Arch. Pharm. Res. Vol. 17, No. 5, pp. 354-359, 1994

Inhibition of C-Terminal O-Methyltransferase by a Rat Liver Cytosolic Peptide

Seung Hee Park and Hyang Woo Lee

College of Pharmacy, SungKyunKwan University, Suwon 440-746, Korea

(Received July 27, 1994)

The activity of S-farnesylcysteine O-methyltransferase was assayed by incubating the enzyme with a synthetic *in vitro* substrate, [N-acetyl-S-*trans, trans*-farnesyl-L-cysteine (AFC)], together with S-adenosyl-L-[*methyl*-^{14}C]methionine. The resulting methylesterification product, [N-acetyl-S-*trans, trans*-farnesyl-L-cysteine (*methyl*-^{14}C) ester (AFCME)], was then analyzed either directly on HPLC or by converting the AFC[*methyl*-^{14}C]ME to [*methyl*-^{14}C] alcohol by base-hydrolysis. Employing these two analytical methods, it was established that a peptide purified from rat liver cytosol fraction [Int. J. Biochem., 25, 1157 (1993)] strongly inhibited the above enzyme activity with IC_{50} of 7.1×10^{-8} M. Also, the S-farnesylcysteine O-methyltransferase from several human colon cancer cells was also equally inhibited by the peptide.

Key words: Peptide inhibitor, S-farnesylcysteine O-methyltransferase

INTRODUCTION

The *ras* oncogenes code for closely related 21 kDa proteins, and they are thought to be involved in cell growth and differentiation (Barbacid, 1987; Rodenhuis *et al.*, 1990; Bos *et al.*, 1987). These proteins are synthesized in the cytoplasm as precursor forms with a C-terminal sequence, Cys-XaaXaaXaa (where -Xaa is any amino acid) and undergo a series of posttranslational modifications at their C-terminals. These modifications increase the hydrophobicity of the proteins, thereby facilitating their association with the inner face of the plasma membrane (Haubruck *et al.*, 1991; Lowy *et al.*, 1993). The C-terminal cysteine is first isoprenylated with farnesyl, then followed by proteolytic removal of three amino acid residues from the C-terminus and by the methylesterification of C-terminal cysteine. The cysteine residues upstream of the farnesylated C-terminal cysteine become palmitoylated, further increasing the membrane affinity and biological activity. Despite the generally well-characterized reaction mechanism, the specific biological function of each of these reactions is not clearly delineated (Lowy *et al.*, 1993).

Because of its potential biological importance, the enzyme responsible for the methylation of $p21^{ras}$ C-terminus has been studied by several groups of investigators (Clarke, 1992, Gibbs, 1991), employing a synthetic *in vitro* substrate, N-acetyl-S-farnesyl-L-cysteine (AFC). AFC is of a small size and is easy to synthesize. Furthermore, the reaction product [N-acetyl-S-farnesyl-L-cysteine methyl ester (AFCME)] is hydrophobic and the methyl ester is labile under basic hydrolysis conditions. These properties make AFC well suited for the assay of C-terminal O-methyltransferase activity. The enzyme which is responsible for methylesterification of *ras* oncogene product p21 is described here as C-terminal O-methyltransferase or S-farnesylcysteine O-methyltransferase interchangeably, since N-acetyl-S-farnesyl-L-cysteine was used as a convenient in vitro substrate for the enzyme assay.

We recently purified and characterized a peptide from rat liver cytosol which inhibited several AdoMet-dependent transmethylation reactions (Park *et al.*, 1993). Therefore, it was worthwhile to study the possibility of inhibition of C-terminal O-methyltransferase by the peptide. This might suggest potential importance of the peptide in modulating the cell growth and differentiation through p21 processing.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 58 mCi/mmol) was purchased from ICN, Irvine, CA. Histone (calf thymus, type II-AS), bovine serum

Correspondence to: Hyang Woo Lee, Department of Biochemistry, College of Pharmacy, SungKyunKwan University, Suwon 440-746, Korea

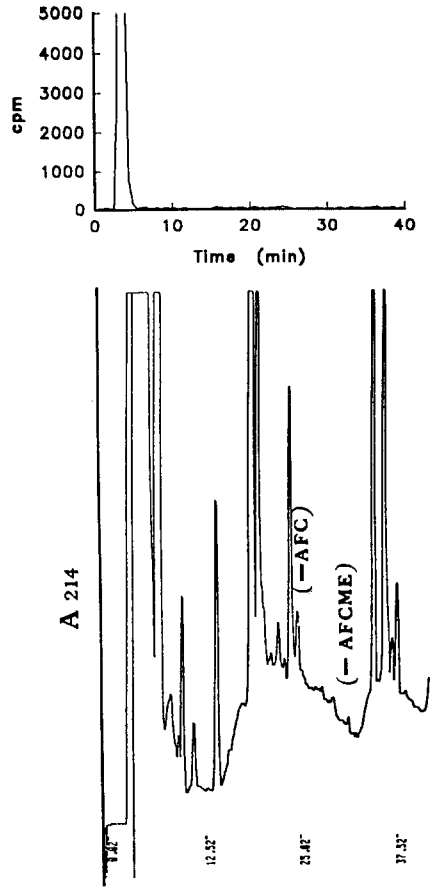

Fig. 1. HPLC analysis of the control incubation mixture (without substrate AFC). The incubation mixture contained all the components except the *in vitro* substrate, AFC. The upper panel indicates the radioactivity profile, and the lower panel A_{214} tracing. No radioactive AFCME (upper panel) or AFC(lower panel) are detectable. The rest of the experimental procedures are described under Methods.

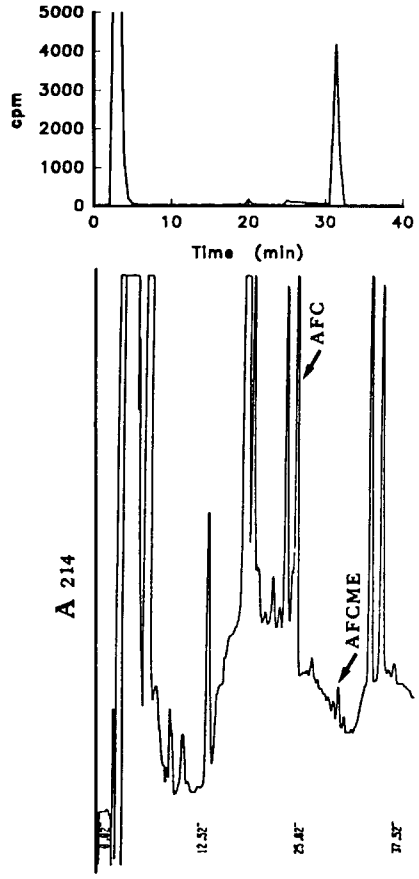

Fig. 2. HPLC analysis of the assay incubation mixture. Contrary to Fig. 1, the incubation mixture contained all the ingredients. A_{214} tracing of AFC (lower panel) and radioactivity peak corresponding to AFCME (upper panel) are seen. Detailed explanations are given under Methods and Fig. 1.

albumin, isoamyl alcohol, acetonitrile, sodium dodecylsulfate(SDS), L-cysteine·HCl, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co., St. Louis, MO. *Trans, trans*-farnesyl bromide was from Aldrich Chemical Co., Milwaukee, WI. The rest of the chemicals were obtained from various commercial sources and were of the highest grade available.

Synthesis of N-acetyl-S-*trans, trans*-farnesyl-L-cysteine (AFC)

One gram of L-cysteine·HCl, 7 ml of ethyl alcohol and 5.7 ml of 2 N NaOH were allowed to react according to the method of Volker *et al.* (1990). After 1 hr in an ice-bath, the pH of the mixture was adjusted to approximately 6 to 7 with 2 N HCl. The precipitate formed (farnesyl-L-cysteine) was filtered and washed with water and ether. Farnesyl-L-cysteine was then allowed to react with acetyl chloride in the presence of pyridine in ether to synthesize N-acetyl derivatives. The resulting N-acetyl-S-*trans, trans*-farnesyl-L-cysteine (AFC) was purified on silica gel open column and further on reverse-phase HPLC. The identity of the compound was confirmed by 300 MHz proton NMR. Further analysis of the compound was performed on HPLC, as described later (Fig. 1 and 2).

Purification of the peptide inhibitor

The peptide inhibitor for the transmethylation reaction was carried out according to the method described recently by Park *et al.*(1993). Twenty grams of fresh rat liver were homogenized in 4 volumes of 10 mM phosphate buffer (pH 7.0), the homogenate was centrifuged at 12,500 g for 20 min, and the supernatant obtained was further centrifuged at 105,000 g for 1 hr. The supernatant was heated at 100°C for 5 min and the coagulated protein was removed at 48,000 g for 15 min. The lyophilized supernatant fraction was dissolved in 2.0 ml of water. The suspension was then loaded onto a Bio Gel P-6 column [1.8 cm (i.d.)×120

cm] and the column was eluted with 25 mM Tris-HCl buffer (pH 6.5). The flow rate was 20 ml/hr. The fractions containing the inhibitory activity against protein methylase II [S-adenosyl-L-methionine (AdoMet): protein-carboxyl O-methyltransferase; EC 2.1.1.77; see below] were pooled and the sample was lyophilized. There were two peaks of inhibitory activity, and the second peak was lyophilized. The lyophilized powder was suspended in 1.0 ml of doubly distilled water and was injected onto an HPLC column [JALGEL ODS column; 2.0 cm (i.d.)×25 cm] which had previously been equilibrated with 0.1% TFA in water. The column was eluted with 0.1% TFA in water for 1 hr and the elution was continued with 0.1% TFA in 5% acetonitrile. The flow rate was 5.0 ml/min and the elution was monitored at 214 nm. The sample obtained was lyophilized, and the lyophilized sample was dissolved in 1.0 ml of doubly distilled water, and was chromatographed a second time on the HPLC as described above: An isocratic elution system was employed with mobile phase of 0.1% TFA in 5% acetonitrile. The final preparation was lyophilized and was employed for the inhibitory activity for both protein methylase II and S-farnesylcysteine O-methyltransferase.

Purification of S-farnesylcysteine O-methyltransferase

Fresh rat brain was homogenized in 7 volumes of sucrose solution (250 mM sucrose, pH 7.4; 10 mM Tris-HCl, 1 mM Na-EDTA) and the homogenate was centrifuged at 14,500 g for 12 min. The supernatant obtained was further centrifuged at 120,000 g for 1 hr and the pellet was suspended in the sucrose solution.

Purification of AdoMet : protein-carboxyl O-methyltransferase (protein methylase II)

The enzyme was purified according to the method of Kim and Paik (1978), up to the stage of DEAE-Cellulose column chromatography. Specific activity of the partially purified enzyme was 7.63 pmol Ado[*methyl*-^{14}C]Met transferred/min/mg enzyme protein.

Enzyme and the peptide inhibitor assays for S-farnesylcysteine O-methyltransferase

Enzyme assay was carried out according to the method of Stephenson and Clarke (1990). Briefly described, an incubation mixture containing 33 mM HEPES (pH 7.6), 33 mM NaCl, 0.2 mM AFC, the enzyme preparation and the peptide inhibitor (without the peptide inhibitor in case of the control) in a total volume of 60 μl was preincubated at 37℃ for 5 min, and it was subsequently incubated for 15 min after addition of 10 μl of 50 μM Ado[*methyl*-^{14}C]Met (138 cpm/pmole). The reaction was terminated with 70 μl of 1.0 N NaOH containing 1% SDS, the reaction mixture was thoroughly mixed, and a 120 μl aliquot (140 μl total) was transferred to a pleated filter paper (1.5× 8 cm, Wattman No. 1). The filter paper was placed on the neck of scintillation vial (20 ml-capacity) containing 10 ml of scintillation cocktail solution, and was left at room temperature for 2 hr (base-hydrolysis). After removing the paper, the amount of volatile $^{14}CH_3OH$ formed was measured. The difference between the radioactivity values in the presence and absence of AFC in the incubation mixture was taken as the means of enzyme activity of S-farnesylcysteine O-methyltransferase.

Identification and quantitation of N-acetyl-S-*trans*, *trans*-farnesyl-L-cysteine methyl ester (AFCME) on HPLC

Further confirmation of AFCME formed from AFC by the action of S-farnesylcysteine O-methyltransferase was carried out by the method of Volker *et al.* (1990). After AFC was allowed to react with S-farnesylcysteine O-methyltransferase as described above, the reaction was terminated with 21.4 μl of 50% trichloroacetic acid (TCA), the mixture was centrifuged at 10,000 g for 3 min, and the supernatant was set aside. The pellet was then washed once with 75 μl of acetonitrile, and the supernatant after centrifugation was combined with the above first supernatant. The presence and amount of AFC[*methyl*-^{14}C]ME was determined by the following HPLC method.

Analysis of AFC[*methyl*-^{14}C]ME on HPLC was carried out under the following conditions. Column: Shodex C_{18} column (4.6 mm i.d.×250 mm); Mobile phase: Solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile); Detection: 214 nm UV absorbance. The sample was injected onto the column preequilibrated with solvent A, the sample was initially eluted for 5 min with 50% solvent B, and the solvent concentration was subsequently raised to 90% in 25 min with a linear gradient system. Finally, the sample was eluted with 90% solvent B up to 40 min running time. The flow rate was 1.0 ml/min and fractions of 30 sec were collected for radioactivity measurement.

Assay for protein methylase II

Enzyme assay for protein methylase II was carried out by the method of Kim (1984). The incubation mixture contained 0.05 M citrate-phosphate buffer (pH 6.0), 1.2 mg of histone (type II-As), and purified enzyme preparation in a total volume of 90 μl and the mixture was preincubated at 37℃ for 5 min. Subsequently, 10 μl of 50 μM Ado[*methyl*-^{14}C]Met was added and the mixture was incubated for 15 min. The reaction was terminated with 100 μl of 0.5 M potas-

Table I. Comparison of results obtained by two analytical methods for S-farnesylcysteine O-methyltransferase activity

Methods used	+AFC	+AFC +Peptide Inhibitor**	Degree of inhibition (%)
$^{14}CH_3OH$ vapor	9.66*	3.59	63
HPLC	10.35	3.14	70

*Results are expressed in specific activity (pmoles of [*methyl*-^{14}C] transferred/min/mg enzyme protein), and are averages of 3 independent determinations. Since a 120 µl portion out of the 140 µl total incubation mixture was used in the $^{14}CH_3$ OH vapor method (see Methods), these numbers were corrected by the factor of 140/120=1.17. The rest of the experimental conditions are described under Methods.
**17.6 pmoles of peptide inhibitor (0.13 µM) was used.

sium borate buffer (pH 11.0) and the mixture was allowed to stand for 5 min. To this mixture, 1.0 ml of isoamyl alcohol was added and the mixture was vortexed and centrifuged. 0.7 ml of the supernatant was transferred to a scintillation vial and radioactivity was measured.

Protein concentration

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.

RESULTS

Standardization of the enzyme assay for S-farnesylcysteine O-methyltransferase

Before investigating the effect of the peptide inhibitor, it is of extreme importance to establish and verify the assay conditions for the S-farnesylcysteine O-methyltransferase activity.

For our enzyme assay procedure, the rat liver microsomal S-farnesylcysteine O-methyltransferase was to employed to catalyze methylesterification of the synthetic *in vitro* substrate AFC with Ado[methyl-^{14}C]Met, resulting in the formation of AFC[*methyl*-^{14}C]ME. The formation of AFC[*methyl*-^{14}C]ME can be determined either directly on HPLC (Figs. 1 and 2) or by the $^{14}CH_3$ OH vapor method (Table I).

Fig. 1 illustrates the elution pattern of AFC and AFCME in the control reaction mixture (without AFC). There is no radioactive AFCME formed (the upper panel). The radioactivity peak appearing at approximately 3 min elution time is most likely due to contaminating unreacted Ado[*methyl*-^{14}C]Met (see also Fig. 2). When the complete reaction mixture which included AFC was incubated, there appeared a distinct radioactivity peak at approximately 31 min elution time (the upper panel of Fig. 2). It is evident from Figs. 1 and 2 that both AFC and AFCME are clearly resolved by HPLC.

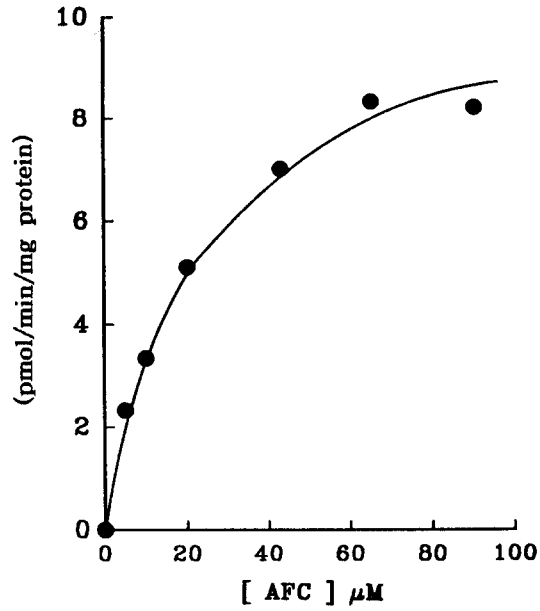

Fig. 3. Effect of AFC concentration on the rate of S-farnesylcysteine O-methyltransferase activity. Total incubation mixture was 70 µl, and enzyme assay was carried out by the $^{14}CH_3$ OH vapor method. Approximate *Km* value for AFC was 2×10^{-5} M. Detailed explanation of the experimental procedures is described under Methods.

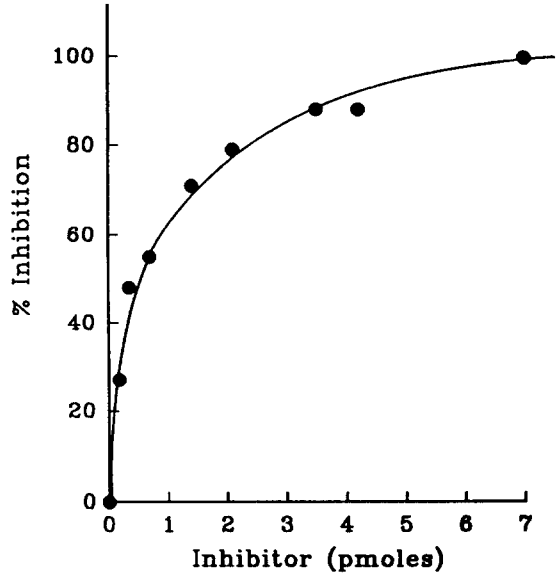

Fig. 4. Inhibitory effect of the rat liver cytosol peptide on AdoMet : protein-carboxyl O-methyltransferase (protein methylase II). Varying amounts of a purified rat liver cytosolic peptide preparation were added to the incubation mixture for protein methylase II (100 µl total). One hundred % of protein methylase II activity was 7.63 pmoles [*methyl*-^{14}C] transferred/min/mg enzyme protein. IC_{50} was 0.48×10^{-8} M.

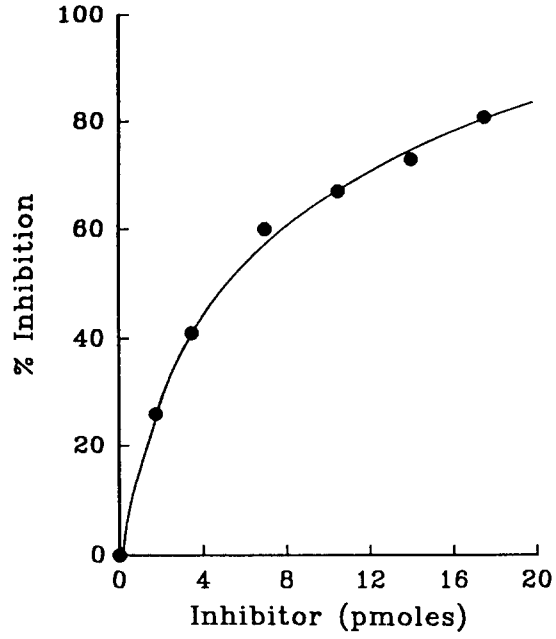

Fig. 5. Inhibitory effect of the rat liver cytosol peptide on S-farnesylcysteine O-methyltransferase. Different amounts of the peptide preparation were added to the total 70 μl of incubation mixture for S-farnesylcysteine O-methyltransferase assay. Rat brain microsomal fraction was used as the source of the enzyme, and 100% enzyme activity indicates 9.9 pmoles of [*methyl*-^{14}C] transferred/min/mg enzyme protein. IC_{50} was found to be 7.1×10^{-8} M.

The S-farnesylcysteine O-methyltransferase activity could also be quantitated by another assay method, the $^{14}CH_3OH$ vapor method. As listed in Table I (2nd column), the results obtained by both HPLC and the $^{14}CH_3OH$ vapor method are very compatible.

Fig. 3 shows the effect of AFC concentration on the enzyme activity. A nearly hyperbolic relationship exists between the concentration of AFC and the initial enzyme velocity. Km value for AFC was determined to be approximately 2×10^{-5} M which was very close to the value reported by Volker (1990).

The effect of the peptide inhibitor on protein methylase II

The peptide inhibitor described in this paper was originally identified as an inhibitor for phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17) (Chiva *et al.*, 1984) and protein methylase II (Hong *et al.*, 1986). In order to confirm the inhibitory activity of this peptide preparation, it was tested with protein methylase II (Fig. 4). It is seen in the figure that almost complete inhibition of the protein methylase II activity was achieved with about 7 pmoles of the peptide in the incubation mixture of 100 μl volume (approximately 7×10^{-8} M). Our earlier observation showed that the *Ki* value was 1.9×10^{-8} M (Park *et al.*, 1993).

Table II. Inhibitory activity of the peptide on S-farnesylcysteine O-methyltransferase of human colon cancer cells

Cancer cell lines	Amount of protein used (μg)*	The peptide inhibitor		Percent inhibition
		without	with	
		(pmol/min/mg enz. protein)		
DLD 1	113	9.09**	3.11	66
HCT 15	116	8.84	3.66	59
HT 29	96	9.17	3.19	65

*Total volume of incubation mixture was 70 μl and 14 pmoles of the inhibitor per assay (0.2 μM) was used.
**Average of two independent determinations.
Human colon cancer cells cultured to 100% confluence were trypsinized and were centrifuged at 5,000 g for 1 min. The pellets were washed with a sucrose buffer solution (250 mM sucrose, 10 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4) and resuspended in the sucrose buffer. The suspensions were sonicated for 30 sec twice and 20 μl of the suspension were used as the enzyme source. Detailed experimental procedures are described under Methods.

The peptide inhibitor on S-farnesylcysteine O-methyltransferase

As shown in Fig. 5, the peptide inhibitor was also highly effective on S-farnesylcysteine O-methyltransferase activity. IC_{50} was found to be around 7.1×10^{-8} M.

In order to further confirm the inhibitory effect of the peptide, the inhibition of S-farnesylcysteine O-methyltransferase by the peptide was investigated by two different assay methods, namely, HPLC and the $^{14}CH_3OH$ vapor method. As shown in Table I, the results obtained by these two methods were very similar. As expected, however, it was observed that, the control blank value (without AFC and the peptide inhibitor) from the HPLC was nil, whereas the $^{14}CH_3OH$ vapor method had a substantial amount of radioactivity. Therefore, the values in Table I were corrected accordingly.

DISCUSSION

We have recently purified from rat liver cytosol a peptide which inhibited AdoMet : protein-carboxyl O-methyltransferase (protein methylase II; EC 2.1.1.77) *in vitro* (Park *et al.*, 1993), (also Fig. 4). The preparation purified through the processes of heat-treatment, Bio Gel column chromatography and repeated HPLC was found to be homogenous, judged by the demonstration of a single peak on the HPLC eluted by both gradient as well as isocratic elution. The molecular weight of the peptide was 2,584 with 29 amino acid residues, glycine accounting for approximately 52% of the total amino acids. Employing protein methylase

II and bovine serum γ-globulin as an *in vitro* substrate, the mode of inhibition was found to be noncompetitive with a *Ki* value of 1.9×10^{-8} M, which indicated the peptide to be a highly effective inhibitor. The *Ki* value for AdoHcy(S-Adenosyl-C-homocysteine), which is a competitive inhibitor for AdoMet, is two orders of magnitude higher 1.03×10^{-6} M.

The peptide inhibitor was also found to inhibit some other AdoMet-dependent methyltransferases such as AdoMet : protein-arginine N-methyltransferase (EC 2.1. 1.23), AdoMet : protein-lysine N-methyltransferase (EC 2.1.1.43), AdoMet : cytochrome c-lysine N-methyltransferase (EC 2.1.1.59), AdoMet : RNA methyltransferase and AdoMet : DNA methyltransferase (Park *et al.*, 19 93). This suggested that this peptide affected generally all the AdoMet-dependent transmethylation reactions. It was, however, noncompetitive with respect to AdoMet, binding the enzyme at a different site from that of AdoMet or AdoHcy (a 'potent' competitive inhibitor for AdoMet). S-Farnesylcysteine O-methyltransferase is one of the posttranslational processing enzymes of *ras* oncogene product p21 protein which requires AdoMet for the reaction (Barbacid, 1987; Bos *et al.*, 1987). Therefore, it was worthwhile to investigate whether the peptide inhibitor could also inhibit the methylesterification of a synthetic *in vitro* substrate for the enzyme, AFC.

Indeed, the peptide inhibitor was highly effective for S-farnesylcysteine O-methyltransferase with an IC_{50} of 7.1×10^{-8} M (Fig. 5). Inhibitory activity of the peptide on S-farnesylcysteine O-methyltransferase was confirmed by two different analytical methods; HPLC and the $^{14}CH_3OH$ vapor method. It is also of interest to observe that the peptide was equally effective in inhibiting the enzyme, regardless of the source of the enzyme (Table II); normal or neoplastic tissues.

The biochemical function of enzymatic methylesterification of *ras* oncogene product p21 has not yet been clarified. Nonetheless, whatever the function may be, there is a good possibility that the peptide might participate in a regulation of cell growth and differentiation exerted by $p21^{ras}$ through the regulation of methylation. Quantitative analysis of the peptide during various cellular conditions such as aging, neoplasia and cellular differentiation might be the first step to be explored.

ACKNOWLEDGEMENTS

This work was supported by a research grant from Korea Science and Engineering Foundation (KOSEF 921-1600-014-2).

REFERENCES CITED

Barbacid, M., ras GENES. *Ann. Rev. Biochem.*, 56, 779-828 (1987).

Bos, J. L., Fearon, E. R. and Hamilton, S. R., Prevalence of ras gene mutation in human colorectal cancer. *Nature*, 327, 293-297 (1987).

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

Chiva, V. A. and Mato, J. M., Inhibition of phospholipid methylation by a cytosolic factor. *Biochem. J.*, 218, 637-639 (1984).

Clarke, S., Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Ann. Rev. Biochem.*, 61, 355-386 (1992).

Gibbs, J. B., Ras C-terminal processing enzymes; New drug targets? *Cell*, 65, 1-4 (1991).

Haubruck, H. and McCormick, F., Ras p21; effects and regulation. *Biochim. Biophys. Acta*, 1072, 215-229 (1991).

Hong, S. Y., Lee, H. W., Desi, S., Kim, S. and Paik, W. K., Studies on naturally occuring proteinous inhibitor for transmethylation reactions. *Eur. J. Biochem.*, 156, 79-84 (1986).

Kim, S. and Paik, W. K., Purification and assay of Protein methylase II(S-Adenosylmethionine: protein-carboxylmethyltransferase; EC 2.1.1.24). In Stein G., Stein J. and Kleinsmith L. (Eds), *Methods in Cell Biology*, Vol. 19, Academic Press, New York, 1978, pp. 79-88.

Kim, S., S-Adenosylmethionine: protein-carboxyl O-methyltransferase (Protein Methylase II). In Wold F. and Moldave K. (Eds), *Methods in Enzymology*, Vol. 106, Academic Press, New York, 1984, pp. 295-309.

Lowy, D. R. and Willumsen, B. M., Function and regulation of RAS. *Ann. Rev. Biochem.*, 62, 851-891 (1993).

Rodenhuis, S. and Slebos, R. J. C., The ras oncogenes in human lung cancer. *Am. Rev. Respir. Dis.*, 142, S27-S30 (1990).

Park, S. H., Lee, H. W., Kim, S. and Paik, W. K., Peptide inhibitor for S-adenosyl-L-methionine dependent transmethylation reactions. *Int. J. Biochem.*, 25, 1157-1164 (1993).

Stephenson, R. C. and Clarke, S., Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesylcysteine containing peptide substrate. *J. Biol. Chem.*, 265, 16248-16254 (1990).

Volker, C., Miller, R. A. and Stock, J. B., S-Farnesylcysteine methyltransferase in the bovine brain. *Methods: A Companion to Methods in Enzymology*, 1, 283-287 (1990).