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# Molecular Cloning and Characterization of *Bacillus cereus O*-Methyltransferase

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**Abstract** Biotransformation is a good tool to synthesize regioselective compounds. It could be performed with diverse sources of genes, and microorganisms provide a myriad of gene sources for biotransformation. We were interested in modification of flavonoids, and therefore, we cloned a putative *O*-methyltransferase from *Bacillus cereus*, *BcOMT-2*. It has a 668-bp open reading frame that encodes a 24.6-kDa protein. In order to investigate the modification reaction mediated by BcOMT-2, it was expressed in *E. coli* as a His-tag fusion protein and purified to homogeneity. Several substrates such as naringenin, luteolin, kaempferol, and quercetin were tested and reaction products were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). BcOMT-2 could transfer a methyl group to substrates that have a 3' functional hydroxyl group, such as luteolin and quercetin. Comparison of the HPLC retention time and UV spectrum of the quercetin reaction product with corresponding authentic 3'-methylated and 4'-methylated compounds showed that the methylation position was at either the 3'-hydroxyl or 4'-hydroxyl group. Thus, BcOMT-2 transfers a methyl group either to the 3'-hydroxyl or 4'-hydroxyl group of flavonoids when both hydroxyl groups are available. Among several flavonoids that contain a 3'- and 4'-hydroxyl group, fisetin was the best substrate for the BcOMT-2.

**Key words:** Biotransformation, flavonoid, *O*-methyltranferase

Biotransformation of natural compounds is an attractive method to prepare regioselective compounds [13]. Either whole organisms [2, 6, 12] or transgenic microbes harboring the engineered gene is used. The use of transgenic microbes that harbors interesting genes from diverse organisms is a well-documented biotransformation method. For example, *E. coli* transformants carrying the biphenyl dioxygenase gene from *P. pseudoalcaligenes* KF707 were used to modify several phenolic compounds [14], and transgenic *E. coli* carrying glutathione *S*-transferase from *Ochrobactrum anthropi* SH35B were used to biotransform fungicide chlorothalonil [7]. In addition, *E. coli* strains carrying plant genes were also used to convert natural compounds [8, 9, 11]. This transgenic approach is expected to be expanded, because of the completion of genome projects from a myriad of organisms. Because the cloning an interesting gene is much easier than before owing to the Genome Project, biological sources will have a great deal of importance.

There have been several target compounds for biotransformation. Enzymatic hydroxylation of several compounds including trimegestrone, codeine, and α-pinene is one of the examples [3]. Recently, the modification of alkaloid

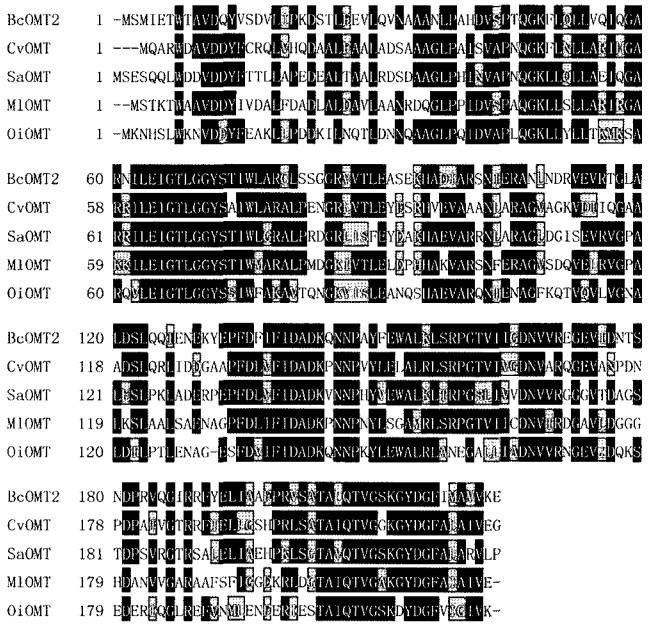


**Fig. 1.** Alignments of BcOMT-2 with other OMTs from several microbes.
BcOMT2, *O*-methyltransferase from *Bacillus cereus*; CvOMT, OMT from *Chromobacterium violaceum*; SaOMT, OMT from *Streptomyces avermitilis*; MlOMT, OMT from *Mesorhizobium loti*, and OiOMT, OMT from *Oceanobacillus iheyensis*.

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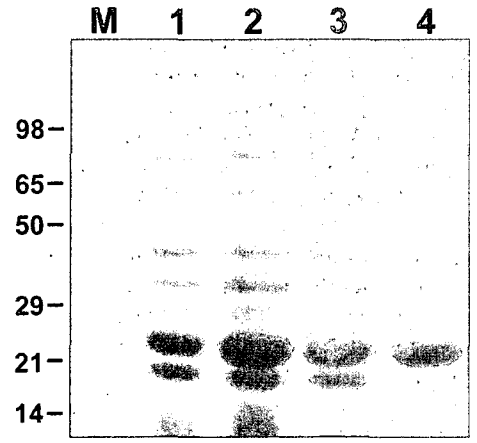



**Fig. 2.** SDS-PAGE of the expressed recombinant BcOMT-2.
M, Standard protein markers; 1, *E. coli* lysate before induction; 2, *E. coli* lysate after induction; 3, Soluble protein after induction; 4, His-tagged affinity-purified protein.

tetrahydroisoquiniline by coclaurine *N*-methyltransferase [11], and flavonoid naringenin by flavonoid 4' *O*-methyltransferase [8] have been reported. In order to employ a transgenic microbe for biotransformation, a functional characterization of the interesting genes is required. Enzymatic methylation in hydroxyl residues of target compounds decreases water solubility and chemical reactivity [10]. On the other hand, *O*-methylation of phenolics such as flavonoid enhances their antimicrobial activity and mutagenicity. We were interested in the modification of flavonoids with transgenic microbes. In the present study, we cloned and characterized a *O*-methyltranferase from *Bacillus cereus*. We also converted flavonoid into a corresponding methylated compound by biotransformation.

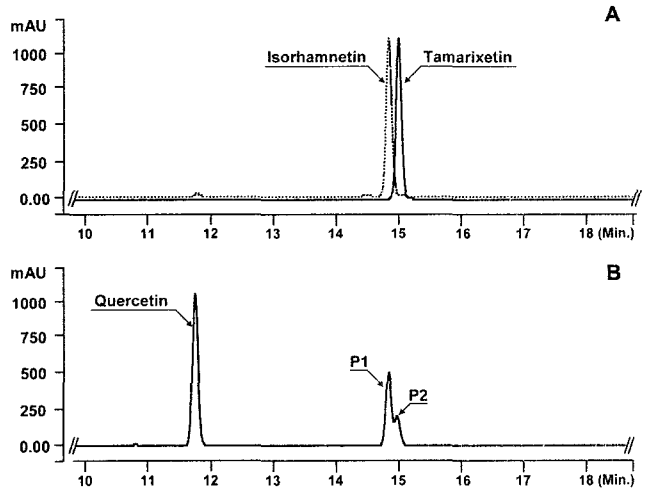



**Fig. 3.** HPLC elution profiles for the reaction products produced from quercetin by recombinant BcOMT-2.
**A.** Authentic 3'-methylated quercetin, isorhamnetin, and 4'-methylated quercetin tamarixetin. **B.** Reaction products P1 and P2.

The genome sequence of *B. cereus* was completed [5] and the annotated *B. cereus* proteins were searched for *O*-methyltransferase (OMT). Four OMTs were found in the genome sequence of *B. cereus*. *BcOMT-2* was cloned by polymerase chain reaction (PCR). Two primers (ATCATATG-AGTATGATTGAGACATGGACGGC as a forward primer and ATGGATCCTCGTTATTCTTTTACGACTGCCAT as a reverse primer) covering the open reading frame of *BcOMT-2* were designed, based on the *BcOMT-2* sequence (GenBank accession number 30022459). The restriction enzyme sites, NdeI and BamHI (underlined), were added to facilitate the cloning process. A polymerase chain reaction (PCR) was performed under the following conditions: 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. The PCR product was sequenced, digested with NdeI and BamHI, and subcloned into pET15b (Novagen, Madison, WI, U.S.A.). BcOMT-2 consisted of a 668-bp ORF, which encodes a 24.6-kDa protein. The BLAST of BcOMT-2 showed more than 70% similarities to OMTs from *Chromobacterium violaceum*, *Streptomyces avermitilis*, *Mesorhizobium loti*, and *Oceanobacillus iheyensis* (Fig. 1). The biological function of none of these is known.

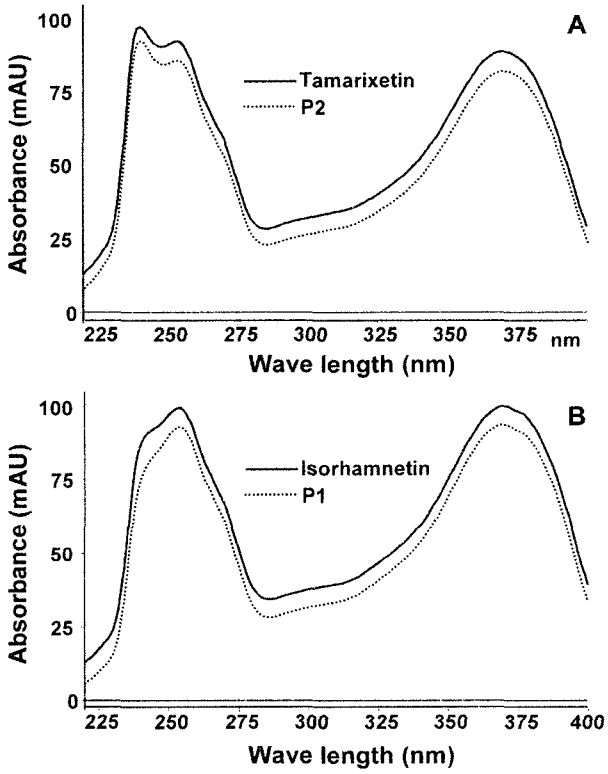
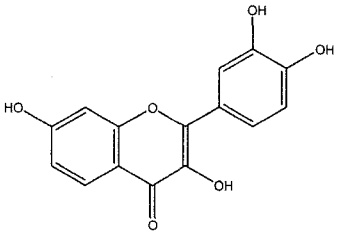
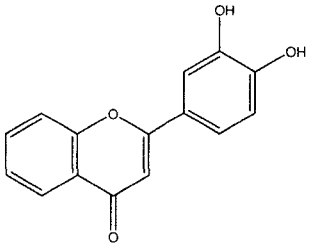
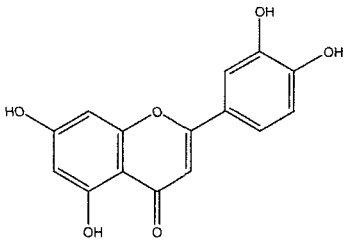
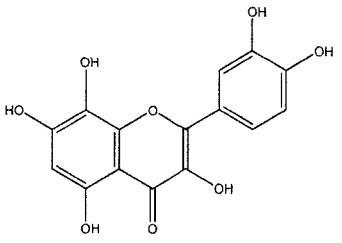
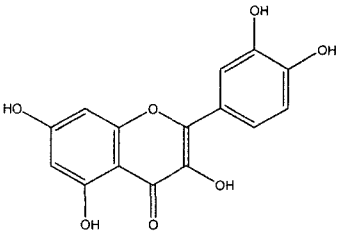



**Fig. 4.** UV spectra for authentic compounds, 3'-methylated quercetin, isorhamnetin, and 4'-methylated quercetin, tamarixetin, and reaction products P1 and P2.
**A.** authentic 3'-methylated quercetin, isorhamnetin (straight line), and reaction product P1 (dotted line). **B.** authentic 4'-methylated quercetin, tamarixetin (straight line), and reaction product P2 (dotted line).

To elucidate the function of BcOMT-2, *BcOMT-2* was expressed in *E. coli*. The *E. coli* transformant carrying *BcOMT-2* was induced by the addition of 1 mM concentration of IPTG, and the cells grew for 4 more hours at 28°C. The cells were harvested and lysed by sonication. The recombinant BcOMT-2 was purified with His-tag affinity chromatography, and SDS-PAGE analysis of the recombinant BcOMT-2 revealed that it was successfully expressed and that it was purified to almost homogeneity (Fig. 2).

Instead of using the purified recombinant BcOMT-2, we carried out the biotranformation with the *E. coli* transformant containing BcOMT-2, because biotransformation does not need the presence of an expensive cosubstrate such as *S*-adenosyl methionine. After induction of the recombinant BcOMT-2, the cells were harvested, resuspended in LB medium containing ampicillin, and adjusted to OD=5.0 at 600 nm. The possible substrates such as apigenin, naringenin, kaempferol, luteolin, and quercetin were added to final concentration of 100 μM. The reaction products were initially analyzed with thin layer chromatography (TLC), as described by Kim *et al.* [8]. Only luteolin and quercetin gave products that had a different $R_f$ value from the corresponding substrates.

**Table 1.** Relative activity of BcOMT-2 toward different substrates.

| Substrate | Structure | Conversion rate (%) |
|---|---|---|
| Fisetin |  | 100 |
| 3'4'-Dihydroxyflavone |  | 81.2 |
| Luteolin |  | 72.7 |
| Gossypetin |  | 60.6 |
| Quercetin |  | 24.4 |

These two flavonoids contain a 3' hydroxyl group, whereas the others do not contain the 3' hydroxyl group, thus suggesting that BcOMT-2 transferred a methyl group into the 3' hydroxyl group. Recently, *O*-methyltransferase from ice plant has been shown to methylate the 3' or 4' hydroxyl group when both hydroxyl groups are available [4]. Thus, we could not exclude the possibility that BcOMT-2 methylates the 4' hydroxyl group when the flavonoid contains both 3' and 4' hydroxyl groups, as found in quercetin and luteolin. The reaction product of quercetin was further analyzed by HPLC under the condition described by Kim *et al.* [8], and it displayed two new peaks with different retention times from the quercetin itself (Fig. 3B). LC/MS analysis of the reaction products showed an increase of 14 Da in both reaction products (data not shown), which corresponds to the addition of a methyl group to the products formed, thus strongly suggesting that BcOMT-2 transfers a methyl group either into the 3' hydroxyl or 4' hydroxyl group. In order to determine the position of methylation, we used methylated quercetin. 3'-Methylated quercetin, isorhamnetin, and 4'-methylated quercetin, tamarixetin, are commercially available. As shown in Figs. 3 and 4A, the retention time and UV spectrum of the first peak were indistinguishable from those of the authentic 3'-methylated quercetin, whereas those of the second peak were identical with those of the 4'-methylated quercetin (Figs. 3, 4B). Taken together, BcOMT-2 methylated flavonoids at 3' or 4', when both hydroxyl groups are available. Based on the peak area of the reaction product, BcOMT-2 appeared to preferentially methylate the 3' hydroxy group about 4-fold.

Substrate preference of BcOMT-2 was examined with 2',3'-dihydroxyflavone, 3',4'-dihydroxyflavone, fisetin, gossypetin, luteolin, and quercetin, by analyzing the amount of reaction product with the peak area in HPLC analysis. All the substrates except 2',3'-dihydroxyflavone yielded products. The substrates that served as substrate had 3' and 4' hydroxyl groups, thus indicating that both 3' and 4' hydroxyl groups are required. Among the substrates tested, the best substrate was fiestin, followed by luteolin, 3',4'-dihydroxyflavone, quercetin, and gossypetin (Table 1).

The endogenous substrate of BcOMT-2 is not known. It is known that some *Bacillus* sp. could metabolize flavonoids and the initial step is methylation [1]. In addition, microbial OMTs are likely to metabolize other phenolic compounds. Thus, BcOMT-2 might be involved in the metabolism of diverse compounds, which would be a valuable tool to survive with limited number of genes against diverse endogenous and exogenous compounds.

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