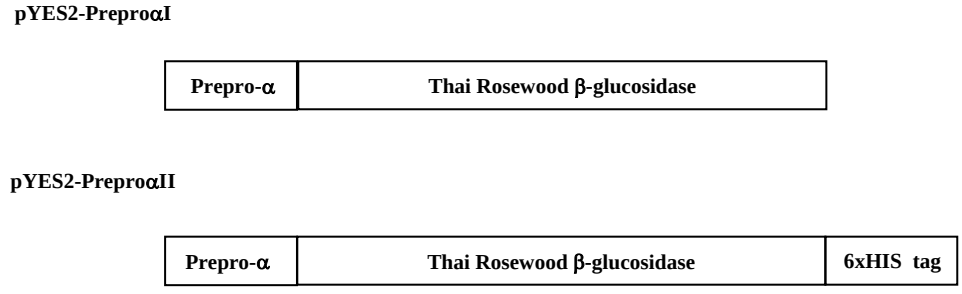


Figure 3.16 Expected proteins from pYES2-PreproαI and pYES2-PreproαII construction and expression of Thai rosewood β-glucosidase in *S.cerevisiae* BJ5462. Preproα refers to the prepro-sequence of *S.cerevisiae* α-factor.

To produce the β-glucosidase, recombinant *S.cerevisiae* yeast was cultured in glucose media at 30°C overnight, then changed to galactose and raffinose media to induce protein expression from the GAL1 promoter of pYES2 plasmid. In induction medium, recombinant Thai rosewood β-glucosidase expression levels from both pYES2-BgluI and pYES2-BgluII plasmid in *S.cerevisiae* were less compared with pYES2 plasmid in *S.cerevisiae*. The level of expression of β-glucosidase in media was measured by *p*-nitrophenol release from *p*NP-β-D-glucoside. As shown in Figure 3.17, the level of recombinant Thai rosewood β-glucosidase was quite low, when compared between 0 h and 24 h, from pYES2-PreproαI and pYES2-PreproαII plasmid in *S.cerevisiae* strain BJ5462. The recombinant Thai rosewood β-glucosidase expression level was highest at 24 h, the activity was 0.1 μmole *p*NP/min/mL of culture for pYES2 plasmid, 0.7 μmole *p*NP/min/mL of culture for pYES2- PreproαI plasmid and 0.8 μmole *p*NP/min/mL of culture for pYES2-PreproαII plasmid expressed in *S.cerevisiae* BJ5462.

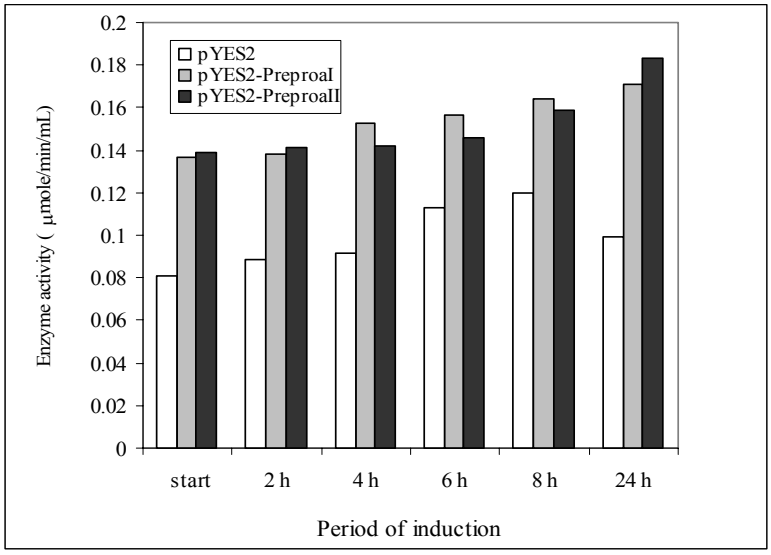


Figure 3.17 β-glucosidase activity in expression media of pYES2 empty plasmid control, pYES2-PreproαI and pYES2-PreproαII in *S.cerevisiae* media. Activity was measured with 1 mM *p*NP-glucoside, 30°C, 30 min.

3.4 Expression of Thai rosewood β-glucosidase enzyme in *Pichia pastoris*

3.4.1 Expression of Thai rosewood β-glucosidase from the pPICZαB-Thrombin-PYG (R519W) construction (C-terminal 6 x histidine tag)

pPICZαB-Thrombin-PYG(R519W) was constructed to incorporate a cDNA encoding a mature Thai rosewood β-glucosidase with a mutation of Arg519 to Trp into pPICZαB-Thrombin. A previous construct in this plasmid without the mutation had resulted in expression of active protein which could not be purified by the C-terminal 6 x histidine tag. The mutation of arginine position 519 to tryptophan

was made to eliminate a Kex2 protease cleavage site and prevent the proteolysis of the C-terminus between the recombinant protein and histidine tag. The expected recombinant protein was to contain the mature protein with a thrombin site and followed by a myc-tag and C-terminal 6xHistidine tag for IMAC purification, as shown in Figure 3.18.

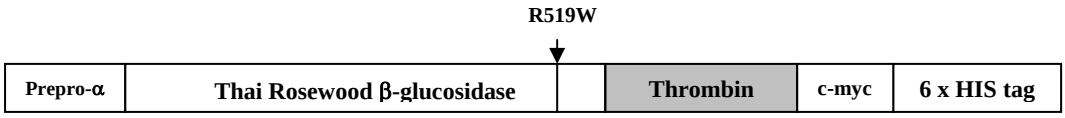


Figure 3.18 Expected protein structure from the pPICZαB-Thrombin-PYG (R519W) construct, which was used for expression in *P.pastoris* GS115. The pPICZαB-Thrombin-PYG (R519W) construct encoded Thai rosewood β-glucosidase cDNA which contained the mutation R519W, a thrombin recognition site, c-myc tag, and C-terminal 6 x histidine tag. Preproα is the *S.cerevisiae* alpha factor prepro peptide sequence.

pPICZαB-Thrombin-PYG (R519W) was transformed into *P.pastoris* GS115, and colonies were screened for enzyme production by small scale expression. Small scale cultures from 14 colonies were induced in 2 mL BMMY with 0.5% methanol and cultured 30°C for 3 days with 0.5% methanol added for induction each 24 h. The level of recombinant protein expression was followed day by day. The transformed clone which gave highest activity was selected for more expression. The culture medium was collected and subjected to IMAC, to determine if it would bind for purification.

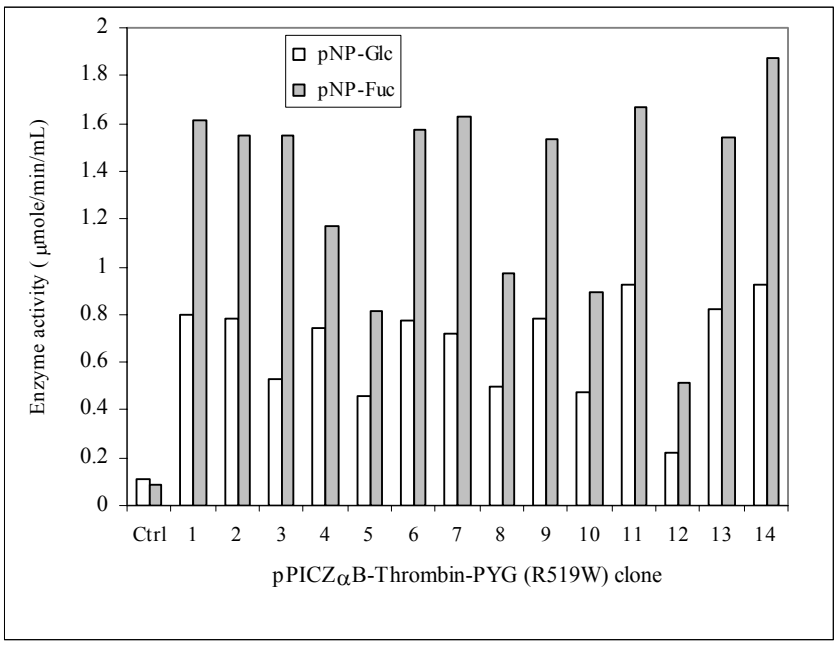


Figure 3.19 β-glucosidase and β-fucosidase activities in 3 days expression media of 14 colonies of *P.pastoris* GS115 transformed with pPICZαB-Thrombin-PYG (R519W) plasmid and one with pPICZαB-Thrombin plasmid as the control (Ctrl).

The level of expression of β-glucosidase and β-fucosidase activities in one-day culture medium were measured. As shown in Figure 3.19, pPICZαB-Thrombin-PYG(R519W) colony no. 14 had highest activity at 0.92 μmole/min/mL of culture medium for *p*NP-glucoside and at 1.87 μmole/min/mL of culture medium for *p*NP-fucoside.

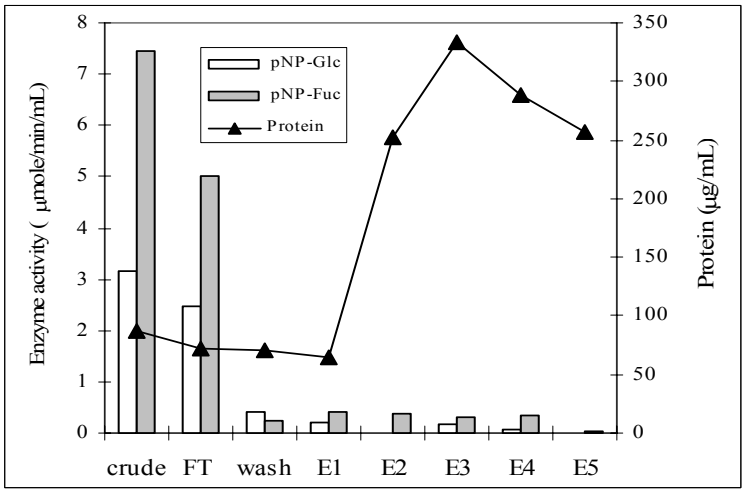


Figure 3.20 Protein and enzyme activity assay of purification fractions from Thai rosewood β-glucosidase expressed from pPICZαB-Thrombin-PYG (R519W) in GS115 *P.pastoris*. Fractions include culture medium (crude), flow-through (FT), wash, and elution fractions (E1-E5) from the HiTrap (immobilized nickel) column.

The culture medium pooled from 14 colonies was subjected to HiTrap (immobilized nickel) column and tested for β-glucosidase and β-fucosidase activities and protein. Compared to the cultured medium, most of the activity was in the flow-through fraction, as shown in Figure 3.20. This suggested that including a 6 x histidine tag at C-terminus might not be suitable for tagging protein for purification. The protein produced from the pPICZαB-Thrombin-PYG construct in *Pichia*, evidently had relatively high activity but could not be purified by IMAC.

Culture medium was concentrated and analyzed by SDS-PAGE. The protein pattern from both pPICZαB-Thrombin plasmid (as control) and pPICZαB-Thrombin-PYG (R519W) clone no. 14 looked similar, as shown in Figure 3.21.

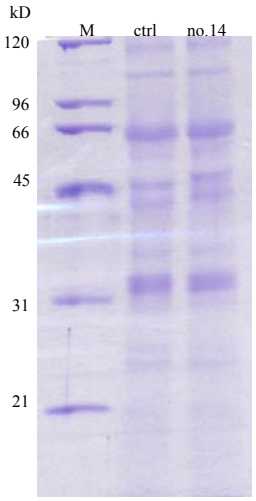


Figure 3.21 10% SDS-PAGE of concentrated culture medium from *Pichia* transformed with pPICZαB-Thrombin plasmid (as control) and pPICZαB-Thrombin-PYG(R519W) clone no. 14. M is the molecular weight protein marker.

Figure 3.22 Immunoblot pattern of concentrated recombinant Thai rosewood β-glucosidase protein expression media: pPICZαB-Thrombin (ctrl) and pPICZαB-Thrombin-PYG(R519W) no. 14 from *P.pastoris* and molecular weight protein marker (M) with polyclonal anti-Thai rosewood β-glucosidase at 1:1000 dilution.

The immunoblot of media from pPICZαB-Thrombin-PYG (R519W) clone no. 14 could not detect the protein but showed non-specific binding to the blot of the concentrated-cultured medium, as shown in Figure 3.22.

The recombinant Thai rosewood β-glucosidase produced from *P.pastoris* was not bound to IMAC, however, its β-glucosidase and β-fucosidase activities were higher, compared with recombinant Thai rosewood β-glucosidase produced from *E.coli* and *S.cerevisiae*, as shown in Figure 3.23. The recombinant Thai rosewood β-glucosidase produced from *P.pastoris* was not much than that higher compared with the activity in *S.cerevisiae*. However, the β-glucosidase and β-fucosidase activities produced from empty plasmid transformed into GS115 *P.pastoris* were less compared with *S.cerevisiae*. Compared to AD494(DE3), BL21(DE3) *E.coli*, and BJ5462 S.*cerevisiae*, *P.pastoris* GS115 was more suitable for the recombinant Thai rosewood β-glucosidase expression, since it gave higher activities of β-glucosidase and β-fucosidase with less background.

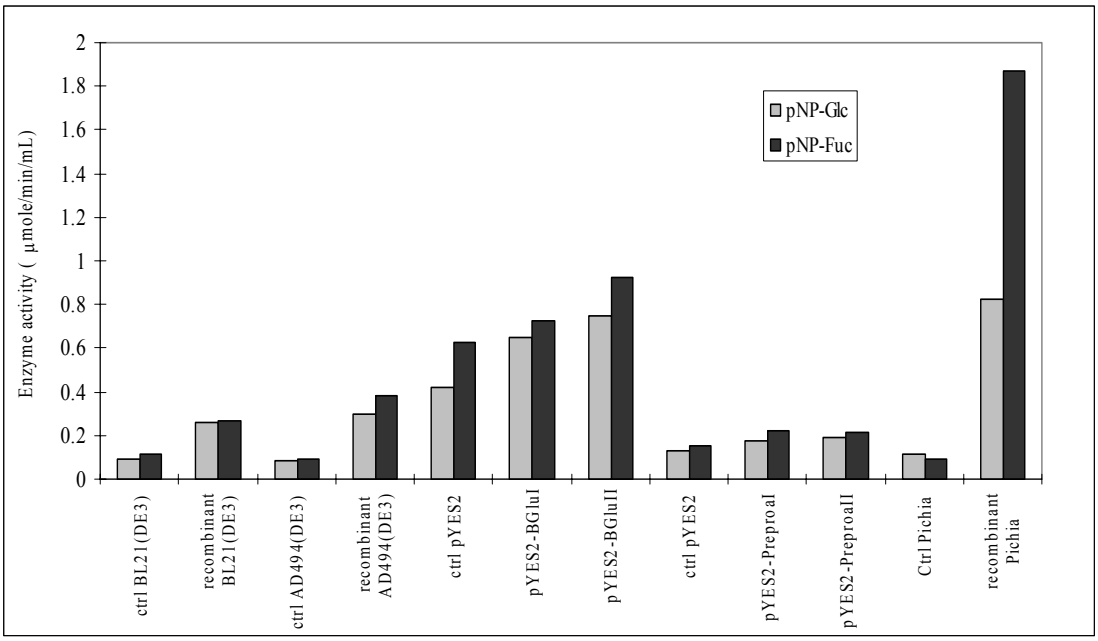


Figure 3.23 Comparison of the β-glucosidase level between control (ctrl) and recombinant of AD494(DE3), BL21(DE3) *E.coli*, BJ5462 *S.cerevisiae* and *P.pastoris* GS115 from the best condition for recombinant Thai rosewood β-glucosidase activity. Activity is given in µmole of *p*NP/min assay/mL of cell lysate or culture medium.

3.4.2 Expression of Thai rosewood β-glucosidase from the pPICZαBNH-PCR (R519W) construct with N-terminus 6 x histidine tag

The pPICZαBNH-PCR (R519W) construct contained coding regions for an N-terminal 6 x histidine tag, which was introduced in to the pPICZαB plasmid (named pPICZαBNH), and the mature Thai rosewood β-glucosidase protein. pPICZαBNH-PCR (R519W) was PCR amplified from 5'AOX and DcbgSacIIRev primers with Platinum *Pfx* polymerase and pPICZαB-Thrombin-PYG (R519W) clone no.5 as template. The PCR product was cloned into pBluescript II SK(+). The

*Pst*I and *Sac*II insert band was purified and ligated with *Pst*I and *Sac*II digested pPICZαBNH to produce the construct named pPICZαBNH-PCR (R519W), as shown in Figure 3.24. The pPICZαBNH-PCR (R519W) plasmid was transformed into GS115 *P.pastoris* competent cells. The transformed colonies were screened by small scale expression with 0.5% methanol at 30°C for 3 days with methanol induction each 24 h.

Figure 3.24 Expected structure of protein expressed from pPICZαBNH-PCR(R519W) in GS115 *P.pastoris*.

The transformed pPICZαBNH-PCR GS115 *P.pastoris* clones were expressed at 30°C for 3 days. The β-glucosidase activity from all colonies were compared as shown in Figure 3.25. The pPICZαBNH-PCR no. 7.4 gave higher activity than the others. However, the expressed activity was only slightly higher than the control. The culture medium was loaded onto a HiTrap (immobilized nickel) column to purify the Thai rosewood β-glucosidase. Most β-glucosidase and β-fucosidase activities were in flow-though, as shown in Figure 3.25.

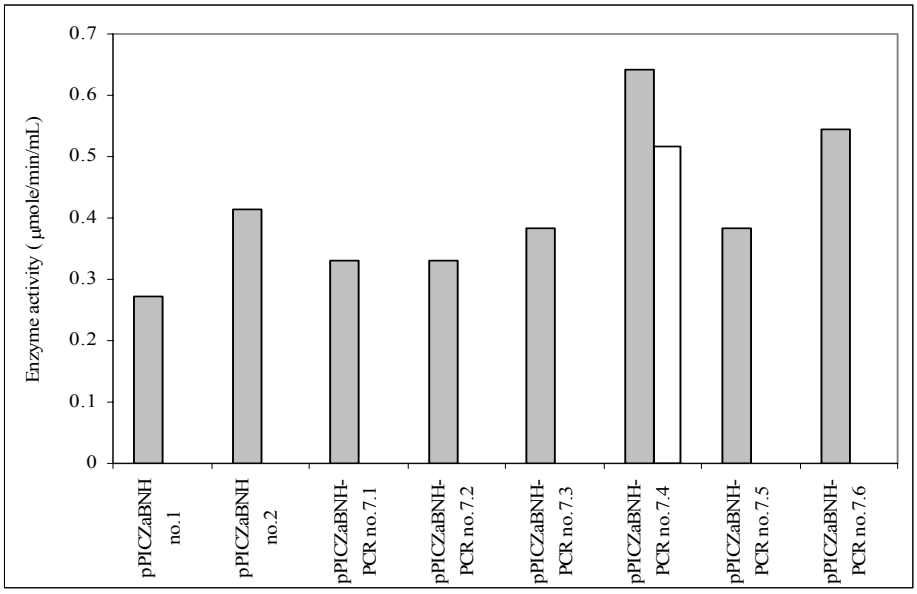


Figure 3.25 β-glucosidase and β-fucosidase activity of recombinant Thai rosewood β-glucosidase protein of 6 clones of *P.pastoris* GS115 transformed with pPICZαBNH-PCR (R519W) plasmid and 2 clones transformed with pPICZαNH plasmid as controls. Activity assays were done with 3 mM *p*NP-Glc (shaded bar) and 3 mM *p*NP-Fuc (white bar).

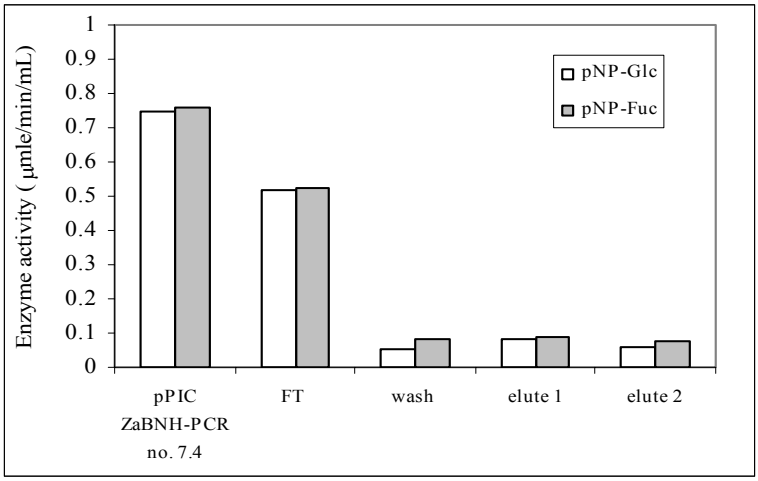


Figure 3.26 Enzyme activity of fractions from purification of Thai rosewood β-glucosidase expressed from pPICZαBNH-PCR (R519W) construct no. 7.4 with 3 mM *p*NP-Glc and 3 mM *p*NP-Fuc. Fractions include concentrated culture medium of pPICZαBNH-PCR (R519W) construct no. 7.4, flow-through (FT), wash, and elution fractions (E1 and E2) from the HiTrap immobilized nickel column.

3.4.3 Expression of Thai rosewood β-glucosidase from the pPICZαBNH-PYG (R519W) construct with N-and C-terminal 6 x histidine tags

*Pst*I and *Xba*I digested pPICZαB-PYG -Thrombin no. 5 was ligated with *Pst*I and *Xba*I digested pPICZαBNH to produce the construct named pPICZαBNH-PYG, as shown in Figure 3.27. The pPICZαBNH-PYG was transformed into GS115 *P.pastoris* and then expression with pPICZαBNH transformed clones.

Prepro-α	6 x HIS tag	Thai Rosewood β-glucosidase	Thrombin	c-myc	6 x HIS tag

Figure 3.27 Expected structure of protein expressed from pPICZαBNH-PCR (R519W) construction and expression in GS115 *P.pastoris*.

The transformed clone no. 6.3, which gave highest activity, was selected for more expression. The result shown that the level of expression of recombinant protein produced from pPICZαBNH-PYG construct was quite low, comparable with control, as shown in Figure 3.28. The β-fucosidase activity was quite lower than β-glucosidase activity. The culture medium was purified by HiTrap (immobilized nickel) column. The result was shown in Figure 3.29. Most β-glucosidase and β-fucosidase activities were in the flow-though.

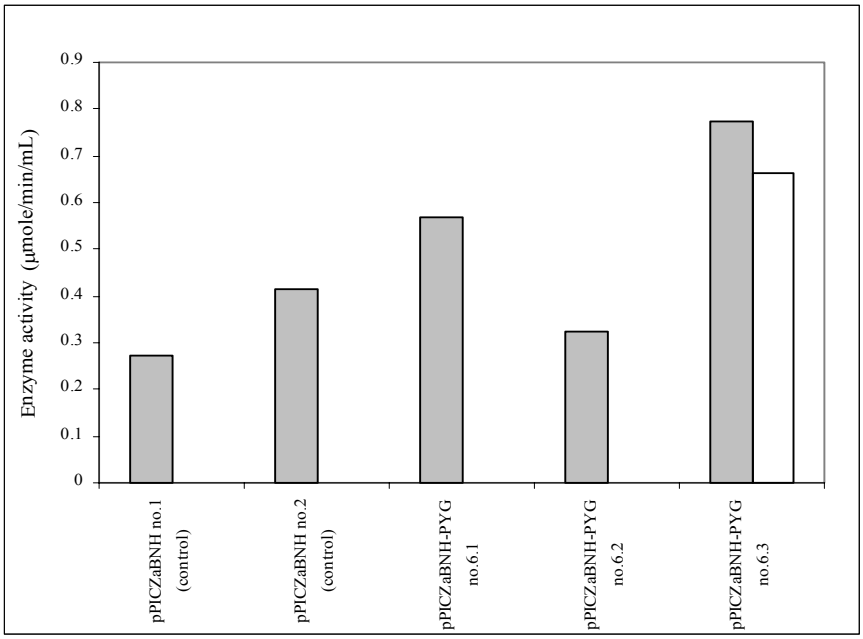


Figure 3.28 β-glucosidase and β-fucosidase activity of recombinant Thai rosewood β-glucosidase protein of 3 colonies of transformed pPICZαBNH-PYG (R519W) plasmid and transformed pPICZαNH plasmid into GS115 as control. Activity assays were done with 3 mM *p*NP-Glc (shaded bars) and 3 mM *p*NP-Fuc (white bar).

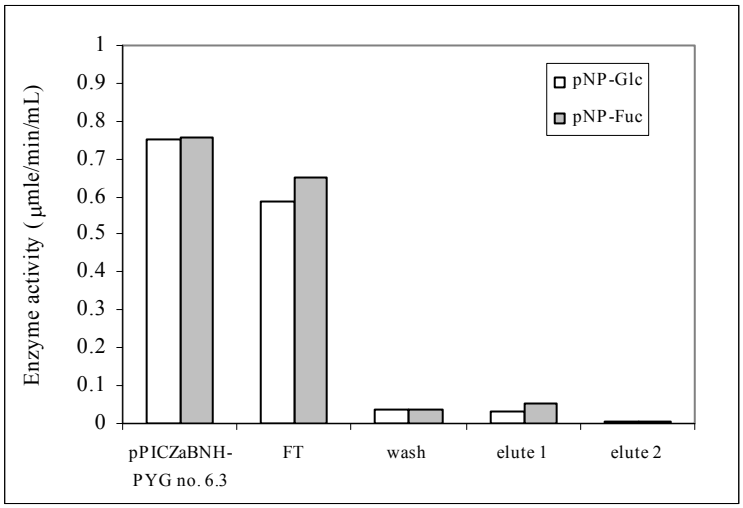


Figure 3.29 Enzyme activity assay profile of purification of Thai rosewood β-glucosidase expressed from pPICZαBNH-PYG (R519W) clone no. 6.3 with 3 mM *p*NP-Glc and 3 mM *p*NP-Fuc. Fractions include concentrated culture medium of pPICZαBNH-PYG (R519W) construct no. 6.3, flow-through (FT), wash, and elution fractions (E1and E2) from the HiTrap immobilized nickel column.

3.4.4 Expression of Thai rosewood β-glucosidase from pPICZαB-Truncated R488 construction

The Thai rosewood β-glucosidase was truncated at arginine 488 corresponding to the last residue in the 1CBG white clover β-glucosidase structure to reduce C-terminal protease susceptible sequence and determine whether the C-terminus was necessary for activity. The insert for pPICZαB-Truncated R488 was amplified from the DalYEXf1 and DcR488StopXbar primers with *Pfu* polymerase and pPICZαB-Thrombin-PYG (R519W) clone no. 5 as template. The insert digested

with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαB to produce the construct named pPICZαB-Truncated R488, as shown in Figure 3.30.

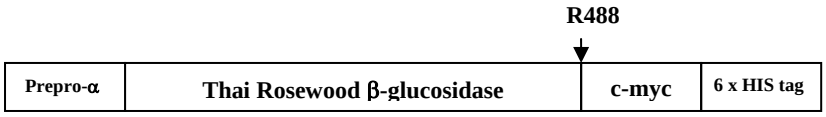


Figure 3.30 Diagram of the protein design of pPICZαB-Truncated R488 construction for expression in GS115 *P.pastoris*.

The pPICZαB-truncated R488 plasmid was transformed into GS115 *P.pastoris* competent cells, and forty zeocin-resistant colonies were screened by small scale expression. The 40 clones of *P.pastoris* containing the pPICZαB-Truncated R488 construct were found to have low activity (<2 μmole of *p*NP/min/mL). Both β-glucosidase and β-fucosidase activity of protein produced from pPICZαB-Truncated R488 construct were higher than the control pPICZαB transformed clones. However, β-fucosidase activity of protein produced from pPICZαB-Truncated R488 construct was lower than β-glucosidase activity.

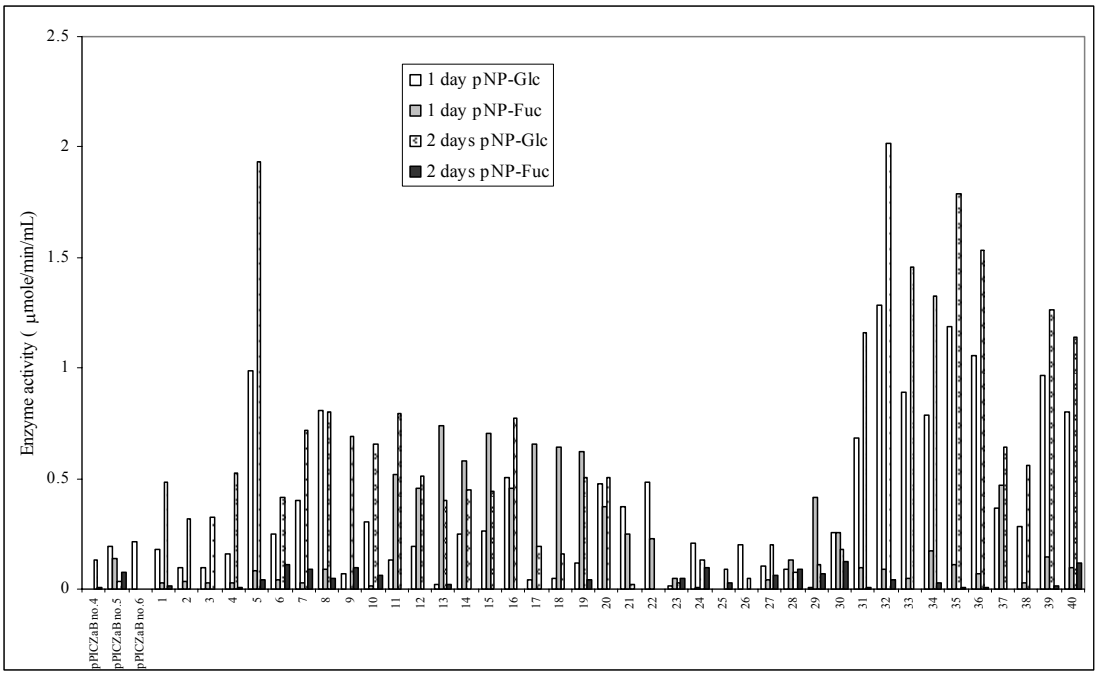


Figure 3.31 β-glucosidase and β-fucosidase activities in recombinant R519 truncated Thai rosewood β-glucosidase expression with pPICZαBNH-Truncated R488 and pPICZαB (control) transformed *P.pastoris* GS115 clones no. 1-40 expression was done for 2 days in BMMY with 1% methanol. pPICZαB-Truncated R488 clones are simply designate 1 to 40, while the controls are pPICZαB no. 4, 5, and 6.

The highest pPICZαBNH-R488 transformed GS115 clone no.5 was selected to express more and attempt purification by HiTrap column, as shown in Figure 3.32. The culture medium was subjected to HiTrap (immobilized nickel) column chromatography and β-glucosidase and β-fucosidase activities and protein were measured in the starting media, flow-through, wash and elution fractions. The recombinant protein contained both N-terminal and C-terminal 6 x histidine tags for

IMAC purification of R488 truncated Thai rosewood β-glucosidase protein. Compared to the cultured medium, most of recombinant enzyme activity was in the flow-through fraction, but some activity in elution fractions.

In the Figures 3.31 and 3.32, the recombinant protein produced from pPICZαBNH-Truncated R488 construct had β-glucosidase activity higher than β-fucosidase activity. The removal of arginine residue 488 might have affected β-fucosidase activity of Thai rosewood β-glucosidase more than β-glucosidase activity despite the fact these activities are in the same active site (looks completely inactive), or, more likely the activity may have come from a different protein.

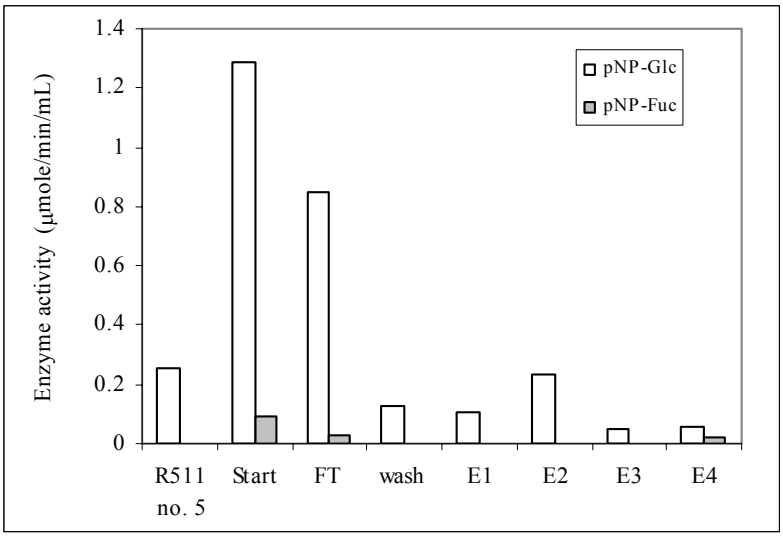


Figure 3.32 Enzyme activity in fractions from immobilized metal affinity chromatography of pPICZαB-Truncated R488 clone no. 5 media. The fractions were BMMY culture medium (start), flow through (FT), wash, and elution fractions (E1-E4) from the HiTrap column.

All fractions from the HiTrap column were analyzed by SDS-PAGE and a band of apparent purified truncated protein was evident at 66 kD, as seen in Figure 3.33. The C-terminal 6 x histidine tagged and truncated protein may have some protease susceptible amino acid sequences which produce protein degradation, causing a low amount to bind with the HiTrap column.

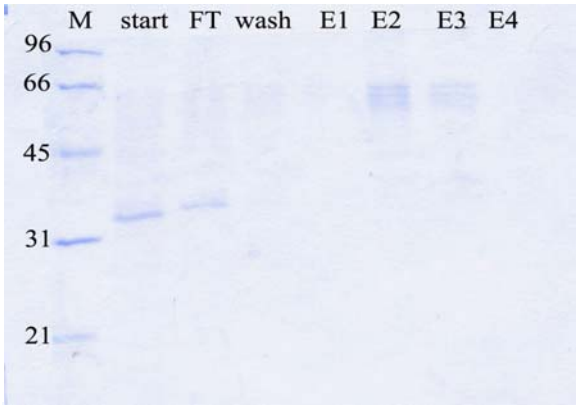


Figure 3.33 10% SDS-PAGE of protein purification of His-tagged protein from pPICZαBNH-Truncated R488 clone no. 5 culture medium. The fractions analyzed are concentrated culture medium (start), flow-through (FT), wash, and elution fractions (E1-E4) from the HiTrap (immobilized nickel) column.

3.4.5 Expression of Thai rosewood β-glucosidase from pPICZαBNH-Truncated N construction (truncated N terminus with N-terminal 8 x Histidine tag)

N-terminal sequencing of Thai rosewood β-glucosidase purified from *Pichia* media, showed the sequence began at glutamate residue 13. The Thai rosewood β-glucosidase was truncated at valine residue 14 to reduce the N-terminal

protease-susceptible sequence. pPICZαBNH8-Truncated N was amplified with DncVPPFpst1f and PMctermI primers and *Pfu* polymerase with the pPICZαB-Thrombin-PYG(R519W) clone no. 5 as template. The PCR product was cloned into pGEMT-easy plasmid as shown in Figure 3.34. Then, the insert was excised with *Pst*I and *Xba*I and ligated with *Pst*I and *Xba*I digested pPICZαBNH8, to produce the construct named pPICZαBNH8-Truncated N, as shown in Figure 3.35. The pPICZαBNH8-Truncated N plasmid was transformed into GS115 *P.pastoris* and transformed colonies were screened by small scale expression.

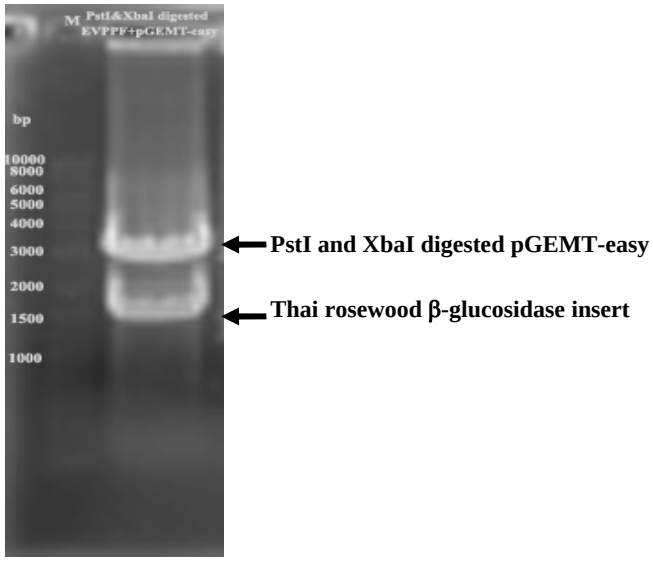


Figure 3.34 0.7% agarose gel shown *Pst*I and *Xba*I digested pGEMT-easy-EVPPF clone no. 1

Figure 3.35 pPICZαBNH8-Truncated N construction and expression in GS115 *P.pastoris*. The Thai rosewood β-glucosidase sequence began at valine residue 14 of the mature protein, as designed by the "VPPF".

```
    pPICZαB                    α-factor signal sequence
ATTATTCGAAACG ATG AGA TTT CTT TCA ATT TTT·····AAA GAA GAA GGG GTA TCT CTC
              Met Arg Phe Pro Ser Ile Phe·····Lys Glu Glu Gly Val Ser Leu

       Kex2▽ signal cleavage site      8 x histidine tag          PstI
GAG AAA AGA GAG GCT GAA GCA GCA CAT CAC CAT CAC CAT CAT CAC CAT GCT GCA
Glu Lys Arg Glu Ala Glu Ala Ala His His His His His His His His Ala AlA
           ΔSTE   ΔSTE cleavage site

 Truncated N-terminal Thai Rosewood β-glucosidase                 R519W
GTT CCT CCA TTC AAC CGA AGC TGT TTT CCT TCA GAT·····AAA CCC AAG TGG
Val Pro Pro Phe Asn Arg Ser Cys Phe Pro Ser Asp·····Lys Pro Lys Trp

                    Stop XbaI            pPICZαB
GGC ATT GAA GGC TTT TGA TCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTC
Gly Ile Glu Gly Phe ***
```

Figure 3.36 Sequence of the pPICZαBNH8-Truncated N construct which was used to express in GS115 *P.pastoris*. The Thai rosewood β-glucosidase sequence began at valine residue 14 and mutated Arg519 to Trp of the mature protein. Truncated N-terminal Thai rosewood β-glucosidase was inserted into *Pst*I and *Xba*I site of pPICZαBNH8 plasmid which contained on 8 x histidine tag followed by *Pst*I site inserted into the original *Pst*I site of the pPICZαB plasmid.

The three colonies transformed with pPICZαBNH8-Truncated N in GS115 *P.pastoris* and three colonies transformed with pPICZαBNH8 in GS115 *P.pastoris* as control were screened for expression for 3 days with 1% methanol added every 24 h. The pPICZαBNH8-Truncated N clone N1 gave highest activity of both β-glucosidase and β-fucosidase activities, as shown in Figure 3.37.

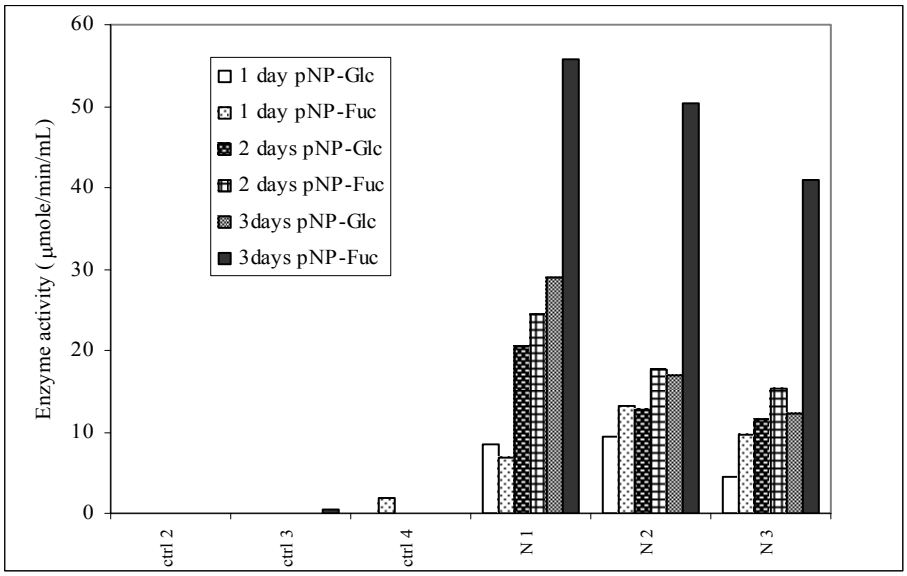


Figure 3.37 β-glucosidase and β-fucosidase activities in media from recombinant truncated N-terminus Thai rosewood β-glucosidase expression with pPICZαBNH8-Truncated N transformed GS115 *P.pastoris* clones N1, N2, and N3 and pPICZαBNH8 transformed as control clones (ctrl1, ctrl2, and ctrl3). Expression was done for 3 days in BMMY with 1% methanol.

The transformed clone N1, which gave highest activity, was selected for larger scale expression. The culture medium was subjected to IMAC to determine the binding of the protein to the column and feasibility of purification. The protein

produced from pPICZαBNH8-Truncated N was purified by HiTrap column chromatography and β-glucosidase and β-fucosidase activities and protein were determined. The recombinant protein produced from the pPICZαBNH8-Truncated N construct was firstly purified by HiTrap (immobilized nickel) column to determine affinity to IMAC. The recombinant protein bound with immobilized nickel resin in HiTrap and could be eluted out in elution fraction no. 2 and some in elution fraction no. 3, as shown in Figure 3.37. From Table 3.1, the percent recovery was calculated from the total activity in all elution fractions and total activity from culture medium (start). The percent recovery of β-glucosidase activity was similar to β-fucosidase activity, but somewhat low. Therefore, BD Talon™ (immobilized cobalt resin) metal affinity resin was also tried. The results of which were described in Section 3.7.

Table 3.1 Purification of recombinant Thai rosewood β-glucosidase protein from *P. pastoris* by HiTrap (nickel) affinity resin. Total enzyme activities in the starting concentrated media, column flow-through (FT), column wash and elution fractions were shown, and recovery was calculated from the amount in the elution fractions vs. the starting media.

Fraction	start	FT	Wash	elution fraction	% recovery
Total *p*NP-β-D-glucoside hydrolysis activity (μmole/min)	91.1	36.3	0	35.6	39
Total *p*NP-β-D-fucoside hydrolysis activity (μmole/min)	186	38.5	0	58.8	31

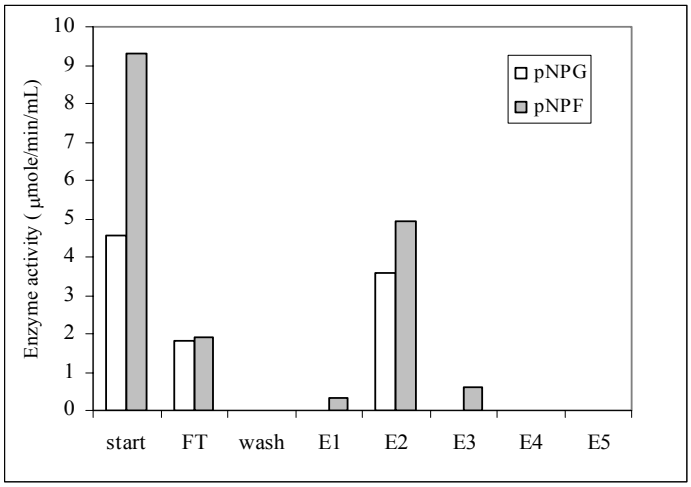


Figure 3.38 Enzyme activities in fractions from IMAC purification of pPICZαBNH8-Truncated N clone N1. Enzyme activities in concentrated culture medium (start), a flow-through (FT), wash, and elution fractions (E1-E5) from the HiTrap (immobilized nickel) column purification were shown. The β-glucosidase (*p*NPG) and β-fucosidase (*p*NPF) activities were shown.

In Figure 3.39, all fractions from BD Talon™ (immobilized cobalt resin) metal affinity resin were assayed β-glucosidase and β-fucosidase activities. Most of β-glucosidase and β-fucosidase activities were in the elution fractions.

Recombinant protein could be purified by HiTrap (immobilized nickel) column and BD Talon™ (immobilized cobalt) metal affinity column. N-terminal 8 x histidine tag and truncated protein could be expressed in active form and had affinity to bind with the IMAC column.

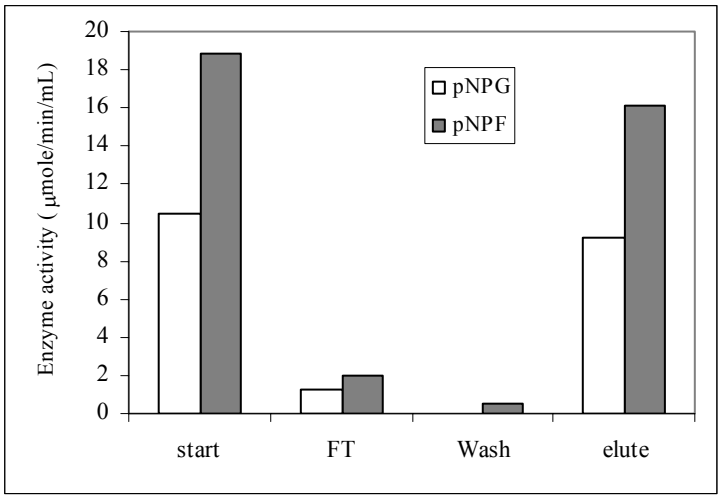


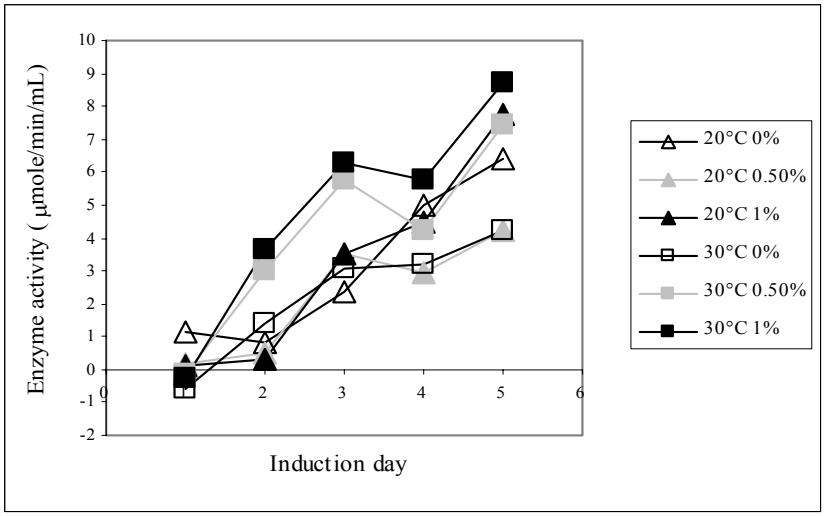
Figure 3.39 Enzyme activities in fractions from IMAC purification of pPICZαBNH8-Truncated N clone N1. Enzyme activities with *p*NP-β-glucoside (pNPG) and *p*NP-β-fucoside (pNPF) substrate in concentrated culture medium (start), flow-through (FT), wash, and elution fractions (eluate) from the BD Talon™ Metal affinity column (immobilized cobalt) purification are shown.

3.5 Optimal conditions for Thai rosewood β-glucosidase expression in *P.pastoris*

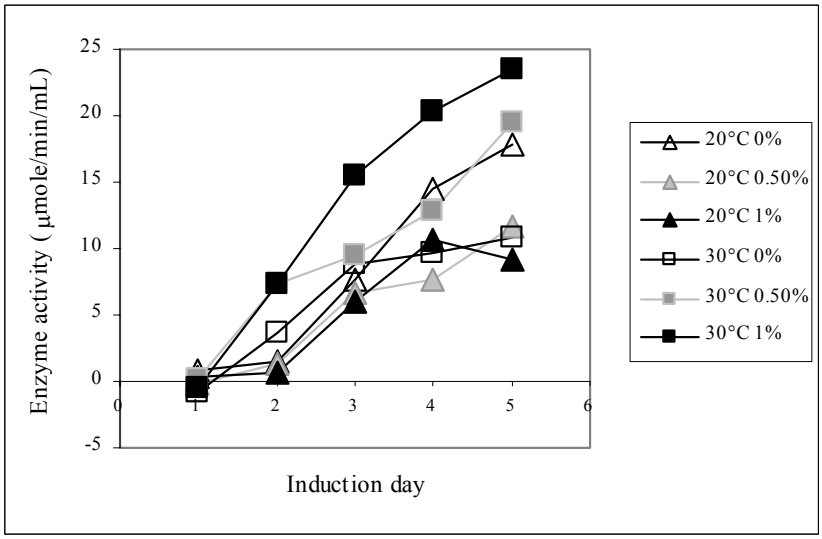
3.5.1 Suitable Temperature and percent of casamino acid for Thai rosewood β-glucosidase enzyme expression in *P. pastoris*

The effects of culture temperature and percent of casamino acids on Thai rosewood β-glucosidase expression in *P.pastoris* were determined. Casamino acids were added to prevent proteolysis by *Pichia* secreted proteases. Casamino acids (0, 0.5 or 1%) were added in BMMY with 1% methanol. The expression was continued at 20°C and 30°C for 5 days with addition of 1% methanol each day and

the β-glucosidase and β-fucosidase activities were measured, as shown in Figure 3.40.



A) *p*-nitrophenol glucoside (*p*NP-Glc)

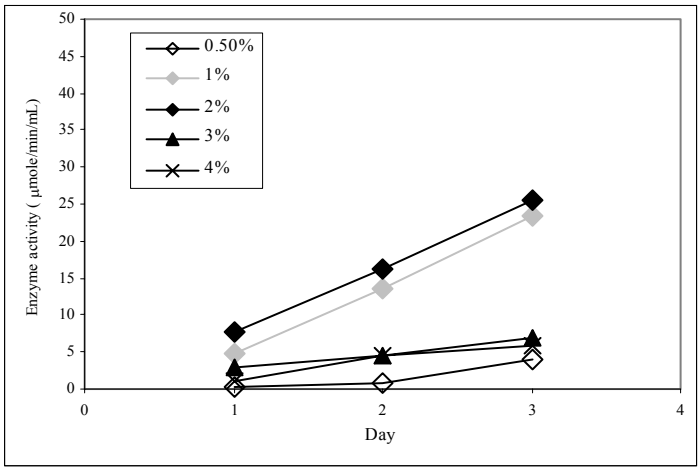


B) *p*-nitrophenol fucoside (*p*NP-Fuc)

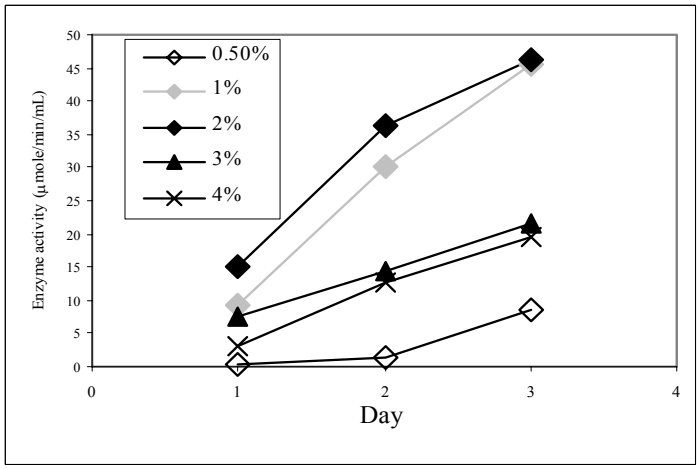
Figure 3.40 Effect of casamino acids and temperature on recombinant Thai rosewood β-glucosidase expression. The legend gives temperature followed by percent of casamino acids for each curve. Enzyme activities were measured with A) *p*-nitrophenol glucoside (*p*NP-Glc) and B) *p*-nitrophenol fucoside (*p*NP-Fuc).

3.5.2 Optimization of percent of methanol for *Pichia* expression

The percent of methanol used for induction was varied from 0.5% to 4% for recombinant protein expression at 30°C for three days of induction, and samples were analyzed each day, as shown in Figure 3.41. Purified recombinant enzyme was assayed for 1 mM *p*NP-Glc and *p*NP-Fuc hydrolysis.



A*) p*-nitrophenol glucoside (*p*NP-Glc)



B*) p*-nitrophenol fucoside (*p*NP-Fuc)

Figure 3.41 Effect of the methanol concentration on recombinant Thai rosewood β-glucosidase levels. The legend gives percent of methanol for each curve. Enzyme activities were measured with A) *p*-nitrophenol glucoside (*p*NP-Glc) and B) *p*-nitrophenol fucoside (*p*NP-Fuc)

In Figure 3.41 A and B, the β-glucosidase and β-fucosidase activities of recombinant Thai rosewood β-glucosidase were increased when the percent of methanol was increased. At 1% and 2% methanol, the activity was not much different. At 4% methanol, the activity was decreased. This might be because higher methanol was toxic to the cells or damaged the proteins. The suitable concentration of methanol for Thai rosewood β-glucosidase enzyme expression in *P.pastoris* was 1% methanol in BMMY with 1% casamino acid.

The optimal conditions for expression of recombinant Thai rosewood β-glucosidase expression were at 30°C in BMMY with 1% methanol and 1% casamino acids for 5 days.

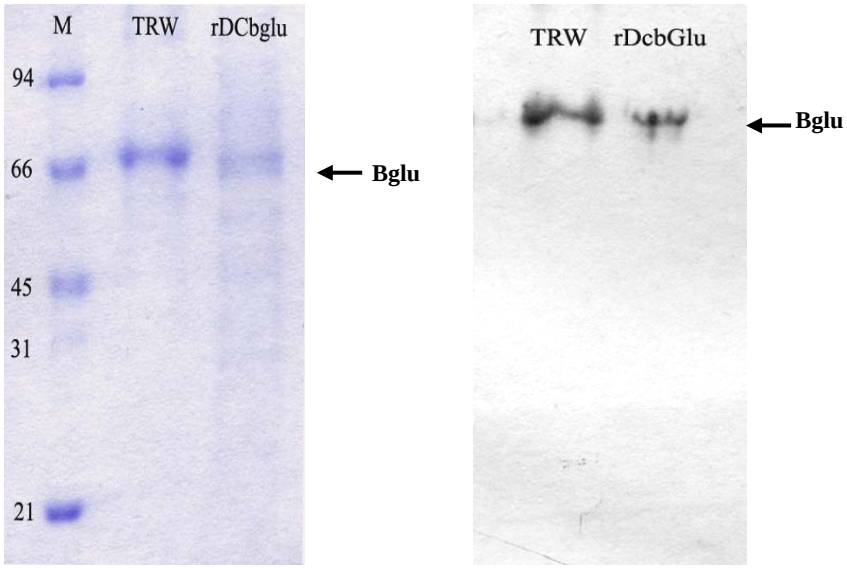
3.6 Purification and characterization of Thai rosewood β-glucosidase from *Pichia pastoris*

The Thai rosewood β-glucosidase enzyme was purified by IMAC and its activity assayed. Only recombinant protein from pPICZαBNH8-Truncated N was able to bind well with BD Talon™ metal affinity resin (Cobalt), as shown in Table 3.2. The percent recovery was calculated from percent of ratio between total activity from elution fraction and culture medium (start). Recovery of recombinant Thai rosewood protein produced from pPICZαBNH8-Truncated N bound to BD Talon™ metal affinity resin (Cobalt) was about 75%.

Table 3.2 Purification of recombinant Thai rosewood β-glucosidase protein from *P. pastoris* by BD Talon™ metal (Cobalt) affinity resin**.** The total protein (μg) and enzyme activities in the starting concentrated media, column flow-through, wash and elution fractions and the percent of protein and activities recovered in the elution fractions were shown.

	start	FT	Wash	elution fraction	% recovery
Total protein (μg)	927	873	0	127	13.7
Total *p*NP-β-D-glucosidase activity (μmole/min)	208	52.2	0	158	76
Total *p*NP-β-D-fucosidase activity (μmole/min)	377	80.5	11.1	278	74

The purified recombinant protein was concentrated 10 fold after desalting by centricon (cutoff 10,000 Da) filtration. The concentrated protein was seen by coomassie blue staining in a 10% SDS-PAGE gel, as shown in Figure 3.42. When Thai rosewood β-glucosidase from seed and concentrated recombinant protein were blotted onto nitrocellulose and probed with polyclonal rabbit anti-Thai rosewood β-glucosidase antiserum, both proteins were seen on x-ray film after developing the western blot. The recombinant Thai rosewood β-glucosidase from pPICZαBNH8-Truncated N clone N1 protein band was slightly lower than Thai rosewood β-glucosidase from seed, as shown in Figure 3.42 A. Both protein bands reacted with polyclonal anti-Thai rosewood β-glucosidase anti-serum, as also shown in Figure 3.42 B.

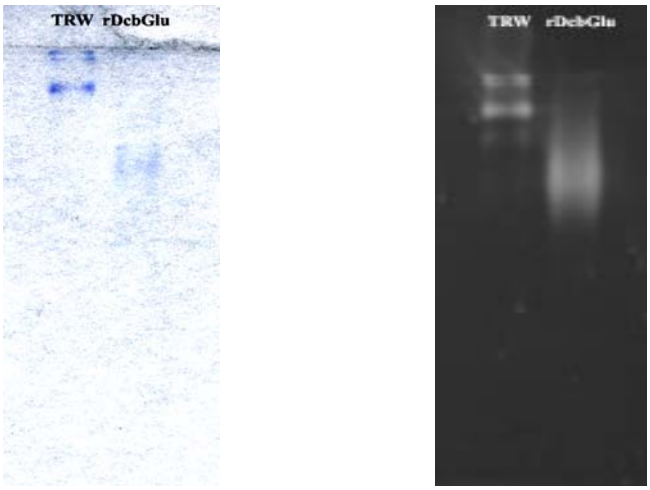


A) B)

Figure 3.42 Comparison of Thai rosewood β-glucosidase proteins from seed and *P.pastoris* by SDS-PAGE. A) Ten percent of SDS-PAGE of Thai rosewood β-glucosidase purified from seed (TRW) and concentrated purified recombinant protein from expression in *P.pastoris* (rDCbglu) with coomassie brilliant blue staining B) Immunoblot of 10% SDS-PAGE gel of Thai rosewood β-glucosidase purified from seed and concentrated purified recombinant protein from expression in *P.pastoris*.

The Thai rosewood β-glucosidase from seed and concentrated recombinant protein were electrophoresed on a native gel and stained with 1 mM 4-methylumbelliferyl-β-glucopyranoside, as shown in Figures 3.42 and 3.43. The β-glucosidase activity of both Thai rosewood β-glucosidase from seed and concentrated recombinant protein were seen on the 7% native gel with the same amount of protein. After activity staining, this native gel was stained with coomassie blue. In the 7% native gel in Figure 3.42, β-mercaptoethanol was not added to the loading buffer. The

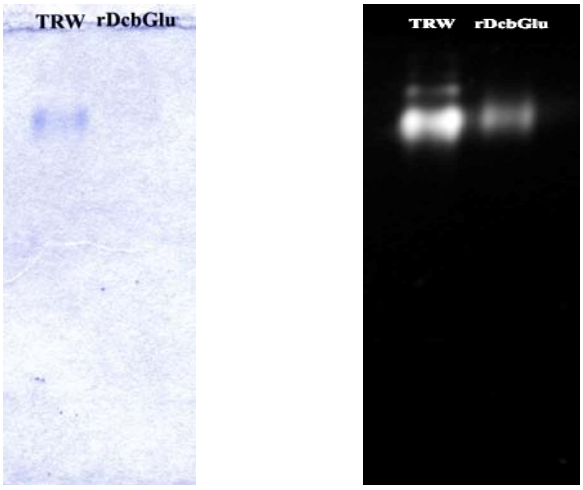
protein from seed (TRW) appeared to form polymer aggregates, as seen by the many bands in both coomassie blue staining and activity staining of the gel. The recombinant protein (rDcbGlu) moved faster than protein from seed.



A) B)

Figure 3.43 Native gel of Thai rosewood β-glucosidase from seed (TRW) and concentrated recombinant protein expression in *P.pastoris* (rDcbglu) without β-mercaptoethanol added to the loading buffer. The 7% polyacrylamide gel was stained with A) coomassie staining gel and B) activity staining of with 1 mM 4-methylumbelliferyl-β-glucopyranoside

In Figure 3.44, β-mercaptoethanol was added to loading buffer. β-mercaptoethanol appeared to reduce polymer formation of protein from seed (TRW) in both coomassie blue staining and activity staining of gel and resulted in the main band migrating at a similar position with the band of recombinant protein.



A) B)

Figure 3.44 7% Native gel of Thai rosewood β-glucosidase from seed (TRW) and concentrated recombinant protein expression in *P.pastoris* (rDcbglu) with β-mercaptoethanol added to the loading buffer. The 7% polyacrylamide gel was visualized by: A) coomassie staining gel and B) activity staining with 1 mM 4-methylumbelliferyl-β-glucopyranoside

The K_m values of recombinant Thai rosewood β-glucosidase were similar to those of native Thai rosewood β-glucosidase, 5.07 mM for *p*NP-β-D-fucoside and 0.55 mM for *p*NP-β-D-glucoside, as shown in Table 3.2. Both the K_m and the k_{cat} for the recombinant enzyme were lower for *p*NP-β-D-fucoside than *p*NP-β-D-glucoside. The K_m/ k_{cat} was higher for *p*NP-β-D-fucoside.

Table 3.3 Comparison of Michaelis-Menten parameters between native Thai rosewood β-glucosidase purified from seed and recombinant enzyme with a truncated N-terminus for *p*NP-β-D-glucoside and *p*NP-β-D-fucoside.

	Substrate	K_m	k_{cat}	k_{cat}/K_m
Native*	*p*NP-β-D-glucoside	5.37±0.09	307±4.6	57,300
	*p*NP-β-D-fucoside	0.54±0.04	151±3.0	283,100
recombinant	*p*NP-β-D-glucoside	5.07±0.07	206±7.0	40,600
	*p*NP-β-D-fucoside	0.55±0.02	156±22	282,000

* values for the native protein from seed are taken from Srisomsap. *et al*., 1996

3.7 pH and Temperature optimum of recombinant Thai rosewood β-glucosidase from pPICZαBNH8 truncated N construction

To examine the effect of pH on recombinant protein activity, purified recombinant enzyme was assayed in 0.1 M sodium phosphate-citrate buffer pH 3.0 to 9.0 with 1 mM *p*NP-Glc or *p*NP-Fuc at 30°C for 10 min.

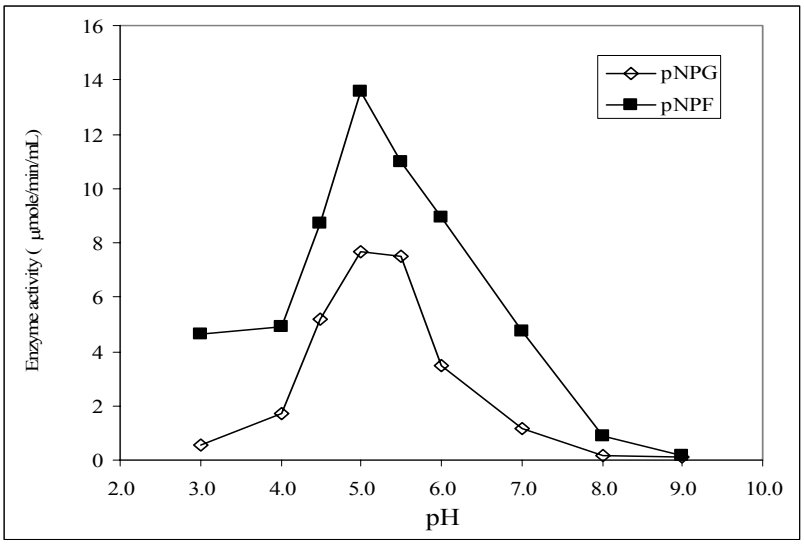


Figure 3.45 Effect of pH on recombinant Thai rosewood β-glucosidase activity. The activities for hydrolysis of pNP-β-D-glucoside (pNPG) and of pNP-β-D-fucoside (pNPF) were assayed at 30°C in buffer at various pH values.

The optimum pH of recombinant Thai rosewood from *P.pastoris* is 5.0 for both β-glucosidase and β-fucosidase activities, as shown in Figure 3.45. At the pH 3 and 4, the β-glucosidase and β-fucosidase activities were 80% lower than the activities at pH 5. At a pH of 4.5, the activity was half maximal. The activity was highest at pH 5.0 and tended to decrease at pH 6.0 and higher. At the pH 8.0 and pH 9.0, the enzyme was nearly inactive.

The temperature was varied to examine the effect of temperature on recombinant β-glucosidase activity. Purified recombinant enzyme was assayed in 0.1 M sodium acetate buffer pH 5.0 with 1 mM *p*NP-Glc or *p*NP-Fuc at appropriate temperature for 10 min.

In the Figure 3.46, the optimal temperature was 60°C, whereas at 20°C and 25°C, the enzyme activity was 5-fold lower. At 70°C, the recombinant protein was likely denatured, which led to a decrease in enzyme activity.

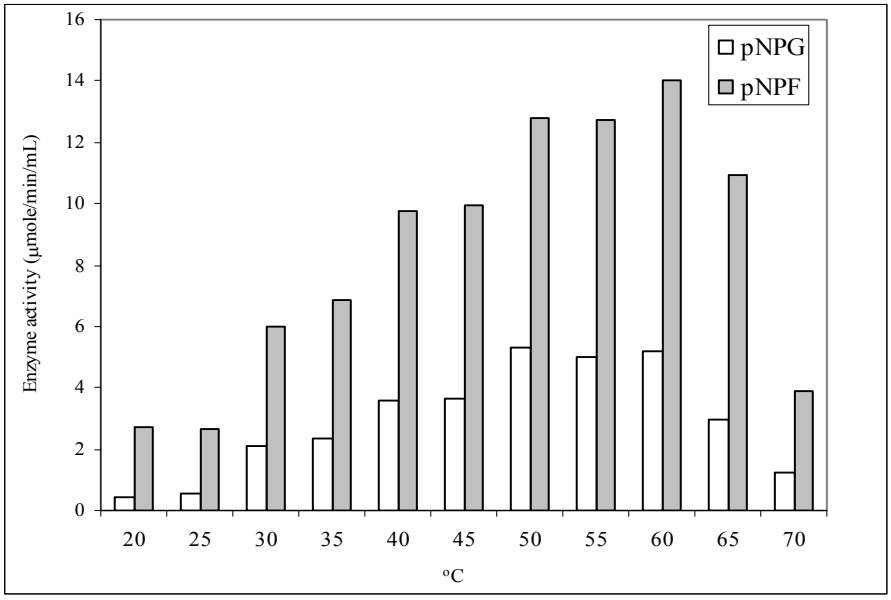


Figure 3.46 Effect of temperature on recombinant Thai rosewood β-glucosidase activity. The purified recombinant enzyme was assayed with 1 mM *p*NP-Glc (pNPG) and *p*NP-Fuc (pNPF) at 20-70°C, 10 min.

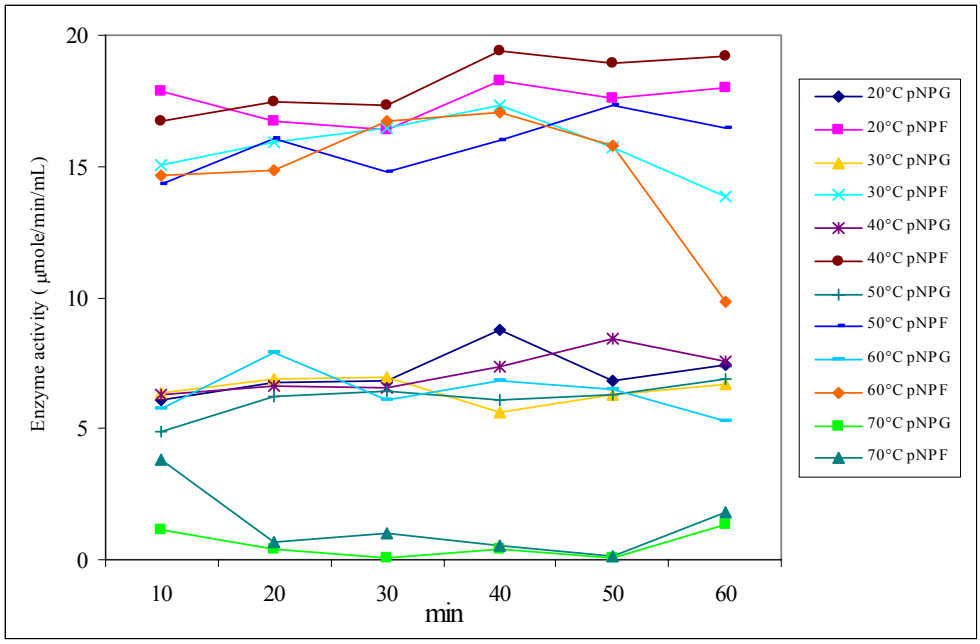


Figure 3.47 Thermostability of Thai rosewood β-glucosidase expressed in *Pichia*. Thermostability was tested by incubating at temperatures from 10-70°C for 10-60 min, then assaying with 1 mM *p*NP-Glc and *p*NP-Fuc at 30°C for 10 min.

The purified Thai rosewood β-glucosidase from pPICZαNH8-truncated N was tested for thermostability, as shown in Figure 3.47. At 70°C, the β-glucosidase/ β-fucosidase activity was decreased. The recombinant Thai rosewood β-glucosidase was stable between 20°C to 50°C for 60 min. At 60°C, recombinant Thai rosewood β-glucosidase was stable for 50 min, and then the activity was decreased by heat after 60 min incubation.

CHAPTER IV

DISCUSSION

Recombinant DNA techniques have been used for obtaining and combining genes from a variety of sources, and expressing these genes in different host cells. The choices of host for the recombinant protein production depend mainly on the properties and the final use of the expressed protein. If the protein consists of multiple subunits or requires post-translational modification, a eukaryotic host is preferred (Jonasson *et al*., 2002).

Previously, Ketudat Cairns *et al*. (2000) tried to express the Thai rosewood β-glucosidase protein in *E.coli*. The insoluble protein produced from *E.coli* was not functional. The expression of Thai rosewood β-glucosidase protein in *P.pastoris* was then expressed with the pPIC9K plasmid with prepro-α-mating factor in GS115 *P.pastoris*. The culture cells were lysed by glass beads and the soluble and fraction, tested along with the media. The β-glucosidase was found in secreted and intracellular fractions. The culture medium of control cells was also tested for β-glucosidase and β-fucosidase, but no activity was found. The recombinant enzyme had similar enzymatic constants to native enzyme purified from seed.

Though the Thai rosewood β-glucosidase expression in *E.coli* and *P.pastoris* has previously done, the amount of active recombinant enzyme produced was low. In the *P.pastoris* expression system with a prepro-α-mating factor secretory signal, the active protein in culture medium was low and protein purification was not convenient.

To develop a reliable recombinant expression system, recombinant systems for expression of Thai rosewood β-glucosidase enzyme in bacteria and yeast were explored. The improvement was intended to allow rapid purification of the enzyme from the recombinant system. The recombinant protein was produced in the *P.pastoris* expression system and its catalytic properties characterized in comparison to the native enzyme purified from seed. The reliable recombinant expression system and rapid purification method for recombinant Thai rosewood β-glucosidase described here can be used for further studies of its structure-function relationship.

4.1 Production of antibodies to Thai rosewood β-glucosidase

In order to aid in detection of Thai rosewood β-glucosidase during expression, polyclonal antibodies against the enzyme were produced. The rabbit anti Thai rosewood β-glucosidase polyclonal antibody was tested with native enzyme purified from seed. The dilution of 1:1000 was selected for use based on the titer.

The rabbit anti Thai rosewood β-glucosidase polyclonal antibody was used to detect protein from various expression systems. In the *E.coli* expression system, the immunoblot result showed nonspecific binding to the *E.coli* cell lysate. In the *P.pastoris* expression system, the immunoblot results also showed only non-specific binding to the concentrated culture medium. However, the immunoblot result of protein purified from seed and concentrated recombinant protein showed binding of the antibody to the band of purified protein. This might be because the impurities of recombinant protein that bound to the antiserum were removed in the purification steps, which left only the concentrated recombinant protein to react with the rabbit

anti Thai rosewood β-glucosidase polyclonal antibody. However, it is also likely that the antibody was not very sensitive at the dilution used, so only in this gel was the protein concentrated enough to detect. In other cases, only general nonspecific background was seen.

4.2 Expression of Thai rosewood β-glucosidase enzyme in *Escherichia coli*

E.coli is one of the most widely used hosts for the production of heterologous proteins and its genetics are far better characterized than those of any other organism (Jonasson *et al*., 2002). *E.coli* has mainly been used for production of proteins which are limited in size and have relatively simple structures. The target recombinant protein produced by *E.coli* may accumulate intracellularly in a soluble form or as inclusion bodies, or the protein may be secreted into the periplasm or culture medium. The selected strategy influences the recombinant protein purification, as well as quality and yield of protein product.

The pET-32a(+) plasmid (Novagen) carries the T7 *lac* promoter to direct the expression of target genes so that transcription does not occur in the absence of induction ('leakiness"). However, in *E.coli* strains containing DE3 prophage, a small amount of T polymerase is produced without induction. This vector encodes the Trx·Tag, His·Tag and S·Tag sequences that allow easy purification. The N-terminal fusion sequences can be removed with thrombin (Trx·Tag and His·Tag) or enterokinase (all three tags). A second His·Tag sequence follows the multiple cloning site of pET-32a(+) plasmid.

Two *E.coli* host strains were tried for expression. BL21(DE3) (Novagen), a protease deficiency strain, is the most widely used host for target gene expression. AD494(DE3) (Novagen), a thioredoxin reductase (*trxB*) mutant that allows disulfide bond formation in the *E.coli* cytoplasm, provides a possible means to produce correctly folded, soluble, disulfide-containing foreign proteins in the *E.coli* cytoplasm.

Thai rosewood β-glucosidase cDNA was cloned into pET-32a(+) and expressed in BL21(DE3) and AD494(DE3) *E.coli.* The optimal IPTG concentration and temperature of expression were determined. The recombinant Thai rosewood β-glucosidase had low amounts of β-glucosidase and β-fucosidase activity and could not be purified by IMAC on the HiTrap immobilized nickel column.

The protein bands from bacterial cell lysates of control and sample were similar. Most of the bacterial cell lysate protein was in the flow-through and elution fractions which contained an increased concentration of imidazole. The elution fractions E3 and E4 had 2 major bands, which corresponded to MW of 66 and 22. These two bands were confirmed to be the recombinant protein by N-terminal sequencing. The two separate protein bands were evidently produced by the degradation of recombinant protein, which had an expected size of about 80 kD. The degradation might be from bacterial proteases which cut the Thai rosewood β-glucosidase's protein sequence to produce the 66 kD protein, since its N-terminal sequence matched thioredoxin. The 22 kD protein might be from thioredoxin protein and some part of the N-terminus of Thai rosewood β-glucosidase. Thai rosewood β-glucosidase's protein sequence appeared to be proteolyticly susceptible at both the N-

terminal and C-terminal ends. This reason might lead to a protein truncated at both the N-terminus and C-terminus.

4.3 Expression of Thai rosewood β-glucosidase in *Saccharomyces cerevisiae*

E.coli is a prokaryote and lacks intracellular organelles, such as the endoplasmic reticulum and the golgi apparatus, that are present in eukaryotes and are responsible for modifications of the proteins being produced. Many eukaryotic proteins can be produced in *E.coli* but are produced in a nonfunctional, unfinished form, since glycosylation and other eukaryotic post-translational modifications do not occur.

Many recombinant proteins expressed in *E.coli* gave inactive protein, insoluble protein or unsatisfactory yield. The recombinant protein was further expressed in other expression systems. Keresztessy *et al*. (1996) cloned linamarase from cassava into the glutathione S-transferase (GST) gene fusion protein expression vector pGEX-2T to produced the construct named pEXCAS and expressed it in *E.coli*. With GroEL co-expression and purification, recombinant protein was active but at low yield with poor stability. In 2001, Keresztessy and colleagues cloned mature cyanogenic β-glucosidase (linamarase) into the vector pYX243 modified to contain the SUC2 yeast secretion signal sequence and expressed active protein in *S. cerevisiae* (Keresztessy *et al*., 2001).

Previously, three β-glucosidase genes (BglA1, BglB1, and BglC1) from the cellulolytic bacterium *Cellulomonanse biazotea* were cloned into pUC18 and

transformed in *E.coli* (Rajoka *et al*., 1998). BglA1, BglB1, and BglC1 were also cloned into pYES2 and transformed into *S.cerevisiae*. The Gal1 promoter of the shuttle pYES2vector enabled the *S.cerevisiae* to produce twice as much β-glucosidase than that supported by the lacZ promoter of pUC18 plasmid in *E.coli*. The enzymatic properties of β-glucosidases from *S.cerevisiae* were the same as the native enzymes from *C. biazotea*.

As a eukaryotic organism, *S.cerevisiae* has post-translational processes similar to other eukaryotes. Therefore, *S.cerevisiae* was used for Thai rosewood β-glucosidase protein expression. Thai rosewood β-glucosidase cDNA was cloned into the pYES2 plasmid, in the constructs named pYES2-BgluI and pYES2-BgluII. pYES2-BgluII contained C-terminal 6 x histidine tag for ease to protein purification. The recombinant protein produced from pYES2-BgluI and pYES2-BgluII had low levels of enzyme activities, only slightly higher than control expression.

Schekman and his collaborators (Novick *et al*., 1981; Schekman and Novick, 1981) have shown that the yeast secretory pathway involves a series of membrane-bound structures that mediate the transfer of exported proteins from their site of synthesis at the endoplasmic reticulum to their site of release at the plasma membrane. As in other organisms, these secreted proteins are synthesized as larger precursors including “signal” sequences, which are cleaved by a membrane-bound protease to yield the mature gene products.

The recombinant Thai rosewood β-glucosidase was produced from pYES2-PreproαI and pYES2-PreproαII constructs in *S.cerevisiae* BJ 5462. The pYES2-PreproαI construct was to produce the mature protein fused to the yeast alpha factor propro-α signal sequence, which should be secreted into the medium. The

recombinant protein from the pYES2-PreproαII construct was to similarly produce the preproα-mature protein fusion with a 6 x histidine tag, which should also be secreted into the medium. However, the recombinant protein from these two constructs was not secreted into the medium. Therefore, the transformed yeast was broken and the cell lysate was centrifuged to separate supernatant. The level of recombinant Thai rosewood β-glucosidase from pYES2-PreproαI and pYES2-PreproαII plasmid in *S.cerevisiae* was quite low, with little difference between 0 h and 24 h induction.

4.4 Expression of Thai rosewood β-glucosidase enzyme in *Pichia pastoris*

Another eukaryotic yeast is the methylotrophic *Pichia pastoris*. *P.pastoris* has been developed to be an outstanding host for the production of foreign proteins since its alcohol oxidase promoter was isolated and cloned (Cregg *et al*., 1987). Its transformation was first reported in 1985 (Cregg *et al*., 1987; Brierley *et al*., 1992). *P.pastoris* is suitable for foreign protein expression for three reasons, it can be easily manipulated by molecular genetic manipulation, and it can express protein at high levels and can perform many higher eukaryotic modifications (Cregg *et al*., 2000). Because of these characteristics some proteins that cannot be expressed efficiently in bacteria or *S.cerevisiae* have been successfully produced in *P.pastoris* (Lin Cereghino *et al*., 2001). For example, since most of *Arabidopsis* GH Family 1 enzymes were glycosylated, *P.pastoris* was chosen as recombinant host for protein expression of this family of proteins (Xu *et al*., 2004).

P. pastoris can utilize methanol as a carbon source in the absence of glucose (Ogata *et al*., 1969). The *P.pastoris* expression system uses the methanol-induced alcohol oxidase (*AOX1*) promoter, which controls the gene that codes for alcohol oxidase I, the enzyme which catalyzes the first step in the metabolism of methanol. This promoter has been characterized and incorporated into a series of *P.pastoris* expression vectors. Since the proteins produced in *P.pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E.coli* expression systems. A number of proteins have been produced using this system, including a tetanus toxin fragment, *Bordatella pertussis* pertactin, human serum albumin and lysozyme (Chen *et al*., 1996; Clare *et al*., 1991; Cregg *et al*., 1993; Digan *et al*., 1989; Tschopp *et al*., 1987).

GS115 (*his4*) is the most commonly used *P.pastoris* strain for transformation and subsequent protein expression studies. Transformation of strain with vectors bearing the wild-type *HIS4* and partial *AOX1* gene allows one to integrate plasmid within the chromosomal *HIS4* gene or *AOX1* locus.

pPICZαB plasmid was used as the expression vector, because it carried the *S.cereviseae* alpha-factor prepropeptide, which allows secretion of recombinant protein into culture medium, a zeocin resistance gene for selection, and a multiple cloning site followed by a myc epitope-6 x histidine fusion tag.

The recombinant Thai rosewood β-glucosidase expressed in *E.coli* and *S.cerevisiae* had low activity both for β-glucosidase and β-fucosidase. Therefore, to develop the optimized recombinant expression system for Thai rosewood β-glucosidase, *P.pastoris* was selected, since it had previously been shown to produce active protein, though the expressed protein was difficult to purify.

The isolation of the recombinant Thai rosewood β-glucosidase enzyme from *P.pastoris* medium was important in order to characterize the catalytic activity of enzyme. A secreted protein follows a specific pathway out of the cell, to which the protein was targeted by the yeast α-factor prepro sequence in pPICZαB plasmid. The constructed plasmids include the addition of a His-tag after the prepro peptide sequence or at the C-terminus. In this case, the secreted Thai rosewood β-glucosidase could be purified by using a metal affinity column to specifically bind the protein from the culture media. All of Thai rosewood β-glucosidase enzymes from the various expression systems were subjected to immobilized metal affinity chromatography (IMAC).

4.4.1 Expression of pPICZα-Thrombin-PYG (R519W) construction

Previous results had shown that Thai rosewood β-glucosidase expressed from pPICZα with a C-terminal His-tag could not be purified by IMAC. pPICZα-Thrombin-PYG (R519W) was constructed with a mutation of the arginine at residue 519 to tryptophan to prevent the proteolysis of the C-terminus at the dibasic Kex2 cleavage site between the rest of the recombinant protein and the 6 x histidine tag. The expectation was to produce the mature protein with a Thrombin cleavage site, followed by a c-myc tag and C-terminal 6 x histidine tag for IMAC purification.

pPICZαB-Thrombin-PYG (R519W) clone no. 14 had the highest β-glucosidase and β-fucosidase activity of the 14 clones tested. When its culture medium was subjected to IMAC, most of the activity was in the flow-through fraction. This suggested that the 6 x histidine tag at the C-terminus might not be

suitable for tagging protein for purification. The recombinant protein might be degraded at a position close to the C-terminal 6 x histidine tag. This may have led to the β-glucosidase and β-fucosidase activities being found mostly in the flow-through fraction. The pPICZαB-Thrombin-PYG (R519W) construct gave protein with high activity, but it could not be purified by IMAC on the HiTrap column. Since the 6 x histidine tag at the C-terminus might be susceptible to release by proteolysis during culture, the 6 x histidine tag was introduced to the N-terminus in further studies.

4.4.2 Expression of pPICZαBNH-PYG (R519W) and pPICZαBNH-PCR (R519W) constructs

The pPICZαBNH-PYG (R519W) construct was intended to produce the Thai rosewood β-glucosidase with an N-terminal 6 x histidine tag, followed by an enterokinase site for removal of the tag after IMAC purification. The pPICZαBNH-PCR (R519W) construct was similarly intended to produce the Thai rosewood β-glucosidase with an N-terminal 6 x histidine tag, followed by an enterokinase site and a C-terminal 6 x histidine tag, as well. The culture media from both had β-glucosidase and β-fucosidase activities. When the culture media were subjected to IMAC, all β-glucosidase and β-fucosidase activities were found in the flow-through fraction. These results indicated that a 6 x histidine tag might not have enough affinity to bind IMAC on the HiTrap column, or there was another protease susceptible site on N-terminus of the Thai rosewood β-glucosidase protein or both.

4.4.3 Expression of Protein from the pPICZαB-Truncated R488 construct

The pPICZαB-Truncated R488 construct was produced to remove the possible proteolysis susceptible site(s) from the C-terminus of the Thai rosewood β-glucosidase protein, and see if the protein was still active. The pPICZαB-Truncated R488 construct would produce Thai rosewood β-glucosidase protein truncated at arginine residue 488 followed by c-myc and 6 x histidine tags.

All 40 colonies containing pPICZαB- Truncated R488 had low activity. From the clone with highest activity, some recombinant protein could be purified by IMAC on the HiTrap column.

All fractions from the HiTrap column were analyzed by SDS-PAGE and some purified truncated protein with an apparent molecular weight below 66 kD was seen in Figure 3.29. The C-terminal 6 x histidine tag and truncated protein may have some protease susceptible amino acid sequences which allowed some protein degradation, resulting in a low amount to binding with the HiTrap column. The lower amount of β-fucosidase activity of these constructs might result from the truncation of the recombinant protein, or might indicate that much of the activity came from native *P.pastoris* β-glucosidase, rather than from Thai rosewood β-glucosidase.

4.4.4 Expression of β-glucosidase from the pPICZαBNH8-Truncated N construction

There was evidence that the recombinant protein had low binding to IMAC even though the 6 x histidine tag was moved to the N-terminus. The N-terminal sequence of the expressed protein, which was purified by traditional

chromatographic methods, began at glutamate residue 13. These results indicated that there might be a protease susceptible sequence at the N-terminus. Therefore, the pPICZαBNH8-Truncated N plasmid was constructed. A forward primer with a *Pst*I site and Thai rosewood β-glucosidase sequence starting at the valine residue 14 codon was designed. The protein produced from the pPICZαBNH8-Truncated N construct should have an N-terminal 8 x histidine tag and an N-terminus truncated at valine residue 14 of Thai rosewood β-glucosidase, as shown in Figures 3.35 and 3.36. The pPICZαBNH8-Truncated N construct produced β-glucosidase with high activity. The recombinant protein could be purified by IMAC on HiTrap (immobilized nickel) column and BD Talon™ (immobilized cobalt) metal affinity columns. All fractions from IMAC columns were analyzed by SDS-PAGE, but the purified truncated protein could only be seen after the fractions were concentrated. Around 75% of recombinant protein produced from pPICZαBNH8-Truncated N could be bound to IMAC. The fact that some protein did not bind indicated that some recombinant protein might still be degraded between the N-terminal 8 x histidine tag and N-terminal part of truncated Thai rosewood β-glucosidase protein. The other point was GS115 *P.pastoris* is common host for *Pichia* expression, but is not protease deficient. To avoid proteolysis, a protease deficient strains could be used as host, such as SMD1168, which is useful for susceptible recombinant proteins.

Following the transformation of *P.pastoris* with the appropriate vector encoding the recombinant gene of interest, multiple clones were screened for relative recombinant protein expression in shake flask culture. Shake flask growth conditions can dramatically affect the expression or stability of the specific protein under investigation (Rosenfeld, 1999). Medium, pH and induction methodologies influence

the expression level of a specific recombinant protein. Shakers can only run in a batch mode, meaning that the growth of the cells is limited by the nutrients present in the medium at the time of inoculation. One advantage for high level expression of recombinant protein using *P.pastoris* is the ability to perform high cell density fermentation (Rosenfeld, 1999). Careful control of medium, dissolved oxygen, pH, temperature, and induction lead to exceptionally high density fermentation (450 g wet cell weight liter). Researchers at New Brunswick Scientific (Edison, NJ, USA) found that by switching from a shaker to a fermentor, protein production in *Pichia* could be increased by over 140% (Chen *et al*., 1996). The ability of *P.pastoris* to secrete large amounts of protein to the culture medium is supported by the secretion of human serum albumin (HSA) at 4 g/L (Barr *et al*., 1992) and tumor necrosis factor α (TNFα) at 10 g/L (Sreekrishna *et al*., 1988 and Sreekrishna *et al*., 1989). The fermentor's fed-batch mode can control methanol flow rates and provide addition of just enough methanol for protein synthesis, while preventing excess methanol addition, which can cause toxicity (Chen *et al*., 1996). For this project, a limited number of clones were screened and the shake flask culture was used, which limited the amount of culture and the level of protein production. Therefore, protein production was less, and the protein was hard to see on SDS-PAGE without concentration. The pPICZαBNH8-Truncated N construct in GS115 should be cultured in a fermentor for fed-batch expression, which should be able to produce more recombinant protein, and screening of more colonies might allow identification of higher producing clones.

After recombinant protein production, subsequent purification of recombinant protein was considered to produce an easy and fast single step purification. An affinity-fusion system was used as the recombinant protein

purification strategy. A powerful purification method involves the use of peptide affinity tags, which are fused to the protein of interest and used for affinity chromatography. Immobilized metal affinity chromatography (IMAC) is used to purify polyhistidine residues. IMAC is based on the interactions between a transition metal ion (Co^{2+}, Cu^{2+}, Ni^{2+}, or Zn^{2+}) immobilized on a matrix and specific amino acid side chains. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC column matrices. Following washing of the matrix material, the peptide containing the polyhistidine sequences can be easily eluted by either adjusting the pH of the column buffer or adding free imidazole to the column buffer (Bornhorst and Falke, 2000). Polyhistidine tags were utilized to purification of recombinant protein from bacterial and yeast expression systems.

Six polyhistidine residues are commonly used in IMAC. Whereas tags of six histidine residues are long enough to interact with IMAC, either shorter or longer affinity tags have been used successfully (Bornhorst and Falke, 2000). Use of the smallest number of histidine residues needed for efficient purification has been advised to minimize possible perturbation of protein function. Polyhistidine tags are commonly placed on either the N or C-terminus of recombinant proteins. Optimal placement of the tags is protein specific. To optimize recombinant protein expression and purification and ensure that polyhistidine tags did not affect protein activity and folding, the recombinant Thai rosewood β-glucosidase was fused with polyhistidine tags, either C-terminal or N-terminal 6 x histidine tag or 8 x histidine tag. The

conclusion of all constructs of recombinant Thai rosewood β-glucosidase in pPICZαB is shown in Figure 4.1. The optimal construct encoded a mature Thai rosewood β-glucosidase with its N-terminus truncated at valine residue 14 linked to an 8 x histidine tag at the N-terminus in the pPICZαB plasmid.

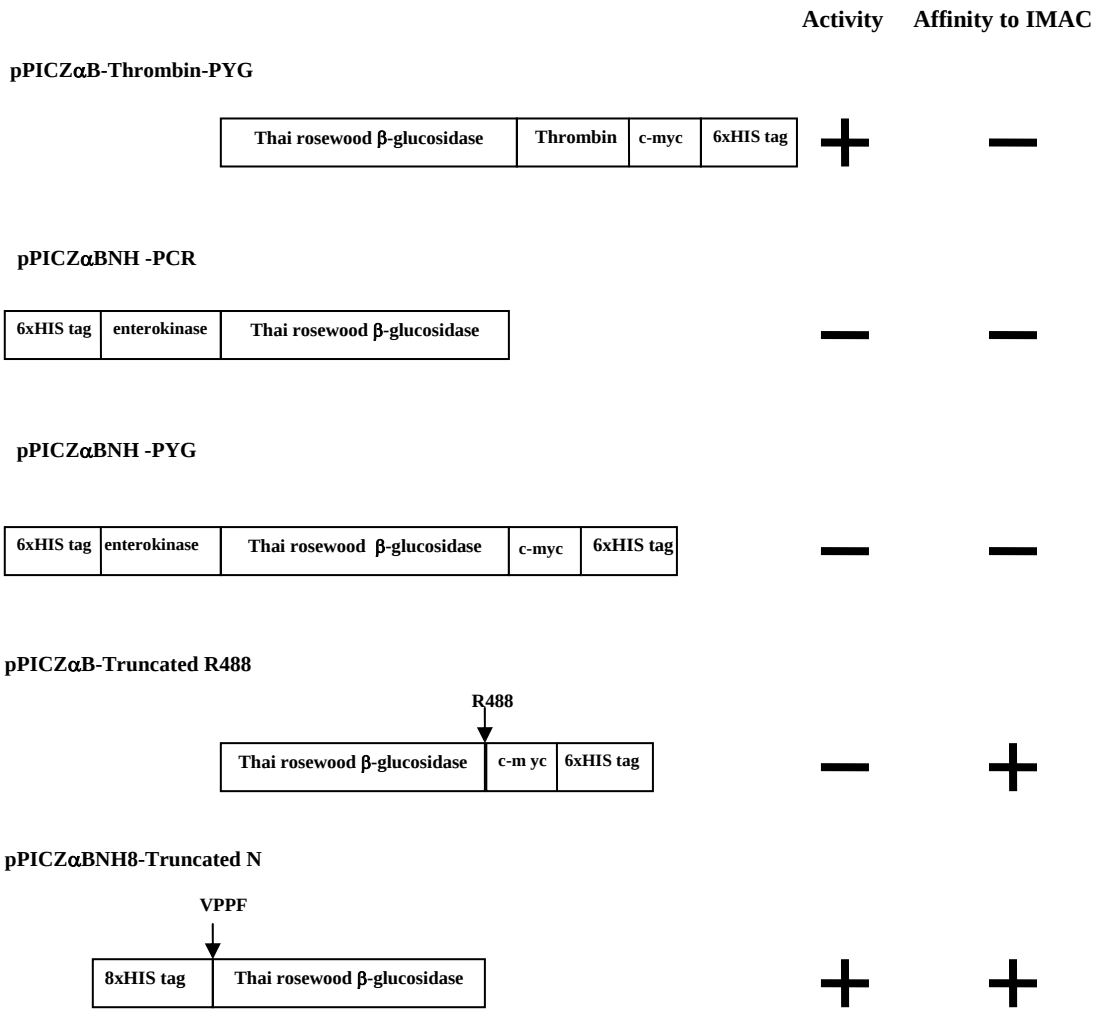


Figure 4.1 Conclusion of all constructs for expression of Thai rosewood β-glucosidase from cDNA in *Pichia*. The maps of the expected mature proteins are shown, Thrombin represents a thrombin cleavage site.

4.5 Conditions for Thai rosewood β-glucosidase enzyme expression in *Pichia pastoris*

4.5.1 Optimal Temperature and percent of casamino acid for Thai rosewood β-glucosidase enzyme expression in *P. pastoris*

Clare *et al*. (1995b) found that the expression level of mouse epidermal growth factor was increased in BMMY with 1% casamino acid and buffering of the medium at pH 6.0, at which pH extracellular proteases were inhibited.

The effect of temperature and percent of casamino acid were determined. The suitable condition for expression recombinant Thai rosewood β-glucosidase expression was at 30°C in BMMY with 1% casamino acid for 5 days.

4.5.2 The appropriate of methanol concentration for *Pichia* expression of β-glucosidase

P.pastoris is a methylotropic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. This reaction generates hydrogen peroxide and takes place within the peroxisome. The majority of alcohol oxidase activity is the product of the *AOX1* gene. The expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically ≥ 30% of total soluble protein in cells grown with methanol (Ellis *et al*., 1985; Koutz *et al*., 1989; Tschopp *et al*., 1987a). For this reason, the percent of methanol was varied for recombinant protein expression at 30°C for three days of induction, with samples analyzed each day.

The suitable methanol concentration for Thai rosewood β-glucosidase enzyme expression in *P.pastoris* was 1% methanol in BMMY with 1% casamino acid. A higher percent of methanol did not raise the recombinant protein production, possibly due to toxicity effects.

4.6 pH and Temperature optimum of recombinant Thai rosewood β-glucosidase enzyme from the pPICZαBNH8-Truncated N construction

The pH was varied to examine the effect of pH on recombinant protein activity. The optimum pH of recombinant Thai rosewood β-glucosidase from *P.pastoris* was 5.0 for both β-glucosidase and β-fucosidase activity. The optimum pH of recombinant Thai rosewood β-glucosidase from *P.pastoris* was the same as native enzyme from seeds.

Temperature was varied to examine the effect of temperature on recombinant protein activity. The optimum temperature was 60°C. The optimum temperature of recombinant Thai rosewood β-glucosidase from *P.pastoris* was also close to that of native enzyme from seeds.

The recombinant enzyme was stable at temperature up to 60°C. At 30°C to 50°C, recombinant enzyme was stable for at least 1 h. The thermostability of recombinant enzyme was useful for applications like reverse-hydrolysis and transglycosylation (Goyal *et al*., 2002).

4.7 Characterization of purified Thai rosewood β-glucosidase enzyme from *Pichia pastoris*

Both Thai rosewood β-glucosidase purified from seed and purified recombinant enzyme were separated on native PAGE and activity stained with 1 mM 4-methylumbelliferyl-β-glucopyranoside. With the β-mercaptoethanol, both proteins migrated at the same position. On the other hand, without the β-mercaptoethanol, Thai rosewood β-glucosidase purified from seed appeared to form aggregates on the 7% native gel and migrate slower than the recombinant enzyme. Without β-mercaptoethanol, the migration of the recombinant enzyme was faster than Thai rosewood β-glucosidase purified from seed. This might be because the recombinant enzyme was a monomer which could migrate faster while the enzyme purified from seed formed tetramers as previously reported (Srisomsap *et al*., 1996) or higher level aggregates. β-mercaptoethanol is reducing agent which cleaves disulfide bonds. Besides cleavage of disulfide bonds, which could affect the quaternary structure, there might also be β-mercaptoethanol covalently bound to the Thai rosewood β-glucosidase purified from seed and the recombinant enzyme purified from *P.pastoris*.

The isolation of the recombinant Thai rosewood β-glucosidase enzyme *P.pastoris* is important in order to characterize the catalytic activity of the enzyme. Since, isolation and purification of Thai rosewood β-glucosidase enzyme from *P.pastoris* was successful using IMAC, the catalytic properties could be evaluated. The enzymatic properties of recombinant protein were close to the native enzyme purified from seeds. The K_m values of the recombinant enzyme were quite close to the K_m values of native enzyme with *p*NP-glucoside and *p*NP-fucoside. The k_{cat}

values of recombinant enzyme were 206 s^{-1} and 156 s^{-1} for *p*NP-glucoside and *p*NP-fucoside, respectively. The k_{cat} values of recombinant enzyme were lower for *p*NP-glucoside, compared to native enzyme (307 s^{-1}). The K_m and k_{cat} values for the recombinant enzyme were slightly lower for the *p*NP-fucoside than *p*NP-glucoside, which is similar to native enzyme purified from seed (Srisomsap *et al*., 1996). The k_{cat}/ K_m values of recombinant enzyme were 40,600 $s^{-1}M^{-1}$ and 282,000 $s^{-1}M^{-1}$ for *p*NP-glucoside and *p*NP-fucoside, respectively. The k_{cat}/ K_m values of recombinant enzyme were lower compared to native enzyme (57,300 and 285,100 $s^{-1}M^{-1}$, respectively). The k_{cat}/ K_m value of the recombinant enzyme for *p*NP-fucoside were closed to the k_{cat}/ K_m value of native enzyme. The small difference in enzymatic properties might be due to the difference of recombinant Thai rosewood β-glucosidase amino acid composition. The recombinant protein contained an N-terminal 8 x histidine and Thai rosewood β-glucosidase with its N-terminus truncated at valine residue 14. The N-terminus 8 x histidine tag and truncation might affect the enzymatic activity, but its effect is relatively small, since the properties of native and truncated proteins are quite similar.

Thai rosewood β-glucosidase was successfully expressed in *P.pastoris* in active form. The recombinant Thai rosewood β-glucosidase protein included an N-terminal 8 x histidine tag and the mature protein with an N-terminal truncation at valine residue 14. The truncated N-terminal Thai rosewood β-glucosidase could be purified by IMAC and its enzymatic properties were characterized. The recombinant enzyme had enzymatic properties similar to native enzyme purified from seed.

CHAPTER V

CONCLUSION

To examine the appropriate recombinant expression system, the Thai rosewood β-glucosidase cDNA was cloned into expression vectors and expressed as heterologous protein in *E.coli*, *S.cerevisiae* and *P.pastoris*. In the *E.coli* expression system, pET-32a(+) was used as the expression vector and expression done in BL21(DE3) and AD494(DE3) *E.coli*. In the *S.cerevisiae* expression system, pYES2 was used as the expression vector and expression was done in *S.cerevisiae* strain BJ5462. In the *P.pastoris* expression system, pPICZαB and GS115 *P.pastoris* were used. When the level of expression was compared, then pPICZαB expression vector with GS115 *P.pastoris* was the most suitable system to use for production of recombinant Thai rosewood β-glucosidase, as it gave the highest β-glucosidase and β-fucosidase activities. Therefore, the pPICZαB expression vector with GS115 *P.pastoris* was improved to increase activity and ease of purification with IMAC.

The pPICZαB-Thrombin plasmid was first constructed with a C-terminal thrombin recognition site and 6 x histidine tag for purification with IMAC, followed by removal of the 6 x histidine tag with thrombin. The recombinant Thai rosewood β-glucosidase could be produced from the construct named pPICZαB-Thrombin-PYG (C-terminal 6x histidine tag), but could not be purified by IMAC. This might be due to a proteolytic site at C-terminal of recombinant protein. Mutation of Kex2 protease

site near the C-terminus did not allow IMAC purification. Thai rosewood β-glucosidase cDNA with a truncated C-terminus at R488 and fused to a C-terminal tag was constructed in pPICZαB to produced the construct named pPICZαB-Truncated R488. The recombinant protein produced from pPICZαB-Truncated R488 had little activity but bound to the HiTrap (immobilized nickel) column and the purified recombinant protein was seen on a coomassie blue stained SDS-PAGE gel. Then, the 6 x histidine tag was added at the N-terminus of Thai rosewood β-glucosidase. The recombinant Thai rosewood β-glucosidase could be produced from this construct named pPICZαBNH-PCR (N-terminal 6 x histidine tag), but could not be purified by IMAC. This might be due to a proteolytic site at the N-terminus of the recombinant protein. The insert from pPICZαB-Thrombin-PYG (R519W) was ligated into pPICZαBNH to produce the construct named pPICZαBNH-PYG (N- and C-terminal 6 x histidine tags). The recombinant Thai rosewood β-glucosidase could not be purified by IMAC, even though it contained both N-terminal and C-terminal 6 x histidine tags.

The final plasmid used, pPICZαBNH8, has an N-terminal 8 x histidine tag put into the pPICZαB plasmid after the yeast prepro-α sequence. The Thai rosewood β-glucosidase cDNA encoding the protein truncated at its N-terminus at valine position 14 was put into pPICZαBNH8 after the tag to produce the construct named pPICZαBNH8-Truncated N. The recombinant protein produced from pPICZαBNH8-Truncated N construct was active and bound to both HiTrap (immobilized nickel) and BD Talon™ (immobilized cobalt) metal affinity resin column. The purified recombinant protein could be seen on a coomassie blue stained SDS-PAGE gel after it

was concentrated by centrifugal filtration. An immunoblot of Thai rosewood β-glucosidase purified from seed and concentrated recombinant enzyme indicated that both proteins could react with polyclonal anti- Thai rosewood β-glucosidase antibody. The recombinant enzyme showed K_m values for *p*NP-glucoside and *p*NP-fucoside (5.07 and 0.55 mM, respectively) similar to the enzyme purified from seeds (5.37 and 0.54 mM, respectively). The recombinant enzyme also showed k_{cat}/ K_m (40,600 $s^{-1}M^{-1}$ and 282,000 $s^{-1}M^{-1}$, respectively) for *p*NP-glucoside and *p*NP-fucoside similar to the enzyme purified from seeds (57,300 and 285,100 $s^{-1}M^{-1}$).

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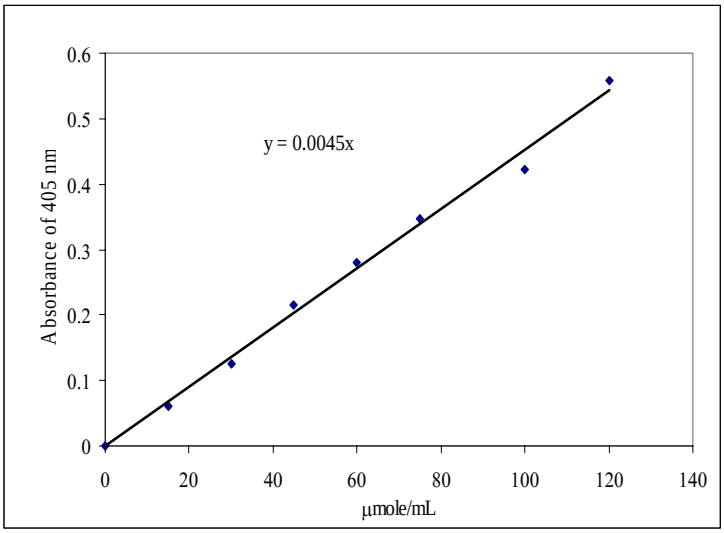
APPENDICES

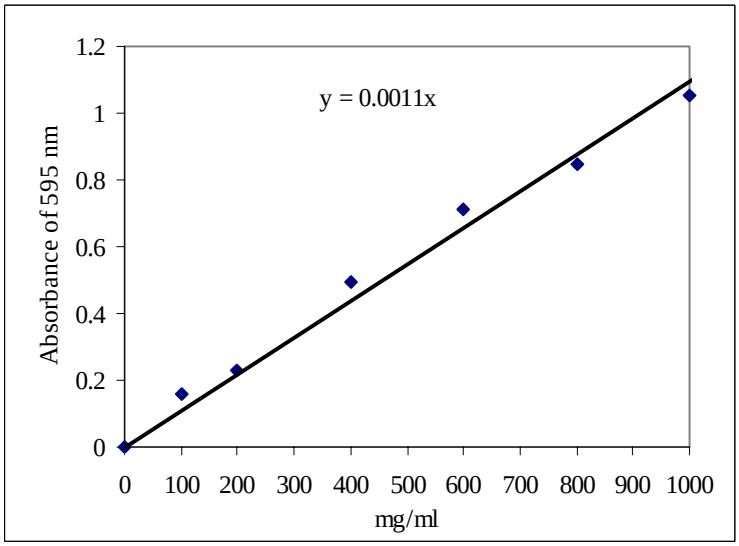
APPENDIX A

STANDARD CURVES

1. Standard curve of *p*-nitrophenol



2. Stand curve of BSA by Bio-Rad Protein Assay

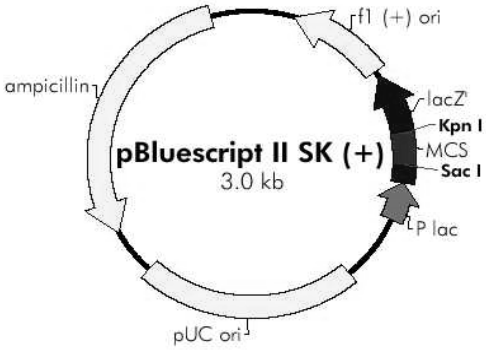


APPENDIX B

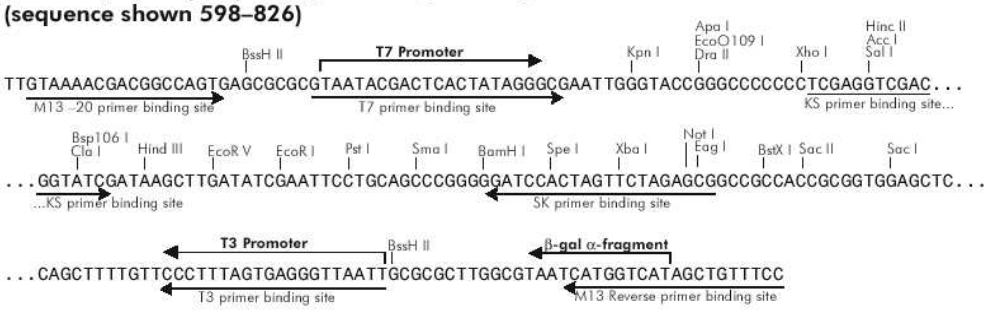
PLASMID MAPS

1. pBlueScript II SK(+)

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833

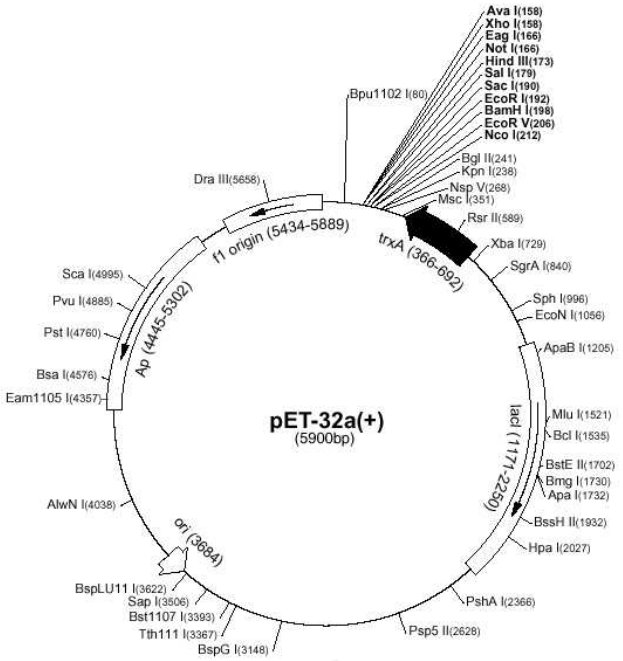


pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



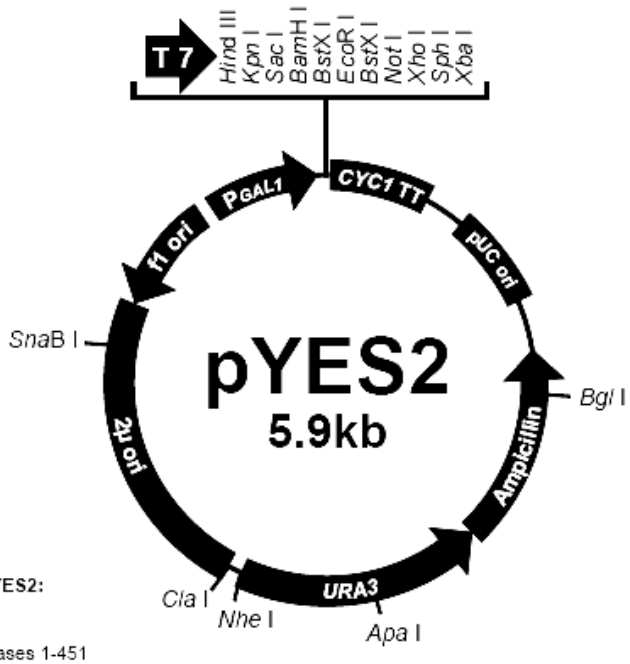
2. pET-32a(+) Vector

The pET-32a(+) vector is designed for cloning and high-level expression of peptide sequences fused with the 109 amino acids of thioredoxin protein (Trx·Tag). Cloning sites are available for producing fusion proteins also containing cleavable His6·Tag and S·Tag sequences for detection and purification. The sequence is numbered by the pBR322 convention, so the T7 expression region is reserved on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.



3. pYES2 vector

The pYES2 vector is designed for optimal gene expression in *Saccharomyces cerevisiae*. pYES2 vector carries the promoter and enhancer sequences from the *GAL1* gene for regulated expression. It contains the *URA3* gene for selection in yeast and 2μ origin for high-copy maintenance.

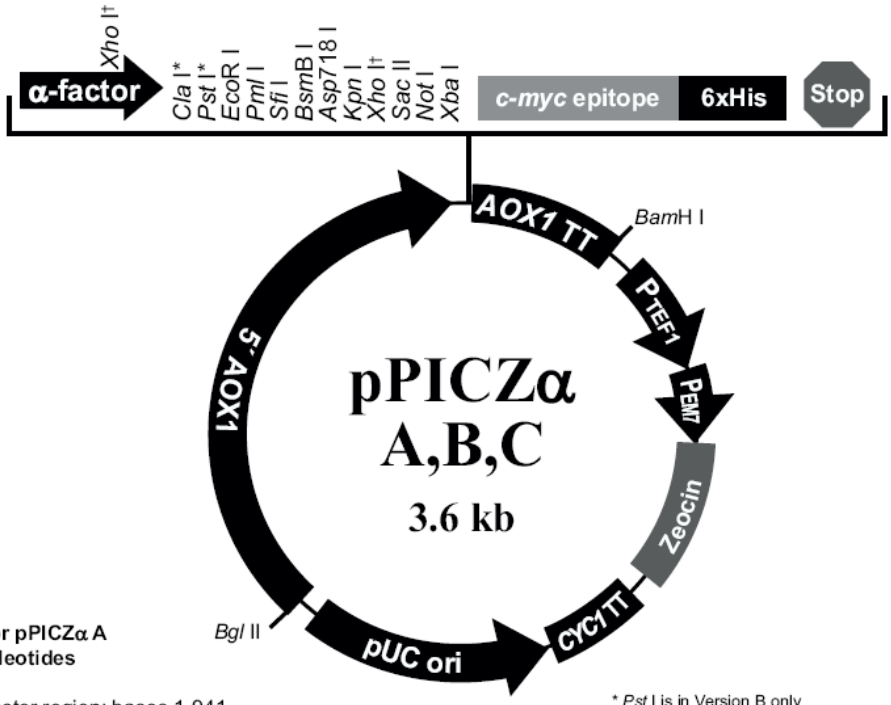


Comments for pYES2:
5856 nucleotides

GAL1 promoter: bases 1-451
T7 promoter/priming site: bases 475-494
Multiple cloning site: bases 501-600
CYC1 transcription terminator: bases 608-856
pUC origin: bases 1038-1711
Ampicillin resistance gene: bases 1856-2716 (C)
URA3 gene: bases 2734-3841 (C)
2 micron (μ) origin: bases 3845-5316
f1 origin: bases 5384-5839 (C)
(C) = complementary strand

4. pPICZαB vector

The pPICZαB vector is designed to express secreted fusion protein, which may also contain C-terminal c-myc and histidine tags which can purified by metal affinity resin. This plasmid contains alcohol oxidase promoter (AOX1) which utilizes methanol. Native *Saccharomyces cerevisiae* α-factor secretion signal allows for efficient secretion of most proteins from *Pichia*.



Comments for pPICZα A
3593 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
α-factor priming site: bases 1144-1164
Multiple cloning site: bases 1208-1276
c-myc epitope: bases 1275-1304
Polyhistidine (6xHis) tag: bases 1320-1337
3´ *AOX1* priming site: bases 1423-1443
AOX1 transcription termination region: bases 1341-1682
TEF1 promoter: bases 1683-2093
EM7 promoter: bases 2095-2162
Sh ble ORF: bases 2163-2537
CYC1 transcription termination region: bases 2538-2855
pUC origin: bases 2866-3539 (complementary strand)

* *Pst* I is in Version B only
Cla I is in Version C only

†The two *Xho* I sites in the vector allow the user to clone their gene in frame with the Kex2 cleavage site, resulting in expression of their native gene without additional amino acids at the N-terminus.

APPENDIX C

SOLUTION PREPARATION

1. Reagents for bacterial culture and competent cell transformation

1.1 LB broth containing antibiotics (1 L)

Dissolve 10 g Tryptone, 5 g Yeast Extract and 5 g NaCl in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Autoclave the solution at 121°C for 15 min. Allow the medium to cool to 50°C before adding antibiotics with concentration recommended for each cloning system and store at 4°C.

1.2 LB plate with 100 μg/mL of ampicillin (1 L)

Dissolve 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g agar in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Sterilize by autoclaving at 121°C for 20 min. Allow the medium to cool to 50°C, then add ampicillin to a final concentration 100 μg/mL. Pour medium into Petri-dishes. Allow the agar to harden, and keep at 4°C.

1.3 LB agar plate with 100 μg/mL of ampicillin, 15 μg/mL kanamycin, 12.5 μg/mL tetracyclin (1 L)

Dissolve 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g agar in 800

mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Sterilize by autoclaving at 121°C for 20 min. Allow the medium to cool to 50°C, then add ampicillin to a final concentration 50 μg/mL, kanamycin to 15 μg/mL, and tetracycline to 12.5 μg/mL. Pour medium into Petri-dishes, allow the agar to harden, and keep at 4°C.

1.4 LB plate with 100 μg/mL of ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above. Then spread 50 μL of 100 mM IPTG and 25 μL of 50 mg/mL X-Gal over the surface of the plates and allow to absorb for 10 min before use.

1.5 LB plate with 25 μg/mL of zeocin

Dissolve 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g agar in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Sterilize by autoclaving at 121°C for 20 min. Allow the medium to cool to 50°C, then add zeocin to 25 μg/mL. Pour medium into Petri-dishes. Allow the agar to harden, and keep at 4°C

1.6 Antibiotics solution stock

Ampicillin (50 mg/mL): dissolve 50 mg ampicillin in 1 mL sterile distilled water.

Kanamycin (30 mg/mL): dissolve 30 mg kanamycin in 1 mL sterile distilled water.

Tetracyclin (12.5 mg/mL): dissolve 12.5 mg tetracyclin in 1 mL absolute alcohol.

Filter sterile all antibiotic solutions and keep at -20°C.

1.7 IPTG stock solution (100 mM)

Dissolve 0.12 g IPTG (isopropyl thio-β-D-galactoside) in distilled water and adjust to 5 mL final volume. Sterilize by filter sterilize and store at -20°C.

1.8 X-gal stock solution

Dissolve 50 mg X-gal in DMF and store in the dark bottle at -20°C.

2. Reagent for competent cell preparation

2.1 $CaCl_2$ Solution (60 mM $CaCl_2$, 10 mM PIPES pH 7.0, 15% glycerol)

To prepare 100 mL solution, mix the stock solutions as follows:

- 6 mL of 1 M $CaCl_2$ (14.7 g/100 mL, filter sterile)
- 10 mL of 100 mM PIPES (piperazine-1,4-bis(2-ethanesulfonic acid), pH 7.0, 3.02 g/100 mL adjust pH with KOH, filter sterile)
- 15 mL of 100% glycerol (autoclave at 121°C, 15 min)

Add sterile distilled water to bring a volume up to 100 mL. Store the solution at 4°C.

2.2 SOC media (1 L)

Dissolve 20 g Tryptone, 5 g Yeast extract, 10 mL 1 M NaCl (5.85 g/100 mL), and 2.5 mL 1 M KCl (7.44 g/100 mL) in distilled water and make to 980 mL final volume. Sterilize by autoclaving at 121°C for 20 min. Allow the medium cool

to room temperature, then add 5 mL of 1 M $MgCl_2 \cdot 6H_2O$ (20.33 g/100 mL), 5 mL of 1 M $MgSO_4 \cdot 7\ H_2O$ (12.30 g/100 mL), and 10 mL of 2 M of glucose (36 g/100 mL) which were filter sterilized. Store the solution at 4°C.

3. Reagents for isolation plasmid DNA (boiling prep)

3.1 STET (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 5% Triton X-100) (100 mL)

Mix 20 mL of 1 M NaCl (sterile), 2 mL of 1 M Tris-HCl, pH 8.0 (sterile), 0.4 mL of 0.5 M EDTA pH 8.0 (sterile), and 5 mL Triton X-100 and adjust the volume to 100 mL with sterile distilled water. Store at room temperature.

3.2 3 M Sodium acetate pH 4.8 (100 mL)

Dissolve 24.6 g sodium acetate in 80 mL distilled water. Adjust pH to 4.8 with glacial acetic acid and the volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

3.3 RNase A (10 mg/mL)

Dissolve 10 mg RNase A in 10 mM Tris-HCl pH 7.4, 15 mM NaCl buffer (sterile). Store at -20°C.

3.4 1 M Tris-HCl pH 7.4 and pH 8.0 (100 mL)

Dissolve 12.11 g of Tris Base in 80 mL distilled water. Adjust pH with HCl to pH 7.4 or 8.0, and then adjust the volume to 100 mL with distilled water and autoclave at 121°C for 20 min. Store at room temperature.

3.5 0.5 M EDTA (pH 8.0) (100 mL)

Dissolve 18.61 g EDTA (disodium ethylene diamine tetraacetate $\cdot 2H_2O$) in 70 mL distilled water. Adjust the pH to 8.0 with NaOH (about 20 g) and the volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

4. Reagents for isolation plasmid DNA (alkaline lysis)

4.1 Resuspension buffer (100 mL)

Mix 5 mL 1 M Tris HCl (pH 8.0) with 0.5 M EDTA (pH 8.0) 2 mL and distilled water up to 90 mL. Sterilize by autoclaving at 121°C for 20 min, then add 1 M glucose 5 mL. Store at 4°C.

4.2 1%SDS/ 0.2 N NaOH

Dissolve SDS 1.6 g in distilled water up to 40 mL as 10% SDS solution.

Dissolve NaOH 4 g in distilled water up to 100 ml as 1 N NaOH.

Mix 10 mL 10% SDS and 1 N NaOH 20 mL add water up to 100 mL. Store at room temperature.

4.3 3 M Sodium acetate, pH 4.8 (100 mL)

Dissolve 24.6 g sodium acetate in 80 mL distilled water. Adjust the pH to 4.8 with glacial acetic acid and the volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

4.4 TE saturated phenol (pH 8.0)

Mix 300 mL TE (pH 8.0), 300 mL melted phenol at 50°C, and 4.5 g 8-hydroxyquinoline. Incubate at 4°C overnight. Remove supernatant. Store at 4°C.

4.5 20% PEG6000/ 2.5 M NaCl

Dissolve 20 g Polyethylene Glycol (PEG) 6000 and 14.61 g NaCl in 80 mL distilled water. Adjust the volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min. Store at 4°C.

5. Reagent for agarose gel electrophoresis

5.1 50 X TAE for agarose gel electrophoresis (1 L)

Mix 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) and adjust the volume to 1 L with distilled water. Store at room temperature.

5.2 6 X DNA loading dye (10 mL)

Mix 0.025 g Bromophenol blue, 0.025 g xylene cyanol and 3 mL of 100% glycerol in distilled water to a 10 mL final volume and store at 4°C.

6. Solutions for protein electrophoresis

6.1 SDS-gel loading buffer (5 X stock) (2.5 M Tris, 10% SDS, 0.5% Bromophenol blue, 50% glycerol)

Dissolve 0.30 g Tris Base, 1 g SDS, 0.05 g Bromophenol blue, 5 mL glycerol and adjust the pH to 6.8 with HCl and the volume to 8 mL with distilled water. Before use, add 20 μL of β-mercapthoethanol to 80 μL of solution mixture. Store at room temperature.

6.2 1.5 M Tris pH 8.8 (100 mL)

Dissolve 18.17 g Tris base in 80 mL distilled water. Adjust pH to 8.0 with HCl and the volume to 100 mL with distilled water. Store at 4°C.

6.3 0.5 M Tris pH 6.8 (100 mL)

Dissolve 6.06 g Tris base in 80 mL distilled water. Adjust the pH to 6.8 with HCl and the volume to 100 mL with distilled water. Store at 4°C.

6.4 30% Acrylamide solution (100 mL)

Dissolve 29 g acrylamide and 1 g *N, N′*-methylene-bis-acrylamide in distilled water to a volume of 100 mL. Mix the solution by stirring for 1 h to be homogenious and filter through Whatman membrane No. 1. Store in the dark bottle at 4°C.

6.5 Tris-Glycine electrode buffer (5 X stock) (1 L)

Dissolve 30.29 g Tris Base, 144 g glycine, 5 g SDS in distilled water. Adjust pH to 8.3 with HCl and the volume to 1 L with distilled water.

6.6 Staining solution with Coomassie brillant blue for protein

Mix 1 g Coomassie brilliant blue R-250, 400 mL methanol, 500 mL distilled water and 100 mL glacial acetic acid and filter through a Whatman No. 1 paper.

6.7 Destaining solution for Coomassie Stain

Mix 400 mL methanol, 100 mL glacial acetic acid, and add distilled water to a final volume of 1000 mL.

6.8 10% (w/v) Ammonium persulfate (1 mL)

Dissolve 100 mg ammonium persulfate in 1 mL distilled water. Store at -20°C.

6.9 10% Separating gel SDS-PAGE (10 mL)

Mix the solution as follow:

1.5 M Tris (pH 8.8)	2.5 mL
Distilled water	3.3 mL
10% (w/v) SDS	0.1 mL
30% acrylamide solution	3.3 mL
10% (w/v) ammonium persulfate	0.1 mL
TEMED	0.004 mL

6.10 5% Stacking gel SDS-PAGE (5 mL)

Mix the solution as follow:

0.5 M Tris (pH 6.8)	1.26 mL
Distilled water	2.77 mL
10% (w/v) SDS	0.05 mL
30% acrylamide solution	0.83 mL
10% (w/v) ammonium persulfate	0.05 mL
TEMED	0.005 mL

7. Solutions for western blotting

7.1 1 X PBS (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl)

Dissolve 0.216 g KH_2PO_4, 1.192 g Na_2HPO_4, 0.199 g KCl, 8 g NaCl in distilled water and add distilled water to 1 L final volume.

7.2 1 X PBST (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, 0.05% Tween 20)

Dissolve 0.216 g KH_2PO_4, 1.192 g Na_2HPO_4, 0.199 g KCl, 8 g NaCl, 0.5 mL Tween 20 in distilled water and make to 1 L final volume.

7.3 1 X TBS (150 mM NaCl, 5 mM Tris-HCl pH 8.0)

Dissolve 2.42 g Tris base and 29.24 g NaCl in 800 mL distilled water and adjust pH to 7.5 with HCl and make-up to 1 L final volume.

7.4 1 X TBST (150 mM NaCl, 5 mM Tris-HCl pH 8.0 with 0.5% Tween 20)

Dissolve 2.42 g Tris base 29.24 g NaCl and 0.05 mL Tween 20 in 800 mL DW and adjust pH to 7.5 with HCl and make-up to 1 L final volume.

7.5 Western blot buffer (25 mM Tris, 192 mM Glycine, 20% MeOH)

Dissolve 3.03 g Tris and 14.4 g glycine in distilled water up to 800 mL final volume, adding 200 mL methanol before use.

7.6 Fast green staining solution

Dissolve 0.1 g Fast green with 20 mL methanol, 5 mL glacial acetic acid, and 75 mL distilled water.

8. Buffers and Reagents for enzyme assay

8.1 10 mM *p*-Nitrophenol (10 mL)

Dissolve 0.0139 *p*-nitrophenol in 50 mM NaOAc pH 5.0 buffer and bring to 10 mL final volume with the same buffer.

8.2 100 mM NaOAc pH 5.0 buffer (100 mL)

Dissolve 0.82 g NaOAc in 80 mL distilled water, and adjust the pH to 5.0 with glacial acetic acid and the volume to 100 mL with distilled water.

8.3 2 M Na_2CO_3 (100 mL)

Dissolve 2.1 g Na_2CO_3 in distilled water, and adjust the volume to 100 mL with distilled water.

9. Yeast cell culture medium and buffers

9.1 YPD (Yeast extract Peptone Dextrose medium)

Dissolve 10 g Yeast extract, 20 g Peptone and 10 g glucose in 1000 ml of distilled water. For plate, add 20 g agar. Autoclave 110°C, 10 min. Store medium at room temperature or cool the medium and pour plates.

9.2 YPD plate with zeocin

Dissolve 10 g yeast extract, 20 g peptone, 10 g glucose and 20 g bacterial agar in 800 ml of distilled water. Adjust the volume to 1 L with distilled water. Sterilize by autoclaving at 110°C for 10 min. Allow the medium to cool to 50°C, then add zeocin to a final concentration 100 μg/mL. Pour medium into Petri-dishes. Allow

the agar to harden, and keep at 4°C

9.3 Minimal medium (MM) plates

6.7 g Yeast nitrogen base without amino acid, 0.1 g L-Leucine, 0.1 g Tryptophan and 20 g agar were dissolved in to 900 mL distilled water, then added up to 1 L. Autoclave 121°C, 15 min. Cool to 50°C and added 100 ml of filter-sterilized 20% glucose

9.4 Minimal medium (MM) or Uracil drop out medium for yeast expression

6.7 g Yeast nitrogen base without amino acid, 0.1 g L-Leucine, 0.1 g Tryptophan, 0.05 L-Proline and 0.05 g L-Histidine were dissolved in to 900 mL distilled water, then added up to 1 L. Autoclave 121°C, 15 min. Cool to 50°C and added 100 ml of filter-sterilized 20% glucose or galactose and 10% raffinose.

9.5 Solution for yeast transformation by Lithium acetate

9.5.1 Solution I (100 mM Lithium acetate in TE pH 8.0)

Dissolved 1.02 g lithium acetate in TE pH 8.0 and add up to 100 mL.

9.5.2 Solution II (100 mM Lithium acetate, 40% Polyethylene Glycol 4000 in TE pH 8.0)

Dissolved 1.02 g lithium acetate and 40 g polyethylene glycol 4000 in TE pH 8.0 and make-up to 100 mL.

9.5.3 DNA carrier (10 mg/ mL sonicated salmon sperm DNA)

APPENDIX D

DNA SEQUENCING CHROMATOGRAM

5′AOX primer

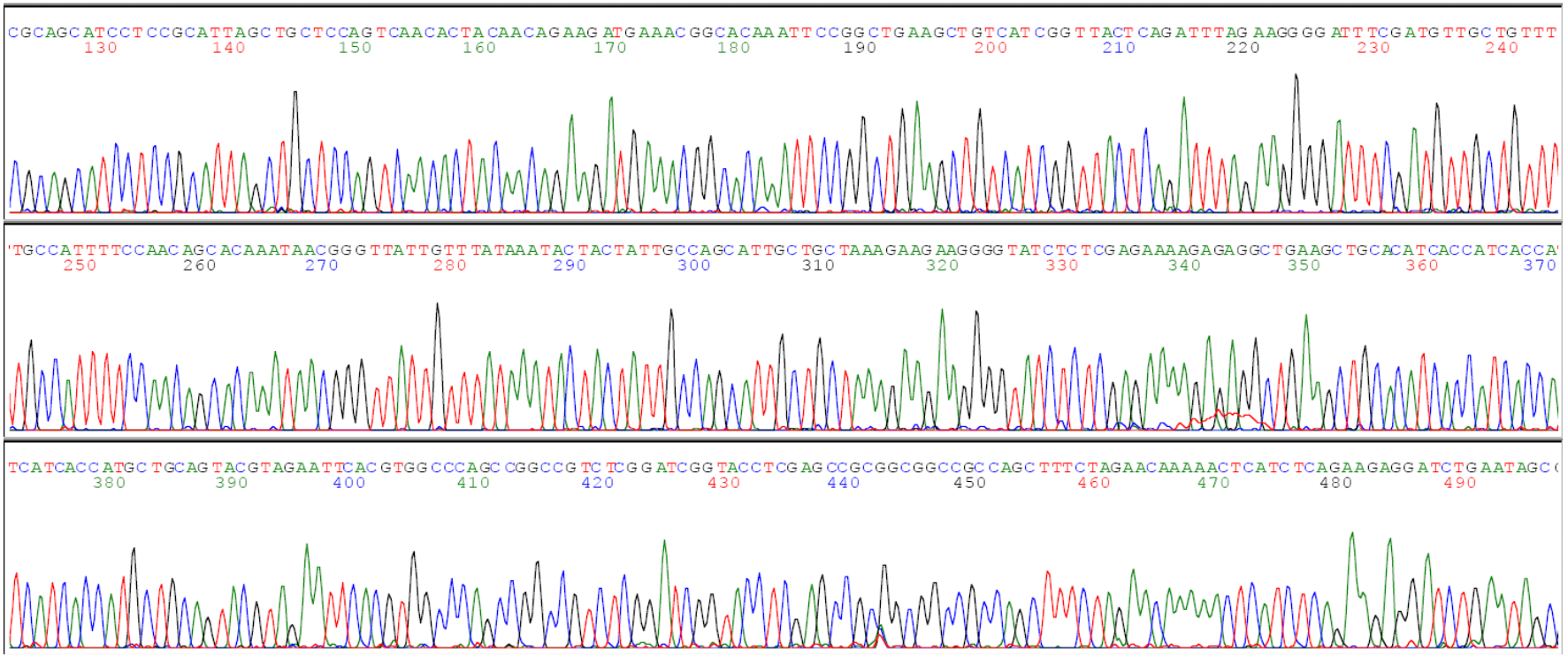


Figure 1 The chromatogram of cDNA sequence of pPICZαBNH8, using 5′AOX primer as sequencing primer.

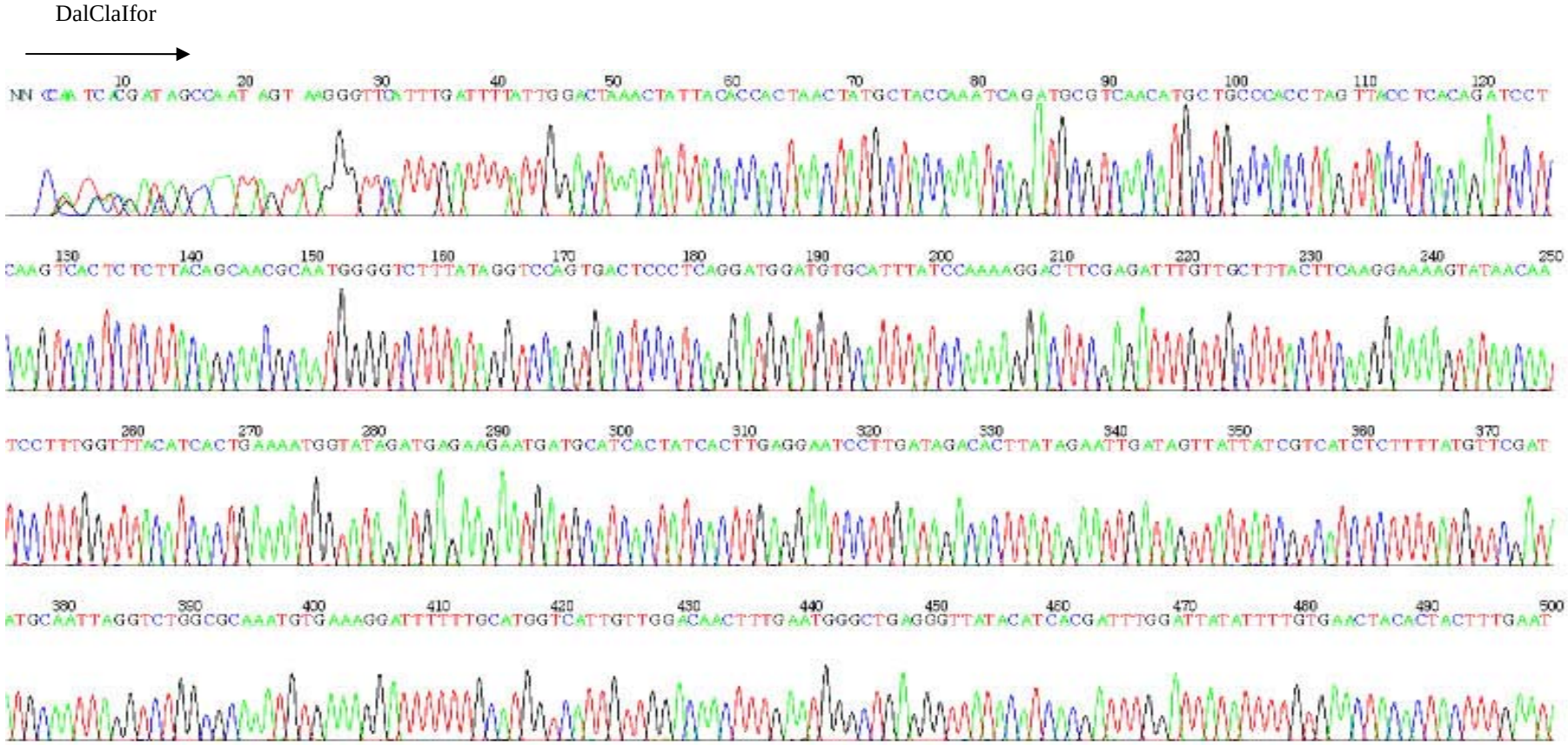


Figure 2 The chromatogram of cDNA sequence of pPICZαB-thrombin-PYG(R519W) clone no.5 , using DalClaIfor primer as sequencing primer.

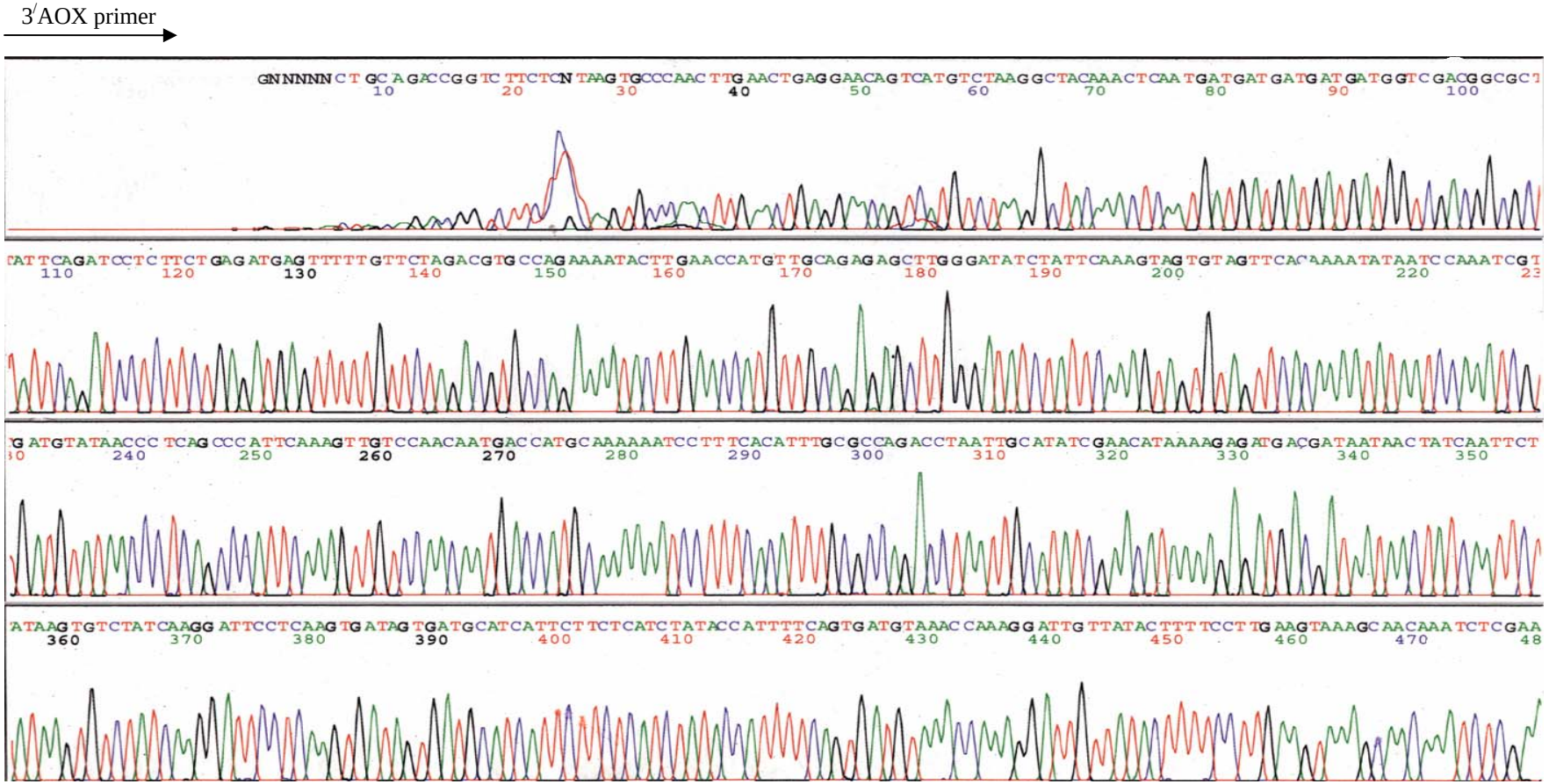


Figure 3 The chromatogram of cDNA sequence of pPICZαB-truncated R488 clone no.5 , using DalClaIfor primer as sequencing primer.

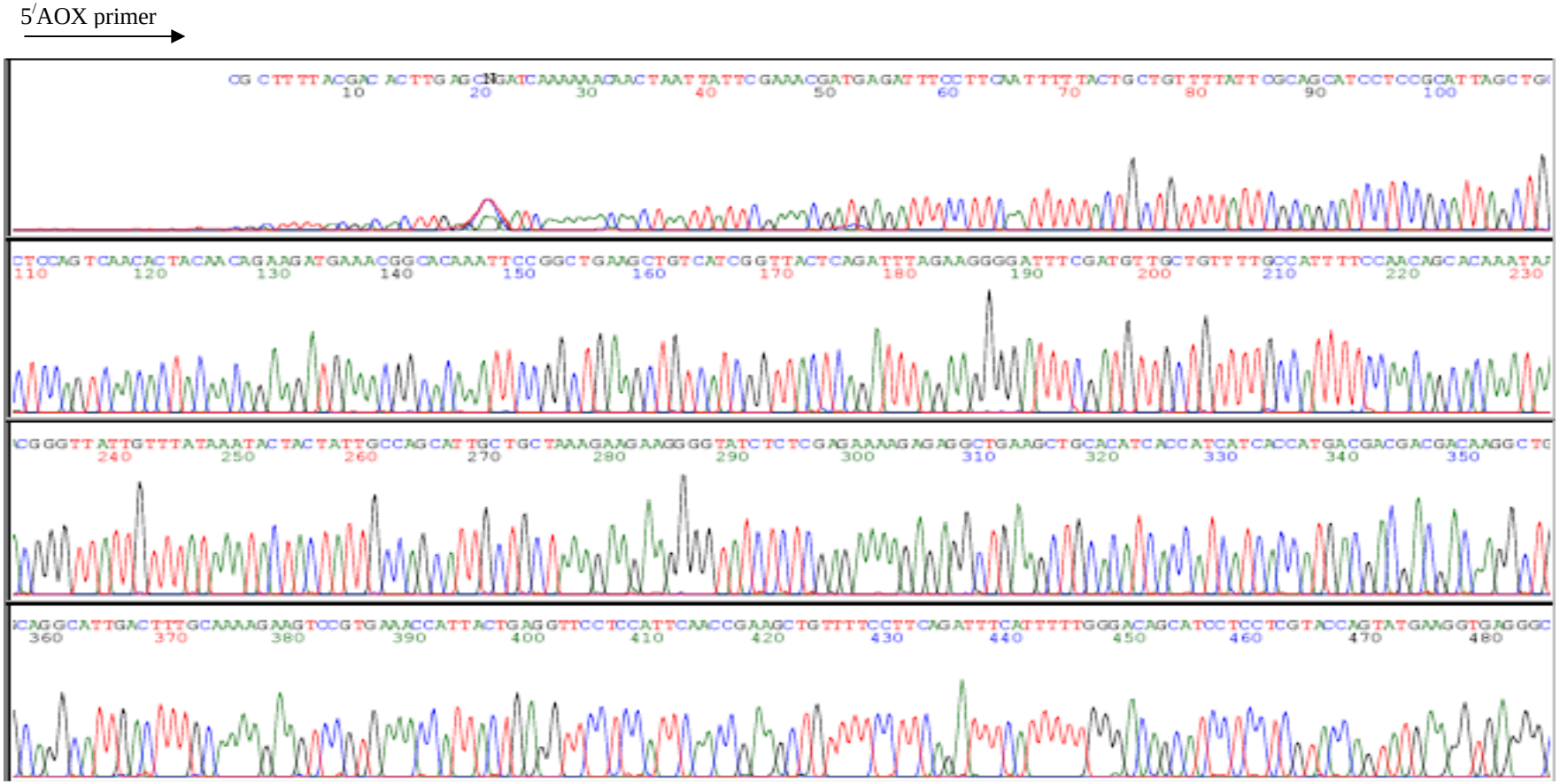


Figure 4 The chromatogram of cDNA sequence of pPICZαBNH-PCR(R519W) clone no6.3 , using 5′AOX primer as sequencing primer.

5′AOX primer

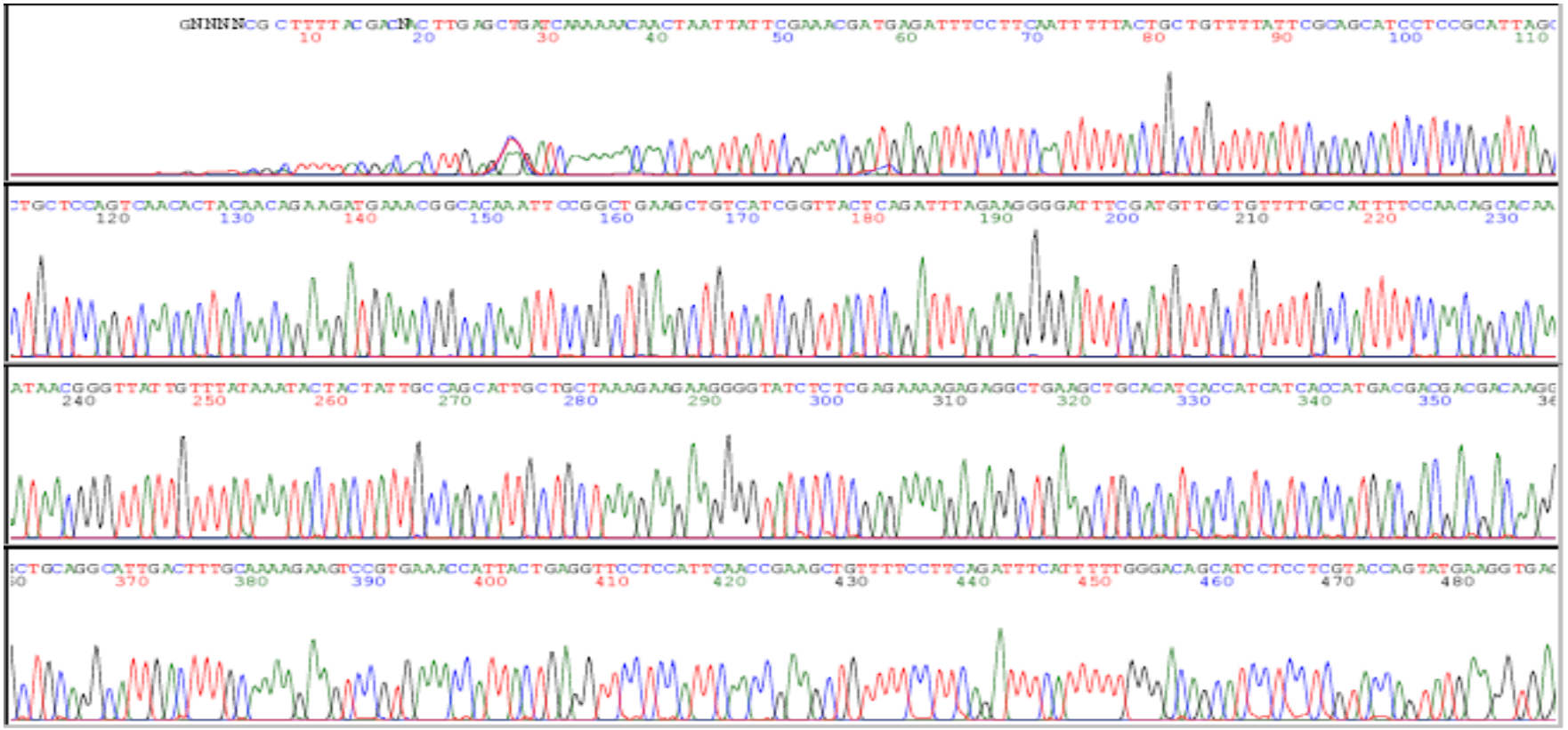


Figure 5 The chromatogram of cDNA sequence of pPICZαBNH-PCR(R519W) clone no7.4 , using 5′AOX primer as sequencing primer.

Figure 6 The chromatogram of cDNA sequence of pPICZαBNH8-Truncated N clone no. 8, using 5′AOX primer as sequencing primer.

CURRICULUM VITAE

NAME Miss Pornphimon Metheenukul

DATE OF BIRTH 22 August 1973

PLACE OF BIRTH Nakhon Ratchasima, Thailand

INSTITUTION ATTENDED

Khon Kaen University, Khon Kaen, Thailand, 1991-1994, Bachelor of Science (Medical Technology)

Mahidol University, Bangkok, Thailand, 1995-1999, Master of Science (Biochemistry)

Sukhothai Thammathirat Open University, Nonthaburi, Thailand, 1994-2001, Bachelor of Public Administration

Suranaree University of Technology, Nakhon Ratchasima, Thailand, 1999-2005, Doctor of Philosophy (Biochemistry)