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The first bacterial β-1,6-endoglucanase from *Saccharophagus degradans* 2-40^T for the hydrolysis of pustulan and laminarin

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Abstract β-1,6-glucan is a polysaccharide found in brown macroalgae and fungal cell walls. In this study, a β-1,6-endoglucanase gene from *Saccharophagus degradans* 2-40^T, *gly30B*, was cloned and overexpressed in *Escherichia coli*. Gly30B, which belongs to the glycoside hydrolase family 30 (GH30), was found to possess β-1,6-endoglucanase activity by hydrolyzing β-1,6-glycosidic linkages of pustulan (β-1,6-glucan derived from fungal cell walls) and laminarin (β-1,3-glucan with β-1,6-branchings, derived from brown macroalgae) to produce gentiobiose and glucose as the final products. The optimal pH and temperature for Gly30B activity were found to be pH 7.0 and 40 °C, respectively. The kinetic constants of Gly30B, V_{max}, K_M, and k_{cat} were determined to be 153.8 U/mg protein, 24.2 g/L, and 135.6 s^{-1} for pustulan and 32.8 U/mg protein, 100.8 g/L, and 28.9 s^{-1} for laminarin, respectively. To our knowledge, Gly30B is the first β-1,6-endoglucanase characterized from bacteria. Gly30B can be used to hydrolyze β-1,6-glucans of brown algae or fungal cell walls for producing gentiobiose as a high-value sugar and glucose as a fermentable sugar.

Keywords β-1,6-endoglucanase · GH30 · Hydrolysis · *Saccharophagus degradans* 2-40^T

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Introduction

β-1,6-glucans, which consist of glucose residues linked by β-1,6 glycosidic linkages, are unique and essential polysaccharides in fungal cell walls. β-1,6 glucans show a branched and amorphous structure whereas β-1,3-glucans form a crystalline and microfibrillar structure (Kollár et al. 1997). For instance, in *Saccharomyces cerevisiae*, β-1,6-glucans covalently link mannoproteins in the outer part of the wall to the major structural polysaccharides found in the inner wall: β-1,3-glucan and chitin (Boisramé and Gaillardin 2009). Moreover, glycosyl phosphatidylinositol (GPI)-anchored proteins are covalently linked to β-1,6-glucans through a component of their anchor (Ruiz-Herrera et al. 2006), which might play a significant role for the biosynthesis and integrity of fungal cell walls (Klis 1994). However, β-1,6-glucan polysaccharides are naturally less abundant than other β-glucan polysaccharides such as β-1,4- and β-1,3-glucans. Pustulan, a compound isolated from the lichen *Lasallia pustulata*, is a typical and well-known β-1,6-glucan (Nishikawa et al. 1970). Laminarin, a β-1,3-1,6-glucan, is the major storage carbohydrate in brown macroalgae. Laminarin consists of β-1,3-glucans in the backbone and β-1,6-glucans in the branches. For example, laminarin from *Eisenia bicyclis*, a brown macroalga, is composed of β-1,3- and β-1,6-glycosidic linkages in a ratio of 2:1 (Shin et al. 2009).

β-1,6-glucanase (EC 3.2.1.75), which randomly hydrolyzes 1,6-glycosidic linkages of β-glucans, was found in glycoside hydrolase (GH) families 5 and 30 in the Carbohydrate Active enZymes database (CAZy; http://www.cazy.org/). The β-1,6-glucanase mechanism of action has been described as lytic against yeast and fungal cell walls in filamentous fungi (Yamamoto et al. 1974) and bacteria (Rombouts and Phaff 1976). β-1,6-glucanase is also used for disassociating cell wall mannoproteins since these proteins are retained in the

protein-glucan compartment through a phosphodiester linkage between a remnant of their glycosylphosphatidylinositol anchor and the β-1,6-glucan polysaccharides (Boisramé and Gaillardin 2009). β-1,6-glucanases are widely distributed among filamentous fungi. Several have been cloned, purified, and characterized, such as BGN16.2 and BGN16.3 from *Trichoderma harzianum* (Lora et al. 1995; Montero et al. 2007), GcnA from *Epichloë festucae* (Bryant et al. 2007), NEG1 from *Aspergillus fumigatus* (Boisramé and Gaillardin 2009), and Tvbgn3 from *Trichoderma virens* (Djonović et al. 2006). However, all these enzymes were found in fungi, and no bacterial β-1,6-glucanase has been reported so far.

The marine bacterium *Saccharophagus degradans* 2-40^T is one of the most versatile oceanic carbohydrate degraders, and can degrade at least ten complex polysaccharides, including cellulose, agarose, alginate, chitin, laminarin, fucoidan, pectin, and xylan (Ekborg et al. 2005; Howard et al. 2003). Various polysaccharide-depolymerizing enzymes such as cellulases, xylanases, agarases, and alginate lyases of *S. degradans* 2-40^T have been identified and characterized (Taylor et al. 2006; Ko et al. 2009; Kim et al. 2010, 2012; Ha et al. 2011; Hutcheson et al. 2011). However, there are no reports of β-1,6-glucanase activity in *S. degradans* 2-40^T. In this study, a β-1,6-glucanase was cloned and overexpressed in *Escherichia coli*, and the enzymatic properties of the recombinant β-1,6-glucanase were characterized. To our knowledge, this is the first report of a β-1,6-glucanase from bacteria.

Materials and methods

Cloning of *gly30B* from *S. degradans* 2-40^T

S. degradans 2-40^T (ATCC 43961) was cultivated in minimal broth containing 23 g/L Instant Ocean Sea Salt (Aquarium Systems, Mentor, OH, USA), 50 mM Tris–HCl, 2 g/L glucose, 1 g/L yeast extract, and 0.5 g/L ammonium chloride at 30 °C for 12 h. Cells were harvested by centrifugation at 4000× *g* for 15 min, and genomic DNA was obtained using a commercial DNA isolation kit (Qiagen, Valencia, CA, USA). The target gene *gly30B* (GenBank ID: ABD82251.1) was amplified by PCR using Solg 2X Taq PCR smart mix 2 (SolGent, Daejeon, Korea), and the primers used were 5'-GCGGGATCCCACCACCACCACCACCACCAATACTGGTTAACCAGCGGTGATCTAAGT-3' (forward) and 5'-GCGCTCGAGGTGGTGGTGGTGGTGGTGATCTATAACTAGCGTTACAACGCTCTGTGC-3' (reverse), with the underlined regions indicating *Bam*HI and *Xho*I restriction sites, respectively. A sequence encoding six histidines was added in the reverse primer to enhance the affinity of the recombinant protein for the HisTrap column used for protein purification (GE Healthcare, Piscataway, NJ). The PCR products of *gly30B* and pET28a + vectors (EMD Millipore, Billerica, MA) were both double digested using *Bam*HI and *Xho*I, the fragments were ligated using T4 DNA ligase (New England BioLabs, Ipswich, MA), and the resulting plasmid harboring the *gly30B* gene was transformed into *Escherichia coli* BL21(DE3) (New England BioLabs, Ipswich, MA).

Expression and purification of the recombinant Gly30B

Recombinant *E. coli* BL21(DE3) harboring *gly30B* was grown in Luria–Bertani broth (BD, Sparks, MD, USA) containing 50 mg/L kanamycin in a 37 °C incubator with shaking at 200 rpm until the absorbance at 600 nm of the culture reached 0.6. The expression of *gly30B* was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C for 18 h with shaking at 180 rpm. Cells were harvested by centrifugation at 4000 × *g* for 15 min, then membranes were disrupted by ultrasonication and were centrifuged again at 16,000 × *g* for 1 h. The cell-free supernatant obtained from the centrifugation was passed through a HisTrap column (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's protocol. Purified recombinant protein was concentrated using an Amicon Ultra Centrifugal filter unit (UFC903024, MW cutoff of 30,000; Millipore, Billerica, MA, USA). The concentration of purified protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

Determination of Gly30B enzymatic activity

To determine the activity of the recombinant Gly30B obtained from *E. coli*, 1.89 nmol of Gly30B was incubated in 100 μL of 20 mM Tris-HCl buffer (pH 6.0) containing 2 % (w/v) pustulan (InvivoGen, San Diego, CA, USA) or laminarin (TCI, Tokyo, Japan) at 40 °C for 30 min. The enzymatic reaction was quenched by immersing the reaction mixture in boiling water for 5 min. Relative enzyme activity was measured by using the dinitrosalicylic acid (DNS) method (Miller 1959) using glucose as a standard. One unit of Gly30B was defined as the quantity of enzyme required to release 1 μmol of reducing sugar (based on glucose) per min in the above enzymatic reaction conditions.

Screening for the substrate specificity of Gly30B

To determine the substrate specificity of Gly30B, 1 % (w/v) of various glucans including pustulan, laminarin, curdlan (Wako, Osaka, Japan), carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA), and xylan (Sigma-Aldrich, St Louis, MO, USA) were incubated with 10.5 μM Gly30B in 20 mM Tris − HCl buffer (pH 6.0) at 40 °C for 30 min. The released reducing sugars were quantified by the DNS method as described above.

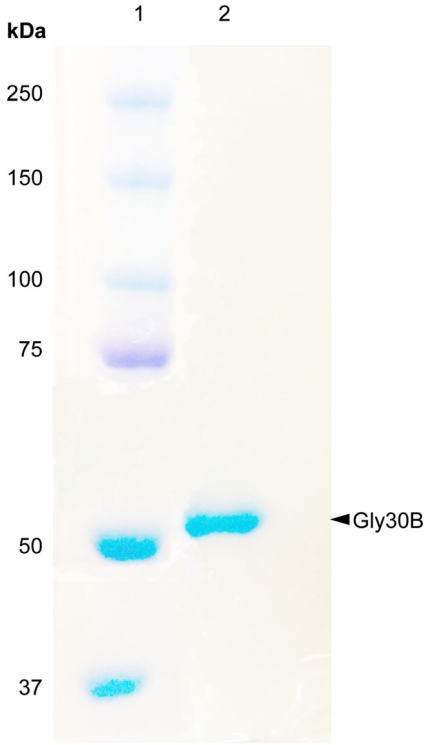

Fig. 1 SDS–PAGE of recombinant Gly30B. *Lane 1*, the protein marker; *lane 2*, the purified Gly30B

Enzyme characterization of Gly30B

To determine the optimum temperature for Gly30B activity, the enzyme was incubated with 2 % (w/v) laminarin as the substrate in 20 mM Tris–HCl buffer (pH 6.0) at temperatures ranging from 20 to 70 °C for 30 min, the enzymatic reactions were quenched in boiling water for 5 min, and the products were quantified by the DNS method. To determine the optimum pH of Gly30B, the enzyme was incubated with 2 % (w/v) laminarin as the substrate in various pH buffers including 20 mM glycine–HCl (pH 2.0 to 4.0), 20 mM sodium acetate (pH 4.0 to 6.0), 20 mM Tris–HCl (pH 6.0 to 9.0), and 20 mM glycine–NaOH (pH 9.0 to 10.0) at 40 °C for 30 min. To avoid the possible pH effect of the DNS analysis, the 2-cyanoacetamide method was used to quantify the post-reaction sugar products. The kinetic parameters (V_{max}, K_M, and k_{cat}) of Gly30B reactions were determined from the Lineweaver–Burk plot, in which the enzyme activity was measured using pustulan and laminarin at concentrations ranging from 0.45 to 9.1 % (w/v) under the optimized pH and temperature determined earlier in the study.

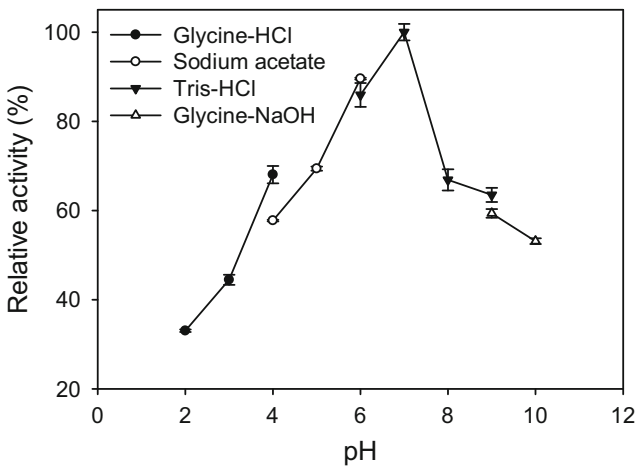

Fig. 2 Effect of pH on the enzymatic activity of Gly30B. Enzymatic reactions were performed with 2 % (w/v) laminarin as a substrate at 40 °C for 30 min at different pHs using various buffers such as 20 mM glycine–HCl (pH 2.0–4.0), sodium acetate (pH 4.0–6.0), Tris–HCl (pH 6.0–9.0), and glycine–NaOH (pH 9.0–10.0). *Error bars* indicate standard deviations of three experimental replicates

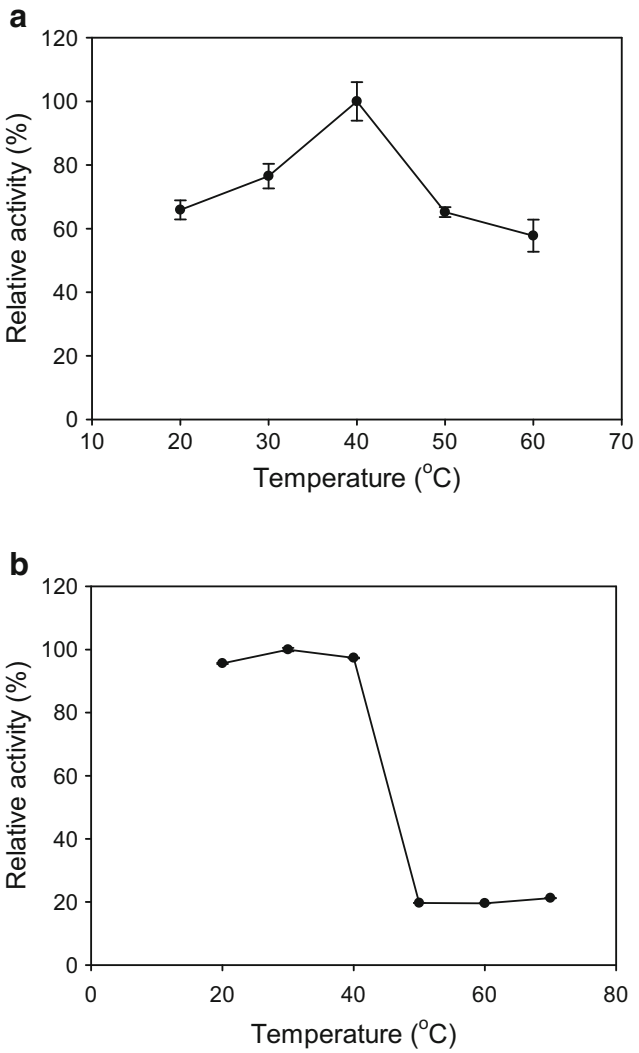

Fig. 3 (**a**) Relative enzymatic activity and (**b**) thermal stability of Gly30B at different temperatures before enzymatic reactions which were performed with 2 % (w/v) laminarin as a substrate at pH 7.0 for 30 min. *Error bars* indicate standard deviations of three experimental replicates

Table 1 Substrate specificity of the recombinant Gly30B

Substrate	Main glycosidic linkage type	Monomeric sugar	Relative enzymatic activity (%)
Pustulan	β-1,6	Glucose	100
Laminarin	β-1,3: β-1,6	Glucose	22.37
Curdlan	β-1,3	Glucose	ND
β-glucan (barely)	β-1,3: β-1,4	Glucose	ND
Xylan	β-1,4	Xylose	ND
Carboxymethyl (CMC)	β-1,4	Glucose	ND

ND not detected

Analyses of the enzymatic reaction products by TLC and HPLC

To monitor the reaction progress of Gly30B over time, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used. For TLC analysis, reaction products were developed on a silica gel 60 plate (Merck, Darmstadt, Germany) with a mobile phase consisting of *n*-butanol/acetic acid/water (3:2:2 by volume), then visualized by incubation with an orcinol reagent (Kawai et al. 2004) followed by heating the TLC plate at 130 °C for 5 min to develop color.

The reaction products were also analyzed using an Agilent 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a gel permeation and ligand exchange column (KS-802, Shodex, Japan) and a refractive index detector (Agilent). HPLC analysis was performed at 80 °C using distilled water as the mobile phase at a flow rate of 0.5 mL/min. Laminaribiose (degree of polymerization (DP) of 2 [DP2]), laminaritriose (DP3), laminaritetraose (DP4), laminaripentaose (DP5), and laminarihexaose (DP6) (all form Megazyme, Wicklow, Ireland) were used as standards.

Results

Amino acid sequence analysis of Gly30B

The gene sequence of *gly30B* (GenBank accession No. ABD82251.1) was obtained from *S. degradans* 2-40^T (NCBI database, http://www.ncbi.nlm.nih.gov/protein/ABD82251.1). The *gly30B* gene encodes a 481-amino acid protein with a calculated molecular mass of 52.8 kDa, and it belongs to GH30 based on its amino acid sequence. This protein shows high amino acid sequence similarity with other GH30 proteins such as glycosyl hydrolase D770_25550 (GenBank accession No. AHM63355.1) from *Flammeovirgaceae bacterium* 311 containing 485 amino acids (52.9 kDa), glucuronoxylanase xynC (GenBank accession No. EMR03163.1) from *Cesiribacter andamanensis* AMV16 containing 490 amino acids (53.2 kDa), glucosylceramidase Q763_08945 (GenBank accession No. KGO81198.1) from *Flavobacterium beibuense* F44-8 with 471 amino acids (52.2 kDa), and glucan endo-1,6-β-glucosidase Pedsa_3076 (GenBank accession No. ADY53615.1) from *Pedobacter saltans* with 481 amino acids (52.8 kDa). The Gly30B amino acid sequence of *S. degradans* 2-40^T was found to be more than 50 % similar to the aforementioned GH30 enzymes.

Overexpression and purification of recombinant Gly30B in *E. coli*

According to the SignalIP prediction (www.cbs.dtu.dk/services/SignalP/), Gly30B contains a signal peptide composed of 36 amino acids. The signal peptide gene from *gly30b* was removed to facilitate the overexpression of the recombinant protein in a heterologous system, *E. coli*. After induction with 0.5 mM IPTG at 16 °C, most of the recombinant protein was expressed in soluble form. The molar mass of the recombinant Gly30B expressed in *E. coli* was estimated to be approximately 52 kDa using SDS-PAGE

Table 2 Effect of various cations on the enzymatic activity of Gly30B

Cation	Relative enzymatic activity (%)
Control	100 ± 0.8
K^+	93.3 ± 1.1
Na^+	94.5 ± 1.4
Mg^{2+}	57.0 ± 2.5
Ca^{2+}	99.0 ± 0.7
Mn^{2+}	85.0 ± 1.2
Ni^{2+}	54.7 ± 1.7
Cu^{2+}	29.4 ± 0.2
Fe^{2+}	61.5 ± 1.9
Co^{2+}	70.9 ± 3.1

The enzymatic activity with no cations was set as 100 %

Experimental data are represented as mean ± standard deviation from triplicate experiments

(Fig. 1), which agreed well with the theoretical molar mass of Gly30B.

Optimal temperature and pH of Gly30B

To investigate the optimal pH and temperature of Gly30B, 2 % (w/v) laminarin as the substrate was incubated with 10.5 μM Gly5M at various pHs (2.0 to 10.0) and temperatures (20 to 70 °C). Optimal enzyme activity was obtained at pH 7.0 in 20 mM Tris−HCl buffer (Fig. 2). Gly30B exhibited less than 40 % relative enzyme activity at pH 2.0 in 20 mM glycine–HCl buffer and approximately 50 % relative activity at pH 10.0 in 20 mM glycine–NaOH buffer. Therefore, pH 7.0 in 20 mM Tris–HCl buffer was determined to be the optimum pH of Gly30B. The optimal temperature of Gly30B was determined to be approximately 40 °C in 20 mM Tris–HCl buffer (pH 7.0) (Fig. 3a), the enzyme showed 60 % relative activity at both 20 and 60 °C. In addition, Gly30B was stable at temperatures below 40 °C after 1 h pre-incubation but was significantly inactivated after pre-incubation at temperatures higher than 40 °C (Fig. 3b).

Kinetic parameters of Gly30B in the hydrolysis of pustulan and laminarin

To determine the kinetic parameters of Gly30B with β-1,6-linked pustulan and β-1,3- and β-1,6-linked laminarin as substrates, enzymatic reactions were performed at pH 7.0 and 40 °C in 20 mM Tris–HCl buffer with different substrate concentrations. Using the Lineweaver–Burk plot (data not shown), V_{max}, K_M, and k_{cat} were determined to be 153.8 U/mg protein, 24.2 g/L, and 135.6 s^{-1} for pustulan and 32.8 U/mg protein, 100.8 g/L, and 28.9 s^{-1} for laminarin, respectively.

Substrate specificity of Gly30B and the cation effect on Gly30B

To investigate the substrate specificity of Gly30B, several glycans with various types of glycosidic linkages were subjected to enzymatic reaction with Gly30B (Table 1). Gly30B showed the highest activity on pustulan that has only β-1,6-glycosidic linkages, but also was capable of hydrolyzing laminarin (22 % relative activity), which contains both β-1,3- and β-1,6-

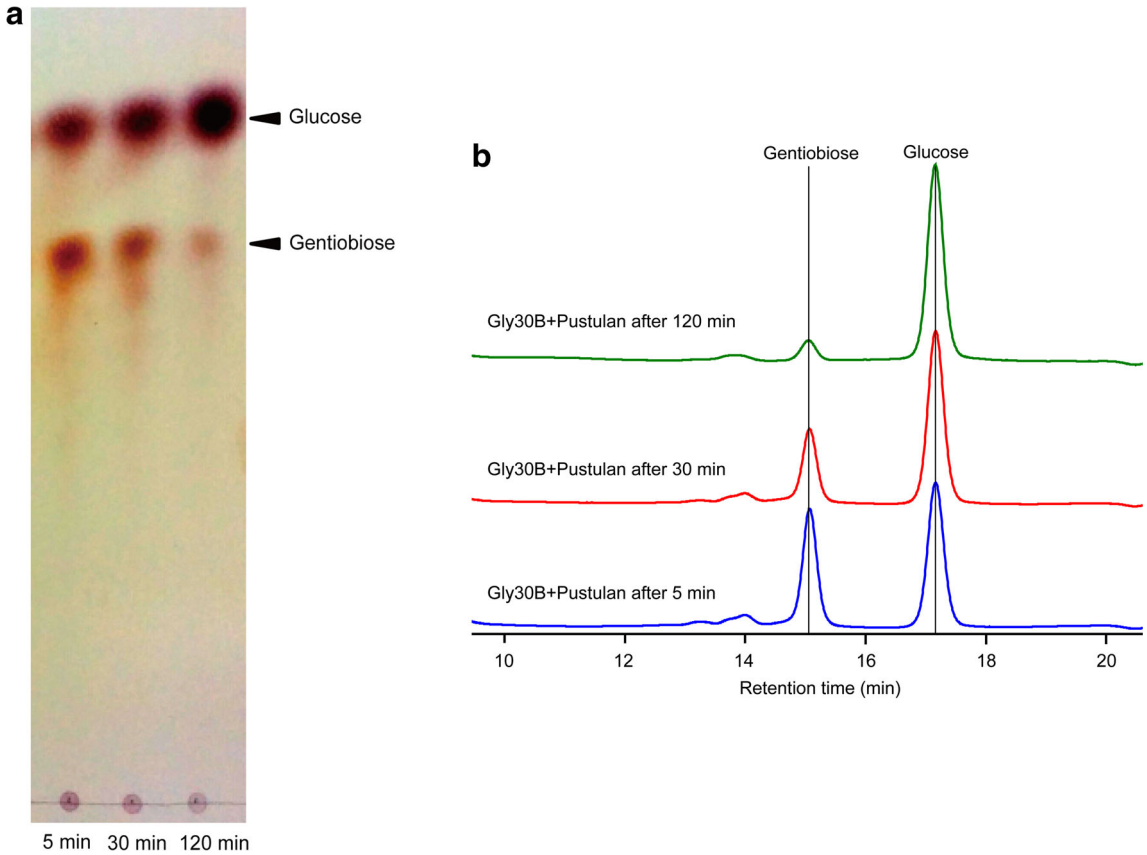

Fig. 4 (**a**) TLC and (**b**) HPLC analyses of enzymatic reaction products from the hydrolysis of pustulan by Gly30B. The reactions were performed at 40 °C in 20 mM Tris–HCl (pH 7.0)

glycosidic linkages. However, Gly30B did not hydrolyze curdlan, a water-insoluble β-1,3-glucan. This suggests that Gly30B has substrate specificity only for β-1,6-glycosidic linkages. Gly30B showed no activity when incubated with cellulose (e.g., Avicel), CM-cellulose, or xylan, indicating that it has no specificity for β-1,4-glycosidic linkages. Enzymatic reactions using various cations showed that the enzymatic activity of Gly30B is not affected by K^+, Ca^{2+}, or Na^+, but the presence of Mg^{2+}, Ni^{2+}, Fe^{2+}, or Cu^{2+} significantly decreased the enzymatic activity of Gly30B. Cu^{2+} was the strongest inhibitor of Gly30B (Table 2).

Analysis of reaction products by TLC and HPLC

The mechanism of action of Gly30B was examined using pustulan and laminarin as the substrates. The enzymatic reaction products obtained from different reaction times were analyzed by TLC and HPLC. When pustulan was used as the substrate, gentiobiose (a disaccharide with a β-1,6-glycosidic linkage) and glucose, were formed in 5 min (Fig. 4a). As the reaction further progressed, gentiobiose was further hydrolyzed into glucose. After allowing the reaction to proceed for 120 min, glucose was formed as the major product, and only a small amount of gentiobiose remained (Fig. 4b).

Unlike when pustulan was used as the Gly30B substrate (Fig. 4), when laminarin was used, the initial spot of laminarin and its oligosaccharides still remained in the final TLC analysis (Fig. 5a). Glucose and gentiobiose were also detected among the final products from laminarin hydrolysis (Fig. 5b). Based on the observations of the hydrolysis of pustulan and laminarin made in this study, Gly30B was found to possess the ability to cleave β-1,6-glycosidic linkages without cleaving β-1,3-glycosidic linkages.

Discussion

In this study, we have characterized a β-1,6-endoglucanase, Gly30B, that originated from *S. degradans* 2-40^T. Gly30B is capable of cleaving β-1,6-glycosidic linkages, resulting in hydrolysis of the backbone of pustulan and the branches of laminarin. This enzyme functions through random hydrolysis, leading to the production of glucose and gentiobiose (DP2 with a β-1,6-glycosidic linkage) as final products.

Gly30B showed significantly higher specific activity compared with previously reported β-1,6-endoglucanases (Takeshi et al. 2013; Cruz et al. 1995). A β-1,6-glucanase from *Penicillium multicolor* hydrolyzed pustulan through three steps including a very slow process of producing oligosaccharides (DPs 3 − 4) then further hydrolyzing DP3 and DP4 into gentiobiose and glucose (Takeshi et al. 2013). β-1,6- endoglucanase from *T. harzianum* also showed slow hydrolysis (~5 h) in producing pustulan oligosaccharides (DPs 2 − 5) without producing glucose (Cruz et al. 1995). Compared

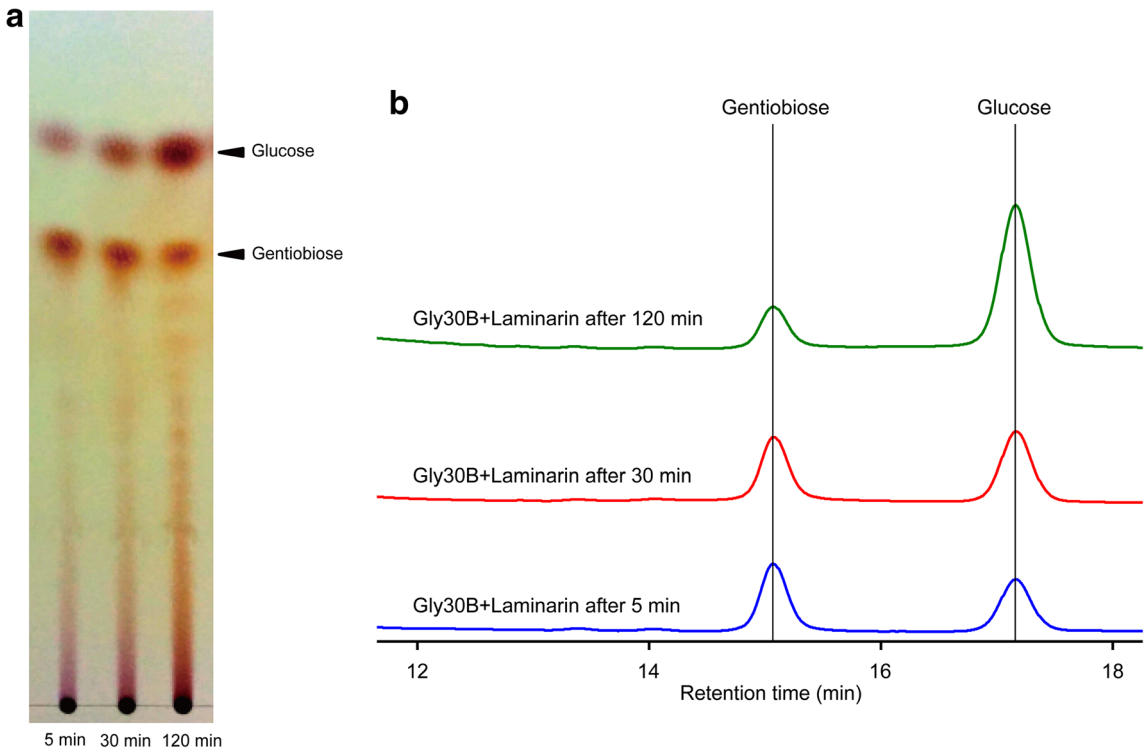

Fig. 5 (**a**) TLC and (**b**) HPLC analyses of enzymatic reaction products from the hydrolysis of laminarin by Gly30B. The reactions were performed at 40 °C in 20 mM Tris–HCl (pH 7.0)

with these enzymes, Gly30B showed much higher activity towards β-1,6-glucan, leading to complete hydrolysis within ~2 h. In addition, due to Gly30B's capability of forming gentiobiose from laminarin in the initial hydrolysis process, this enzyme has the potential to produce high-value gentiobiose (Gibson and Roberfroid 1995) from brown macroalgae that contain laminarin.

In the CAZy database, Gly30B was categorized into GH30, which is derived from GH5 (St John et al. 2010). Enzyme activities currently assigned within GH30 include glucosylceramidase (EC 3.2.1.45), β-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37), and β-1,6-endoglucanase (EC 3.2.1.75). Until now, all the characterized β-1,6-endoglucanases were from eukaryotes, mainly fungi such as *E. festucae* (Bryant et al. 2007), *T. harzianum* (Lora et al. 1995), and *T. virens* (Djonović et al. 2006). A phylogenetic tree has been established by aligning all the characterized β-1,6-endoglucanases (EC 3.2.1.75) including Gly30B (Fig. 6). According to sequence alignment results (data not shown), all the selected candidates contain an Asn-Glu-Pro (NEP) sequence with the Glu residue as the active site (Sakaguchi et al. 1999). Among all the characterized β-1,6-endoglucanases so far, Gly30B from *S. degradans* 2-40^T is the only one found in bacteria.

S. degradans 2-40^T can grow using laminarin as a sole carbon source; both laminarinase and amylase activities were detected (Ensor et al. 1999). Eight putative laminarinase genes were reported in *S. degradans* 2-40^T, all of which belong to GH16 except Lam81A. Gly30B has a locus tag of sde_2994 in the *S. degradans* 2-40^T genome and is located adjacent to several putative β-glycosidases such as Gly30A (sde_2992), Gly5K (sde_2993), and Gly5L (sde_2996), which are predicted to have β-1,6-endoglucanase or β-1,3-exoglucanase activities according to the activities of their GH families. Gly30A, Gly30B, and Gly5L all contain a lipobox at the C terminal signal peptide (LipoP 1.0, http://www.cbs.dtu.dk/services/LipoP/), indicating these enzymes are surface-associated through acylation. Therefore, this microorganism is capable of debranching laminarin through the function of these enzymes.

In conclusion, we have characterized Gly30B cloned from *S. degradans* 2-40^T, which is determined as a β-1,6-endoglucanase in GH30. To our knowledge, Gly30B is the first β-1,6-endoglucanase found in bacteria. This enzyme exhibited complete hydrolysis of β-1,6-glycosidic linkages, mainly producing glucose and gentiobiose. According to the function of this enzyme, Gly30B can be used to hydrolyze

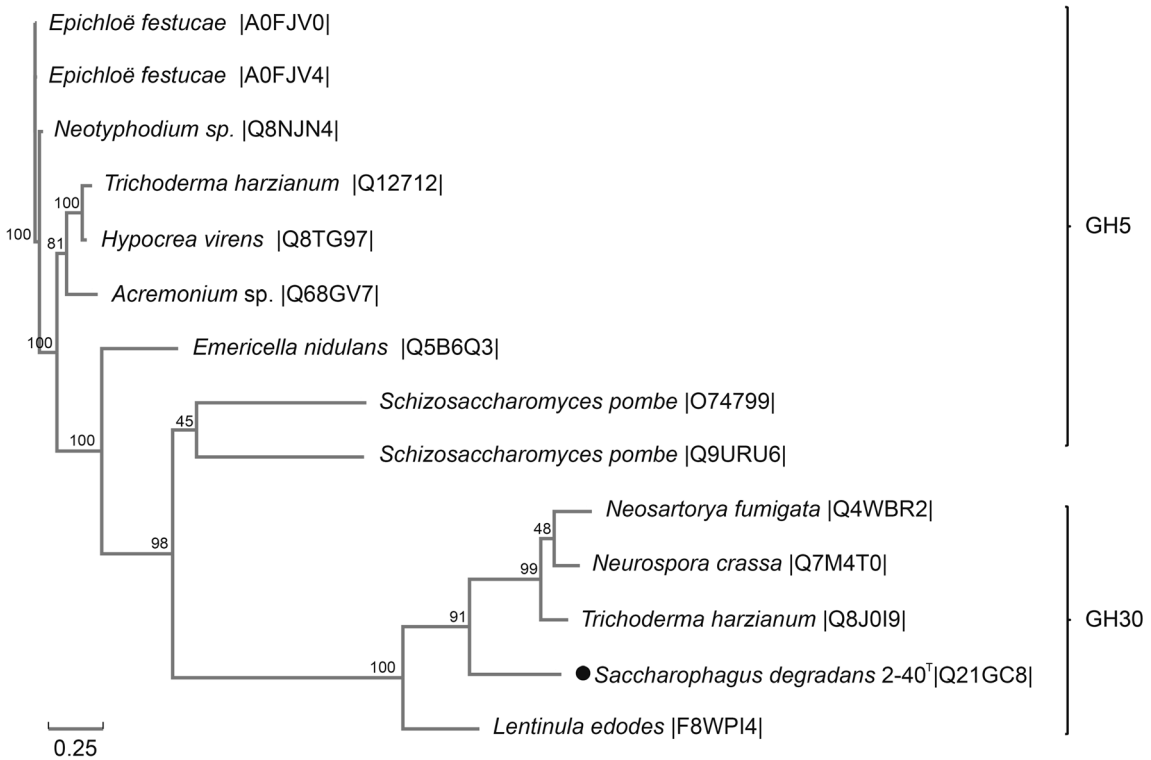

Fig. 6 Phylogenetic analysis of Gly30B (ABD82251.1) and other characterized β-1,6-endoglucanases (EC 3.2.1.75). This tree is based on protein sequence alignments of the entire amino acid sequences obtained from the CAZy and UniProt databases. For the bootstrap analyses in this tree were performed by using the neighbor-joining method using MAFFT (version 7. http://mafft.cbrc.jp/alignment/software/). Bootstrap values based on 100 replications are shown next to the nodes

β-1,6-glucans like laminarin from brown macroalgae and fugal cell walls for the production of gentiobiose or glucose.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

Boisramé A, Gaillardin C (2009) Heterologous expression and characterization of a β-1,6-glucanase from *Aspergillus fumigatus*. Appl Microbiol Biotechnol 82:663–669

Bryant MK, May KJ, Bryan GT, Scott B (2007) Functional analysis of a β-1,6-glucanase gene from the grass endophytic fungus *Epichloë festucae*. Fungal Genet Biol 44:808–817

Cruz J, Pintor-Toro JA, Benitez T, Llobell A (1995) Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J Bacteriol 177: 1864–1871

Djonović S, Pozo MJ, Kenerley CM (2006) Tvbgn3, a β-1,6-glucanase from the biocontrol fungus *Trichoderma virens*, is involved in mycoparasitism and control of *Pythium ultimum*. Appl Environ Microbiol 72:7661–7670

Ekborg NA, Gonzalez JM, Howard MB, Taylor LE, Hutcheson SW, Weiner RM (2005) *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. Int J Syst Evol Microbiol 55:1545–1549

Ensor L, Stosz SK, Weiner RM (1999) Expression of multiple insoluble complex polysaccharide degrading enzyme systems by a marine bacterium strain 2-40. J Ind Microbiol Biotechnol 23:123–126

Gibson SR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412

Ha SC, Lee S, Lee J, Kim HT, Ko H-J, Kim KH, Choi I-G (2011) Crystal structure of a key enzyme in the agarolytic pathway, α-neoagarobiose hydrolase from *Saccharophagus degradans* 2-40. Biochem Biophys Res Commun 412:238–244

Howard MB, Ekborg NA, Taylor LE, Weiner RM, Hutcheson SW (2003) Genomic analysis and initial characterization of the chitinolytic system of *Microbulbifer degradans* strain 2-40. J Bacteriol 185:3352–3360

Hutcheson SW, Zhang H, Suvorov M (2011) Carbohydrase systems of *Saccharophagus degradans* degrading marine complex polysaccharides. Mar Drugs 9:645–665

Kawai R, Igarashi K, Kitaoka M, Ishii T, Samejima M (2004) Kinetics of substrate transglycosylation by glycoside hydrolase family 3 glucan (1→3)-β-glucosidease from the white-rot fungus *Phanerochaete chrysosporium*. Carbohydr Res 339:2851–2857

Kim HT, Lee S, Lee D, Kim H-S, Bang W-G, Kim KH, Choi I-G (2010) Overexpression and molecular characterization of Aga50D from *Saccharophagus degradans* 2-40: an exo-type β-agarase producing neoagarobiose. Appl Microbiol Biotechnol 86:227–234

Kim HT, Chung JH, Wang D, Lee J, Woo HC, Choi I-G, Kim KH (2012) Depolymerization of alginate into a monomeric sugar acid using Alg17C, an exo-oligoalginate lyase cloned from *Saccharophagus degradans* 2-40. Appl Microbiol Biotechnol 93:2233–2239

Klis FM (1994) Review: cell wall assembly in yeast. Yeast 10:851–869

Ko JK, Jung MW, Kim KH, Choi I-G (2009) Optimal production of a novel endo-acting β-1,4-xylanase cloned from *Saccharophagus degradans* 2-40 into *Escherichia coli* BL21(DE3). New Biotechnol 26:157–164

Kollár R, Reinhold BB, Petráková E, Yeh HJ, Ashwell G, Drgonová J, Kapteyn JC, Klis FM, Cabib E (1997) Architecture of the yeast cell wall: β(1→6)-glucan interconnects mannoprotein, β(1→3)-glucan, and chitin. J Biol Chem 272:17762–17775

Lora JM, De la Cruz J, Llobell A, Benítez T, Pintor-Toro JA (1995) Molecular characterization and heterologous expression of an endo-β-1,6 glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol Gen Genet 247:639–645

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Montero M, Sanz L, Rey M, Llobell A, Monte E (2007) Cloning and characterization of *bgn16.3*, coding for a β-1,6-glucanase expressed during *Trichoderma harzianum* mycoparasitism. J Appl Microbiol 103:1291–1300

Nishikawa Y, Tanaka M, Shibata S, Fukuoka F (1970) Polysaccharides of lichens and fungi. IV Antitumour active *O*-acetylated pustulan-type glucans from the lichens of *Umbilicaria* species. Chem Pharm Bull 18:1431–1434

Rombouts FM, Phaff HJ (1976) Lysis of yeast cell walls. Lytic β-1,6-glucanase from *Bacillus circulans* WL-12. Eur J Biochem 63:109–120

Ruiz-Herrera J, Elorza MV, Valentín E, Sentandreu R (2006) Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. FEMS Yeast Res 6:14–29

Sakaguchi K, Okino N, Izu H, Ito M (1999) The Glu residue in the conserved Asn-Glu-Pro sequence of endoglycoceramidase is essential for enzymatic activity. Biochem Biophys Res Commun 260:89–93

Shin HJ, Oh SJ, Kim SI, Kim HW, Son J-H (2009) Conformational characteristics of β-glucan in laminarin probed by terahertz spectroscopy. Appl Phys Lett 94:1119111–1119113

St John FJ, Gonzalez JM, Pozharski E (2010) Consolidation of glycosyl hydrolase family 30: a dual domain 4/7 hydrolase family consisting of two structurally distinct groups. FEBS Lett 584:4435–4441

Takeshi H, Yasuna K, Shuji U, Taichi U (2013) Mode of action of a β-(1→6)-glucanase from *Penicillium multicolor*. Carbohydr Res 366: 6–16

Taylor LE, Henrissat B, Coutinho PM, Ekborg NA, Hutcheson SW, Weiner RM (2006) Complete cellulase system in the marine bacterium *Saccharophagus degradans* strain 2-40^T. J Bacteriol 188: 3849–3861

Yamamoto S, Kobayashi R, Nagasaki S (1974) Purification and properties of an endo β-1,6-glucanase from *Rhizopus chinensis* R-69. Agric Biol Chem 38:1493–1500