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Enzymatic activity of cell-free extracts from *Burkholderia oxyphila* OX-01 bio-converts (+)-catechin and (−)-epicatechin to (+)-taxifolin

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This study characterized the enzymatic ability of a cell-free extract from an acidophilic (+)-catechin degrader *Burkholderia oxyphila* (OX-01). The crude OX-01 extracts were able to transform (+)-catechin and (−)-epicatechin into (+)-taxifolin via a leucocyanidin intermediate in a two-step oxidation. Enzymatic oxidation at the C-4 position was carried out anaerobically using H_2O as an oxygen donor. The C-4 oxidation occurred only in the presence of the 2R-catechin stereoisomer, with the C-3 stereoisomer not affecting the reaction. These results suggest that the OX-01 may have evolved to target both (+)-catechin and (−)-epicatechin, which are major structural units in plants.

Key words: burkholderia; (+)-catechin; (+)-taxifolin; (−)-epicatechin; enzymatic reaction

Catechin is a flavonoid and a secondary metabolite of plants. It is a major precursor of condensed tannin, which is the second-most abundant aromatic plant biomass following lignin.[1)] Catechin plays an important role as a defensive material in plants,[2)] and is secreted from the roots to maintain a network of micro-organisms in the rhizosphere.[3)] There is significant interest in how catechin, which is produced in large quantities by plants, is decomposed and recycled by soil micro-organisms.

In 2011, a bacterium identified as *Burkholderia oxyphila* OX-01 was isolated from an acidic forest soil and was shown to grow on (+)-catechin as a sole carbon source under acidic conditions (pH 3.5).[4)] Prior to that research, only one study of bacterial (+)-catechin degradation to taxifolin under acidic conditions by a *Burkolderia* species had been published.[5)] In that paper, we identified that *Burkholderia* sp. KTC-1 metabolizes (+)-catechin via laucocyanidin and taxifolin.[5)] However, we did not clarify how (+)-catechin was being converted to taxifolin by enzymatic reaction. This work builds upon that previous research to look more closely at the mechanisms involved in the transformation. Furthermore, the stereospecificity of the enzymatic reaction against various cathechin isomers was also evaluated. Understanding the function of the catechin metabolic enzymes in OX-01 will potentially contribute to our overall understanding of the cycling of flavonoids in forest ecosystems.

Materials and methods

Micro-organism, medium and culture conditions. *Burkholderia oxyphila* OX-01 (NBRC 105797 =DSM 22550) was isolated from an acidic forest soil in Tsukuba, Japan, and grown in W medium[6)] containing 0.2% (+)-catechin as the sole carbon source at pH 3.5 (adjusted with HCl). The culture medium was filter-sterilized using a Millex-GV filter unit (0.22 μm, Millipore Corp., Bedford, MA, USA) and OX-01 was then grown in stationary culture, or shaken at 28 °C (50 mL of media in a 200 mL Erlenmeyer flask). For stationary culture, 15 g of agar (Nacalai Tesque, Inc., Kyoto, Japan) was added to 1.0 L of the above media.

Preparation of crude OX-01 extract. Cells in the mid-log growth phase were harvested by centrifugation at 8000 *g* for 20 min at 4 °C. The resulting pellet was washed twice with 50-mM phosphate buffer (pH 7.0), and resuspended in 2 ml of the same buffer. The suspension was then homogenized using a French press (Ohtake Seisakusho, Tokyo, Japan) at 4 °C. Unbroken cells were removed by centrifugation (13,000 *g* for 10 min at 4 °C). The supernatant was stored at 4 °C and directly used throughout the experiments.

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Measurement of enzymatic activity of crude OX-01 extract. During preliminary analysis, a reaction mixture containing 0.002% (+)-catechin in 50-mM phosphate buffer (pH 7.0) was prepared. Into 1 mL of this solution, 25 µL of crude extract solution was added and the UV spectra of the sample were evaluated at 28 °C. As control, the same crude extract solution, which was boiled for 15 min to inactivate the enzymes and then centrifuged at 13,000 *g* for 10 min at 4 °C, was employed.

HPLC analysis of enzymatic reaction product. One mL of the crude OX-01 extract was added to 9 mL of the reaction mixture. At 20-min intervals, 1 mL of the reaction solution was collected and then soon frozen with liquid nitrogen to stop the reaction. The frozen samples were lyophilized. Acetonitrile (0.5 mL) was added to the lyophilized sample and the mixture was centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was passed through a Minisart RC15 syringe filter (Sartorius AG, Göttingen, Germany) and then subjected to HPLC analysis using a C-18 column (Inertsil ODS-3, 5 µm column, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan) with detection by UV/Vis detector (UV970, JASCO Corp., Japan) set at 280 nm. Exactly 20 µL of sample was injected and eluted for 15 min at a flow rate of 1.0 mL/min of solvent A (10 mM H_3PO_4 sol.) with a linear gradient from 10 to 50% of solvent B (acetonitrile).

Incorporation of ^{18}O from $H_2{}^{18}O$. The reaction was initiated by the addition of 10 µL of the crude OX-01 extract into the reaction mixture containing 100 µL of 100 mM phosphate buffer (pH 7.0), 2 µL of 0.2% (+)-catechin and 100 µL of $^{18}O_2$-labeled water (99 atom%^{18}O; Taiyo Nippon Sanso Co., Tokyo, Japan) or non-labeled water. The mixtures were incubated for 15 h at 30 °C after which the product was extracted with ethyl acetate and then evaporated. The residue was dissolved in 50 µL of pyridine and treated with 50 µL of BSA + TMCS + TMSI 3:2:3 (Supelco) for 30 min at 65 °C to prepare trimethylsilyl (TMS) derivatives. Two µL of this solution was subjected to GC–MS analysis (1200 Quadrupole Mass Spectrometers, Varian Inc., California, USA) to assess the incorporation of the labeled water. A factor-four VF-5MS column (30 m × 0.25 mm ID, DF 0.25 mm) was used as the stationary phase. The temperature of the mobile phase was increased from 180 to 300 °C at a rate of 10 °C/min

Analysis of substrate-specific enzymatic activity of crude OX-01 extract. Four stereoisomers (+)-catechin, (−)-catechin, (+)-epicatechin, and (−)-epicatechin reacted with the crude OX-01 extract. All substrates were purchased from Nagara Science (Gifu, Japan). The enzyme reaction and measurement of UV spectra were conducted as described in a previous study by Matsuda et al.[5]. Briefly, the reaction mixtures containing 0.002% of catechin stereoisomer in 50-mM phosphate buffer (pH 7.0) were prepared. For measurement of the UV spectra, 25 µL of the crude OX-01 extract was added to 1 mL of the different reaction mixtures and then incubated at 30 °C.

To elucidate the structure of the reaction product, the reaction mixture was extracted with ethyl acetate and purified using silica gel column chromatography with chloroform/ethyl acetate/formic acid (10:8:1) as the mobile phase. The purified product was analyzed by NMR spectroscopy. NMR spectra were measured at 600 MHz with a JNM-ECX-600 instrument (JEOL, Akishima, Japan). CD_3OD was used as solvent and as internal standard for ^{1}H-NMR at δ3.30 ppm.

Results

Analysis of the (+)-catechin metabolic pathway by a crude OX-01 extract reaction

Preliminary evaluation of the enzymatic activity of crude OX-01 extracts from *Burkholderia oxyphila* OX-01 for (+)-catechin degradation has revealed positive results. As shown in Fig. 1(A), the UV spectra of the treated sample showed striking shifts in the absorbance values relative to the initial sample to indicate the chemical conversion of (+)-cathechin. The treated sample also displayed an increase in absorbance at 325 nm suggesting the formation of a new compound. In this connection, the isolation of the enzyme by ammonium sulfate precipitation as well as gel permeation chromatography was attempted. Unfortunately, despite our significant efforts, product isolation was found to be extremely difficult as even a slight indication of enzymatic activity was not detected. Even attempts to incorporate possible cofactors in the reaction were unsuccessful. In any case, with the major goal of describing the degradation pathway of (+)-catechin in natural environments, the observed activity prompted us to further evaluate various parameters in relation to its activity against this compound.

To elucidate the pathway for (+)-catechin metabolism in *B. oxyphila* OX-01, the intermediate and by-products of the action of the crude OX-01 extract at different reaction times were analyzed by HPLC. Figure 2 shows the time course of enzymatic reaction with (+)-catechin. The peak representing (+)-catechin rapidly decreased and was completely undetected after 60 min. This was accompanied by the appearance of a new major peak representing taxifolin, which increased with the decline of (+)-catechin peak. In addition, another peak that was identified in our previous study,[5] which represents leucocyanidin, was also found to increase initially and then subsequently decreased during the reaction process. These results suggest that (+)-catechin was being converted into (+)-taxifolin via a leucocyanidin intermediate by the crude OX-01 extract obtained from OX-01.

Effect of dissolved oxygen and incorporation of ^{18}O from $H_2{}^{18}O$

More detailed evaluation of the mechanism for enzymatic reaction was conducted to determine the oxygen donor for (+)-taxifolin formation. Initially, the possible contribution of dissolved oxygen in generating carbonyl groups was considered. For this purpose, the crude

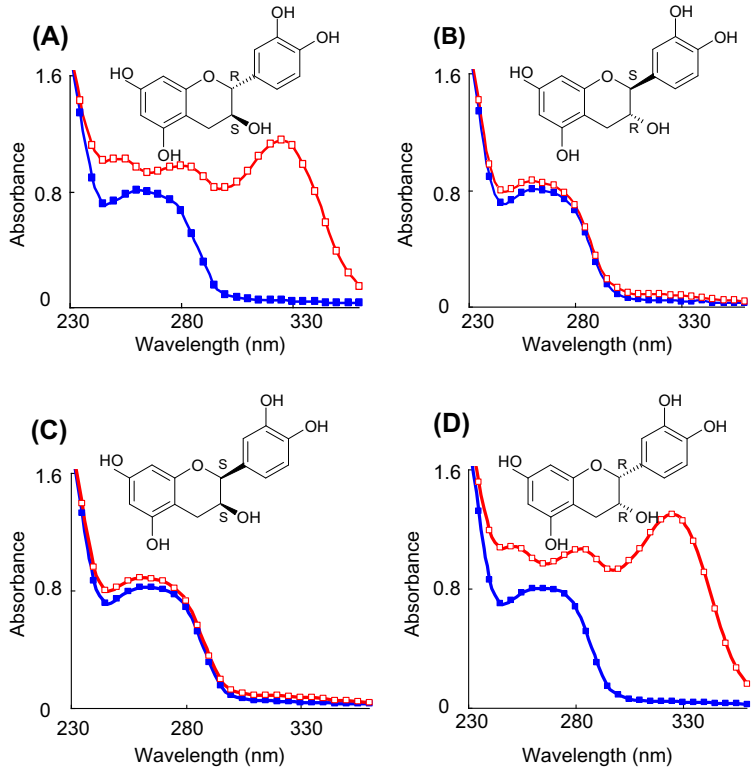

Fig. 1. Substrate specific properties of the C-4 oxidative activity of crude enzyme. The crude enzyme of OX-01 reacted with (+)-catechin (A), (−)-catechin (B), (+)-epicatechin (C), and (−)-epicatechin (D).
Note: Squares show the UV spectra of the substrate (■) and the reaction product (□).

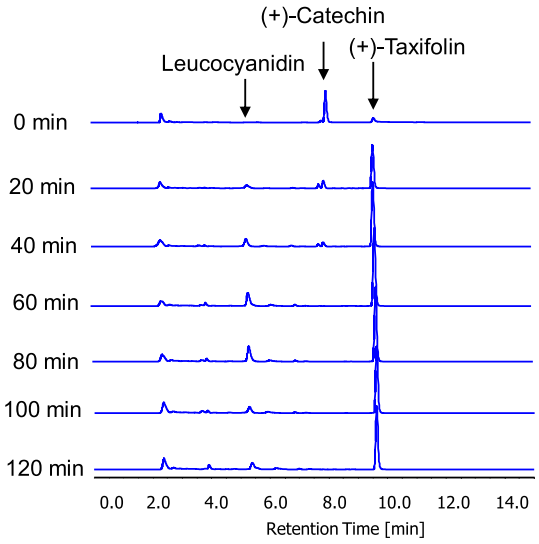

Fig. 2. HPLC analysis of reaction products from (+)-catechin by a crude enzyme mixture from OX-01.
Note: The time on the left of each chromatogram represents the reaction time.

OX-01 extract reaction was initiated after bubbling nitrogen through the mixture to eliminate traces of oxygen in the system. Despite this condition, UV analysis of the reacted sample showed an increase in absorbance at 325 nm indicating that carbonyl formation was not affected by the dissolved oxygen in the system (data not shown).

Next, we determined whether water is directly being utilized for the oxidation of (+)-catechin. This was evaluated by isotope probing using labeled and non-labeled $H_2{}^{18}O$ during enzymatic reaction. The TMS-modified products were analyzed by GC–MS. The major product, trimethylsilyl-taxifolin (TMS-taxifolin), was detected at 18.3 min albeit at different mass values (*m/z*). The reaction product with non-labeled H_2O was detected as the *m/z* 664.5 (Fig. 3(A)) molecular ion. On the other hand, the reaction product with $H_2{}^{18}O$ was detected as two *m/z* 664.3 and 666.4 molecular ions (Fig. 3(B)). One of the fragment ions (fragment I) derived from the A-ring including the C-4 carbonyl group was detected as (*m/z* 296.1 and 298.1, data are not shown). Another fragment ion (fragment II) derived from the B-ring did not incorporate the $H_2{}^{18}O$.

Stereoselectivity of catechin 4-carbonylation

To understand the stereoselectivity of carbonyl formation activity, crude OX-01 extract reactions were carried out using four different catechin stereoisomers as substrates. After 3 h, both the UV spectra of the reaction mixtures containing (−)-epicatechin and (+)-catechin displayed the characteristic peak at about 325 nm (Fig. 1(A) and (D)). The UV spectra of the reaction mixtures containing (−)-catechin and (+)-epicatechin showed that these enantiomers were not modified (Fig. 1 (B) and (C)).

Unexpectedly, both products from (+)-catechin and (−)-epicatechin showed same ^{1}H NMR spectra as follows, δ: 6.95 (1H, d, J = 2.0 Hz, H-2′), 6.84 (1H, dd, J = 2.0, 8.0 Hz, H-6′), 6.79 (1H, d, J = 8.0 Hz, H-5′), 5.92 (1H, d, J = 2.0 Hz, H-6), 5.87 (1H, d, J = 2.0 Hz, H-8), 4.90 (1H, d, J = 11.3 Hz, H-2), 4.49 (1H, d, J = 11.3 Hz, H-3). These spectra were

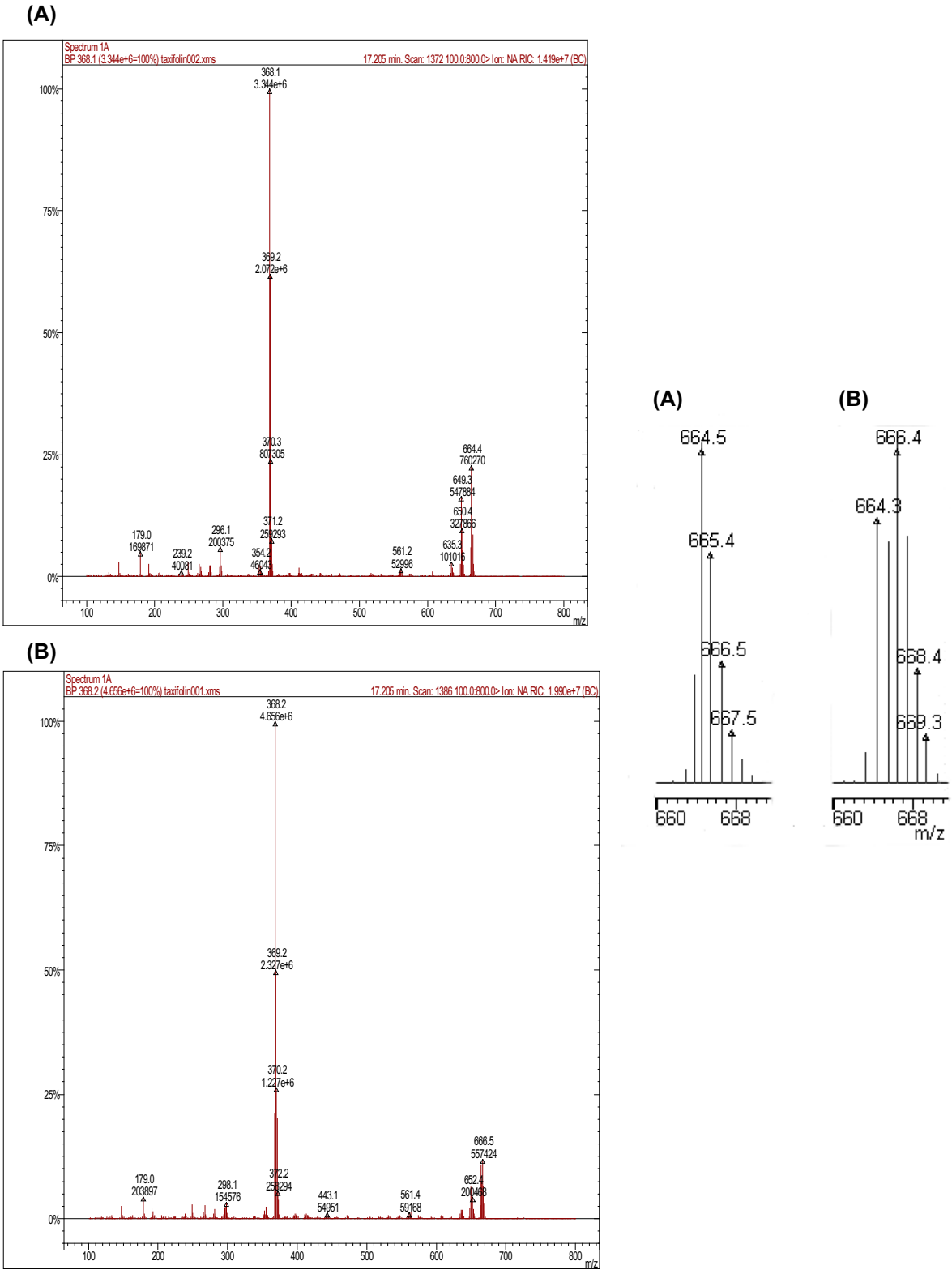

Fig. 3. Mass spectra of taxifolin, the products of the crude OX-01 extract reaction from (+)-catechin. Reaction products (taxifolin) of (+)-catechin generated by the crude OX-01 extract in non-labeled water (A), and in ^{18}O labeled water (B).

annotated as (+)-taxifolin. Therefore, these results suggest that the epimerizing reaction occurs during the conversion of (−)-epicatechin to (+)-taxifolin.

Discussion

In a previous study, a novel (+)-catechin-degrading bacterium, designated as *Burkholderia oxyphila* OX-01, was isolated from an acidic forest soil.[4] In this work, a crude OX-01 extract was demonstrated to convert (+)-catechin into (+)-taxifolin via a leucocyanidin intermediate. The enzymatic conversion of (+)-catechin was the same as the enzymatic reaction of *Burkholderia* sp. KTC-1 that was previously reported by Matsuda et al.[5]. The primary enzyme reactions for (+)-catechin of OX-01 and KTC-1 were apparently the same and thus a common bacterial function may be extant in at

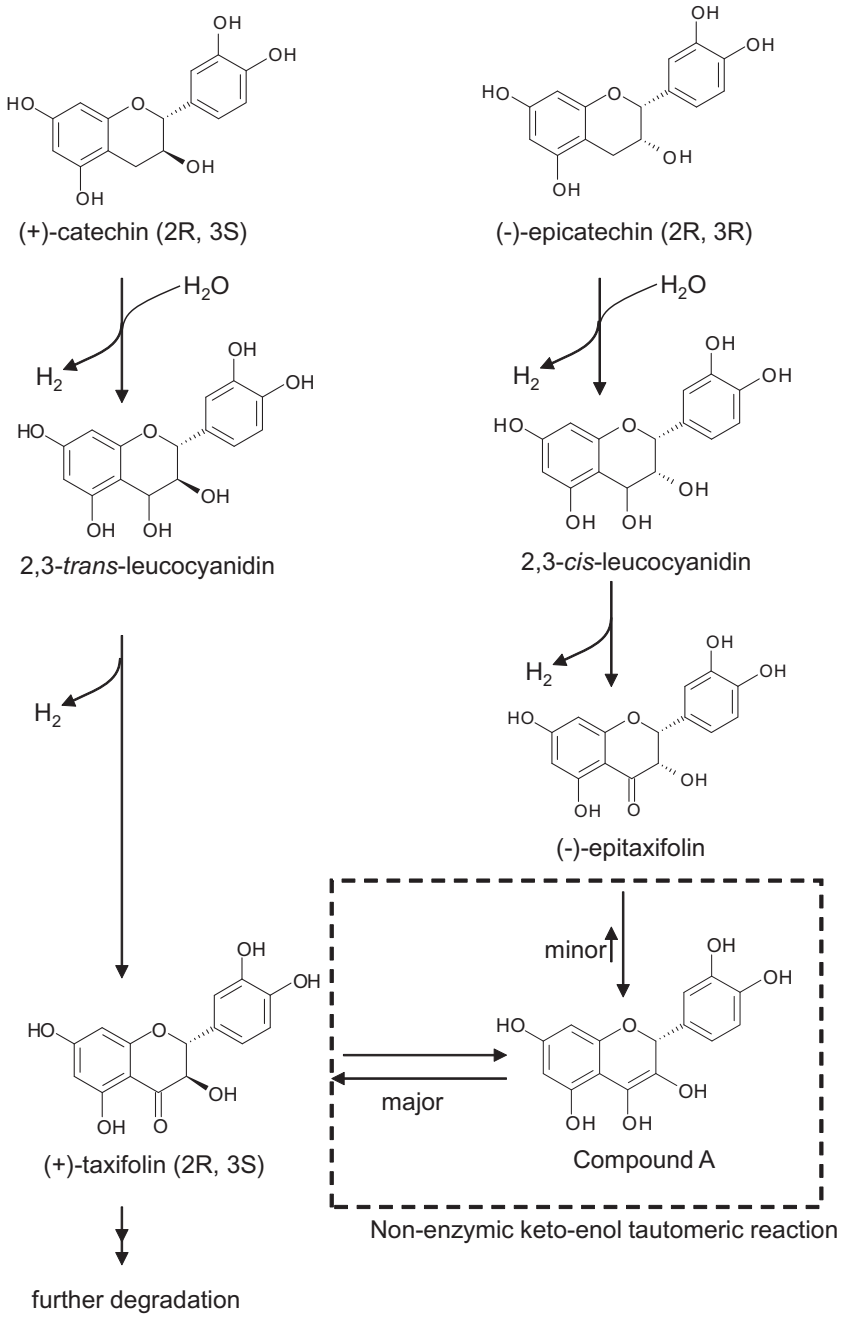

Fig. 4. Proposed metabolic pathway for (+)-catechin and (−)-epicatechin to (+)-taxifolin by crude enzyme of OX-01.

least these two species permitting the conversion of (+)-catechin under acidic conditions. Furthermore, Jeffery et al.[7,8] reported the A-ring fission of taxifolin by (+)-catechin-degrading bacteria isolated from rat feces, but they did not elucidate the metabolic pathway from (+)-catechin to taxifolin. It must be emphasized that the enzymatic function of OX-01 was clearly different from the (+)-catechin dioxygenase reaction that generates protocatechuic acid and phloroglucinol carboxylic acid from (+)-catechin via a single-step oxidation, as reported by Mahadevan et al.[9–15]. It is also distinct from very recent findings on the biotransformation of four cathechin stereoisomers via ring cleavage at the C-ring oxygen by isoflavone-metabolizing bacteria in anaerobic condition, which was found accelerated by the presence of hydrogen.[16,17]

To determine the oxygen donor for (+)-catechin conversion, experiments were performed under several conditions. No relationship was found between dissolved oxygen and the extent of (+)-catechin oxidation activity when incubations were conducted using the crude OX-01 extract. Moreover, GC–MS analysis of the reaction mixture containing $H_2{}^{18}O$, showed that ^{18}O was incorporated into (+)-taxifolin as the carbonyl oxygen indicating that H_2O is the oxygen donor in (+)-catechin 4-hydroxylation. These results suggest that the oxidation of (+)-catechin by OX-01 is anaerobic reaction using water as the oxygen donor.

Enzymatic carbonyl formation into C-4 occurred not only with (+)-catechin but also with (−)-epicatechin (Fig. 4). Enzymatic activity on (−)-catechin or (+)-epicatechin was not observed. The (+)-catechin is a 2R,

3S structure and (−)-epicatechin is a 2R, 3R structure indicating that C-4 carbonylation by OX-01 is specific to the 2R-stereoisomer.

The ^{1}H NMR spectrum of reaction products transformed from (−)-epicatechin was consistent with the spectrum of (+)-taxifolin and not (−)-epitaxifolin. This result indicates that the isomerization occurs at C-3 position. (Figure 4) shows the proposed conversion pathway of (+)-catechin and/or (−)-epicatechin into (+)-taxifolin in the presence of a cellular extract of OX-01. This crude OX-01 extract not only converted (+)-catechin to (+)-taxifolin but also was capable of converting (−)-epicatechin to (+)-taxifolin. The (−)-epicatechin was converted to (−)-epitaxifolin via a 2,3-*cis*-leucocyanidin intermediate because the stereoisomers at the C-3 position were stable when the 2,3-*cis*-leucocyanidin was formed.[18] The (−)-epitaxifolin converged with (+)-taxifolin as a more stable structure via compound A through a non-enzymatic keto–enol tautomeric reaction.

In aerobic bacteria, (−)-epicatechin metabolism has not been reported. Almost all of the flavanols in free form in the leaves, such as tea leaves, have been found to be in the (+)-catechin (2R, 3S) structure. However, flavanol units observed in bark as condensed tannins were of the (+)-catechin and (−)-epicatechin (2R, 3R) structure.[19] In our work, (−)-catechin and (+)-epicatechin were unable to react with the crude OX-01 extract, but it should be noted that these forms are rare in nature. There are however, some bacterium that can degrade (−)-catechin. Wang et al.[20] reported that a bacterium isolated from the rhizosphere of the (−)-catechin-producing plant, *Rhododendron formosanum*, could degrade (−)-catechin and was capable of using (−)-catechin as a sole carbon source. The catechin oxidative enzymes of OX-01, which lack the ability to react with the (−)-catechin, may have a different evolutionary origin and ecological function from that of the (−)-catechin-degrading bacteria described by Wang.

Jeremy et al.[21] reported that procyanidin oligomers, constructed of catechin units, automatically decomposed to catechin monomers under acidic conditions. In acidic forest soil containing leaf litter and various other plant constituents, part of the condensed tannin also would be automatically decomposed to catechins. This suggests that the evolutionary targets for the OX-01 catechin oxidative enzymes were not only (+)-catechin in leaves and nutshells as a free form, but also the more recalcitrant (−)-epicatechins present in bark as a condensed tannin polymer. A more detailed analysis of the catechin degradation abilities in OX-01 will contribute to the understanding of how flavonoids and condensed tannins, produced in great quantities by plants are recycled by soil micro-organisms under acidic conditions.

The (+)-taxifolin is not only of ecological interest but is also of proven and potential medical interest. This compound is not mutagenic and less toxic than the similar flavonoid quercetin.[22] Numerous reports on (+)-taxifolin demonstrate its many medicinal applications including anti-cancer [23] and antioxidant activity.[24] In addition, Sato et al.[25] recently reported that (+)-taxifolin could inhibit amyloid β aggregations, an observation of potential relevance to the progression of Alzheimer's disease. These and others reports suggest that (+)-taxifolin will continue to be a compound of significant pharmaceutical interest. If we can utilize the enzyme function of OX-01 effectively, (+)-taxifolin could be produced from (+)-catechin and/or (−)-epicatechin, both of which are abundant plant flavonoids and easily recovered by simple extraction. Therefore, we believe this study will contribute not only to a better understanding of microbial degradation of catechin in forest ecosystems, but will also provide insights into processes which may enable the economically favorable production of (+)-taxifolin for commercial use.

Author contributions

Yuichiro Otsuka, Motoki Matsuda, Tomonori Sonoki, Kanna Sato-Izawa, Barry Goodell, Jody Jelison, Hitoshi Murata, and Masaya Nakamura designed this study. Yuichiro Otsuka, Motoki Matsuda, and Hitoshi Murata performed the experiment. Yuichiro Otsuka, Motoki Matsuda, Barry Goodell, Jody Jellison, and Ronald R. Navarro wrote this manuscript. All the authors reviewed and approved the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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