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Enzymatic Synthesis of 4-Hydroxyphenyl β-D-Oligoxylosides and Their Notable Tyrosinase Inhibitory Activity

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We have purified and characterized an oligoxylosyl transfer enzyme (OxtA) from *Bacillus* sp. strain KT12. In the present study, a N-terminally His-tagged recombinant form of the enzyme, OxtA(H)^{E}, was overproduced in *Escherichia coli* and applied to the reaction with xylan and hydroquinone to produce 4-hydroxyphenyl β-D-oligoxylosides, β-(Xyl)$_n$-HQ (n = 1–4), by one step reaction. The obtained β-(Xyl)$_n$-HQ inhibited mushroom tyrosinase, which catalyzes the oxidation of L-DOPA to L-DOPA quinine, and the IC$_{50}$ values of β-Xyl-HQ, β-(Xyl)$_2$-HQ, β-(Xyl)$_3$-HQ, and β-(Xyl)$_4$-HQ were 3.0, 0.74, 0.48, and 0.18 mM respectively. β-(Xyl)$_4$-HQ showed 35-fold more potent inhibitory activity than β-arbutin (4-hydroxyphenyl β-D-glucopyranoside), of which the IC$_{50}$ value was measured to be 6.3 mM. Kinetic analysis revealed that β-(Xyl)$_2$-HQ, β-(Xyl)$_3$-HQ, and β-(Xyl)$_4$-HQ competitively inhibited the enzyme, and the corresponding K_i values were calculated to be 0.20, 0.29, and 0.057 mM respectively.

Key words: transxylosylation; tyrosinase inhibitor; 4-hydroxyphenyl β-oligoxylosides

Tyrosinase (EC 1.14.18.1) is a rate-limiting enzyme in melanin synthesis that catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenyl L-alanine (L-DOPA), followed by oxidation of L-DOPA to L-DOPA quinone.[1,2] The product is finally transformed to a black pigment, melanin, with the aid of both enzymatic and non-enzymatic reactions. Melanin formation can protect the skin from ultraviolet-induced skin injury, but excessive melanin production has hyperpigmenting effects such as sunburn, freckles, lentigo, and melasma.[3] Melanin pigments are also found in the mammalian brain. The excessive generation of L-DOPA quinone and dopamine, which are enzymatically produced from L-DOPA, can irreversibly alter protein functions, inducing dopaminergic neurodegeneration in the mammalian brain, and contribute to the neurodegeneration associated with Parkinson's disease.[4,5] In addition, the browning reaction on foods is undesired in the food industry. Therefore, tyrosinase inhibitors are of extensive interest in medicine, and the cosmetics and food industries.

As popular tyrosinase inhibitors, hydroquinone[6,7] and β-arbutin (4-hydroxyphenyl β-D-glucopyranoside)[8,9] are used to prevent deposition of melanin pigments. However, the hydroquinone is an irritating agent inducing ochronosis[10] and can cause permanent leucoderma in long-term use.[11] Animal studies and *in vitro* cell studies have demonstrated that the hydroquinone is a possible carcinogenic compound.[12–14] On the other hand, β-arbutin is known as a harmless, water-soluble compound.[15] Tyrosinase inhibitors with more effective, less irritating, and water-soluble properties are still to be desired.

Recently we isolated a novel enzyme which can catalyze a β-oligoxylosyl transfer reaction to polyhydric phenol.[16] This enzyme showed transxylosylation activity toward catechol and hydroquinone with xylan as xylosyl donor. We named it OxtA (oligoxylosyl transfer enzyme A). The N-terminal amino acid sequences of OxtA showed similarities with endo-1,4-β-xylanhydrolases that belong to the family 11 of the glycosyl hydrolase classification.[17]

In this study, we constructed a system to overproduce the N-terminally His-tagged recombinant OxtA protein, OxtA(H)^{E}, in *E. coli* and applied the protein to the production of oligoxylosyl hydroquinones, which were

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DP, degree of polymerization; D_2O, deuterium oxide; FABMS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; HRESIMS, high resolution electrospray ionization mass spectrometry; IC_{50}, 50% inhibitory concentration; NMR, nuclear magnetic resonance; N-terminal, amino-terminal; OxtA, oligoxylosyl transfer enzyme; OxtA(H)^{E}, His-tagged recombinant OxtA protein produced in *Escherichia coli*; TLC, thin-layer chromatography; β-(Xyl)$_n$-Cat, 2-hydroxyphenyl β-oligoxyloside; β-(Xyl)$_n$-HQ, 4-hydroxyphenyl β-oligoxyloside; β-Xyl-HQ, 4-hydroxyphenyl β-xyloside; β-(Xyl)$_2$-HQ, 4-hydroxyphenyl β-xylobioside; β-(Xyl)$_3$-HQ, 4-hydroxyphenyl β-xylotrioside; β-(Xyl)$_4$-HQ, 4-hydroxyphenyl β-xylotetraoside

designed as candidates for novel tyrosinase inhibitors. Inhibition assay of the derived 4-hydroxyphenyl β-oligoxylosides with tyrosinase revealed that all the compounds designed possessed a notable inhibitory effect against a mushroom tyrosinase.

Materials and Methods

Overproduction and purification of the recombinant OxtA protein. To construct an overproduction system for the recombinant oligoxylosyl transfer enzyme OxtA, the coding region of the gene was first amplified, based on the high identity level (91%) of the N-terminal amino acid sequence of OxtA with the *Bacillus subtilis* xylanase (NC000964).[16] A set of primers (5′-ATGTTTAAGTTTAAAAAGAATTTCTTAGTT-3′ and 5′-TTACCACACTGTTACGTTAGAACTTCCACT-3′) were designed according to the nucleotide sequence of the *Bacillus subtilis* xylanase gene. PCR was done using the genomic DNA of *Bacillus* sp. strain KT12 as the template. The amplified DNA fragment was inserted into the plasmid vector pGEM®-T Easy (Promega, Medison, WI) to obtain plasmid pGEM/oxtA, which was subjected to analysis of the nucleotide sequence. An expression plasmid that carries a gene encoding the OxtA protein without the signal sequence, and tagged with six histidine residues (His-tag) at the N-terminus, was thereafter constructed as follows: Primers 5′-CTCGAGGCAGCTGGCACAGATTAC-3′ and 5′-GGATCCTTACCACACTGTTACGTTAG-3′, in which the *Bam*HI and *Xho*I sites respectively are underlined were designed to amplify the part of the *oxtA* gene that encodes the part of the OxtA protein without the putative signal peptide. The part of the *oxtA* gene was amplified with the primer set using pGEM/oxtA as a template. The PCR product was cloned in plasmid vector pGEM-T Easy to obtain plasmid pGEM/oxtAR. The nucleotide sequence of the cloned fragment was ascertained, and the *Bam*HI-*Xho*I fragment of pGEM/oxtAR was then ligated to the corresponding sites of pET15b (Novagen, Darmstadt, Germany). The resulting plasmid, pET15b/OxtA, was introduced into *E. coli* BL21(DE3) (Novagen). The recombinant OxtA protein was overproduced in soluble form and purified in accordance with Novagen's instructions. The His-tagged recombinant OxtA protein obtained was used in this study. The His-tagged protein was designated OxtA(H)^E to distinguish it from the native OxtA protein.

Enzyme assays. β-xylanase activity of OxtA toward xylan from beech wood (Sigma, St. Louis, MO) was determined at 40 °C, as reported previously.[16] The reaction was terminated by heating at 100 °C for 10 min. The amount of xylooligosaccharides released into the solution was measured by the Somogyi–Nelson method.[18] One unit of hydrolysis activity corresponded to 1 μmol of reducing sugar (expressed as D-xylose) released per min. The transxylosylation activity of OxtA toward catechol was determined at 40 °C. The amount of 2-hydroxyphenyl β-oligoxylosides was analyzed by HPLC.[16] One unit of transxylosylation activity corresponded to 1 μmol of 2-hydroxyphenyl β-oligoxylosides (expressed as the total amount of β-$(Xyl)_{2-4}$-Cat) release per min. The buffer systems for measuring xylanolytic and transxylosylation activities were as follows: 50 mM glycine-HCl buffer (pH 1.3, 2.0, and 3.0), 50 mM sodium citrate buffer (pH 3.0, 4.0, and 5.0), 100 mM sodium acetate buffer (pH 5.0), 50 mM potassium phosphate buffer (pH 6.0, 7.0, and 8.0), 50 mM Tris–HCl buffer (pH 7.0, 8.0, 9.0, and 10.0), or 50 mM glycine-NaOH buffer (pH 10.0, 11.0, 12.0, and 13.5).

TLC analysis. TLC was performed on Silica gel-60 plates (Whatman, Maidstone, UK) and Silica gel-60 F_{254} (Merck, Darmstadt, Germany) using EtOAc:AcOH:H_2O (3:1:1) as a solvent. Spots were visualized at 254 nm using a SLUV-6 UV-Handy Lamp (As One, Osaka) and spraying with 20% sulfuric acid in MeOH, followed by heating at 120 °C for 7 min.

Preparation of 4-hydroxyphenyl β-D-oligoxylosides. 4-Hydroxyphenyl β-oligoxylosides, β-$(Xyl)_n$-HQ, were prepared as follows. OxtA(H)^E (100 U of xylanase activity) was added to 100 ml of 20 mM potassium phosphate buffer (pH 8.0) containing 5.0 g (4.5 mmol) of hydroquinone (Sigma) and 5.0 g of xylan (beach wood, Sigma) and incubated at 40 °C for 24 h, and the products were periodically analyzed by HPLC and TLC. For convenience of purification of the reaction products, the buffer concentration was lowered over that used for the enzymatic characteristics, which was originally set to 50 mM. The reaction mixture, incubated for 24 h, was centrifuged at 8,000 × *g* for 20 min, and 400 ml of EtOAc was added to the supernatant. The water layer was concentrated and lyophilized. The residue containing xylosylated products was purified by active carbon column chromatography (Wako, Tokyo) and each product was subsequently eluted with H_2O, 10, 20% EtOH, and 40% acetone. Two fractions, eluted with 20% EtOH and 40% acetone, were collected and concentrated under diminished pressure. Each residue was dissolved in a small amount of MeOH and purified by silica gel column chromatography (gradually eluted by EtOAc:MeOH:H_2O = 50:2:1 → 34:2:1 → 17:2:1 → 15:2:1 → 10:2:1) to afford four fractions containing transxylosylated products. Crystallization of two fractions, obtained by elution with EtOAc:MeOH:H_2O = 34:2:1 and 17:2:1, afforded compounds 2 and 3 respectively. The other two fractions, eluted with EtOAc:MeOH:H_2O = 50:2:1 and 15:2:1, were further purified on an Shodex Asahipak NH2P-50 4E column (4.6 i.d. × 250 mm, Showa Denko, Tokyo) using a linear gradient (80–70%) of MeCN in water to give compounds **1** and **4**. The structures of compounds **1**–**4** were identified by MS data and ^{1}H-NMR spectra.

Mass and ^{1}H-NMR analysis of the products. A FABMS spectrum was obtained on JEOL JMS-AX50 (Jeol, Tokyo). HRESIMS spectra were recorded in positive ion mode on a Bruker micro TOF (Bruker Daltonics, Bremen, Germany). ^{1}H-NMR spectra were recorded with a model JNM-ECA500 (500 MHz, JEOL, Tokyo) at 25 °C, for solution in D_2O with *tert*-BuOH (1.23 ppm), or (3-trimethylsilyl)-propane sulfonic acid sodium salt (0 ppm), as the internal standard. The splitting patterns are reported as d (doublet), t (triplet), m (multiplet), dd (double of doublets), and dt (double of triplets).

*4-Hydroxyphenyl β-D-xylopyranoside (**1**).* ^{1}H-NMR (500 MHz, D_2O): δ 6.85 and 7.01 (d × 2, 4H, J = 8.9 Hz, Ar*H*), 4.91 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1), 3.97 (dd, 1H, $J_{4,5e}$ = 5.5 Hz, $J_{5a,5e}$ = 11.5 Hz, H-5e), 3.65–3.71 (m, 1H, H-4), 3.48–3.54 (m, 2H, H-2 and H-3), 3.38 (t, 1H, $J_{4,5a}$ = 10.8 Hz, H-5a); FABMS: m/z: 242 (M^+), 281 (M^+ + K).

*4-Hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**2**).* ^{1}H-NMR (500 MHz, D_2O): δ 6.85 and 7.01 (d × 2, 4H, J = 9.0 Hz, Ar*H*), 4.92 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1), 4.45 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 4.10 (dd, 1H, $J_{4,5e}$ = 5.3 Hz, $J_{5a,5e}$ = 11.9 Hz, H-5e), 3.97 (dd, 1H, $J_{4',5'e}$ = 5.4 Hz, $J_{5'a,5'e}$ = 11.9 Hz, H-5′e), 3.83 (dt, 1H, $J_{3,4}$ = 9.0 Hz, $J_{4,5eq}$ = 5.3 Hz, H-4), 3.60–3.66 (m, 2H, H-3 and H-4′), 3.53 (t, 1H, $J_{1,2}$ = $J_{2,3}$ = 7.7 Hz, H-2), 3.40–3.48 (m, 2H, H-5a and H-2′), 3.24–3.32 (m, 2H, H-3′ and H-5′a); HRESIMS: calcd for $C_{16}H_{22}NaO_{10}$ (M^+ + Na): 397.1111, found: m/z 397.1096.

*4-Hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**3**).* ^{1}H-NMR (500 MHz, D_2O): δ 6.85 and 7.01 (d × 2, 4H, J = 8.9 Hz, Ar*H*), 4.92 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1), 4.43–4.48 (d × 2, 2H, J = 7.6 and 7.7 Hz, H-1′ and H-1″), 4.09–4.12 (m, 2H, H-5e and H-5′e), 3.94–3.98 (dd, 1H, $J_{4'',5''e}$ = 5.4 Hz, $J_{5''a,5''e}$ = 11.6 Hz, H-5"e), 3.75–3.85 (m, 2H, H-4 and H-4′), 3.23–3.66 (m, 10H, sugar-*H*); HRESIMS: calcd for $C_{21}H_{30}NaO_{14}$ (M^+ + Na): 529.1533, found: m/z 529.1539.

*4-Hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**4**).* ^{1}H-NMR (500 MHz, D_2O): δ 6.85 and 7.02 (d × 2, 4H, J = 8.9 Hz, Ar*H*), 4.93 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1), 4.43–4.49 (m, 3H, H-1′, H-1″, and H-1‴), 3.23–4.11 (m, 20H, sugar-*H*); HRESIMS: calcd for $C_{26}H_{38}NaO_{18}$ (M^+ + Na): 661.1956, found: m/z 661.1960.

Quantitative analysis of 4-hydroxyphenyl β-D-oligoxylosides. The concentration of 4-hydroxyphenyl β-D-oligoxyloside was determinated by HPLC. Samples were centrifuged at 8,000 × *g* for 10 min at 4 °C, passed through a 0.20-μm membrane filter, and subjected to an HPLC

system (Hitachi, Tokyo) with an L-7400 type UV detector (Hitachi) and an Shodex Asahipak NH2P-50 4E column (4.6 i.d. × 250 mm, Showa Denko). The HPLC system was used under the following conditions: mobile phase, MeCN:H_2O (5:1); flow rate, 0.6 ml/min; absorbance, 280 nm; sample volume, 5 µl; and temperature, 40 °C.

Tyrosinase inhibition assay. The effects of the purified β-$(Xyl)_n$-HQ on a reaction catalyzed by a mushroom tyrosinase were evaluated *in vitro*. A series of various concentrations (0.025–10 mM) of β-$(Xyl)_n$-HQ was set in a reaction premixture containing L-DOPA (0.25–1.5 mM) as substrate in 100 mM sodium phosphate buffer (pH 6.8). The substrate was freshly prepared before the experiment. The reaction was initiated by adding 30 µl of the enzyme (30 U/reaction) at 25 °C, and the amounts of L-DOPA quinone produced in the reaction mixture were determined spectrophotometrically at 475 nm (OD_{475}) at 10 s intervals. IC_{50} is the concentration of a drug that inhibits a standard reaction at the 50% level. K_i values were calculated from shared non-linear regression fits of substrate-velocity curves taken at different concentrations of the inhibitors. The data was analyzed using Enzyme Kinetics Module software (Hulinks, Tokyo).

Results

Production and purification of recombinant OxtA

The N-terminal amino acid sequence of the OxtA protein exhibited a striking identity (91%) with the *Bacillus subtilis* xylanases belonging to family 11 of the glycosyl hydrolase classification.[16] Based on the high identity level with the *B. subtilis* xylanase, we successfully amplified the gene for the OxtA protein (see "Materials and Methods" for details). The deduced amino acid sequence of OxtA without the putative signal sequence was identical to that of the endo-xylanase (AAD10834), a family 11 of the glycosyl hydrolase classification, from *Bacillus* sp.[19] Two glutamic acid residues that are active centers in family 11 xylanses[20] were conserved in OxtA. The recombinant OxtA protein $OxtA(H)^E$, which was N-terminally His-tagged, was overproduced in a soluble form in *E. coli* and purified (Fig. 1). The purified $OxtA(H)^E$ protein was subjected to enzymatic characterization, without removing the His-tag. $OxtA(H)^E$ catalyzed the transxylosylation reaction to catechol (33 U/mg) in 100 mM sodium acetate buffer (pH 5.0), and hydrolyzed xylan (0.46 U/mg) in the same buffer. $OxtA(H)^E$ was thus found to exhibit transxylosylation and xylanolytic activities, as the native OxtA did.[16] The pH dependence on $OxtA(H)^E$, however, differed from that on the native OxtA. $OxtA(H)^E$ exhibited the highest transxylosylation activity (79.2 U/mg) in 50 mM potassium phosphate buffer (pH 8.0) (Fig. 2), whereas the native OxtA exhibited constant transxylosylation activity in a pH range, 4–11.[16] The N-terminally attached His-tag in the $OxtA(H)^E$ might affect the mode of pH dependence.

Synthesis and identification of oligoxylosyl hydroquinones by recombinant OxtA

The enzymatic reaction was performed as follows: A mixture of 5 g of xylan, 5 g of hydroquinone, 100 ml of 20 mM potassium phosphate buffer (pH 8.0), and the purified $OxtA(H)^E$ protein containing 500 U of xylanase activity was incubated in a shake flask with stirring at 40 °C for 24 h. The xylosylated hydroquinones produced were purified by two or three chromatographic steps, as described in "Materials and Methods," to afford different compounds **1** (25 mg), **2** (626 mg), **3** (210 mg), and **4** (39.9 mg) (Fig. 3).

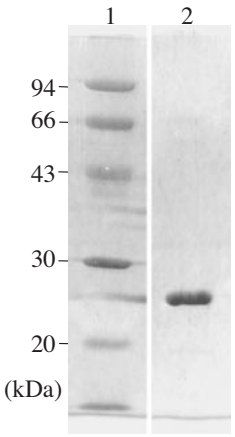

Fig. 1. SDS–PAGE of the Recombinant OxtA Protein $OxtA(H)^E$.

Lane 1, Protein size markers (phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (20 kDa)); lane 2, the purified $OxtA(H)^E$ protein. The gel was stained with Commassie Brilliant Blue R-250.

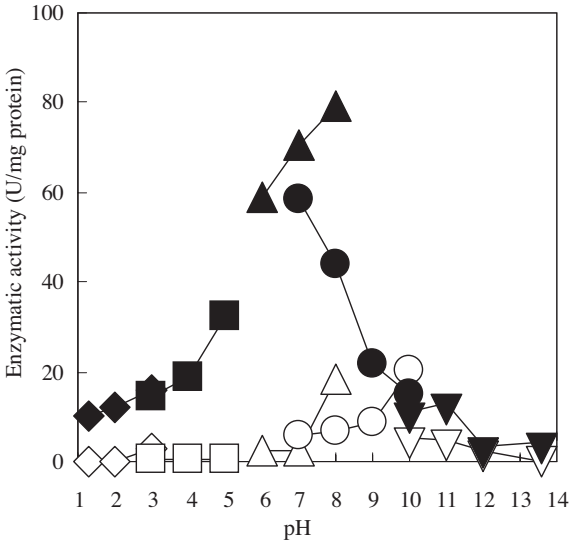

Fig. 2. Effects of pH on the Enzymatic Activity of the $OxtA(H)^E$ Protein.

Transxylosylation (filled symbols) and hydrolytic activities (open symbols) were measured under standard assay conditions in the following buffers: ◆ and ◇, 50 mM glycine-HCl buffer (pH 1.3, 2.0, and 3.0); ■ and □, 50 mM sodium citrate buffer (pH 3.0, 4.0, and 5.0); ▲ and △, 50 mM potassium phosphate buffer (pH 6.0, 7.0, and 8.0); ● and ○, 50 mM tris-HCl buffer (pH 7.0, 8.0, 9.0, and 10.0); ▼ and ▽, 50 mM glycine-NaOH buffer (pH 10.0, 11.0, 12.0, and 13.5).

Compounds **1**–**4** were identified by MS and ^{1}H-NMR spectroscopy. Compound **1** gave a $[M + K]^+$ signal at m/z 281 by FABMS. A doublet signals at δ4.9 ($J_{1.2} =$ 7.3 Hz, β-linkage) was observed by ^{1}H-NMR spectroscopy. Compound **1** was determined to be 4-hydroxyphenyl β-xyloside[21] from the molecular ion peak at m/z 281 $[M + K]^+$ obtained by FABMS and a doublet signal at δ4.9 ($J_{1.2} = 7.3$ Hz, β-linkage) observed by ^{1}H-NMR spectroscopy. The other compounds **2**–**4** showed no molecular-related ion peak on FABMS, while their HRESIMS spectra gave $[M + Na]^+$ signals at m/z 397.1096, 529.1539, and 661.1960, respectively. The structures of compounds **2**–**4** were analyzed by ^{1}H-NMR. In a previous study, 2-hydroxyphenyl β-

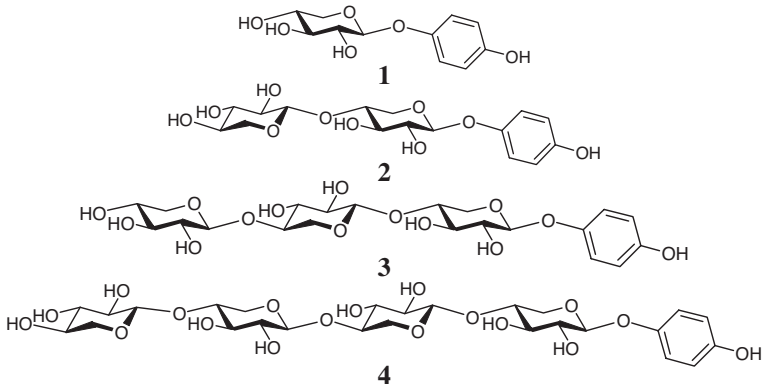

Fig. 3. Chemical Structures of 4-Hydroxyphenyl β-D-Oligoxylosides (Compounds **1**–**4**).

oligoxylosides were fully characterized by spectroscopic data (^{1}H- and ^{13}C-NMR, DQF-COSY, HMBC, HSQC, DPFGSE-TOCSY, and DPFGSE-ROESY).[22] The data were applied to the structural determination of compounds **2**–**4**. The ^{1}H-NMR spectra gave peaks in a range of δ3.2–4.5, characteristic of hexoside ring protons, very similar to the signals of the 2-hydroxyphenyl β-oligoxylosides[16] and *p*-nitrophenyl β-oligoxylosides.[23] Two doublet signals at δ6.9 and δ7.0 ($J = 8.9$ Hz) were assigned to the aromatic protons. A doublet signal at δ4.9 ($J_{1.2} = 7.3$–7.8 Hz, β-linkage) was assigned to the anomeric proton of the xylopyranosyl residue at the reducing end attached to the hydroquinone. The other doublet signals at δ4.4–4.5 ($J_{1.2} = 7.6$–7.9 Hz, β-linkage) were assigned to the anomeric protons of β-xylosides at the internal and the non-reducing end. These NMR and MS data confirmed that the structures of compounds **1**–**4** were 4-hydroxyphenyl β-D-xylopyranoside (**1**, β-Xyl-HQ), 4-hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**2**, β-(Xyl)$_2$-HQ), 4-hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**3**, β-(Xyl)$_3$-HQ), and 4-hydroxyphenyl β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranosyl-(1-4)-β-D-xylopyranoside (**4**, β-(Xyl)$_4$-HQ).

Effects of concentration of recombinant OxtA

Transxylosylation of hydroquinone with the OxtA(H)^E protein containing 0.1, 1.0, and 5.0 U/ml of xylanase activity occurred in 5.0 ml of 20 mM potassium phosphate buffer (pH 8.0). These reaction mixtures were incubated at 40 °C for 0–72 h, and the enzyme reaction, yielding β-(Xyl)$_{2–4}$-HQ, was periodically analyzed by HPLC (Fig. 4). The concentration of β-(Xyl)$_{2–4}$-HQ increased concurrently after the OxtA(H)^E protein was added. Production of β-(Xyl)$_2$-HQ increased sharply with increasing OxtA(H)^E protein concentrations, although β-(Xyl)$_4$-HQ decreased slightly with the OxtA(H)^E protein, which showed 5 U/ml of xylanase activity. These data indicate that β-(Xyl)$_2$-HQ is hardly hydrolyzed by the OxtA(H)^E protein. After 24 h of incubation, the yields of β-(Xyl)$_2$-HQ, β-(Xyl)$_3$-HQ, and β-(Xyl)$_4$-HQ were 33, 13, and 1.4 mM respectively, and conversion reached 10% based on the amount of hydroquinone supplied (the transfer rate). Production of the β-(Xyl)$_{2–4}$-HQ by the OxtA(H)^E protein was more than that of β-(Xyl)$_{2–4}$-Cat by the native OxtA protein from *Bacillus* sp. KT12 (4.2% of the transfer rate).[16]

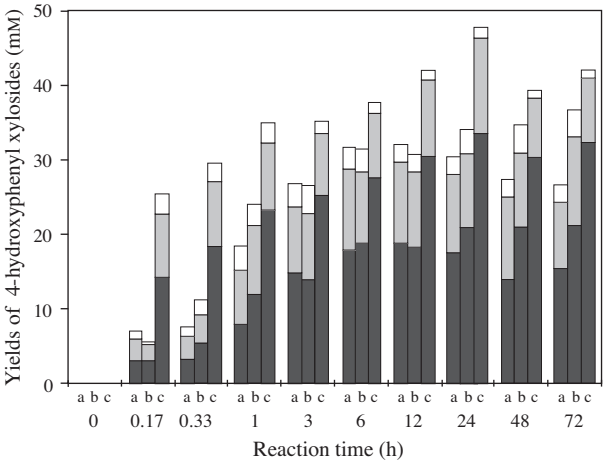

Fig. 4. Time Course of Transxylosylation to Hydroquinone by the OxtA(H)^E Protein.

Xylosylation of hydroquinone was monitored in 20 mM potassium phosphate buffer (pH 8.0) containing 5% (w/v) of xylan, 5% (w/v) of hydroquinone, and the purified OxtA(H)^E protein corresponding to 0.1 (a), 1.0 (b), and 5.0 U/ml (c) of xylanase activity. Reaction mixtures were incubated at 40 °C for 0–72 h and the products were periodically analyzed by HPLC. The compositions of the reaction products are shown as follows: ■, β-(Xyl)$_2$-HQ; ▩, β-(Xyl)$_3$-HQ; □, β-(Xyl)$_4$-HQ.

Inhibition of mushroom tyrosinase by 4-hydroxyphenyl β-D-oligoxylosides

To investigate the biological significance of the prepared β-(Xyl)$_n$-HQ, the IC$_{50}$ and K_i values against mushroom tyrosinase were measured. The IC$_{50}$ values of β-Xyl-HQ, β-(Xyl)$_2$-HQ, β-(Xyl)$_3$-HQ, β-(Xyl)$_4$-HQ, and the β-arbutin were 3.7, 0.74, 0.48, 0.18, and 6.3 mM respectively (Fig. 5). β-(Xyl)$_4$-HQ showed 35-fold more active inhibition than β-arbutin. According to Lineweaver-Burk plots, the K_i values of β-(Xyl)$_2$-HQ and β-(Xyl)$_3$-HQ were calculated to be 0.20 and 0.29 mM respectively, and β-(Xyl)$_4$-HQ showed the highest inhibitory activity ($K_i = 0.057$ mM, Fig. 6, Table 1). The inhibition types of compounds 1–4 to the enzyme were competitive.

Discussion

We constructed a system to overproduce the His-tagged recombinant OxtA, OxtA(H)^E, in *E. coli*, and applied the protein to the production of oligoxylosyl hydroquinones. The purified OxtA(H)^E showed high

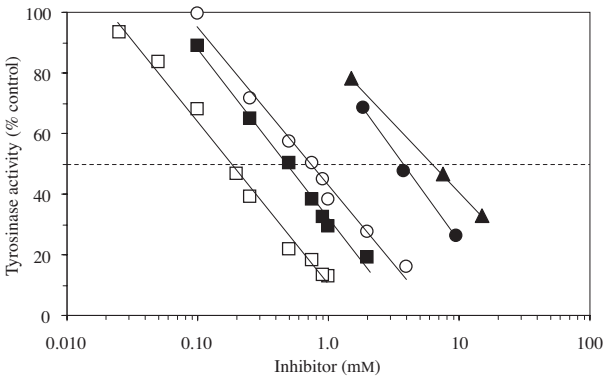

Fig. 5. Inhibitory Effects of 4-Hydroxyphenyl β-D-Oligoxylosides on Mushroom Tyrosinase.

Tyrosinase activity was measured using 1.0 mM L-DOPA as the substrate. The results are expressed as the percentages of inhibition by ●, β-Xyl-HQ; ○, β-$(Xyl)_2$-HQ; ■, β-$(Xyl)_3$-HQ; and □, β-$(Xyl)_4$-HQ; and ▲, β-Glc-HQ (β-arbutin) with respect to the untreated control. Fifty percent inhibition is indicated by a dotted line. Inhibitor concentrations are logarithmically plotted.

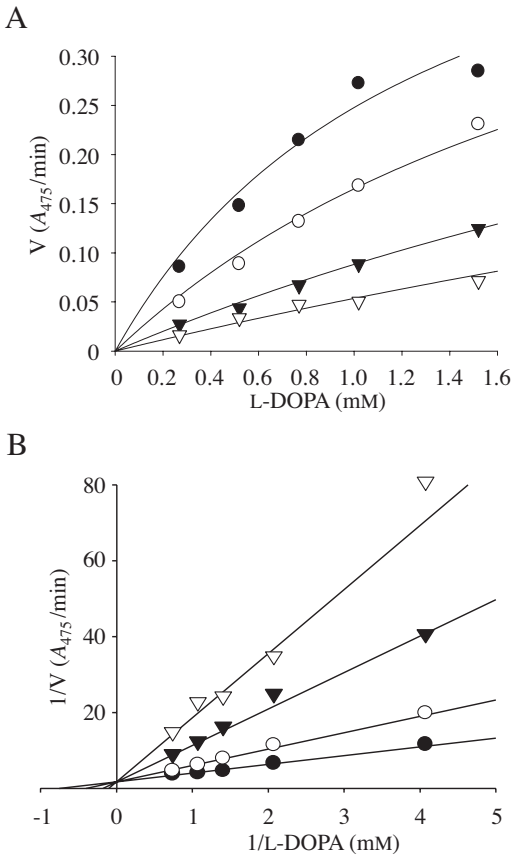

Fig. 6. Kinetics of Mushroom Tyrosinase Inhibition by β-$(Xyl)_4$-HQ.

A series of substrate-velocity plots were taken at increasing concentrations of L-DOPA as substrate in the presence of β-$(Xyl)_4$-HQ. β-$(Xyl)_4$-HQ were added as follows: ●, 0 mM; ○, 0.05 mM; ▼, 0.18 mM; and ▽, 0.36 mM in reaction mixture. Michaelis-Menten (A) and Lineweaver-Burk (B) plots for the inhibition of mushroom tyrosinase by β-$(Xyl)_4$-HQ are shown.

transxylosylation activity. The deduced amino acid sequence of $OxtA(H)^E$ showed a complete similarity to xylanases belonging to family 11 of glycosyl hydrolase. The family 11 enzymes catalyze a hydrolysis reaction by a double displacement mechanism, in which two glutamic acid residues act at the active center.[20] The catalytic mechanism of the glycosynthases has been explained in terms of a double displacement mechanism as well.[24–26] The OxtA protein might also catalyze the transxylosylation reaction in the same manner.

The $OxtA(H)^E$ obtained produced β-$(Xyl)_n$-HQ by the transxylosylation reaction in one step. $OxtA(H)^E$ produced β-$(Xyl)_2$-HQ as the major product, together with β-$(Xyl)_3$-HQ and β-$(Xyl)_4$-HQ. The transxylosylation reaction by $OxtA(H)^E$ could be an endo-type reaction in view of the characteristics of family 11 enzymes,[20] which have a *syn*-protonation mechanism at the active site. $OxtA(H)^E$ can not hydrolyze β-$(Xyl)_2$-HQ. This accords with the fact that family 11 enzymes are inactive on aryl cellobiosides.[20]

In the effort to develop water-soluble and more effective tyrosinase inhibitors, glycosylated hydroquinones such as β-arbutin are lead compounds.[27–29] For example, Moon *et al.*[28] synthesized a series of arbutin β-glucosides and showed their notable inhibitory activity to a mushroom tyrosinase. They reported that 4-hydroxyphenyl β-D-glucopyranosyl-(1-4)-β-D-glucopyranoside (IC_{50} = 10 mM) showed lower inhibitory activity than 4-hydroxyphenyl β-D-glucopyranoside (IC_{50} = 6 mM).[27] Our present data showed the opposite tendency: 4-hydroxyphenyl β-D-oligoxylosides showed stronger inhibitory activity than 4-hydroxyphenyl β-D-xyloside. Obviously, the inhibitory activity increased with the molecular weight in our case. The β-$(Xyl)_4$-HQ was found to have a 35-fold more inhibitory effect than arbutin. The strong activity originates in its high affinity with the enzyme (a low K_i value). The xylotetraoside moiety of β-$(Xyl)_4$-HQ interact with this enzyme to enhance the affinity.

In addition, aryl β-xylosides are known to penetrate through cell membranes and to activate a biosynthesis of chondroitin and dermatan sulfates.[30–32] These glycosaminoglycans are recognized by various extracellular matrix proteins, cytokines, growth factors, enzymes, and inhibitors, and are responsible for diverse biological functions. Hence, we think that the β-oligoxylosides prepared in the present study have much potential for biomedical applications.

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Table 1. Inhibitory Effects of β-Oligoxylosyl Hydroquinones and β-Arbutin on a Mushroom Tyrosinase

	β-Xyl-HQ	β-$(Xyl)_2$-HQ	β-$(Xyl)_3$-HQ	β-$(Xyl)_4$-HQ	β-Glc-HQ
IC_{50} (mM)	3.7	0.74	0.48	0.18	6.3
K_i (mM)	—	0.20	0.29	0.057	—

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