3D Structure of *Bacillus halodurans O*-Methyltransferase, a Novel Bacterial *O*-Methyltransferase by Comparative Homology Modeling

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Bacillus halodurans O-methyltransferase (BhOMT) is a S-adenosylmethionine (SAM or AdoMet) dependent methyltransferase. Three dimensional structure of the BhOMT bound to S-adenosyl-L-homocysteine (SAH or AdoHcy) has been determined by comparative homology modeling. BhOMT has 40% sequence identity with caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) from alfalfa. Based on x-ray structure of CCoAOMT, three dimensional structure of BhOMT was determined using MODELLER. The substrate binding sites of these two proteins showed slight differences, but these differences were important to characterize the substrate of BhOMT. 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. These flavonoids form stable hydrogen bondings with K211, R170, and hydroxyl group at 3'-position in the B-ring has stable electrostatic interaction with Ca^{2+} ion in BhOMT. This study will be helpful to understand the biochemical function of BhOMT as an *O*-methyltransferase for flavonoids.

Key Words : Methyltransferase, Flavonoids, Homology modeling, *In silico* screening, Docking study

Introduction

The transfer of methyl groups from S-adenosyl-L-methionine (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 S-adenosyl-L-methionine (AdoMet) dependent *O*-methyltransferases (OMTs) (EC 2.1.1) is a common modification in natural product biosynthesis.[3-5] Today, more than 100 methyltransferases (MTs) have been identified, but it is estimated that hundreds of MTs remain to be discovered. Identification of new MTs is an important study to decipher a variety of actions.

Over the past two decades, the studies of bacteria have been focused on three main areas: Enzymology, physiology and molecular genetics of alkaliphilic microorganisms to clarify their mechanisms of adaptation to alkaline environments, particularly *Bacillus* strains.[6-8] *Bacillus halodurans* (*B. halodurans*) is the second *Bacillus* species. *B. halodurans O*-methyltransferase (BhOMT) was identified six years ago, but its three dimensional structure and function have not been reported yet.[9] BhOMT is composed of 223 residues and has about 40% sequence identity with caffeoyl-CoA 3-*O*-methyltransferase from alfalfa. Caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) has been isolated and characterized from a number of different plants including alfalfa, and is a SAM dependent *O*-methyltranferase involved in lignin biosynthesis.[10,11] Lignin and etherified phenolic compounds provide structural support for the cell walls of conducting tissues and treachery elements and were critical in the evolution of higher plants from their aquatic progenitors.[10] CCoAOMT has been shown to play an important role in lignin biosynthesis. Immunolocalization and tissue printing studies in various plant species have shown that the expression of CCoAOMT is highly correlated with lignifying tissues.

CCoAOMT methylates caffeoyl and 5-hydroxyferuloyl CoA thioesters with an *in vitro* kinetic preference for caffeoyl CoA as shown in Figure 1. CCoAOMT contains a well-ordered active site and a divalent metal-binding site, which is suggestive of a metal dependent catalytic mechanism. Particularly, a calcium ion is observed in the active site surrounded by an octahedral arrangement of hydroxyl and carboxyl ligands. The crystal structure of alfalfa (Medicago sativa) CCoAOMT is known as a complex with the reaction products S-adenosine-L-homocystein (SAH or AdoHcy), feruloyl CoA and a calcium ion.[10] The structure of BhOMT is expected to be similar to that of CCoAOMT, because the amino acid sequence identity is about 40%.

The role of *O*-methyltransferase in microorgarnisms is not well known yet. However, it is well known that many bacterial methyltransferase are involved in antibiotics biosynthesis, as well as in the methylation of other compounds. In this study, three dimensional structure of BhOMT was determined by comparative homology modeling. The crystal structure of alfalfa CCoAOMT was used as a template

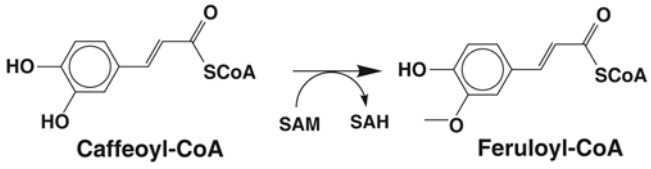


Figure 1. CCoAOMT catalyzed methylation reaction.

protein for homology modeling. By comparison of active sites of these two proteins, we characterized the structural feature and substrate binding model of BhOMT.

One of the largest classes of polyphenolic compounds are the flavonoids. This group of plant pigments is largely responsible for the colors of many fruits and flowers, and over 3,000 flavonoids have been characterized and classified according to the chemical structure. Many flavonoids have low toxicity in mammals and some of them are widely used in medicine for several diseases. Flavonoids are often hydroxylated in position 3, 5, 7, 2', 3', 4', and 5'.[12] Here, we collected twenty-two naturally-occurring flavonoid based on the subclass of flavonoids and BhOMT substrate was characterized by docking study.

Methods

Template Proteins. The amino acid sequences of the BhOMT was retrieved from Expasy[13]. Alfalfa caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) was used as a template protein, and deposited in Protein Data Bank at the 2.7 Å resolution (PDB entry: 1SUS.pdb). 1SUS is a complex structure with SAH and feruloyl-CoA. The three dimensional structure of SAH was adapted from template protein, CCoAOMT.

Comparative Homology Modeling. We determined the three dimensional structure of BhOMT using comparative homology modeling based on the x-ray structure of CCoAOMT from alfalfa. The structurally conserved regions (SCRs) were determined by pairwise sequence alignment with the Insight/Homology module as shown in Figure 2. For a given alignment, five comparative models of the target sequence were built by MODELLER,[14] applying the default model building routine 'model' with fast refinement. This procedure is advantageous because one can select the best model from several candidates. Furthermore, the variability among the models can be used to evaluate the reliability of the modeling.[15,16] Energy minimization (steepest descent and conjugated gradient algorithms; gradient on energies less than 1 kcal/mol used as convergence criteria) and molecular dynamics (MD) simulations at 300 K were performed using the consistent valence force field. These processes were performed using the program InsightII/Discover. The qualities of these models were analyzed by PROCHECK.[17] These molecular modeling procedures were carried out on an OCTANE R12000 Silicon Graphics workstation.

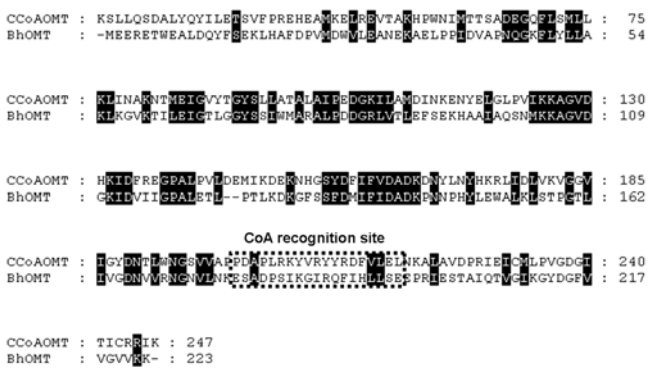


Figure 2. Sequence alignment of template protein (CCoAOMT) and target protein (BhOMT). Highly conserved residues between CCoAOMT and BhOMT families are depicted in black. CoA recognition site was marked with dotted lines and substrate binding site was marked with black lines.

Ligands Docking. Twenty-two naturally-occurring flavonoids were collected considering subclass of flavonoids (flavones, flavonols, flavonones, isoflavones and flavan-3-ols). Nomenclature of flavonoids is listed in Table 3. Docking study was performed for these flavonoids and BhOMT determined by comparative homology modeling in order to find specific ligand of BhOMT using AutoDock.[18] The Lamarckian Genetic Algorithm (LGA) of the AutoDock 3.05 was used for docking experiments. Distance-dependent function of the dielectric constant was used for the calculation of the energetic maps and all other parameters were used by default value. MD simulations[19] were performed for the final docking structures in the canonical ensemble (NVT) at 300 K using the program InsightII/Discover. All atoms of the system were considered explicitly, and their interactions were computed using the consistent valence force field. A distance cutoff of 10 Å was used for van der Waals interactions and electrostatic interactions. The time step in the MD simulations was 1 fs and MD simulation was performed for 2ns. Coordinates were saved every 1 ps.

Results and Discussion

Five models of BhOMT were generated by MODELLER and optimized using energy minimization and MD simulation. Energy and RMSD for five BhOMT models were listed in Table 1. Template protein structure was represented in Figure 3(A), and the lowest energy model of BhOMT was represented in Figure 3(B) and structural alignment of BhOMT and CCoAOMT is shown in Figure 3(C). Three dimensional structures of structurally conserved regions (SCRs) were very similar to each five models of BhOMT. In order to select the best model, we checked the structural validity of BhOMT by PROCHECK. The torsion angles (φ and ψ) of 85.3% in the generated models had values within the most favored regions and only 0.5% of the residues had values within disallowed regions. The overall G-factor which is a measure of the overall normality of the structure is 0.42.[20] Low G-factors indicate that residues have unlikely conformations. The overall value is obtained from an aver-

Table 1. RMSD and energies of five BhOMTs predicted by MODELLER

	RMSD with CCoAOMT	Energy (kcal)
BhOMT1	0.13219	1021.52
BhOMT2	0.19270	1142.40
BhOMT3	0.16218	1076.77
BhOMT4	0.21371	1090.13
BhOMT5	0.25617	1095.33

Table 2. Residues on ligand binding site of BhOMT and CCoAOMT

	Site	CCoAOMT	BhOMT
Residues	Metal binding site	Thr63	Val42
		Glu67	Gln46
		Asp163	Asp140
		Asp189	Asp166
		Asn190	Asn167
	Substrate binding site	Met61	Ile40
		Gly138	Gly171
		Asp165	Asp142
		Trp193	Arg170
		Asn194	Asn171
		Arg206	Ser183
		Tyr208	Lys185
		Tyr212	Gln189

age of G-factors for all residues in the structure. X-ray structure of CCoAOMT has a resolution of 2.7 Å and a G-factor of 0.31. In Ramachandran plot, the stereochemical quality of a protein model can be judged by the use of φ, ψ scatter plots, with incorrect structures generally having a much larger fraction of residues lying in disallowed regions.[21]

The overall structure of BhOMT was very similar to that of CCoAOMT. The structural difference between BhOMT and CCoAOMT appears only at loop region, which is a CoA recognition site as denoted by dotted lines in Figure 2 and Figure 4. SAH (or SAM) binding site of two proteins is highly conserved while the CoA recognition site showed differences, resulting from poor sequence identity (less than 10%) of these regions in BhOMT and CCoAOMT. Sequence identity of CoA recognition site of BhOMT and CCoAOMT was very low, but the important residues in CCoAOMT are similar to those in BhOMT, such as Asn194 (CCoAOMT)/ Asn171 (BhOMT).

Ligand binding site of CCoAOMT is divided into two important regions, one is metal binding site and the other is substrate binding site. As listed in Table 2, the side chain oxygens of Thr63, Glu67, Asp163, Asp189, and Asn190 in CCoAOMT are involved in the chelation of Ca^{2+}, with the coordination complex completed by the 3-methoxy oxygen atom of feruloyl-CoA and a water molecule. The residues in BhOMT corresponding to these residues in CCoAOMT are Gln46, Asp140, Asp166, and Asn167.

In Table 2, we compared the residues in the substrate binding site between these two OMTs. Three aromatic residues on CCoAOMT including Trp193, Tyr208 and Tyr212 sequester phenyl ring of substrate. Met61, Asp165, and Asn194 complete the rest of the substrate-binding pocket. In the case of BhOMT, three aromatic residues were substituted with charged residues, such as Arg170, Lys185, and Gln189. The possibility to have hydrogen bond interactions was increased, while the ability to have hydrophobic interactions was decreased. This is the main difference between the substrate binding of BhOMT and CCoAOMT. The surface models of ligand binding site of two OMTs are shown in Figure 4. As shown in Figure 4(A), feruloyl CoA is the substrate of CCoAOMT and hydrophobic interactions are important in substrate binding site of CCoAOMT. As shown Figure 4(B), hydrophilic residues in substrate binding sites of BhOMT are depicted in blue color.

Since there are differences in substrate binding sites of these two proteins, we carried out ligand docking study using AutoDock to find the possible ligands of BhOMT. Twenty-two standard and well known flavonoids were selected for the docking study.[12] Only four flavonoids out of 24 flavonoids, such as quercetin, fisetin, myricetin, and luteolin were well docked in ligand binding site of BhOMT with low energy. Docking energy between twenty-two flavonoids and BhOMT is shown in Table 3. Docking energy of these four flavonoids ranked more than 6 kcal/mol lower than the rest of the flavonoids. Molecular dynamics simulation was performed for docking models in order to determine the optimal structures. Docking structures of BhOMT and flavonoids represented in Figure 5. It is known that in metal dependent methyltranferase, 3',4'-dihydroxyl flavonoids, such as quercetin, has electrostatic interaction with metal ion in active site. As shown in Figure 5, 3'-OH of flavonoids has a stable electrostatic interaction with Ca^{2+} ion. By this stable electrostatic interaction, flavonoids occupy an advantageous

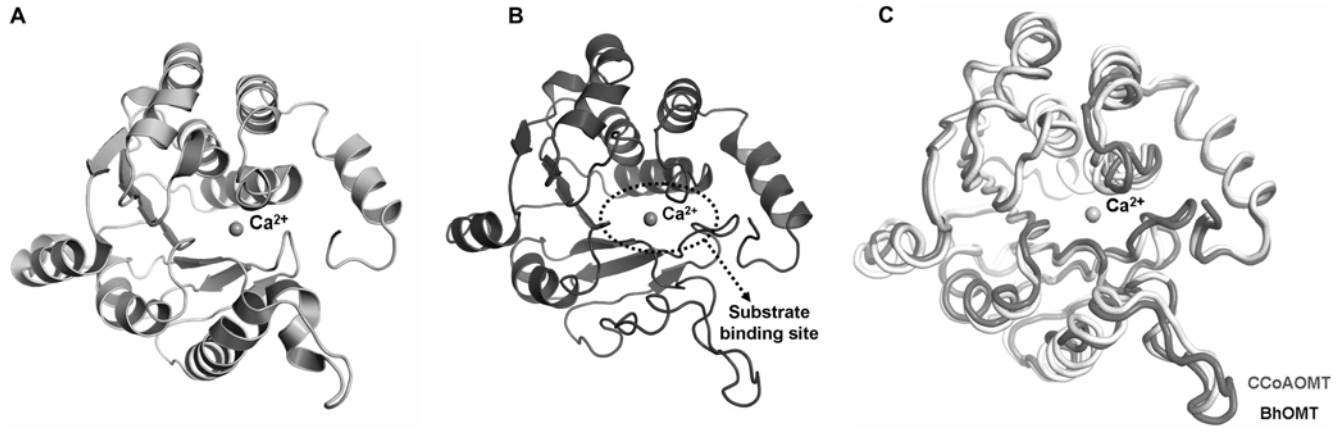


Figure 3. 3D Structure of BhOMT. (A) X-ray structure of CCoAOMT. (B) The lowest energy model of BhCOMT. (C) Structural alignment of BhOMT and CCoAOMT.

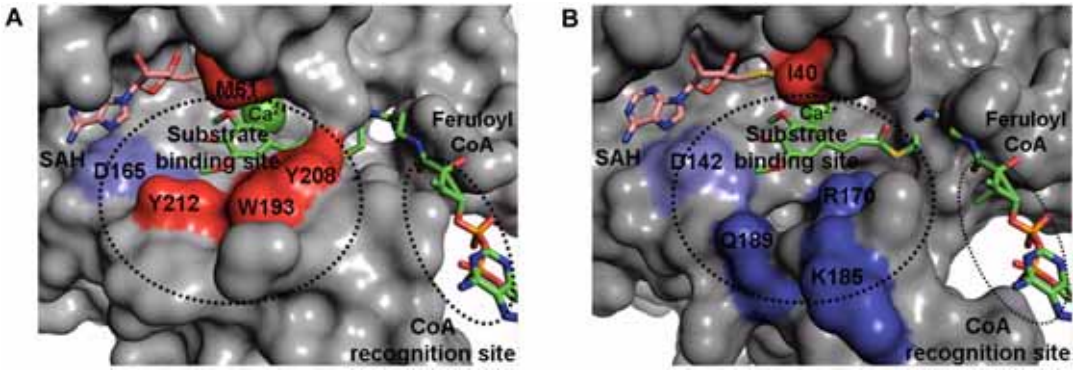
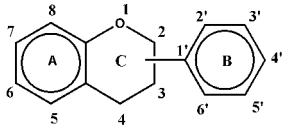


Figure 4. Representation of docking model of OMTs and substrates. (A) Surface model of ligand binding site of CCoAOMT. (B) Surface model of ligand binding site of BhOMT. The ligand binding site includes a Ca^{2+}, SAH and feruloyl CoA.

Table 3. Classification of flavonoids based on the position of their substituents and docking energy between twenty-two flavonoids and BhOMT



Class of Flavonoids		Position of Substituents							Docking Energy (kcal/mol)
		3	5	7	2'	3'	4'	5'	
Flavones	Chrysin	H	OH	OH	H	H	H	H	3.14
	Techtochrysin	H	O-Me	OH	H	H	H	H	4.01
	Baicalein	H	OH	OH	H	H	H	H	3.32
	Apigenin	H	OH	OH	H	H	OH	H	−12.25
	Luteolin	H	OH	OH	H	OH	OH	H	−23.52
	Diosmetin	H	OH	OH	H	OH	O-Me	H	−2.71
	Tangeretin	H	O-Me	O-Me	H	H	O-Me	H	5.55
Flavonols	Galangin	OH	OH	OH	H	H	H	H	−7.25
	Kaempferol	OH	OH	OH	H	H	OH	H	−11.99
	Quercetin	OH	OH	OH	H	OH	OH	H	−25.12
	Myricetin	OH	OH	OH	H	OH	OH	OH	−19.11
	Fisetin	OH	H	OH	H	OH	OH	H	−27.34
	Morin	OH	OH	OH	OH	H	OH	H	−7.81
	Kaempferide	OH	OH	OH	H	H	OMe	H	3.78
	Rhamnetin	OH	OH	O-Me	H	OH	OH	H	−12.64
Flavonones	Pinocembrin	H	OH	OH	H	H	H	H	−1.74
	Naringenin	H	OH	OH	H	H	OH	H	−10.32
	Eriodictyol	H	OH	OH	H	OH	OH	H	5.90
	Hesperitin	H	OH	OH	H	OH	O-Me	H	−10.51
Isoflavones	Genistein	–	OH	OH	H	H	OH	H	12.55
	Genistin	–	OH	O-Glu	H	H	OH	H	18.43
	Daidzin	–	H	O-Glu	H	H	OH	H	16.12
Flavan-3-ols	Catechin	OH	OH	OH	H	OH	OH	H	−2.26
	Gallocatechin	OH	OH	OH	H	OH	OH	OH	3.78

-O-Me = Methoxy -O-Glu = Glucosyl -O-R'=Alkoxy

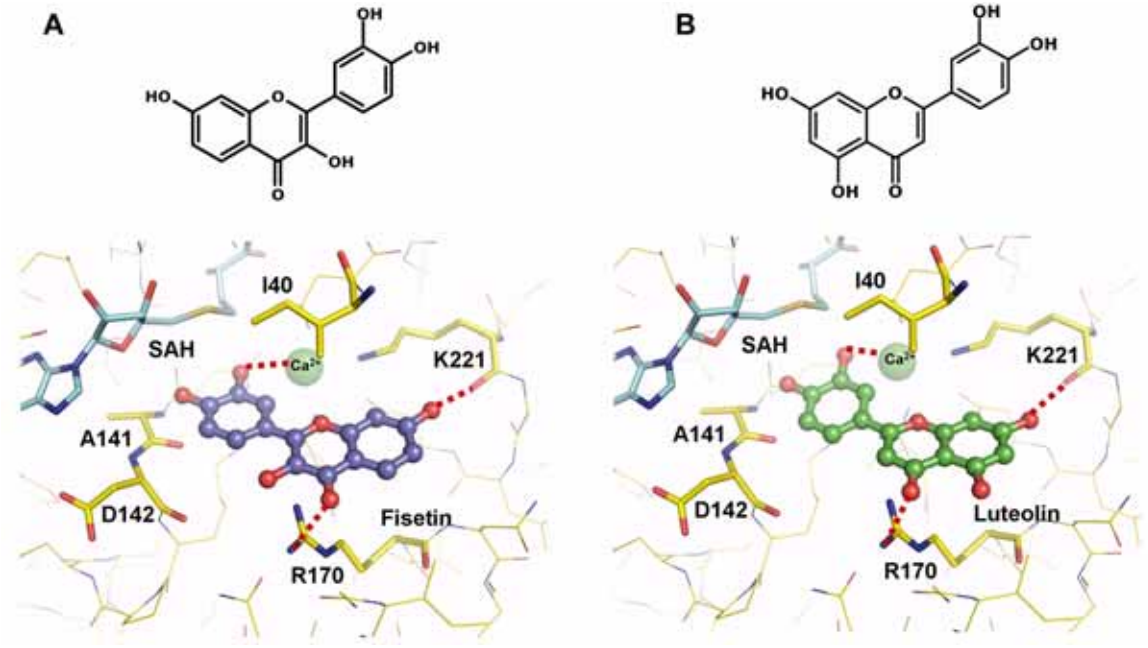


Figure 5. Docking models of flavonoid and BhOMT. (A) Docking model of fisetin and BhOMT. (B) Docking model of luteolin and BhOMT.

position for accepting a methyl group from SAM at 3'- or 4'-OH. There is a hydrogen bond between backbone carbonyl oxygen of K211 in BhOMT and 7-OH of these four flavonoids, and one between the amide side chain of R170 and carboxyl oxygen of flavonoids. Particularly, R170 was a residue which was substituted for aromatic amino acid in CCoAOMT. It can be proposed that these hydrogen bonds participate in stabilization of docking structure. Therefore, we can propose that hydrophilic interaction with the substrate is more important in BhOMT than in CCoAOMT. Four flavonoids have several common features. For example, they have two hydroxyl groups on 3'- and 4'-position, and a double bond on C2-C3 of C-ring. These common features favored those hydrogen bonding interactions with BhOMT because of two hydroxyl groups at 3' and 4' positions were conducive to make strong hydrogen bonds with BhOMT and structural rigidity of C-ring resulting from the double bond character between C2 and C3 position make it possible for the carboxyl oxygen at C4 to form stable hydrogen bonds with R170 in BhOMT. In Table 3, other group of flavonoids which have only one hydroxyl group at 4' position, such as apigenin, kaempferol, and naringenin, was deficient in hydrogen bonds to bind to BhOMT compared with four lowest energy flavonoids. Therefore, these results imply that hydrogen bonds play an important role for ligand to bind to BhOMT. Another group of flavonoids such as rhamnetin and hesperitin which have methoxy groups at 3' or 4' position can not form a stable hydrogen bond with BhOMT and metal ion. Docking models of fisetin and luteolin, with BhOMT that had lowest docking energy are shown in Figure 5. Two hydrogen bonds with BhOMT and electrostatic interaction with Ca^{2+} ion are depicted in this figure. Therefore, docking study showed clearly which flavonoids can be the candidates of substrates for BhOMT.

The role of *O*-methyltransferase in microorganisms is not well known yet. However, it is well known that many bacterial methyltransferase are involved in antibiotics biosynthesis as well as in the methylation of other compounds.[22] Flavonoids can be the substrate for the *O*-methyltransferase. According to recent research on *Streptomyces coelicolo* A3, *O*-methyltransferase also favors ortho-dihydroxyl flavones as substrates.[23] We can propose that flavonols such as quercetin, fisetin, and myricetin, and flavone such as luteolin may act as substrates of BhOMT and may play important roles in biochemical functions in BhOMT. Further study will be performed to prove these possibilities.

Conclusion

Three dimensional structure of *Bacillus halodurans O*-methyltransferase (BhOMT) was determined by comparative homology modeling. The x-ray structure of alfalfa caffeoyl 3-*O*-methyltransferase (CCoAOMT) was used as a template protein. We evaluated the structural validity of BhCOMT by PROCHECK and we confirmed three dimensional structure of BhOMT was reasonable. By using structural and sequential analysis, we confirmed that the three dimensional structure of these two OMTs were very similar. In particular, metal binding site of these two OMTs were identical and substrate binding site showed small difference resulting from the poor sequence identity and structural flexibility. Substrate binding site of CCoAOMT consists of several aromatic residues, while that of BhOMT consists of charged residues. On the other hand, ligand binding site of BhOMT showed stronger hydrophilic environment than that of CCoAOMT. This implies that the substrate of two proteins

might be very different. Four flavonoids such as quercetin, fisetin, myricetin, and luteolin were docked well into the ligand binding site of BhOMT and have much lower docking energies than the other flavonoids. These four flavonoids have stable hydrogen bondings with K211 and R170, and have stable electrostatic interaction with Ca^{2+} ion in BhOMT. Common features such as hydroxyl groups at both 3' and 4' positions, and structural rigidity of C-ring resulting from the double bond characters between C2 and C3 positions are characterized as important factors for substrates of BhOMT and strong hydrogen bonds between ligands and BhOMT are necessary. Further study will be carried out in order to understand the biochemical function of BhOMT as an *O*-methyltransferase for these flavonoids.

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