Expression, Purification and Activity Assay of the Recombinant Protein of Catechol-O-Methyltransferase from Chinese White Shrimp (*Fenneropenaeus chinensis*)

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Abstract: Problem statement: We have previously cloned a gene of Chinese white shrimp Catechol O-Methyltransferase (designated Fc-COMT) and characterized the gene expression pattern. In this study, expression and purification as well as activity assay of the recombinant Fc-COMT was further conducted. Approach: Using pET-30a (+) as a prokaryotic expression vector, the recombinant Fc-COMT was expressed in the supernatant of *Escherichia coli* lysate and easily purified by His-Bind resin chromatography. SDS-PAGE analysis showed that the molecular mass of recombinant Fc-COMT was approximately 30,000 Da, in good agreement with the software-predicted molecular weight. The enzymatic activity of recombinant Fc-COMT was tested using Dihydroxybenzoic Acid (DHBAc) as a substrate. Results: The methyl products of DHBAc, Vanillic Acid (VA) and Isovanillic Acid (IVA), were detected in the enzymatic reaction mixture with recombinant Fc-COMT by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS). Conclusion: The recombinant Fc-COMT has catalytic activity of transferring methyl group from S-Adenosyl-L-Methionine (SAM) to the 3′ hydroxyl or 4′ hydroxyl group of benzyl ring of DHBAc.

Key words: *Fenneropenaeus chinensis*, recombinant Fc-COMT, VA, HPLC-MS

INTRODUCTION

Catechol-O-Methyltransferase (COMT, E.C.2.1.1.6.) is one of O-methyltransferases that catalyse the formation of methoxylated products by transferring one methyl group from S-adenosyl-L-methionine to the hydroxyl group of molecules containing a catechol moiety in the presence of Mg²⁺ (Axelrod and Tomchick, 1958). Thus, COMT can inactivate catecholamines and other catechol-type compounds including many catechol-containing xenobiotics and drugs (Mannisto *et al.*, 1992; Bonifacio *et al.*, 2002).

Researchers have achieved a lot in COMT studies including gene cloning (Bertocci *et al.*, 1991), gene expressions (Matsumoto *et al.*, 2003; Tilgmann and Ulmanen, 1996), gene functions (Chen *et al.*, 2004), enzyme kinetics (Bonifacio *et al.*, 2002) and enzyme inhibitors (Mannisto *et al.*, 1992), most of which were from mammal COMTs, especially from human COMT. For example, it has been found that the sequence variations of the COMT gene and the COMT activity level were associated with cancer risk and schizophrenia susceptibility in human (Karayiorgou *et al.*, 1998; Cheng *et al.*, 2005). COMT inhibitors have also been developed as adjuvant drugs in the treatment of Parkinson's disease (Schrag, 2005). So, COMT is involved in the studies of pharmacology and etiology for some diseases.

Since human COMT was reported in 1958, COMTs have been found in invertebrates (Guldberg and Marsden, 1975). However, no crustacean COMT, except for Chinese white shrimp (*F. chinensis*) COMT (Fc-COMT), has yet been reported. We have previously cloned a Fc-COMT gene and characterized the gene expression pattern (Li *et al.*, 2006). The sequence data of the Fc-COMT gene has also been submitted to the GenBank databases under accession number DQ091255. In order to further analyze the enzymatic activity of the Fc-COMT, this study was conducted.

COMT activity may be estimated from the reaction products that were made by enzymatic O-methylation of substrates after termination of the enzymatic reaction (Axelrod and Tomchick, 1958). It was known that the substrates of COMT were various, some of which were...
endogenous like catecholamines, dopamine, epinephrine and catecholestrogens, the others were exogenous including 3,4-Dihydroxybenzoic Aldehyde (DHBAlD), 3,4-Dihydroxybenzoic Acid (DHBAc) and 3,4-Dihydroxybenzoic Alcohol (DHBAlc) (Axelrod and Tomchick, 1958; Koh et al., 1991). However, in most in vitro cases, COMT preferred DHBAc to endogenous catecholamines, dopamine and epinephrine as substrate (Pihlavisto and Reenila, 2002). A wide variety of COMT enzyme assays have also been developed such as spectrophotometric assay (Borchardt, 1974), radioassay (Gulliver and Tipton, 1978), fluorometric method (Okada et al., 1981) and so on. Of which high-performance liquid chromatography was high-sensitivity and double-quick for COMT enzyme assays (Pihlavisto and Reenila, 2002). Additionally, mass spectrometric can be used to identify the reaction products in the COMT enzyme assay (Vilbois et al., 1994). High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) therefore was employed to analyze the reaction products of purified recombinant Fc-COMT using DHBAc as substrate after termination of the enzymatic reaction.

MATERIALS AND METHODS

Chemicals: Restriction enzymes EcoRI, XhoI and Taq polymerase were purchased from TaKaRa Biotechnology Company (Dalian, China). T4 DNA ligases and DNA purification kit from Sangon Company (Shanghai, China). pET-30a(+) vector, monohydrogen phosphate (NaH2PO4), magnesium chloride, disodium phosphate buffer (pH7.4) from Sigma (St. Louis, MO, USA). Phosphoric acid, (Italy). S-Adenosyl-L-Methionine iodide (SAM) was product of Amersham Biosciences Company (Buckinghamshire, England).

Construction of Fc-COMT expression vector: Using the primer pair Met-Ex-F1 (5’-TACTCAGAATTCTGTCTTCTCTGCCAGATTTAC-3’) and Met-Ex-R1 (5’-TACTCAGGAGGAGATGTTACCTATCGA-3’), the ORF of the Fc-COMT gene was amplified from shrimp cDNA library. Underlined bases are the restriction enzyme sites of EcoRI and XhoI PCR reaction conditions included predenaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 57°C for 45 sec, 72°C for 1 min and an extension at 72°C for 10 min. The PCR product was separated on an 1% agarose gel. A band of about 700 bp was purified by DNA purification kit.

Both the purified cDNA fragment of the Fc-COMT gene and pET-30a(+) vector were cut individually by EcoRI and XhoI The digested products were separately purified using gel purification kit and then were linked into the expression vector pET-30a(+)/FcCOMT using T4 DNA ligases. The expression vector was transformed into E. coli DH5α cells to verify the sequence correct by restriction analysis and sequencing.

Expression and purification of recombinant Fc-COMT: The obtained expression plasmid pET-30a(+)/FcCOMT was transformed into E. coli BL21(DE3) cells fertilized on LB plate with 50 µg mL−1 kanamycin at 37°C overnight. Single white clone was selected to be cultured in 5 mL LB (50 µg mL−1 kanamycin) liquid overnight. The overnight culture (1 mL) was inoculated into 100 mL of fresh LB medium. When bacteria grew to a density of OD600 = 0.6, the recombinant protein was induced by addition of isopropyl β-D-Thiogalactopyranoside (IPTG) to a final concentration of 0.4 mmol L−1. Cells were harvested after 5 h subsequently culturing and resuspended in PBS containing 0.2% Triton X-100. Following cell sonication, the cell lysate was centrifuged at 10,000 g for 10 min at 4°C and the supernatant and pellet were collected respectively and used for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1974).

Following the manufacturer’s instructions, the recombinant Fc-COMT was purified using His-Bind resin (Novagen, Madison, WI). The purified protein was subjected to 12.5% SDS-PAGE analysis and quantified by Bradford (1976) assay, then aliquoted and stored at -80°C until its activity assay.

Activity assay of recombinant Fc-COMT using HPLC-MS: Using DHBAc as substrate, the enzymatic reaction products of Vanillic Acid (VA) and Isovanillic Acid (IVA) were detected by HPLC-MS according to methods previously described (Li et al., 2004; Reenila and Rauhala, 2009; Reenila et al., 1995).

Briefly, stock solution of standard VA and DHBAc were prepared as follows. First VA was dissolved in 100 µL acetonitrile, then was adjusted to 2 mmol L−1 with 10 mmol L−1 sodium phosphate buffer (pH7.4) and...
stored at -20°C. VA can be diluted to µmol L\(^{-1}\) work concentration with super-pure water. About 1 mmol L\(^{-1}\) of DHBAc can be prepared with super-pure water, stored at -20°C and diluted to 200 µmol L\(^{-1}\) with super-pure water when use.

According to the literature (Reenila et al., 1995), reaction mixture (1 mL) contained 5 mmol L\(^{-1}\) MgCl\(_2\), 200 µmol L\(^{-1}\) DHBAc, 200 µmol L\(^{-1}\) SAM and 30 µg recombinant Fc-COMT in 100 mmol L\(^{-1}\) sodium phosphate buffer (pH 7.4). Sample without enzyme was run as control. After 20 min incubation at 37°C in a dark shaking water bath, 200 µmol L\(^{-1}\) SAM were added and left there for another 20 min. Reaction was stopped by the addition of 100 µL of 4 mol L\(^{-1}\) perchloric acid and keeping in ice-bath for 10 min. Protein precipitate was removed by centrifugation of 6000 g at 4°C for 10 min. Supernatant was filtered by 0.22 µm filter and then 20 µL of reaction solution was used to HPLC analysis.

HPLC analysis was carried out according to the report (Li et al., 2004) using N2000 HPLC system that consisted of an LC-10ATUP liquid chromatography (SHIMADZU), an SPD-10AUP UV-VIS detector (SHIMADZU) and an reverse-phase C\(_{18}\) column (5 µm, 250 × 4.60 mm i.d.). The mobile phase was 15% acetonitrile with a flow rate of 1.0 mL min\(^{-1}\), which was adjusted to pH3.0 with acetic acid. The column temperature was maintained at 30°C. Peaks of chromatogram were scanned by the UV detector at 260 nm along with aliquots (20 µL) of the samples injected by an autosampler.

HPLC-MS assay used API 4000™ LC/MS/MS System (Applied Biosystems, US) with Electrospray Inoization (ESI) in negative-ionization mode. The mass scan ranged from m/z 50-500.

RESULTS

Expression and purification of recombinant Fc-COMT: When sequencing analysis confirmed that the DNA sequence and protein sequence of pET30a(+)FcCOMT were correct, the recombinant plasmid was transformed into E. coli cells and recombinant Fc-COMT was induced by IPTG. As a result, the recombinant Fc-COMT band of 30 kDa was observed by SDS-PAGE analysis. The protein reached it’s the highest amount after being inducted for 4 h (Fig. 1). Also, the recombinant Fc-COMT existed in the supernatant of E. coli lysate in soluble form and easily purified by the His-Bind resin chromatography (Fig. 2).

Activity assay of recombinant Fc-COMT: Under HPLC analysis, VA of 0.4 µmol L\(^{-1}\) was underdetectable, but 16 µmol L\(^{-1}\) VA gave a single peak of chromatogram that had 10 mv height and an retention time of 9.4 min (Fig. 3a), also, the mixture of 200 µmol L\(^{-1}\) DHBAc and 10 µmol L\(^{-1}\) VA showed no interfered peak of chromatogram, in which the VA peak had the same retention time to that VA peak in Fig. 3a and the retention time of the DHBAc peak was 5.2 min (Fig. 3b). The chromatogram of reaction mixture as control containing 200 µmol L\(^{-1}\) DHBAc and 200 µmol L\(^{-1}\) SAM without recombinant Fc-COMT gave several peaks, of which the height of DHBAc peak was 130 mv.

![Fig. 1: SDS-PAGE analysis and Coomassie Blue staining of bacterial protein extracts induced by IPTG. Lane 1 is molecular mass markers. Lane 2 is crude protein extracts of bacteria of non-induction; Crude extracts of bacteria cells induced for 5, 4, 3, 2 and 1 h from lane 3 to lane 7 (protein loading is 40 µg well\(^{-1}\), 12.5% gel)](image1)

![Fig. 2: SDS-PAGE analysis and Coomassie Blue staining of recombinant protein purified. Lane 1: Crude extract of uninduced bacteria cells; Lane 2: Crude extract of induced bacteria cells. Soluble and insoluble protein fraction of bacteria cells induced for 4 h in Lane 3 and Lane 4 respectively. Recombinant protein purified by His•Bind affinity column in Lane 5. Lane 6 for molecular mass markers (protein loading is 15 µg well\(^{-1}\), 12.5% gel)](image2)
Fig. 3: The activity assay of recombinant Fc-COMT by HPLC. (a) Chromatogram of standard VA, whose concentration is 16 µm. (b) Chromatogram of standard VA of 10 µm and 200 µm DHBAc. (c) Chromatogram of the control containing 200 µM DHBAc and 200 µM SAM without enzyme. (d) Chromatogram of reaction solution with 30 µg recombinant Fc-COMT except for 200 µM DHBAc and 200 µM SAM. Peak 1 and peak 2 stand for standard VA and DHBAc respectively. Peak 3 and Peak 4 stand for two methyl products of DHBAc. Injection volume was 20 µL. Reaction mixture were scanned by UV detector.

Fig. 4: Mass spectrum of peak 3 in Fig. 3

No peak was observed in the retention time from 9.4-17 min (Fig. 3c). However, as compared with the control, two kinds of new peaks appeared in the chromatogram of reaction mixture containing 30 µg recombinant Fc-COMT, the main new-peak was at retention time of 9.4 min and had 20 mv peak height and the smaller at 10.4 min (height 2.3 mv). In addition, the height of DHBAc peak decreased to 96 mv (Fig. 3d).

Using HPLC-MS to scan the reaction mixture, the mass spectra of the main new-peak with retention time of 9.4 min in HPLC analysis was m/z 167.3 of its [M-H] peak (Fig. 4). Then the main new-peak should stand for a kind of substance with molecular weight of 168.3.

**DISCUSSION**

According to previous report (Li et al., 2006), the Fc-COMT gene contained a single Open Reading Frame (ORF) of 666 bp encoding a protein of 221 amino acids with the predicted molecular weight of 24.57 kDa. When the ORF of the Fc-COMT gene was constructed into prokaryotic expression vector pET30a (+), the recombinant Fc-COMT will have a His tag of about 5 kDa at its N terminal. So, It can be speculated that the 30 kDa induced protein should be the fusion of the Fc-COMT and the His tag in Fig. 1.

Because COMT can catalyze the transferring of methyl group from SAM to the 3’ hydroxyl or 4’ hydroxyl group of benzyl ring of DHBAc, two types of methyl products of 4-hydroxy-3-methoxybenzoic acid (VA) and 3-hydroxy-4-Methoxybenzoic Acid (IVA) should be formed respectively (Tuomainen et al., 1996). Here, the master maps of Fig. 3 (Fig. 5) have revealed either standard VA or substrate DHBAc all gave a single peak of chromatogram and their retention times were 9.4 and 5.2 min respectively (Fig. 5a, 5b). This indicated the standard VA and the substrate DHBAc all had high purity. In the control chromatogram (Fig. 5c) there was no product peak from the retention time of 9.4-17 min.
Fig. 5: The activity assay of recombinant Fc-COMT by HPLC. (a) Chromatogram of standard VA, whose concentration is 16 µm; (b) Chromatogram of standard VA of 10 µM and 200 µm DHBAc; (c) Chromatogram of the control containing 200 µm DHBAc and 200 µm SAM without enzyme; (d) Chromatogram of reaction solution with 30 µg recombinant protein except for 200 µm DHBAc and 200 µM SAM. Peak 1 and peak 2 stand for standard VA and DHBAc respectively. Peak 3 and peak 4 stand for two methyl products of DHBAc. Injection volume was 20 µL. Reaction mixture were scanned by UV.

Fig. 6: Mass spectrum of peak 3 in Fig. 5d

While in the reaction chromatogram two of new peaks, marked 3 and 4, appeared at the retention time of 9.4 min and 10.4 min respectively and the height of DHBAc peak had declined by 34 mv (Fig. 5d). So it was supposed that
the DHBAc had been methylated by the recombinant Fc-COMT and the two of new peaks may be product-peaks.

By comparing, the peak 3 was bigger than Peak 4 and had the same retention time as standard VA, in addition, when VA was added to the reaction mixture, the big peak became higher and no additional peak was observed (data not shown). The big peak may be stand for a kind of product, VA. HPLC-MS further validated the product had a molecular weight of 168.3 that was the same as VA (Fig. 6). Taken together, the peak 3 should stand for the reaction product VA, while that peak 4 with retention time 10.4 min (height 2.3 mv) should stand for the second product of IVA. Since the methyl products of DHBAc, VA and IVA, have been detected in the reaction mixtures with the recombinant Fc-COMT by HPLC-MS, the recombinant Fc-COMT has COMT activity.

CONCLUSION

This research details the expression and purification as well as activity assay of recombinant Fc-COMT. The recombinant Fc-COMT existed in the supernatant of E. coli lysate in soluble form and was easily purified by the His-Bind resin chromatography. Two types of methyl products of DHBAc, VA and IVA, were detected in the enzymatic reaction mixtures with recombinant Fc-COMT by HPLC-MS. Therefore, we can conclude the recombinant Fc-COMT has been successfully expressed and purified from E. coli strain and the recombinant Fc-COMT has catalytic activity of transferring methyl group from SAM to the 3' hydroxyl or 4' hydroxyl group of benzyl ring of DHBAc.

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