Arch Microbiol (2005) 184: 215–224
DOI 10.1007/s00203-005-0038-8

ORIGINAL PAPER

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Degradation of rice bran hemicellulose by *Paenibacillus* sp. strain HC1: gene cloning, characterization and function of β-D-glucosidase as an enzyme involved in degradation

Received: 16 April 2005 / Revised: 24 August 2005 / Accepted: 31 August 2005 / Published online: 5 October 2005
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Abstract A bacterium (strain HC1) capable of assimilating rice bran hemicellulose was isolated from a soil and identified as belonging to the genus *Paenibacillus* through taxonomical and 16S rDNA sequence analysis. Strain HC1 cells grown on rice bran hemicellulose as a sole carbon source inducibly produced extracellular xylanase and intracellular glycosidases such as β-D-glucosidase and β-D-arabinosidase. One of them, β-D-glucosidase was further analyzed. A genomic DNA library of the bacterium was constructed in *Escherichia coli* and gene coding for β-D-glucosidase was cloned by screening for β-D-glucoside-degrading phenotype in *E. coli* cells. Nucleotide sequence determination indicated that the gene for the enzyme contained an open reading frame consisting of 1,347 bp coding for a polypeptide with a molecular mass of 51.4 kDa. The polypeptide exhibits significant homology with other bacterial β-D-glucosidases and belongs to glycoside hydrolase family 1. β-D-Glucosidase purified from *E. coli* cells was a monomeric enzyme with a molecular mass of 50 kDa most active at around pH 7.0 and 37°C. Strain HC1 glycosidases responsible for degradation of rice bran hemicellulose are expected to be useful for structurally determining and molecularly modifying rice bran hemicellulose and its derivatives.

Keywords Rice bran · Hemicellulose · Degradation · *Paenibacillus* · β-D-glucosidase

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Abbreviations RB: Rice bran · TLC: Thin-layer chromatography · X-Glc: 5-Bromo-4-chrolo-3-indoryl-β-D-glucopyranoside · *p*-NP: *p*-nitrophenyl · HPLC: High-performance liquid chromatography · KPB: Potassium phosphate buffer · SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis · aa: Amino acids · acc no: Accession number · *bglA*: Strain HC1 β-D-glucosidase gene · BglA: Strain HC1 β-D-glucosidase

Introduction

Rice bran (RB), the outer skin of grain, occupies about 10% of unpolished rice by weight. RB is a typical biomass formed during rice milling process and approximately 4.5 million tons is produced annually worldwide (Wang et al. 2003). Oil extraction currently is the only means enabling RB to be used practically (Sugano and Tsuji 1996), although RB contains nutritionally, chemically, biologically, and pharmaceutically important classes of compounds (Nam et al. 2005), e.g., dietary fibers, vitamins, and minerals (nutritional factors); tocopherols, tocotrienols and orysanols (antioxidative, hypocholesterolemic and antitumor activities) (Iqbal et al. 2003; Qureshi et al. 2000; Saunders 1990; Sheetharamaiah and Chabdrasekhara 1988); phenols such as caffeic acid and tricin (cancer chemopreventives); and triterpene alcohol and sterol ferulates (antiinflammentaries) (Akihisa et al. 2000). Furthermore, RB hemicellulose inhibits 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats (Aoe et al. 1993) and arabinoxylan derived from RB hemicellulose shows anti-human immunodeficiency virus through inhibition of p24 antigen production and syncytia formation (Ghoneum 1998).

Hemicellulose, a tough fibrous material with xylan as a major component, is the second most common polysaccharide in nature and is recently being focused on in bioconversion to such useful materials as fuels and

chemicals (Saha 2003). RB hemicellulose, a heteropolysaccharide, has xylan as its backbone and several sugars as side chains (Ghoneum 1998) (Fig. 1). Heteropolysaccharide and its derivatives show biochemical and biotechnological properties based on their structural complexity, some of which are applicable in the food, agricultural, chemical, and medical industries (Giavasis et al. 2000; Hashimoto et al. 2002; Onsøyen 1996). The complete structure of RB hemicellulose and its derivatives with biological activities remain unclear, however, because the sugar composition of the RB hemicellulose model differs significantly from that determined experimentally (Shibuya and Iwasaki 1985). Structures of polysaccharide and its derivatives are usually determined by spectrophotometry, infrared spectrometry, NMR, X-ray crystallography, etc. Enzymes acting on polysaccharide are also useful for determining its structure due to their high substrate specificity.

We have focused on depolymerization of heteropolysaccharides (alginate, gellan, and xanthan) by bacteria such as *Sphingomonas* and *Paenibacillus*, and obtained useful enzymes for the structure determination and molecular design of saccharides with biological activities (Hashimoto et al. 2002). Xanthan treated with xanthan lyase, for example, tends to form a sol even in the presence of guar gum, and may be useful in food and chemical applications. Oligosaccharides derived from polysaccharides frequently exhibit physiological functions. Oligoalginates depolymerized with alginate lyase enhance the proliferation and/or differentiation of Bifidobacteria (Akiyama et al. 1992) and some plant cells (Yonemoto et al. 1993). This makes it possible to use oligoalginates as growth factors for Bifidobacteria in the food industry or as biochemical fertilizers in agriculture. Depolymerized products of alginate obtained with alginate lyase and gellan obtained with gellan lyase also promote the differentiation and proliferation of human epithelial cells (Hashimoto et al. 2002). Thus, the information on the biodegradation pathway of heteropolysaccharides in bacteria and related enzymes will provide invaluable insights into enzyme use for structure determination and into improvements in poly- and oligosaccharides having great potential for advanced biotechnological uses.

This paper deals with the biodegradation of RB hemicellulose by a *Paenibacillus* species, a potent producer of enzymes useful for the determination and modification of structures of RB hemicellulose and its derivatives.

Materials and methods

Materials

Rice bran produced from unpolished Japanese rice during milling process was used in this study. Hemicellulose was prepared from RB after lipids and lignins were removed with organic solvents and extracted with sodium hydroxide as described elsewhere (Siegel 1968). Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Bio Inc., Kyoto, and Toyobo Co., Tokyo, Japan, respectively. DEAE-Sepharose CL-6B and Sepharose CL-6B were purchased from Amersham Biosciences, Uppsala, Sweden. Butyl Toyopearl 650 M was purchased from Tosoh Co. Silica gel 60/Kieselguhr F_{254} thin-layer chromatography (TLC) plates were purchased from E. Merck, Darmstadt, Germany. 5-Bromo-4-chrolo-3-indoryl-*β*-D-glucopyranoside (X-Glc), *p*-nitrophenyl (*p*-NP)-glycosides, and gentiobiose were purchased from Nacalai Tesque Co., Kyoto, Japan. Pectin was purchased from Wako Pure Chemicals Co., Osaka, Japan. Beechwood xylan and cellopentaose were purchased from Sigma Chemical Co., St. Louis, MO, USA.

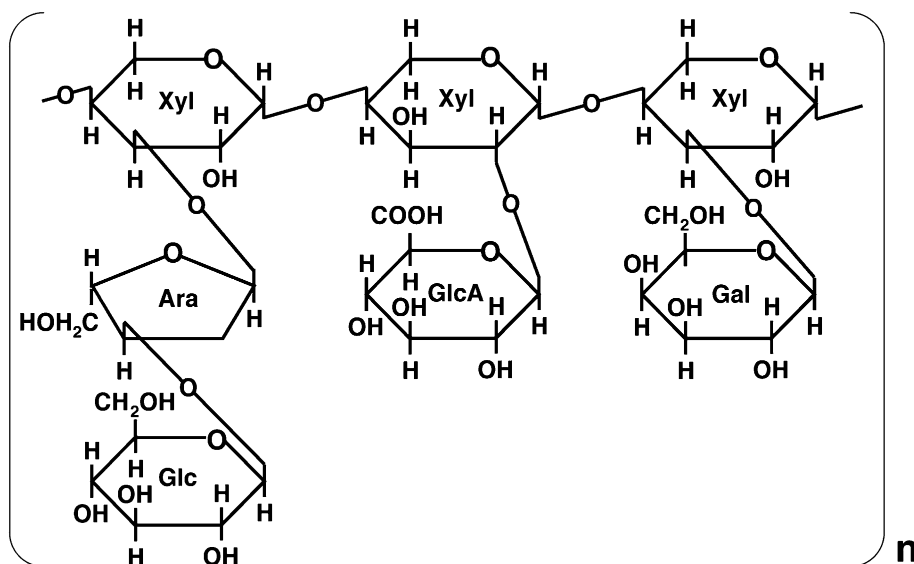

Fig. 1 RB hemicellulose model (Ghoneum 1998). *Ara* arabinose; *Gal* galactose; *Glc* glucose; *GlcA* glucuronic acid; *Xyl* xylose

Sugar composition analysis of RB hemicellulose

Rice bran hemicellulose was hydrolyzed in 2 M of trifluoroacetic acid at 100°C for 6 h. After hydrolysis, trifluoroacetic acid was evaporated under vacuum. Hydrolysates were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu SCL-6B controller chromatograph (Shimadzu Co, Kyoto, Japan) with a Shimadzu LC-9A pump and RF-10AXL fluorescence monitor. Hydrolysates were separated by HPLC with a post-column reaction with 1% arginine and 3% borate using ion-exchange column chromatography for neutral sugars (4.6×150 mm, TSK-gel sugar AXG, Tosoh Co.) and for uronic acid (4.6×250 mm, Shim-pack ISA07, Shimadzu Co.) using 0.5 and 1 M of potassium borate (pH 8.7) at flows of 0.5 and 0.8 ml/min. Elution was conducted at 70°C and reaction at 150°C. To detect sugar derivatives with arginine, we used an excitation wavelength of 320 nm and an emission wavelength of 430 nm.

Microorganisms and culture conditions

To screen RB hemicellulose-degrading microbes, microbial cells in a soil sample were aerobically cultured at 30°C for 48 h in liquid RB hemicellulose medium consisting of 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% Na_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, and 0.4% RB hemicellulose (pH 7.2). To isolate microbes, cells were grown at 30°C on plates of the above RB hemicellulose medium solidified with agar (1.5%). To study the effects of carbon sources on the production of RB hemicellulose-degrading enzymes, RB hemicellulose in the medium (20 ml) was replaced with xylan, pectin, or glucose (0.4%). To isolate chromosomal DNA from strain HC1, the bacterium was aerobically cultured at 30°C for 24 h in LB medium (Sambrook et al. 1989). To screen for the *β*-D-glucosidase producer, *E. coli* strain DH5α cells with the strain HC1 genomic DNA library were grown at 37°C on LB plates supplemented with 50 μg/ml of ampicillin and 40 μg/ml of X-Glc. To purify *β*-D-glucosidase, *E. coli* cells harboring the strain HC1 enzyme gene were aerobically cultivated at 37°C for 24 h in 6 l of LB medium with 50 μg/ml of ampicillin.

Bacterium identification

Morphological and biochemical analysis/properties of the isolated bacterium were determined based on API20NE identification (API System S.A., Montalieu, France). A 16S rDNA sequence analysis was conducted using a MicroSeq Kit (Applied Biosystems, Foster City, CA, USA). The genomic DNA of strain HC1 was isolated as described elsewhere (Ausubel et al. 1987). A 16S rRNA gene of the bacterium was amplified by PCR using genomic DNA as a template, and two oligonucleotides as primers recommended by the manufacturer of MicroSeq Kit. BLAST and CLUSTALW programs, assisted by GenomeNet on the Internet (http://www.genome.jp/), were used for DNA/protein sequence similarity search and multiple sequence alignment, respectively.

Thin-layer chromatography

Products derived from RB hemicellulose after treatment with bacterial enzymes were analyzed by TLC with a solvent system of 1-butanol-acetic acid-water (3:2:2, v/v) and, after spraying with 10% (v/v) sulfuric acid in ethanol, visualized by heating TLC plates at 110°C for 5 min.

Preparation of enzyme sources

Cells grown at 30°C for 48 h were harvested by centrifugation at 6,000*g* and 4°C for 10 min, and the resulting culture fluid was used as an extracellular enzyme source. Collected cells were washed in 20 mM of potassium phosphate (KPB) (pH 7.0), then resuspended in the same buffer. Cells were ultrasonically disrupted (Insonator 201 M, Kubota, Tokyo, Japan) at 0°C and 9 kHz for 5 min, and the clear solution obtained on centrifugation at 13,000*g* and 4°C for 20 min was dialyzed against 20 mM KPB (pH 7.0) overnight. The dialysate was used as an intracellular enzyme source.

Enzyme and protein assays

α-L-Arabinofuranosidase, *β*-D-galactosidase, *β*-D-glucosidase, *β*-D-glucuronidase, and *β*-D-xylosidase were assayed using *p*-NP-sugar derivatives as substrates (Hashimoto et al. 1996). Briefly, the reaction mixture of 0.5 ml consisting of 0.4 mM of *p*-NP sugar derivative, 50 mM of KPB (pH7.0), and enzyme was incubated at 30°C for 10 min, 1 ml of 0.25 M Na_2CO_3 was added, then the amount of *p*-nitrophenol released was determined by measuring absorbance at 400 nm. Xylanase was assayed using beechwood xylan as a substrate, and enzyme activity was determined by measuring reducing sugars released from the substrate by the Somogyi-Nelson method (Somogyi 1952). One unit of each enzyme activity was defined as the amount of enzyme required to release 1 μmol of product from the substrate per min. Protein was determined by the method of Bradford (Bradford 1976), with bovine serum albumin as a standard.

Construction of genomic DNA library

Strain HC1 genomic DNA was partially digested with *Sau*3AI. Fragments of 2 to 6 kb were purified using GeneClean II kit (BIO 101, Inc., CA, USA) and ligated

into *Bam*HI-digested pUC118 cloning vector (Takara Bio Inc.). *E. coli* strain DH5α cells were transformed with ligated DNAs.

DNA sequence and DNA manipulation

Nucleotide sequences of 16S rRNA and β-D-glucosidase genes were determined by dideoxy-chain termination using automated DNA sequencer model 377 (Applied Biosystems) (Sanger et al. 1977), and deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers (acc nos) AB198337 and AB198338, respectively. Subcloning, transformation, and gel electrophoresis were conducted as described elsewhere (Ausubel et al. 1987; Sambrook et al. 1989).

Purification of β-D-glucosidase from *E. coli* cells

Unless otherwise specified, all operations were conducted at 0 to 4°C. *E. coli* cells harboring the pUC118 plasmid with an insertion of the enzyme gene were grown in 6 l of LB medium (1.5 l/flask), collected by centrifugation at 6,000*g* and 4°C for 5 min, washed with 20 mM of KPB (pH 7.0), then resuspended in the same buffer. Cells were ultrasonically disrupted (Insonator 201 M) at 0°C and 9 kHz for 10 min, and the clear solution obtained on centrifugation at 15,000*g* and 4°C for 20 min was used as the cell extract. After supplementation with 1 mM phenylmethylsulfonyl furoride and 0.1 μM pepstatin A, the cell extract was subjected to ammonium sulfate fractionation. The precipitate (50–70% saturation) was collected by centrifugation at 15,000*g* and 4°C for 20 min, dissolved in 20 mM of KPB (pH 7.0), then applied to a DEAE-Sepharose CL-6B column (4.2 by 38 cm) previously equilibrated with 20 mM of KPB (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (0–1.0 M) in 20 mM of KPB (pH 7.0) (1 l), and 10 ml fractions were collected every 10 min. Active fractions, eluted with 0.43 M of NaCl, were saturated with ammonium sulfate (30%) and applied to a Butyl-Toyopearl 650 M column (2.7 by 9 cm) previously equilibrated with 20 mM of KPB (pH 7.0) saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient of ammonium sulfate (30-0%) in 20 mM of KPB (pH 7.0) (300 ml), and 3 ml fractions were collected every 3 min. Active fractions, eluted with 20 mM of KPB (pH 7.0) saturated with ammonium sulfate (20%), were combined and subjected to Sepharose CL-6B (2.7 by 68 cm) previously equilibrated with 20 mM of KPB (pH 7.0) containing 0.15 M of NaCl. The enzyme was eluted with the same buffer and 3 ml fractions were collected every 6 min. The enzyme was eluted between fraction nos 65 and 70. These fractions were combined, dialyzed against 20 mM of KPB (pH 7.0), then used as the purified enzyme.

Determination of molecular mass

The molecular mass of β-D-glucosidase was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and gel permeation chromatography (Sepharose CL-6B).

N-terminal amino acid sequence

The N-terminal amino acid (aa) sequence of β-D-glucosidase purified from *E. coli* cells was determined by Edman degradation with a Procise 492 protein sequencing system (Applied Biosystems).

Optimal pH and temperature, and thermal stability of β-D-glucosidase

Experiments were conducted using the purified enzyme and *p*-NP-β-D-glucopyranoside as a substrate. (1) Optimal pH: Reactions were conducted at 30°C for 10 min in the following 50 mM buffers: sodium acetate, potassium phosphate, sodium Hepes, and glycine-sodium hydroxide. (2) Optimal temperature: Reactions were conducted for 10 min at different temperatures in 50 mM of KPB (pH 7.0). (3) Thermal stability: after the enzyme was preincubated at different temperatures for 10 min, residual activity was measured at 30°C for 10 min in 50 mM of KPB (pH 7.0).

Effects of various compounds on β-D-glucosidase activity

Reactions were conducted for 10 min at 30°C and pH 7.0 (KPB) in the presence or absence (control) of the compounds examined.

Results and discussion

Sugar composition of RB hemicellulose

Although, in the RB hemicellulose model, glucosylarabinose, glucuronic acid and galactose are shown to be linked to the xylan backbone as side chains (Ghoneum 1998) (Fig. 1), RB hemicellulose used in this work contained almost equal amounts of arabinose and xylose as main components, and small amounts of glucose and galactose as follows: xylose, 45.8%; arabinose, 42.5%; galactose, 4.7%; glucose, 4.1%; galacturonic acid, 1.5%; rhamnose, 1.1%; ribose, 0.3%; fructose, glucuronic acid and mannose, undetectable. This sugar composition in RB hemicellulose almost coincided with that described previously (Shibuya and Iwasaki 1985), so RB hemicellulose here is thought to be categorized into arabinoxylan with branched chains consisting of glucose and galactose.

Isolation and identification of RB hemicellulose-degrading bacterium

To obtain RB hemicellulose-degrading enzymes useful for the structural determination and molecular modification of the polysaccharide and its derivatives, we attempted to isolate microbes assimilating the polysaccharide. Although several microbes formed large colonies on hemicellulose plates, only two (strains HC1 and 2) grew well on the liquid medium (Fig. 2a). Since strain HC1 cells could grow on the polysaccharide most sufficiently (Fig. 2b) and release low-molecular-weight sugars from the polysaccharide, strain HC1 was thought to be a potent producer of RB hemicellulose-degrading enzymes. Strain HC1 was a flagella-forming bacterium, and identified as belonging to the genus *Paenibacillus* or *Bacillus* through morphological and biochemical analysis as follows: shape, rod (0.8×2.0 µm); Gram staining, variable; spore formation, positive; colony, smooth, round, opaque and cream; culture temperature at 37°C, growth; culture temperature at 45°C, no growth; catalase, positive; oxidase, positive; acid/gas formation from glucose, negative; and O/F test from glucose, negative. To determine the phylogenic position of the bacterium, the nucleotide sequence of the 16S rRNA gene (1,514 bp) was determined. The gene for 16S rRNA exhibited the highest identity score (99%) with that of *P. pabuli* (acc no AB073191) and high similarity with those of *P. xylanilyticus* (98%) (acc no AY427832), *P. amylolyticus* (98%) (acc no AY509234), and *P. illinoisensis* (98%) (acc no AB073192). Paenibacilli are known to degrade various polysaccharides including chitin, chitosan, xylan, pectin, glucan and curdran, and strain HC1 is also phylogenetically close to our *Paenibacillus* sp. strain GL1 isolated as a gellan/xanthan-degrading bacterium (Hashimoto et al. 2002). The phylogenic tree through multiple sequence alignment of 16S rDNAs indicates that strain HC1 belongs to the genus *Paenibacillus* and is a member of paenibacilli assimilating polysaccharides (data not shown).

RB hemicellulose-degrading enzymes in strain HC1

Since sufficient growth of strain HC1 was observed when cultured in the medium containing RB hemicellulose as a sole carbon source, the activities of possible RB hemicellulose-degrading enzymes, such as xylanase, α-L-arabinofuranosidase, β-D-galactosidase, β-D-glucosidase, β-D-glucuronidase, and β-D-xylosidase, were determined (Table 1). These enzymes were scarcely expressed in the pectin and glucose media. Extracellular xylanase was produced in hemicellulose and xylan media and some intracellular exoglycosidases were inducibly expressed in the presence of RB hemicellulose. α-L-Arabinofuranosidase and β-D-glucosidase were significantly induced in the hemicellulose medium, while β-D-galactosidase and β-D-xylosidase activities were found in hemicellulose and xylan media. Degradation of RB hemicellulose by these enzymes was confirmed through TLC analysis (Fig. 3). No low-molecular-weight compounds were observed before the reaction (Fig. 3, lanes 1 and 2). After the incubation of RB hemicellulose with the extracellular enzyme fraction, products were detected as smeared ladders (Fig. 3, lane 4), indicating that extracellular xylanase endolytically depolymerized RB hemicellulose into saccharides with various degrees of polymerization. Several clear spots corresponding to authentic sugars such as glucose and xylose were, however, observed in the reaction products resulting from the mixture of the hemicellulose and intracellular enzyme fraction (Fig. 3, lane 3). This indicates that intracellular exoglycosidases such as glucosidase and xylosidase act on RB hemicellulose and release the constituent monosaccharides, suggesting that strain HC1 excretes xylanase acting on the main chain of RB hemicellulose and degrades resultant oligosaccharides by intracellular exoglycosidases after their incorporation into cells. As the first step in modifying RB hemicellulose, β-D-glucosidase was further analyzed since only glucose is shown not to be linked to the main chain of the polysaccharide, although the polysaccharide model is thought to be somewhat inaccurate (Fig. 1).

Molecular cloning and sequence analysis of β-D-glucosidase gene

A gene responsible for β-D-glucosidase was screened for in the genomic DNA library of strain HC1, which was constructed in *E. coli* strain DH5α cells having no

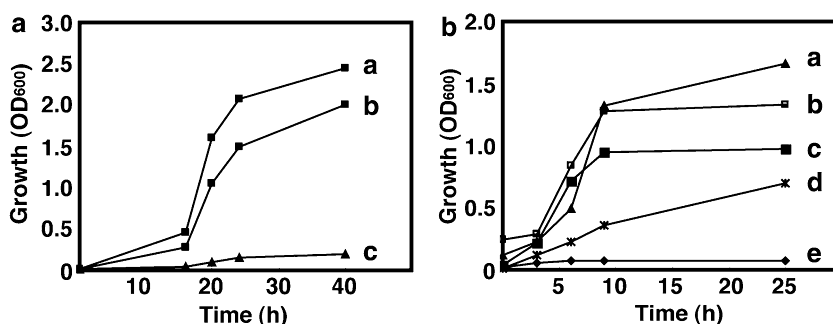

Fig. 2 Polysaccharide assimilation by bacteria. **A** Growth at 30°C of bacteria on RB hemicellulose. *a* strain HC1; *b* strain HC2; *c* strain HC3. **B** Growth at 37°C of strain HC1 on polysaccharides. *a* RB hemicellulose; *b* xylan; *c* pectin; *d* curdran; *e* no polysaccharide. Bacterial growth was determined by measuring turbidity at 600 nm

Table 1 Expression of RB hemicellulose-degrading enzymes in strain HC1 cells

Enzyme	Specific activity (U/mg)[a]							
	RB hemicellulose		Xylan		Pectin		Glucose	
	Extra	Intra	Extra	Intra	Extra	Intra	Extra	Intra
Xylanase	1743	297	521	142	10.1	ND	11.0	ND
β-D-Xylosidase	ND	20.0	ND	28.0	ND	0.2	ND	0.2
β-D-Glucosidase	ND	39.4	ND	4.2	ND	0.3	ND	0.2
β-D-Galactosidase	ND	60.4	ND	60.0	ND	0.3	ND	0.6
β-D-Glucuronidase	ND	ND	ND	ND	ND	ND	ND	ND
α-L-Arabinofuranosidase	ND	9.5	ND	2.5	ND	0.1	ND	0.1

[a]Cells were grown on various carbon souces, and extra- and intracellular enzyme activity was assayed under condition described in Materials and methods
ND not detected

β-D-glucosidase activity. Several clones colored blue on LB medium containing X-Glc used as the substrate. Positive clones were purified and confirmed to have genomic DNA fragments of strain HC1 in cloning vector pUC118. Judging from the restriction enzyme maps of fragments, clones could be categorized into a single group. Through subcloning of genomic DNA fragments, each (about 2.5 kb fragment) of them was found to be essential for β-D-glucosidase activity, and the gene was named *bglA*. The nucleotide sequence of the *Sau*3AI-treated strain A1 genomic DNA fragment (2.5 kb) containing the β-D-glucosidase gene (*bglA*) was determined. The gene (1,347 bp) contained an ORF encoding a protein (BglA: 448 aa) with a molecular weight of 51,381, and was found to show the highest identity (73%) with *P. polymyxa* β-D-glucosidase A (Gonzalez-Candelas et al. 1990) (Fig. 4). Because sequences homologous with those of a bacterial consensus promoter (Hawley and McClure 1983) existed in the 5′-region upstream of the initiation codon of *bglA*, *bglA* transcription is considered to be directed by the endogeneous strain HC1 promoter. A predicted ribosome-binding site (Shine-Dalgarno sequence, GGAGGA) (Shine and Dalgarno 1974) existed just before the start codons of *bglA*. A hairpin structure containing a stem composed of 14 nucleotides was observed downstream from the stop codon of *bglA*. The structure exhibited free energy of –15.9 kcal and may function as transcriptional terminators in a Rho factor-independent manner.

Purification and characterization of β-D-glucosidase expressed in *E. coli* cells

To characterize BglA, the enzyme was purified from *E. coli* cells harboring pUC118 with an insertion of the *Sau*3AI fragment (2.5 kb). BglA was purified 52.9-fold from the *E. coli* transformant with a recovery of 27.9% (Table 2). The purified enzyme was confirmed to be homogeneous by SDS-PAGE (Fig. 5A) and native gradient PAGE (data not shown). Properties of the enzyme from *E. coli* cells are as follows:

1. *Molecular mass*. The molecular mass of BglA was determined to be 50 kDa by SDS-PAGE (Fig. 5a)comparable to the theoretical one (51,381 Da) deduced from the predicted amino acid sequence of BglA. The molecular mass in the native form was determined to be 50 kDa by gel permeation chromatography (Fig. 5b), indicating that BglA is a monomeric enzyme.
2. *pH and temperature*. BglA was most active at pH 6.0–7.0 and 37°C, and more than 50% of enzyme activity was lost after incubation at 40°C for 10 min (data not shown).
3. *Metal ions and other compounds*. The activity of BglA was determined in the presence and absence of various compounds (Table 3). Metal ions (Ca^{2+}, Co^{2+}, and Mg^{2+}), a chelator (EDTA), and sugars (L-fucose, D-glucuronic acid, D-mannose, L-rhamnose, and D-xylose) had no appreciable effects on enzyme activity.

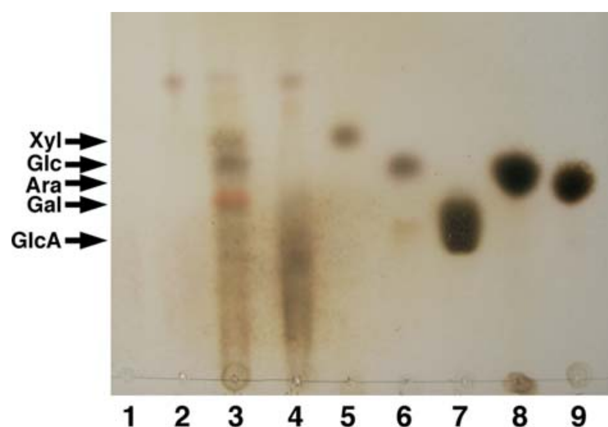

Fig. 3 Degradation of RB hemicellulose by strain HC1 enzymes. RB hemicellulose was incubated with an extracellular or intracellular enzyme source, and products were analyzed by TLC staining with sulfuric acid. *Lane 1* mixture of hemicellulose and intracellular enzyme source (reaction time, 0 h); *lane 2* mixture of hemicellulose and extracellular enzyme source (reaction time, 0 h); *lane 3* mixture of hemicellulose and intracellular enzyme source (reaction time, 24 h); *lane 4* mixture of hemicellulose and extracellular enzyme source (reaction time, 24 h); *lane 5* authentic xylose; *lane 6* authentic glucose; *lane 7* authentic glucuronic acid; *lane 8* authentic arabinose; *lane 9* authentic galactose

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BglA(strain HC1)   MTIFQFPQDFRWGTATASYQIEGAAQEGGRGVSIWDTFARTPGKVFNGDNGDVACDSYHR
BglA(P.polymyxa)   MTIFQFPQDFMWGTATAAYQIEGAYQEDGRGLSIWDTFAHTPGKVFNGDNGNVACDSYHR
Bgl (B.circulans)  MSIHMFPSDFKWGVATAAYQIEGAYNEDGRGMSIWDTFAHTPGKVKNGDNGNVACDSYHR
BglA(strain GL1)   MASIQFPKDFVWGTATASYQIEGAYNEDGRGMSIWDTFSRTPGKVVNGDTGDIACDSYHR
                   *:   **.** **.***:****** :*.***:******::***** ***.*::*******

BglA(strain HC1)   YEEDIELMKKLGINTYRFSIAWPRIIPDGDGEINREGLDFYHRFVDKLLEAGIEPFCTLY
BglA(P.polymyxa)   YEEDIRLMKELGIRTYRFSVSWPRIFPNGDGEVNQEGLDYYHRVVDLLNDNGIEPFCTLY
Bgl (B.circulans)  VEEDVQLLKDLGVKVYRFSISWPRVLPQGTGEVNRAGLDYYHRLVDELLANGIEPFCTLY
BglA(strain GL1)   YEEDIALLKNLGVKAYRFSIAWPRIYPDGDGELNQKGLDYYAKVIDGLLAAGIEPCVTLY
                    ***: *:*.**:..****::***: *:* **:*: ***:* :.:* *   ****  ***
                                                                     ▼
BglA(strain HC1)   HWDLPQTLEDIGGWGNRRTVDAFVKYAEVIFKEFSGKINFWLTFNEPWCIAFLSNLLGIH
BglA(P.polymyxa)   HWDLPQALQDAGGWGNRRTIQAFVQFAETMFREFHGKIQHWLTFNEPWCIAFLSNMLGVH
Bgl (B.circulans)  HWDLPQALQDQGGWGSRRITIDAFAEYAELMFKELGGKIKQWITFNEPWCMAFLSNYLGVH
BglA(strain GL1)   HWDLPQALQDKGGWDNRDTIRAFVRYAETAFKAFGGKVKQWITFNETWCVSFLSNYIGAH
                   ******:*:* ***..* *: **..:**  *: : **:: *:****.**::**** :* *

BglA(strain HC1)   APGNKDLQTSINVAHGLLVAHGKAVQSFRRLGTTGQIGIAPNVCWAEPYSKSPEDQAACD
BglA(P.polymyxa)   APGLTNLQTAIDVGHHLLVAHGLSVRRFRELGTSGQIGIAPNVSWAVPYSTSEEDKAACA
Bgl (B.circulans)  APGNKDLQLAIDVSHHLLVAHGRAVTLFRELGISGEIGIAPNTSWAVPYRRTKEDMEACL
BglA(strain GL1)   APGNTDLQLAVNVAHNCMVAHGEAVKAFRALGISGEIGTTHNLYWFEPYTTKPEDVAAAH
                   *** .:** :::*.*  :**** :*  ** ** :*:** : *  *  **  . **  *.

BglA(strain HC1)   RSIALNTDWFLDPIYKGAYPQFMVDWFAEAGATVPIQEGDMEIISQPIDLLGINYYTMGI
BglA(P.polymyxa)   RTISLHSDWFLQPIYQGSYPQFLVDWFAEQGATVPIQDGDMDIIGEPIDMIGINYYSMSV
Bgl (B.circulans)  RVNGWSGDWYLDPIYFGEYPKFMLDWYENLGYKPPIVDGDMELIHQPIDFIGINYYTSSM
BglA(strain GL1)   RNRAYNNEWFMDPTFKGQYPQFMVDWFKGKGVEVPIQPGDMETIAQPIDFIGVNFYSGGF
                   *  .   :*:::* : * **:*::**:   *   **  ***: * :***::*:*:*: ..
                                                                           ▼
BglA(strain HC1)   NRFNP--EAGVLQSEEVDMGLTKTDIGWPVESRGLYEFMHY-LQKYGNVDVYITENGACI
BglA(P.polymyxa)   NRFNP--EAGFLQSEEINMGLPVTDIGWPVESRGLYEVLHY-LQKYGNIDIYITENGACI
Bgl (B.circulans)  NRYNPGEAGGMLSSEAISMGAPKTDIGWEIYAEGLYDLLRYTADKYGNPTLYITENGACY
BglA(strain GL1)   GRYKE--GEGLFDCEEVQVGFDKTFMDWNVYAEGLYKVLSWVHEEYGDVPIYITENGACY
                   .*::      *.:..* :.:*   * :.* : :.***..: :  ::**:  :********

BglA(strain HC1)   NDDLENG-KINDDRRIAYYEQHLAQIHRIINDGINLKGYMAWSLMDNFEWAEGYRMRFGL
BglA(P.polymyxa)   NDEVVNG-KVQDDRRISYMQQHLVQVHRTIHDGLHVKGYMAWSLLDNFEWAEGYNMRFGM
Bgl (B.circulans)  NDGLSLDGRIHDQRRIDYLAMHLIQASRAIEDGINLKGYMEWSLMDNFEWAEGYGMRFGL
BglA(strain GL1)   EDELTQEGRVHDAKRADYFKKHFIQCHRLIESGVPLKGYFAWSLLDNFEWAEGYVKRFGI
                   :* :     :::* :*  *    *: *  * *..*: :***: ***:*********  ***:

BglA(strain HC1)   VHVDYRSLVRTPKESFYWYQNVIKNNWVETRI
BglA(P.polymyxa)   IHVDFRTQVRTPKESYYWYRNVVSNNWLETRR
Bgl (B.circulans)  VHVDYDTLVRTPKDSFYWYKGVISRGWLDL--
BglA(strain GL1)   VYTDYKTLKRYPKDSYRFIQSVIENDGFEA--
                   ::.*: :  * **:*: : :.*:... .:
```

Fig. 4 Alignment of aa sequences of β-D-glucosidases using the CLUSTALW program. BglA (strain HC1), β-D-glucosidase of strain HC1 (acc no AB198338); BglA (*P. polymyxa*), β-D-glucosidase A of *P. polymyxa* (acc no M60210); Bgl (*B. circulans*), β-D-glucosidase of *B. circulans* (acc no M96979); BglA (strain GL1), β-D-glucosidase A of strain GL1 (acc no AB009410). Identical and similar amino acid residues in the four kinds of β-D-glucosidase are denoted by *asterisks* and *dots*, respectively. *Arrow heads* indicate catalytic Glu residues

Thiol reagents [DTT, glutathione (reduced form), 2-mercaptoethanol, iodoacetic acid, and *N*-ethylmaleimide] showed no inhibition of the enzyme reaction, suggesting that a sulfhydryl group play no crucial role in the catalysis. Metal ions (Cu^{2+}, Fe^{2+}, and Mn^{2+}) partially inhibited enzyme activity, $HgCl_2$ (1 mM) was a potent inhibitor for enzyme activity. The enzyme activity was partially inhibited in the presence of D-glucose and D-galactose. Both sugars possibly can be accommodated in the active site of the enzyme.

4. *Substrate specificity*. The substrate specificity of BglA was determined (Table 4). BglA was highly active on *p*-NP-β-D-glucopyranoside and *p*-NP-β-D-fucopyranoside, and slightly active on *p*-NP-β-D-galacto-

Table 2 Expression in *E. coli* cells and purification of strain HC1 β-D-glucosidase

Step[a]	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	2,840	4,004	1.41	100	1
Ammonium sulfate	626	1,966	3.14	49.1	2.2
DEAE-Sepharose CL-6B	167	1,820	10.9	45.5	7.7
Butyl-Toyopearl 650M	33.8	1,680	49.7	42.0	35.2
Sepharose CL-6B	15.0	1,119	74.6	27.9	52.9

[a]Purification is detailed in Materials and methods

pyranoside and RB hemicellulose. Other *p*-NP-sugars were not substrates for BglA.

5. *Mode of action*. Cellopentaose was incubated with BglA and resultant products were analyzed on the TLC plate (Fig. 6). Cellopentaose was at first converted to glucose and cellotetraose and gradually gave rise to cellotriose and cellobiose, indicating that BglA acts on the substrate exolytically.
6. *Kinetics*. The K_m of BglA for *p*-NP-β-D-glucopyranoside was determined to be 9.1 mM and *V*max to be 52.4 μmol/min/μg.
7. *N-Terminal amino acid sequence*. The N-terminal amino acid sequence of BglA expressed in *E. coli* cells was determined to be NH_2-TIFQ corresponding to ^{2}TIFQ^{5} of BglA, showing that the enzyme is post-translationally processed by methionine aminopeptidase of *E. coli*.

We, thus, isolated a bacterium (strain HC1) assimilating RB hemicellulose and identified it as a *Paenibacillus* species. To the best of our knowledge, this is the first bacterium growing on RB hemicellulose as a sole carbon source, although *Streptomyces actuosus* strain A-151 has been reported to grow on RB as a sole carbon source (Wang et al. 2003). A large number of bacteria able to assimilate various polysaccharides are categorized into the genus *Paenibacillus* (Dasman et al. 2002; Finnegan et al. 2003; Hosoda et al. 2003; Ito et al. 2003; Kaulpiboon et al. 2004; Nankai et al. 1999; Rivas et al. 2005; Ruijssenaars et al. 1999; Sakiyama et al. 2001; Takeda et al. 2000; van der Maarel MJ et al. 2000), suggesting that paenibacilli have characteristic polysaccharide-depolymerization systems consisting of diverse polysaccharide-degrading enzymes, hydrolases and lyases. Strain HC1 also produced various enzymes responsible for degrading RB hemicellulose, indicating that bacterial enzymes are useful for the structural determination and molecular modification of RB hemicellulose and its derivatives.

One such enzyme, β-D-glucosidase (BglA), and its gene were isolated and characterized. BglA highly resembles in primary structure with the β-D-glucosidases grouped into glycoside hydrolase family GH1 on the carbohydrate active enzyme (CAZY) database (http://afmb.cnrs-mrs.fr/CAZY/index.html). Family GH1 enzymes are shown to adopt an $(\alpha/\beta)_8$ barrel as a basic frame and have two catalytic acidic residues as acid/base and nucleophile catalysts (Sanz-Aparicio et al. 1998). These catalytic residues are well conserved among family GH1 enzymes. Homology modeling using *P. polymyxa* β-D-glucosidase A (BglA) as a search model (Peitsch

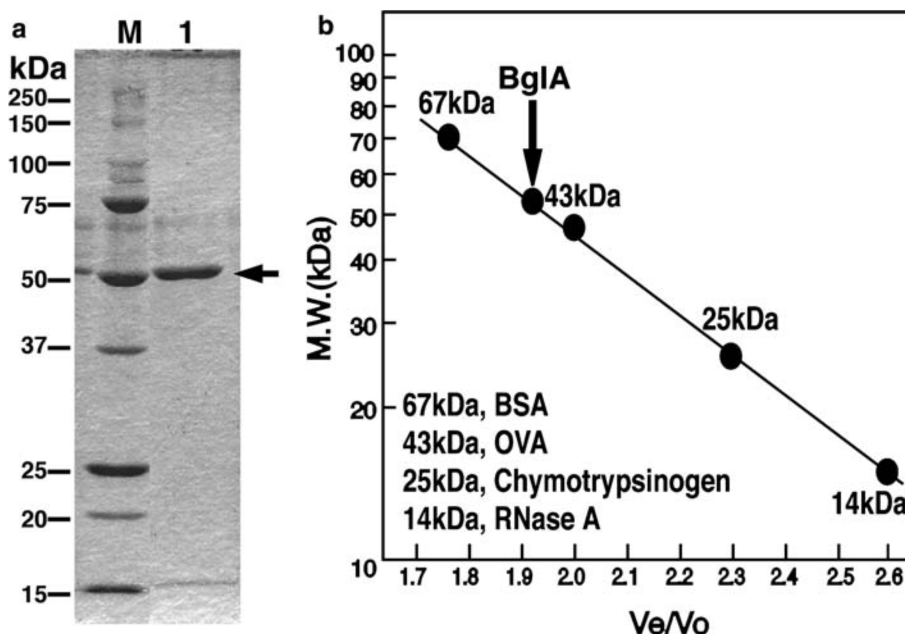

Fig. 5 Molecular mass determination of strain HC1 β-D-glucosidase. **A** SDS-PAGE, followed by protein staining with Coomassie brilliant blue. *Lane* M, Molecular mass (*MW*) standards (from *top*): standards with molecular masses of 250, 150, 100, 75, 50, 37, 25, 20, and 15 kDa; *lane 1*, purified enzyme from *E. coli* cells. **B** Gel permeation chromatography. The standard proteins used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (14 kDa). MW was plotted versus elution volume/void volume. The void volume was determined with blue dextran 2000 (2,000 kDa). The elution position of the purified enzyme (BglA) is indicated by an *arrow*

Table 3 Effects of various compounds on strain HC1 β-D-glucosidase activity

Compound	Concentration (mM)	Activity[a] (%)
None (control)		100
Metals		
$CoCl_2$	1	114
$ZnCl_2$	1	111
$MgCl_2$	1	99
$CaCl_2$	1	88
$MnCl_2$	1	60
$FeCl_2$	1	37
$CuCl_2$	1	21
$HgCl_2$	1	1
Thiol reagents		
Glutathione (reduced)	1	96
Iodoacetic acid	1	95
Dithiothreitol	1	93
2-Mercaptoethanol	1	92
N-ethylmaleimide	1	74
Chelator		
EDTA	1	105
Sugars		
D-Xylose	1.5	102
L-Fucose	1.5	92
L-Rhamnose	1.5	91
D-Mannose	1.5	90
D-Glucuronic acid	1.5	88
D-Galactose	1.5	54
D-Glucose	1.5	24

[a]Reactions were conducted for 10 min at 30°C and pH 7.0 (KPB) in the presence or absence (control) of the above compounds. The activity of the control was relatively taken as 100%

Table 4 Substrate specificity of strain HC1 β-D-glucosidase

Substrate	Activity[a] (%)
p-NP-β-D-glucopyranoside	100
p-NP-β-D-fucopyranoside	94.7
p-NP-β-D-galactopyranoside	6.4
RB hemicellulose	5.3
p-NP-α-D-glucopyranoside	2.1
p-NP-β-D-xylopyranoside	1.1
p-NP-β-D-glucuronate	0.4
p-NP-α-L-arabinoside	ND
p-NP-β-L-arabinoside	ND
p-NP-α-D-mannopyranoside	ND
p-NP-β-D-mannopyranoside	ND

[a]Reactions were conducted for 10 min at 30°C and pH 7.0 (KPB). Activity toward *p*-NP-β-D-glucopyranoside was taken as 100% *ND* not detected

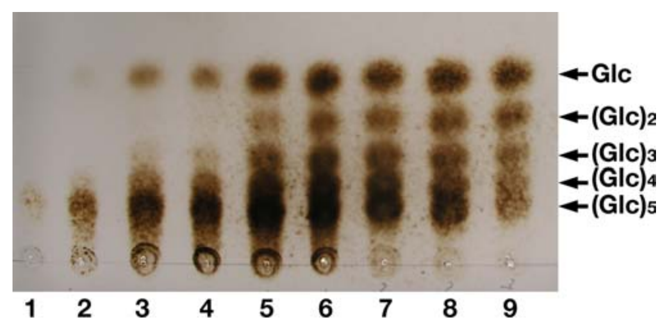

Fig. 6 Mode of action of strain HC1 β-D-glucosidase. Cellopentaose was incubated with the enzyme and resultant products were subjected to TLC analysis. Reaction times (h) are 0 (*lane 1*), 0.16 (*lane 2*), 0.5 (*lane 3*), 1 (*lane 4*), 2 (*lane 5*), 3 (*lane 6*), 4 (*lane 7*), 5 (*lane 8*), and 6 (*lane 9*)

1996) demonstrated that strain HC1 BglA shows a similar $(\alpha/\beta)_8$ barrel structure (data not shown). Based on the aa sequence alignment, the enzyme is suggested to have Glu166 as acid/base and Glu352 as nucleophile catalysts (Fig. 4). Although strain HC1 BglA shows a significant identity with β-D-glucosidases of *P. polymyxa* (73%) (Gonzalez-Candelas et al. 1990), *Bacillus circulans* (65%) (Paavilainen et al. 1993), and strain GL1 (59%) (Hashimoto et al. 1998) (Fig. 4), differences exist in substrate specificity for *p*-NP-β-D-galactopyranoside and K_m for *p*-NP-β-D-glucopyranoside between strain HC1 and *P. polymyxa* enzymes, in optimal temperature between strain HC1 and *B. circulans* enzymes, and in optimal pH and inhibition by glucose between strains HC1 and GL1 enzymes.

Strain HC1 BglA released a small amount of sugar from RB hemicellulose compared to *p*-NP-β-D-glucopyranoside (Table 4). This low activity of the enzyme on RB hemicellulose is probably due to the native structure of RB hemicellulose. In the case of assimilation of gellan and xanthan by strain GL1, we have already identified the complete depolymerization pathways of polysaccharides and β-D-glucosidase can act on substrates after treatment with other glycosidases (Hashimoto et al. 2002). The intrinsic structure of RB hemicellulose thus must be determined. Together with the biological activity assay of RB hemicellulose treated with β-D-glucosidase, we are now determining the RB hemicellulose structure and identifying the depolymerization pathway of RB hemicellulose in strain HC1.

Acknowledgements This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.M. and W.H. and by the Program of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan to K.M. A part of this work was also supported by the Iijima foundation of Japan.

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