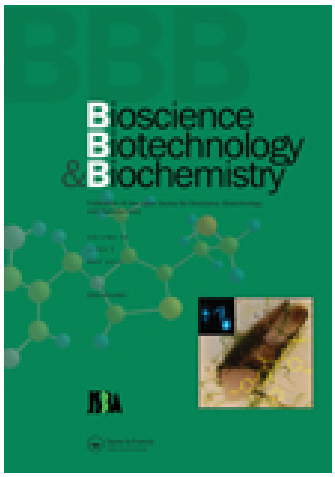

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Masanobu Kirita[a], Yoshihisa Tanaka[a], Motoyuki Tagashira[a], Tomomasa Kanda[a] & Mari Maeda-Yamamoto[b]

[a] Research & Development-Production Headquarters, Asahi Breweries Limited, Moriya-shi, Japan

[b] National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba-shi, Japan

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Masanobu Kirita[1,*], Yoshihisa Tanaka[1], Motoyuki Tagashira[1], Tomomasa Kanda[1] and Mari Maeda-Yamamoto[2]

[1]*Research & Development-Production Headquarters, Asahi Breweries Limited, Moriya-shi, Japan;* [2]*National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba-shi, Japan*

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An enzyme catalyzing the methylation of phenolic hydroxyl groups in polyphenols was identified from mycelial cultures of edible mushrooms to synthesize *O*-methylated polyphenols. Enzyme activity was measured to assess whether methyl groups were introduced into (−)-epigallocatechin-3-*O*-gallate (EGCG) using SAM as a methyl donor, and (−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3″Me), (−)-epigallocatechin-3-*O*-(4-*O*-methyl)-gallate (EGCG4″Me), and (−)-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3″,5″diMe) peaks were detected using crude enzyme preparations from mycelial cultures of *Flammulina velutipes*. The enzyme was purified using chromatographic and two-dimensional electrophoresis. The purified enzyme was subsequently analyzed on the basis of the partial amino acid sequence using LC–MS/MS. Partial amino acid sequencing identified the 17 and 12 amino acid sequences, VLEVGTLGGYSTTWLAR and TGGIIIVDNVVR. In database searches, these sequences showed high identity with *O*-methyltransferases from other mushroom species and completely matched 11 of 17 and 9 of 12 amino acids from five other mushroom *O*-methyltransferases.

Key words: *Flammulina velutipes*; *O*-methyltransferase; (−)-epigallocatechin-3-*O*-gallate; EGCG

Dietary polyphenols, generally contained in our diet, contribute to human health and prevent disease. Low bioavailability and instability of metabolic functions hamper the potential of several native polyphenols[1,2]; however, *O*-methylated polyphenols have improved bioavailability and metabolic stability.

In a previous study, the *O*-methylated (−)-epigallocatechin-3-*O*-gallate (EGCG), (−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3″Me) was isolated from 'Benifuuki' cultivars of *Camellia sinensis* L.[3] After drinking *O*-methylated EGCG-rich green tea containing 43.5 mg of EGCG and 8.5 mg of EGCG3″Me, the AUC (area under the drug concentration time curve; min l g mL^{-1}) of EGCG was 6.72 ± 2.87 and EGCG3″Me was 8.48 ± 2.54 in healthy human volunteers. Though the dose of EGCG was 5.1 times the dose of EGCG3″Me, the AUC of EGCG3″Me was higher than that of EGCG's.[4] The antiallergic effects of EGCG and other *O*-methylated EGCGs were measured using histamine release assays in bone marrow-derived mouse mast cells, and the order of potencies was (−)-3′-*O*-methyl-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3′,3″,5″triMe) = (−)-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3″,5″diMe) > EGCG3″Me > EGCG.[5] The inhibition of histamine release was related to an increase in the number of methyl groups in EGCG, particularly the galloyl moiety. The metabolic stability of the methylated flavones 7-methoxyflavone, 7,4′-dimethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4′-trimethoxyflavone in pooled human liver S9 fractions was investigated which showed their intestinal absorption in caco-2 cells to be more effective than that of unmethylated flavones.[6] However, these *O*-methylated polyphenols are present at extremely low levels in plants.

These reports indicate that the bioavailability and efficacy of polyphenols are improved by methylation of the phenolic hydroxl group. Therefore, enzymes catalyzing such polyphenol-methylating reactions may be useful tools for enhancing the efficacy of naturally occuring polyphenols. In plants, Caffeoyl-Coenzyme A *O*-methyltransferases (CCoAOMT) play important roles in the biosynthesis of lignin.[7,8] In our previous report, *O*-methyltransferase isolated from tea catalyzed the methylation of EGCG, which is highly homologous to CCoAOMT.[5] Similarly, mushroom including white rot fungi have the ability to degrade lignin; this degradation involves *O*-methyltransferases. In *Phanerochaete chrysosporium*, this multistep lignin degredation pathway has been shown to involve oxidation, reduction, and methylation reactions.[9,10] Therefore, we predicted that such mushroom species would express enzymes that catalyze the methylation of polyphenols. However,

*Corresponding author. Email: masanobu.kirita@asahibeer.co.jp
Abbreviations: EGCG, (−)-epigallocatechin-3-*O*-gallate; SAM, *S*-adenosyl-L-methionine; CCoAOMT, Caffeoyl-Coenzyme A *O*-methyltransferas; PAGE, polyacrylamide gel electrophoresis.

till date, very few reports demonstrate methylase activities in mushrooms such as white rot fungi. Among these studies, 3-*O*- and 4-*O*-methyltransferases were purified from *P. chrysosporium*.[11,12] The 3-*O*-methyltransferases catalyze the methylation of isovanillic acid, whereas 4-*O*-methyltransferases catalyzes the methylation of acetovanilline using *S*-adenosyl-L-methionine (SAM) as a donor. Therefore, we searched for novel enzymes in mycelial cultures of edible mushroom that can be applied as functional foods. Subsequently, we demonstrated the potential for large-scale synthesis of *O*-methylated EGCGs using crude enzyme preparations.

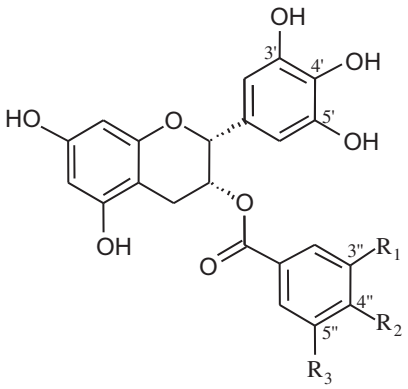
Materials and methods

Reagents. All chemicals and solvents were of high or high performance liquid chromatography (HPLC) grade. EGCG3″Me, (−)-epigallocatechin-3-*O*-(4-*O*-methyl)-gallate (EGCG4″Me), and EGCG3″,5″diMe were prepared according to a previous method with minor modifications (Fig. 1).[5] Edible mushrooms were purchased from a Japanese supermarket.

Isolation and mycelial culture of edible mushrooms. Stalks of edible mushrooms were cut, and mushroom surfaces were disinfected with 0.5% sodium hypochlorite solution for 15 min. Internal stalks were cut after rinsing with sterile distilled water and placed on potato dextrose agar medium at 25 °C to isolate mycelia. Isolated mycelia were cultured in a broth containing 0.02% glucose, 0.01% peptone, 0.002% yeast extract, 0.002% KH_2PO_4, and 0.001% $MgSO_4$ at 28 °C while shaking. Edible mushroom species included *Lentinus edodes*, *Lyophyllum shimeji*, *Grifola frondosa*, *Flammulina velutipes*, *Hypsizygus marmoreus*, *Pleurotus ostreatus*, *Armillaria mellea* subsp. *nipponica*, *Pleurotus cornucopiae* var. *citrinopileatus*, *Pleurotus eryngii*, and *Pleurotus nebrodensis*.

Assay of O-methyltransferase activity. Mycelial cultures were harvested and sonicated in a suspension buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% glycerol. Supernatants were collected and centrifuged to extract the crude enzyme, which was assayed for *O*-methyltransferase activity by assessing EGCG methylation rates. The 3-mL reaction mixture contained 100 mM Tris–HCl (pH 7.4), 0.2 mM $MgCl_2$, 0.25 mM EGCG, 0.5 mM SAM as a methyl donor, and 1.5 mL of crude enzyme solution. The mixture was incubated at 37 °C for 14 h, and the reaction was stopped by the addition of 1 N HCl. The reaction mixture was extracted with 5 mL of ethyl acetate, and centrifugation was used to collect the organic phase, which was dried under N_2 gas and resuspended in 30% methanol containing 1% ascorbic acid. Enzyme activity was determined using HPLC or LC–TOF–MS according to a previously described method.[5]

Enzyme purification. To purify the enzyme, mycelial cultures of *F. velutipes* were lyophilized and ground and subsequently resuspended in the above suspension buffer. After sonication, the supernatant was collected by centrifugation and fractionated using 60–80% ammonium sulfate saturation. The precipitate was suspended in suspension buffer and desalted using PD-10 columns (GE Healthcare, Japan). The sample was loaded onto a DEAE column (HiPrep 16/10, GE Healthcare, Japan) that had been previously equilibrated with 20 mM Tris–HCl (pH 7.5) containing



Compounds	R_1	R_2	R_3
(−)-epigallocatechin-3-*O*-gallate (EGCG)	OH	OH	OH
(−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3"Me)	OCH_3	OH	OH
(−)-epigallocatechin-3-*O*-(4-*O*-methyl)-gallate (EGCG4"Me)	OH	OCH_3	OH
(−)-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3",5"diMe)	OCH_3	OH	OCH_3

Fig. 1. Chemical structures of EGCG and *O*-methylated EGCGs.

1 mM DTT. The enzyme was eluted at a flow rate of 2 mL min^{-1} using a linear gradient of 0–1 M NaCl in the same buffer. Active fractions were combined, desalted, and concentrated by ultrafiltration. Subsequently, the sample was prepared with 1.5 M ammonium sulfate and loaded on to a Phenyl Sepharose HP column (Hiload 26/10, GE Healthcare, Japan) that had been previously equilibrated with 100 mM phosphate buffer (pH 7.5) containing 1 mM DTT and 1.5 M ammonium sulfate. Elution was performed at a flow rate of 2 mL min^{-1} using a linear gradient of 1.5–0 M ammonium sulfate. Active fractions were collected, desalted, and concentrated as above. For further purification, an aliquot of the eluate from the Phenyl Sepharose HP column was loaded onto an anion exchange column (TSK-gel BioAssist Q, 4.6-mm i.d. × 5 cm; TOSHO corporation, Japan) that had been previously equilibrated with 20 mM Tris–HCl (pH 7.5) containing 1 mM DTT. The enzyme was eluted at a flow rate of 1 mL min^{-1} using a 0–0.5 M linear gradient of NaCl in the same buffer. The active fractions were collected, desalted, and concentrated as above. Each purified sample was subsequently analyzed using 12% SDS-PAGE.

SDS-PAGE, 2D gel electrophoresis, and trypsin digestion. Active fractions from the Phenyl Sepharose HP column were separated using 12% SDS-PAGE and 2D gel electrophoresis. Bands of interest were excised from SDS-PAGE gels. For 2D gel electrophoresis, isoelectric focusing was performed using an Ettan IPGphor3 IEF System (GE Healthcare, Japan). Immobiline Dry Strip linear immobilized pH gradient gel strips (7 cm) with a pH range of 3–10 (GE Healthcare, Japan) were rehydrated in a solution containing 9 M urea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes (pH 3–10), 0.002% bromophenol blue, and 20 µg of protein. Proteins were focused at 500 V for 1 h, 1000 V for 1 h, 8000 V for 2 h, and 8000 V for 12 h at 20 °C. After focusing, Immobiline Dry Strips were embedded onto 4–20% SDS-PAGE gels. Protein spots that confirmed active fractions were excised from 2D electrophoresis gels. These excised proteins were purified and subjected to in-gel trypsin digestion according to a previously reported method.[13)]

LC–MS/MS peptide sequence analysis. Purified protein samples from SDS-PAGE or 2D gel electrophoresis were analyzed using LC–MS/MS. LC–MS/MS was performed using a nano-LC system paradigm (AMC Inc., Camden) with a MS system LTQ (Thermo Fisher Scientific K.K., Japan). LC–MS/MS was performed using a Magic C18, 3-µm, 200-Å column (0.2 mm i.d. × 50 mm, Michrom Bioresources, CA) at a flow rate of 1.5 µL min^{-1}. Mixture of A (0.1% (v/v) formic acid/2% (v/v) acetonitrile) and B (0.1% (v/v) formic acid/90% (v/v) acetonitrile were used as the mobile phase. The initial eluent was 5% mobile phase B, followed by a linear gradient from 5 to 45% B for 20 min. Survey full scan spectra were acquired in the *m/z* range of 400–2000. Data were analyzed using Bioworks software version 3.1 (Thermo Fisher Scientific K.K., Japan).

The *de novo* sequence analysis was performed using a Serveyo-LC-system (Thermo Fisher Scientific K.K., Japan) with a MS system LTQ-orbitrap mass spectrometer (Thermo Fisher Scientific K.K., Japan). LC–MS/MS was performed using a Zorbax SB-300 column (0.075 mm i.d. × 50 mm, Agilent Technologies, Japan) at a flow rate of 200 µL min^{-1}. Mixtures of A (0.1% (v/v) formic acid) and B (80% (v/v) methanol containing 0.1% (v/v) formic acid) were used as mobile phases. The initial eluent used was 5% mobile phase B, followed by a linear gradient from 5 to 55% B for 65 min, 100% B for 10 min, and finally 5% B for 15 min.

Optimal pH and temperature of the enzyme. Optimal pH and temperature were assessed using 0.25 mL of crude enzyme and 0.05 mM EGCG as a substrate, in a solution containing 2.5 mM $MgCl_2$, 0.04% ascorbic acid, and 0.5 mM SAM. Optimal pH was determined in the range of 3–10 using 20 mM acetate buffer (pH, 3.0–5.5), 20 mM phosphate buffer (pH, 6.0–7.0), and 20 mM Tris–HCl (pH, 7.5–10). The mixture was incubated at 37 °C for 6 h. The optimal temperature was determined by incubation with 20 mM phosphate buffer (pH 7.0) for 6 h and *O*-methylated EGCGs were analyzed using HPLC.

Culture of mycelia in a jar fermentor and production of EGCG3″Me. Mycelia were cultured in 300 mL Erlenmeyer flasks containing 150 mL of 0.02% glucose, 0.01% peptone, 0.002% yeast extract, 0.002% KH_2PO_4, and 0.001% $MgSO_4$. The culture was prepared in a rotary shaker at 130 rpm for 6 days at 20 °C. Cultured mycelia were inoculated into a 3-L working volume in a 5-L jar fermentor (B. E. Marubishi Co., Ltd., Japan) of 165-mm diameter and 265-mm height. Fermentation was carried out at 50 rpm using a standard turbine-type impeller at 20 °C and an aeration rate of 1.5 vvm for 5 days. Mycelial cultures were lyophilized before measuring enzyme activity.

Results

O-methyltransferase activity assays

Methylation activity for EGCG was determined in mycelial cultures of edible mushroom. In these experiments, EGCG3″Me, EGCG4″Me, and EGCG3″,5″diMe peaks were detected in *F. velutipes* crude enzyme preparations at retention times that matched those of *O*-methylated EGCGs' standards (Fig. 2). Theoretical values of molecular weights for these *O*-methylated EGCGs were confirmed using LC–TOF-MS. No peaks corresponding to *O*-methylated EGCGs were detected in enzyme extracts from any of the other mushroom species.

Enzyme purification and peptide sequence analyses

Enzyme purification protocols are summarized in Table 1. After sonication, supernatants from mycelial cultures were fractionated using 40%–80% ammonium sulfate saturation in 10% increments. After desalting, enzyme activity was determined in each fraction.

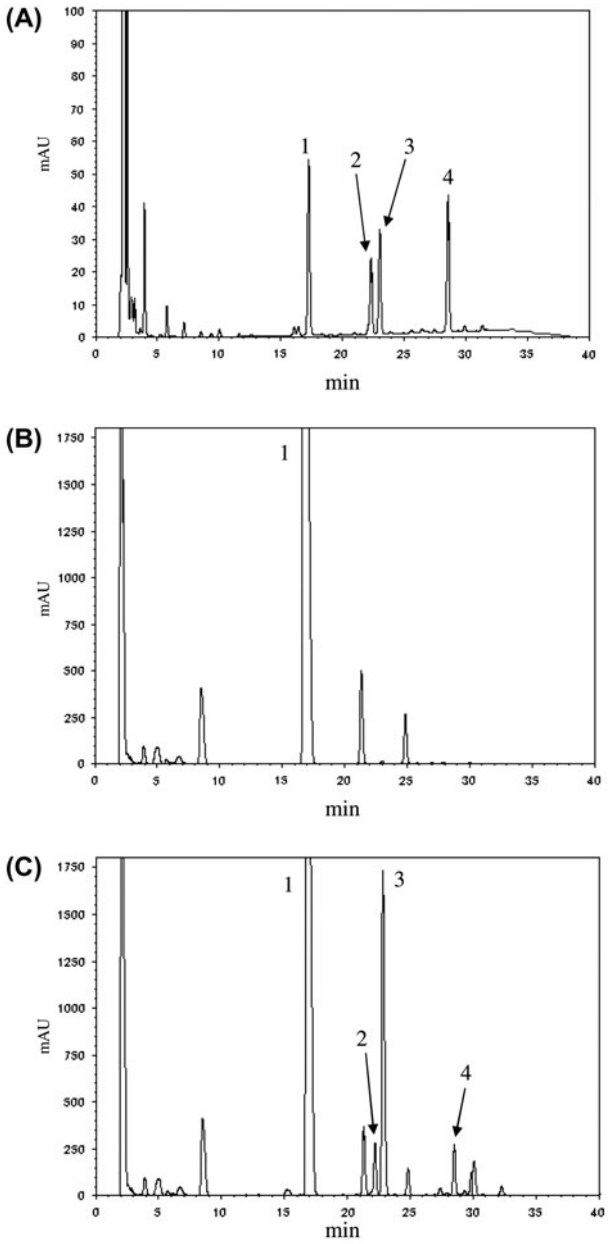


Fig. 2. HPLC analysis of the enzymatic reaction products using crude enzyme from *F. velutipes*.

Note: (A) Standard catechins. Peak identification: 1, EGCG; 2, EGCG4″Me; 3, EGCG3″Me; 4, EGCG3″,5″diMe. (B) Enzymatic reaction products. But enzyme was added after thermal denaturation. (C) Enzymatic reaction products.

Fractions from 60%–80% ammonium sulfate saturations contained more than 90% of total EGCG *O*-methylase activity and were collected and further purified. After loading onto anion exchange columns (BioAssist Q), a few bands were detected on SDS-PAGE gels. Although only a small amount of sample was purified after anion exchange chromatography, the same bands were excised from the SDS-PAGE gels after elution from Phenyl Sepharose HP column. Bands of interest were analyzed for partial amino acid sequences using LC–MS/MS after treatment with in-gel trypsin digestion. In database searches, the identified amino acid sequence VLEVGTLGGYSTTWLAR was highly homologous with *O*-methyltransferase sequences from *Laccaria bicolor* S238 N-H82 (XP_001878803.1; Fig. 3). This *L. bicolor O*-methyltransferase had a molecular weight of 25.3 kDa and an isoelectric point of 5.24. Similarly, the sequence of *F. velutipes* was confirmed from an excised SDS-PAGE band of approximately 24–25 kDa. To further purify the enzyme, active fractions from the Phenyl Sepharose HP column were separated using 2D gel electrophoresis and spots of similar molecular weight and isoelectric point were excised from the marginal zone as above (Fig. 4). The above amino acid sequence was confirmed in one of these protein spots, and subsequent *de novo* sequence analyses identified the sequence TGGIIIVDNVVR. Database searching indicated a high degree of homology with another region of *O*-methyltransferase from *L. bicolor*. Taken together, these results suggest purification of an EGCG *O*-methyltransferase (Fv-OMT) from *F. velutipes*.

Optimal pH and temperature for enzyme activity

The optimal pH and temperature for enzyme activity were determined using the *O*-methylated EGCG synthesis index. The enzyme was stable for 6 h in a pH range of 6.5–8.5 at 37 °C (Fig. 5(A)) and its activity was greatest at pH 7.0. The enzyme was active for 6 h between 20 and 42 °C at pH 7.0 (Fig. 5(B)), and the optimal temperature was 37 °C. Enzymatic activity dramatically decreased as the temperature was increased from 42 °C. Each ratio of EGCG3″Me, EGCG4″Me, and EGCG3″,5″diMe production by pH and temperature was same.

EGCG3″Me production

Mycelium pellets were grown in a jar fermentor and 49 g of lyophilized mycelium was collected. Subsequently, 50 mg of lyophilized mycelium was sonicated in 3 mL of suspension buffer, and 1 mL of this enzyme suspension produced 150.7 μM EGCG3″Me in 14 h (71.2 μg).

Table 1. Purification of the Fv-OMT from *F. velutipes*.

Step no.		Total protein (mg)	Yield (%)	Purification fold	Total activity (unit)	Specific activity (unit/mg)
1	Crude extract	173.28	100.0	1	715.92	4.13
2	60–80% ammonium sulfate saturation	21.00	91.8	7.58	657.35	31.30
3	Anion-exchange chromatography (DEAE)	9.48	46.6	8.52	333.96	35.20
4	Hydrophobic interaction chromatography (Phenyl Sepharose HP)	2.19	16.7	13.24	119.84	54.70
5	Anion-exchange chromatography (BioAssist Q)	0.07	0.9	21.93	6.52	90.56

Note: One unit of enzyme activity is defined as the amount of enzyme producing the formation of 1 mmol of EGCG3″Me, following 14 h of incubation at 37 °C.

Fv-OMT1	V	L	E	V	G	T	L	G	G	Y	S	T	T	W	L	A	R
L.bicolor	I	L	E	V	G	T	L	G	G	Y	S	S	I	W	L	A	R
T.versicolor	I	L	E	V	G	T	L	G	G	Y	S	T	I	W	F	A	R
D.squalens	V	L	E	V	G	T	L	G	G	Y	S	A	I	W	L	A	R
C.cinerea okayama	I	L	E	V	G	T	L	G	G	Y	S	T	I	W	L	A	R
S.hirsutum	V	L	E	V	G	T	L	G	G	S	S	A	I	C	F	A	R

Fv-OMT1	T	G	G	I	I	I	V	D	N	V	V	R
L.bicolor	K	G	G	V	I	I	V	D	N	V	V	R
T.versicolor	R	G	G	I	I	I	V	D	N	V	I	R
D.squalens	K	G	G	I	I	I	V	D	N	V	V	R
C.cinerea okayama	S	G	G	V	I	I	V	D	N	V	V	R
S.hirsutum	K	G	G	I	I	I	V	D	N	V	V	R

Fig. 3. Comparison of partial amino acid sequences of *O*-metyltransferase with the mushroom species *L. bicolor*, *T. versicolor*, *D. sequalens*, *C. cinerea okayama*, and *S. hirsutum*.

Note: Completely matched residues are indicated with gray boxes.

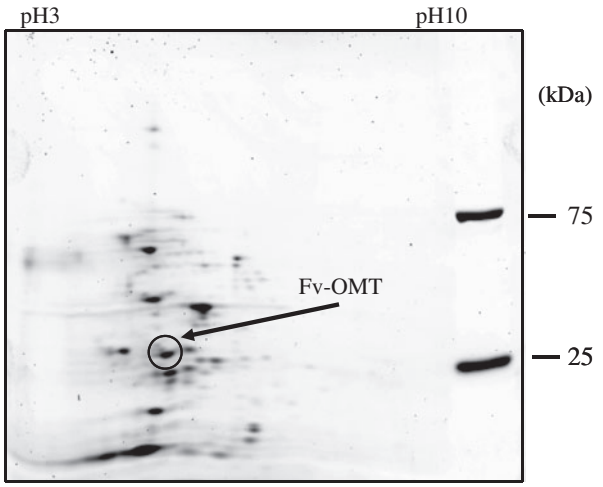


Fig. 4. Representative 2D gel electrophoresis of Fv-OMT in active fractions from phenyl sepharose columns.

Note: Proteins were stained with SYPRO Red Protein (Takara Bio Inc., Japan). The immobilized pH gradient (pH, 3–10) is indicated on top. Molecular mass markers are indicated on the right. The arrow mark indicates the spot from which *O*-methyltransferase amino acid sequence was confirmed.

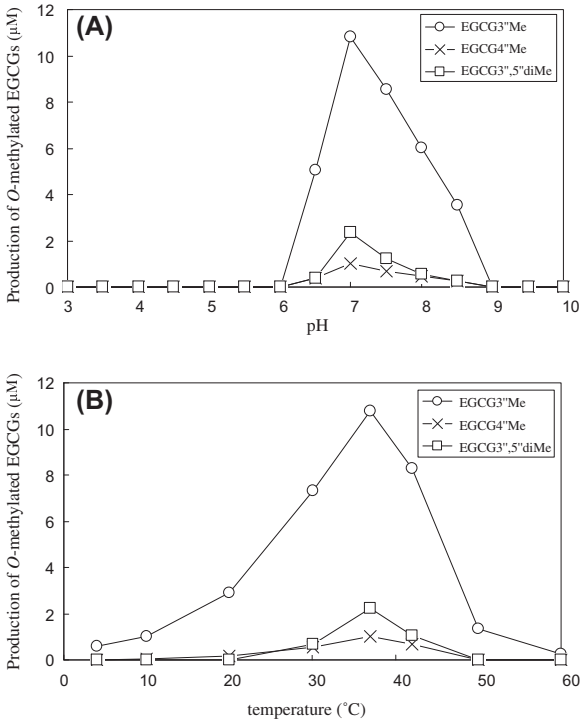


Fig. 5. Optimal pH (A) and Temperature (B) of Fv-OMT.

Note: Activity of the enzyme is indexed according to the production of EGCG3″Me, EGCG4″Me, and EGCG3″,5″diMe.

Discussion

We identified, isolated, and measured the activity of an enzyme from *F. velutipes* that methylates EGCG. Although three kinds of columns were used to purify this enzyme, multiple bands were identified on SDS-PAGE gels. Therefore, candidate bands were excised and amino acid sequences were determined using LC–MS/MS and *de novo* sequence analyses. The partial amino acid sequence VLEVGTLGGYSTTWLAR had high identity with *O*-methyltransferases from other mushrooms that were identified in database searches. In comparisons with five other mushroom species, 11 of 17 amino acids completely matched and the other residues were extremely similar. SAM-dependent methyltransferases of plants belonging to the CCoAOMT group are categorized into two major classes.[14] Class I and II enzymes comprise 231–248 and 344–383 amino acids, respectively. Mushroom *O*-methyltransferases, which showed high homology with the *O*-methyltransferase idenitified in this study, comprised 222–234 amino acids. Joshi et al.[14] identified several conserved regions among plant enzymes of the CCoAOMT family. Partial amino acid sequences of Fv-OMT were positioned in the motif E (GVXTGYS) that shows some similarity with the putative SAM-binding domains. Hence, the sequence GTLGGYS of Fv-OMT may serve as a SAM-binding domain.

The other partial Fv-OMT amino acid sequence TGGIIIVDNVVR showed high identity with sequences from other mushrooms, and 9 of 11 amino acids were completely mached. This sequence was positioned in motif A (LVXXGGXI) of the plant CCoAOMT family, which also shows some homology to putative SAM-binding domains. Amino acid sequencing of this region in the other five mushrooms indicated the common sequence (L/M)VRXGG(I/V)I. Similarly, the partial Fv-OMT sequence TGGIIIVDNVVR may include a SAM-binding domain. On the basis of these sequence homologies, we speculate that Fv-OMT comprises

approximately 230 residues and has a molecular weight of 25 kDa.

Only a few reports demonstrate methylation activities in mushrooms such as white rot fungi. Among these, Coulter et al. and Jeffers et al. purified 3-*O*- and 4-*O*-methyltransferases from *P. chrysosporium*[12,15] and indicated dimeric and monomeric proteins of 36 and 54 kDa, respectively. In experiments using several benzoic acids, the substrate specificity of 3-*O*-methyltransferase was highly restricted to hydroxyl groups at the 3rd position. In contrast, 4-*O*-methyltransferase catalyzed methylation at both 2nd and 4th positions. The present data indicate that Fv-OMT has a distinctly different molecular weight and low substrate preference for hydroxyl group positions. Nonetheless, the optimal pH for Fv-OMT activity was between 6.5 and 8.5, similar to that of *P. chrysosporium* 3-*O*-methyltransferase's.[12] However, the optimal temperature for Fv-OMT activity was between 20 and 42 °C, whereas that of 3-*O*-methyltransferase of *P. chrysosporium* was between 15 and 55 °C. Fv-OMT was denatured at 50 °C.

Mycelia from Erlenmeyer flasks cultures were pelleted, but when fermentation was conducted at 200 rpm, mycelia were extremely finely sheared and gave no pellet, and the ensuing enzyme preparation failed to produce EGCG3″Me. In contrast, fermentation at 50 rpm produced mycelium pellets from Erlenmeyer flask cultures and enzymatic activity was obtained. Hence, formation of the pellet was important for fermentation.

In conclusion, we searched for enzymes that methylate EGCG and identified Fv-OMT from *F. velutipes*. Subsequently, we characterized the partial amino acid sequence of Fv-OMT and determined optimal catalytic pH and temperature. EGCG3″Me was synthesized using the enzyme prepared from mycelial fermentation. This enzyme may be a useful tool for the production of *O*-methylated polyphenols and elucidation of their function. In future studies, we will isolate the gene for Fv-OMT using degenerated primers that will be designed on the basis of partial amino acid sequences and will confirm the methylation activities for other polyphenols.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.912117.

Acknowledgments

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