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Purification and Characterization of a Cysteine Protease Produced by Pathogenic Luminous *Vibrio harveyi*

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Abstract. The purification and characterization of an extracellular protease produced by pathogenic luminous *Vibrio harveyi* strain 820514, originally isolated from diseased tiger prawn (*Penaeus monodon*), was presented in this paper. The purification steps included ammonium sulfate precipitation, with columns of hydrophobic interaction chromatography and anion exchange on fast protein liquid chromatography. The protease is an alkaline cysteine protease, heat labile, inhibited by iodoacetamide, iodoacetic acid, N-ethylmaleimide, *p*-chloromercuribenzoate, and *p*-chloromercuribenzene-sulfonic acid, and showed maximal activities at pH 8 and 50°C, having a molecular mass of 38 kDa as estimated by SDS-PAGE and gel filtration column. In addition, the protease was also completely inhibited by $CuCl_2$ and $HgCl_2$, but not or only partially inhibited by other inhibitors tested. Furthermore, 2-mercaptoethanol was the most effective reducing agent in the activation of the enzyme. The present protease is the first cysteine protease found in *Vibrio* species.

Vibrio harveyi is commonly isolated from various habitats, i.e., warm marine waters, surfaces of marine animals, light organs of certain marine fish and cephalopods, and intestine of aquatic animals [3, 36, 42–44, 48]. Recently, the virulence of this species has been recognized in a small but growing list of marine animals especially in cultured penaeids in Asia and Australia [2, 6, 15, 17, 22, 25, 29, 30, 38, 40, 51, 54]. Mass mortalities of *Penaeus monodon* and larvae or juveniles associated with luminous vibrios have been observed in hatcheries or farms in Australia [40], India [17], Indonesia [54], Thailand [15], the Philippines [2, 25], and Taiwan [6, 29, 51].

Various extracellular proteases produced by a number of *Vibrio* species isolated from sea water, fish, and shellfish have been isolated and examined with regard to their enzymatic properties and/or virulence. Most of these studies are on the enzymes (mainly proteases) from *Vibrio anguillarum*, since its foremost importance is as a pathogen in fish [8, 9, 14, 31, 33, 53]. Two extracellular proteases produced by *V. alginolyticus* NCMB 1339 and one metalloprotease produced by *V. alginolyticus* S3y have also been described to be toxic to larval *Ostrea edulis* [34, 35] and juvenile *Epinephelus malabaricus* [26], respectively. Three extracellular alkaline metal-chelater-sensitive proteases produced by *V. harveyi* isolated from sea water have also been reported [10, 11]. However, no information is available concerning the extracellular protease(s) produced by pathogenic *V. harveyi* isolated from diseased penaeids.

In the present paper, an exoprotease has been purified from the extracellular products of *Vibrio harveyi* strain 820514 to apparent homogeneity by a combination of ammonium sulfate precipitation, hydrophobic interaction chromatography, and anion-exchange chromatography on fast protein liquid chromatography. The purified protease appears to be a cysteine protease by virtue of the inhibition of enzyme activity by iodoacetamide, iodoacetic acid, N-ethylmaleimide, *p*-chloromercuribenzoate, and *p*-chloromercuribenzene-sulfonic acid, and the partial or complete resistance to several inhibitors of serine protease, acid protease, and metalloprotease. The relationship of this protease to previously identified *Vibrio* proteases and cysteine proteases from other organisms is discussed.

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Materials and Methods

Bacteria and extracellular products (ECP). Luminous *V. harveyi* 820514, originally isolated from diseased tiger prawn (*Penaeus monodon*) in Taiwan in 1993, was used in this study [29]. Stock cultures of strain 820514 were grown on tryptic soy agar (TSA; Oxoid, Basingstoke, supplemented with 1.5% NaCl) for 24 h at 27°C. Two swabs of these bacteria suspended in 5 ml phosphate-buffered saline (PBS) pH 7.2 were spread onto TSA (+1.5% NaCl) overlaid with sterile cellophane and grown for 24 h at 27°C. The ECP was harvested as previously described [27]. Briefly, 15 ml of PBS was added to the surface of the cellophane overlaying TSA (+1.5% NaCl) and spread completely. The harvested bacterial suspension was then centrifuged at 25,000 *g* for 60 min at 4°C; the pellet was discarded. The supernatant fluids were passed through a 0.22-µm filter (Millipore, Bedford), and the ECP was stored in 1-ml aliquots at −70°C. Total protein was measured by the method of Bradford [5] with bovine serum albumin as standard. Protease activity was measured by hide powder azure (HPA) digestion as previously described [27]. Briefly, the enzyme solution (0.1 ml) was incubated and shaken with 25 mg HPA in 2.4-ml PBS at 37°C for 15 min. On addition of 2.5 ml 10% trichloroacetic acid (TCA) and after centrifugation, the absorbance of the supernatant was measured at 600 nm. Blanks were prepared by addition of 10% TCA to substrate prior to the proteolytic assay. One unit of protease activity is an increase in absorbance of 0.01 unit. Caseinase, gelatinase, and phospholipase activities were detected by placing samples in wells cut in agarose (1% in PBS, pH 7.2) containing 0.2% (wt/vol) sodium caseinate, gelatin, and lecithin, respectively, and incubating in humidified chamber for 24 h at 25°C. Hemolytic activity was measured by a standard microtitration method with sheep erythrocytes as described previously [27].

Purification of extracellular protease. To purify the extracellular protease, proteins in the ECP of *V. harveyi* 820514 were precipitated with 90% saturated ammonium sulfate (Serva, Heidelberg) for 60 min at 4°C, collected by centrifugation at 25,000 *g* for 60 min at 4°C, suspended in 50 ml deionized distilled water, and dialyzed against 5 liters of deionized distilled water overnight at 4°C. This dialyzed sample was then mixed with an equal volume of 2 M ammonium sulfate in 100 mM phosphate buffer, pH 7.0, then fractionated by fast protein liquid chromatography (Pharmacia, Uppsala) with a hydrophobic interaction chromatography (HIC, Phenyl Sepharose High Performance, Pharmacia) column equilibrated with 1 M ammonium sulfate in 50 mM phosphate buffer pH 7.0. Fractions (4 ml) were eluted with 50 mM phosphate buffer, pH 7.0, at a rate of 1 ml/min. The fractions possessing protease activity were pooled, dialyzed against deionized distilled water overnight at 4°C, lyophilized, and then resuspended in 2 M ammonium sulfate in 100 mM phosphate buffer (pH 7.0) prior to a further application to the same HIC-FPLC column with the same protocol. The fractions possessing protease activity were again pooled, dialyzed, and lyophilized as described above, and resuspended in 20 mM Tris (hydroxmethyl) methylamine (Tris buffer, pH 8.0) prior to fractionation by FPLC-anion exchange column (Q Sepharose High Performance, Pharmacia) equilibrated with 20 mM Tris buffer (pH 8.0). Fractions (3 ml) were eluted with a sodium chloride gradient at a rate of 1 ml/min. The fractions possessing protease activity were again pooled, dialyzed, and lyophilized as described above, and resuspended in 20 mM Tris buffer (pH 8.0) and fractionated by FPLC-anion exchange column (RESOURCE Q, Pharmacia) equilibrated with 20 mM Tris buffer (pH 8.0). Fractions (2 ml) were eluted with a sodium chloride gradient at a rate of 1 ml/min. The fractions possessing protease activity were again pooled, dialyzed, and lyophilized as described above, resuspended in 20 mM Tris buffer (pH 8.0), and fractionated by FPLC-anion exchange column (Mono Q, Pharmacia). Fractions (1 ml) were again eluted with a sodium chloride gradient at a rate of 1 ml/min. Aliquots of 1 ml in Eppendorf were stored at −70°C.

Molecular mass determination. Phastgel gradient (8–25%) polyacrylamide (Pharmacia) was employed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and used to check the purity of fractions and determine the molecular mass of protease. Pharmacia low-molecular-mass calibration kits were used as marker proteins. Electrophoresis was conducted on the PhastSystem (Pharmacia) according to the recommendations of the manufacturer. After electrophoresis, the gels were stained with Coomassie brilliant blue R 250 (Pharmacia). For molecular mass determination by gel filtration chromatography, the purified protease from the FPLC-anion exchange column was lyophilized, resuspended in PBS (pH 7.2), and 200 µl applied to an FPLC Superose 12 gel filtration column (Pharmacia) that had been calibrated with standard marker (blue dextran, 2000 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; ribonuclease, 13.7 kDa; Pharmacia). The sample was eluted with PBS at a rate of 1 ml/min.

Protease zymogram. Purified protease was electrophoresed as the protocol described above for SDS-PAGE except that 2-mercaptoethanol was absent in the sample buffer (non-reduced) and the sample was not heated. After electrophoresis, the gels were stained with silver stain reagent (Pharmacia) for protein staining or overlaid with gels containing 1% agarose and 0.3% sodium caseinate in PBS for 2 h at 25°C for protease zymogram. Following removal of the overlay, the zymogram gels were stained with Coomassie brilliant blue R 250.

Optimum pH, optimum temperature, and thermostability. For determination of optimum pH, the purified protease preparation was added to various PBS buffer (vol/vol, 1:9) with pH adjusted from 3 to 10 with 1 *N* HCl and/or 1 *N* NaOH, and then incubated for 2 h at 4°C prior to the protease activity assay (in duplicate) as described above. The highest protease activity was used as control (100% of relative activity). For determination of optimum temperature, aliquots of the purified protease were incubated with HPA and assayed (in duplicate) as described above except that the enzymatic activities were determined at 5, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 80°C, separately. The highest protease activity was used as control (100% of relative activity). For determination of thermostability, aliquots of the purified protease were incubated for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80°C, separately, and then directly put into an ice water bath prior to the protease activity assay (in duplicate) as described above. The protease activity of the sample incubated at 4°C was used as control (100% of relative activity). Aliquots of the purified protease were further lyophilized, and the autodigestion of the enzyme was checked in SDS-PAGE as described above.

Enzyme inhibition. The effects of antipain (Sigma, St. Louis), L-cysteine (Sigma), dithiothreitol (Merck, Frankfurt), ethylenediamine tetraacetic acid (EDTA; Sigma), ethylene glycol-bis(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid (EGTA; Sigma), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane(E-64; Boehringer Mannheim GmbH, Mannheim), iodoacetamide (Serva, Heidelberg), iodoacetic acid (Sigma), leupeptin (Boehringer Mannheim GmbH), 2-mercaptoethanol (Serva), N-ethylmaleimide (Serva), *p*-chloromercuribenzoate (PCMB; Sigma), *p*-chloromercuribenzene-sulfonic acid (PCMBs; Sigma), pepstatin A (Serva), phenyl-methanesulfonyl fluoride (PMSF; Sigma), *N*α-*p*-tosyl-l-lysine-chloromethyl ketone (TLCK; Sigma), *N*-tosyl-l-phenyl-alanine chloromethyl ketone (TPCK; Sigma), sodium dodecyl sulfate (SDS; Serva), $CaCl_2$ (Serva), $CdCl_2$ (Merck), $CuCl_2$ (Merck), $HgCl_2$ (Merck), and $ZnCl_2$ (Merck) on the protease activity were examined. The concentration of each reagent was indicated in Table 2. After incubation at 37°C for 60 min, the changes of protease activity were determined by HPA assay as described above.

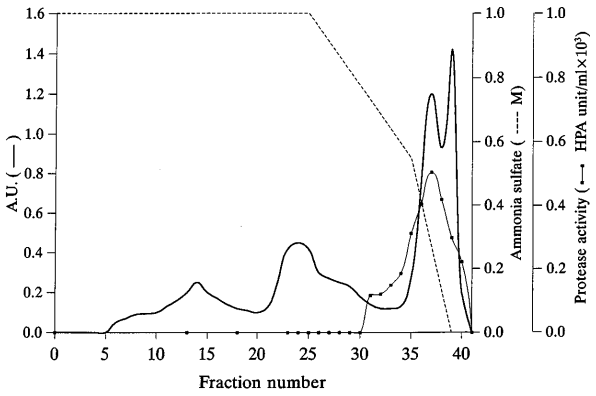

Fig. 1. Profile of FPLC-hydrophobic interaction chromatography (1st FPLC-HIC) of extracelluar products (ECP) of strain 820514. Sample was applied to Phenyl Sepharose High Performance column and eluted with stepped gradient of 1 to 0 M ammonium sulfate in 50 mM phosphate buffer pH 7.0 at a rate of 1 ml/min.

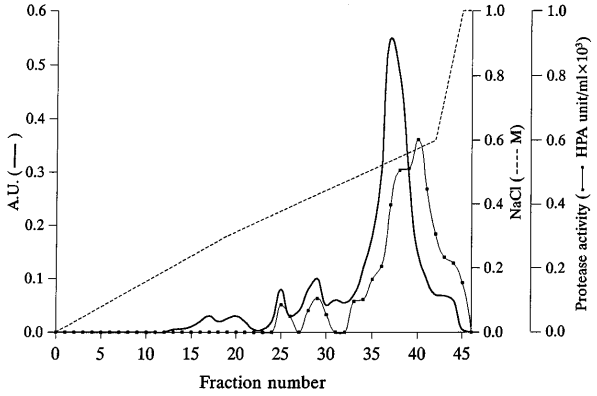

Fig. 2. Profile of FPLC-anion exchange chromatography of pools of fractions 18–25 from the 2nd FPLC-HIC. Sample was applied to Q Sepharose High Performance (QHP) column and eluted with stepped gradient of 0 to 1 M NaCl in 20 mM Tris buffer pH 8.0 at a rate of 1 ml/min.

Results

ECP of *V. harveyi* strain 820514. The ECP of strain 820514 were harvested after 24 h of incubation of the culture at 27°C. The ECP showed caseinase, gelatinase, and lecithinase activities in tests on the agar plates. The protease (HPA digestibility) and hemolytic activities of the ECP were 551 units/mg protein and 24 hemolytic units/mg protein, respectively.

Purification of extracellular protease. ECP proteins of strain 820514 eluted as two major and two minor peaks in the first FPLC-HIC, with protease activity eluting between 0 and 0.6 M ammonium sulfate (Fig. 1). Fractions (34–40) possessing protease activity were pooled, dialyzed, lyophilized, and eluted as two major and one minor peak in the second FPLC-HIC, with protease activity eluting between 0.10 M and 0.55 M ammonium sulfate (data not shown). Fractions (18–25) possessing protease activity were pooled, dialyzed, lyophilized, and eluted as one major and two minor peaks in the FPLC-Q Sepharose High Performance, with protease activity eluting between 0.5 M and 0.8 M NaCl (Fig. 2). Fractions (34–45) possessing protease activity were again pooled, dialyzed, lyophilized, and eluted as three major and two minor peaks in the FPLC-RESOURCE Q, with protease activity eluting between 0.5 and 0.9 M NaCl (data not shown). Fractions (46–59) possessing protease activity were again pooled, dialyzed, lyophilized, and eluted as one major and four minor peaks in the FPLC-Mono Q, with protease activity eluting between 0.3 M and 0.5 M NaCl (Fig. 3). Fractions 10–14 collected from the major peak produced a single polypeptide band (Figs. 3, 4) in SDS-PAGE. The purification of the protease is summarized in Table 1. A purification of approximately 12-fold with a yield of 4.4% was achieved.

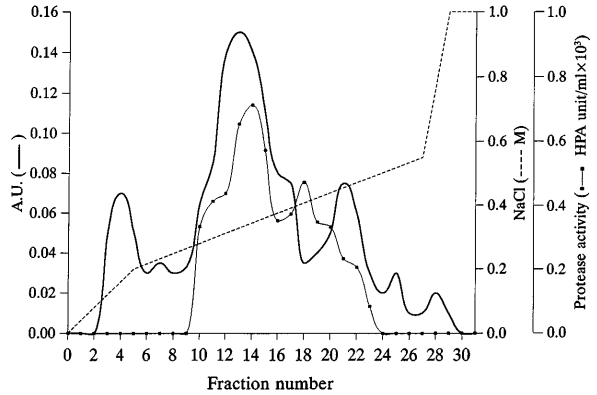

Fig. 3. Profile of FPLC-anion exchange chromatography of pools of fractions 46–59 from the RESOURCE Q column. Sample was applied to Mono Q column and separated as described in Fig. 2.

Molecular mass determination. The protein profiles of crude ECP and pools of proteolytic fractions in each purification step are shown in Fig. 4A. Pools of fractions 10–14 collected from FPLC-Mono Q produced only one single 38-kDa polypeptide band in SDS-PAGE and, therefore, termed purified protease. Autodigestion of the purified protease apparently occurred during lyophilization, since nearly no band was stained in SDS-PAGE (Fig. 4A). The molecular mass of the purified protease was further confirmed by an estimation made by passage through an FPLC-Superose gel filtration, and also determined to be about 38 kDa (Fig. 5).

Protease zymogram. Pools of fractions 10–14 collected from FPLC-Mono Q also produced only one single polypeptide band in non-reduced SDS-PAGE, and this band exhibited caseinase activity in its protease zymogram (Fig. 4B).

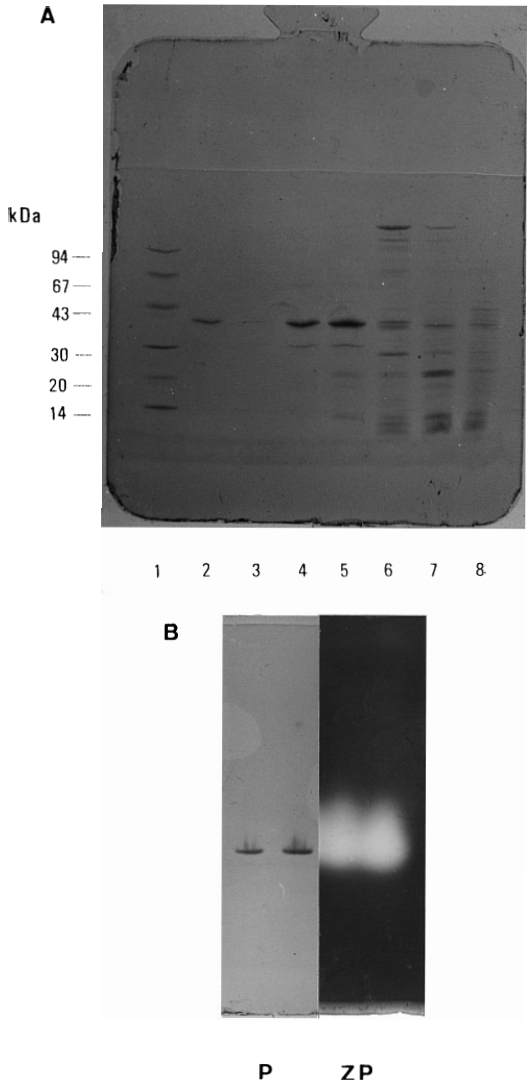

Fig. 4. (A) Protein profile of each purification step on SDS-PAGE (Phastgel, 8–25% gradient). Lane 1, marker proteins (1.0 µg); lane 2, purified protease from FPLC-Mono Q (0.1 µg); Lane 3, purified protease after further lyophilization (0.1 µg); lane 4, pool of fractions 46–59 of FPLC-RESOURCE Q (1 µg); lane 5, pool of fractions 34–45 of FPLC-Q Sepharose High Performance (1 µg); lane 6, pool of fractions 18–25 of the 2nd FPLC-HIC (1 µg); lane 7, pool of fractions 34–40 of the 1st FPLC-HIC (1 µg); lane 8, ECP (1 µg). The Phastgel was stained with Coomassie brilliant blue R 250. (B) SDS-PAGE (non-reduced, Phastgel gradient 8–25%) and protease zymogram (casein agar overlaying gel) of the purified protease. Lane P, purified protease, stained with silver stain reagent; lane ZP, zymogram of purified protease, stained with Coomassie brilliant blue R 250.

Optimum pH, optimum temperature, and thermostability. The purified protease showed maximal activities at pH 8.0 and at 50°C (Fig. 6). For thermostability study, the relative protease activities of protease aliquots incubated at temperatures above 60°C were lower by more than 30% when compared with the activity when incubated at 4°C (Fig. 6), indicating that the purified protease was heat labile. In addition, the purified protease was autodigested after further lyophilization (Fig. 4A), suggesting that the enzyme was not stable even at low temperature.

Protease inhibition. As shown in Table 2, the purified protease was completely inhibited by iodoacetamide, iodoacetic acid, *N*-ethylmaleimide, PCMB, and PCMBs, indicating that the enzyme is a cysteine protease. In addition, the protease activity was also completely inhibited by $CuCl_2$ and $HgCl_2$, and only partially inhibited by $CdCl_2$, $ZnCl_2$, antipain, EGTA, E-64, pepstatin A, PMSF, and TLCK. However, activation of the enzyme activity was obtained with L-cysteine (1.5 mM), dithiothreitol, leupeptin, 2-mercaptoethanol (1.5 mM), higher concentrations (i.e., 5, 10 mM) of PCMB, TPCK, SDS, and $CaCl_2$. A better inhibition of protease activity by PCMB was obtained only in low concentration (i.e., 1 mM), but in contrast the inhibition by L-cysteine or 2-mercaptoethanol was obtained in high concentration (10 mM). Furthermore, nearly half of the protease activity was inhibited by EDTA.

Discussion

Previous studies of the proteases from *V. harveyi* found that there are three types of metal-chelater-sensitive, alkaline proteases produced by the organism with a molecular mass of 84 kDa (tetramer of 21 kDa) [10], 49 kDa, and 46 kDa (dimer of 23 kDa) [11]. However, the bacteria used in those studies were sea water isolates. In our present study, we were able to purify the 38-kDa protease, determined on SDS-PAGE and FPLC-Superose 12 gel filtration (Fig. 4, 5), from extracellular products of pathogenic *V. harveyi* isolated from diseased tiger prawn (*Penaeus monodon*) by column chromatography on FPLC-HIC and FPLC-anion exchange (Table 1). The purified protease revealed homogeneity on SDS-PAGE (Fig. 4A,B), and its enzymatic activity was shown in a matching zymogram gel with casein as substrate (Fig. 4B).

The purified protease exhibited optimum enzyme activities at pH 8.0 and at 50°C; these results are similar to the results reported by Fukasawa et al. [10, 11]. The enzyme showed a fairly wide range of optimum pH from 6 to 10 (Fig. 6), indicating that the production of this protease by the pathogenic *V. harveyi* might play a certain important role in marine environment and/or in tiger prawn. The purified protease was inactivated at temperatures above 60°C (Fig. 6) and was completely autodigested during further lyophilization (Fig. 4A), suggesting that the enzyme is heat labile.

In enzyme inhibition examination, the purified protease was completely inhibited by certain cysteine protease inhibitors: iodoacetamide, iodoacetic acid, N-ethylma-

Table 1. Summary of purification of the protease from the extracellular products (ECP) of luminous *Vibrio harveyi* strain 820514

Purification step	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)
Crude ECP	115.6	63,696	551	1.0	100.0
90% ammonium sulfate fraction	39.0	29,796	764	1.4	47.0
Hydrophobic interaction chromatography (Phenyl Sepharose High Performance)					
First application	9.4	13,470	1,433	2.6	21.0
Second application	5.8	12,342	2,128	3.8	19.0
Anion exchange chromatography					
Q Sepharose High Performance	4.1	10,849	2,646	4.8	17.0
RESOURCE Q	1.8	9,756	5,420	9.8	15.3
Mono Q	0.4	2,821	6,716	12.2	4.4

[a] Hide powder azure was used as the substrate for protease assay.

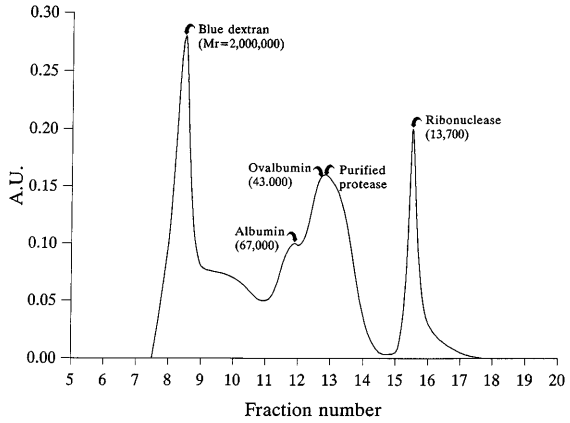

Fig. 5. Profile of purified protease eluted from FPLC-Superose 12 gel filtration column. Elution was with PBS, pH 7.2, at a rate of 1 ml/min. The column was calibrated with standard markers (Pharmacia), and points of peak elution are indicated. The molecular mass of blue dextran, bovine serum albumin, ovalbumin, and ribonuclease are 2000, 67, 43, and 13.7 kDa, respectively.

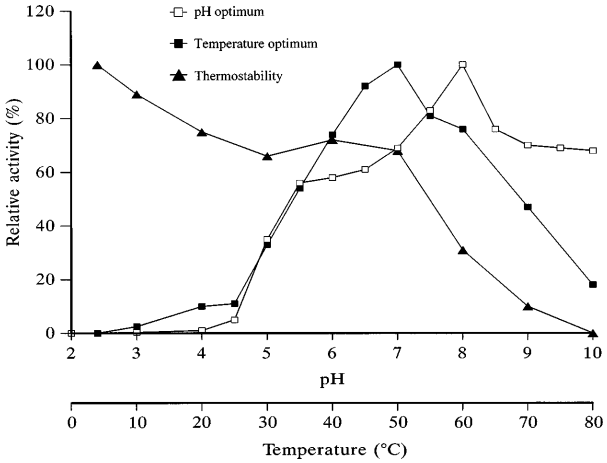

Fig. 6. Profiles of pH optimum, temperature optimum, and thermostability of the purified protease. For pH optimum (-□-), the PBS buffer is in the pH range of 3–10 and hide powder azure (HPA) was used as substrate; for temperature optimum (-■-), protease activities were assayed at 5, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 80°C, separately, and HPA was used as substrate; for thermostability (-▲-), aliquots of the enzyme were incubated for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80°C, separately, and then directly put into an ice/water bath prior to the HPA assay.

leimide, PCMB, and PCMBs; but was not or only partially inhibited by other protease inhibitors (i.e., acid protease: pepstatin A; metalloprotease: EDTA and EGTA; serine protease: PMSF) (Table 2). It was also completely inhibited by $CuCl_2$ and $HgCl_2$, while only partially inhibited by $CdCl_2$, $ZnCl_2$. Compared with the results reported by Fukasawa et al. [10, 11], our purified protease was similarly inhibited by $CuCl_2$ and $HgCl_2$, but was only partially inhibited by EDTA, $ZnCl_2$, and $CdCl_2$. Therefore, our present cysteine protease may not be the same protease(s) reported by these authors [10, 11]. Activation of the enzyme activity could be obtained with some reagents, i.e., L-cysteine (1, 5 mM), dithiothreitol, leupeptin, 2-mercaptoethanol (1, 5 mM), higher concentrations (5, 10 mM) of PCMB, TPCK, SDS, and $CaCl_2$ (Table 2); 2-mercaptoethanol (5 mM) (a cysteine protease enhancer) was the most effective reducing agent, while the other two cysteine protease enhancers, L-cysteine and dithiothreitol, were not so effective compared with 2-mercaptoethanol (5 mM). Low levels (1, 5 mM) of L-cysteine or 2-mercaptoethanol were able to activate the enzyme, while 10 mM might denature the enzyme as also demonstrated by Pike et al. [39] in a study on cysteine proteases from *Porphyromonas gingivalis*. However, the reason that PCMB can activate the protease activity at higher concentration of itself, in spite of its inhibitory effect at lower concentration, is still unknown.

The cysteine proteases (also termed thiol protease, sulfhydryl protease, or hydrolase) in mammals include several intracellular proteolytic enzymes (cathepsins B, H, L, and S) [1, 18, 45, 46], but the best-known members of this class are the plant proteases such as papain,

Table 2. Effects of various concentration of reagents on the activity of *Vibrio harveyi* protease.

Reagent	Concentration (mM)	Relative activity (% of control)
None	0	100
Cysteine protease inhibitor:		
E-64	1	100
	5	69
Iodoacetamide	10	25
Iodoacetic acid	10	0
N-Ethylmaleimide	10	0
p-Chloromercuribenzoate	1	28
	5	105
	10	120
p-Chloromercuribenzene-sulfonic acid	5	45
	10	0
Acid protease inhibitor:		
Pepstatin A	5	63
Metalloprotease inhibitor:		
EDTA	5	50
	10	60
EGTA	5	90
Serine protease inhibitor:		
Phenylmethanesulfonyl fluoride	5	66
Broad-spectrum protease inhibitor:		
Antipain	1	100
	5	82
Leupeptin	5	123
TLCK	5	77
TPCK	5	103
Other reagent:		
L-Cysteine	1	115
	5	153
	10	30
Dithiothreitol	1	116
	2	103
2-Mercaptoethanol	1	203
	5	300
	10	74
Sodium dodecyl sulfate	5	106
Divalent metal ion:		
$CaCl_2$	5	106
$CdCl_2$	5	79
$CuCl_2$	5	9
$HgCl_2$	5	1
$ZnCl_2$	5	85

EDTA: ethylenediamine tetraacetic acid.
EGTA: ethylene glycol-bis(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid.
E-64: *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.
TLCK: N-α-*p*-tosyl-L-lysine-chloromethyl ketone.
TPCK: N-tosyl-L-phenyl-alanine chloromethyl ketone.

bromelain, ficin, and ginger proteases (from papaya, pineapple, fig, and ginger, respectively) [1, 37]. Although compounds E-64, leupeptin, and antipain are well known to be effective inhibitors of the papain family, these three compounds failed to effectively inhibit the purified cysteine protease, indicating that the present enzyme is a protease different from the papain family cysteine proteases.

Apart from the cysteine proteases found in animals and plants, the enzymes have also been demonstrated to play important roles in the disease process of some infectious agents such as viruses (picornavirus family) [50, 55], bacteria (*P. gingivalis* and *Streptococcus pyogens*) [4, 13, 39], parasites (*Entamoeba histolytica*, Myxosporidian parasite, *Plasmodium falciparcum*, and *Trichomonas vaginalis*) [19–21, 41, 47, 49, 52], in Alzheimer's disease [28], and in apoptosis (programmed cell death, mammalian interleukin-1 β-converting enzyme (ICE)-like proteases) [16, 23, 24, 32]. However, no such cysteine protease has ever been found to be produced by *Vibrio* species. As the majority of proteases previously studied in *Vibrio* species are metalloproteases [9, 14, 26, 31, 33–35, 53] and serine proteases [7, 12], and only a few non-vibrio (e.g., *P. gingivalis*, and *Strep. pyogens*) bacterial cysteine proteases [4, 13, 39] have been studied so far, therefore, we conclude that our present study is the first isolation and characterization of a cysteine protease from *Vibrio* species known to date, and thus may contribute to the further understanding of bacterial cysteine proteases.

Since the present purified protease was found to be different from the cysteine proteases previously studied in many respects, it was extensively characterized in inhibitor studies. Inhibition and activation studies showed that it was a cysteine protease and that 2-mercaptoethanol was the most effective reducing agent in the activation of the enzyme. The enzyme was, therefore, suggested to be a new cysteine protease found in the ECP of *V. harveyi*.

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