EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RICE BETA-GLUCAN EXOGLUCANASE

Akkarawit Prawisut

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การศึกษาคุณสมบัติและหน้าที่ของเอนไซม์เบต้ากลูแคนเอกโซกลูคาเนส จากข้าว

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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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AKKARAWIT PRAWISUT : EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RICE BETA-GLUCAN EXOGLUCANASE. THESIS ADVISOR : PROF. JAMES R. KETUDAT-CAIRNS, Ph.D. 111 PP.

GLYCOSIDE HYDROLASE/β-GLUCAN EXOHYDROLASE/ RECOMBINANT EXPRESSION/ RICE/ SUBSTRATE SPECIFICITY

Glycoside hydrolase family 3 (GH3) enzymes play important roles in several fundamental biological processes. Higher plant GH3 enzyme amino acid sequences generate a phylogenetic tree with two distinct groups. One group contains the β-D-glucan glucohydrolases-like enzymes, while the second group contains β-D-xylosidase-like enzymes. Within the glucohydrolase-like branch of rice GH3 proteins, the one most similar to barley Exo1 exoglucanase was designated OsExo1. A cDNA optimized for expression of this protein in yeast was synthesized and cloned into the pPICZαBNH8 expression vector. The recombinant protein was extracted and purified by immobilized metal affinity chromatography (IMAC). Similarly, the cDNA for the rice GH3 β-exoglucanse-like gene most similar to barley Exo2, was cloned and used to express recombinant enzyme, designated OsExo2. This protein was expressed in *Escherichia coli* strain Origami B(DE3) as a fusion protein with N-terminal thioredoxin and His_6 tags from the pET32a expression vector. The recombinant protein was extracted and purified by IMAC, phenyl sepharose chromatography and gel filtration chromatography. Both rice GH3 enzymes could hydrolyze the polysaccharides laminarin, barley (1,3;1,4)-β-D-glucan, and lichenan and the synthetic glycosides *p*NP-β-D-glucopyranoside and *p*NP-β-D-xyloside. Moreover, OsExo2 hydrolyzed *p*NP-β-

D-fucopyranoside, *p*NP-β-D-galactopyranoside, and *p*NP-α-L-arabinoside at a relatively low rate. It also hydrolyzed cellobiose with a higher catalytic efficiency (k_{cat}/K_M 25.0 $mM^{-1}s^{-1}$) than the other oligosaccharides substrates tested, although the values for cellotriose (k_{cat}/K_M 20.5 $mM^{-1}s^{-1}$) and laminaritriose (k_{cat}/K_M 21.0 $mM^{-1}s^{-1}$) were similar. Among the substrates tested, rice OsExo1 had similar activity to OsExo2. Based on these observations and their rather broad expression pattern, we propose that rice β-exoglucanase might be involved in the turnover or modification of cell walls in various tissues in rice plants.

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อัครวิทย์ ประวิสุทธิ์ : การศึกษาคุณสมบัติและหน้าที่ของเอนไซม์เบต้ากลูแคนเอกโซกลูคาเนสจากข้าว (EXPRESSION, PURIFICATION AND CHARACTERIZTION OF RICE BETA-GLUCAN EXOGLUCANASE). อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.เจมส์ เกตุทัต-คาร์นส์, 111 หน้า.

เอนไซม์ไกลโคไซด์ไฮโดรเลสกลุ่มที่ 3 มีหน้าที่สำคัญหลายอย่างในกระบวนการทางชีวภาพขั้นพื้นฐาน ซึ่งในพืชชั้นสูงสามารถวิเคราะห์วิวัฒนาการชาติพันธุ์ของเอนไซม์ออกได้เป็น 2 กลุ่ม โดยกลุ่มแรกประกอบด้วยเอนไซม์ที่มีคุณสมบัติของเอนไซม์เบต้ากลูแคนกลูโคไฮโดรเลส และกลุ่ม 2 ประกอบด้วยเอนไซม์ที่มีคุณสมบัติของเอนไซม์เบต้าไซโลซิเดส ยีนของเอนไซม์ไกลโคไซด์ไฮโดรเลสกลุ่มที่ 3 ของข้าวที่มีความคล้ายคลึงกับเอนไซม์เบต้าเอกโซกลูคาเนสของบาร์เลย์ (HvExo1) เรียกว่า OsExo1 ได้ถูกสังเคราะห์ขึ้นในพลาสมิด pPICZαBNH8 เพื่อใช้ในการผลิตเอนไซม์ตัวดังกล่าวในยีสต์ เมื่อเอนไซม์ OsExo1 ถูกผลิตออกมาแล้ว เอนไซม์จะถูกปล่อยออกมาจากเซลล์ยีสต์มายังอาหารเลี้ยงเชื้อ แล้วถูกนำมาแยกให้บริสุทธิ์ด้วยวิธี immobilized metal affinity chromatography (IMAC) เอนไซม์ที่ได้ศึกษาอีกตัวหนึ่งเรียกว่า OsExo2 โดย cDNA ของเอนไซม์ดังกล่าวได้ถูกโคลน และตัดต่อเข้าไปในพลาสมิด pET32a/DEST จากนั้นพลาสมิดดังกล่าวได้ถูกนำเข้าไปใน *Escherichia coli* เพื่อนำไปใช้ในการผลิตเอนไซม์โดยมี thioredoxin และ His6 tags ต่อที่ปลาย N-terminal ของเอนไซม์ เอนไซม์ OsExo2 ที่ผลิตออกมาถูกนำมาทำให้บริสุทธิ์ด้วยวิธี IMAC ตามด้วย phenyl sepharose และ gel filtration ตามลำดับ จากการศึกษาพบว่าทั้งเอนไซม์ OsExo1 และ OsExo2 สามารถย่อยสับสเตรทได้แก่ ลามินาริน บาร์เล่ย์-เบต้า-ดี-กลูแคน และลิชิแนน รวมถึงสามารถย่อยกลูโคไซด์สับสเตรทได้แก่ *p*NP-glucoside และ *p*NP-xyloside เฉพาะในเอนไซม์ OsExo2 ที่สามารถย่อยสับสเตรท *p*NP-fucoside *p*NP-galactoside และ *p*NP-arabinoside รวมทั้ง เอนไซม์ OsExo2 ยังสามารถย่อยเซลลูโลส ได้เร็วกว่าโอลิโกแซคคาไรด์สับสเตรทตัวอื่น ๆ (k_{cat}/K_M, 25.0 $mM^{-1}s^{-1}$) แม้ว่าค่าความสามารถย่อยเซลลูโลส (k_{cat}/K_M 20.5 $mM^{-1}s^{-1}$) และลามินาริไตรโอส (k_{cat}/K_M 21.0 $mM^{-1}s^{-1}$) จะมีค่าใกล้เคียงกัน การทดสอบการย่อยสับสเตรทของเอนไซม์ OsExo1 พบว่าคล้ายกับการย่อยสับสเตรทของเอนไซม์ OsExo2 จากผลการทดลองและจาก

รูปแบบการแสดงออกของยีนที่ค่อนข้างกว้าง พบว่าเอนไซม์เบต้าเอกโซกลูคาเนสจากข้าวน่าจะเกี่ยวข้องกับกระบวนการสร้างกลับคืนหรือการปรับแต่งของผนังเซลล์ในข้าว

สาขาวิชาเคมี ลายมือชื่อนักศึกษา _____

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LIST OF ABBREVIATIONS

ABTS	2,2′-azinobis-3-ethylbenthaiazolinesulfonic acid
AIM	Auto-induction media
APS	Ammonium persulfate
bis-acryalmide	N,N-methylene-bis-acrylamide
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxynucleic acid
CV	Column volume
DMSO	Dimethyl sulfoxide
DP	degree of polymerization
EDTA	Ethylenediamine tetraacetate
EtOAc	Ethyl acetate
GH	Glycoside hydrolase
GH3	Glycoside hydrolase family 3
HPLC	High performance liquid chromatography
IMAC	Immobilized metal affinity chromatography

LIST OF ABBREVIATIONS (Continued)

IPTG	Isopropyl thio-β-D-galactoside
kDa	Kilo Dalton(s)
LB	Luria-Bertani lysogeny broth
MeOH	Methanol
MW	Molecular weight
MWCO	Molecular weight cut off
NaOAc	Sodium acetate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGO	peroxidase-glucose oxidase
PMSF	Phenylmethylsulfonyl fluoride
*p*NP	para -Nitrophenyl
*p*NPGlc	*p*NP-β-D-glucopyranoside
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis with SDS
TB	Terrific broth
TEMED	Tetramethylenediamin
Tris	Tris-(hydroxymethyl)-aminoethane

LIST OF ABBREVIATIONS (Continued)

UV	Ultraviolet
v/v	volume by volume
w/v	weight by volume
YNB	Yeast nitrogen base
YPDS	Yeast extract peptone dextrose medium with sorbitol

CHAPTER I

INTRODUCTION

1.1 Glycoside Hydrolase

Glycoside hydrolases (EC 3.2.1-), also called glycosidases, are enzymes that catalyze the hydrolysis of the glycosidic linkages of glycosides, leading to the formation of a sugar and the corresponding free aglycone. Glycoside hydrolases transfer the glycosidic bond to a water molecule, while transglycosidases transfer this glycosidic bond to the OH group of another glycosyl residue or to another nucleophile (Figure 1.1). The glycoside hydrolases act in many essential steps of life, including hydrolysis of structural or storage polysaccharides, defense against pathogens, invasion of certain pathogens into cells, turnover of cell surface carbohydrates, etc. (Henrissat et al., 1995). Glycoside hydrolases can be classified in many different ways, such as endo/exo acting (Figure 1.2), enzyme commission (EC) number, mechanistic classification and on the basis of sequence similarity determined by amino acid sequence alignments and hydrophobic cluster analyses (Henrissat, 1991; Henrissat and Bairoch, 1993; Cantarel et al., 2009).

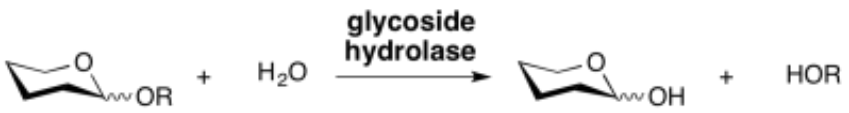

Figure 1.1 Glycoside hydrolase activity (www.cazypedia.org).

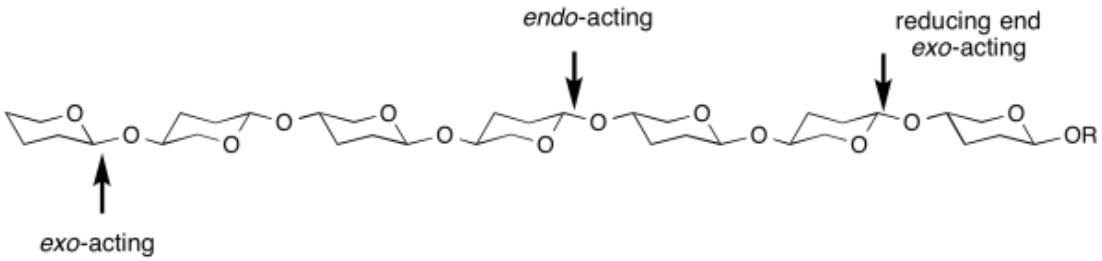

Figure 1.2 Endo/exo-acting glycoside hydrolases (www.cazypedia.org).

1.2 Glycosidase Mechanism

The hydrolysis of glycosidic bonds by glycosidase enzymes have two most common mechanisms, which are distinguished by the retained or inverted configuration of the anomeric carbon of the product. (Sinnott, 1990; McCarter and Withers, 1994). Glycosidase enzymes use general acid/ base catalysis, which involves two acidic catalytic residues in the active site that act as a general acid or proton donor and a general base or nucleophile (Koshland, 1953).

Inverting glycosidases (Figure 1.3a) catalyze hydrolysis via a single oxo-carbenium ion- like transition state and no covalent enzyme intermediate is formed during catalysis. The two carboxyl groups in inverting glycosidases are usually two carboxylic acid residues (Asp or Glu), which are located on opposite sides of the active site (Zechel and Withers, 2000; Rempel and Withers, 2008). One of the carboxylic acids is deprotonated allowing it to remove a proton from the incoming nucleophile (water in the hydrolysis mechanism, and this carboxylic acid acts as a general base. The other carboxylic acid acts as a general acid residue to protonate the aglycone oxygen atom, thereby assisting in cleavage of the glycosidic bond to release the leaving group. The reaction starts with the carboxyl group that acts as a general base extracting a proton to

activate the nucleophilic water molecule to attack the anomeric carbon, while another carboxyl group acts as a general acid, protonating the departing oxygen atom in a concerted fashion as the bond is cleaved (Koshland, 1953). The retaining mechanism (Figure 1.3b) also uses a pair of essential carboxylic acid residues (Asp or Glu) located on opposite sides of the active site cleft entrance. In the first step, called glycosylation, the glycosidic oxygen is protonated by one of the carboxylic acid residues, which acts as the general acid, while the other carboxylate group acts as a nucleophile and attacks the anomeric carbon, resulting in the formation of a covalently linked glycosyl-enzyme intermediate. In the second step, called deglycosylation, the carboxylate residue that first acted as acid catalyst now acts as a base to receive a proton from the incoming nucleophile, the water molecule in the case of hydrolysis, which attacks at the anomeric center to cleave the covalent intermediate and displace the protein from the sugar.

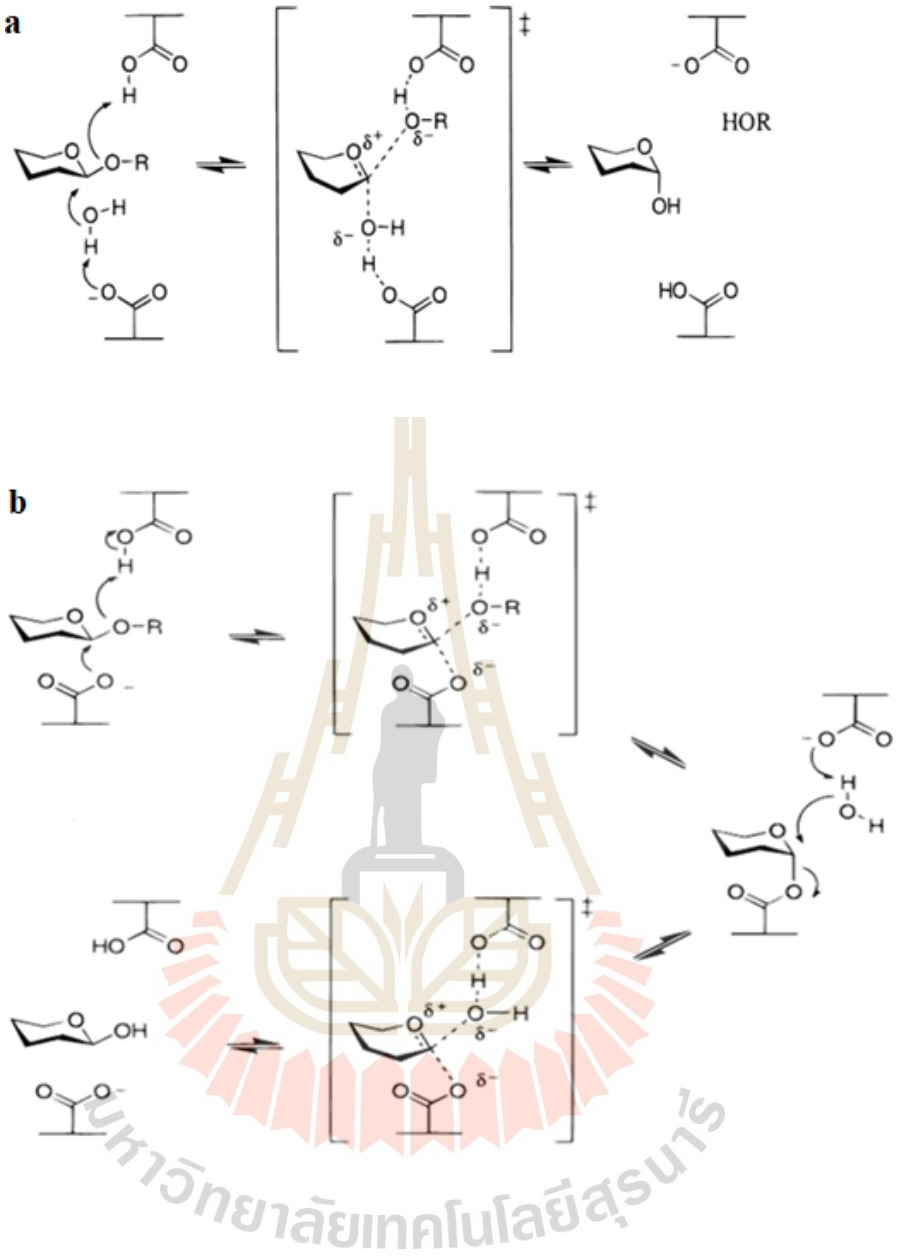

Figure 1.3 General mechanisms of inverting and retaining glycosidases. Mechanism of (a) inverting glycosidases and (b) retaining glycosidases (Zechel and Withers, 2000).

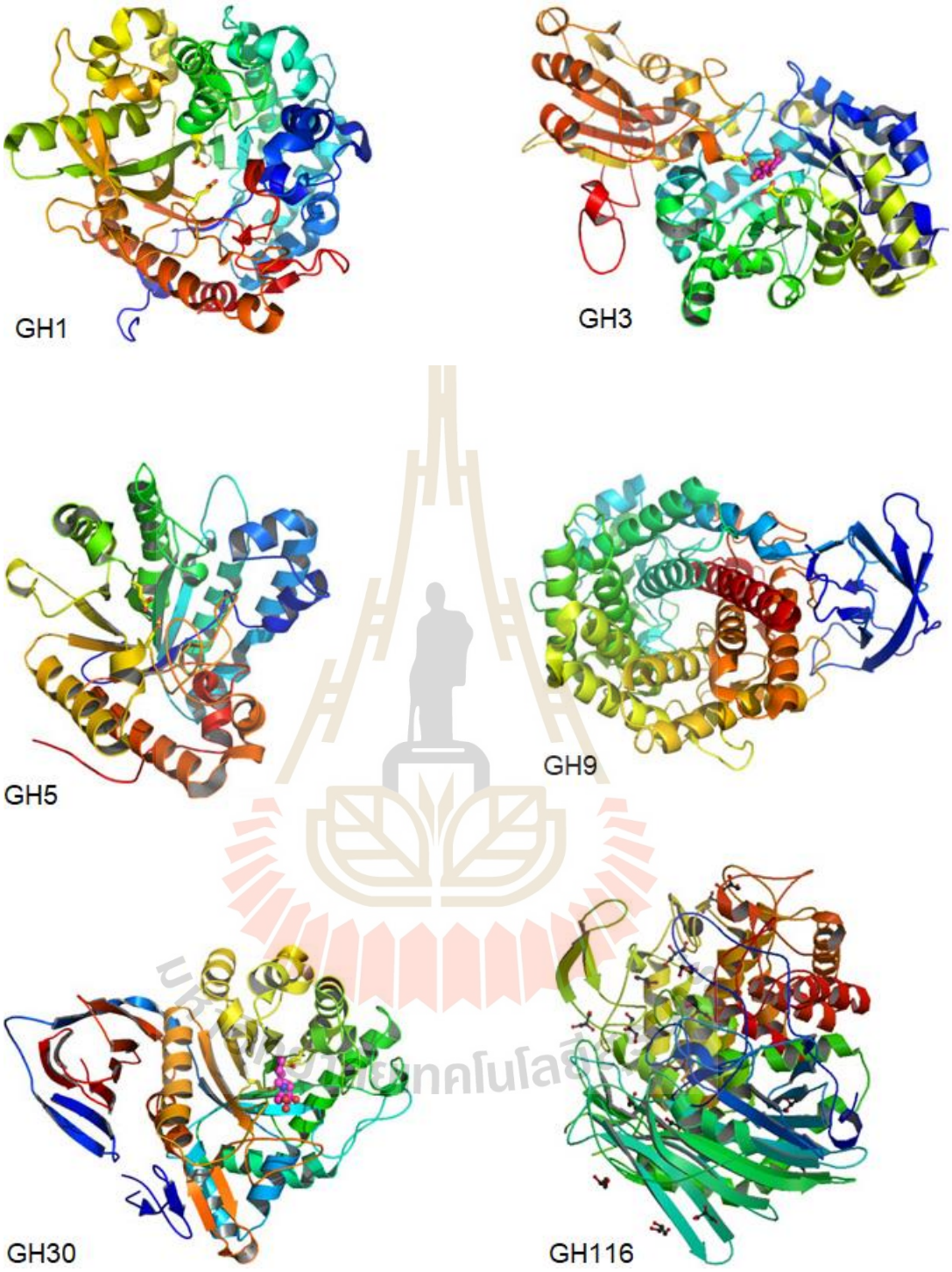

Figure 1.4 Structures of β-glucosidases from different GH families (Ketudat-Cairns and Esen, 2010; Charoenwattanasatien et al., 2016).

1.3 β-Glucosidase

β-Glucosidases (E.C.3.2.1.21) are enzymes that catalyze the hydrolysis of the β-glycosidic bond between the reducing side of glucose and an aryl- or alkyl-moiety or an oligosaccharide, releasing β-D-glucose and an aglycon. These enzymes play important roles in many biological mechanisms, such as chemical defense (Morant et al., 2008), lignification (Escamilla-Trevino et al., 2006), cell wall modification (Hrmova and Fincher, 2001) and phytohormone activation (Lee et al., 2006). β-Glucosidases have been classified into glycoside hydrolase (GH) families GH1, GH2, GH3, GH5, GH9, GH30, and GH116, based on their amino acid sequences (Cantarel et al., 2009; Ketudat Cairns and Esen, 2010). The families GH1, GH3, GH5, and GH30 belong to the Clan GH-A, and they all have similar $(\beta/\alpha)_8$-barrel domains that contain their active site. GH9 and GH116 enzymes have catalytic domains with $(\alpha/\alpha)_6$-barrel structures, while GH3 enzymes have two-domain modular structures surrounding the active site. The first domain folds into an $(\alpha/\beta)_8$ or triose phosphate isomerase (TIM)-barrel conformation and the second domain forms an $(\alpha/\beta)_6$ sandwich (Hrmova et al., 2001; Hrmova et al., 2002) (Figure 1.4).

1.4 Rice β-glucosidases

In rice (*Oryza sativa L*), 40 glycoside hydrolase family GH1 genes were identified, 34 of which at least are likely to encode functional rice β-D-glucosidases (Opassiri et al., 2006). Several β-D-glucosidases have been characterized from rice seedlings (Akiyama et al., 1998; Opassiri et al., 2003). The GH1 β-glucosidase BGlu1 or Os3BGlu7 is highly expressed in rice flower and germinating shoots, and showed broad substrate specificity toward natural and synthetic substrates (Opassiri et al.,

2003). BGlu1 can hydrolyze β-1,3- and β-1,4- linked oligosaccharides and pyridoxine 5′-O-β-D-glucoside, and also has high tranglucosylation activity with pyridoxine acceptor (vitamin B6) to synthesize pyridoxine 5′-O-β-D-glucoside (Opassiri et al., 2004). The transglycosylation activity of BGlu1 predominantly adds β-1,4-linked glucosyl residues and the E414G glycosynthase mutant of BGlu1 is able to synthesize long β-1,4-linked gluco-oligosaccharides of at least 11 glucosyl residues (Homalai et al., 2007). BGlu1 can hydrolyze *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) faster than p-nitrophenyl β-D-mannopyranoside (*p*NPMan), in contrast to a closely related β-mannosidase from barley (*Hordeum vulgare*) (rHvBII) and rice Os7BGlu26 (Kuntothom et al., 2009). While all of these enzymes hydrolyze cellooligosaccharides with hydrolytic rates that increase with the degree of polymerization from 3-6, only rHvBII hydrolyzes cellobiose with a higher k_{cat}/K_M value than cellotriose (Hrmova et al., 1998; Opassiri et al., 2004; Kuntothom et al., 2009).

Although many GH1 enzymes from rice have been characterized. No reports on characterization of rice enzymes from glycoside hydrolase family 3 (GH3) have yet appeared.

1.5 Glycoside hydrolase family 3

Glycoside hydrolase family 3 enzymes include over 25,000 entries in the CAZy database. The members of this family are distributed predominantly in eubacteria, fungi, and plants, and are scarce in archaea and apparently absent in animals (Cantarel et al., 2009). These enzymes have been classified as β-D-glucosidases (including exoglucanases), α-L-arabinofuranosidases, β-D-xylopyranosidases and N-acetyl-β-D-glucosaminidases (Harvey et al., 2000). In fact, the putative N-acetyl-β-D-glucosa-

minidases have recently been demonstrated to be phosphorylases. The family GH3 members play important roles in fundamental biological processes, for example the microbial degradation of plant residues, the modification of structures of glycosides, bacterial antibiotics and plant-derived antifungal molecules, the turnover, recycling and remodeling of cellular components in bacteria, fungi and plants, and the modification of host-pathogen interactions during microbial infection of plants (Cournoyer and Faure, 2003; Hrmova and Fincher, 2001). The enzymes have a broad range of substrate specificities. For example, there are characterized bifunctional enzymes in the family that have both α-L-arabinofuranosidase and β-D-xylo-pyranosidase activities (Lee et al., 2003). The family GH3 glucan β-D-glucosidases from barley are broad specificity exo-hydrolases that remove single glucosyl residues from the non-reducing ends of a range of β-D-glucans, β-D-oligoglucosides and aryl β-D-glucosides, including (1,3)-β-D-glucans, (1,4)-β-D-glucans, (1,3;1,4)-β-D-glucans and (1,6)-β-D-glucans, *p*-nitrophenyl-β-D-glucoside, certain cyanogenic β-D-glucosides and some β-D-oligoxyloglucosides (Hrmova and Fincher, 1998). The family GH3 enzymes have a retaining catalytic mechanism, which removes single monosaccharide units from the nonreducing end of their substrates, with retention of anomeric configuration (Koshland, 1953; Hrmova et al., 1996) (Figure 1.5). In the barley Exo1 exoglucanase, the catalytic nucleophile is Asp285, which is located in a highly conserved GFVISDW motif in plant family GH3 β-D-glycosidases. The catalytic acid is E491, which is conserved in plant family GH3 exoglucanase-like enzymes, but is present only in closely related members of the GH3 family (Harvey et al., 2000; Hrmova et al., 2001).

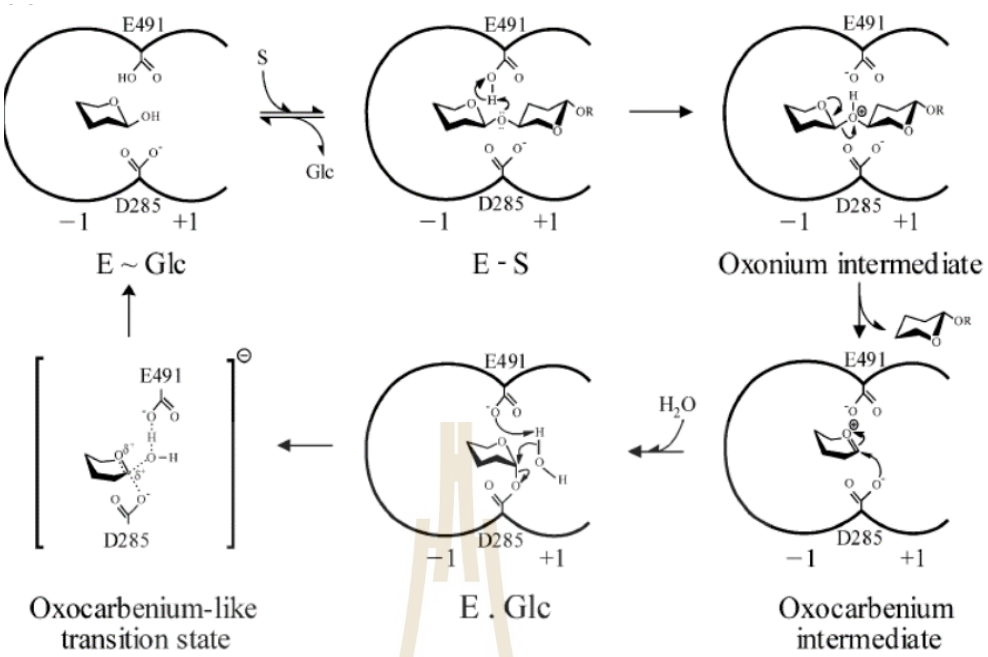

Figure 1.5 Catalytic mechanism of barley Exo1, a plant family GH3 retaining β-D-glucan glucohydrolase (Hrmova et al., 2001).

The structural models of GH3 family have been reported (Table 1.1 and Table 1.2). The structure of Exo1 β-glycosidase from barley has a two-domain structure, connected by a helix-like linker (Varghese et al., 1999). The first domain folds into an $(\beta/\alpha)_8$ TIM-barrel conformation followed by the second domain that forms an $(\alpha/\beta)_6$ sandwich (Varghese et al., 1999; Hrmova et al., 2001). The structure of β-hexosaminidase from *Vibrio cholerae* (NagZ) has one domain comprising 340 amino acids that adopts a $(\beta/\alpha)_8$ TIM barrel fold (Stubbs et al., 2007). The structure of β-hexosaminidase from *Bacillus subtilis* has two domains similar to barley Exo1 (Berman, 2008). The structure of recombinant thermostable β-glucosidase 3B from *Thermotoga neapolitana* (TnBgl3B; EC 3.2.1.21) was the first example of a family

GH3 enzyme with a three-domain structure. The structure is composed of a $(\beta/\alpha)_8$ TIM-barrel domain, followed by a five-stranded α/β sandwich domain, and a C-terminal fibronectin type III (FnIII) domain of unknown function (Pozzo et al., 2010). A homo-tetrameric structure of β-glucosidase from *Kluyveromyces marxianus* was described, in which the monomeric structure is composed of N-terminal $(\beta/\alpha)_8$-fold-like domain, an $(\alpha/\beta)_6$-sandwich domain, a C-terminal domain and a PA14 domain, for which a carbohydrate-binding role was proposed (Yoshida, 2010). GH3 structures that have recently been described are similar to one of these models.

Table 1.1 Glycoside hydrolase family 3 structures from prokaryotes (www.cazy.org).

Protein Name	**Organism**	**PDB**
β-N-acetylglucosaminidase	*Aspergillus aculeatus* F-50	4IIB
BACINT_00768	*Bacteroides intestinalis* DSM 17393	5TF0
β-glucosidase	*Bacteroides ovatus* ATCC 8483	5JP0
β-1,2-glucosidase	*Bacteroides thetaiotaomicron* VPI-5482	5XXL
β-N-acetylhexosaminidase	*Beutenbergia cavernae* DSM 12333	5BU9
BAD_1194	*Bifidobacterium adolescentis* ATCC 15703	5WAB
β-glucosidase	*Bifidobacterium longum* subsp. longum KACC 91563	5Z9S

Table 1.1 Glycoside hydrolase family 3 structures from prokaryotes (www.cazy.org) (Continued).

Protein Name	**Organism**	**PDB**
BCN122_I0864 (NagZ)	*Burkholderia cenocepacia* GIMC4560:Bcn122	6DTE
β-hexosaminidase 1	*Burkholderia cenocepacia* J2315	
cg3158 (NagA2)	*Corynebacterium glutamicum* ATCC 13032	5IOB
DR1333/DR_1333	*Deinococcus radiodurans* R1	3TEV
β-glucosidase EmGH1	*Erythrobacter marinus*	5Z87
β-1,2-glucosidase/β-1,3-glucosidase	*Listeria innocua* Clip11262	4ZO6
β-N-acetylglucosaminidase	*Mycobacterium tuberculosis* H37Rv	6GFV
MSMEG_0361	*Mycolicibacterium smegmatis* MC2 155	4YYF
β-glucosidase	*Paenibacillus barengoltzii* CAU904	5WUG
exo-1,3/1,4-β-glucanase	*Pseudoalteromonas* sp. BB1	3F93
β-N-acetylglucosaminidase	*Pseudomonas aeruginosa* PAO1	5G1M
glucan 1,4-β-glucosidase	*Saccharopolyspora erythraea* NRRL 2338	5M6G
β-N-acetylglucosaminidase	*Salmonella enterica* subsp. enterica serovar Typhimurium str. LT2	4GVF

Table 1.1 Glycoside hydrolase family 3 structures from prokaryotes (www.cazy.org) (Continued).

Protein Name	**Organism**	**PDB**
β-xylosidase (BxlA)	*Streptomyces thermoviolaceus* OPC-520	cryst
macrolide β-glycosidase/β-glucosidase (DesR)	*Streptomyces venezuelae*	4I3G
SYNPCC7002_A0075	*Synechococcus* sp. PCC 7002	3SQL
β-xylosidase (Xyl3)	*Thermotoga maritima* MSB8	cryst
β-N-acetylglucosaminidase	*Thermotoga maritima* MSB8	3WO8
β-glucosidase B (Bgl3B)	*Thermotoga neapolitana* DSM 4359	2WT3
β-N-acetylglucosaminidase (CbsA)	*Thermotoga neapolitana* KCCM41025	5BZA
β-glucosidase B/laminaribiase (BglB)	*Thermotoga neapolitana* Z2706-MC24	cryst
β-glucoside phosphorylase BglP	uncultured bacterium	5VQD
β-N-acetylhexosaminidase	*Vibrio cholerae* O1 biovar El Tor str. N16961	1TR9

Table 1.2 Glycoside hydrolase family 3 structures from eukaryotes (www.cazy.org).

Protein Name	**Organism**	**PDB**
β-glucosidase 1	*Aspergillus aculeatus* F-50	4IIB
β-glucosidase 1	*Aspergillus fumigatus* Af293	5FJI
β-xylosidase	*Aspergillus nidulans* FGSC A4	6Q7I
β-glucosidase (Cel3A)	*Aspergillus oryzae* RIB40	5FJJ
β-glucosidase (BglD)	*Aspergillus oryzae* RIB40	5YOT
exo-β-1,3-1,4-glucanase (ExoI)	*Hordeum vulgare* subsp. vulgare	1EX1
β-glucosidase I	*Kluyveromyces marxianus* NBRC1777	3ABZ
β-glucosidase (Cel3A)	*Neurospora crassa* OR74A	5NBS
β-glucosidase (Cel3A)	*Rasamsonia emersonii* IMI 392299	4D0J
β-glucosidase (tomatinase; B2Tom)	*Septoria lycopersici*	cryst
β-N-acetylglucosaminidase	*Rhizomucor miehei* CAU432	4ZM6
β-xylosidase (Xyl3A)	*Trichoderma reesei*	5A7M
β-glucosidase1 (Cel3A)	*Trichoderma reesei* QM9414	3ZYZ

Table 1.3 Glycoside hydrolase family 3 structures from unclassified organisms (www.cazy.org).

Protein Name	**Organism**	**PDB**
β-glucosidase (JMB19063)	compost metagenome	3U48
broad-specificity β-glucosidase/β-xylosidase (GlyA1) (partial)	metagenome	5K6M

The structures of the GH3 enzymes yield clues to the intricacies of their mechanisms. For instance, Streltsov et al. (2019) recently examined product and substrate pathways along the catalytic cycle of the plant GH3 exo-hydrolase HvExoI, and how the displacement of the glucose trapped in the active site after the previous round of hydrolysis leads to processive hydrolysis of oligo- and polysaccharide substrates. The results revealed the glucose product displacement route and how each hydrolytic event, including glucose release, is precisely coordinated with the incoming substrate association and hydrolysis. The glucose product modifies its binding pattern and thereby causes the transient formation of a lateral cavity, which serves as a route for glucose departure from the active site. This departure allows the nonreducing glucosyl residue of the substrate to enter for the next catalytic round. This process allows for substrate-product assisted processive catalysis through multiple hydrolytic events without release of the polymer or oligomer substrate from HvExoI.

The substrate binding and product displacement routes proceed through stages that are well coordinated, as illustrated in Figure 1.6 (Streltsov et al., 2019). For a disaccharide substrate (Figure 1.6 a), after the disaccharide bound in the −1 and +1

subsites (step 1) is hydrolyzed the aglycon diffuses away (step 2), but the glucose released from the nonreducing end remains non-covalently trapped and oscillates between the −1 and +1 subsites. Glucose is consolidated to the −1 subsite after an incoming substrate binds (step 3). Glucose modifies its binding to active site residues and exits via a lateral opening that is briefly formed near the catalytic site (step 4); after which the next hydrolytic cycle can begin. In the case of a polysaccharide, this mechanism enables substrate-product assisted processive catalysis (Figure 1.6b). After the non-reducing and penultimate glucosyl residues bind in a productive mode at the −1 and +1 subsites (step 1), the non-reducing glucose residue is cleaved off, with the remainder of the substrate attached to the enzyme (step 2). The glucose left in the -1 subsite modifies its binding patterns and is released via the opening to the side of the active site (step 3). In this case, the next hydrolytic cycle continues with the same polysaccharide, where the polysaccharide, shortened by one glucosyl residue advances into the catalytic site after uninterrupted binding. The structural basis of this substrate-product assisted processive catalysis is shown in Figure 1.6c, which illustrates how the substrate enters from the front side of the active site, while the glucose product escapes via the transient cavity in the back side.

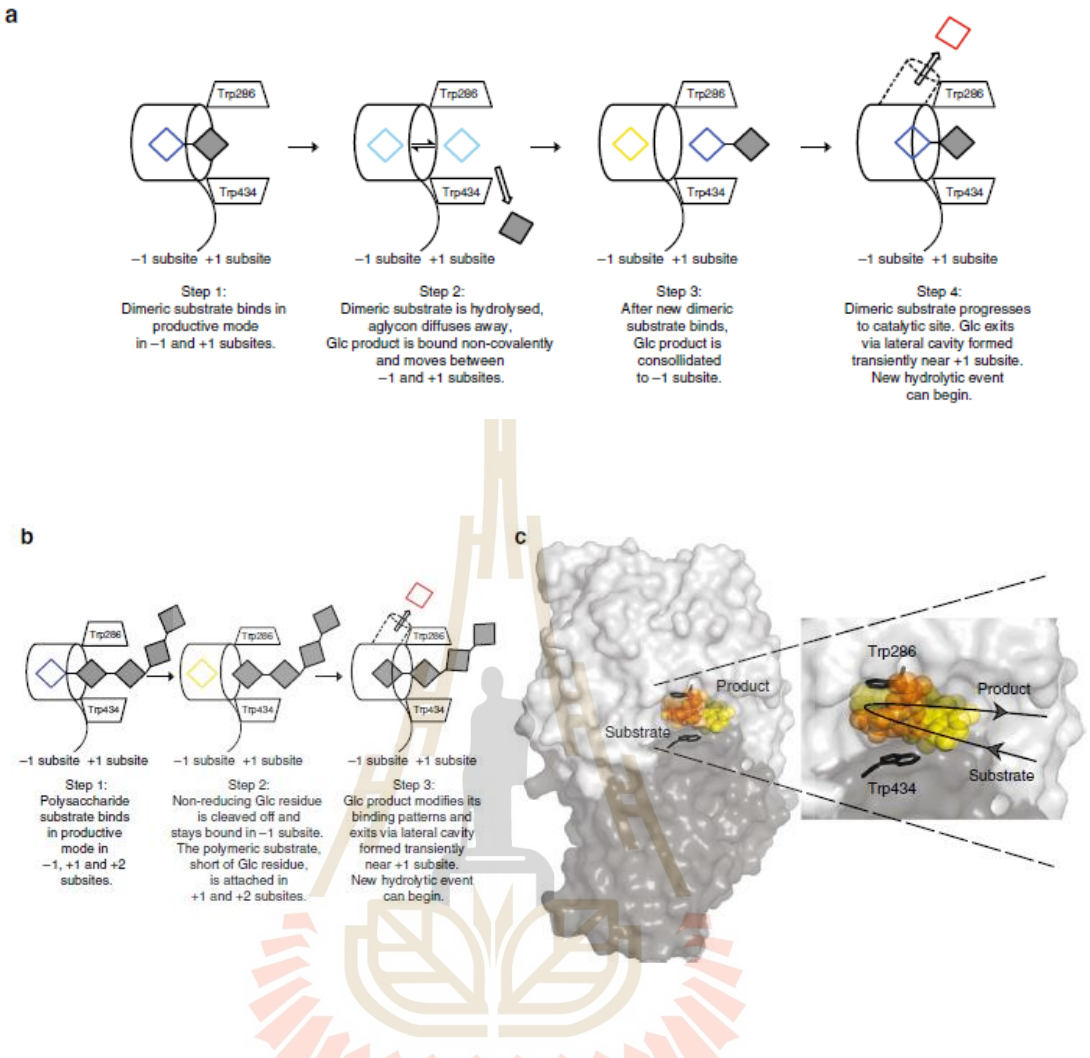

Figure 1.6 The mechanism of substrate- product assisted processive catalysis by HvExoI (Streltsov et al., 2019). (a) Process for hydrolysis of disaccharides. (b) Processive hydrolysis of a polysaccharide. (c) Image of the active site cleft of Exo1 showing the routes for entrance of the new substrate and exit of the trapped glucose product.

Phylogenetic clusters have been identified within GH3, and in higher plant enzymes they generate a phylogenetic tree with two distinct groups. One group contains the β-D-glucan exo-glucohydrolases-like enzymes, the second group contains β-D-xylosidase-like enzymes (Hrmova et al., 2002) (Figure 1.7). Sixteen japonica rice genome loci encode GH3 enzymes of these; five rice GH3 β-exoglucanase-like enzymes and one apparent gene fragment are located in the β-D-glucan glucohydrolases-like enzymes group that includes HvExo1. However, the functions and structures of rice GH3 β-exoglucanase have not yet been characterized.

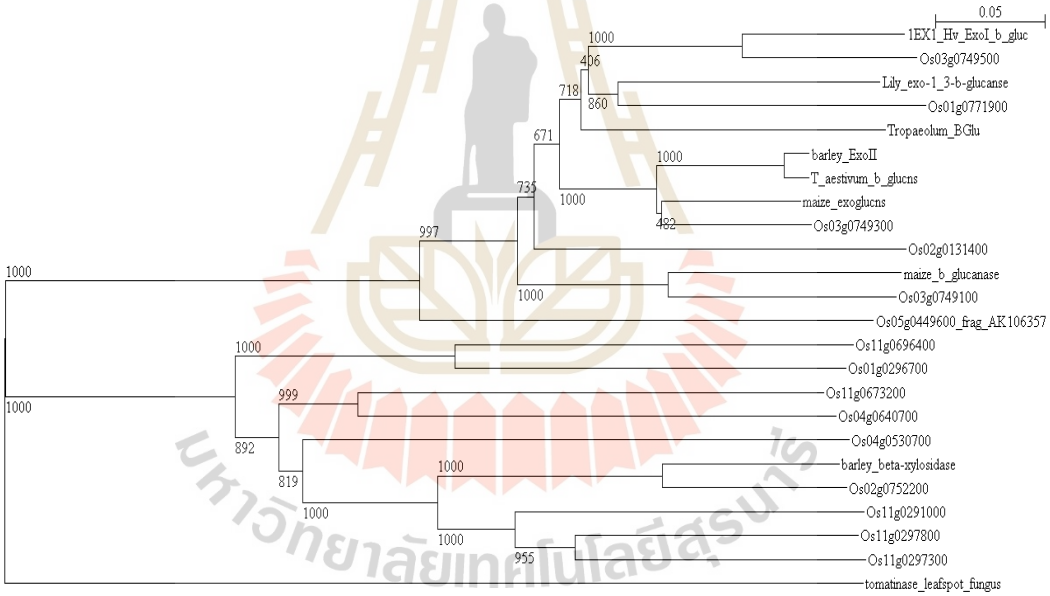

Figure 1.7 Phylogenetic tree of plant family 3 glycoside hydrolases.

1.6 Function of glycoside hydrolase family 3 in plants

The broad distribution of family GH3 members in various kingdoms suggests that they play key roles in fundamental biological processes. For example, the β-D-glucan exohydrolases from barley are thought to play biological roles in the degradation of the barley $(1\rightarrow3)$, $(1\rightarrow4)$-β-D-glucans during endosperm mobilization and in cell elongation (Hrmova and Fincher, 2001). In barley, two isoenzymes were isolated from germinated seedlings and are designated isoenzymes Exo1 and Exo2 (Hrmova et al., 1996). Both enzymes hydrolyze (1→3)-β-glucan and laminarin, but also hydrolyze (1→3), (1→4)-β-glucan and 4-nitrophenyl β-D-glucoside. Two other enzymes with β-glucosidase activity, designated βI and βII, were also purified from the extracts. Although all these enzymes hydrolyzed 4-nitrophenyl β-glucoside, only Exo1 and Exo II released glucose from polysaccharides, indicating that their substrate specificities and action patterns were more typical of polysaccharide exohydrolases of the (1→4)-β-glucan glucohydrolase type. The isoenzymes Exo1 and Exo2 can hydrolyze of a range of polymeric β-D-glucans, β-1 inked oligo-D-glucosides, and aryl β-D-glucosides (Table 1.4). The possible functions of these enzymes include acting to complete the conversion of cell-wall polysaccharides to glucose in the germinated grain, remodeling polysaccharides during the auxin-induced elongation of cells in growing coleoptiles and hydrolyzing (1→3)-β-glucosyl linkages in (1→3)- and (1→3); (1→6)-β-glucans of the type commonly found in fungal cell walls (Hrmova and Fincher, 1998). Andriotis et al., reported that starch degradation in the endosperm is dependent on cell wall degradation, which permeabilises the walls and thus permits rapid diffusion of amylolytic enzymes (Andriotis et al., 2016), so the breakdown of endosperm cell wall β-glucans is critical for cereal seed germination.

β-D-Glucan exohydrolases were also purified from the cell walls of developing maize, and were found to hydrolyze the non-reducing terminal glucosyl residue from (1→3)-β-D-glucans, but also hydrolyzes (1→2)-, (1→6)-, and (1→4)-β-D-glucosyl units in decreasing order of activity (Kim et al., 2000). They were proposed to function coordinately with the pathogenesis-related endoglucanase as part of the defense against fungal pathogens, act in removal of the 1→3-linked glucan callose and/or be associated with cellulose biosynthesis, which occurs at the wall membrane interface.

In *Solanum torvum*, a GH3 β-glucosidase (torvosidase) was purified from the young leaves. This enzyme was highly specific for cleaving steroid glycosides and did not hydrolyze oligosaccharides, unlike the GH3 exoglucanases mentioned above (Arthan et al., 2006). It was proposed that the torvoside–torvosidase combination may act as a defense mechanism in *S. torvum* by releasing a toxic aglycone to deter herbivores.

Table 1.4 Relative rates of hydrolysis of β-1inked poly- and oligosaccharides and aryl β-D-glucosides by barley β-glucan exohydrolase isoenzymes Exo1 and Exo2 (Hrmova and Fincher, 1998).

Substrate	**Relative rate (%)**	
	Exo1	**Exo2**
Polysaccharides		
laminaran		
L. digitata	100	100
L. hyperborea	50	75
E. bicyclis	9	10
CM-pachyman (*P. cocos*)	40	34
CM-(1→3;1→6)-β-glucan (*S. cerevisiae*)	25	29
(1→3;1→4)-β-glucan (*Hordeum vulgare*)	10	14
(1→3;1→4)-β-glucan (lichenin)		
U. barbata	11	27
C. islandica	18	49
SIII polysaccharide (*S. pneumoniae*)	2	5
Oligosaccharides		
sophorose	55	54
laminarabiose	70	75
cellobiose	14	20
gentiobiose	36	20
4-nitrophenyl β-D-glucoside	10	56
2-nitrophenyl β-D-glucoside	5	12

1.7 Protein Expression Systems

Proteins have been recombinantly expressed in many systems, ranging from bacteria and yeast to animal and plant cells and even whole animals and plants. However, the single cell organism systems of bacteria and plants represent the simplest systems with greatest ease of protein purification, so they were the starting point for our expression.

1.7. 1 Expression of proteins in bacterial systems

Bacteria act as rapid and simple systems for expressing recombinant proteins, due to their short doubling time and simple media. The most widely used host system is *Escherichia coli*, since there is ample knowledge about its genetics, genome sequence and physiology. Genetic manipulation of *E. coli* is easy and it also grows to high densities and is suitable for large-scale fermentations (Sezonov et al., 2007). However, multi- domain eukaryotic proteins expressed in bacteria often are non- functional because the cells are not equipped to accomplish the required post- translational modifications or facilitate their molecular folding. Also, many proteins become insoluble as inclusion bodies that are often very difficult to recover without denaturants and subsequent protein-refolding procedures (Thermo Fisher Scientific, 2019).

In *E. coli* expression, the T7 system is the most popular approach for producing proteins. In this system, an expression vector containing a gene of interest cloned downstream of the T7 promoter is introduced into a T7 expression host. T7 expression hosts such as DE3 strains or T7 Express strains carry a chromosomal copy of the phage T7 RNA polymerase gene, usually under the control of a modified *Lac* promoter. When inducer is added, T7 RNA polymerase is expressed and transcribes the gene of interest found after the T7 promoter (Tabor, 2001).

1.7.2 Expression of proteins in yeast systems

Yeast expression systems allow efficient production of secreted and intracellular target proteins. In addition, yeast provides the advantage of eukaryotic post-translational modifications, which may be beneficial for some recombinant proteins (Baghban et al., 2019). Several yeast protein expression systems exist in various species, such as *Saccharomyces cerevisiae* and *Pichia pastoris*. A major component of any yeast expression system is the expression vector (Gomes et al., 2019). Vectors that integrate into the host chromosome are most widely used, because of their stable transfer to the next generation in the absence of a selection. However, episomal expression vectors, which do not integrate into the genome, exist for some yeast systems. Expression vectors typically contain a strong yeast promoter/terminator and a yeast selectable marker cassette. Many yeast expression vectors include the ability to optionally clone a gene downstream of an efficient secretory system-targeting sequence that efficiently directs a heterologous protein to be secreted from the cell.

A growing number of natural and engineered yeast strains are becoming available for protein expression. Strains have been described that increase yield of secreted proteins, improve the performance of certain affinity tags, reduce proteolysis, define the composition of N-glycans, and permit non-native amino acids to be incorporated into proteins (New England Biolabs, 2019; Gibco Education, 2015).

Pichia pastoris is a methylotrophic yeast, which can produce large amounts of recombinant proteins and has effective synthesize eukaryotic post-translationally modified proteins (Cregg et al., 2000). High levels of expression are often achieved by methanol induction of the alcohol oxidase 1 (AOX) promoter in *P. pastoris*. Researchers have achieved much higher yields of active plant glycoside hydrolases,

such as barley α- amylase (Juge et al., 1996) and Thai rosewood β-D-glucosidase (Ketudat Cairns et al., 2000), in *P. pastoris* than in *S. cerevisiae*. High-level expression of barley β-D-glucan exohydrolase HvExo1 in recombinant *P. pastoris* was accomplished from a codon- optimized cDNA, at the lowered expression temperature of 20 °C, to produce a high amount of enzyme (Luang et al., 2010). For instance, expression in a *P. pastoris* expression system was used to produce an enzyme that could not be produced in *E. coli* in this thesis.

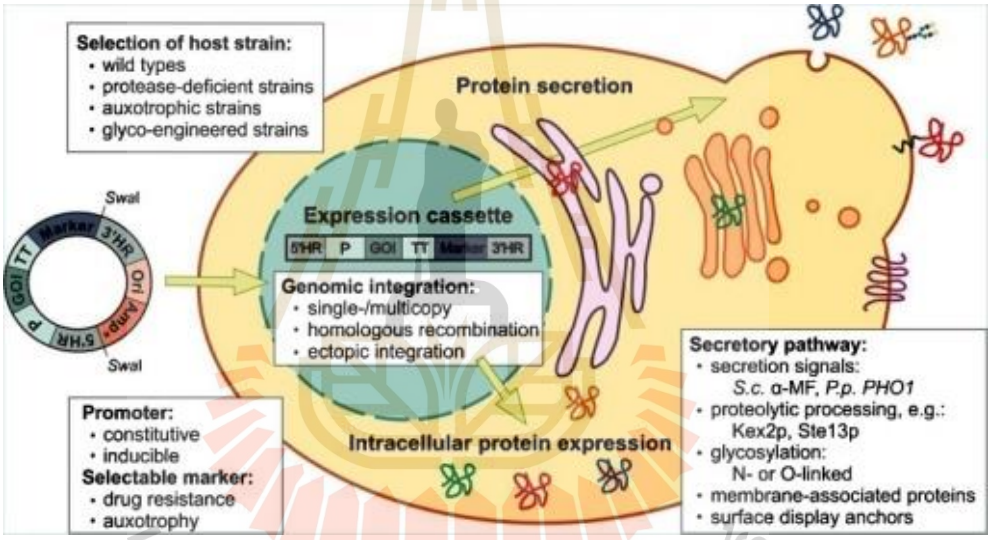

Figure 1.7 General considerations for heterologous gene expression in *P. pastoris*. Methylotrophic yeasts have two major features: (1) they are capable of growing to high cell densities even in unsophisticated fermentation processes; (2) their high demand for methanol- oxidizing enzymes during growth on alcohol endows them with very strong and strictly regulated promoters. These features make it possible for methylotrophic yeasts to be used not only in the process development for the commercial production of feed protein (single cell protein), but also as production systems for high value and scientifically interesting recombinant proteins. (Ahmad et al., 2014)

1.8 pET expression system

The pET vectors were originally constructed by Studier and colleagues (Studier and Moffatt, 1986). It is a powerful system for cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell (Moffatt and Studier, 1987; Rosenberg et al., 1987; Studier et al., 1990). T7 RNA polymerase is a very active enzyme that synthesizes RNA at a rate several times that of *E. coli* RNA polymerase and it terminates transcription less frequently. The pET vectors contain a T7 promotor upstream of the gene to be expressed (Tabor, 2001). The pET expression system is used in combination with *E. coli* (DE3) strains or with T7 phage infection to produce the T7 polymerase (Studier and Moffatt, 1986). Most often, they are produced in strains of bacteria containing the DE3 prophage, which includes a T7 polymerase gene under control of the lac_{UV5} promoter. The lac_{UV5} promoter, similar to the wild type promoter of the lac operon, can be induced by allolactose (generated from lactose by β-galactosidase inside the cell) and its molecular mimic isopropyl β-D-1-thiogalacto-pyranoside (IPTG) (Neubauer et al., 1992).

1.9 Auto-induction media

IPTG is a very strong inducer of the *lac* promoter and its derivatives that is not metabolized by *E. coli* (Gombert and Kilikian, 1998; Neubauer et al., 1992). Strong induction of transcription by IPTG may put a high metabolic burden on the cells via rapid production of the induced mRNA and protein, which may result in the formation of inactive aggregates of the recombinant target protein, called inclusion bodies. Thus,

lactose has been studied as alternative inducer. Lactose was found to be as effective as IPTG, to increase cell fitness, to reduce inclusion bodies formation, and to enhance the formation of soluble recombinant product.

Auto-induction media contain glucose, lactose, and glycerol, and are a simple and efficient approach for high-throughput protein expression in *E. coli* with lac promoter-derived expression systems (Studier, 2005). Its principle is based on the fact that the glucose blocks the activation of the lac operon, preventing the induction of the lac promoter by lactose before the depletion of glucose.

The presence of 0.05% glucose in auto-inducing media blocks induction by lactose in the early stage of growth so effectively that even strains containing plasmids to express target proteins that are highly toxic to the host cell can grow and maintain functional plasmid until induction (Studier, 2005). In the absence of glucose, amino acids appear to modulate or prevent induction of transcription of the genes for target proteins by lactose in the early stages of growth. Having a carbon and energy source other than lactose to support continued growth and production of target protein after induction enhances high-level production of target proteins from T7 expression strains. T7 RNA polymerase is so active that induction can direct most transcription and translation to the target protein (Studier, 2005).

1.10 Research objectives

The objectives of this study included:

1. To express active recombinant rice β-D-glucan exohydrolase 1(OsExo1).

2. To express active recombinant rice β-D-glucan exohydrolase2 (OsExo2).

3. To purify the recombinant OsExo1 and OsExo2 in order to characterize the activities of each specific enzyme in terms of its substrate specificity and biochemical properties.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmids, bacterial and yeast stains

The accession AK065044 cDNA clone plasmid that encodes the full-length precursor of OsExo2 was provided by the Rice Genome Resource full-length cDNA project (Kikuchi et al., 2003). A cDNA optimized for OsExo1 expression in *Pichia pastoris* (Genbank accession number AK073110) was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The vectors used for cloning and expression included pENTR™/D-TOPO (Invitrogen, CA, USA), pET32a (+) (Novagen, WI, USA), and pPICZαB(NH8) (Toonkool et al., 2006). The *Escherichia coli* stain DH5α was used for cloning and the Origami (DE3) strain was used for protein expression in *E. coli*. *P. pastoris* strain SMD1168H was used to express the OsExo1 protein.

2.1.2 Chemicals and reagents

Tryptone, yeast extract, sodium dodecyl sulfate (SDS), acrylamide, N, N′, N″, N‴- tetramethylethylenediamine (TEMED), ammonium persulfate(APS), Triton X-100, lysozyme, sodium hydroxide (NaOH), sodium acetate (NaOAc), sodium chloride (NaCl), sodium carbonate (Na_2CO_3), ethylene diamine tetra acetic acid (EDTA), glacial acetic acid, methanol, acetonitrile, HPLC-grade water, ethanol, 2,2′-azinobis (3-

ethylbenthaiazolinesulfonic acid) (ABTS), 3,3′,5,5′-tetramethyl benzidine (TMB), isopropyl thio-β-D-galactoside (IPTG), β-mercaptoethanol (BME), *p*-nitrophenol-β-D glucoside (*p*NPGlc), *p*NP-β-D-fucoside, *p*NP-α-L-arabinoside, *p*NP-β-D-galactoside, *p*NP-β-D-xyloside,*p*NP-β-D-mannoside, *p*NP-β-D-cellobioside, Coomassie brilliant blue R250, phenylmethyl-sulfonylfluoride (PMSF), calcium chloride ($CaCl_2$), ethidium bromide (EB), ammonium sulfate ($(NH_4)_2SO_4$), potassium dihydrogen phosphate (KH_2PO_4), manganese sulfate ($MnSO_4$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), copper sulfate pentahy-drate ($CuSO_4 \cdot 5H_2O$), calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), dipotassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), ammonium chloride (NH_4Cl), and potassium chloride (KCl) were purchased from variety of suppliers. Cellooligosaccharides with degrees of polymerization (DP) 3-6 and laminari-oligosaccharides of DP 2-6 were purchased from Segu-gaku Corp. (Tokyo, Japan) or Megazyme (Bray, Ireland), and Superscript first-strand cDNA synthesis system for RT-PCR and 100 bp DNA ladder were purchased from Invitrogen (Carlsbad, CA, USA).

2.2 General methods

2.2.1 Preparation of *E. coli* competent cells

Glycerol stocks of DH5α and XL1-Blue were streaked on LB plates without antibiotic. Origami(DE3) was streaked on an LB plate containing 15 µg/ml kanamycin and 12.5 µg/ml tetracycline and incubated at 37 °C for 16-18 hours. A single colony was picked and inoculated into 5 ml of LB broth with shaking at 37 °C, 200 rpm for

16-18 hours. One hundred microliters of starter culture were transferred to 100 ml of LB broth and shaken at 37 °C, 200 rpm until the optical density at 600 nm (OD600) reached 0.4-0.6. The cell culture was chilled on ice for 5 minutes in sterile polypropylene tube and collected at 4,000 rpm at 4 °C for 10 minutes. The cell pellets were resuspended in 10 ml ice-cold sterile 0.1 M $CaCl_2$ and centrifuged to collect the cell pellets again. Finally, the pellets were resuspended with 1 ml of 0.1 M $CaCl_2$ containing 15% glycerol and 50 µl aliquots were stored at -80 °C.

2.2.2 Transformation of plasmids into competent cells

An aliquot of frozen competent cells was thawed 5 minutes on ice, then 1 µl of cloning or expression plasmids (20-100 ng) or ligation reactions were mixed with fresh or thawed competent cells. The reaction was incubated on ice for 30 minutes. The plasmid was transformed by heat shocking the cells at 42 °C for 45 seconds and quickly chilled on ice for 5 minutes. Two hundred microliters of LB broth was added to the transformed competent cells, which were then incubated at 37 °C for 1 hour. The transformed cells were spread on LB agar containing appropriate antibiotics and incubated at 37 °C overnight.

2.2.3 Plasmid isolation by alkaline lysis method

A single colony of transformed recombinant bacteria was picked into 5 ml of LB broth and incubated at 37 °C with shaking at 200 rpm for 16-18 hours. The cultured cells were collected by centrifugation at 10,000xg, 1 minute. The supernatant was removed and the cells were resuspended in 100 µl of lysis buffer I (50 mM glucose, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). Then, 200 µl of freshly prepared lysis buffer II (0.2 N NaOH, 1% (w/v) SDS) was added and the tube was inverted 4-6 times. After that, 150 µl of ice-cold lysis buffer III (3 M potassium acetate, pH 4.8) was added and

the tube was mixed by inverting 4-6 times. The alkaline lysis reaction was incubated on ice for 5 minutes and the clear solution containing the plasmids was separated from the cell debris by centrifugation at 13,000xg, 10 minutes. The supernatant was transferred to a new tube and the DNA was precipitated with 2 volumes of absolute ethanol for 10 minutes at 4 °C. The precipitated DNA was collected by centrifugation at 13,000xg for 10 minutes. After removing the supernatant, the pellet was washed with 500 µl of 70% ethanol and centrifugation was repeated, followed by removal of the ethanol. The left-over solution was removed by speed vacuum. Then, the DNA pellet was resuspended in 100 µl TE buffer containing 2 µg RNase A and incubated at 37 °C for 10 minutes. The RNase A-treated plasmids were further purified by adding 70 µl of ice-cold precipitation solution (20% PEG 6000, 2.5 M NaCl) and chilled on ice for 1 hour. The precipitated DNA was collected by centrifugation at 13,000xg for 10 minutes. The supernatant was removed and the pellet was washed by adding 0.5 ml of 70% ethanol and inverting the tube twice, after that the ethanol solution was removed and the tube dried by speed vacuum. Finally, the DNA was re-dissolved with 30 µl of TE buffer or sterile water.

2.2.4 QIAGEN plasmid miniprep

The QIAprep® spin miniprep kit (QIAGEN) was used to purify recombinant plasmid DNA according to the manufacturer's instructions. A single colony was picked and inoculated in 5 ml LB broth with appropriate antibiotics and cultured at 37 °C overnight. The cultured cells were pelleted by centrifugation at 10,000xg for 1 minute. The cell pelleted was resuspended completely in 250 µl P1 buffer (100 mg/ml RNase A in 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). Two hundred fifty microliters of P2 buffer (200 mM NaOH, 1% (v/v) SDS) was added to the resuspended cells, and mixed

by inverting the tube gently 4-6 times until the solution became viscous and slightly clear. After that, 350 µl of P3 buffer (3 M potassium acetate, pH 5.5) was added and mixed immediately, to avoid localized precipitation, by inverting the tube gently 4-6 times. The solution was centrifuged at 12,000 rpm for 10 minutes to compact the white pellet. The supernatant was applied to a QIA prep column by pipetting and centrifuging at 12,000 rpm for 1 minute, and then the flow-through was discarded. To protect against nuclease activity or decrease carbohydrate content, 0.5 ml of PB buffer (1.0 M potassium acetate, pH 5.0) was added to the column, which was then centrifuged at 12,000 rpm for 1 minute. The column was washed 2 times by applying 0.75 ml PE buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol) and centrifuging at 12,000 rpm for 1 minute. The flow-through was discarded, and the column was centrifuged for an additional 1 minute to remove residual wash buffer. Lastly, the column was placed in a new 1.5 ml microtube and 50 µl distilled water was added to the center of column. The column was allowed to stand for 1 minute, and centrifuged at 12,000 rpm for 1 minute to elute the plasmid DNA.

2.2.5 Agarose gel electrophoresis for DNA

The purified plasmids and PCR products were checked by agarose gel electrophoresis. One percent agarose gels were prepared in TAE buffer (40 mM Tris-HCl, pH 8.0, 40 mM acetic acid, 1 mM EDTA, pH 8.0) or in TBE buffer (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2.5 mM EDTA, pH 8.0). The DNA samples were mixed 5:1 with 6X loading dye (0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 30% (v/v) sterilized glycerol). Agarose gel electrophoresis was performed in a Pharmacia GNA- 100 Gel Electrophoresis Apparatus (GE Healthcare, Buckinghamshire, UK) at a constant voltage of 120 V for 30 min. The DNA bands on

the agarose gel were detected by staining with ethidium bromide (0.1 μg/ml) 30 seconds and destained in distilled water for 5 minutes. The DNA bands were visualized by UV light transillumination with a Fluoro-S TM MultiImager (Bio-Rad). The sizes of the DNA bands were estimated by comparing their migration with those of 1 kb or 100 bp ladder (Fermentas, Burlington, ON, Canada).

2.2.6 Purification of DNA bands from gels

The correct size DNA bands that had been separated on agarose gel electrophoresis were purified with a HiYield™ Gel/PCR DNA fragments extraction kit (RBC Bioscience Corp., Taiwan). The agarose gel containing the target DNA band was sliced with a blade cutter and not more than 300 mg was transferred to a microtube. The agarose gel purification was done according to the manufacturer's instructions.

2.2.7 Protein analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile and the apparent molecular weights of proteins in various fractions were determined by SDS-PAGE, as described by Laemmli (1970). The 12% SDS-PAGE separating gel consisted of 12% (w/v) acrylamide, 375 mM Tris- HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% TEMED, while the 4% stacking gel consisted of 4% (w/v) acrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% TEMED. Protein samples were mixed 5:1 with 6X loading buffer (50 mM Tris-HCl, pH 6.8, 10% SDS, 0.2 mg/ml bromophenol blue, 50% glycerol, 20% β-mercaptoethanol) and boiled for 5 minutes to denature proteins. Twenty microliters of protein samples were loaded into sample wells, and electrophoresed through the polymerized gel at 170 V with Tris-glycine electrode buffers (50 mM Tris base, 125 mM glycine and 0.1% SDS, pH 8.3) until the dye front

reached the bottom of the gel plate. The gels were subsequently stained in staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, and 10% (v/v) acetic acid in water for 30 minutes and destained with destaining solution (40% (v/v) methanol and 10% (v/v) acetic acid) for 1-2 hours. The molecular masses of protein bands were determined by comparing to standard low molecular weight protein markers (GE Healthcare, Uppsala, Sweden), which consist of 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 kDa).

2.2.8 Determination of protein concentration

The protein concentration was determined by the Bio-Rad Bradford assay (Hercules, CA, USA). The bovine serum albumin (BSA) was used as a standard ranging from 0.1-5 µg. Each concentration was mixed with 200 µl of Bio-Rad protein assay solution and made up to 1 ml with distilled water. The mixture was incubated at room temperature for 10 min. The absorbance was measured at a wavelength of 595 nm (A595) with the Protein Bradford program of a NanoDrop 2000 spectrophoto-meter (Thermo Scientific, MA, USA).

2.2.9 Preparation of *P. pastoris* SMD1168H competent cells

A glycerol stock of *P. pastoris* strain SMD1168H was streaked on a yeast extract peptone dextrose (YPD) plate without antibiotic, which was then incubated at 28 °C for 2-3 days. A single colony was inoculated into 5 ml YPD broth and grown at 28 °C with 220 rpm overnight. The starter culture was transferred into 500 ml YPD broth and grown until the OD600 reached 1.3-1.5. The cells were collected by centrifugation at 1,500xg for 5 minutes at 4 °C. The pellet was washed 2 times in 500 ml and 250 ml of ice-cold sterile water and collected by centrifugation at 1,500xg for 5 minutes at 4 °C

each time. Next, the pellet was resuspended with 20 ml of ice-cold 1 M sorbitol and centrifuged at 1,500xg for 5 minutes at 4 °C. Finally, the pellet was resuspended and kept in 1 ml of ice-cold 1 M sorbitol and 80 µl aliquots per tube was used for transformation.

2.3 Cloning, Expression and Purification of OsExo1

2.3.1 Cloning of optimized OsExo1 cDNA into the pPICZαBNH8 Vector

An optimized OsExo1 was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The plasimid containing the optimized OsExo1 cDNA was transformed into DH5α competent cells and spread onto LB agar containing 50 µg/ml ampicillin. The colonies were picked and inoculated into LB media containing 50 µg/ml ampicillin and incubated at 37 °C overnight. The plasmid was extracted with a QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany). The optimized OsExo1 and pPICZαBNH8 plasmid (Toonkool et al., 2006) were cut with *Pst*I and *Xba*I. The gel purified OsExo1 insert and pPICZαBNH8 plasmid were ligated and transformed into DH5α bacteria, as described in section 2.2.2, and colonies selected on 25 µg/ml zeocin. Colonies were picked and plasmid DNA extracted, as described in section 2.2.4. The recombinant gene insert was checked by cutting the plasmid clones with *Pst*I and *Xba*I and evaluating the products by agarose gel electrophoresis, and the sequence confirmed by automated DNA sequencing at Macrogen Corp. (Seoul, Korea).

2.3.2 DNA preparation and electroporation

The optimized pPICZαBNH/OsExo1 plasmid was linearized with *Sac*I and the reaction was incubated at 37 °C overnight. Linearization of the plasmid was checked

by electrophoresis of 2 µl of reaction on a 1% agarose gel. The restriction enzyme was inactivated by heating at 65 °C for 10 min. Linear DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol, and then incubating at -20 °C for 30 minutes. Precipitated DNA was collected by centrifugation at 12,000xg for 10 minutes. The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged at 12,000xg for 10 min. All ethanol was removed by inverting the tube on tissue paper for 10 min. The DNA pellet was dissolved in 5-10 µl of sterile HPLC water.

The linearized recombinant pPICZαBNH/OsExo1 vector (5-10 µg) was add to a microcentrifuge tube containing 80 µg of *P. pastoris* competent cells. The cell mixture was transferred to a pre-cooled 0.1 cm electroporation cuvette. The cuvette with the cells was incubated on ice for 5 minutes. Then, linearized recombinant pPICZαBNH/ OsExo1 vector was transformed into *P. pastoris* competent cells by electroporation (Bio-Rad) with the parameters of 1.5 kV, 25 µF and 400 Ω (Pichia manual, Invitrogen). Immediately after electroporation 1 ml containing 500 µl of YPD and 500 µl of 1 M sorbitol was added to the electroporated cells, they were mixed by pipetting and they were incubated at 30 °C for 1 hour without shaking. Then 100, 200 and 500 µl of the transformation mixture were spread on Yeast Extract Peptone Dextrose medium with sorbitol (YPDS) plates containing 100 µg/ml zeocin. The YDPS plate was incubated at 28 °C for 3-5 days. The transformed colonies were picked and selected again by streaking them on a YPD plate containing 250 µg/ml zeocin.

2.3.3 Expression of OsExo1

A single colony that had been selected on a 250 µg/ml zeocin YPD plate was inoculated into 100 ml of buffered glycerol-complex medium (BMGY) medium

containing 100 µg/ml zeocin and grown in a shaking incubator (220 rpm) at 28 °C until the cell culture OD_{600} reached 2-3. Cells were harvested by centrifugation at 3000xg for 5 minutes at 20 °C and resuspended in 200 ml of buffered methanol-complex medium (BMMY) medium at the final OD_{600} of 1. The protein expression was induced by adding methanol to 1% (v/v) final concentration every 24 hours for 5 days at 20 °C, and the activity in the media was checked every day.

2.3.4 Purification of OsExo1

The protein was purified from the culture broth after removal of the cells by centrifugation. The pH of the culture broth with secreted protein was adjusted to 8.0 with 1 M Na_2CO_3 and it was loaded onto an immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare,Buckinghamshire, United Kingdom) charged with Ni^{2+}, and the column was washed with 5 column volumes of 50 mM Tris-HCl, pH 8.0, then the protein was eluted with gradient of 250 mM imidazole, 300 mM NaCl in 50 mM Tris-HCl, pH 8.0.

2.4 Cloning, Expression and Purification of OsExo2

2.4.1 Cloning of OsExo2

OsExo2 was cloned from the Genbank accession number AK065044 cDNA clone plasmid provided by the Rice Genome Resource full-length cDNA project (Kikuchi et al., 2003). A fragment of the cDNA that encoded the predicted mature rice OsExo2 gene was amplified with with *Pfu* DNA polymerase and the OsExo2 MatF (5′ CACCATGGCTCAGTATGTCAAGTATAAGG-3′) and OsExo2 StopR (5′-AAAGCTTGCCGCATCAGCTGCTGTG-3′) primers. The PCR product of mature

OsExo2 was combined with pENTR-D-TOPO from a pENTR™ Directional TOPO® Cloning Kit, in a 2:1 molar ratio of purified PCR product:TOPO vector, to which were added 1 µl of salt solution and sterile water to 6 µl final volume. The topoisomerase reaction was incubated at room temperature overnight, and the mixture was used to transform chemically competent DH5α *E. coli*. The transformed competent cells were spread onto an LB plate containing 15 µg/ml kanamycin, then incubated at 37 °C overnight. The colonies that had grown overnight were picked, and extracted by the QIAGEN minipreparation method (Section 2.2.4). The cDNA insert sequences of the plasmids with proper sized inserts were determined by automated DNA sequencing at Macrogen Corp. (Seoul, South Korea).

The recombinant pENTR-D-TOPO plasmid containing the mature OsExo2 was recombined with pET32a/DEST (Opassiri et al., 2006) by LR Clonase recombination reaction to insert the OsExo2 into pET32a/DEST. The LR recombination reaction was transformed into *E. coli* competent cells by the $CaCl_2$ heat shock method (section 2.2.2) and selected on LB-agar containing 50 µg/ml ampicillin at 37 °C overnight. The colonies were picked and inoculated into 5 ml LB media containing 50 µg/ml ampicillin and incubated at 37 °C, shaking at 200 rpm overnight. The recombinant plasmids were extracted from the cultures by the alkaline lysis method (section 2.2.3).

2.4.2 Expression screening of OsExo2

The circular recombinant pET32a/DEST plasmid containing the gene of OsExo2 (pET32a/ DEST/ OsExo2) was transformed into *E. coli* Origami(DE3) , Rosettagami(DE3), and BL21(DE3) competent cells by heat shock (section 2.2.2) and the cells were selected on an LB plate containing 15 µg/ml kanamycin, 12.5 µg/ml tetracycline and 50 µg/ml ampicillin for *E. coli* strain Origami(DE3); containing 34

µg/ml chloramphenicol, 15 µg/ml kanamycin, 12.5 µg/ml tetracycline, and 50 µg/ml ampicillin for *E. coli* strain Rosettagami(DE3), or containing 50 µg/ml ampicillin for *E. coli* BL21(DE3). The culture plates were incubated at 37 °C for overnight. One colony of transformed cells were picked and used to inoculate 10 ml LB medium containing appropriate antibiotics. After culture of the cells overnight, 2% of the overnight starter culture were inoculated in LB medium containing appropriate antibiotics at 37 °C for 4 hours while shaking at 200 rpm.

The expression conditions were optimized by varying the IPTG concentration (0.1, 0.2, 0.3, 0.4, and 0.5 mM) and induction temperatures (15 °C, 20 °C, and 25 °C), the cells were collected after 18 hours of incubation by centrifugation for 10 minutes at 3,000xg. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 200 µg/ml lysozyme, 1% Triton-X100, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.25 mg/ml DNaseI), then sonicated and incubated at room temperature for 30 minutes. The soluble protein was separated from cell debris by centrifugation at 12,000xg for 20 minutes at 4 °C.

The protein expression levels were determined by checking the activity to hydrolyze *p*NPGlc in the soluble fractions of the cell lysates, and the protein expression patterns were evaluated by SDS-PAGE.

2.4.3 Expression of OsExo2

A colony containing pET32a/DEST/OsExo2 in *E. coli* Origami(DE3) cells was picked and used to inoculate 100 ml LB medium containing 15 µg/ml kanamycin, 12.5 µg/ml tetracycline and 50 µg/ml ampicillin, and the resulting culture was grown at 37 °C overnight. The starter culture was added to 2% to a large scale (4 liters total) of auto-

induction medium (AIM) and grown continuously at 37 °C with shaking at 200 rpm until the OD_{600} reached 0.8-1. The culture was transferred to 15 °C for 24 hours to allow the protein production to be induced at low temperature. Cells were collected by centrifugation at 4500 rpm for 10 min at 4 °C, and the pellet was stored at -80 °C.

2.4.4 Purification of OsExo2

The frozen pellet was thawed at room temperature. The cells were resuspended with extraction buffer (20 mM Tris-HCl, pH 8.0, 200 µg/ml lysozyme, 1% Triton-X100, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.25 mg/ml DNaseI), sonicated and incubated at room temperature for 1 hour. Soluble protein was separated from cell debris by centrifugation at 12,000xg for 20 minutes at 4 °C. Recombinant OsExo2 was purified with 3 steps. First, crude protein was immediately loaded into a Ni^{2+} (IMAC) resin with equilibration buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and the recombinant protein was eluted with a gradient of imidazole 0-260 mM (Imidazole in equilibration buffer), the fractions with activity were pooled. Next, the recombinant protein was mixed with NaCl to 2 M NaCl and loaded onto a Phenyl Sepharose column (5 ml), which was eluted with a gradient of 2-0 M NaCl, followed by a second gradient of 0-20% ethylene glycol in 50 mM Tris-HCl, pH8, with a flow rate of 0.5 ml/min. The fractions containing activity to hydrolyze *p*NPGlc were pooled and concentrated with a 30 kDa molecular-weight cutoff ultrafiltration membrane. Then, 0.5 ml of concentrated protein was loaded onto a Superdex 200 gel filtration column that had been equilibrated with 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, and run at a flow rate of 0.3 ml/min in the same buffer. Finally, protein fractions were pooled and kept on ice.

2.5 Enzyme assay, pH and temperature optimum and stability studies

The optimum pH values of the enzymes were determined in 140 µl of reactions containing 1 mM *p*NPGlc in citrate-phosphate universal buffer with pH values in the range of 3-9 at 0.5 pH unit increments. The enzyme activity was assayed at 30 °C for 15 minutes and the reaction stopped by adding 70 µl of 2 M sodium carbonate. Enzyme activity was measured as *p*-nitrophenol released, based on the absorbance at 405 nm.

The optimum temperature for enzyme activity was determined by pre-incubating the purified enzyme at temperatures ranging from 10 °C to 65 °C at 5 °C increments for 10 minutes, and then pre-incubated enzyme was incubated with 1 mM *p*NPGlc in 50 mM buffer at the optimum pH, in a reaction volume of 140 µl at the same temperatures for 30 minutes, and then 70 µl of 2 M Na_2CO_3 was added to stop the reaction, and the enzyme activity was measured, as described above.

The thermostability of the purified enzyme was determined by pre-incubating the enzyme in 50 mM sodium acetate, pH 5.0, at temperatures between 20-80 °C for 15 min. Then, the enzyme was immediately cooled down on ice for 3 minutes. Enzyme activity was assayed in 140 µl of the reaction containing 1 mM *p*NPGlc at 30 °C for 15 minutes. The reaction was stopped with 70 µl of 2 M sodium carbonate and the *p*-nitrophenol released measured via its absorbance at 405 nm.

2.6 Substrate specificity

The purified enzyme activity was assayed with 1 mM substrate, in 50 mM sodium acetate, pH 5.0, at 30 °C for 15 minutes. The activity was tested for synthetic substrates,

oligosaccharides and polysaccharides. The synthetic substrates consisted of *p*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-L-arabinopyranoside, and *p*NP-β-D-cellobioside, which were assayed in 140 µl reaction volumes, and the reactions stopped with 70 µl of 2 M sodium carbonate.

The peroxidase/glucose oxidase-based assay (PGO assay), was used to measure glucose released in reactions with oligosaccharides and polysaccharides. The reaction of oligosaccharides (laminarioligosaccharides DP 2-5, cellooligosaccharide DP 2-5) and polysaccharides (laminarin, barley (1,3;1,4)-β-D-glucan, and lichenan) were assayed in 50 µl reactions, which were stopped by boiling at 100 °C for 5 minutes. Then 40 µl of a reaction was placed in a microtiter plate well and 50 µl of 2,2′-azinobis (3-ethylbenthaiazolinesulfonic acid) (ABTS) and 100 µl of PGO were added to each reaction. The reactions were incubated at 37 °C for 30 minutes. The glucose release was measured from the absorbance at 450 nm compared to a glucose standard curve generated in the same buffer and PGO reaction conditions.

2.7 Enzyme kinetics

Kinetic parameters of purified protein were determined in triplicate reactions for hydrolysis of pNP-glucoside, oligosaccharide and polysaccharide (laminarin, barley (1,3;1,4)-β-D-glucan and lichenan). Initially, a time course was run for each substrate and condition to determine the amount of enzyme and time that gave a linear time course, indicating initial velocity. The substrate concentrations used covered a from one third to three times the apparent K_M or broader. The K_M and V_{max} values were calculated

by fitting the rate of product formation and substrate concentrations from non-linear regression analysis of Michaelis-Menten plots with Grafit 5.0 (Erithacus Software, Horley, Surrey, U.K.). The apparent catalytic rate constant (k_{cat}) values are calculated by dividing the maximum velocity (V_{max}) by the total amount of enzyme protein in the reaction.

2.8 Effects of EDTA, metal salts and inhibitors on enzyme activity

The effects of EDTA, metal ions and inhibitors on OsExo1 and OsExo2 activity were studied by pre-incubating purified enzyme with 1 mM concentration of EDTA and metal ions, 20 µM and 40 µM of 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoroglucopyranoside, 1 mM and 5 mM of glucono-δ-lactone inhibitors in 50 mM sodium acetate, pH 5.0, at 30 °C for 30 minutes, and then enzyme activity to hydrolyze 5 mM *p*NPGlc at 30 °C for 30 minutes was determined. The enzyme activity was detected as described above the absorbance compared with the reaction preincubated and incubated in the same way without inhibitor.

CHAPTER III

RESULTS

3.1 Cloning and expression of OsExo2

The cDNA encoding the mature OsExo2 protein was amplified from the Genbank accession number AK065044 cDNA clone plasmid provided by the Rice Genome Resource full-length cDNA project (Kikuchi et al., 2003) and ligated into the pET32a expression vector (Figure 3.1).

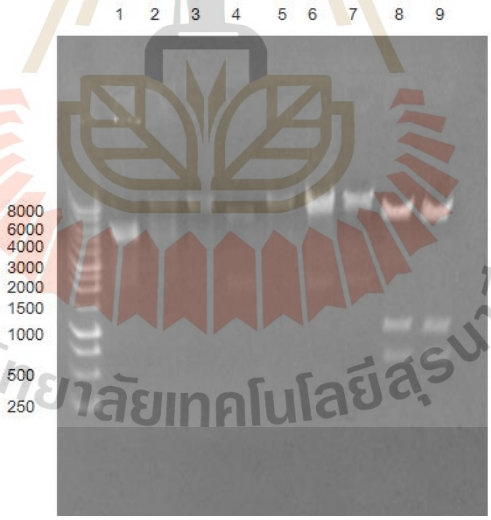

Figure 3. 1 Recombinant pET32a/ DEST plasmid containing the OsExo2 cDNA (pET32a/ DEST/ OsExo2). The plasmid was separated on 1% agarose gel electrophoresis and stained with ethidium bromide. Lane M, Thermo Scientific GeneRuler 1kb DNA ladder, Lanes 1-9, pET32a/DEST/OsExo2 plasmid after cut with *EcoR*I.

Expression of OsExo2 was tested with the recombinant pET32a/DEST/OsExo2 plasmids in *E. coli* strains OrigamiB(DE3) (Figures 3.2-3.4), Rosetta-gami(DE3) (Figures 3.5-3.7) and BL21(DE3) (Figure 3.8-3.10). The concentration of IPTG used for induction was varied from 0 to 0.5 mM at 15 to 25 °C for 18 h. The activity of this enzyme could be detected in soluble cell lysates of OrigamiB(DE3) with *p*NPGlc substrate. So, OrigamiB(DE3) was used as host cells for OsExo2 expression. OsExo2 enzyme could be expressed at all concentrations of IPTG. However, cells induced with 0.1 mM IPTG had the highest activity to hydrolyze *p*NPGlc. Thus, the *E. coli* strains OrigamiB(DE3) was used to express OsExo2 enzyme and induced with 0.1 mM IPTG at 20 °C. However, SDS-PAGE showed that the protein band of OsExo2 was found at low concentration in the soluble cell lysate and was mostly in the insoluble pellet. So, Auto-induction media was used to over express OsExo2 in *E. coli* to increased protein yields.

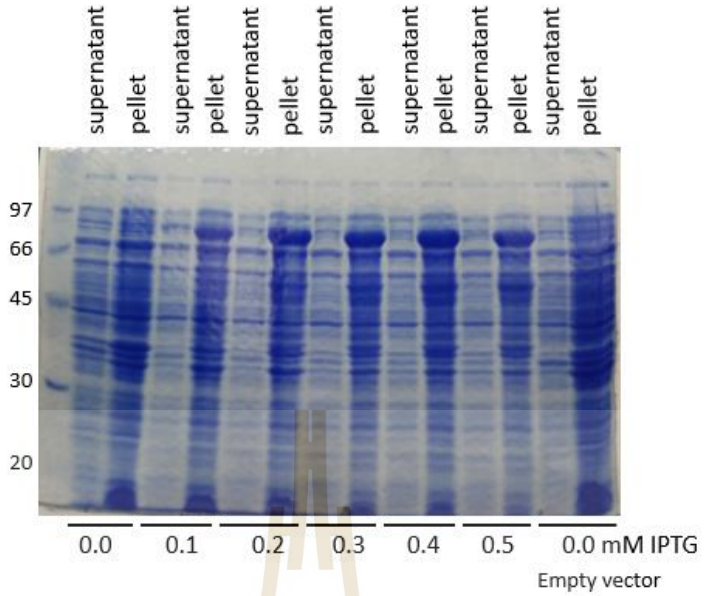

Figure 3.2 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Origami B(DE3) induced with 0 to 0.5 mM IPTG at 15 °C for 18 hours.

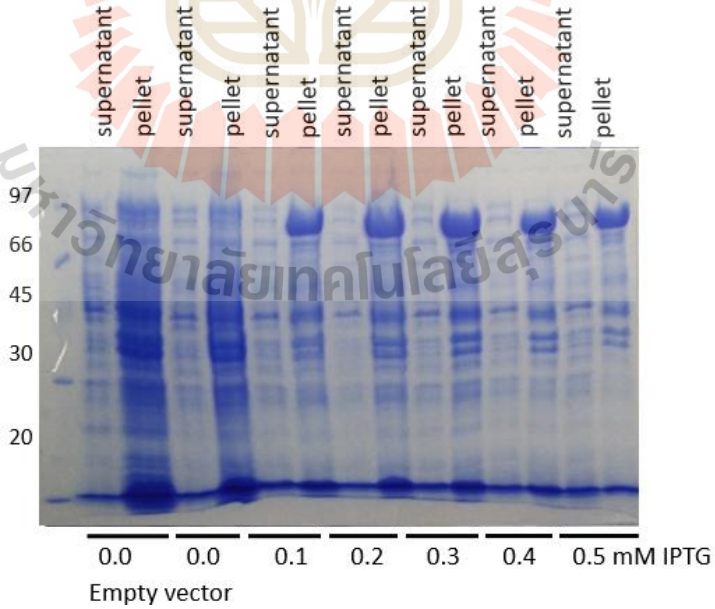

Figure 3.3 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Origami B(DE3) induced with 0 to 0.5 mM IPTG at 20 °C for 18 hours.

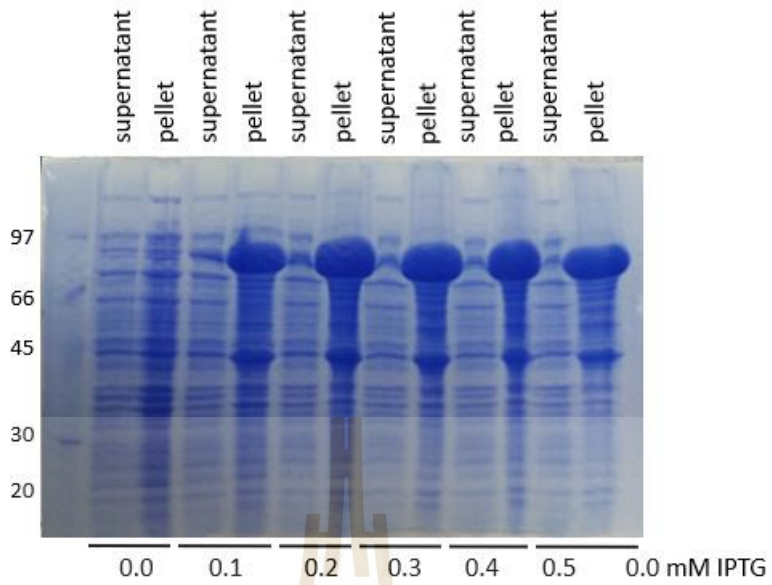

Figure 3.4 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Origami B(DE3) induced with 0 to 0.5 mM IPTG at 25 °C for 18 hours.

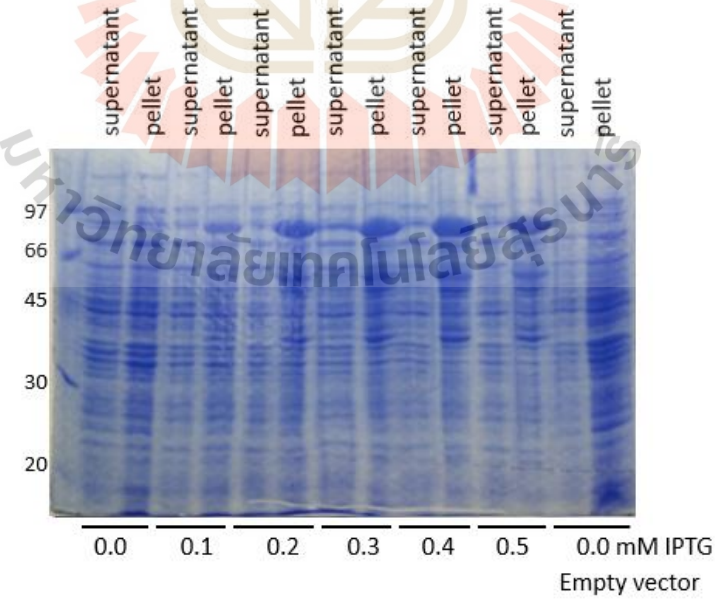

Figure 3.5 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Rosetta-gami(DE3) induced with 0 to 0.5 mM IPTG at 15 °C for 18 hours.

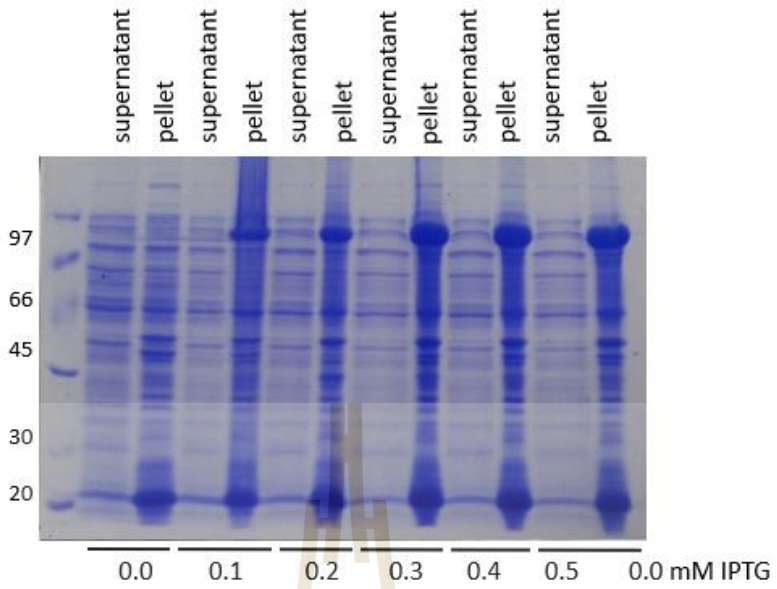

Figure 3.6 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Rosetta-gami(DE3) induced with 0 to 0.5 mM IPTG at 20 °C for 18 hours.

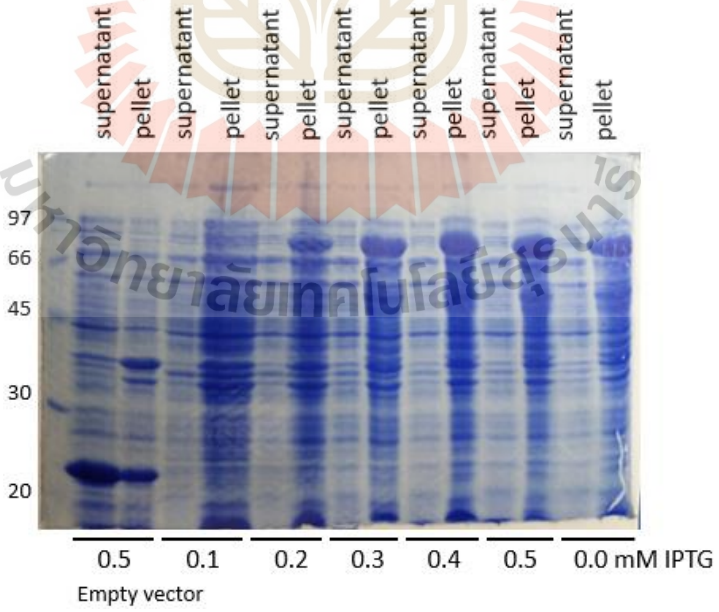

Figure 3.7 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Rosetta-gami(DE3) induced with 0 to 0.5 mM IPTG at 25 °C for 18 hours.

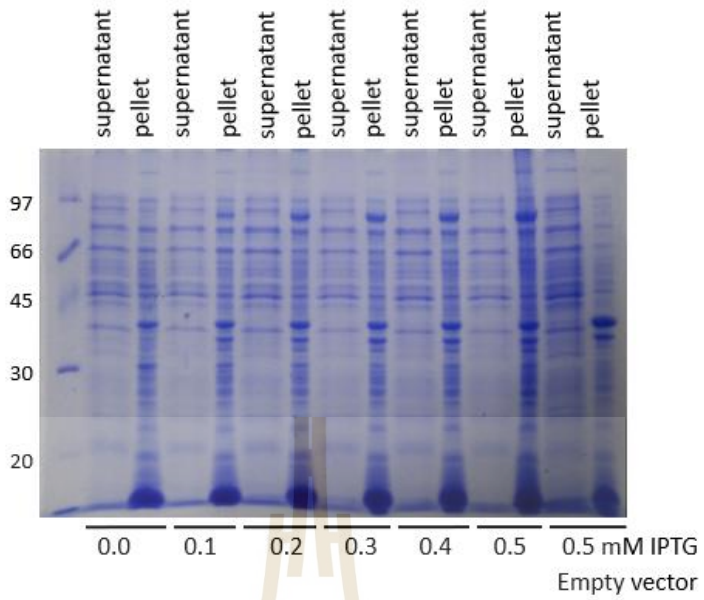

Figure 3.8 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in BL21(DE3) induced with 0 to 0.5 mM IPTG at 15 °C for 18 hours.

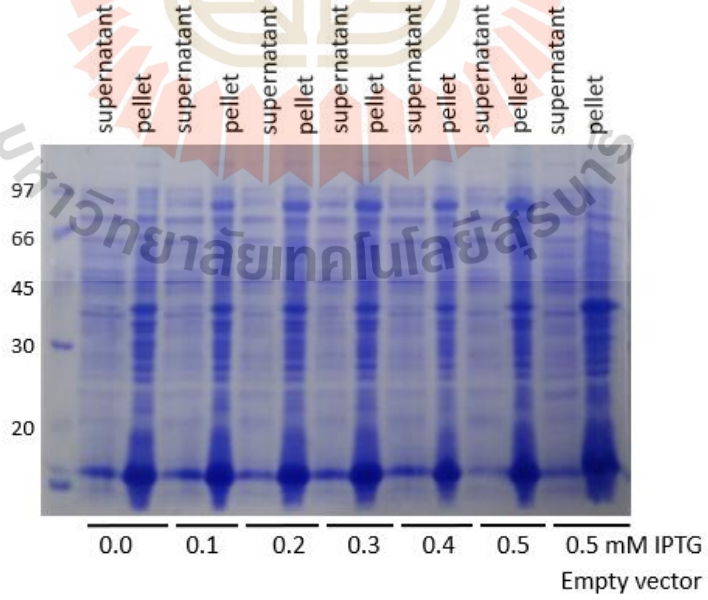

Figure 3.9 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in BL21(DE3) induced with 0 to 0.5 mM IPTG at 20 °C for 18 hours.

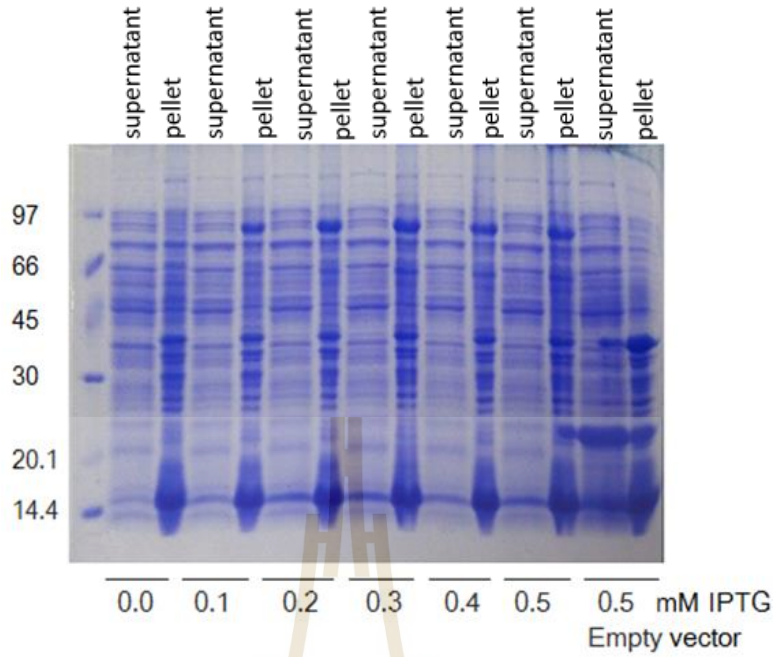

Figure 3.10 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in BL21(DE3) induced with 0 to 0.5 mM IPTG at 25 °C for 18 hours.

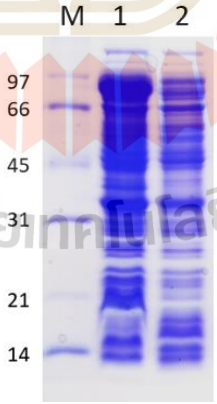

Figure 3.11 SDS-PAGE analysis of pET32a/DEST/OsExo2 expression in Origami B(DE3) with auto-induction medium (AIM). Cultures were induced at 15 °C for 24 hours. Lane M, Bio-Rad low molecular weight markers; Lane 1, insoluble protein from Origami B(DE3); Lane 2, soluble protein from Origami B(DE3).

3.2 Cloning and expression of OsExo1

A gene optimized for OsExo1 (Genbank accession number AK073110) expression in *Pichia pastoris* was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The optimized OsExo1 cDNA was inserted into the pPICZαBNH plasmid (Toonkool et al., 2006), which was then linearized with *Sac*I and transformed into *P. pastoris* by electroporation (Figure 3.12).

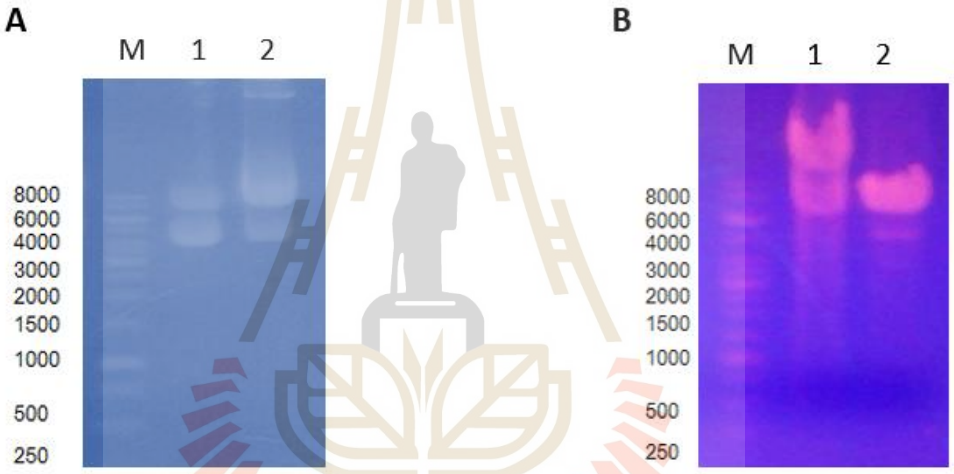

Figure 3.12 Verification of the pPICZαBNH/ OsExo1 plasmid by agarose gel electrophoresis. The 1% agarose gels were stained with ethidium bromide. A. The pPICZαBNH/ OsExo1 plasmid. Lane M is Thermo Scientific GeneRuler 1kb DNA ladder and Lanes 1 and 2 are pPICZαBNH/ OsExo1 plasmid. B. The linearized recombinant pPICZαBNH/ OsExo1 plasmid before and after digestion with *Sac*I and ethanol precipitation. Lane M is Thermo Scientific GeneRuler 1 kb DNA ladder and Lane 1 uncut and Lane 2 linearized recombinant pPICZαBNH/OsExo1.

The pPICZαBNH/OsExo1 plasmid was cloned into *P. pastoris* strain SMD1168H, and protein expression was induced with 1% (v/v) methanol for 6 days at 20 °C. The activity for hydrolysis of *p*NPGlc in the media of the 7 clones was checked. Clone 7 had the highest activity, which was maximal at 5 days induction (Figure 3.13).

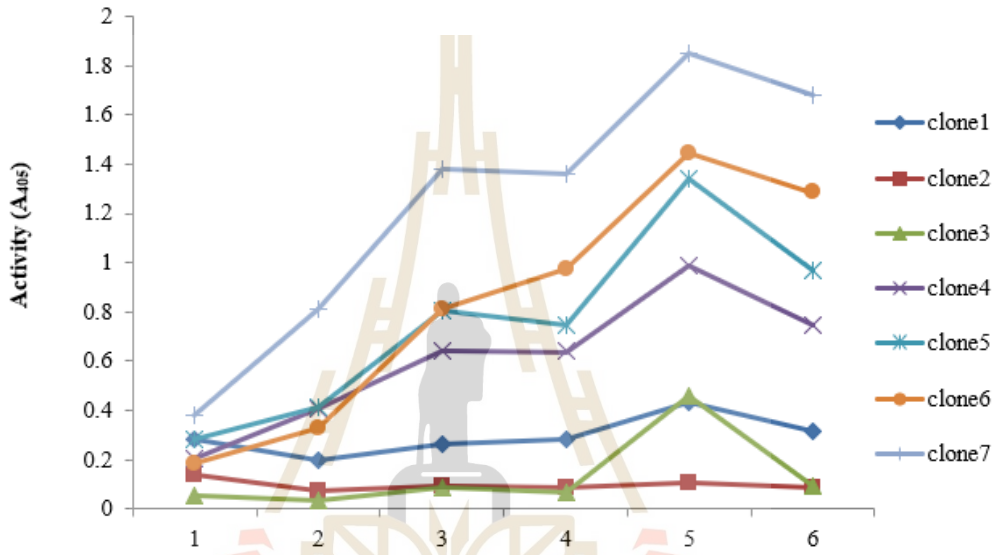

Figure 3.13 The *p*NPGlc hydrolysis activity in the media of each of the clones expressing OsExo1 over 6 days. The activity was determined by incubating 50 µl of media from induced cultures with 5 mM *p*NPGlc, in 50 mM NaOAc, pH 5.0, at 30 °C for 30 minutes. The activity is plotted as OD_{405} versus the day.

3.3 Purification of OsExo2

After expression in the autoinduction system from Origami B(DE3) containing pET32a/OsExo2, recombinant OsExo2 containing thioredoxin and 6x histidine-tag was purified and concentrated from host proteins by Ni^{2+} IMAC (Figure 3.14). The fractions containing activity were pooled, and OsExo2 was further purified by phenyl sepharose

column chromatography (Figure 3.15). The fractions containing activity were analyzed on SDS-PAGE and concentrated in a 30 kDa molecular-weight cutoff ultrafiltration membrane. Finally, OsExo2 was purified by gel filtration chromatography over a Superdex S200 gel filtration column, the fractions containing activity were analyzed on SDS-PAGE (Figure 3.16). The OsExo2 was produced at approximately 90 kDa and approximately 90% pure, as judged by SDS-PAGE (Figure 3.16) and the specific activity of OsExo2 was increased by about 36-fold by the end of the purification (Table 3.1). A comparison of the protein in the pooled fractions after each step is shown in Figure 3.17.

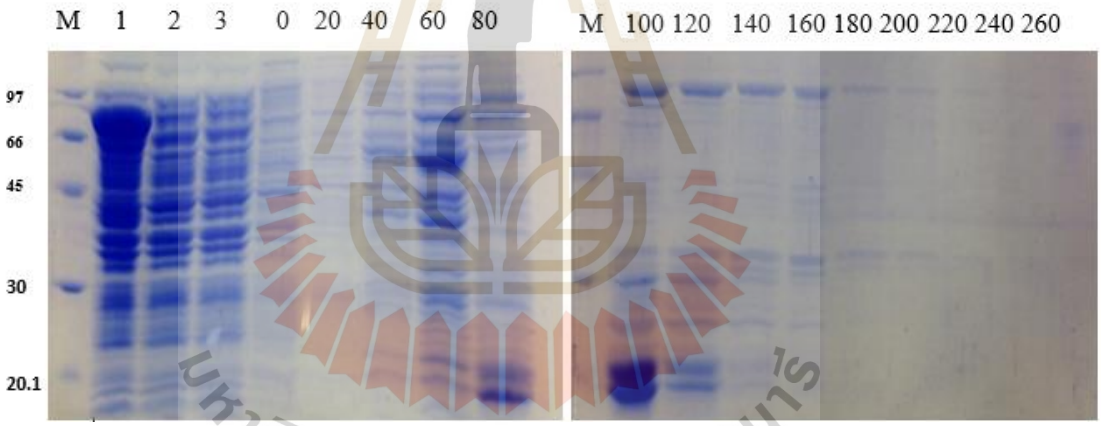

Figure 3.14 SDS-PAGE analysis of OsExo2 purified by immobilized affinity chromatography (IMAC). Lane M, Bio-Rad low molecular weight markers; lane 1, cell pellet; lane 2, crude soluble protein extract of induced Origami B(DE3) cells; lane 3, flow-through fraction of proteins that passed through the column. Further lanes are labeled by the imidazole concentration (in mM) at which the samples in them were eluted.

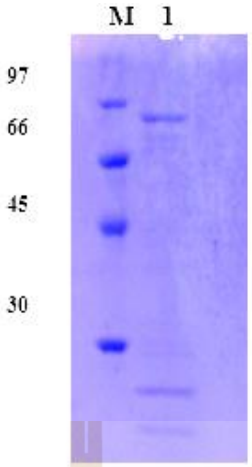

Figure 3.15 SDS-PAGE analysis of OsExo2 purified by phenyl sepharose chromatography. M, Bio-Rad low molecular weight markers; lane 1, OsExo2 purified by Phenyl Sepharose chromatography from the pool of IMAC fractions that contained activity to hydrolyze *p*NPGlc.

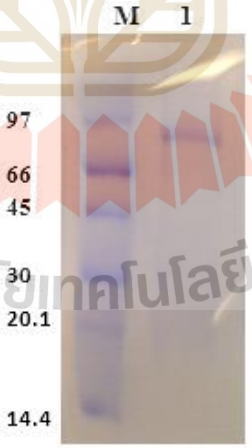

Figure 3.16 SDS-PAGE analysis of OsExo2 purified by S200 gel filtration chromatography after IMAC and Phenyl Sepharose chromatography. Lane M, Bio-Rad low molecular weight markers; lanes 1, pool of fractions which contained activity to 1 mM *p*NPGlc.

Table 3.1 OsExo2 enzyme specific activity and yields during purification.

Purification Step	**Protein concentration (mg/ml)**	**Total volume (ml)**	**Total protein (mg)**	**Total activity**	**Specific activity**	**Fold Purification**	**Percent Yield**
Crude	10.45	150	1,568	20.31	0.013	1	100
IMAC	1.25	27	33.75	9.33	0.28	21.5	45.9
Phenyl Sepharose	3.27	1	3.27	1.48	0.45	34.6	7.28
Gel filtration	1.76	1	1.76	0.82	0.47	36.2	4.03

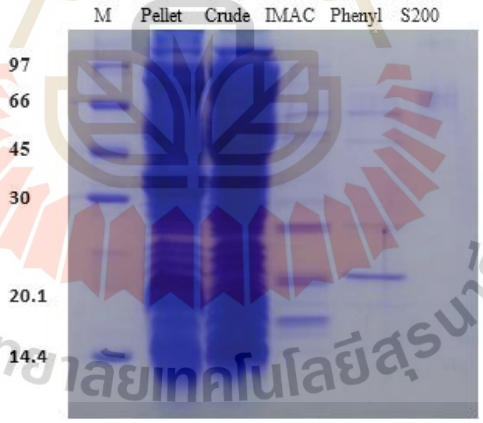

Figure 3.17 SDS-PAGE analysis of OsExo2 purified by IMAC, phenyl sepharose chromatography and S200 gel filtration chromatography. Lane M, Bio-Rad low molecular weight markers; Pellet is the insoluble fraction of the bacterial cells from the expression, Crude is the soluble fraction extracted from those cells, IMAC, Phenyl and S200 are the pools of fractions containing activity from IMAC, phenyl sepharose and gel filtration chromatography steps, respectively.

3.4 Purification of OsExo1

The clone with the highest β-glucosidase activity from the optimized OsExo1 cDNA was used to produce the secreted, N-terminally His_8-tagged protein in *P. pastoris*, and the recombinant protein was induced and purified from the media by IMAC. β-Glucosidase activity was detected in the media, and a broad protein band was detected at about 90 kDa on the SDS-PAGE gel (Figure 3.18 lane 2). To test whether the broadness of the protein band was due to glycosylation, the protein was deglycosylated by digestion with endoglycosidase H and a single band of approximately 70 kDa was detected on a Coomassie-stained SDS-PAGE gel (Figure 3.18 lane 3).

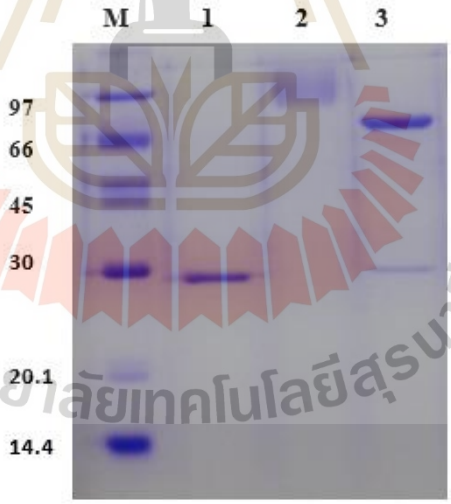

Figure 3.18 SDS-PAGE analysis of OsExo1 protein expressed in *Pichia pastoris*. Lane M, standard protein marker; Lane 1, endoglycosidase H; Lane 2, OsExo1 protein before deglycosylation; Lane 4, OsExo1 protein after deglycosylation with endoglycosidase H.

3.5 Effect of pH and temperature on the activity and stability of OsExo2

As shown in Figure 3.19, the OsExo2 activity versus pH profile was a bell-shaped curve, with the activity increasing from pH 3.0 to 4.5, where it reached the maximum and maintained approximately 90% activity at pH 5.0, then rapidly decreased from pH 5.0 to 6.5. OsExo2 was not significantly active above pH 7. Therefore, the activity of OsExo2 at temperatures between 30 °C and 65 °C was determined at pH 5. The highest activity was achieved at 40 °C, with a rapid drop in the activity at 45 °C (Figure 3.20). For the temperature stability of OsExo2, the activity was assayed after incubation at temperatures over the range between 10 and 80 °C for 15 minutes (Figure 3.21). The OsExo2 was stable at the temperature range between 10 °C to 30 °C. Above this temperature range, the activities of OsExo2 decreased and it appeared to be denatured within 15 minutes at 60 °C and showed no activity after incubation at higher temperatures.

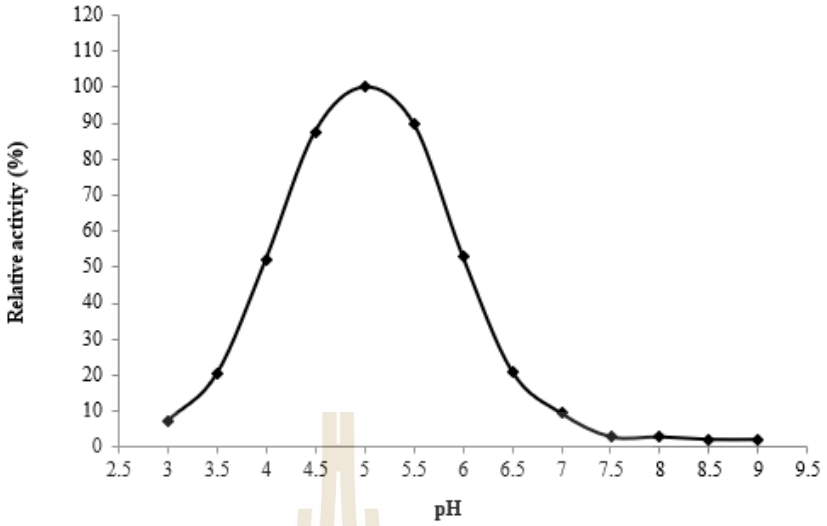

Figure 3.19 Relative activity versus pH profiles of OsExo2. The purified enzyme was incubated with 1 mM *p*NPGlc substrate in 50 mM universal citrate-phophate buffers with pH over the range of 3.0 to 9.0.

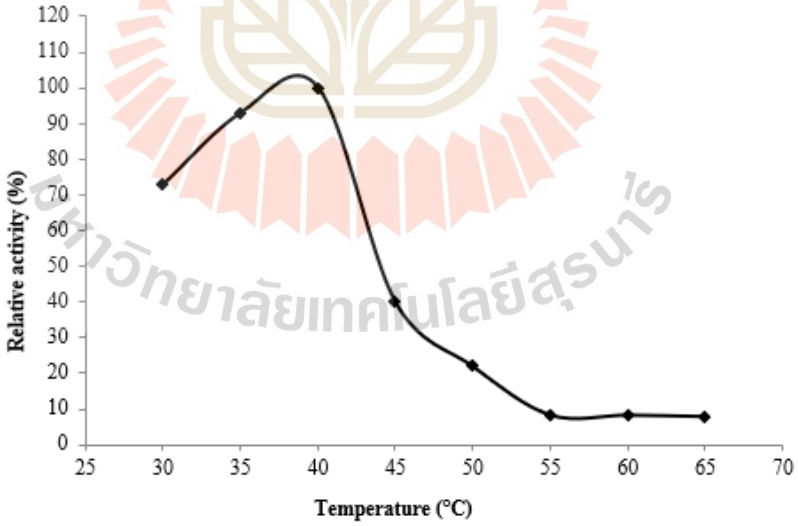

Figure 3.20 Temperature optimum of OsExo2. The activity was assayed with 1 µg of enzyme in 50 mM sodium acetate, pH 5, at the specified temperatures for 30 minutes.

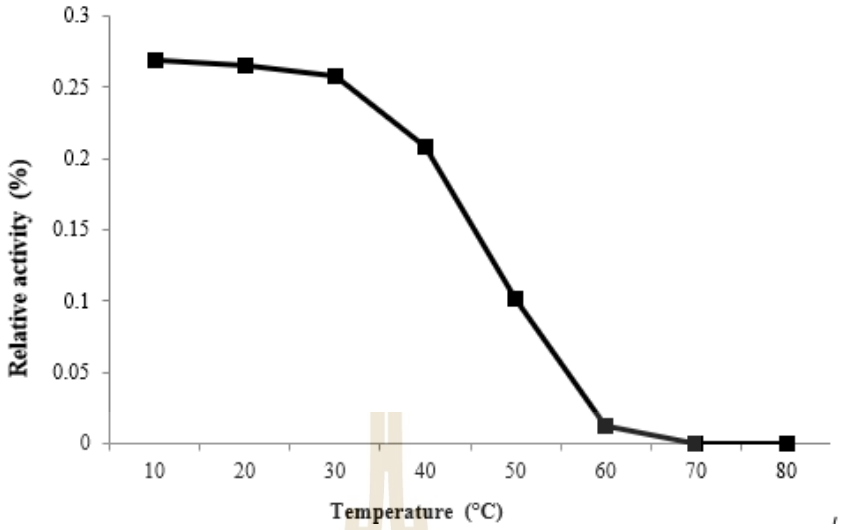

Figure 3.21 Thermostability of OsExo2. The OsExo2 was pre-incubating in 50 mM sodium acetate, pH 5.0, at the indicated temperatures (10-80 °C) for 15 minutes. Enzyme activity was determined at 30 °C by adding 1 mM final concentration of *p*NPGlc.

3.6 Effect of pH and temperature on the activity and stability of OsExo1

As shown in Figure 3.22, the optimum pH for OsExo1 expressed in *P. pastoris* was found to be at pH 5. The enzyme lost nearly half of its activity when the pH was decreased to 4.0 or increased to 6.5. The temperature optimum for the enzyme was 50 °C (Figure 3.23), but >80% maximal activity was seen from 45-70 °C. Since the enzyme was pre-incubated at the temperature before the assay and showed relatively high activity up to 70 °C, the temperature optimum at 50 °C may indicate the stability of the substrate-enzyme and energy of the transition state complex, rather than the stability of the enzyme itself. However, in the determination of the temperature

stability, OsExo1 was stable when preincubated 15 min at temperatures in the range between 10 °C to 30 °C, but lost activity after preincubation at higher temperatures, and had little activity after preincubation at 60 °C and higher (Figure 3.24).

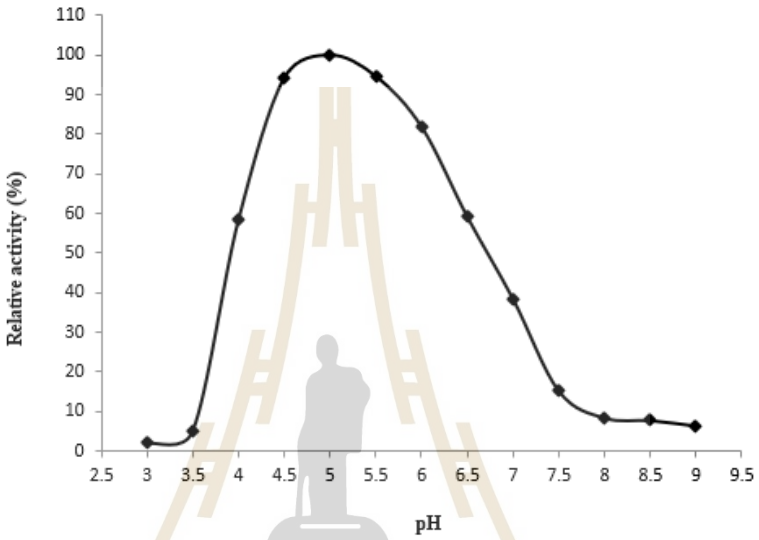

Figure 3.22 Activity versus pH profile of OsExo1. The purified enzyme was incubated with 5 mM *p*NPGlc in 100 mM universal citrate-phosphate buffers ranging from pH 3.0 to 9.0 at 30 °C for 15 minutes., and reactions were stopped by adding 70 µl of 2 M sodium carbonate. The p-nitrophenolate product was then monitored by the A_{405}.

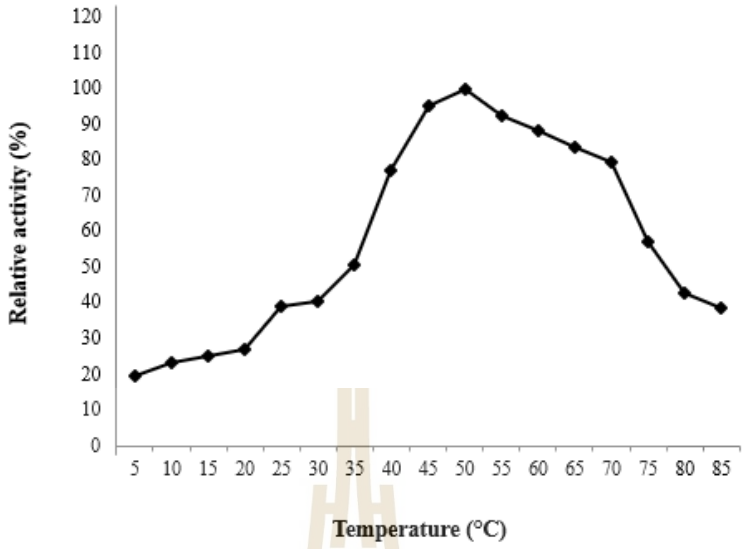

Figure 3.23 Temperature optimum of OsExo1. The activity was assayed by pre-incubating enzyme in 50 mM sodium acetate buffer, pH 5, at the specified temperatures for 15 min to bring the reactions up to temperature, then the enzyme activity was assayed by adding 5 mM *p*NPGlc and incubating for 15 minutes at the same temperature as the preincubation.

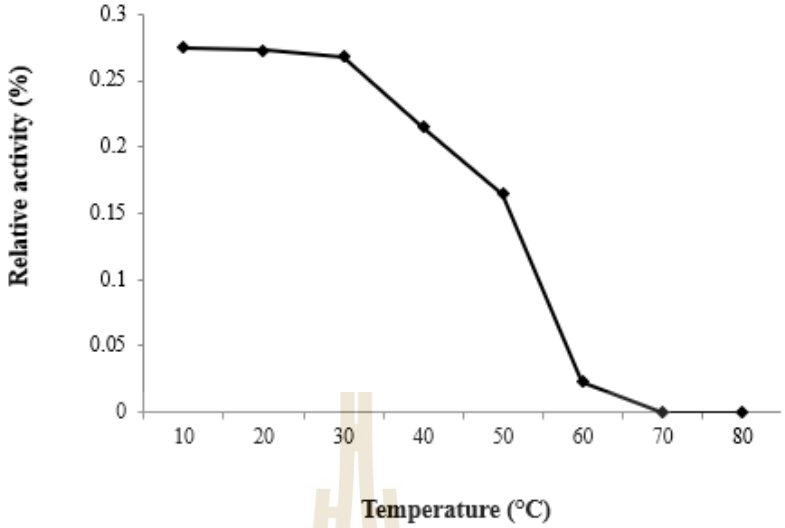

Figure 3.24 Thermostability of OsExo1. The OsExo1 was pre-incubating in 50 mM sodium acetate, pH 5.0, at temperatures between 10-80 °C for 15 minutes. Enzyme activity was determined at 30 °C by adding 5 mM final concentration of *p*NPGlc and incubating 15 minutes.

3.7 Effects of EDTA, metal salts and inhibitors on OsExo2 activity

The effects of several metal ions and EDTA, 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoroglucoside, glucono δ-lactone on OsExo2 activity are show in Table 3.2. No or little inhibition of OsExo2 activity by EDTA, Zn^{2+} and Ca^{2+} was observed. The enzyme activity was decreased 30-40% by 1 mM and 10 mM Ni^{2+}, while 1 mM and 5 mM δ-glucono lactone decreased the activity by 32% and 58%, respectively. Hg^{2+} had a greater effect on OsExo2, decreasing activity by approximately 82% at 1 mM. The concentration of 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside, which would completely inhibit many β-glucosidases, had no significant effect on OsExo2 activity.

Table 3.2 Effects of EDTA, metal salts and inhibitors on OsExo2 activity.

Metal ions/inhibitors	Concentration	Relative activity (%)
Control	-	100
EDTA	1 mM	94.1
$HgCl_2$	1 mM	18.3
$NiSO_4$	1 mM	70.2
	10 mM	61.5
$ZnSO_4$	1 mM	96.5
$CaCl_2$	1 mM	92.1
2,4-dinitrophenyl-β-D-2-deoxy	20 μM	95.4
-2-fluoro-glucopyranoside	40 μM	93.1
Glucono δ-lactone	1 mM	68.3
	5 mM	42.8

3.8 Effects of EDTA, metal salts and inhibitors on OsExo1 activity

The effects of several metal ions and EDTA, 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucoside, glucono δ-lactone on OsExo1 activity are show in Table 3.3. No or little inhibition of OsExo2 activity by EDTA, Zn^{2+} and Ca^{2+} was observed. The enzyme activity was decreased by approximately 20 and 40% by 1 mM and 5 mM glucono δ-lactone, respectively. Similar to the results with OsExo2, 40 μM 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside, which would completely inhibit many β-glucosidases, had no significant effect on OsExo1 activity.

Table 3.3 Effects of EDTA, metal salts and inhibitors on OsExo1 activity.

Metal ions/inhibitors	Concentration	Relative activity (%)
Control	-	100
EDTA	1 mM	93.4
$ZnSO_4$	1 mM	92.5
$CaCl_2$	1 mM	90.2
2,4-dinitrophenyl-β-D-2-deoxy -2-fluoro-glucopyranoside	20 μM	92.3
	40 μM	91.5
Glucono δ-lactone	1 mM	79.3
	5 mM	61.4

3.9 Substrate specificity of OsExo2

The substrate specificities of the purified recombinant OsExo2 enzymes were determined by testing different *p*NP-glycosides, oligosaccharides and polysaccharide substrates, as shown in Table 3.4. OsExo2 hydrolyzed β-D-glucoside fastest and had low activity to β-D-galactoside, α-L-arabinoside, β-D-xyloside and β-D-fucoside, respectively. To evaluate hydrolysis of oligosaccharides, OsExo2 could hydrolyze different β (1,3) and β (1,4) - linked gluco- oligosaccharides, which laminari-oligasaccharides hydrolyzed faster than cello- oligosaccharides. Among the polysaccharides tested, OsExo2 hydrolyzed laminarin best, followed by lichenan and barley (1,3;1,4)-β-D-glucan, respectively.

Table 3.4 Relative activities of OsExo2 on polysaccharides, oligosaccharides and synthetic substrates.

Substrate	Relative activity (%)
Polysaccharide	
Laminarin (*L. digitata*)	100
Barley (1,3;1,4)-β-D-glucan	65.2
Lichenan (*C. islandica*)	71.0
Oligosaccharides	
Laminaribiose	100
Laminaritriose	98.3
Laminaritetraose	97.1
Laminaripentaose	98.3
Cellobiose	91.2
Cellotriose	83.8
Cellotatraose	57.4
Cellopentaose	69.4
Synthetic substrates	
*p*NP-β-D-glucopyranoside	100
*p*NP-β-D-galactopyranoside	2.3
*p*NP-α-L-arabinopyranoside	2.0
*p*NP-β-D-xylopyranoside	1.9
*p*NP-β-D-fucopyranoside	1.7

3.10 Substrate specificity of OsExo1

The substrate specificity of the purified recombinant OsExo1 enzyme was assessed by testing different *p*NP-glycosides, oligosaccharides and polysaccharide substrates as shown in Table 3.5. Like OsExo2, the OsExo1 hydrolyzed β-D-glucoside fastest and had low activity to β-D-xyloside. Moreover, OsExo1 hydrolysed pNP-β-D-cellobioside, but did not hydrolyze β- D- galactoside, α- L- arabinoside, and β- D-fucoside. OsExo1 could hydrolyze different β-(1,3) and β-(1,4) - linked gluco-oligosaccharides, and among these hydrolyzed laminari- oligasaccharides faster than cello- oligosaccharides. Among the polysaccharides tested, OsExo1 hydrolyzed laminarin best, followed by lichenan and barley (1,3;1,4)-β-D-glucan, respectively.

Table 3.5 Relative activities of OsExo1 on polysaccharides, oligosaccharides and synthetic substrates.

Substrate	Relative activity (%)
Polysaccharide	
Laminarin (*L. digitata*)	100
Barley (1,3;1,4)-β-D-glucan	63.2
Lichenan (*C. islandica*)	70.4
Oligosaccharides	
Laminaribiose	100
Laminaritriose	97.2
Laminaritetraose	96.0
Laminaripentaose	97.0
Cellobiose	95.4
Cellotriose	93.1
Cellotatraose	73.7
Cellopentaose	71.5
Synthetic substrates	
*p*NP-β-D-glucopyranoside	100
*p*NP-β-D-galactopyranoside	NA
*p*NP-α-L-arabinopyranoside	NA
*p*NP-β-D-xylopyranoside	6.1
*p*NP-β-D-fucopyranoside	NA
*p*NP-β-D-cellobioside	44.3

"NA" means no activity detected.

3.11 Kinetic parameters of OsExo2

The kinetic parameters of OsExo2 for hydrolysis of pNPGlc, laminaribiose, laminaritriose, cellobiose, cellotriose, laminarin, barley (1,3;1,4) - β- D- glucan and lichenan, were determined, as shown in Table 3.6. Among the polysaccharide substrates, OsExo2 hydrolyzed lichenan with a k_{cat}/K_M of 20 $Lg^{-1}s^{-1}$, followed by laminarin and barley β- D- glucan, with k_{cat}/K_M values of 15 $Lg^{-1}s^{-1}$ and 10 $Lg^{-1}s^{-1}$, respectively. The OsExo2, had a catalytic efficiency value (k_{cat}/K_M) for cellobiose that was 1. 7- fold higher than that for laminaribiose, and was higher than that for *p*NPGlc about 8.6-fold.The catalytic efficiency values (k_{cat}/K_M)for cellotriose and laminaritriose were not significantly different and were about 7-fold higher than that for *p*NPGlc.

Table 3.6 Kinetic parameters for OsExo2 hydrolysis of *p*NPGlc, oligosaccharide and polysaccharide substrates.

Substrate	**Apparent Kinetic Parameters**		
	K_M	k_{cat} (s^{-1})	k_{cat}/K_M
*p*NPGlc	0.226±0.012 (mM)	0.647 ± 0.011	2.9 ($mM^{-1}s^{-1}$)
Cellobiose	0.0155±0.0009 (mM)	0.352 ± 0.006	25.0 ($mM^{-1}s^{-1}$)
Laminaribiose	0.039±0.003 (mM)	0.50 ± 0.01	14.0 ($mM^{-1}s^{-1}$)
Cellotriose	0.0154±0.0006 (mM)	0.315 ± 0.003	20.5 ($mM^{-1}s^{-1}$)
Laminaritriose	0.0133±0.0007 (mM)	0.276 ± 0.003	21.0 ($mM^{-1}s^{-1}$)
Laminarin	0.0376±0.0016 (g/L)	0.572 ± 0.007	15.0 ($Lg^{-1}s^{-1}$)
Barley β-D-glucan	0.046±0.004 (g/L)	0.463 ± 0.016	10.0 ($Lg^{-1}s^{-1}$)
Lichenan	0.0154±0.0006 (g/L)	0.315 ± 0.003	20.0 ($Lg^{-1}s^{-1}$)

3.12 Expression of enzymes OsExo1 and OsExo2 in rice

The Rice Expression Profile Database (RiceXpro) provides the expression profile of nearly all rice genes, based on microarray RNA expression analysis. It was queried to uncover the expression patterns of OsExo1 and OsExo2, as shown in Figure 3.25 and Figure 3.26. From the analysis, both genes are fairly broadly expressed, but they are most highly expressed in roots in the vegetative growth stage. Both genes are also expressed early in endosperm development, although little or no OsExo2 expression is observed late in endosperm development.

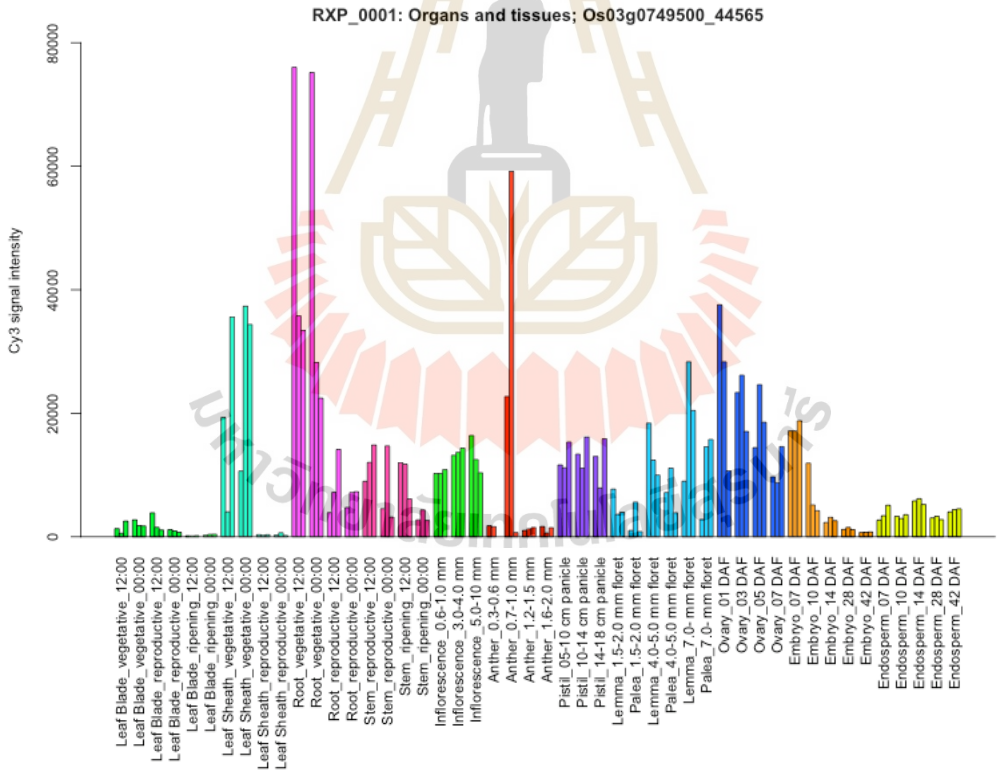

Figure 3.25 Expression profile of OsExo1 in different organs and tissues. The data were collected from the Rice Expression Profile Database, which is based on microarray RNA expression analysis.

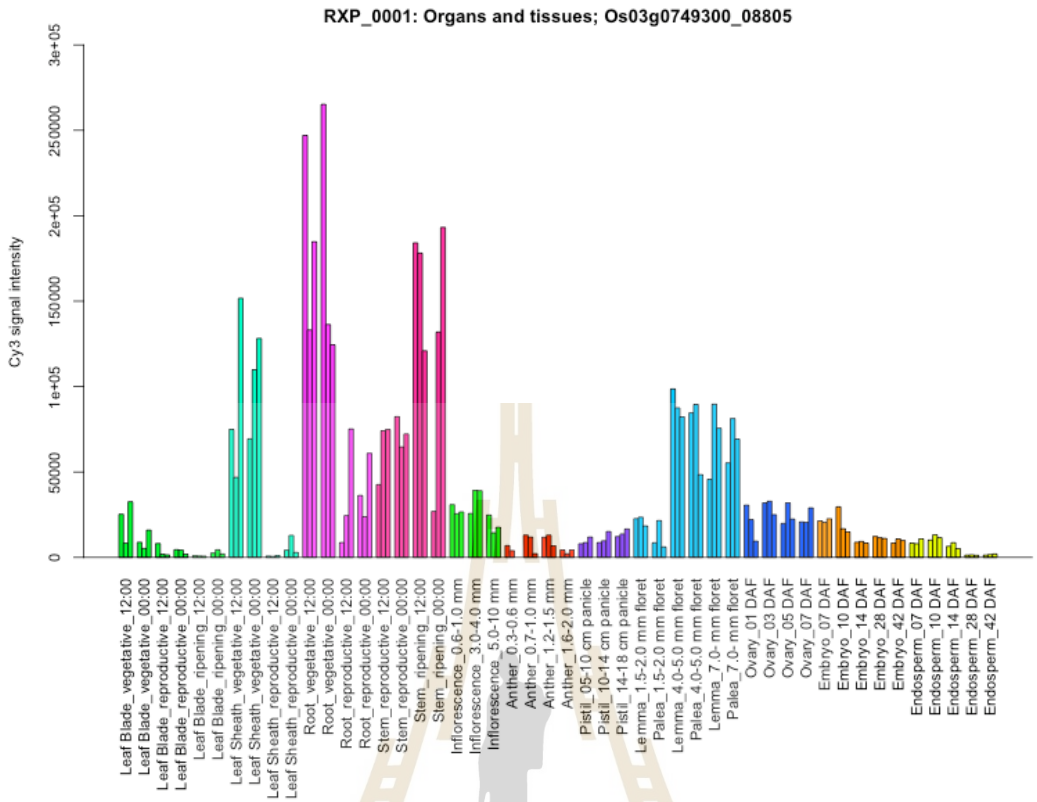

Figure 3.26 Expression profile of OsExo2 in different organs and tissues. The data were collected from the Rice Expression Profile Database, which is based on microarray RNA expression analysis.

CHAPTER IV

DISCUSSION

4.1 Expression and purification of OsExo2

To produce mature OsExo2 in *E. coli*, the cDNA encoding the mature OsExo2 was cloned into the pET32a/DEST expression vector, as was previously successful with several family GH1 rice β-glucosidases (Opassiri et al., 2003; 2006; Kuntothom et al., 2009), though not successful for the family GH3 enzyme barley Exo1 (Luang et al., 2010). The predicted molecular weight and isoelectric point (pI) of the OsExo2 protein were approximately 67.7 kDa and 7.23, respectively. The OsExo2 protein with N-terminal thioredoxin and His6 tags was express in several *E. coli* strains and induction conditions. For IPTG induction systems, it was optimally expressed in *E. coli* strain Origami B(DE3) induced with 0.1 mM IPTG at 20 °C for 18 h, and significant *p*NPGlc hydrolysis activity was detected in the crude soluble cell extract. However, most of the OsExo2 protein was observed in the insoluble fraction of the cell lysates, and less of the protein could be detected in the soluble cell lysate, suggesting that this OsExo2 was not expressed well in soluble form. So, I attempted to use auto-induction for expression in *E. coli* to increased protein yields. The amount of protein in the soluble fraction appeared to be significantly increased, but despite the improved production, the yield in the soluble fraction still appeared to be much less than in the insoluble fraction.

The protein was purified by 3 steps, including IMAC, phenyl sepharose and gel filtration chromatography to produce a protein of approximately 90 kDa as judged by

SDS-PAGE. It was still difficult to consistently produce high purity protein due to the low expression levels, which resulted in a high ratio of contaminating proteins to the protein of interest.

4.2 Expression and purification of OsExo1

To study the activity of OsExo1, a cDNA optimized for expression of OsExo1 (Genbank accession number AK073110) in *P. pastoris* was synthesized. The optimized OsExo1 cDNA was used to produce a secreted, N-terminally His-tagged protein in *P. pastoris*, with an expected molecular weight (MW) of 67.6 kDa and pI of 6.89. A basically following the strategy described by Luang et al. (2010) for recombinant expression of barley Exo1. The β-glucosidase activity was detected in the media with *p*NPGlc substrate and a band of protein was detected at about 90 kDa on the SDS-PAGE gel. The protein purified from the pichia media by IMAC showed a broad band, suggesting glycosylation. After deglycosylation with endoglycosidase H, a single band of approximately 70 kDa was detected on a Coomassie-stained SDS-PAGE gel. As noted above, this expression system and purification system were quite similar to that of barley Exo1, which gave similar results (Luang et al., 2010).

4.3 Substrate specificity of OsExo2

The hydrolysis specificity of OsExo2 was determined by varying the substrate. The enzyme can hydrolyze several *p*NP-glycosides, including *p*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-D-xylopyranoside and *p*NP-β-D-fucopyranoside. OsExo2 hydrolyzed β-D-glucoside fastest and had low activity to β-D-galactoside, β-D-xyloside, α-L- arabinoside and β-D-fucoside.

OsExo2 could hydrolyze different β-(1,3)- and β-(1,4)-linked glucooligosaccharides, and polysaccharides, including barley (1,3;1,4)-β-D-glucan, laminarin and lichenan. For hydrolysis of oligosaccharides, the enzyme showed higher catalytic efficiency specificity constant (k_{cat}/K_m) than with pNPGlc, in that cellobiose, cellotriose and laminaritriose were hydrolyzed at around 7-fold higher rates than *p*NP-β-D-Glc, and laminaribiose was hydrolyzed at a rate around 5-fold higher than *p*NPGlc.

By comparing the kinetic parameters of OsExo2 for polysaccharide substrate, the OsExo2 also hydrolyzed lichenan faster than barley (1,3;1,4)-β-D-glucan and laminarin, with at around 2 and 1.3-fold higher rates than barley (1,3;1,4)-β-D-glucan and laminarin, respectively.

4.4 Substrate specificity of OsExo1

The OsExo1 protein was tested for hydrolysis of *p*NP-glycosides, oligosaccharides and polysaccharides. OsExo1 hydrolyzed glucoside substrates, including *p*NPGlc and *p*NP-β-D-cellobioside, but could not hydrolyze β-D-galactoside, β-D-fucoside and α-L-arabinoside. This specificity is similar to that seen with rHvExo1 (Luang et al., 2010). However, OsExo1 can hydrolyze β-D-xyloside, which rHvExo1 was not reported to hydrolyze. The oligosaccharides and polysaccharides were hydrolyzed by OsExo1 with different relative activities. To compared with oligosaccharide substrate, OsExo1 had relative activity with laminari-oligosaccharide higher than cello-oligosaccharide. For polysaccharides, OsExo1 activity was highest with laminarin, followed by lichenans and barley (1,3;1,4)-β-D-glucan, respectively.

A comparison of the relative activities of OsExo1 and OsExo2 with the two β-glucan exohydrolases purified from germinated barley (Hrmova and Fincher, 1996,

1998), assigned the names HvExo1 and HvExo2, is shown in Table 4.1. Similar to native HvExo1 and HvExo2, the two-rice enzyme could hydrolyze polysaccharides substrates and preferred (1,3)-β-glucan, laminaran followed by the mixed linkage (1,3;1,4)-β-glucans from barley-β-glucan and lichenan from *C. islandica*. The relatively broad specificity for linkage type in polysaccharide substrates was confirmed in studies on the hydrolysis of oligosaccharides. The β- 1,3- cellooligosaccharides and β- 1,4- laminarioligosaccharides are all hydrolysed by two rice enzymes, as they were by the barley enzymes (Hrmova and Fincher, 1998). The glycone specificity of OsExo1 and OsExo2 for the glycosyl moiety of substrates was examined using *p*NP-glycosides. As noted above, the two rice enzymes have high specificity for β-glucosyl residues and very low rates or not hydrolyse α-L-arabinoside, β-D-galactoside, β-D-xyloside, and β-D-fucoside, which was similar to the barley enzymes where no hydrolysis of *p*NP-glycosides other than *p*NPGlc was reported. Although, OsExo1 and OsExo2 could hydrolyze *p*NP-β-glucosides, the enzymes are classified as β-glucan exohydrolases rather than β-glucosidases because they can also release glucose from polysaccharide substrates, as described for the barley enzymes (Hrmova et al., 1996; 1998; 2002).

Table 4.1 Comparable of the relative activities of OsExo1, OsExo2, HvExo1 and HvExo2.

Substrate	Relative activity (%)			
	OsExo1	**OsExo2**	**HvExo1**[a]	**HvExo2**[a]
Polysaccharide				
Laminarin (*L. digitata*)	100	100	100	100
Barley (1,3;1,4)-β-D-glucan	63.2	65.2	10	14
Lichenan (*C. islandica*)	70.4	71.0	18	49
Oligosaccharides				
Laminaribiose	100	100	70	75
Laminaritriose	97.2	98.3	NM	NM
Laminaritetraose	96.0	97.1	NM	NM
Cellobiose	95.4	91.2	14	20
Cellotriose	93.1	83.8	NM	NM
Cellotetraose	73.7	57.4	NM	NM
Cellopentaose	71.5	69.4	NM	NM
Synthetic substrates				
*p*NP-β-D-glucopyranoside	100	100	10	56
*p*NP-β-D-galactopyranoside	NA	2.3	NA	NA
*p*NP-α-L-arabinopyranoside	NA	2.0	NA	NA
*p*NP-β-D-xylopyranoside	6.1	1.9	NA	NA
*p*NP-β-D-fucopyranoside	NA	1.7	NA	NA
*p*NP-β-D-cellobioside	44.3	NM	NA	NA

[a]The data are from Hrmova and Fincher, 1998. “NA” means no activity detected. “NM” mean not measured

4.5 Effect of pH and temperature on enzyme activity

The pH optimum profile of OsExo1 and OsExo2 was similar to rHvExo1 (glycosylated and N-deglycosylated forms) (Table 4.2), which showed bell-shaped pH versus activity curves, and highest activities detected at pH 5.0 (Luang et al., 2010). The OsExo1 and OsExo2 had pH optima of 4.5-5.0, suggesting that they could act in acidic compartments, such as the apoplast. The temperature stabilities of OsExo1 and OsExo2 were similar to that of the N-deglycosylated rHvExo1, which was stable in the range between 10 and 30 °C, while the temperature stability of glycosylated rHvExo1 was stable only at the temperature range between 10 and 20 °C. These results of the glycosylated rHvExo1 were different from the report of Koseki et al. (2006), for which the N-glycosylation of asparagines in the catalytic domain increased the thermostability of *Aspergillus kawachii* α-L-arabinosidase. Since OsExo1 and OsExo2 were both stable for 15 minutes at 10-30 °C and OsExo1 was produced in *E. coli*, where it would not be glycosylated, glycosylation does not seem to be critical for stability of the rice isoenzymes either, although the thermostability of deglycosylated OsExo1 should be tested in the future to compare the effect within the same enzyme.

Above 30 °C, the activities of OsExo1, OsExo1 and both forms of rHvExo1 were decreased. The activity decreased by 50% for the glycosylated and N-deglycosylated rHvExo1 forms at about 34 and 39 °C, respectively, and both rHvExo1 forms were apparently denatured at 50 °C with no activity (Luang et al., 2010). In comparison, the activities of OsExo1 and OsExo2 decreased by 50% at approximately 50 °C and they each had a little activity at 60 °C. This result demonstrated that the OsExo1 and OsExo2 are apparently more stable than the rHvExo1.

The temperature optimum for the OsExo1 enzyme was 50 °C (Figure 3.17), but >80% maximal activity was seen from 45-70 °C. Since the enzyme was pre-incubated at the temperature before the assay and showed relatively high activity up to 70 °C, the temperature optimum at 50 °C may indicate the stability of the substrate-enzyme and energy of the transition state complex, rather than the stability of the enzyme itself. Nonetheless, this interpretation is not consistent with the temperature stability test experiments, which suggested that the enzymes could be denatured at much lower temperatures, or that cooling after heating was destabilizing to them.

Table 4.2 Comparable the catalytic properties of OsExo1 and OsExo2 with rHvExo1 (glycosylated and N-deglycosylated).

Properties	**OsExo1**	**OsExo2**	**glycosylated rHvExo1[a]**	**N-deglycosylated rHvExo1[a]**
pH optimum	5.0	5.0	5.0	5.0
Temperature optimum (°C)	50.0	40.0	nm[b]	nm[b]
Temperature stability (°C)	10.0-30.0	10.0-30.0	10.0-20.0	10.0-30.0

[a] the data from Luang et al., 2010

[b] 'nm' indicates 'not measured'

4.6 Inhibition of OsExo1 and OsExo2

The OsExo1 and OsExo2 enzymes had similar inhibition profiles with the metal ions and inhibitors tested, although the set of ions and β-glucosidase inhibitors tested

was not comprehensive (Table 4.3). No or little inhibition of OsExo1 and OsExo2 activity was seen with EDTA, Zn^{2+} and Ca^{2+}. The activity of OsExo2 was inhibited by Hg^{2+}, which has been reported as a strong inhibitor of several β-glucosidases (Esen A., 1992; Opassiri et al., 2003). It was also inhibited by Ni^{2+}, which decreased the relative activity to about 60 to 70% of the reaction without metal ion added (Table 4.3). This inhibition of OsExo2 by these metal ions was comparable to other characterized β-glucosidases (Ketudat Cairns and Esen, 2010). Metal ions can be associated to the proteins by acting as electron pair acceptors and by charge-charge interactions with negative ions. They can activate or inhibit the enzymatic activity by interacting with amine or carboxylic acid group of the amino acids (Ishida et al., 1980).

Inhibition of OsExo1 and OsExo2 hydrolysis of *p*NPGlc by the presence of glucono δ–lactone, a competitive inhibitor of native HvExoI (Hrmova et al., 1996; 2001; 2002) was tested. Glucono δ-lactone is an analogue of the oxocarbenium ion-like transition state that could inhibit the activity of other glucose-active enzymes, such as rice Os3BGlu7 β-glucosidase (Opassiri et al., 2003), native HvExo1 (Hrmova et al., 1996; 2001; 2002) and rHvExo1 (Lung et al., 2010). It also inhibited the activities of OsExo1 and OsExo2. Unlike native HvExo1 and rHvExo1, OsExo1 and OsExo2 were not significantly inhibited by the covalent inhibitor 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside. 2,4-Dinitrophenyl-β-D-2-deoxy-2-fluorogluco-pyranoside is generally a strong inhibitor because electron withdrawal by the 2-fluoro group destabilizes the oxocarbenium ion-like transition state for both the glycosylation and deglycosylation half reactions of the retaining mechanism. The 2,4-dinitrophenolate is an excellent leaving group that allows the glycosylation step to proceed, but the deglycosylation step often cannot proceed, resulting in a stable covalent intermediate.

(Withers et al., 1987; Williams and Withers, 2000; Rempel and Withers, 2008). The lack of inhibition of OsExo1 and OsExo2 may suggest that they are not binding the inhibitor or that the deglycosylation step is relatively rapid, even in the presence of 2-fluoroglucoside.

Table 4.3 Comparable of the effects of EDTA, metal salts and glucosidase inhibitors on OsExo1 and OsExo2 activity.

Metal ions /inhibitors	**Relative activity (%)**	
	OsExo1	**OsExo2**
Control	100	100
EDTA (1 mM)	93.4	94.1
$HgCl_2$ (1 mM)	nm	18.3
$NiSO_4$ (1 mM)	nm	70.2
$NiSO_4$ (10 mM)	nm	61.5
$ZnSO_4$ (1 mM)	92.5	96.5
$CaCl_2$ (1 mM)	90.2	92.1
2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside (20 μM)	92.3	95.4
2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside (40 μM)	91.5	93.1
Glucono δ-lactone (1mM)	79.3	68.3
Glucono δ-lactone (5mM)	61.4	42.8

'nm' indicates 'not measured'

4.7 Expression patterns and likely functions of OsExo1 and OsExo2

The expression patterns of OsExo1 and OsExo2 showed that they are most highly expressed in roots in the vegetative growth stage and also expressed endosperm development (Figure 3.25 and Figure 3.26), which are tissues that undergo cell-wall degradation or remodeling from vegetative to reproductive stage. Hrmova and Fincher (2001) reported that the β-D-glucan exohydrolases from barley likely play biological roles in the degradation of the barley (1→3), (1→4)-β-D-glucans during endosperm mobilization and in cell elongation. The native barley Exo1 and Exo2 isolated from germinated seedlings could hydrolyze (1→3)- β-glucan (lami-narin), and (1 →3), (1→4)-β-glucan, which suggested that the possible functions of these enzymes include acting to complete the conversion of cell-wall polysaccharides to glucose in the germinated grain, remodeling polysaccharides during the auxin-induced elongation of cells in growing coleoptiles and hydrolyzing (1→3)-β-glucosyl linkages in (1→3)- and (1→3);(1→6)-β-glucans of the type commonly found in fungal cell walls (Hrmova and Fincher, 1998). Kim et al. (2000) showed that two isoforms of the maize β-D-glucan exohydrolases are associated with cell walls, while a third is tightly bound to the plasma membrane. In barley the majority of β-D-glucan exohydrolase activity can be extracted from homogenates of coleoptiles, a finding that makes strong binding to either cell walls or to plasma membranes unlikely. Nevertheless, the enzymes are extracellular and could be trapped in the cell wall matrix and released when the cell walls are broken down by homogenization (Harvey et al., 2001).

The relatively broad substrate specificities of OsExo1 and OsExo2 have higher efficiency to hydrolyze (1,3;1,4)-β-glucan similar with the native HvExo1 and HvExo2 suggesting that, one possible functions of their enzymes include acting to complete the

conversion of cell-wall polysaccharides to glucose, which would then be available as an energy source for the developing seedling (Hrmova et al., 1996; Hrmova and Fincher, 1998).

A second possible function of enzyme could be in the auxin-induced elongation of cells in growing coleoptiles or during growth of other tissues, such as roots during the vegetative stage. The (1,3;1,4)-β-glucan of the coleoptile cell wall has been reported to be an exoglucanase substrate, which decreases significantly during coleoptile growth (Sakurai et al., 1987; Gibeaut and Carpita, 1991) in a process that has been linked to wall 'loosening' and auxin-induced cell elongation (Labrado and Nevins, 1996). Based on the expression pattern of OsExo1 and OsExo2, they would likely have a more general role in release of glucose from cell wall polysaccharides broken down in the process of cell wall remodeling during growth.

Another possible role enzyme could be in defense against fungi, to hydrolyze the (1,3)-β-glucosyl linkages in (1,3)- and (1,3;1,6)-β-glucans found in fungal cell walls (Wessels, 1993). The (1,3)-β-glucan-endohydrolases (EC 3.2.1.39) are considered pathogenesis-related proteins and are thought to protect plants from fungal infection through their hydrolysis of the (1,3;1,6)-β-glucans found in fungal cell wall (Hoj and Fincher, 1995). The rice β-glucan exohydrolases characterized here are also able to hydrolyze (1,3)-β-glucans, so it is possible that OsExo1 and OsExo2 could act in together with the endohydrolases to degrade cell walls of invading fungi, as suggested for the barley enzymes.

CHAPTER V

CONCLUSION

This thesis describes the recombinant expression, purification and characterization of two rice glycoside hydrolase family GH3 enzymes named OsExol and OsExo2, according to their similarity to the previously characterized barley enzymes Exol and Exo2. To our knowledge, these are the first rice GH3 exoglucanases to be studied, and this is the first time to report the expression of a plant GH3 enzyme in *E. coli*, in the case of OsExo2. Because OsExo2 was more thoroughly characterized, the description starts from OsExo2.

The OsExo2 work started from cloning the natural gene into an expression vector, followed by expression, purification and characterization. The plasmid containing the AK065044 cDNA provided by the Rice Genome Resource full-length cDNA project was used to amplify the natural gene encoding the predicted mature OsExo2 protein. The purified gene encoding mature OsExo2 was cloned into pET32a/DEST expression vector to express an OsExo2 protein with N-terminal thioredoxin and His_6 tags in *E. coli* strain Origami B(DE3). Although OsExo2 could be expressed in bacteria by induction with IPTG, the target protein was found at low concentration in the soluble cell lysate and was mostly in the insoluble pellet. So, the best expression was obtained in E. coli by growth in Auto-induction media with induction at 15 °C for 24 hours. The OsExo2 protein was purified from the host by Ni^{2+} IMAC, hydrophobic interaction

chromatography over a phenyl sepharose column and gel filtration chromatography over an S200 column, respectively.

Since we could not express OsExo1 in *E. coli*, OsExo1 was expressed in a yeast expression system, as previously reported for other plant enzymes (e. g. Ketudat Cairns et al., 2000; Hrmova et al., 2009, Luang et al., 2010). Expression in the yeast *P. pastoris* has been reported to produce plant enzymes in active forms. The predicted mature OsExol could be expressed from an optimized gene in *P. pastoris*, from a recombinant pPICZαBNH8 plasmid. In this system, OsExol was produced as a secreted protein from *P. pastoris* strain SMD1168H at 20 °C for 5 days with induction by methanol to 1% (v/v) final concentration. OsExo1 enzyme was successfully purified from the culture medium to produce a highly glycosylated protein after a one step purification by IMAC from the Pichia media.

Upon N-deglycosylation with endoglycosidase H, the protein appeared as a single, apparently homogeneous band on SDS-PAGE. However, the specific activity of OsExol was decreased compared to the native glycosylated form. So, the glycosylated OsExol was used for characterization.

The optimal pH values for both OsExol and OsExo2 were found to be at pH 5.0 and activity of both enzymes decreased in high pH, with little activity above pH 7.0, suggesting that these enzymes are active in somewhat acid conditions, although they lose their activity in the highly acidic range. This pH optimum suggests that the OsExo1 and OsExo2 exoglucanases may act in acidic compartments, such as the apoplast around plant cell walls. The optimum temperature of the OsExol enzyme was 50 °C, but it was thermostable for only 15 minutes over the range of 20-50 °C. The temperature

optimum for OsExo2 enzyme was 40 °C, but it was maintained activity for 15 minutes over the range of 20-50 °C, similar to OsExol.

Among *p*NP-glycosides, OsExol and OsExo2 hydrolyzed *p*NP-β-D-gluco-pyranoside (*p*NPGlc) by far the best, followed by *p*NP-β-D-xyloside, respectively. However, OsExo2 also had a little activity to hydrolyze *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside and *p*NP-a-L-arabinoside, while OsExol could not hydrolyze these substrates.

OsExol and OsExo2 was able to hydrolyze β-(1,3), β-(1,4), and β-(1,6)-linked disaccharides and polysaccharides, including laminarin, barley (1,3;1,4)-β-D-glucan and lichenan, but with differences in the relative activities on these substrates between them. The rate of hydrolysis of oligosaccharides were independent on the degree of polymerization (DP) for cellooligosaccharides and laminarioligosaccharides with DP of 2-5 and highest activity with laminaribiose. For the soluble glucan polysaccharides, the relative activities of the two enzymes did not differ, in that they both hydrolyzed laminarin best, followed by lichenan and barley (1,3;1,4) -β-6-D-glucan, respectively. OsExol and OsExo2 had the activity on a variety of β-linked glucoside substrates, and oligosaccharide and polysaccharide substrates, similar to rHvExo1. The fact that both enzymes could hydrolyze β-1,3-, β-1,4, or β-1,6-linked gluco-oligosaccharides and polysaccharides, suggests the enzymes may play a role in cell wall degradation during tissue growth and remodeling or defense against fungal pathogens containing cell wall glucans.

In terms of the kinetic parameters for hydrolysis of oligosaccharide substrates, OsExo2 has high catalytic efficiency toward cellobiose (k_{cat}/K_M of 25.0 $mM^{-1}s^{-1}$),

followed by laminaritriose (k_{cat}/K_M of 21 $mM^{-1}s^{-1}$), cellotriose (k_{cat}/K_M of 20.5 $mM^{-1}s^{-1}$) and laminaribiose (k_{cat}/K_M of 14.0 $mM^{-1}s^{-1}$), respectively. For the polysaccharide substrates, OsExo2 had high catalytic efficiency toward lichenan (k_{cat}/K_M of 20.0 $Lg^{-1}s^{-1}$) followed by laminarin (k_{cat}/K_M of 15 $Lg^{-1}s^{-1}$) and barley (1,3;1,4)-β-D-glucan (k_{cat}/K_M of 10.0 $Lg^{-1}s^{-1}$). The trend of these kinetic parameters differs from that for rHvExol, which has high catalytic efficiency for barley (1,3;1,4)-β-D-glucan, followed by laminarin.

The inhibition study showed that OsExol and OsExo2 activities were inhibited by glucono-δ-lactone at 1 and 5 mM, while 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside, which is a strong inhibitor for other β-glucosidases and rHvExol, had little effect on the activity of both enzymes. OsExo2 activity was inhibited nearly completely by mercuric ion at 1 mM, while nickel at 1 and 10 mM had partial inhibition effects on the activity of the enzyme.

The expression profile of OsExol and OsExo2 in various rice organs showed that both genes are most highly expressed in roots in the vegetative growth stage, and were expressed in a range of other tissues such as, leaf sheath and root, and also expressed early in endosperm development. This result suggested that these enzymes may apparently function in both vegetative and reproductive tissues, possibly in cell wall remodeling, although their exact role is not clear.

In summary, this research supports the 1dea that OsExol and OsExo2 might be involved in the turnover or modification of cell walls in rice plants. First, successful recombinant expression and purification allowed us to see that OsExol and OsExo2 enzymes have higher efficiency to hydrolyze β-1,3-, β-1,4-, or β-1,6-linked gluco-oligosaccharides and polysaccharides containing β-1,3-glucosyl linkages. Second, the

pH optimum of OsExol and OsExo2 are in the acidic range, consistent with a role in the apoplast or acidic vacuole. Last, the OsExol and OsExo2 expression was found to occur in the tissues that undergo cell-wall degradation or remodeling from vegetative to reproductive stage.

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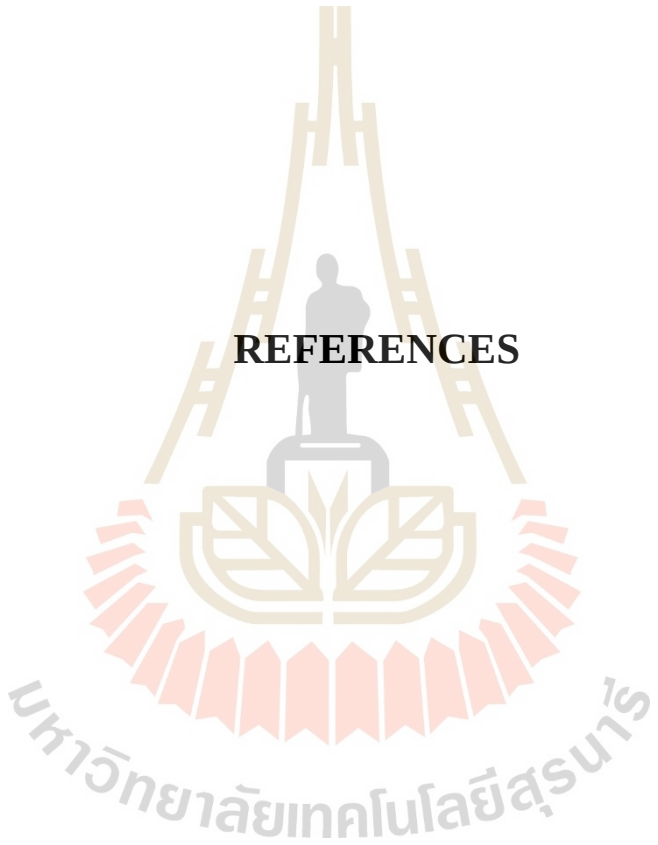

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APPENDIX

APPENDIX

CHEMICAL PREPARATIONS

1 M Tris-HCl (500 ml)

1. Dissolve 60.55 g Tris base in 300 ml of dH_2O.
2. Adjust the pH to the desired value with concentrated HCl.
3. Bring up the volume to 500 ml with dH_2O.

1 M Sodium acetate (200 ml)

1. Dissolve 27.22 g sodium acetate in 100 ml of dH_2O.
2. Add 6 ml of glacial acetic acid.
3. Adjust the pH to the desired value with 10 *N* NaOH.
4. Bring up the volume to 200 ml with dH_2O.

0.5 M EDTA, pH 8 (500 ml)

1. Resuspend 93.05 g Na_2•EDTA•$2H_2O$ (disodium dihydrate) in about 400 ml of dH_2O.
2. Add about 9 g solid NaOH.
3. Once all the NaOH dissolves, slowly adjust the pH with NaOH.
4. Bring up the volume to 500 ml with dH_2O.

Note: EDTA will not completely dissolve until the pH reaches 8.

4 *N* NaOH (200 ml)

Dissolve 32 g NaOH in a final volume of 200 ml dH_2O.

50X TAE buffer (1 L)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Bring up the volume to 1 L.

6X DNA loading sample buffer (10 ml)

Glycerol	3 g
Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g

Bring up the volume to 10 ml and store at 4 °C.

SDS-PAGE preparation (30% gel)

Acrylamide gel solution (100 ml)

acrylamide	29.4 g
bis-acrylamide	0.6 g

Separating gel (15% gel)

dH_2O	2.9 ml
2 M Tris-HCl, pH 8.8	2 ml
10% SDS	0.1ml
30% acrylamide gel solution	5 ml

10% $(NH_4)_2S_2O_8$	50 µl
TEMED	5 µl

Stacking gel (4% gel)

dH_2O	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% SDS	0.1 ml
30% acrylamide gel solution	1.3 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

10X Running buffer (1 L)

Tris base	30 g
Glycine	144 g
SDS	10 g

Bring up the volume to 1 L.

5X Sample buffer

SDS	1.0 g
Glycerol	5.0 ml
Bromophenol blue	25 mg
Tris base	242 mg
HCl	0.35 ml (adjust the pH to 6.8)
2-Mercapoethanol	1.0 ml

Bring up the volume to 10 ml and store at 4 °C.

Coomassie blue stain (1 L)

Methanol	500 ml
Acetic acid	100 ml
Coomassie blue	0.5 g
dH_2O	400 ml

Mix on stir plate until all coomassie blue is dissolved.

De-stain (1 L)

Methanol	400 ml
Acetic acid	100 ml

Bring up the volume to 1 L.

10% SDS (100 ml)

10 g SDS into 100 ml, heat to 68 °C for solubility, pH ~6.6.

50 mg/ml ampicillin (20 ml)

1. Weigh 1 g of ampicillin.
2. Bring up the volume to 20 ml by DI water and filter sterilizes (0.22 µm), store at -40 °C.

34 mg/ml chloramphenicol (20 ml)

1. Weigh 0.68 g of chloramphenicol.
2. Bring up the volume to 20 ml by 70% ethanol and filter sterilizes (0.22 µm), store at -40 °C.

15 mg/ml kanamycin (20 ml)

1. Weigh 0.3 g of kanamycin.
2. Bring up the volume to 20 ml by DI water and filter sterilizes (0.22 µm), store at -40 °C.

12.5 mg/ml tetracycline (20 ml)

1. Weigh 0.25 g of tetracycline.
2. Bring up the volume to 20 ml by 70% ethanol and filter sterilizes (0.22 µm), store at -40 °C.

1 M IPTG (10 ml)

1. Weigh 2.38 g of IPTG (MW = 238.3 g/mol).
2. Bring up the volume to 10 ml and filter sterilizes (0.22 µm), store at -40 °C.

LB broth (1 L)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Dissolve components in 1 L of dH_2O and sterilize by autoclaving at 15 psi, at 121 °C for 15 minutes.

LB agar (200 ml)

Tryptone	2 g
Yeast extract	1 g
NaCl	1 g
Agar	4 g

Dissolve components in 200 ml of dH_2O and sterilize by autoclaving at 15 psi, at 121 °C for 15 minutes.

Auto-induction media (AIM)

1. Terrific broth (TB)

12 g tryptone, 24 g yeast extract, 9.4 g potassium phosphate dibasic, and 2.2 g potassium phosphate monobasic. Add DI water to ~800 ml, autoclave, and top-up with sterile DI water to 1 L.

2. AIM solution I, 50x stock

Weigh NH_4Cl (MW 53.49) 133.72 g, Na_2SO_4 (MW 142.04) 35.51 g, mix and dissolve in 1 L of DI water, and filtrate. Final concentrations are 2500 mM NH_4Cl and 250 mM Na_2SO_4.

3. AIM solution II, 50x stock

Weigh D-glucose (MW 180.16) 7.5 g, measure 100% glycerol (density 1.261 g/ml) 476 ml, mix and dissolve in 1 L of DI water, and filtrate. Final concentrations are 0.75 w/v % D-glucose, 60 w/v % glycerol.

4. AIM solution III, 25x stock

Weigh alpha-lactose monohydrate (MW 306.31) 150 g, dissolve in 1 L water, and autoclave. Final concentration is 15 w/v % alpha-lactose.

5. AIM solution IV, 500x stock

Weigh $MgSO_4$ (MW 120.415) 120.415 g, dissolve in 1 L water, and filtrate. Final concentration is 1000 mM $MgSO_4$.

To assemble 1 L AIM

- 918 ml of TB
- 20 ml of AIM solution I
- 20 ml of AIM solution II
- 40 ml of AIM solution III
- 2 ml of AIM solution IV

Note:

- Depending on the Terrific broth formula, the composition of AIM stock solution I and II may vary, so as to keep the concentrations of each component constant [12 g tryptone, 24 g yeast extract, 50-100 mM Na or K phosphate, 50 mM NH_4Cl, 5 mM Na_2SO_4, 0.0015 w/v % D-glucose, 0.6 w/v % alpha-lactose, 12 w/v % glycerol, and 2 mM $MgSO_4$]. The pH of the media should be neutral (7.0-7.2).

- Weights of chemicals are based the most common hydration type (anhydrous for all the salts and glucose, monohydrate for alpha-lactose). Double check the molecular weight of each chemicals to make sure you take the same type, if you want to follow the calculation. Otherwise, calculate yourself.

- Sterilization of solutions is achieved by either autoclave at 121 °C for 20 min, or filtration through 0.22 μm filter.

BMGY and BMMY - Buffered Glycerol-complex Medium and Buffered Methanol-complex

Media (1 L)

1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB 4× 10-5% biotin, 1% glycerol or 0.5-1% methanol.

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 mL water for BMGY and 795 mL water for BMMY, autoclave at 121 °C for 20 min.
2. 500X Biotin (0.02% biotin)

   Dissolve 20 mg biotin in 100 mL of water and filter sterilize. Store at 4 °C. The shelf life of this solution is approximately one year.
3. 10X Glycerol

   Mix 100 mL of glycerol with 900 mL of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.
4. 10X YNB (13.4% yeast nitrogen base with ammonium sulfate without amino acids)

   Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1,000 mL of water. Heat the solution to dissolve YNB completely in water.

   Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate. Filter sterilize and store at 4 °C. The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch.

5. 1M Potassium phosphate buffer, pH 6.0

Combine 132 mL of 1 M K2HPO4, 868 mL of 1 M KH2PO4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

To assemble 1 L AIM

- 100 mL 1 M potassium phosphate buffer, pH 6.0
- 100 mL 10X YNB
- 2 mL 500X B
- 100 mL 10X GY
- For BMMY, add 100% methanol directly to culture flasks to reach a final concentration of 0.5-1%.

Note: For expression of laccases, 0.1-0.2 mM copper sulfate should be added to BMMY medium (2-4 mL 500X Cu_2SO_4 to 1L of BMMY).

YPDS + Zeocin™ Agar - Yeast Extract Peptone Dextrose Medium with Sorbitol (0.5 l)

1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agar, and appropriate concentration of Zeocin.

1. 10X Dextrose (20% dextrose)

Dissolve 200 g of D-glucose in 1,000 mL of water. Autoclave for 15 minutes or filter sterilize (recommended). The shelf life of this solution is approximately one year.

2. 1M Sorbitol

Dissolve 18.2 g sorbitol in 100mL water. Autoclave for 15 minutes or filter sterilise.

3. Dissolve: 5 g yeast extract, 91 g sorbitol, 10 g of peptone 10 g, and agar in 450 ml of water, sterilize by autoclave.

4. Add 100 ml of 20% dextrose (filter-sterilize dextrose before use).

5. Cool solution to ~60°C and adds the appropriate amount of Zeocin™ from a 100 mg/ml stock solution.

Note: It is necessary to include Zeocin™ in the medium for selection of Pichia transformants only. Zeocin™ may be omitted from the medium when performing expression studies. Store YPDS slants or plates containing Zeocin™ in the dark at 4 °C. If stored away from light, the shelf life is 1-2 months.

Ni-NTA resin regeneration

1. Put all of the resin into a big column.
2. Wash with 3 CV water.
3. Wash with 3 – 5 CV of 0.5 M NaOH.
4. Wash with water 5 CV make sure pH turn back to 6 – 7.
5. Wash with 6 M guanidine hydrochloride and 25 mM imidazole, 2 CV each.
6. Wash with 5 CV water immediately.
7. Wash with 2 CV 100 mM EDTA.
8. Wash with more than 5CV water.
9. Re-charge the resin with 2 – 3 CV 100 mM $NiSO_4$ and keep resin in buffer for a couple hours with shaking.

10. Wash the resin with more than 10 CV water.

11. Wash with 2 CV 20% ethanol.

12. Wash with 2 CV 50% ethanol.

13. Wash with 2 CV 70% ethanol.

14. Wash with 2 CV 50% ethanol.

15. Wash with 2 CV 20% ethanol.

Stock the resin in 20% ethanol in 4 degree.

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