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Kinetics of substrate transglycosylation by glycoside hydrolase family 3 glucan (1→3)-β-glucosidase from the white-rot fungus *Phanerochaete chrysosporium*

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Abstract—To elucidate the interaction between substrate inhibition and substrate transglycosylation of retaining glycoside hydrolases (GHs), a steady-state kinetic study was performed for the GH family 3 glucan (1→3)-β-glucosidase from the white-rot fungus *Phanerochaete chrysosporium*, using laminarioligosaccharides as substrates. When laminaribiose was incubated with the enzyme, a transglycosylation product was detected by thin-layer chromatography. The product was purified by size-exclusion chromatography, and was identified as a 6-*O*-glucosyl-laminaribiose (β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-Glc) by ^{1}H NMR spectroscopy and electrospray ionization mass spectrometry analysis. In steady-state kinetic studies, an apparent decrease of laminaribiose hydrolysis was observed at high concentrations of the substrate, and the plots of glucose production versus substrate concentration were thus fitted to a modified Michaelis–Menten equation including hydrolytic and transglycosylation parameters (K_m, K_{m2}, k_{cat}, k_{cat2}). The rate of 6-*O*-glucosyl-laminaribiose production estimated by high-performance anion-exchange chromatography coincided with the theoretical rate calculated using these parameters, clearly indicating that substrate inhibition of this enzyme is fully explained by substrate transglycosylation. Moreover, when K_m, k_{cat}, and affinity for glucosyl-enzyme intermediates (K_{m2}) were estimated for laminarioligosaccharides (DP = 3–5), the K_m value of laminaribiose was approximately 5–9 times higher than those of the other oligosaccharides (DP = 3–5), whereas the K_{m2} values were independent of the DP of the substrates. The kinetics of transglycosylation by the enzyme could be well interpreted in terms of the subsite affinities estimated from the hydrolytic parameters (K_m and k_{cat}), and a possible mechanism of transglycosylation is proposed.

Keywords: Glycoside hydrolase family 3; Glucan (1→3)-β-glucosidase; Laminaribiose; Substrate inhibition; Transglycosylation

1. Introduction

Many glycoside hydrolases exhibit transglycosylation activity.[1–4] This occurs through a double-displacement mechanism of retaining glycoside hydrolases (GHs), that is, an enzyme–substrate complex is first formed and the glycosidic linkage of the substrate is cleaved via protonation of the leaving aglycone moiety.[5] The cleavage of the linkage affords a glycosyl-enzyme intermediate that can decompose with different possible

Abbreviations: GH, glycoside hydrolase; DP, degree of polymerization; BGL, glucan (1→3)-β-glucosidase; *p*NPG, *p*-nitrophenyl-β-D-glucoside; HPAEC, high-performance anion-exchange chromatography; ESI, electrospray ionization.

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outcomes. Although attack of water on the glycosyl-enzyme intermediate releases a hydrolyzed sugar, a ternary transglycosylation complex will form if the water molecule is replaced by a competing acceptor (carbohydrates, alcohols, etc.). When the second substrate molecule acts as an acceptor, substrate transglycosylation occurs. The rate of substrate transglycosylation thus increases, while the rate of hydrolysis decreases, in proportion to the concentration of the acceptor.

The extracellular β-glucosidase (BGL; EC 3.2.1.21) from the white-rot fungus *Phanerochaete chrysosporium* is a retaining glycoside hydrolase, and has been classified into GH family 3 according to the Carbohydrate-Active enZymes (CAZy) server [Coutinho and Henrissat, URL: <http://afmb.cnrs-mrs.fr/CAZY/>].[6,7] BGL was first isolated and characterized as a cellobiase responsible for the hydrolysis of cellobiose, which is a major product of cellulose degradation by cellulase.[8–10] In a previous study, we investigated the substrate specificity of BGL using a variety of compounds with β-glucosidic linkages, and clarified that the BGL is primarily a glucan (1→3)-β-glucosidase (EC 3.2.1.58).[11] Moreover, during hydrolysis of laminarioligosaccharides with degrees of polymerization (DP) of 2–5, apparent substrate inhibition was observed only when the DP was more than three. Because β-glucosidases from various animals, plants, fungi, and bacteria catalyze transglycosylation,[12–16] and BGL is one of the retaining glycoside hydrolases, a possible contribution of transglycosylation to the substrate inhibition should be considered. Although several reports have correlated transglycosylation with substrate inhibition of retaining glycoside hydrolases,[17–20] the relationship between them has not been quantitatively established.

In this study, therefore, a detailed kinetic analysis of hydrolysis and substrate transglycosylation was performed for *P. chrysosporium* BGL, and the relationship between the reaction processes and substrate inhibition was clarified. Moreover, we discuss the mechanisms at the catalytic site of BGL, based on the estimated kinetic parameters of hydrolysis and transglycosylation reactions.

2. Experimental

2.1. Preparation of *P. chrysosporium* glucan (1→3)-β-glucosidase

P. chrysosporium glucan (1→3)-β-glucosidase (BGL) was over expressed in the methylotrophic yeast *Pichia pastoris*, as described in a previous paper.[7] Crude enzyme solution was obtained by ammonium sulfate precipitation (70% saturation) of 4-d culture filtrate. The solution was applied to a Toyopearl HW-40C gel filtration column (44 × 200 mm; Tosoh, Tokyo, Japan) for desalting and dialysis against 20 mM sodium acetate buffer, pH 6.0. The fractions containing *p*-nitrophenyl-β-D-glucoside (*p*NPG; Seikagaku Corporation, Tokyo, Japan) hydrolyzing activity were concentrated, and applied to a QAE-Toyopearl 550C column (44 × 170 mm; Tosoh) equilibrated with the same buffer. *p*NPG-hydrolyzing activity was assayed by monitoring the release of *p*-nitrophenol from *p*NPG at 30 °C in 100 mM sodium acetate buffer, pH 4.5, as described previously.[7] BGL was eluted in the void volume under these conditions. This fraction was further purified on a QAE-Toyopearl 550C column equilibrated with 20 mM Tris–HCl buffer, pH 8.0, containing 0.02% sodium azide. The enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in 1500 mL. The purified BGL gave a single band on SDS-PAGE. The concentration of BGL was determined from the absorbance at 280 nm ($\varepsilon_{280} = 119\,cm^{-1}\,mM^{-1}$) and the specific activity of BGL was almost identical to that of purified wild-type BGL.[7]

2.2. Enzyme assay

Enzyme activity was assayed by monitoring the release of glucose from laminarioligosaccharides (DP 2–5; Seikagaku Corporation) after incubation with BGL according to our previous report;[7] 20 mM laminaribiose was incubated with 60 nM BGL at 30 °C in 10 mM sodium acetate buffer, pH 4.5, for 20 min, then the solution was boiled for 5 min to terminate the reaction. To confirm the transglycosylation activity of BGL, 1.0 μL of the reaction mixture was applied to precoated silica gel 60 TLC plates (Merck, Darmstadt, Germany), which were developed with EtOAc/CH_3COOH/water (3:2:1, by vol).[21] The products were detected with orcinol reagent (1% orcinol in 10% H_2SO_4 dissolved in ethanol) as described previously.[22]

High-performance anion-exchange chromatography (HPAEC; model D-300 BioLC; Dionex, Sunnyvale, CA, U.S.A.) with a CarboPac PA1 column (4 × 250 mm) was used to estimate the amount of the transglycosylation product in the reaction mixture. The column was equilibrated with 100 mM NaOH and the reaction products were eluted with a linear gradient of 0–500 mM sodium acetate in 100 mM NaOH at flow rate of 1 mL/min in 30 min.

2.3. Isolation of transglycosylation product

To determine the chemical structure of the transglycosylation product, 6.5 mL of the reaction mixture was applied to a Toyopearl HW-40S gel filtration column (22 × 900 mm; Tosoh),[23,24] equilibrated and eluted with water at the flow rate of 0.5 mL/min. The fractions containing transglycosylation product were freeze-dried and further purified in an HPLC system with a column of LiChrospher 100 NH_2 (4 × 250 mm, 5 μm; Merck), equil-

ibrated and eluted with 70% acetonitrile at the flow rate of 1 mL/min.

2.4. NMR and MS analyses

The ^{1}H NMR spectrum of the transglycosylation product was obtained on a Bruker JNM 600 spectrometer operated at 600.13 MHz. Spectra were obtained at 298 K in 99.9% D_2O (Wako Pure Chemicals, Osaka, Japan). *t*-BuOH was added to the sample as an internal reference standard for ^{1}H NMR chemical shifts (1.23 ppm). Electrospray ionization (ESI) mass spectrometry (MS) analysis was performed with Apex II 70e Fourier-transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA, U.S.A.) operated in the negative-ion mode.

2.5. Steady-state kinetic parameters

Scheme 1 shows the reaction process and related parameters for hydrolysis and substrate transglycosylation by a double-displacement glycoside hydrolase. This gives rise to the modified Michaelis–Menten rate Eqs. 1–3.[18,25] The four parameters in the equations are given in terms of individual rate constants in the Eqs. 4–7.

$$v_{P1} = (k_{cat}K_{m2}S + k_{cat2}S^2)/(K_mK_{m2} + K_{m2}S + S^2) \quad (1)$$

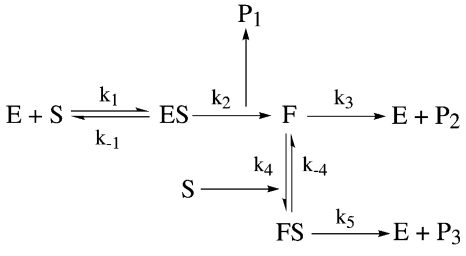
$$v_{P2} = k_{cat}S/(K_m + S + S^2/K_{m2}) \quad (2)$$

$$v_{P3} = k_{cat2}S^2/(K_mK_{m2} + K_{m2}S + S^2) \quad (3)$$

$$K_m = k_3(k_2 + k_{-1})(k_5 + k_{-4})/(k_1k_2k_5 + k_1k_3k_5 + k_2k_4k_5 + k_4k_5k_{-1} + k_1k_2k_{-4} + k_1k_3k_{-4}) \quad (4)$$

$$k_{cat} = k_1k_2k_3(k_5 + k_{-4})/(k_1k_2k_5 + k_1k_3k_5 + k_2k_4k_5 + k_4k_5k_{-1} + k_1k_2k_{-4} + k_1k_3k_{-4}) \quad (5)$$

$$K_{m2} = (k_1k_2k_5 + k_1k_3k_5 + k_2k_4k_5 + k_4k_5k_{-1} + k_1k_2k_{-4} + k_1k_3k_{-4})/\{k_1k_4(k_2 + k_5)\} \quad (6)$$



Scheme 1. Steady-state kinetic scheme for hydrolysis and substrate transglycosylation by a double-displacement GH.

$$k_{cat2} = k_2k_5/(k_2 + k_5) \quad (7)$$

During both hydrolysis and the transglycosylation reaction of laminaribiose, glucose is produced as P1 and P2, whereas glucose is released as P2 from other laminarioligosaccharides. Therefore, plots of glucose production from laminaribiose were fitted to the modified Michaelis–Menten Eq. 8, given by the sum of v_{P1} and v_{P2}.

$$v_{glucose} = (2k_{cat}K_{m2}S + k_{cat2}S^2)/(K_mK_{m2} + K_{m2}S + S^2) \quad (8)$$

All kinetic parameters were calculated using DeltaGraph 4.0.5 (SPSS Inc. Chicago, IL, U.S.A.) and KaleidaGraph 3.08 (Synergy Software, Reading, PA, USA).

Subsite affinities of *P. chrysosporium* BGL were calculated using hydrolytic kinetic parameters K_m and k_{cat} obtained from the reaction with laminarioligosaccharides of DP 2–5. The calculation procedures, using equations for the subsite analysis of *exo*-acting enzyme,[26] are summarized below. Consider an active site consisting of x subsites, labeled −1, +1 to +4. The subsite affinity A_i of the subsite labeled i (i = +2 to +4) can be calculated from Eq. 9.

$$A_n = RT\{\ln(k_{cat}/K_m)_{n+1} - \ln(k_{cat}/K_m)_n\} \quad (9)$$

The parameter $(k_{cat}/K_m)_n$ was determined for oligosaccharides of DP n. R is the gas constant and T is the absolute temperature. The values A_{-1} and k_{int} were extrapolated from a plot of $\exp(A_{n+1}/RT)$ versus $(1/k_{cat})_n$.

$$\exp(A_{n+1}/RT) = \{k_{int}/(1/k_{cat})_n - 1\}\exp(A_{-1}/RT) \quad (10)$$

The parameter k_{int} is the intrinsic catalytic rate constant. Finally, A_{+1} was calculated from Eq. 11.

$$(1/K_m)_n = (0.018)\exp(A_{+1}/RT)[\exp\{(A_{-1} + A_{+2} + \cdots + A_n)/RT\} + \exp\{(A_{+2} + A_{+3} + \cdots + A_{n+1})/RT\}] \quad (11)$$

3. Results

3.1. Detection and identification of transglycosylation product

Laminaribiose was incubated with *P. chrysosporium* BGL and the reaction was followed by TLC (Fig. 1). The hydrolytic product (glucose) and the substrate (laminaribiose) gave spots with R_f values of 0.57 and 0.45, respectively. In addition, there was another product with an R_f value of 0.32, clearly suggesting that transglycosylation had occurred. When the reaction mixture was subjected to size-exclusion chromatography, the peak

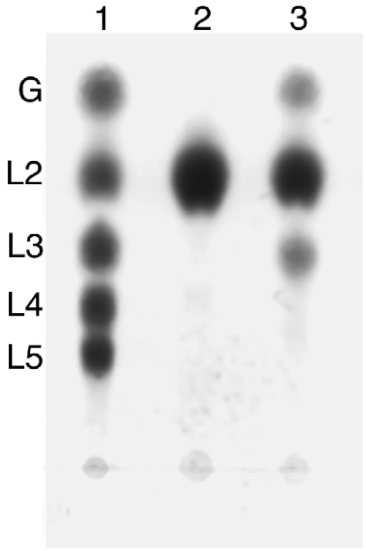


Figure 1. TLC analysis of laminaribiose with and without incubation with BGL. Lane 1, standards of glucose and laminarioligosaccharides (G-L5); lane 2, laminaribiose; lane 3, laminaribiose incubated with BGL. Incubation was carried out in 10 mM sodium acetate, pH 4.5, as described in Section 2.

of the transglycosylation product appeared earlier than that of the substrate (data not shown) and incubation of each fraction with BGL gave only glucose on the TLC plate, suggesting that the transglycosylation product was composed of β-linked glucoses. In the ^{1}H NMR spectrum (Fig. 2), α and β anomeric protons, as well as β-(1→3)-linked (IIα and βH-1) and β-(1→6)-linked (IIIα and βH-1) glucosidic protons, were observed. Integration of the signals suggested that the product is a glucose trimer. Signals characteristic of a β-(1→6)-glucosidic residue were detected and all other signal assignments were consistent with published data.[15,27] Moreover, the ESI-MS spectrum of the transglycosylation product gave the corresponding ion peaks at *m*/*z* 503.1624, $[M-H]^{-}$; *m*/*z* 521.1737, $[M+OH]^{-}$; *m*/*z* 539.1393, $[M+Cl]^{-}$, calculated for 503.1618, 521.1729, 539.1393, respectively (data not shown). The transglycosylation product was thus identified as a 6-*O*-glucosyl-laminaribiose, β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-Glc (Fig. 2).

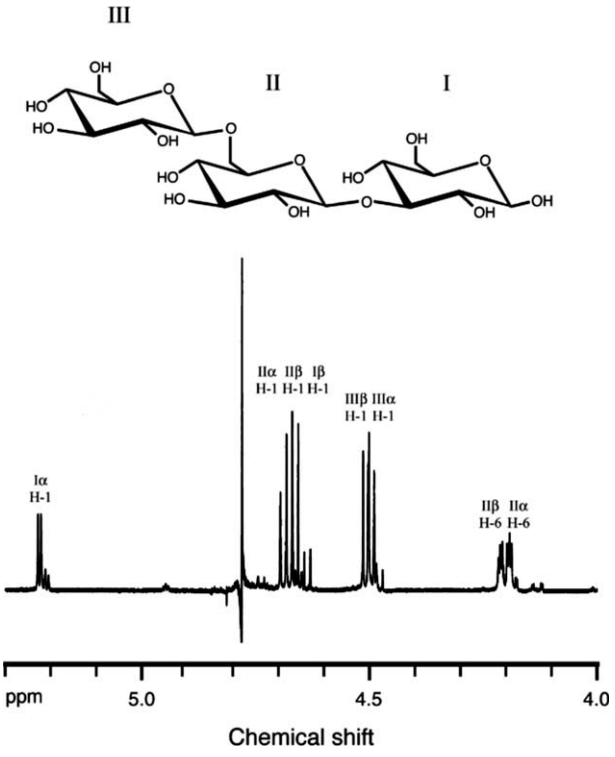


Figure 2. Chemical structure of the transglycosylation product (upper) and its ^{1}H NMR spectrum (lower). Iα, IIα, IIIα, Iβ, IIβ, and IIIβ represent anomers of the I, II, and III glucose residues of the transglycosylation product, respectively.

3.2. Kinetic analysis of substrate transglycosylation

To determine the kinetic parameters for the hydrolytic and transglycosylation reactions, glucose production by BGL was monitored at various laminaribiose concentrations. As shown in Figure 3A, glucose production

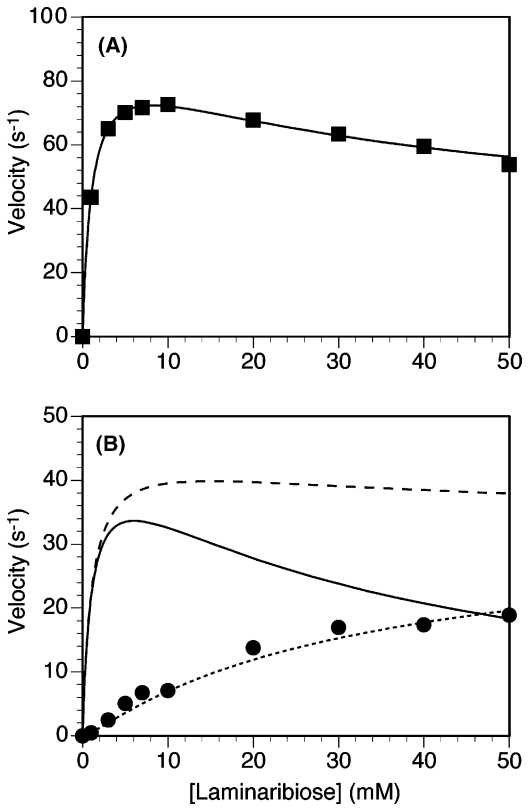


Figure 3. Plots of laminaribiose concentration versus velocity of glucose (A) and 6-*O*-glucosyl-laminaribiose (B) production. Filled square, production rate of glucose; filled circles, production rate of 6-*O*-glucosyl-laminaribiose; dashed line, simulated curve for glucose from the reducing end; solid line, simulated curve for glucose from the non-reducing end; dotted line, simulated curve for transglycosylation product.

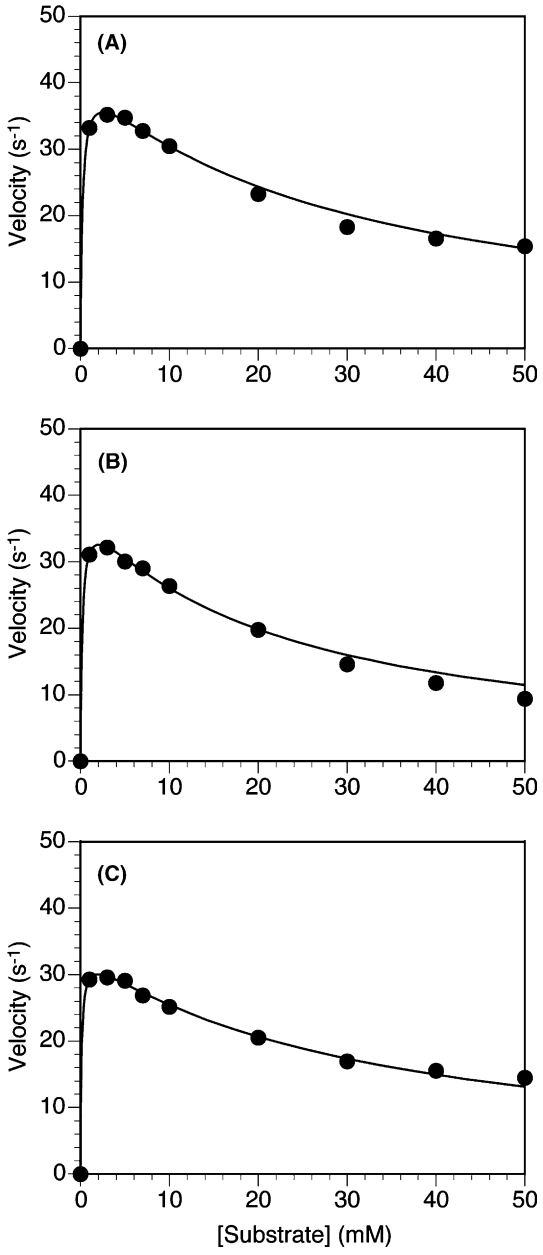


Figure 4. Effect of DP of laminarioligosaccharides on velocity of glucose production catalyzed by BGL. Production rate of glucose for laminaritriose (A), laminaritetraose (B), and laminaripentaose (C) were estimated as described in Section 2.

increased in proportion to the increase of substrate concentration up to 10 mM, whereas it decreased at higher substrate concentrations. When Eq. 8 was applied to the glucose production in Figure 3A, the kinetic parameters for hydrolysis and transglycosylation were estimated as $K_m = 1.08\,mM$, $k_{cat} = 45.6\,s^{-1}$, $K_{m2} = 34.1\,mM$, and $k_{cat2} = 33.3\,s^{-1}$. Furthermore, by substituting the obtained parameters into Eqs. 1–3, theoretical production rates for P1 (glucose from the reducing end unit of laminaribiose), P2 (glucose from the non-reducing end unit of laminaribiose), and P3 (the transglycosylation product) were calculated (Fig. 3B). At low concentrations of the substrate, the rates of P1 and P2 production were synchronous, indicating that the laminaribiose was fully hydrolyzed without transglycosylation. However, a difference between them appeared with an increase of substrate concentration, because of the increase of P3 production. The theoretical rate of P3 production well fitted the production of 6-*O*-glucosyl-laminaribiose estimated by HPAEC (R^2 value 0.974).

The catalytic rates of BGL were also monitored at various concentrations of laminarioligosaccharides (DP = 3–5), as shown in Figure 4. In the case of these substrates, a glucose unit is only produced from the non-reducing end of the substrate as P2 in Scheme 1. Therefore, the glucose production was fitted to Eq. 2, and the kinetic parameters for hydrolysis of laminarioligosaccharides were compared with those of laminaribiose. As shown in Table 1, k_{cat} and K_{m2} values for all substrates were in similar ranges, whereas the K_m value for laminaribiose was 5–9 times higher than those for laminarioligosaccharides. Based on the hydrolytic parameters (k_{cat} and K_m) for these substrates, a subsite affinity map was generated, as shown in Figure 5. Subsite binding affinity at subsite +1 was $20.6\,kJ\,mol^{-1}$, the highest value among the subsites. Although the apparent binding energies at subsites −1 and +2 were 6.54 and $3.75\,kJ\,mol^{-1}$, respectively, the affinity was almost zero at subsites +3 and +4.

4. Discussion

Many retaining glycoside hydrolases have been shown to possess transglycosylation activities in addition to hydrolytic activity.[1–4] In the case of GH family 3 glucan (1→3)-β-glucosidase from the white-rot fungus *P. chrysosporium*, substrate transglycosylation also occurs simultaneously with hydrolysis. The transglycosylation

Table 1. Steady-state kinetic parameters of BGL for laminarioligosaccharides[a]

	K_m (mM)	k_{cat} (s^{-1})	K_{m2} (mM)	k_{cat2} (s^{-1})
Laminaribiose	1.08 ± 0.01	45.6 ± 0.1	34.1 ± 3.5	33.3 ± 3.2
Laminaritriose	0.223 ± 0.006	41.8 ± 0.2	28.3 ± 0.8	
Laminaritetraose	0.205 ± 0.007	39.0 ± 0.3	20.9 ± 0.6	
Laminaripentaose	0.119 ± 0.006	33.7 ± 0.2	32.0 ± 1.2	

[a] Values are given as means with standard deviations.

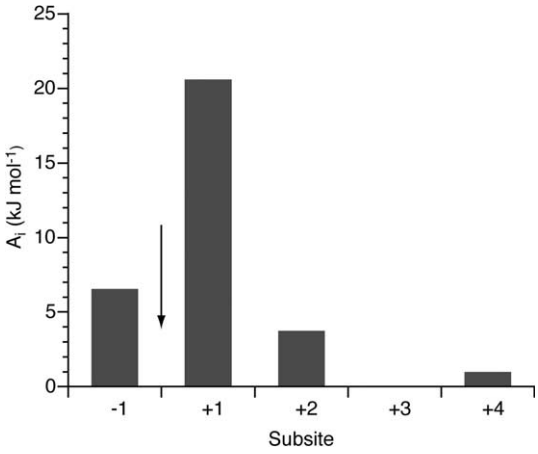


Figure 5. Subsite affinity map of *P. chrysosporium* BGL based upon the hydrolytic parameters (K_m and k_{cat}) for laminaribiose and laminarioligosaccharides. The arrow indicates the position of the hydrolytic site. Subsites are labeled from −1 to +4, where the −1 subsite binds a non-reducing glucosyl residue of the substrate.

process is regioselective and the product from laminaribiose was identified as 6-*O*-glucosyl-laminaribiose. This result indicated that glucose from the non-reducing unit of the first laminaribiose molecule was transferred to the *O*-6 position of the non-reducing unit of the second laminaribiose. In a study on transglycosylation by *Trichoderma viride* β-glucosidase, the same transglycosylation product was obtained when a high concentration of laminaribiose was used as a substrate.[15] With cellobiose as a substrate, moreover, *Aspergillus niger* β-glucosidase also formed 6-*O*-glucosyl-cellobiose as a transglycosylation product,[14] suggesting that transglycosylation at the *O*-6 position is a common tendency in the fungal β-glucosidases. Recently, the three-dimensional structures of GH family 3 barley β-glucan glucohydrolase I (ExoI) with thiocellobiose and thiolaminaribiose have been solved by Hrmova and co-workers, and the structures with sophorose (β-D-Glc*p*-(1→2)-β-D-Glc) and gentiobiose (β-D-Glc*p*-(1→6)-β-D-Glc) were also predicted.[28–30] In this enzyme, the glucose unit at −1 (non-reducing end) is locked tightly by many hydrogen bonds and the conformation is conserved among four glucose dimers.[30] On the other hand, the glucose unit at subsite +1 (reducing end) is fixed by the hydrophobic sandwich of double tryptophan residues and the site is loose enough to accept various β-linked glucose dimers. Because ExoI shows quite similar substrate specificities to *P. chrysosporium* BGL toward the four substrates, the two enzymes might have some similarity in the catalytic site.[11,30] In *P. chrysosporium* BGL, therefore, the active site +1 might be loose enough to accept not only β-(1→3) but also β-(1→6)-linked glucose, and the high reactivity of the C-6 hydroxyl group compared to hydroxyl groups at other positions might favor transglycosylation at O-6.

In substrate transglycosylation, the glycosyl part of the substrate is transferred to a hydroxyl-containing compound other than water, and the reaction inhibits hydrolysis proportionally with an increase in substrate concentration. Transglycosylation can thus mimic substrate inhibition in the reaction of retaining glycoside hydrolases, and the activity can be estimated from the accumulation of hydrolytic products. In the present study, therefore, kinetic analysis was performed for the hydrolytic and transglycosylation reactions to elucidate the quantitative relationship between substrate inhibition and substrate transglycosylation. In previous kinetic studies of BGL, we demonstrated that substrate inhibition was observed only when laminarioligosaccharides (DP 3–5) were used as substrates, but not in the case of laminaribiose.[11] However, glucose production was clearly decreased at substrate concentrations of more than 10 mM laminaribiose, as shown in Figure 3A. Moreover, the theoretical transglycosylation rate simulated using the kinetic parameters obtained from the data shown in Figure 3A well fitted the production rate of 6-*O*-glucosyl-laminaribiose measured by HPAEC. These results clearly suggest that substrate inhibition also occurred when laminaribiose was used as a substrate, and the inhibition is fully explained by the transglycosylation reaction producing 6-*O*-glucosyl-laminaribiose. Because both P1 and P2 in Scheme 1 are glucose when glucose dimers are used as substrates, their hydrolytic rate is commonly estimated by using half the value of glucose concentration in the reaction mixture. As shown in Figure 3B, however, the apparent difference between the rates of P1 and P2 production increases proportionally to the substrate concentration, indicating that the transglycosylation cannot be neglected in calculating kinetic parameters.

Comparing the hydrolytic and transglycosylation parameters for laminaribiose and laminarioligosaccharides (DP 3–5), the K_{m2} values were independent of the DP of substrates, whereas the K_m value of laminaribiose was approximately 5–9 times higher than those of the other oligosaccharides. Moreover, a subsite affinity map of *P. chrysosporium* BGL determined from the hydrolytic parameters for laminarioligosaccharides showed a similar pattern to those of some β-glucosidases and ExoI, that is, apparent binding was detected at subsites −1 to +2, and the affinity at subsite +1 was the highest.[30,31] In the case of *P. chrysosporium* BGL, the subsite affinities at −1 and +2 are almost the same, suggesting that non-productive binding, in which glucose residues are positioned at subsites +1 and +2, may occur as well as productive binding (−1 and +1) during laminaribiose hydrolysis. However, non-productive binding hardly occurs when laminarioligosaccharides with DP of more than 3 are used as substrates, because binding affinities beyond subsite +2 are close to zero. In contrast to the hydrolytic reaction, the transglycosylation reac-

tion could be affected by the affinity between the substrate and the enzyme–glucose complex (K_{m2}). Because subsite −1 has already been filled with glucose in the intermediate of BGL, the non-reducing end glucose unit of the transglycosylation acceptor will bind at +1. The DP of the acceptor thus hardly affects the affinity between enzyme–glucose complex and the acceptor, because subsite affinities at more than +2 are close to zero. This might account for the similar K_{m2} values of laminaribiose and laminarioligosaccharides. Therefore, strong binding at +1, which permits various β-linkages between −1 and +1, plays a key role in transglycosylation by *P. chrysosporium* BGL.

The present study demonstrates that BGL of *P. chrysosporium* catalyzes substrate transglycosylation, and the product derived from laminaribiose was identified. Moreover, steady-state kinetic analysis indicated that substrate inhibition of hydrolysis depends upon the transglycosylation. Although the physiological function of the extracellular *P. chrysosporium* BGL has been considered only in terms of the hydrolytic activity, the transglycosylation ability also needs to be taken into account. Further kinetic and structural analyses will be needed to understand the transglycosylation mechanism and its true function.

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References

1. Chipman, D. M.; Pollock, J. J.; Sharon, N. *J. Biol. Chem.* **1968**, *243*, 487–496.
2. Usui, T.; Hayashi, Y.; Nanjo, F.; Sakai, K.; Ishido, Y. *Biochim. Biophys. Acta* **1987**, *923*, 302–309.
3. Bardales, R. M.; Bhavanandan, V. P. *J. Biol. Chem.* **1989**, *264*, 19893–19897.
4. Eneyskaya, E. V.; Golubev, A. M.; Kauchurin, A. M.; Savel'ev, A. N.; Neustroev, K. N. *Carbohydr. Res.* **1998**, *305*, 83–91.
5. Withers, S. G. *Carbohydr. Polym.* **2001**, *44*, 325–337.
6. Li, B.; Renganathan, V. *Appl. Environ. Microbiol.* **1998**, *64*, 2748–2754.
7. Kawai, R.; Yoshida, M.; Tani, T.; Igarashi, K.; Ohira, T.; Nagasawa, H.; Samejima, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1–7.
8. Deshpande, V.; Eriksson, K.-E.; Pettersson, B. *Eur. J. Biochem.* **1978**, *90*, 191–198.
9. Smith, M. H.; Gold, M. H. *Appl. Environ. Microbiol.* **1979**, *37*, 928–942.
10. Lymar, E. S.; Li, B.; Renganathan, V. *Appl. Environ. Microbiol.* **1995**, *61*, 2976–2980.
11. Igarashi, K.; Tani, T.; Kawai, R.; Samejima, M. *J. Biosci. Bioeng.* **2003**, *95*, 572–576.
12. Gopalan, V.; Vander Jagt, D. J.; Libell, D. P.; Glew, R. H. *J. Biol. Chem.* **1992**, *267*, 9629–9638.
13. Akiyama, T.; Kaku, H.; Shibuya, N. *Phytochemistry* **1998**, *48*, 49–54.
14. Watanabe, T.; Sato, T.; Yoshioka, S.; Koshijima, T.; Kuwahara, M. *Eur. J. Biochem.* **1992**, *209*, 651–659.
15. Kono, H.; Kawano, S.; Tajima, K.; Erata, T.; Takai, M. *Glycoconj. J.* **1999**, *16*, 415–423.
16. Parry, N. J.; Beever, D. E.; Owen, E.; Vandenberghe, I.; Beeumen, J. V.; Bhat, M. K. *Biochem. J.* **2001**, *353*, 117–127.
17. Li, X.; Calza, R. E. *Biochim. Biophys. Acta* **1991**, *1080*, 148–154.
18. Savel'ev, A. N.; Ibatyllin, F. M.; Eneyskaya, E. V.; Kachurin, A. M.; Neustroev, K. N. *Carbohydr. Res.* **1996**, *296*, 261–273.
19. Auguilera, B.; Ghauharali-van der Vlught, K.; Helmond, M. T. J.; Out, J. M. M.; Donker-Koopman, W. E.; Groener, J. E. M.; Boot, R. G.; Renkema, G. H.; van der Marel, G. A.; van Boom, J. H.; Overkleeft, H. S.; Aerts, J. M. F. G. *J. Biol. Chem.* **2003**, *278*, 40911–40916.
20. Zechel, D. L.; Reid, S. P.; Stoll, D.; Nashiru, O.; Warren, R. A. J.; Withers, S. G. *Biochemistry* **2003**, *42*, 7195–7204.
21. Hrmova, M.; Fincher, G. B. *Carbohydr. Res.* **1998**, *305*, 209–221.
22. Farkas, V.; Maclachlan, G. *Carbohydr. Res.* **1988**, *184*, 213–219.
23. Kondo, H.; Nakatani, H.; Hiromi, K. *Agric. Biol. Chem.* **1981**, *45*, 2369–2370.
24. Shintate, K.; Kitaoka, M.; Kim, Y.-K.; Hayashi, K. *Carbohydr. Res.* **2003**, *338*, 1981–1990.
25. Brumer, H., III; Sims, P. F. G.; Sinnott, M. L. *Biochem. J.* **1999**, *339*, 43–53.
26. Hiromi, K.; Nitta, Y.; Numata, C.; Ono, S. *Biochim. Biophys. Acta* **1973**, *302*, 362–375.
27. Petersen, B. O.; Krah, M.; Duus, J. O.; Thomsen, K. K. *Eur. J. Biochem.* **2000**, *267*, 361–369.
28. Varghese, J. N.; Hrmova, M.; Fincher, G. B. *Structure* **1999**, *7*, 179–190.
29. Hrmova, M.; Varghese, J. N.; Gori, R. D.; Smith, B. J.; Driguez, H.; Fincher, G. B. *Structure* **2001**, *9*, 1005–1016.
30. Hrmova, M.; Gori, R. D.; Smith, B. J.; Fairweather, J. K.; Driguez, H.; Varghese, J. N.; Fincher, G. B. *Plant Cell* **2002**, *14*, 1033–1052.
31. Yazaki, T.; Ohnishi, M.; Rokushika, S.; Okada, G. *Carbohydr. Res.* **1998**, *298*, 51–57.