Molecular Modeling and Site Directed Mutagenesis of the *O*-Methyltransferase, SOMT-9 Reveal Amino Acids Important for Its Reaction and Regioselectivity

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SOMT-9 is an *O*-methyltransferase that utilizes quercetin to produce 3'-methoxy quercetin. In order to determine which amino acids of SOMT-9 are important for this reaction and its regioselectivity, molecular docking experiments followed by site directed mutagenesis were performed. Molecular modeling and molecular docking experiments identified several amino acid residues involved in metal binding, AdoMet binding, and substrate binding. Site-directed mutagenesis showed that Asp188 is critical for metal binding and that Lys165 assists other metal binding residues in maintaining quercetin in the proper position during the reaction. In addition, Tyr207 was shown to play an important role in the determination of the regioselectivity and Met60 was shown to be involved in formation of the hydrophobic pocket necessary for substrate binding. The molecular modeling and docking experiments discussed in this study could be applicable to future research including prediction of substrate binding and regioselectivity of an enzyme.

Key Words : *O*-Methyltransferase, Molecular modeling, Quercetin, Site-directed mutagenesis

Introduction

Enzymatic *O*-methylation is catalyzed by *O*-methyltransferases (OMTs) and involves the transfer of the methyl group of *S*-adenosyl-*L*-methionine (AdoMet) to the hydroxyl group of an acceptor molecule, resulting in the formation of a methyl ether derivative and *S*-adenosyl-*L*-homocysteine (AdoHcy) as products. Plant OMTs can be divided into two classes based on the primary amino acid sequence and substrate specificity.[1,2] The class I plant OMTs represented by a caffeoyl CoA OMT (CCoAOMT) have a low molecular weight (around 28-kDa) and exhibit specificity for CoA thioesters such as caffeoyl-CoA, ferulic CoA, and sinapic CoA, all of which are precursors for the biosynthesis of lignin. The class II plant OMTs have higher molecular weight (approximately 40-kDa) and include caffeic acid OMT (COMT) and OMTs that methylate flavonoids, alkaloids, and other secondary metabolites. Recently, a new class of OMTs that have a low molecular weight but utilize both caffeoyl-CoA and flavonoids has been identified.[3] These OMTs have been used for the regioselective syntheses of flavonoids and other chemicals.[4-8] Genome projects from various organisms including plants and microorganisms have identified various OMTs. Although quite often the amino acid sequence homology among OMTs did not provide the actual substrate and the position of methylation, characterization of individual genes must be carried out one by one. Structural analysis of several OMTs including caffeic acid OMT (COMT),[9] isoflavone OMT IOMT),[10] and caffeoyl-CoA OMT (CCoAOMT)[11] provides a faster way to predict substrate and regioselectivity of OMTs through molecular modeling studies. However, while molecular modelings of OMTs have been carried out,[12-14] evaluation of modeled structures by site-directed mutagenesis has not yet been performed.

SOMT-9 is an OMT from soybean that utilizes several flavonoids containing vincinal dihydroxyl groups.[15] In order to determine the amino acids important for this reaction and its regioselectivity, molecular modeling of SOMT-9 was conducted using CCoAOMT as a template for identifying important amino acids. Here we report the identity of several metal binding amino acid residues that are critical for the enzymatic reaction of SOMT-9 and we also show that the amino acid Tyr207 has an essential role in determining the regioselectivity of SOMT-9.

Methods

Molecular modeling of SOMT-9. The structure of SOMT-9 was constructed using Modeller 8.2 (Accelrys, San Diego, USA). Alfalfa CCoAOMT (PDB accession no. 1SUS.pdb) was chosen as a template for molecular modeling. To refine the 3D structure of SOMT-9, energy minimization and molecular dynamics were performed. The protein was embedded in a 5 Å shell of 1271 water molecules. For energy minimization, the force field was cvff provided by Accelys, and the steepest descents were performed until a maximum derivative of 0.001 kcal/molÅ was reached. Following energy minimization, molecular dynamics were performed using a computational Grid system (http://www.mgrid.or.kr) at 300 K, 1 atm for 1ns with 1 fs each step. Output conformers were collected at every 400 fs. The energy profile was analyzed using Analysis/InsightII. Of a total of 2500 conformers, 20 conformers with every 50 ps were chosen. The selected conformers were then superimposed, and the resulting structures showed a root mean square derivation (RMSD) of 0.8 Å. Of the 20 conformers, one showing the lowest total energy was evaluated by

Table 1. Primers used for site-directed mutagenesis of SOMT-9 and relative activity of the mutants

Mutations		Relative activity (%)
M60L	CCACTGAACATCctgGCAACACCAGC	102 ± 5
M60G	CCACTGAACATCgggGCAACACCAG	62 ± 2
D162G	TTCGTGggtGCTGATAAGGACAAT	95 ± 2
K165L	TTTCGTGGATGCTGATctgGACAATTACTTGAA	7 ± 2
D188G	TGATCGGATACggtAACACCCTATG	0
N189I	TGATCGGATACGATatcACCCTATGGGCT	0
Y207G	CCATTGATGGATggcATTAAGCCTCTT	115 ± 2

*Mutagenized positions are shown in lower-cases.

PROCHECK.[16] The substrate used for the docking study was built using Sybyl 7.2 (Tripos, St.Louis, MO) on a Pentium IV 3.2 Ghz Linux PC. A Gasteiger-Huckel charge was given to the substrate, which was then subjected to energy minimization by the conjugate gradient algorithm using a Tripos force field. The minimization process was forced to stop either when the iteration number reached 1,000 or when the convergence criteria were met (maximum root mean square gradient 0.05 kcal/mol). AdoMet was extracted from CCoAOMT and attached to the SOMT-9 protein using the conformation of AdoMet in CCoAOMT as a template. All docking experiments were performed using the FlexX module (Tripos).

Site directed mutagenesis and expression of the mutant in *E. coli*. Site directed mutagenesis was performed using QuickChange II XL Site-directed Mutagenesis Kit (Stratagene) according to manufactures' instructions. The primers used for mutagenesis are listed in Table 1. Mutagenesis was confirmed by DNA sequencing.

Transformed cells containing wild type or mutant SOMT-9 were grown in LB medium containing 50 μg/mL ampicillin. Cultures were grown until the absorbance at 600 nm reached 0.6. At this point, IPTG was added at the final concentration 0.1 mM, and the culture was incubated for an additional 5 hrs at 28 °C. Cells were harvested and washed with PBS buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), resuspended in the same buffer, and then lysed by sonication. The expressed protein was purified using a GST affinity chromatography column.

Enzyme assay. Purified wild type or mutant SOMT-9 (50 μg) was incubated with 60 μM SAM and 50 μM substrate at 37 °C for 1 h and then extracted twice with ethyl acetate and the ethyl acetate was evaporated completely. The evaporated samples were dissolved in dimethylsulfoxide and analyzed by high performance liquid chromatography as described in Kim *et al.*[5]

Results and Discussion

SOMT-9 showed 84.3% similarity in amino acid to CCoAOMT from alfalfa and the structure of CCoAOMT was determined using X-ray chrystallography.[11] The structure of SOMT-9 was built using CCoAOMT (1SUS.pdb) of alfalfa as a template. As is the case with other OMTs, SOMT-9 exists as a homodimer.[9-11] However, in our molecular modeling experiments, a monomer of SOMT-9 was used. CCoAOMT uses calcium ion as a cofactor[9] but magnesium ion was as effective as calcium ion. The importance of the metal ion for the enzymatic activity of SOMT-9 was tested *in vitro*. Removal of metal ions from SOMT-9 by the addition of EDTA at the final concentration of 1 mM resulted in a complete loss of enzymatic activity. The loss of activity was recovered by the addition of magnesium, iron, or calcium at the final concentration of 3 mM to the reaction mixture (Table 2), indicating that metal ion is critical for the enzymatic reaction of SOMT-9. For *in silico* experiments, the magnesium ion was used for building the structure of SOMT-9. In addition, the crystal structure of CCoAOMT included AdoHcy instead of AdoMet, so that in the docking experiments of SOMT-9 and its ligands rather than AdoHcy, was embedded. Based on a superimposition of SOMT-9 obtained from the homology modeling on CCoAOMT, the position of the metal ion was determined. The residues nearest to the metal ion were the carboxyl groups of side chain from Asp162, Asp188, and Asp189 (Fig. 1), which are all conserved metal-binding amino acid residues. The substrates, AdoHcy and flavonoid, were docked to the SOMT-9 model using FlexX. Amino acid residues that showed interaction with AdoHcy are listed in Table 3.

Table 2. Effect of metal ion on quercetin methylation by SOMT-9

Metal	Relative activity (%)*
Mg^{2+}	120
Fe^{2+}	115
Ca^{2+}	120
EDTA(no metal)	3

*One mM of EDTA was added to the each enzyme and the mixture was incubated for 15 min. Then, 3 mM of metal was added to the mixture with substrates. Enzyme before addition of EDTA was served as a control.

Molecular docking experiments with quercetin revealed that two amino acids, Met60 and Tyr207, formed a hydrophobic pocket in which the A ring of quercetin fits (Fig. 2). Based on the docking experiment, Tyr207 appeared to play a role in locating 3'-hydroxyl group near the co-substrate AdoMet. The movement of the 4'-hydroxyl group toward AdoMet was hindered by Tyr207 as the residue overlapped with the A ring of quercetin. Thus, it is obvious that Tyr207 is critical for the regioselectivity of SOMT-9 (Fig. 3A). Additional residues that were found to interact with quer-

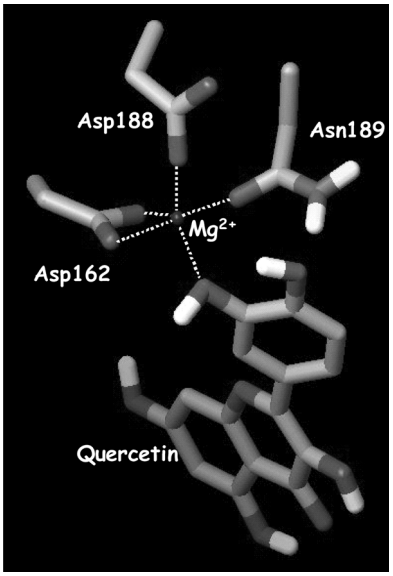

Figure 1. Metal binding amino acid residues of SOMT-9. The side chains of Aps162, Asp188, and Asp189 interact with Mg^{+2}. In addition, Mg^{+2} interacts with the 3'-hydroxyl group of substrate quercetin.

Table 3. Interactions between AdoHcy and SOMT-9

AdoHcy	distance	residues
adenosine NH2	3.85	Asp164(COO)
	2.82	Tyr171(OH)
adenosine 2'-OH(H)	3.38	Asp110(COO)
adenosine 3'-OH(O)	1.85	Asn112(NH2)
methionine NH2	5.76	Thr62(OH)
	6.54	Ser92(OH)
	6.54	Asp162(COO)
methionine OH(O)	4.29	Thr62(OH)
	5.62	Ser92(COO)
methionine OH(H)	5.58	Glu66(OH)

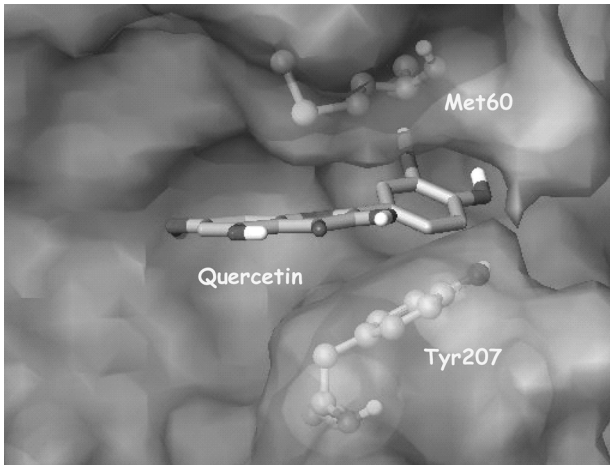

Figure 2. Substrate binding pocket of SOMT-9. Met60 and Tyr207 are critical residues needed to form the hydrophobic pocket in which the A ring of quercetin fits. In addition, Tyr207 plays a critical role in determining the regioselectivity of SOMT-9.

cetin are listed in Table 3. It is interesting to note that Asp162 interacted with the 3'-OH and 4'-OH of quercetin, and the NH_2 of methionine of AdoHcy. Likewise, Lys165, Asp188, and Tyr207 all played an important role in the interaction between quercetin and AdoHcy.

In order to evaluate the validity of the modeled structure, site-directed mutagenesis of SOMT-9 was carried out. Based on the molecular structure and reaction mechanisms, metal binding amino acids and hydrophobic pocket were hypothesized to be important for the reaction. Thus, we selected for mutagenesis those amino acids thought to be important for the metal binding and hydrophobic pocket formation. Asp162, Asp188, and Asn189, which were predicted to be involved in metal binding, were mutagenized. Lys165 was also mutagenized as it was predicted to assist Asp122 and

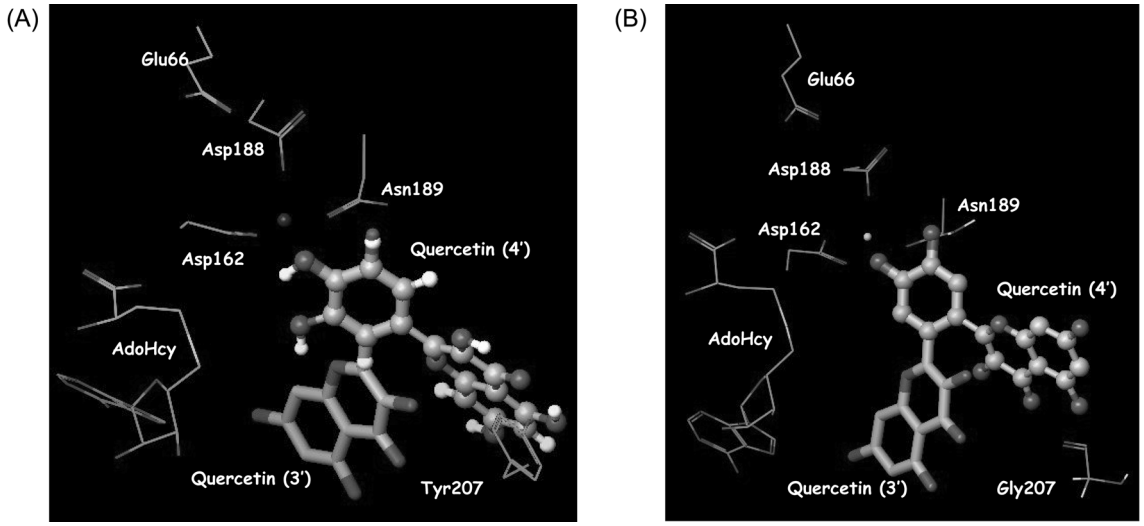

Figure 3. Role of Tyr207 for determination of regioselectivity. Two configurations (3' and 4') of substrate are shown. A, wild type SOMT-9 in which 207th amino acid is tyrosine. Tyr207 prevents the A ring of the quercetin from rotating into a position favouring methylation of the 4'-hydroxyl group and thus only 3'-methylated product is produced; B, mutant SOMT-9 (Tyr207gGy) in which 207th amino acid is glycine. The A ring of quercetin in Tyr207Gly was able to rotate because of disappearance of steric hindrance, thereby allowing methylation of both the 3' and 4'-hydroxyl groups.

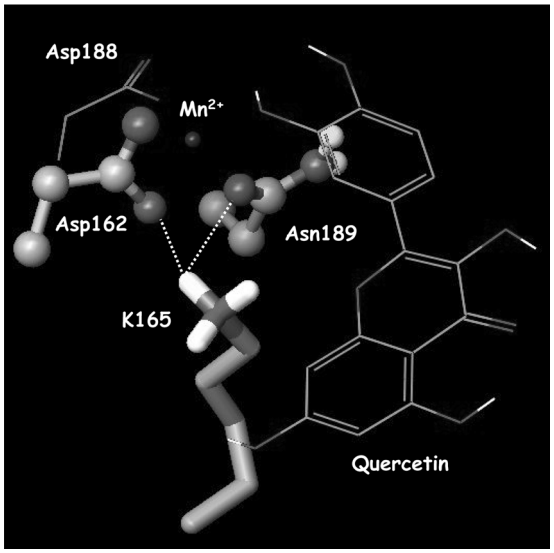

Figure 4. Role of Lys165 in stabilization of metal binding amino acids. Lys165 has a role in the proper positioning of the two metal binding amino acids, Asp162 and Asn189 through hydrogen bonds.

Asp189 (metal binding amino acids) in maintaining the proper configuration of the two metal binding amino acids through hydrogen bonds. Lastly, Tyr207 and Met60 were mutagenized, as both are involved in the formation of hydrophobic pocket and the former is likely to be important for the regioselectivity of SOMT-9. All of the mutant proteins were expressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein and were purified using a GST affinity column.

First, site-directed mutations were carried out in the three metal binding amino acids (Fig. 1). Mutation of Asp162Gly did not change the reactivity of SOMT-9, whereas mutation of Asp188 to Gly resulted in an almost complete loss of activity. In addition, mutation of Asn189 into isoleucine also resulted in a complete loss of enzymatic activity. The discrepancy between the reactivity of the two mutants can be explained by the location of the backbone carbonyl group. Met42 is located 4.3 Å away from the metal ion. The carbonyl group of Met42 could serve to neutralize the positive charge of the metal ion through the formation of bridge with water. However, around Asp188, the backbone carbonyl groups are not close enough to substitute the side chain of Asp188 or Asn189.

Lys165 was predicted to have a role in the proper positioning of the two metal binding amino acids, Asp162 and Asn189 through hydrogen bonds (Fig. 4). Consistent with our hypothesis, mutation of Lys165 to either Leu or Gly resulted in an almost complete loss of the activity, suggesting that the hydrogen bonds between Lys165 and the two metal binding amino acids are indeed critical for the enzymatic activity of SOMT-9.

Among the putative substrate binding amino acids, Tyr207 and Met60 were predicted to form a hydropobic pocket for the phenolic groups of flavonoids. Based on the result from the mutagenesis, it appears that the two residues have different roles. Met60 is located to the opposite side of Tyr206,

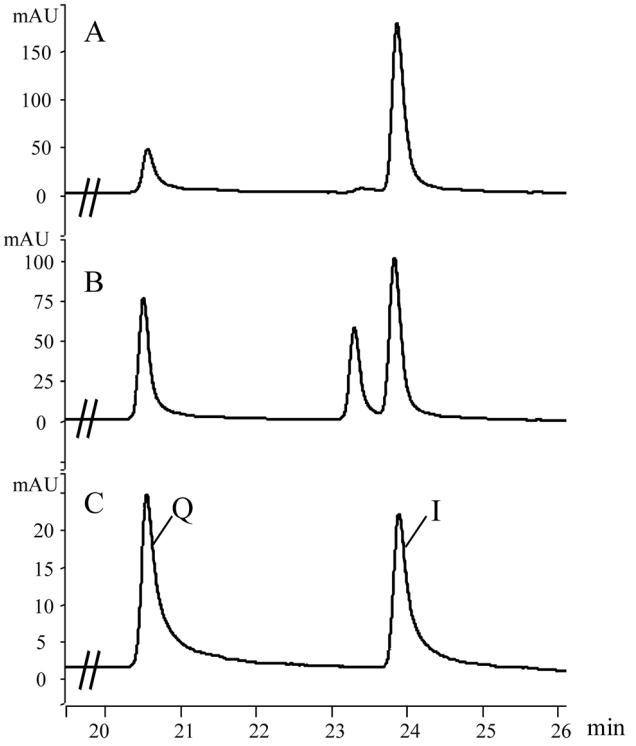

Figure 5. HPLC analysis of wild type and mutant Tyr205Gly reacted with quercetin. A, reaction product of wild type SOMT-9. Only one methylation product (3'-methoxy quercetin) could be observed; B, reaction product of Tyr205Gly. Two products (3'-methoxy and 4'-methoxy quercetin) were observed; C, authentic quercetin (Q) and 3'-methoxy quercetin (isorahmnetin, I).

conceivable that while a mutation of met60 to a similar amino acid such as leucine may not alter the reactivity of SOMT-9 but changing of Met60 to a smaller amino acid such glycine would change the reactivity due to the size of hydrophibic pocket. As expected, mutation of Met60 to Leu did not significantly change enzyme reactivity (less than 10%), whereas mutation of Met60 to Gly reduced the reactivity of SOMT-9 by 40% (Table 1). On the other hand, Tyr207, which is located close to A ring of quercetin, was thought to be involved in determination of regioselectivity of SOMT-9 based on the molecular docking experiment. Mutation of Tyr207 to Gly did not change the reactivity of SOMT-9 but rather changed the regioselectivity. As shown in Figure 5, analysis of the reaction product of wild type enzyme with quercetin resulted in a single peak identified as 3'-*O*-methyled quercetin (Fig. 5A). However, the reaction product obtained with the Tyr207Gly mutation resulted in two distinct peaks. From the modeled structure of SOMT-9, we hypothesized that Tyr207 prevents the A ring of the quercetin from rotating into a position favouring methylation of the 4'-hydroxyl group (Fig. 3A), and thus only 3'-methylated product is produced (Fig. 5A). When we conducted molecular docking experiments with the mutant Tyr205Gly, the A ring of quercetin was able to rotate because of disappearance of steric hindrance, thereby allowing methylation of both the 3' and 4'-hydroxyl groups (Fig. 5B).

Based on molecular modeling and docking experiments,

the reaction mechanism of SOMT-9 could be predicted. First, the flavonoid substrate goes into the substrate binding pocket formed by hydrophobic amino acids like Met60 and Tyr207; this hydrophobic pocket also has a role in determining the regioselectivity of SOMT-9. Once the flavonoid is located in the pocket, the metal ion serves as a base removing a proton from the hydroxyl group of the flavonoids. Thus, proper configuration of the metal ion in the enzyme is indispensable for the activity. Our mutagenesis results clearly demonstrated the importance of these amino acids to the enzymatic activity of SOMT-9. The approach might be applicable for the prediction of substrate and regioselectivity of other enzymes, and aid efforts to engineer new regioselectivities of existing enzymatic reactions.

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