

Characteristics of transxylosylation by β -xylosidase from *Aspergillus awamori* K4[☆]

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Received 23 May 2005; received in revised form 22 August 2005; accepted 22 August 2005

Available online 13 September 2005

Abstract

The *Aspergillus awamori* K4 β -xylosidase gene (*XawI*) sequence was deduced by sequencing RT-PCR and PCR products. The ORF was 2,412 bp and the predicted peptide was 804 amino acids long, corresponding to a molecular weight of 87,156 Da. The mature protein was 778 amino acids long with a molecular weight of 84,632 Da. A homology search of the amino acid sequence revealed that it was very similar to the *Aspergillus niger* β -xylosidase gene with only five amino acid differences. K4 β -xylosidase had the same catalytic mechanism as family 3 β -glucosidases, involving Asp in region A. At an early stage in the reaction with xylobiose and xylotriose, the hydrolysis rate was much lower than the transxylosylation rate, decreasing gradually as the substrate concentration increased, whereas the transxylosylation rate increased greatly. *Aspergillus awamori* K4 β -xylosidase had broad acceptor specificity toward alcohols, hydroxybenzenealcohols, sugar alcohols and disaccharides. A consensus portion involving the hydroxymethyl group of the acceptor was confirmed in the major transfer products 1(4)-*O*- β -D-xylosyl erythritol, (2-hydroxyl)-phenyl-methyl- β -D-xylopyranoside, 6*S*-*O*- β -D-xylosyl maltitol (S: sorbitol residue) and 6*G*-*O*- β -D-xylosyl palatinose (G: glucosyl residue). This might suggest that the methylene in the hydroxymethyl group facilitates base-catalyzed hydroxyl group attack of the anomeric center of the xylosyl–enzyme intermediate.

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Keywords: β -xylosidase; Transglycosylation; *Aspergillus*; Glucosidase; Xylooligosaccharide

1. Introduction

β -Xylosidase (EC 3.2.1.37) hydrolyzes xylooligosaccharides such as xylobiose and xylotriose to xylose by recognizing the xylosyl residue at the nonreducing terminal and cleaving the β -1,4 glycosidic linkage [1]. It is a useful enzyme for producing xylose from the xylan of hemicellulose, one of the most abundant biomass resources. β -Xylosidases are presently classified into five families: 3, 39, 43, 52, and 54 [2–4]. The catalytic action of β -xylosidases is generally accepted to be a double-displacement mechanism involving a glycosyl–enzyme intermediate, similar to the mechanism of glycosidases [5–7]. Two essential amino acids play important roles in the enzymatic action of β -xylosidases. The first acts as a nucleo-

phile, attacking the anomeric center of the substrate to form the covalent glycosyl–enzyme intermediate. The second then acts as a general acid/base catalyst, deprotonating the water molecule and attacking the anomeric center to cleave the covalent binding and release the xylose. These processes are performed through oxocarbenium ion transition states. The mechanism of and essential amino acids in the active site have been studied and discussed with regard to some β -xylosidases [8–10]. Family 39 and 52 β -xylosidases possess Glu-277 and Glu-160 and Glu-335 and Asp-495 as the nucleophile and acid/base residues, respectively. On the other hand, many fungal β -xylosidases have partial similarity to family 3 glucosidases with the amino acid sequence corresponding to the active site being highly conserved [11–15]. However, the three-dimensional structure and catalytic mechanism of family 3 glycosidases, including β -xylosidases, remain largely unknown.

Some β -xylosidases show transxylosylation at high substrate concentrations. When the xylobiose of the substrate is also used as the acceptor, higher molecular weight xylooligo-

[☆] The nucleotide sequence of *XawI* is presented in the DDBJ/GenBank with the accession number AB154359.

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saccharides with a higher DP value are synthesized [16]. The enzyme transfers the xylosyl residue to xylobiose, thus synthesizing xylotriose, and the transfer product is used as an acceptor to produce the higher molecular weight (higher DP value) xylooligosaccharides. *Aspergillus* sp. β -xylosidase was shown to synthesize *p*-nitrophenyl β -1,4-D-xylooligosaccharides (chain length: DP 2–7) in transglycosylation with *p*-nitrophenyl β -D-xylopyranoside [17]. Transxylosylation also proceeds in cases where an acceptor, such as an alcohol, is added to the reaction mixture. *Aspergillus niger* and *Trichoderma viride* β -xylosidases synthesize alkyl β -xylosides from xylobiose or methyl- β -D-xyloside, as substrates, and alcohols, as acceptors, through such transxylosylation reactions [18,19]. *Aspergillus pulverulentus* β -xylosidase even transfers the xylosyl residue to the toxic hydroquinone diphenol [20]. These fungi produce β -xylosidases with high transferring activity and secrete related enzymes such as xylanase and α -L-arabinofuranosidase. Details of the enzymatic mechanism of transxylosylation, such as the action of catalytic groups in the active site and acceptor specificity, remain largely unknown. It is thought that a specific regular chemical structure might be necessary with regard to the acceptors.

Previously, we reported the characteristics of β -xylosidase from *Aspergillus awamori* K4 [21]. The optimal enzymatic conditions were pH 5 and 60 °C, and the molecular weight was estimated to be 115,000 Da by SDS-PAGE. This enzyme has a high xylosyl-transferring activity and broad acceptor specificity for transxylosylation. In particular, trehalose, sorbitol and mannitol were shown to be good acceptors, producing 6G-*O*- β -D-xylosyl trehalose, 6-*O*- β -D-xylosyl sorbitol and 1(6)-*O*- β -D-xylosyl mannitol as the major transfer products, respectively. Elucidation of the catalytic mechanism in the active site and the recognition of acceptors for transxylosylation would be very useful for synthesis of new oligosaccharides. In this study, the DNA sequence of *Aspergillus awamori* K4 β -xylosidase was analyzed and the transxylosylation properties of the enzyme were investigated to obtain information regarding the mechanism of transxylosylation with various acceptors.

2. Materials and methods

2.1. Materials

Xylobiose, xylotriose and xylooligosaccharides (xylotriose 3.8%, xylobiose 68.3% and xylose 27.9%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), oat spelt xylan was obtained from Sigma-Aldrich Ltd. (USA), and erythritol was supplied by Nikken Chemicals Co. Ltd. All other chemicals were of reagent grade.

2.2. Production and purification of β -xylosidase by liquid culture

The K4 strain was cultivated in liquid medium (400 ml) containing 1% oat spelt xylan, 0.5% yeast extract, and 0.2% disodium hydrogenphosphate 12-hydrate in a 2L flask at 30 °C and 140 rpm for 3 days. The mycelium pellet grown in the liquid culture was separated, washed by filtration and freeze-dried. The dried pellet was then powdered, and a 4% suspension was lysed by 0.5% yatalase (Oozeki Ltd., Japan) at pH 5 and 40 °C for 24 h. The β -xylosidase released from the strain by digestion was separated into the supernatant by centrifugation at 1500 $\times$ g for 10 min. The enzyme was precipitated by adding 75% (v/v) cold acetone (–20 °C) and separated by filtration. The precipitate

was dissolved in 15 ml 0.1 M acetate buffer (pH 5) and subjected to the following column chromatography steps at 4 °C. The enzyme solution (15 ml) was first subjected to gel filtration chromatography on a Sephadex G-25 column (ϕ 2.5 $\times$ 50 cm) pre-equilibrated with 10 mM sodium acetate buffer (pH 5). The proteins were eluted with the same buffer at a flow rate of 1.3 ml/min and fractions were collected every 3 min. Next, fractions showing β -xylosidase activity were pooled and subjected to anion-exchange chromatography on a Super Q Toyopearl column (ϕ 1.0 $\times$ 50 cm; Toso Ltd., Japan) pre-equilibrated with 10 mM sodium acetate buffer (pH 5). The proteins were eluted with a linear gradient of 0 to 0.6 M NaCl at a flow rate of 0.16 ml/min. The β -xylosidase fractions, eluted at around 0.5 M NaCl, were pooled and subjected to gel filtration chromatography on a Sephacryl S-200 column (ϕ 1.0 $\times$ 100 cm). The proteins were eluted with the same buffer at a flow rate of 0.1 ml/min and fractions were collected every 15 min. At this chromatography step, K4 β -xylosidase was completely isolated from other enzymes [21].

2.3. Determination of β -xylosidase activity

p-Nitrophenyl- β -D-xylopyranoside (1 mM) was incubated with K4 β -xylosidase at pH 5 and 40 °C for 10 min. The reaction was stopped by adding 1 M Na₂CO₃. The concentration of nitrophenol released into the solution was measured by the absorbance at 400 nm. One unit was defined as the amount of enzyme that could produce 1 μ mol nitrophenol/min.

2.4. Transxylosylation

Xylobiose or xylotriose (0.2–6.4%) was incubated with 0.2 U/ml K4 β -xylosidase at pH 5 and 40 °C for 30 min. The reaction was stopped by incubation for 5 min in boiling water then subjected to high performance liquid chromatography (HPLC) analysis after dilution and filtration through a 0.22- μ m membrane filter. In experiments where an acceptor was added, 3.2% xylobiose (substrate) and 4 or 8% acceptor were incubated with K4 β -xylosidase at 0.2, 1.0, or 1.8 U/ml at pH 5 and 40 °C for 24 h.

2.5. Analysis of sugars

Xylooligosaccharides and transfer products were analyzed by HPLC under the following two conditions: (A) column: ϕ 4.6 $\times$ 250 mm NH2P-50 (Asahi Chemical Industry Co. Ltd.), temperature: 25 °C, mobile phase: acetonitrile–water (24:76 to 70:30 v/v), flow rate: 1.0 ml/min; and (B) column: ϕ 7 $\times$ 250 mm GL-C610 (Hitachikasei Ltd.), temperature: 60 °C, mobile phase: water, flow rate: 1.0 ml/min. The eluted sugars were detected using a Hitachi model L-3300 differential refractive index monitor.

For analysis of the chemical structure of the transfer products, larger amounts of the reaction components were required. Xylooligosaccharides (10%) and acceptors (10%) were incubated with K4 β -xylosidase (0.2 U/ml) at pH 5 and 40 °C for 48 h. Each reaction was stopped by incubation for 5 min in boiling water then subjected to HPLC separation after filtration through a 0.22 μ m membrane filter. An aliquot (0.1–0.25 ml) was injected onto an NH2P-50 column (ϕ 10 $\times$ 250 mm) under the following conditions: temperature: 25 °C, mobile phase: acetonitrile–water (24:76 or 70:30 v/v), flow rate: 2.0 ml/min. The separated elution peak was fractionated and freeze-dried and the transfer product was identified by ¹H- and ¹³C-NMR analysis. ¹H- and ¹³C-NMR spectra were recorded at 400 MHz using a model AT 300 K NMR analyzer (Nihon Bruker Ltd.). Each sample (1%) was dissolved in deuterium oxide and the chemical shifts were calibrated using the signal of acetone as an internal standard.

2.6. N-terminal amino acid sequencing of peptides from β -xylosidase

Purified β -xylosidase was reduced to disrupt disulfide linkages. The freeze-dried enzyme was dissolved in 0.5 ml buffer containing 7 M guanidine hydrochloride, 10 mM EDTA and 0.5 M Tris–HCl (pH 8.5). One milligram of dithiothreitol was then added and incubated at 25 °C for over 5 h. The released thiol groups were modified by adding 4-vinylpyridine (2 μ mol) and the resultant mixture was incubated at 25 °C for 30 min. The mixture was then dialyzed against 0.1% trifluoroacetic acid (TFA) and the modified enzyme was

freeze-dried. The enzyme was dissolved in 0.1 M acetic acid and incubated with 0.02–0.1 mg/ml TPCK-treated trypsin at 37 °C for 15 h. The digested peptides were separated by HPLC under the following conditions: column: $\phi 4.6 \times 250$ mm TSKgel Octyl-80 Ts (Toso Ltd., Japan), temperature: 25 °C, mobile phase: (A) 0.1% TFA and (B) 2-propanol-acetonitrile (70:30, v/v) containing 0.08% TFA (the composition of B in a mixture of A and B was changed from 10 to 50% over 60 min with a linear gradient), flow rate: 1.0 ml/min, detector: model SPD-7A UV spectrophotometer (Shimadzu Ltd.). The separated peptide peaks were fractionated and subjected to N-terminal amino acid sequencing by automatic Edman degradation using a Procise 491 protein sequencing system (Applied Biosystems Ltd.).

2.7. mRNA purification and RT-PCR

Aspergillus awamori K4 was cultured in medium containing 1% oat spelt xylan, 0.5% yeast extract and 0.2% disodium hydrogenphosphate 12-hydrate at 40 °C and 140 rpm for 32 h. Total RNA was extracted from the mycelium pellet using a cationic surfactant followed by a lithium chloride-based method (Catrimox-14 RNA Isolation Kit; Takara Biomedical Ltd.). mRNA was purified using Oligo(dT) 30 immobilized in latex (Oligotex-dT30 mRNA Purification Kit; Takara Biomedical Ltd.). Reverse-transcription polymerase chain reaction (RT-PCR) was performed with mRNA using combinations of seven designed primers: forward: 5'-GTCATGTGCGCCTACAACGCC-3' (VMCAYNA), 5'-GGATACGCTCCAGCGACTGC-3' (GYVSSDC), 5'-GTCCTTCTCAAGA-ACTCCAAC-3' (VLLKNSN), 5'-GTTTCTGCACTTCTCTGGGCGGA-TAC-3' (VSALLWGGY), and 5'-ATGGCGCACTCAATGTCTCGT-3' (MAHMSR); reverse: 5'-GTATCCGCCCGAGAGAAGTGCAGAAAC-3' (VSALLWGGY), 5'-GTAGAATAACCCGTGGCCGAA-3' (FGHGLFY), and 5'-CTCCTTCCCCGCGCACTTCAGCAC-3' (VLKWPGE). Reverse transcription (RT) was performed for 30 min at 50 °C then PCR was performed under the following conditions: denaturation for 5 min at 99 °C followed by 50 cycles of 30 s at 94 °C, 30 s at 60 °C and 2.5 min at 72 °C, and final extension for 5 min at 72 °C.

2.8. PCR of genomic DNA

Genomic DNA isolation from *Aspergillus awamori* K4 was performed according to a standard procedure [22]. 3' and 5' noncoding regions of the β -xylosidase gene were amplified by PCR using two primers (5'-CGTCAA-GATTGAGGCGCGAGAT-3' and 5'-TAGTATAGTAGGAGCTATGCT-3') designed on the basis of noncoding regions of the *Aspergillus niger* β -xylosidase gene [14], and used for PCR with the primers within the ORF.

2.9. Nucleotide sequencing

Each RT-PCR product was excised from 0.6% agarose gel after electrophoresis and sequenced using a BigDye Terminators v1.1 Cycle Sequencing Kit (Applied Biosystems Ltd.) and ABI PRISM 377XL DNA sequencer (Applied Biosystems Ltd.).

3. Results

3.1. Nucleotide sequence of K4 β -xylosidase

β -xylosidase was obtained from *Aspergillus awamori* K4 in liquid culture and purified by several chromatography steps. The molecular mass was estimated to be 115,000 Da by SDS-PAGE [21]. The purified β -xylosidase was digested into peptides with trypsin then some of these peptides were separated by HPLC using a TSKgel Octyl-80Ts column. Increasingly, hydrophobic peptides were eluted later by graduation of the organic mobile phase. Six major peaks were detected by HPLC (data not shown), and the fractionated peptides were analyzed using a protein sequencer. The amino

acid sequences of three peptides were determined as VSALLWGGYPGQSGGFALR, AYPP, and LYTT. The longest amino acid sequence was almost conserved in β -xylosidases from other fungi [11–15], and was especially similar to that from *Aspergillus niger*.

RT-PCR was performed to obtain the *Aspergillus awamori* β -xylosidase gene using primers designed based on the nucleotide sequence of *Aspergillus niger* β -xylosidase due to the similarity between the peptide fragments. The *Aspergillus niger* β -xylosidase gene shows similarities to four regions (A, B, C, and D) of the family 3 glucosidases [14,23]. Seven primers for RT-PCR were prepared on the basis of the consensus region of the *Aspergillus niger* β -xylosidase gene (accession number Z 84377) [14]. Five of the designed primers corresponded to VMCAYNA (region A), GYVSSDC (region A), VLLKNSN (region B), VSALLWGGY (region C), and FGHGLFY (region D), respectively. The remaining two primers corresponded to MAHMSR and VLKWPGE, which are outside the ORF of *Aspergillus niger* β -xylosidase. DNA fragments were amplified using all forward-reverse primer pairs, detected after agarose gel electrophoresis and sequenced. The two sequences outside the ORF were confirmed by sequencing PCR-amplified DNA with ORF-specific primers and genomic DNA of *Aspergillus niger* [14].

The sequence of the *Aspergillus awamori* K4 β -xylosidase gene (named *XawI*) was deduced by sequencing all the RT-PCR products. The ORF consisted of 2412 bp and the predicted peptide was 804 amino acids in length, corresponding to a molecular weight of 87,156 Da. The three amino acid sequences determined by peptide analysis were contained in this ORF. Taking a potential signal peptide of 26 amino acids into consideration [24], the mature protein was thought to be 778 amino acids long with a molecular mass of 84,632 Da. A homology search of the amino acid sequence revealed that it was similar to that of β -xylosidase gene sequences from *Aspergillus niger*, with DNA sequence identities of 98% (2377/2412) [14] and 94% (2282/2412) [11]. From this homology, the DNA amplified by RT-PCR was identified as the K4 β -xylosidase coding gene. The molecular mass of the native enzyme (115,000 Da) was larger than that of the determined sequence because of glycosylation. In the 5' noncoding region of *XawI*, five CreA consensus binding sites were found at 57, 94, 204, 464, and 547, repressing enzyme expression by glucose [25]. Moreover, two GGCTAAA sequences were found at 443 and 457, the binding site for the transcriptional activator: XlnR, which regulates xylanolytic enzyme gene expression [26].

3.2. Transxylosylation by β -xylosidase

Xylopentaose, xyloetraose, and xylotriose were produced during transxylosylation with xylobiose (data not shown). The xylosyl residue was transferred to xylobiose and then to xylotriose of the transfer products. In general, transglycosylation by a hydrolase is improved in the presence of high concentrations of the acceptor and substrate due to a decrease in water molecules, whereas the hydrolyzing activity of the hydrolase is depressed. The initial rates of transxylosyla-

tion and hydrolysis by β -xylosidase were investigated for two substrates, xylobiose and xylotriose. As shown in Fig. 1, at an early stage, the transxylosylation and hydrolysis rates of K4 β -xylosidase showed different profiles in relation to the initial concentration of the substrate. The hydrolysis rate was much lower and decreased gradually as the substrate concentration increased. On the contrary, the transxylosylation rate increased greatly as the substrate concentration increased.

3.3. Acceptor specificity of β -xylosidase

The acceptor specificity of transxylosylation by β -xylosidase was investigated using various acceptors such as alcohols, phenols, hydroxybenzylalcohols, sugar alcohols and disaccharides, as shown in Table 1. Transfer and hydrolysis ratios were calculated from the concentration of xylose and xylotriose produced and residual xylobiose [21]. In the case of monatomic alcohols, the enzyme more often transferred the xylosyl residue to the hydroxyl group of the first carbon than to that of the second carbon in alcohols. These alcohols did not inhibit the enzyme. Of the phenols, transxylosylation was higher with hydroxybenzylalcohol, dihydroxybenzene and phenol in turn. With phenol, the degradation of xylobiose was greatly decreased by enzyme inhibition. Although the transferring activities to phenol and dihydroxybenzene were small, β -xylosidase showed high acceptor specificity and high transferring activity to *o*-hydroxybenzylalcohol.

As shown in Table 1, with respect to transxylosylation with sugar alcohols, erythritol (four carbon atoms) was a good acceptor, as were sorbitol and mannitol (six carbon atoms). Palatinitol and maltitol, which were produced by reduction of their disaccharides, were also relatively efficiently used for transxylosylation. *myo*-Inositol with its ring structure did not act as an acceptor. Of the disaccharides, palatinose and trehalose

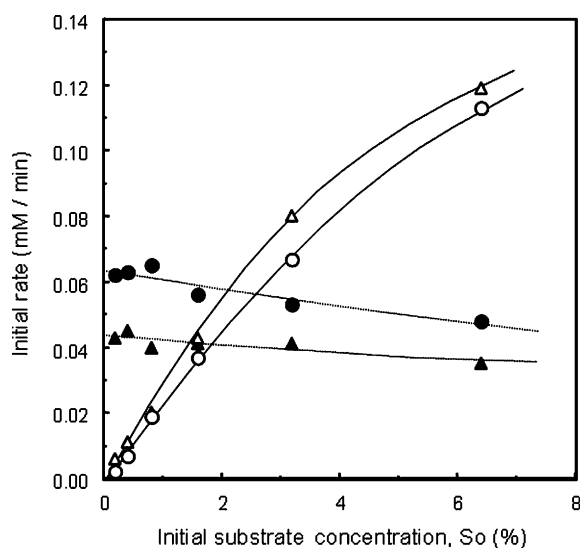


Fig. 1. Relationships between the initial rates of transxylosylation and hydrolysis and initial substrate concentration. Xylobiose or xylotriose (0.2–6.4%) was incubated with K4 β -xylosidase (0.2 U/ml) at pH 5.0 and 40 °C for 30 min. Initial hydrolysis rates: $\blacktriangle$ (xylobiose) and $\bullet$ (xylotriose); initial transxylosylation rates: $\triangle$ (xylobiose) and $\circ$ (xylotriose).

Table 1

Transxylosylation by K4 β -xylosidase to various acceptors

Acceptor	Transxylosylation ratio (%)		Hydrolysis ratio (%)	Degradation of xylobiose (%)
	to acceptor	to substrate		
None	–	9.9	90.1	75.1
<i>Alcohols</i> ^a				
Methanol	56.5	1.0	42.5	92.4
Ethanol	66.5	0.8	32.7	94.7
1-Propanol	73.9	0.5	25.6	95.5
2-Propanol	52.3	0.6	47.1	94.5
1-Butanol	55.8	0.7	43.5	93.2
2-Butanol	45.8	0.9	53.3	91.7
2-Methyl-1-Propanol	62.2	0.9	36.9	91.7
2-Methyl-2-Propanol	7.7	1.8	90.5	86.7
<i>Phenols and hydroxybenzylalcohols</i> ^b				
Phenol	0.0	51.8	48.2	34.7
1,2-Dihydroxybenzene	5.2	6.4	88.4	74.2
1,3-Dihydroxybenzene	12.8	3.8	83.4	80.8
1,4-Dihydroxybenzene	23.2	9.5	67.3	67.0
<i>o</i> -Hydroxybenzylalcohol	52.2	0.5	47.3	94.4
<i>m</i> -Hydroxybenzylalcohol	40.2	0.6	59.2	94.2
<i>p</i> -Hydroxybenzylalcohol	35.0	0.5	64.5	94.6
<i>Sugar alcohols</i> ^c				
Erythritol	61.4	0.0	38.6	100
Xylitol	44.2	0.0	55.8	100
Sorbitol	58.5	0.0	41.5	100
Mannitol	68.0	0.0	32.0	100
Dulcitol	22.5	0.0	77.5	100
Maltitol	22.0	0.0	78.0	99.5
Lactitol	10.3	0.0	89.7	85.9
Palatinitol	30.5	0.0	65.5	100
<i>myo</i> -Inositol	15.8	0.0	84.2	95.9
<i>Disaccharides</i> ^d				
Trehalose	49.5	1.8	98.2	93.5
Isomaltose	34.6	0.0	50.5	92.2
Palatinose	44.2	0.0	65.4	93.0
Maltose	0.8	0.0	99.2	87.1

Condition of reaction: ^a3.2% xylobiose, 8% acceptor, 1.0 U/ml enzyme, ^b3.2% xylobiose, 4% acceptor, 1.8 U/ml enzyme, ^c3.2% xylobiose, 8% acceptor, 1.8 U/ml enzyme, ^d3.2% xylobiose, 8% acceptor, 0.2 U/ml enzyme.

were favorable acceptors for β -xylosidase. Although K4 β -xylosidase had broad acceptor specificity for transxylosylation, its reactivity depended on the chemical structure of the acceptor.

Next, the major transfer products of some of these acceptors (*o*-hydroxybenzylalcohol, erythritol, palatinose and maltitol) were purified and their structures were determined by ¹H- and ¹³C-NMR. The transfer products of mannitol, sorbitol and trehalose were 1(6)-*O*- β -D-xylosyl mannitol, 6-*O*- β -D-xylosyl sorbitol and 6G-*O*- β -D-xylosyl trehalose, respectively [21]. Fig. 2 shows the HPLC chart of the NH2P-50 column with reaction mixture containing erythritol as the acceptor. Three transfer products, designated PE-1, PE-2 and PE-3, were detected in turn, of which PE-2 was the major product. According to its retention time, PE-3 was thought to have been synthesized by transfer of the xylosyl residue to PE-1 or PE-2. In the same manner, the transfer products of *o*-hydroxybenzylalcohol, maltitol and palatinose were separated into PO-1

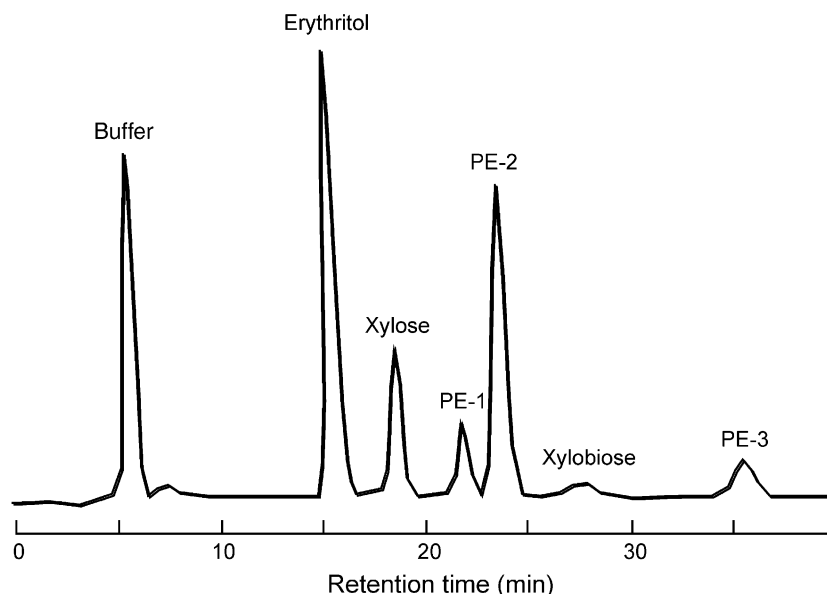


Fig. 2. Separation of transfer products with erythritol as the acceptor. Xylooligosaccharides (10%) and erythritol (10%) were incubated with K4 β -xylosidase (0.2 U/ml) at pH 5.0 and 40 °C for 48 h. The sugars were separated using a NH2P-50 column with a mobile phase of water:acetonitrile (24:76; the concentration at which a change in refractive index was observed). The initial peak at 6 min is that of the buffer in the reaction mixture. PE-1, PE-2, and PE-3 are the transfer product peaks.

(major product) and PO-2, PM-1 (major product) and PM-2, and PP-1 (major product) and PP-2, respectively. PO-2, PM-2, and PP-2 seemed to be secondary transfer products according to their retention time on HPLC, similar to the case of PE-3. Table 2 shows the chemical shifts of each transfer product on ^{13}C -NMR. With all transfer products, the signal related to C1 in the β -xylosyl residue shifted to a lower magnetic field, indicating that the β -xylosyl residue binds to the acceptors with its β -anomeric proton. In PE-1 and PE-2, the signals related to C1(C4) and C2(C3) shifted to a lower magnetic

Table 2
 ^{13}C -NMR chemical shifts of transfer product and related sugars in D_2O

Sample	Residue	Chemical Shifts (δ (ppm))						
		C1	C2	C3	C4	C5	C6	CH_2OH
β -Xylose	β -Xylp	96.2	73.6	75.4	68.8	64.7	—	—
<i>o</i> -HBA		153.4	126.1	129.2	120.1	129.1	115.2	59.2
PO-1	β -Xylp	101.7	72.6	75.3	68.8	64.7	—	—
	<i>o</i> -HBA	154.0	122.6	130.8	120.1	129.9	115.4	66.7
PO-2	β -Xylp	101.6	72.5	75.2	68.8	64.8	—	—
	β -Xylp	101.4	72.3	76.0	73.4	64.8	—	—
	<i>o</i> -HBA	154.0	122.6	130.8	120.1	129.9	115.4	66.8
Erythritol		62.2	71.5	71.5	62.2			
PE-1	β -Xylp	102.9	72.7	75.2	68.8	64.8		
	Erythritol	62.1	79.4	70.4	60.6			
PE-2	β -Xylp	103.1	72.6	75.1	68.8	64.7		
	Erythritol	62.1	70.6	71.3	70.2			
Maltitol	α -Glcp	100.1	71.2	72.2	68.9	72.0	59.9	
	Sorbitol	61.8	71.1	70.0	81.4	72.4	62.3	
PMa-1	β -Xylp	102.8	72.5	75.2	68.8	64.7		
	α -Glcp	100.1	71.2	72.3	69.3	72.0	59.9	
	Sorbitol	61.8	70.4	70.0	81.1	72.0	68.9	
Palatinose	α -Glcp	101.3	71.0	72.6	69.1	71.5	60.1	
	β -Fruf	62.1	97.8	74.8	74.0	78.5	67.4	
PP-1	β -Xylp	103.1	72.6	75.2	68.8	64.7		
	α -Glcp	101.3	70.4	72.5	68.9	70.9	68.2	
	β -Fruf	62.1	97.9	74.8	74.0	78.5	67.5	

field, respectively. Each carbon signal with the erythritol and xylosyl residues was a double signal. In erythritol, this appeared to be caused by rotation of each carbon with a hydroxyl group. In PO-1 and PO-2, the signal related to the carbon in the hydroxymethyl group on the benzene ring shifted, and PO-2 showed signals for two xylosyl residues. In PM-1 and PP-1, only the signals of C6 of the sorbitol residue in maltitol and glucosyl residue in palatinose shifted, respectively.

In the ^1H -NMR analysis, a signal for the β -anomeric proton xylopyranoside was detected in all transfer products (data not shown). From these data, PO-1 and PO-2 were identified as (2-hydroxyl)-phenyl-methyl- β -D-xylopyranoside and (2-hydroxyl)-phenyl-methyl- β -D-xylopyranoside- β -D-xylopyranoside, respectively, and PE-1, PE-2, PM-1 and PP-1 as 2(3)-*O*- β -D-xylosyl erythritol, 1(4)-*O*- β -D-xylosyl erythritol, 6*S*-*O*- β -D-xylosyl maltitol (S: sorbitol residue), and 6*G*-*O*- β -D-xylosyl palatinose (G: glucosyl residue), respectively. Fig. 3 shows the chemical structures of the major transfer products. In analyses of the minor products PE-3, PM-1, and PP-2, many of the signals detected were incomplete, and thus the structures could not be determined.

4. Discussion

The DNA sequence of *Aspergillus awamori* K4 β -xylosidase had four conserved regions related to family 3 β -glucosidases as well as other fungal enzymes. Moreover, its molecular weight was almost the same as those of β -xylosidases from *Aspergillus niger* and *oryzae* [12,14]. In particular, the amino acid sequence of K4 β -xylosidase was very similar to that of the *Aspergillus niger* enzyme [14], with only five amino acid differences. This indicates that K4 β -xylosidase hydrolyzes substrates via the same mechanism as

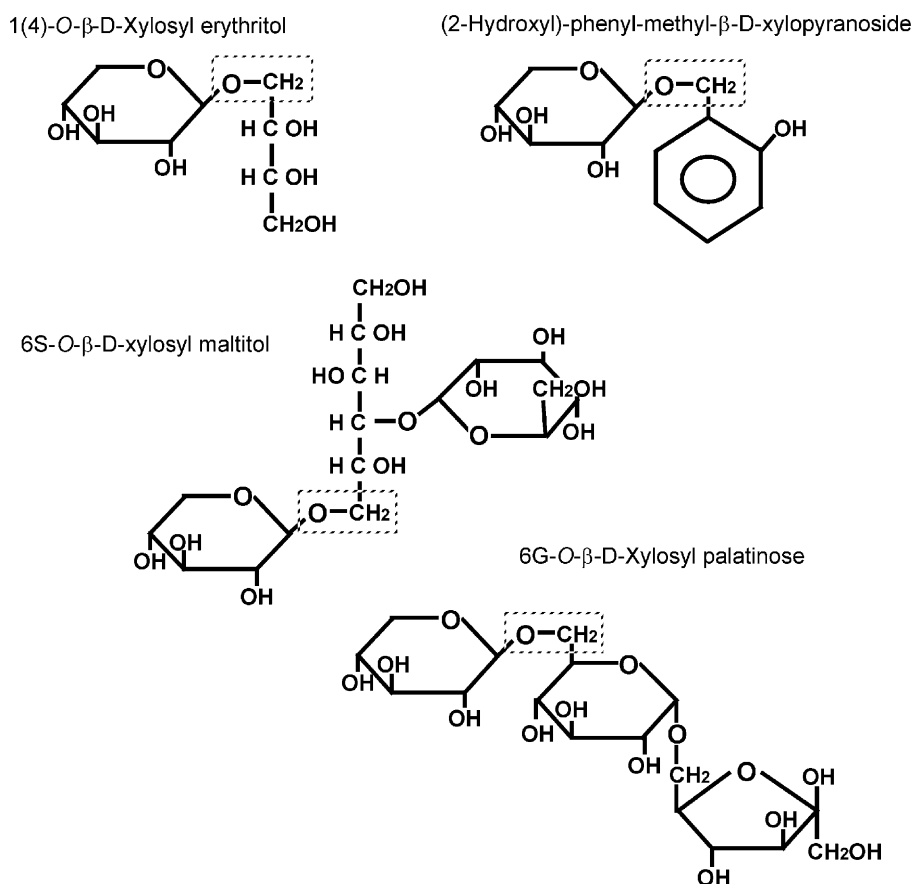


Fig. 3. Chemical structures of the major transfer products obtained with erythritol as the acceptor during transxylosylation of xylooligosaccharides by K4 β -xylosidase. The major transfer products with each acceptor were 1(4)-O- β -D-Xylosyl erythritol, (2-hydroxyl)-phenyl-methyl- β -D-xylopyranoside, 6S-O- β -D-xylosyl maltitol (S: sorbitol residue) and 6G-O- β -D-Xylosyl palatinose (G: glucosyl residue).

family 3 β -glucosidases, involving an Asp residue in the region A active site.

In general, the reaction mechanism of glycosidases can be explained by a double-displacement action [5–7]. The substrate (S) binds to the enzyme (E) to form an ES complex in which a glycosyl–enzyme intermediate is formed by an acid-catalyzed attack on the anomeric center by an enzymatic nucleophile (Asp). A water molecule is then base-catalyzed by other amino acid residues, attacking the anomeric center of the glycosyl–enzyme intermediate, causing release of the glucose product from the enzyme. The double-displacement mechanism has been verified in family 3 glucosidases and a number of essential amino acids have been identified [27–29]. In K4 β -xylosidase, at an early stage in the reaction with xylobiose and xylotriose (Fig. 1), the hydrolysis rate was much lower and decreased gradually as the substrate concentration increased, whereas the transxylosylation rate increased greatly. The hydrolysis rate was almost dependent on the substrate concentration, suggesting that the degree of base-catalyzed attack of the water molecule was very small. On the other hand, when the substrate (xylobiose or xylotriose) binds to the subsite adjacent to the glycosyl residue of the intermediate, the base-catalyzed hydroxyl group at the C4 position of the terminal xylosyl residue is thought to take up a more

suitable position for attacking the anomeric center, resulting in transxylosylation.

When other substances, such as ethanol, were used as the acceptor in reaction mixture containing xylobiose, the transxylosylation proceeded more efficiently and the transfer product could not be easily hydrolyzed, resulting in its accumulation in the reaction mixture. This finding suggests that the hydroxyl group of the ethanol molecule takes up a better position in the catalytic site than the water molecule. Primary alcohols such as methanol, ethanol, propanol, and butanol were shown to be good acceptors. *Aspergillus niger* β -xylosidase also synthesized alkyl β -xylosides from these alcohols [19] with both primary and secondary alcohols being effectively used as acceptors. However, *Aspergillus awamori* K4 β -xylosidase preferentially transferred the xylosyl residue to primary alcohols rather than secondary alcohols. This action is thought to be attributable to the influence of steric hindrance of the hydroxyl group in alcohols. Interestingly, the optimal alkyl chain length of the primary alcohols for transxylosylation was shown to be that of 1-propanol (C3). It appears that with a shorter alkyl chain, the hydroxyl group does not attach as strongly to the active site, whereas with a longer alkyl chain, the alkyl chain cannot approach the active site due to the high hydrophobicity. In particular, a linear chain appears to be the most appropriate structure, since 2-methyl 1-propanol (1-

tertiary butanol) was barely used as an acceptor in contrast to 1-propanol.

Agrobacterium tumefaciens β -glucosidase also shows β -xylosidase activity with high transxylosylation, and efficiently transfers the xylosyl residue to primary alcohols [30]. Watt et al. suggested that there is a hydrophobic portion in the enzymatic active site that interacts hydrophobically with the alkyl chain in the alcohols. Thus, a longer alkyl chain length is effective for transxylosylation. However, with *Aspergillus awamori* K4 β -xylosidase, which is a family 3 glycosidase along with the *Agrobacterium tumefaciens* enzyme, it is difficult to explain the hydrophobic interaction because hydrophilic sugar alcohols and disaccharides also acted as good acceptors.

Hydroxybenzylalcohols with a hydroxymethyl group were better acceptors than diphenols with the same benzene ring. Enzyme activity also decreased during transxylosylation with diphenols (data not shown), suggesting that although the benzene ring inhibits the anomeric center of the intermediate xylosyl residue, the chemical structure of the hydroxymethyl group is of the required length for attack. It is thought that this deactivation occurs due to hydrophobic binding in the active site and is depressed by substitution of the hydroxymethyl group. It is interesting that *o*-hydroxybenzylalcohol was the most appropriate acceptor for transxylosylation, and that the xylosyl residue was transferred to the hydroxymethyl group. Their position on the benzene ring is thought to be an important factor in transxylosylation.

Sugar alcohols of smaller size were more effectively used as acceptors. The hydroxyl group at the terminal of the linear chain structure attacks the intermediate xylosyl residue, resulting in efficient synthesis of 1(4)-*O*- β -D-xylosyl erythritol, 6-*O*- β -D-xylosyl sorbitol, and 1(6)-*O*- β -D-xylosyl mannitol through transxylosylation. With maltitol produced by the reduction of maltose, the hydroxyl group at the terminal end of the sorbitol residue binds to the xylosyl residue. Incidentally, *myo*-inositol, which has six carbon atoms similar to sorbitol and mannitol, was not a good acceptor. Its ring structure was thought to be inappropriate due to steric hindrance. With the disaccharides trehalose [21] and palatinose, the hydroxyl group at C6 of the glycosyl residue binds to the xylosyl residue. Furthermore, in both cases, the hydroxymethyl group of the acceptor is an important structure for transxylosylation. *Aspergillus awamori* K4 β -xylosidase was shown to have broad acceptor specificity for transxylosylation with regard to molecular size, chemical structure, and hydrophobic–hydrophilic properties. The consensus portion of the hydroxymethyl group was, however, confirmed in its major transfer products, 1(4)-*O*- β -D-xylosyl erythritol, (2-hydroxyl)-phenyl-methyl- β -D-xylopyranoside, 6S-*O*- β -D-xylosyl maltitol (S: sorbitol residue) and 6G-*O*- β -D-xylosyl palatinose (G: glucosyl residue) (Fig. 3). In the other major products, 6-*O*- β -D-xylosyl sorbitol, 1(6)-*O*- β -D-xylosyl mannitol and 6G-*O*- β -D-xylosyl trehalose [21], the hydroxymethyl groups were also used for binding to the xylosyl residue during transxylosylation. During hydrolysis by K4 β -xylosidase, base-catalyzed attack of the water molecule of the intermediate xylosyl residue was

inhibited in the active site. In contrast, the methylene in the hydroxymethyl group of the acceptor is thought to facilitate the base-catalyzed hydroxyl group attack of the anomeric center of the xylosyl–enzyme intermediate. In any case, the structures of other regions of the acceptors were not clearly recognized as shown in Fig. 3. It is thought that a pocket exists in the active area that can accept various types of molecular substances. Furthermore, identification of amino acids and their actions in the catalytic site must be studied in order to clarify the transglycosylation mechanism to various acceptors.

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