A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing-end-xylose releasing exo-oligoxylanase*

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ABSTRACT

The gene encoding family 8 glycoside hydrolases (GH8) from *Bacillus halodurans* C-125 (BH2105), an alkalophilic bacterium with a known genomic sequence, was expressed in *Escherichia coli*. The protein was expressed with the intact N-terminal sequence, suggesting that it did not possess a signal peptide and that it was an intracellular enzyme. The recombinant enzyme showed no hydrolytic activity on xylan whereas it had been annotated as xylanase Y. It hydrolyzed xylooligosaccharide whose degree of polymerization is greater than or equal to 3 in an exo-splitting manner with anomeric inversion, releasing the xylose unit at the reducing end. Judging from its substrate specificity and reaction mechanism, we named the enzyme reducing-end-xylose releasing exo-xylanase (Rex). Rex was found to utilize only the β-anomer of the substrate to form β-xylose and α-xylooligosaccharide. The optimum pH of the enzymatic reaction (6.2-7.3) was found in the neutral range, a range beneficial for intracellular enzymes. The genomic sequence suggests that *B. halodurans* secretes two endo-xylanases and possesses two α-arabinofuranosidases, one α-glucuronidase, and three β-xylosidases intracellularly in addition to Rex. The extracellular enzymes supposedly hydrolyze xylan into arabino/glucurono-xylooligosaccharides that are then transported into the cells. Rex may play a role as a key enzyme in intracellular xylan metabolism in *B. halodurans* by cleaving xylooligosaccharides that were produced by the action of other intracellular enzymes from the arabino/glucurono-xylooligosaccharides.
INTRODUCTION

Endo β-1,4 xylanases (EC 3.2.1.8) are glycoside hydrolases (GH) that catalyze the degradation of xylan, a main component of hemicelluloses. The enzyme has been classified mainly into GH family 10 and 11 based on the amino acid sequences (CAZy: http://afmb.cnrs-mrs.fr/CAZY/). GH10 is known to have a (α/β)8 barrel structure to be classified as clan GH-A (1-8) while GH11 has a Jelly-roll structure to be classified as clan GH-C(9-14). Although GH10 and GH11 xylanases have different 3D structures, both of these enzymes can produce xylooligosaccharides from xylan in an endo-splitting manner with anomeric retention.

Recently, a GH8 endo β-1,4 xylanase was found in a culture supernatant of *Pseudoalteromonas haloplanktis* and characterized (15). Although, the GH8 xylanase also hydrolyzes the β-1,4 glycosidic bond of xylan in an endo-splitting manner, its reaction proceeds with anomeric inversion. The GH8 family contains various endo glycoside hydrolases, such as chitosanase (EC.3.2.1.132), endoglucanase (EC.3.2.1.4), and licheninase (EC.3.2.1.73) as well as endo β-1,4 xylanase. These enzymes also hydrolyze corresponding polysaccharides with anomeric inversion. The 3D structures of two GH8 enzymes, the endo β-1,4 xylanase from *P. haloplanktis* and an endoglucanase from *Clostridium thermocellum*, were found to have an (α/α)8 barrel structure (clan GH-M) (3,16).

The GH8 endo β-1,4 xylanase from *P. haloplanktis* has the highest amino acid identity (32.6%) with the protein encoded by the BH2105 gene (GenBank accession number: BAB05824) of *Bacillus halodurans* C-125(15), which is annotated as “xylanase Y”. *B. halodurans* C-125 is a xylanase-producing alkalophilic bacterium (17) whose genomic sequence is available (18). According to the annotation of the genomic sequence, the alkalophilic microbe supposedly possesses three endo β-1,4 xylanases (GH8, GH10 and GH11). Enzymatic characterizations of two secreted xylanases, XynA (BH2120, GH10) and XynN (probably BH0899, GH11), indicate that XynA exhibits a broad optimal pH range for activity (pH 4-10) whereas XynN (pH 4-6) is active only at neutral acidity (17,19). No other endo-xylanases have been reported in *B. hadolurans* C125. Thus, we planned to express the
BH2105 protein on *Escherichia coli* and characterize the properties of the enzyme.

In this study, we found that the BH2105 protein was not an endo β-1,4 xylanase, but has a novel activity of hydrolyzing xylooligosaccharides, releasing xylose from their reducing ends. This result prompted us to characterize the unique enzymatic properties (substrate specificity and reaction mechanism) in detail. Here, we describe the characterization of the newly found enzyme named reducing-end-xylose releasing exo-oligoxylanase (Rex1).
EXPERIMENTAL PROCEDURES

Materials.

The *B. halodurans* C-125 (9153) strain was obtained from the Japan Collection of Microorganisms (Wako, Japan). Restriction endonucleases were obtained from New England BioLabs (Beverly, MA, USA) and the DNA polymerase from *Thermococcus kodakaraensis* KOD1 was obtained from Toyobo (Osaka, Japan). Birchwood xylan (BWX) was prepared by lyophilizing the water soluble fraction of birch wood xylan (Fluka, Buchs, Switzerland) as described previously (20). Chitosan (Sigma-Aldrich, St Louis, MO, USA), curdlan (Wako Pure Chemicals, Osaka, Japan), lichenan (Sigma-Aldrich) and carboxyl methyl cellulose (Nacalai Tesque, Kyoto, Japan) were used as purchased. Cellotriose (G3), cellopentaose, laminaripentaose, chitopentaose, and chitosanpentaose were purchased from Seikagaku Kogyo (Tokyo, Japan). Xylooligosaccharides (Xn: n = degree of polymerization) were purchased from Megazyme (Wicklow, Ireland). *p*-Nitrophenyl-β-D-xylopyranoside (X-PNP) was purchased from Sigma-Aldrich. *p*-Nitropenyl-β-D-xylobioside was prepared as previously described (21). β-(1→4) Hetero-D-glucose and D-xylose-based disaccharides and trisaccharides (G-X, X-G, G-X-X, X-X-G, G-X-G, X-G-G, G-G-X and X-G-X [Abbreviation indicates the monosaccharide unit from the non-reducing end: G; glucose, X; xylose]) were prepared as described previously (22). 1,5-Anhydroxylotriitol (1-deoxy-xylotriose, X3-de) was synthesized by reducing acetobromoxylotriose, prepared by acetylation and bromination of X3 with pyridine-acetic anhydride and TiBr4, respectively, followed by reduction with lithium aluminum hydride as described for 1,5-anhydro-D-glucitol (23,24). Other reagents were of analytical grade and were obtained commercially.

DNA manipulation

Recombinant DNA techniques and agarose gel electrophoresis were performed as described by Sambrook et al. (25) Plasmid DNA was prepared using a QIAprep Spin Plasmid Kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was carried out in the
appropriate buffer at concentrations of 1-10 units/µg of DNA for 0.5-16 h at 37°C. Completion of the reaction was confirmed by agarose-gel electrophoresis. A QIAEX Agarose Gel Extraction Kit (Qiagen) was used for the extraction and purification of DNA from agarose gels.

**Nucleotide sequence analysis**

The nucleotide sequence was determined by the dideoxynucleotide chain termination method using an automated DNA sequencer (Model 310A, Applied Biosystems, Foster City, CA, USA) with a dRhodamine Terminator Kit (Perkin-Elmer, Freemont, CA, USA). At least three independent clones of each PCR product were sequenced. Sequence data was analyzed by using GENETYX MAC software Version 11.0 (GENETYX Software Development Co., Ltd., Tokyo, Japan).

**Expression of BH2105 in Escherichia coli**

The gene encoding BH2105 was amplified from the genomic DNA of *B. halodurans* C-125 by the polymerase chain reaction, using the forward primer, 5'-CCT TCC ATG GAG AAA ACG ACA GAA GGT GCA TTT -3' (containing an *Nco*I site; bold characters) and the reverse primer, 5'-GAA CTC GAG GTG TTC CTC TCT TGG CCC TCA G -3' (containing an *Xho*I site; denoted by bold type). Because of the addition of the *Nco*I site, the second amino acid residue coded was changed into Glu from Lys. The amplified fragment was digested by the corresponding restriction enzymes. The digested fragment was ligated into pET28b (Novagen, Madison, WI, USA) at the corresponding sites, generating the plasmid pET28b-BH2105 encoding the BH2105 protein, with the His×6 sequence added to its C-terminal end. Next, pET28b-BH2105 was electroporated into *Escherichia coli* BL21GOLD(DE3) cells, and positive colonies were selected. Resulting transformants were incubated in Luria broth (100 ml) containing 0.05 mg/ml kanamycin at 37°C until the optical density, at 600 nm, reached a level of 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to give a final concentration of 1 mM and the cultures were incubated for 24 h at 25°C. The BH2105 protein expressed was extracted from the wet cells (1 g) in 5 ml of 50 mM
sodium phosphate buffer (pH 8.0) using a sonicator (Branson Model 250D Sonifier; Branson, Danbury, CT, USA).

Purification of recombinant BH2105

The cell-free extract was loaded onto a Ni-NTA agarose (Qiagen) column (1 × 3 cm) and the enzyme was eluted with a stepwise gradient of imidazole (1, 10 mM; 2, 20 mM; 3, 250 mM) in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl. The fraction containing the BH2105 protein was desalted using a PD-10 column (Amersham Pharmacia Biotech, Upsalla, Sweden). Next, the protein solution was loaded onto a Q-Sepharose column (2.5 × 4 cm) and the enzyme was eluted with a stepwise gradient of NaCl (1: 0.05 M; 2: 0.2 M; 3: 0.3 M) in 50 mM sodium phosphate buffer (pH 7.2). The appropriate fractions were collected and purity was checked by SDS-PAGE (26). A 10 kDa protein ladder (Gibco-BRL, Grand Island, NY, USA) was used as a standard molecular marker for SDS-PAGE.

Protein concentrations were determined from the absorbance, at 280 nm, based on the theoretical molar absorption coefficients (106,210 M⁻¹cm⁻¹) determined from the amino acid compositions of BH2105 (27). The N-terminal amino acid sequence of the purified recombinant BH2105 protein was determined using a G1000A protein sequencer (Hewlett Packard, Palo Alto, CA, USA).

Enzyme assay

The enzyme activity was routinely determined by measuring the increase in xylose during the hydrolysis of X3. The enzymatic reaction was carried out in 50 mM sodium phosphate buffer (pH 7.1) containing various concentrations of X3 at 40°C. Periodically, a portion of the reaction mixture was boiled for 5 min to inactivate the enzyme and the concentration of xylooligosaccharides (X1-X6) was quantified by high performance ion exchange chromatography (HPIC) on a CARBOPAC PA1 column (4 × 250 mm, Dionex, Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (DX-3, Dionex).
Chromatography was performed with a linear gradient of 0-0.2 M sodium acetate in 0.1 M NaOH for 20 min at a flow rate of 1 ml/min.

**Effect of pH and temperature on enzymatic activity**

Enzymatic activity was measured under standard conditions of X3 (0.5 mM) hydrolysis while pH of the reaction mixture was changed with each 50 mM buffer. The pH-stability was determined by incubating the enzyme at 30°C for 30 min at each pH, followed by measuring activity under standard conditions. The buffer systems used were sodium acetate (pH 3.5-5.5) and sodium phosphate (pH 6.0-8.0), TAPS (pH 8.0-9.0) and CAPS (pH 9.7-11.0). The final pH values of the reaction solution were determined after addition of the enzyme and the substrates. The optimum temperature of activity was measured for 10 min under standard conditions except for temperature. The thermostability was determined by incubating the enzyme at each temperature for 30 min in 50 mM sodium phosphate buffer (pH 7.1), followed by measuring the activity under the standard conditions at 40°C.

**Analysis of the products**

The reaction products from various substrates were separated by thin layer chromatography (TLC) on a silica gel 60 F254 plate (5.0 × 7.5 cm; Merck, Darmstadt, Germany) with a solvent system of acetonitrile/water (4:1, v/v). Sugars were detected by baking after dipping the plate in 5% sulfuric acid in methanol. When necessary, the amounts of the products were quantified by using HPLC as described above. The amounts of reducing sugar liberated in the hydrolysis of polysaccharides by the enzyme were determined using the DNS method (28) or the copper-bicinchoninic acid method (29).

**Analysis of the anomeric form of the products**

The anomeric form of the hydrolytic product from X3 and X4 (50 mM) was determined by using an isocratic HPLC method (30) described below. The enzymatic reaction was carried out in 25 mM sodium phosphate buffer (pH 7.1) at 25°C with an enzyme
concentration of 5.5 μM. After incubation for 1 min and 25 min, an aliquot (10 μl) of the reaction solution was immediately loaded onto a TSK-GEL AMIDE-80 column (4.6 × 250 mm, Tosoh, Japan), and eluted with acetonitrile-water (7:3 v/v) at a flow rate of 1.5 ml/min at 25°C, separating the xyloooligosaccharides anomers. The initial substrate and products were detected using a refractive index monitor (RI model 504, GL Science, Tokyo, Japan). The retention times of α- and β-xylose were confirmed by loading a solution of α-xylopyranose immediately after preparation, while the retention times of anomers of xylobiose were confirmed by loading the products of 1 min and 25 min hydrolyses of phenyl-β-xylobioside by Cex (31), a family 10 xylanase that forms β-xylobiose as its initial product. In addition, the retention times of xyloooligosaccharides were evaluated by the proportion of the equilibrated anomers (α:β = approximately 4:6) using an equilibrated solution of xyloooligosaccharides. For all xyloooligosaccharides, the α-anomers had shorter retention times than the corresponding β-anomers.

**Kinetic analysis**

To determine the apparent kinetic parameters, X3-X6 were subjected to the hydrolysis in 50 mM sodium phosphate buffer (pH 7.1) at 40°C. The initial rates were measured as the increase in xylose by using HPIC, as described above. The kinetic parameters were calculated by regressing the experimental data (substrate concentration range: 0.2-3K_m) with the Michaelis-Menten equation by the curve-fit method using Kaleidagraph™ ver. 3.51 (Synergy Software).

**Site directed mutagenesis**

Site directed mutagenesis for E70A, D128A, D263A and D128A/D263A was performed using the PCR-overlap extension method (32). The following mutagenetic oligonucleotide primers were used (the mismatched bases are underlined): 5’-CTC GAT GTG CGG ACT GCA GGA ATG TCC TAC-3’ (E70A), 5’-GCC CCA CCA GCT CCG GCC GGA
GAG GAA TAT TTT-3’ (D128A), 5’-CAC TTT TTT AGC GCT TCT TAT CGT GTG GCT-3’ (D263A). The mutated enzymes were prepared and purified as described above.
RESULTS

Characterization of recombinant BH2105

The recombinant BH2105 protein was expressed in *E. coli* BL21(GOLD) and purified to yield a 45-kDa protein on SDS-PAGE. The enzyme was purified 2.7-fold from the cell free extract. The N-terminal sequence of the purified protein was Met-Glu-Lys-Thr-Thr-Glu-Gly-Ala-Phe-Tyr, corresponding to the deduced amino acid sequence from the starting codon. This sequence information suggested that the enzyme has no signal peptide.

This enzyme did not hydrolyze birch wood xylan, even at high concentrations (10 mg/ml), as shown by measuring the increase in reducing value, clearly indicating that it is not an endo β-1,4-xylanase even though it was annotated as xylanase Y. Furthermore, the enzyme did not hydrolyze any other polymeric substrates for GH8 enzymes (chitosan, lichenan, curdlan, and carboxymethyl cellulose) determined by increases in reducing value. Various pentasaccharides (xylopentaose, cellopentaose, laminaripentaose, chitopentaose and chitosanpentaose) were examined for the substrate and the enzyme showed hydrolytic activity only on xylopentaose, producing initially X1 and X4, and finally X1 and X2. To obtain further information on the hydrolysis of xylooligosaccharides (Xn: n = 2 to 6), the products were analyzed on TLC. In the initial stage, the enzyme released X1 and X(n-1) from Xn and the final products were X1 and X2, when n≥3. It hydrolyzed X2 into X1 at a much slower rate than that of X3 (less than 0.01% rate). On the other hand, the enzyme exhibited no activity on X-PNP and X2-PNP, even at extended incubation time, suggesting the possibility of hydrolysis at the reducing end. Oligosaccharides larger than the initial substrates in the enzymatic reaction were not detected, indicating that the enzyme had no transglycosidation activity.

Enzymatic properties as a function of pH and temperature were determined with the X3 hydrolysis (Fig. 1). The optimum pH for activity was between 6.2 and 7.3 and the enzyme was completely stable between pH 5.0 and 9.8 at 30°C for 30 min. The enzyme was stable at temperatures up to 40°C and the optimal temperature was 50°C.
Substrate specificity: recognition of sugar residue

To determine the position of hydrolysis and further substrate specificity of the enzyme, various derivatives of X3 were examined. When β-(1→4) D-glucose and D-xylose-based trisaccharides (G-X-X, X-X-G, G-X-G, X-G-G, G-G-X X-G-X, and G3) were examined as the substrate, the enzyme hydrolyzed only G-X-X, X-X-G and G-X-G at the linkage of the reducing end side, judging from the products, with much slower rate than X3 (Table 1). The reducing end specificity is completely different from that of β-xylosidase, which liberates xylose from the non-reducing end. The substitution of the middle xylose unit into glucose caused complete loss of activity, indicating that the sugar moiety located at the -1 subsite (Fig. 2) must strictly be xylose. Substitution at the reducing end and the non-reducing end caused drastic decreases in activity, indicating that both the +1 and -2 subsites (Fig. 2) strongly prefer xylose, but was not as strict as the subsite -1. Judging from the specificity, the enzyme is very specific to homo xylooligosaccharides. It hydrolyzed X3-de into X2 and 1,5-anhydroxylitol (X-de) at a rate 3.2% of the hydrolysis of X3 (Table 1). This result again confirms that the enzyme hydrolyzes the reducing end glycosyl linkage. The result also suggests that the enzyme recognizes one of the anomeric hydroxyl groups at the reducing end. The small hydrolysis rates for the derivatives of xylotriose were due to their higher $K_m$ values evidenced by their linear S-v relations in the range lower than 2.6 mM, whereas $K_m$ for X3 was 2.4 mM.

Anomeric hydroxyl group recognition by the enzyme

The anomeric composition of the degradation products of X3 and X4 by the BH2105 protein were analyzed by HPLC. Figure 3 shows the HPLC profiles of the hydrolytic products from X3 and X4 obtained with the enzymes. The standard equilibrium ratio of $\alpha$:$\beta$ anomers for X3 and X4 were approximately 4:6 (Fig. 3).

As shown in Fig. 3A, the enzyme produced $\beta$-anomer of X1 and $\alpha$-anomer of X2 from X3 in the reaction for 1 min. Furthermore, the $\alpha$-anomer of X3 was the predominant anomer remaining in the reaction. Small amounts of $\alpha$-X1 and $\beta$-X2 were also observed,
which may have been due to the mutarotation of the initial products over the short term. This result strongly suggests that the enzyme hydrolyzed only the β-anomer of X3 at the linkage of the reducing-end side with anomeric inversion to form α-X2 and β-X1. The preference of the β-anomer explains the reason why the enzyme hydrolyzed X3-de at a much slower rate. After 25 min of the reaction, the substrate (X3) disappeared and the anomeric composition of the product (X and X2) reached equilibrium. This result suggests that the enzyme hydrolyzed α-X3 after the mutarotation that converted α-X3 into β-X3.

In the case of X4 hydrolytic reaction after 1 min, β-X1 and α-X3 were produced with a decrease in β-X4 (Fig. 3B). Again, small amounts of the opposite anomers were also observed, which were probably due to mutarotation. It should be noted that X2, the hydrolytic product of X3, was hardly detected even though half of X4 had already been hydrolyzed in the reaction, suggesting that the enzyme did not act on the α-anomer. The α-X4 remaining and the α-X3 produced must be converted into their β-anomers before the hydrolysis. After 25 min of reaction, the products were equilibrated X1 and X2. The hydrolytic mechanism is schematically summarized in Fig. 4.

**Kinetic property**

The S-v curve of X3-X6 hydrolysis by the enzyme indicates a typical Michaelis-Menten type relationship, the kinetic parameters were summarized in Table 2. $K_m$ value increased with the increase in degree of polymerization while $k_{cat}/K_m$ decreased with the increase in $K_m$. These results suggest that X3 is the most suitable substrate for the enzyme and that the role of the enzyme is to hydrolyze smaller xylooligosaccharides. Subsites downstream of subsite -2 (Fig. 2) may be postulated but such subsites must have negative binding energies.

**Mutational analysis**

The BH2105 amino acid sequence analysis indicated a 32.6% identity with *P. haloplanktis* GH8 xylanase (GenBank accession number: AJ427291). As found in the sequence alignment between these enzymes, the catalytic residues (E70, D128 and D263:
BH2105 numbering) proposed for the xylanase were strongly conserved in the BH2105 amino acid sequence (16). Therefore, we examined the enzymatic activity of alanine mutants (E70A, D123A and D263A) and found that, as expected, the hydrolytic activity of E70A and D263A were $10^{-4}$ orders less that of wild type (Table 3). On the other hand, D128A retains slightly higher activity than that of E70A and D263A, although still approximately three hundredth of wild type (Table 3). The residues were predicted to act as an acid catalyst (E70) and a base catalyst (D263), and to stabilize the $^{2,5}B$ conformation of the sugar moiety bound at subsite -1 (D123) (3,16). These results suggest that these amino acid residues are conserved and are essential for the catalytic reaction of the enzyme.
DISCUSSION

In this report, we determined that the GH8 glycosidase from *B. halodurans* C-125 has novel enzymatic properties, specifically: (i) the enzyme hydrolyze xylooligosaccharides but not xylan; (ii) the enzyme releases xylose from the reducing end of a xylooligosaccharide in an exo-splitting manner; and (iii) the enzyme strictly recognizes the β-anomeric hydroxyl group at the reducing end of the substrate. Considering the reaction mechanism and the substrate specificity, we propose that the name of the enzyme be reducing-end-xylose releasing exo-oligoxylanase (Rex). Unique enzymatic properties and the role of Rex are discussed below.

**Reaction mechanism of Rex**

Rex is a GH8 enzyme found to be an exo-enzyme recognizing reducing ends. It shows 32% amino acid sequence identity with endo-β-1,4 xylanase from *P. haloplanktis*, with at least six subsites at the substrate binding groove, both sides of which are open (15,16). Considering the substrate specificity of Rex, presuming three subsites (-2, -1, and +1) should be enough to explain the results. Also, the substrate binding groove should be close to the upstream subsite +1 (Fig. 2) to be such an exo enzyme. The structural analysis of Rex will reveal the real mechanism of the exo hydrolysis.

It is interesting to note that Rex essentially recognizes β-anomeric hydroxyl group at the reducing end of the substrate (Fig. 3, 4). To our knowledge, such a strict substrate recognition of the anomeric hydroxy group opposite to the catalytic position has been reported only with maltose phosphorylase (33) and cellobiose phosphorylase (34,35). The reducing end sugar residue has one fewer hydroxyl group than the non-reducing end sugar residue, if the anomeric hydroxyl group is not taken into account. In the case of Rex as the reducing-end specific enzyme, the recognition of the anomeric hydroxyl group is probably necessary to increase the recognition position for fixing the reducing-end xylose unit.

The anomeric specificity of Rex necessitates the mutarotation of the products required for the next step, as shown in Figs. 3 and 4. Under the conditions used in the experiments shown in Fig. 3, mutarotation would be the rate limiting step to complete the
cleavage of X4 into X2 + 2 X1. A mutarotase for xylooligosaccharides may accelerate the Rex reaction in vivo. However, the mutarotation seems fast enough to complete the hydrolysis within 25 min (Fig. 3B), suggesting that it is probably not the rate-limiting step of the Rex reaction in vivo, because the enzyme concentration in vivo is not expected to be so high.

The reducing-end specific monomer-forming exo-glycosylase is not so widely known. To our knowledge, this activity has been reported only with two amylolytic enzymes, one from Escherichia coli K12 (36) and one from Thermotoga maritina (37). Although the enzymes released glucose from the reducing end of the substrate, the enzymes could also release p-nitrophenol from p-nitrophenyl α-maltooligosaccharides (such as G2-PNP or G5-PNP) and could hydrolyze cyclodextrins. Thus, these enzymes have some endo-hydrolytic activities and are not complete reducing-end specific exo-glycosylases. In contrast, Rex does not hydrolyze p-nitrophenyl xylobioside at all, indicating that it specifically liberates the reducing-end sugar moiety.

It is necessary to mention the confusing nomenclature of these reducing-end specific enzymes. The term “α-glucosidase” was used to describe amylolytic enzymes that release glucose from maltooligosaccharides (36,37). However, in our opinion, this term is incorrect for these enzymes because they hydrolyze the linkage at the reducing end, an α- glycoside of a maltooligosaccharide, releasing glucose from the reducing end, but they do not hydrolyze real α-glucosides, meaning a linkage at the non-reducing end. We propose that the term monosaccharide glycosidase (e.g. glucosidase or xylosidase) should be used for enzymes that liberate the corresponding monosaccharide from the non-reducing end, as clearly stated in Enzyme Nomenclature (URL: http://www.chem.qmul.ac.uk/iubmb/enzyme/). Thus, careful naming of the reducing end specific exo-glycosylase is required. Considering this, Rex should not be named a β-xylosidase, thus we gave the name of “reducing-end-xylose releasing exo-oligoxylanase.”

Roles of Rex in xylan metabolism in B. halodurans C-125

Our research reveals that Rex has no signal peptide and that neutral pH is optimal
for hydrolytic activity, indicating that the enzyme is an intracellular enzyme of \textit{B. halodurans} C-125. To understand the role of Rex in xylan metabolism of \textit{B. halodurans} C-125, related enzymes are listed in Table 4. The microbe possesses 11 possible genes encoding xylan-related enzymes. However, none of the genes of the related enzymes seemed to form an operon with the gene of Rex. The locations of the enzymes were judged as to whether they had signal peptides predicted using computational analysis (URL: http://www.cbs.dtu.dk/services/SignalP/). As shown in Table 4, signal peptides were predicted with all the acetylxylan esterases and endo-xylanases, suggesting that they were extracellular enzymes. All of the other enzymes, \(\alpha\)-arabinofuranosidases, \(\alpha\)-glucuronidase, Rex and \(\beta\)-xylosidases, were predicted not to have signal peptides, suggesting that they were intracellular enzymes.

Many microorganisms are known to secrete \(\alpha\)-arabinofuranosidase and \(\alpha\)-glucuronidase as well as endo-xylanase, which digest xylan into unsubstituted xyloooligosaccharides or xylose (38,39). However, \textit{B. halodurans} seems to secrete enzymes to digest xylan only up to arabino/glucurono-xyloooligosaccharides, according to the predictions. The proposed pathway of xylan metabolism in \textit{B. halodurans} C125 is illustrated in Fig. 5. Xylans, with \(\alpha\)-arabinofuranosyl and/or \(\alpha\)-glucuronyl side chains, are hydrolyzed to form arabino/glucurono-xyloooligosaccharides, with a larger degree of polymerization in the main \(\beta\)-1,4 xylosyl chain, due to intact side chains, compared with the xylan metabolism systems of other microorganisms. The resulting oligosaccharides are then incorporated into the cell by an unidentified transporter system. Inside the cells, the side chains are removed by \(\alpha\)-arabinofuranosidases and \(\alpha\)-glucuronidases to give unsubstituted xyloooligosaccharides, followed by the hydrolysis of Rex and \(\beta\)-xylosidases from the reducing end and the non-reducing end, respectively. Although it has not been shown experimentally, Rex probably does not directly hydrolyze xyloooligosaccharides having side chains, as suggested by the finding that the GH8 endoxylanase from \textit{P. haloplanktis}, which is expected to have a similar substrate binding groove due to its high sequence similarity to Rex, does not produce short xyloooligosaccharides having side chains from arabino/glucurono-xylans (15). It is likely that a larger degree of xyloooligosaccharide polymerization, caused by the extracellular
hydrolysis of xylan such that the decoration remains intact, requires the action of intracellular Rex to utilize the xylooligosaccharides more efficiently.

From another point of view, such intracellular enzymes may play a role in *B. halodurans* C125 strategies to acquire alkalophilic features. Even though this microbe lives under alkaline conditions, the inside of the cell retains a neutral environment (40). To minimize changes that promote enzymatic function in alkaline conditions, *B. halodurans* C125 may have adopted a strategy that sequesters such enzymes in the intracellular compartment, making it unnecessary to develop alkalophilic enzymes.

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FOOTNOTES

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1 The abbreviations used: Rex, reducing-end-xylose releasing exo-oligoxylanase; Xn, xylooligosaccharide whose degree of polymerization is n.
LEGENDS TO FIGURES

Fig. 1. Enzymatic activity and stability at various pH and temperatures.
A: The effect of pH on activity (open circles) and stability (solid circles). B: The effect of temperature on activity (open circles) and stability (solid circles).

Fig. 2. Schematic drawing of BH2105 subsite structure.
Numbers mean the subsites.

Fig. 3. Anomer analysis of the hydrolytic products from X3 and X4.
A: X3 degradation, B: X4 degradation. For experimental methods see text.

Fig. 4. Reaction mechanism of X3 and X4 hydrolysis by the BH2105 protein

Fig. 5. Schematic proposed pathway for metabolism of xylan in B. halodurans C-125.
Numbers in circles indicate the group of enzymes. The enzymes in each group are given in Table 4.
Tables and Figures

Table 1. Activity of BH2105 toward various trisaccharides

| Substrate          | Product       | Specific Activity (s⁻¹) |
|--------------------|---------------|------------------------|
| X-X-X (X₃)        | X-X + X       | 84.0 (100%)            |
| X-X-X-de (X₃-de)  | X-X + X-de    | 2.7 (3.2%)             |
| G-X-X             | G-X + X       | 0.94 (1.1%)            |
| X-X-G             | X-X + G       | 0.42 (0.5%)            |
| G-X-G             | G-X + G       | 0.003 (0.004%)         |
| X-G-G             | -             |                        |
| G-G-X             | -             |                        |
| X-G-X             | -             |                        |
| G-G-G (G₃)        | -             |                        |

*: not detectable.

[S]₀ = 2.6 mM. The products were analyzed by HPIC.
Table 2. Kinetic parameters of xylooligosaccharides hydrolysis catalyzed by BH2105

| Substrate | $k_{\text{cat}}$ (s$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ (s$^{-1}$ mM$^{-1}$) |
|-----------|--------------------------|------------|---------------------------------|
| X3        | 163±5                    | 2.4±0.2    | 66.9±3.5                       |
| X4        | 162±6                    | 5.0±0.4    | 32.3±1.4                       |
| X5        | 73±2                     | 4.4±0.3    | 16.7±0.5                       |
| X6        | 175±19                   | 18.5±1.3   | 18.5±1.3                       |
Table 3. Specific activity of BH2105 mutants

| Enzyme   | Specific activity (s\(^{-1}\)) | Relative activity (mutant / wild type) |
|----------|---------------------------------|----------------------------------------|
| wild type| 84.0                            | 1.0                                    |
| E70A     | 0.0088                          | 1.1 \times 10^{-4}                     |
| D128A    | 0.29                            | 3.5 \times 10^{-3}                     |
| D263A    | 0.019                           | 2.3 \times 10^{-4}                     |

Substrate: X3 (2.6 mM)
Table 4. A list of enzymes involved in xylan degradation in *B. halodurans* C-125

| Enzyme                                           | Locus   | Family*  | Signal peptide | Group** |
|--------------------------------------------------|---------|----------|----------------|---------|
| Acetylxylan esterase                              | BH3326  | CE7      | yes            | 1       |
| Acetylxylan esterase                              | BH3902  | CE4      | yes            | 1       |
| Endo-β-1,4 xylanase                              | BH0899  | GH11     | yes            | 1       |
| Endo-β-1,4 xylanase                              | BH2120  | GH10     | yes            | 1       |
| α-L-arabinofuranosidase                          | BH1861  | GH51     | no             | 2       |
| α-L-arabinofuranosidase                          | BH1874  | GH51     | no             | 2       |
| α-glucuronidase                                  | BH1061  | GH67     | no             | 2       |
| Reducing-end-xylose releasing exo-oligoxylanase  | BH2105  | GH8      | no             | 3       |
| Xylosidase                                       | BH1068  | GH39     | no             | 4       |
| Xylan β-1,4-xylosidase                           | BH1867  | GH43     | no             | 4       |
| Xylan β-1,4-xylosidase                           | BH3683  | GH43     | no             | 4       |

*GH, glycoside hydrolase; CE, carbohydrate esterase; for further details see CAZy web site (URL: http://afmb.cnrs-mrs.fr/CAZY/).

** The group numbers correspond to the enzymes in Fig. 5.
Fig. 1 Honda et al.
Fig. 2 Honda et al.
Fig. 3 Honda et al.
Mutarotation (non-enzymatic)

Inverting hydrolysis (enzymatic)

Mutarotation (non-enzymatic)

Inverting hydrolysis (enzymatic)

Fig. 4 Honda et al.
Out
Xylan

Transporter

In
Arabino/glucurono-xylooligosaccharides

Arabinose and glucuronic acid

Xylooligosaccharides

Xylose

Xylobiose

Fig. 5 Honda et al.