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# Inhibitory effect of arginine-derivatives from ginseng extract and basic amino acids on protein-arginine N-methyltransferase

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**Summary.** Protein-arginine N-methyltransferase (protein methylase I) catalyzes methylation of arginyl residues on substrate protein posttranslationally utilizing S-adenosyl-L-methionine as the methyl donor and yields $N^G$-methylarginine residues. Arginyl-fructose and arginyl-fructosyl-glucose from Korean red ginseng were found to inhibit protein methylase I activity in vitro. This inhibitory activity was shown to be due to arginyl moiety in the molecules, rather than that of carbohydrates. Several basic amino acids as well as polyamines were also found to inhibit protein methylase I activity. Interestingly, the intensity of the inhibitory activity was correlated with the number of amino-group in polyamines, thus, in the order of spermine > spermidine > putrescine > agmatine-sulfate, with $IC_{50}$ at approximately 15 mM, 25 mM, 35 mM, and 50 mM, respectively. On the other hand, neutral amino acids or NaCl did not inhibit the enzyme activity. Lineweaver-Burk plot analysis of the protein methylase I activity in the presence of arginine and spermidine indicated that the inhibition was competitive in nature in respect to protein substrate, with the $K_i$ values of 24.8 mM and 11.5 mM, respectively.

**Keywords:** Amino acids – Protein-arginine methyltransferase – Inhibitors – Ginseng extract – Arginine derivatives – Basic amino acids – Polyamines

**Abbreviations:** AdoMet, S-adenosyl-L-methionine; PM I, protein methylase I; Arg-Fru, arginyl-fructose; Arg-Fru-Glu, arginyl-fructosyl-glucose; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; hnRNP, heterogeneous ribonuclear particle; TCA, trichloroacetic acid; EDTA, ethylenediamine tetraacetic acid

## Introduction

Protein methylase I (PM I, S-adenosylmethionine: protein-arginine N-methyl-transferase, EC.2.1.1.23) transfers methyl group of S-adenosyl-L-

methionine (AdoMet)[1] to arginine residues of protein substrate yielding $N^G$-methylated arginine derivatives (Paik and Kim, 1980; Kim et al., 1990). The enzyme is widely distributed in nature both in eukaryotes and prokaryotes. The enzyme has been purified from mammalian organs and has identified two subtypes, namely myelin basic protein-specific and nuclear protein/histone-specific PM I (Ghosh et al., 1988; Rajpurohit et al., 1994; Rawal et al., 1994). While the former class of enzyme only methylates arginine-107 in myelin basic protein (MBP) (Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971; Ghosh et al., 1988), the latter methylates arginines-193, -205, -217 and -224 of heterogeneous ribonuclear particle (hnRNP) protein A1 (Williams et al., 1985; Kim et al., 1997) and also histone, although the precise location of the arginine residue methylated in histone has not yet been identified. Since histone is readily available commercially, the protein is widely used as the methyl acceptor substrate for routine enzyme assay (Lee et al., 1977; Paik et al., 1997). Utilizing several chemically synthesized oligopeptides, we have investigated chain length and amino acid sequence motifs of the methyl accepting substrate for PM I and found that the minimum chain length of hexapeptide with Gly-**Arg**-Gly sequence is the most favorable structural requirement (Ghosh et al., 1990; Rawal et al., 1995).

Panax ginseng is one of the well studied herbs in oriental countries for over thousand years and believed to be effective in maintaining youth and prolonging life span. Several active compounds extracted from ginseng have been characterized to be consistently effective on the cardiovascular and central nervous systems (Brekhman and Dardymov, 1969) and also as an antioxidant (Kim et al., 1996). Partially purified fraction from petroleum-ether extract of Panax ginseng root exhibited cytotoxic effect against several human cancer cell lines (Hwang, 1993). Furthermore, Rg1 fraction purified from ginsenosides was shown to protect pulmonary vascular endothelium against free radical-induced injury by promoting release of nitric oxide (Kim et al., 1992). Recently, Matsuura et al. (1994) have extracted ninhydrin positive substances from water extract of Korean red ginseng and their chemical structures were identified as argininyl-fructose (Arg-Fru) and arginyl-fructosyl-glucose (Arg-Fru-Glu) (Matsuura et al., 1994; Takaku et al., 1996). The latter compound was shown to inhibit maltase activity (Matsuura et al., 1994). In the present study we report that these arginyl-derivatives act as inhibitor for PM I (protein-arginine methyltransferase) in vitro. Several basic amino acids and polyamines were also found to inhibit PM I activities.

## Materials and methods

### *Materials*

S-Adenosyl-L-[*methyl*-$^3$H]methionine (specific activity, 77.0 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Histone (type II-AS; calf thymus), $\gamma$-globulin, trichloroacetic acid (TCA), Sephadex G-200, spermine, spermidine, putrescine, taurine, adenosine, agmatine sulfate and various amino acids were purchased from Sigma chemical Co., St. Louis, MO. DE-52 was from Whatman Ltd., Maidstone, U.K.

and Centriprep 10 was from Amicon, Inc., Beverly, MA. All other chemicals were purchased from various commercial sources and were of the highest grade available.

### *Assay for protein methylase I*

Protein methylase I (S-Adenosylmethionine: protein-arginine N-methyltransferase) activity was measured as described (Gosh et al., 1988; Rawal et al., 1994) with slight modification. Total incubation mixture, 0.125 ml, containing 50 mM potassium phosphate buffer (pH 7.6), 0.025 ml protein substrate (histone type II-AS, 40 mg/ml), 0.04 mM AdoMet (1208 dpm/pmol; diluted with unlabeled AdoMet) were incubated at 37°C for 15 min. The reaction was terminated by the addition of 2.5 ml of 15% TCA and 4 mg $\gamma$-globulin as a carrier protein. The mixture was centrifugated at 3,000 rpm for 10 min, and the resulting precipitates were washed 3 more times with TCA. Finally, the precipitates were suspended into 5 ml Scintillation cocktail solution and counted for the radioactivity incorporated. All the reactions were carried out in duplicate. Protein concentration was estimated by the Coomassie Blue method of Bradford (1976) modified by Pierce Chemical Co (Cat. No. 23200) using bovine serum albumin as the standard.

### *Purification of protein methylase I*

PM I was purified according to the procedures described by Rawal et al. (1994). All procedures were carried out at 4°C unless otherwise stated. Frozen rat liver (38 g) was thawed and homogenized in 4 vol of precooled buffer containing 5 mM sodium phosphate (pH 7.4), 5 mM EDTA and 0.32 M sucrose in a Teflon-glass homogenizer. The homogenate was centrifugated at 100,000 × g for 60 min, and the supernatant was taken for subsequent purification.

*DE-52 chromatography*: Fourteen milliliters of the supernatant (300 mg protein) were mixed with an equal volume of 10 mM Mops buffer (pH 7.4) containing 2 mM EDTA, 0.5 mM PMSF and 10 mM 2-mercaptoethanol (Buffer A), and loaded onto a DE-52 column (2.6 i.d. × 11 cm) which had been preequilibrated with Buffer A. After eluting the unabsorbed proteins, the column was washed with Buffer A containing 0.1 M NaCl until the absorbance at 280 nm was less than 0.05. The proteins were then eluted with a linear salt gradient consisted of 100 ml of each Buffer A plus 0.1 M NaCl in the first chamber and Buffer A plus 0.25 M NaCl in the second. Fractions of 2 ml were collected at a flow rate of 20 ml/hr. Enzyme activity was determined using 0.03 ml aliquots from each fractions. The fractions containing enzyme activity were pooled and concentrated by centriprep 10.

*Sephadex G-200 chromatography*: The concentrated enzyme preparation (0.7 ml) was applied to a column of Sephadex G-200 (1.5 i.d. × 110 cm) which had been preequilibrated in Buffer A containing 2% glycerol. Fractions of 2.5 ml were collected at a flow rate of 5 ml/hr. Enzyme activity was determined using 0.02 ml aliquots from each fractions. The fractions containing high enzyme activity were pooled. Specific activity of the pooled fraction at this stage of purification was 35.8 pmol methyl-group transferred/min/mg enzyme protein.

### *Purification and assay for protein methylase II*

Protein methylase II (S-adenosylmethionine: protein-carboxyl O-methyltransferase; EC. 2.1.1.24) was purified from calf brain essentially same as described (Kim, 1984) by the use of S-adenosylhomocysteine-affinity chromatography, and the enzyme activity was assayed by the method of isoamyl alcohol extraction.

*Preparation of arginyl derivatives*

Arg-Fru and Arg-Fru-Glu were prepared by Maillard reaction as described (Matsuura et al., 1994; Takaku et al., 1996) and further purified on amino acid analyzer according to the method (Matsuura et al., 1994). Both compounds were shown to be a single peak on the analyzer (see Fig. 1 of ref. Matsuura et al., 1994).

## Results

*Effect of arginyl-fructose and arginyl-fructosyl-glucose on protein methylase I activity*

PM I purified by DE-52 and Sephadex G-200 chromatography, with the specific activity of 35.8 pmol methyl-group transferred/min/mg protein, was used to investigate the effect of several compounds on its enzyme activity. This preparation is suited for the present study, since it did not contain any endogenous substrate nor natural inhibitor for PM I (Rawal et al., 1994). The reaction was linear under the assay condition for almost 30 min at 37°C, thus, all the enzyme assays were carried out for 15 min in the present study. Figure 1A shows the relative percent activities of PM I in the presence of various concentrations of testing compounds; $IC_{50}$ (inhibition concentration to inhibit 50% of activity) for Arg-Fru, Arg-Fru-Glu and Arg were shown to be 45, 55, and 60 mM, respectively (closed symbols). However, glucose or fructose per se did not show any inhibition (open symbols) and the inhibition caused by arginine is comparable to that by Arg-Fru and Arg-Fru-Glu, suggesting that arginine moieties in these arginine derivatives are likely to be responsible for the enzyme inhibition.

*Effect of basic amino acid on protein methylase I activity*

Because arginine showed an inhibitory activity on PM I, other basic amino acids have been also tested. As shown in Table 1 and Fig. 1B, in the presence of histidine and lysine at the concentration of 40 mM, the activity has been reduced to 56.1% and 74.3% of the control, respectively, which is about the same extent of inhibition caused by arginine (54.7%). A possibility of salt effect has been considered since the inhibitory concentrations of the compounds are rather high; however, an inclusion of NaCl up to 60 mM did not show any significant inhibition (Table 1), indicating that a possible salt effect can be ruled out. In addition, several neutral amino acids (glycine, threonine, leucine and methionine) at the concentration of 60 mM each did not show any significant changes in the activities (Table 1), suggesting that the inhibitory effect is indeed exerted by the basic amino acids. Additionally, we have examined an effect of free arginine on protein methylase II (protein-carboxyl O-methyltransferase) activity which methyl-esterifies free carboxyl groups of protein. In the presence of 20 mM and 40 mM arginine, 105% and 107% of the methylase II activity were shown, indicating that the inhibitory effect of arginine is specific to enzymatic methylation on arginyl residue.

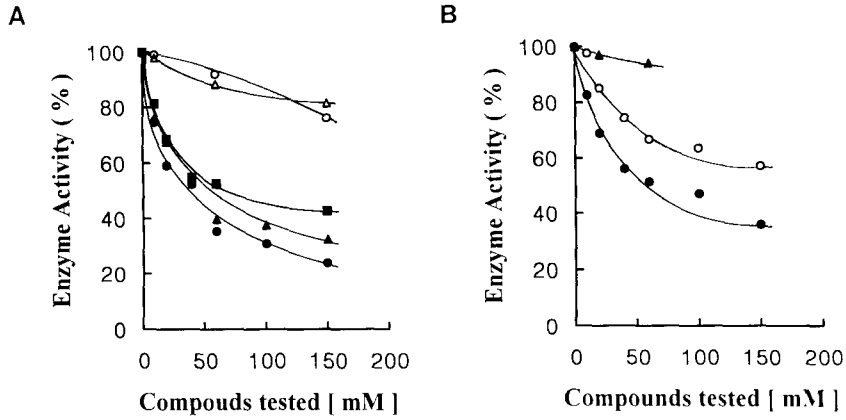


**Fig. 1.** Effect of arginine derivatives on protein methylase I activity. Assay conditions for protein methylase I activity were the same as Table 1. The indicated amount of various compounds to be tested were added to the reaction mixture. Symbols indicate in panel **A**: ■ for arginine; ● for Arg-Fru; ▲, Arg-Fru-Glu; ○, fructose; and △, glucose. Panel **B**: ● for histidine; ○, lysine; and ▲, NaCl

**Table 1.** Effect of amino acids on protein methylase I activity

| Compound tested | Relative enzyme activity (%) | | | | |
|---|---|---|---|---|---|
| | 0mM | 10mM | 20mM | 40mM | 60mM |
| Basic amino acids | | | | | |
| Arginine | 100 | 81.0 | 68.4 | 54.7 | 52.6 |
| Histidine | 100 | 82.5 | 68.9 | 56.1 | 51.4 |
| Lysine | 100 | 97.6 | 85.1 | 74.3 | 66.8 |
| Neutral amino acids | | | | | |
| Glycine | 100 | | 96.6 | | 90.4 |
| Threonine | 100 | | 100.8 | | 87.8 |
| Leucine | 100 | | 100.5 | | |
| Methionine | 100 | | 101.5 | | 97.2 |
| Salt, NaCl | 100 | | 97.0 | | 94.1 |

Protein methylase I activity was measured in a standard reaction mixture containing 1 mg histone, 60$\mu$M Ado[*mehyl*-$^3$H]Met and the Sephadex G-200 purified protein methylase I preparation (11$\mu$g) at pH 7.6 as described in "Materials and methods". The values are shown as percent activities when the activity without addition of the testing compound was taken as 100%. All the compounds to be tested were dissolved in distilled water and pH of the solutions were carefully adjusted to 7.6 with either NaOH or HCl prior to the addition into the reaction mixture.

### *Effect of polyamines on protein methylase I*

While only basic amino acids showed inhibitory activity on PM I, agmatine, the decarboxylated arginine, and polyamines containing several amino groups were tested for their inhibitory activities. Figure 2 shows that agmatine-sulfate exhibited an inhibitory activity, about the same or slightly weaker than that by

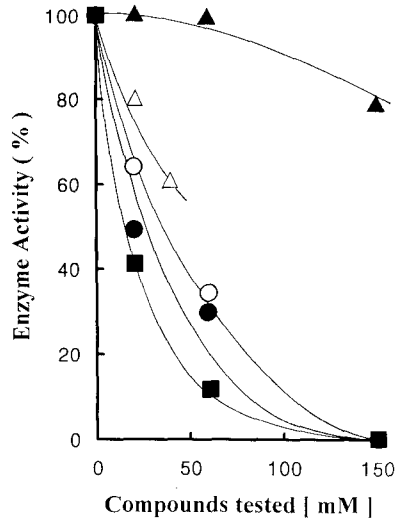


**Fig. 2.** Effect of polyamines on protein methylase I activty. Protein methylase I activity was determined same as Table 1. The indicated amount of various compounds were added to the reaction mixture. Symbols indicate; ■ for spermine; ●, spermidine; ○, putrescine; ▲, taurine; and △, agmatine sulfate

arginine (54.7% vs. 61.7% at 40mM,). Since the guanidino-group of agmatin is likely to be conjugated with sulfate under the assay condition, $\alpha$-amino group of the molecule can only contribute a positive charge. Interestingly, all polyamines tested were also effective inhibitors, and the extent of inhibition is well correlated with the number of the amino groups. From Fig. 2, $IC_{50}$ can be estimated to be 60mM for agmatine-sulfate, 35mM for putrescine (two amino-group), 25mM for spermidine (3 amino-group), and 15mM for spermine (four amino-group), respectively. On the other hand, taurine did not inhibit PM I activity significantly. This compound is amphoteric in nature, thus, under the assay condition of pH 7.6, its effective charge will be nil.

### *Kinetic analyses of inhibition by arginine and spermidine*

To understand type of inhibition caused by basic compound on PM I, initial velocity patterns under the varied concentrations of histone as substrate at various fixed concentrations of arginine or spermidine were investigated (Fig. 3A and B). The lines appear to converge at a point on the 1/V axis indicating that the nature of inhibition was competitive in respect to histone for both inhibitors. The numerical values of the constants were obtained graphically by replotting the slopes and intercepts of the reciprocal plots vs. the reciprocal concentrations of substrate, histone. The $K_i$ values for arginine and spermidine were calculated to be 24.8 mM and 11.5 mM, respectively.

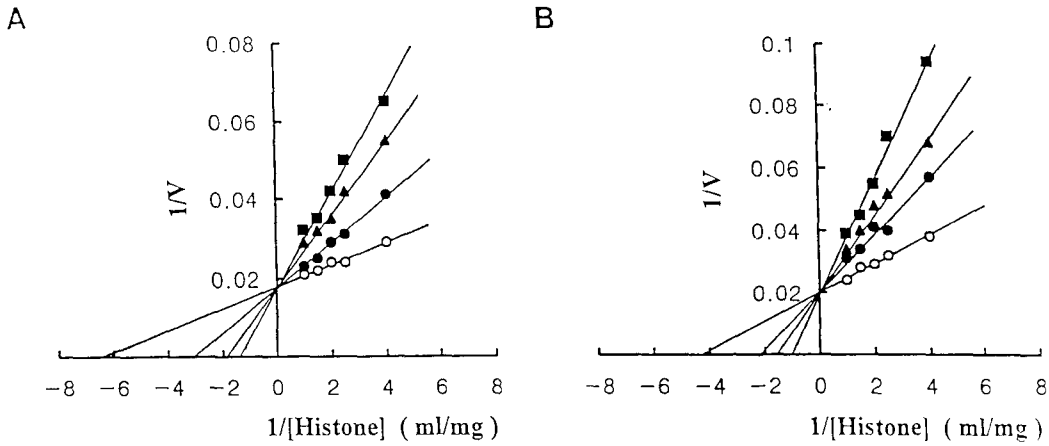


**Fig. 3.** Lineweaver-Burk plot of protein methylase I in the presence of inhibitor. Protein methylase I activity was assayed as described under Table 1 in the presence of the indicated amount of arginine (**A**) or spermidine (**B**) as the inhibitor using PM I preparation (16.5$\mu$g) with the varied concentrations of histone as the substrate. Panel **A** indicates arginine as inhibitor; ○-○ for no addition; ●-● for 20 mM; ▲-▲ for 50 mM and ■-■ for 100 mM, respectively. *Panel* **B**, spermidine as inhibitor; ○-○, no addition; ●-●, 10 mM; ▲-▲, 20 mM; and ■-■, 30 mM, respectively. The $K_i$ values for arginine and spermidine were calculated to be 24.8 mM and 11.5 mM, respectively. Histone concentrations were calculated based on the molecular weight of histone as 14,400. Initial velocity ($V$) is defined as pmole of [methyl-$^3$H]group incorporated into substrate per min per mg enzyme protein

## Discussion

Ginseng is one of the most misterious herbs for over thousand years, since its efficacy has been implicated to human health, although the biochemical mechanism is not well understood in spite of the fact that many laboratories are under the active investigation. However, anti-proliferative activity of the water soluble ginsenoside or saponin, and also the petroleum-ether extract of ginseng roots in cancer cells in cultures are well documented (Brekhman and Dardymov, 1969; Hwang, 1993). Recently, ginsenoside has been shown to cause vasodilatation in preconstricted lungs due to an increased nitric oxide synthesis (Kim et al., 1992). From the dialysate of ginseng roots water-extract, Matsuura et al. (1994) have isolated several new arginyl-derivatives from red ginseng which has been processed by steam-drying and known to be far better than white ginseng in pharmacological effects (Hwang et al., 1987). In contrast to it, the white ginseng prepared by sun-drying, contained negligible amount of these arginyl-derivatives. Free amino acid composition of the water extract of red ginseng also showed the most unusual profile in that arginine is the major and predominant amino acid, while other amino acids are present at extremely lower concentration (Matsuura et al., 1994).

In the present study, we report that arginine derivatives from Korean red ginseng, namely, Arg-Fru and Arg-Fru-Glu, inhibit protein-arginyl N-methyltransferase (protein methylase I) *in vitro*. This inhibitory activity is most likely

exerted by arginyl moiety, since fructose or glucose did not show significant inhibitory activity, while free arginine itself exhibited about the same extent of inhibition as the arginyl-derivatives (Fig. 1 and Table 1). Since free arginine is not a substrate for protein arginine methyltransferase but only specific arginyl residues in the substrates are susceptible to undergo this posttranslational modification, the inhibitory nature of free arginine, although its inhibition constant is rather high, may be due to its structural complimentarity to the protein substrate binding site of the enzyme. This assumption is supported by the fact that another class of protein methyltransferase, protein-carboxyl O-methyltransferase (protein methylase II) which methyl esterifies only free carboxyl groups of protein, is not inhibited by free arginine. On the other hand, the inhibitory activities of basic amino acids and several polyamines on PM I are not readily understandable. However, it might be due to an electronegativity of amino nitrogen of the inhibitors which may create alterations in overall microenvironment unfavorable for transmethylation reaction.

Several powerful inhibitors for AdoMet-dependent transmethylation have been reported; for example, S-adenosyl-L-homocysteine (Duerre, 1962; Zappia et al., 1969), and its analogues, such as SIBA [5′-S-(2-methyl-propyl)-adenosine], 5′-S-methylthio-adenosine (Oliva et al., 1980; Lawrence and Robert-Gero, 1990), sinefungin, a nucleoside antibiotics isolated form Streptomyces graseolus, and adenosine from wheat germ (Gupta et al., 1982). Also, proteinous inhibitors for methyltransferases are present in mammalian organs. Chiva and Mato (1984) have partially purified a proteinous inhibitor from rat liver cytosol which inhibits phosphatidylethanolamine methyltransferase. Subsequently, the similar proteinous inhibitor composed of 29 amino acids with chromophore has been also isolated from rat liver (Hong et al., 1986; Park et al., 1993). All of these inhibitors compete with AdoMet binding site, and therefore, inhibit not only PM I, but also act on many other transmethylation reactions.

On the other hand, inhibitors that act on methyl-acceptor subsrate site has not been well studied, except that myelin basic protein (MBP) which has been shown to inhibit histone-specific PM I from calf brain (Park et al., 1986). This basic protein is the only known methyl acceptor for MBP-specific PM I: It is noted that molecular and catalytic properties of these two subclasses of PM I's are quite different from each other (Ghosh et al., 1988; Rajpurohit et al., 1994). In investigating endogenous substrate for PM I, a synthetic peptide substrate for PM I, R3 (19-mer peptide), has been used as an inhibitor to suppress the methylation of endogenous substrate in tissue culture (Najbauer et al., 1993). The presently descirbed Arg-Fru, Arg-Fru-Glu, basic amino acids differ from the above peptide inhibitor although $K_i$ values are high. In view of the fact that PM I reaction is a bisubstrate reaction with sequential Bi-Bi mechanism being AdoMet as the first substrate and histone as the second substrate (Lee et al., 1977), it is conceivable that these two binding sites are sterically separated, and combination of two different types of inhibtors might be very effective in inhibiting the protein methylase I activity in a synergistic manner.

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