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Characterization of a *Paenibacillus woosongensis* β-Xylosidase/ α-Arabinofuranosidase Produced by Recombinant *Escherichia coli*

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A gene encoding the β-xylosidase/α-arabinofuranosidase (XylC) of *Paenibacillus woosongensis* was cloned into *Escherichia coli.* This *xylC* gene consisted of 1,425 nucleotides, encoding a polypeptide of 474 amino acid residues. The deduced amino acid sequence exhibited an 80% similarity with those of both *Clostridium stercorarium* β-xylosidase/α-*N*-arabinosidase and *Bacillus cellulosilyticus* α-arabinofuranosidase, belonging to the glycosyl hydrolase family 43. The structural gene was subcloned with a C-terminal His-tag into a pET23a(+) expression vector. The His-tagged XylC, purified from a cell-free extract of a recombinant *E. coli* BL21(DE3) Codon Plus carrying a *xylC* gene by affinity chromatography, was active on *para*-nitrophenyl-α-arabinofuranoside (pNPA) as well as *para*-nitrophenyl-β-xylopyranoside (pNPX). However, the enzymatic activities for the substrates were somewhat incongruously influenced by reaction pHs and temperatures. The enzyme was also affected by various chemicals at different levels. SDS (5 mM) inhibited the enzymatic activity for pNPX, while enhancing the enzymatic activity for pNPA. Enzyme activity was also found to be inhibited by addition of pentose or hexose. The Michaelis constant and maximum velocity of the purified enzyme were determined for hydrolysis of pNPX and pNPA, respectively.

Keywords: *Paenibacillus woosongensis*, β-xylosidase/α-arabinofuranosidase, characterization, recombinant *Escherichia coli*

Xylan, a major component of hemicellulose, is a highly branched β-1,4-linked D-xylose polymer with substituents that include acetyl, arabinosyl, and the glucuronyl groups. Complete degradation of xylan requires the action of several types of enzymes; endo-β-1,4-xylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and ferulic acid esterase. Among these enzymes, endoxylanase and β-xylosidase have the most important activities in the degradation of xylan. Endoxylanase degrades xylan into xylooligosaccharides by attacking internal xylosidic linkages on the xylan backbone, and β-xylosidase subsequently hydrolyzes xylobiose and short xylooligosaccharides into D-xylose by endwise attack. Xylose is regarded as a fermentative sugar for the production of valuable products, including xylitol, xylulose, and ethanol [21]. β-Xylosidase is therefore important for the complete hydrolysis of xylan.

β-Xylosidases are produced by a variety of bacteria and fungi. With some exceptions [6], the majority of bacterial β-xylosidases are intracellular, whereas those of fungi are cell-bound or extracellular. β-Xylosidases are currently classified into families 3, 30, 39, 43, 52, 54, and 116 of the glycosyl hydrolases (GHs), based on their amino acid sequence similarities. These enzymes are monomeric [8, 15], dimeric [2, 6, 12, 14, 19], or tertrameric [11] components. Some β-xylosidases were named β-xylosidase/α-arabinofuranosidase because they are bifuctional proteins showing both activities of β-xylosidase and α-arabinofuranosidase [4, 12]. Recently, β-xylosidases have been intensively studied with respect to their structures and catalytic residues from *Selenomonas ruminantium* [4] and *Geobacillus stearothermophilus* [1], although several enzymes were purified and characterized from other bacterial strains of genera *Aeromonas* [14], *Bacillus* [5, 19], *Clostridium* [11, 13], *Geobacillus* [10, 18], *Leifsonia* [9], *Prevotella* [3], *Streptomyces* [15], *Thermoanaerobacter* [12], *Thermoanaerobacterium* [8], and *Thermotoga* [20].

From 2005, about 15 novel species of genus *Paenibacillus*, able to degrade xylan, had been isolated and identified. Some xylanases were characterized from *Paenibacillus* stains including *P. barcinonensis* [16] and *P. curdlanolyticus* [17]. To our knowledge, neither β-xylosidase nor α-arabinofuranosidase has been biochemically investigated from *Paenibacillus* strains, although their genes were predicted from the genome sequence of *Paenibacillus* sp. JDR-2 (GenBank Accession No. ABKS01000022). We

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have previously reported the isolation of *P. woosongensis* from forest soil [7]. The novel species exhibits enzymatic activities suitable for hydrolyzing polysaccharides such as cellulose, xylan, and mannan. In this work, a *P. woosongensis* β-xylosidase/α-arabinofuranosidase gene (*xylC*) was cloned into *Escherichia coli*, with the enzyme then being characterized from the recombinant *E. coli*.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Media

P. woosongensis KCTC 3953 (DSM 16971) was used as the source of the gene coding for β-xylosidase/α-arabinofuranosidase (XylC), *E. coli* DH5α as the host for recombinant plasmids, and *E. coli* BL21(DE3) Codon Plus as the host for gene expression. *E. coli* was cultured at 37°C in an LB broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter, pH 7.0). Ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) were used for the selection and culture of *E. coli* transformants. *P. woosongensis* was cultured at 37°C in tryptic soy broth (TSB; 17 g of tryptone, 3 g of soytone, 2.5 g of dextrose, 5 g of NaCl, 2.5 g of K_2HPO_4 per liter, pH 7.2). Plasmid pUC19 was used for all cloning and sequencing experiments, and pET23a(+) was used as the expression vector for the *xylC* gene.

DNA Manipulation and DNA Sequencing

The genomic DNA was isolated from *P. woosongensis* cells grown exponentially in TSB using the genomic DNA preparation kit from Solgent Co. (Deajeon, Korea). The genomic DNA was partially digested with *Sau*3AI, and DNA fragments, ranging from 1.5 to 10 kb, were isolated from an agarose gel. The *Sau*3AI-generated chromosomal DNA fragments were introduced into the dephosphorylated *Bam*HI site of pUC19, and the ligation mixture was transformed into *E. coli* DH5α. The nucleotide sequence of the cloned DNA fragments was determined with a DNA sequencer (ABI Prism 377, Perkin Elmer Co., Foster City, CA, U.S.A.). To construct a recombinant plasmid for high expression of a *xylC* gene in *E. coli*, the gene was subcloned into pET23a(+). Oligonucleotide primers were designed for the amplification of the gene by PCR according to its nucleotide sequences as follows: forward primer (CATTACATATGACAAAAC AAGGTTTGAATCC) and reverse primer (ATTCACTCGAGTTCC AGCGTGAACGAGGCCA). The recognition sites for suitable restriction enzymes were added to the forward (*Nde*I site is underlined) and the reverse (*Xho*I site is underlined) primers. In addition, the reverse primer was designed to have a no-stop codon for preparing a gene product fused with the six-His tag of pET23a(+) at its C terminus. The PCR amplification was performed in a final volume of 50 μl, with the reaction mixtures containing the cloned gene (10 ng), 40 pmol of each primer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs, PCR buffer, and 2.5 U of *Pfu* DNA polymerase for 30 cycles in the process of 25 s at 95°C and 40 s at 58°C, and 3 min at 72°C. *Nde*I- and *Xho*I-generated DNA fragments of the PCR product were introduced into the same sites of pET23a(+), and the resulting plasmid was named pEAY51.

Expression and Purification of the His-tagged Enzyme in *E. coli*

The recombinant *E. coli* BL21(DE3) Codon Plus cell harboring plasmid pEAY51 was grown at 37°C in an LB medium containing antibiotics. When the cultures reached an optical density of 0.6 at 600 nm, IPTG was added for a final concentration of 0.5 mM. After induction for 2 h at 37°C, the cells were harvested and resuspended in a cell lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), disrupted by sonication, and centrifuged to obtain the cell-free extracts. The cell-free extract was applied to a Ni-NTA column, which was equilibrated with the same buffer. After removing the unbound and weakly bound proteins with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the His-tagged enzyme was eluted with an elution buffer containing 250 mM imidazole. The active fractions were pooled and dialyzed against a 10 mM sodium phosphate buffer (pH 6.5).

Enzyme Assays and Kinetic Analysis

XylC activity was determined by measuring the *para*-nitrophenol (pNP) released from pNP-β-D-xylopyranoside (pNPX) or pNP-α-L-arabinofuranoside (pNPA). Reaction mixtures of 0.5 ml, containing a 1 mM substrate in 50 mM sodium phosphate buffer (pH 6.5 for pNPX, pH 7.0 for pNPA), were incubated for 10 min at 50°C (for pNPX) or 45°C (for pNPA), respectively. The reaction was stopped by the addition of 1 ml of 1 M sodium carbonate. The absorbance was measured at 405 nm. Steady-state kinetic measurements were performed at optimal temperatures and pHs for pNPX and pNPA by varying the concentrations of the substrates (0.125−4 mM). The K_m and V_{max} values were calculated from the initial rate of pNP liberation. The enzyme activity for hydrolyzing xylans was determined by measuring the amount of reducing sugars. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of pNP or reducing sugar per minute. Protein concentrations were determined by the Bradford method.

Effects of pH, Temperature, and Various Reagents on Enzymatic Activity

The effect of pH on the reaction rate was determined by measuring the enzyme activity for pNPX and pNPA at a fixed temperature (45°C) and various pHs with 50 mM of sodium citrate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), and Tris (pH 8.0 to 9.0) buffers. The activity of the purified enzyme was also assayed with a 50 mM sodium phosphate buffer (pH 6.5 or 7.0) at various temperatures, ranging from 30°C to 65°C. To investigate the effects of chemicals on XylC activity, both β-xylosidase and α-arabinofuranosidase activities were measured by adding several reagents (5 mM) into the standard reaction mixture. For examining the effect of sugar on enzyme activity, hydrolysis reactions for pNPX and pNPA were performed after the addition of monosaccharides to the standard reaction mixture, with the final concentrations ranging from 25 mM to 400 mM.

Results and Discussion

Cloning and Nucleotide Sequence of a *P. woosongensis xylC* Gene

E. coli transformants containing *P. woosongensis* genomic DNA were transferred to LB agar plates for overnight incubation. Soft agar [0.7% (w/v)] containing 0.2 mM 4-methylumbelliferyl β-D-xylopyranoside (MUX) was overlaid on the plates for screening *E. coli* clones that exhibited β-

xylosidase activity. After incubation for 2 h at 40°C, one colony capable of hydrolyzing MUX was selected through the observance of intense fluorescence at 360 nm, the result of liberated 4-methylumbelliferone.

The complete 1,768-bp nucleotide sequence of *P. woosongenesis* genomic DNA on the recombinant plasmid was determined (GenBank Accession No. HQ404312). The nucleotide sequence revealed that the open reading frame (ORF) was composed of 1,425 base pairs coding for a polypeptide of 474 amino acid residues with a molecular mass of 52,852 Da. When the deduced amino acid sequence of the *P. woosongensis* XylC was compared with the sequences of other proteins in the NCBI database using the BLAST search program, the former enzyme displayed homologies of approximately 80% with β-xylosidase/α-arabinofuranosidase of *C. stercorarium* (GenBank Accession No. P48790), and with α-arabinofuranosidases of *B. cellulosilyticus* DSM 2522 (EFC16922), *C. cellulolyticum* H10 (ACL75373), and *C. papyrosolvens* DSM 2782 (EEU60867). However, XylC exhibited no amino acid similarity with β-xylosidase/α-arabinofuranosidase predicted through the genome sequence of *Paenibacillus* sp. JDR-2 (EDS50275) (data not shown). These enzymes consist of a single catalytic domain belonging to GH family 43.

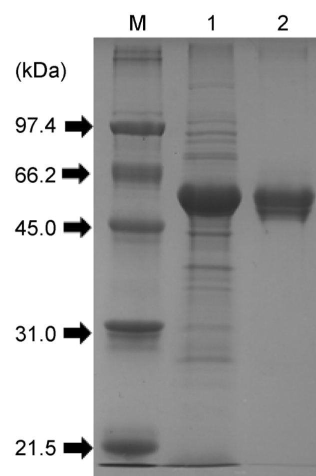

Fig. 1. SDS−PAGE of the purified His-tagged XylC from recombinant *E. coli.*

Lane M, the molecular mass markers; Lane 2, cell-free extract; Lane 3, the purified enzyme. Molecular size is shown in kilodaltons to the left side of the gel.

Production and Purification of His-tagged XylC in *E. coli.*

Many fusion proteins were constructed and produced by recombinant *E. coli* for the simple purification of the enzymes. The His-tagged XylC (HtXylC), containing a six-histidyl tail at its C terminus, was constructed as previously described. The fused protein could not be expressed effectively by IPTG in *E. coli* BL21(DE3), but could be highly expressed in *E. coli* BL21(DE3) Codon Plus transformed with rare tRNA genes. The enzyme was produced in a soluble form from the recombinant *E. coli* cells grown at 37°C as well as from those grown at 25°C (data not shown).

The HtXylC was purified to homogeneity of above 90% from the cell-free extract in a single step using Ni-NTA resin (Fig. 1). The purified enzyme displayed a single protein band, corresponding to a molecular mass of 53−54 kDa, through the use of SDS−PAGE. The molecular

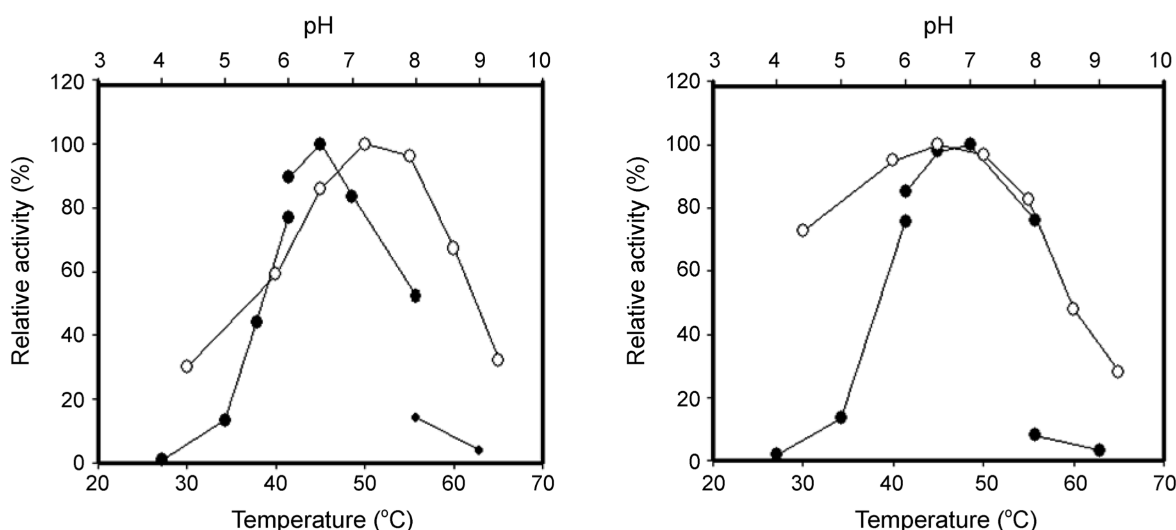

Fig. 2. Temperature and pH optima of the purified His-tagged XylC for hydrolyzing pNPX (left) and pNPA (right).

The temperature profile (-○-) was obtained by measuring the enzyme activities at different temperatures with fixed pHs 6.5 for pNPX hydrolysis and 7.0 for pNPA hydrolysis. The pH profile (-●-) was obtained by measuring the enzyme activities at various pHs with constant temperatures of 50°C for pNPX hydrolysis and 45°C for pNPA hydrolysis. Buffers (50 mM) used were as follows: sodium citrate (pH 4−6), sodium phosphate (pH 6−8), and Tris (pH 8−9).

mass of the purified enzyme agrees with that of the HtXylC predicted by the nucleotide sequence of the *xylC* gene. The XylC is smaller than many bacterial β-xylosidases estimated as having molecular masses of 75 to 180 kDa by SDS−PAGE [9, 14]. β-Xylosidase of *Thermoanerobacterium saccharolyticum* B6A-RI (55 kDa) has been reported as having a molecular mass similar to that of XylC [8].

Reaction Properties of the Purified His-tagged XylC

Two substrates, pNPX and pNPA, were used to investigate the effects of reaction temperatures and pHs on enzymatic activity. Purified HtXylC had the highest activity on pNPX at 50°C and with a pH of 6.5, with a greater than 80% activity at any pH between 6.0 and 7.0, whereas the enzyme showed maximal activity toward pNPA at 45°C and at a pH of 7.0 (Fig. 2). The relative enzyme activity was approximately 52% for pNPX and 76% for pNPA at pH 8.0. The HtXylC has an optimal temperature similar to β-xylosidase of *B. pumilus* [5], but its optimal temperature is seen to be very much lower than those of many β-xylosidases [2, 6, 8, 10, 12, 20]. β-Xylosidase of *Thermoanaerobacter ethanolicus* was also reported to exhibit its maximal activity for pNPX and pNPA at different pHs and temperatures [12]. The thermostability and pH stability of purified HtXylC were examined by measuring the residual activity for pNPX hydrolysis after preincubating at various temperatures (30 to 45°C) or pHs (5.0 to 9.0) without the substrate. As shown in Fig. 3, the enzyme was stable up to 30°C for 3 h, and it retained 83% of its maximal activity at 40°C after 3 h of incubation. However, the stability of the enzyme decreased rapidly at temperature above 45°C, indicating the XylC is thermolabile. An extremely thermostable β-xylosidase of *Thermotoga maritima* was known to have a half-life of over 22 min at 95°C [20].

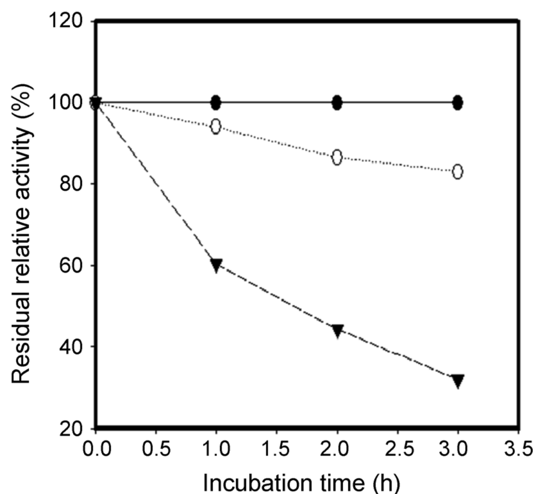

Fig. 3. Thermostability of the purified His-tagged XylC.
The thermostability was determined by measuring the residual activities after preincubation for 3 h at different temperatures: 30°C (-●-), 40°C (-○-), 45°C (-▼-).

Table 1. Substrate specificity of the purified His-tagged XylC.

Substrates	Specific activity (mU/mg protein)
pNP-β-D-xylopyranoside	659.90
pNP-α-L-arabinofuranoside	347.29
pNP-α-L-arabinopyranoside	0.06
pNP-β-D-galactopyranoside	0.65
pNP-β-D-glucopyranoside	ND
pNP-β-D-mannopyranoside	ND
pNP-α-D-galactopyranoside	ND
pNP-α-D-glucopyranoside	ND
pNP-β-D-glucuronide	ND
pNP-β-D-cellobioside	ND
Oat spelt xylan	>0.03
Birchwood xylan	<0.03

ND, Not detected.

Although the deduced amino acid sequence of XylC was found to be highly homologous with those of β-xylosidase/α-arabinofuranosidase and α-arabinofuranosidases as described above, HtXylC was more active on pNPX than on pNPA, as shown in Table 1. The enzyme hydrolyzed pNPA with 52% of the activity toward pNPX, but exhibited a very low activity against pNP-α-L-arabinopyranoside (pNPAP) and pNP-β-D-galactopyranoside. Since pNPX is a substrate for β-xylosidases and the pNPA is a substrate for α-arabinofuranosidase, XylC is assumed to be a bifuctional enzyme showing β-xylosidase and α-arabinofuranosidase activities. On the other hand, the xylosidase of *T. ethanolicus* has been reported to have specific activity of above 5-folds greater for pNPA and above 4-folds greater for pNPAP than for pNPX [12]. β-Xylosidases of both *P. ruminicola* 23 [3] and *T. maritima* [20] could hydrolyze pNPA with below 20% of their activity toward pNPX. Mutagenesis of the putative

Table 2. Effects of metal ions and other reagents on enzymatic activity.

Effector (5 mM)	Relative hydrolyzing activity (%) for	
	pNPX	pNPA
None	100	100
KCl	101	107
$NiCl_2$	120	109
$MgCl_2$	171	145
$MnCl_2$	187	133
$CaCl_2$	120	106
$CuCl_2$	83	88
$FeCl_2$	57	12
$FeCl_3$	170	49
EDTA	79	68
SDS	70	112

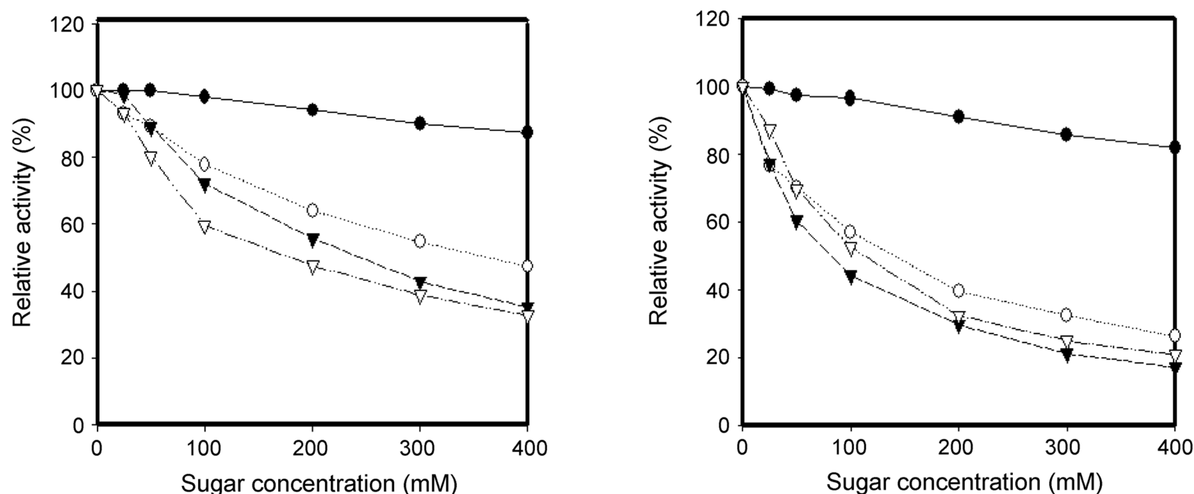

Fig. 4. Effects of sugars on the β-xylosidase (left) and α-arabinofuranosidase (right) activities of the purified His-tagged XylC.
The relative activity was determined by measuring β-xylosidase activity for pNPX (1 mM) and α-arabinofuranosidase activity for pNPA (1 mM) in the presence of various concentrations of each sugar, including D-glucose (-●-), D-xylose(-▼-), D-ribose (-○-), and L-arabinose (-▽-), respectively.

catalytic amino acids residues was found to affect to a similar extent both the pNPX and pNPA hydrolyzing activities of β-xylosidase from *S. ruminantium*, suggesting that the two substrates share the same active site [4]. In addition, the HtXylC displayed very low levels of enzymatic activity toward xylans from oat spelt and birchwood.

The kinetic parameters, K_m and V_{max}, of the HtXylC for pNPX and pNPA were examined by steady-state kinetic analysis. The K_m was estimated to be 1.1 mM for pNPX and 8.5 mM for pNPA, indicating that the enzyme exhibits a higher affinity for pNPX, whereas V_{max} was estimated to be 1.4 µmol/min/mg for pNPX and 3.1 µmol/min/mg for pNPA. The β-xylosidases of *C. stercorarium* [11] and *T. ethanolicus* [12] also showed a higher affinity for pNPX than for pNPA. This K_m value of HtXylC for pNPX is moderate in comparison with the K_m values of other β-xylosidases ranging from 0.038 to 6.2 mM [2, 6, 9, 10, 11, 12, 13, 14, 20].

Effects of Metal Ions and Other Reagents on Enzymatic Activity

The effects of various reagents, including metal salts, EDTA, and SDS on HtXylC activity were investigated (Table 2). When the enzyme activity of the XylC was measured for the hydrolysis of pNPX and pNPA in the presence of reagents, respectively, the activities were affected differently by them according to the substrates. The enzymatic activities for hydrolyzing both substrates were noticeably increased by metals such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} at different levels. However, the enzyme activity for hydrolyzing pNPX was enhanced by SDS and inhibited by Fe^{3+}, whereas the activity toward pNPA was inhibited by SDS and increased by Fe^{3+}. The Fe^{2+} ion inhibited the pNPX hydrolyzing activity much more than the pNPA hydrolyzing activity. In addition, the enzymatic activities toward both substrates were similarly decreased by EDTA and Cu^{2+}. It was reported that Cu^{2+} and Fe^{2+} inhibited the pNPX hydrolyzing activity of other β-xylosidases [6, 9, 15]. However, Ca^{2+} and Mg^{2+} did not have an effect on *B. thermantarcticus* β-xylosidase [6], and only marginally inhibited the *G. pallidus* enzyme for hydrolysis of pNPX [10], differing from HtXylC.

Most of the glycosidases studied so far have been inhibited by sugars, an end-product of the enzymatic reaction catalyzed by them, and have also been inhibited by other monosaccharides [18]. To investigate the effects of end-products of hydrolysis reactions on enzyme activity, the pNPX and pNPA hydrolyzing activities of the HtXylC were determined in the presence of various concentrations of D-glucose, D-ribose, D-xylose, and L-arabinose, respectively. As shown in Fig. 4, the hydrolyzing activities for both pNPX and pNPA were inhibited greater by pentoses than by glucose. The enzyme activity decreased severely with increasing concentrations of sugar. The pNPA hydrolyzing activity was inhibited by pentoses to a much greater extent than pNPX hydrolyzing activity, whereas both activities of HtXylC were similarly decreased by glucose. This study thus represents the first examination and characterization of the biochemical properties of β-xylosidase/α-arabinofuranosidase for the genus *Paenibacillus*.

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