รหัสโครงการ SUT3-304-54-12-29

รายงานการวิจัย

การโคลนและการศึกษาการทำงานของ Os1BGlu4 เบตากลูโคไซเดสจากข้าว

Cloning and Functional Characterization of Rice Os1BGlu4 Beta-Glucosidase

ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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Assoc Prof. Mariena Ketudat-Cairns

Head of the project

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บทคัดย่อ

เบต้ากลูโคซิเดส (β-D-glucopyranosideglucohydrolases, E.C. 3.2.1.21) เป็นเอนไซม์ที่สามารถย่อยสลายพันธะไกลโคซิดิก และปลดปล่อยหมู่ไกลโคซิลออกจากไกลโคไซด์ และโอลิโกแซคคาไรด์ในจีโนมของข้าวพบยีนเบต้ากลูโคซิเดสทั้งหมด 34 ยีน จากการวิเคราะห์ลำดับโปรตีนโดยอาศัยความสัมพันธ์เชิงวิวัฒนาการ พบว่า Os1BGlu4 จากข้าวจัดอยู่ในกลุ่มเดียวกันกับ BGLU42 จาก *Arabidopsis* และ latex cyanogenic β-glucosidase จาก *Heveabrasiliensis* และเป็นอิสระจากกลุ่มอื่น ๆ เพื่อให้เข้าใจถึงบทบาทหน้าที่ของเอนไซม์ Os1BGlu4 จึงทำการผลิตโปรตีนลูกผสม (recombinant Os1BGlu4; rOs1BGlu4) ใน *Escherichia coli* สายพันธุ์ Origami B(DE3) พบว่าเวลา 16 ชั่วโมง ที่อุณหภูมิ 20 องศาเซลเซียส เป็นสภาวะที่เหมาะสมในการผลิตโปรตีนลูกผสมนี้ โดยไม่จำเป็นต้องมีตัวกระตุ้น (isopropyl β-D-1-thiogalactopyranoside; IPTG) การวิเคราะห์ทางชีวเคมี พบว่าที่ค่าพีเอช 6.5 และอุณหภูมิ 45 องศาเซลเซียส เป็นสภาวะที่เหมาะสมสำหรับการทำงานของเอนไซม์ การวิเคราะห์ความจำเพาะของเอนไซม์ rOs1BGlu4 ต่อสารตั้งต้น พบว่าในระดับขั้นโพลีเมอไรเซชัน (degree of polymerization; DP) ของเอนไซม์ลูกผสมนี้มีความสามารถในการย่อยสลายพันธะ β-(1, 3)-linked oligosaccharides ที่ระดับ 2-3 และพันธะ β-(1, 4)-linked oligosaccharide ที่ระดับ 3-4 การศึกษาความสามารถในการย่อยสลายซับสเตรท โดยอาศัยพื้นฐานของตัวแปรทางจลนศาสตร์ พบว่าเอนไซม์นี้สามารถย่อยสลาย *p*NP-glucopyranoside (*p*NPG) และ *p*NP-fucopyranoside ได้อย่างมีประสิทธิภาพ นอกจากนี้การวิเคราะห์ด้วยโครมาโตกราฟีแบบแผ่นบาง (thin layer chromatography; TLC) ยังแสดงให้เห็นว่าเอนไซม์ชนิดนี้สามารถย่อยสลายสารตั้งต้นจากธรรมชาติ เช่น salicin esculin และ para-coumaryl alcohol glucoside ได้ ในการศึกษาลำดับการย่อยสลายของเอนไซม์โดยใช้ *p*NP-cellobioside เป็นสารตั้งต้น พบว่าเอนไซม์นี้สามารถทำงานได้เมื่อสารตั้งต้นประกอบไปด้วยกลูโคสตั้งแต่ 2 โมเลกุล ขึ้นไป การศึกษาทรานไกลโคซิเลชั่น พบว่าที่ความเข้มข้น *p*NPG ในระดับสูงเอนไซม์ rOs1BGlu4 มีความสามารถในการส่งต่อกลุ่มของกลูโคสจาก *p*NPG ไปสู่เอทานอลและ *p*NPG ได้ จากการศึกษาสารยับยั้งการทำงานของเอนไซม์นี้ แสดงให้เห็นว่า $HgCl_2$ delta-glucono-lactone และ $FeCl_3$ มีอิทธิพลในการยับยั้งการทำงานของเอนไซม์ rOs1BGlu4

Abstract

Beta-glucosidases (β-D-glucopyranoside glucohydrolases, E.C. 3.2.1.21) are enzymes that hydrolyze glycosidic bonds to release nonreducing terminal glucosyl residues from glycosides and oligosaccharides. Thirty-four active rice β-glucosidase genes had been identified in the rice genome. Protein sequence based phylogenetic analysis showed that Os1BGlu4 along with *Arabidopsis* BGlu42 and *Heveabra-siliensis* latex cyanogenic β-glucosidase represented an independent cluster. To help narrow the possible functions of Os1BGlu4, recombinant Os1BGlu4 (rOs1BGlu4) was expressed in *E. coli* OrigamiB(DE3). The optimized expression conditions showed that 16hr incubation time at 20^{o}C without isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer were the optimum condition for the expression. Biochemical analyses showed that pH 6.5 and 45^{o}C were optimum conditions for the hydrolysis activity of rOs1BGlu4. The rOs1BGlu4 efficiently hydrolyzed β-(1,3)-linked oligosaccharides of degree of polymerization (DP) 2-3 and β-(1, 4)-linked oligosaccharide of DP 3-4. The rOs1BGlu4 can hydrolyze *para*nitrophenyl-β-D-glu-copyranoside (*p*NPG) and *p*NP-β-D-fucopyranoside efficiently, based on the kinetic parameters. Hydrolysis of natural substrates salicin, esculin and para-coumarylalcohol glucoside by rOs1BGlu4 can be detected by thin layer chromatography (TLC). The study of *p*NP-cellobioside sequential hydrolysis showed that the initial hydrolysis was between the two glucosyl moieties. The transglycosylation studies showed that a high concentration of pNPG, rOs1BGlu4 has the ability to transfer the glucose group of *p*NPG to ethanol and *p*NPG. The inhibition study revealed that $HgCl_2$, delta-glucono-lactone and $FeCl_3$ strongly inhibited the hydrolysis activity of rOs1BGlu4.

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CHAPTER I

INTRODUCTION

1. Importance and background of research project

Beta-glucosidases are enzymes mainly belong to GH1 and catalyze the hydrolysis of the β-glucosidic linkages between two carbohydrate moieties or a carbohydrate and an aglycone moiety (http://www.cazy.org/fam/GH1.html). These enzymes have been found in all living organisms and play many functions. Plant β-glucosidases contribute to variety of physiology mechanisms including lignification, cell wall degradation, chemical defense, activation of phytohormones, responses to biotic or abiotic stress as well as plant secondary metabolism (Dharmawardhana *et al*., 1995; Forslund *et al*., 2004; Sue *et al*., 2000; Brzobohaty *et al*., 1993; Kristoffersen *et al*., 2000; Leah *et al*., 1995; Morant *et al*., 2008). However, the understanding about rice β-glucosidases is still not complete. In rice, 40 β-glucosidase genes have been found and 34 GH1 genes are expressed in a range of organs and stages of rice, based on the cDNA and EST sequences in public databases (Opassiri *et al*., 2006). To date, only a few rice β-glucosidases have been characterized for their expression, physiology, biochemical properties, and structure (Akiyama *et al*., 1998; Opassiri *et al*., 2003, 2004, 2006, 2010; Chuenchor *et al*., 2008, 2010; Seshadri *et al*., 2009; Kuntothom *et al*., 2009, 2010; Wakuta *et al*., 2010).

Bioinformatics analysis indicated that almost all rice β-glucosidase ORFs, except *Os*1*bglu*4 were predicted to have signal peptides ranging in length from 18 to 44 amino acids, which would target them to the secretory pathway. Os1BGlu4 would be the only β-glucosidase to be localized in the cytosol. Therefore, the important of Os1BGlu4 the only GH1 of rice in the cytosol should be investigate. In this research the *Os*1*bglu*4 was cloned and express in *Escherichia. coli* and then the recombinant protein was characterized.

2. Research objectives

The objectives of this research project were:

2.1 Cloning of rice *Os*1*bglu*4 gene.

2.2 Recombinant expression of Os1BGlu4 β-glucosidase and test the kinetic parameter on variety of substrates to determine the substrate specificity. The temperature and pH optimum of the enzyme were also evaluated.

3. Scope

This research tried to elucidate the rice *Oryza sativa* β-glucosidase 4 (*Os*1*bglu*4) gene function. The main content includes the amplification of the *Os*1*bglu*4 gene from the rice cDNA library. Then the gene was cloned in to prokaryotic expression vector in order to express the recombinant protein. The protein was then purified and studied. Numbers of natural and synthetic substrates were test with the recombinant Os1Bglu4 protein to try to determine the function of the enzyme. The enzymatic parameters, including K_m, V_{max}, k_{cat}, etc. were also determined.

4. Conceptual framework

The knowledge of the substrate specificities and enzyme kinetics can help elucidate the function of the Os1BGlu4 enzyme.

5. Benefits of the research and expected beneficiaries

5.1 Benefits in addressing the problems of the institution

This research benefited SUT by addressing the need for graduate student training and production of international publications and meeting presentations to improve the standing of SUT in the academic community. This project was expected to have at least one international publication and a few meeting presentations. It had provided excellent training for one Ph.D. student.

5.2 Generation of new knowledge

Currently, the roles of cytoplasmic β-glucosidase are still unknown, so this research has tried to fill an important gap in the understanding of rice plant. The knowledge generated was used as the basis for further study of the regulation of the genes and their effects on plant growth, development and stress response. This understanding of basic rice biology allows basic knowledge approach to rice breeding in the future.

5.3 Providing knowledge to the people

Aside from serving as a training project for graduate students, this project had provided important knowledge of the inner workings of rice, which is an important part of Thai culture.

Discovery of new knowledge in this field had attract the attention and interests of the press and the general citizen, allowing them to acquire new knowledge about how rice grows in the Thailand Research Expo organized by NRCT in the year 2012.

5.4 Production of knowledge for business

Although this product has not immediate benefit expected for businesses, it has provide knowledge that will allow farmers and rice mills to improve their businesses in the future.

5.5 Use in product production

It is hoped that in the future, the knowledge gained can be used to improve the production of rice by either allowing production of rice with appropriate growth for cultivation and harvesting or improving rice seed germination rates to allow more production from less seed.

5.6 Use in improving product quality

The knowledge can be used for production of higher quality rice seed in the future. Currently, it will provide for high quality graduates of SUT.

5.7 Benefits to target groups

Plant scientists have benefit from this work by gaining new knowledge in the regulation of cytoplasmic β-glucosidase and rice growth and development. This knowledge will be transferred to molecular breeders. In the end, this should provide knowledge for improvement of rice varieties, which will ultimately benefit the farmers and the rice consuming public.

6. Background and literature review

β-Glucosidases (EC. 3.2.1.21) are glycosyl hydrolases (GH) which are found widely in all types of organisms (bacteria, archaea, and eukaryote). They play important roles in fundamental biological processes (Esen, 1993). These enzymes hydrolyze the β-O-glycosidic bond at the anomeric carbon of glucose moieties at the non reducing end of carbohydrate or glycoside molecules.

Besides β-glucosidases, plant GH1 members include β-mannosidases, β-thioglucosidases (Burmeister *et al*., 1997), and disaccharidases, such as primeverosidase (Mizutani *et al*., 2002), as well as hydroxyisourate hydrolase, which hydrolyzes the internal bond in a purine ring rather than a glycosidic bond (Raychaudhuri and Tipton, 2002).

The completion of *Oryza sativa* L. spp. *japonica* Rice Genome Project and the complementary indica rice (*Oryza sativa* L. spp. *indica*) genome project by the Beijing Genomic Institute (BGI) have allowed genome-wide analysis of gene families in this important crop (Yu *et al*., 2002). Forty GH1

genes were identified from rice databases, including 2 possible endophyte genes, 2 likely pseudogenes, 2 gene fragments, and 34 apparently competent rice glycosidase genes.

Bioinformatics analysis indicated that almost all rice β-glucosidase ORFs were predicted to have signal peptides, which would target them to the secretory pathway. However, Os1BGlu4, the only rice GH1 β-glucosidase sequence without signal peptide, clustered with *Arabidopsis* BGlu42 and *Hevea brasiliensis* latex cyano-genic β-glucosidase in an independent cluster (Opassiri *et al.* 2006). So, it was interesting to determine the substrate specificity and characteristics of a representative member in this cluster, which can enrich the knowledge of the GH1 β-glucosidases and help, narrow the possible biological function of Os1BGlu4. Therefore, in this research the *Os*1*bglu*4 was cloned and express in *E. coli* and then the recombinant protein was characterized.

There are many factors that affect soluble recombinant β-glucosidase expression, such as temperature, chaperonin proteins, induction time and IPTG application and expression host strains. It has also been suggested that an increasing growth temperature is one parameter to promote aggregation of a recombinant protein as an inclusion body (Strandberg *et al.* 1991, Chrunyk *et al.* 1993). Cicek and Esen (1998) expressed the maize β-glucosidases, rGlu1 and rGlu2, from the pET21 vector in *E. coli* strain BL21 pLysS and indicated that a higher percentage of total expressed β-glucosidase was soluble when the cultures were grown and induced at room temperature (25°C) than at 37°C. Higher temperatures (30°C and 37°C) did not favor production of higher active protein, while lower temperatures (20°C and 25°C) favored production of Os3BGlu6 in the active form (Seshadri 2008). Appropriate induction temperature promoted the correct folding of the protein (Dinner *et al.* 1999) and increased the percentage of the soluble protein. So far, many β-glucosidases have been produced and induced at 20-30°C (Opassiri *et al.* 2003, Opassiri *et al.* 2004, Opassiri *et al.* 2007, Chuenchor *et al.* 2008, Kuntothom *et al.* 2009, Seshadri *et al.* 2009, Opassiri *et al.* 2010).

It has been demonstrated that the folding of many proteins can be facilitated by proteins called molecular chaperones, and the aggregation of overexpressed protein may be prevented by fusion with small protein molecules, such as Thioredoxin (Trx), Glutathione S-transferase (GST), Maltose binding protein (MBP), The *E. coli* heat shock protein complex (GroEL/ES) and small ubiquitin-like modifier (SUMO), have also been reported to used with several proteins (Liu *et al.* 2005, Purbey *et al.* 2006). Among these small proteins, the Trx, in the pET32 vector system, has been extensively used in the expression of β-glucosidases (Cicek and Esen 1998, Opassiri *et al.* 2003, Opassiri *et al.* 2006, Opassiri *et al.* 2007, Kuntothom *et al.* 2009, Gomez *et al.* 2010, Zhang *et al.* 2011).

In the expression of recombinant protein using the pET systems, IPTG should be added into the medium so the *Lac* operon can initiate the expression of T7 polymerase. However, different IPTG concentrations result in variable amounts of active protein. Many β-glucosidases, for example Os3BGlu8, Os3BGlu7, Os7BGlu26, Os4BGlu12, can be induced to express at the IPTG concentration from 0.1-0.5mM. There were no significant differences in the activity of Trx-Os4bglu12 expressed in *E. coli* in the presence of 0.1-0.5mM IPTG at 20, 25 and 30^{o}C, for 8-16hr. The expression of pET32a(+)-*Os*3*bglu*7 (BGlu1) was induced in the presence of 0.4mM IPTG at 20^{o}C for 8hr (Opassiri *et al.* 2003). Strangely, in the expression of rice Os3BGlu6, after 16hr of incubation at 20^{o}C, soluble extracts of induced cells with 0.4mM IPTG and with no addition of IPTG had similar β-glucosidase activity, which means the protein can be expressed well even without the IPTG inducer (Seshadri 2008).

Many *E. coli* strains can be used as the host strains for pET vector protein expression system, such as BL21(DE3), Origami(DE3), Origami B(DE3), Rosetta(DE3), Rosettagami(DE3). Among these *E. coli* strains, Origami(DE3) had been used for expressing Os3BGlu7, Os3BGlu8 and Os7BGlu26 (Kuntothom *et al.* 2009). Origami B(DE3) had been used for the expression of Os4BGlu12 and GH5BG (Opassiri *et al.* 2006, Chantarangsee *et al.* 2007, Opassiri *et al.* 2007). The *E. coli* strain Rosetta(DE3) was used for the expression of two intracellular β-glucosidases belonging to the glycoside hydrolase family 1 from the basidiomycete *Phanerochaete chrysosporium* (Tsukada *et al.* 2006).

Bioinformatics analysis has shown that among all rice β-glucosidase, the Os1BGlu4 is the only one that does not contain any signal peptides which would indicate that the protein should not be targeted to the secretory pathway. Os1BGlu4 would be the only β-glucosidase to be localized in the cytosol. Therefore, the important of Os1BGlu4 was investigated. In this research the *Os*1*bglu*4 was cloned and express in *Escherichia. coli* and then the recombinant protein was characterized.

CHAPTER II

EPERIMENTAL METHODS

1. Os1BGlu4 Cloning

Rice cDNA was prepared for *Os*1*blgu*4 gene amplification. The PCR reaction contained 1X buffer, 2mM $MgCl_2$, 0.4mM dNTP, 0.4µM forward primer 3bglu4ATG_f (CACCATGGGG AGCACGGGGCGC), 0.4µM reverse primer 5bglu4pET32 (AGGGAATTCCTAGTTCATGTCAGC), 0.05U/µL *Taq* DNA polymerase, 0.05U/µL *Pfu* DNA polymerase, and 1µL cDNA template. The amplification condition was maintained at 94°C for 4min and then 30 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 1min and 50sec, and a final extension step at 72°C for 10min. The PCR products were purified by QIA quick extraction kit (QIAGEN).

2. Expression vector construction

The PCR products were digested with *Nco*I and *Eco*RI and purified, and then ligated into pET32a(+), which had been digested with the same restriction enzymes, and transformed into DH5α *E. coli* by electroporation. The cells were selected on 100µg/mL ampicillin LB-agar plate. Colony PCR was used to check for positive clones with gene specific primers. The recombinant plasmid (pET32a(+)*Os*1*blgu*4) from positive clones were extracted using QIAGEN Plasmid Prep Kits. The recombinant plasmid was sequenced to confirm the correct reading frame and sequences.

3. Recombinant protein expression in Origami B(DE3) *E. coli*

To produce recombinant thioredoxin-Os1BGlu4 fusion protein, the plasmid pET32a(+) *Os*1*blgu*4 was transformed into Origami B(DE3) *E. coli* by electroporation and selected the right clones on LB plate containing 100µg/mL ampicillin, 15µg/mL kanamycin and 12.5µg/mL tetracycline at 37°C. The positive clone was grown overnight in LB broth containing the same antibiotics at 37°C with shaking at 200rpm. The fresh starter culture was inoculated into LB broth containing the same antibiotics and then incubated at 37°C with shaking at 200rpm for 3hr until the optical density of 600nm reached 0.5-0.6. IPTG final concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5mM were added to the culture flasks to induce the expression of recombinant protein. The cultures were shaken 200rpm at 20°C, 25°C and 30°C with different induction times of 4, 8, 12 and 16hr. The induced cultures were

chilled on ice for 10min and then centrifuged at 4,000 x *g* for 10min at 4°C. The cell pellets were kept at -70°C until analysis.

4. Extraction and purification of recombinant protein

The cell pellets were thawed and resuspended in freshly prepared extraction buffer (50mM phosphate buffer, pH 8.0, 200µg/mL lysozyme, 1% Triton-X 100, 1mM PMSF). The resuspended cells were incubated at room temperature for 30min, and then the soluble proteins were recovered by centrifugation at 12,000rpm, 4°C for 10min. The soluble protein fractions were kept on ice for protein purification in the next step. An aliquot of the supernatants (10µL) were subjected to protein analysis by SDS-PAGE. The soluble protein fractions extracted from the above step was purified by immobilized metal affinity chromatography (IMAC) on BD TALON cobalt resin. Ten milliliter of soluble protein fractions were loaded onto a 2mL bed volume of cobalt resin, which was pre-equilibrated with 8 bed volumes of equilibration buffer (50mM NaH_2PO_4, 300mM NaCl, pH 8.0). The column containing bound recombinant proteins were washed with 4 bed volumes of equilibration buffer and then with 8 bed volumes of washing buffer (10mM immidazole in an equilibration buffer). The bound protein fractions were eluted from a column with 4 bed volumes of elution buffer (250mM immidazole in an equilibration buffer). The protein fractions were kept at 4°C.

5. SDS-PAGE analysis of protein expression

Protein samples were mixed with 1/4 volume of 5X loading buffer and boiled for 5min to denature proteins. Then, 10µL of each samples were loaded into 15% SDS polyacrylamide gels, and electrophoresed at a constant voltage of 120V for 80min. The gels were stained in staining solution for 30min and destained in destaining solution for 1hr. The molecular mass of protein bands was estimated by comparison to the Fermentas Protein Molecular Weight Markers.

6. Protein concentration determination and activity assay

The purified protein concentration was determined by protein assay kit, using bovine serum albumin (BSA) as the standard. Zero point eight milliliters of diluted protein samples were mixed with 0.2mL protein assay solution. The reactions were incubated at room temperature for 10min and the

absorbance of 595nm was measured.

The 10µL of purified Os1BGlu4 samples were incubated with 1mM *p*-nitrophenyl-β-D-glucoside (*p*NPG) in 50mM sodium acetate, pH 5.0, in a volume of 100µL at 37°C for 20min, and then 70µL of 1M Na_2CO_3 were added to stop the reaction, the liberated *p*NP were measured at 405nm (Opassiri *et al*., 2006).

7. Optimum pH and pH stability

To determine the pH optimum of Trx-Os1BGlu4 enzyme, buffers ranging from pH 3.5 to 10.5 (formate pH 3.5-4; sodium acetate pH 4.5-5.5; sodium phosphate pH 6-8.5; and CAPS pH 9-10.5), at 0.5 pH increment at the same buffer concentration were set up for measuring the pH optimum of enzyme activity.

The pH stability for the Trx-Os1BGlu4 were determined by incubating the enzymes in buffers ranging from pH 4 to 10 as above at increments of 1.0 pH unit for 10min, 1, 3, 6, 12 and 24hr at room temperature. After incubation, the enzyme was diluted 20 fold in 50mM buffer at the optimum pH, pH 5.0, and the aliquots of enzyme were assayed for activity with 1mM *p*NPG, and the products released were measured (Opassiri *et al*., 2006).

8. Optimum temperature and thermostability

The optimum temperature for enzyme activity was determined by incubating the Trx-Os1BGlu4 with 1mM *p*NPG in 50mM buffer at the optimum pH, pH 5.0, in a reaction volume of 100µL at temperatures ranging from 5-90°C at 5°C increments for 20min, and then 50µL of 1M Na_2CO_3 were added to stop the reaction, and measured the activity as above.

Thermostability of the enzyme was measured by incubating enzyme in 50mM buffer at the optimum pH, at different temperatures in the range of 20-70°C at 10°C intervals for 15, 30, 45 and 60min. Then, in a reaction volume of 100µL, the enzyme samples were assayed with 1mM *p*NPG in 50mM sodium acetate, pH 5.0, at 37°C for 20min.

9. Activity assays and kinetics study

9.1 Substrate specificity assay

The enzyme activity on various substrates were determined by either (1) the *p*-nitrophenol (pNP) liberated from the pNP derivatives of monosaccharides or disaccharides, or (2) glucose released from natural or artificial substrates. All substrate solutions were prepared in 50 mM sodium acetate, pH 5.0. The aglycone specificity of Os1BGlu4 β-glucosidase were tested with synthetic substrates, pNP-glycosides (pNP-β-D-glucoside, pNP-β-L-fucoside, pNP-α-D-glucoside, pNP-β-D-cellobioside, pNP-β-D-mannoside, pNP-α-L-arabionoside, pNP-β-D-xyloside, pNP-β-D-fucoside, pNP-β-D-galactoside). Trx-Os1BGlu4 enzyme was incubated with pNP glycoside substrate in 50mM buffer at the optimum pH for 5min at 37°C. Then, 50μL of 0.4M sodium carbonate were added to stop the reaction and the absorbance of the liberated *p*NP were measured at 405nm.

The Os1BGlu4 was also tested with polysaccharides and oligosaccharides. In the assay, 1-5μg enzyme were incubated separately with 0.5% (w/v) laminarin and cellulose, including laminaripentaose, laminaritetraose, laminaritriose, laminaribiose, laminari, cellohexaose, cellopentaose, cellotetraose, cellotriose and cellobiose. In 50mM buffer at the optimum pH, at 37°C for 30-60min. The reactions were stopped by boiling and the increase of glucose was measured colorimetrically by the peroxidase/glucose oxidase assay (Opassiri *et al*., 2006).

The products of Trx-Os1BGlu4 hydrolysis of cello- and laminari-oligosaccharides were detected by TLC. In a 50μL reaction mixture, 5μL enzyme was incubated with 5mM substrate in 50mM buffer at the optimum pH for 30min at 37°C. A 5μL of the reactions mixture were spotted on silica-gel 60 F254 plates and chromatographed vertically with solvent consisting of ethylacetate, acetic acid and water (2:1:1, by volume). The products were detected by spraying with developer solution (ethanolic 10% H_2SO_4) and baked at 120°C for 5min to visualize the sugar.

9.2 Kinetic parameter determination

Kinetic parameters, K_m and V_{max} of purified Trx-Os1BGlu4 with pNP-glycosides and oligosaccharides were determined in triplicate reactions. The initial velocity of hydrolysis for each substrate was initially determined using various protein concentrations and incubation times (5-20min) to find conditions that yield 0.1 to 1.0 absorbance units. The rates versus times were plotted and an appropriate time and enzyme amount were chosen for kinetic studies. The reactions containing buffer at 5-7 different substrate concentrations ranging from 0.1-4 fold the apparent K_m value were pre-

incubated at 37°C for 10min, and then the reactions were started by adding appropriately dilution of enzyme. The reactions were incubated as above at 37°C for 5-15min, depending on each substrate, to establish the initial velocity (V_0).

One unit of enzyme activity was defined as the amount of enzyme that produced 1μmole of product per min. Note that, the activity values for disaccharides were determined by dividing the amount of glucose released by two, since two glucose molecules were released per molecule of disaccharides hydrolyzed. The micromoles of the product formation for oligosaccharides was defined in terms of total glucose released, though oligosaccharides may also had more than one glucose released per substrate molecule due to sequential cleavage. The kinetic parameters were calculated by nonlinear regression of the Michaelis-Menten curves with the Grafit program.

10. The inhibition chemicals study

The inhibition of Os1BGlu4 activity by many chemicals was studied and 1mM *p*NPG was used as substrate. Various inhibitors were mixed with substrate in 50mM buffer at the optimum pH followed by adding Os1BGlu4 β-glucosidase and incubated for 10min at 37°C. The reactions were stopped by adding 70μL of 1M Na_2CO_3 and the absorbance was read at 405nm.

CHAPTER III

RESULTS AND DISCUSSION

1. Recombinant protein expression conditions

The coding sequence of *Os*1*bglu*4 gene was amplified from 14 day rice seedlings cDNA library. A single intense band near 1.5 kb was observed (Figure 1). The pET32a(+) expression system was chosen to produce recombinant plasmid (pET32a(+)*Os*1*bglu*4)

Figure 1 PCR product amplified with gene specific primers. Lane 1, DNA marker, lanes 2 and lane 3, PCR product.

After sequence confirmation, the pET32a(+)*Os*1*bglu*4 was introduced into competent Origami B(DE3) by electroporation. The transformed bacteria were induced to express the protein, in order to obtain large amount of active protein, IPTG concentration, induction time and induction temperature were investigated to optimize the expression of the protein. The results showed that, even though the amount of cell from 10°C and 20°C treatments were lower than that of 30°C (Figure 2), the crude extract had higher 'activity' (Figure 3). This indicated that the rOs1BGlu4 is expressed better at 20°C. The activity of the enzyme decrease when expressed at 30°C. This phenomenon was also observed in the expression of Os3BGlu6 (Seshadri *et al.* 2009). This is caused by the higher percentage of insoluble

protein which were not folded properly due to fast expression under higher temperature (Baneyx 1999, Baneyx and Mujacic 2004).

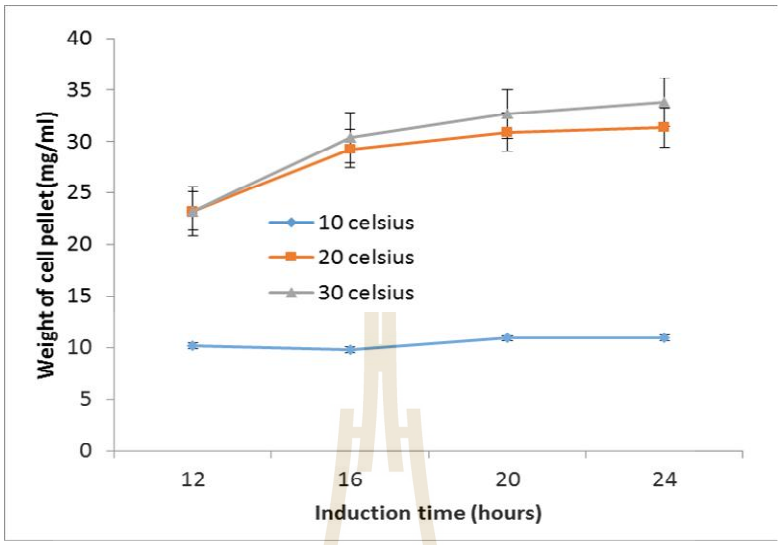

Figure 2 The effect of induction time and induction temperature to the weight of cell pellet.

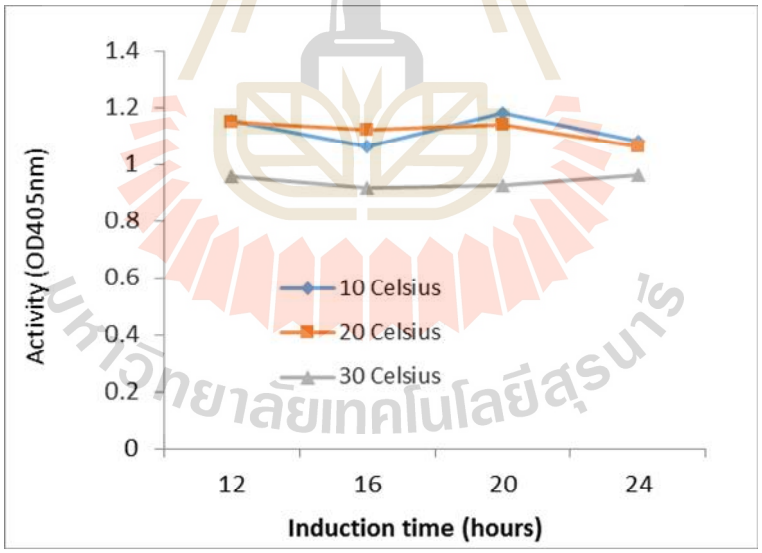

Figure 3 The effect of induction time and induction temperature to the 'activity' of crude Trx-His6-rOs1BGlu4. The 100µL reaction included 10µL of crude protein (1g cell pellet/3ml extraction buffer), 1mM *p*NPG and 50mM sodium acetate buffer; pH 5.0, the reaction was incubated at 30°C for 15min. The *p*NP release was measured at OD 405nm.

The induction time had no significant effect to the activity of 6His-Trx- rOs1BGlu4 from 12-24hr (Figure 3). The IPTG concentration had no significant effect on the weight of cell pellet in the 10°C treatment (Figure 4), because the cell growth at 10°C treatment was very low. In contrast, the cell growth of the 20°C and 30°C treatments was inhibited by the addition of IPTG. Most proteins expressed in the pET system need IPTG to induce the expression of the protein. The concentration is between 0.1-0.5mM in the expression of some rice β-glucosidases despite the 1mM concentration recommended by the manufacture (Opassiri *et al.* 2006, Jeng *et al.* 2011). However, IPTG was not needed for the expression of Trx-His6-rOs1BGlu4. The IPTG however, inhibited the growth of the cells instead, which resulted in the decrease in the weight of cell pellet (Figure 4). Moreover, the presences of IPTG have no significant effect on the specific activity of Trx-His6-rOs1BGlu4 (Figure 5). This phenomenon was also observed during the expression of Os3BGlu6 (Seshadri *et al.* 2009).

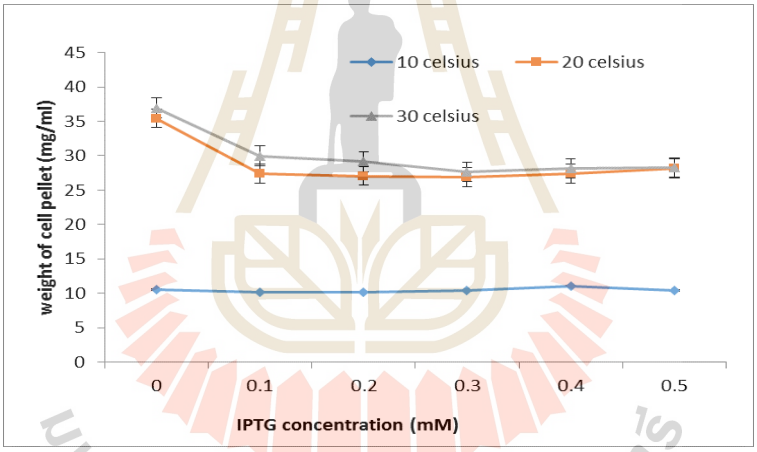

Figure 4 The effect of IPTG concentration to the weight of the cell pellet. The 100µL reaction included 10µL of crude protein (1g cell pellet/3 ml extraction buffer), 1mM *p*NPG and 50mM sodium acetate buffer; pH 5.0, the reaction was incubated at 30°C for 15min. The *p*NP release was measured at OD 405nm.

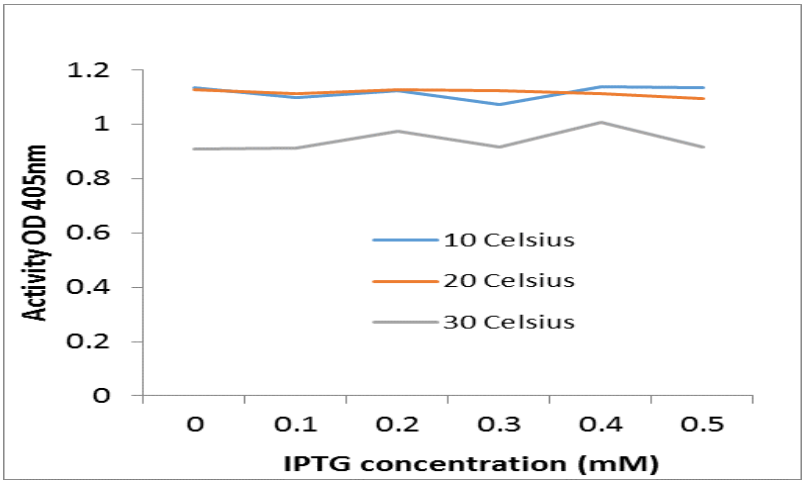

Figure 5 The effect of IPTG concentration to the 'activity' of crude Trx-His6- rOs1BGlu4.

2. Recombinant protein extraction and purification

Purification of the recombinant protein was designed to allow easy and fast, single step purification. The 6His tag in the pET system facilitates the use of affinity chromatography. The recombinant Trx-His6-rOs1BGlu4 was purified by IMAC on BD Talon™ (immobilized cobalt) metal affinity column to obtain approximately 85% pure protein. And an intense band at 66kD was observed on SDS-PAGE (Figure 6). After concentrated and changing the buffer, the concentration of Trx-His6-rOs1BGlu4 was 3.4mg/mL. Approximately 2.8mg of purified Trx-His6-rOs1BGlu4 could be obtained per liter of bacterial expression culture. The purified Trx-His6-rOs1BGlu4 was cut by the enterokinase and the recombinant Os1BGlu4 (rOs1BGlu4) which is about 55kD, and the thioredoxin tag were released (Figure 7). The size of rOs1BGlu4 by experimental estimate is almost identical to the predicted molecular weight (55.3kD). After a second IMAC purification step, the pure rOs1BGlu4 was obtained as a single band on SDS-PAGE. The buffer of rOs1BGlu4 was changed to 20mM Tris-Cl, pH 8.0, the concentration was 2.2mg/mL. This rOs1BGlu4 was aliquotted and kept in -20°C and used to characterize the biochemical properties.

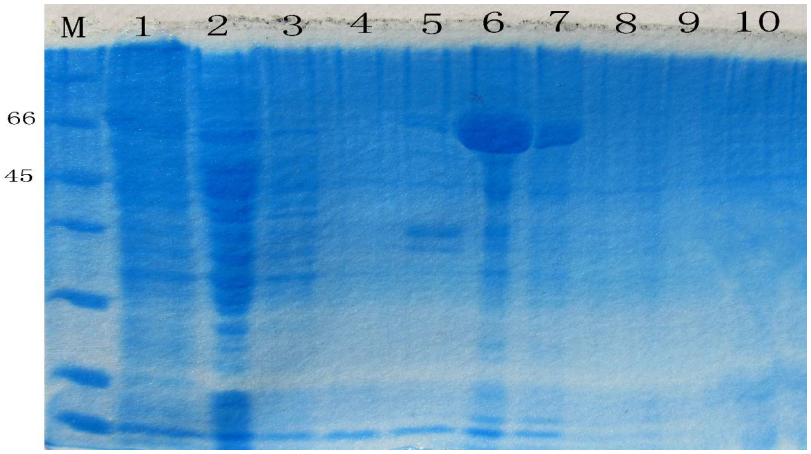

Figure 6 SDS-PAGE profiles of Trx-His6-rOs1BGlu4 purification using IMAC. M, standard marker (Bio-RAD), lane 1, crude protein, lane 2, flow through, lane 3, solution washed by W0, lane4, solution washed by W0+5 mM imidazole, lane 5, solution washed by W0+20mM imidazole, lane 6, solution washed by W0+50mM imidazole, lane 7, solution washed by W0+100mM imidazole, lane 8, solution washed by W0+250mM imidazole, lane 9, solution washed by 500mM imidazole, lane 10, solution washed by 50mM MES, pH5.0.

Figure 7 SDS-PAGE profiles of Trx-His6-rOs1BGlu4 recombinant protein expressed in Origami B(DE3) after incubation at 20°C for 16hr. M, standard protein marker (Bio-RAD), Lane 1, crude Trx-His6-rOs1BGlu4, lane 2, purified Trx-His6-rOs1BGlu4, lane 3, Trx-His6-rOs1BGlu4 digested by enterokinase, lane 4, purified rOs1BGlu4.

3. Optimum pH and pH stability

When the pure rOs1BGlu4 was obtained, the rOs1BGlu4 was characterized. The results showed that rOs1BGlu4 was most active at pH 6.5 when assayed with 1mM *p*NPG for 10min (Figure 8). When compared with others pH buffers, rOs1BGlu4 had higher activity at pH 6.0-7.0 buffers. The rOs1BGlu4 had almost no activity when the enzyme was assayed in buffers with pH below 5.0. The enzyme activity also decreased dramatically at pH above 8.0. The activity of rOs1BGlu4 had similar trend between the two sets of the buffer (Figure 8 and 9). The pH optima of most β-glucosidases ranged between pH 4 and 7.5, depending on their source and cellular location, and they tend to be stable over a range of pH from 4 to 9 (Ketudat-Cairns and Esen 2010). The pH optimum of the purified rOs1BGlu4 was pH 6.5, which is reasonable even it is different to many others β-glucosidases (Os3BGlu8, pH 5.0, Os7BGlu26, pH 4.5, Os3BGlu7, pH 5.0, rHvBII, pH 4.0) (Esen 1993, Hrmova *et al.* 1998, Opassiri *et al.* 2003). This pH is related to the pH environment of the catalytic reaction, since Os1BGlu4 was predicted to localize to the cytoplasm, the pH of which should be near neutral pH 7 (Berrin *et al.* 2002).

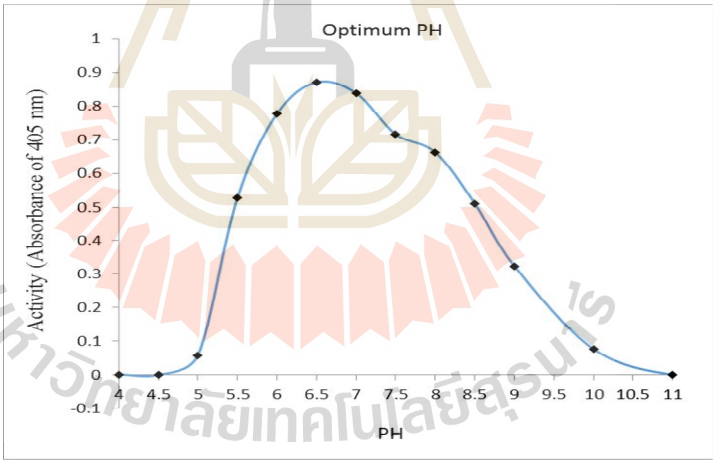

Figure 8 The activity versus pH profile for rOs1BGlu4 over the pH range of 4.0-11 (formate pH 3.5-4.0; sodium acetate pH 4.5-5.5; sodium phosphate pH 6.0-7.5; Tris pH 8.0-9.5 CAPS pH10.0-11.0). rOs1BGlu4 (0.25μg) was assayed with 1mM *p*NPG in different 50mM pH buffers at 30°C for 10min.

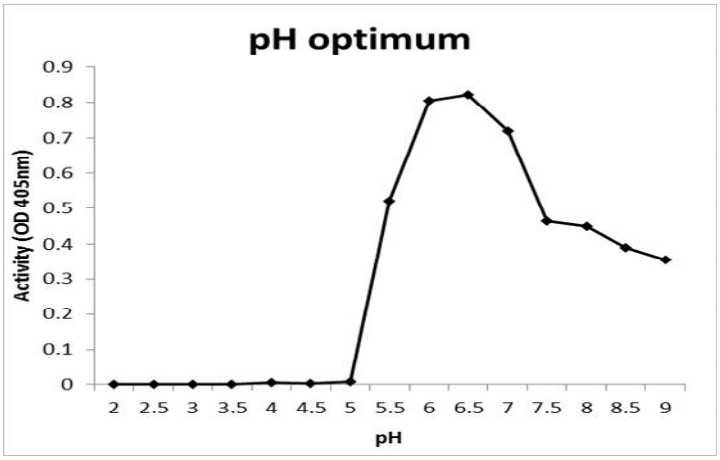

Figure 9 The pH optimum for rOs1BGlu4 over the pH range of 2.0-9.0 (50mM citric acid and disodium hydrogen phosphate buffers with pH ranging from pH 2.0 to pH 9.0). Os1BGlu4 (0.25μg) was assayed with 1mM *p*NPG in different pH buffers at 30°C for 10min.

The rOs1BGlu4 was relatively stable over the pH range of 6.0-8.0, when incubated for up to 24hr (Figure 10). As the time increased from 10min to 24hr, the activity of rOs1BGlu4 decreased in all the pH buffers, but the decreased of the activity was relatively low in pH 6.0-8.0 when compared with the other pH buffers. The pH 7-8 and temperature 0-4°C are the normal storage condition for many proteins, when major protease contaminants have been removed (Ketudat-Cairns and Esen 2010). As with other proteins, pH extremes, co-purifying proteases, and microbial contamination may result in degradation, although many β-glucosidases are resistant to proteases due to their tightly folded core structure (Ketudat-Cairns and Esen 2010). The purified rOs1BGlu4 showed two bands on the SDS-PAGE occasionally (Figure 11), which may result from internal cleavage but leaves the fold intact (Ketudat-Cairns and Esen 2010).

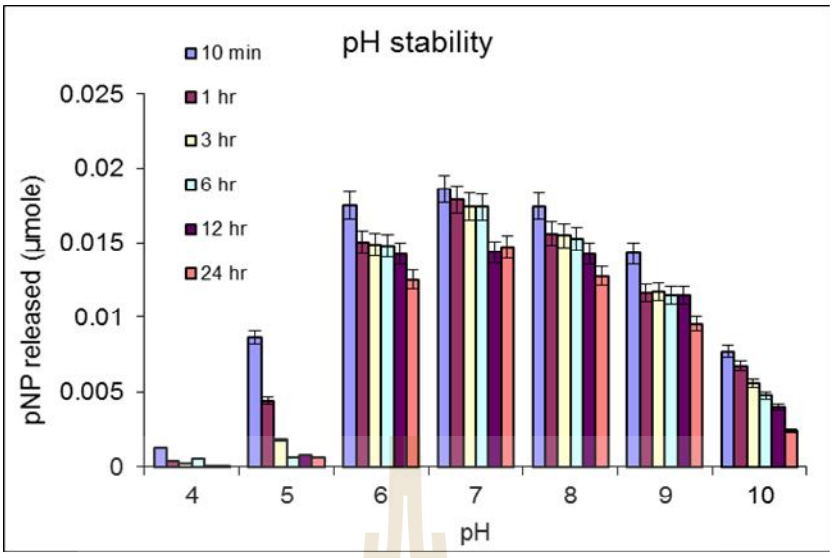

Figure 10 The pH stability of rOs1BGlu4 after incubation for 10min to 24hr at 30°C over the pH range of 4.0-10.0 (formate pH 4.0; sodium acetate pH 5.0; sodium phosphate pH 6.0-8.0; CAPS pH 9.0-10.0). Aliquots of enzyme in each pH buffer were sampled at the designated times and diluted 5 fold and assayed in 50mM phosphate buffer, pH 6.5, and incubated with 1mM *p*NPG at 30°C for 10min.

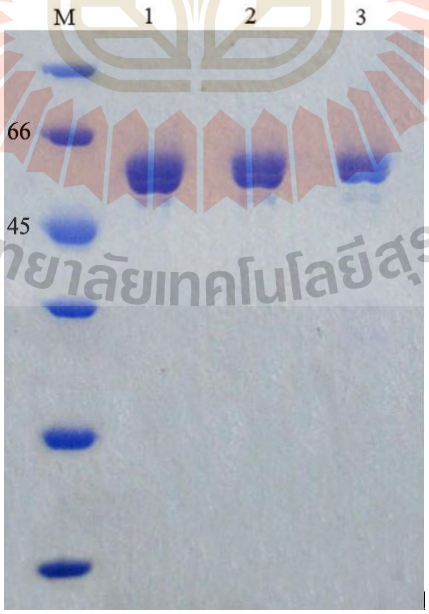

Figure 11 SDS-PAGE profile of purified rOs1BGlu4 showed two bands. M, protein marker, lane 1-3, purified rOs1BGlu4.

4. Optimum temperature and thermostability

The temperature optimum for rOs1BGlu4 was determined by incubating the enzyme with 1mM *p*NPG for 10min at different temperatures ranging from 5-90°C. The activity of rOs1BGlu4 at different temperature was considered to be of normal distribution (Figure 12), the peak was 45°C. As the temperature increased from 5-45°C, the activity of rOs1BGlu4 increased correspondingly, as the temperature continued to increase from 45-90°C, the activity of rOs1BGlu4 decreased gradually. The optimum point was 45°C, which corresponded to the results that many β-glucosidases that have temperature optima near 50°C (Konno *et al.* 1996, Akiyama 1998, Riou *et al.* 1998). However some β-glucosidases have higher optimal temperatures, such as the Thai rosewood and *Dalbergia nigrescens* β-glucosidases, which have a temperature optima of 60 and 65°C, respectively (Srisomsap *et al.* 1996, Chuankhayan *et al.* 2005). Because high activity at temperatures above the extremes of the enzyme's natural environment is not physiologically relevant and these temperatures may result in rapid heat denaturation, assays are often run at 30-40°C (Ketudat-Cairns and Esen 2010).

The thermostability study was performed by incubating the enzyme with the phosphate buffer pH 6.5, at temperatures ranging from 20-60°C for 10-60min, and then aliquots of rOs1BGlu4 was assayed with 1mM *p*NPG at pH 6.5 for 10min. Figure 13 showed that the enzyme was stable at 20°C and 30°C in the 1hr incubation. The rOs1BGlu4 lost about 20% of its activity when incubated at 40°C for 20min, rOs1BGlu4 was unstable at 50 and 60°C, 70% of the activity was lost with the 50°C treatment after only in 10min incubation, 83% of the activity was lost in 20min. At 60°C, only 10min incubation resulted in almost complete loss of rOs1BGlu4 activity.

For rOs1BGlu4, the activity decreased even after 10min incubation at 40, 50 and 60°C, which indicated that irreversible inactivation of the enzyme, occurred at temperatures higher than 40°C. However, the Os4BGlu12 and BGlu1 have been shown to be more stable than Os1BGlu4. The irreversible inactivation of the 2 enzymes happened at 50°C (Opassiri *et al.* 2003, 2006).

The maximum temperature for a given enzyme depends on a balance between the rate of the catalytic reaction and enzyme denaturation. β-Glucosidases from different organisms might have different optimum temperatures and stability, which would reflect different interactions stabilizing the enzymes (Dixon and Webb 1979). According to above analysis, 30°C was used as the standard incubation temperature in this experiment.

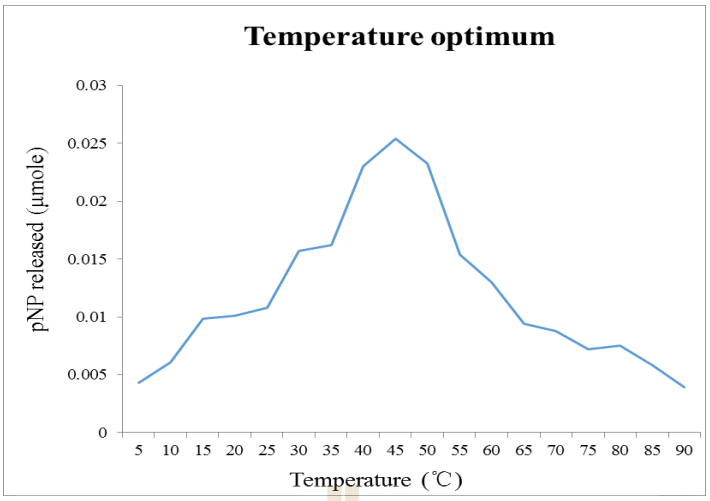

Figure 12 Activity of rOs1BGlu4 over the temperature range from 5-90°C. rOs1BGlu4 (0.25µg) was assayed with 1mM *p*NPG for 10min at the designated temperature.

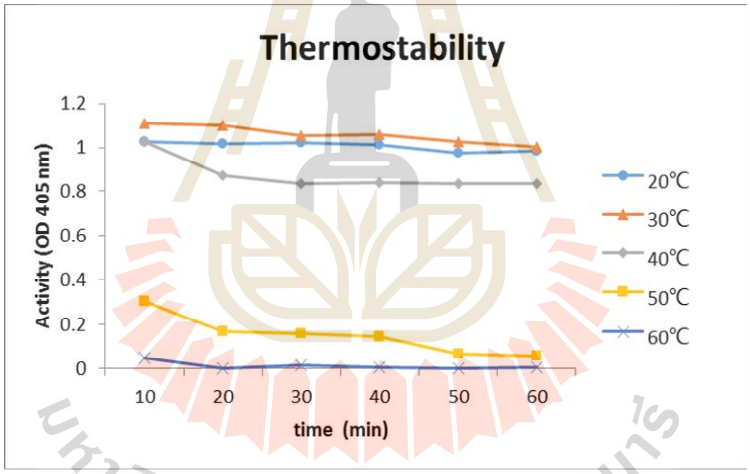

Figure 13 Thermostability of rOs1BGlu4 for 10-60min at 20-60°C. Concentrated rOs1BGlu4 were incubated in the phosphate buffer (pH 6.5) from 20-60°C, aliquots of the enzyme (0.25µg) at designated time were assayed with 1mM *p*NPG at 30°C for 10min.

5. Activity assays and kinetics study

5.1 Substrate specificity assay

The activity of the purified rice Os1BGlu4 β-glucosidase towards natural and artificial glycosides was characterized. Hydrolysis of *p*NP-glycosides with different glycone moieties was used

to assess glycone specificity of rOs1BGlu4. The release of *p*NP was measured according to the *p*NP standard curve. The activity of the purified rice rOs1BGlu4 towards *p*NP-glycosides was summarized in Table 1. Among the artificial *p*NP-glycosides, rOs1BGlu4 hydrolyzed the *p*NPG with relatively high efficiency, and *p*NP-β-D-fucopyranoside was hydrolyzed at 82% of the rate of *p*NPG. The rOs1BGlu4 hydrolyzed *p*NPG (k_{cat}/K_m, 17.92, s^{-1} mM^{-1}) and *p*NP-β-D-fucopyranoside (k_{cat}/K_m, 9.34, s^{-1} mM^{-1}) with high efficiency. Besides, rOs1BGlu4 hydrolyzed *p*NP-β-D-galactopyranoside, *p*NP-β-D-cellobioside, *p*NP-α-L-arabionopyranoside, *p*NP-β-D-mannopyranoside and *p*NP-β-D-xylopyranoside and at 4.32%, 3.39%, 2.4%, 1.8% and 1.0% the rate of *p*NPG, respectively. Hydrolysis of *p*NP-α-D-glucopyranoside, *p*NP-α-L-fucopyranoside, *p*NP-N-acetyl-β-D-glucosaminide, *p*NP-β-D-maltoside, *p*NP-β-L-fucopyranoside and 2,4-dinitrophenyl-2-deoxy-2- fluoro-β-D-glucopyranoside was not detectable.

Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess the glycone specificity of rOs1BGlu4, and the results showed that the rOs1BGlu4 was not stringent at the + 1 subsite, where the non-reducing glycosyl moiety is bound. This phenomenon is similar to many GH1 and GH3 β-glucosidases, such as the rice Os3BGlu7 (Opassiri *et al.* 2003) and rice Os4BGlu12 and GH5BG enzymes (Opassiri *et al.* 2006, 2007).

Substrate specificity of rOs1BGlu4 towards various kinds of oligosaccharides was determined. The enzyme activity was assayed by incubating 0.125μg enzyme with 1mM substrates in pH 6.5 phosphate buffer, at 30°C for 20min. The released glucose was oxidized by PGO solution; the OD 405nm was measured and compared with the glucose standard. The results are summarized in the Table 2. The rOs1BGlu4 hydrolyzed the β-1,3-linked oligosaccharide laminaribiose and laminaritriose, but not laminaritetraose, laminaripentaose. β-1,4-linked oligosaccharide cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose can be hydrolyzed at different rates. The rOs1BGlu4 cannot hydrolyze chitopentaose and the β-1,6-linked disaccharide gentiobiose. rOs1BGlu4 showed high hydrolytic efficiency with β-(1, 3)-linked oligosaccharides with DP of 2-3. The hydrolysis rates toward while those of cellotriose and cellotetraose are similar, about 70% of the rate of laminaribiose, cellopentaose and cellohexaose were about 28% of the rate of laminaribiose. By contrast, cellobiose is the most poorly hydrolyzed substrate. On the TLC profile, rOs1BGlu4 showed hydrolytic activity towards laminari-oligosaccharides and cello-oligosaccharides, but no measurable transglycosylation activity at the concentrations tested (Figure 14).

Table 1 Relative activities of purified rOs1BGlu4 in the hydrolysis of *p*NP-derivatives.

No.	Substrate	Activity[a](µmole/min/mg)	Relative activity[b] (%)
1	pNP-β-D-glucopyranoside	5.69	100.00
2	pNP-β-D-fucopyranoside	4.6	80.94
3	pNP-β-D-galactopyranoside	0.25	4.32
4	pNP-β-D-cellobioside	0.19	3.39
5	pNP-α-L-arabinopyranoside	0.14	2.40
6	pNP-β-D-mannopyranoside	0.1	1.80
7	pNP-β-D-xylopyranoside	0.06	1
8	pNP-α-D-galactopyranoside	n.d[c].	n.d.
9	pNP-α-D-mannopyranoside	n.d.	n.d.
10	pNP-β-L-arabinopyranoside	n.d.	n.d.
11	pNP-β-D-maltoside	n.d.	n.d.
12	pNP-α-L-fucopyranoside	n.d.	n.d.
13	pNP-N-acetyl-β-D-glucosaminide	n.d.	n.d.
14	2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside	n.d.	n.d.
15	pNP-α-D-galactopyranoside	n.d.	n.d.

[a]The assay contained 1mM substrate in 50mM sodium phosphate pH 6.5 buffer at 30°C

[b] Percentage activity relative to *p*NP released from *p*NP-β-D-glucopyranoside.

[c] n.d. means not detected

The cello-oligosaccharides and laminari-oligosaccharides were reported to be hydrolyzed by β-glucosidases which may be involved in cell-wall related processes. For example, rice Os3BGlu7 (BGlu1), Os3BGlu8, and Os7BGlu26 and Os4BGlu12 (Kuntothom *et al.* 2009, Opassiri *et al.* 2010). Although, Os1BGlu4 was predicted to be localized in the cytoplasm, surprisingly, it can hydrolyze the cello-oligosaccharide with DP 2-6 and laminari-oligosaccharides with DP 2-3 (Figure 14). Since Os1BGlu4 can hydrolyze the oligosaccharides, the hydrolysis activity of Os1BGlu4 was compared with some cell-wall remolding related β-glucosidases which have been characterized before.

Table 2 Relative activities of purified rOs1BGlu4 in the hydrolysis of oligosaccharides.

No.	Substrate	Activity[a] (µmole/min/mg)	Relative activity[b] (%)
1	Laminaribiose	1.81[c]	100.00
2	Laminaritriose	1.50	82.77
3	Laminaritetraose	n.d.[d]	n.d.
4	Laminaripentaose	n.d.	n.d.
5	Cellobiose	0.10	5.52
6	Cellotriose	1.27	69.96
7	Cellotetraose	1.39	76.91
8	Cellopentaose	0.53	29.22
9	Cellohexaose	0.48	26.61
10	Gentiobiose	n.d.	n.d.
11	Chitopentaose	n.d.	n.d.

[a] The assay contained 1 mM substrate in 50 mM sodium phosphate, pH 6.5, at 30 °C

[b] Percentage activity relative to glucose released from laminaribiose.

[c] The released glucose of laminaribiose and cellobiose was divided by 2 since one cut produced two glucose molecules.

[d] Means not detected.

Natural substrate specificity of rOs1BGlu4 hydrolysis was determined to study the possible natural substrate in rice. Some commercially available natural substrates were tested at 1mM final concentration (Table 3). The product was loaded onto the TLC plate. The result indicated that salicin, esculin and *para* coumaryl alcohol glucoside (*p*CAG) can be hydrolyzed by rOs1BGlu4 (Figure 15). As judged from the TLC plate, the glucose released from the esculin at the identical time is more than that from salicin and *p*CAG. That suggested that esculin can be hydrolyzed more efficiently than salicin and *p*CAG.

The rOs1BGlu4 was predicted to be a cyanogenic β-glucosidase and clustered with a *Hevea brasiliensis* latex cyanogenic β-glucosidase (Opassiri *et al.* 2006), but the plant cyanogenic glucosides, linamarin, and its precursor amygdalin were not hydrolyzed by rOs1BGlu4 (Table 3).

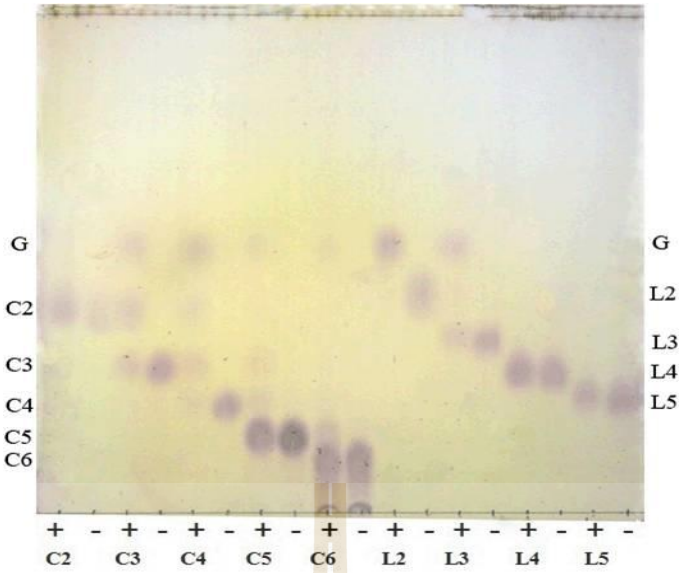

Figure 14 Hydrolysis products of rice Os1BGlu4 with cello-oligosaccharides and laminari-oligosaccharides and detected by TLC. In each 50µL reaction, 0.125µg Os1BGlu4 was incubated with 1mM oligosaccharide in 50mM phosphate buffer, pH 6.5, at 30°C for 20min. Samples were incubated with (+) and without (-) enzyme. Then, 2µL of the reaction was loaded onto the TLC plate. The plate was detected with staining method as described in method 3.3.11.3. G, C2, C3, C4, C5, C6 stand for glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, respec- tively, L2, L3, L4, L5 stand for the laminaribiose, laminaritriose, lamina- ritetraose, laminaripentaose, respectively.

The Os3BGlu8 could only hydrolyse esculin among the tested natural substrates, Os3BGlu6 can hydrolyze esculin, *p*CAG and salicin, the same as the rOs1BGlu4, so, the co-expression of these genes was checked to determine whether they are co-expressed. When *Os*1*bglu*4 expression was set to 1, the co-expression of Os3Bglu6 is 0.58 (close to the threshold 0.6 significant level), but the *Os*3*bglu*8 is not co-expressed (http://genecat.mpg.de). That possibly means the *Os*3*bglu*6 was co-expressed together with the *Os*1*bglu*4 to implement a similar function by hydrolyzing similar glycosides.

Since the natural substrate is similar, the hydrolysis activity of Os3BGlu6 was compared with rOs1BGlu4 extensively. Os3BGlu6 hydrolyzed *p*NP-β-D-fucopy-ranoside (k_{cat}/K_m =67 s^{-1} mM^{-1}) and *p*NPG (k_{cat}/K_m = 6.2 s^{-1} mM^{-1}), compared with rOs1BGlu4 hydrolyzed *p*NP-β-D-fucopyranoside (k_{cat}/K_m =9.3 s^{-1} mM^{-1}), *p*NPG (k_{cat}/K_m = 17.9 s^{-1} mM^{-1}).

Table 3 Commercially available natural substrates.

No.	Substrate	Glucose	No.	Substrate	Glucose
1	Salicin	+	14	n-octyl-β-D-glucoside	-
2	Esculin	+	15	GA_4 glucose ester	-
3	Linamarin	-	16	α-lactose	-
4	D-amygdalin	-	17	Sinigrin monohydrate	-
5	Trans-zeatin glucoside	-	18	Maltose	-
6	Daidzin	-	19	Metyl-β-D-glucopyranoside	-
7	Genistin	-	20	Arbutin	-
8	Naringin	-	21	Palatinose	-
9	Queretin-3-glucoside	-	22	mangiferin	-
10	*p*-CAG	+	23	lactulose	-
11	Coniferin	-	24	epigenin-7-glucoside	-
12	Indoxyl-β-D-glucoside	-	25	uridine	-
13	n-hepty-β-D-glucoside	-			

'+' stands for glucose was detected, '-' stands for glucose was not detected

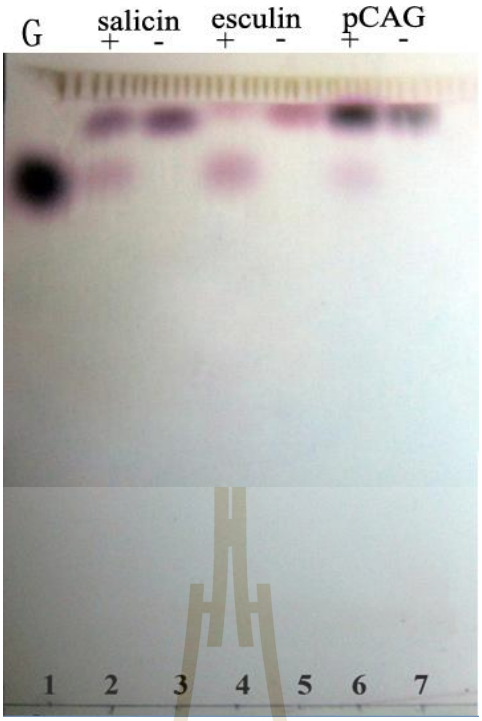

Figure 15 Hydrolysis products of rOs1BGlu4 with natural substrates. In 50μL reactions, rOs1BGlu4 (0.125μg) was incubated with 1mM natural substrate in 50mM phosphate buffer, pH 6.5, at 30°C for 30min. Two microliters of the reaction was loaded onto the TLC plate. Carbohydrates on the plate were detected as described in 3.3.11.3. Lane 1, glucose standard, lane 2, salicin+rOs1BGlu4, lane 3, salicin control reaction, lane 4, esculin+rOs1BGlu4, lane 5, esculin control reaction, lane 6, pCAG+rOs1BGlu4, lane 7, *p*CAG control reaction.

5.2 Kinetic parameter determination

The kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) of rOs1BGlu4 enzyme in the hydrolysis of various *p*NP-β-D-glycosides were determined and the data were summarized in Table 4. K_m value measures affinity of the enzyme for substrate. The lower K_m value, the less substrate was needed to saturate the enzyme. The k_{cat} gives a direct measure of the catalytic production of product under optimum conditions. *p*NP-β-D-glucoside can be hydrolyzed by rOs1BGlu4 efficiently, with the k_{cat}/K_m value 17.92 $s^{-1}mM^{-1}$. *p*NP-β-D-fucoside can be hydrolyzed about 2-fold less efficiently than *p*NPG by rOs1BGlu4. However, the K_m value of *p*NP-β-D-fucopyranoside and *p*NPG were similar at about 0.71±0.02 mM, which indicated the k_{cat} value of *p*NPG is 2-fold that of *p*NP-β-D-fucopyranoside. The *p*NP-β-D-cellobioside can be hydrolyzed slowly, with the catalytic efficiency (k_{cat}/K_m) value 3.92 s^{-1} mM^{-1} in terms of *p*NP release. *p*NP-β-D-mannoside, *p*NP-α-L-arabinopyranoside and *p*NP-β-D-

galactopyranoside were hydrolyzed very slowly, with similar k_{cat}/K_m value about 0.5 s^{-1} mM^{-1}. The *p*NP-α-L-arabinopyranoside has the lowest K_m value, that means this substrate easily saturates to the rOs1BGlu4, but the bond between the *p*NP and the L-arabinose is difficult to cleave.

Table 4 Apparent kinetic parameters of rice rOs1BGlu4 in the hydrolysis of *p*NP-derivatives.

Substrate	**k_{cat} (S^{-1})**	**K_m (mM)**	**k_{cat}/K_m (S^{-1} mM^{-1})**
*p*NP-β-D-glucoside	12.76±0.18	0.71±0.02	17.92
*p*NP-β-D-fucoside	6.61±0.080	0.71±0.02	9.34
*p*NP-β-D-cellobioside	2.06±0.062	0.53±0.03	3.92
*p*NP-α-L-arabinoside	0.52±0.0043	0.38±0.02	0.43
*p*NP-β-D-galactoside	3.16±0.065	7.33±0.32	0.43
*p*NP-β-D-mannoside	1.25±0.025	2.24±0.03	0.56

The kinetic parameters of rOs1BGlu4 enzyme in the hydrolysis of various oligosaccharides were determined and the data were summarized in Table 5. The rOs1BGlu4 hydrolyzed laminaribiose most efficiently, with the k_{cat}/K_m value of 12.45$s^{-1}$$mM^{-1}$, followed by the cellotetraose, the k_{cat}/K_m value is 8.73s^{-1} mM^{-1}. Laminaritriose, cellotriose, cellopentaose and cellohexaose can be hydrolyzed with the gradually decreasing efficiencies. The cellobiose was hydrolyzed most slowly, with the k_{cat}/K_m value only 0.03s^{-1} mM^{-1}. In contrast, laminaribiose can be hydrolyzed 415-fold more efficiently than cellobiose.

Rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 hydrolyzed cello-oligosaccharides with increasing efficiency as the degree of polymerization (DP) increased from 2 to 6, while Os4BGlu12 showed little increase in activity with DP of 4-6 (Ketudat-Cairns and Esen 2010). By contrast, the hydrolysis rate of rOs1BGlu4 had a relatively big difference, the k_{cat}/K_m increased from cellobiose to cellotetraose, and decreased from cellotetraose to cellohexaose. The cellotetraose can be hydrolyzed at 8 fold higher efficiency than the cellohexaose, which indicated that the rOs1BGlu4 has four subsites for binding of the glucosyl group.

The kinetic parameters of rOs1BGlu4 enzyme in the hydrolysis of esculin were determined. The hydrolysis product of esculin was esculetin, a yellow compound, with the absorbance at 405nm. Therefore, the standard curve of esculetin was set up. The kinetic parameters of hydrolysis

activity towards esculin was measured, the results showed that the k_{cat} is 2.13±0.04s^{-1}, the K_m is 0.55±0.02mM and the catalytic efficiency, k_{cat}/K_m is 3.86$s^{-1}mM^{-1}$, the esculin was hydrolyzed about 3.8 fold less efficiency than *p*NPG.

Table 5 Apparent kinetic parameters of rOs1BGlu4 in the hydrolysis of oligosaccharide.

Substrate	k_{cat} (S^{-1})	K_m (mM)	k_{cat}/K_m (S^{-1} mM^{-1})
Laminaribiose	4.67±0.09	0.38±0.02	12.45
Laminaritriose	3.37±0.55	0.6±0.03	5.63
Cellobiose	0.58±0.01	19.0±0.5	0.03
Cellotriose	2.74±0.06	0.59±0.03	4.64
Cellotetraose	2.27±0.03	0.26±0.01	8.73
Cellopentaose	2.15±0.03	1.07±0.04	2.01
Cellohexaose	1.08±0.02	1.1±0.05	0.99

6. The inhibition chemicals study

The effects of selected chemicals on rOs1BGlu4 hydrolysis activity were determined by adding to 10mM final concentration of various possible inhibitors in the substrate. The same reaction without the inhibitor was used as the control. The same reaction components without the substrate were used as a blank. The results were shown in Table 6. $HgCl_2$, delta-glucono-lactone, $FeCl_3$, 1%SDS and $CuSO_4$ had strong inhibitory effects on the activity of rOs1BGlu4. The $HgCl_2$, delta-glucono- lactone, $FeCl_3$ were able to inhibit almost 100% of the hydrolysis activity of rOs1BGlu4. Besides, 1%SDS also had strong inhibitory effect on rOs1BGlu4. The salts $PbCl_2$ and $CuSO_4$ also have relatively strong inhibitory effects. The EDTA, $CoSO_4$ and $MnSO_4$ had no inhibitory effects on the hydrolysis activity of rOs1BGlu4. The rest of the chemicals tested in the experiment have the inhibitory effects to the hydrolysis activity of rOs1BGlu4 ranging from 38% to 7%.

In order to figure out the inhibition effect of the strong inhibitors, the strong inhibitors were diluted, and the relative activity was measured (Table 7). The results indicated that $HgCl_2$ is a very strong inhibitor to the hydrolysis activity of rOs1BGlu4, when the concentration deceased to 0.05mM, the inhibitory effect was still 100%. The activity recovered to 18% eventually when the concentration was diluted to 0.01mM. For the delta-glucono-lactone, 62% of the activity was recovered when it was diluted to 0.1mM.

Table 6 The activity of Os1BGlu4 when 10mM of various inhibitor exist with 1mM *p*NPG in 50mM sodium phosphate, pH 6.5 at 30°C. The same reaction without the inhibitor was used as the CK. The same reaction components without the substrate were used as a blank.

No.	Inhibitor	Activity	Relative activity remaining (%)
1	CK	8.1	100
2	$HgCl_2$	0	0
3	Delta-glucono-lactone	0.02	0
4	$FeCl_3$	0.12	2
5	1%SDS	0.43	5
6	$CuSO_4$	2.49	31
7	$PbCl_2$	4.16	51
8	L-Arabinose	4.96	61
9	Imidazole	5.92	73
10	Urea	5.91	73
11	D-Mannose	5.91	73
12	LiCl	5.88	73
13	$NiSO_4$	6.4	79
14	$ZnCl_2$	6.36	79
15	D-Glucosamine	6.82	84
16	D-Xylose	6.9	85
17	$CdCl_2$	6.98	86
18	$MgCl_2$	7.16	88
19	D-Galactose	7.29	90
20	D-Glucose	7.55	93
21	$CaCl_2$	7.8	96
22	KCl	7.88	97
23	L-Histidine	8.01	99
24	$MnSO_4$	8.11	100
25	$CoSO_4$	8.16	101
26	EDTA	8.35	103

Table 7 The activity of rOs1BGlu4 when different concentrations of inhibitors were present together with 1mM *p*NPG in 50mM sodium phosphate, pH 6.5, at 30°C.

Inhibitor	**Concentration (mM)**	**Relative activity (%)**
Delta-glucono-lactone	10.0	0
	1.0	14
	0.1	62
	0	100
$HgCl_2$	10.0	0
	1.0	0
	0.1	0
	0.05	0
	0.01	18
	0	100

CHAPTER IV

CONCLUSION

The recombinant thioredoxin-Os1BGlu4 (Trx-His6-rOs1BGlu4) fusion protein was functionally expressed in Origami B(DE3). Sixteen hour incubation at 20°C, without IPTG inducer produced high amount of Trx-His6-rOs1BGlu4. The biochemical characterization showed that the optimum pH for the hydrolysis by rOs1BGlu4 was 6.5. The rOs1BGlu4 was stable over the pH range of 6.0-8.0 during the 24hr incubation. The optimum hydrolysis temperature was 45°C. The rOs1BGlu4 was stable at 20 and 30°C after 1hr incubation.

The rOs1BGlu4 efficiently hydrolyzed β-(1, 3)-linked oligosaccharides with DP of 2 and 3, and β-(1,4)-linked oligosaccharide with DP of 3 and 4. Cellopentaose and cellohexaose can be hydrolyzed with less efficiency. The laminari-oligosaccharides with DP more than 3 can not be hydrolyzed by the rOs1BGlu4. The rOs1BGlu4 can hydrolyze *p*NPG efficiently; the pNP-fucopyranoside was hydrolyzed with about 50% hydrolysis efficiency of the *p*NPG. Based on the kinetic parameters, others *p*NP-derivatives can be hydrolyzed with low efficiency. According to the TLC results, salicin, esculin and *p*-CAG can be hydrolyzed by rOs1BGlu4.

The effects of selected chemicals on rOs1BGlu4 hydrolysis activity were determined. The hydrolysis activity of rOs1BGlu4 was strongly inhibited by $HgCl_2$, delta-glucono-lactone and $FeCl_3$.

The Os1BGlu4 was predicted to localize to the cytoplasm, but it may still be involved in cello- and lamimari-oligosaccharides hydrolysis and might contribute to the formation of lignin by monolignol/glucoconjugate equilibrium or to pathogen and/or herbivore resistance.

This research has shown that rOs1BGlu4 could be expressed in *E. coli*. The characterization of this rOs1BGlu4 indicated that it can hydrolyze several synthetic and natural substrates. Further investigation in Os1BGlu4 over expressed and knock down transgenic plans might give more clue on the role of this enzymes in the rice plant.

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Chen, R. and Ketudat-Cairns, M. (oral presentation). Amplification of Rice Os1BGlu4 Beta-Glucosidase cDNA and Expression in *Escherichia coli* Proceeding of the 3rd Graduate conference Suranaree University of Technology 21-23 November 2010

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Education:

1984 High School Diploma
Math and Science Major
Chulalongkorn University Demonstration School
G.P.A. 3.89

1988 B.Sc. Biology (Plant Science and Technology)
Minor in Chemistry
Chiang Mai University, Thailand
G.P.A. 3.24

1995 Ph.D. Biology (Plant Molecular Biology and Genetic Engineering)
University of California San Diego, USA
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Awards, Scholarships & International Training courses:

1988 Prof. Dr. Dhab Nelanithi Foundation Award

1988-1990 Scholarship from Institute for Promotion of Teaching Science and Technology (IPST), Thailand

1990-1991 Scholarship from Biology Department UCSD, USA

1991-1992 Graduate Student Fellowship UCSD, USA

1992-1995 Genetics Training Grant from National Institute of Health (NIH), USA

1995 28/8-13/10 International Training Program (ITP) in Biotechnology at Gesellschaft fur Biotechnologische Forschung (GBF), Braunschweig,Germany

2000 21/9 ICRO-UNESCO International Training course on RNA and Biotechnology at Chinese Academic of Science, Shanghai, China

2006 Best paper of the year 2005 Award from Bioprocess and Biosystems Engineering Journal

Experiences:

1988-1995 Research Assistance, UCSD

1989-1994 Teaching Assistance, UCSD

1990-1995 Teaching Assistance Trainer, Department of Biology, UCSD

1995-1998 Instructor, Suranaree University of Technology

1995 4/11-16/12 Head of Business Center for WorldTech'95, Thailand

1997 Secretariat of the JSPS/NRCT Biotechnology Conference, Thailand

1998- 2000 Secretariat of the SUT Biotechnology Graduate Curriculum Development

1998- 2010 Assistant Professor, Suranaree University of Technology

1998- present Thai Society for Biotechnology, committee (7 terms)

2001- 2004 National Bio-safety Subcommittee (Microorganism)

2001- 2003 National Graduate Biotechnology Curriculum Development Project (Thai Society for Biotechnology, TSB & National Science and Technology Development Agency, NSTDA)

2001- present Institute of Agricultural Technology committee (5 terms)

2001- 2009 SUT Academic Senate member (4 terms)

2002- 2006 Department Chair, School of Biotechnology, Institute of Agricultural Technology, SUT

2010- present Associate Professor, Suranaree University of Technology

2011- 2013 SUT Academic Senate member

Membership: Thai Society for Biotechnology (Society committee 1998-2012, 7 terms)

Thai Society for Genetics

American Society of Plant Biology and Plant Physiology

Research Interested: Recombinant Protein Productions

Rice Functional Genomics

Rice Glycosyl Hydrolases

Molecular Biology of Cloned Animal and Stem cells

Reviewer for J. of Biotechnology, Food Control**,** Cellular Reprogramming, J. of the Science of Food and Agriculture, African Journal of Biotechnology, African Journal of Microbiology Research, Science Asia, etc.

International Publications:

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