J. Biochem. Mol. Biol. Vol. 30, No. 6, pp. 410~414 November 30, 1997

Purification and Characterization of Protein Carboxyl *O*-Methyltransferase from Porcine Spleen

Sungpil Yoon, Minsik Son, Jeung-Whan Han[1], Hyang Woo Lee[1] and Sungyoul Hong*

Department of Genetic Engineering, College of Life Science and Natural Resources
[1]Laboratory of Biochemistry, College of Pharmacy, Sung Kyun Kwan University, Suwon 440-746, Korea.
(Received October 8, 1997)

Abstract : We purified a protein carboxyl *O*-methyltransferase (protein methylase II) from porcine spleen to homogeneity. The molecular weight of the porcine spleen protein methylase II (ps-PM II) was estimated to be 27,500 daltons on SDS-PAGE. Amino acid sequence of N-terminal 28 residues for ps-PM II was identified. Amino-terminal three amino acid residues of ps-PM II were deleted when compared to those of other protein carboxyl methyltransferases. S-Adenosyl-L-homocysteine competitively inhibits ps-PM II with a K_i value of 1.63×10^{-7} M. Myelin basic protein exhibited the highest methyl-accepting capacity among the proteins tested.
Key words : porcine spleen, protein carboxyl *O*-methyltransferase, purification

Protein-carboxyl *O*-methyltransferases (S-adenosyl methionine:protein-carboxyl *O*-methyltransferase; EC 2.1.1.24; Protein methylase II; PM II) transfer the active methyl group from S-adenosyl-L-methionine (AdoMet) to the carboxyl side chains in methyl acceptor proteins (Paik *et al.*, 1980; Paik *et al.*, 1990). Since one of the protein carboxyl methylases (PCM), PM II was firstly purified in 1970 (Kim *et al.*), several PCMs have been identified in different sources and referred to under a variety of different names such as "protein carboxyl methyltransferase," "protein carboxylmethylase," or "protein L-isoaspartate (D-aspartate) O-methyltransferase (PIMT; EC 2.1.1.77)" (Aswad, 1995). A single gene is localized in human chromosome 6 and multiple transcripts arising through alternative splicing have been identified. The human PIMT gene consists of 8 exons interrupted by 7 introns, spanning a genomic region of approximately 60 kb (DeVry, 1996). The molecular weight of mammalian PCM is believed to be 24,000-28,500 in all tissues, although there is one report that the ox brain enzyme is 34,000. The isoelectric point of PCM appears to vary over the range of 4.9-6.5 depending on the tissue source, and some tissues have more than one isoelectric form. In calf brain, Kim *et al.* (1978) found evidence of four isoelectric forms and speculated that they might represent isozymes of PCM.

Proposed functions in conjunction with the eukaryotic PCM were roles in exocrine secretion, hormone storage, calmodulin activity, sperm motility, and leukocyte chemotaxis (Barten *et al.*, 1990). Recently, this enzyme is believed to methylate abnormal aspartic acid residues such as D-aspartyl and L-isoaspartyl residues, on a wide variety of damaged proteins (Clarke, 1985; Galletti *et al.*, 1995). Protein carboxyl methyltransferase activity has been demonstrated in all vertebrate tissues so far examined, the activities of the enzyme being present in erythrocytes (Boivin *et al.*, 1995), brain, testis (Ingrosso *et al.*, 1989), hypothalamus tissues (Paik *et al.*, 1971; Diliberto *et al.*, 1976), plants (Brennan *et al.*, 1993), and in bacteria (Li *et al.*, 1992). As the changes in its activity have been observed during aging and disease conditions (Kondo *et al.*, 1996; Najbauer *et al.*, 1996), mounting evidences of the physiological significance of PCM have been excavated. Among them, it was reported that the knock-out mice showed significant growth retardation and succumbed to fatal seizures at an early age (Kim *et al.*, 1997). These results suggest that the repair enzyme is essential for normal growth and for normal central nervous system function.

A prerequisite for the understanding of cellular mechanisms associated with PCM is the characterization of purified PCMs from different tissues. Complete purification and characterization of the protein carboxyl methylase from spleen has not been achieved, though the existence of the enzyme in calf spleen was first reported by Liss *et al.* (1969). In an effort to probe the functions of protein carboxyl methylation in the mammalian immune system, an attempt has been made to purify

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

protein methylase II from porcine spleen.

Materials and Methods

Materials

S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 58 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.

Measurement of enzyme activity

Two kinds of assay methods modified from Kim and Paik (1984) were employed. For rapid identification of active fractions in chromatography, we used the methanol extraction method. The reaction mixture was composed of 20 ml of 0.25 M citrate-phosphate buffer, pH 6.0, 20 ml of γ-globulin (30 mg/ml in water), and 50 ml of enzyme preparation. The mixture was preincubated for 3 min at 37°C, then the reaction was initiated by adding 10 ml of [methyl-^{14}C]AdoMet (9×10^{-5} M, 11.6 mCi/mmole). The reaction was carried out for 15 min at 37°C and was terminated by adding 100 ml of borate buffer (pH 11). Then, the tube was reincubated at 37 for 5 min and then cooled in an ice bath. The mixture was then vigorously mixed after 1 ml of isoamylalcohol was added, and centrifuged for 10 min at 12000 rpm. After centrifugation, 1 ml portion of the isoamylalcohol extract was counted on a liquid scintillation counter with 5 ml of Bray's solution.

Second, we used the trichloroacetic acid (TCA) precipitation method for specific activity of PM II. The reaction mixture was composed of 40 ml of 0.25 M citrate-phosphate buffer, pH 6.0, 20 ml of γ-globulin (30 mg/ml in water), and 30 ml of enzyme preparation. The mixture was preincubated for 3 min at 37°C, and then the reaction was initiated by adding 10 ml of [methyl-^{14}C]AdoMet. The reaction was carried out for 10 min at 37°C and terminated by the addition of 400 ml of 15% (w/v) TCA. Precipitates were washed by repeated centrifugation and resuspension with 15% TCA, chloroform:ether:ethanol mixture (1:2:2, v/v), and 98% ethanol. Finally, the radioactivity in the precipitate was counted on a liquid scintillation counter with 5 ml of Bray's solution. One unit of the enzyme activity is defined as the amount of 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. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Preparation of protein methylase II

A modification of the method of Kim *et al.* (1978) was used. All procedures were carried out at 4°C unless otherwise stated. Fresh porcine spleen was trimmed of fat tissue and homogenized in 4 volume of 0.3 M sucrose. The homogenate was centrifuged first at 39,000 g for 30 min and then at 105,000 g for 60 min. The supernatant was then brought to 70% ammonium sulfate saturation by the addition of solid crystals over a period of 30 min. The precipitate was recovered by centrifugation at 39,000 g for 30 min, then dissolved in 20 mM Tris-HCl, 0.2 mM EDTA, and 15 mM 2-mercaptoethanol buffer, pH 8.0 (buffer A), then dialyzed overnight against the same buffer A.

The dialyzed sample was applied to a DEAE-Sephacel column previously equilibrated with buffer A. Enzyme fractions were eluted with buffer A containing 0.05 M NaCl at a flow rate of 72 ml/h. Active fractions were pooled and sedimented by 80% ammonium sulfate saturation. The precipitate was dissolved in a minimum amount of buffer A and dialyzed overnight against the same buffer.

The dialyzed sample was adjusted to pH 6.2 by a dropwise addition of cold 0.5 N acetic acid, and applied to a AdoHcy-Sepharose-4B affinity column, which was equilibrated with 5 mM sodium phosphate buffer (pH 6.2, 5 mM EDTA, 2.4 mM 2-mercaptoethanol). After centrifugation at 39,000 g, the supernatent was applied to the column. The flow rate was kept at 30 ml/h. The enzyme was then eluted with 5 mM sodium phosphate buffer containing 0.02 mM AdoMet at a flow rate of 30 ml/h. One ml fractions were collected in test tubes containing 0.1 ml of 50 mM sodium borate (pH 9.3, 50 mM EDTA). The collected fractions containing PM II activity were pooled and concentrated by ultrafiltration using an Amicon apparatus with a YM-10 (molecular cutoff : 10,000 Da) membrane.

The concentrated enzyme preparation was chromatographed on a Sephadex G-100 column previously equilibrated with buffer A and eluted with buffer A at a flow rate of 8 ml/h, collected, pooled and concentrated.

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970) with 10% acrylamide for the running gel and 4% for the stacking gel at a constant current of 35 mA for 2 h at room temperature. Protein samples were solu-

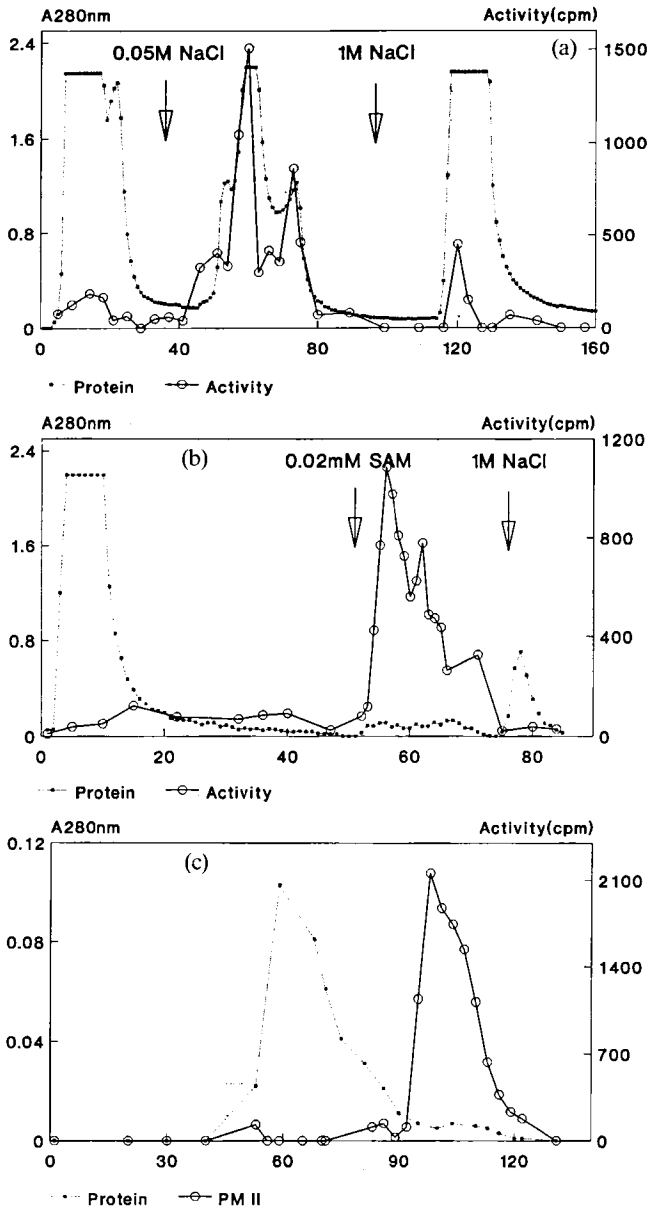

Fig. 1. Purification of porcine spleen protein methylase II by DEAE-Sephacel chromatography (a), AdoHcy-Affinity chromatography (b), and Sephadex G-100 chromatography (c). (a) Dialysate was applied to a DEAE-Sephacel column (5×25 cm). Aliquots of 10 ml were collected at each fraction. The incubation and assay conditions were the same as described in the method section (methanol extraction method). (b) Dialysate was adjusted to pH 6.2 and applied to a AdoHcy-Sepharose-4B affinity column (2×10 cm), which was equilibrated with 5 mM sodium phosphate buffer (pH 6.2). The flow rate of the column was kept at 30 ml/h. Fractions of 5 ml were collected into test tubes. Each test tubes contained 10% of 50 mM sodium borate (pH 9.3, 50 mM EDTA). (c) The concentrated enzyme preparation ultrafiltration was chromatographed on a Sephadex G-100 column (1.2×150 cm) and eluted 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 2.4 mM 2-mercaptoethanol at a flow rate 8 ml per h. Aliquotes of 1 ml each were collected at each fraction.

bilized with reducing SDS sample buffer.

Amino-terminal amino acid sequencing

The purified protein methylase II was subjected to SDS-PAGE and electroblotted on a PVDF membrane for 3 h at 200 V using 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8.3) (Towbin *et al.*, 1979). After staining with Coomassie Blue, the blot was air dried, and the PM II band was cut out. Amino-terminal amino acid sequence analysis of the PM II band was performed on an Applied Biosystems 473A protein sequencer equipped with an on-line analyzer for phenylthiohydanton-derivatized amino acids. The amino-terminal sequences were compared to an NBRF-PIR database.

Results

Purification of PM II from porcine spleen

Porcine spleen PM II was purified by DEAE-Sephacel chromatography (Fig. 1.a), AdoHcy-Sepharose-4B affinity chromatography (Fig. 1.b), and Sephadex G-100 chromatography (Fig. 1.c). The specific activity of the purified enzyme was 1436 U per mg of protein, and a 957 fold purification and yield of 1.9% was achieved (Table 1). SDS-PAGE analysis of the purified enzyme revealed a single polypeptide band at 27.5 kDa (Fig. 2)

Kinetic properties of cytosolic protein methylase II

The purified PM II has a K_m value of 1.2×10^{-6} M and V_{max} of 4074.7 pmoles of methyl-^{14}C/min./mg enzyme for γ-globulin. A lineweaver-Burk plot of the methylation inhibited by AdoHcy was of a competitive type. The K_i value for AdoHcy was 1.63×10^{-7} M (Fig. 3).

Substrate specificity of protein methylase II

Table 2 shows the relative capacity of various protein substrates. Myelin basic protein marked the highest

Table 1. Purification of porcine spleen protein methylase II[a]

Purification step	Total protein (mg)	Total activity (unit[b])	Specific activity (unit/mg)	Purification (-fold)	Yield (%)
Homogenate	15928	23399	1.5	1	100
105K supn	12888	21136	1.6	1.1	90.3
70% Amm. Sulf.	6802	20837	3.1	2.1	89.1
DEAE Sephacel	836	8627	10.3	21.1	36.9
Affinity	2.4	667	278	185.3	2.9
Sephadex G-100	0.3	439	1436	957.3	1.9

[a]280 g of porcine spleen was used.
[b]The incubation and assay conditions were the same as described in the method section (TCA method).
unit: pmoles of methyl-^{14}C groups incorporated to the acceptor substrate per minute at pH 6.0.

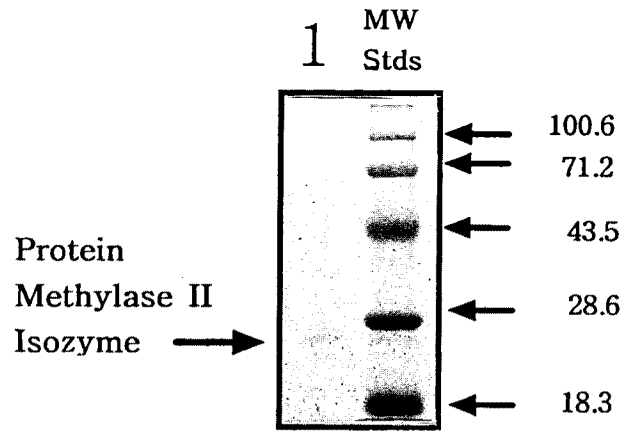

Fig. 2. SDS-PAGE of purified porcine spleen protein methylase II. SDS-PAGE was performed in 10% acrylamide using concentrated enzyme described in the text. Lane MW Stds, molecular size standard: prestained low-molecular markers were used; Phosphorylase B (100.6 kDa), BSA (71.2 kDa), O-valbumin (43.5 kDa), Carbonic anhydrase (28.6 kDa) and β-Lactoglobulin (18.3 kDa). Lane 1: purified PM II.

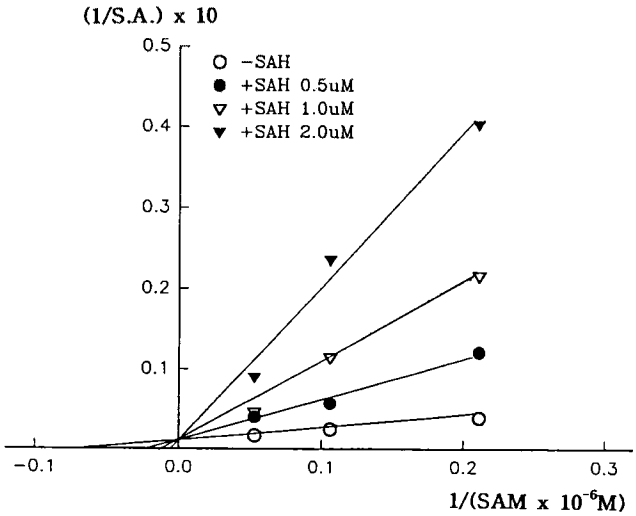

Fig. 3. Inhibitory effect of various concentrations of S-adenosyl-L-homocysteine. Lineweaver-Burk plot of inhibition of protein methylase II by S-adenosyl-L-homocysteine. The incubation and assay conditions were the same as described in the method section (TCA method). The purified protein methylase II (1.25 mg) was used as enzyme source. S.A is expressed as pmoles of methyl-14C groups transferred per min per mg enzyme protein. The variable substrate was S-adenosyl-methionine.

Table 2. Substrate specificity for porcine spleen protein methylase II

Substrate protein[a]	Relative activity (%)
γ-Globulin (bovine)	100.0
Myelin basic protein (bovine brain)	467.1
Histone (calf thymus)	159.1
Serum albumin (bovine)	35.7
Cytochrome c (horse heart)	23.4
Carbonic anhydrase (bovine erythrocyte)	23.3
Hemoglobin (goat)	41.8
Hemoglobin (horse)	33.2
Hemoglobin (dog)	23.0
Hemoglobin (human)	11.5

The incubation and assay conditions were the same as described in the method section (TCA method).
[a]80 μg of each substrate protein and 2.5 μg of the enzyme were used.

methyl accepting capacity among those tested.

Identification of the N-terminal 28 amino acids

We were able to obtain sequence information on amino-terminal 28 residues of the ps-PM II (Table 3). The N-terminal sequence analysis provided a sequence of porcine spleen protein methylase II starting with serine. The results of the NBRF-PIR database search with the N-terminal sequence of the psPM II revealed significant similarities to other PCMs.

Discussion

The existence of protein methylase II in spleen was reported and purification was attempted from calf spleen almost 28 years ago (Liss *et al.*, 1969), but purification of protein methylase II from spleen has not been achieved until now. We purified ps-PM II to homogeneity using γ-globulin as a substrate. The molecular weight of the enzyme is 27,500 daltons. Molecular weights of PCMs from bovine brain, human erythrocytes, equine erythrocytes, rat erythrocytes, calf brain, and rat brain range from 24,000 to 28,500 daltons. Thus the molecular weight of ps-PM II is similar to the other PCMs

Table 3. Comparison of amino-terminal amino acid sequence of porcine spleen protein methylase II with the published sequences of bovine, human and rat protein carboxyl methyltransferases

	Amino-terminal sequence			
	1	11	21	31
Porcine spleen[a]	---SGGASHS	ELIHNLRKNG	IIKTDKVFEV	M
Rat brain	AWKSGGASHS	ELIHNLRKNG	IIKTDKVFEV	M
Bovine brain	AWKSGGASHS	ELIHNLRKNG	IIKTDKVFEV	M
Human erythrocyte	AWKSGGASHS	ELIHNLRKNG	IIKTDKVFEV	M

[a]The N-terminal amino acid sequence of porcine spleen protein methylase II reported in this table has been submitted to the SWISS-PROT Data Bank with Accession No.P80895.

from mammalian tissues. Gel filtration on HPLC indicated that protein methylase II is a monomeric globular protein.

Porcine spleen protein methylase II has a broad substrate specificity (Table 2). The best substrate for the enzyme is myelin basic protein followed by histone. On the other hand, hemoglobin is a low methyl accepting substrate. Differences in substrate capacity for highly conservative hemoglobins of different sources suggest that a minute difference in three dimensional structures might influence accessibility of the enzyme for the substrates. S-adenosyl-L-homocysteine was known to be a potent inhibitor for most of methyltransferases. AdoHcy was shown to be a competitive inhibitor for ps-PM II (Fig. 3).

As shown in Table 3, sequence analysis of the N-terminal 28-amino acid residues of ps-PM II showed 100% identity from the 4th residue to the 31st residue of other PCMs. The high level of similarity suggests that ps PM II has a structure-function relationship and is evolutionarily related. While the N-termini of PCMs from erythrocyte and brain are modfied by acetylation, spleen PCM is not acetyltaed at its amino terminus. In addition, the critical difference of the psPM II from other PCMs is a deletion in the first three residues (A-W-K-). We have two possible assumptions on the reasons for the deletion. One is a consequence of organ specific polymorphism due to alternative splicing of a single gene transcript or unidentified post-translational modification in spleen. The other is digestion of the first three residues during the purification procedure. Several possibilities exist in terms of the biological significance of the N-terminal peptide. For example, this region in PIMT might be related to the stability of the enzyme. Another possibility is that the N-terminal region may interact with a cellular structure or specific substrate in the spleen (Mizobuchi, 1994).

Aswad and Deight (1983) separated two of the major isozymes from bovine brain by DEAE-cellulose chromatography. The first isozyme (I) that eluted earlier had an apparent isoelectric point of 6.5 , while the second (II) was comprised of components with isoelectric points of 5.5 and 5.6. They found that the purified isozymes had very similar catalytic and structural properties. On the other hand, the analogous protein carboxyl methyltransferase has been reported as a single molecular species.

It now appears that eucaryotic PCMs are much more heterogeneous than was previously thought. This heterogeneity could be associated with biological functions of protein carboxyl methylation in eucaryotic cells, and has raised the possibility that PCMs constitute a family of enzymes that modulate a variety of regulatory and nonregulatory proteins. This hypothesis can reconcile with previously described data which on the one hand point to the involvement of PCM-catalyzed reaction in neurosecretion, leukocyte chemotaxis, sperm motility, and cell differentiation and, on the other, suggest their involvement in the repair of age-damaged proteins.

Acknowledgement

This work was supported by Faculty Research Fund, Sung Kyun Kwan University.

References

Aswad, D. W. and Deight, E. A. (1983) *J. Neurochem.* **40**, 1718.

Aswad, D. W. (1995) Deamidation and Isoaspartate Formation in Peptides and Proteins, CRC Press Inc., Boca Raton, FL.

Barten, D. M. and O'Dea, R. F. (1990) *Life Sci.*, **47**, 181.

Boivin, D., Bilodeau, D. and Beliveau, R. (1995) *Biochem. J.* **309**, 993.

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.

Brennan, T. V. and Clarke, S. (1993) *Biochem. Biophys. Res. Commun.* **193**, 1031.

Clarke, S. (1985) *Annu. Rev. Biochem.* **54**, 479.

Diliberto, E. J. and Axelrod, J. (1976) *J. Neurochem.* **26**, 1159.

DeVry, C. G., Tsai, W. and Clarke, S. (1996) *Arch. Biochem. Biophys.* **335**, 321.

Galletti, P., Ingrosso, D., Manna, C., Clement, G. and Zappia, V. (1995) *Biochem. J.* **306**, 313.

Ingrosso, D., Fowler, A. V., Bleibaum, J. and Clarke, S. (1989) *J. Biol. Chem.* **264**, 20131.

Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S., Young, S. G. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 6132.

Kim, S. and Paik, W. K. (1970) *J. Biol. Chem.*, **245**, 1806.

Kim, S., Nochumson, S., Chin, W. and Paik, W. K. (1978) *Anal. Biochem.* **84**, 415.

Kim, S. (1984) *Methods Enzymol.* **106**, 295.

Kondo, T., Shirasawa, T., Itoyama, Y. and Mori, H. (1996) *Neurosci. Lett.* **209**, 157.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Li, C. and Clarke, S. (1992) *J. Bacteriol.*, **174**, 355.

Liss, M., Maxam, A. M. and Cuprak, L. J. (1969) *J. Biol. Chem.* **294**, 1617.

Mizobuchi, M., Murao, K., Takeda, R. and Kakimoto, K. (1994) *J. Neurochem.* **62**, 322.

Najbauer, J., Orpiszewski, J. and Aswad, D. W. (1996) *Biochemistry* **35**, 5183.

Paik, W. K., Lee, H. W. and Lawson, D. (1971) *Exp. Gerontol.* **6**, 271.

Paik, W. K. and Kim, S. (1980) Protein Methylation, John Wiley and Sons, New York.

Paik, W. K. and Kim, S. (1990) in Protein Methylation (Paik, W. K. and Kim, S., eds.) pp. 23-31, CRC Press, Boca Raton, Florida.

Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350.