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High-performance liquid chromatographic assay for farnesyl-protein transferase activity with dabsylated peptide

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Abstract

An HPLC assay for farnesyl-protein transferase activity using a dabsylated peptide is described. The substrates used were a synthetic dabsylated nonapeptide, N-dabsyl-L-serinyl-L-methioninyl-L-glycinyl-L-leucinyl-L-prolinyl-L-cysteinyl-L-valinyl-L-valinyl-L-methionine, corresponding to the C-terminal peptide sequence of human N-Ras p21 without the N-terminal serine, and farnesyl diphosphate. The product was separated from the substrates on a reversed-phase C_{18} column, using gradient elution with acetonitrile (0.05% trifluoroacetic acid)–water (0.1% trifluoroacetic acid) and was detected at 436 nm. The addition of the farnesyl group to the peptide was confirmed by MS and NMR. Enzymatic reaction was ascertained from the dependences on time, on the protein of the enzyme source and on the substrates. The reaction was specifically inhibited by L-cysteinyl-L-valinyl-L-valinyl-L-methionine, the tetrapeptide corresponding to the "CAAX" motif. The limit of detection was 2 pmol per 100-μl reaction mixture. The farnesyl-protein transferase activity can quantitatively be measured up to 200 μg cytosolic protein in human liver. This method provides a convenient and quantitative assay for crude materials, such as tissue homogenate from clinical samples, without the use of radioactive probes and large amounts of Ras protein.

1. Introduction

Farnesylation plays an important role in post-translational modification to express the functions of many proteins, such as small G proteins. Farnesyl-protein transferase (FPTase)[1], which catalyzes this reaction, introduces a farnesyl group from farnesyl diphosphate (FPP), which is an intermediate metabolite in the cholesterol pathway, to the cysteine residue at the COOH-terminus of the protein substrate with a thioether linkage. The protein substrates possess a com-

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[1] Abbreviations used: FPTase, farnesyl-protein transferase; SMGLPCVVM, L-serinyl-L-methioninyl-L-glycinyl-L - leucinyl-L-prolinyl-L-cysteinyl-L-valinyl-L-valinyl-L-methionine; Dab-SMGLPCVVM, N-L-dabsyl-L-serinyl-L-methioninyl-L-glycinyl-L-leucinyl-L-prolinyl-L-cysteinyl-L-valinyl-L-valinyl-L-methionine; CVVM, L-cysteinyl-L-valinyl-L-valinyl-L-methionine; SVVM, L-serinyl-L-valinyl-L-valinyl-L-methionine; TKCVIM, L-threoninyl-L-lysinyl-L-cysteinyl-L-valinyl-L-isoleucinyl-L-methionine; CVLS, L-cysteinyl-L-valinyl-L-leucinyl-L-serine; FPP, farnesyl diphosphate; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMF, dimethylformamide; SIMS, secondary-ion mass spectroscopy.

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mon tetrapeptide sequence referred to as the "CAAX" motif in the COOH terminus, where C is a cysteine, A is any aliphatic amino acid, and X is the C-terminal amino acid, such as methionine, serine, cysteine or alanine [1,2]. The addition of the CAAX sequence to the C-terminus changes a protein into a substrate for farnesylation [3]. The enzyme is inhibited by tetrapeptides corresponding to the CAAX sequence. Thus, this CAAX motif appears to be the sole recognition site for FPTase [1,2]. A peptide containing the CAAX sequence at the COOH terminus can also be a substrate for farnesylation [4].

In Ras p21, farnesylation is the first step in the posttranslational modification following cleavage of the three C-terminal amino-acid residues (AAX) and methylation of the resulting C-terminal farnesylated cysteine. It is the key step in the expression of its function which leads to localization of the Ras protein in the plasma membrane and yields the essential components of receptor-mediated signaling pathways controlling cell proliferation and certain differentiation processes [2,5–7]. Mutated Ras p21 and overexpressed Ras p21 stimulate cell proliferation and induce transformation [5]. Enhanced expression of Ras p21 is frequently detected in human tumors [8]. Farnesylation is essential for efficient cell transformation by oncogenic forms of the Ras protein [9]. Thus inhibitors of FPTase may serve as antitumor agents [10] and the exploitation of its potent inhibitors is progressing [11–13]. Ras protein is not unique to neoplasms, but it is also important in physiologically normal processes. For instance, the expression of Ras p21 protein is related to the process of hepatic regeneration [14,15]. Ras protein can reveal the relation to cell proliferation and differentiation and FPTase regulates the expression of the functional ras protein, but little is known about the role of the enzyme in the pathogenesis of diseases related to cell proliferation and differentiation, e.g. carcinogenesis and regeneration. In order to investigate its pathophysiological role, a convenient and quantitative assay is required for crude materials such as tissue homogenate from clinical samples.

At present, the assay for FPTase activity is mainly performed with Ras p21 protein and tritium-labeled FPP [1]. This conventional assay needs large amounts of Ras p21 protein for the quantitative measurement of crude samples. The background counts of tritium in the precipitates tend to increase with increasing protein content. Thus, this conventional assay is not favorable for quantitative measurement in crude samples. The immunoprecipitation assay performed according to Moores et al. [16] is sensitive, but the response becomes nonlinear with Ras p21 protein [17]. Recently, simpler assays for screening FPTase inhibitors have been reported. Hara et al. [11] used a yeast strain with a conditional deficiency in the GPA-1 gene for a microbial screening. Pompliano et al. [18] measured intramolecular fluorescence enhancement due to farnesylation of N-dansylated Ras peptide. These assays are more suitable for semiquantitative than for quantitative measurements.

In the present paper, we describe an HPLC assay for FPTase activity. In their assay Pompliano et al. [17] used [^{3}H]FPP and the peptide CVLS as substrates and the product [^{3}H]farnesyl-CVLS was isolated from the reaction mixture by reversed-phase HPLC and counted. However, we used the synthetic dabsylated nonapeptide corresponding to the C-terminal peptide sequence of human N-Ras p21 without the N-terminal serine as substrate instead of the Ras p21 protein. This peptide can be detected in the visible range. This method offers a convenient quantitative assay for crude materials, such as tissue homogenate from clinical samples.

2. Experimental

2.1. Equipment

The same HPLC system was used for both the purification of the dabsylated Ras peptide used as the substrate and the assay of the farnesylated product. The system consisted of Gilson HPLC pumps (Model 305, 306), a Gilson manometric module (Model 805), a Gilson dynamic mixer (Model 811C), a Gilson sample injector (Model

231) with a 200-μl sample loop, a Gilson dilutor (Model 401), and a Gilson fraction collector (Model 201). Separation was performed on a reversed-phase C_{18} column (TSKgel OSD-80TM, 150 × 4.6 mm I.D.). Detection was accomplished at 436 nm with a linear UV-Vis detector (UVIS 204). The recording of signals from the detector and the integration were performed with a Sony personal computer (QuarterL PCX-320), Epson printer (VP-960), Gilson system interface module (Model 508B), and computer program for HPLC system (Gilson 712 HPLC).

Mass spectra were determined with a JEOL JMS-HX/HX110A. NMR spectra were obtained with a Varian Unity-400.

2.2. Chemicals

The following chemicals were purchased from commercial sources: 4-dimethylaminoazobenzene-4′-sufonyl chloride (dabsyl chloride) from Dojin Chemical Lab.; Tris, Hepes, $NaHCO_3$, $NaCO_3$, $CuSO_4$, $MgCl_2$, $ZnCl_2$, TCA, DTT, Triton X-100, HPLC-grade water, acetonitrile, acetone, and ethanol, amino acid analaytical grade of TFA from Wako Pure Chemical Industries; leupeptin, benzamizine, pepstatine, PMSF from Sigma; farnesol from Aldrich; and the protein assay kit from Bio-Rad. Synthetic peptides (SMGLPCVVM, CVVM, SVVM) and FPP were kindly provided by Kyowa Hakko Kogyo. All reagents were of the highest purity available.

2.3. Enzymes

Human kidney for the purification of FPTase by ion-exchange chromatography was obtained at autopsy. Partially purified FPTase was prepared according to a partially modified procedure of Reiss et al. [1]. Briefly, a 30–50% ammonium sulfate fraction of the cytosol was dialyzed overnight and chromatographed on a Mono Q 5/5 column (Pharmacia L.K.B. Biotechnology) in 50 m*M* Tris (pH 7.5) containing 20 μ*M* $ZnCl_2$ and 10 m*M* $MgCl_2$. Fractions eluting at 0.25–0.30 *M* NaCl contained the major FPTase activity. These fractions were pooled, divided into aliquots and stored at −35°C. The cytosol sample of human liver tissue for study of the protein dependence of the enzymatic activity was prepared from surgically resected materials. Informed consent was obtained from all patients.

2.4. Preparation of dabsylated Ras peptide

In order to selectively dabsylate the terminal amino group of the Ras peptide and not the thiol group or the alcohol group, dabsylation was performed after dimerization of the peptide by disulfide bonds which protected the thiol group and after pH adjustment under the following conditions [19]: 100 μl of 1 m*M* Ras peptide (SMGLPCVVM) in 0.13 *M* sodium carbonate–sodium bicarbonate buffer (pH 8.8) containing 5 μl of 1 m*M* copper sulfate was allowed to react at 70°C in a water bath for 15 min. The reaction mixture was adjusted to pH 6.1 with 9 μl of 1.2 *M* HCl. Next, 100 μl of 5 n*M* dabsyl chloride solution in acetone was added. The Eppendolf tube containing the mixture was tightly stoppered and the mixture allowed to react at 70°C in a water bath for 15 min. After the precipitate was redissolved and centrifuged at 8800 *g* for 5 min, 50 μl of 100 m*M* DTT in 0.13 *M* sodium carbonate–sodium bicarbonate buffer (pH 8.8) was added to 100 μl supernatant of the reaction mixture. The resulting mixture was applied to a C_{18} reversed-phase HPLC column (see section 2.6). The separated fraction of dabsylated peptide was evaporated under dry nitrogen gas. The powder obtained was used as substrate.

2.5. Enzymatic reaction and removal of proteins

The reaction mixture typically contained the following concentrations of components in a final volume of 80 μl: 50 m*M* Hepes–NaOH (pH 7.4), 50 μ*M* $ZnCl_2$, 10 m*M* $MgCl_2$, 2.5 μ*M* pepstatine, 2 m*M* PMSF, 25 m*M* benzamizine, 2.5 μ*M* leupeptin, 2% Triton X-100, 1 m*M* DTT, 125 μ*M* FPP, 80 μ*M* Dab-SMGLPCVVM and the enzyme. In inhibition assays, the final reaction mixture contained 0.1–100 μ*M* CVVM

or 5.0–1800 μM SVVM. Since FPP was soluble in 25 mM ammonium bicarbonate–70% ethanol, a 5-mM FPP solution was prepared. However, Dab-SMGLPCVVM is slightly soluble in acetone. Thus, first 2 μl of acetone and then 16 μl of 10% Triton X-100/200 mM Hepes–NaOH buffer (pH 7.4) were added to 6.4 nmol of Dab-SMGLPCVVM powder in an Eppendolf tube for one assay, the mixture being stirred vigorously. The amount of Dab-SMGLPCVVM was estimated by the peak intensity in HPLC after purification. To the mixture the following were added: 2 μl of 5 mM FPP solution, 4 μl of 200 mM Hepes buffer (pH 7.4), 2 μl of 200 mM DTT, and protease inhibitors (1 μl of 200 mM pepstatine and 160 μM PMSF in ethanol, 2 μl of 1 M benzamizine and 2 μl of 50 mM leupeptin). Finally, the enzyme was added to start the reaction. After incubation for 1 h at 37°C, the reaction was stopped by cooling on ice and adding 1 μl of 10 mM CVVM. Immediately, 10 μl of ethanol were added four times, 10 μl of acetone twice, and 10 μl of TCA–acetone (80:20, w/v) solution twice, the mixture being stirred vigorously, to remove proteins. The mixture was then centrifuged at 8800 g for 5 min. The supernatant of this mixture was centrifuged again under the same conditions. The amount of product in the resulting supernatant was measured by HPLC (see section 2.6). The tube was stoppered and cooled on ice to prevent evaporation of the solvent in each process. To investigate the kinetic behavior of Dab-SMGLPCVVM and FPP and the inhibition by tetrapeptides, 3.5 μg of partially purified enzyme from human kidney was used in one assay. To examine the time dependence, the same amount of the enzyme was used. Protein dependence was tested with various amounts of the cytosolic fraction of human liver tissue.

2.6. Chromatographic procedures

All HPLC runs were performed at a flow-rate of 1.0 ml/min at room temperature. A 100-μl sample from a total volume of 160 μl was applied to the C_{18} reversed-phase HPLC column using the autosampler. The substrate and product were eluted with a gradient of acetonitrile (with 0.05% TFA)–water (with 0.1% TFA) as follows: 5 ml of water, 25 ml linear gradient of 0–100% acetonitrile and 5 ml of 100% acetonitrile. Each peak was detected at 436 nm. The amounts of substrate and product were estimated by assuming that the molecular extinction coefficient of dabsyl chloride was the same as that of its derivatives ($\varepsilon = 2.7 \times 10^4\ M^{-1}\ cm^{-1}$ in acetone at 436 nm).

2.7. MS and NMR

To identify the product, the peak fractions with retention times of 23 min (the substrate peptide) and 29.5 min (the product) were collected and the solvent was evaporated. The resulting residues, suspected to be the substrate and product, were subjected to MS and NMR.

Mass spectra were observed by SIMS procedure. Samples were dissolved in DMF. For the matrix, m-nitrobenzylalcohol–glycerol (6:4, v/v) was used. The accelerating voltage was 10 kV.

Proton NMR measurements were carried out at 399.95 MHz at 30°C. For NMR measurement, the substrate peptide and the product were suspended in 99.9% D_2O, lyophilized from D_2O and then dissolved in acetone-d_6. Chemical shifts are expressed in parts/million (ppm), with the residual signal of the solvent being used as the internal standard (2.17 ppm).

3. Results and discussion

3.1. Separation with HPLC and identification

The dabsylated Ras C-terminal peptide, Dab-SMGLPCVVM, reacted with FPP to give a farnesylated product that could be detected in the visible range (Fig. 1). Complete separation of the product from the substrate was achieved by HPLC with a visible range detector on a reversed-phase C_{18} column using a gradient concentration of acetonitrile (with 0.05% TFA)–water (with 0.1% TFA) (Fig. 2). The retention times of substrate and product were approximately 23 min (72% acetonitrile) and 29.5 min

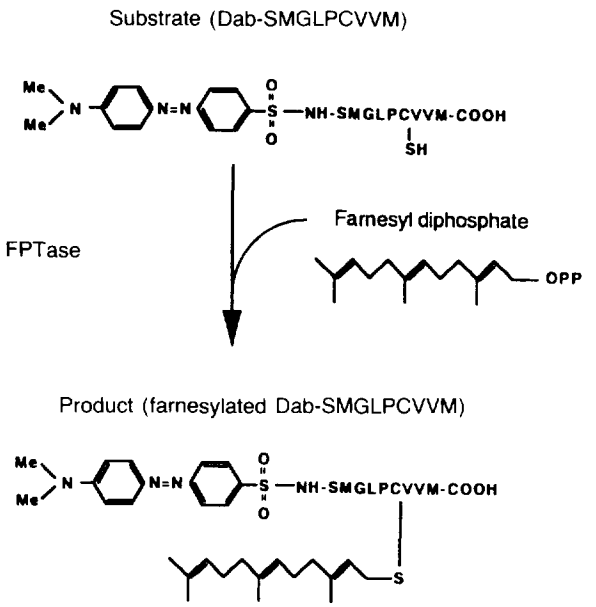

Fig. 1. Reaction scheme for the incorporation of farnesyl group from FPP to Dab-SMGLPCVVM by FPTase.

(95% acetonitrile), respectively. To identify the peaks, each peak fraction was collected and the solvent evaporated under a stream of dry nitrogen gas. MS and NMR measurements were carried out with the powders obtained from the fractions. Mass spectra of the substrate and the product showed quasimolecular ion peaks $[M+H]^+$ at 1223.5 and 1427.7 corresponding to Dab-SMGLPCVVM (M + H: $C_{53}H_{83}O_{13}N_{12}S_4$) and farnesylated Dab-SMGLPCVVM (M + H: $C_{68}H_{107}O_{13}N_{12}S_4$), respectively, in agreement with the calculated values. In NMR spectra, the CH_3 and CH protons corresponding to those of farnesol were detected only for the product and not for the substrate peptide. The CH_2 peak could not be confirmed because of impurities. The powder of farnesylated Dab-SMGLPCVVM was used as the authentic product to confirm the retention time, calibrate the amount of product and estimate its recovery from the reaction mixture (see section 3.3.). The data of the amino acid analysis of the substrate agreed with the predicted values (data not shown). The nonapeptide may possess a subtle balance between hydrophilicity and hydrophobicity which enables one to perform the dabsylation, the enzymatic reaction, the removal of protein and also the separation by HPLC. If the proline is changed to a glycine, the peptide becomes hardly soluble in water (data not shown).

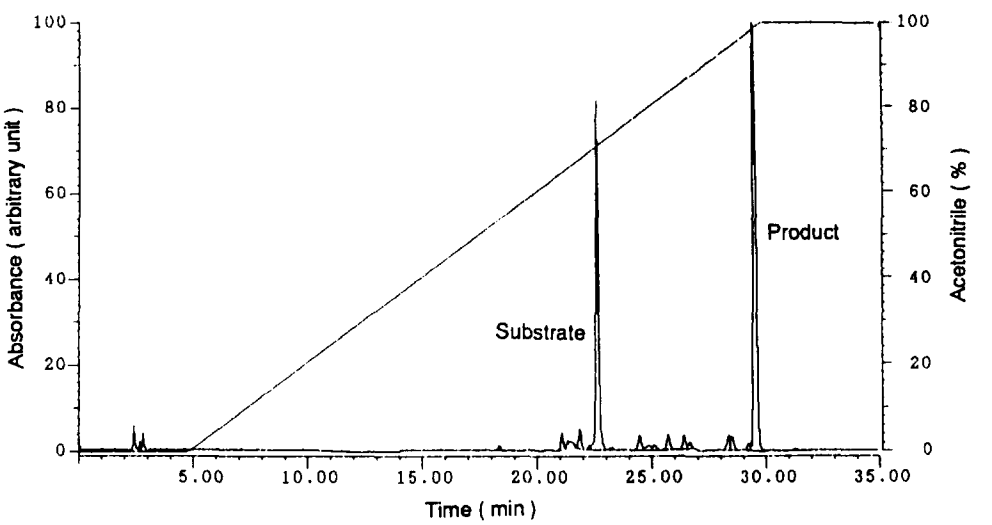

Fig. 2. HPLC of the reaction mixture. The substrate (Dab-SMGLPCVVM) and the product (farnesylated Dab-SMGLPCVVM) were eluted with gradient concentration of acetonitrile (with 0.05% TFA)–water (with 0.1% TFA).

3.2. Effects of Dab-SMGLPCVVM and FPP

The effect of Dab-SMGLPCVVM or FPP in the enzymatic reaction was investigated using the assays under the standard conditions described in the Experimental section without varying the substrate concentration. The results are shown in Fig. 3. The FPTase activity was nearly saturated at 80 μM Dab-SMGLPCVVM and 125 μM FPP, which were used under the standard assay conditions. The apparent Michaelis constants for Dab-SMGLPCVVM and FPP from Hanes' plots were 22 μM and 1.2 μM, respectively. These values are larger than the Michaelis constants for the assay with Ras p21 protein and tritium-labeled FPP [1,16,17,20]. The affinity of the substrates for the enzyme under the assay conditions may differ from that under the conditions of the conventional assay. This difference may be caused by the use of the dabsylated peptide as substrate instead of the Ras p21 protein and/or by other proteins, detergents, and other components in the reaction mixture. More purified FPTase, using affinity chromatography, coupled with C-terminal K-Ras peptide TKCVIM [1] gave almost the same K_m value as the present data (data not shown). This means that the high K_m may have been caused by the detergent (Triton X-100) rather than by other proteins.

3.3. Time and protein dependence

The amount of product increased linearly with time at least until 90 min (Fig. 4A). The dependence on the concentration of the cytosolic fraction of human liver tissue is shown in Fig. 4B. A quantitative assay can be performed for 13–200 μg of protein. The mean specific activity and standard deviation were 0.25 ± 0.01 nmol/h/mg. These data showed that the assay is reproducible at various protein concentrations. The detection limit (signal-to-noise ratio 3:1) for the product was about 2 pmol per 100-μl injection volume. The sensitivity was not higher than that for the method with radio-labeled substances [1], but as has been shown, our method can serve as a practical assay of crude materials. To estimate the loss due to removal of proteins, the following experiments were carried out. Product recovery was determined by comparing the amount of authentic farnesylated Dab-SMGLPCVVM in the incubation mixture with and without FPP. Mixtures containing a certain amount of authentic farnesylated Dab-SMGLPCVVM under the standard assay conditions were prepared and incubated for 60 min at 37°C with or without the enzyme. The amount of product in the mixture after removal of proteins was measured. The recovery was about

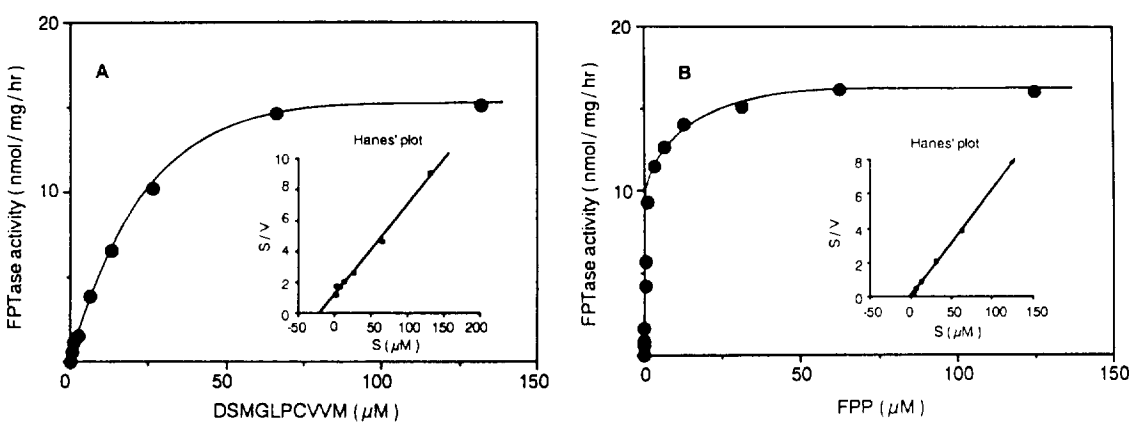

Fig. 3. Effects of (A) Dab-SMGLPCVVM and (B) FPP on FPTase activity with Michaelis–Menten type representation as a function of each substrate concentration. Hanes' plots of the same data are shown in the insets where V is FPTase activity and S is Dab-SMGLPCVVM concentration in (A) or FPP concentration in (B).

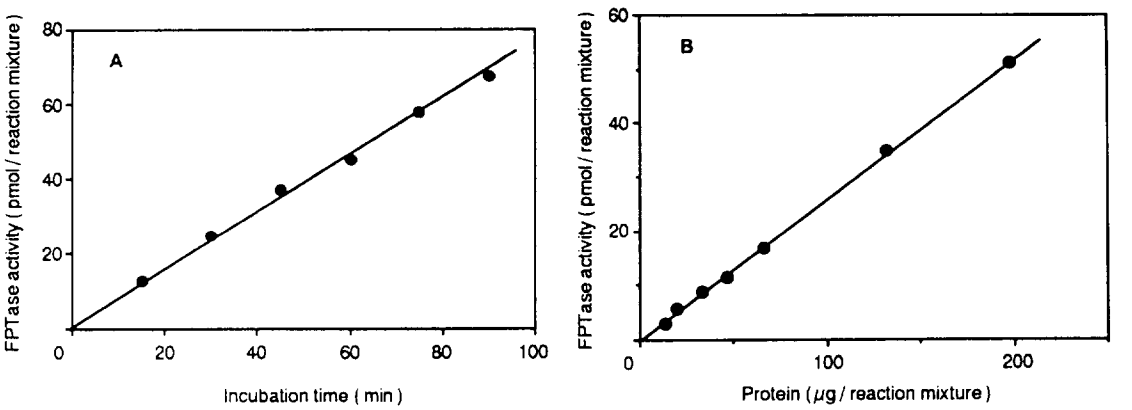

Fig. 4. Time and protein dependences of FPTase activity. (A) Plot of farnesylated Dab-SMGLPCVVM versus incubation time with 3.5 μg of purified FPTase of human kidney. (B) Plot of farnesylated Dab-SMGLPCVVM versus the amount of cytosolic protein of human liver tissue (incubation time was 60 min).

99.5% when 50 μg cytosol protein was used, indicating that adsorption of the product to proteins in the reaction mixture was negligible.

3.4. Inhibition by tetrapeptides

The inhibition of FPTase activity by tetrapeptides was determined in order to confirm the specific affinity of the enzyme to the peptide substrate (Fig. 5). IC_{50} values were about 3 μM in CVVM and 150 μM in SVVM. This shows that CVVM corresponding to the CAAX motif inhibited the FPTase activity more effectively. The IC_{50} is larger than reported values [1,16,17]. This may also be due to differences in incubation conditions (see section 3.2). This inhibition study also shows that our assay can be used to screen potential inhibitors of FPTase.

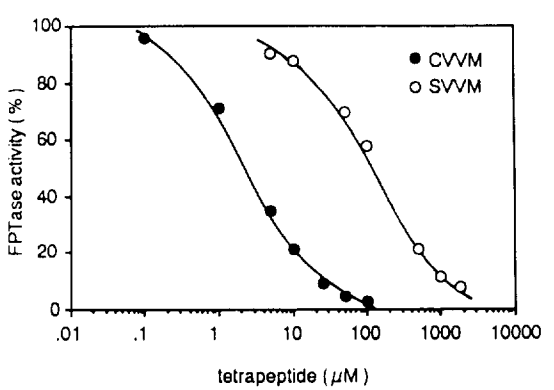

Fig. 5. Inhibition of FPTase activity by tetrapeptides (CVVM, SVVM). FPTase activity was shown with the percentage against the FPTase activity without the tetrapeptides.

4. Conclusions

The HPLC assay for FPTase activity with dabsylated peptide was shown to be a quantitative and reliable method. The direct detection of the farnesylated product makes it more reliable than the conventional assay using precipitation. The reliability and reproducibility of a quantitative assay are the most important aspects in the processing of samples with a limited availability such as clinical materials. We expect this method to be useful for the assay of FPTase activity of crude samples.

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