Botanical Studies (2012) 53: 197-206.

Molecular cloning and functional analysis of bergaptol-O-methyltransferase from *Angelica dahurica* (Bai Zhi) and using it to efficiently produce bergapten in *E. coli*

Shu-Chin LO[1,2], Pei-En CHUNG[2], and Co-Shine WANG[1,*]

[1]*Graduate Institute of Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan*
[2]*Department of Agricultural Chemistry Division, Taiwan Agricultural Research Institute, Taichung 41362, Taiwan*

(Received September 19, 2011; Accepted February 3, 2012)

ABSTRACT. Bai Zhi (*Angelica dahurica*), a Chinese herb, has long been used as a face cream for skin-whitening purposes. One of the known skin-whitening components, 8-hydroxybergapten is a hydroxylated product of bergapten that is converted from bergaptol by bergaptol 5-O-methyltransferase (BMT) in Bai Zhi. The complementary DNA of BMT was cloned from Bai Zhi root using a pair of degenerate primers designed from the highly conserved regions of other plant O-methyltransferases (OMTs). RT-PCR analysis indicated that a single band of DNA fragment corresponding to *AdBMT* sequence was obtained. The tandem 5′- and 3′-rapid amplification of cDNA ends via polymerase chain reaction was used to obtain the full-length cDNA sequences. The *AdBMT* cDNA contains an open reading frame of 1,080 bp encoding a BMT polypeptide of 359 amino acids with a calculated molecular mass of 39 kDa and a calculated pI of 5.9. Sequence alignment revealed the considerable sequence similarity of AdBMT to those of other plant OMTs. The AdBMT sequence contains conserved region I-V, similar to other plant OMTs. His-tagged AdBMT was expressed in *E. coli* and partially purified by ammonium sulfate precipitation. The recombinant AdBMT is most active in potassium phosphate buffer at pH 7.5 and 35°C. The enzyme does not require a divalent cation for activity and the addition of Cu^{2+}, Ni^{2+}, and Co^{2+} at concentrations even as low as 0.1 mM severely inhibits enzyme activity. A simple and efficient production of bergapten in the *E. coli* culture overexpressing AdBMT was performed. The bergapten yield is approximately 13-fold higher than that produced by enzymes in the ammonium sulfate-purified fraction. With the supply of bergaptol in the medium, *E. coli* cells can be used as a potential bioreactor to produce bergapten.

Keywords: Bai Zhi; Bergapten; Bergaptol 5-O-methyltransferase; cDNA cloning; Enzyme activity.

Abbreviations: BMT, bergaptol 5-O-methyltransferase; **cDNA,** complementary DNA; **EDTA,** ethylenediaminetetraacetic acid; **IPTG,** isopropyl β-D-thiogalactopyranoside; **NTA,** nitrilotriacetic acid; **OMT,** O-methyltransferases; **PAGE,** polyacrylamide gel electrophoresis; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **RACE**, rapid amplification of cDNA ends; **SAM,** S-adenosyl-L-methionine; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **UPLC,** ultra performance liquid chromatography.

INTRODUCTION

Methylation by *S*-adenosyl-L-methionine (SAM) dependent *O*-methyltransferases (OMTs) is a common modification in natural product biosynthesis. In plants, *O*-methylation is also required for linear furanocoumarin biosynthesis. Furanocoumarins are synthesized from the phenylpropanoid pathway as phytoalexins or defense-related substances in response to microbial infection (Tietjen et al., 1983; Kuete et al., 2007; Alexander et al., 2008). The most abundant linear furanocoumarins are psoralen, bergapten, xanthotoxin and isopimpinellin, all of which are bioactive compounds. The biosynthetic pathway of these bioactive furanocoumarins was outlined by Hehmann et al. (2004) although the sequence of hydroxylations and O-methylations of psoralen leading to isopimpinellin has not been established. Of those bioactive compounds, bergapten is produced from bergaptol by bergaptol 5-O-methyltransferase (BMT, Figure 1) and the corresponding OMTs were identified from elicitor-treated *Ammi majus* cells (Hehmann et al., 2004) and more recently, from *Glehnia littoralis* cell cultures (Ishikawa et al., 2009). Since BMT is constitutively expressed in *G. littoralis* cell cultures, it is possible that cell cultures can be used as a potential bioreactor to produce bergapten by supply of psolaren. Bergapten is further converted into 8-hydroxybergapten

*Corresponding author: E-mail: cswang2@dragon.nchu.edu.tw; Tel: 886-4-22840328 ext. 771; Fax: 886-4-22853527.

that may inhibit mushroom tyrosinase. The inhibition of mushroom tyrosinase by 8-hydroxybergapten was reported to be superior to kojic acid and arbutin, two tyrosinase inhibitors widely used as skin-whitening agents in cosmetics (Piao et al., 2004).

Plant OMTs can be categorized into two major groups (Joshi and Chiang, 1998). Group I includes a class of OMTs with low molecular masses (23 to 27 kDa) and this group of OMTs is Mg^{2+}-dependent. The Group II consists of higher molecular mass OMTs (38 to 43 kDa) that do not require Mg^{2+} for catalytic activity. Prominent Group II members include caffeic acid, flavonoid, coumarin, and alkaloid OMTs (Frick and Kutchan, 1999; Dong et al., 2003). The BMT enzymes also belong to Group II. Several identified plant OMT genes encode a universal OMT signature composed of five highly conserved regions, two of which (regions I and IV) are believed to be involved in SAM and metal binding, respectively (Ibrahim et al., 1998; Kopycki et al., 2008). Despite the fact that several plant OMT genes have already been cloned, more such sequences are required to obtain a complete picture.

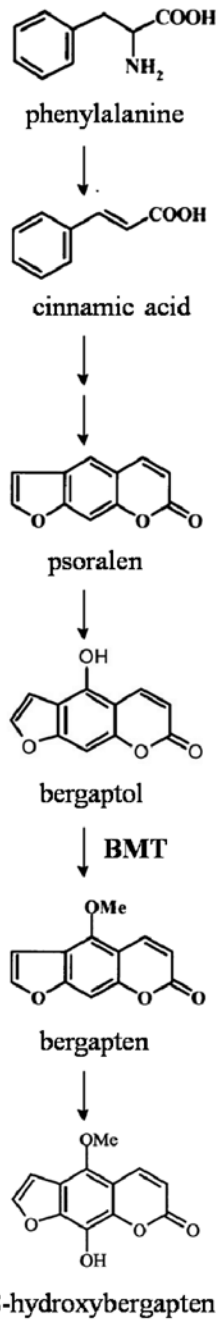

Figure 1. Biosynthesis pathway of bergapten and 8-hydroxybergapten.

The dried root of *Angelica dahurica* (Umbelliferae), also known as Bai Zhi, is an important Chinese traditional herb. Many coumarins isolated from Bai Zhi exhibit antimicrobial activities (Kwon et al., 1997). Interestingly, 8-hydroxybergapten, a furanocoumarin from Bai Zhi, has potent inhibitory activities against mushroom tyrosinase (Piao et al., 2004). Thus, Bai Zhi may potentially be used to treat abnormal pigmentation disorders and applied to skin-whitening compositions in the cosmetic industry. Since 8-hydroxybergapten is a skin-whitening component, the objective of the present study is to identify BMT from *A. dahurica*, a key enzyme to produce bergapten as one of the two major steps towards the production of 8-hydroxybergapten, a skin-whitening agent. The development of *E. coli* as a bioreactor to produce bergapten which is superior to the use of cell culture is also discussed.

MATERIALS AND METHODS

Plant material

Plants of Bai Zhi [*Angelica dahurica* (Fish.) BENTH. *et* HOOK] were grown in the field. Roots were collected and immediately frozen in liquid nitrogen. All the plant material was stored at -80°C until use.

cDNA cloning

Total RNA was isolated from 3 month-old roots of *A. dahurica*. The cDNA fragments were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification using a pair of degenerate primers designed from two highly conserved sequences of plant OMTs. The 5′-primer (5′-gtg/tgatgttggc/tggtggg/a/cactgga/t-3′) resides in conserved region I while the 3′-primer (5′-ggg/a/tgcatcg/t/cc/tca/gac/tg/a/cacgtga/ggg-3′) in conserved region II as indicated in Figure 2. The generated cDNA fragments were cloned in pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced to confirm their identities. The tandem 5′- and 3′-rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) was done according to the user manual of SMART^{TM} RACE cDNA amplification kit (CLONTECH Laboratories, Inc., Mountain View, CA, USA). The complete DNA sequence was determined from both strands of cloned inserts with an ABI 3730 XL DNA analyzer (Foster City, CA, USA) by Mission Biotech Co. Ltd. (Taipei, Taiwan). Sequence alignment was achieved using the Vector NTI Suite 8 program (InforMax, Inc., Bethesda, MD, USA) and the homology search was done with the BLAST program (Altschul et al., 1997).

Overexpression of AdBMT and kinetic conversion of bergaptol into bergapten in *E. coli*

The full-size coding *AdBMT* cDNA as a template and a pair of gene-specific primers AdBMT-over-f (5′-GTCGACCATATGGCAGAAATGAAAACTAG-3′) containing a *Sal*I site, and AdBMT-over-r (5′-GCGGCCGCCTTCGAAAATTCCATAATC-3′) containing a *Not*I site were used for PCR amplifications. The 1,077 bp *AdBMT* fragment was therefore cloned into the *Sal*I/*Not*I-cut pET32a expression vector (Novagen, Madison, WI, USA). After digestion with *Nde*I, the resulting 6.5 kb-fragment was eluted and self-ligated to produce an AdBMT expression plasmid designated pET32a-AdBMT that expressed AdBMT with His-tag at the C-terminus. The pET32a-AdBMTa was transformed into *E. coli* BL21. After inoculation, the bacterial cells were grown in the LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 170 mM NaCl, pH 7.0) containing ampicillin (50 μg/ml) at 37°C until an OD_{600} between 0.6-0.8 was reached. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to give a final concentration of 1 mM and cells were then incubated at various indicated temperature conditions for additional 16 h. Samples of cells were harvested by centrifugation and either used for further protein isolation and enzyme purification or resuspended in 25 ml of LB medium containing ampicillin (50 μg/ml) and 100 μM bergaptol and incubated for an additional 24 h at 25°C. After centrifugation, the supernatant was used to extract bergapten for measurement by ultra performance liquid chromatography (UPLC). Bergapten yield was normalized on the basis of equal portions of *E. coli* cells (wet weight). Only the AdBMT-overexpressing *E. coli* grown at the optimum induction temperature was taken for the kinetic analysis. The incubation time was extended from 24 to 48 h.

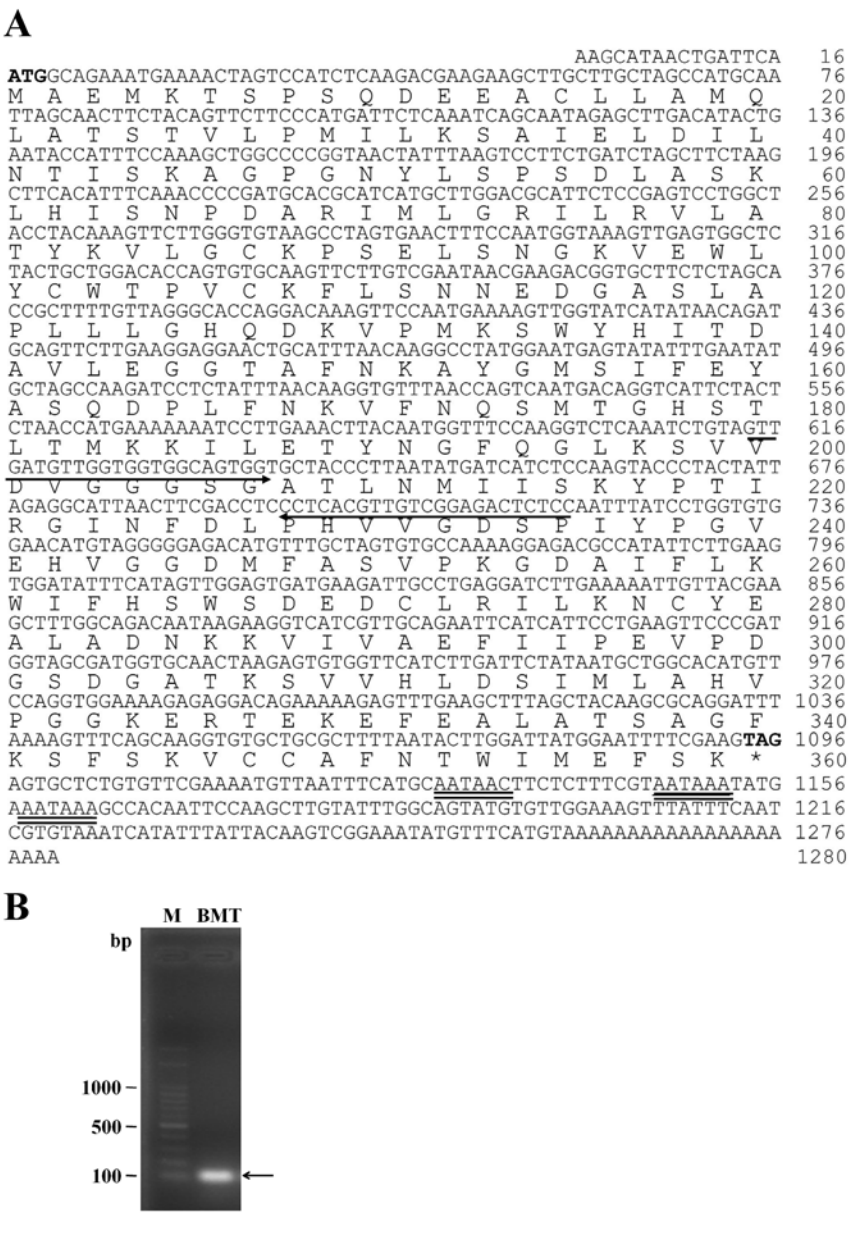

Figure 2. Nucleotide and predicted amino acid sequences of *AdBMT* cDNA identified from *A. dahurica*. (A) The numbers of the nucleotide sequence and amino acid sequence are indicated on the right. The bold letters in the nucleotide sequence indicate start and stop codons. The two degenerate primers are indicated by arrows. The putative polyadenylation signals are double underlined. The translation end is marked with an asterisk; (B) RT-PCR was performed on total RNA isolated from *A. dahurica* root. The sequence of *AdBMT* was amplified using a pair of indicated degenerate primers.

Ultra performance liquid chromatography

After centrifugation, 5 ml aliquots of the supernatant were taken and mixed with ethyl acetate (1 ml) and centrifuged at 16,000 ×*g* for 2 min. The organic layer was collected and evaporated in vacuum after which the residue was dissolved in methanol (0.3 ml). The bergaptol and bergapten were then measured at indicated time intervals by UPLC.

The reaction product was analyzed using a Waters ACQUITYTM UPLC system (Waters Crop, Milford, MA, USA) that contains a cooling autosampler, a column oven enabling temperature control of analytical column and a photodiode array detector. For each injection, 5 μl of methanol-dissolved product was loaded onto a BEH C18 (2.1 mm × 18 cm, 1.7 μm) column and eluted with a water/acetonitrile mix (70/30) by UPLC pump at a flow rate of 0.25 ml/min. The column was maintained in an oven at 40ºC. Detection was at 254 nm. The reaction product was determined and quantitated in duplicate. The pure (authentic) bergaptol (ChromaDex, Inc., Irvine, CA, USA) and bergapten (Sigma-Aldrich Chemical Co., St Louis, MO, USA) were used as standard chemicals. Data were collected and processed by chromatographic software MassLynx (Waters Crop, Milford, MA, USA).

SDS-PAGE and immunoblotting

For protein fractionation, the cell pellets collected from 16°C were resuspended in 200 mM potassium phosphate buffer (pH 7.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA), sonicated and centrifuged at 16,000 ×*g* for 5 min at 4ºC. Total protein in the supernatant was mixed with 2x sample buffer [2% (w/v) sodium dodecyl sulfate (SDS) and 4% (v/v) 2-mercaptoethanol] and fractionated by 12% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was either stained with 0.25% Coomassie blue R-250, 10% acetic acid, and 5% ethanol for 4 h, and destained with 10% acetic acid and 20% ethanol or electroblotted onto nitrocellulose (0.45 μm, Sartorius Stedim, Biotech, Goettingen, Germany). The membrane was blocked at 37°C for 1 h in a solution containing 10 mM Tris-HCl, pH 7.5, 0.05% Tween-20, 150 mM NaCl and 5% nonfat dry milk. The membrane was incubated with anti-His-tag antibody with a 1:1,000 dilution for 2 h at 4°C after which the membrane was washed, incubated with alkaline phosphatase-conjugated secondary antibody for 1 h at 4°C. The locations of antigen-antibody complex were visualized by color development catalyzed by alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate substrate and nitro blue tetrazolium in a carbonate buffer (100 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8). Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

Enzyme purification and activity assays

For enzyme purification, the cell pellets collected from 16°C were resuspended in 200 mM potassium phosphate buffer (pH 7.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA), sonicated and centrifuged at 16,000 ×*g* for 5 min at 4°C. Because Ni-nitrilotriacetic acid (NTA) column is not suitable for BMT purification (Hehmann et al., 2004), total protein in the supernatant was fractionated by ammonium sulfate precipitation from which a fraction of 40-50% saturation was collected. Protein in this fraction was dissolved in 200 mM potassium phosphate buffer (pH 7.5) and desalted through PD-10 columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The ammonium sulfate-fractionated protein was further concentrated by centricon-30 (Millipore, Billerica, MA, USA) and its concentration was determined. Each fraction was subjected to enzyme activity assay.

Assay of BMT (EC 2.1.1.69) activity was as previously reported (Hehmann et al., 2004) with some modifications. Briefly, BMT activity in vitro was routinely measured at 35ºC in a volume of 200 μl containing 200 mM potassium phosphate buffer, pH 7.5, 1.25 mM bergaptol as a substrate (Indofine Chemical Co, Somerville, NJ, USA), and 9.6 μg of purified protein which corresponds to 2.0 nkat BMT activity (specific activity 0.21 kat/kg). The reaction was started with the addition of SAM (5 mM) and terminated after 90 min incubation by adding 30 μl of 1N HCl. The reaction mixture was extracted with ethyl acetate (0.5 ml) and centrifuged at 16,000 ×*g* for 2 min. The organic layer was collected and evaporated in vacuum after which the residue was dissolved in methanol (0.3 ml). The reaction product (bergapten) was then determined and quantitated using UPLC in duplicate. The extract of cells with empty vector was used as a negative control.

pH and temperature optimum and metal requirement of AdBMT

The optimal pH for enzyme activity was determined by incubations at 35°C for 90 min in different buffers (200 mM) ranging between 4.0 and 10.0 (acetate, pH 4.0-5.5; phosphate, pH 5.5-9.0; Tris-HCl, pH 7.0-8.5; carbonate, pH 8.5-10.0). The optimum temperature for enzyme activity was determined between 16°C and 42°C in 200 mM potassium phosphate buffer (pH 7.5) for 90 min. The effect of various metal ions at 0.1 and 1.5 mM concentrations on enzyme activity was determined in 200 mM potassium phosphate buffer (pH 7.5) at 35°C for 90 min. The addition of 5.4 mM EDTA in the buffer was used as a control. Three independent experiments in duplicate were carried out for each analysis.

Reactions were assayed under conditions in which product formation was linear for both time (15-120 min) and the concentration of purified protein (1.8-16 μg). The apparent Km and Vmax values were determined by Lineweaver-Burk plots using approximately 9.6 μg desalted protein extract and the adjustment of a serial dilutions of the substrates both bergaptol (0.625~7 mM) and SAM (1.25 mM~7.5 mM) in the enzyme reaction as described above.

RESULTS

Cloning and characterization of BMT cDNA from Bai Zhi

To obtain the BMT cDNA from Bai Zhi, total RNA isolated from Bai Zhi root was used as a template. A pair of degenerate oligonucleotide primers were designed from highly conserved regions of other plant BMT sequences as indicated in Figure 2A. Using RT-PCR amplifications, a single band of 107 bp DNA fragment corresponding to *AdBMT* sequence was obtained (Figure 2B). Because the cDNA size was partial, the method of 5′- and 3′-RACE-PCR was used to obtain the full length of 1,259 bp *AdBMT* cDNA (accession no. JN585954) excluding the poly (A) tail.

The *AdBMT* cDNA contained an open reading frame of 1080 bp encoding a polypeptide of 359 amino acids (Figure 2A) with a calculated molecular mass of 39 kDa and a calculated pI of 5.9. Assessment of its hydropathy profile (Kyte and Doolittle, 1982) showed that the AdBMT polypeptide did not have a strong hydrophobic region near the N-terminus, possibly indicating the absence of a signal peptide (data not shown). In the 3′-untranslated region, a variant AATAAC and two putative AATAAA consensus motifs of polyadenylation were located at 130, 113, and 102 bp upstream from the site of polyadenylation.

The predicted amino acid sequence of *AdBMT* was used to search protein databases. Sequence alignment revealed that the AdBMT polypeptide, in which all five conserved regions I-V (Ibrahim et al., 1998) were found, shared 28%-91% identity with plant OMTs. and identified the least with human OMT (13%) (Figure 3). AdBMT exhibits high sequence similarity with GlBMT (91% identity) and AmBMT (84% identity). Regions I and IV had been shown by X-ray diffraction to be involved in SAM and metal binding, respectively (Vidgren et al., 1994). In addition to the common signature of these highly conserved regions (regions I-V) characterized for SAM-dependent OMTs, motifs A and B were considered to govern the substrate specificity (Joshi and Chiang, 1998; Schroder et al., 2002).

Overexpression of recombinant AdBMT in *E. coli*

The open reading frame sequence of *AdBMT* was subcloned into an expression vector, pET32a, and overexpressed in *E. coli*. To determine the conditions for high functional AdBMT expression, cultures of the transformants were induced at various temperatures for 16 h in the presence of 1 mM IPTG. The *E. coli* cells were harvested by centrifugation and resuspended in LB medium for 24 h incubation with 100 μM bergaptol, and the formation of the product (bergapten) was quantified by UPLC as a biotransformation assay for cellular AdBMT activity. The overexpressed AdBMT in the cell may convert bergaptol into bergapten, which could be identified and quantitated by UPLC. Bergapten production significantly increased as the induction temperatures decreased (Figure 4). The maximal amount of bergapten was produced in incubations at 16°C, indicating the most suitable setting for highest AdBMT activity among the test temperatures. Therefore, 16ºC was chosen for further investigation. Controls with an empty vector were conducted in a parallel manner at each incubation temperature; no bergapten was detected in any of the control experiments (Figure 4).

The protein from each preparation was further separated by SDS-PAGE (Figure 5A). A 39-kDa protein was observed in cells harboring pET32a-BMT (lanes A2), whereas it was not detected in cells harboring empty pET32a (lane A1), suggesting that the recombinant AdBMT (with His-tag) was successfully expressed in *E. coli*. The AdBMT protein was immunologically detected using anti-His-tag antibody, as indicated by an arrow (Figure 5B, lane 2). The sizes of the detected proteins were all in good agreement with the expected size of 39 kDa predicted from the *AdBMT* sequence. The 17.5-kDa band produced by a pET32a vector was used as a control (Figure 5, lane 1).

Characterization of AdBMT

Because Hehmann et al. (2004) reported that the BMT activity of *A. majus* in the bacterial extracts was very labile and could not enable the extensive purification, the AdBMT extract isolated from the *E. coli* cells was thus, subjected to ammonium sulfate fractionation (40%-50% saturation) and subsequently desalted and concentrated for biochemical characterization.

As shown in Figure 6, bergapten formation was linear for both indicated time (15-120 min) and concentrations of purified protein (1.8-16 μg). Therefore, 9.6 μg of purified protein (specific activity 0.21 kat/kg) and 90 min of reaction time at 35ºC were taken to measure the optimal pH of AdBMT activity. It should be noted that the bergapten production reached a maximum level (36 μg) after 2-h incubation (Figure 6B). Significant activity of the recombinant AdBMT was observed from pH 6.5-9.0 with an optimum pH of around 7.5 either in potassium phosphate or in Tris-HCl buffer (Figure 7A). At pH 7.5, the AdBMT activity in the potassium phosphate buffer was active over a broad temperatures ranging from 16 to 42°C with an optimum temperature of around 35°C (Figure 7B). It should be noted that without the addition of SAM (5 mM), no bergapten was observed in the reaction mixture. The kinetic analysis revealed that the Km value of AdBMT to bergaptol is 0.56 mM whereas the Km value of AdBMT to SAM is 10.68 mM.

Plant OMTs can be categorized into two major classes, Class I and Class II. Class I is Mg^{2+}-dependent, while Class II does not require Mg^{2+} for catalytic activity (Joshi and Chiang, 1998; Kopycki et al., 2008). The effect at 0.1 or 1.5 mM concentrations of Mg^{2+} on enzyme activity was examined in the potassium phosphate buffer (pH 7.5) at 35°C for 90 min. No matter which concentration of Mg^{2+} was applied to the incubation, the production of bergapten

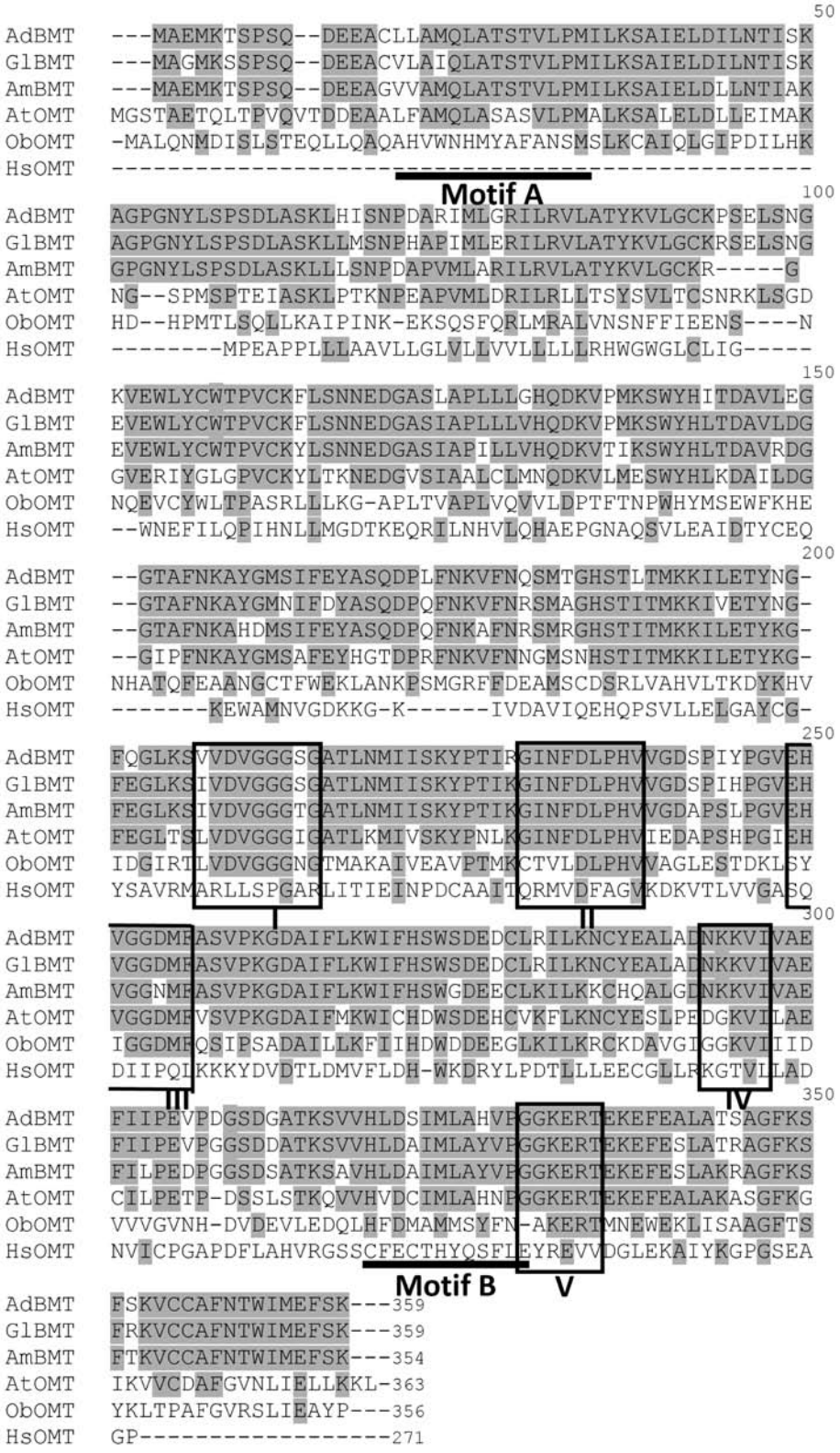

Figure 3. Amino acid alignment of the AdBMT protein sequence with other BMTs and OMT-related proteins. A dash in the sequence indicates a gap introduced to maintain good alignment. The five highly conserved regions (regions I-V) are indicated by a box. The motifs A and B are indicated by an underline. The two BMTs are GlBMT (AB363638), a bergaptol O-methyltransferase of *Glehnia littoralis*, and AmBMT (AY443006), a bergaptol O-methyltransferase of *Ammi majus*. The OMT-related proteins include AtOMT (NM_124796), a quercetin 3-O-methyltransferase of *Arabidopsis thaliana*; ObOMT (AB530137), a chavicol O-methyltransferase of *Ocimum basilicum*; and HsOMT (Z26491), a catechol O-methyltransferase of *Homo sapiens*.

was similar to that in the medium containing EDTA, a metal chelator, as a control, suggesting that AdBMT does not require Mg^{2+}. This result is consistent with that of AmBMT, whose catalytic activity is reported to be Mg^{2+}-independent (Hehmann et al., 2004).

The effect of several other metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, and Ca^{2+}) on enzyme activity was also examined. Significant inhibition of AdBMT activity was observed in the presence of Cu^{2+} (99% and 100%), Ni^{2+} (71% and 100%), and Co^{2+} (50% and 64%) (Figure 7C). The addition of Cu^{2+} and Ni^{2+} up to 1.5 mM completely inhibited AdBMT activity. Fe^{2+} and Zn^{2+} only slightly affected AdBMT activity at 0.1 mM concentration. However, the addition of Mn^{2+}, Fe^{3+}, and Ca^{2+} barely affected AdBMT catalytic activity (Figure 7C).

Efficient production of bergapten in the culture overexpressing AdBMT

The conversion kinetics of bergaptol into bergapten in the culture overexpressing AdBMT was further examined. After the *E. coli* cells were grown at 16°C for 16 h, the cells were harvested by centrifugation and resuspended in LB medium, When bergaptol was added to give a final concentration of 100 μM, the cells were incubated at 25°C for 48 h (Figure 8). For bergaptol conversion, 48 h was used instead. After a short reaction period of 2 h, the production of bergapten reached 180 μg, which corresponds to 60% of the maximal level observed after 24 h if the maximum level of bergapten production at 24 h was

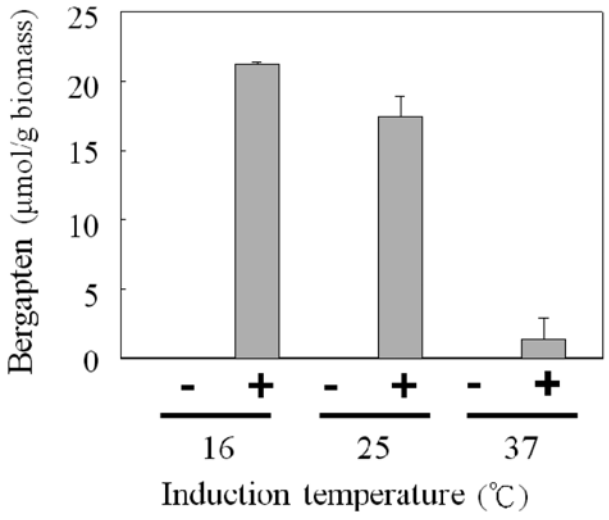

Figure 4. Estimation of the functional AdBMT overexpressed in *E. coli*. Fifty milliliters of cell culture overexpressing AdBMT and an empty vector were induced in the presence of 1 mM IPTG at the indicated temperatures for 16 h. The cells from each preparation were harvested by centrifugation, resuspended in 25 ml of LB and incubated at 25°C for 24 h with the addition of bergaptol to achieve a final concentration of 100 μM. The product (bergapten) in the reaction medium was estimated by UPLC. Bergapten is normalized on the basis of an equal amount of *E. coli* cells. Three independent experiments in duplicate were carried out for each analysis.

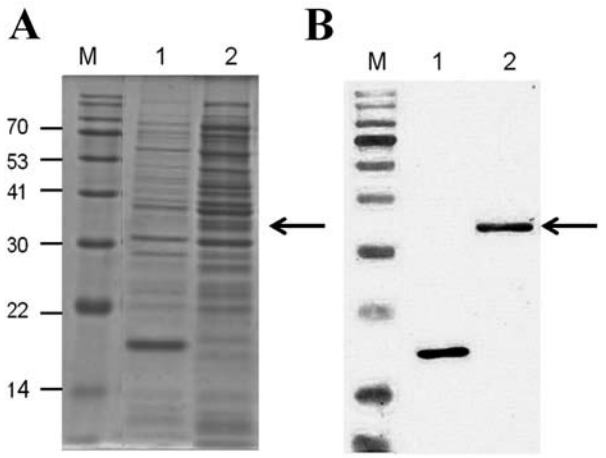

Figure 5. Overexpression of AdBMT in *E. coli*. The cells harboring empty pET32a (1) or pET32a-BMT (2) plasmids were expressed in the presence of 1 mM IPTG for 16 h at 16°C. The total protein was extracted from each preparation. An equal volume of protein was separated by SDS-PAGE and either stained with Coomassie blue (A) or electroblotted onto nitrocellulose and immunologically detected using anti-His-tag antibody (B). M indicates marker proteins (70, 53, 41, 30, 22, and 14 kDa). The arrows indicate the overexpressed AdBMT.

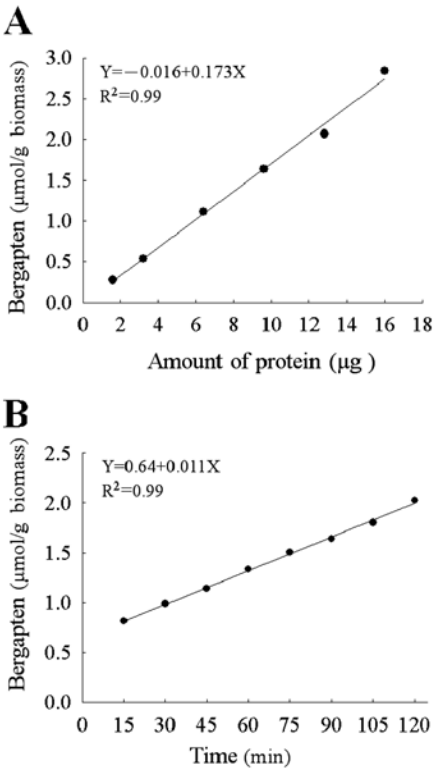

Figure 6. Bergapten production was linear for both indicated concentrations of purified protein and time period of reaction. Bergapten was estimated in a volume of 200 μl at 35°C (A) for 90 min with the addition of various amounts of purified protein or (B) for various indicated time intervals with 9.6 μg of purified protein (specific activity 0.21 kat/kg) isolated from AdBMT-overexpressed *E. coli* cells. Bergapten is normalized on the basis of an equal amount of *E. coli* cells.

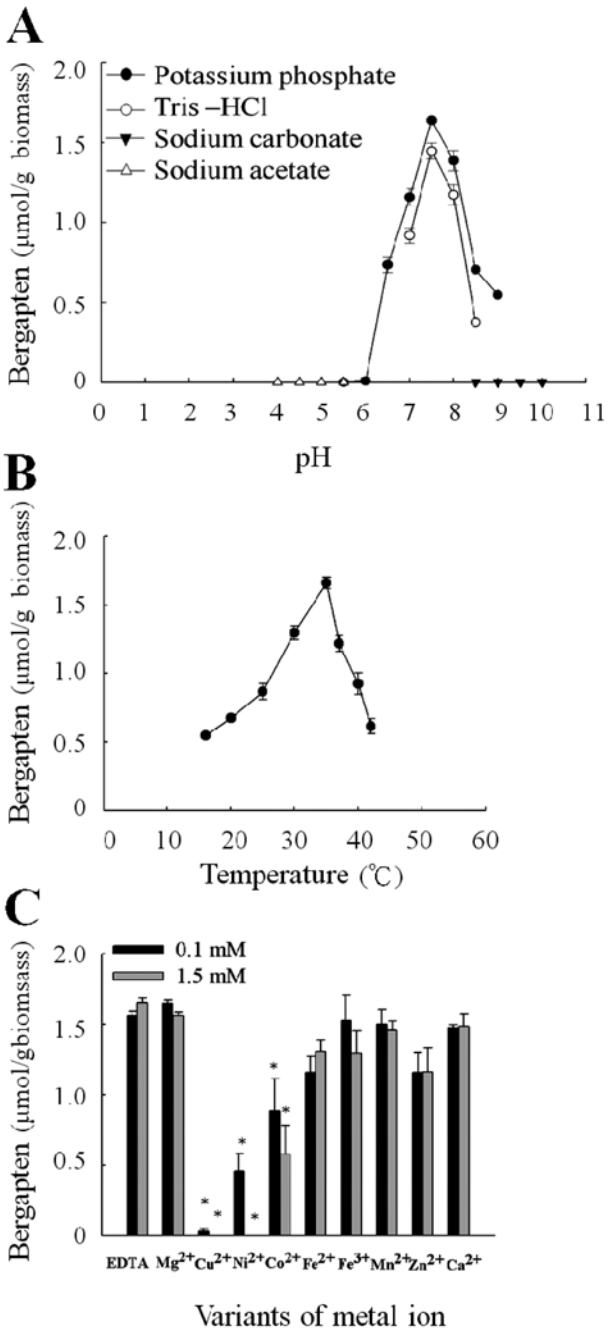

Figure 7. Effects of pH, temperature and metal ions on the recombinant AdBMT activity. Approximately 9.6 μg of ammonium sulfate-fractionated protein were added into (A) 200 mM of various indicated buffers which cover a wide pH range between pH 4.0 and 10.0, (B) into 200 mM potassium phosphate buffer (pH 7.5) and incubated at indicated temperatures from 16 to 42°C, or (C) into 200 mM potassium phosphate buffer (pH 7.5), all of which contain various metal ions at 0.1 and 1.5 mM concentrations, and incubated at 35°C, respectively. EDTA, a metal chelator, was added to the buffer as a control. Bergapten is normalized on the basis of an equal amount of *E. coli* cells. Three independent experiments in duplicate were carried out for each analysis. The asterisks indicate the significance of the difference from the control value determined by the Student's t-test (*$P < 0.01$). The error bars represent SD.

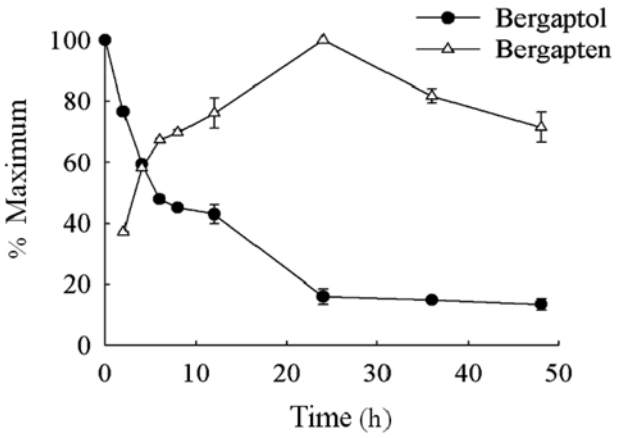

Figure 8. Efficient production of bergapten in the culture overexpressing AdBMT. Fifty milliliters of cell culture overexpressing AdBMT were induced in the presence of 1 mM IPTG at 16°C for 16 h. The cells were harvested by centrifugation, resuspended in 25 ml of LB medium and incubated at 25°C for 48 h with the addition of bergaptol to achieve a final concentration of 100 μM. Various time-intervals were taken to estimate the concentration of bergapten and bergaptol in the medium by UPLC. Bergapten is normalized on the basis of an equal amount of *E. coli* cells. The maximum production of bergapten at 24 h is indicated as 100%. Three independent experiments in duplicate were carried out for each analysis.

counted as 100% (Figure 8). It is worthy of noting that under the same reaction time (i.e. 2 h) the production of bergapten was 5-fold higher than that produced by the ammonium sulfate-purified AdBMT fraction (36 μg) derived from the same amount of cells. When the incubation time extended to 24 h, the production of bergapten reached a maximum yield of 460.1 μg, which was approximately 13-fold higher than that produced by the ammonium sulfate-precipitated AdBMT fraction that is also derived from the same amount of cells (Figure 8). Afterwards, the concentration of the bergapten gradually decreased to 71% of conversion after 48 h of incubation.

DISCUSSION

Bai Zhi, a Chinese herb, has long been used as a face cream for skin-whitening purposes. The mechanism of skin-whitening compositions was not scientifically proven until Piao et al. (2004) reported that ethyl acetate extracts of *A. dahurica* that had potential inhibitory activity against mushroom tyrosinase. Tyrosinase is a rate-limiting enzyme that converts tyrosine to 3,4-dihydroxyphenylalanine and subsequently oxidizes it to form dopaquinone, which leads to the ultimate formation of melanin. Considering that tyrosinase inhibitors prevent the formation of melanin, they may result in reductions in skin darkness. The active skin-whitening material isolated from Bai Zhi root was identified as 8-hydroxybergapten (Piao et al., 2004), which is a compound converted from bergapten by one-step hydroxylation. Bergapten, however, is the product from bergaptol

catalyzed by BMT, a key enzyme of the furanocoumarin biosynthesis pathway.

A earlier report indicated that BMT is labile and thus, the method of ammonium sulfate fractionation is adopted for enzyme purification (Hehmann et al., 2004). Accordingly, AdBMT was routinely isolated from *E. coli* cell culture and purified by ammonium sulfate fractionation. A simple and efficient method is developed to produce bergapten in *E. coli* (Figure 8). The production of bergapten in vivo has several major advantages. First, given the same amount of *E. coli* cells, a large amount of bergapten can be produced in the fluid. After a short reaction period of 2-h (Figure 8), the production of bergapten in vivo is 5-fold higher than that produced by the ammonium sulfate-purified AdBMT fraction derived from the same amount of cells. More importantly, the incubation of the medium can be further extended to 24 h to reach maximal production of bergapten, but not for the reaction that is conducted in vitro because the exogenous SAM and added AdBMT may be labile. The production of bergapten in vivo is, thus, 13-fold higher than that produced by the ammonium sulfate-purified AdBMT fraction (Figure 8). These results suggest that the ammonium sulfate-purified AdMBT fraction significantly decreases its enzyme activity probably due to the processes of protein extraction and purification. Second, SAM is no longer necessary to be added into the reaction in vitro because *E. coli* can self-supply the cofactor for methylation and thus, greatly reduce production costs. Third, the purification of BMT from the cells is unnecessary. *E. coli* can utilize the supplemented bergaptol and convert it into bergapten, which is finally released to the medium although the secretory mechanism of bergapten into the medium is unclear. Given the fact that bergapten can be detected in the medium, the isolation and purification of bergapten is thus greatly simplified. It is consistent with a previous report published by Ishikawa et al. (2009), who indicated that bergapten can be produced in the fluid of *G. littoralis* cell suspension cultures. However, the production of bergapten in *E. coli* cell culture is more convenient than that produced in the fluid of *G. littoralis* cell suspension because of the preparation of *E. coli* is easy and widely used.

The amino acid sequence of AdBMT shares high sequence similarity with the BMT sequences from *G. littoralis* and *A. majus*. In general, all plant OMTs contain five highly conserved regions (regions I-V) characterized for SAM-dependent OMTs (Vidgren et al., 1994; Ibrahim et al., 1998). The two motifs A and B are considered to control the substrate specificity (Joshi and Chiang, 1998; Schroder et al., 2002). The existence of highly conserved motifs A and B in these three BMT may reflect that they utilize the same substrate (i.e., bergaptol). This is supported by the fact that AmBMT from *A. majus* also uses bergaptol as a substrate. However, these common structural features are hardly found in animal OMTs (i.e., human).

AdBMT is classified as a member of Class II OMTs (Kopycki et al., 2008) and its enzyme activity is Mg^{2+}-independent (Figure 7C). However, the addition of Cu^{2+}, Ni^{2+}, and Co^{2+} at concentrations even as low as 0.1 mM severely inhibited enzyme activity. Similar results were described for heterologous OMTs (Morishige et al., 2000; Hehmann et al., 2004). In addition, the loss of enzyme activity may also due to the following purification processes such as elution and each concentrated step. Given that Ni^{2+} ions inhibit BMT activity, the Ni-NTA column commonly used to purify protein is not suitable for BMT purification. Thus, ammonium sulfate precipitation is adopted for the purification of AdBMT from protein extracts of AdBMT-overexpressing cells.

In conclusion, the *BMT* cDNA was identified from Bai Zhi and overexpressed in *E. coli*. The optimum conditions of AdBMT were determined in potassium phosphate buffer, and it was found that the enzyme does not require Mg^{2+} for catalytic activity. A simple and efficient production of bergapten in the culture fluid was developed. With the supply of bergaptol in the medium, *E. coli* cells can be used as a potential bioreactor to produce bergapten.

Acknowledgments. This work was supported by National Science Council Grant NSC98-2311-B-005-002-MY3 in Taiwan to C.-S. Wang.

LITERATURE CITED

Alexander, N.J., S.P. McCormick, and J.A. Blackburn. 2008. Effects of xanthotoxin treatment on trichothecene production in *Fusarium sporotrichioides*. Can. J. Microbiol. **54:** 1023-1031.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389-3402.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

Dong, C., D. Anzellotti, R.K. Ibrahim, N.P. Huner, and F. Sarhan. 2003. Daphnetin methylation by a novel O-methyltransferase is associated with cold acclimation and photosystem II excitation pressure in rye. J. Biol. Chem. **278:** 6854-6861.

Frick, S. and T.M. Kutchan. 1999. Molecular cloning and functional expression of O-methyltransferases common to isoquinoline alkaloid and phenylpropanoid biosynthesis. Plant J. **17:** 329-339.

Hehmann, M., R. Lukacin, H. Ekiert, and U. Matern. 2004. Furanocoumarin biosynthesis in *Ammi majus* L. Cloning of bergaptol O-methyltransferase. Eur. J. Biochem. **271:** 932-940.

Ibrahim, R.K., A. Bruneau, and B. Bantignies. 1998. Plant O-methyltransferases: molecular analysis, common signature and classification. Plant Mol. Biol. **36:** 1-10.

Ishikawa, A., T. Kuma, H. Sasaki, N. Sasaki, Y. Ozeki, N. Ko-

bayashi, and Y. Kitamura. 2009. Constitutive expression of bergaptol O-methyltransferase in *Glehnia littoralis* cell cultures. Plant Cell Rep. **28:** 257-265.

Joshi, C.P. and V.L. Chiang. 1998. Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. Plant Mol. Biol. **37:** 663-674.

Kopycki, J.G., D. Rauh, A.A. Chumanevich, P. Neumann, T. Vogt, and M.T. Stubbs. 2008. Biochemical and structural analysis of substrate promiscuity in plant Mg^{2+}-dependent O-methyltransferases. J. Mol. Biol. **378:** 154-164.

Kuete, V., R. Metuno, B. Ngameni, A.M. Tsafack, F. Ngandeu, G.W. Fotso, M. Bezabih, F.X. Etoa, B.T. Ngadjui, B.M. Abegaz, and V.P. Beng. 2007. Antimicrobial activity of the methanolic extracts and compounds from *Treculia obovoidea* (Moraceae). J. Ethnopharmacol. **112:** 531-536.

Kwon, Y.S., A. Kobayashi, S.I. Kajiyama, K. Kawazu, H. Kanzaki, and C.M. Kim. 1997. Antimicrobial constituents of *Angelica dahurica* roots. Phytochemistry **44:** 887-889.

Kyte, J. and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:** 105-132.

Morishige, T., T. Tsujita, Y. Yamada, and F. Sato. 2000. Molecular characterization of the S-adenosyl-Lmethionine: 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase involved in isoquinoline alkaloid biosynthesis in *Coptis japonica*. J. Biol. Chem. **275:** 23398-23405.

Piao, X.L., S.H. Baek, M.K. Park, and J.H. Park. 2004. Tyrosinase-nhibitory furanocoumarin from *Angelica dahurica*. Biol. Pharm. Bull. **27:** 1144-1146.

Schröder, G., E. Wehinger, and J. Schroeder. 2002. Predicting the substrates of cloned plant O-methyltransferases. Phytochemistry **59:** 1-8.

Tietjen, K.G., D. Hunkler, and U. Matern. 1983. Differential response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. 1. Identification of induced products as coumarin derivatives. Eur. J. Biochem. **131:** 401-407.

Vidgren, J., L.A. Svensson, and A. Liijas. 1994. Crystal structure of catechol O-methyltransferase. Nature **368:** 354-358.

白芷佛手柑內醇甲基轉移酶之分子選殖和功能分析以及利用大腸桿菌有效率的量產佛手柑內酯

羅淑卿[1, 2]　鍾佩恩[2]　王國祥[1]

[1] 國立中興大學 生物科技學研究所

[2] 行政院農業委員會 農業試驗所農業化學組

白芷中草藥古早以來具有面霜潤膚之美白功效。其中已知的一個美白成分，氫氧基化佛手柑內酯 (8-hydroxybergapte) 是佛手柑內酯 (bergapten) 氫氧基化的產物，而佛手柑內酯是由佛手柑內醇甲基轉移酶 (bergaptol 5-O-methyltransferase, BMT) 轉化佛手柑內醇 (bergaptol) 而成。從來自於其他植物 BMT 高保留區的序列中設計一對退化引子 (degenerate primers)，再從白芷根中選殖出單一的 *AdBMT* DNA 片段。利用 5′- 與 3′-RACE-PCR 對白芷 DNA 片段進行聚合酶連鎖增幅反應以取得全長的 cDNA 序列。此 AdBMT 可編譯區爲 1,080 個核苷酸，可轉譯出 359 個胺基酸之蛋白質，預測分子量爲 39 kDa。經序列比對分析顯示 AdBMT 和其它植物 O-methyltransferases 具有相當程度的相同度 (identity)，序列中含有的 I-V 區域全部保留。將 AdBMT 轉形至大腸桿菌可表達出含 His-tag 的融合蛋白質，進一步硫酸胺鹽沈澱以局部純化蛋白質。AdBMT 於 pH 7.5 之磷酸鉀緩衝液和 35°C 的反應溫度下的活性最佳，且其活性不須要二價離子的存在。AdBMT 的活性受到 Cu^{2+}、Ni^{2+} 及 Co^{2+} 等離子的嚴重抑制，即使濃度低至 0.1 mM。在培養液中含有 *AdBMT* 基因之大腸桿菌可簡便有效率的轉化佛手柑內醇 (bergaptol) 成大量的佛手柑內酯，其產量比經過硫酸胺鹽純化之酵素所能催化的量還多出 13 倍。大腸桿菌可成爲有潛力的生物反應器，以生產佛手柑內酯。

關鍵詞：白芷；佛手柑內酯；佛手柑內醇甲基轉移酶；cDNA 選殖；酵素活性。