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Partial Purification and Characterization of a CAAX-Motif-Specific Protease from Bovine Brain Using a Novel Fluorometric Assay[1]

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Proteolytic trimming of isoprenylated proteins, including Ras, at the C-terminal CAAX motifs is a key event in their activation. However, the protease responsible for the proteolysis has not been well characterized yet. In this study, we established a novel assay method for the enzyme using a fluorescent substrate, dansyl(Dns)-KSKTKC(*S*-farnesyl)-VIM, with which we can assess the proteolytic activity with high sensitivity and more easily than by the former assay methods using radio-labeled substrates. Using this assay method, we purified the protease 104-fold from bovine brain microsomal membranes by Sepharose CL-6B gel filtration and DE-52 chromatography. The partially purified enzyme was shown to be an endoprotease specific to the farnesylated peptide and to have a K_m value of 1.0 μM for Dns-KSKTKC(*S*-farnesyl)VIM. *o*-Phenanthroline and zinc chloride strongly inhibited the activity. Interestingly, however, *m*- and *p*-phenanthrolines were as effective as *o*-phenanthroline, indicating that the inhibition by *o*-phenanthroline is not simply due to its chelating action. The molecular mass of the protease was deduced to be 480 kDa by gel filtration. The enzymatic activity was lost during further attempts at chromatographic purification, but was partially recovered by mixing the chromatographic fractions which had apparently lost the activity. These results suggest that this protease consists of multiple subunits.

Key words: CAAX-motif-specific protease, endoprotease, isoprenylated protein, novel fluorometric assay, Ras.

A number of proteins, including Ras, which are involved in signal transduction at cellular membranes contain a CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid) motif at their C-termini (*1, 2*). This motif is a target for a series of posttranslational modifications that includes isoprenylation at the cysteine residue, proteolytic removal of the three terminal amino acids, and *S*-(5′-adenosyl)-4-methionine(AdoMet)-dependent methylation of the newly exposed α-carboxyl group of the cysteine residue (Fig. 1). These modifications are important not only for membrane association but also for interaction with other proteins. For example, the γ-subunit of a heterotrimeric G protein and a fungal mating factor require a fully modified CAAX motif at their C-termini to interact with the α-subunit and the receptor, respectively (*3, 4*). In the case of Ras, farnesylation is apparently essential for its function, such as activation of Raf (*5*), adenylyl cyclase (*6*), hSOS1 (*7*), SmgGDS (*8*), and RalGDS (*9*), but the physiological roles of the C-terminal proteolysis and methylation have not yet been clarified. A study on the membrane-binding abilities of fully or partially modified Ras suggests that proteolysis and methylation may play an important role in the Ras function (*10*). Moreover, methylation is a reversible reaction, and so the molecular interaction may be regulated through methylation and demethylation (*11, 12*). Thus, these results indicate that the proteolysis of farnesylated Ras may be important for regulation of its molecular interaction. However, the protease that recognizes the isoprenylated CAAX sequence to cleave the C-terminal three amino acids (AAX) has not been identified, although the enzymes responsible for isoprenylation and methylation of the CAAX motif have been genetically or biochemically identified (*1, 13*). To understand the physiological role of proteolysis of the CAAX motif, it is important to identify and characterize the protease.

The present study was initiated to shed light on the role of this proteolysis through isolation and characterization of the protease. Since the enzyme was present only in a very small amount and was not very stable, a simple, rapid, and

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Abbreviations: AdoMet, *S*-(5′-adenosyl)-L-methionine; BCA, bicinchoninic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DFP, diisopropyl fluorophosphate; Dns, dansyl; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; Fmoc, 9-fluorenylmethoxycarbonyl; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; TFA, trifluoroacetic acid; TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone.

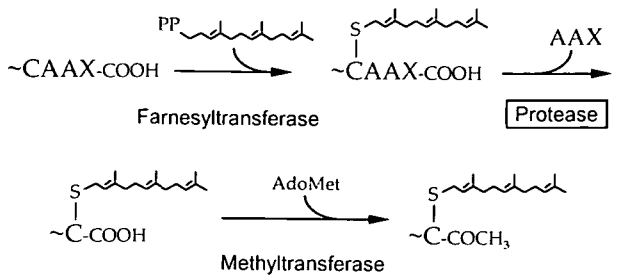

Fig. 1. **Posttranslational modifications of the CAAX motif of Ras.** C, cysteine; A, aliphatic amino acid; X, any amino acid.

sensitive assay method was required. Therefore we first developed such an assay method using a fluorescent substrate, dansyl(Dns)-KSKTKC(*S*-farnesyl)VIM to measure the fluorescence of the proteolytically processed substrate, Dns-KSKTKC(*S*-farnesyl). This assay method is highly sensitive and simpler than those using radiolabeled substrates to measure the radioactivity of proteolytically processed fragments (*14*). With this method at hand, we solubilized the protease from bovine brain microsomal membranes with sodium deoxycholate, and partially purified it by Sepharose CL-6B gel filtration and DE-52 chromatography, and investigated its properties.

EXPERIMENTAL PROCEDURES

Materials—Bovine brains were obtained from a local slaughterhouse. All-*trans*-farnesyl bromide was from Sigma. Acetonitrile, dicyclohexylcarbodiimide, Dns-chloride, 9-fluorenylmethoxycarbonyl (Fmoc)-amino acids, 1-hydroxybenzotriazole, and trifluoroacetic acid (TFA) were from Wako Pure Chemicals (Osaka). DE-52 was from Whatman Chemical Separation (Tokyo). Sepharose CL-6B was from Pharmacia Biotech. *p*-Alkoxybenzylalcohol resin was from Peptide Institute (Kyoto). Peptide synthesis tubes were from Kokusan Chemicals (Tokyo). All other reagents used were of analytical grade. Reverse-phase HPLC analyses were performed in a Shimadzu LC 10A system with a J'sphere ODS-H80 column (YMC, Kyoto). Amino acid composition analyses were done in an Applied Biosystems protein delivatizer/analyzer (420A/130A) equipped with an automated acid hydrolysis apparatus. Amino acid sequence analyses were done with an Applied Biosystems protein sequencer model 477A. Mass spectra were measured in a Bruker matrix associated laser desorption/ionization-time of flight mass spectrometer.

Syntheses of Dns-Peptides and Analogues—Dns-KSKTKC(*S*-farnesyl)VIM was synthesized as follows. Side-chain-protected KSKTKCVIM was synthesized on *p*-alkoxybenzylalcohol resin by using Fmoc-amino acids with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide in a Kokusan peptide synthesis tube (*15*). Protecting groups used were the *t*-butoxycarbonyl group for lysine, the *t*-butyl group for serine and threonine, and the triphenylmethyl group for cysteine. The α-amino group of the N-terminus was dansylated by reaction with 2 equivalents of dansyl chloride in 0.2 M sodium bicarbonate for 3 h (*16*). The dansylated peptide was released from the resin and deprotected with TFA in the presence of conventional scavengers for 90 min. Dns-KSKTKCVIM was precipitated in diethyl ether, purified by reversed-phase HPLC with an acetonitrile gradient in 0.1% TFA, and lyophilized. The thiol group of Dns-KSKTKCVIM was then farnesylated with all-*trans*-farnesyl bromide in the presence of guanidine carbonate (*17*). Dns-KSKTKC(*S*-farnesyl)VIM was purified by reversed-phase HPLC with an acetonitrile gradient in 0.1% TFA, and lyophilized. The structure of the product was confirmed by mass spectroscopy and amino acid analysis. Dns-KSKTKC(*S*-farnesyl) was also synthesized in the same way.

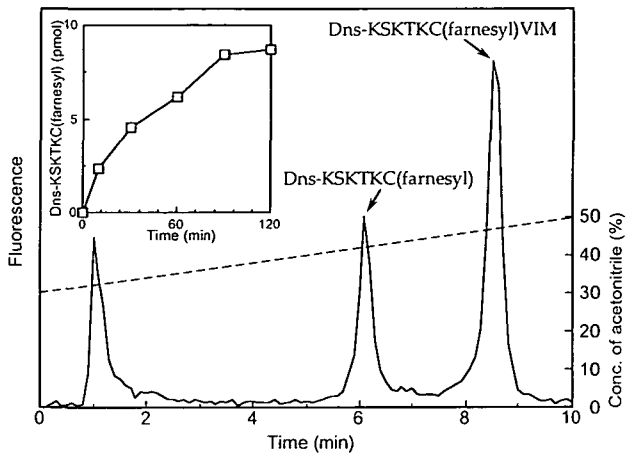

Fig. 2. **The proteolytic activity toward the fluorescent substrate.** HPLC analysis of the proteolytic cleavage of the substrate. Twenty picomoles of Dns-KSKTKC(*S*-farnesyl)VIM was incubated with 20 μg protein of the bovine brain microsomes (see "EXPERIMENTAL PROCEDURES") at 37°C for 1 h in 60 μl of 20 mM Tris-Cl (pH 7.5). The reaction was quenched by adding 20 μl of 0.4% TFA in acetonitrile, and the mixture was analyzed by reverse-phase HPLC (column, YMC J'sphere ODS-H80, 4.6×75 mm) with fluorescence detection (excitation at 329 nm and emission at 520 nm). The column was equilibrated with 35% acetonitrile/0.1% TFA/water and eluted with a gradient of acetonitrile (35–55% in 10 min) at a flow rate of 1.0 ml/min. The peak of Dns-KSKTKC(*S*-farnesyl), which was confirmed by mass spectroscopy, was eluted at a retention time of 6.0 min, which was identical with that of the chemically synthesized Dns-KSKTKC(*S*-farnesyl) (data not shown). The time course of hydrolysis of Dns-KSKTKC(*S*-farnesyl)VIM is shown in the inset. The enzymatic reaction was carried out in a manner similar to the above except for quenching of the incubation at each time point. The generated Dns-KSKTKC(*S*-farnesyl) was measured in terms of its fluorescence.

KSKTKCVIM and KSKTKC(*S*-farnesyl)VIM were synthesized in a similar way to the dansylated peptides except for omission of the dansylation step. The structures were confirmed by mass spectroscopy and amino acid sequence analyses.

Protease Activity Assay—The protease activity was assayed as follows. Ten microliters of the enzyme solution was mixed with 50 μl of 0.2 mM Dns-KSKTKC(*S*-farnesyl)VIM and 20 mM Tris-Cl, pH 7.5. The enzymatic reaction was allowed to proceed for 1 h at 37°C and then quenched by adding 20 μl of 0.4% TFA in acetonitrile. Cleavage of the substrate was detected by reverse-phase HPLC as described in the legend to Fig. 2. The protease activity was quantitated from the fluorescence intensity (excitation at 329 nm and emission at 520 nm) of the generated peak, which was confirmed to be Dns-KSKTKC(*S*-farnesyl) by mass spectroscopy, the retention time

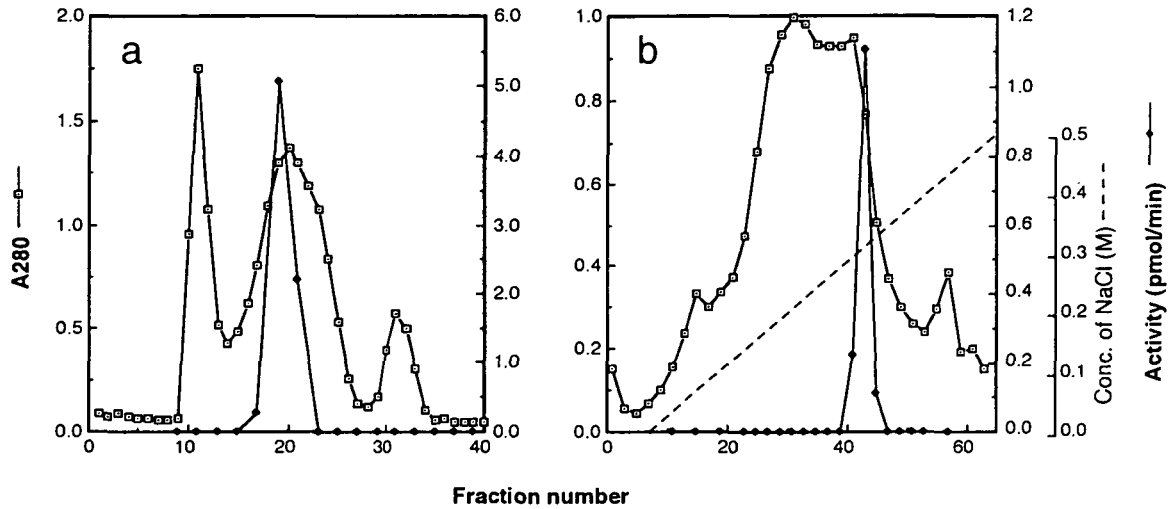

Fig. 3. **Chromatographic purification of the protease.** a: Sepharose CL-6B gel filtration. Seven milliliters of the solubilized membrane fraction was applied to a Sepharose CL-6B column (1.3×70 cm) equilibrated with buffer A (20 mM Tris-Cl/0.1% sodium deoxycholate/2 mM 2-mercaptoethanol at pH 7.5) containing 0.2 M NaCl and eluted with buffer A at a flow rate of 30 ml/h. Fraction size, 7 ml. The molecular mass of the protease was estimated using the calibration curve obtained with the molecular mass standard proteins. The activity was eluted between the elution positions of thyroglobulin (669 kDa) and ferritin (440 kDa). b: DE-52 ion exchange chromatography. The active fractions obtained by Sepharose CL-6B gel filtration was dialyzed against buffer A and applied to a DE-52 column (1.0×8 cm) equilibrated with buffer A. The column was eluted with a gradient of NaCl (0-0.5 M) in a total volume of 70 ml at a flow rate of 10 ml/min. Fraction size, 1.5 ml.

being identical with that of chemically synthesized Dns-KSKTKC(*S*-farnesyl).

Preparation and Solubilization of the Bovine Brain Microsomal Fraction—All the procedures were carried out at 4°C. Bovine brain (250 g) (about half of a single adult brain) was minced and homogenized in a Waring blender with 2.5 volumes of 20 mM Tris-Cl buffer, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, and 0.5 mM DTT. The homogenate was centrifuged at 10,000×*g* for 10 min, and the supernatant fraction was centrifuged at 100,000×*g* for 90 min. The pellet obtained by the former centrifugation was used as the crude nuclear/mitochondria fraction, while that obtained by the latter centrifugation was suspended in 4 volumes of 20 mM Tris-Cl buffer, pH 7.5, and used as the microsomal membrane fraction. To solubilize the microsomal membranes, a 1/9 volume of 10% sodium deoxycholate was added to the above membrane fraction. The suspension was stirred overnight with a magnetic stirrer and centrifuged at 100,000×*g* for 70 min. The supernatant was used as the solubilized membrane fraction.

Purification of the Protease—Seven milliliters of the solubilized microsomal fraction was chromatographed on a Sepharose CL-6B column (1.3×70 cm). The chromatography was performed as described in the legend to Fig. 3a. Twenty milliliters of the active fractions was dialyzed against buffer A (20 mM Tris-Cl, pH 7.5, 2 mM 2-mercaptoethanol, and 0.1% sodium deoxycholate) and applied to a DE-52 column (1.0×8.0 cm) equilibrated with buffer A. The column was washed and eluted with a gradient of NaCl (0 to 0.5 M) in a total volume of 70 ml of buffer A. The fractions eluted at 0.4-0.45 M NaCl contained the enzymatic activity. These fractions were stored at −30°C and used as the partially purified enzyme fraction.

Electrophoresis—SDS-PAGE was performed in 8% polyacrylamide gel by the method of Laemmli (*18*), followed by silver staining.

Determination of Protein—Protein was determined by measuring the absorbance at 280 nm of the sample solution or by the method of Smith *et al.* (*19*) using the BCA reagents with bovine serum albumin as a standard.

RESULTS

Novel Assay Method for the Proteolytic Activity—The proteolytic activity was assayed by using a fluorescent substrate, Dns-KSKTKC(*S*-farnesyl)VIM, which contains the C-terminal 9-residue sequence of human K-Ras-4B (*20*). After incubation with bovine brain microsomes, a peak of Dns-KSKTKC(*S*-farnesyl), confirmed by mass spectroscopy, was generated and detected by reverse-phase HPLC analyses (Fig. 2). The peak increased in a time-dependent manner. The activity was determined by measuring the fluorescence intensity of Dns-KSKTKC(*S*-farnesyl). This assay method is not only simple and brief, but also highly sensitive and can measure the activity using approximately 10 fmol of substrate, Dns-KSKTKC(*S*-farnesyl).

Purification of the Protease from Bovine Brain—Bovine brain was used as the source of the protease because it is known to be rich in isoprenylated proteins (*21*). It was homogenized and submitted to subcellular fractionation by centrifugation. The activity was detected in the microsomal membrane fraction and the crude nuclear/mitochondria fraction, whereas no activity was detected in the cytosolic fraction. We used the microsomal membrane fraction because it had the highest relative activity (data not shown). To solubilize the microsomal membranes, we tested various detergents (Table I). Sodium deoxycholate was most effective for solubilizing the activity and sodium cholate was also effective, whereas Lubrol PX, Triton X-100, Nikkol, *n*-heptyl-β-D-thioglucoside, and *n*-dodecyltrimethylammonium bromide were not so effective. The

TABLE I. **Effects of detergents on solubilization and activity of the protease.** The bovine microsomes were suspended in 4 volumes of 20 mM Tris-Cl buffer, pH 7.5, containing 1% detergent, homogenized manually and incubated for 2 h at 4°C. The mixtures were then centrifuged at 100,000 × *g* for 70 min. The enzyme activities of the supernatants and pellets were assayed as described in "EXPERIMENTAL PROCEDURES."

Detergent	Activity (pmol/min)		
	Supernatant	Pellet	Total
No detergent	96	820	910
Sodium deoxycholate	640	340	980
Sodium cholate	480	460	940
n-Heptyl-β-D-glucoside	360	400	760
Triton X-100	190	140	330
Lubrol	0.0	120	120
Nikkol	0.0	0.0	0.0
n-Dodecyltrimethylammonium bromide	0.0	0.0	0.0

TABLE II. **Partial purification of the protease.**

Purification step	Protein (mg)	Total activity (pmol/min)	Relative activity (pmol/min/mg)	Purification (-fold)	Recovery (%)
Microsomes	730	37	0.051	1	100
Solubilizate	200	32	0.16	3.1	86
Sepharose CL-6B	48	28	0.58	11	76
DE-52	1.8	9.5	5.3	104	26

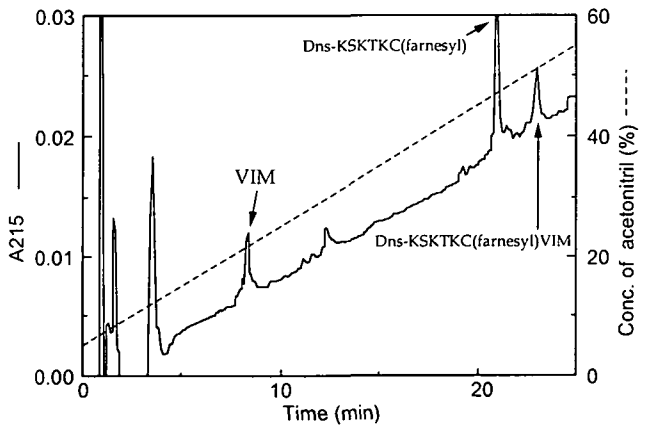

Fig. 4. **Identification of the C-terminal fragment of the substrate released by the protease.** Approximately 10 μg protein of the partially purified protease was incubated with 300 pmol of Dns-KSKTKC(*S*-farnesyl)VIM in 60 μl of 20 mM Tris-Cl for 24 h. The reaction was quenched by adding 20 μl of 0.4% TFA in acetonitrile, and the mixture was applied to reverse-phase HPLC (column, YMC J'sphere ODS-H80, 4.6 × 75 mm; UV detection at 215 nm; flow rate, 1.0 ml/min). The column was equilibrated with 5% acetonitrile/0.1% TFA/water and eluted with a gradient of acetonitrile 5-55% in 25 min. The newly generated peak at the retention time of 8.5 min was identified as VIM by amino acid sequence analysis.

enzyme fraction solubilized by sodium deoxycholate was partially purified by Sepharose CL-6B gel filtration and DE-52 anion exchange chromatography (Fig. 3). The activity was eluted as a single peak in each step. The results of partial purification of the protease are shown in Table II. The purification was 104-fold from the microsomal membrane fraction.

We attempted to purify the protease further by using

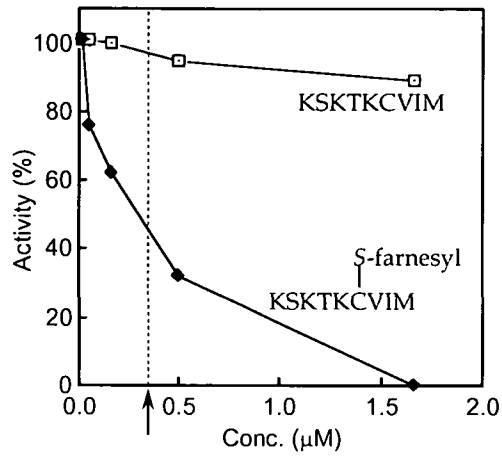

Fig. 5. **Competition by a peptide containing a C-terminal farnesylated CAAX sequence.** The proteolytic activity was assayed in the presence of various concentrations of KSKTKC(*S*-farnesyl)-VIM or KSKTKCVIM as described in "EXPERIMENTAL PROCEDURES," except that the concentration of the substrate was 0.33 μM (shown by a vertical arrow).

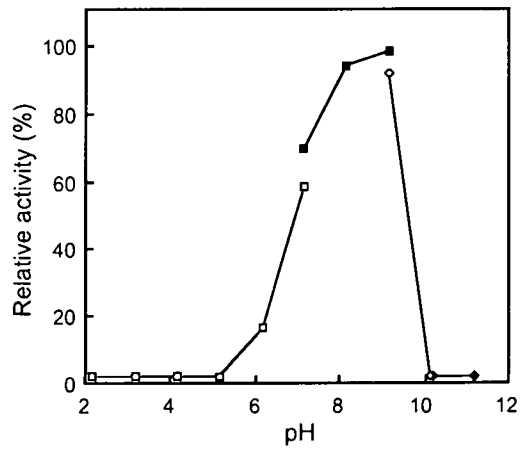

Fig. 6. **pH dependence of the proteolytic activity.** The activity of the partially purified protease was assayed as described in "EXPERIMENTAL PROCEDURES" except that different buffers were used: 0.1 M citric acid-sodium phosphate buffer (□), 0.1 M Tris-Cl buffer (■), Gly-NaOH buffer (◇), and 0.1 M sodium phosphate-NaOH buffer (◆). The activity in 0.1 M Tris-Cl buffer at pH 9.0 was taken as 100%.

various chromatographic methods including ion exchange, adsorption, hydrophobic, and gel filtration chromatographies. DE-52 was the most effective among the anion exchangers tested, including Mono Q, QAE-Sepharose, DEAE-Toyopearl, *etc.*, whereas the protease was not bound to cation exchangers, such as Mono S and CM-Sepharose. The activity was lost upon hydroxyapatite and various hydophobic chromatographies. Gel filtration chromatography was generally effective. Upon DE-52 rechromatography of the partially purified fraction, however, the activity was lost completely, although approximately 5% of the activity was recovered by mixing all the fractions from the chromatography. Further purification of the enzyme has not been achieved.

TABLE III. **Effects of various protease inhibitors on the activity.** The partially purified protease was incubated with each inhibitor for 2 h in 20 mM Tris-Cl, pH 7.5, and then assayed as described in "EXPERIMENTAL PROCEDURES." The activity without inhibitors was taken as 100%.

Inhibitor	Conc. (M)	Activity (%)	Inhibitor	Conc. (M)	Activity (%)
None	–	100	Aprotinin	10^{-5}	105
o-Phenanthroline	10^{-3}	0	SBTI	10^{-4}	95
	10^{-4}	80	Chymostatin	10^{-4}	100
	10^{-5}	100	Leupeptin	10^{-4}	105
Phosphoramidon	10^{-5}	100	Antipain	10^{-5}	111
EDTA	10^{-1}	95	TLCK	10^{-4}	103
EGTA	10^{-1}	105	TPCK	10^{-4}	116
$ZnCl_2$	10^{-4}	0	PCMB	10^{-4}	95
	10^{-5}	88	Iodoacetic acid	10^{-4}	111
	10^{-6}	90	E-64	10^{-5}	100
$CoCl_2$	10^{-4}	95	Pepstatin	10^{-6}	100
DFP	10^{-4}	121	Bestatin	10^{-5}	97
PMSF	10^{-5}	103			

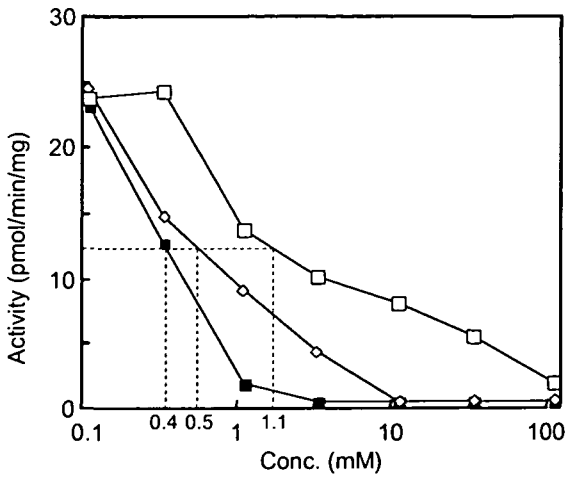

Fig. 7. **Effects of *o*-, *m*-, and *p*-phenanthrolines on the activity.** Inhibition by *o*- (■), *m*- (◇), and *p*-phenanthroline (□) at various concentrations was analyzed in the same manner as described in the footnote of Table III.

Properties of the Protease—The C-terminal fragment released by the proteolytic activity was shown to be a tripeptide, VIM (Fig. 4), indicating that this protease is an endoprotease. The activity of the partially purified protease toward Dns-KSKTKC(*S*-farnesyl)VIM was competed out by the farnesylated peptide KSKTKC(*S*-farnesyl)-VIM, with similar affinity, but not by the non-farnesylated peptide KSKTKCVIM (Fig. 5). The K_m and V_{max} values for Dns-KSKTKC(*S*-farnesyl)VIM calculated from the Lineweaver-Burk plot were 1.0 μM and 14 pmol/min/mg protein, respectively (data not shown). The pH dependency of the proteolytic activity is shown in Fig. 6; the optimal pH for the activity was approximately 9.0. The molecular mass was estimated to be 480 kDa by the gel filtration on Sepharose CL-6B (Fig. 3a).

The effects of various protease inhibitors are shown in Table III. *o*-Phenanthroline and zinc chloride remarkably inhibited the activity. However, none of the other typical inhibitors of serine proteases (*i.e.*, DFP, PMSF, chymostatin *etc.*), cysteine protease (*i.e.*, iodoacetic acid, PCMB, and E-64), aspartic proteases (*i.e.*, pepstatin), metallo proteases (*i.e.*, EDTA, phosphoramidon *etc.*), and aminopeptidases (*i.e.*, bestatin) was effective. The inhibition by 1.0 mM *o*-phenanthroline was not reversed by addition of 0.01 to 1 mM zinc chloride. To investigate whether *o*-phenanthroline acted as a metal ion chelator, we also tested the effects of *m*- and *p*-phenanthrolines, which are non-chelating isomers of *o*-phenanthroline (Fig. 7). The concentrations of *o*-, *m*-, and *p*-phenanthrolines giving 50% inhibition of the proteolytic activity were 0.4, 0.5, and 1.1 mM, respectively.

DISCUSSION

Previously, two kinds of radiometric assay methods were used for the CAAX-motif-specific protease. One is a direct method using a radio-labeled substrate, such as [^{14}C]acetyl-C(*S*-farnesyl)VIM (*22*), propionyl-GSPC(*S*-farnesyl)-V[^{14}C]LM (*23*), acetyl-C(*S*-[^{3}H]farnesyl)VIS (*24*), *etc.* The proteolytic activity toward these substrates was analyzed by HPLC with an on-line radioactivity monitor to measure the radioactivity of the hydrolysis products. Although this assay method is as sensitive as the present assay method, it requires expensive materials and equipment for the synthesis of radioactive substrates and analysis of the radio-labeled products, respectively, and needs special precautions. Another assay method is an indirect one; it uses yeast STE14 methyltransferase, which transfers the methyl group of AdoMet to the carboxyl group of the C-terminal farnesylated cysteine (*25*). The proteolytic activity is assessed by measuring the radioactivity of the [^{14}C]methyl group of [*methyl*-^{14}C]AdoMet transferred to proteolytically processed peptide substrates. It does not require synthesis and HPLC analysis of radioactive products, but is not highly sensitive and is not suitable for kinetic analyses of the protease activity due to its indirect nature. Furthermore, it requires a specific enzyme and the usual precautions for radioactivity. In contrast, the present assay method needs no special equipment or precautions, requiring only the synthesis of the fluorescent substrate and the use of an HPLC apparatus with an on-line fluorescence detector. The analysis of the fluorescent product is not only highly sensitive, but also simple, rapid, and quantitative.

The proteolytic activity was detected in the membrane fractions, but not in the cytosolic fraction. This result is consistent with previous reports (*26-29*), suggesting that Ras and the other proteins with a C-terminal CAAX motif are proteolytically processed on cellular membranes. Among the detergents tested, sodium deoxycholate and sodium cholate were most effective for solubilization, whereas Lubrol PX, Triton X-100, Nikkol, *n*-heptyl-β-D-thioglucoside, and *n*-dodecyltrimethylammonium bromide were not only ineffective for solubilization, but also appeared to strongly inhibit and/or destroy the activity. These detergents are thought to act at least partly as inhibitors of the protease because their straight aliphatic chains may act as analogs of the isoprenyl group of the substrate.

The protease was partially purified from bovine brain by using Sepharose CL-6B and DE-52 chromatographies (*i.e.*, 104-fold from the microsomal membrane fraction and 34-fold from the solubilized membrane fraction). Attempts

at further purification have been unsuccessful so far; the activity of the partially purified enzyme was lost completely upon DE-52 rechromatography. Interestingly, however, approximately 5% of the activity was recovered by mixing all the fractions from the chromatography (data not shown). Furthermore, the molecular mass of the protease, which was estimated to be 480 kDa by gel filtration (Fig. 3a), is relatively high for a single polypeptide chain. These results suggest that the protease consists of multiple subunits and that they are dissociated under certain conditions of chromatography.

During our studies, two reports appeared on partial purification of the CAAX-motif-specific protease using a radiometric assay method (*30, 31*). Akopyan *et al.* (*30*) partially purified a CAAX-motif-specific protease from pig brain microsomal membranes. They solubilized the protease by simple freezing and thawing and reported that the detergents they tested were not very effective, including *n*-octyl-β-D-glucopyranoside and CHAPS. The enzyme was partially purified about 40-fold from the solubilized membrane fraction by chromatography on DEAE Trysacryl M and Sephacryl S-200. They estimated the molecular mass of the enzyme to be about 70 kDa. On the other hand, Chen *et al.* (*31*) effectively solubilized the protease from bovine brain microsomal membranes with CHAPSO, and partially purified it about 4.3-fold from the solubilized membrane fraction by chromatography on Resource Q and Superose 12. They estimated the molecular mass to be about 641 kDa. Thus, the enzyme obtained in the present study more closely resembles the enzyme obtained by Chen *et al.* than that obtained by Akopyan *et al.* in the requirement of detergent for solubilization and molecular mass (present enzyme, ~480 kDa).

Akopyan *et al.* (*30*) reported that the optimum pH of their enzyme using propionyl-Gly-Ser-Pro-Cys(*S*-farnesyl)-Val-Leu-Met as a substrate was 6.2-7.5, that the K_m and V_{max} values were 32.5 μM and 60.8 nmol/min/mg protein, respectively, and that the enzyme was strongly inhibited by *o*-phenanthroline (10 mM), PCMB (0.1 mM), chymostatin (0.1 mM), and zinc chloride (0.1 mM). They concluded that the enzyme resembles endopeptidase 24.15, a thiol-dependent zinc metallopeptidase. On the other hand, Chen *et al.* (*31*) reported that the K_m and V_{max} values were 0.65 μM and 1.96 nmol/min/mg protein, respectively, with *N*-acetyl-Cys(*S*-farnesyl)-Val-Ile-Met as a substrate and that the enzyme was strongly inhibited by PCMB (0.1 mM) and TPCK (1 mM), but not by *o*-phenanthroline (1 mM) or chymostatin (0.3 mM). They concluded that the enzyme is a thiol protease. The present enzyme is optimally active at pH 9.0 and this differs significantly from the pH optimum of the enzyme obtained by Akopyan *et al.* The K_m value of the present protease was 1.0 μM, which indicates that the affinity of the protease for the farnesylated peptide is very high. This value is rather close to that obtained by Chen *et al.* On the other hand, the V_{max} value of the present enzyme was apparently lower than the other enzyme preparations. The present enzyme is also different from the enzyme obtained by Akopyan *et al.* (*30*) in that it was not inhibited by PCMB (1 mM) or chymostatin (0.1 mM), which strongly inhibited the latter. Further, the present enzyme is different from the enzyme obtained by Chen *et al.* (*31*) in that it was strongly inhibited by *o*-phenanthroline (1 mM), but not by PCMB (0.1 mM) or TPCK (0.1 mM), whereas the latter was inhibited strongly by PCMB (0.1 mM) and TPCK (0.1 mM), but not by *o*-phenanthroline (1 mM).

Thus, there are several discrepancies in properties among the enzyme preparations. This may be partly due to the differences in species, degree of purification, and various experimental conditions used. On the whole, the present enzyme appears to be more similar to, though not identical with, the enzyme obtained by Chen *et al.* than that obtained by Akopyan *et al.* There may be several similar proteases present in the microsomal membranes, although we have observed only a single activity peak upon chromatography of the solubilized fraction. In this connection, it may be worthy of note that Chen *et al.* observed a second minor activity peak with M_r ~60 kDa on gel filtration chromatography.

In the present study, it appeared that *o*-phenanthroline did not inhibit the activity simply through its metal-chelating action. Planar molecules, such as *o*-phenanthroline, are known to bind nonspecifically to proteins (*32*). Furthermore, the reaction of PCMB with certain SH groups in proteins often destroys higher-order structure (*33*). Assuming that the present protease consists of multiple subunits, such reagents as *o*-phenanthroline and PCMB may destroy the higher-order structure of the protease under certain conditions, although under the conditions used in the present study, PCMB was not very inhibitory. Therefore, the present protease is thought to possess a characteristic structure and catalytic mechanism. Further studies, including extensive purification and characterization are necessary to obtain definite conclusions as to its identity and roles in the pathways of cellular signal transduction.

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