Characterization of Extracellular Proteases from Alkalophilic *Vibrio* sp. Strain RH530

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An alkalophilic *Vibrio* sp. RH530 showing high proteolytic activity was isolated from soil samples by enrichment culture. The activity staining using gelatin SDS-polyacrylamide gel electrophoresis (PAGE) revealed that the strain produced an alkaline major protease (Apr B) with a size of 27 kDa, and at least six minor proteases. The apparent sizes of four of the minor proteases were approximately 45, 28, 22 and 19 kDa. Apr B and five of the minor proteases were inhibited by serine protease inhibitors including PMSF and DFP, suggesting that they are serine proteases. One of the minor proteases was inhibited by metalloprotease inhibitors, not by serine protease inhibitors, indicating it to be a metalloprotease. Furthermore, the activities of Apr B and Prt 3 were not inhibited by SDS in the reaction mixture. The production of Apr B and some of the minor proteases was specifically affected by culture temperature (30 to 37°C) and pH (7 to 10). The production of Apr B, Prt 2, Prt 5 and Prt 6 was mainly affected by culture temperature, while Prt 4 by culture pH. Prt 1 and Prt 3 were not affected by neither of these factors.

KEY WORDS □ ***Vibrio* sp., gelatin SDS-PAGE, extracellular protease, protease inhibitor, SDS-resistancy**

Microbial extracellular proteases are produced from various microorganisms including genus *Bacillus* (27, 28, 32), *Streptomyces* (22), *Serratia* (25, 32), *Vibrio* (7, 8) and *Pseudomonas* (10, 11). Of these, those from Gram-positive bacteria have been extensively studied mainly due to their quantity production, and the informations for the expression, processing and industrial application have been markedly accumulated. In Gram-negative bacteria, on the other hand, their toxicity and poor ability to secrete proteins have limited their studies mainly to the genetic level, and comparatively little is known of them.

Many bacterial species, but paticularly those belonging to the *Vibrionaceae* and *Pseudomonaceae* secrete numerous proteins, including toxins, hemolysins and proteases, which many contribute to the pathogenic properties of these orgnisms (2, 4-6, 9, 16, 22, 29). Like the elastolytic and alkaline proteases of *Pseudomonas aeruginosa*, which are crucial for the tissue destruction seen in opporunistic infections by this bacteria (10, 11, 23, 26, 30), some of the *Vibrio* proteases are well known or at least suspected as causative agents of tissue damage (5, 6, 14, 17, 20). In this study, an alkaline *Vibrio* sp. RH530 was isolated from soil samples by enrichment culture. The number and approximate molecular weights of the extracellular proteases from this strain was determined using gelatin SDS-PAGE. The biochemical properties including the interaction with various inhibitors and the resistancy to SDS were also investigated.

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MATERIALS AND METHODS

Media and culture condition

Isolates were routinely cultivated on LSC agar medium which contains (per liter) 10g of Bacto-tryptone, 5g of yeast extract, 10g sodium chloride, and 15g of Bacto-agar. After autoclaving and cooling, sterile 20% (wt/vol) sodium carbonate and sterile 15% skim milk (wt/vol) were added to the medium to final concentration of 2% and 1.5%, respectively. The LCC agar medium which contained 1% casein instead of skim milk was also used. The casein was solubilized by dissolution to 0.01 N NaOH prior to autoclaving. The pH of the casein medium was adjusted after autoclaving by addition of appropriate volume of sterile

sodium carbonate.

Strain isolation and characterization

Vibrio sp. RH530 was isolated by the following procedure. An soil sample (1g) was suspended in 3.0 ml of distilled water by vigorous vortexing. A 1.0 ml portion of the soil suspension was plated directly onto alkaline LSC- or LCC-agar medium (pH 10) and incubated at 30°C. After a 24 hr-incubation, individual colonies were picked and purified by streaking three times onto alkaline LSC-agar plates. In situ protease production was demonstrated by the clearing of opaque milk proteins in the area surrounding isolated colonies growing on the surface. The purified isolates were analysed by microscope and Gram-staining. The other physiological properties were analysed API 20E kit (Analytab products Inc.).

Enzyme assay

Protease activity was measured by the method of Yanagida *et al.* (1986). The 2.5 ml of prewarmed 1% casein (vitamine-free, Fluka) in 50 mM borate buffer (pH 9.0) and 0.5 ml of enzyme solution appropriately diluted with the same buffer were mixed and incubated at 37°C for 10 min. The reaction was stopped by the addition of stopping mixture (0.11 M trichloroacetate, 0.22 M acetic acid and 0.33 M sodium acetate), and centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was subjected to optical density determination at 280 nm on a spectrophotometer. One unit of the protease activity (pu) was defined as an amount of enzyme which caltalyzes an increase in absorbance of 0.1 per 10 min by the result of tyrosin liberation.

PAGE

The gelatin SDS-PAGE in slab gels containing SDS and gelatin as a copolymerized substrate (13) was used for the detection of the protease activity. Cultures were sedimented by centrifugation and 1.0 ml supernatant samples were mixed with 0.1 ml SDS (25% wt/vol) and 0.1 ml glycerol, and incubated at 37°C for 30 min. PAGE was carried out at 4°C on gelatin SDS-polyacrylamide gel. Each lane was loaded with 0.05~5 μg protein. After electrophoresis, the gels were washed in Triton X-100 for 1 hr at 4°C to remove the SDS and restore enzyme activity. The gels were incubated in 0.1 M Tris-HCl buffer (pH 8.0) for 3 hr at 37°C, and stained for protein with 1% (wt/vol) amido black. The bands of proteolytic activity were revealed as areas depleted of gelatin.

For the detection of protease species with bacic isoelectric points, proteins were separated on the conventional continuous SDS- or native PAGE (pH 6.8) with the polarity of the gels reversed. The proteolytic activity which is retained under these conditions was detected by placing the polyacrylamide gels on skim milk agar. Bands of clearing corresponding to skim milk hydrolysis appeared after 1 hr incubation at 37°C.

Table 1. *Characteristics of Vibrio sp. RH530.*

Test	Result	Test	Result
Cell morphology	rod	Gelatinase	+
motility	high	oxidase	+
Gram staining	−	catalase	+
VP test	+	Acid production from	
β-galactosidase	−	Glucose	+
Arginine dehydrolase	+	Mannitol	+
Lysine decarboxylase	−	Inositol	−
Ornithine decarboxylase	−	Sorbitol	+
Citrate (carbon source)	−	Rhamnose	−
H_2S production	−	Sucrose	+
Urease	−	Melibiose	−
Tryptophane deaminase	−	Amygdalin	−
Indole production	−	Arabinose	−

Protease inhibitor assays

The effects of the following protease inhibitors on the activities of the proteases were determined by adding the inhibitors to the gelatin gels during incubation in Triton X-100 and 0.1 M Tris-HCl buffer: DFP (1 mM), PMSF (0.5 to 2 mM), EDTA (2 to 5 mM), o-phenanthroline (1 mM), trypsin inhibitor (chicken egg white type II-O) (0.1 mg/ml), soybean trypsin inhibitor (0.1 mg/ml), E-64 (1 mM), PCMB (1 mM), pepstatin (1 mM), elastatinal (10 μg/ml), mercaptoethanol (20 mM), DTT (20 mM) and Histidine (0.5%). Inhibition of protease activity by the divalent cations from $CaCl_2$, $MgCl_2$, $MgSO_4$, LiCl, ZnCl and $HgCl_2$, all at 1 mM, was also determined.

RESULTS AND DISCUSSION

Characteristics of *Vibrio* sp. RH530

The isolate was a highly motile, rod shaped, Gram-negative bacterium analysed by microscope and Gram-staining. The characteristics of isolate RH530 analysed by API 20E kit are listed in Table 1.

Time courses of protease production from *vibrio* sp. RH530

The culture profiles of *Vibrio* sp. RH530 grown on LSC medium (pH 10) at 30°C and 37°C are shown in Fig. 1. The production of the proteases was started when the growth reached late log phase, and total protease activity reached maximum at stationary phase. At 30°C, both of the cell density and protease productivity were higher than those at 37°C. The pH of the culture, although not controlled, did not fall below pH 9.5 (data not shown).

Resolution of multiple protease species by gelatin-PAGE

The extracellular proteases produced by *Vibrio*

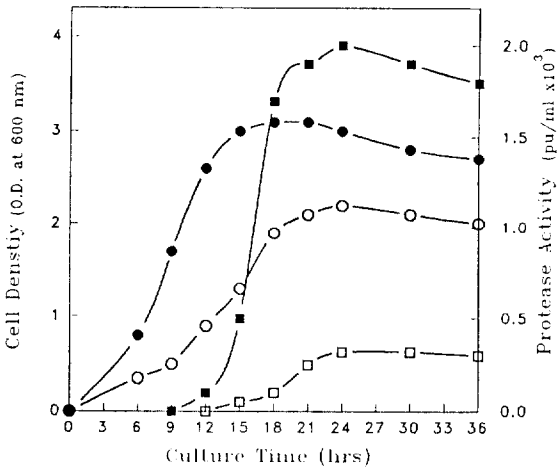

Fig. 1. *Time courses of growth and extracellular protease production by Vibrio sp. RH530. Cells were cultured in LSC-medium (pH 10) at 30°C and 37°C, respectively.*
Symbols: (●) and (○), growth of *Vibrio* sp. RH530 at 30°C and 37°C, respectively; (■) and (□), protease activity in the culture medium, at 30°C and 37°C, respectively.

sp. RH530 were examined by gelatin-PAGE. At least six bands of protease activity were obtained (Fig. 2A, and 2B). The apprarent molecular weights of these proteases were approximately 45, 28, 22 and 19 kDa for Prt 3, Prt 4, Prt 5 and Prt 6, respectively. The molecular weights of Prt 1 and Prt 2 which had relatively low mobility on gelatin-PAGE were difficult to determine. Interestingly, the major protease (Prt 7) with a size of 27 kDa on conventional SDS-PAGE (data not shown) exhibited mobility toward the anode in the same buffer system, that it could be detected only by reversing the polarity on both of SDS (data not shown) and native-polyacrylamide gel (Fig. 2C), indicating Prt 7, designated Apr B, was extremely basic protein. Furthermore, all attempts to concentrate Apr B led to a considerable decrease in activity like the proteases from *Erwinia chrysanthemi* (4).

The production of the proteases was affected by both of temperature and pH of the culture medium. The production of Apr B was much reduced at 37°C (Fig. 2C), suggesting that the reduction of total protease activity at 37°C (Fig. 1) is mainly due to that of Apr B. This result agrees to previous report that the production of some alkaline exoproteases was specifically regulated and depressed at 37°C (Fukushima *et al.*, 1989). When cells were cultured at 37°C, the production of Apr B was higher at pH 9 than pH 7 (Fig. 2C, lane a and b). When cells were cultured at 30°C, the pH of the medium in the range of 7 to 10 did not affect the production of Apr B (Fig. 2C, c and d), suggesting that the production of Apr B are mainly affected by culture temperature.

The production of Prt 1 and Prt 3 was affected

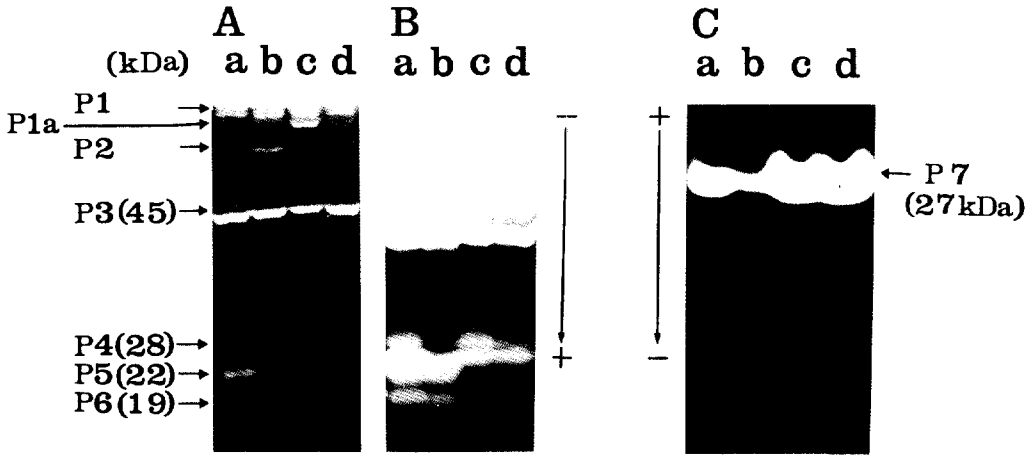

Fig. 2. *Resolution of multiple species of proteases from Vibrio sp. RH530.*
Cells were grown in LSC-medium (pH 10) (lane a and c of each panels) or LB-medium (pH 7) (lane b and d of each panels) at 37°C (lane a and b) or 30°C (lane c and d). Panels A and B represent gelatin-PAGE of culture supernatants with (A) 50-fold dilution, and (B) no dilution. Panel C represents skim milk-overlay analysis after conventional SDS-PAGE of 20-fold diluted culture supernatant. The polarity of the gel shown in panel C is reversed from that of panel A and B to allow detection of protease species with basic isoelectric points. The arrows indicate the positions of proteases. The aparrent molecular weights of each of proteases are shown in the parentheses.

Table 2. *Effect of protease inhibitors and salts on the activity of extracellular proteases produced by Vibrio sp. RH530.*

Inhibitor	Prt 7	Prt 1	Prt 2	Prt 3	Prt 4	Prt 5	Prt 6
DFP, PMSF, trypsin inhibitor[a]	+	−	+	+	+	+	+
EDTA, O-phenanthroline	−	+	−	−	−	−	−
PCMB, E-64, pepstatin, Elastatinal, soybean trpsin inhibitor	−	−	−	−	−	−	−
$CaCl_2$, $MgCl_2$, $MgSO_4$	−	ND	ND	−	−	−	−
$HgCl_2$	−	+	+	+	+	+	+
$ZnCl_2$	−	ND	ND	−	+	+	+
LiCl	−	ND	ND	−	−	−	+
Mercaptoethanol, DTT	−	+	+	−	−	+	+

[a]Chicken egg white type II-O (Sigma)
After gelatin-PAGE the inhibitors were added to the gelatin gels during incubation in 0.1 M Tris buffer for 3 h at 37°C. The gels were then stained with amido black.
+, Inhibition of protease activity after gelatin-PAGE; −, No inhibition of protease activity after gelatin-PAGE.

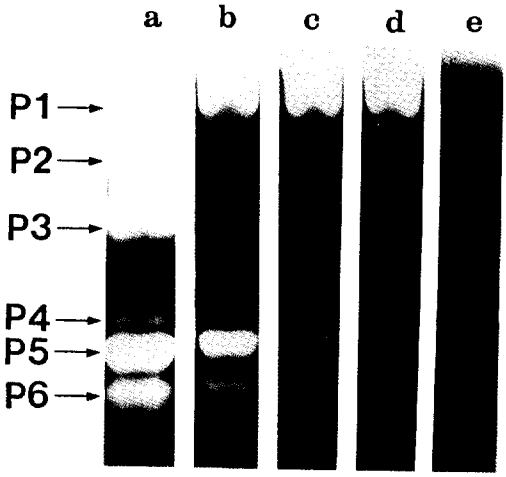

Fig. 3. *Effect of PMSF and EDTA on the activity of proteases from Vibrio sp. RH530.*
Cells were cultured in LSC-medium (pH 10) at 37°C. (a) positive control preparation; (b-c) preparation treated with: (b) PMSF (0.5 mM); (c) PMSF (1 mM); (d) PMSF (2 mM); (e) PMSF (2 mM) and EDTA (2 mM).

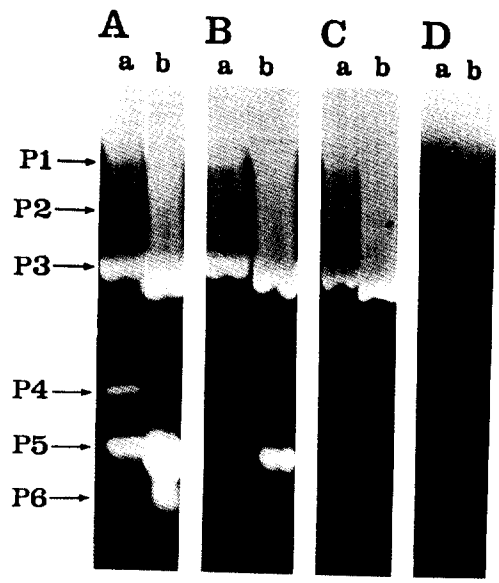

Fig. 4. *Effect of SDS on the activity of proteases from Vibrio sp. RH530.*
Cells were grown in LSC-medium (pH 10) (lane a of each panels) or LB-medium (pH 7) at 37°C, and culture supernatants were applied to gelatin-PAGE. (A) positive control preparation; (B-C) media preparation treated with: (B) SDS (0.1%), and (c) SDS (1%); (D) media preparation fixed immediately after gelatin-PAGE.

by neither pH nor temperature (Fig. 2A), but the others, Prt 2, Prt 4, Prt 5 and Prt 6 were significantly affected by these factors (Fig. 2A and 2B). The production of Prt 2, Prt 5 and Prt 6 was much increased when cultured at 37°C than at 30°C, indicating it to be mainly affected by culture temperature. On the other hand, Prt 4 showed higher activity when cultured at pH 9 than pH 7, indicating it be mainly affected by culture pH (Fig. 2B). Interestingly, when cells were cultured in LSC medium (pH 10) at 30°C, a band of proteolytic activity (Prt 1a) was specifically induced (Fig. 2A, lane c). Although this band of protease repeatedly appearred on gelatin-PAGE at this condition, the property of the activity was difficult to investigate.

Effect of inhibitors

The effect of various inhibitors and salts on the activities of proteases was investigated after

gelatin-PAGE by adding the inhibitors to the gelatin gels during incubation (Table 2 and Fig. 3). Apr B (data not shown) and all the minor proteases except Prt 1 (Table 2 and Fig. 3) were inhibited by 2 mM PMSF or DFP, but by neither of EDTA nor o-phenanthroline (data not shown), suggesting that they are serine proteases. The sensitivity of proteases to PMSF was variable (Fig. 3). Apr B (data not shown), Prt 2, Prt 4, Prt 5, and Prt 6 were partially inhibited by 1 mM PMSF, and nearly completly inhibited by 2 mM PMSF, while Prt 3 was completely inhibited even below 1 mM PMSF (Fig. 3). On the other hand, Prt 1 was not inhibited by PMSF (Fig. 3) nor DFP (Table 2), but by EDTA (Fig. 3, lane c) or o-phenanthroline (Table 2), suggesting it to be a metalloprotease. Any of the proteases were not inhibited by E-64, elastatinal nor pepstatin. $HgCl_2$ inhibited all the proteases except Apr B, whereas $CaCl_2$, $MgCl_2$, and $MgSO_4$ had no effect. LiCl inhibited Prt 6, and $ZnCl_2$ inhibited Prt 4, 5 and 6. Mercaptoethanol and DTT inhibited Prt 1, 2, 5, and 6, suggesting that they may require a disulfide bond for proteolytic activity. Histidine has been known to inhibit the microbial collagenases (7). The addition of histidine during incubation did not effect the activities of any of proteases, suggesting that they are true proteases, not collagenases (Table 2).

SDS-resistancy of the proteases

SDS-resistancy of the proteases were investigated by adding SDS to gelatin gels during soaking in Triton X-100 and incubation in 0.1 M Tris-HCl buffer (Fig. 3). Apr B was resistant to 2% SDS (data not shown) and Prt 3, to 1% SDS, suggesting that these proteases are SDS resistant proteases. Prt 5 was partially resistant to 0.1% SDS, but completly inhibited by 1% SDS. Prt 4 and Prt 6 were completly inhibited by 0.1% SDS, suggesting these two proteases are SDS-sensitive (Fig. 3, lane b). SDS-resistancy of Prt 1 and Prt 2 were difficult to determine due to their low mobility in gelatin gels. Recently some of SDS-resistant proteases were reported from *Vibrio alginolyticus* (7) and thermophilic archaebacterium *Pyrococcus furiosus* (3). This SDS-resistancy of Apr B and Prt 3 may be useful in studing the proteolytic mechanism of proteases.

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초 록: 호 염기성 *Vibrio* sp. 균주 RH530의 세포외 단백질 분해효소의 특성

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단백질 분해효소 생산균주인 염기성 *Vibrio* sp. 균주 RH530을 선별배양법을 이용하여 분리하였다. Gelatin SDS-전기영동을 이용한 활성염색법을 통하여, 본 균주가 분자량이 27 kDa인 알카리성 주요 단백질 분해효소(Apr B)와 적어도 6종의 미량 단백질 분해효소를 분비함을 밝혔다. 이들 미량효소 중 4종의 대략적인 분자량은 45, 28, 22 그리고, 19 kDa으로 측정되었다. 단백질 분해효소 저해제를 처리한 결과, Apr B 및 5종의 미량 효소들은 serine 계열의, 1종의 미량 효소는 metallo 계열의 단백질 분해효소로 밝혀졌다. 이 효소들의 SDS-저항성을 연구한 결과 Apr B와 Prt 3는 각각 2% 및 1%의 SDS 농도에서도 활성을 잃지 않았다. 이들 효소들의 생산성은 배양시 온도와 pH에 의하여 크게 영향을 받았다. Apr B, Prt 2, Prt 5 그리고, Prt 6는 배양온도, Prt 4는 배양 pH에 의해서 영향을 받았으며, Prt 1과 Prt 3는 이들의 영향을 크게 받지 않았다.