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Cloning, Expression, and Characterization of Protein Carboxyl *O*-methyltransferase from Porcine Brain

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Protein carboxyl *O*-methyltransferase (E.C.2.1.1.24) may play a role in the repair of aged protein that is spontaneously incorporated with isoaspartyl residues. The porcine brain carboxyl *O*-methyltransferase was cloned in the pET32 vector, and overexpressed in *E.coli* (BL21) that harbors pETPCMT, which encodes 227 amino acids, including tagging proteins at the N-terminus. The protein sequence of the cloned porcine brain PCMT (r-pbPCMT) shares a 98% identity with that of human erythrocyte PCMT and rat brain PCMT. It is 100% identical with that of bovine brain. The r-pbPCMT was purified using Ni-NTA affinity chromatography and digested by enterokinase in order to remove the protein tags. Then Superdex 75HR gel filtration chromatography was performed. The r-pbPCMT exhibited similar *in vitro* substrate specificities with the PCMT that was purified from porcine brain. The molecular weight of the enzyme was estimated to be 24.5 kDa on the SDS polyacrylamide gel electrophoresis. The K_m value was 1.1×10^{-7} M for *S*-adenosyl-L-methionine. *S*-adnosyl-L-homocysteine was a competitive type of inhibitor with the K_i value of 1.38×10^{-4} M. The enzyme has optimal activity at pH 6.0 and 37°C. These results indicate that the expressed enzyme is functionally similar to the natural protein. It also suggests that it may be a suitable model to further understand the function of the mammalian enzyme.

Keywords: Cloning, Expression, Porcine brain protein carboxyl *O*-methyltransferase

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Introduction

The methyl esterification of side-chain carboxyl groups of the glutamate or aspartate residues of protein substrates that utilize S-adenosyl-L-methionine (AdoMet) as a methyl donor is catalyzed by protein carboxyl-*O*-methyltransferase (PCMT: S-adenosyl methionine: protein-carboxyl *O*-methyltransferase, EC 2.1.1.24; Protein methylase II; PM II), yielding protein-methyl ester and methanol (Paik and Kim, 1971; 1980). Since Kim and Paik (1970) originally purified this enzyme from calf thymus, PCMT activity has been observed in the pituitary gland (Diliberto and Axelrod, 1974), adrenal gland (Diliberto and Axelrod, 1976), brain (Iqubal and Steenson, 1976), and erythrocyte of mammals (Kim, 1974). The enzyme was also identified in a variety of organisms. These include bacteria (Fu *et al.*, 1991; Ichigawa and Clarke, 1998), nematodes (Kagan and Clarke, 1995), and plants (Mudgett and Clarke, 1993). In these organisms, the enzymes catalyze the transfer of a methyl group from AdoMet to the abnormal α-carboxyl group of L-isoaspartyl or D-aspartyl residues (Janson and Clarke, 1980; OConner and Clarke, 1983). It is then known by a different name and EC number - protein L-isoaspartyl methyltransferase (PIMT, EC 2.1.1.77) (Aswad, 1995).

It was recently reported that PCMT has an important role in disease states and biological phenomenon, including apoptosis (Huebscher *et al.*, 1999; Shimizu *et al.*, 2000). In Alzheimers disease and human cataractous lens, the level of PCMT of normal tissues was different than those of diseased tissues (Kodama *et al.*, 1995; Kondo *et al.*, 1996). Since protein isomerization of L-isoaspartate residues causes structural changes and enhances the aggregation process in modified proteins, this post-translational modification may be one of the progression factors in Alzheimer's Disease (AD) (Shimizu *et al.*, 2000). During the aging of rat tubulin, the accumulation of isoaspartate sites was identified *in vivo* and *in vitro* (Najbauer *et al.*, 1996). PCMT may repair damaged or aged

proteins that accumulate isoaspartyl residues. In a study using knockout mice, the deficiency of this enzyme resulted in fatal progressive epilepsy, retardation of growth, and fetal seizures (Kim *et al.*, 1997; Yamamoto *et al.*, 1998). Therefore, it was proposed that PCMT might be essential for normal growth and function of the central nervous system.

The molecular weights of mammalian PCMTs were reported to be between 24 and 28 kDa in all of the tissues that were examined, except for the enzyme from an ox's brain, which was 34 kDa (Iqubal and Steenson, 1976). The protein repair enzymes exist in a number of isoforms with different isoelectric points between 4.9 and 7.4, depending on the tissue sources. Some tissues have more than one isoelectric form, but no clear functional differences among the isozymes have been reported to date. A single gene is localized in the human chromosome 6. Multiple transcripts, arising through alternative splicing, have been identified. The human PCMT gene consists of 8 exons that are interrupted by 7 introns, spanning a genomic region of approximately 60 kb (DeVry, 1996).

We purified PCMTs from the porcine testis (Jung *et al.*, 1995), spleen (Yoon *et al.*, 1997), and brain (Park *et al.*, 1999). The cDNA of PCMT has been cloned from a wide variety of organisms in eukaryotes and prokaryotes. These include humans (Ingrosso *et al*,. 1989; Takeda *et al.*, 1995), bovine (Henzel *et al.*, 1989), rats (Mizobuchi *et al.*, 1994), wheat germ (Mudgett and Clarke, 1993), *E.coli* (Fu *et al.*, 1991), and *Thermotoga maritima* (Ichigawa and Clarke, 1998). But, the cDNA of the porcine brain PCMT has not been cloned.

In this paper, we report the following: (i) The cloning of the gene for the porcine brain PCMT (pbPCMT), (ii) The nucleotide sequence of the pbPCMT gene and its deduced amino acid sequence, (iii) Comparison of the amino acid sequence of pbPCMT with those of other PCMT, (iv) The expression of the gene, purification, and characterization of the recombinant porcine brain PCMT (r-pbPCMT).

Materials and Methods

Materials S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 56 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK). S-adenosyl-L-homocysteine (AdoHcy), S-adenosyl-L-methionine (AdoMet, iodine salt), γ-globulin, bovine serum albumin, histone, myelin basic protein, cytochrome C, hemoglobin, and low molecular markers were obtained from the Sigma Chemical Co. (St. Louis, USA). Superdex 75HR was from Pharmacia LKB (Uppsala, Sweden). Ni-NTA Superflow was obtained from Qiagen Inc. (CA, USA).

Enzyme assay for PCMT The trichloroacetic acid (TCA) precipitation method (Kim and Paik, 1971) was employed for the specific activity of protein methyltransferase. The reaction mixture was composed of 30 μl of a 0.25 M citrate-phosphate buffer, pH 6.0, 20 μl of histone (30 mg/ml in water) and 20 μl of an enzyme preparation. The mixture was preincubated for 5 min at 37°C. Then the reaction was initiated by adding 5 μl of [^{14}C-methyl]AdoMet (35 mM, 126.75 cpm/pmole). After the reaction was stopped by the addition of 15% TCA, the precipitate was washed 3 times with TCA, once with a mixture of chloroform, ether and ethanol (1 : 2 : 2), and once with ethanol. The radioactivity in the precipitate was counted on a liquid scintillation counter (LS 6500: Beckman, Palo Alto, CA, USA). One enzyme unit is defined as the amount of the enzyme that catalyzes the transfer of one pmole of ^{14}C-methyl to the methyl acceptor substrate per minute at pH 6.0 and 37°C. Specific activity is expressed as units of enzyme per milligram of protein. The protein concentration was determination by Bradfords method (1976) using bovine serum albumin as a standard.

Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) Total RNA from tissue was isolated by the guanidine thiocyanate extraction method (Chomczynski and Sacchi., 1987). Reverse transcription was performed following the BRL protocol for Superscript™ II reverse transcriptase (GIBCO BRL, Grand Island, NY, USA). The sense and antisense primers for PCMT were 5'-CTGCTCCGAGTGTGCTTAGC-3' (P9) and 5'-ACCCTTTCACTTGCATGTG-3' (P10), respectively. A polymerase chain reaction (PCR) was performed using the SuperTaq (SuperBio, Seoul, Korea), initial denaturation at 94°C with an amplification profile of each cycle that consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 62°C, and elongation for 40 sec at 70°C. This was followed by a final extension for 7 min at 72°C.

DNA sequencing The cloned PCR product was sequenced on both strands by the dideoxynucleotide chain termination method using the Top DNA sequencing kit (Bioneer, Seoul, Korea), according to the manufacturers instructions. Sequence data were analyzed using DNASIS and NCBI BLAST similarity searching program.

Construction of expression vector and expression in BL21 cells For the expression of pbPCMT in *E. coli*, the PCMT gene was subcloned into plasmid pET32b. Two oligonucleotide primers pbMT-s (5'-NNNNCATATGGCCTGGAAATCCGGC-3') and pbMT-a (5'-NNNNCTCGAGTCACTTCCACCTGGACCAC-3') were synthesized. Each contained the *Nde*I and *Xho*I sites, respectively (underlined in the sequences). The resulting expression plasmid was named pETPCMT. It contained the pbPCMT gene that was fused to an additional sequence at the N-terminus, encoding Trx · Tag, His · Tag, and S · Tag, and an enterokinase cleavage site that facilitated purification. The *E. coli* strain BL21 that carried pETPCMT was cultured at 37°C in a LB medium that contained 100 μg/ml ampicillin. When the A_{600} of the culture was about 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 300 μM, then incubated at 30°C for 3 h. The cells were collected by centrifugation (Supra 25K: Hanil, Korea) and stored at −80°C.

Homogenization and Ni-NTA Superflow affinity chromatography The cell pellet was thawed on ice and resuspended with a lysis buffer (50 mM NaH_2PO_4 , pH 8.0, 300 mM NaCl, 10 mM imidazole). Lysozyme was added to 1 mg/ml and incubated on ice for 30 min. The resuspended cells were disrupted by sonication (Ultrasonic processor VCX-550/750: Sonic and Material Newtown, CT, USA) and centrifuged for 30 min at 10,000 × g to remove intact cells and cell debris. The supernatant was loaded onto a Ni-NTA Superflow column that was previously equilibrated with a

lysis buffer at a flow rate of 1 ml/min. The activity fraction was pooled from 50 to 100 mM imidazole elution and concentrated by Ultrafiltration (Ultrafiltration equipment, 8050: Amicon, Beverly, MA, USA).

Recombinant Enterokinase digestion The concentrated enzyme fractions were digested with recombinant enterokinase (rEnterokinase) (Novagen, Madison, WI, USA). Ten units of rEnterokinase were incubated with 30 mg of r-pbPCMT in a buffer that contained 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 2 mM $CaCl_2$ for 18 h at 20°C.

Superdex 75HR gel filtration chromatography The enterokinase digested r-pbPCMT was loaded onto a FPLC Superdex 75HR column (20 × 300 mm, Pharmacia LKB Biotechnology, Sweden) that was previously equilibrated with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 2 mM $CaCl_2$ at a flow rate of 0.75 ml/min. The enzyme eluted fractions were pooled and concentrated with Centricon 10.

Molecular weight of the purified PCMT The molecular weight of the purified PCMT was determined on SDS-PAGE by a modification of Laemmli's method (1970).

***In vitro* substrate specificity** The relative activities of various commercially available pure proteins as a methyl acceptor for PCMT were examined by the TCA assay method.

Results

Molecular cloning of a cDNA encoding PCMT from porcine brain pons To isolate genes from porcine brain pons, RT-PCR was performed. Fig. 1 shows the nucleotide and deduced amino acid sequences of the porcine brain PCMT (pbPCMT). The nucleotide sequence of the clone will appear under the accession number AF239700 in the GenBank nucleotide sequence database. Fig. 1 shows that the pbPCMT protein contains 227 amino acids, which include the RWK, the C-terminal sequence of the PCMT isozyme I. Fig. 2 represents the alignment of the cDNA sequence of porcine brain with that of other organisms. The protein sequence of pbPCMT shares a 98% identity with PCMT from the human erythrocyte and rat brain, and 100% identical with bovine brain. Three regions (motif I, II, and III) that are known to be conserved among methyltransferases (Kagan and Clarke, 1994) are also conserved in pbPCMT.

Purification of recombinant porcine brain PCMT (r-pbPCMT) The porcine brain PCMT was overexpressed in *E. coli*, the BL21 strain that harbors pETPCMT. This plasmid encodes a fusion enzyme with an additional 14 kDa tag at the N-terminus, including Trx · Tag, His · Tag, and S · Tag sequences (described in Materials and Methods). The cytosolic extract was applied to a Ni-NTA affinity column in order to isolate the tagged proteins. A peak of activity was eluted from the column at an approximate imidazole concentration of 50 mM. A SDS-PAGE analysis of the Ni-NTA pooled activity fractions revealed a single major protein with a molecular mass of approximately 45 kDa, including 14 kDa tag (Fig. 3). After digestion with rEnterokinase, 14 kDa tag was removed by a Superdex 75HR gel filtration chromatography (Fig. 3). The purified enzyme was identified on SDS-PAGE, and the properties were studied. The molecular weight of the enzyme was 24.5 kDa, based on a SDS polyacrylamide gel electrophoresis (Fig. 3).

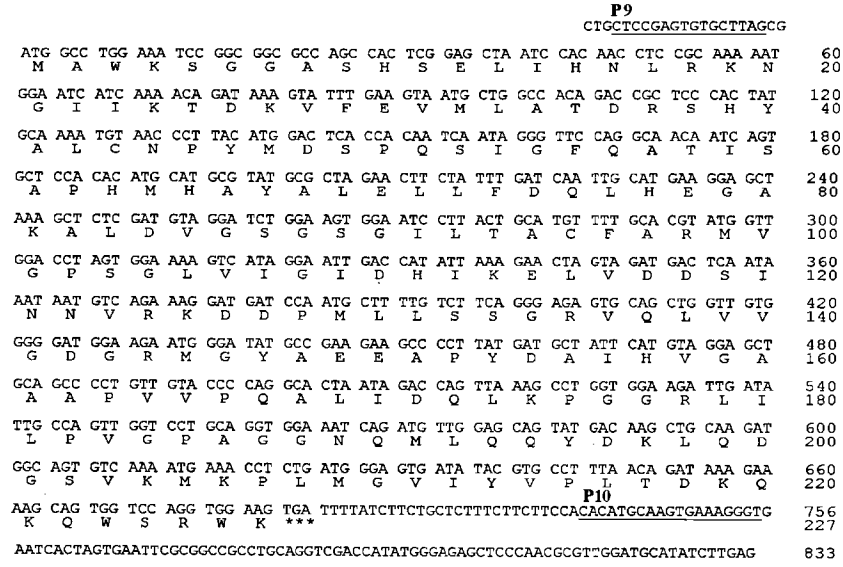

Fig. 1. Nucleotide and deduced amino acid sequences of the porcine brain PCMT. Asterisks indicate the stop codon. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence databases with the accession number AF239700. The primers for cloning are underlined for sense 5'-CTGCTCCGAGTGTGCTTAGC-3' (P9) and antisense 5'-ACCCTTTCACTTGCATGTG-3' (P10).

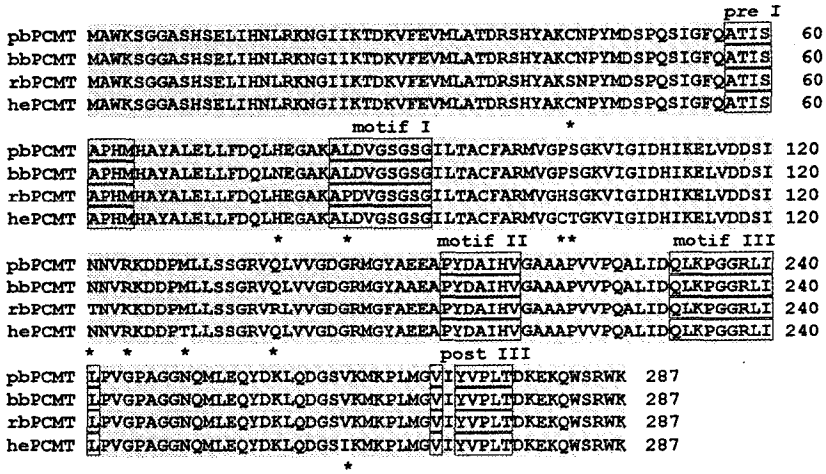

Fig. 2. Comparison of deduced amino acid sequence of PCMT of porcine brain with those of other organisms. The sequence of the porcine brain PCMT (pbPCMT) is shown compared with those of the bovine brain PCMT (bbPCMT), human erythrocyte (hePCMT), and rat brain PCMT (rbPCMT). Three regions (motif I, II, and III) indicated the three AdoMet-binding motifs. The sequence motifs, designated 'pre I' and 'post III', are described in the text. Identical amino acids between pbPCMT and others are indicated by stippled boxes.

Kinetic properties of r-pbPCMT The K_m value was 1.1 $\times 10^{-7}$ M for AdoMet (Fig. 4). A Lineweaver-Burk plot of the methylation that was inhibited by AdoHcy was a competitive type of inhibitor, and the K_i value was 1.38×10^{-4} M (Fig. 5). The enzyme had optimal activity at pH 6.0 and 37°C (data not shown).

Substrate specificity of r-pbPCMT Table 1 shows the relative specificities that were obtained *in vitro* with various substrates as the methyl-acceptor proteins. The myelin basic protein (MBP) was the best methyl acceptor among the substrates tested.

Discussion

To date, cDNA of PCMT was cloned in a wide variety of organisms in eukaryotes and prokaryotes. This includes humans (Takeda *et al.*, 1995), bovine (Henzel *et al.*, 1989), rats (Mizobuchi *et al.*, 1994), wheat germ (Mudgett and Clarke, 1993), *E.coli* (Fu *et al.*, 1991), and *Thermotoga maritima* (Ichigawa and Clarke, 1998). In this paper, the cDNA of the porcine brain PCMT isozyme I was sequenced for the first time. The isozymes of PCMT were purified and characterized from bovine brain (Aswad *et al.*, 1983) and porcine brain (Park *et al.*, 1999). The two isozymes had similar substrate specificities and displayed different isoelectric points-7.9 for PCMT I and 5.3 for PCMT II. Porcine brain has PCMT I that is approximately three times as much as PCMT II. Also, the two isozymes of PCMT differ at the C-terminal sequence, where the RWK sequence of isozyme I is replaced by a -RDEL sequence in isozyme II (MacLaren *et al.*, 1992; Potter *et al.*, 1992). Since -RDEL, like -KDEL, serves as an effective endoplasmic reticulum retention signal, it is proposed that the isozyme II serves to repair damaged proteins within the endoplasmic reticulum, or perhaps within some other specialized compartment of the cell. The PCMT isozyme I exists in cytosolic fraction and might have roles in repairing the damaged proteins in cytosol. Fig. 2 represents the alignment of the amino acid sequence of porcine brain with those of other organisms. All aligned proteins possess the three conserved S-adenosyl-L-methionine (AdoMet) and S-adenosyl-L-homocysteine (AdoHcy) binding motifs that are characteristic of most AdoMet-dependent methyltransferases (Kagan and Clarke, 1994), which are also conserved in pbPCMT. Two additional protein motifs, designated pre I and post III, are conserved in protein carboxyl-*O*-methyltransferases. These motifs have not yet been identified in other types of methyltransferase, thus, they may be determinants of the substrate specificity for the L-isoaspartyl or D-aspartyl residues. The protein sequence of pbPCMT shares a 98%, 49%, and 100% identity with that of the human erythrocyte, wheat germ, and bovine brain, respectively. The pbPCMT displays a 28% amino acid sequence identity to the *E.coli* PCMT, and a 23% amino acid identity to the *Thermotoga maritima* PCMT (data not shown). Such conservation in sequences suggests that this enzyme catalyzes a fundamental role in both the prokaryotic and eukaryotic cells.

Purification of recombinant porcine brain PCMT (r-pbPCMT) The porcine brain PCMT was overexpressed in *E. coli*, the BL21 strain that harbors pETPCMT. This plasmid encodes a fusion enzyme with an additional 14 kDa tag at the N-terminus, including the Trx · Tag, His · Tag, and S · Tag sequences (described under Materials and Methods). A SDS-PAGE analysis of the Ni-NTA pooled activity fractions revealed a single major protein with a molecular mass of approximately 45 kDa, including 14 kDa tag (Fig. 3). After

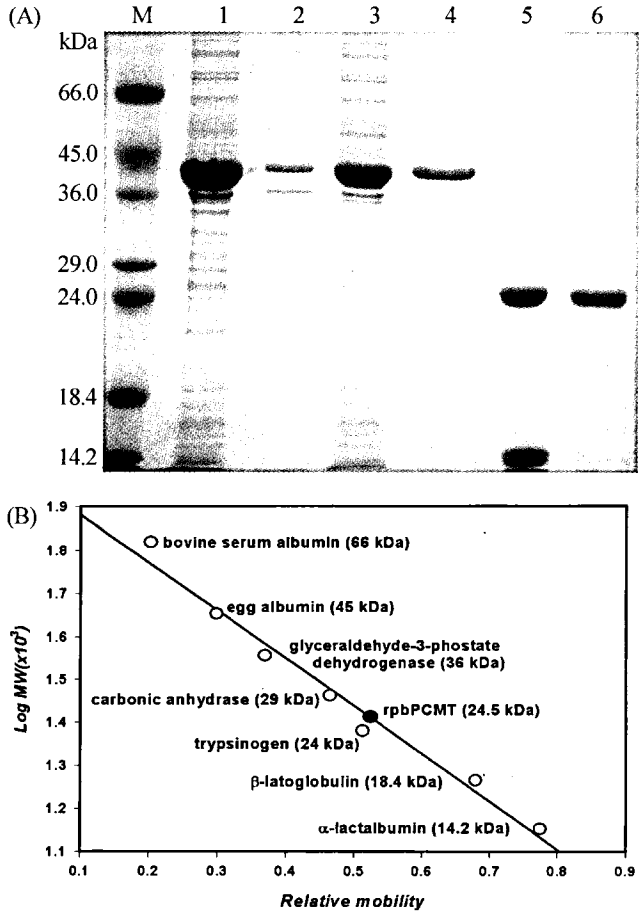

Fig. 3. Expression of the r-pbPCMT by BL21 carrying pETPCMT, as analyzed by SDS-PAGE and the calibration curve of purified r-pbPCMT. (A) Electrophoresis was carried out on a 15% acrylamide gel. Lanes: M, protein standards; 1, transformed BL21 whole cells; 2, insoluble fraction of transformed cells; 3, cytosolic fraction of transformed cells; 4, Ni-NTA affinity chromatography purified fusion protein; 5, rEnterokinase digested fusion proteins; 6, Superdex 75 HR purified fraction. (B) A calibration curve of SDS-PAGE was obtained by plotting the relative mobility of the standard proteins against the logarithms of molecular weights.

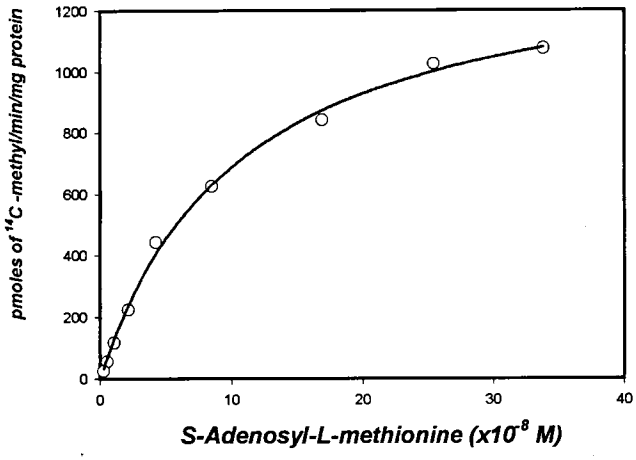

Fig. 4. Effect of Ado [^{14}C-methyl]Met concentration on r-pbPCMT activity. The reaction mixture that contained 10 μg of the purified r-pbPCMT and histone (0.5×10^{-3} M) was incubated under the conditions described in the Methods Section.

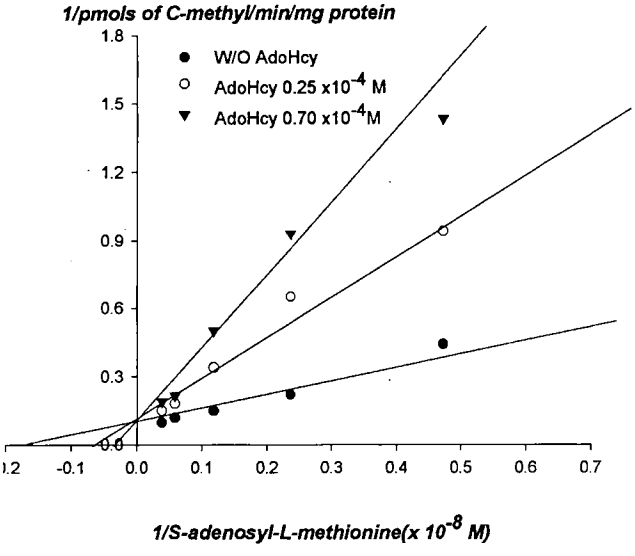

Fig. 5. Lineweaver-Burk plot of inhibition of r-pbPCMT by AdoHcy. The r-pbPCMT activity was measured with 10 μg of purified enzyme using 0.5×10^{-3} M histone as a substrate at the pH 6.0 in the absence and presence of AdoHcy, as indicated.

digestion with rEnterokinase, 14 kDa tag was removed by Superdex 75HR gel filtration chromatography (Fig. 3). The purified enzyme was identified on SDS-PAGE, and the properties were studied. The molecular weight of the enzyme was 24.5 kDa, based on a SDS polyacrylamide gel electrophoresis (Fig. 3). The molecular weights of protein carboxyl methyl transferase from bovine brain, bovine erythrocytes, human erythrocytes, equine erythrocytes, rat kidney cortex, rat erythrocyte, calf brain, and rat brain (Diliberto and Axelrod, 1976; Polastro *et al.*, 1978; Olive *et al.*, 1980; Ingrosso *et al.*, 1989; Sato *et al.*, 1989) range from 24 kDa to 28.5 kDa. The molecular weight of the pbPCMT is the same as bovine brain PCMT (Henzel *et al.*, 1989)

Kinetic properties of r-pbPCMT The K_m value was 1.1×10^{-7} M for AdoMet (Fig. 4). A Lineweaver-Burk plot of the methylation that was inhibited by AdoHcy was shown to a competitive type of inhibitor. The K_i value was 1.38×10^{-4} M (Fig. 5). The enzyme had optimal activity at pH 6.0 and 37°C (data not shown). The optimal pH value is very close to the optimum pH for the porcine brain methyltransferase (pH 6.2). It is a little different from the PCMT of the prokaryotic origin (Ichikawa and Clarke, 1998). This result indicates that the expressed enzyme was functionally similar to the natural protein. It also suggests that it may be a suitable tool for proving the function of protein carboxyl *O*-methylation.

Table 1. Substrate specificity of PCMT.

Substrate [1)]	Relative activity (%)
Myelin basic protein (Bovine)	318
γ-Globulin (Bovine)	100
Histone (type II-AS from calf thymus)	267
Hemoglobin (Human)	9
Serum albumin (Bovine)	3
Cytochrome C	3

[1)]30 μg of substrate and 8.47×10^{-8} M of Ado[^{14}C-methyl]Met was used.

Substrate specificity of r-pbPCMT Table 1 shows the relative specificities obtained *in vitro* with various substrates as methyl-acceptor proteins. Among the substrates that we tested, the myelin basic protein (MBP) was the best methyl acceptor. In 1987, Shapira *et al.* reported that L-aspartate undergoes racemization in the MBP of aging humans. The studies on the biological meaning of L-isoasparte and D-aspartate in MBP, and the conversion of L-isoaspartyl and D-aspartyl residues to normal L-aspartyl residues by PCMT *in vivo,* will further our understanding of the function of PCMT on MBP. Histones were also methylated by pbPCMT. The addition of methyl groups to histone proteins was discovered more than three decades ago. Recently, the biological significance of lysine, and the arginine methylation of histone-related transcriptional regulation and chromosome condensation, was elucidated (Chen *et al.*, 1999; Nakayama *et al.*, 2001). Further studies on the protein carboxyl *O*-methylation of histone *in vivo*, and the type of histones being methylated, might provide the possible role of PCMT on histone-related functions.

In this work, the cloned gene of the porcine brain L-isoaspartyl-*O*-methyl transferase was expressed, purified, and characterized. These results indicated that the cloned gene and expressed enzyme are structurally and functionally similar to its natural porcine brain PCMT. It is suggested that the *E. coli* expressed protein can be a suitable model for further understanding of the functions of the PCMT.

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