Carbohydrate Research 366 (2013) 6–16

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Mode of action of a β-(1→6)-glucanase from *Penicillium multicolor*

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ARTICLE INFO

Article history:
Received 16 September 2012
Received in revised form 5 November 2012
Accepted 6 November 2012
Available online 15 November 2012

Keywords:
β-(1→6)-Glucanase
Hydrolytic manner
β-(1→6)-Glucan
Gentiooligosaccharide
Transglycosylation

ABSTRACT

β-(1→6)-Glucanase from the culture filtrate of *Penicillium multicolor* LAM7153 was purified by ammonium sulfate precipitation, followed by cation-exchange and affinity chromatography using gentiotetraose (Gen_4) as ligand. The hydrolytic mode of action of the purified protein on β-(1→6)-glucan (pustulan) was elucidated in real time during the reaction by HPAEC-PAD analysis. Gentiooligosaccharides (DP 2-9, Gen_{2-9}), methyl β-gentiooligosides (DP 2-6, Gen_{2-6} β-*O*Me), and *p*-nitrophenyl β-gentiooligosides (DP 2-6, Gen_{2-6} β-*p*NP) were used as substrates to provide analytical insight into how the cleavage of pustulan (DP 320) is actually achieved by the enzyme. The enzyme was shown to completely hydrolyze pustulan in three steps as follows. In the initial stage, the enzyme quickly cleaved the glucan with a pattern resembling an *endo*-hydrolase to produce a short-chain glucan (DP 45) as an intermediate. In the midterm stage, the resulting short-chain glucan was further cleaved into two fractions corresponding to DP 15-7 and DP 2-4 with great regularity. In the final stage, the lower oligomers corresponding to DP 3 and DP 4 were very slowly hydrolyzed into glucose and gentiobiose (Gen_2). As a result, the hydrolytic cooperation of both an *endo*-type and saccharifying-type reaction by a single enzyme, which plays a bifunctional role, led to complete hydrolysis of the glucan. Thus, β-(1→6)-glucanase varies its mode of action depending on the chain length derived from the glucan.

1. Introduction

β-(1→6)-Glucan, which consists of glucose residues joined in a β-(1→6)-linkage, is a structurally important component of the cell wall of yeasts and fungi. It is produced in budding yeasts as a link between cell wall proteins and the main β-(1→3)-glucan/chitin polysaccharides.[1] However, the polymer is much less abundant in nature than other β-glucans, such as β-(1→4)- or β-(1→3)-glucan. Pustulan from *Umbilicaria papullosa* is well known as a typical linear β-(1→6)-glucan.[2] On the other hand, β-(1→6)-glucanase (EC 3.2.1.75) belongs to glycosyl hydrolase families 5 and 30 (http://www.cazy.org/).[3–6] Although β-(1→6)-glucanases are widely distributed among fungi,[7] few of them have been purified and characterized.[8–17] As a result, there are a few studies on the hydrolytic mode of action of β-(1→6)-glucanase on β-(1→6)-glucan.[18–20] In general, enzymatic degradation of polysaccharides is considered to consist of *endo*-acting enzymes, which randomly hydrolyze glycosidic linkages in the polymers, and processive *exo*-acting enzymes, which degrade the polysaccharides from the polymer ends. For example, cellulose[21] and chitin[22] are hydrolyzed by the synergistic action of corresponding *endo*- and *exo*-acting enzymes. In starch hydrolysis, the reaction is performed through liquefaction (by an *endo*-type enzyme) and saccharification (by an *exo*-type enzyme).[23] During the first step, starch is solubilized in water and partially hydrolyzed with an α-amylase. In the second step, saccharifying enzymes such as β-amylase or glucoamylase transform the liquefied starch into final products that can be specific oligosaccharides such as glucose or maltose.

The present paper describes the hydrolytic mode of action of β-(1→6)-glucanase on pustulan based on kinetic and product analysis, together with specific purification of the enzyme and the methods for synthesizing a series of gentiooligosides using its transglycosylation activity.

2. Results

2.1. Purification of β-(1→6)-glucanase based on gentiotetraose (Gen_4)-immobilized affinity chromatography

To purify *Penicillium multicolor* β-(1→6)-glucanase, the crude enzyme preparation was fractionated by ammonium sulfate precipitation. The desalted fraction was applied to cation-exchange chromatography using CM-Sepharose Fast Flow, yielding a single peak of β-(1→6)-glucanase activity that was completely devoid of β-glucosidase activity. This fraction was then subjected to substrate affinity chromatography on a Gen_4-coupled TOYOPEARL AF-Amino-650M column (Fig. 1A). β-(1→6)-Glucanase activity

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http://dx.doi.org/10.1016/j.carres.2012.11.002

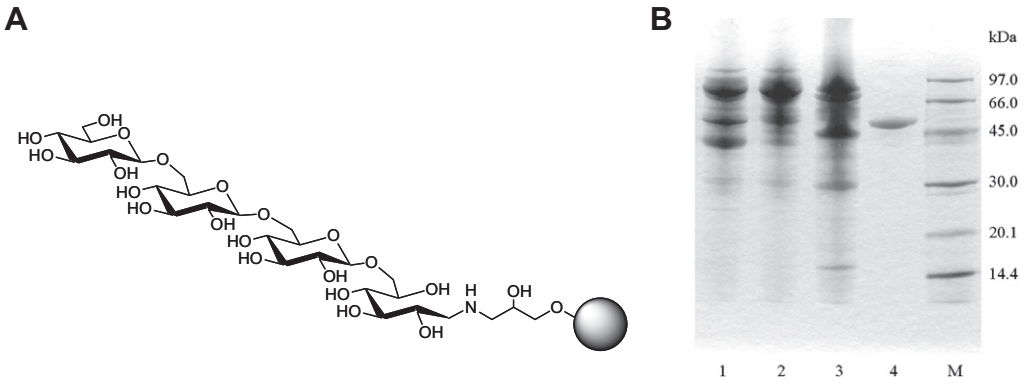


Figure 1. (A) Structure of the Gen_4-coupled affinity gel. (B) SDS–PAGE of purified *P. multicolor* β-(1→6)-glucanase using a 12.5% gel under reducing conditions. Proteins were stained with CBB. Lane 1, crude enzyme; Lane 2, ammonium sulfate precipitation; Lane 3, cation-exchange elute; Lane 4, affinity elute. Numbers on the right indicate the molecular mass of the protein standards. (Lane M).

Table 1
Summary of the purification and yield of β-(1→6)-glucanase from *P. multicolor*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	73.0	49.1	0.7	100.0	1.0
$(NH_4)_2SO_4$ precipitation	29.7	33.3	1.1	67.8	1.7
CM Sepharose F.F.	0.8	3.7	4.5	7.6	6.7
Affinity	0.007	1.1	164.1	2.2	244.3

was strongly adsorbed on the column, and was subsequently easily eluted as a single peak by a salt gradient. The fraction was highly purified (244-fold relative to the crude enzyme preparation) with a specific activity of 164.1 U/mg (Table 1). It gave a single protein band by SDS–PAGE with a molecular mass of 51 kDa (Fig. 1B). Furthermore, the protein was analyzed by MALDI-TOF mass spectrometry, and a peak with an *m/z* value of 49,909 was observed. This suggests that the enzyme does take a monomer but not a unit structure. The isoelectric point (pI) was not determined because it was beyond the limit of detection (pI <3.5) (data not shown). The optimum temperature for the purified enzyme was 50 °C, and heat inactivation occurred above 60 °C. The optimum pH was pH 4.0, and more than 90% of the maximum enzyme activity was maintained in the pH range 2.0–9.0 at 4 °C and 24 h of incubation. The hydrolytic activity of the enzyme was prominent only for pustulan (a linear β-(1→6)-glucan) (Table 2). Little activity was detected toward soluble starch, carboxymethyl cellulose, or curdlan, enabling us to conclude that the enzyme is a specific β-(1→6)-glucanase.

Table 2
Substrate specificity of purified β-(1→6)-glucanase toward selected glucans and oligosaccharides

Substrate	Linkage type	Activity (U/mg)
Pustulan	β-1,6	164.1
Yeast glucan	β-1,3:β-1,6	1.4
Soluble starch	α-1,4:α-1,6	0
Carboxymethyl cellulose	β-1,4	0
Curdlan (*Alcaligenes faecalis*)	β-1,3	0
Gen_8	β-1,6	4.2

2.2. Synthesis of methyl β-gentiooligosides and *p*-nitrophenyl β-gentiooligosides

A series of methyl β-gentiooligosides (DP 2-6, Gen_{2-6} β-*O*Me) and *p*-nitrophenyl β-gentiooligosides (DP 2-6, Gen_{2-6} β-*p*NP) were prepared as control samples for analyzing the hydrolytic mechanism of the purified β-(1→6)-glucanase on the β-(1→6)-glucan linkage. We have recently reported that gentiooligosaccharides (DP 3-9) can be prepared from the starting substance Gen_2 by the sequential transglycosylation reactions of β-glucosidase and β-(1→6)-glucanase.[24] By following the reported methodology, a series of methyl β-gentiooligosides (DP 2-6) were enzymatically prepared in a similar way.

Gen_2 β-*O*Me was first synthesized from methyl β-D-glucoside (Glc β-*O*Me), which serves as both the donor and acceptor substrate, through the transglycosylation reaction of *Hypocrea jecorina* β-glucosidase. The resulting Gen_2 β-*O*Me was then used as a starting substance for obtaining methyl β-gentiooligosides (DP 3-6), utilizing the above-purified β-(1→6)-glucanase. The transglycosylation reaction was carried out at a high substrate concentration of Gen_2 β-*O*Me (4 M), which served as both the donor and acceptor. After terminating the enzyme reaction, the reaction mixture was subjected to pyridylamination with 2-amino-pyridine to give pyridylaminated gentiooligosyl derivatives because it proved difficult to separate the desired methyl β-gentiooligosides from gentiooligosaccharide by-products by chromatography. The reaction solution was applied to TOYOPEARL HW-40S chromatography to remove the excess of 2-aminopyridine. The fractions that showed color development with phenol-sulfuric acid were further subjected to charcoal-Celite chromatography with a linear gradient of ethanol (Fig. 2A and Scheme 1). The chromatogram showed six fractions (MG_1–MG_6), numbered according to their order of elution. Fractions MG_1 and MG_2 were recovered as Glc β-*O*Me and Gen_2 β-*O*Me, respectively. Fractions MG_3–MG_6 were obtained as the desired products Gen_3 β-*O*Me, Gen_4 β-*O*Me, Gen_5 β-*O*Me, and Gen_6 β-*O*Me in yields of 7.0%, 4.3%, 1.5%, and 0.8%, respectively. The structures of the synthesized products were analyzed by ^1H, ^{13}C NMR (Table 3), and ESI-MS.

A series of *p*-nitrophenyl β-gentiooligosides (DP 2-6) were prepared in a similar way to the methyl β-gentiooligosides. *p*-Nitrophenyl β-D-glucoside (Glc β-*p*NP) was used as a starting material for the synthesis of Gen_2 β-*p*NP. The reaction was performed by using the transglycosylation activity of *H. jecorina* β-glucosidase with a high monomer concentration (100 mM). The reaction

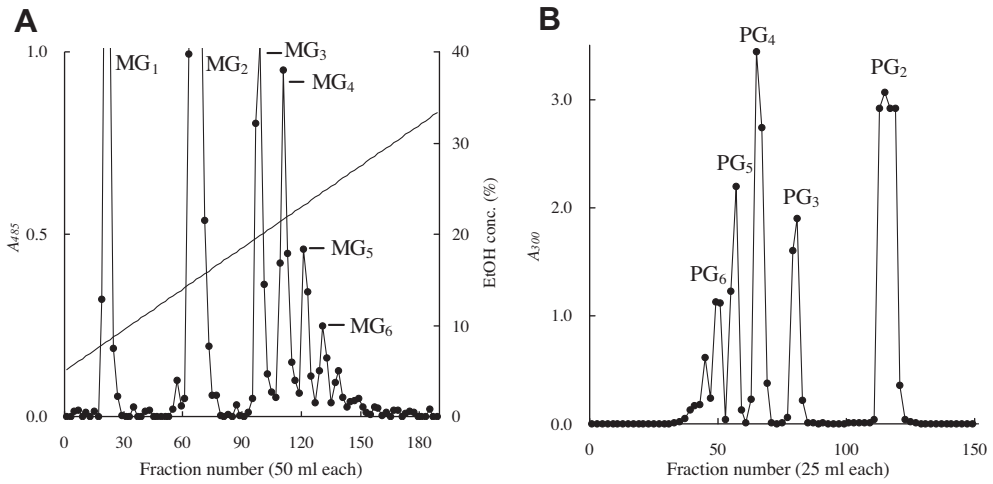


Figure 2. (A) Charcoal-Celite chromatography of transglycosylation products formed by the action of β-(1→6)-glucanase on methyl β-gentiobioside. The elution positions of MG_1 and MG_2–MG_6 correspond to methyl β-D-glucoside and methyl β-gentiooligosides (DP 2-6), respectively. The straight line indicates ethanol concentration (%). (B) TOYOPEARL HW-40S chromatography of transglycosylation products formed by the action of β-(1→6)-glucanase on gentiotriose and *p*-nitrophenyl β-gentiobioside. The elution positions of PG_2–PG_6 correspond to *p*-nitrophenyl β-gentiooligosides (DP 2-6), respectively.

mixture was applied to TOYOPEARL HW-40S chromatography to obtain the target dimer in 15.2% yield. The resulting Gen_2 β-*p*NP was used for the synthesis of Gen_{3-6} β-*p*NP. The gentiooligosides (DP 3-6) were synthesized by a β-(1→6)-glucanase-catalyzed transglycosylation reaction from the Gen_3 donor to a Gen_2 β-*p*NP acceptor. In this case, low temperature (4 °C) was effective for raising the transglycosylation rate (data not shown). The reaction mixture was applied to TOYOPEARL HW-40S chromatography. The chromatogram showed five peaks designated PG_2–PG_6 (Fig. 2B). Fraction PG_2 was recovered as Gen_2 β-*p*NP (57.4% recovery). Fractions PG_3 and PG_4 were obtained as the target products Gen_3β-*p*NP and Gen_4 β-*p*NP in yields of 2.1% and 14.2%, respectively. Fractions PG_5 and PG_6 were each further purified by reverse-phase ODS chromatography to remove the gentiooligosaccharide by-products. The target products Gen_5 β-*p*NP and Gen_6 β-*p*NP were obtained in yields of 2.6% and 1.7%, respectively. The structures of the synthesized products were identified by ^1H, ^{13}C NMR (Table 4), and ESI-MS analyses. The synthetic gentiooligosaccharides and gentiooligosides were used to analyze the mechanism of action of β-(1→6)-glucanase on β-(1→6)-glucan as described below.

2.3. Hydrolysis of methyl β-gentiooligosides, *p*-nitrophenyl β-gentiooligosides, and gentiooligosaccharides: bond cleavage frequency

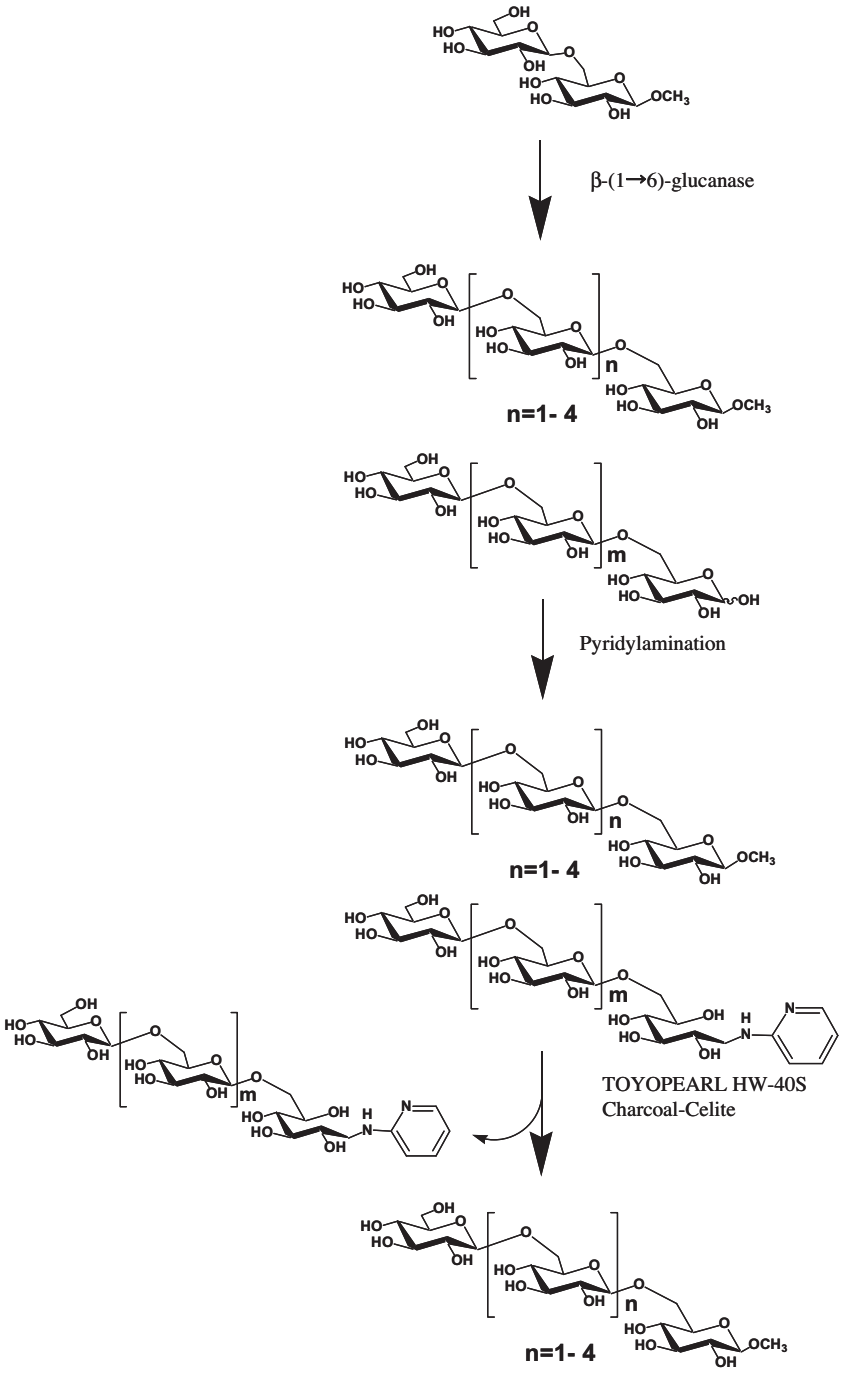
The mode of action of the purified β-(1→6)-glucanase on the gentiooligosides and gentiooligosaccharides prepared above were examined. The cleavage frequencies and relative hydrolytic rate of the enzyme were measured on the basis of the initial velocity of the hydrolytic reaction and are summarized in Tables 5 and 6.

2.3.1. Methyl β-gentiooligosides and *p*-nitrophenyl β-gentiooligosides

The cleavage frequencies and relative hydrolytic rate of the purified β-(1→6)-glucanase on methyl β-gentiooligosides (DP 2-6) were determined by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (Table 5). As compared with Gen_3 β-*O*Me, Gen_{4-6} β-*O*Me acted as fairly good substrates. For Gen_3 β-*O*Me, the second glucosidic bond (bond 2) from the methyl group was cut preferentially. The major cleavage of Gen_4 β-*O*Me occurred at the second (bond 2) and third (bond 3) glucosidic bonds from the methyl group. The hydrolytic pattern of Gen_5 β-*O*Me was substantially similar to that of Gen_6 β-*O*Me: the major cleavages occurred at bonds 2, 3, and 4 in approximately equal proportion. It should be noted that the non-reducing end glucosyl residues of Gen_{4-6} β-*O*Me were either not or scarcely cleaved by the enzyme. Gen_3 β-*O*Me was hydrolyzed slowly to form Glc β-*O*Me and Gen_2 β-*O*Me in the ratio 91:9. The presence of a methyl group at the reducing end did not seem to prohibit the reaction. These results indicate that methyl β-gentiooligosides are cleaved through the terminal glycoside, and the number of the susceptible bond varies with the chain-length of substrate. Thus, the cleavage gradually shifts from bond 2 to bonds 3 and 4 with increasing DP. The cleavage frequencies of *p*-nitrophenyl β-gentiooligosides (DP 2-6) were similarly analyzed by HPLC. For Gen_4 β-*p*NP, the third bond (bond 3) from the *p*-nitrophenyl group was cut preferentially. The major cleavage of Gen_5 β-*p*NP occurred at bonds 3 and 4, whereas bond 2 was not completely cleaved. The hydrolytic pattern of Gen_6 β-*p*NP, which was found to be the best substrate for the enzyme, was substantially similar to that of Gen_5 β-*p*NP. Notably, glucose was not detected during any of the reactions with Gen_{4-6} β-*p*NP. This indicates that the non-reducing-end glucosyl terminus of the derivatives with DP 4 or higher is not subject to hydrolysis. The purified β-(1→6)-glucanase acted slightly on Gen_3 β-*p*NP (2% hydrolytic activity relative to 100% for Gen_6 β-*p*NP), which was a poor substrate. By contrast, the cleavage point of derivatives with DP 4 or higher shifted such that the enzyme kept away from the *p*-nitrophenyl group at the terminus. In all the tested substrates, release of *p*-nitrophenol was not observed by the HPLC-based assay and the result also confirmed colorimetrically by a standard end-point assay (data not shown). As a result, the relative enzyme activity for Gen_{4-6} β-*p*NP markedly increased in comparison to that for Gen_3 β-*p*NP.

2.3.2. Gentiooligosaccharides

The enzymatic cleavage of gentiooligosaccharides (DP 2-6) and the relative rate of hydrolysis by the purified enzyme were analyzed by HPAEC-PAD. However, the position of cleavage in the reducing oligosaccharides was not localized from the hydrolysate of the oligomers, because of the lack of aglycon. Taking into account the data for β-gentiooligosides, the frequencies of cleavage of gentiooligosaccharides with DP 4 or higher were proposed on the basis of the assumption that the glucosyl residue at the non-reducing end was not hydrolyzed by the enzyme (Table 6). The major cleavage of Gen_4 occurred at the first (bond 1) and



Scheme 1. Synthesis and purification of methyl β-gentiooligosides.

second (bond 2) glucosidic bonds from the reducing terminus, and the activity was much lower relative to that of Gen_5, suggesting that gentiooligomers of DP 5 and higher are good substrates. Regarding Gen_5 and Gen_6, there was less cleavage at bond 1 as the DP increased, with cleavage shifting to bonds 2 and 3/4, respectively. Gen_2 was found to act only slightly as a substrate.

2.4. Hydrolytic mechanism of β-(1→6)-glucanase on pustulan

2.4.1. NMR analysis

To elucidate the mechanism of pustulan hydrolysis by the enzyme, the stereochemistry of the hydrolysis was investigated by ^{1}H NMR analysis. Figure 3 shows spectra of the time course of the hydrolysis reaction in the 4.6–5.3 ppm region. Before hydrolysis, no signal was observed (Fig. 3A). During the early stage of hydrolysis, doublets at 4.66–4.68 ppm (J = 8 Hz), characteristic of H-1β from gentiooligosaccharides with different chain lengths, appeared and rapidly increased in intensity over time (Fig. 3B and C). Instead of these signals, a small doublet at 5.25 ppm (J = 4 Hz), characteristic of H-1α from the corresponding gentiooligosaccharides, appeared at 1 h after addition of the enzyme and gradually increased over time (Fig. 3C–E). These observations suggest that the β-anomer is initially formed and then easily converted into the α-anomer by mutarotation.

2.4.2. HPAEC-PAD analysis

The manner of the hydrolysis of β-(1→6)-glucan (pustulan) by the purified β-(1→6)-glucanase was analyzed in real time by

Table 3
^{13}C-chemical shifts of methyl β-gentiooligosides (DP 2-6) in D_2O solution (30 °C)

Compound	Residue[a]	Chemical shift (δ)						
		C-1	C-2	C-3	C-4	C-5	C-6	CH_3
Gen_2 β-OMe	CH_3							60.2
	1	106.2	75.87	78.5	72.3	77.7	71.4	
	2	105.7	75.91	78.5	72.5	78.7	63.6	
Gen_3 β-OMe	CH_3							60.2
	1	106.2	75.87	78.5	72.3	77.7	71.6	
	2	105.74	75.87	78.4	72.2	77.8	71.4	
	3	105.69	75.93	78.5	72.5	78.7	63.6	
Gen_4 β-OMe	CH_3							60.2
	1	106.2	75.88	78.5	72.3	77.70	71.6	
	2	105.7	75.88	78.4	72.3	77.74	71.6	
	3	105.7	75.88	78.4	72.3	77.77	71.5	
	4	105.8	75.93	78.5	72.5	78.8	63.6	
Gen_5 β-OMe	CH_3							60.3
	1	106.2	75.89	78.53	72.3	77.7	71.6	
	2	105.7	75.89	78.45	72.3	77.7	71.6	
	3	105.7	75.89	78.45	72.3	77.7	71.6	
	4	105.84	75.89	78.45	72.3	77.7	71.5	
	5	105.77	75.94	78.53	72.5	78.8	63.6	
Gen_6 β-OMe	CH_3							60.3
	1	106.2	75.88	78.5	72.3	77.7	71.6	
	2	105.7	75.88	78.4	72.3	77.7	71.6	
	3	105.7	75.88	78.4	72.3	77.7	71.6	
	4	105.8	75.88	78.4	72.3	77.7	71.6	
	5	105.8	75.88	78.4	72.3	77.7	71.5	
	6	105.8	75.92	78.5	72.5	78.8	63.6	

[a] Numbers indicate the position of the glucose residues; the glucose residue that is attached to the methyl group is represented as 1.

HPAEC-PAD. Pustulan was shown to have an average degree of polymerization ($\bar{DP}$) of 320, within the DP 200–550 range (Fig. 4A). In the early stage (after 3 min) of incubation, the glucan was quickly hydrolyzed to form a broad peak corresponding to $\bar{DP}$ 45 in DP 15–100 range, which was designated a short-chain glucan (Fig 4B). After 10 min of incubation, the peak corresponding to $\bar{DP}$ 45 had decreased to afford a main broad peak of $\bar{DP}$ 15 with minor peaks of DP 2-4 (Fig. 4C). After 30 min of incubation, the resulting oligomer ($\bar{DP}$ 15) was further distributed into two peak groups corresponding to $\bar{DP}$ 10 and DP 2-4 (Fig. 4D). One should remark that the peak of DP 2-4 increased in proportion with great regularity over time, while a low level of glucose was detected during the reaction. The resulting oligomers ($\bar{DP}$ 10) were slowly degraded over time to two peak groups corresponding to $\bar{DP}$ 7 and DP 2-4 after 2 h of incubation (Fig. 4E). In the final stage of the reaction, the cleavage of oligomers (DP 3-4) into glucose and Gen_2 proceeded extremely slowly, and it took prolonged incubation (96 h) to reach this stage (Fig. 4F).

Lastly, the hydrolytic profiles of Gen_8 and Gen_9 (Fig. 5) as reference compounds were compared with the time course of the hydrolysis of pustulan (Fig. 4). Gen_8 was hydrolyzed in the order of DP 2 and DP 6 > DP 3 and DP 5 > DP 4, and Gen_9 in the order of DP 2 and DP 7 > DP 3 and DP 6 > DP 4 and DP 5 (Fig. 5). This indicates that the major cleavages occur at bonds 2, 3, and 4 through a reducing terminus. The cleavage pattern was substantially similar to that of $\bar{DP}$ 10 derived from pustulan (Fig. 4D).

3. Discussion

The aim of the present work was to elucidate the hydrolytic mode of action of purified β-(1→6)-glucanase on a typical linear β-(1→6)-glucan pustulan. For this purpose, the enzyme was purified from the culture filtrate of *P. multicolor*. In this process, substrate affinity chromatography using Gen_4 as a ligand was shown to be a powerful technique for the purification of β-(1→6)-glucanase. The highly purified enzyme (244-fold purified, 164.1 U/mg) gave a single protein band at 51 kDa by SDS–PAGE and was used for analysis of its hydrolytic mode of action on pustulan. The enzyme showed activity only against substrates with a β-(1→6)-glycosidic linkage.

Table 4
^{1}H- and ^{13}C-chemical shifts of *p*-nitrophenyl β-gentiooligosides (DP 2-6) in D_2O solution (25 °C)

Compound	Residue[a]	Chemical shift (δ)											
		C-1	C-2	C-3	C-4	C-5	C-6	*o*-ph	*m*-ph	*p*-ph	C–O	H-1	$J_{1,2}$[b]
Gen_2 β-*p*NP	*p*NP							119.4	128.9	145.4	164.4		
	1	102.2	75.5	78.2	72.0	78.3	71.2					5.29	6.7
	2	105.6	75.9	78.5	72.5	78.7	63.6					4.48	8.0
Gen_3 β-*p*NP	*p*NP							119.5	129.0	145.4	164.4		
	1	102.1	75.6	78.2	72.2	78.1	71.7					5.29	5.5
	2	105.8	75.94	78.4	72.2	77.7	71.3					4.50	8.0
	3	105.6	75.89	78.5	72.5	78.7	63.6					4.46	7.9
Gen_4 β-*p*NP	*p*NP							119.5	129.0	145.4	164.5		
	1	102.1	75.5	78.2	72.16	78.1	71.7					5.30	7.6
	2	105.7	75.92	78.4	72.20	77.7	71.6					4.51	7.9
	3	105.8	75.88	78.4	72.3	77.7	71.5					4.48	7.9
	4	105.7	75.86	78.5	72.4	78.7	63.6					4.46	8.0
Gen_5 β-*p*NP	*p*NP							119.5	129.0	145.5	164.5		
	1	102.2	75.6	78.2	72.19	78.1	71.7					5.31	7.4
	2	105.73	75.91	78.4	72.25	77.72	71.6					4.51	7.9
	3	105.80	75.87	78.4	72.33	77.72	71.8					4.49	8.3
	4	105.83	75.83	78.4	72.21	77.75	71.4					4.47	8.0
	5	105.68	75.93	78.5	72.5	78.7	63.6					4.51	7.9
Gen_6 β-*p*NP	*p*NP							119.5	129.1	145.5	164.5		
	1	102.2	75.6	78.2	72.20	78.1	71.70					5.31	7.7
	2	105.74	75.91	78.4	72.25	77.72	71.65					4.51	7.4
	3	105.8	75.87	78.4	72.34	77.72	71.8					4.49	9.2
	4	105.8	75.84	78.4	72.21	77.74	71.65					4.48	8.0
	5	105.8	75.93	78.4	72.31	77.8	71.4					4.52	7.6
	6	105.71	75.87	78.5	72.5	78.7	63.6					4.52	7.6

[a] Numbers indicate the position of the glucose residues; the glucose residue that is attached to *p*-nitrophenyl group is represented as 1.
[b] $J_{1,2}$, coupling constants given in Hz.

Table 5
Frequency and relative activity of the β-(1→6)-glucanase-catalyzed hydrolysis of methyl β-gentiooligosides and *p*-nitrophenyl β-gentiooligosides

Compound	Frequency of hydrolysis[a] (%)	Relative activity[b] (%)
Gen_2 β-*O*Me	100 G – G – CH_3	4
Gen_3 β-*O*Me	9 91 G – G – G – CH_3	13
Gen_4 β-*O*Me	5 39 56 G – G – G – G – CH_3	67
Gen_5 β-*O*Me	31 30 39 G – G – G – G – G – CH_3	81
Gen_6 β-*O*Me	12 31 30 27 G – G – G – G – G – G – CH_3	100
Gen_2 β-*p*NP	100 G – G – *p*NP	1
Gen_3 β-*p*NP	39 61 G – G – G – *p*NP	2
Gen_4 β-*p*NP	90 10 G – G – G – G – *p*NP	22
Gen_5 β-*p*NP	38 62 G – G – G – G – G – *p*NP	39
Gen_6 β-*p*NP	25 29 46 G – G – G – G – G – G – *p*NP	100

[a] The frequency of the enzymatic cleavage of the indicated glycosidic linkages was estimated by measuring the amount of lower methyl β-gentiooligosides and *p*-nitrophenyl gentiooligosides liberated from the corresponding methyl β-gentiooligosides and *p*-nitrophenyl β-gentiooligosaccharides, respectively.
[b] The hydrolytic rate of Gen_6 β-*O*Me and Gen_6 β-*p*NP was arbitrarily set to 100. G, glucose residue.

Table 6
Frequency and relative activity of the β-(1→6)-glucanase-catalyzed hydrolysis of gentiooligosaccharides

Compound	Frequency of hydrolysis[a] (%)		Relative activity[b] (%)
Gen_2	100 G – G*		<1
Gen_3	a A G – G – G*	a + A = 100 (a < A)	15
Gen_4	68 32 G – G – G – G*		21
Gen_5	a A 22 G – G – G – G – G*	a + A = 78 (a < A)	77
Gen_6	a 22 A 13 G – G – G – G – G – G*	a + A = 65 (a < A)	100

[a] The frequency of the enzymatic cleavage of the indicated glycosidic linkages was estimated by measuring the amount of lower gentiooligosaccharides liberated from the corresponding gentiooligosaccharides.
[b] The hydrolytic rate of Gen_6 was arbitrarily set at 100. G, glucose residue; G*, glucose residue with a reducing end.

The present method for obtaining a series of gentiooligosides (DP 3-6) starting from pre-prepared gentiooligoside (DP 2) incorporated a simple one-pot reaction process and ensured the regioselectivity of glycosylation, although it was not always sufficient. The resulting synthetic methyl β-gentiooligosides and *p*-nitrophenyl β-gentiooligosides were very useful as substrates for providing analytical insight into how the enzyme actually cleaves pustulan. From the cleavage frequencies, it was shown that methyl β-gentiooligosides (DP 2-6) and *p*-nitrophenyl β-gentiooligosides (DP 2-6) were cleaved through the terminal glycoside and that the number of the susceptible bond varied with the chain length of substrate. However, the cleavage pattern of methyl β-gentiooligosides (DP 4-6) was somewhat different from that of *p*-nitrophenyl β-gentiooligosides (DP 4-6). For example, the cleavage of Gen_5 β-*O*Me occurred in almost equal proportion at bonds 2, 3, and 4, whereas Gen_5 β-*p*NP was preferentially cleaved at bonds 3 and 4, but not cleaved at all at bond 2. This indicates that the presence of a methyl group does not influence enzyme action, whereas that of a *p*-nitrophenyl group shifts the cleavage position for one glucosyl residue to the non-reducing end. Taking these data into account, the cleavage frequencies of gentiooligosaccharides were proposed on the basis of the assumption that the glucose residue at the non-reducing terminus of gentiooligosaccharides with DP 4 or higher is not hydrolyzed by the enzyme. The cleavage pattern of gentiooligosaccharides is approximated to that of Gen_n β-*O*Me rather than Gen_n β-*p*NP. Thus, cleavage at bond 1 gradually decreases with extension of the DP and the cleavage location shifts to bonds 2 and 3.As mentioned above, our purpose was to elucidate the mechanism of the hydrolysis of pustulan by β-(1→6)-glucanase based on kinetic and product analyses. Enzymatic hydrolysis of glycosidic bonds usually occurs with two possible stereochemical outcomes namely, inversion or retention of the anomeric configuration at the cleavage site.[25] The hydrolytic mechanism of the enzyme was first evaluated by ^{1}H NMR. In the initial stage of the reaction, the β-anomer was formed and was then easily converted into the α-anomer by mutarotation. This indicates that the hydrolysis of pustulan occurs with retention of the anomeric configuration. Pitson et al.[14] have reported that β-(1→6)-glucanase from *Acremonium persicinum* acts with retention of the anomeric center, releasing products in the β-configuration. Furthermore, the pathway of the hydrolysis of pustulan by the purified enzyme could be proposed on the basis of HPAEC-PAD analysis of the time course of hydrolysate production (Scheme 2). The enzyme was shown to achieve complete hydrolysis of the glucan in three steps as follows. In the initial stage, the enzyme cleaved the glucan with a pattern resembling that of an *endo*-hydrolase to quickly produce a short-chain glucan ($\bar{D}P$ 45) as an intermediate after 3 min of incubation. In the midterm stage, the resulting short-chain glucan was further cleaved into two peak fractions corresponding to DP 15-10 and DP 2-4 within 30 min. During this process, it can be envisaged how the enzyme initially attacks the glucan so quickly. Notably, the relative hydrolytic activity of the enzyme toward Gen_8 was 3, whereas that toward pustulan was 100, representing a 33-fold difference. Once the chain length of DP 15-10 was reached, the preferred cleavage occurred at bonds 2, 3, and 4 from the reducing terminus of the chain to afford DP 2-4 with great regularity. Only a low level of glucose was detected despite the subsequent reaction. In the midterm stage, the cleavage pattern of $\bar{D}P$ 10 derived from pustulan (Fig. 4D) was substantially similar to those of Gen_8 and Gen_9 (Fig. 5). In the final stage of the reaction, the lower oligomers (DP 3 and DP 4) underwent hydrolysis extremely slowly to produce glucose and Gen_2 as the main products, because of the lower hydrolytic activity of the enzyme on Gen_3 and Gen_4. It took prolonged incubation to reach this stage (Fig. 4F). In this way, the enzyme varies its mode of action depending on the chain length derived from the glucan during the whole course of the reaction. Montero et al.[20] demonstrated that a β-(1→6)-glucanase from *Trichoderma harzianum*, namely BGN16.3, has an

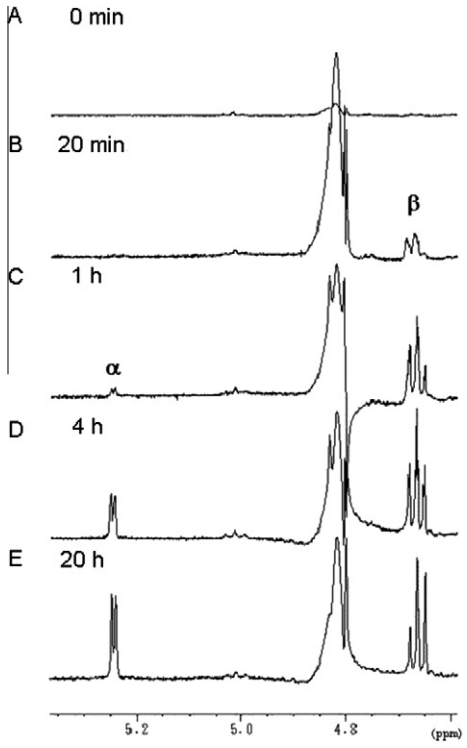


Figure 3. ^{1}H NMR spectra of the time course of pustulan hydrolysis by the purified β-(1→6)-glucanase. (A) Initial substrate, (B) 20 min, (C) 1 h, (D) 4 h, and (E) 20 h incubation after addition of the enzyme. α and β indicate signals from H-1α and H-1β protons, respectively, of gentiooligosaccharides produced by pustulan hydrolysis by the enzyme. The enzyme reaction was performed in a 3-mm sample tube at 25 °C. Pustulan was dissolved in D_2O (0.65%, w/v) and then the enzyme solution (10 μL, 72 mU) was added.

endo-hydrolytic mode of action by HPLC analysis of the products from pustulan.

In conclusion, β-(1→6)-glucanase from *P. multicolor* was purified and the hydrolytic manner of the enzyme toward β-(1→6)-glucan was elucidated in real time during the whole course of the reaction. Longer polymers are initially hydrolyzed by an *endo*-hydrolytic mode of action. In the subsequent reaction, the resulting short-chain glucan is hydrolyzed by a pattern resembling a saccharifying-hydrolase to produce DP 2-4 with great regularity; ultimately, DP 2-4 are cut up to produce glucose and Gen_2. As a result, it has been revealed that the hydrolytic cooperation of both an *endo*-type and a saccharifying-type reaction of a single enzyme, which plays a bifunctional role, leads to complete hydrolysis of the β-(1→6)-glucan pustulan.

4. Experimental

4.1. Materials

A crude enzyme preparation from *P. multicolor* IAM7153 (Institute of Molecular and Cellular Biosciences, The University of Tokyo) was kindly supplied by Amano Enzyme, Inc. (Gifu, Japan). Cellulase from *H. jecorina* was purchased from NagaseChemteX Co. (Osaka, Japan). Gentiobiose, methyl β-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, glucan from *Saccharomyces cerevisiae*, carboxymethylcellulose, and curdlan from *Alcaligenes faecalis* were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pustulan was purchased from Calbiochem (San Diego, CA, USA) and prepared for the removal of insoluble material as follows: the pustulan was dissolved in water (10 mg/mL) and boiled for 10 min. After cooling, the resulting precipitate was removed by centrifugation (5000*g*, 15 min). The supernatant was then lyophilized and used as substrate for the β-(1→6)-glucanase assay. The lyophilized pustulan was also fractionated by 50% ethanol precipitation to afford a water-soluble pustulan for hydrolytic analysis. All other commonly used chemicals were obtained from commercial sources.

4.2. Analytical methods

MALDI-TOF mass spectra were obtained on an AutoFlex instrument (Bruker Daltonics, Bremen, Germany). The spectra were measured in positive linear mode with 2,5-dihydroxybenzoic acid (10 mg/mL) in H_2O/ethanol (70:30) as the matrix solution. The

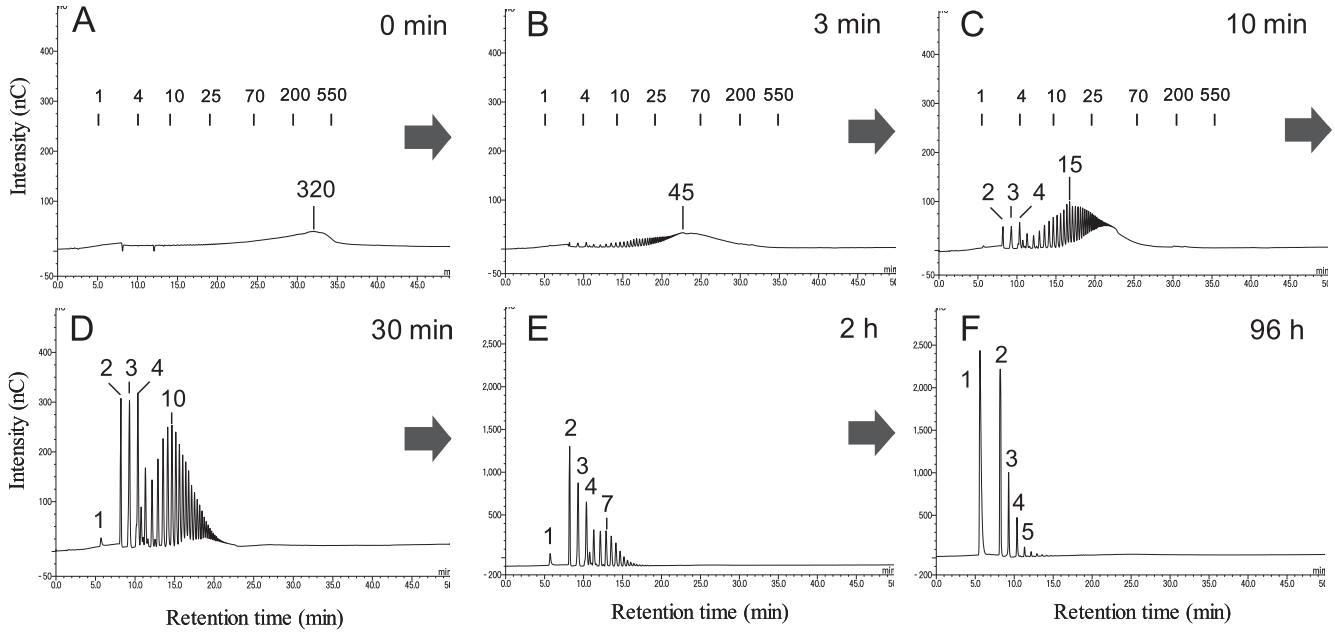


Figure 4. HPAEC-PAD analysis of the hydrolytic products of pustulan obtained with the purified β-(1→6)-glucanase. (A) Initial substrate, (B) 3 min, (C) 10 min, (D) 30 min, (E) 2 h, and (F) 96 h after incubation after the addition of enzyme. Numbers indicate the DP of gentiooligosaccharides.

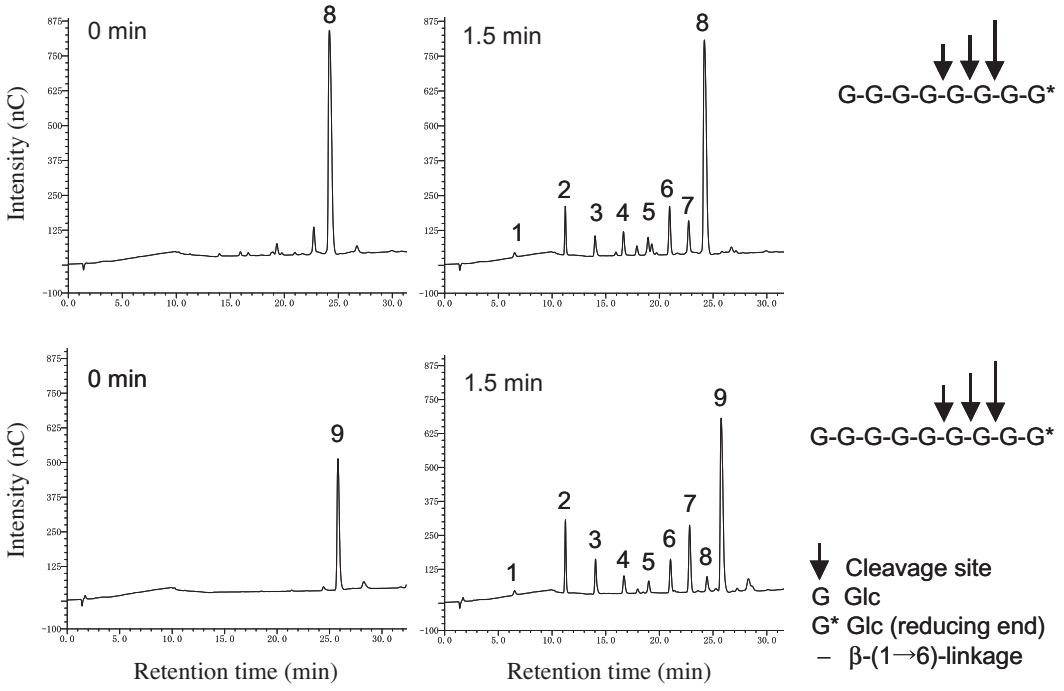


Figure 5. HPAEC-PAD analysis of the hydrolytic products of Gen_8 (top) and Gen_9 (bottom) obtained with the purified β-(1→6)-glucanase. Left, initial substrate; right, 1.5 min after the addition of enzyme. Numbers indicate the DP of gentiooligosaccharides.

Scheme 2. Proposed mechanism of the hydrolytic action of β-(1→6)-glucanase from *P. multicolor* on pustulan.

sample solutions were mixed with the matrix solution (1:4 v/v), and then a 1-μL droplet was applied to a stainless target plate and dried at room temperature. A mass calibration procedure was carried out before the analysis of samples by using protein calibration standard II (Bruker Daltonics). The HPLC analysis was carried out on an Asahipak GS-220 HQ column (7.5 × 300 mm, Japan) with a Jasco Intelligent system liquid chromatograph and detection at 300 nm. The bound material was eluted with H_2O at a flow rate of 0.9 mL/min at 40 °C. HPAEC-PAD analysis was carried out using a DIONEX DX-500 system (Dionex, Sunnyvale, CA) fitted with a PAD (ED-40) and a CarboPac PA1 column (4.0 × 250 mm). Bound material was eluted by the following gradients. Condition 1: 0–40 min, the ratio of eluent A was decreased from 60% (v/v) to 45% (v/v), the ratio of eluent B was increased from 0% (v/v) to 15% (v/v), and eluent C was kept at 40% (v/v); 40–50 min, 100% (v/v) eluent B; 50–60 min, 60% (v/v) eluent A, and 40% (v/v) eluent C. Condition 2: 0–50 min, the ratio of eluent A was decreased from 60% (v/v) to 0% (v/v), the ratio of eluent B was increased from 0% (v/v) to 60% (v/v), and eluent C was kept at 40% (v/v); 50-60 min, 100% (v/v) eluent B; 60–70 min, 60% (v/v) eluent A, and 40% (v/v) eluent C. In both conditions, eluents A, B, and C were deionized water, 0.1 M NaOH containing 1 M sodium acetate, and 0.2 M NaOH, respectively. The flow rate was 1.0 mL/min. ^1H and ^{13}C NMR spectra of each sample in D_2O were recorded on a JEOL JNM-LA 500 spectrometer. Chemical shifts (δ) were expressed relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an external standard. ESI-MS spectra were recorded on a JMS-T100LC mass spectrometer. The samples were injected in methanol (0.2–0.5 mg/mL) directly into the spectrometer.

4.3. Enzyme assays

β-(1→6)-Glucanase was assayed as follows. A mixture containing 180 μL of substrate (5 mg/mL of pustulan) in 50 mM sodium acetate buffer (pH 5.5) and 20 μL of an appropriate amount of enzyme was incubated for 15 min at 40 °C. The reducing sugars

produced were determined directly by Somogyi-Nelson's method.[26,27] One unit was determined as the amount of enzyme catalyzing the formation of 1 μmol of D-glucose per min from the substrate under the conditions of the assay. β-Glucosidase was measured as follows: 100 μL of 10 mM *p*-nitrophenyl β-D-glucoside solution, 250 μL of 100 mM sodium acetate buffer (pH 4.0), and 125 μL of H_2O were premixed. Then, enzyme solution (25 μL) was added to the solution, which was kept at 40 °C for 15 min. One-tenth of the reaction mixture was removed at 5-min intervals and immediately transferred to a microplate containing 50 μL of 1 M Na_2CO_3 to stop the reaction, and the *p*-nitrophenol liberated was determined spectrophotometrically at 420 nm. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min.

4.4. Preparation of Gen_4-coupled gel

Gen_4-coupled Toyopearl AF-Amino-650M was prepared as follows: Gen_4 (580 mg in 2 mL of 0.2 M K_2HPO_4) was added to preswollen gel (1 g) and then $NaCNBH_3$ (200 mg) was added. The gel in the solution was incubated at 60 °C overnight with gentle agitation, and then washed with water (20 mL) three times and sodium borate buffer (20 mL, pH 8.2) three times. For acetylation of the free amino group, 0.2 M sodium acetate (8 mL) and acetic anhydride (4 mL) were added to the gel and allowed to react for 30 min at 0 °C. Next, acetic anhydride (4 mL) was added to the solution containing the gel, which was then kept for 30 min at room temperature with gentle agitation. The gel was washed successively with H_2O, 0.1 M NaOH, and H_2O (three times for each) and packed into a column (1 mL).

4.5. Purification of β-(1→6)-glucanase

Unless otherwise indicated, all steps were carried out at 4 °C. The *P. multicolor* crude enzyme (720 mg) was dissolved in 50 mM sodium acetate buffer (pH 5.5), brought to 60% saturation with solid ammonium sulfate, and left to stand for 30 min. The precipitate was removed by centrifugation (5000 g, 10 min). The supernatant was brought to 80% saturation with solid ammonium sulfate and left to stand for 30 min. The precipitate was dissolved in 50 mM sodium acetate buffer (10 mL, pH 5.5), dialyzed against H_2O, and lyophilized. The enzyme powder (80 mg) was dissolved in 20 mM sodium acetate buffer (1 mL, pH 4.0), applied to a CM-Sepharose Fast Flow column (2.5 × 30 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with the same buffer (200 mL) at a flow rate of 1 mL/min. The eluent was collected in 10-mL fractions. β-(1→6)-Glucanase activity was measured by a standard assay using each fraction as an enzyme solution. Fractions showing β-(1→6)-glucanase activity (tubes 6–10) were pooled and concentrated with an Amicon PM-30 membrane. The concentrated enzyme fraction was applied to a Gen_4-coupled affinity column (1 mL) equilibrated with 20 mM sodium acetate buffer (pH 5.5) containing 45 mM NaCl. After the column was washed with 20 mL of equilibration buffer, the adsorbed material was eluted with a linear gradient from 45 to 800 mM NaCl in the same buffer over a volume of 20 mL, and then with 800 mM NaCl in the same buffer at a flow rate of 0.1 mL/min. The eluent was collected in 0.5-mL fractions. Fractions showing β-(1→6)-glucanase activity (tubes 52–54) were combined and concentrated with a Nanosep 10k Omega membrane. The enzyme purity was checked by SDS–PAGE.

4.6. Characterization of β-(1→6)-glucanase

The effect of pH on the activity of the purified β-(1→6)-glucanase was measured under the standard conditions of pustulan hydrolysis while changing the pH of the 50 mM buffers used in the reaction mixture. The pH stability was determined by incubating the enzyme at 4 °C for 24 h at each pH, and then measuring the activity under the standard conditions. The buffers used were Tris–HCl (pH 2.0–3.0), sodium acetate (pH 3.0–6.0), and sodium phosphate (pH 7.0–9.0). The optimum temperature of the activity was measured under the standard conditions at various temperatures (4–80 °C). Thermostability was determined by incubating the enzyme at each temperature for 1 h in 50 mM sodium acetate buffer (pH 5.5), and then measuring the activity under the standard conditions.

4.7. Enzymatic synthesis of methyl β-gentiooligosides (DP 2-6, Gen_{2-6} β-*O*Me)

Glcβ-*O*Me (15 g, 77.2 mmol) was dissolved in 21.2 mL of 100 mM sodium acetate buffer (pH 4.0) and β-glucosidase (17.4 mL, 776 U) in crude cellulase from *H. jecorina* was added. The solution was incubated without stirring for 23 h at 40 °C, and the reaction was terminated by boiling for 5 min. The reaction mixture was applied to a charcoal-Celite column (4.5 × 50 cm) equilibrated with water. The column was washed with 1.5 L of water and eluted with a linear gradient of 0% (3 L) to 7.5% (3 L) ethanol (flow rate 7 mL/min; a fraction size 50 mL/tube). Elution was monitored at 485 nm, with carbohydrate content determined by the phenol–H_2SO_4 method. The eluate (550–4115 mL) was concentrated and applied to charcoal-Celite chromatography again. The eluate (850–1550 mL) was concentrated and lyophilized, and afforded 770 mg of Gen_2 β-*O*Me[28] in 5.6% yield based on the Glcβ-*O*Me added. As an acceptor and a donor, the synthetic Gen_2 β-*O*Me (627 mg, 1.8 mmol) was dissolved in 340 μL of 50 mM sodium acetate buffer (pH 4.0) and purified β-(1→6)-glucanase (100 μL, 28.3 mU) was added. After the solution was incubated for 60 h at 50 °C, the reaction mixture was boiled for 15 min. To remove reducing sugar in the reaction mixture by chromatography, pyridylamination was performed as follows: Solution A was prepared by dissolving 0.5 g of 2-aminopyridine in 380 μL of 35% HCl. Solution B was prepared by dissolving 40 mg of $NaBH_3CN$ in Solution A and adding 100 μL of H_2O. Solution A was added to the reaction mixture, kept at 100 °C for 15 min, and then 295 μL of solution B was added. The mixture was incubated for 24 h at 70C. After pyridylamination, the mixture was loaded onto a TOYOPEARL HW-40S column (4.5 × 90 cm) equilibrated with 25% methanol at a flow rate of 1.5 mL/min. The eluate (1125–2475 mL) was collected, evaporated to remove methanol, and loaded onto a charcoal-Celite column (4.5 × 90 cm) equilibrated with H_2O. The column was washed with 1.5 L of water and then eluted with a linear gradient of 5% (5 L) to 35% (5 L) ethanol (flow rate, 7 mL/min; fraction size, 50 mL/tube). The eluate showed six main peaks, MG_1 (tubes 18–26), MG_2 (tubes 62–73), MG_3 (tubes 95–103), MG_4 (tubes 108–115), MG_5 (tubes 120–125), and MG_6 (tubes 129–134), which were concentrated and lyophilized. Fraction MG_1 was recovered as Glc β-*O*Me (142.3 mg) and fraction MG_2 was recovered as Gen_2 β-*O*Me (242.2 mg, 38.6% recovery). Fractions MG_3, MG_4, MG_5, and MG_6 gave Gen_3 β-*O*Me (63.4 mg, 7.0%), Gen_4 β-*O*Me (51.5 mg, 4.3%), Gen_5 β-*O*Me (22.9 mg, 1.5%), and Gen_6 β-*O*Me (15.0 mg, 0.8%), respectively.

The structures of synthetic Gen_2–Gen_6 β-*O*Me were evaluated by ^1H and ^{13}C NMR analyses in D_2O. Characteristic signals were commonly observed in the region 4.20–4.55 ppm. H-1β signals of the products were observed at 4.39–4.42 ppm (d, $J_{1,2}$ = 8 Hz). The peaks of internal anomeric protons were observed as overlapping signals at 4.55–4.51 ppm (d, $J_{1,2}$ = 8 Hz), characteristic of the β-(1→6)-linkage from the corresponding methyl β-gentiooligosides. Furthermore, overlapping signals at 4.21–4.25 ppm were observed. The signals were indicative of low-field shifts of internal H-6 protons. In addition, peaks of methyl group were observed at

3.58–3.60 ppm (s, 3H). The structures of these methyl β-gentiooligosides were further confirmed by ^{13}C NMR analysis, as shown in Table 3. Each peak could be assigned to the corresponding carbon atom of a methyl β-oligoside with a β-(1→6) linkage. No signals derived from other linkages were detected. In addition, ESI-MS analysis of Gen_2 β-*O*Me, Gen_3 β-*O*Me, Gen_4 β-*O*Me, Gen_5 β-*O*Me, and Gen_6 β-*O*Me showed molecular ions at *m/z* 379.12125 (Calcd for $C_{13}H_{24}NaO_{11}$, 379.12163), 541.17498 (Calcd for $C_{19}H_{34}NaO_{16}$, 541.17445), 703.22668 (Calcd for $C_{25}H_{44}NaO_{21}$, 703.22728), 865.28050 (Calcd for $C_{31}H_{54}NaO_{26}$, 865.28010), and 1027.33524 (Calcd for $C_{37}H_{64}NaO_{31}$, 1027.33292), respectively, arising from the $[M+Na]^+$ ions.

4.8. Enzymatic synthesis of *p*-nitrophenyl β-gentiooligosides (DP 3-6, Gen_{3-6} β-*p*NP)

Glc β-*p*NP (1356 mg, 4.5 mmol) was dissolved in 33.7 mL of 83 mM sodium acetate buffer (pH 4.0) and β-glucosidase (11.3 mL, 24.9 U) in crude cellulase from *H. jecorina* was added. The mixture was incubated without stirring for 6 h at 40 °C. The reaction was terminated by boiling for 15 min, and the solution was loaded onto a Toyopearl HW-40S column (6 × 100 cm) equilibrated with 25% (v/v) methanol at a flow rate of 1.5 mL/min. The eluate was collected in 25-mL fractions and monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group). Peak fractions (3150–3400 mL) containing the target product were pooled, concentrated, and lyophilized. Gen_2 β-*p*NP[29] (158.1 mg) was obtained in 15.2% yield based on the initial substrate. The synthetic Gen_2 β-*p*NP (278 mg, 0.6 mmol) and Gen_3 (757 mg, 1.5 mmol) were dissolved in 2.8 mL of 55 mM sodium acetate buffer (pH 5.5) and *P. multicolor* β-1,6-glucanase solution (0.2 mL, 2.4 U) was added. The mixture was incubated for 116 h at 4 °C. The reaction was terminated by boiling for 15 min and loaded onto a Toyopearl HW-40S column (6 × 100 cm) equilibrated with 25% methanol at a flow rate of 1.5 mL/min (25 mL/tube). The chromatogram showed five peaks eluted in the following order: PG_6 (1700–1800 mL), PG_5 (1850–1950 mL), PG_4 (2075–2200 mL), PG_3 (2450–2550 mL), and PG_2 (3300–3525 mL). Fractions PG_4, PG_3, and PG_2 were individually concentrated and lyophilized to afford Gen_4 β-*p*NP (66.9 mg), Gen_3 β-*p*NP (7.7 mg), and Gen_2 β-*p*NP (159.7 mg), respectively, in 14.2%, 2.1%, and 57.4% yields based on the acceptor. PG_6 and PG_5 were separately concentrated and loaded onto a reverse-phase ODS column (1.5 × 3.1 cm) equilibrated with H_2O. After washing the column with H_2O (30 mL), the adsorbed glucoside was eluted with methanol (30 mL), and the eluate was collected in 2-mL fractions and monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group). Peak fractions (32-50 mL) were individually combined, concentrated, and lyophilized to afford Gen_6 β-*p*NP (7.8 mg) and Gen_5 β-*p*NP (14.9 mg) in 1.7% and 2.6% yields, respectively, based on the acceptor.

The structures of synthetic Gen_2-Gen_6 β-*p*NP were evaluated by ^1H NMR and ^{13}C NMR as in Table 4. ESI-MS analysis of Gen_2 β-*p*NP, Gen_3 β-*p*NP, Gen_4 β-*p*NP, Gen_5 β-*p*NP, and Gen_6 β-*p*NP showed molecular ions at *m/z* 486.12527 (Calcd for $C_{18}H_{25}NNaO_{13}$, 486.12236), 648.17548 (Calcd for $C_{24}H_{35}NNaO_{18}$, 648.17518), 810.22788 (Calcd for $C_{30}H_{45}NNaO_{23}$, 810.22800), 972.28091 (Calcd for $C_{36}H_{55}NNaO_{28}$, 972.28083), and 1134.33385 (Calcd for $C_{42}H_{65}NNaO_{33}$, 1134.33365), respectively, arising from the $[M+Na]^+$ ions.

4.9. Action of β-(1→6)-glucanase

The hydrolytic activity of purified β-(1→6)-glucanase was evaluated by using several glucans and an oligosaccharide (Gen_8) under standard conditions. Before the assay, Gen_8 was dissolved in H_2O and then the reducing power in the solution was adjusted by H_2O to match that of the pustulan solution (5 mg/mL). The hydrolysate and enzyme activity on these substrates was determined by measuring the amount of reducing sugar by the Somogyi-Nelson method.[26,27] Methyl β-gentiooligosides (DP 2-6), *p*-nitrophenyl β-gentiooligosides (DP 2-6), and gentioligosaccharides (DP 2-6) were used to analyze the frequency of the enzymatic cleavage of the β-(1→6)-linkage and relative hydrolytic rate. Each substrate was dissolved in H_2O (25 μL, 10 mM) and mixed with 25 mM sodium acetate buffer (200 μL, pH4.0) and then added to the enzyme solution (25 μL, 59 mU). The reaction was conducted at 40 °C. The amount of each product formed at an early stage (within 27% hydrolysis) from the initial substrate during incubation with the enzyme was analyzed by HPAEC-PAD (Gen_{2-6} β-*O*Me and Gen_{2-6}, condition 1) and HPLC (Gen_{2-6} β-*p*NP). From the peak areas on the chromatogram of the digest of each substrate with enzyme, the amount of the products was calculated. The amount of each product increased linearly with time in the initial stage of the reaction. On the basis of these data, the frequency and relative hydrolytic rate of the β-(1→6)-glucanase-catalyzed cleavage of glycosidic linkages were determined.

The pattern of pustulan hydrolysis by the enzyme was analyzed as follows: purified pustulan was dissolved in H_2O (900 μL, 5 mg/mL) and then added to enzyme solution (100 μL, 53 mU) in 50 mM sodium acetate buffer (pH 5.5). The reaction was performed at 40 °C and monitored by HPAEC-PAD analysis (condition 2). The hydrolytic profile of Gen_8 and Gen_9 was analyzed as follows: each substrate was dissolved in H_2O (25 μL, 10 mM), mixed with 25 mM sodium acetate buffer (200 μL, pH 4.0) and then added to enzyme solution (25 μL, 59 mU). The reaction was conducted at 40 °C. The amount of each product formed at an early stage (Gen_8; 10% hydrolysis, Gen_9; 35% hydrolysis) was analyzed by HPAEC-PAD (condition 1)

DP of pustulan was estimated as follows: purified pustulan was hydrolyzed by β-(1→6)-glucanase and the hydrolysate was analyzed by HPAEC-PAD (condition 2). Standard curve was generated from the retention time of clearly resolved peaks and corresponding DPs that have less than 56. Higher DPs were calculated from the extrapolated standard curve.

Acknowledgments

We thank Amano Enzyme, Inc. (Gifu, Japan) for the gift of the enzyme preparation from *P. multicolor*. This work was supported by a research grant (Agribusiness) from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.11.002.

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