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Identification and characterization of a novel (−)-*vibo*-quercitol 1-dehydrogenase from *Burkholderia terrae* suitable for production of (−)-*vibo*-quercitol from 2-deoxy-*scyllo*-inosose

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Abstract (−)-*vibo*-Quercitol is a deoxyinositol (1L-1,2,4/3,5-cyclohexanepentol) that naturally occurs in oak species, honeydew honey, wines aged in oak barrels, and *Gymnema sylvestre* and is a potential intermediate for pharmaceuticals. We found that (−)-*vibo*-quercitol is stereoselectively synthesized from 2-deoxy-*scyllo*-inosose by the reductive reaction of a novel (−)-*vibo*-quercitol 1-dehydrogenase found in the proteomes of *Burkholderia*, *Pseudomonas*, and *Arthrobacter*. Among them, *Burkholderia terrae* sp. AKC-020 (40-1) produced a (−)-*vibo*-quercitol 1-dehydrogenase appropriate for synthesizing (−)-*vibo*-quercitol with a high diastereomeric excess. The enzyme was strongly induced in *Bu. terrae* cells when quercitol or 2-deoxy-*scyllo*-inosose was used as carbon source in the culture medium. The enzyme is NAD(H)-dependent and shows highly specific activity for (−)-*vibo*-quercitol and *myo*-inositol among the substrates tested. The enzyme gene (*qudh*) was obtained by PCR using degenerate primers based on the *N*-terminal and internal amino acid sequences of the purified enzyme, followed by thermal asymmetric interlaced PCR. The *qudh* gene showed homology with inositol 2-dehydrogenase (sharing 49.5% amino acid identity with IdhA from *Sinorhizobium meliloti* 1021). We successfully produced several recombinant (−)-*vibo*-quercitol 1-dehydrogenases and related enzymes identified by genome database mining using an *Escherichia coli* expression system. This revealed that *scyllo*-inositol dehydrogenase (IolX) in *Bacillus subtilis* can catalyze the reduction of 2-deoxy-*scyllo*-inosose to yield *scyllo*-quercitol, a stereoisomer of (−)-*vibo*-quercitol. Thus, we successfully identified two enzymes to produce both stereoisomers of deoxyinositols that are rare in nature.

Keywords (−)-*vibo*-Quercitol 1-dehydrogenase (2-deoxy-*scyllo*-inosose reductase) · (−)-*vibo*-Quercitol · *scyllo*-Quercitol · *Burkholderia terrae* · *scyllo*-Inositol dehydrogenase (IolX) · Enzymatic synthesis

Introduction

(−)-*vibo*-Quercitol (1L-1,2,4/3,5-cyclohexanepentol) is a deoxyinositol that naturally occurs in *Quercus* (oak) species (Anderson 1972), honeydew honey (Sanz et al. 2004), wines aged in oak barrels (Carlavilla et al. 2006), and *Gymnema sylvestre* (Asclepiadaceae), which is popularly known as "Gurmar" (Potawale et al. 2008). It is a deoxy analog of *myo*-inositol (1,2,3,5/4,6-cyclohexanehexol), the most abundant cyclohexanehexol in nature. This compound is partly converted into hexaphosphate of inositol (phytic acid) in plants and is an essential compound that serves as a phosphatidylinositol moiety in eukaryotic cell membranes and as a secondary messenger. *myo*-Inositol catabolism has been well studied in microorganisms such as *Bacillus subtilis* by Yoshida et al. (2008), and the *iolABCDEFGHIJ* operon is responsible for *myo*-inositol degradation. *myo*-Inositol 2-dehydrogenases (EC 1.1.1.18), including IolG from *Ba. subtilis*, have been characterized in several

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microorganisms (Walker 1975; Ramaley et al. 1979; Jiang et al. 2001; Yoshida et al. 2012). To our knowledge, however, there have been no detailed reports concerning the catabolism of deoxyinositol, (−)-*vibo*-Quercitol and its stereoisomers, including (+)-*proto*-quercitol (1L-1,3,4/2,5-cyclohexanepentol) and *scyllo*-quercitol (2-deoxy-*myo*-inositol, 1,3,5/2,4-cyclohexanepentol), which are potential chiral synthons for drug candidates (Ogawa et al. 1999; Ogawa and Kanto 2007; Wacharasindhu et al. 2009; Kuno et al. 2011).

Takahashi et al. (1999) reported the biotransformation of *myo*-inositol using *Salmonella typhimurium* and produced three quercitols from fermentation broth by using a combination of ion-exchange chromatography and recrystallization. The major products were (−)-*vibo*-quercitol (35% yield), (+)-*epi*-quercitol (11%), and (+)-*proto*-quercitol (5%). They proposed the following biotransformation mechanism: initial oxidation from *myo*-inositol to *scyllo*-inosose, followed by dehydration and reduction to quercitols. However, it has remained difficult to obtain pure chiral quercitols as a raw material for pharmaceuticals.

Yamauchi and Kakinuma (1995) reported on 2-deoxy-*scyllo*-inosose synthase (BtrC), which has a key role in the biosynthetic pathway of the aminoglycoside antibiotic butirosin from *Bacillus circulans* (Dion et al. 1972). This research group also identified the gene cluster (*btrA-X*) for butirosin biosynthesis in *Ba. circulans* (Ota et al. 2000; Kudo et al. 2005). The enzyme BtrC catalyzes the multistep carbocycle-forming reaction of glucose-6-phosphate in the presence of NAD^+ and Co^{2+} as cofactors, which enables the microbial production of 2-deoxy-*scyllo*-inosose (DOI) from D-glucose (Kogure et al. 2007; Miyazawa and Matsumoto 2015). Recently, DOI has been identified as a carbohydrate for green chemistry, for example, in the syntheses of catechol via reductive dehydration (Kakinuma et al. 2000) and of some carbasugar derivatives (Ogawa and Kanto 2007). Thus, we have focused on the biosynthesis of useful deoxyinositols from DOI by microbial or enzymatic reduction.

In this study, we describe the isolation of quercitol-assimilating microorganisms by enrichment culture and identification of a novel (−)-*vibo*-quercitol 1-dehydrogenase (2-deoxy-*scyllo*-inosose reductase) (QUDH) from *Burkholderia terrae* AKC-020 (40-1), which is suitable for (−)-*vibo*-quercitol production from DOI. This research included the characterization, cloning, and heterologous expression of the *qudh* gene in *Escherichia coli.* Moreover, we clarified that one of the QUDH orthologs, *scyllo*-inositol dehydrogenase (IolX; Morinaga et al. 2010), can catalyze the reduction of DOI to give *scyllo*-quercitol (1,3,5/2,4-cyclohexanepentol), a stereoisomer of (−)-*vibo*-quercitol (Fig. 1). Thus, we present a series of possible enzymatic processes for producing two chiral quercitols.

Materials and methods

Screening of microbial strains for the assimilation of quercitol as a carbon source

The medium of the agar plates for screening microorganisms able to assimilate quercitol consisted of a 0.3% (*w*/*v*) (−)-*vibo*- and *scyllo*-quercitol mixture, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.1% NaCl, 0.02% $MgSO_4{\cdot}7H_2O$, and 1.5% agar in tap water (pH 7.0; medium A). The (−)-*vibo*- and *scyllo*-quercitol mixture was added to the medium after sterile filtration. Soil samples collected from fertile fields in Toyama were mixed with 10 mL of saline solution, and then 0.1 mL of the mixture was spread onto agar plates. The quercitol-assimilating microorganisms that were able to form colonies on the agar plate were aerobically cultivated at 30 °C for 1–2 days. The bacterial cultures obtained by monocolony isolation on LB agar medium containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0) were maintained at 4 °C on a LB-quercitol agar medium consisting of a 0.3% (*w*/*v*) (−)-*vibo*- and *scyllo*-quercitol mixture, 0.5% tryptone, 0.25% yeast extract, and 0.25% NaCl (pH 7.0; medium B).

Each strain isolated as described above was cultured in medium B in a test tube that was constantly shaken for 1 day at 30 °C in a total volume of 2 mL. The cells grown in the 2-mL culture were collected by centrifugation (20,000×*g*, 1 min), suspended in 1 mL of 50 mM potassium phosphate buffer (KPB; pH 7.0), and then disrupted with an ultrasonic oscillator (Ultra Sonic Disrupter UD-200; Tomy Corp., Tokyo, Japan) for 150 s in total (five disruptions of 30 s each, followed by a 30-s interval for cooling). After centrifugation (10,000×*g*, 5 min), the supernatant was used as a crude enzyme solution.

Enzyme assay

QUDH activity was assayed spectrophotometrically at 25 °C as a reductive reaction of DOI by measuring the decrease in the absorbance of NADH (or NADPH) at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). The reaction mixture consisted of 5 μmol of DOI, 0.3 μmol of NADH, 50 μmol of KPB (pH 7.0), and 10 μL of enzyme solution, in a total reaction volume of 1.0 mL. The oxidative reaction of QUDH was also measured at 340 nm in 1.0 mL of reaction mixture containing 5 μmol of (−)-*vibo*-quercitol or other polyols as a substrate, 1 μmol of NAD^+, 100 μmol of glycine-NaOH buffer (pH 9.0), and 10 μL of enzyme solution. The blank contained buffer instead of substrate. One unit of enzyme was defined as the amount that converted 1 μmol of NADH in 1 min under these conditions.

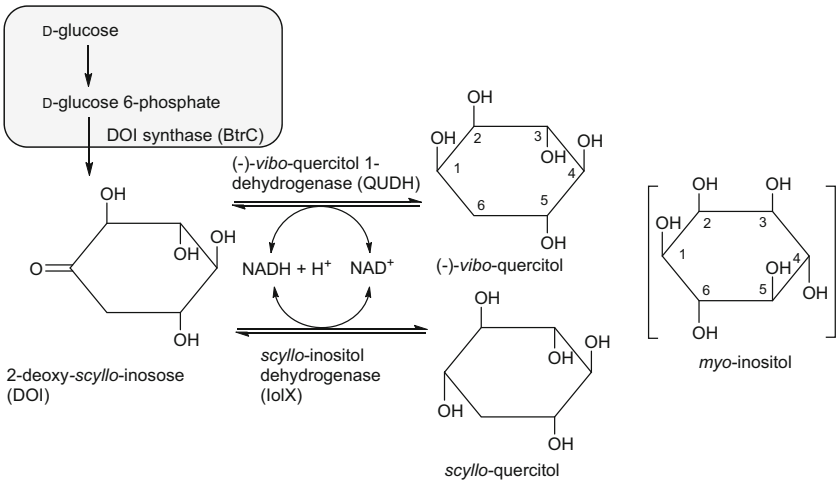

Fig. 1 Bioreduction of 2-deoxy-*scyllo*-inosose (DOI) to (−)-*vibo*-quercitol or its stereoisomer *scyllo*-quercitol and their chemical structures, including naturally abundant *myo*-inositol. The gray box denotes the simple scheme of DOI biosynthesis from D-glucose

Purification and physicochemical characterization of QUDH from *Bu. terrae* AKC-020 (40-1)

Bu. terrae sp. AKC-020 (40-1) was deposited for a patent application as NITE P-01745 at the National Institute of Technology and Evaluation (NITE), Kisarazu, Chiba, Japan. The strain was grown in LB medium in a test tube at 30 °C for 24 h with shaking. Then, 1 mL of culture broth was transferred into 100 mL of fresh medium consisting of 0.5% (*w*/*v*) DOI, 0.4% tryptone, and 0.2% yeast extract (pH 7.0) in a 500-mL shaking flask and aerobically cultured at 30 °C for 24 h with shaking. The cells collected from the 100-mL culture were resuspended in 20 mL of buffer and disrupted on ice with an ultrasonic oscillator (Ultra Sonic Disruptor UD-200) for 300 s (in five disruptions of 30 s each followed by a 30-s interval for cooling). After centrifugation (at 15,000×*g* for 10 min), the supernatant was used as a crude enzyme solution. All the purification procedures were carried out at 0–4 °C in 20 mM sodium phosphate buffer (pH 7.0) unless otherwise stated. Solid ammonium sulfate was added to the crude enzyme solution to reach 30% saturation, and the precipitate was removed by centrifugation (at 15,000×*g* for 10 min). The supernatant was applied to a Butyl-Toyopearl 650 M column (25 × 65 mm; Tosoh Corp., Tokyo, Japan) that had been equilibrated using the same buffer. The enzyme was eluted with a linear 30–0% aqueous ammonium sulfate gradient in buffer. Fractions with high enzyme activity were dialyzed against a 20-mM 3-(*N*-morpholino)propanesulfonic acid-NaOH (MOPS) buffer (pH 7.0) and applied to a RESOURCE Q 1 mL column (GE Healthcare Japan, Tokyo, Japan) equipped with an ÄKTA purifier (GE Healthcare). The enzyme was eluted using a linear 0–0.5-M aqueous NaCl gradient in MOPS buffer. Fractions with high enzyme activity were concentrated using a Centriprep YM-30 filter unit (30,000 Da molecular weight cutoff; Merck Millipore, Darmstadt, Germany). The enzyme solution was applied to a TSK-Gel3000SW gel filtration column (21.5 × 300 mm; Tosoh) that had been equilibrated with sodium phosphate buffer containing 0.3 M NaCl (pH 6.0) and eluted at a flow rate of 1.0 mL/min.

The purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an ATTO AE-6530 mini-slab size electrophoresis system with an e-PAGEL E-T10L 10% precast gel (ATTO Corp., Tokyo, Japan), which was performed at 20 mA according to the method described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie brilliant blue G-250. The *N*-terminal and internal amino acid sequences were determined by APRO Life Science Institute (Tokushima, Japan).

The molecular mass of the enzyme was determined by analytical high-performance liquid chromatography (HPLC) with a TSK-Gel G3000SW column (7.5 × 300 mm; Tosoh) at a flow rate of 0.5 mL/min with 50 mM Tris-HCl (pH 7.0) containing 0.1 M NaCl. The molecular mass of the native enzyme was determined by comparing the retention time of QUDH with that of the MW-Marker (Oriental Yeast Co., Tokyo, Japan).

Protein concentration was estimated using the Bradford method calibrated with bovine serum albumin as a standard (Bio-Rad Protein Assay Kit; Bio-Rad, Hercules, CA).

HPLC analysis of deoxyinositols

The reaction mixture for evaluating the DOI reduction consisted of 10 μmol of DOI, 1.0 μmol of NAD^+, 100 μmol of sodium formate, 200 μmol of KPB (pH 7.0), 0.2 units of formate dehydrogenase (Wako Pure Chemicals, Osaka, Japan), and 0.1 unit of QUDH, in a total reaction volume of 1.0 mL. The reaction was performed at 30 °C with incubation in a 2.0-mL tube for 2 h. After filtration, the solution was subjected to HPLC analysis using a Shimadzu Prominence HPLC (Shimadzu Corp, Kyoto, Japan) equipped with a

Shodex SUGAR KS-801 ligand exchange column (8 × 300 mm; Showa Denko, Tokyo, Japan) and a SUGAR KS-G guard column, with a Shodex RI-71 refractive index detector (Showa Denko) at 70 °C using distilled water as the mobile phase following degassing. The flow rate was 1.0 mL/min with retention times of 8.7 min for *scyllo*-quercitol, 9.3 min for (−)-*vibo*-quercitol, and 9.8 min for DOI.

Cloning and DNA sequencing of the *qudh* gene

Genomic DNA was prepared from *Bu. terrae* AKC-020 (40-1) cells grown in LB medium using standard techniques for DNA manipulation (Sambrook and Russell 2001) to obtain partial fragments of the QUDH-coding gene. PCR was performed using genomic DNA as a template. The combination of the degenerate primers for amplifying *qudh* fragments was designed from the *N*-terminal and internal amino acid sequences of QUDH and sequences conserved among inositol dehydrogenases in microorganisms. This approach was selected because the *N*-terminal amino acid sequence (MIRIAVLGAG) was highly similar to those of bacterial inositol dehydrogenases and glycerol-3-phosphate dehydrogenases. All oligonucleotide primers used to clone the QUDH-coding gene are shown in Table S1, and all nucleotide sequences were determined using a Capillary DNA Sequencer 3130 (Applied Biosystems, Tokyo, Japan) to perform Sanger DNA sequencing of both strands. We were able to amplify an approximately 900-bp DNA fragment using the primers DOIRdgnF1 (ATGATHMGNATHGCNGT) and DOIRdgnR8 (GCRTCNACRAANGCYTC). We then amplified an approximately 700-bp DNA fragment, which was similar to the inositol dehydrogenase gene by nested PCR using this 900-bp DNA fragment as a template with the primers DOIRdgnF1 and DOIRdgnR7Q (CKYTGRTCRTANCCRTA). To obtain the neighboring regions of the QUDH-coding gene, thermal asymmetric interlaced PCR (TAIL-PCR; Liu and Whittier 1995) was performed using the primers based on the deduced partial nucleotide sequences and arbitrary primers. The full length of the DNA fragment of *qudh* was finally amplified by PCR using the genomic DNA as a template and the primers DOIRtopF2 (TACGGCGTGGAACTCATC) and DOIRbotR2 (TGAGTGATACCAAGACATGCC). The sequence of the PCR fragment was confirmed. Then, to construct the expression vector, the DNA fragment of *qudh* was amplified by PCR using the genomic DNA as a template and the primers DOIRexF1Bam (AGAGGATCCAATGATTCGAATCGCCGTACT, where GGATCC is a *Bam*HI site) and DOIRexR1Hind (AGAGAAGCTTCACCTTGACGGCTTTGC, where AAGCTT is a *Hin*dIII site). The amplified fragments were digested with *Bam*HI and *Hin*dIII and cloned into the corresponding sites of plasmid pETduet-1 (Merck Japan, Tokyo, Japan) to generate the pETduet-QUDH vector. The *qudh* gene of this vector was under the control of the T7 promoter and was fused to a His × 6-tag at the *N*-terminus. *E. coli* BL21(DE3) (Takara Bio, Kyoto, Japan) was used as the host cell. The transformants possessing pETduet-QUDH were grown on MagicMedia™ (Thermo Fisher Scientific Inc., Yokohama, Japan) containing 50 μg/mL of ampicillin at 30 °C.

Genome database mining and the construction of expression vectors

A database homology search was performed using BLAST (National Center for Biotechnology Information, Bethesda, MD). Microbial genes that shared 36–79% amino acid sequence identity with *Bu. terrae* QUDH were selected from databases and chemically synthesized to optimize translational codon usage in *E. coli*. The initiation codon of GTG in *Ba. subtilis iolX* was substituted with ATG. Each synthesized DNA fragment was digested with *Bam*HI and *Hin*dIII and cloned into the same sites of plasmid pET21b (+) (Merck) to generate each expression vector. *E. coli* BL21(DE3) transformant cells possessing each vector were grown in MagicMedia™ containing 50 μg/mL of ampicillin at 30 °C in the same manner as *E. coli* BL21(DE3) (i.e., pETduet-QUDH).

Chemicals

2-Deoxy-*scyllo*-inosose (DOI), (−)-*vibo*-quercitol, and (−)-*vibo*- and *scyllo*-quercitol mixtures were acquired from Asahi Kasei Chemicals Corp. (Tokyo, Japan), and (+)-*proto*-quercitol (1L-1,3,4/2,5-cyclohexanepentol), *scyllo*-inositol (1,3,5/2,4,6-cyclohexanehexol), and 2-deoxy-D-glucose were purchased from Tokyo Kasei Corp. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries.

Nucleotide sequence accession number

The *qudh* sequence was registered at the DNA Data Bank of Japan (DDBJ) under accession number LC259984.

Results

Screening of microbial strains for QUDH production

Several microorganisms that were able to assimilate the (−)-*vibo*- and *scyllo*-quercitol mixture as a sole carbon source were easily obtained from the soil samples. We observed that 42 of the 109 cultural microorganisms isolated were able to rapidly grow in a (−)-*vibo*- and *scyllo*-quercitol-containing liquid medium. Most of these quercitol-assimilating microorganisms demonstrated varying levels of DOI-reducing activity using NADH or NAPDH. Many of these strains exhibited high QUDH (i.e., DOI reducing) activity and high

diastereomeric excess of the produced quercitol. These included strains AKC-020 (40-1), 40-42, 81-8, 83-4, 85-1, 85-4, 86-4, 93-1, 96-3, and 97-1, which were selected for preliminary taxonomic identification based on 500 bp of their 16S ribosomal DNA (rDNA) sequences. As Table 1 indicates, strains AKC-020 (40-1), 40-42, 89-3, and 97-1 belong to the genus *Burkholderia*; strains 81-8, 83-4, 85-1, 85-4, 86-4, and 96-3 to *Pseudomonas*; and strain 93-1 to *Arthrobacter*. Moreover, all the QUDHs found in these microorganisms exhibited the stereoselectivity to generate (−)-*vibo*-quercitol but not *scyllo*-quercitol from DOI (Fig. 1, Table 1).

Identification of the isolated microorganism

The AKC-020 (40-1) strain inferred to be *Burkholderia* sp. was a rod-shaped Gram-negative bacterium (0.8–0.9 × 1.2–2.0 μm) with the following physiological features: pale yellow colonies on LB agar, growth at 37 °C (−), positive for motility, negative for oxidase activity, positive for catalase activity, no spore formation, (−/−) for acid and gas formation from glucose, and (−/−) in the O-F test. In addition, an analysis of the 16S rDNA sequence (1000 bp) from AKC-020 (40-1) performed by Techno Suruga Lab (Shizuoka, Japan) showed that the sequence from the strain was a perfect match with 16S rDNA from *Bu. terrae.* This sequence result combined with the physiological properties of the culture confirmed the identity of the microorganism as *Bu. terrae* strain AKC-020 (40-1). The genera of the preliminarily identified strains *Pseudomonas* sp. 86-4 and *Arthrobacter* sp. 93-1 were also confirmed to be correct by 16S rDNA sequencing (1000 bp) and physiological features, but they were not classified to the species level.

Production and purification of QUDH from *Bu. terrae*

The enzyme production by *Bu. terrae* AKC-020 (40-1) was strongly induced by the addition of DOI or quercitol and slightly induced by *myo*-inositol in the culture media. DOI was chosen as a carbon source for *Bu. terrae* culture because it was available at a relatively low cost. QUDH was strongly produced under the optimized culture medium containing DOI and negligibly produced in the absence of DOI or quercitol (Fig. S1). Thus, the enzyme production was inducible, and the combination of DOI (0.5–1.0%), tryptone (0.4%), and yeast extract (0.2%) in the medium was the most suitable for generating QUDH in the culture.

The enzyme was stable throughout the purification procedures. Therefore, the process of purifying QUDH from the crude extract was conducted by standard methods including ammonium sulfate fractionation and a combination of chromatographic procedures, including hydrophobic, anion exchange, and gel filtration techniques (Table 2). The enzyme was purified by 24.6-fold, and the yield was 4.5%.

SDS-PAGE indicated that the purified enzyme was homogeneous (Fig. 2a). The *N*-terminal and internal peptide sequences of QUDH were MIRIAVLGAGRI and AELEAFVDALNTN, respectively, which indicated that the sequence of the enzyme was quite similar to those of the previously reported inositol dehydrogenase and glycerol-3-phosphate dehydrogenase enzymes.

Table 1 2-Deoxy-*scyllo*-inosose reducing activity of various quercitol-assimilating microorganisms

Strain	Culture medium[a]	Activity (U/mL culture)	Conversion yield (%)[b]	Absolute configuration	Diastereomeric excess (% *de*)
Burkholderia terrae AKC-020 (40-1)	A	0.41	42.0	(−)-*vibo*	90.7
Burkholderia terrae 40-4-2	A	0.59	68.5	(−)-*vibo*	79.4
Burkholderia sediminicola 89-3	A	0.94	49.2	(−)-*vibo*	74.0
Burkholderia sp. 97-1	B	0.55	35.6	(−)-*vibo*	88.6
Pseudomonas sp. 81-8	A	1.73	54.7	(−)-*vibo*	83.4
Pseudomonas sp. 83-4	A	0.16	70.7	(−)-*vibo*	82.5
Pseudomonas sp. 85-1	A	0.50	53.9	(−)-*vibo*	84.0
Pseudomonas sp. 85-4	A	1.08	54.5	(−)-*vibo*	82.6
Pseudomonas sp. 86-4	A	0.69	75.4	(−)-*vibo*	87.7
Pseudomonas sp. 96-3	A	1.05	57.6	(−)-*vibo*	85.3
Arthrobacter sp. 93-1	YA	0.16	41.9	(−)-*vibo*	82.0

[a] Refer to "Materials and methods"; medium YA is supplemented with 0.5% (*w*/*v*) yeast extract to medium A

[b] Conversion yield = quercitol (mM)/2-deoxy-*scyllo*-inosose (mM) × 100 in the reaction mixture after incubation. The modified reaction mixture consists of 60 μmol of DOI, 1.0 μmol of NAD^+, 200 μmol of sodium formate, 200 μmol of KPB (pH 7.0), 0.2 units of formate dehydrogenase, and 0.5 units of QUDH, in a total reaction volume of 1.0 mL for a 3-h incubation at 30 °C

Table 2 Summary of purification of QUDH from *Burkholderia terrae* AKC-020 (40-1)

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Culture extract	37.6	78.7	2.09	1	100
$(NH_4)_2SO_4$ precipitation (30%)	18.6	74.7	4.02	1.9	95
Butyl TOYOPEARL650M	0.4	15.8	39.5	18.9	20
RESORCE Q	0.24	11.2	46.7	22.3	14
TSK-Gel G3000SW	0.07	3.6	51.4	24.6	4.6

Enzymatic properties of QUDH

Molecular mass and subunit structure

The molecular mass of native QUDH was estimated to be 130.9 kDa according to analytical HPLC with a TSK-Gel G3000SW column. SDS-PAGE revealed a single band corresponding to a molecular mass of 37.0 kDa (Fig. 2a). This suggested that QUDH is a homotetrameric enzyme. As described later, QUDH consists of 330 amino acids, and its molecular mass was estimated to be 36.2 kDa. Therefore, the theoretical molecular mass of native QUDH was inferred to be 144.8 kDa.

Effect of pH on enzyme activity

The effect of pH on enzyme activity at a final buffer concentration of 0.2 M was measured in citrate buffer (pH 5.0–6.0), KPB (pH 6.0–8.0), Tris-HCl buffer (pH 7.5–9.0), glycine-NaOH buffer (pH 8.5–10.0), and CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)-NaOH buffer (pH 10.0–11.5). The maximum activity of the reductive reaction of DOI was 100% at pH 8.0 and 60% at both pH 7.0 and 9.5 (Fig. 3a). In contrast, the optimal pH for the oxidative reaction of (−)-*vibo*-quercitol was 9.5 (Fig. 3b).

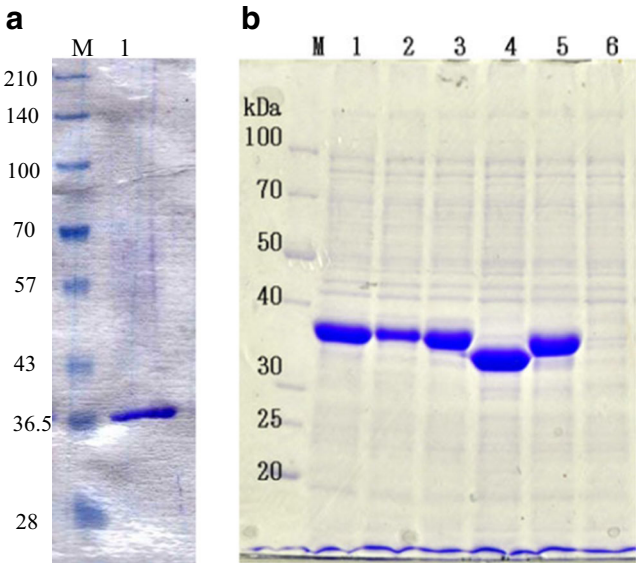

Fig. 2 SDS-PAGE analysis of **a** purified and **b** recombinant QUDH and its homologous enzymes, which were expressed in *E. coli* BL21(DE3). In **a**, lane M contained molecular weight standards (prestained XL-Ladder, APRO Science, Tokushima, Japan), and lane 1 contained the purified QUDH from *Burkholderia terrae*; in **b**, lane M contained molecular weight standards, and lanes 1–6 contained the following crude enzyme solutions from recombinant *E. coli* cells: (1) QUDH/*B. terrae*, (2) IolG/*Burkholderia* sp., (3) Idh/*Pantoea* sp., (4) Idh/*Pseudomonas synxantha*, (5) Idh/*Sinorhizobium fredii*, and (6) IolX/*Bacillus subtilis*

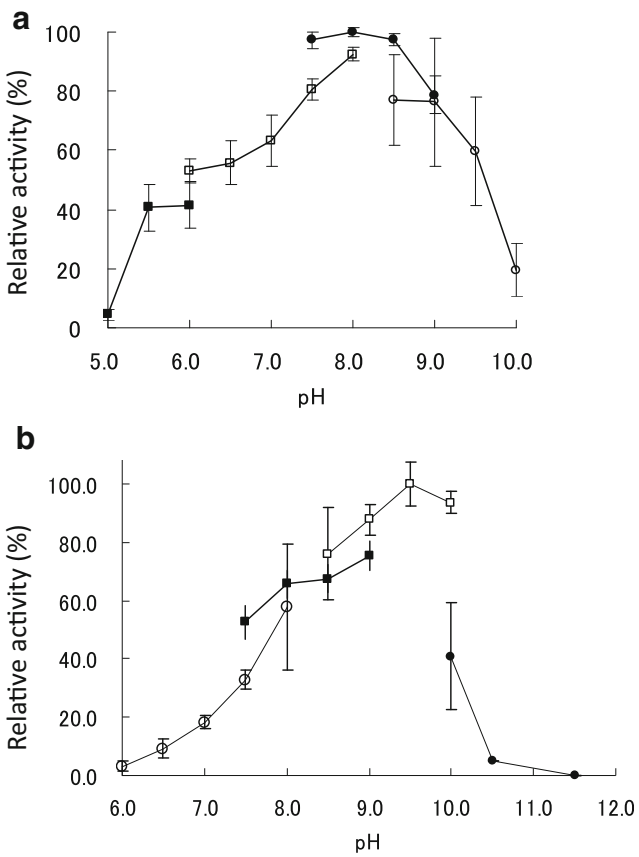

Fig. 3 Effect of pH on the activity of purified QUDH on **a** the reductive reaction of DOI and **b** the oxidative reaction of (−)-*vibo*-quercitol. In **a**, closed squares indicate citrate buffer (pH 5.0–6.0), open squares indicate KPB (pH 6.0–8.0), closed circles indicate Tris-HCl buffer (pH 7.5–9.0), and open circles indicate glycine-NaOH buffer (pH 8.5–10.0), while in **b**, open circles indicate KPB (pH 6.0–8.0), closed squares indicate Tris-HCl buffer (pH 7.5–9.0), open squares indicate glycine-NaOH buffer (pH 8.5–10.0), and closed circles indicate CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)-NaOH buffer (pH 10.0–11.5). The bars indicate the standard deviations from three measurements

Effect of temperature on enzyme activity and stability

The optimal temperature of the QUDH reaction was measured using the purified enzyme for 1 min at temperatures ranging from 20 to 70 °C. The enzyme assay indicated a maximum activity at 50 °C, with 50 and 6% of this maximum activity achieved at 40 and 60 °C, respectively (Fig. S2).

The thermal stability of the enzyme was measured by incubating the purified enzyme at each tested temperature for 30 min in 50 mM KPB (pH 7.0). The enzyme was stable after incubations below 30 °C, with 53 and 11% of the original activity maintained after incubations at 35 and 40 °C, respectively.

Substrate specificity and kinetic parameters

The activities and kinetic parameters of the purified enzyme were measured for various substrates and coenzymes (Table 3). QUDH used NADH as a coenzyme, and 28.5% of this activity level was observed when NADH was substituted with NADPH. Therefore, QUDH is an NADH-dependent oxidoreductase. The K_m values of QUDH toward DOI and NADH in the reductive reaction were calculated from the Lineweaver-Burk plot to be 0.41 and 0.04 mM, respectively. The K_m values of the enzyme toward (−)-*vibo*-quercitol and NAD^+ in the oxidative reaction were 1.60 and 0.11 mM, respectively. In all cases, regular saturation curves of the activity versus the substrate concentration were observed. As summarized in Table 3, the k_{cat} value of QUDH toward DOI (70.1 s^{-1}) was sufficient to catalyze the reduction of DOI to produce (−)-*vibo*-quercitol.

Table 4 shows the specific activity for the oxidative reaction of QUDH with NAD^+ for some cyclitols, which are cycloalkanes containing a hydroxyl group on each of three or more ring atoms, as well as cyclohexanepolyols, sugars, and alcohols. Among the various substrates tested, QUDH catalyzed the oxidation of only (−)-*vibo*-quercitol and *myo*-inositol, the latter at 41% of the activity for (−)-*vibo*-quercitol; these somewhat similar activity levels are probably due to the structural similarity of *myo*-inositol with (−)-*vibo*-quercitol (Fig. 1). The substrate specificity of QUDH was very strict, with limited cyclitols serving as a substrate. *myo*-Inositol dehydrogenase generally catalyzes the oxidation of D-glucose and D-xylose (Ramaley et al. 1979; Daniellou et al. 2005), but QUDH never catalyzed the oxidation of either compound. Moreover, (+)-*proto*-quercitol, a stereoisomer of (−)-*vibo*-quercitol and *scyllo*-inositol, did not serve as a substrate. Thus, QUDH was identified as a novel NAD^+-dependent (−)-*vibo*-quercitol 1-dehydrogenase, which was supported by its ability to efficiently reduce DOI into (−)-*vibo*-quercitol (Fig. 1).

Cloning the *qudh* gene

The *qudh* gene was successfully amplified from the genomic DNA of *Bu. terrae* AKC-020 (40-1) and cloned into *E. coli* cells according to the procedures described in the "Materials and methods." The PCR-based amplification procedures utilized the *N*-terminal amino acid sequence of QUDH and the conserved regions of inositol dehydrogenases followed by TAIL-PCR. The amplified DNA fragment was 3012 bp and contained an open reading frame that consisted of 993 nucleotides corresponding to 330 amino acid residues with a calculated molecular mass of 36,195 Da (Fig. 4). A comparison of the *qudh* sequence revealed that it contains the *N*-terminal and internal amino acid sequences confirmed in native QUDH (i.e., MIRIAVLGAGRI and AELEAFVDALNTN). The theoretical p*I* of QUDH was calculated to be 5.45. QUDH shared relatively high amino acid identities with bacterial inositol dehydrogenases from *Sinorhizobium meliloti* (*idhA*; 49.5% identity; accession number, Q68965; Kohler et al. 2010), *Sinorhizobium fredii* (*idhA*; 48.9% identity; accession number, Q9EZV8; Jiang et al. 2001), *Lactobacillus casei* (*iolG*; 24.3% identity; accession number, A5YBJ7; Yebra et al. 2007), and *Ba. subtilis* (*iolG*; 24.0% identity; accession number, P26935; Fujita et al. 1991). Motif analysis with Motif Search (GenomeNet, http://www.genome.jp/tools/motif/) indicated that QUDH contains an NAD/P(H)-binding Rossmann fold domain (at amino acid positions 2–119) with the well-known glycine-rich phosphate binding loop (GXGXXG, at amino acid positions 8–13) and C-terminal α/β domain (at amino acid positions 133–220) observed within the oxidoreductase family.

Table 3 Kinetic parameters of QUDH from *Burkholderia terrae* AKC-020 (40-1)

Substrate	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})[a]	k_{cat}/K_m (s^{-1} mM^{-1})
2-Deoxy-*scyllo*-inosose (DOI) (pH 7.0)	0.41 ± 0.02	116.3 ± 8.0	70.1 ± 4.8	171.0 ± 12.0
NADH (pH 7.0)	0.04 ± 0.003	–	–	–
(−)-*vibo*-Quercitol (pH 9.0)	1.60 ± 0.08	16.5 ± 0.8	9.9 ± 0.5	6.2 ± 0.38
NAD^+ (pH 9.0)	0.11 ± 0.004	–	–	–

[a] Value per subunit

Table 4 Substrate specificity of QUDH compared with *myo*-inositol 2-dehydrogenase and *scyllo*-inositol dehydrogenase

Substrate	QUDH (U/mg)	*myo*-Inositol dehydrogenase[a]	*scyllo*-Inositol dehydrogenase (IolX; U/mg)[b]
(−)-*vibo*-Quercitol	13.9	–	ND
(+)-*proto*-Quercitol	ND	–	ND
myo-Inositol	5.7	34.4	0.07
scyllo-Inositol	ND	–	0.39 (6.4)[c]
1,2,3-Cyclohexanetriol (*cis*-,*trans*-mixture)	ND	–	ND
trans-1,2-Cyclohexanediol	ND	–	ND
1,3-Cyclohexanediol	ND	–	ND
Cyclohexanol	ND	–	ND
D-Glucose	ND	8.6	0.32
2-Deoxy-D-glucose	ND	< 0.1	ND
D-Xylose	ND	4.3	0.25
Sorbitol	ND	–	ND
1,2-Propanediol	ND	–	ND
2-Propanol	ND	–	ND

ND not detected

[a] Data from Ramaley et al. (1979)

[b] Activities were measured using the crude extract of recombinant IolX in *E. coli* cells. The control experiment was performed using a crude extract of *E. coli* cells without the plasmid expressing *iolX*

[c] V_{max} value reported by Morinaga et al. (2010)

Genome mining and synthesis of quercitol stereoisomers by QUDH and IolX

We performed genome mining based on the QUDH sequence to confirm whether QUDH homologs can produce (−)-*vibo*-quercitol from DOI. The microbial genes that shared 36–79% inferred amino acid sequence identity with *Bu. terrae* QUDH, including putative genes, were selected and chemically synthesized to optimize the translational codon usage in *E. coli*. The corresponding genes were inositol 2-dehydrogenase or its related genes from *Burkholderia* sp. SJ98, *Pantoea* sp. At-9b CGD2M, *Pseudomonas synxantha* BG33R, and

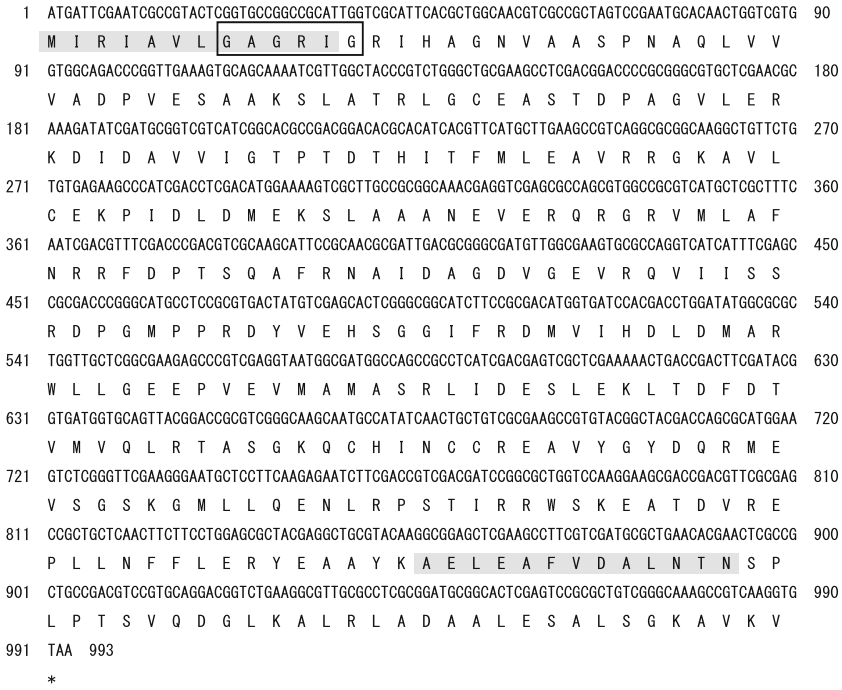

Fig. 4 Nucleotide and amino acid sequences of QUDH. The amino acid sequence (*N*-terminal and internal) within the shaded areas is identical to those determined by protein sequencing. The asterisk indicates the *qudh* termination codon. The box denotes the glycine-rich phosphate binding loop in the Rossmann fold of the NAD/P(H)-binding domain

Sinorhizobium fredii USDA191 as well as *iolX* from *Ba. subtilis* 168. These genes were successfully expressed in *E. coli* with the pET21b (+) vector except *iolX* (Fig. 2b). The expression level of IolX was insufficient to be detected by SDS-PAGE, although enzymatic activity was observed in the cell-free extract.

The production of (−)-*vibo*-quercitol from DOI was tested using recombinant QUDH orthologs as a biocatalyst with an NADH-regenerating system using formate dehydrogenase. As summarized in Table 5, three QUDH homologs from *Burkholderia* sp., *Pantoea* sp., and *P. synxantha* were able to stereoselectively reduce DOI into (−)-*vibo*-quercitol with high conversion yields and with almost the same diastereomeric excess (*de*) of products (89–91%) as exhibited by *Bu. terrae* QUDH. Notably, *scyllo*-inositol dehydrogenase (IolX) from *Ba. subtilis* (Morinaga et al. 2010) exhibited the opposite stereoselectivity for DOI, yielding *scyllo*-quercitol with an excellent *de* value of more than 99%.

Discussion

Various bacterial strains able to grow on quercitol as the sole carbon and energy source were screened for the production of enzymes suitable for the bioreduction of DOI into chiral quercitols. DOI-reducing activity that yielded quercitol was observed for 42 isolates among the total 109 strains of microorganisms isolated, suggesting that our approach was a suitable screening method. Eleven strains were selected as candidate producers of DOI-reducing enzymes. We confirmed that these isolates belong to the genera *Burkholderia*, *Pseudomonas*, and *Arthrobacter*. By comparing the enzyme activity, cofactor dependency, and diastereomeric excess of the produced (−)-*vibo*-quercitol, we selected *Bu. terrae* AKC-020 (40-1) for the production of a DOI-reducing enzyme (i.e., DOI reductase). The initial results also suggested that many soil microorganisms have the ability to assimilate not only inositol but also deoxyinositols.

We successfully purified the QUDH enzyme from *Bu. terrae* AKC-020 (40-1) cells grown on DOI and characterized it in detail. DOI and quercitol strongly induced QUDH production. Under the optimal culture conditions, the enzyme activity level reached more than 0.7 units/mL culture broth (Fig. S1). The purified enzyme catalyzed the reduction of DOI to yield (−)-*vibo*-quercitol with 90.7% *de*. The *de* value of the product (89.0%) of the recombinant QUDH (Table 5) was slightly lower than that of the native purified enzyme (90.7%). This small discrepancy might be caused by the His-tag of the recombinant enzyme. Table 4 shows the substrate specificity of the oxidative reaction of the enzyme compared with that of *Ba. subtilis* inositol 2-dehydrogenase (Ramaley et al. 1979; Daniellou et al. 2005) and *scyllo*-inositol dehydrogenase (IolX; Morinaga et al. 2010). The substrate specificity of the enzyme indicated that it has high activity for (−)-*vibo*-quercitol, although it possesses less activity for *myo*-inositol and no activity for D-glucose, D-xylose, and other quercitol stereoisomers tested. These results suggested that the isolated QUDH enzyme is different from inositol 2-dehydrogenase and should be defined as a novel NAD^+-dependent (−)-*vibo*-quercitol 1-dehydrogenase. The low K_m value of 0.41 mM and high k_{cat} value of 70.1 s^{-1} for DOI reduction as well as the K_m value of 1.60 mM and k_{cat} value of 9.9 s^{-1} for (−)-*vibo*-quercitol oxidation supported our enzyme identification.

We cloned the *qudh* gene by PCR amplification from genomic DNA based on the *N*-terminal and internal amino acid sequences as well as the conserved sequences of inositol 2-dehydrogenase-related enzymes. Such sequences were confirmed in the amino acid sequence of the cloned *qudh* gene (Fig. 4). A protein similarity BLAST search using the QUDH sequence showed that the highest identity shared was 79% with a putative inositol dehydrogenase in *Burkholderia* sp. Therefore, we speculated that these enzymes that have been tentatively annotated as inositol dehydrogenase might more accurately be designated as (−)-*vibo*-quercitol 1-dehydrogenases like QUDH. As shown in Table 5, some *qudh*

Table 5 Production of quercitol by recombinant QUDH and its homologs originating from several microorganisms

Enzyme/origin	Amino acid identity shared with QUDH (%)	Molecular weight of subunit/ accession no.	(−)-*vibo*-Quercitol conc. (mM)	Conversion yield (%)	Diastereomeric excess (% *de*)
QUDH/*Burkholderia terrae*	100	36,195/LC259984	9.9	99	89.0
IolG/*Burkholderia* sp.	79	36,308/ZP_10026200.1	9.9	99	90.3
Idh/*Pseudomonas synxantha*	68	36,618/ZP_10145400.1	9.9	99	90.8
Idh/*Pantoea* sp.	58	36,160/YP_004119064.1	9.9	99	89.1
Idh/*Sinorhizobium fredii*	49	34,648/AAG44816.1	2.8	28	81.7
IolX/*Bacillus subtilis*	36	37,483/NP_388965.1	9.6 (*scyllo*-quercitol)	96	> 99

homologs that share amino acid sequence identities of 79–46% with *qudh* indicated QUDH activity and produced (−)-*vibo*-quercitol from DOI. These data support the hypothesis that these *qudh* homologs have been inappropriately identified as inositol dehydrogenases. Moreover, we confirmed that *scyllo*-inositol dehydrogenase (IolX), which shares an amino acid sequence identity of 38% with QUDH, possesses the opposite stereoselectivity with QUDH and yields *scyllo*-quercitol with an excellent *de* (>99%). Thus, we identified two useful biocatalysts to produce (−)-*vibo*-quercitol and *scyllo*-quercitol from DOI via a coupling reaction of a NADH-regenerating system such as formate dehydrogenase and formate, which suggests a promising bioprocess for producing pure chiral quercitols for pharmaceuticals. In addition, it should be possible to directly produce quercitol stereoisomers from D-glucose by using *E. coli* as a cell factory, in which 2-deoxy-*scyllo*-inososе synthase (BtrC) or its homologs with high activity (Konishi and Imazu 2010) can effectively produce DOI from glucose-6-phosphate, in combination with QUDH or *scyllo*-inositol dehydrogenase (IolX). This process would be useful for supplying chiral quercitols for pharmaceuticals because it permits skipping the step of purifying DOI from the fermentation broth.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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