Flavonoids as Substrates of *Bacillus halodurans O*-Methyltransferase

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Bacillus halodurans O-methyltransferase (BhOMT) is an s-adenosylmethionine dependent methyltransferase. In our previous study, three dimensional structure of the BhOMT has been determined by comparative homology modeling and automated docking study showed that two hydroxyl groups at 3'- and 4'-position in B-ring and structural rigidity of C-ring resulting from the double bond characters between C2 and C3 of flavonoid, were key factors for interaction with BhOMT. In the present study, BhOMT was cloned and expressed. Binding assay was performed on purified BhOMT using fluorescence experiments and binding affinity of luteolin, quercetin, fisetin, and myricetin were measured in the range of 10^7. Fluorescence quenching experiments indicated that divalent cation plays a critical role on the metal-mediated electrostatic interactions between flavonoid and substrate binding site of BhOMT. Fluorescence study confirmed successfully the data obtained from the docking study and these results imply that hydroxyl group at 7-position of luteolin, quercetin, fisetin, and myricetin forms a stable hydrogen bonding with K211 and carboxyl oxygen of C-ring forms a stable hydrogen bonding with R170. Hydroxyl group at 3'-and 4'-position in the B-ring also has strong Ca^{2+} mediated electrostatic interactions with BhOMT.

Key Words : Methyltransferase, Flavonoids, Binding assay, Docking, Divalent metal

Introduction

The transfer of methyl groups from s-adenosyl-L-methione (SAM or AdoMet) to methyl acceptor substrates is related to extensive reactions in nature.[1,2] Methyl conjugation is an important pathway in the metabolism of many drugs, xenobiotics, neurotransmitters, and hormones. Methylation by SAM dependent *O*-methyltransferases (OMTs) (E.C. 2.1.1) is a common modification in natural product biosynthesis.[3-5] Plant OMTs play roles in pigmentation of flowers and fruits,[6] defense against phytopathogens,[7,8] protection against UV light,[9] and regulation of auxin transport,[10] while the role of OMT in microorgarnisms is not well known yet.[11] However, it is well known that many bacterial methyltransferases are involved in antibiotics biosynthesis, as well as in the methylation of other compounds[12] and it is designated as a group of Mg^{2+}-dependent enzymes.[13]

In our previous study, three dimensional structure of *Bacillus halodurans O*-methyltransferase (BhOMT) was determined by comparative homology modeling. The crystal structure of alfalfa CCoAOMT was used as a template protein for homology modeling,[14] and automated docking study showed that four flavonoids, quercetin, fisetin, myricetin, and luteolin which have two hydroxyl groups simultaneously at 3'- and 4'-position in the B-ring and structural rigidity of C-ring resulting from the double bond characters between C2 and C3, were well docked as ligands of BhOMT.

Here, binding assay was performed using fluorescence experiments to characterize the substrates of BhOMT. In order to investigate the substrate preference depending on the metal cofactor, fluorescence quenching experiment was performed on BhOMT at different concentrations of divalent cations.[13] These results provide a strategy to investigate the interactions as well as metal dependency between substrates and BhOMT.

Methods

Chemicals. The flavonoids were purchased from Sigma Chemical Co. and INDOFINE Chemical Company, Inc., and deuterated dimethyl sulfoxide (DMSO-d_6) and deuterium oxide were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.).

Cloning of BhOMT. The mdmC gene, encoding a *O*-methyltransferase was amplified from *Bacillus haloduras* C-125 genomic DNA. The sense primer 5'-cggtacatcgtctgaacgct-3' and the antisense primer 5'-tttttctccagcttggggtct-3' were designed based on the sequence of Genbank accession number 57596592. At the 5'-end of each primer, a restriction site (NdeI site for sense primer and BamHI site for antisense primer) was attached to facilitate the cloning. PCR was performed under the following condition: 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1min at 72 °C. The resulting PCR product was sequenced and cloned into NdeI and BamHI site of pET-15b vector (Novagen, Madison, WI, USA). The ligation mixture was transformed to *E. coli* DH5α competent cell.

Expression and Purification of BhOMT. The pET-15b/BhOMT plasmid was brought into the expression host *E. coli* BL21 (DE3). Transformed cells were grown on Luria-Bertani (LB) agar plates containing 50 μg/mL ampicillin. SDS-PAGE analysis was used to select a colony for over-

expression of BhOMT with an *N*-terminal polyhistidin tag. One colony was used to inoculate 50 mL of LB medium with 50 μg/mL ampicillin, and grown overnight in 37 °C shaking incubator. 10 mL of the fully grown culture was mixed with 1-liter of fresh LB medium with 50 μg/mL, and grown at 37 °C until optical density reached 1.0 at 600 nm. The culture was induced with 1 mM IPTG and was grown 5 more hours at 30 °C.[15] The cells were harvested and the cell pellet was stored at −80 °C. All lysis and purification processes were carried out at 4 °C. A frozen pellet was resuspended and lysed by ultrasonication in buffer A containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl buffer. The cell lysate was centrifuged and the supernatant was loaded onto a Hitrap chelating column (GE Healthcare) that had been preequilibrated with buffer A. The column was washed with buffer A and then the bound material eluted with linear gradient from 0 to 600 mM imidazole. The BhOMT-containing fraction were pooled and concentrated with a AmiconUltra (Millipore) by exchanging buffer B, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 50 mM NaCl (pH 7.5).[16] At each stage of purification SDS-PAGE analysis was used to identify BhOMT containing fraction.

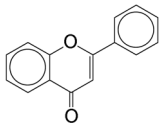
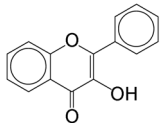
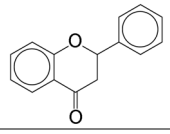
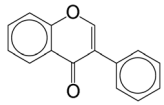
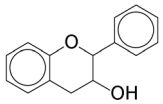
Fluorescence Analysis. Experiments were performed at 25 °C on a RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). BhOMT (10 μM) was dissolved in 25 mM HEPES buffer including 50 mM NaCl, 2 μM $CaCl_2$ and 20 mM SAM. Each flavonoid was titrated to a final protein at a protein:ligand mole ratio of 1:10. The sample was contained in a 2 mL thermostated cuvette with excitation and emission path lengths of 10 mm. Samples were excited at 290 nm, and emission spectra were recorded for light scattering effects from 290 to 500 nm.[17] We estimated K_d using the following equation:[18]

$$\log\left(\frac{F_0 - F}{F}\right) = \log\frac{1}{K_d} + n \log [inhibitor]$$

F_0 and F represent fluorescence intensity from BhOMT at 342 nm in the absence and presence of inhibitor, respectively, while n is the number of inhibitor binding sites on the protein.

Metal-dependent Fluorescence Quenching. Purified BhOMT was dialyzed against 25 mM HEPES, 50 mM NaCl, and 5 mM EDTA for 12 h. The protein was then concentrated approximately 5 mg/mL. To determine the metal dependency of BhOMT, the reaction mixtures were made in 2 mL volumes containing 25 mM HEPES, 50 mM NaCl (pH 7.5), 20 uM SAM, 10 uM BhOMT with 0.0-2.0 mM divalent cations.[16] The reaction mixtures were incubated at 37 °C for 30 min.

Table 1. The docking energy and binding constant between flavonoids and BhOMT

Class of Flavonoid		Substituents of 3'- and 4'-position		Binding Constatnt (M^{-1})	Docking Energy (kcal/mol)
		3'	4'		
Flavones	Chrysin	H	H	6.9	3.14
	Baicalein	H	H	2.7×10^5	3.32
	Apigenin	H	OH	3.7×10^3	−12.25
	Luteolin	OH	OH	4.2×10^7	−23.52
Flavonols	Kaempferol	H	OH	1.6×10^3	−11.99
	Quercetin	OH	OH	8.7×10^7	−25.12
	Myricetin	OH	OH	6.2×10^7	−19.11
	Fisetin	OH	OH	3.1×10^7	−27.34
	Morin	H	OH	4.3×10^5	−7.81
Flavanones	Naringenin	H	OH	4.8×10^4	−10.32
	Eriodictyol	OH	OH	3.9×10^6	5.90
	Taxifolin	OH	OH	8.7×10^6	2.72
Isoflavones					
	Genistein	H	OH	4.8×10^4	12.55
Flavan-3-ols					
	Catechin	OH	OH	8.9×10^2	−2.26

Results and Discussion

BhOMT was subcloned into *E. coli* expression vector pET-15b and expressed in *E. coli* BL21 (DE3) cells and the yield of BhOMT was about 20 mg per liter in LB broth. The BhOMT contains four tryptophan residues (positions 7, 27, 75, and 152). Fluorescence quenching experiment was performed to estimate the binding constant by monitoring Trp75 near the substrate binding site of BhOMT.[18,19]

In our previous docking study, we concluded that hydroxyl group at both 3' and 4' positions, and structural rigidity of C-ring resulting from the double bond characters between C2 and C3 positions are important factors for substrates of BhOMT.[14] Docking energy of luteolin, fisetin, myricetin, and quercetin to BhOMT were very low as listed in Table 1. Changes in the fluorescence intensity with increase of concentration of flavonoids are attributed to the formation of a complex of protein and flavonoids. As listed in Table 1, the binding affinity of luteolin, fisetin, myricetin, and quercetin to BhOMT were very high, $4.2 \times 10^7\ M^{-1}$, $3.1 \times 10^7\ M^{-1}$, $6.2 \times 10^7\ M^{-1}$, $8.7 \times 10^7\ M^{-1}$, respectively. Results from the binding assay in the present study agreed well with our previous docking study. Naringenin, apigenin, and kaempferol which were deficient in a double bond at C2-C3 position of C-ring or OH group at 3' position showed medium binding affinity, 10^3~10^4.

Table 2. The extent of fluorescence quenching of BhOMT by binding of quercetin in the presence of Ca^{2+} or Mg^{2+}

	% of fluorescence quenching referenced to the value with 2 mM cation
BhOMT + 5 mM EDTA	53
BhOMT + 0.5 mM Ca^{2+}	93
BhOMT + 1 mM Ca^{2+}	100
BhOMT + 2 mM Ca^{2+}	100
BhOMT + 0.5 mM Mg^{2+}	94
BhOMT + 1 mM Mg^{2+}	100
BhOMT + 2 mM Mg^{2+}	100

Crystal structure of CCoAOMT revealed that calcium ion is involved in its O-methyltransfer catlaysis.[17] We expected

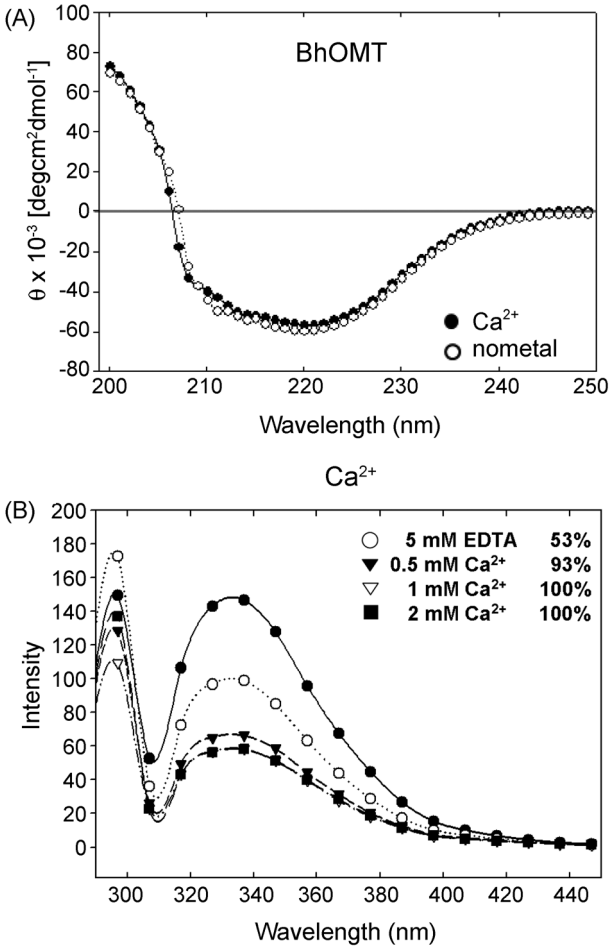


Figure 1. Metal dependencies of BhOMT. (A) CD spectra of BhOMT in the presence of Ca^{2+} (●) and in the absence of Ca^{2+} (○). (B) Fluorescence spectra of BhOMT in the presence of Ca^{2+}. Samples were excited at 290 nm, and emission spectra were recorded for light scattering effects from 290 to 450 nm. All assays were performed in the presence of 20 uM SAM and 80 uM quercetin. Amount of metals are indicated as follows: no metal (●), 5 mM EDTA (○), 0.5 mM (▼), 1 mM (▽), and 2 mM (■).

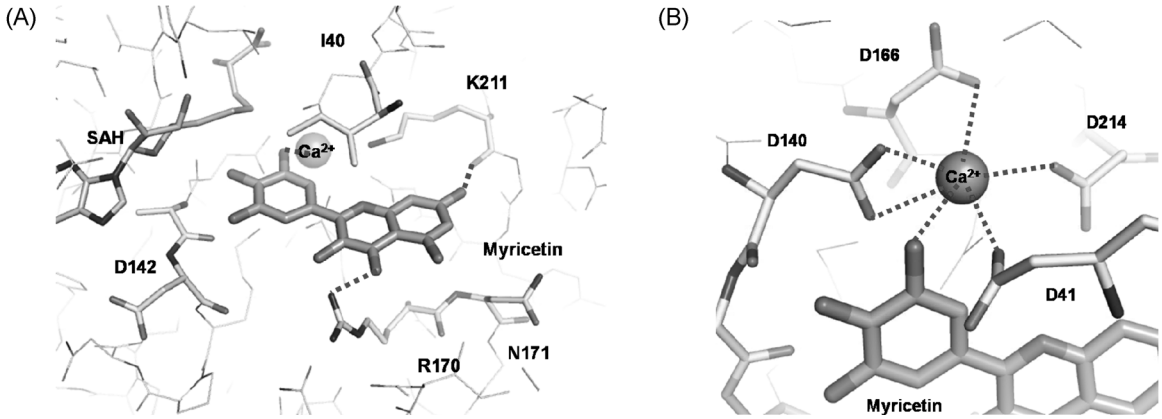


Figure 2. Docking model of myricetin and BhOMT. The ligand binding site includes a $Ca^{2+}$and SAH. Hydrogen bondings are denoted by dotted lines. (A) Docking model of myricetin and BhOMT. (B) Calcium mediated electrostatic interactions of BhOMT.

that these four flavonoids with lowest docking energy should have strong metal-mediated electrostatic interactions with BhOMT.[14] In order to investigate the metal dependency of BhOMT, the extent of fluorescence quenching of BhOMT was examined by stripping the metal ion from BhOMT with EDTA and then replacing with the putative Ca^{2+} or Mg^{2+}. As listed in Table 2, the binding affinity of quercetin to BhOMT was not influenced by the type of metal ions, but influenced by the amount of metal. As shown in CD spectrum (Figure 1A), the overall structure of BhOMT was not affected by existence of metal ions. However, the extent of fluorescence quenching of BhOMT was significantly low in the absence of divalent cations, while the extent of fluorescence quenching by the binding of quercetin to BhOMT was 100% in the presence of Ca^{2+} higher than 1 mM (Figure 1B).

Figure 2 shows the docking model of myricetin and BhOMT and the Ca^{2+} mediated electrostatic interaction between myricetin and BhOMT. As shown in Figure 2B, the Ca^{2+} ion is coordinated with carboxyl oxygens of the side chains of four aspartic acids, D41, D140, D166, and D214. Hydroxyl group at 3'-position in the B-ring of myricetin also participated in this metal mediated electrostatic interaction. Hydroxyl group at 7-position of luteolin, quercetin, fisetin, and myricetin forms a stable hydrogen bonding with K211 and carboxyl oxygen of C-ring forms a stable hydrogen bonding with R170. From these results, we confirmed that interactions between flavonoid and BhOMT are enhanced by metal-mediated electrostatic interactions in substrate binding site of BhOMT. Therefore, fluorescence quenching experiments confirmed successfully the results of docking study of BhOMT.

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