Doctoral Dissertation (Shinshu University)

Studies on enzymatic properties of *Trichoderma reesei* β-glucosidases and their role in cellulase induction

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Contents

Abbreviations

1. Terms

GH	Glycoside hydrolase family
BGL	β-Glucosidase
CBH	Cellobiohydrolase
EG	Endoglucanase
WT	Wild type
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresism
TLC	Thin-layer chromatography
HPLC	High performance liquid chromatography
PDA	Potato dextrose agar medium

2. Carbohydrate

Glc	Glucose
G2	Cellobiose
G3	Cellotriose
G4	Cellotetraose
Lam	Laminaribiose
Sop	Sophorose
Gen	Gentiobiose
pNPG	*p*-Nitrophenyl-β-D-glucoside
CMC	Carboxymethyl cellulose

Chapter 1

Introduction and review of literature

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1.1. Enzymatic lignocellulosic biomass degradation by fungi

Lignocellulosic biomass, which is composite of cellulose, hemicellulose, lignin, pectin and other minor constituents, is considered as the most abundant renewable bioresource. If complete digestion is succeeded in biomass, numerous and diverse applications such as biofuel production were expected [1,2]. However, its complex structure hampers efficient enzymatic conversion to soluble sugars because many kinds of enzymes such as multiple cellulases, xylanase and other hydrolytic enzymes derived from a large number of microorganisms are needed for complete digestion. In addition it is well known that pre-treatment before enzyme digestion is usually required for biomass utilization. Especially lignin is the toughest component in woody biomass, and the structure of lignin has varied significantly in biomass origin such as herbaceous plant, coniferous (softwood) and hardwood. Therefore the mechanism of degradation of lignocellulosic biomass from different origin is also different in some fungi. According to these mechanisms, the biomass degrading fungi are generally classified into three groups (white rot, brown rot and soft rot). White rot fungi are very special which can degrade native lignin, mainly in hard wood, and they have the ability for cleaving the bond between C_{α} and C_{β} atoms in lignin. The brown rot fungi that can attack on coniferous wood and only cellulose and hemicellulose are degraded, but not the lignin. Both of the white rot and brown rot are most exist in basidiomycetes. The soft rot fungi are distributed among both asco- and basidiomycetes. This type of the fungus prefer to act on biomass which has the low content of lignin [3,4].

Cellulose, which is a polymer composed of glucose residues with β-1,4 linkages, takes the highest proportion in lignocellulosic biomass [5,6]. Therefore cellulose degradation is a major process that contributes its conversion to useful products. In cellulose degradation under mild condition, the hydrolytic enzymes which are the mixture of cellulases, mainly are cellobiohydrolase (CBH, EC 3.2.1.91) and

endoglucanase (EG, EC 3.2.1.4) as well as β-glucosidase (BGL, EC 3.2.1.21) have the key for the effective digestion. CBHs can degrade cellulose chain in a sequential manner and produce cellobiose (G2, β-D-1,4-linked disaccharide) mainly, whereas EGs act randomly on the cellulose chains and can increase the number of reducing and non-reducing end of cellulose chain. The combined action of these two classes of enzymes shows synergistic effect and produces the end product, cellobiose. BGL releases glucose (Glc) from cello-oligosaccharide, mainly G2 [7], which prevents product inhibition of CBH and EG activities (Fig. 1-1) [8].

Since a large amount of cellulase and other types of enzymes such as oxidoreductase were required for complete cellulose digestion, commercial enzyme preparations from fungus is limited and numerous fungal species are not used for industrial manufacturing purposes. For example, *Irpex lacteus* is one type of the wood-rotting fungi, which is well studied as a capable producer of lignocellulose degrading enzymes. However Driselase from *I. lacteus* is used as only feed additive, because the productivity of cellulase is quiet low, resulting high cost of enzyme preparation. Furthermore some other fungus such as *Aspergillus niger* and *Humicola insolens*, are used only limited application for food processing and textile finishing processes. The low levels of protein production by *Penicillium* species [9–11] also hampers their use in industrial fermentations. Whereas *Trichoderma* species are used in wide application even in bioethanol production, because they produce sufficient levels of cellulase through a well-characterised mechanism [12]. For example, a robust system for producing cellulase by *Trichoderma reesei* has been established in large industrial scale [13, 14,15].

1.2. Induction of cellulolytic enzymes in *Trichoderma reesei*

Secretion of cellulolytic enzymes by microorganisms is needed for the efficient degradation of lignocellulose in nature. Several studies have proposed that cellulases

and hemicellulase are not produced in the absence of plant biomass because most cellulases are not constituent enzymes and produced only in the presence of an inducer. However, in general, natural substrates, biomass components are usually insoluble, therefore it is hard to induce cellulase. Major cellulases in *T. reesei*, Cel7A and Cel7B, are constitutively expressed at low levels even in the absence of an inducer, and they can produce low-molecular-weight or soluble inducers if biomass component exist in neighbour. In contrast, their expression is induced 1000-fold larger in the presence of cellulose [16]. Evidence indicates that an extremely low level of constitutive cellulase synthesis initiates the degradation of cellulose that produces a small amount of oligosaccharides, which trigger further induction [17].

The identity of the inducer(s) of cellulase and hemicellulase synthesis by *T. reesei* is a longstanding mystery. Previous studies have found that a large amount of cellulases are produced when the culture medium contains cellulose or plant cell wall. Nevertheless, presence of cellulose-derived product as inducer is suggested because the insoluble molecules are not incorporated to fungal cells [18]. Further studies suggested that G2 is the major soluble product from cellulose by *T. reesei* cellulase, and from these results it is considered G2 can induce cellulase production. This assumption is later evidenced in many fungal species [19] and also in the resting mycelia of *T. reesei* [20,21]. Oligosaccharides derived from cellulose degradation, such as β-D-1,4-linked disaccharides (cellobiose, G2), β-D-1,6-linked disaccharides (gentiobiose, Gen) [22] and β-D-1,2-linked disaccharides (sophorose, Sop) [23–25], have been proved to be a inducer for cellulase production in *T. reesei*. Moreover a previous study found that Sop induces cellulase levels 2800-fold higher than G2 [25], thus it is considered as the most potential inducer for cellulase induction in *T. reesei*. Some related studies figured out that optimum concentration of Sop for cellulase induction in *T. reesei* should be 1 mM since the Sop could hydrolysis by BGL into two molecular of Glc, which might act on the catabolite repression [26,27]. In addition, it is reported that Sop could form by the cell wall or cell membrane binding BGL in *T. reesei* [28]. It is suggested β-D-

galactopyranosyl-(1,4)-D-glucopyranose (lactose) is the only economically feasible inducer of cellulase production [29,30], and there are reports of its effects on *T. reesei* as well as on other fungi [31,32]. Evidence indicates that β-galactosidase produces the β-D-galactose anomer from lactose and that induction is further regulated by the generation of β-D-galactose via a catabolic pathway [33]. Moreover cellobiono-1,5-lactone is reported as another inducer, which may use the common receptor with Sop generated through the same mechanism [34,35]. However further studies are still requires to determine the mechanism of this induction in details.

1.3. Transcriptional regulation of cellulolytic enzymes

The filamentous fungus can use various carbon sources including the cellulose, hemicellulose and pectin. The genetic expressions of enzymes related to those metabolisms are regulated depending on the conditions of their surroundings. Generally, their transcriptions are promoted by the positive and negative transcriptional regulators. By far some studies are proved that some transcription factors are located in downstream pathway of cellulose, G2, Sop and Glc metabolism (Fig. 1-1).

1.3.1. Xylanase regulator 1 (*xyr1*)

The transcriptional activator Xlyr for regulation of cellulase and hemicellulose genes were first discovered in the filamentous fungus *A. niger*. Xlyr is a binuclear zinc finger protein that is classified as a transcription factor. The homolog, Xyr1found in *T. reesei* [36,37], which binds to a GGCTAA-like motif and mainly controls the expression of *xyn1*, *xyn2*, *bxl1*, *cbh1*, *cbh2*, *egl1* and *bgl1* [37]. Deletion of *xyr1* (*Δxyr1*) leads to reduction or complete loss of the induction of the genes encoding these proteins, indicating that Xyr1 is a key regulator of the induction of cellulase and hemicellulase transcription in *T. reesei*.

1.3.2. Activator of cellulases 1 (*ace1*)

A yeast-based screening was used to isolate Ace1 from by *T. reesei.* Ace1 contains three Cys_2His_2-type zinc fingers and binds the sequence 5′-AGGCA-3′ presenting on eight sites in the *cbh1* promoter. Deletion of *ace1* from the *T. reesei* genome (*Δace1*) increases the expression of the genes encoding cellulase and xylanase that are induced by cellulose and Sop, indicating that *ace1* negatively regulates cellulase and hemicellulase expression in *T. reesei*. Moreover, *Δace1* strain has been reported to produce cellulases at a level 20-fold higher than the wild strain in the presence of cellulose or Sop [38].

1.3.3. Activator of cellulase 2 (*ace2*)

A yeast-based screening was also used to isolate from Ace2 from *T. reesei*. Ace2 is also a zinc binuclear cluster protein that binds in the sequence motif of 5′-GGCTAATAA-3′ or 5′-GGGTAA-3′ sequences in the promoters of *cbh1* and *xyn2* [39]. Deletion of *ace2* reduces the expression of the main cellulase genes that are induced in cultures containing cellulose but not Sop, indicating that Ace2 activates the expression of cellulase and hemicellulase in the presence of cellulose, although the induction mechanism is different, in part, from that of Sop [39,40]. Furthermore, Ace1 and Ace2 can regulate the activity of the key activator XlnR.

1.3.4. β-Glucosidase regulator (*bglR*)

Bglr, which was isolated from a *T. reesei* mutant strain PC-3-7 [41], contains a conserved $Zn(II)_2Cys_6$ binuclear cluster domain and a fungal-specific transcription domain or middle homology region. Deletion of *bglR* increases cellulase expression with G2. The mechanism involves the expression of certain BGL (except Cel3A), indirect regulation of Glc production, consequently decrease the transcription of *cre*.

1.3.5. Catabolite repression proteins (*cre1*)

Readily metabolisable carbon sources such as Glc, xylose and fructose repress the expression of genes required to utilise other carbon sources. The mechanism is called Glc repression or carbon catabolite repression. The regulation of carbon catabolite repression in numerous filamentous fungi is mediated by the Cys_2His_2-type transcription factor CreA/CREI that binds to the CreA/CREI motif (5′-SYGGRG-3′) [42,43]. In *T. reesei*, numerous genes encoding cellulase, hemicellulase and pectinase are regulated by the Cre including Cre1 and Cre2, which are studied to determine their effects on the transcription of genes encoding cellulase and hemicellulase in the presence of different inducers [42]. For example, deletion of *cre1* or *cer2* increases *cbh1* mRNA levels under repressing conditions [42,44]. Furthermore, *xyr1* transcription is repressed by *cre1* [37].

1.4. Variety of β-glucosidases from microorganisms

1.4.1. β-Glucosidase isozyme

BGL can cleave the β-1,4-glycosidic bond linking glucosyl residues or aglyconic linkage. In filamentous fungi, BGLs that mediate cello-oligosaccharides digestion or glycoside metabolism are classified into the glycoside hydrolase family 1 (GH1) and glycoside hydrolase family 3 (GH3). The hydrolysis reaction is occurring by the general acid and nucleophile of two Glu residues, which can catalyze the hydrolysis of the glycosidic bond between the anomeric carbon and the glycosidic oxygen, which the anomeric conformation of the Glc is retained.

The BGLs that belong to GH1 family distribute in archaebacterial, bacteria and fungi. Usually they have a classical $(\beta/\alpha)_8$-TIM barrel structure, and are anomer retention type enzymes. Most of the GH1 BGLs contain a NEP motif (Asn-Glu-Pro) in the catalytic acid/base and a TENG motif (Thr-Glu-Asn-Gly) in the catalytic nucleophile. The catalytic residues (two molecules of Glu) are located in the number 4

and number 7 of a C-terminal of a β-strand, thus also widely separated in a 4/7 super family. Moreover, the BGLs belonging to GH1 are generally intracellular, and have a relatively small molecular mass and cleave not only β-linked oligosaccharides but also act as β-galactosidases.

The BGLs belonging to GH3 are distributed to bacteria, fungi and plants. The crystal structures are represented by domains one and two together, and an alternating repeat of eight α-helices and eight parallel β-strands in a α/β barrel sandwich are present in many GH3 BGLs. Moreover most of GH3 BGLs are extracellular, and can remove glycosyl residues from the non-reducing ends of substrates. Catalysis occurs via a classical Koshland double-displacement mechanism with retaining the anomeric configuration of products. This type of BGLs usually show a broad specificities which can do action on not only β-1,4-linked oligosaccharide but also β-1,3-linked and β-1,6-linked one [45]. Furthermore, most GH3 BGLs are larger molecular masses than those of GH1. Both of the GH1 and GH3 BGLs are the same mechanism as retaining enzyme, and thus to be able to catalyse transglycosyl reaction.

1.4.2. Both of hydrolysis and transglycosyl reactions by BGLs

Transglycosylation might be considered similar to hydrolytic reaction which incorporated hydroxyl group of the sugar (acceptor) instead of water molecule [46–48]. In reverse reaction of hydrolysis, the condensation reactions are the reactions synthesizing a new glycosyl bond between different molecules. Occurring in a donor glycoside is first cleaved by the enzyme and an enzyme-glycosyl intermediate is formed, and finally reacts with a nucleophile other than water (e.g. monosaccharide, disaccharide or alkyl-alcohol) to yield new products as shown in Fig. 1-2 [49].

1.4.3. β-Glucosidases of *Trichoderma reesei*

The *T. reesei* genome database v.2.0 (http://genome.jgipsf.org/Trire2/Trire2.home.html) and the Carbohydrate Active enZymes (CAZy) database [50,51] show that *T. reesei* genome may encode at least 10 BGL isozymes, including two of GH1 and eight of GH3 families [52]. Their protein ID and a neighbour-joining phylogram are shown in Fig. 1-3. Among these isozymes, Cel1A is an intracellular enzyme that is induced in medium supplemented with cellulose or Sop [53,54]. Previous studies have shown that Cel1A catalyzes transglycosylation and condensation reactions that generate cello-oligosaccharides such as cellotriose and cellotetraose as well as a variety of isomeric gluco-disaccharides, including Sop [53]. Cel1B is another intracellular BGL that is induced by cellulose [54]. Cel3A is reported as the sole extracellular enzyme induced synchronously with cellulolytic enzymes in the cellulase hyper-producing strain RL-P37, but it is not inducible in the QM6a strain [54]. Deletion mutants of *cel3a*, *cel1a* or *cel1b* exhibit considerable delays in cellulase production when cultivated on cellulose [21,55–57]. Furthermore, it is reported that mutant strain having multiple copies of *cel3a* produces more cellulase than the parental strain [56]. These results indicate that the activities of the three BGL isoforms have influence the induction and production of cellulase in *T. reesei*.

1.5. Purpose of this study

The possibility about the inducer formation by BGLs in *T. reesei* are propose in the last century, and it is suggested the function of them were not only metabolism of G2 but also the trigger on the cellulase induction. As *T. reesei,* however, produced more than 10 isozymes and it is difficult to get purified enzyme, the function of individual BGL were remained to be solved. Recently, with the publication of the whole genome sequence of *T. reesei*, the researcher have paid more attention to the function of BGLs again.

In this research the author was focused on explore the properties of BGL isozymes from *T. reesei*, especially their behaviour on Sop formation, which is the candidate of positive inducer. If the key enzyme among BGL isozymes are found out, this results make helpful for the improvement of cellulase productivity, and it is benefit for promoting efficient biomass conversion.

From the above purpose, the author prepared with 10 purified recombinant BGL isozymes form *T. reesei* using *E. coli* expression system, and then performed a systematic comparison of their catalytic properties. In order to identify the key enzyme for Sop formation, the kinetic parameters on gluco-disaccharides have been determined. Further more the abilities of transglycosylation by BGL isozymes were evaluated. In addition, the targeted BGLs were further investigated *in vivo* of their effects on cellulase induction, by deletion of the corresponding BGLs encoding genes. At the last part of this dissertation, a mutagenesis of Cel1A, the key enzyme that determined in this work was constructed using site-directed mutagenesis to improve not only the formation of Sop but also the Glc sensitivity. Finally the author discussed the induction mechanism of enzymes in *T. reesei,* especially the contribution of BGL for cellulase induction.

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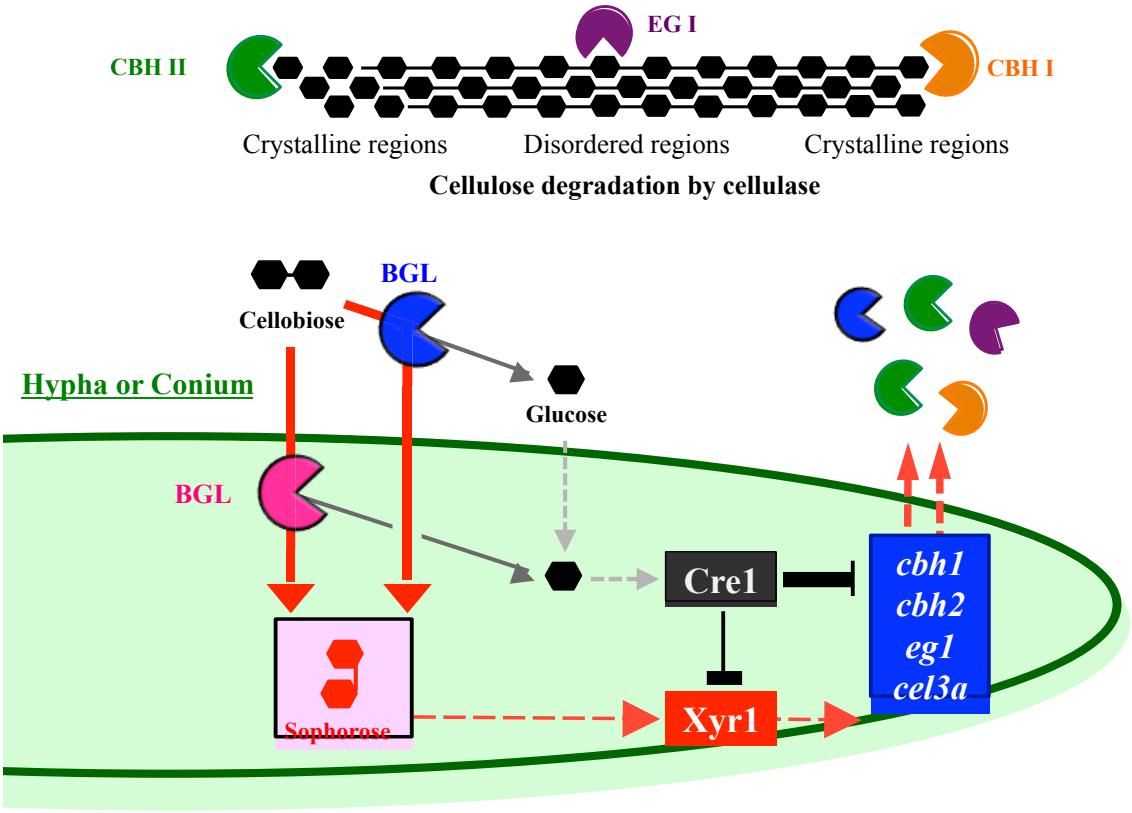


Fig. 1-1 The cellulose degradation is performed by different types of cellulases and BGL synergise to degrade crystalline cellulose to Glc. CBHs attack on the crystalline regions, EGs act on the disordered regions and BGLs hydrolysis G2 to Glc as the end product. The cellulase induction is performed by Sop which synthesized via the BGL transglycosylation reaction intracellular or extracellular. The synthesized Sop might act on the positive transcription regulator Xyr1 to promote the cellulase production and this induction behaviour is repress by the catabolite repressor protein Cre1.

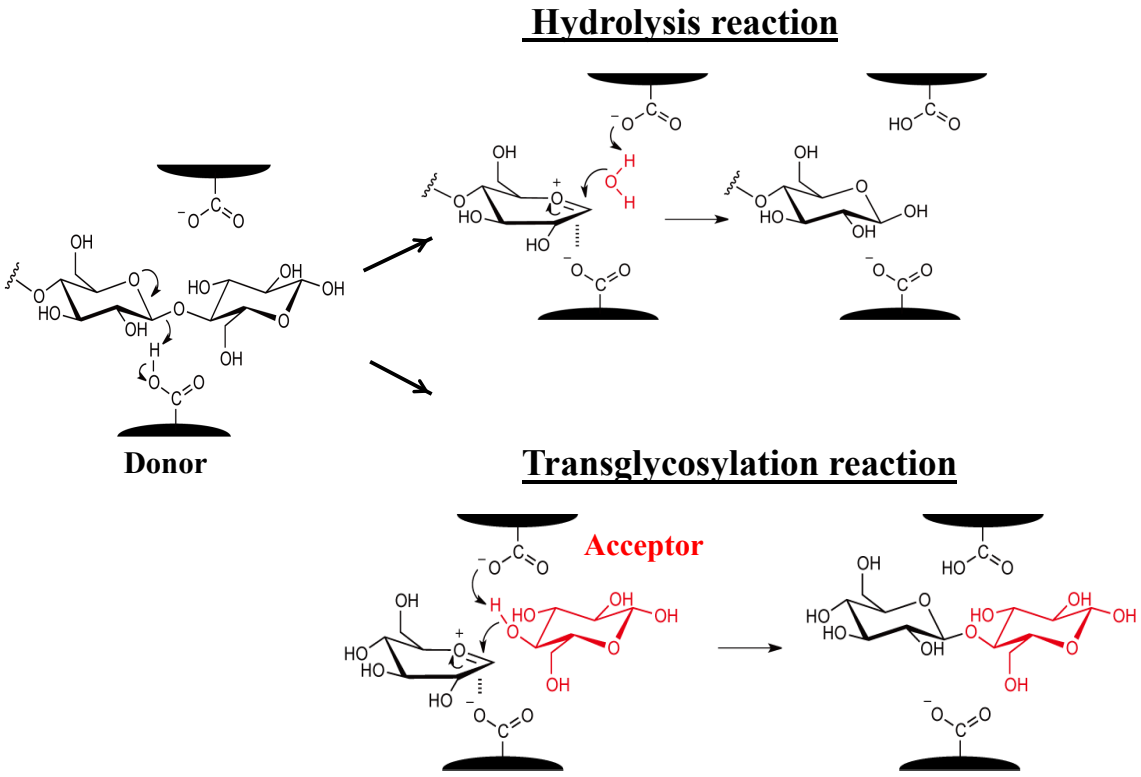
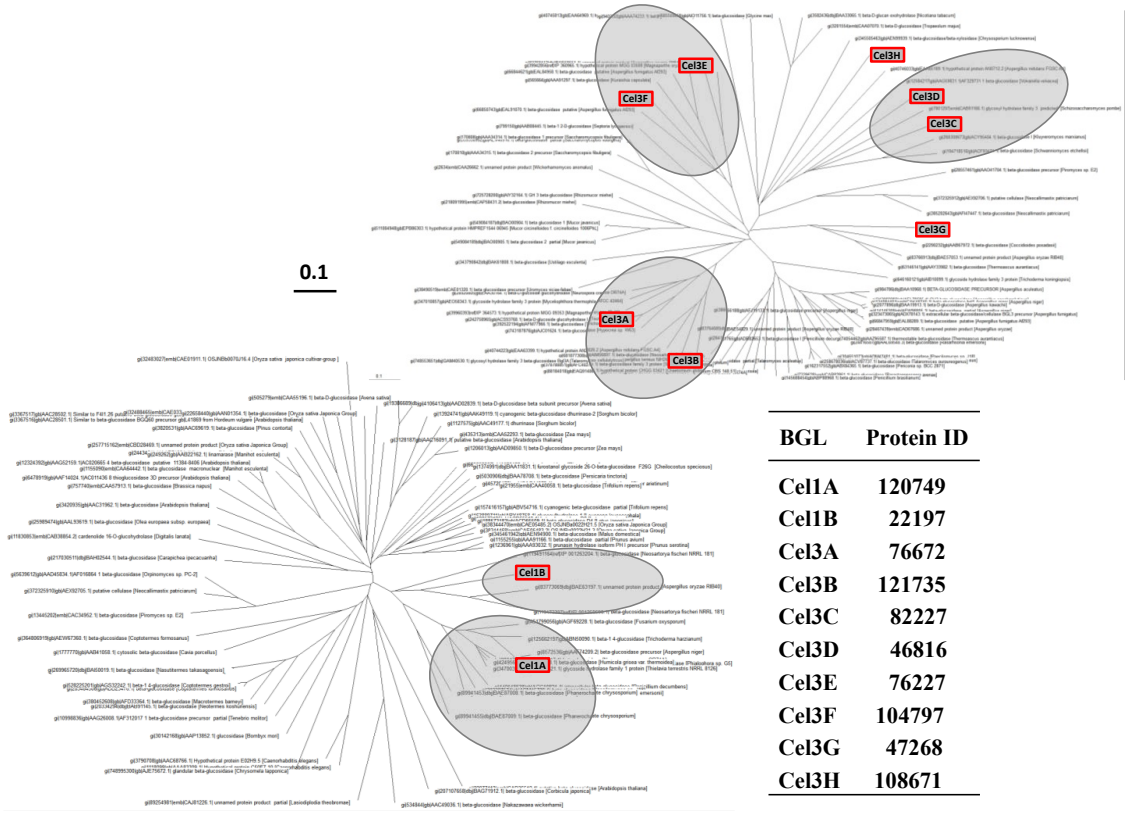


Fig. 1-2 Mechanism of hydrolysis and transglycosylation reactions by BGL using G2 as substrate.



BGL	Protein ID
Cel1A	120749
Cel1B	22197
Cel3A	76672
Cel3B	121735
Cel3C	82227
Cel3D	46816
Cel3E	76227
Cel3F	104797
Cel3G	47268
Cel3H	108671

Fig. 1-3 Neighbour-joining phylogram of BGLs in microorganisms. *T. reesei* BGLs are shown in red box, their protein ID was assigned based on the *T. reesei* genome database v2.0.

Chapter 2

Comparison of catalytic properties including transglycosylation ability of multiple β-glucosidases in *Trichoderma reesei*

Chapter 2: Comparison of catalytic properties including transglycosylation ability of multiple β-glucosidase in *Trichoderma reesei*

2.1. Introduction

T. reesei, is known as one of the best producer of cellulose-degrading enzymes, it thus applied to convert cellulose into G2 and Glc [1–3]. Some previous studies evidenced the cellulase production of *T. reesei* could induced by cellulose and its fragments such as G2 [4]. However, although G2 is believed to be primarily responsible for cellulase production, its induction ability is considerably lower than that of either of the positional isomers Gen, 1,6-β-glucobiose or Sop, 1,2-β-glucobiose [4]. Further, the BGLs in *T. reesei* are supposed to produce cello-oligosaccharides via a transglycosylation reaction catalysed using the products of cellulose hydrolysis as substrate [5–6]. This supposition was moreover supported by Vaheri, he reported that enzymes from cell extraction showed more transglycosylation activity than those from culture supernatant, and the products were further analysed including Sop, Gen and Lam. All these findings suggest that intracellular BGLs not only play a role in the hydrolysis of cello-oligosaccharides but also affect the formation of isomeric glucosides associated with cellulase induction. Thus, elucidation of BGL functions in *T. reesei* would be of interest, particularly for their transglycosylation abilities and role in cellulose decomposition.

In this chapter, ten putative BGL isozymes in *T. reesei* were heterologously expressed in *Escherichia coli* and *Aspergillus oryzae*, after purification their pH and temperature profiles as well as substrate specificities were investigated. To identify BGLs responsible to Sop formation, their transglycosylation and condensation abilities were evaluated. For understanding of the preference of the tansglycosylation products by different BGLs, their kinetics parameters towards the generated sugars and the substrate were furthermore investigated.

2.2. Materials and methods

2.2.1. Strains and plasmid vectors

T. reesei strain QM9414 (NBRC 26921) was used as a source of BGL genes. *E. coli* DH5α was used for the routine propagation of all plasmids. *E. coli* Rosetta-Gami 2 (DE3) pLysS strain and pET-23d (+) vector (Merck, Darmstadt, Germany) were used to express GH1 BGLs. *A. oryzae* (niaD$^-$) and the high-level expression vector pNAN8142 were provided by Ozeki Corporation (Hyogo, Japan) for the expression of GH3 BGLs.

2.2.2. Nucleotide sequences and expressions of β-glucosidases

To prepare recombinant enzymes, 10 predicted BGL genes were identified in the *T. reesei* genome database, v.2.0 (http://genome.jgipsf.org/Trire2/Trire2.home.html). Their protein IDs are presented in Fig. 2-1. N-terminal sequences of Cel3C and Cel3D were considered to be insufficient in length. Their full-length cDNAs were amplified using the 5′-RACE system, and appropriate N-terminals were predicted by comparing the amino acid sequences of GH3 BGLs from other sources.

T. reesei conidia (10^6) were grown in a 300-mL baffled flask on a rotary shaker (200 rpm) at 30°C in 50 mL of a minimum medium [7] containing 2% Glc as the carbon source. After incubation for 3 days, mycelia were harvested, washed with distilled water and transferred into fresh medium supplemented with 1% microcrystalline cellulose (Merck) and 1 mM Sop (Sigma-Aldrich, St. Louis, MO) as carbon sources. Mycelia were then incubated for 24 h under the same conditions.

Total RNA (176 μg) was prepared from 0.1 g of wet mycelium by grinding in liquid nitrogen with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesised using a PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan) with 10 μg of the total RNA as a template. To construct expression vectors, cDNAs encoding BGLs were amplified by PCR with PrimeSTAR® HS DNA polymerase (TaKaRa) using the first-strand cDNA as a template. The pairs of PCR primers used are listed in Table 2-1. After

denaturation at 95°C for 3 min, cycling conditions were 98°C for 10 s, 55°C for 10 s and 68°C for 3 min for 30 cycles, followed by a final extension at 68°C for 3 min. The amplified fragments treated with the restriction enzymes corresponded to the recognition sites located on the PCR primers. For the predicted intracellular BGLs, the digested cDNA fragments were directly ligated with the corresponding sites of pET-23d (+) (cel1A and cel1B) and pNAN8142 (cel3C and cel3D). The other BGLs containing the deduced signal peptide were expressed in *A. oryzae* as a secreted enzyme in culture supernatant mediated by the host Taka-amylase A signal peptide (AoAmyA, GenBankAB021876) rather than the native *T. reesei* signal peptides, as described below [8]. The DNA fragment encoding the AoAmyA signal peptide was amplified by PCR using genomic DNA as the template, with AmyA-sig-F and AmyA-sig-R primers (Table 2-1) following the same PCR conditions described above.

The amplified fragment was digested with *Hin*d III and *Sma* I. The two resulting DNA fragments encoding the signal peptide and BGL were simultaneously ligated into pBluescript II SK (Stratagene, La Jolla, CA) digested with *Hin*d III and *Spe* I. After fidelity was confirmed by sequencing, the whole insert (from the *Hin*d III or *Xho* I site to the *Spe* I site) was transferred into pNAN8142 digested by the same restriction enzymes. The resulting plasmids that served to express GH1 BGL were transferred into the Rosetta-Gami 2 (DE3) pLysS strain. The plasmids for the expression of GH3 BGLs were also transferred into *A. oryzae* (niaD-) using the method by Gomi et al. (1987) [9]. To obtain *A. oryzae* transformants in a homokaryotic state, conidia were selected on Czapek–Dox medium containing sodium nitrate as the sole nitrogen source.

2.2.3. Production and purification of recombinant β-glucosidases

The *E. coli* strains harbouring the expression vectors for GH1 BGLs were grown in 100 mL of LB liquid medium (Nakarai Tesque, Kyoto, Japan) supplemented with 100 μg/mL ampicillin in a 1-L baffled flask on a rotary shaker (150 rpm) at 25°C. 1 mM of isopropyl-β-D-thiogalactopyranoside was added as an inducer when the absorbance at 660 nm reached a

value of 0.6. After a further 12 h growth, the cells were harvested by centrifugation (13,000×*g* for 5 min) and resuspended in 15 mL of 20 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl. After ultrasonication (VCX-130; Sonics & Materials, Inc., CT) for 10 min on ice, cell debris was removed by centrifugation (13,000×*g* for 5 min). Protein purification was performed with the supernatant using a column packed with 15 mL of TALON metal affinity resin (TaKaRa) equilibrated with the same buffer. The proteins adsorbed onto the column were eluted with 200 mM imidazole in the same buffer. Tractions displaying BGL activity were pooled and concentrated on a Vivaspin 20-10K (GE Healthcare, Piscataway, NJ), and the buffer was substituted with 0.1 M acetate buffer (pH 5.5).

The transformants of *A. oryzae* strain inoculated with 10^6 conidia were grown in 200 mL of medium containing 2% potato starch, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ in a 1-L baffled flask. After shaking for an optimum culture time of 10 days at 120 rpm and at 30°C, the supernatant was passed through a G3 glass filter (Asahi Glass Co., Tokyo, Japan). Proteins were precipitated by the addition of $(NH_4)_2SO_4$ at up to 80% saturation and were collected by centrifugation. The precipitate was dissolved in 30 mL of 5 mM acetate buffer (pH 5.5) and subjected to the chromatographic procedures summarised in Fig. 2-2 under the following conditions:

(i) Hydrophobic chromatography

A TOYOPEARL Butyl-650M (Tosoh, Tokyo, Japan) column (2.3 × 19 cm) was equilibrated with 20 mM acetate buffer (pH 5.5) containing 1 M $(NH_4)_2SO_4$. Proteins adsorbed onto the column were eluted with 400 mL of a decreasing gradient of $(NH_4)_2SO_4$ (1–0 M) in the same buffer.

(ii) TOYOPEARL HW-50S gel filtration

The enzyme solution concentrated to 2 mL was loaded on a TOYOPEARL HW-50S (Tosoh) column (1.6 × 48 cm) equilibrated with 50 mM acetate buffer (pH 5.5). It was eluted

with the same buffer at a flow rate of 2 mL/h.

(iii) DEAE Sepharose anion exchange chromatography

Before chromatography, the enzyme solution was dialysed against 50 mM phosphate buffer (pH 7.0). The dialyzate was then applied onto a DEAE Sepharose Fast Flow (GE Healthcare) column (5 × 15 cm) equilibrated with the same buffer. After non-adsorbed proteins were completely eluted, adsorbed proteins were eluted with 300 mL of a linear gradient from 0 to 1 M NaCl in the same buffer.

(iv) Bio-Gel P-100 gel filtration

A column (1.6 × 35 cm) was filled with a Bio-Gel P-100 (Bio-Rad, Hercules, CA) in 50 mM acetate buffer (pH 5.5). The enzyme solution concentrated to 2 mL was subjected to chromatography in the same buffer at a flow rate of 1.6 mL/h.

(v) CM Sepharose cation exchange chromatography

The enzyme solution dialysed against 50 mM acetate buffer (pH 6.0) was applied onto a CM Sepharose Fast Flow (GE Healthcare) column (5 × 15 cm) equilibrated with the same buffer. After the column was washed with the same buffer, proteins were then eluted with 200 mL of a linear gradient from 0 to 1 M NaCl in the same buffer.

(vi) Hydroxyapatite chromatography

The enzyme solution was first dialysed against 20 mM phosphate buffer (pH 6.0) and then applied onto a column (5 × 15 cm) of hydroxyapatite (Seikagaku Kogyo) equilibrated with the same buffer. Elution was performed with a stepwise gradient of the same buffer in a concentration range of 0.05–0.2 M.

(vii) Q-Sepharose HP anion exchange chromatography

Chromatography was performed using a fast protein liquid chromatography system (Bio-Rad). The enzyme solution dialysed against 20 mM Tris-HCl (pH 7.5) was loaded onto a pre-packed column (Q-Sepharose HP, 1.6 × 10 cm; GE Healthcare) equilibrated with the same buffer. After the column was washed, elution was performed with an increasing gradient of NaCl (0–1 M) of the same buffer at a flow rate of 35 mL/h.

(viii) Sephacryl S-200 gel filtration

Enzyme solution (2 mL) was loaded onto a pre-packed column of Sephacryl S-200 (1.6 × 35 cm; GE Healthcare) washed with 50 mM phosphate buffer (pH 7.0) using a Bio-Rad FPLC system at a flow rate of 1.5 mL/h. During all purification steps, the collected fractions were assayed for BGL activity; purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4. Determination of β-glucosidases activity

BGL activity was determined on pNPG (Sigma-Aldrich) and cello-oligosaccharides. In the assay on pNPG, BGLs were incubated with 1.25 mM pNPG in 100 mM acetate buffer (pH 5.5) in a total volume of 0.4 mL at 40°C. The reaction was stopped by the addition of 2 mL of 0.5% Na_2CO_3, and the released *p*-nitrophenol was determined at 420 nm. BGL activity on cello-oligosaccharides was performed under the same conditions as described above, but the reaction was terminated by 5 min of boiling. After cooling, the liberated Glc was determined using glucose oxidase-mutarotase reagent (Glucose CII-Test Wako; Wako Pure Chemical, Tokyo, Japan) according to the manufacturer's instructions. One activity unit is defined as the amount of enzyme releasing 1 μmol of product per min. Protein content was also measured by the Lowry method [10] using BSA as a standard.

Kinetic parameters in hydrolysis were determined using Lineweaver–Burk plots after the measurement of initial reaction velocities at various substrate concentrations [11]

2.2.5. Assays of transglycosylation and condensation abilities

The transglycosylation ability of BGLs was measured using the substrate G2, the main product in the enzymatic degradation of cellulose. The reaction mixture (1 mL), containing 1% or 10% (w/v) G2, 0.1 M acetate buffer (pH 5.5) and 50 μg of the purified BGL was incubated at 30°C for 72 h. Aliquots of the reaction mixtures taken at the time intervals were denatured by heat and filtered (0.45 μm filter; Merck). Filtrates were analysed for sugar composition by high-performance liquid chromatography (HPLC) using a GL-7410 pump (GL Science, Tokyo, Japan), a LC-2000 plus fluorescence detector (JASCO, Tokyo, Japan) and a UK-Amino UKA36 column (0.3 × 25 cm; Imtakt Co., Kyoto, Japan). The elution was performed at a rate of 0.4 mL/min at 60°C with a gradient shown in Table 2-2. After separation, the eluent was mixed with a detection regent (phenylhydrazine/acetic acid/H_3PO_4 (88%) (6/180/220, v/v) at a rate of 0.2 mL/min and then placed on an on-line post-reaction coil (0.5 mm × 6 m) at 140°C in a CRB-6A reaction chamber (Shimadzu, Kyoto, Japan) to convert the sugars into fluorescent derivatives. Compounds exhibiting fluorescence after excitation at 330 nm were determined at 470 nm (emission). Good separation of all isomeric diglucosides linked with β-glycosidic bonds as well as cello-oligosaccharides was achieved under these conditions. Transglycosylation ability was expressed as the percentage of G2 converted into different sugars. This value (%) is calculated from the scheme as follows:

$$\frac{\text{total amount of the transfer products} \times 100}{\text{amount of G2 decreased}}$$

2.3. Result

2.3.1. Nucleotide and deduced amino acid sequences of β-glucosidases

The *T. reesei* genome contains 10 genes that encode known and putative BGLs. The products of eight enzymes, namely Cel3A, Cel3B, Cel3C, Cel3D, Cel3E, Cel3F, Cel3G and Cel3H, are classified as GH3, whereas the remaining two enzymes Cel1A and Cel1B are

classified as GH1. During the isolation and sequencing of cDNAs, we noticed that the start codons and exon-/intron junctions for three genes were not properly located. Accordingly, the corrected sequences were deposited in the database under new numbers, namely LC002807 for cel3*D*, LC002808 for cel3*G* and LC002809 for cel3*H*. The signal peptides of the 10 deduced BGLs were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The six BGLs Cel3A, Cel3B, Cel3E, Cel3F, Cel3G and Cel3H showed D-scores as high as those of typical extracellular cellulolytic enzymes of *T. reesei* belonging to GH5, GH6, GH7 and GH61 (Fig. 2-1). However, the values of the four remaining enzymes Cel3C, Cel3D, Cel1A and Cel1B were considerably lower, in agreement with their intracellular localisation.

2.3.2. Heterologous expression and purification of β-glucosidases

Cel1A and Cel1B, which belong to GH1, were efficiently expressed as soluble proteins in *E. coli*. The six GH3 BGLs (Cel3A, Cel3B, Cel3E, Cel3F, Cel3G and Cel3H) were expressed at high levels as extracellular proteins in 5-day cultures of the host *A. oryzae*. The remaining GH3 enzymes, namely Cel3C and Cel3D, were expressed in 10-day cultures as secreted proteins at levels that were lower but sufficient for further purification.

All 10 enzymes, except Cel3H, exhibited hydrolytic activity towards both pNPG and G2. The recombinant Cel3H (99 kDa) was an inactive species without hydrolytic activity against pNPG, cello- and xylo-oligosaccharides. This finding is in accordance with its low sequence similarity with other BGLs. Thus, Cel3H was eliminated from subsequent experiments. Finally, the nine recombinant enzymes Cel1A, Cel1B, Cel3A, Cel3B, Cel3C, Cel3D, Cel3E, Cel3F and Cel3G were purified (Fig. 2-2 and from Fig. 2-2-1 to Fig. 2-2-7) to homogeneity and were further characterised.

2.3.3. pH and temperature profiles

The purified recombinant BGLs expressed in *A. oryzae* appeared as single bands on SDS-PAGE with larger molecular masses than those calculated because of glycosylation (Fig. 2-3 and Table 2-3). As shown in Table 2-3, the maximum activity of all BGLs was found in the acidic pH region (pH 3.5−6.5). It should be noted that the predicted intracellular enzymes (Cel1A, Cel1B, Cel3C and Cel3D) exhibited the highest activity in a narrow pH range of 5.5−6.5. The pH stability of all enzymes was highest in the pH range 3.0−8.0. Among them, Cel3B showed wide pH stability (pH 3.0−8.0) and high temperature stability (80% of activity at 50°C). Most enzymes became inactivated after incubation for 30 min at 40°C, but the stability of Cel1B was considerably lower.

2.3.4. Substrate specificity

Specific activities of the recombinant BGLs were determined on pNPG, cello-oligosaccharides and positional isomers of G2, namely Lam, Gen and Sop (Table 2-4). For the Cel3C, Cel3D, Cel3F, Cel3G and Cel1B exhibited the highest activity on pNPG, these BGLs are thus behaved as aryl β-glucosidases, on the other hand Cel3A, Cel3B and Cel3E are showed typical for exo-β-1,4-glucanases [12] for their high specific activity not only against pNPG and G2 but also against Lam, Gen and Sop; however, they differed in their preference for disaccharides. For example, Cel3A showed high specific activity against Sop and Lam, and Cel3B and Cel3E preferentially hydrolysed Lam and Gen. Another property of these BGLs was a higher specific activity for longer cello-oligosaccharides. The highest reactivity of Cel1A was shown against G2 (23 U/mg), indicating that Cel1A is a typical cellobiase responsible for the conversion of G2, the main product of cellulases. Cel1A also showed high specific activity against Lam (14 U/mg) but very low activity against Gen.

2.3.5. Transglycosylation and condensation

Of the nine *T. reesei* BGLs, only four (Cel3A, Cel3B, Cel3E and Cel1A) catalysed

efficient transglycosylation reactions; the TLC results are summarised in Fig. 2-4. Both of the result in TLC (Fig. 2-4) and HPLC (Fig. 2-5a) were shows that G3 was the main transfer product of these enzymes. As the reaction proceeded, isomeric β-glucobioses started to appear. The GH3 BGLs, particularly Cel3B and Cel3E, synthesised Gen. Cel1A preferentially produced Sop and Lam, both at a conversion of approximately 10%. Simultaneously, G2 consumption was also calculated. Fig. 2-5b shows a continuous G2 consumption by Cel3A, Cel3B and Cel3E, and its sudden cessation in the case of Cel1A. Importantly, Cel1A was also capable of converting 3.6% of G2 to Sop at 1% G2 concentration (Fig. 2-5c).

2.3.6. Kinetic parameters

Comparison of the kinetic parameters of BGLs on various substrates illustrates the accumulation of transfer products during G2 hydrolysis (Table 2-5 and Fig. 2-6). The results show that G3 is a preferred substrate for Cel3A, Cel3B and Cel3E with high k_{cat}/K_m values. This inference explains the rapid disappearance of G3 as a transitionally accumulated product in glycosyl transfer reactions (Fig. 2-4 and Fig. 2-5a). Other transfer products such as isomeric glucobioses persist in the reaction mixtures for a longer time, with the enzyme showing lower K_m and k_{cat} values for those products. Examples of this situation are Gen produced by Cel3A, Cel3B and Cel3E as well as Sop and Lam accumulated by Cel1A.

2.3.7. Glucose inhibition of β-glucosidases

An important property of BGLs with respect to saccharification of cellulose at the stage of cello-oligosaccharides is their tolerance to inhibition by Glc. Fig. 2-7a shows the effect of different concentrations of Glc on the rate of hydrolysis of pNPG and G2. The three enzymes Cel3A, Cel3B and Cel3E showed similar sensitivity to Glc. Their activity on both substrates was almost completely abolished at a Glc concentration of 100 mM. The most Glc-tolerant enzyme was Cel1A exhibiting up to 30–40% activity even at a concentration of 100 or 200 mM Glc. The presence of Glc promoted transglycosylation reactions of the three

Glc-most sensitive BGLs. Cel3E reacted differently with the formation of transfer products decreasing with increasing Glc concentration (Fig. 2-7b).

2.4. Discussion

Reducing the cost of cellulolytic enzymes or increasing the efficiency of their saccharification capabilities remains a challenge for scientists investigating the bioconversion of plant biomass [13]. It has long been known that in one of the best natural cellulase producers, namely *Trichoderma* sp., cellulase synthesis can be triggered by a positional isomer of G2, β-1,2-glucobiose [12,14], called Sop. It has been repeatedly demonstrated that Sop is a product of glycosyl transfer reactions catalysed by BGLs. To identify the *T. reesei* enzyme responsible for the synthesis of the β-1,2-linkage between two Glc molecules is not easy because the *T. reesei* genome contains 10 different genes encoding BGLs. Products of several of these genes have already been identified and characterised, and some of them have been proposed to generate Sop [5]. To the best of our knowledge, the present study is the first to attempt the comparison of catalytic properties of all 10 BGL isozymes, including those not previously examined, with the aim to identify the key enzyme of *T. reesei* responsible for cellulose breakdown and Sop formation as well as the best candidate for enzyme engineering. Products of 10 *T. reesei* BGL genes were obtained by heterologous expression, purified to homogeneity and examined for properties relevant to Sop formation and cellulose saccharification. The gene sequences also provided information on the probable cellular localisation of the enzymes. Five BGL genes encoding Cel3A, Cel3B, Cel3E, Cel3F and Cel3G contained signal peptides directing the proteins out of the cells or to organelles. None of the genes contained sequences encoding amino acid sequences anchoring the enzymes in the cell membrane or cell walls. Given that no signal sequences were found in BGL genes encoding Cel3C, Cel3D, Cel1A and Cel1B, these enzymes are most probably intracellular. Based on the determination of specific activities towards various substrates (Table 2-4), among these intracellular BGLs, only the intracellularly located Cel1A appears to play a

major role in the hydrolysis of cello-oligosaccharides or fragments of other types of β-glucans (such as laminarin, pustulan and lichenan) transported into cells through the plasma membrane.

Addition to the Cel3H, the other nine studied BGLs exhibited similar pH optima and stabilities but differed in specific activities towards various substrates, efficiently hydrolysed pNPG but only four showed high specific activities on G2, longer cello-oligosaccharides and Glc dimers with other than β-1,4-glycosidic linkage. These four enzymes were two extracellular BGLs Cel3A and Cel3B and two intracellular BGLs Cel3E and Cel1A. Cel3A, Cel3B and Cel3E are GH3 enzymes exhibited increases in polymerization degree, a property of BGLs compatible with exo-β-1,4-glucanases. Cel1A showed the highest specific activity on G2, but this activity decreased considerably with longer cello-oligosaccharides. This intracellular enzyme behaves as a typical cellobiase, the physiological role of which is the4 intracellular cleavage of cellobiose pumped into the cell interior by membrane transport systems [15]. An additional feature distinguishing Cel1A from the Cel3A exoglucanase-type BGLs was among the β-linkage disaccharide its high specific activity on Lam and only very low activity on Gen.

Cel1A, Cel3A, Cel3B and Cel3E hydrolysing cello-oligosaccharides showed pronounced glycosyl transfer activity at high concentrations of G2. All enzymes incubated with a 10% G2 transitionally accumulated high levels of G3, which later also became a subject of hydrolysis to Glc. In agreement with the low specific activity of Cel1A on higher cello-oligosaccharides, G3 was formed as a principal tranglycosylation product of this enzyme from G2. The trimer formed transitionally disappeared from the Cel1A reaction mixture more slowly than the rate of disappearance with regard to the other three enzymes. Another unique catalytic property of Cel1A was the highest level of transglycosylation to Sop, which exceeded 10% at 10% G2 concentration and 3.6% at 1% G2 concentration. It is hypothesised Cel1A is the Sop-generating enzyme inside the cell. Of the four BGLs hydrolysing cello-oligosaccharides, namely Cel3A, Cel3B, Cel3E and Cel1A, Cel1A showed

the highest tolerance to inhibition by Glc when tested on either pNPG or G2. Thus, Cel1A appears to be the most suitable candidate for site-directed mutation and directed evolution towards higher activity, thermal stability and eventually higher efficiency of Sop synthesis.

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Table 2-1 List of PCR primers used for amplification of cDNAs

Primer name	Sequence (5'–3')	Target
Cel3A-F	GCCCGGGAGTCACTCAACATCGGG	Cel3AΔsig
Cel3A-R	ACTAGTCTACGCTACCGACAGAGTGC	Cel3A
Cel3B-F	CCGATATCTACCCGCCTCCTCACTC	Cel3BΔsig
Cel3B-R	ACTAGTTCATGGCAGGCGGGCGCTCAG	Cel3B
Cel3C-F	AAGCTTATGGCTGATATTGATGTTGAGGC	Cel3C
Cel3C-R	ACTAGTTTACACGCCAGACCACCAATA	Cel3C
Cel3D-F	AAGCTTATGATGGGTTTTGACGTGGAGGA	Cel3D
Cel3D-R	ACTAGTCTACAGCCCCATCCAGAATCG	Cel3D
Cel3E-F	CTGATATCAAAGGCGTCTCGCAAAT	Cel3EΔsig
Cel3E-R	ACTAGTCTAAATATGCAGTTTTCCACTGAG	Cel3E
Cel3F-F	CAGATATCGCAGAAAAGGTCATCAC	Cel3FΔsig
Cel3F-R	ACTAGTTCAGATGTCCAGCGTCCCATTGA	Cel3F
Cel3G-F	ATCCCGGGACTCTGGCAGAAAAGAC	Cel3GΔsig
Cel3G-R	ACTAGTCTACACAGGCCCAACAACACCAGTCTC	Cel3G
Cel3H-F	AGGATATCTTTTGGAACTTTGGTCG	Cel3HΔsig
Cel3H-R	ACTAGTCAACCAACGCACTGCAGCGTCACATTC	Cel3H
Cel1A-F	ACCATGGTGCCCAAGGACTTTCAGTGG	Cel1A
Cel1A-R	AAGCTTCGCCGCAATCAGCTCGTCAAA	Cel1A
Cel1B-F	CCCATGGCCGAGTCGCTAGCTCTG	Cel1B
Cel1B-R	AAGCTTTGCCGCCACTTTAACCCTCT	Cel1B
AmyA-sig-F	CAGAAAGCTTTTATGATGGTCGCGTGG	AmyA-sig
AmyA-sig-R	AGTCCCCGGGCGTTGCAGCCAAAGCAG	AmyA-sig

Restriction sites introduced were underlined. Δsig corresponds the cDNA sequence except the deduced signal peptide.

Table 2-2 Gradient program of HPLC analysis for determination of sugars

Analytical time (min)	Eluent A (%)	Eluent B (%)
0	100	0
30	100	0
31	85.2	14.8
36	85.2	14.8
54	56.8	43.2
59	56.8	43.2
60	100	0
79	100	0

Elution A was using 88% (v/v) acetonitrile, elution B was HPLC grade water. The gradient was performed rectilinear.

Table 2-3 Characteristics for pH and temperature of recombinant BGLs

BGL	Mw[1] ($\times 10^3$)	Mw[2] ($\times 10^3$)	pH optimum	Stability[3] (>80%)	Temp optimum	Stability[4] (>80%)
Cel3A	76.4	88	4.5	3.0–6.5	70ºC	<50ºC
Cel3B	92.2	108	3.5	3.0–8.0	60ºC	<60ºC
Cel3C	91.0	100	6.0	5.5–6.5	50ºC	<40ºC
Cel3D	91.1	90	6.0	6.0–7.0	40ºC	<50ºC
Cel3E	80.5	104	5.0	3.0–8.0	60ºC	<40ºC
Cel3F	82.7	130	4.0	3.5–8.0	60ºC	<40ºC
Cel3G	93.9	120	4.5	4.4–5.0	50ºC	<40ºC
Cel1A	52.2	55	6.5	5.5–6.5	40ºC	<40ºC
Cel1B	55.0	58	5.5	5.0–6.5	40ºC	<30ºC

(1) Molecular weights (Mw) were calculated on the basis of cDNA sequences, and (2) estimated by SDS-PAGE analysis. (3) Stability was determined after the enzyme was incubated at various pHs at 4°C for 24 h or (4) incubated at each temperature for 30 min at the optimum pH. Activity was determined under standard conditions using pNPG as the substrate.

Table 2-4 Substrate specificity of nine recombinant BGLs

BGL	Specific activity (U/mg)						
	pNPG	G2	G3	G4	Sop	Lam	Gen
Cel3A	41±1.70	17±0.31	38±2.40	41±0.79	24±0.68	26±2.0	7.9±0.79
Cel3B	36±1.20	33±3.60	36±2.20	36±1.50	22±0.34	21±0.65	42±3.20
Cel3C	110±5.10	0.32±0.03	0.16±0.02	0.16±0.03	1.4±0.08	1.7±0.24	0.02±0.01
Cel3D	35±1.60	0.06±0.01	0.07±0.02	0.04±0.00	0.04±0.00	0.04±0.02	0.06±0.00
Cel3E	6.5±0.08	8.6±0.14	11±0.81	13±0.55	4.8±0.34	13±0.48	6.8±0.40
Cel3F	12±1.60	1.3±0.07	0.64±0.10	1.4±0.12	2.0±0.05	1.9±0.13	2.3±0.05
Cel3G	29±1.70	0.79±0.04	0.25±0.01	0.63±0.03	7.5±0.18	2.8±0.10	0.30±0.04
Cel1A	2.9±0.71	25±0.85	18±0.93	2.6±0.08	6.6±0.18	14±1.9	1.2±0.02
Cel1B	0.48±0.22	0.14±0.06	ND	0.03±0.01	1.0±0.00	0.86±0.01	0.03±0.00

Activity was determined under standard conditions using pNPG as the substrate.

ND indicates not hydrolyzed. Values are the mean of three independent replicates.

Table 2-5 Kinetic parameters of the purified BGLs for the hydrolysis of oligosaccharides

	Cel3A			Cel3B			Cel3E			Cel1A		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
G2	**0.37±0.17**	**13.79±4.65**	**37.3**	**0.31±0.04**	**6.71±0.83**	**21.6**	**0.79±0.09**	**7.94±1.69**	**10.1**	**1.11±0.25**	**78.42±15.06**	**70.6**
G3	**0.20±0.06**	**39.48±10.23**	**197.4**	**0.30±0.04**	**89.89±6.67**	**299.6**	**0.52±0.05**	**15.23±1.10**	**29.3**	**1.17±0.71**	**43.97±24.56**	**37.6**
Sop	**0.25±0.04**	**28.03±4.3**	**112.1**	**0.55±0.05**	**125.97±7.62**	**229.0**	**2.66±0.10**	**15.25±3.22**	**5.7**	**0.56±0.14**	**13.70±2.09**	**24.5**
Lam	**1.1±0.46**	**43.61±15.57**	**39.6**	**0.26±0.03**	**167.80±14.37**	**645.4**	**0.45±0.04**	**14.31±1.29**	**31.8**	**1.1±0.31**	**40.54±8.21**	**37.0**
Gen	**1.22±0.32**	**5.55±1.12**	**4.5**	**0.13±0.02**	**120.95±14.2**	**930.4**	**0.40±0.02**	**9.83±0.455**	**24.6**	**3.78±2.1**	**0.05±0.02**	**0.01**

Data shown are the mean of three independent replicates. The vertical bars indicate the SD.

Enzyme	Protein Id		D-score	Locali-Zation
Cel5A	120312	MNKSV-APLLLAASILYGGAVA ↓ QQTVWG	0.856	+
Cel6A	72567	MIVGILTTLATL-ATLAAS ↓ VPLEER	0.788	+
Cel7A	123989	MYRKLAVIT--AFL-AT-ARA ↓ QSACTL	0.683	+
Cel7B	122081	MAPSVTLPLTTAILAIARLVAA ↓ QQPGTS	0.811	+
Cel61A	73643	MIQKLSNLLVTALAVA-TGVVG ↓ HGHIND	0.621	+
Cel3A	76672	MRYRTAAALALATGPFARA ↓ DSHSTS	0.316	+
Cel3B	121735	MKTLSVFAAALLAA-VAEA ↓ NPYPPP	0.807	+
Cel3E	76227	MRLCDLSSLASWVLVTVALPSSGAA ↓ AKGVSQ	0.707	+
Cel3F	104797	MVAVKQ--IALLAGLAH-WADA ↓ AEKVIT	0.644	+
Cel3G	47268	MTSFHDGVKLSTVTCVLSGLVALGSA ↓ GPTAAS	0.599	+
Cel3C	82227		0.134	-
Cel3D	46816		0.103	-
Cel3H				+
Cel1A	120749		0.129	-
Cel1B	22197		0.114	-

Fig. 2-1 Alignment of the predicted signal peptides from the secreted enzymes and BGLs. Their N-terminal sequences were subjected to the SignalP 4.1 program (http: //www.cbs.dtu.dk/services/SignalP/). The D-score is used to discriminate signal peptides from non-signal peptides. The black boxes indicate hydrophobic residues. Arrows indicate actual or deduced cleavage sites. Predicted intracellular enzymes with low D-scores are Cel1A, Cel1B, Cel3C and Cel3D.

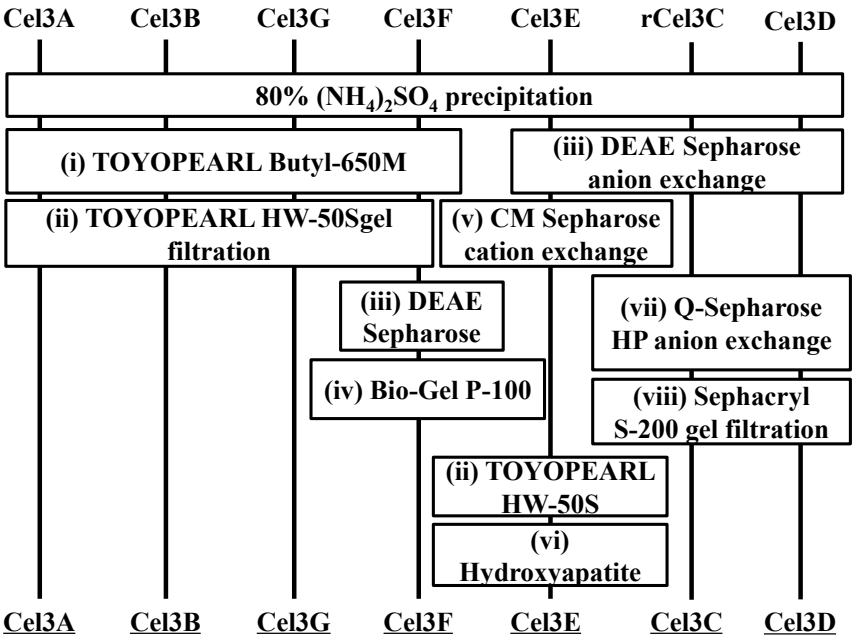
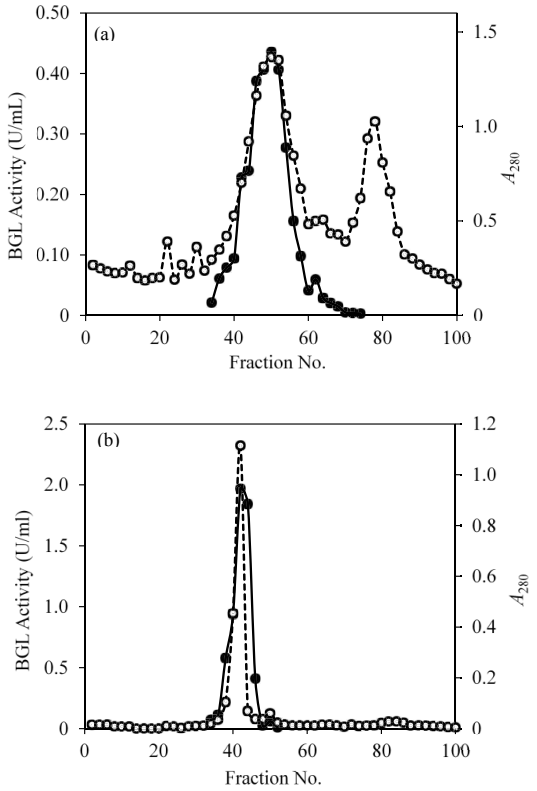
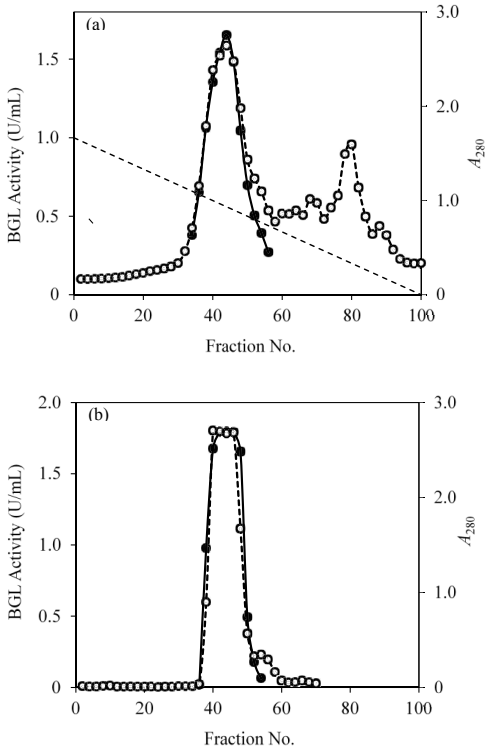


Fig. 2-2 Purification procedures for recombinants BGLs expressed in *A. oryzae.*



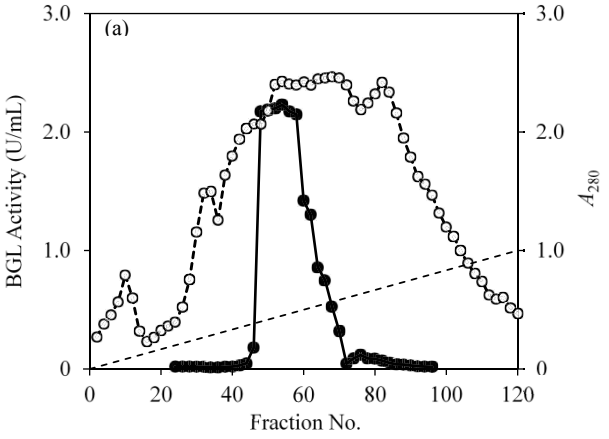
	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**1,210**	**806**	**1.50**	**1.0**	**100**
80% $(NH_4)_2SO_4$ precipitation	**1,180**	**600**	**2.00**	**1.3**	**97.0**
TOYOPEARL-Butyl 650M	**285**	**11.9**	**24.0**	**16**	**24.0**
TOYOPEARL HW-50S	**187**	**4.60**	**41.0**	**20**	**16.0**

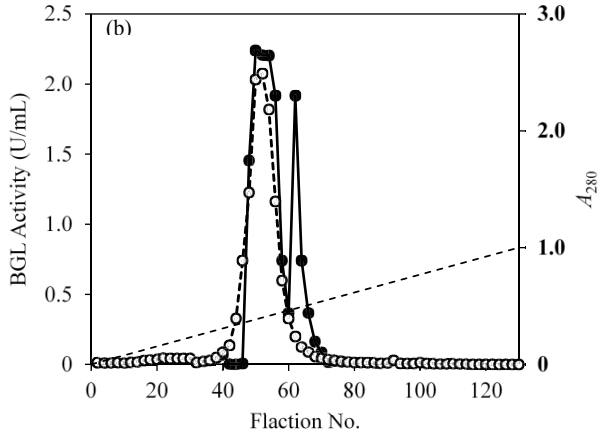
Fig. 2-2-1 Purification procedures for Cel3A, columns used in orders as (a) TOYOPEARL Butyl 650M (b) Toyopearl HW-50S. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity under the tested under the standard assay condition.

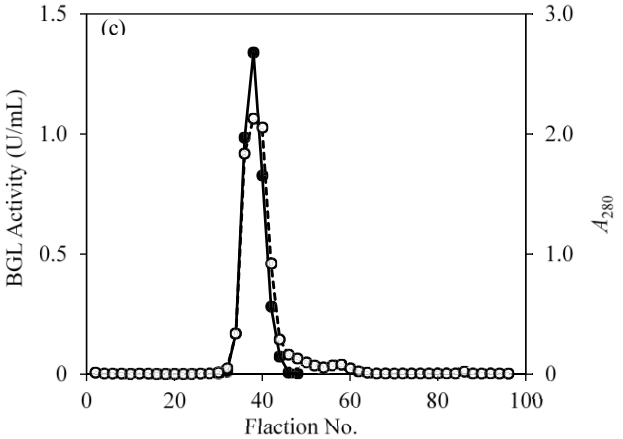


	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**7,000**	**1,800**	**3.90**	**1.0**	**100**
80% $(NH_4)_2SO_4$ precipitation	**6,280**	**410**	**15.3**	**3.9**	**90.0**
TOYOPEARL Butyl 650M	**4,414**	**112**	**39.4**	**10**	**63.0**
TOYOPEARL HW-50S	**3,231**	**90.0**	**36.0**	**9.1**	**46.0**

Fig. 2-2-2 Purification procedures for Cel3B, columns used in orders as (a) TOYOPEARL Butyl 650M, (b) TOYOPEARL HW-50S. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.

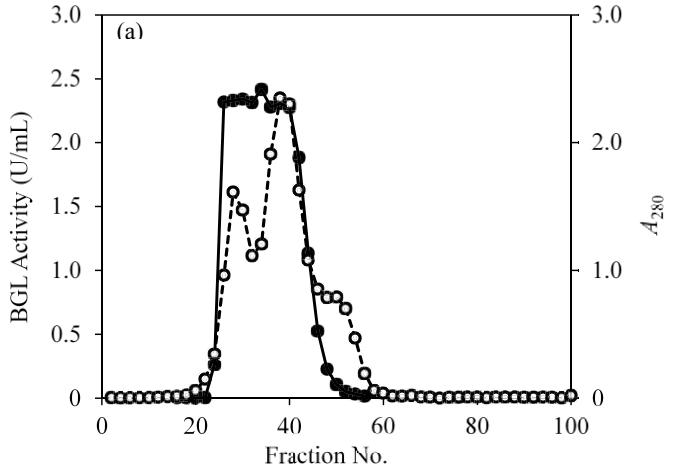

(a)
BGL Activity (U/mL)
A_{280}
Fraction No.

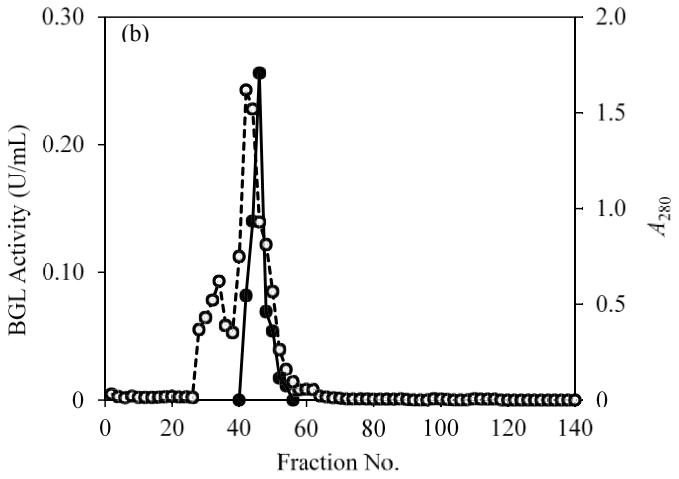

(b)
BGL Activity (U/mL)
A_{280}
Flaction No.

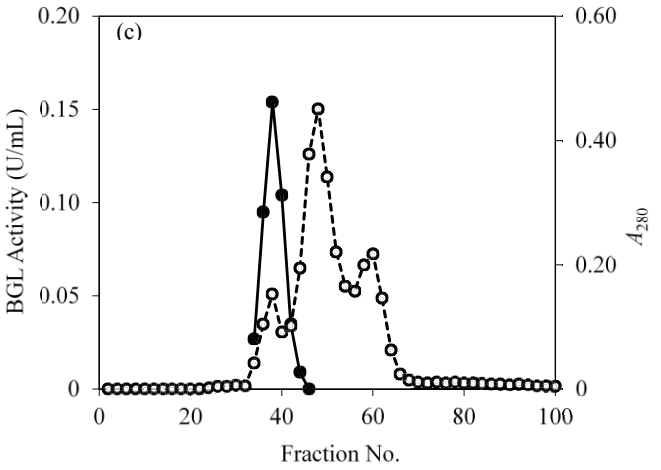

(c)
BGL Activity (U/mL)
A_{280}
Flaction No.

	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**13,711**	**5,763.5**	**2.40**	**1.00**	**100**
80% $(NH_4)_2SO_4$ precipitation	**12,340**	**1,647.0**	**7.50**	**3.10**	**90.0**
DEAE Sepharose	**7,581.0**	**174.50**	**43.4**	**18.0**	**55.3**
Q-Sepharose	**5,681.0**	**103.20**	**55.1**	**30.0**	**41.4**
Sephacry S200HR	**1,400.0**	**13.100**	**110**	**44.6**	**10.2**

Fig. 2-2-3 Purification procedures for Cel3C, columns used in orders as (a) DEAE Sepharose, (b) Q-Sepharose, (c) Sephacry S200HR. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.

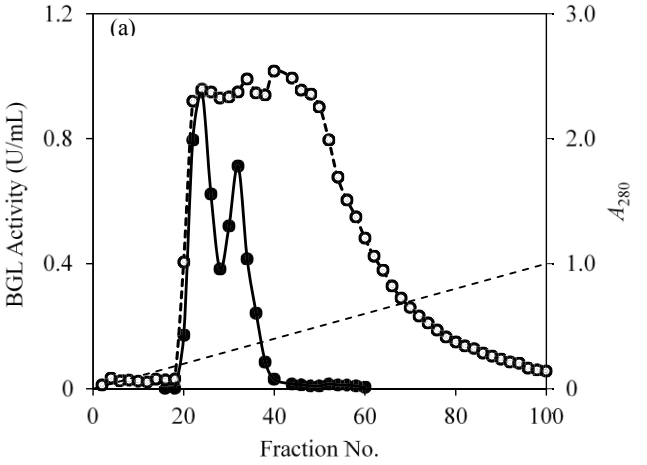

(a)
BGL Activity (U/mL)
A_{280}
Fraction No.
3.0
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100

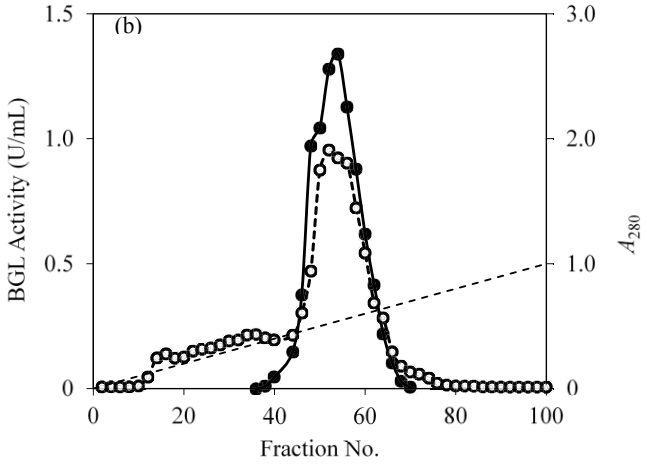

(b)
BGL Activity (U/mL)
A_{280}
Fraction No.
0.30
0.20
0.10
0
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140

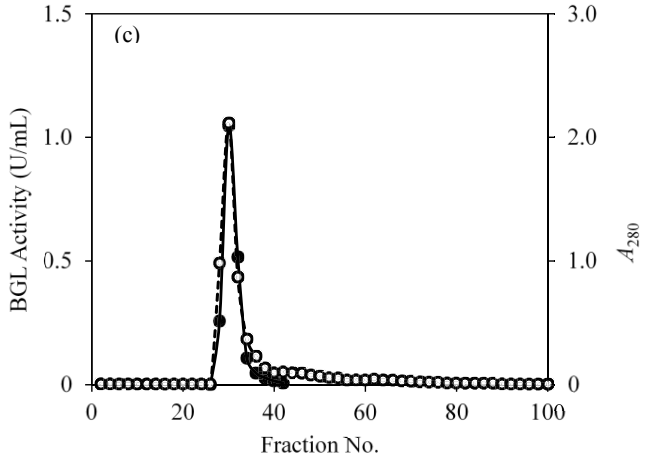

(c)
BGL Activity (U/mL)
A_{280}
Fraction No.
0.20
0.15
0.10
0.05
0
0.60
0.40
0.20
0
0
20
40
60
80
100

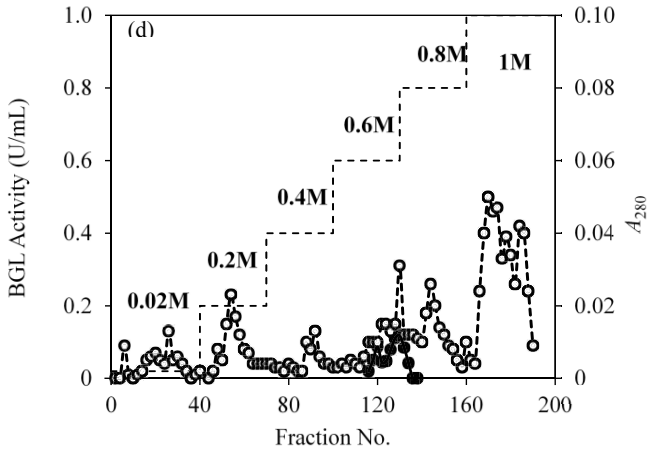
	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**304**	**27.4**	**4.85**	**1.00**	**100**
80% $(NH_4)_2SO_4$ precipitation	**224**	**187**	**4.90**	**1.01**	**73.7**
DEAE Sepharose	**156**	**187**	**8.03**	**1.65**	**51.2**
Q-Sepharose	**108**	**3.30**	**33.0**	**6.80**	**12.0**
Sephacry S200HR	**98.0**	**2.80**	**35.0**	**7.20**	**10.2**

Fig. 2-2-4 The purification procedure for Cel3D, columns used in orders as (a) DEAE Sepharose, (b) Q-Sepharose, (c) Sephacry S200HR. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.


(a)
BGL Activity (U/mL)
A_{280}
Fraction No.
0
0.4
0.8
1.2
1.0
2.0
3.0
0
20
40
60
80
100

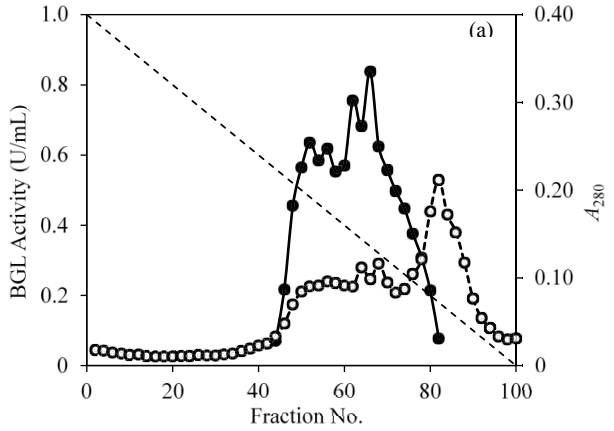

(b)
BGL Activity (U/mL)
A_{280}
Fraction No.
0
0.5
1.0
1.5
1.0
2.0
3.0
0
20
40
60
80
100

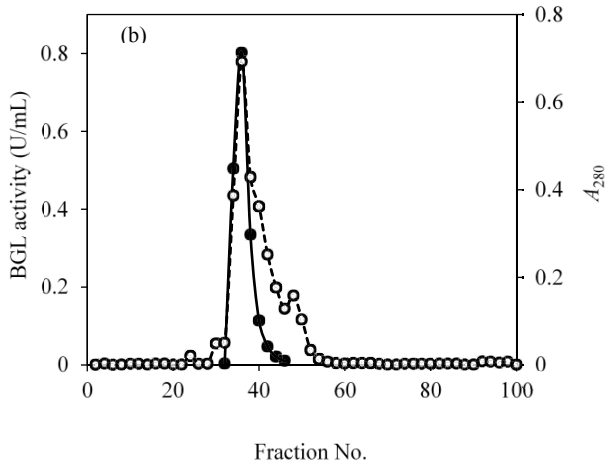

(c)
BGL Activity (U/mL)
A_{280}
Fraction No.
0
0.5
1.0
1.5
1.0
2.0
3.0
0
20
40
60
80
100

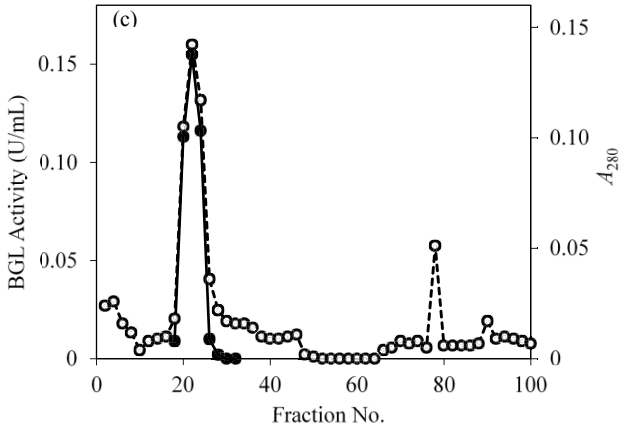


	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**722.0**	**4,400**	**0.16**	**1.00**	**100**
80% $(NH_4)_2SO_4$ precipitation	**393.0**	**620**	**0.63**	**4.00**	**14**
DEAE Sepharose Fast Flow	**328.0**	**220**	**1.5**	**9.40**	**5.0**
CM Sepharose	**71.7**	**42**	**1.7**	**10.6**	**1.0**
TOYOPEARL HW-50S	**23.0**	**17**	**1.4**	**8.40**	**0.40**
Hydrixyapatite	**0.840**	**0.13**	**6.5**	**41.5**	**0.03**

Fig. 2-2-5 The purification procedure for Cel3E, columns used in orders as (a) DEAE Sepharose, (b) CM Sepharose, (c) TOYOPEARL HW-50S, (d) Hydrixyapatite. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.

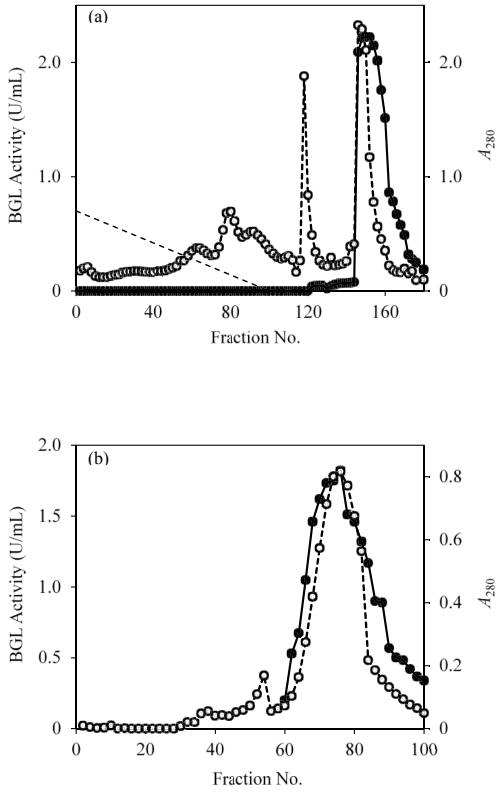

(a)
BGL Activity (U/mL)
A_{280}
Fraction No.


(b)
BGL activity (U/mL)
A_{280}
Fraction No.


(c)
BGL Activity (U/mL)
A_{280}
Fraction No.

	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**127**	**685**	**0.19**	**1.0**	**100**
80% $(NH_4)_2SO_4$ precipitation	**113**	**141**	**0.80**	**4.2**	**20**
TOYOPEARL Butyl 650M	**36.5**	**7.80**	**4.7**	**24**	**1.1**
TOYOPEARL HW-50S	**33.9**	**4.90**	**6.9**	**36**	**0.71**
DEAE Sepharose Fast Flow	**21.4**	**1.80**	**12**	**63**	**0.26**
Bio-Gel P-100	**14.5**	**1.20**	**12**	**61**	**0.17**

Fig. 2-2-6 The purification procedure for Cel3F, columns used in orders as (a) TOYOPEARL Butyl 650M, (b) TOYOPEARL HW-50S, (c) DEAE Sepharose, (d) Bio-Gel P-100. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.



	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	**3,130**	**2,750**	**1.1**	**1.0**	**100**
80% $(NH_4)_2SO_4$ precipitation	**1,500**	**1,056**	**1.4**	**1.3**	**38.4**
DEAE Sepharose Fast Flow	**1,390**	**53.5**	**26**	**24**	**2**
TOYOPEARL HW-50S	**1,090**	**37.6**	**29**	**26**	**1.3**

Fig. 2-2-7 The purification procedure for Cel3G, columns used in orders as (a) DEAE Sepharose, (b) TOYOPEARL HW-50S. The empty circle is responds for protein concentration at absorbance of A_{280}, the solid circle is responds for BGL activity. The dotted line described the gradient concentration.

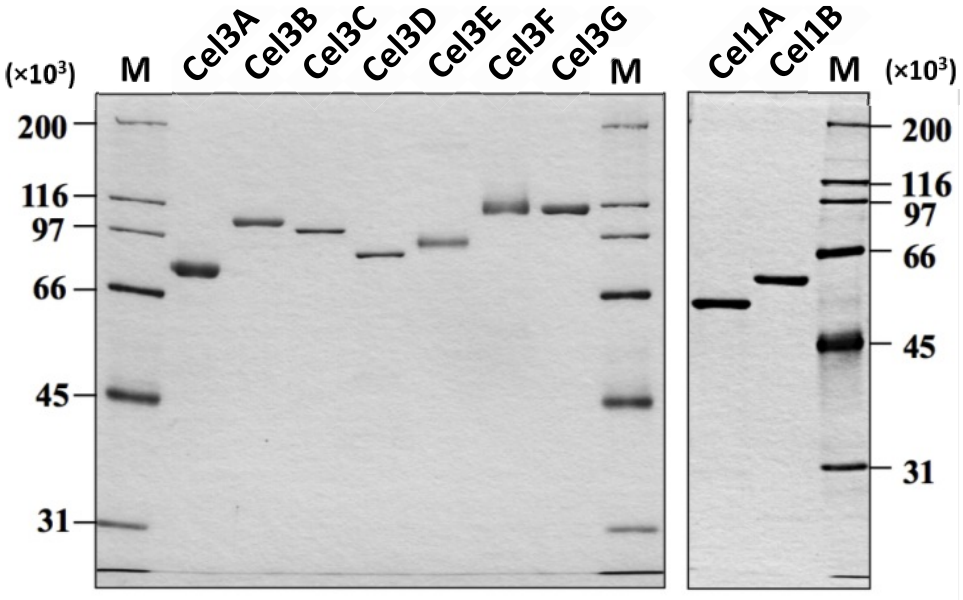
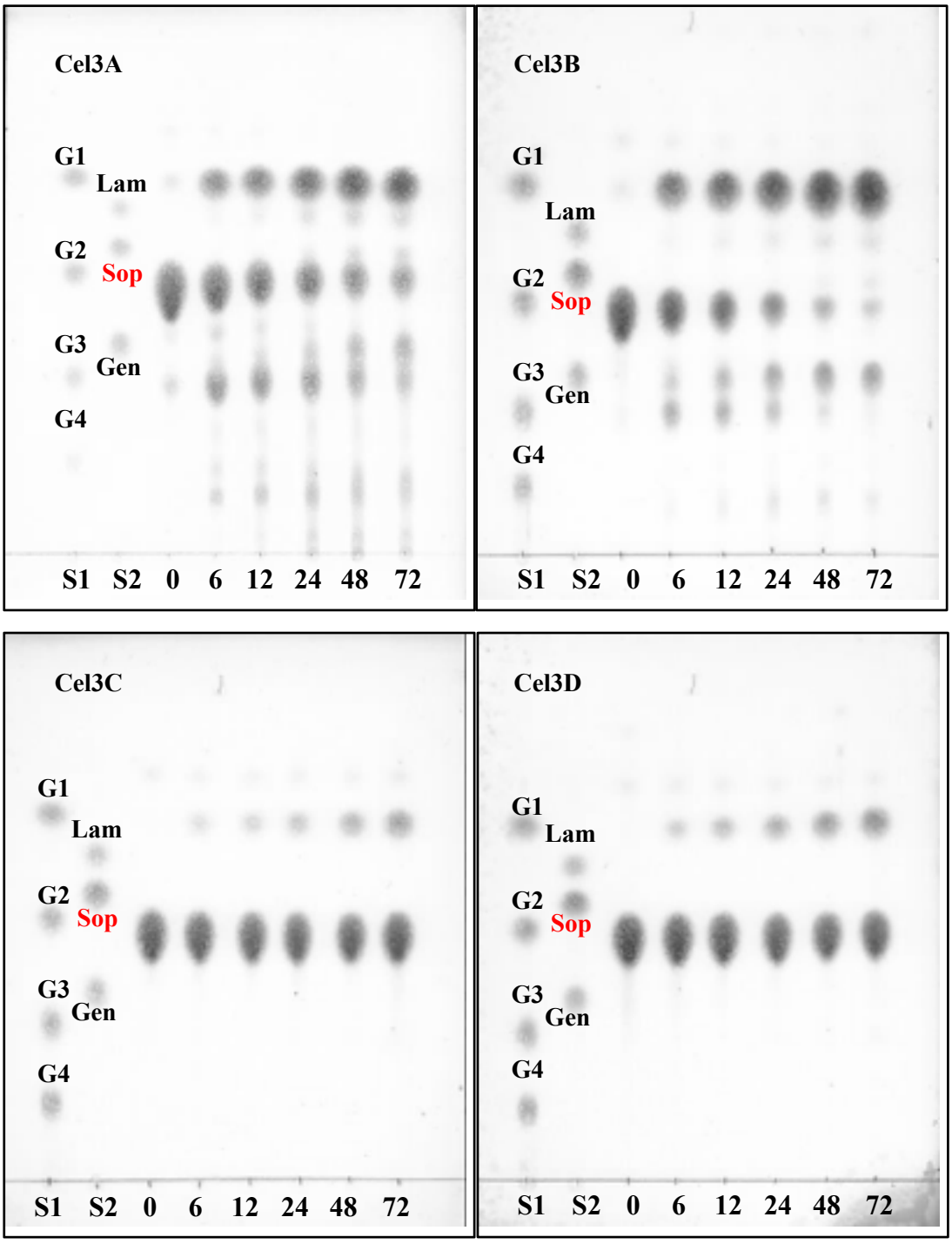
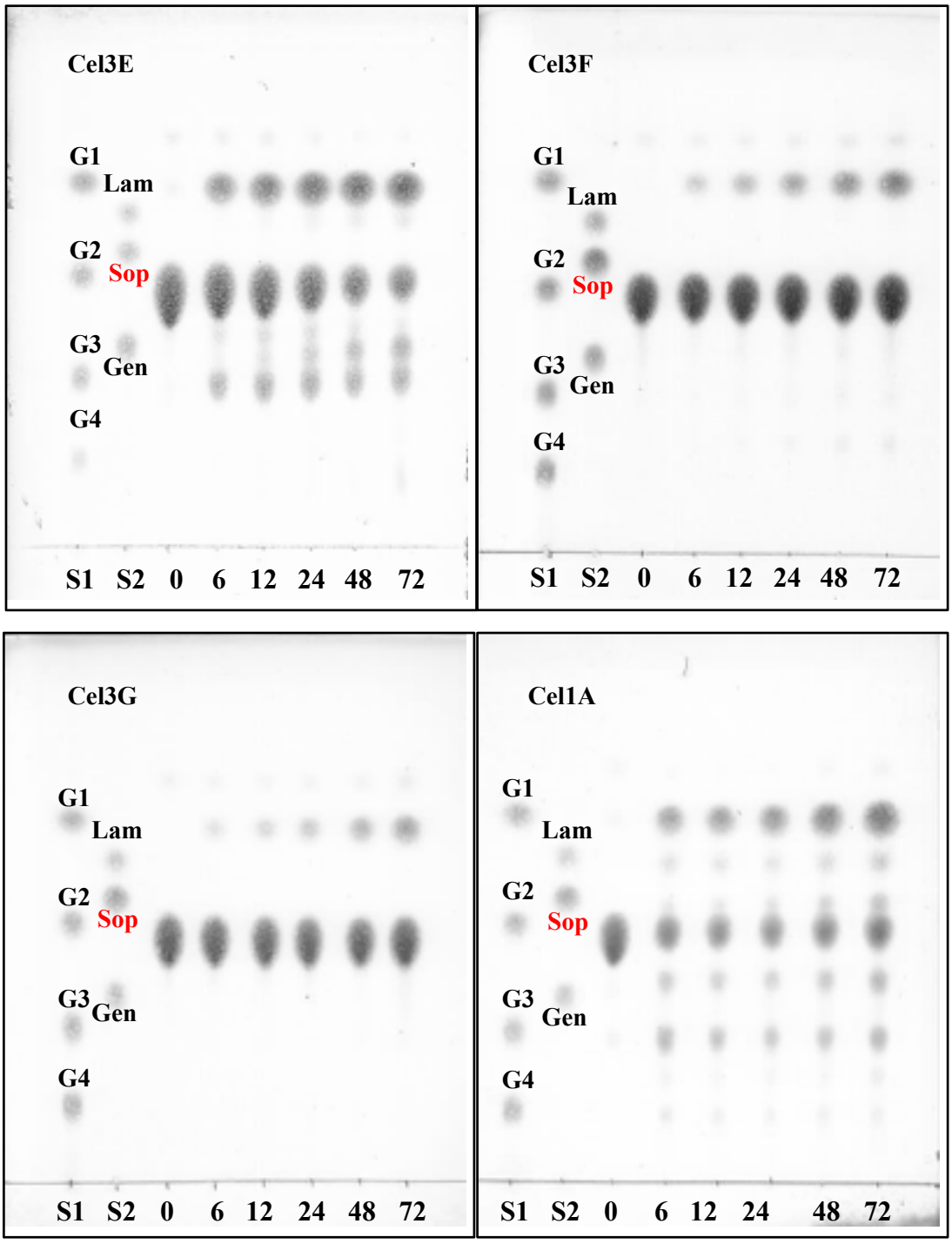


Fig. 2-3 SDS-PAGE analysis of the recombinant BGLs. The purified BGLs belonging to GH3 and GH1 were subjected to a 8% and a 10% polyacrylamide gels respectively. M is represent for molecular weight markers.


Cel3A
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel3B
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel3C
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel3D
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72


Cel3E
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel3F
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel3G
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72
Cel1A
G1
Lam
G2
Sop
G3
Gen
G4
S1 S2 0 6 12 24 48 72

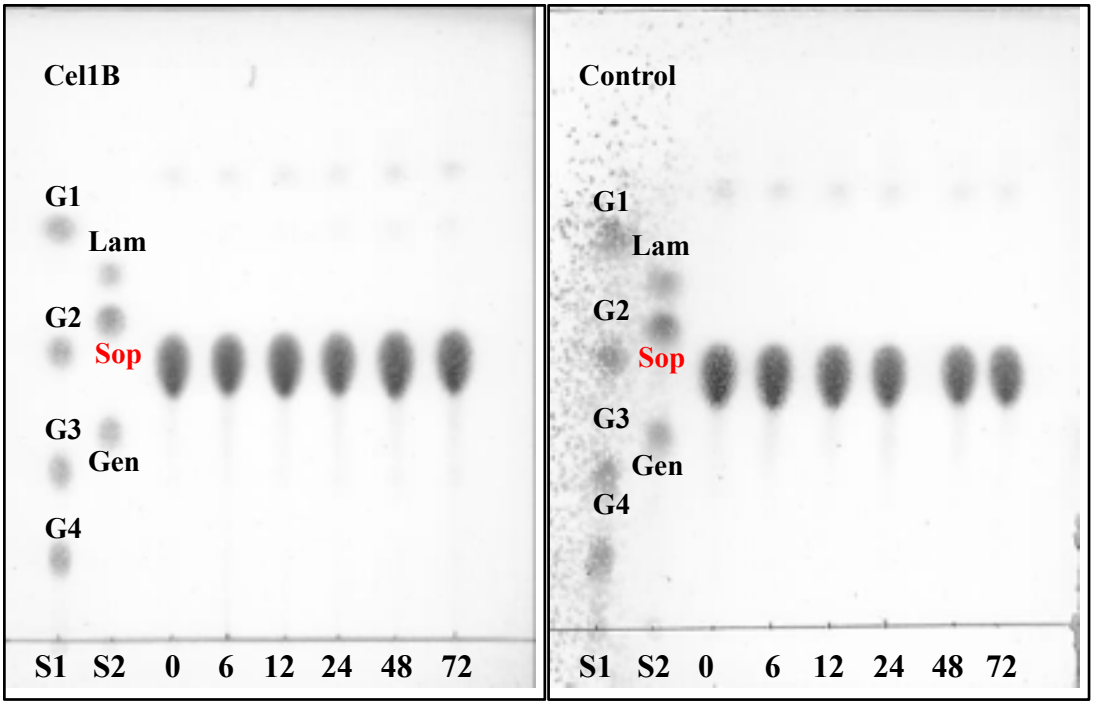
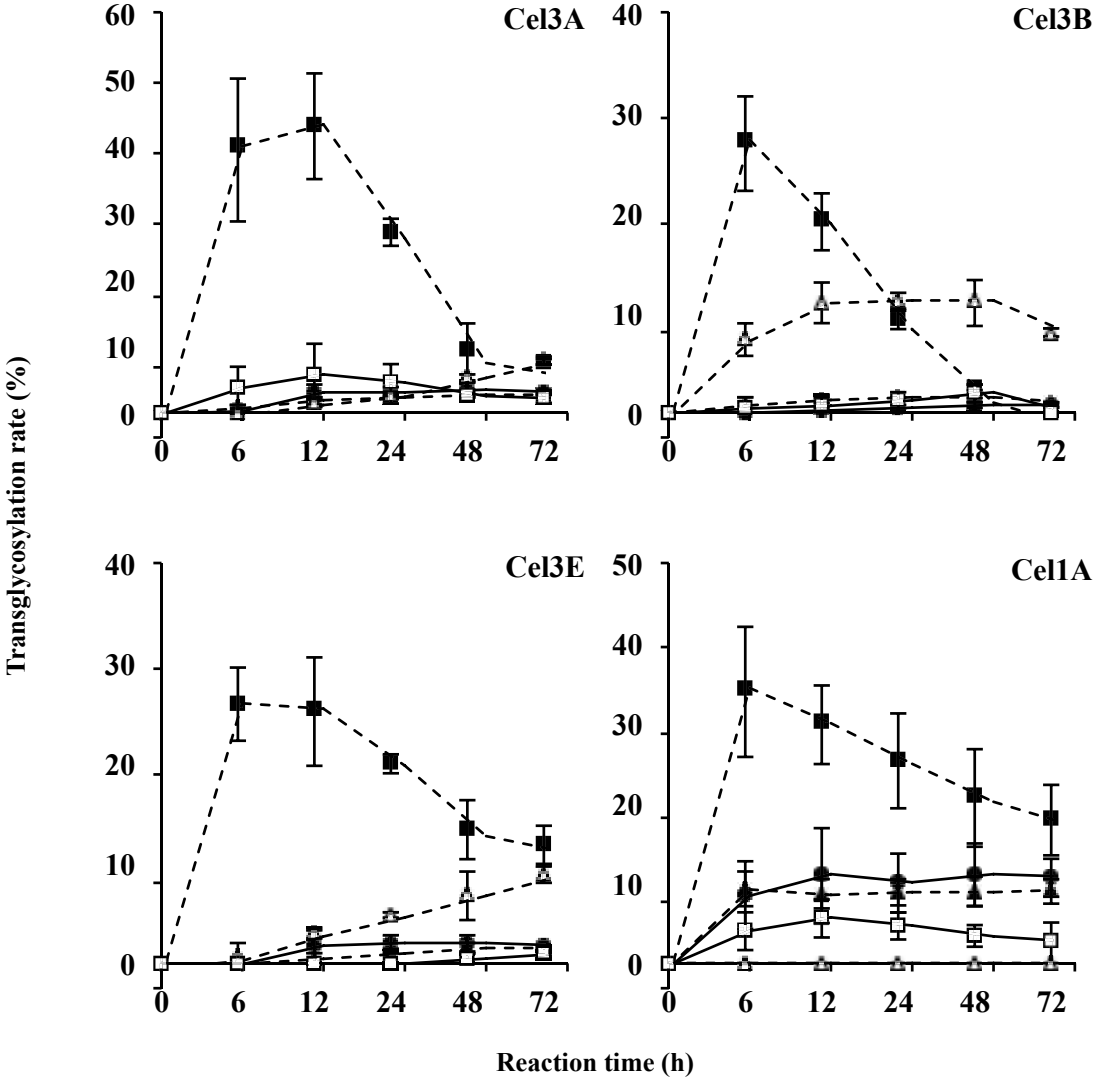
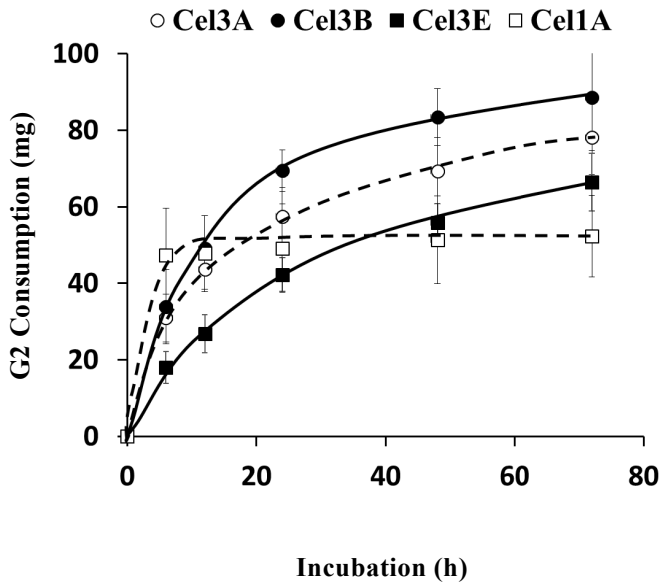


Fig. 2-4 TCL analysis of BGLs of the transglycosylation products by using 10% G2 as substrate. The spots were carried out by 1 μL of the reaction mixture.

(a)


Cel3A
Cel3B
Cel3E
Cel1A
Transglycosylation rate (%)
Reaction time (h)
0
6
12
24
48
72

(b)



(c)

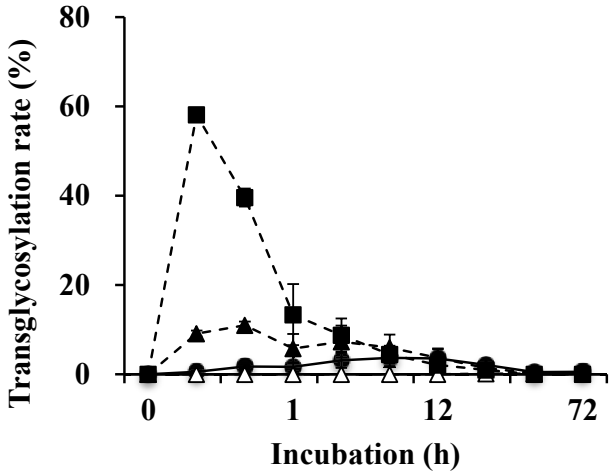
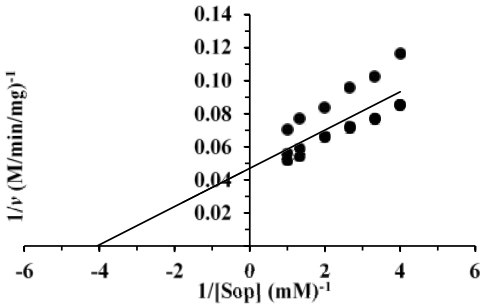
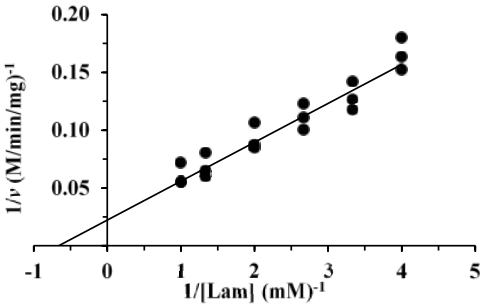
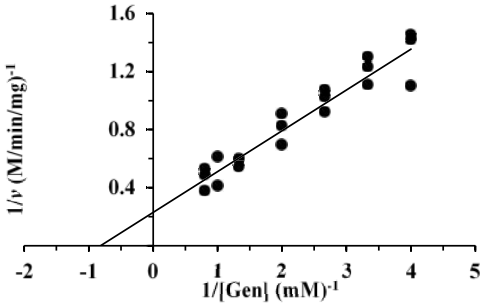


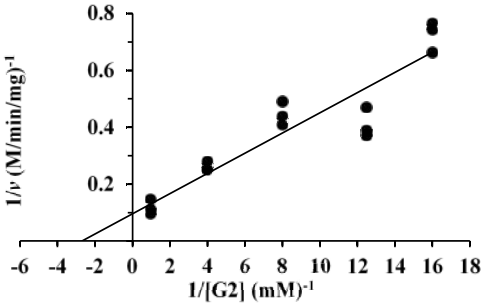
Fig. 2-5 Sugar composition of transglycosylation products. (a) Composition of transglycosylation products produced from 10% G2 by Cel3A, Cel3B, Cel3E and Cel1A. Solid circle indicates Sop; Solid triangle indicates Lam; Empty triangle indicates Gen; Empty hourindicates G3; Solid square indicates G4. (b) G2 consumption of the reaction by time course calculation using 10% G2 as substrate. (c) Composition of transglycosylation products produced from 1% G2 by Cel1A, the symbol present as these of 10% G2. Vertical bars indicate standard deviations of three independent replicates.

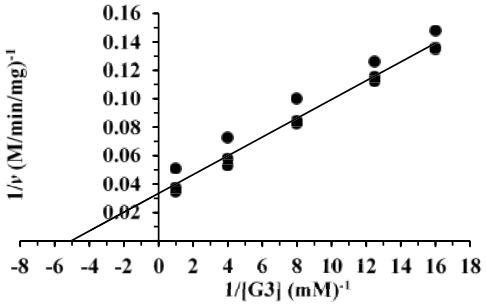
Cel3A


1/v (M/min/mg)-1
1/[Sop] (mM)-1

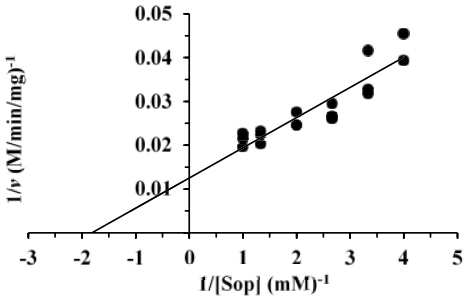

1/v (M/min/mg)-1
1/[Lam] (mM)-1

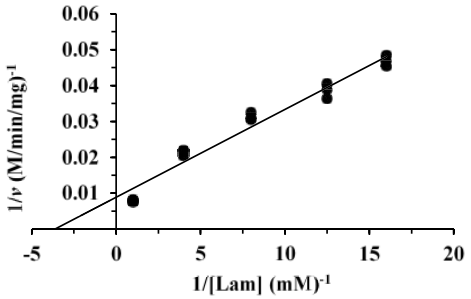

1/v (M/min/mg)-1
1/[Gen] (mM)-1

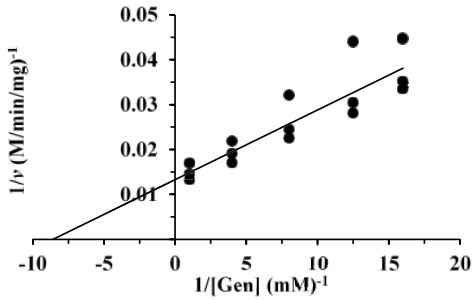

1/v (M/min/mg)-1
1/[G2] (mM)-1

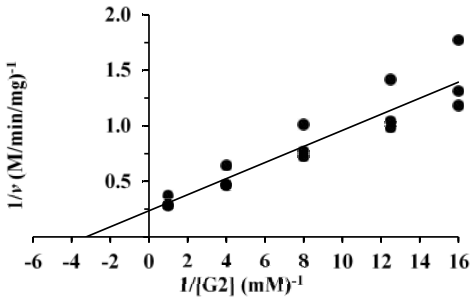

1/v (M/min/mg)-1
1/[G3] (mM)-1

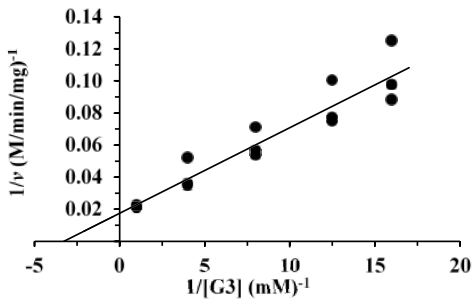
Cel3B


0.05
0.04
0.03
0.02
0.01
1/v (M/min/mg)-1
-3 -2 -1 0 1 2 3 4 5
1/[Sop] (mM)-1

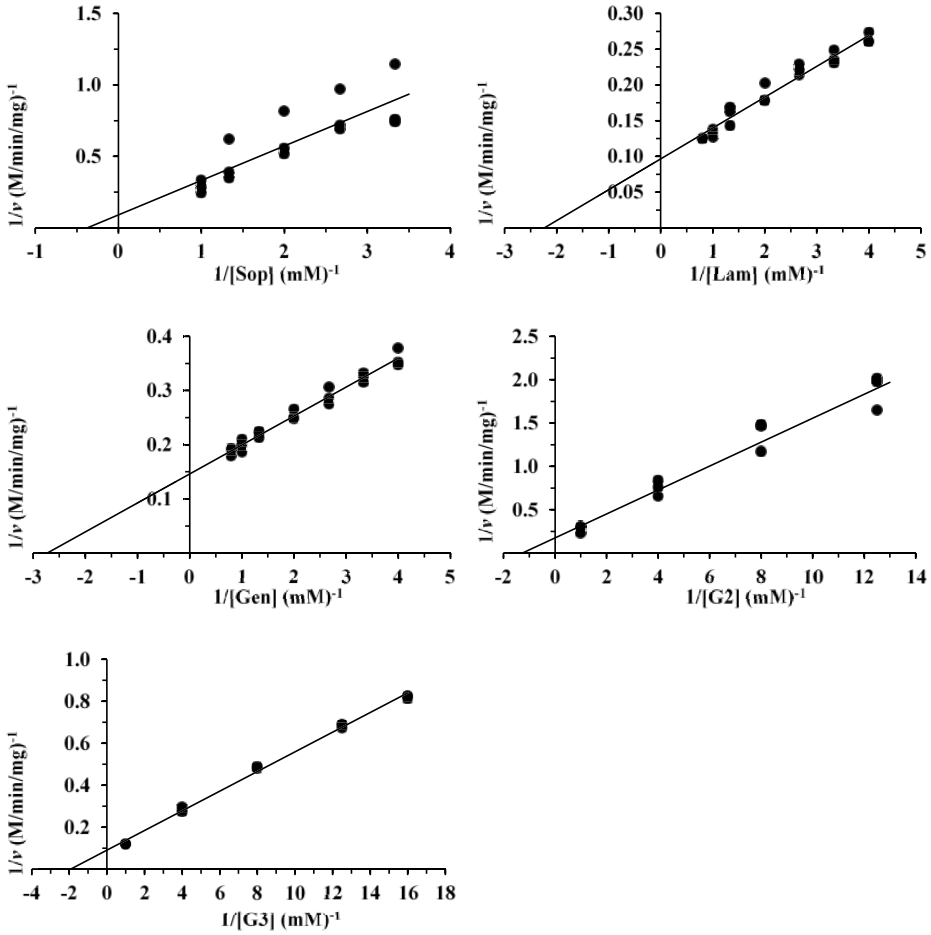

0.06
0.05
0.04
0.03
0.02
0.01
1/v (M/min/mg)-1
-5 0 5 10 15 20
1/[Lam] (mM)-1


0.05
0.04
0.03
0.02
0.01
1/v (M/min/mg)-1
-10 -5 0 5 10 15 20
1/[Gen] (mM)-1


2.0
1.5
1.0
0.5
1/v (M/min/mg)-1
-6 -4 -2 0 2 4 6 8 10 12 14 16
1/[G2] (mM)-1


0.14
0.12
0.10
0.08
0.06
0.04
0.02
1/v (M/min/mg)-1
-5 0 5 10 15 20
1/[G3] (mM)-1

Cel3E



Cel1A

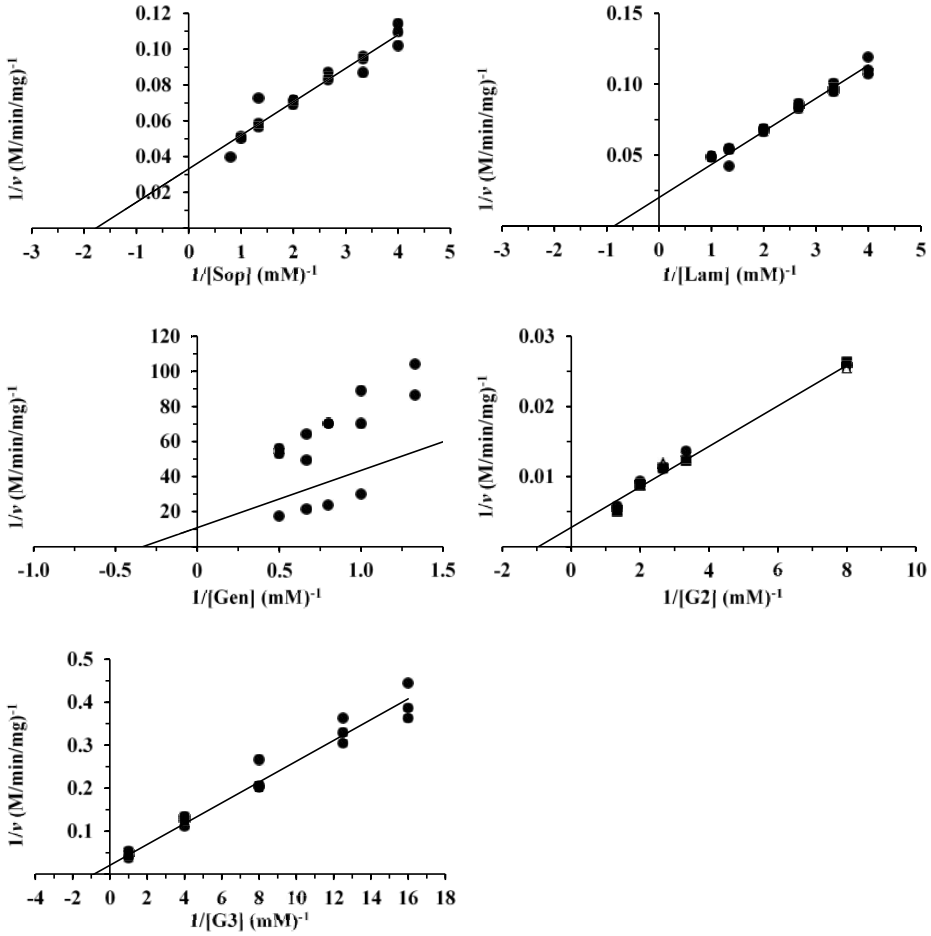


Fig. 2-6 Lineweaver-Burk plot of BGLs towards disaccharides. The three sports responds for parallel independent experiments. The liner was describes the average value on each substrate concentration.

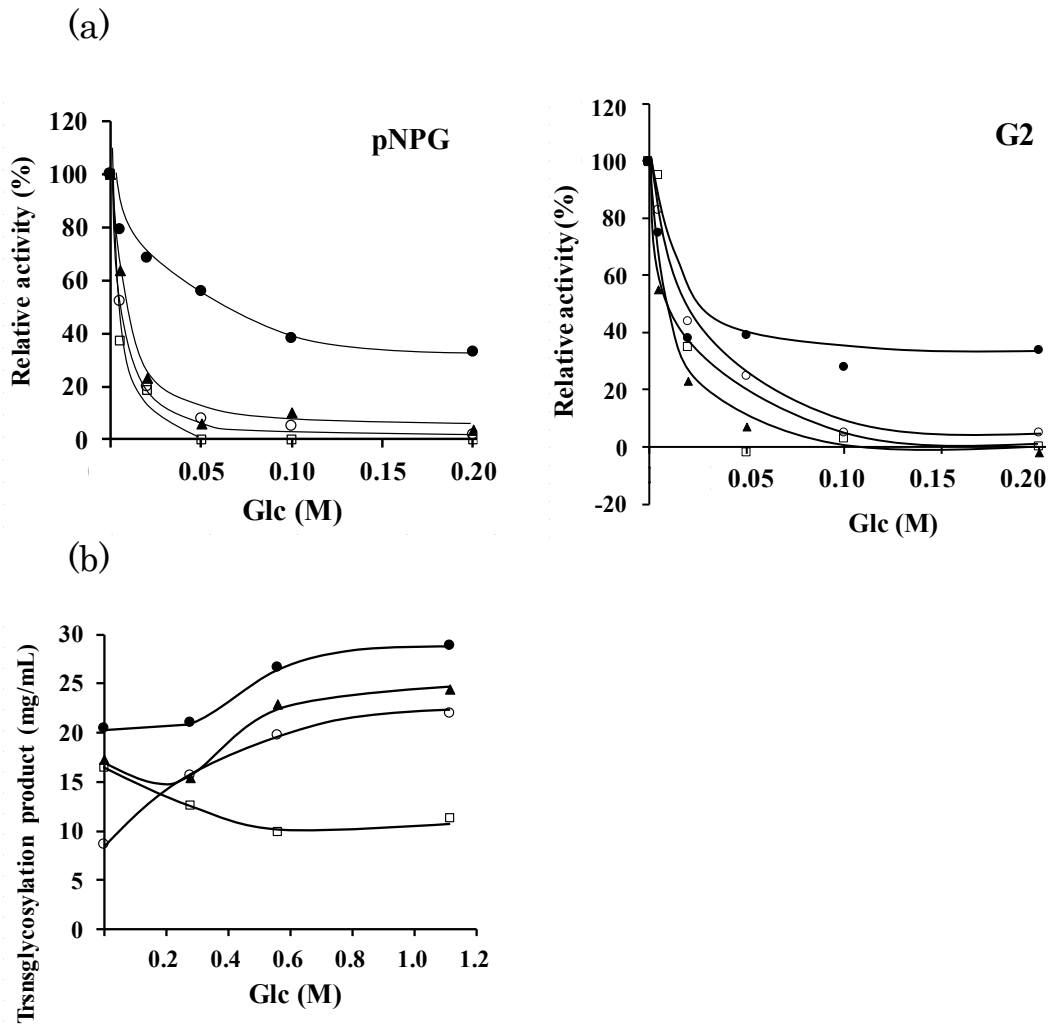


Fig. 2-7 Effect of Glc inhibition on BGL activities against pNPG and G2 (a). Total amount of the transglycosylation products from 10% of G2 after incubation for 72 h was measured by using HPLC system. Data plotted are averages for 2 independent samples. Solid triangle indicates Cel3A, empty circle indicates Cel3B, Empty square indicates Cel3E and solid circle indicates Cel1A.

Chapter 3

Impact of knockout of *cel1a* or *cel3a* genes on cellulase induction in *Trichoderma reesei*

Chapter 3: Impact of knockout of *cel1a* or *cel3a* genes on cellulase induction in *Trichoderma reesei*

3.1. Introduction

As we mention in previously charters, *Trichoderma* sp. fungi are important producers of enzymes for plant biomass digestion. However, it still remains importnat to improve cellulase production for industrial applications [1–3]. Sophorose (Sop) induces cellulase secretion in *Trichoderma* sp. more effectively than other known inducers [4–8]. It is also known that *T. reesei* cell extracts contain BGL capable of synthesizing Sop [9]. Addition of deoxynojirimycin, a β-glucosidase (BGL) inhibitor, to the *T. reesei*, significantly supressed the cellulase production [10] suggesting another possible involvement of BGLs in the process of enzyme production. Cellulase genes in *T. reesei* are expressed simultaneously, indicating these genes might controlled in common regulators [11–13]. Recent studies reported that the absence of *xyr1* gene deprivates the induction ability of most cellulase inducers of *T. reesei*, including Sop [14–17], which suggested that the *xyr1* gene, encoding a downstream cellulase regulator, which could accept the signal transduction from Sop in the process of cellulase induction.

The Cel1A and Cel3A in *T. reesei* were shown to be two important proteins for Sop formation. However, the relationship between BGLs, Sop formation, cellulase transcription regulators and cellulase induction itself, have not been investigated. With the aim to clarify this relationship, in this chapter we constructed two *T. reesei* mutants in which genes *cel1a* (*Δcel1a*) and the *cel3a* (*Δcel3a*) were deleted. Their growth and cellulase production was compared with the wild-type (WT) strain.

3.2. Materials and Methods

3.2.1. Strains, fugal growth, and culture conditions

T. reesei QM9414 (ATCC 26921) and its mutant strains were maintained on Difco™ Potato Dextrose Agar plates (PDA, Becton, Dickinson and Company, NJ, USA). Spores of each strain were collected in 0.8% NaCl containing 0.05% Tween 20 and were stored at 4°C until use. To determine cellulase production, 1.5×10^9 spores were inoculated into 150 mL of the modified Mandels and Andreotti medium containing 2% D-sorbitol, 1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.2% Bacto™ Peptone (Becton-Dickinson and Company, Franklin Lakes, NJ), 0.06% $MgSO_4$, 0.06% $CaCl_2$, and 0.02% trace element solution containing 250 μg/L $FeSO_4 \cdot 7H_2O$, 80 μg/L $MnSO_4 \cdot H_2O$, 70 μg/L $ZnSO_4 \cdot H_2O$, and 100 μg/L $CaCl_2 \cdot 2H_2O$), which was sterilized by passage through a 0.2 μm membrane filter (ADVANTEC, Toyo, Japan). Cells were pre-cultured for 48 h to achieve mycelial growth, and mycelia were then collected using a 1G3 filter (SANSYO, Tokyo, Japan) to wash twice in the same culture medium lacking D-sorbitol. Mycelia were then inoculated into fresh culture medium containing 1% (w/v) Avicel (PH-101; Sigma-Aldrich, MO, USA to induce cellulase. Cells were cultured at 30°C in the dark on a rotary shaker at 220 rpm. Sop was generously provided by Prof. M. Nakajima (Tokyo University of Science) and Prof. Nakai (Niigata University) [18–19].

3.2.2. Disruption constructs for *cel1a* and *cel3a* genes of *Trichoderma reesei*

Escherichia coli DH5α cells were used for routine propagation of all plasmids, and pBluescript SK (+) II was used as the vector for constructing all mutant strains. *T. reesei* transformation was performed using the pPTR II DNA vector (TaKaRa, Shiga, Japan), all PCR amplification were use PrimeSTAR® HS DNA polymerase (TaKaRa). To construct mutant *cel1a* cassette, three fragments were amplified using Cel1A-LF/Cel1A-LR and Cl1A-RF/Cel1A-RR (Table 3-1) from *T. reesei* chromosomal DNA, using *ptr*A-F and

*ptr*A-R from the pPTR II DNA vector. The resulting fragments cel1A-R, cel1A-L, and ptrA were inserted into pBluescript II SK (+) via the *Eco*R V site to produce the respective plasmids pBS-cel1A- R, pBS-cel1A-L, and pBS-*ptr*A. Fragments of cel1A-R and cel1A-L were further released by digestion with *Xho* I/*Hin*d III or *Spe* I/*Not* I respectively, and were then ligated into corresponding digested sites of pBS-*ptr*A to obtain pBS-Cel1A-*ptr*A (5.8 kbp; Fig. 1a). Mutant *cel3a* cassette was constructed identically using the primers for Cel3A-LF/Cel3A-LR and Cl3A-RF/Cel3A-RR to produce pBS-Cel3A-*ptr*A (5.7 kbp). Subsequently, mutant cassette fragments were further amplified using PCR with the universal primers M13 F/R. *T. reesei* cell were then transformed with mutant cassettes following purification by ethanol precipitation and condensation to 1 μg/μL.

3.2.3. Transformation of *Trichoderma reesei* using gene deletion cassettes

Transformations were performed using the PEG method as described previously [20], except that 1% VinoTaste (Novozymes A/S, Copenhagen) was used to obtain protoplast cultured at 30°C. After transformation, protoplasts were spread onto agar media containing 0.2-g/mL pyrithiamine hydrobromide (Sigma-Aldrich, MO, USA), and were cultured for 4 days at 30°C. Single colonies were then transferred into the same medium containing 0.4 g/mL pyrithiamine hydrobromide (Sigma-Aldrich) for secondary selection. Selected colonies were then maintained on PDA plates and gene knockout was determined using PCR with KOD FX Neo (TOYOBO, Osaka, Japan), extracts of genomic DNA from mycelia, and corresponding primers according to the manufacturer's protocol (Table 3-1).

3.2.4. RNA extraction, reverse transcription and cDNA synthesis

Fungal mycelia were harvested using a 1G3 filter (SANSYO) and were immediately frozen in liquid nitrogen and crushed using a mortar. Total RNA was extracted from 0.1 g of crushed mycelium using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the

manufacturer's protocol. After purification, 2 μg of total the RNA was reverse transcribed using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa).

3.2.5. Analysis of gene expression by quantitative real-time PCR

Quantitative real-time PCR was performed with a LightCycler System (Roche Applied Science, Penzberg, Germany) in a final volume of 10 μL using SYBR Premix Ex Taq II (Tli RNaseH Plus) Kits (TaKaRa) with 0.4 μM forward and reverse primers and 1 μL cDNA samples diluted to measurable concentrations. Thermal cycling was conducted under the following conditions: 30 sec at 95°C, followed by 45 cycles of 10 sec at 95°C, 10 sec at 55°C, and 45 sec at 72°C. Assays were performed in quadruplicate with genomic DNA controls. The specificity of PCR analyses was tested using melting curve analyses and relative expression levels were calculated using the ratio of the C_q value for sample genes to that of the house-keeping gene *act1* (actin). A threshold line curve was generated for each sample gene and *act1* by calculating the log (normalized C_q value) for different cycles using various genomic DNA template concentrations. The C_q values for each sample and *act1* were calculated according to the threshold line. All samples were analysed in triplicate in three independent experiments. The primers used in this study are listed in Table 3-1.

3.2.6. Determination of enzyme activity and protein

Total cellulase activity was determined using the dinitrosalicylic acid (DNS) method [21] with Whatman No. 1 filter paper (1 × 3 cm) as a substrate. The reaction mixture (2 mL) contained 1 mL of 100 mM sodium acetate buffer (pH 5.0) and 0.6 mL of H_2O and was incubated at 30°C for 5 min prior to addition of 0.4 mL samples of culture supernatants. Sugar production was determined using Glc as a standard. BGL activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) or G2 as substrates [22]. Protein concentrations were determined using the Lowry method [23] with BSA as a standard.

3.2.7. Detection of Cel1A using western blotting

The secretion of BGL Cel1A was followed using Western blotting. After time intervals mycelium isolated from 10 mL medium aliquots by filtration (G3) and then frozen in liquid nitrogen. The intracellular proteins were isolated grinding the frozen mycelium in a mortar followed by extraction with 40 mL of 50 mM sodium acetate buffer (pH 5.5) containing 0.4 mL protease ArrestTM (GBioscience, MO, USA). 10 µl of the samples were subjected to SDS-PAGE in 10% gels. Proteins were then transferred to PVDF membranes (Bio-Rad, USA) at 15 V for 45 min and immunodetection of Cel1A was performed with a rabbit polyclonal antibody (dilution 1:1000) that was raised against the Cel1A amino acid sequence VMFRALPK (amino acids 153–160), which lacked homology to sequences of the other BGLs in *T. reesei* (Hokkaido System Science Co., Ltd., Japan). IgG goat anti-rabbit antibody conjugated with horse radish peroxidase was used as a secondary antibody (Promega, Co., Madison, USA, dilution to 1:2500) and EzWestBlue (ATTO) was used as substrate for visualization of Cel1A.

3.3. Results

3.3.1. Disruption of the *cel1a* and *cel3a* gene from *Trichoderma reesei*

Central portion of the coding region of *cel1a* or *cel3a* containing the catalytic residues, were replaced with the pyrithiamin resistant gene (*ptr*A). A scheme of the disruption is shown in Fig. 3-1a. After transformation, 17 colonies for *cel3a* and 67 colonies for *cel1a* were obtained from the pyrithiamin ager solid medium and these colonies were used to comfirm their genes deletion. The full length of the deletion cassettes of *Δcel3a* and *Δcel1a* were verified using PCR with the primers Δcel1A-F/Δcel1A-R or Δcel3A-F/Δcel3A-R, respectively. Sizes of the resulting fragments corresponded to those of the deletion cassettes (5.8 kbp for *Δcel1a* and 5.7 kbp for *Δcel3a*). Internal region lengths of the deletion cassettes were also confirmed by PCR using the primer sets Δcel1A-F/ptrA-R3 for *cel1a* (2.21 kbp) and Δcel3A-F/ptrA-R3 for *cel3a* (2.24 kbp; Fig. 3-1b). As a result, one kockout mutant

strain from the 17 colonies for deletion the of *cel3a* gene, two knockout mutant strain from the 67 colonies for deletion of *cel1a* gene were showed gave the expecting PCR results.

3.3.2. Fungi growth

The effects of knockout of *cel3a* and *cel1a* genes on cell growth were examined on solid medium containing Glc, G2, or carboxymethylcellulose (CMC) as carbon sources. The mutants grew similarly as the WT strain. An exception was a slower growth on CMC of the mutant lacking *cel1a* (Fig. 3-2)

3.3.3. Cellulase production by *Δcel3a* and *Δcel1a* on Avicel

Pattern of the protein secretion of the WT strain and its *Δcel1a* and *Δcel3a* mutants was compared using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) during growth on Avicel. The mutants secreted the same type of proteins as the WT strain, however, in lower amounts and at much later time (Fig. 3-3a). The secretion was most severely influenced by the absence of the *cel1a* gene. The first proteins started to appear in the growth medium after 96 h. The delay in secretion was less pronounced in the case of *cel3a* mutant. A simultaneous determination of cellulase activity in the medium showed that cellulose activity corresponded well to the pattern of protein secretion. Long delay of cellulase appearance and its low volumetric activity was observed with the *Δcel1a* strain. Interesting to mention that almost no activity of BGL was secreted by the mutant *Δcel3a*. After 168 h growth, BGL activity in the medium of the WT stain and mutants *Δcel1a* and *Δcel3a* was 0.037, 0.027 and 0.004 U/mL using pNPG as substrate, respectively (Fig. 3-3b).

3.3.4. Transcription levels of cellulase and the relevant regulators on Avicel

Transcriptional level of genes of cellulases and genes of their transcriptional regulators during growth on Avicel were determined by PCR (Fig. 3-4). WT strain and the *Δcel3a* mutant exhibited two distinct transcriptional maxima for BGL genes (*cel3a*, *cel3b*, *cel3e* and *cel1a*), cellulase genes (*cbh1*, *cbh2*, *eg1*and *eg2*), and positive (*xyr1*, *ace2* and *bglR*) and negtive (*cre1* and *ace1*) regulators. The first sharp maximum appeared transitionally around 12 h of cultivation, and the second one, not so sharp, after 96 h, after which the level of mRNAs started to decline. The absence of *cel3a* gene brought about considerable decrease of transcription level of the followed genes particularly in the time of appearance of the second maximum. This decrease shows some correlation with the observed cessation of further increase of cellulase activity in the medium (Fig. 3-3b). The major difference in transcription pattern in comparison to that in the WT strain and *Δcel3a* mutant was observed with the mutant *Δcel1a*. Deletion of *cel1a* lead to the first sudden maximum of the transcription of the genes of all followed enzymes did not appear at all. The transcription in the *Δcel1a* mutant showed only one maximum around 48 h. The *Δcel3a* exhibited slight decreases in the expression of cellulase genes and transcriptional regulators at the later phase of cultivation, suggesting that *cel3a* was involved in the later stage induction.

3.3.5. TLC analysis on the culture products

Time-course comparison of products of cellulose hydrolysis in the growth media of the WT strain and its mutants on Avicel by TLC pointed to one interesting fact. G2 was clearly observed only in the growth medium of *Δcel3a* strain, which supported the view that Cel3A BGL, missing in this mutant, could be responsible for G2 hydrolysis in the extracellular space (Fig. 3-5). To the *Δcel1a* strain, which was showed not or less Glc accumulation all through cultivation, this result was agreed with in the deficiency on the cellulase secretion and cellulase activity analyses in this strain (Fig. 3-3).

3.3.6. Cellulase production and induction in *Δcel1a* by sophorose

The mentioned results imply that Cel1A is an important intracellular BGL that is likely to be associated with Sop formation. Therefore, it was of interest to examine the effect of Sop on the obstructed cellulase induction in the *Δcel1a* mutant. As can be seen in Fig. 3-6, The addition of Sop to Avicel growth medium of the mutant led to complete recovery of the transcription of the cellulase genes and transcription regulator genes (*xyr1* and *cre1*) not influenced in the WT strain. The SDS-PAGE analyses of the secreted protein in the medium with Sop clearly indicated the elimination of the long lag phase of appearance of extracellular proteins (Fig. 3-7), observed in the absence of Sop (Fig. 3-3).

3.3.7. Cel1A production in *Trichoderma reesei*

To determine Cel1A protein response periods in *T. reesei*, an anti-Cel1A rabbit antibody was prepared and its specificity for Cel1A was confirmed using all nine BGL isozymes in *T. reesei* (data not shown). Subsequent Western blotting analyses of the cell extract suggested that Cel1A could be detected in the cells only during early phase of cultivation on Avicel (Fig. 3-8). Fig. 3-8 also shows that the mutant *Δcel1a* are free the corresponding protein of Cel1A.

3.4. Discussion

G2 is believed to be the main fragment of enzymatic cellulose hydrolysis which triggers the cellulose synthesis in *T. reesei* [6,11]. Sop, the positional isomer of G2, is known to be a more efficient cellulase inducer than G2. Several aspects of this behavior of Sop remain unknown, despite Sop was found to enhance transcription of the cellulose regulator genes such as the activator *xyr1* and the repressor *cre1* [13–17, 26–27]. For instance, methods have not been introduced to quantify Sop concentration inside the cells, an important parameter to understand the mechanism of Sop-induced cellulose induction. BGLs are responsible for G2 hydrolysis, but also for the formation of inducers such as Sop

[22, 28–30]. In our previous study dedicated to multiple BGLs of *T. reesei*, BGLs Cel1A and Cel3A were suggested to be the key enzymes producing Sop from G2 [22].

Recently, various molecular and genetic approaches have been used to overexpress or disrupt cellulase genes to elucidate their function in biosynthesis of cellulolytic enzymes. In this study, the knockout of *cel3a* gene resulted in a lower level of secreted cellulolytic enzymes, however, the enzymes appeared in the medium without any significant delay. This correlated well with the pattern of mRNA expressions of the genes coding for BGLs, cellulases, and Xyr1 in the *Δcel3a* strain. A similar result was also reported in a *Δcel3a* mutant of the hyper-producing strain *T. reesei* RL-P37. Its cellulase secretion and activity in the presence of Avicel was reduced but not abolished [31].

Cel1A is the major intracellular BGL in *T. reesei*, which has been implicated for Sop formation [22]. Cel1A catalyzed quite efficiently the formation of Sop from G2 in the presence of Glc [22, 30]. A similar situation occurred with a cellulase hyper-producing strain PC-3-7 from the *T. reesei* QM9414 which has a single-nucleotide mutation within the *cel1a* encoding gene. This strain showed decreased activity of BGL on G2, but a clear increase of the transglycosylation activity to afford Sop [32] when compared with a *T. reesei* strain QM9414. Additionally, simultaneously absent *cel1a*, *cel1b* and *cel3a* genes have been studied gave more serious cellulase induction lag than single deletion of *cel1a* gene [33]. With the observations in this work, it could be explained for simultaneously absent of *cel1a* and *cel3a* genes lead to both of the rapid and the later cellulase induction lags. Together with all the results, the Cel1A is the key BGL to response to biosynthesis of Sop, and regulation the initial cellulase induction by acting on Xyr1.

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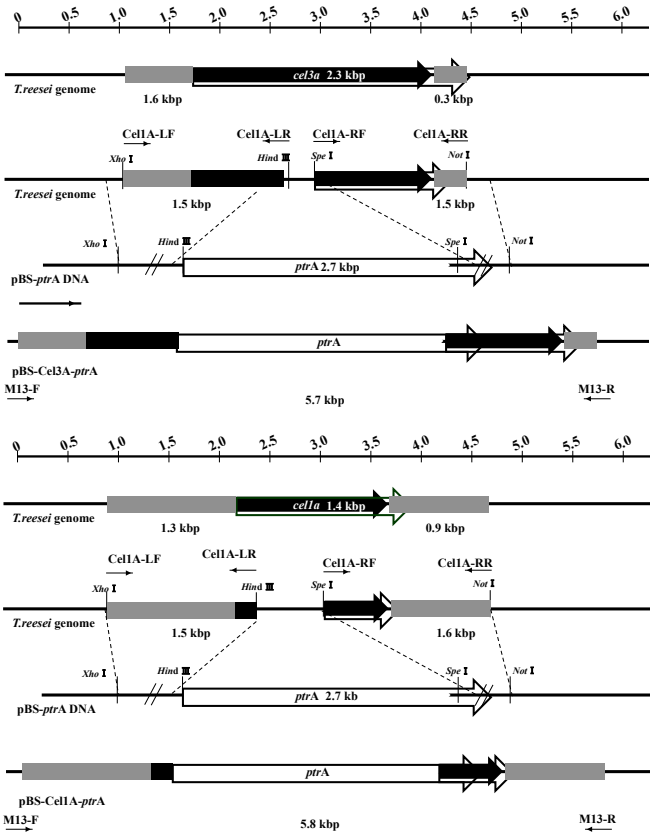
Table 3-1 Primers used for cloning and transcriptional analyses

Primer name	Sequence (5′–3′)
Primers used to construct disruption cassettes	
*ptr*A-F	TAAGCTTCACCAGCGTTTCTGGG
*ptr*A-R	CCCTACCCGGATATCTAGTCCCC
Cel1A-LF	ACTCGAGTAGCACTCGGCTGGAAT
Cel1A-LR	CAAGCTTGCCGGGCTGCGCGCA
Cel1A-RF	CACTAGTTCTTCACGGCCTGGTTCG
Cel1A-RR	CGCGGCCGCGCGAAGCACCCAAA
Cel3A-LF	GCTCGAGGCTTCCCCAGGTTCGC
Cel3A-LR	CAAGCTTTGGACAGTCGTGTGCTGT
Cel3A-RF	GACTAGTTCTGCTCAAGAATGACGC
Cel3A-RR	AGCGGCCGCGGAGCGGAGGATGTC
ΔCel1A-F	GTGTTGTCAAGGAACATGCGGC
ΔCel1A-R	GCGCGGACGAAGTCGCAAAGAA
ΔCel3A-F	GGTTGGACCACCTTCTTGTTCCG
ΔCel3A-R	CAGGGTAAAGTACATGTTGGTGCC
*ptr*A-R3	TGGGAACGAACACCGGCCCACGCC
pPTRII RV	AATACCGCACAGATGCGTAAGG
Primers used for the transcriptional analysis	
cel1a-FW	ACGGCTACGTGACGAGGTTT
cel1a-RV	CATCTTCAGCATCCCTTTGC
cel3a-FW	AACGTTCAACATCCGACGAC
cel3a-RV	GTCACAGGTCAACCGCCTTC
cel3b-FW	ACTGGGACACGAAGAAGCAG
cel3b-RV	CATTCGCGTCACATCTTGTC
cel3e-FW	GTGGGTTATTCAGGAGGGTGA
cel3e-RV	GAACACTAGCCCAGGAGATCG
eg2-FW	CCAAGAGGTTGTAACCGCAATC
eg2-RV	ACCGGAGTTGTCTGAGTCCAAG
cbh2-FW	TGCCTCGAATGGCGAATACT
cbh2-RV	GAGGTTGGTCACCAGGTTGG
cbh1-FW	CTTTACCCTCGATACCACCAAG
cbh1-RV	CCTCAGCTGTGCAGTAATCATC
xyr1-FW	TCACAGTCGCATCTCCACTC
cbh1-FW	CTTTACCCTCGATACCACCAAG
cbh1-RV	CCTCAGCTGTGCAGTAATCATC
xyr1-FW	TCACAGTCGCATCTCCACTC
xyr1-RV	CCTGGCAGCAATAAGAGAGC
ace1-FW	TATCTCACCGACCGCGAACA
ace1-RV	AGGCGAACCTCGTCAGATGG
ace2-FW	CTGCTCTCTGTTGTCGTGGA
ace2-RV	TCCTTGAACCTGGACCACTC
cre1-FW	ACGTCGATGGCTTGATGCAC
cre1-RV	TGCCACGGCCATAGTGAGAA
cre2-FW	TTTGTGCGCAATTTCTTTGG
cre2-RV	GCCTTATCCACGCTTTCGTC
swo1-FW	TCCTCATTCGCAGTCTCCTT
swo1-RV	TGGATACATGGTTCCGGATT
act1-FW	TCCCGCCTTCTATGTCTCCA
act1-RV	AGCCATGTCAACACGAGCAA

Primers used in the construction of BGL gene deletion cassettes, and the transcriptional analysis in quantitative real-time PCR. The introduced restriction sites are indicated in underlined.

(a)



(b)

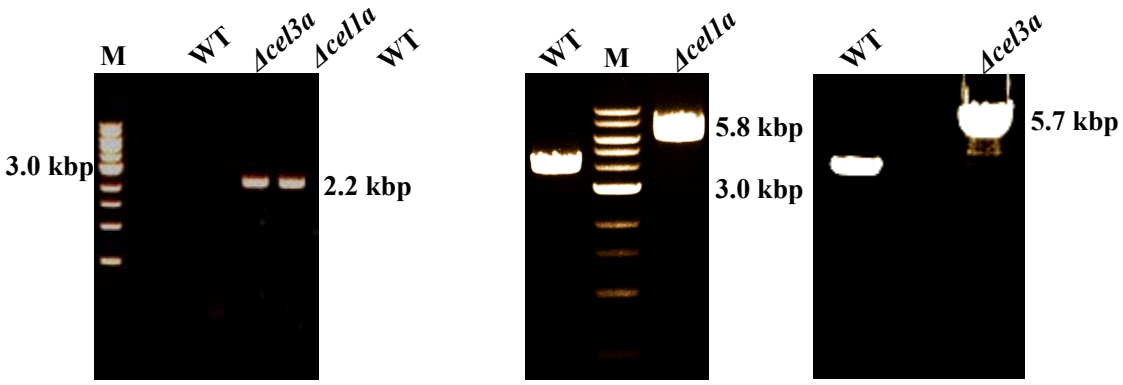


Fig. 3-1 Construction of the disruption cassettes for *cel3a* and *cel1a*; (a) Determination of full-length disruption cassettes for *cel3a* and *cel1a* using M13-F and M13-R; (b) *Δcel1a* and *Δcel3a* were amplified as 2.21 and 2.24 kbp fragments, respectively, and corresponded with expected internal sizes. As expected, outside sizes of *Δcel1a* and *Δcel3a* were amplified as 5.8 kbp and 5.7 kbp.

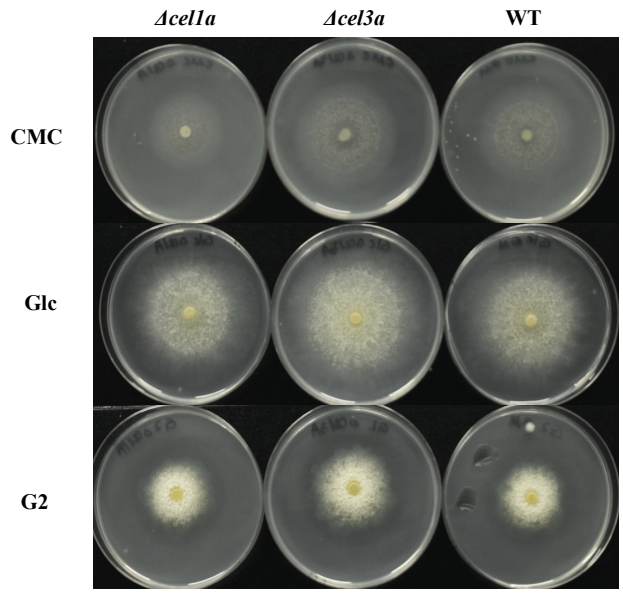


Fig. 3-2 Growth comparison of the WT strain and the BGL gene deletion strains (*Δcel1a* and *Δcel3a*) on a solid modified Mandels and Andreotti medium with additional of 1% Glc, G2, or CMC respectively. After 4 day cultivation (under the condition of light, 30°C, standing), their hypha diameter sizes were compared.

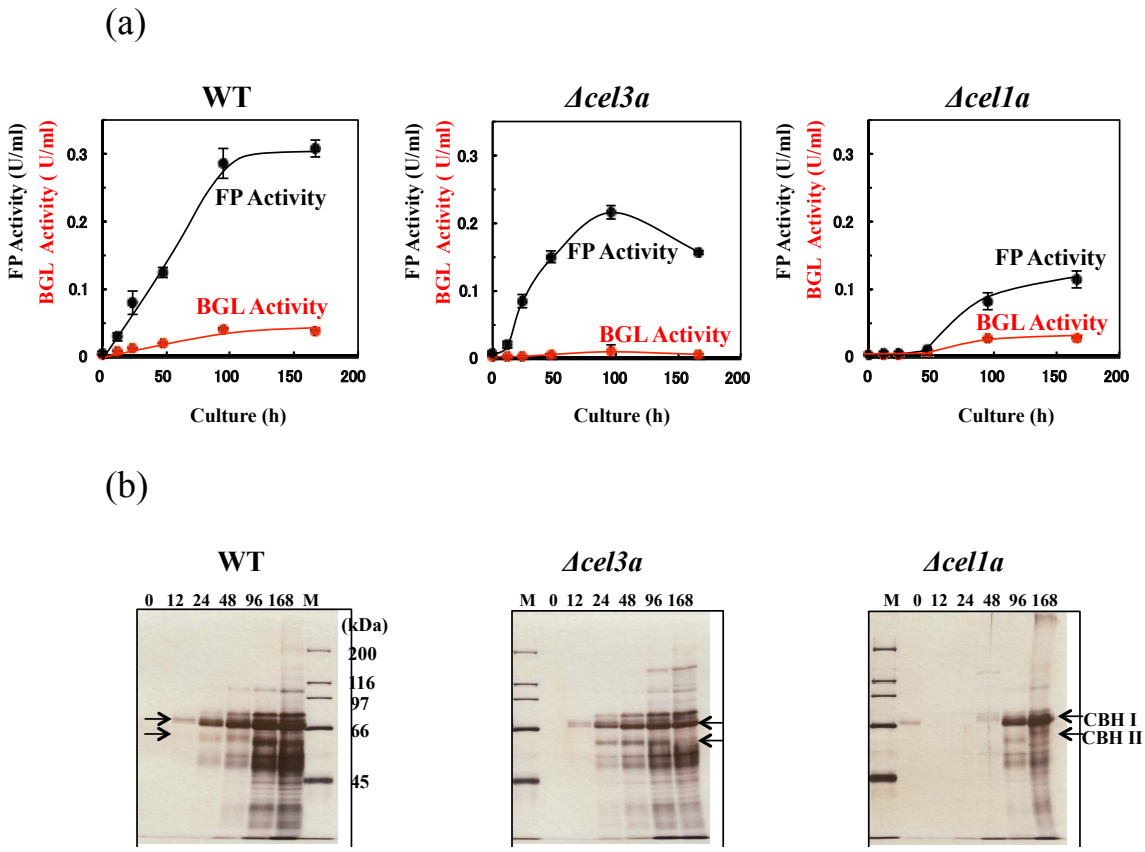


Fig. 3-3 (a) Cellulase and BGL activities were compared between mutant and the WT strains using filter paper or pNPG as substrate. (b) SDS-PAGE analyses of mutant and WT *T. reesei* strains; Culture supernatants were subjected to 10% polyacrylamide gel electrophoresis; M, molecular weight marker.

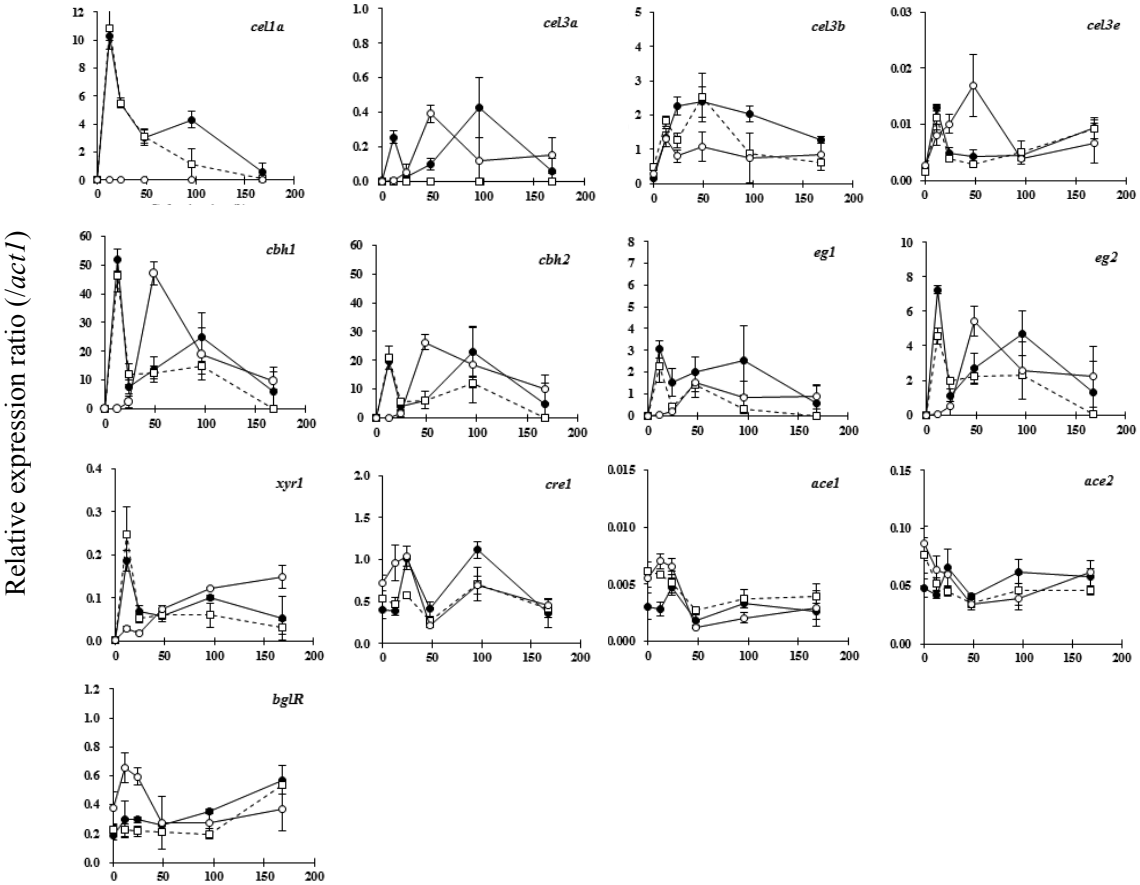


Fig. 3-4 Time-course transcription levels for the WT and the BGL gene deletion strains with cultivation on 1wt% Avicel. The solid circle is represents WT strain, the empty circle represents *Δcel1a* strain, and the empty square is represents *Δcel3a* strain.

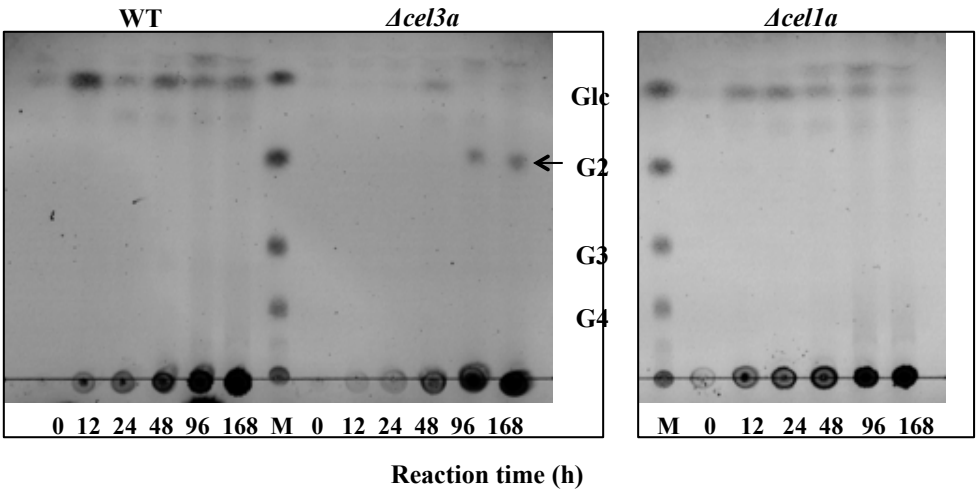


Fig. 3-5 Hydrolysis Products from culture supernatants were analysed using thin-layer chromatography (TLC) with 60 mL of the supernatant loaded on a silica gel plate (Silica Gel 60 Å; Whatman). Chromatography was performed using a solvent system comprising 2-propanol:1-butanol:water (v/v/v) = 60:15:12, and carbohydrate spots were detected using the sulfuric acid baking method after spraying with 30% sulfuric acid.

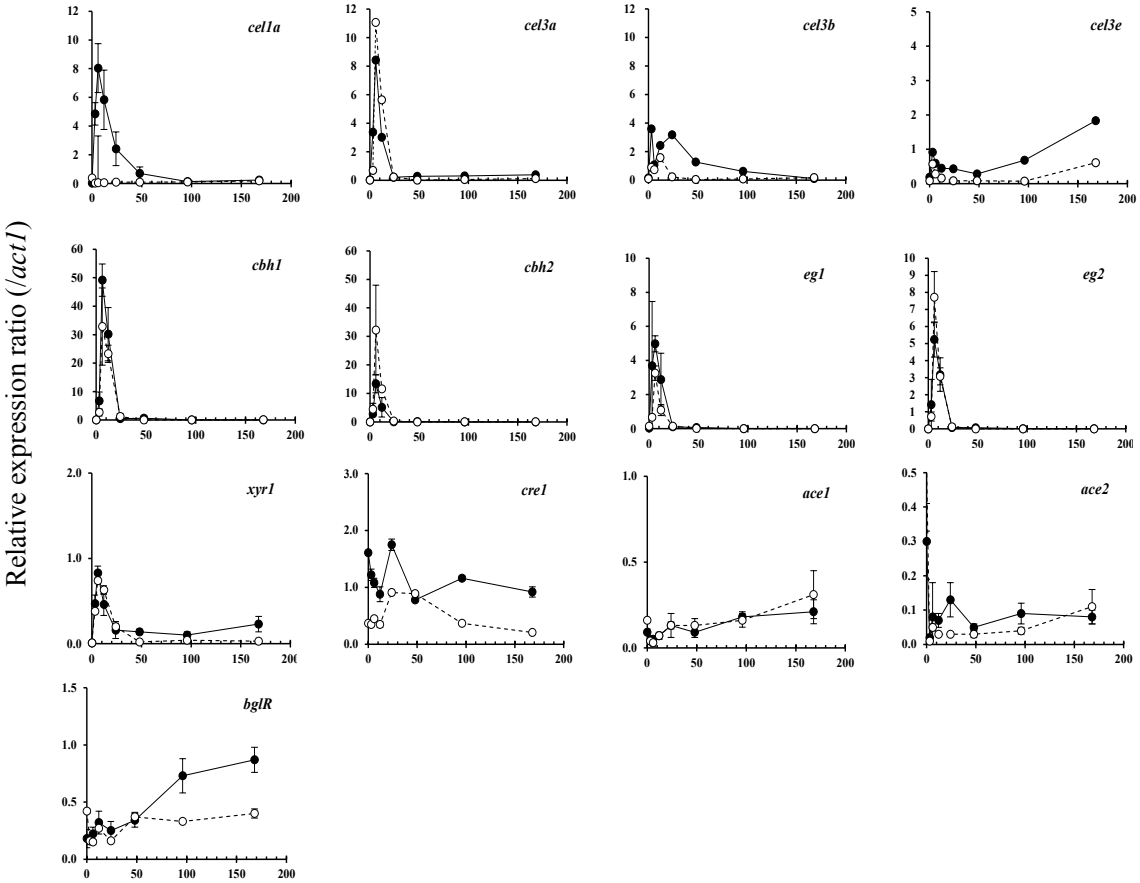


Fig. 3-6 Time-course transcription levels for the WT and the mutant strains on 1wt% Avicel with 1 mM Sop. The solid circle is represents for the WT strain, the empty circle represents for *Δcel1a* strain.

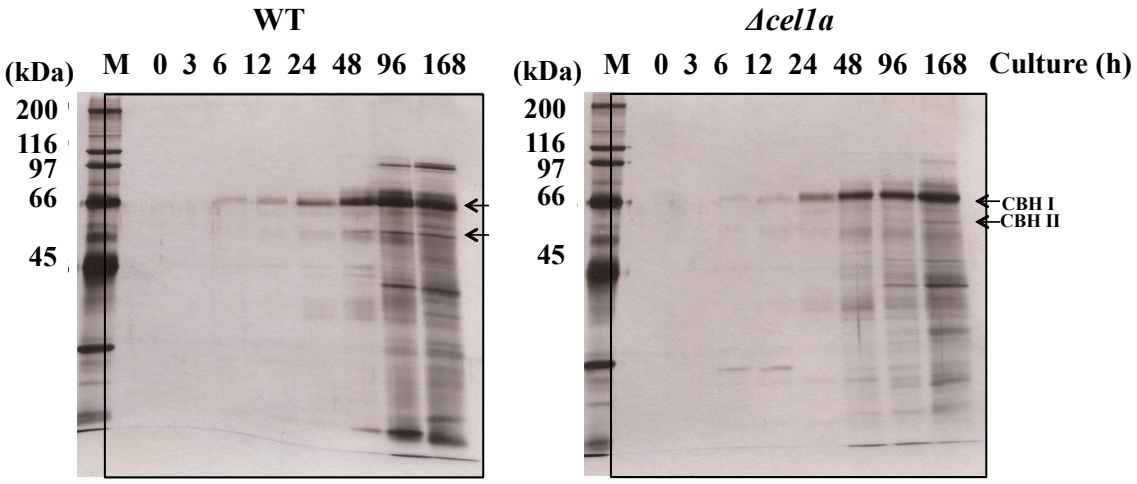


Fig. 3-7 The SDS-PAGE analysis of the protein seceation on cultivation upon 1wt% Avicel with 1 mM Sop. The culture supernatant samples were intervals from WT strain and the *Δcel1a* strain.

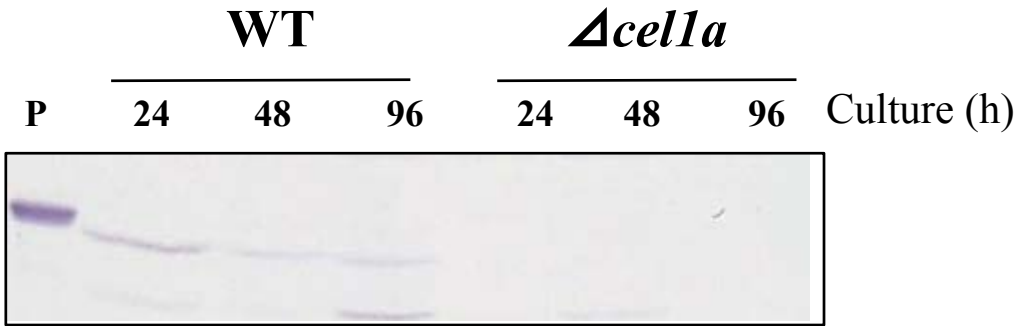


Fig. 3-8 Western blotting of Cel1A from intracellular extracts with proteinase inhibitor from the WT and *Δcel1a* cultures grown on 1wt% Avicel. P represents positive control using a purified recombinant protein of Cel1A.

Chapter 4

Improvement in glucose sensitivity and stability of *Trichoderma reesei* β-glucosidase using site-directed mutagenesis

Chapter 4: Improvement in glucose sensitivity and stability of *Trichoderma. reesei* β-glucosidase using site-directed mutagenesis

4.1. Introduction

BGLs are important for the biomass degradation process because it responsible for the final saccharification step in producing fermentable sugar, additionally contribute to prevention of the product inhibition of exo- and endoglucanases during the degradation of cellulosic biomass [1-7]. Most BGLs produced from fungi or bacteria are commonly inhibited by Glc [8]. This is caused by the competitive binding of substrate and Glc to the same active site on the enzyme. As mentioned previously, Cel1A is the special BGL for Sop synthesis in *T. reesei*, however it also defect on Glc inhibition and showed a K_i approaching 50 mM during the hydrolysis of pNPG [9–11]. Recently several Glc tolerant or -stimulated BGLs belonging to the glycoside hydrolase family 1 or 3 have been isolated and characterized [12–22]. Among them, *Humicola insolens* BGL (HiBGL) specifically shows significant homology to the amino acid sequence of Cel1A with 73% identity and 82% similarity (Fig. 4-1a). This enzyme exhibits high Glc tolerance and enhances 1.8 fold activity at 50 mM Glc or 100 mM xylose [10,15,23]. Comparison of the crystal structures of Cel1A [9] and HiBGL [10] makes plan the experimental strategy for mutagenesis. Around the active site entrance, seven amino acid residues different to each other can be observed (Fig. 4-1b). These are considered important in shaping the entrance and cavity along the active site tunnel. Especially, three of these amino acids (W168, L173, and F348) in HiBGL were reported to correspond with subsite +2 and trap the Glc molecule by hydrophobic interaction. Additionally, W168 and L173 of HiBGL were found to restrict the width of the active site and maintain a narrow cavity [10]. In this chapter, for improvement in hydrolysis activity and transglycosylation activity in present of Glc I tried to substitute the corresponding amino acids L167, P172, and P338 in Cel1A by creating L167W/P172L and

L167W/P172L/P338F mutants of Cel1A. They were named as 167/172 and 167/172/338 mutants, their properties especially the Glc sensitivity and Glc tolerance are investigated.

4.2. Materials and Methods

4.2.1. Cloning and site-directed mutagenesis

To construct expression vectors, cDNAs encoding Cel1A were amplified by PCR with PrimeSTAR® HS DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) using first-strand cDNA as a template. Primer pairs (Cel1A-FW and Cel1A-RV) containing additional *Nco* I and *Hin*d III sites are listed in Table 4-1. The amplified cDNA was inserted into the same sites of pET23d (+) vector (Merck, Darmstadt, Germany) to encode a Histidine-tag fusion at the C-terminus. Mutagenesis was performed by overlapped PCR using PrimeSTAR® Max DNA polymerase (TaKaRa) with the aforementioned resultant plasmid. Forward and reverse primers are also listed in Table 4-1, in which the nucleotide sequences contain the mutation and partially overlapped. Expression vectors for double and triple mutations were also constructed by repetitive mutagenesis in the same way. The resulting amplicon was introduced into *Escherichia coli* DH5α, and the mutated plasmid was then prepared using QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). All nucleotide sequences of the expression plasmids were confirmed by sequencing.

4.2.2. Expression and purification

Each plasmid for expression was introduced into the *E. coli* Origami B (DE3) pLysS strain. The resultant single colony was grown in 100 mL of LB liquid medium (Nakalai Tesque, Kyoto, Japan) supplemented with 100 μg/mL of ampicillin in a 1-L baffled flask on a rotary shaker (160 rpm) at 25°C. Furthermore, 1 mM of isopropyl β-D-thiogalactopyranoside was added as an inducer when the absorbance reached 0.6 at 660 nm. After the next 12 h of growth, the cells were harvested by centrifugation (13,000×*g* for 5 min) and were then re-suspended in 15 mL of 20 mM phosphate buffer (pH 7.5)

containing 0.1 M NaCl. After ultrasonication for 10 min on ice, cell debris was removed by centrifugation (13,000×*g* for 5 min). Protein purification was then conducted with the supernatant using a column packed with 15 mL of the TALON metal affinity resin (TaKaRa) equilibrated with the same buffer. The proteins adsorbed to the column were eluted with 200 mM imidazole in the same buffer. The imidazole in the pooled enzyme solution was removed, and the buffer was substituted with 20 mM acetate buffer (pH 5.5) by gel filtration using a PD-10 column (GE Healthcare). The purities of the recombinant BGLs were confirmed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (Fig. 4-2).

4.2.3. Enzyme assay

BGL activity was assayed in a 0.4 mL of reaction mixture containing 1.25 mM pNPG, 100 mM acetate buffer (pH 5.5), and a suitable amount of enzyme. After incubation for 0–30 min at 30°C, 2 mL of 0.5% Na_2CO_3 was added. The absorbance of the released *p*-nitrophenol was determined at 420 nm. Activity units (U) were calculated from an initial velocity, and 1 U was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min. Protein content was measured by the Lowry method [23] using bovine serum albumin as the standard.

Parameters of Glc inhibition for hydrolysis (K_i) were calculated from Dixon plots [26] using initial velocities. Kinetic parameters for hydrolysis were also determined using the Lineweaver–Burk plots after measuring initial reaction velocities at various substrate concentrations [27].

The transglycosylation activity was carried out, in the total volume of 1.0 mL by using 10% G2 as substrate under the same condition as described in chapter 2. The accumulation Sop was detected by HPLC in the same methord as described in charpter 2.

4.2.4. Effects of pH and temperature on activity and stability

Optimum pH and temperature were measured under the conditions described in the

enzyme assay except where the buffer was substituted with Britton–Robinson's wide range buffer and the reaction temperatures were shifted to 30–65°C, respectively. Stability for pH and temperature were also determined under the same assay conditions after the enzyme was incubated at various pH for 6 h at 4°C and at various temperatures for 30 min at pH 5.5.

4.2.5. Melting temperature of WT and 167/172 mutant

Melting temperature was determined using Protein Thermal Shift Dye Kit (Thermo Fischer Scientific, MA, USA) according to the instruction's manual. Melting curve was created to trace the fluorescence (Ex/Em=580 nm/610 nm) from 40 to 70°C at an increase rate of 0.05°C per second. Melting temperature (T_m) was then obtained from a differential curve calculated from the melting curve.

4.3. Results

4.3.1. Glucose tolerance and stimulation of Cel1A mutants

The mutants of amino acids 167/172 and 167/172/338 had significantly increased specific activities (6.8 U/mg for 167/172 and 4.6 U/mg for 167/172/338) compared with those of wild-type Cel1A (WT, 2.8 U/mg) (Table. 4-2). Furthermore, the activities of the mutant enzymes showed continued to be stimulated with an increasing Glc concentration, the maximum stimulation activity reached up to 2.0 (the 167/172 mutant) and 1.3 fold (the 167/172/338 mutant) at 50 mM Glc (Fig. 4-3). In contrast, the activity of the two mutants showed gradually decreased above a Glc concentration of 50 mM. However, both mutants showed Glc tolerance with the same 50% of inhibitory concentration (IC_{50}) of 600 mM, which was markedly higher than the IC_{50} value of 50 mM of WT (Fig. 4-3).

4.3.2. pH and temperature effects on activity and stability

Compared to the WT Cel1A, both of the mutants showed expanded the optimum pH in the acidic region and exhibited high activity in the pH range of 5.0–7.0 (Fig. 4-4a).

The pH stabilities showed retaining 80% residual activities at a wider pH range of 4.0–9.0 (167/172 mutant) and 7.5–9.5 (167/172/338 mutant) compared to the narrow range of WT Cel1A 6.0–7.3 (Fig. 4-4b). On the other hand, the optimum temperature of both mutants shifted to 55°C, which was higher than that of 30–40°C of WT (Fig. 4-4c), The thermal stabilities of the two mutants were also increased to 50°C compared with that of WT, which was 40°C (>80% of the residual activity) (Fig. 4-4d). Further these data are well corroborated to the T_m values which shifted from 52°C for WT to 59°C for 167/172 and 167/172/338 for 57°C respectively (Fig. 4-5).

4.3.3. Kinetic studies

To elucidate the catalytic properties, a kinetic analysis was performed on the 167/172 mutant using pNPG as the substrate. K_m of the 167/172 mutant (0.23 mM) was much smaller than that of WT (2.48 mM), moreover the V_{max} of the 167/172 mutant (13.3 U/mg) was higher than that of WT (7.7 U/mg) showed in Table. 4-2 and Fig. 4-6a, Fig. 4-6b. Also to our attention, the markedly decreased K_m leading to the catalytic efficiency (V_{max}/K_m) of the 167/172 mutant increased as 19 fold as that of WT Cel1A. Additionally, the mutant showed competitive inhibition in the presence of Glc at low concentrations of substrate (<0.25 mM), When Glc concentration was increased, substrate inhibition was gradually prevented. At 50 mM Glc (the maximum concentration for stimulation), the substrate inhibition was showed completely disappeared (Fig. 4-6c). Further the Dixon plots analysis revealed that the inhibitory parameter (K_i) of the 167/172 mutant was nearly identical to 50 mM, as the same value of that in WT (Fig. 4-7). Also, the Dixon plots pointed out the substrate inhibition that appeared in the 167/172 mutant was not observed in WT Cel1A (Fig. 4-6c).

4.3.4. Transglycisylation

For an understanding on whether the 167/172 mutant improved in the transglycosylation activity for formation of Sop, transglycosylation products from 10% G2

was detected with 50 mg/mL purified 167/172 mutant in the same reaction condition as described chapter 2. The Fig. 4-8 indicating the maximum amount of Sop formation was observed at 72 h showed 7.1 mg/mL Sop from the consumed G2. It is noteworthy that this result is much higher value than the Sop synthesized by the WT Cel1A 5.5 mg/mL (chapter 2 Fig. 2-5a).

4.4. Discussion

To our knowledge, the *Trichoderma* species is an important fungi for cellulase production, however its BGL production is considerable low, and that poor properties on Glc tolerance and thermostability comparing with the BGL in *Aspergillus* species and the *penicillin* species and *Humicola insolens*. In this chapter, based on amino acid sequence of HiBG, two mutants BGL 167/172 mytant Cel1A and 167/172/338 mutant Cel1A were constructed and purified. By analysis their hydrolysis activity in present of Glc, The 167/172 mutant Cel1A showed a similar Glc tolerance and stimulation levels with the that of HiBG under the same Glc concentration (1.8 fold stimulation at 50 mM Glc) [23], however the 167/172/338 mutant Cel1A showed decreased the Glc tolerance and stimulation levels (1.3 fold stimulation at 50 mM Glc). This result pointed out that both of W167 and L172 resides are indispensable for Glc stimulation, because the single mutant Cel1A with W167 or with L172 was not observed in this behave [24]. Interesting, the 167/172/338 mutant Cel1A did not showed any benefit to obtain higher activity in present of Glc than the that with 167/172 , however in the case of HiBG, it was reported that the amino acid F348 is also corresponds with make a hydrophobic binding to Glc, here I think this result could be explained by the differ location and orientation of P338 in Cel1A to the F348 in HiBGL (Fig. 4-1).

Analysis of the properties of pH and temperature behave, the 167/172 mutant Cel1A showed has the optimum pH of the two mutant BGLs expanded in the acidic region and the mixmum activity were appearance different from the pH range of HiBGL (6.0–6.5) [23]. it should be notice of that the 167/172 mutant Cel1A kept activity at the pH range of 4.0–7.0

and was higher than the activity of the 167/172/338 mutant and HiBGL. Moreover both of the mutants also significantly improved the optimum temperature and thermostability compared to the WT Cel1A (Fig. 4-4c and Fig. 4-4d). A similar result of this phenomenon was also reported by the single mutant of Cel1A (L167W or P172L) from Lee et al. Further, when analysis the T_m values, both of the single mutants the L167W or the P172L increased a similar T_m values as I showed in 167/172 mutant Cel1A (L167W for 54.9 and P172L for 54°C) [24]. that all higher than the 167/172/338 mutant Cel1A, suggesting both the L167W and P172L mutations synergistically increased thermostability but it seems that the additional P338F mutation was not necessary, although we were unable to clarify the mechanism for how the thermostability was increased, whereas in the present study the 167/172/338 mutant is less elegant for all of the properties than the 167/172 mutant.

Some recent reports suggested that the BGLs that with Glc tolerant often accompany Glc stimulation [12,14–22,25–27]. Among these BGLs, Td2F2 enhances the hydrolytic activity to pNPG with an increase in Glc concentration and this activity increasing is suggested contributed by transglycosylation, which leading to the formation of disaccharides such as sophorose [27]. For an understanding on whether the Glc stimulation is concerned with the transglycosylation, we thus test the mutant transglycosilation activity towards pNPG, the results showed that both mutants in this study accumulated no transfer products at 1.25 mM pNPG. Thus I concluded that these mutants enhance hydrolytic activity in the presence of Glc is not relevant with the transglycosylation activity. The result of transglycosylation using G2 as substrate indicating the transglycosylation would happen a higher concentration of substrate.

Souza et al. suggested that Glc stimulation in HiBGL is induced by the conformational change of the enzyme binding to Glc at the modular-binding site [28]. Our data do not disprove their estimations, but we have further clarified the mechanism of Glc stimulation (Fig. 4-8). Substrate inhibition is induced by another substrate molecule binding to a secondary site, the non-active site, on the enzyme. The introduced mutation

(L167W/P172L) might create a secondary substrate-binding site. The binding of the substrate to this site inhibits the hydrolysis forming ESS* complex (* indicate the binding to the created secondary site). When Glc is present (<50 mM), the substrate at the secondary site might be replaced by Glc forming ESG* complex as reported in case of HiBGL [10] and glucose stimulation was caused by relieve the substrate inhibition. When glucose concentrations are elevated to over 50 mM, most of the subsites (-1 and +1) and the secondary site are occupied by Glc leading to EG, EGS* or EGG* complexes. As a result, a competitive Glc inhibition was caused as shown in WT (Fig. 4-9).

The mutagenesis of only two amino acid residues (167/172) in Cel1A improved Glc tolerance, glucose stimulation, and pH and thermal stabilities, while also markedly increasing the specific activity. All these properties would be a great advantage in the degradation of cellulose together with cellulases. In addition to this, Cel1A is an important enzyme, which converts G2 to Sop, a powerful inducer of cellulase from *T. reesei* [31,32]. We believe that the mutant enzyme introduced into *T. reesei* enhances both cellulase induction and production.

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Table 4-1 Primers used for amplification of cDNA and mutant construction

Primer name	**Sequence (5′–3′)**
Cel1A-FW	ACCATGGTGCCCAAGGACTTTCAGTGG
Cel1A-RV	CAAAGCTTCGCCGCAATCAGCTCGT
L167W-F	ACGAGCCGTGGTGCTCGGCCATCC
L167W-R	AGCACCACGGCTCGTTGAAGGTGA
P172L-F	CATCCTGGGCTACGGCTCCGGCAC
P172L-R	CCGTAGCCCAGGATGGCCGAGCAC
P338F-F	CGCAGTCCTTCTGGCTGCGCCCCT
P338F-R	GCCAGAAGGACTGCGTCTCGGGGC

The introduced restriction sites are indicated as small letters. The overlapped sequence is underlined.

Table 4-2 Kinetic parameters comparison of wt and the mutant (167/172) strains

Parameters and property	WT	167/172 mutant [1)]
Specific activity (U/mg)	2.8	6.8
V_{max} (U/mg)	7.7 ± 1.62	13.3 ± 0.34
K_m (mM)	2.48 ± 0.75	0.23 ± 0.01
Purification (fold)	3.06	57.7
K_i for Glc (mM)	50	50
Substrate inhibition	−	+
IC_{50} (mM)	50	600

1) Parameters were calculated regardless of substrate inhibition.

(a)

```
HiBGL MSLPPDFKWGFATAAYQIEGSVNEDGRGPSIWDTFCAIPGKIADGSSGAVACDSYKRTKEDIALLKELGANSYRF :  75
Cel1A M-LPKDFQWGFATAAYQIEGAVDQDGRGPSIWDTFCAQPGKIADGSSGVTACDSYNRTAEDIALLKSLGAKSYRF :  74

Cel1A SISWSRIIPEGGRGDAVNQAGIDHYVKFVDDLLDAGITPFITLFHWDLPEGLHQRYGGLLNRTEFPLDFENYARV : 149
HiBGL SISWSRIIPLGGRNDPINQKGIDHYVKFVDDLIEAGITPFITLFHWDLPDALDKRYGGFLNKEEFAADFENYARI : 150

Cel1A MFRALPKVRNWITFNEPLCSAIPGYGSGTFAPGRQSTSE----------PWTVGHNILVAHGRAVKAYRDDFKPA : 214
HiBGL MFKAIPKCKHWITFNEPWCSAILGYNTGYFAPGHTSDRSKSPVGDSAREPWIVGHNILIAHARAVKAYREDFKP- : 224

Cel1A SGDGQIGIVLNGDFTYPWDAADPADKEAAERRLEFFTAWFADPIYLGDYPASMRKQLGDRLPTFTPEERALVHGS : 289
HiBGL TQGGEIGITLNGDATLPWDPEDPADIEACDRKIEFAISWFADPIYFGKYPDSMRKQLGDRLPEFTPEEVALVKGS : 299

Cel1A NDFYGMNHYTSNYIRHRSSPASADDTVGNVDVLFTNKQGNCIGPETQSPWLRPCAAGFRDFLVWISKRYGYPPIY : 464
HiBGL NDFYGMNHYTANYIKHKTGVPPEDDFLGNLETLFYNKYGDCIGPETQSFWLRPHAQGFRDLLNWLSKRYGYPKIY : 374

Cel1A VTENGTSLKGESDLPKEKILEDDFRVKYYNEYIRAMVTAVELDGVNVKGYFAWSLMDNFEWADGYVTRFGVTYVD : 439
HiBGL VTENGTSLKGENDMPLEQVLEDDFRVKYFNDYVRAMAAAVAEDGCNVRGYLAWSLLDNFEWAEGYETRFGVTYVD : 449

Cel1A YENGQKRFPKKSAKSLKPLFDELIAAA   466
HiBGL YANDQKRYPKKSAKSLKPLFDSLIRKE   476
```

Subsite +1 and -1 　 Catalytic residues

Amino acid residues located in entrance of active site

(b)

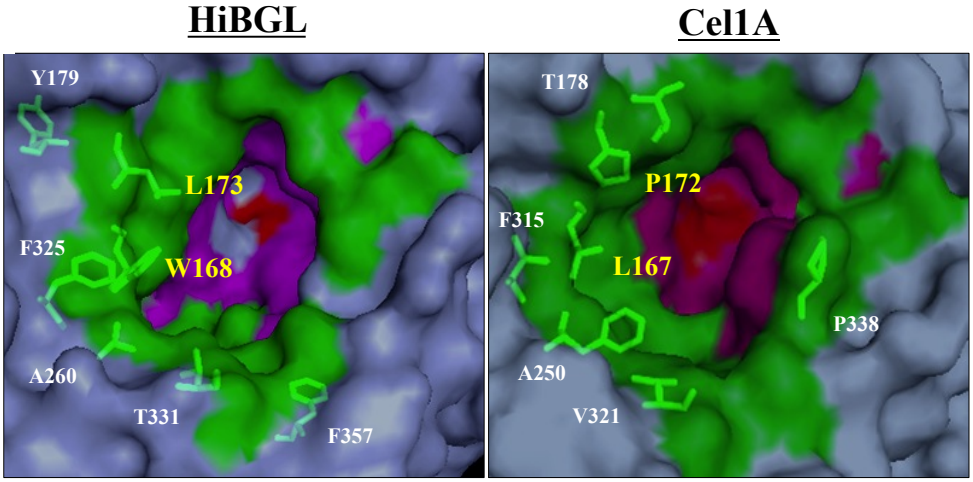

Fig. 4-1 Comparison on the amino acid residues of active-site entrance. (a) Sequence alignment of Cel1A and HiBG. The focused amino acid residues are colored. (b) Comparison of the active-site entrances between Cel1A and HiBGL. The colores corresponds with those of Fig. 4-1 (A). The different amino acid residues (side chain) between Cel1A and HiBGL are indicated as wireframes with amino acid No.

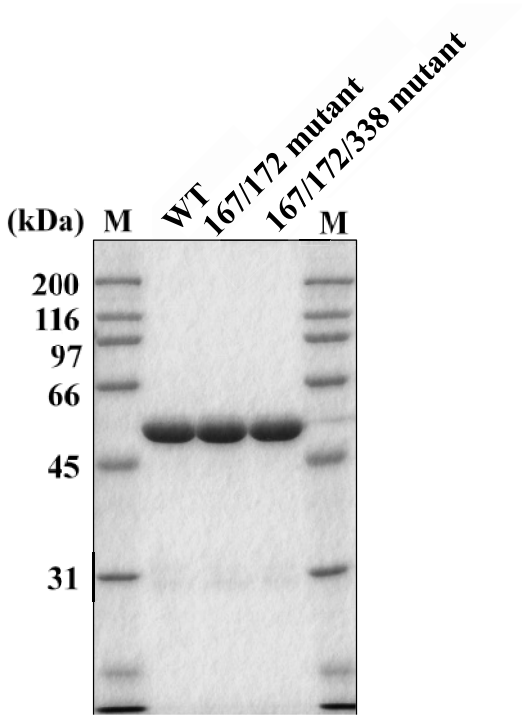

Fig. 4-2 SDS-PAGE analysis of the purified WT, 167/172 and 167/172/338 mutants. The recombinant enzymes (5 μg each) were subjected. M, molecular mass markers.

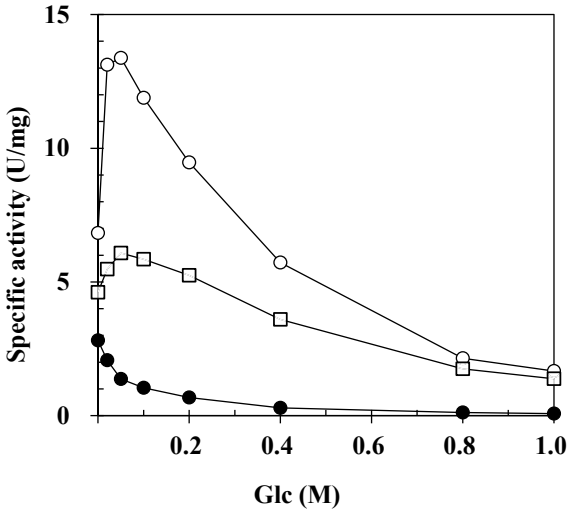

Fig. 4-3 Effect of Glc on BGL activity. Hydrolytic activities at 1.25 mM of pNPG were determined in the presence of Glc. Solid circle indicates WT; empty circle indicates 167/172 mutant; empty square indicates 167/172/338 mutant. Plots are averages of independent triplicates.

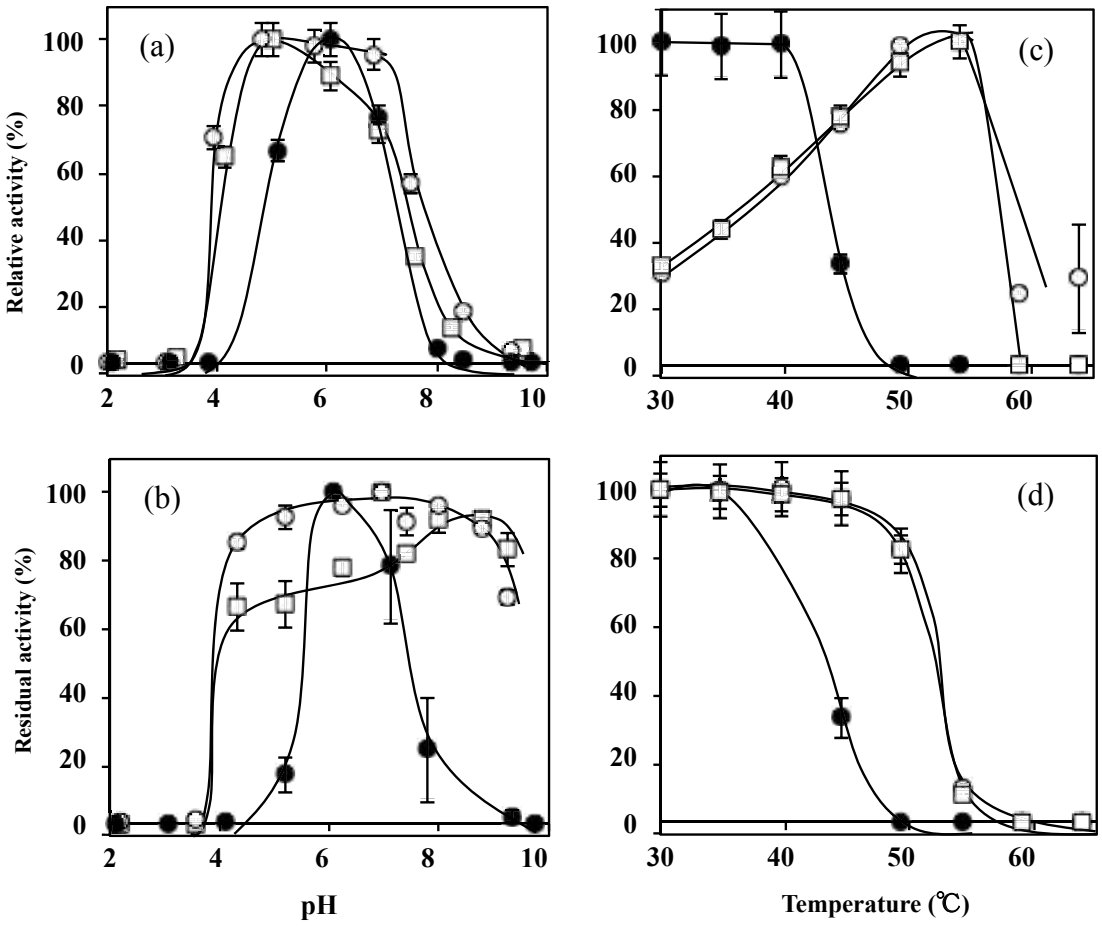

Fig. 4-4 pH and Temperature profiles of WT, 167/172 and 167/172/338 mutants. (a) Optimum pH, (b) pH stabilitiy, (c) Optimum temperature and (d) Thermal stabilities. Solid circle indicates WT; empty circle indicates 167/172 mutant; empty square indicates 167/172/338 mutant. Plots are averages of independent triplicates.

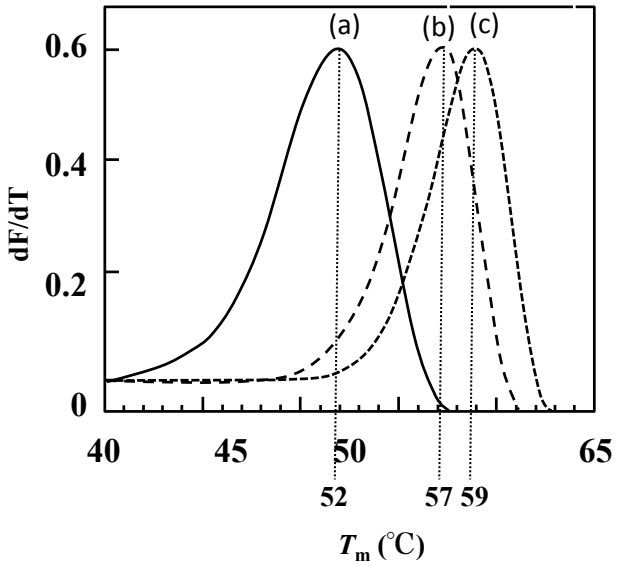

Fig. 4-5 Melting temperatures of WT and 167/172 mutant and 167/172/338 mutant. Melting temperature curve was showed (a) indicates WT, (b) indicates 167/172/338 mutant, (c) indicates 167/172.

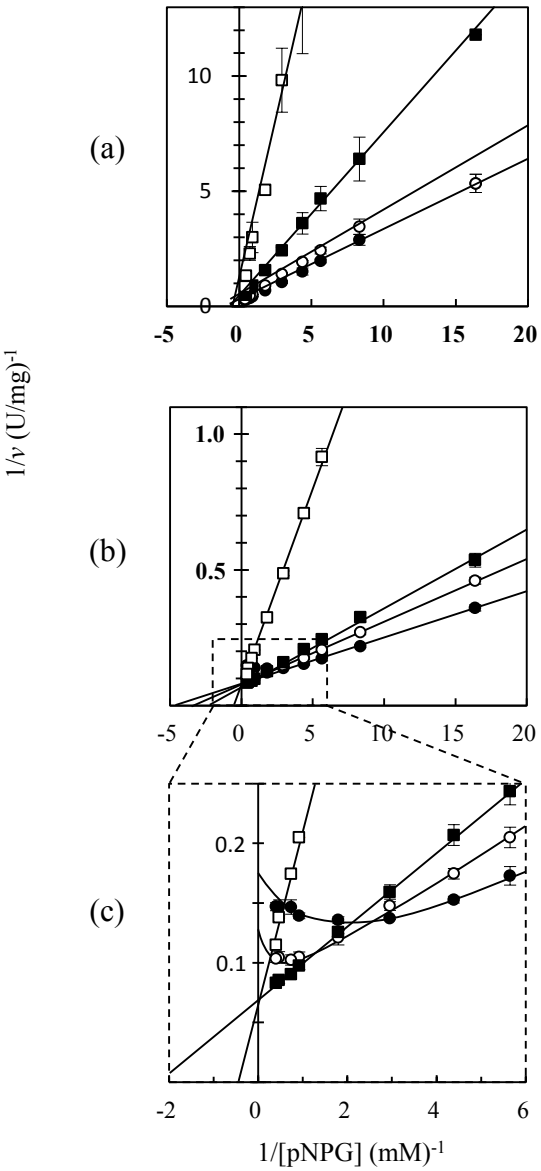

Fig. 4-6 Kinetic analysis of WT and the 167/172 mutant in the presence of Glc. (a) indicates WT, (b) indicates 167/172 mutant, (c) enlarged view of Fig. 4-6b at high concentration of substrate. Glc concentrations were shown, solid circle indicates non-Glc addition; empty circle indicates addition of 10 mM Glc; solid square indicates addition of 50 mM Glc empty square indicates addition of 400 mM Glc. Vertical bars indicate the standard deviations of three independent replicates.

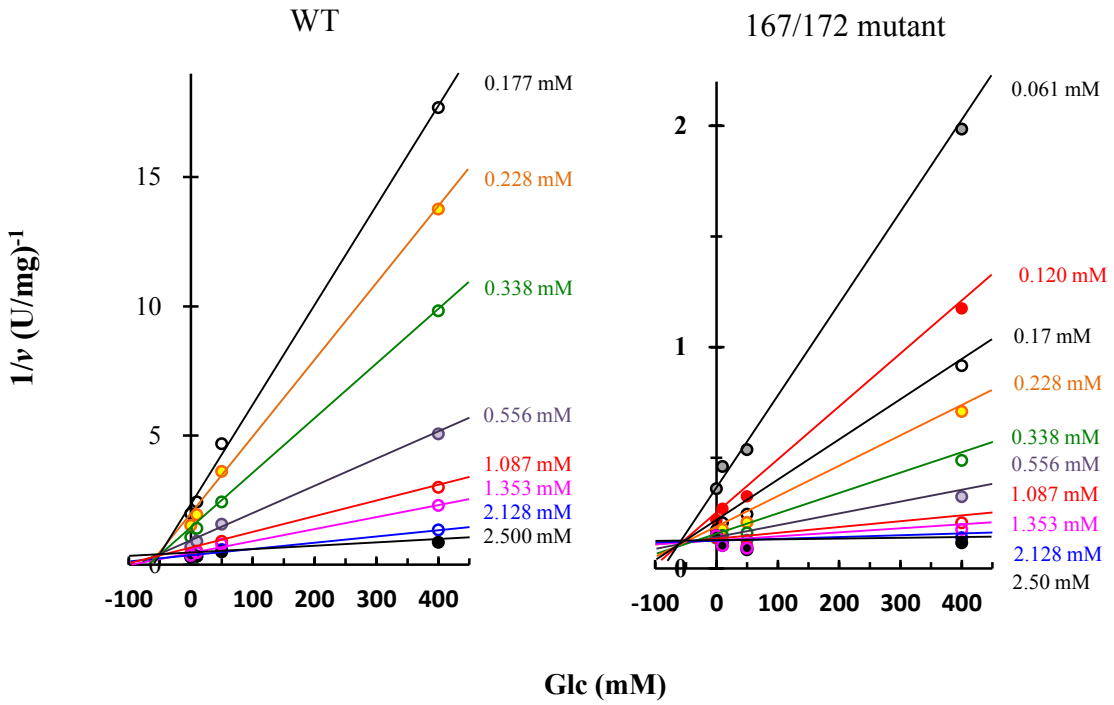

Fig. 4-7 Dixon plots analysis for determination of parameters in Glc-inhibition. The same data from Fig. 4-3 were plotted. Plots are averages of three independent replicates.

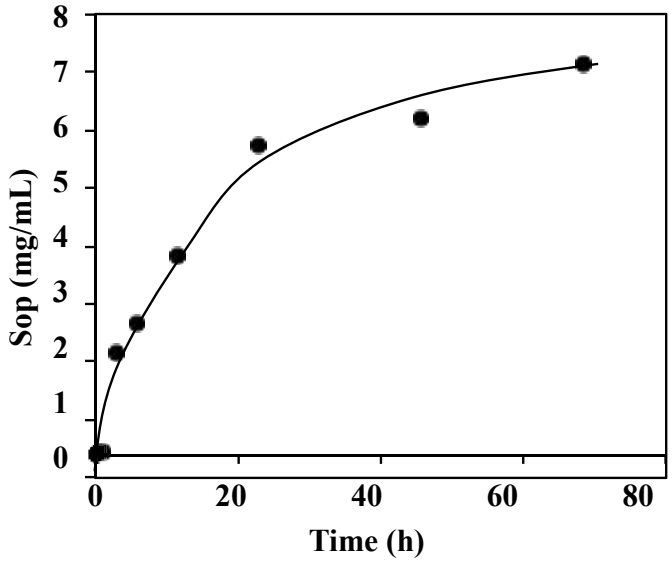

Fig. 4-8. Time-course of Sop formation by the 167/172 mutant Cel1A, the transglycosylation reaction carried out by using 50 μg/mL purified enzyme with 10% G2 as substrate.

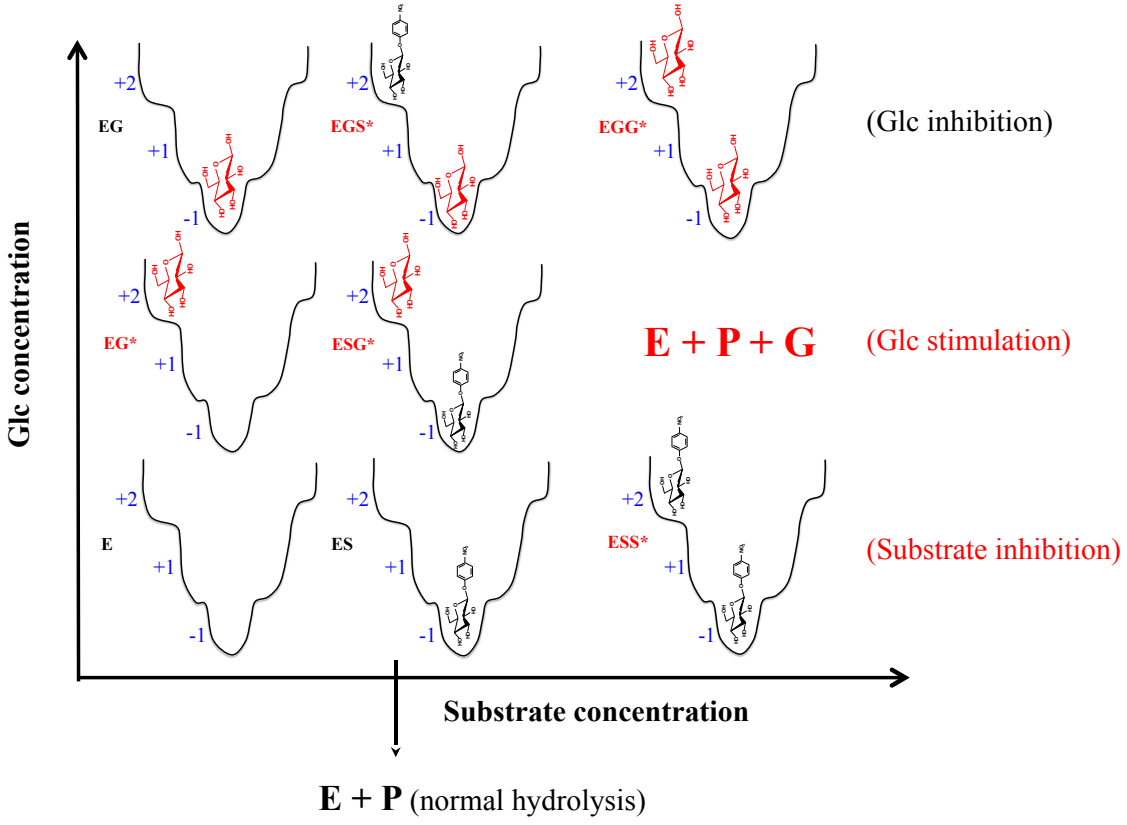

Fig. 4-9 Schematic representation of the reaction mechanisms of the WT Cel1A and 167/172 mutant Cel1A. E indicates enzyme; S indicates substrate; G indicates Glc. The reactions shown in red occur only in the 167/172 mutant. Asterisks indicate the binding to the subsite +2 in 167/172 mutation Cel1A.

Chapter 5

General conclusion

Chapter5: General conclusion

The most important objectives of this dissertation is identified Cel1A is the most capable on Sop formation BGL in *T. reesei*. Also, determined Cel1A is the substance BGL for occurring cellulase repaid induction mediumed by Xyr1. Furthermore it is essential to achieve an improvement of Glc sensitivity in Cel1A mutant by using genetic modification.

Improved production of cellulose-degrading enzymes is important for promoting efficient biomass conversion. *T. reesei* has been developed into one of the most prolific cellulase producers at an industrial scale. The *T. reesei* genome contains 10 different genes encoding BGLs, which catalyzes the release of glucose from cello-oligosaccharides and glycosides. Moreover, BGL produces isomeric glucosides including Sop via a transglycosylation reaction and cellulase in *T. reesei* are potently induced by a precious disaccharide, Sop. However, the properties of BGLs, especially those responsible for Sop synthesis have rarely been investigated. Elucidating the functions of BGLs, including their transglycosylation, would be of interest for understanding its role in cellulase induction and production, bringing efficient decomposition of cellulose.

Chapter 1 provides the background for this research and my overall research objective.

Chapter 2 describes the identification of 4 of 10 β-glucosidases (Cel1A, Cel3A, Cel3B and Cel3E), which were capable of catalyzing transglycosylation reactions and the formation of G3 and isomeric glucobioses, including Sop. Interestingly, Cel1A and Cel3A showed a high rate of conversion of G2 into Sop, exhibiting 10% and 3.2% conversion ratio, respectively, with 10% G2 as substrate. To explain why the Cel1A

ability to selectively accumulate Sop, kinetic studies were performed to verify the results. Cel3A, Cel3B and Cel3E clearly showed lower k_{cat} for Gen than for other transfer products, whereas Cel1A showed lower k_{cat} for Sop and Lam. Therefore, both BGLs were suspected to be involved in Sop synthesis followed to *in vivo* cellulase induction.

Chapter 3 describes the construction of two mutant strains, which were deleted *cel1a* and *cel3a* genes, respectively. Their influences were detected based on their contribution to the expression of cellulase genes cultivation on cellulose or supplemented with Sop. Results suggested that compared to the wild-type *T. reesei*, the mutant strain lacking *cel3a* showed a decrease in cellulase production during the later phases of the cultivation, that were considered owing to the lack of a major extracellular BGL Cel3A, might result in G2 accumulation. However, the deletion of *cel1a* resulted in a serious lag in repaid cellulase induction, which shifting the cellulase expressions from 12 to 96 h. The results of the transcriptional analysis also supported that the absence of *cel1a* resulted in the loss of early induction of cellulase genes in the wild-type strain. Additionally Sop showed efficiently rescue the induction defect in absent of *cel1a*. As mentioned above, Cel1A is the best Sop synthesizer in *T. reesei*, although Sop synthesis *in vivo* provided insufficient evidence, the lag in cellulase induction owing to a deletion of *cel1a* might be involved in the lack of Sop synthesis. Thus Cel1A might responsible for the expressions of cellulase genes in the early culture phase.

Chapter 4 describes the creation of a highly functional Cel1A mutant by mutagenesis of only 2 amino acid residues. BGL not only produces glucose but also plays an important role in the release of product inhibition of cellulases by degradation

of G2. However, most β-glucosidases are strongly inhibited by glucose, decreasing hydrolysis and transglycosylation activities. The Cel1A mutant (L167W/P172L) showed high glucose tolerance (50% of inhibitory concentration=650 mM), even glucose stimulation (2.0 fold at 50 mM glucose) to the wild-type Cel1A. Kinetic studies also revealed that the glucose stimulation was caused by resolved in substrate inhibition which in particular shown in Cel1A mutant.

To conclusion, based on comparison of β-glucosidases transglycosylation ability, Cel1A and Cel3A were targeted in high conversion of capable of synthesis sophorose. *In vivo*, deletion of *cel1a* gene particular present in cellulase induction defect, suggesting Cel1A might play a role in Sop synthesis. Last, a high functional Cel1A mutant was constructed, expecting to offer great advantages in formation of sophorose as well as the degradation of cellulosic biomass together with cellulases.

In future perspective, the desired BGL should functional by less ability of hydrolysis activate towards Sop and high ability of Sop formation. Cel1A is thought to be a tool for increasing the production levels of cellulases in *T. reesei*. The simple model for the cellulase induction is showed as Fig. 5-1. In this process, based on the results in this study, overexpression of Cel1A (mutant Cel1A) is thought the way for increase the intracellular Sop formation, therefore it could promote the expression of Xyr1 to increase the cellulase production. On the other hand, deletion of the BGL which most hydrolysis Sop is an alternative consideration for maintain a certain amount of Sop.

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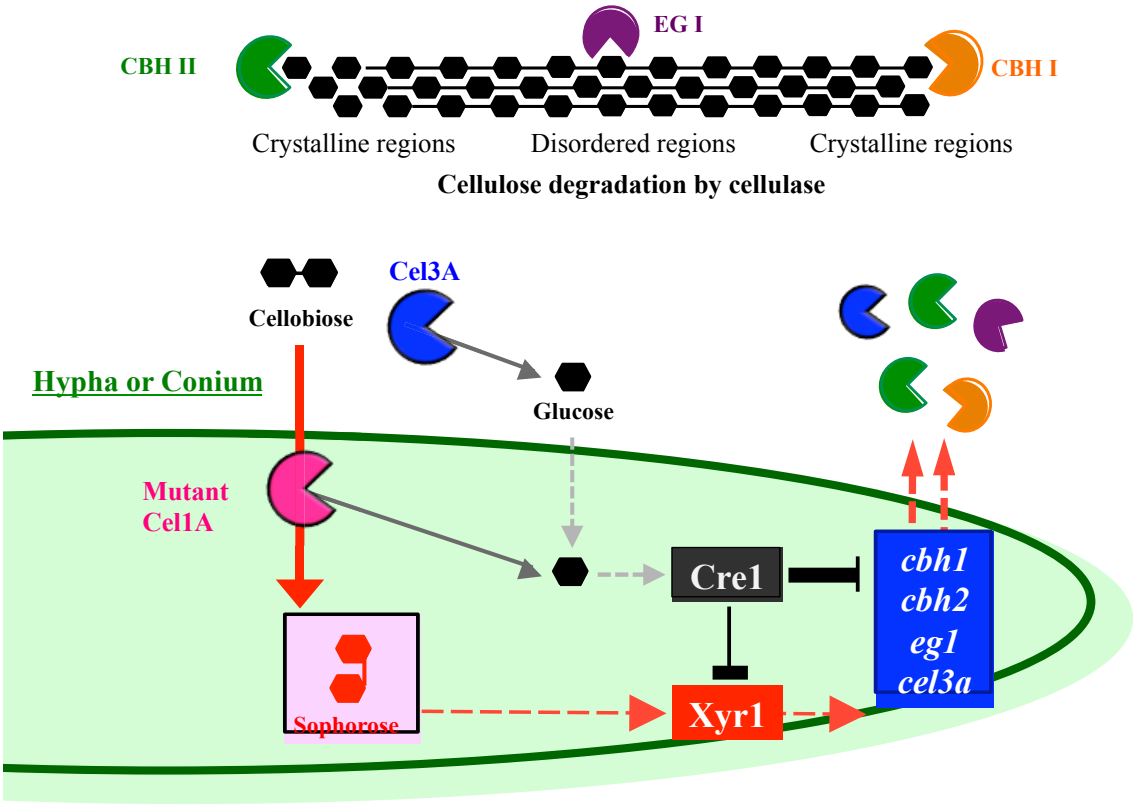

Fig. 5-1 The Model of Inducer formation and cellulase induction

Table 1 Studies on BGL properties from different strains of *Trichoderma* fungi

Trichoderma strain	BGL	Isolation strategies	reference
T. citrinoviride	Extracellular BGL	Protein purification, biochemical and proteomic characterzation	[1]
T. reesei	BGLII	Activity site residues mutational studies	[2]
T. reesei QM9414	BGLI	Entrance of Activity site residues mutational studies	[3]
T. reesei strain X3AB1	BGLI	Expression the BGL from A. aculeatus in *T. reesei*	[4]
T. reesei	BGLI	Molecular cloning expresion in *E. coli*	[5]
T. reesei	BGLII	Molecular cloing and expression in *A. oryzae*	[6]
T. reesei	All BGLs	Molecular cloing and expression in *E. coli* or *A. oryzae*	[7]
T. resei RL-P37	BGLI	Knock out the BGLI encoding gene	[8]
T. reesei QM9414	BGLI, BGLII, Cel1B	Double Knock out the BGLII and Cel1b encoding gene Triple Knock out the BGLI, BGLII and Cel1b encoding genes	[9]

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