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Two Distinct Isozymes of Repair Protein Carboxyl *O*-Methyltransferase from Porcine Brain

Inho Park, Minsik Son, Youngjin Son, Hyung In Moon†, Jeung-Whan Han†, Hyang Woo Lee† and Sungyoul Hong*

Department of Genetic Engineering, College of Life Science and Natural Resources, and
† College of Pharmacy, Sungkyunkwan University, Suwon, 440-746 Korea

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Protein carboxyl *O*-methyltransferase (PCMT) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to free carboxyl groups of methyl-accepting substrate proteins. Two isozymes were separated by DEAE-Sephacel chromatography from porcine brain cytosol and designated PCMT I and II. Isozymes I and II were further purified by adenosyl homocysteine-Sepharose 4B and Superose HR 12 chromatography. The molecular weights of the purified PCMT I and II were determined by mass spectrometry to be 20,138 Da and 25,574 Da, respectively. The two enzymes displayed different isoelectric points; 7.9 for PCMT I and 5.3 for PCMT II. Isozymes I and II exhibited similar substrate specificities when tested with various methyl-accepting proteins. Myelin basic protein, a component of myelinated neurons, was found to be an excellent methyl-accepting substrate for both PCMT isozymes with different K_m values, 21.1 μM for PCMT I and 10.6 μM for PCMT II. The PCMT activity and methyl-accepting capacity displayed similar distribution in the various brain regions with an exception of the lower values in the cerebellum. The overall distribution may relate to a general function of protein repair by PCMT in the brain.

Keywords: Characterization, Porcine brain, Protein carboxyl *O*-methyltransferase isozymes, Purification.

Introduction

Methyl esterification of side-chain carboxyl groups of glutamate or aspartate residues of protein substrates utilizing *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; 1990). 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). PCMT was also identified in a variety of organisms, including bacteria (Fu *et al.*, 1991; Ichigawa and Clarke, 1998), nematodes (Kagan and Clarke, 1995), and plants (Mudgett and Clarke, 1993), where it catalyzes the transfer of a methyl group from AdoMet to the α-carboxyl group of L-isoaspartyl or D-aspartyl residues (Janson and Clarke, 1980; O'Conner and Clarke, 1983) and is known by a different name and EC number, protein L-isoaspartyl methyltransferase (PIMT, EC 2.1.1.77) (Aswad, 1995). We have also purified PCMTs from porcine testis (Jung *et al.*, 1995) and spleen (Yoon *et al.*, 1997). This enzyme converts D-aspartyl or L-isoaspartyl residues of abnormal protein to L-aspartyl residues. Accordingly, it has been postulated that PIMT is correlated with aging processes or disease states (Kondo *et al.*, 1996; Najbauer *et al.*, 1996).

It was recently reported that PCMT has an important role in various abnormal conditions of mammalian organisms. In Alzheimer's disease and human cataractous lens, the levels of PCMT were found to be different between normal and diseased tissues (Kodama *et al.*, 1995; Kondo *et al.*, 1996). During the aging of rat tubulin, the accumulation of isoaspartate sites was identified *in vivo* and *in vitro* (Najbauer *et al.*, 1996). It was suggested that PCMT repairs the damaged and aged proteins that accumulate isoaspartyl residues. In a study using knockout mice, deficiency of this enzyme caused the accumulation of altered proteins leading to retardation of growth and

* To whom correspondence should be addressed.
Tel: 82-331-290-7862; Fax: 82-331-290-7870
E-mail: syhong@yurim.skku.ac.kr

fetal seizures (Kim *et al.*, 1997). Thus, it was proposed that PCMT might be essential for normal growth and central nervous system function.

The molecular weights of mammalian PCMTs were reported to be between 24,000 Da and 28,000 Da in all tissues examined, except for the enzyme from ox brain, which was 34,000 Da (Iqubal and Steenson, 1976). The protein repair enzymes exist in a number of isoforms with different isoelectric points. The isoelectric points of PCMT appear to vary between 4.9 and 7.4 depending on the tissue sources. Some tissues have more than one isoelectric forms, but no clear functional differences among the isozymes have been reported to date.

To characterize isozymes of the brain, we attempted to purify PCMTs to homogeneity from porcine brain. The molecular mass of the purified isozymes was measured using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS); substrate specificity and kinetic parameters were also determined. In addition, the PCMT activity and methyl-accepting capacity were compared in nine regions of porcine brain.

Materials and Methods

Materials S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 55 mCi/mmol) was purchased from Amersham (Buckinghamshire, U.K.). S-adenosyl-L-homocysteine (AdoHcy), S-adenosyl-L-methionine (AdoMet, iodide salt), γ-globulin, bovine serum albumin, histone, myelin basic protein, cytochrome C, hemoglobin, carbonic anhydrase, and Sepharose-4B-1,6-diaminohexane were obtained from Sigma Chemical Co. (St. Louis, USA). DEAE-Sephacel and Sephadex G-100 were from Pharmacia LKB (Uppsala, Sweden). All other reagents were of the highest grade available.

Carboxyl methyltransferase assay A modification of the method of Kim and Paik (1971) was applied for the enzyme assay. A typical assay mixture consisted of 30 μl of 0.25 M citrate-phosphate buffer (pH 6.2), 30 μl of γ-globulin (10 mg/ml in citrate-phosphate buffer), and 30 μl of the enzyme source in a final volume of 90 μl in a 1.5-ml polyethylene microfuge tube. After preincubation for 5 min at 37°C, the reaction was initiated by adding 5 μl of [methyl-^{14}C]-AdoMet (35 μM, 129.83 cpm/pmol). The mixture was incubated for 15 min at 37°C and the reaction was terminated by the addition of 100 μl of 0.25 M sodium borate buffer (pH 11.5). The tube was reincubated for another 5 min at 37°C and allowed to cool for 1 min in an ice bucket. One ml of isoamyl alcohol was added and mixed for 15 s with a vortex mixer, and the isoamyl alcohol and water layers were separated by centrifugation in a table top centrifuge for 10 min. A 700-μl aliquot of the supernatant was placed in a scintillation vial containing 5.0 ml of scintillation cocktail and radioactivity was measured with an LS 6500 scintillation counter (Beckman, Palo Alto, USA). One unit of enzyme activity is defined as the amount of enzyme required to transfer 1 pmol of methyl groups per minute under the above conditions. The Bradford method (1976) was used to determine the concentration of protein with bovine serum albumin as a standard.

Subcellular fractionation of porcine brain All procedures were carried out at 4°C. Fresh porcine brain was obtained from a local slaughter house in Suwon. The brain was cut into small pieces and homogenized in 5 vol of buffer A consisting of 20 mM Tris, 0.2 mM EDTA, and 15 mM 2-mercaptoethanol, pH 8.0, using a Waring blender. The homogenate was filtered through double layers of cheese cloth and centrifuged at 1000 × *g* for 30 min. The resulting supernatant was centrifuged at 18,000 × *g* for 30 min (Supra 25K: Hanil, Inchon, Korea). The 18,000 × *g* supernatant was ultracentrifuged at 105,000 × *g* in a SW28 rotor for 90 min (L8-80M: Beckman, Palo Alto, USA). The supernatant, designated the cytosolic fraction, was brought to 70% saturation with solid ammonium sulfate at 4°C. The protein pellet was collected by centrifugation at 6000 × *g* for 60 min, resuspended in buffer A and dialyzed (cutoff, 10 kDa) against 10 vol of buffer A for 48 h with two changes of buffer.

DEAE-Sephacel chromatography of the cytosolic fraction The dialyzed cytosolic protein was loaded at a flow rate of 1 ml/min onto a DEAE-Sephacel column (24 × 110 mm) previously equilibrated with buffer A. The PCMT isozyme fractions were eluted with buffer A containing 0.05 M NaCl. The active peaks of PCMT isozymes were monitored by enzyme assay. The peak fractions of PCMT I and II were independently concentrated by ultrafiltration using an Amicon apparatus (8050: Amicon Corp., Beverly, USA) with a YM-10 filter, and further purified by affinity and gel filtration procedures.

AdoHcy-Sepharose 4B affinity chromatography The pooled fraction obtained from DEAE-Sephacel chromatography was adjusted to pH 6.2 by dropwise addition of 1 N acetic acid, and denatured proteins were removed by centrifugation at 3000 × *g* for 15 min. The supernatant was loaded at a flow rate of 1 ml/min onto a 12 × 100-mm AdoHcy-Sepharose 4B affinity column (Kim *et al.*, 1978) previously equilibrated with buffer B consisting of 5 mM sodium phosphate, 5 mM EDTA, and 2.4 mM 2-mercaptoethanol, pH 6.2. The active fractions were eluted with buffer B containing 0.02 mM AdoMet. Aliquots of 4 ml/tube were collected into test tubes containing 1 ml of 5 mM sodium borate buffer, pH 9.3. The enzyme fractions were concentrated by ultrafiltration using an Amicon apparatus.

Superose HR 12 gel filtration chromatography The fractions containing PCMT activity from the affinity column were combined, concentrated, and loaded onto an FPLC Superose HR 12 column (20 × 300 mm, Pharmacia LKB Biotechnology, Sweden) previously equilibrated with 10 mM phosphate buffered saline containing 1 mM EDTA and 0.15 M NaCl, pH 7.4 at a flow rate of 0.4 ml/min. The fractions that demonstrated PCMT activity were pooled and concentrated with Centricon 10 (cutoff 10 kDa).

Determination of purity and molecular weight of PCMT on SDS-PAGE and mass spectrometry The purity and the molecular weight of PCMT isozymes were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a 12% separating gel and 5% stacking gel (Mini-protean II electrophoresis system: Bio-Rad, Hercules, USA). The enzyme solution was heated at 100°C for 3 min after being mixed at a ratio of 4:1 (v/v) with sample buffer containing

60 mM Tris·HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 14.4 mM 2-mercaptoethanol, 25% (v/v) glycerol, and 0.1% (w/v) bromophenol blue. Electrophoresis was conducted for 90 min at 100 V. Gels were stained in 0.1% Coomassie blue R-250 made up in 50% methanol:10% acetic acid and destained in 10% methanol:10% acetic acid.

The molecular weights of the purified PCMT isozymes were also determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager Biospectrometry Workstation: Perseptive Biosystems, Framighan, USA) at Kyunggi Pharmaceutical Research Center (Sungkyunkwan University). Both lyophilized PCMT I and PCMT II were mixed with 5 vol each of matrix, cyano-4-hydroxycinnamic acid (10 mg/ml).

***In vitro* substrate specificity** The relative activities of various commercially available methyl-accepting proteins for PCMT were compared by the methanol extraction method. The relative activities are based on the activity of the enzyme with γ-globulin.

Isoelectric focusing Isoelectric focusing (IEF) was carried out over the pH range of 3–10 at 4°C under native conditions using a Novex precasted gel system (X-cell mini cell: Novex, San Diego, USA). The gels contained 5% polyacrylamide without urea. The sample was mixed at a 1:1 ratio (v/v) with sample buffer containing 40 mM arginine, 40 mM lysine, and 30% (v/v) glycerol. The cathode buffer, 20 mM arginine and 20 mM lysine, and anode buffer, 7 mM phosphoric acid, were completely degassed before IEF. Electrophoresis was conducted for 1 h at 100 V, for 1 h at 200 V, and for 30 min at 500 V. Standard markers were lentil lectin (pI: 8.0), human hemoglobin C (7.5), equine hemoglobin (7.0), bovine carbonic anhydrase (6.0), β-lactoglobulin B (5.1), and phycocyanin (4.6). After focusing, the gels were placed in 3.5% sulphosalicyclic acid/10% trichloroacetic acid overnight. Gels were stained in 0.01% Coomassie blue R-250. The destaining solution was composed of 10% methanol:10% acetic acid.

Kinetic properties of PCMT for myelin basic protein Various concentrations (0–80 μM) of myelin basic protein (MBP) were prepared and the PCMT activity at each concentration was measured by methanol extraction assays as described above. The specific activity was calculated from the enzyme activity and protein concentration. Values of K_m and V_{max} were calculated from the Lineweaver-Burk plot.

PCMT activities and methyl-accepting capacity (MAC) in the regions of porcine brain Nine regions of porcine brain were dissected: cerebrum, cerebellum, medulla, pons, hippocampus, midbrain, hypothalamus, thalamus, and striatum. The separated regions were homogenized in 4 vol of buffer A. The PCMT activity and methyl-accepting capacity of each region were measured by methanol extraction assays.

Results and Discussion

Purification of PCMT isozymes from porcine brain Overall, we achieved a 3140-fold purification of PCMT I and a 1807-fold purification of PCMT II from the cytosolic fraction of porcine brain (Table 1). We designated the two peaks of enzyme activity eluted from the DEAE-Sephacel column with buffer A containing 0.05 M NaCl as PCMT I and PCMT II (Fig. 1). The chromatographic data indicated that total cytosolic PCMT consists of 66% of type I and 34% of type II with a recovery of 19.4 % for PCMT I and 10.1% for PCMT II.

Fractions of each peak were eluted from the AdoHcy-Sepharose 4B affinity column (Fig. 2). The PCMT isozymes were eluted with buffer B containing 0.02 mM AdoMet with yields of 1.9% for PCMT I and 0.9% for PCMT II. When PCMT I and II were further purified on a Superose HR 12 gel filtration column, isozyme I was eluted at 38.2 min and isozyme II at 36.5 min (Fig. 3).

Physical properties of the purified isozymes We found that the porcine brain has two isoforms of PCMT as in other eukaryotic tissues reported to date (Ingrosso and Clarke, 1991; Potter *et al.*, 1992). The purified PCMT isozymes appeared as single bands on SDS-PAGE (Fig. 4).

Table 1. Purification of PCMT isozymes from porcine brain[a]

Purification step		Total protein (mg)	Total activity (unit)	Specific activity[b] (unit/mg proteins)	Purification (fold)	Recovery (%)
Porcine brain cytosol		506.86	7564.11	14.92	1.00	100.00
DEAE-Sephacel	Isozyme I	40.68	1467.95	36.09	2.42	19.40
	Isozyme II	24.15	760.93	31.51	2.11	10.06
AdoHcy-Sepharose 4B	Isozyme I	0.05	145.03	2900.23	194.15	1.88
	Isozyme II	0.09	62.32	692.48	46.35	0.86
Superose HR 12	Isozyme I	0.02	62.79	3140.00	210.19	0.79
	Isozyme II	0.01	18.09	1806.61	120.95	0.26

[a] A total of 82 g of porcine brain was used.

[b] An enzyme unit is defined as the amount of enzyme that catalizes the transfer of one pmole of [^{14}C]methyl to the methyl acceptor substrate per min at pH 6.2 and 37°C. Specific activity is expressed as units of enzyme per mg of protein.

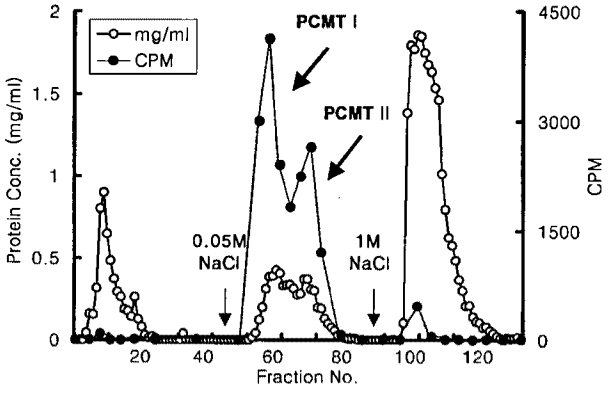

Fig. 1. Separation of PCMT I and PCMT II by DEAE-Sephacel chromatography. The dialysate was applied to the DEAE-Sephacel column (40 × 110 mm). The column was washed with 20 mM Tris·HCl (pH 8.0) containing 0.2 mM EDTA, 15 mM 2-mercaptoethanol, and eluted with 0.05 M NaCl in the buffer at a flow rate of 1 ml/min.

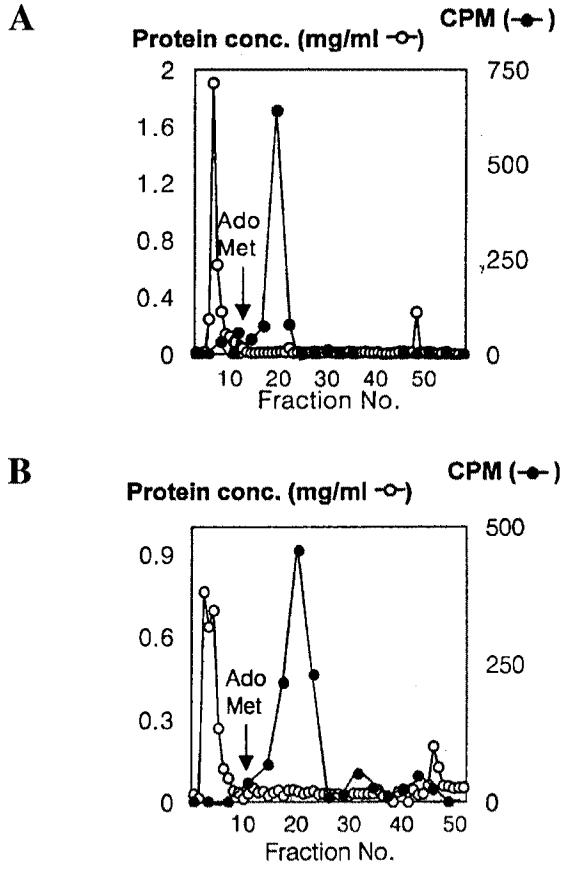

Fig. 2. AdoHcy-Sepharose 4B affinity chromatography. DEAE-Sephacel separated porcine brain PCMT isozymes were applied to the affinity column (1.2 × 120mm). The isozymes were adjusted to pH 6.2 with 1 N acetic acid. The column was washed with 5 mM phosphate buffer (pH 6.2) containing 5 mM EDTA, 2.4 mM 2-mercaptoethanol, and eluted with 0.02 mM AdoMet in the buffer at a flow rate of 1 ml/min. A, isozyme I; B, isozyme II.

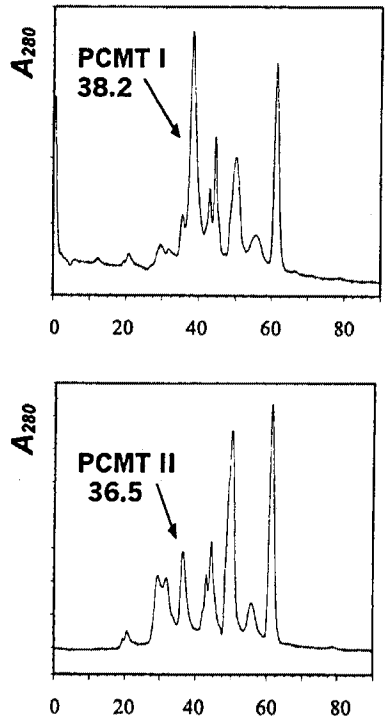

Fig. 3. Gel filtration on FPLC Superose HR 12 column. Affinity purified porcine brain PCMT isozymes were applied onto a Superose HR 12 FPLC column (20 × 300mm). The column was washed with 10 mM PBS containing 1 mM EDTA, 0.15 M NaCl, and eluted at a flow rate of 0.4 ml/min.

Although both isozymes I and II exhibited a major band at molecular mass of 27,000 Da on SDS-PAGE, analysis of native PCMT with MALDI-MS gave major mass signals with an average molecular masses of 20,137.6 Da for PCMT I and 25,574.2 Da for PCMT II (Fig. 5). The mass difference was reflected in the retention times of porcine PCMT isozymes on the Superose HR 12 gel filtration column (Fig. 3). The porcine PCMTs we purified showed a higher molecular weight of 27,000 Da on SDS-PAGE than the PCMTs from bovine brain and human erythrocytes, which are reported to be approximately 24,500 Da. The mass of the bovine isozyme I, as determined by electrospray mass spectrometry, was 24,478 Da. The PCMT II, with a mass of 24,521 Da, was only 43 Da heavier than PCMT I. The two major isoforms, PCMT I and II, from bovine brain are believed to result from differential splicing of a single gene transcript leading to differences in sequence only at the C-terminus, isozyme I being 226 amino acids with –SRWK and isozyme II being 227 residues with –SRDEL (MacLaren *et al.*, 1992). The C-terminal –RDEL are known to serve as an endoplasmic reticulum retention signals for several proteins, suggesting that isozyme II might be specialized for the methylation of isoaspartyl proteins as they transport through the ER (Aswad,1995). However, the mass difference of porcine PCMTs is 5437 Da, which

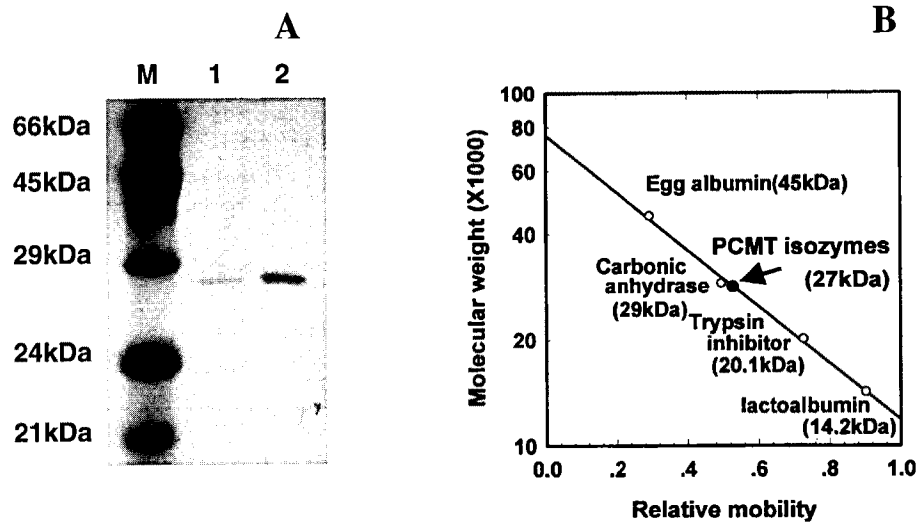

Fig. 4. Molecular weight determination of PCMT isozymes on SDS-PAGE. A. 12% acrylamide gel was used. Lane M, molecular weight markers; Lane 1, PCMT I; Lane 2, PCMT II. B. Calibration curve obtained by SDS-PAGE using egg albumin, carbonic anhydrase, trypsin inhibitor, and α-lactoalbumin as standards.

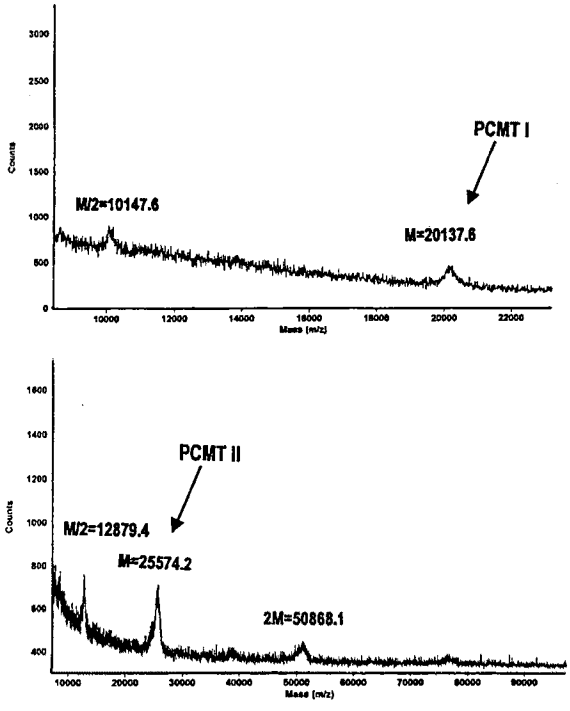

Fig. 5. MALDI mass spectra of native PCMT isozymes. PCMT I and PCMT II were applied on a Voyager Biospectrometry Workstation. The native spectrum gives major broad peaks with indicated average mass values.

corresponds to 49 amino acid residues, calculated using the average mass of one amino acid residue as 110 Da. The function and the reason for the large mass difference of the porcine isozymes remain to be explained.

The isoelectric points (pI) of PCMT I and II from porcine brain were determined to be 7.9 and 5.3, respectively (Fig. 6), which explains the differential elution of the two isoforms during anion-exchange chromatography. The pI difference of the porcine isozymes is greater than that of the bovine enzymes, being 6.5 for PCMT I and 5.6 for isozyme II. Therefore, we suggest that the difference in pI might be caused by different sequences other than the proposed C-terminus.

Substrate specificity of PCMT I and PCMT II Table 2 shows the relative specificities obtained *in vitro* with various substrates as methyl-acceptor proteins. The relative K_m values obtained were very similar for PCMT I and II as reported previously (Diliberto and Axelrod, 1974), with PCMT II exhibiting higher specificity than PCMT I for all substrates. This indicates that there are no large differences in substrate specificity between the two isozymes. Of particular interest was the finding that the best methyl acceptor for the PCMT isozymes was MBP among the substrates we have tested, while the best methyl-acceptor for bovine enzymes was synapsin I, a basic protein associated with synaptic vesicles. Our data is supported by the reported presence of a methylation site in MBP (Jun *et al.*, 1985). Myelin basic protein is a protein associated with myelinated nerves throughout the central nervous system. Since PCMT has been implicated in the degeneration of neurons (Kondo *et al.*, 1996), it seems valuable to further probe the carboxymethylation of MBP.

Distribution of PCMT activities and methyl-accepting capacity in the different regions of porcine brain A comparison of PCMT activity and methyl-accepting capacity in various parts of porcine brain is shown in Fig. 7. The hippocampus and hypothalamus displayed

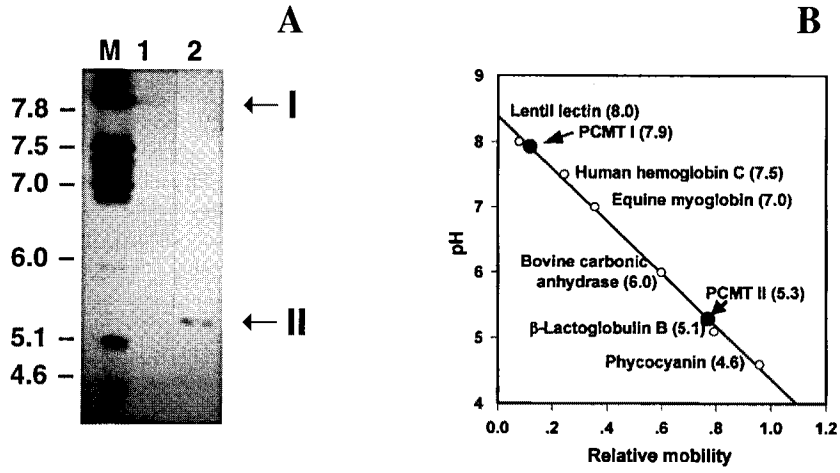

Fig. 6. A. Isoelectric focusing of PCMT isozymes. A. Isoelectric focusing was carried out using precasted gel. Lane M, pI standard markers (4.45–9.6); Lane 1, PCMT I; Lane 2, PCMT II. B. Calibration curve for isoelectric points obtained by plotting the relative mobility of the pI standards against pH.

Table 2. *In vitro* substrate specificity of PCMT isozymes.

Substrate	Relative Activity (%)[a]		
	PCMT I	PCMT II	II/I
Myelin basic protein (bovine brain)	390	553	1.42
γ-globulin (bovine)	100	100	1.00
Histone (calf thymus)	40	55	1.38
Albumin (human)	27	36	1.33
Albumin (pig)	7	10	1.43
Cytochrome C (bovine heart)	18	23	1.28
Hemoglobin (human)	14	22	1.57
Aspartic acid	2	0	–
Glutamic acid	0	0	–

[a] Sixty μmoles of each substrate were used for the enzyme assay. Relative activity is based on the activity with γ-globulin.

higher specific activities and the cerebellum the lowest. Methyl-accepting capacity was greater in the pons, midbrain, and hypothalamus than that in other sections and was the lowest in the cerebellum. These results suggest that both PCMT and its methyl-acceptor substrates share similar patterns of distribution throughout the brain. The activity of PCMT and MAC displayed similar distribution in the brain parts with the exception of the cerebellum. The general distribution may relate to the functional significance of protein repair of this enzyme in the brain. The distribution of isozymes I and II could be further probed by immunolocalization of the PCMT in neurons throughout the porcine brain parts.

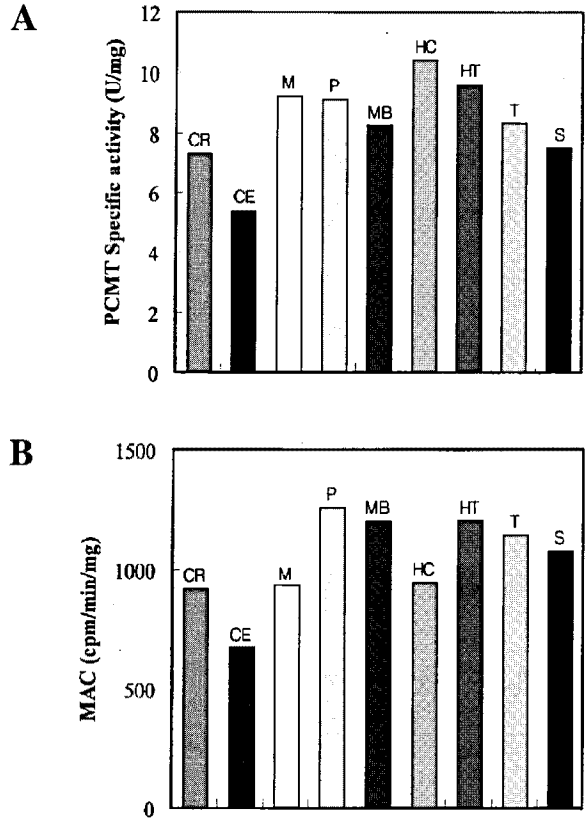

Fig. 7. A. Specific activities of PCMT in porcine brain regions. The enzyme reaction was carried out with nine major regions of porcine brain by the methanol extraction method, using 0.3 mg of γ-globulin as a substrate protein. Cerebrum (CR), cerebellum (CE), medulla (M), pons (P), hippocampus (HC), midbrain (MB), hypothalamus (HT), thalamus (T), and striatum (S). B. Methyl-accepting capacity (MAC) in porcine brain regions. The enzyme reaction was carried out using 2.4 μg of purified PCMT as an enzyme source. MAC was determined by total cpm/min/mg substrate protein.

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References

Aswad, D. W and Deight, E. A. (1983) Purification and characterization of two distinct isozymes of protein carboxymethylase from bovine brain. *J. Neurochem.* **40,** 1718–1726.

Aswad, D. W. (1995) *Deamidation and Isoaspartate Formation in Peptides and Proteins.* pp. 32–36, CRC Press, Boca Raton, Florida.

Axelrod, J. and Daly, J. (1965) Pituitary gland: Enzymatic formation of methanol from *S*-adenosyl-L-methionine. *Science* **150,** 892–893.

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

Diliberto, E. J. Jr. and Axelrod, J. (1974) Characterization and substrate specificity of a protein carboxymethylase in pituitary gland. *Proc. Natl. Acad. Sci. USA* **71,** 1701–1704.

Diliberto, E. J. Jr. and Axelrod, J. (1976) Subcellular distribution of protein carboxymethylase and its endogenous substrates in the adrenal medulla: Possible role in excitation-secretion coupling. *Proc. Natl. Acad. Sci. USA* **73,** 4050–4054.

Fu, J. C., Ding, L. and Clarke, S. (1991) Purification, gene cloning, and sequence analysis of an L-isoaspartyl protein carboxyl methyltransferase from *Escherichia coli. J. Biol. Chem.* **266,** 14562–14572.

Gilbert, J. M., Fowler, A., Bleibaum, J. and Clarke, S. (1988) Purification of homologous protein carboxyl methyltransferase isozymes from human and bovine erythrocytes. *Biochemistry* **27,** 5227–5233.

Ichigawa, J. K. and Clarke, S. (1998) A highly-active protein repair enzyme from an extreme thermophile: The L-isoaspartyl methyltransferase from *Thermotoga maritima. Arch. Biochem. Biophys.* **358,** 222–231.

Ingrosso, D. and Clarke, S. (1991) Human erythrocyte D-aspartyl/L-isoaspartyl methyltransferases: enzymes that recognize age-damaged proteins. *Adv. Exp. Med. Biol.* **307,** 263–276.

Iqubal, M. and Steenson, T. (1976) Purification of protein carboxylmethylase from ox brain. *J. Neurochem.* **27,** 605–612.

Janson, C. A. and Clarke, S. (1980) Identification of aspartic acid as a site of methylation in human erythrocyte membrane proteins. *J. Biol. Chem.* **255,** 11640–11643.

Jun, G. J., Hong, S., Lee, H. W., Kim, M. H., Tuck, M., Kim, S. and Paik, W. K. (1985) Enzymatic carboxyl-methyl esterification of myelin basic protein. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **18,** 152–156.

Jung, K., Kwon, M., Lee, H. Y., Lee, H. W. and Hong, S. (1995) Purification and characterization of protein Methylase II from porcine testis. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **28,** 149–154.

Kagan, R. M. and Clarke, S. (1995) Protein L-isoaspartyl methyltransferase from the nematode *Caenorhabditis elegans*: genomic structure and substrate specificity. *Biochemistry* **34,** 10794–10806.

Kim, E., Loewenson, J. D., MacLaren, D. C., Clarke, S. and Young, S. G. (1997) Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc. Natl. Acad. Sci. USA* **94,** 6132–6137.

Kim, S. (1974) *S*-adenosylmethionine: Protein carboxyl methyltransferase from erythrocyte. *Arch. Biochem. Biophys.* **161,** 652–657.

Kim, S. and Paik, W. K. (1970) Purification and properties of protein methylase II. *J. Biol. Chem.* **245,** 1806–1813.

Kim, S. and Paik, W. K. (1971) New assay method for protein methylase II activity. Suitable for trichloroacetic acid-soluble substrate, based on distillation of methanol. *Anal. Biochem.* **42,** 255–261.

Kim, S., Nochumson, S., Chin, W. and Paik, W. K. (1978) A rapid method for the purification of *S*-adenosylmethionine: Protein carboxyl *O*-methyltransferase by affinity chromatography. *Anal. Biochem.* **84,** 415–422.

Kodama, T., Mizobuchi, M., Taketa, R., Torikai, H., Shinomiya, H. and Ohashi, Y. (1995) Hampered expression of isoaspartyl protein carboxyl methyltransferase gene in the human cataractous lens. *Biochim. Biophys. Acta* **1245,** 269–272.

Kondo, T., Shirasawa, T., Itoyama, Y. and Mori, H. (1996) Embryonic genes expressed in Alzheimer's disease brains. *Neurosci. Lett.* **209,** 157–160.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

MacLaren, D. C., Kagan, R. M. and Clarke, S. (1992) Alternative splicing of the human isoaspartyl protein carboxyl methyltransferase RNA leads to the generation of a C-terminal -RDEL sequence in isozyme II. *Biochem. Biophys. Res. Commun.* **185,** 277–283.

Mudgett, M. B and Clarke, S. (1993) Characterization of plant L-isoaspartyl methyltransferases that may be involved in seed survival: purification, cloning, and sequence analysis of the wheat germ enzyme. *Biochemistry* **32,** 11100–11111.

Najbauer, J., Orpiszewski, J. and Aswad, D. W. (1996) Molecular aging of tubulin: Accumulation of isoaspartyl sites *in vitro* and *in vivo. Biochemistry* **35,** 5183–5190.

O'Conner, C. M. and Clarke, S. (1983) Methylation of erythrocyte membrane proteins at extracellular and intracellular D-aspartyl sites *in vivo. J. Biol. Chem.* **258,** 8485–8492.

Paik, W. K. and Kim, S. (1971) Protein methylation. *Science,* **174,** 114–119.

Paik, W. K. and Kim, S. (1980) *Protein Methylation.* pp. 119–126, John Wiley & Sons, New York.

Paik, W. K. and Kim, S. (1990) *Protein Methylation.* pp. 24–31, CRC Press, Boca Raton.

Potter, S. M., Johnson B. A., Henschen, A., Aswad, D. W., and Guzzetta, A. W. (1992) The type II isoform of bovine brain protein L-isoaspartyl methyltransferase has an endoplasmic reticulum retention signal (...RDEL) at its C-terminus. *Biochemistry* **31,** 6339–6347.

Yoon, S., Son, M., Han, J.-W., Lee, H. W. and Hong, S. (1997) Purification of protein carboxyl *O*-methyltransferase from porcine spleen. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30,** 410–414.