論文の内容の要旨

応用生命工学専攻
平成 24 年度博士課程入学
氏　　名　李　宜海
指導教員名　有岡　学

論文題目

**A study on β-glucosidases derived from wood-feeding insect and symbiotic protist of termite**
**（材食性昆虫およびシロアリ腸内原生生物に由来するβ-グルコシダーゼに関する研究）**

**Introduction**

Wood-feeding insects and termites evolved from the same ancestor, and share a similar digestive system with high efficiency for decomposition of lignocellulose. These organisms, together with their symbiotic protists which live in their guts and contribute to entire degradation of cellulose ingested by their endosymbiosis host, could be new sources of cellulolytic enzymes for development of the second generation biofuel derived from lignocellulosic biomass.

Cellulose is a linear polymer consisting of β-1,4-linked D-glucopyranosyl unit and contributes to about 20–40% of dry weight of the plant primary cell walls. In the sequential degradation of cellulose by cellulases, endoglucanase (EG; endo-1,4-β-glucanase; EC 3.2.1.4) firstly cleaves the cellulose chains in its amorphous regions, then cellobiohydrolase (CBH; exo-1,4-β-glucanase; EC 3.2.1.91) attaches to cellulose chain ends and hydrolyzes cellulose chains typically into disaccharide units (cellobiose), and β-glucosidase (BG) completes the final step by cleaving cellobiose into glucose. The recently-discovered lytic polysaccharide monooxygenases (LPMOs) which cleave the crystalline regions of cellulose possibly have synergistic effects with cellulases. BG can alleviates the product inhibition of EG and CBH by cellobiose, whereas most BGs suffer product inhibition by glucose. Commercial cellulases are mostly from filamentous fungi *Trichoderma*, especially *T. reesei*. However, *Trichoderma* spp. are characterized to have low yield of BG and thus high titers of BG supplements are required in the commercial cellulase cocktails. Therefore, screening of new powerful BG is of great importance to improve the efficiency of commercial bioethanol production from cellulose.

BG is an enzyme that hydrolyzes the terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose. The activities of BGs are not limited to the β-1,4 glucosidic linkages, but also apply to β-1,2, β-1,3, and β-1,6 linkages. Furthermore, many of BGs are reported to be associated with the activities of β-D-galactosidase, α-L-arabinosidase, β-D-xylosidase, and β-D-fucosidase. On the basis of the homology of amino acid sequences, BGs are classified into the glycoside hydrolase (GH) families 1, 3, 5, 9, 30, and 116 in the Carbohydrate-Active enZYmes database (CAZy). In terms of glycosyl-transferring types, BGs are classified into 2 groups, based on whether they invert or retain the configuration of the anomeric carbon (C1). Aside for BGs in GH9, all BGs belong to the retaining type, in which the reaction is

mediated by two carboxyl groups in the catalytic center, with one acting as a nucleophile and the other an acid/base catalysts, respectively, and perform catalysis via a double-displacement mechanism (i.e. glycosylation and deglycosylation). In the GH1 BG, both the catalytic nucleophile and acid/base residues are glutamic acids, while in the GH3, aspartic acid plays a role as a catalytic nucleophile. In this study, PaBG1b, a powerful GH1 BG from the gregarious wood-feeding cockroach *Panesthia angustipennis spadica*, and RsBG, a GH3 β-glucosidase-like protein from the hindgut symbiotic protist of the lower termite *Reticulitermes speratus*, were heterologously expressed in the methylotrophic yeast *Pichia pastoris*, purified, and biochemically characterized.

**Chapter 1. Heterologous expression of PaBG1b in *P. pastoris***

The full-length cDNA of *pabg1b* encodes a putative GH1 BG of 502 amino acids with a KR dibasic amino acid pair located at 7 amino acids downstream from the amino terminus of the mature polypeptide. The expression of recombinant PaBG1b was initially conducted by using the episomal expression plasmid pBGP3, which harbors the autonomous replication sequence and the constitutive glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter. PaBG1b was designed to be fused with c-myc-epitope and hexahistidine ($His_6$) tags at its N-terminus, which allows detection of the recombinant protein by Western blot analysis, and purification via Ni-NTA column chromatography. The expression of PaBG1b was evidenced by Western blot analysis after 4 days of culture. BG activity assay also confirmed that PaBG1b was successfully expressed in the culture supernatant of pBGP3-PaBG1b transformant. The immunoblot bands of PaBG1b was found to fade out from the fifth day of culture, suggesting that PaBG1b was susceptible to proteolytic degradation and/or lost its N-terminal tags by the Kex2 endoprotease cleavage at the KR site.

To increase the production level of PaBG1b, an integration-type plasmid, pPICZα, in which the expression of heterologous protein is achieved by the inducible alcohol oxidase 1 (*AOX1*) promoter, was employed. In this case, the tags were moved to the C-terminus. Time-course analysis showed that the expression of PaBG1b was induced on the first day of induction by methanol and the yield of PaBG1b in the culture supernatant reached the highest after 6 days of induction. Western blot analysis showed that the bands of PaBG1b remained clear and intense even the culture was extended to 7 days, which implied that the protein tags at the C-terminal were stable.

**Chapter 2. Purification and biochemical characterization of PaBG1b**

PaBG1b in the culture supernatant was purified though three successive steps including ammonium sulfate precipitation, followed by Ni-NTA and anion exchange chromatographies. Ni-NTA column combined with imidazole elution is a simple and quick approach for purification of target proteins fused with $His_6$ tag. However, imidazole was found to strongly inhibit the BG activity of PaBG1b such that the inhibition could hardly be reversed either through dialysis or buffer exchange. Fortunately, substitution of sodium phosphate buffer with Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) buffer and instantly removing imidazole through dialysis successfully recovered the activity of purified product, although Tris

itself was also an competitive inhibitor of PaBG1b as described below.

To clarify the molecular nature of PaBG1b, deglycosylation analysis was conducted by treating purified PaBG1b with glycopeptidase F (GPF) or endoglycosidase H (Endo H). SDS-PAGE analysis showed that PaBG1b was *N*-glycosylated and two closely-migrating bands were present. N-terminal amino acid sequence analysis suggested that incomplete cleavage at the KR dibasic amino acid pair in the mature region of PaBG1b by Kex2 generated two different N-termini.

PaBG1b demonstrated the maximum specific activity of 45.5 U/mg and $V_{max}$ of 59.9 U/mg with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) as a substrate, whereas it displayed high specific activity of 338.5 U/mg and $V_{max}$ of 436.7 U/mg towards cellobiose. PaBG1b was also found to have relatively high catalytic efficiency with $k_{cat}/K_m$ of 109.8 $mM^{-1} \cdot s^{-1}$ for cellobiose. Thermostability and pH stability analyses demonstrated that PaBG1b is a mesophilic enzyme with an optimum temperature of 45°C and favors slightly acidic condition (optimum pH at pH5.5). PaBG1b was capable of degrading diverse saccharides and aryl-glycosides. PaBG1b displayed the ability of (but might be not limit to) degrading cello-oligosaccharides up to cellohexaose, and did not show transglycosylation activity under the condition tested. PaBG1b has a moderate tolerance to glucose ($K_i$=200.3 mM), and was not inhibited by cellobiose up to 100 mM. These properties endow PaBG1b with advantages in terms of conversion of cellulose to glucose. Although in general metal cations are not required for the catalytic activity of BGs, $Al^{3+}$ and $Cu^{2+}$ were found to stimulate the activity of PaBG1b, whereas $Zn^{2+}$, $Ni^{2+}$, $Fe^{3+}$, and $Fe^{2+}$ significantly inhibited the activity. To elucidate the interactions of PaBG1b with inhibitors, imidazole and Tris, the inhibition constants ($K_i$s) at pH 5.5 were analyzed, and determined as 4.3 mM and 5.9 mM, respectively. These results indicate that both imidazole and Tris are modest competitive inhibitors for PaBG1b with comparable affinity. Inactivation of PaBG1b under standard Ni-NTA affinity chromatography condition was suggested to be due to a specific binding of imidazole with PaBG1b under alkaline pH. Although PaBG1b displayed high activity towards cellobiose, the activity is approximately half of the native enzyme. One of the possible reasons was that the expression products were comprised of two kinds of polypeptides with different N-termini.

**Chapter 3. Heterologous expression in *P. pastoris* and purification of RsBG**

The full-length cDNA of *rsbg* encodes a polypeptide of 573 amino acids which is composed of two domains: the BglX domain (periplasmic BG and related glycosidases) and the GH3 C-terminal domain. However, the putative catalytic nucleophile residue of RsBG is glutamic acid (E275) rather than aspartic acid which is almost completely conserved in the canonical GH3 BG orthologues. Although BGs in GH1 employ two glutamic acids as the nucleophile and acid/base catalysts, the orientation is reversed to that of GH3 BGs in that the nucleophile is located C-terminal to the acid/base residue in GH1. Furthermore, unlike most GH1 BGs that consist of only one common N-terminal $(\beta/\alpha)_8$ TIM barrel domain, all of nine structurally-characterized GH3 β-glucosidases are multidomain proteins. Thus RsBG shares part of the features of both GH3 and GH1 BGs, but to be precise, it belongs to neither of them.

To elucidate the biochemical properties of RsBG, pBGP3-based expression system was employed.

Expression of RsBG was confirmed by Western blot analysis after 3 to 4 days of culture. BG assay demonstrated, however, that the activity of RsBG-transformant was only a little higher than the control strain harboring the empty vector. Furthermore, RsBG purified by Ni-NTA column chromatography showed no activity. To increase the production level of RsBG, pPICZα was employed to produce N-terminally-tagged RsBG. Although the culture supernatant of RsBG transformant exhibited slightly higher activity than that of the control strain, again the purified RsBG did not exhibit any activity towards either natural or artificial substrates of BG. Considering the possibility that Ni-NTA purification caused inactivation of RsBG, traditional purification techniques were employed. RsBG was purified through ammonium sulfate precipitation followed anion exchange chromatography, but the purified RsBG did not display any activity. RsBG without tags was also expressed, but the assay result showed that the BG activity of culture supernatant of the transformant was similar to that of the transformant expressing tagged RsBG. These results demonstrate that RsBG does not function as a BG. Finally, to examine if the presence of an extra methylene group in the side chain of putative catalytic nucleophile, i.e. glutamic acid (E275) of RsBG, compared to aspartic acid in the counterpart of other GH3 BGs, hindered the access of the substrates, a mutation study was conducted to substitute glutamic acid with aspartic acid (E275D mutation). However, the expression of RsBG E275D mutant failed and the product was not observed presumably due to degradation.

## Conclusion

In this study, two proteins from different organisms were successfully expressed in *P. pastoris* by using various expression settings. Biochemical characterization of PaBG1b revealed features potentially suitable for commercial purposes. Both imidazole and Tris were shown to be competitive inhibitors with the similar level of affinity towards PaBG1b at pH 5.5. However, they exhibited different inhibition potency in the pH shifting experiments. As the inhibition by Tris was easily recovered through dilution and restoring the pH to acidic pH, the inhibition by imidazole, at least when applied at pH 8.0, was hardly recovered. It might be attributable to a specific binding of imidazole and PaBG1b at alkaline pH. Intensive studies of PaBG1b including the structural analysis would be informative to clarify the key residues important for the catalytic function, substrate specificity, thermostability, and glucose tolerance, etc.

RsBG was successfully expressed in *P. pastoris* and the culture supernatant of transformants displayed limited but consistently higher activity compared to that of the control stain. However, no activity was detected in the purified RsBG preparation. These results might imply that the precise role of RsBG is not a function as a BG, but might be a product of a pseudogene gene, or a protein capable of boosting the activities of other BGs. Further studies are surely needed to elucidate the unexpected and unusual function(s) of RsBG distinct from those of canonical GH3 BGs.