Purification and Characterization of a Secreted Protease from the Pathogenic Marine Bacterium *Vibrio anguillarum*†

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ABSTRACT: *Vibrio anguillarum* is a pathogenic marine bacterium which causes the disease vibriosis in salmonid fish, which is characterized by a fatal hemorrhagic septicemia accompanied by massive tissue destruction. In this paper, the purification of the major caseinolytic extracellular protease from *V. anguillarum* is presented. The purification steps include ammonium sulfate precipitation, DEAE-Sepharose chromatography, Sephacryl S-200 chromatography, and DEAE high-pressure liquid chromatography. The purified protease migrates with M_r = 38 000 upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A slightly larger protease of M_r 40 000 is also separated by this procedure, but accounts for only a minor fraction of the caseinolytic activity. The M_r 38 000 protease displays a broad pH activity profile in the neutral to basic range. It is not inhibited by serine, cysteine, or acid protease inhibitors, but is inhibited by EDTA and 1,10-phenanthroline, suggesting that it is a metalloprotease. The activity of the EDTA-inactivated protease could be partially restored by the addition of Ca^{2+} and Zn^{2+} together. The molecular weight and inhibition data show some similarities with proteases isolated from other *Vibrio* species such as *Vibrio cholerae* and *Vibrio vulnificus*.

Vibrio anguillarum is a causative agent of the disease vibriosis in salmonid fish. This disease is characterized by a rapid, fulminating septicemia and widespread tissue damage, which is usually accompanied by death in 2–3 days. As such, it has become a major economic problem in marine aquaculture. The identification of bacterially encoded virulence factors may give insight into the mechanisms of pathogenesis for this disease. At least one virulence factor has been identified which is critical for the establishment of the disease. The bacterium possesses a highly efficient iron uptake system which allows it to survive in the iron-deficient host environment (Crosa, 1980). This system is encoded by a plasmid, pJM1, and produces an iron-binding siderophore, anguibactin (Actis et al., 1986), and a cognate outer membrane receptor for iron–anguibactin complexes, OM2 (Actis et al., 1988). This system is required for bacterial virulence, as mutations in it cause a 10^6-fold increase in LD_{50} (Crosa, 1980). However, other potential virulence factors are much less well documented.

The widespread tissue damage which accompanies vibriosis suggests the involvement of extracellular products from the bacterium which degrade host tissues. In particular, bacterial proteases are likely candidates as causative agents of tissue damage. Bacterial proteases are important virulence factors in many diseases. The elastolytic and alkaline proteases of *Pseudomonas aeruginosa* are crucial for the tissue destruction seen in opportunistic infections by this bacterium (Pavlovskis & Wretlind, 1979; Howe & Iglewski, 1984). Indeed, proteases are also produced by a number of *Vibrio* species. *Vibrio vulnificus* produces a protease which activates plasma prekallikrein to kallikrein, resulting in severe edema at the site of infection (Miyoshi et al., 1987a). In addition, this bacterium also produces an elastolytic protease (Kothary & Kreger, 1985) and a collagenase (Smith & Merkel, 1982) which may contribute to the extensive tissue necrosis observed in wound infections. Extracellular protease activity is essential even in noninvasive diseases such as classical cholera, in which protease-deficient mutants in *Vibrio cholera* are 100-fold less virulent than the wild type (Schneider & Parker, 1978).

In this paper, the major caseinolytic protease from *V. anguillarum* strain 514 has been purified from culture supernatants to apparent homogeneity by a combination of ammonium sulfate precipitation, DEAE-Sepharose chromatography, Sephacryl S-200 chromatography, and DEAE high-pressure liquid chromatography. The purified protease appears to be a metalloprotease by virtue of its inhibition by EDTA and 1,10-phenanthroline, and its lack of susceptibility of several inhibitors of serine, cysteine, and acid proteases. The relationship of this protease to previously identified proteases from *Vibrio* species is discussed.

Materials and Methods

Bacterial Growth Conditions. *Vibrio anguillarum* strain 514 was grown on tryptic soy agar plates (DIFCO) supplemented with 1% NaCl and 1% nonfat dry milk (Carnation) at 26 °C. Starter cultures of 134 mL of tryptic soy broth (DIFCO) supplemented with 1% NaCl were grown from isolated protease-positive colonies for 24 h at 22 °C with vigorous shaking. Four 2-L flasks containing 1 L of tryptic soy broth supplemented with 1% NaCl were each inoculated with 33.3 mL from the starter culture and grown for 24 h at 22 °C with vigorous shaking. The culture medium was centrifuged at 5000 rpm for 15 min at 4 °C in a JA-10 rotor (Beckman) to pellet the cells; then the supernatant was chilled to 4 °C for subsequent processing.

Casein–Agarose Protease Assay. A variation of the method of Schumacher and Schill (1972) as modified by Bjerrum et al. (1975) was used to quantitate the proteolytic activities of the purification steps. Casein–agarose was made with 150 mM NaCl/10 mM HEPES (pH 7.4)/0.1 mM $MgSO_4$/0.01 mM $CaCl_2$/0.02% NaN_3 buffer using 1% agarose (BRL) and 1% nonfat skim milk (Carnation) and poured into petri dishes at

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0.15 mL/cm^2. One-microliter samples were placed in 2-μL wells and incubated at 26 °C for 24 h. For the standard curve, the diameter of the zone of clearing was plotted as a function of the log of the amount of standard trypsin added (0.25–1.0 μg, 10000 BAEE units/mg; Sigma). The diameter of the zone of clearing for the experimental samples was compared to the standard curve generated by using trypsin. The result was expressed as the equivalent amount of trypsin required to give the same diameter zone of clearing; 1 unit of activity corresponds to the amount of protease which gave the same diameter zone of clearing as 1 mg of trypsin.

Protein Assay. Protein determinations were performed by using the method of Bradford (1976) using bovine γ-globulin as standard (Bio-Rad).

Protease Purification. All steps were performed at 4 °C unless otherwise noted.

(*A*) *Ammonium Sulfate Precipitation.* Four liters of chilled culture medium was made 2.0 M with ammonium sulfate (BRL) and stirred for 1 h. The medium was centrifuged at 5000 rpm for 1 h in a JA-10 rotor. The supernatant was removed, and the pellets were resuspended in a total of 30 mL of 20 mM Tris, pH 7.4. The resuspended pellets were dialyzed against 2 L of 20 mM Tris, pH 7.4, with one change of buffer. The final volume was 67.5 mL.

(*B*) *DEAE-Sepharose Chromatography.* The 67.5-mL dialyzed ammonium sulfate pellet was applied to a 2.5 × 6 cm DEAE-Sepharose column (Sigma) equilibrated with 20 mM Tris, pH 7.4, at 49 mL/h. The resin was washed with 20 column volumes (600 mL) of the same buffer and eluted by reverse flow in 0.5 M NaCl/20 mM Tris, pH 7.4, at 32 mL/h. One-milliliter fractions were collected and pooled as indicated in the text.

(*C*) *Sephacryl S-200 Chromatography.* The pooled fractions from DEAE-Sepharose were applied to a 2.5 × 50 cm Sephacryl S-200 column (Sigma) equilibrated in 137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.87 mM $CaCl_2$/0.49 mM $MgCl_2$, pH 7.4 (dPBS), at 9 mL/h; 4.7-mL fractions were collected and assayed for caseinolytic activity on casein–agarose as described above. Fractions with peak activity were pooled as indicated in the text. The final volume recovered was 27 mL.

(*D*) *TSK (Toyo Soda Kogyo)-DEAE-5-PW High-Pressure Liquid Chromatography.* The 27-mL pool from the S-200 column was concentrated on a 1.5-mL DEAE-Sepharose column at 2 mL/h after dialysis in 2 L of 20 mM Tris, pH 7.4. The resin was washed with 8 column volumes of the same buffer and eluted with 0.5 M NaCl/20 mM Tris, pH 7.4. The eluate was dialyzed at 4 °C in 20 mM Tris, pH 7.4 (pH 7.4 at 22 °C; buffer A). A 7.5 × 75 mm TSK-DEAE-5-PW column (Bio-Rad) was equilibrated in buffer A using a high-pressure liquid chromatography (Beckman). The dialyzed sample (3 mL) was applied to the column in three 1-mL injections and washed with buffer A for 10 min and then eluted with a linear gradient of 0–10% B (buffer B = 1 M NaCl/20 mM Tris, pH 7.4 at 22 °C) in 80 min. Two-minute fractions were collected (1 mL) and assayed for caseinolytic activity on casein–agarose. Fractions containing the peak activity of the M_r 40000 protease (3 mL) were pooled separately from the M_r 38000 protease (10 mL) and stored at −70 °C. In some isolations, it was necessary to repeat this chromatographic step on each of the pooled proteases to remove contaminating proteins.

High Powder-Azure Protease Assay. A modification (Howe & Iglewski, 1984) of the method of Rinderknecht et al. (1968) was used to quantitate the effect of pH and inhibitors on the activity of the M_r 38000 protease. Ten milligrams of hide powder-azure (Sigma) was incubated with gentle rocking for 1–2 h at 37 °C in 1 mL of buffer containing 0–0.5 μg of the protease. For the pH activity profile in Figure 5, the buffer was 150 mM NaCl/10 mM acetate/10 mM Bis-Tris/10 mM Tris/10 mM CAPS at the indicated pH values from 4.0 to 10.0. For the inhibitor assays, the inhibitors (Sigma) were incubated at the concentrations indicated in Table II for 15 min at 4 °C with the protease prior to the assay in 150 mM NaCl/10 mM HEPES (pH 7.4)/0.1 mM $MgSO_4$/0.01 mM $CaCl_2$/0.02% NaN_3. For the reconstitution experiments shown in Table III, the protease was incubated with 5 mM EDTA in a 25-μL volume for 15 min at 4 °C and then reconstituted with 10 mM metal salts ($CaCl_2$ and/or $ZnCl_2$) for a further 15 min at 4 °C. The mixture was brought to 1 mL in 150 mM NaCl/10 mM HEPES, pH 7.4, for the assay. The undigested hide powder-azure was removed by centrifugation in an Eppendorf microfuge for 5 min at 4 °C, and the absorbance of the supernatant was read at 595 nm. The remaining activity was calculated based on a standard curve using the purified M_r 38000 protease. The absorbance of the supernatant at 595 nm was plotted as a function of the amount of protease added (0–0.5 μg). The absorbance of the superntants which contained inhibitors was compared to the standard curve and expressed as the percent of the original activity which remained.

Polyacrylamide Gel Electrophoresis. Samples taken from each of the purification steps were electrophoresed on 10% T/2% C polyacrylamide gels as previously described (Farrell et al., 1986) according to the methods of Ornstein (1964) and Davis (1964), as modified by Laemmli (1970). Standard protein markers were from Bio-Rad. Gels were stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma) in 5:1:5 methanol/acetic acid/water (v/v/v) and destained in 5% ethanol/7% acetic acid.

Results

Protease Purification. Initial studies showed that *V. anguillarum* strain 514 grown on casein-containing tryptic soy agar plates secreted an extracellular protease which rapidly digested the casein. The secretion of this protease was not dependent on the presence of casein, and so could be harvested from cultures grown in tryptic soy broth. The conditions for the growth of *V. anguillarum* at 22 °C for 24 h allowed for the production of enough protease activity to make purification feasible.

The protease activity was maximally precipitated from the cleared tryptic soy broth growth medium by 2.0 M ammonium sulfate, which also served to concentrate the protease activity from the dilute broth. The protease activity in the browth was too dilute to measure by using the casein–agarose assay (Schumacher & Schill, 1972), so that calculations of yield and fold purification were based on the 2.0 M ammonium sulfate precipitate.

Experiments indicated that the protease behaved as an acidic molecule and bound to DEAE-Sepharose under near-neutral conditions (Figure 1). Although the summary of the purification (Table I) shows that chromatography of the protease on DEAE-Sepharose resulted in a net loss of activity and a slight loss of specific activity, this step was necessary to eliminate contaminating proteins which eluted with the protease on the next purification step. However, none of the protease activity was detectable in the unadsorbed flow-through from the DEAE-Sepharose column.

Sephacryl S-200 was used to further fractionate the eluate from the DEAE-Sepharose step. This resulted in the highest

Table I: Purification of *Vibrio anguillarum* Proteases

purification step	total protein (mg)	total activity (trypsin equiv)	specific activity (trypsin equiv/mg)	purification (x-fold)	yield (%)
ammonium sulfate	77	6.4	0.084	1.0	100
DEAE-Sepharose	38	2.4	0.063	0.8	37
Sephacryl S-200	2.8	3.3	1.2	14	52
TSK-DEAE-5-PW (M_r 38 000)	0.92	0.49	5.3	63	7.6
TSK-DEAE-5-PW (M_r 40 000)	0.32	0.12	3.9	46	1.9

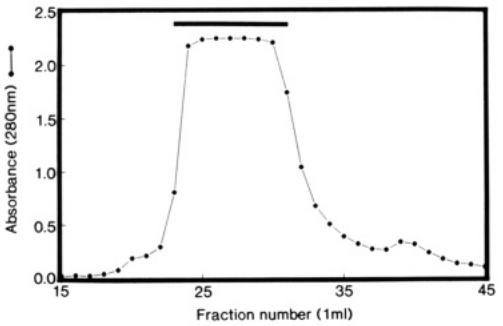

FIGURE 1: DEAE-Sepharose chromatography of ammonium sulfate precipitated culture medium. The 2.0 M ammonium sulfate pellet (67.5 mL) was dialyzed in 20 mM Tris, pH 7.4, and applied to a 2.5 × 6 cm DEAE-Sepharose column equilibrated in the same buffer at 49 mL/h at 4 °C. The resin was washed with 20 column volumes of the same buffer and eluted by reverse flow in 0.5 M NaCl/20 mM Tris, pH 7.4, at 32 mL/h. One-milliliter fractions were collected and pooled as indicated by the bar. No activity was detected in the flow-through wash. Protein (●—●) was monitored by the absorbance at 280 nm.

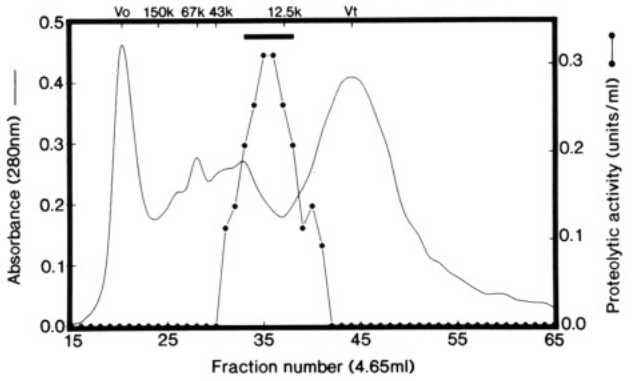

FIGURE 2: Sephacryl S-200 chromatography of the DEAE-Sepharose eluate. The pooled fractions 24–31 indicated in Figure 1 were applied to a 2.5 × 50 cm Sephacryl S-200 column equilibrated in dPBS at 9 mL/h at 4 °C; 4.7-mL fractions were collected and pooled as indicated by the bar. Protein (—) was monitored by the absorbance at 280 nm, and protease activity (●—●) was assayed by the hydrolysis of casein–agarose.

fold purification step in the isolation procedure, with the bulk of the protein eluting prior to the protease activity (Figure 2). (The large peak of absorbance near V_t did not show any Coomassie-staining proteins upon gel electrophoresis.) The activity assay for this step showed that a higher amount of total activity was recovered than was applied to the resin. Several explanations for this are possible, including the removal of inhibitory substances by the resin or the activation of protease zymogens. It is clear that the resulting pool of protease activity is depleted of many high molecular weight proteins (Figure 4, compare lanes 3 and 4). The total protein load was also reduced to a point where a high-resolution, low-capacity method could be used as the last step in the purification.

Final purification to electrophoretic homogeneity was achieved by using high-pressure liquid chromatography on a TSK-DEAE-5-PW ion-exchange resin (Figure 3). This step resulted in the separation of two proteins with protease activity. In addition, a small amount of activity appeared in the

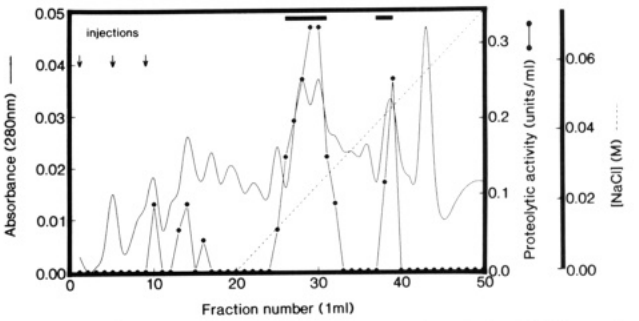

FIGURE 3: TSK-DEAE-5-PW chromatography of the S-200 pool. The pooled fractions 33–38 (27.8 mL) indicated in Figure 2 were concentrated on a 1.5-mL DEAE-Sepharose column after dialysis in 20 mM Tris, pH 7.4, by elution with 0.5 M NaCl/20 mM Tris, pH 7.4, at 4 °C. The eluate was dialyzed at 4 °C in 20 mM Tris, pH 7.4 (pH 7.4 at 22 °C; buffer A). The dialysate (3 mL) was applied in three 1-mL injections to a 7.5 × 75 mm TSK-DEAE-5-PW column equilibrated in 20 mM Tris, pH 7.4, at 22 °C. The resin was washed at 0.5 mL/min with buffer A for 10 min and then eluted with a linear gradient of 0–10% B (buffer B = 1 M NaCl/20 mM Tris, pH 7.4 at 22 °C) in 80 min (- - -). Two-minute fractions were collected (1 mL) and pooled as indicated by the bars. Protein (—) was monitored by the absorbance at 280 nm, and the protease activity (●—●) was assayed by the hydrolysis of casein–agarose.

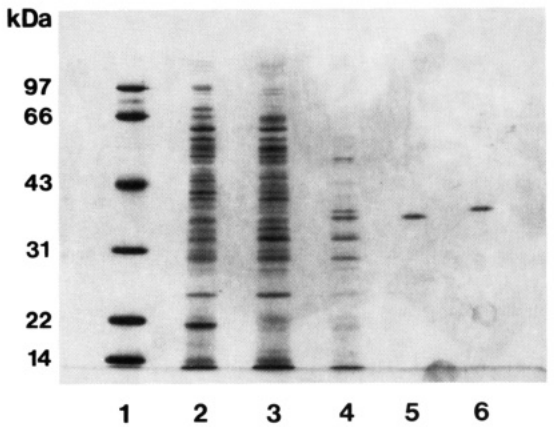

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purification steps. The pooled samples from each of the purification steps were subjected to electrophoresis and stained with Coomassie Brilliant Blue R-250: lane 1, molecular weight markers; lane 2, 2.0 M ammonium sulfate precipitated culture medium; lane 3, DEAE-Sepharose eluate; lane 4, Sephacryl S-200 pooled fractions; lane 5, TSK-DEAE-5-PW pooled M_r 38 000 protease; lane 6, TSK-DEAE-5-PW pooled M_r 40 000 protease.

unadsorbed flow-through fractions, but this accounted for only a minor amount of the total protease activity. The most abundant protease migrated with M_r 38 000, and the second most abundant protease migrated with M_r 40 000 (Figure 4); the final recovery of each protease was 7.6% and 1.9%, respectively, of the total starting ammonium sulfate precipitate (Table I). The more abundant M_r 38 000 protease was chosen for further characterization.

Protease Activity Assays. The proteolytic activity of the M_r 38 000 protease was determined at several pH values and in the presence of different protease inhibitors. For these assays, a different protease activity assay was required, since

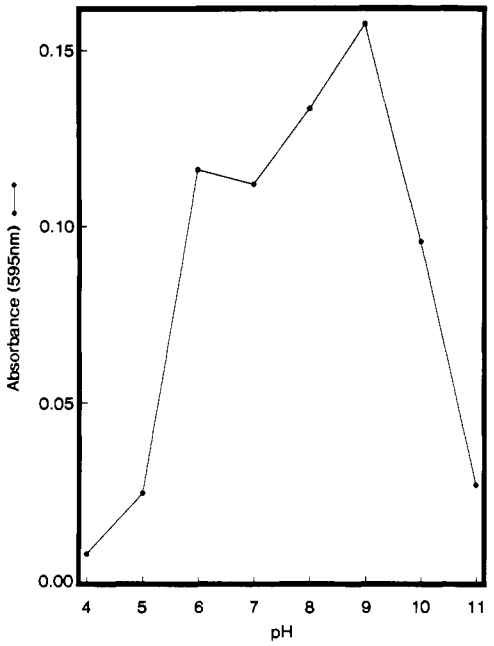

FIGURE 5: Activity of the M_r 38 000 protease at different pH values. The purified protease was incubated in 1 mL of 150 mM NaCl/10 mM acetate/10 mM Bis-Tris/10 mM Tris/10 mM CAPS (adjusted to the indicated pH values) with 10 mg of hide powder-azure as described under Materials and Methods. The reaction mixture was microfuged 5 min at 4 °C, and the supernatant was assayed for its absorbance at 595 nm (●—●). The absorbance of supernatants lacking the protease was subtracted from each data point. The values are the average of duplicate determinations.

Table II: Effect of Protease Inhibitors on the M_r 38 000 Protease[a]

inhibitor	concentration (mM)	activity (%)
none	0	100
EDTA	5.0	9 ± 1
EGTA	5.0	32 ± 0
iodoacetamide	1.0	92 ± 19
leupeptin	0.1	112 ± 8
pepstatin A	0.1	97 ± 11
1,10-phenanthroline	1.0	0
phenylmethanesulfonyl fluoride	1.0	109 ± 7

[a] 0–0.5 μg of purified protease was incubated with the inhibitor in 1 mL of 150 mM NaCl/100 mM HEPES (pH 7.4)/0.1 mM $MgSO_4$/0.01 mM $CaCl_2$/0.02% NaN_3. Ten milligrams of hide powder-azure was added and incubated as described under Materials and Methods. The reaction mixture was then microfuged 5 min at 4 °C, and the supernatant was assayed for its absorbance at 595 nm. The values are the average of duplicate determinations (±SEM).

one of the inhibitors (EDTA) caused a zone of clearing in the absence of protease activity in the casein–agarose assay. An insoluble hide powder-azure assay (Rinderknecht et al., 1968) was therefore used to measure proteolytic activity.

(*A*) *pH Activity Profile.* The protease displayed a broad pH activity profile in the neutral to basic range (Figure 5). Very little activity was seen at or below pH 5.0, but a sharp increase was seen at pH 6.0. The activity continued to be >60% of maximum up to pH 10, with a sharp decline at pH 11.0. The pH activity curve was quite similar to the activity profile seen with a protease produced by *Vibrio cholerae* 01 and non-01 types (Honda et al., 1989).

(*B*) *Protease Inhibitor Assays.* The inhibitor studies (Table II) showed that the protease was insensitive to a number of inhibitors, including the broad-spectrum inhibitor leupeptin. Its insensitivity to phenylmethanesulfonyl fluoride indicated that it is not a serine protease. In addition, iodoacetamide had little effect on its activity, suggesting that the protease is a not a sulfhydryl protease. Pepstatin A, an acid protease inhibitor, also did not significantly affect its activity. The partial inhibition by EGTA may indicate that calcium ions are necessary for full activity, as seen in the reconstitution assays to be described below. In contrast, EDTA decreased its activity by 91%, consistent with a divalent metal ion cofactor. In addition, no proteolytic activity was observed following treatment with the metal ion chelator 1,10-phenanthroline, strongly suggesting a metal ion cofactor requirement for the protease. On the basis of this inhibition by EDTA and 1,10-phenanthroline and lack of inhibition by classical inhibitors of serine, cysteine, and acid proteases, we propose that the M_r 38 000 protease is a metalloprotease.

Table III: Effect of Metal Ions on EDTA-Inhibited M_r 38 000 Protease[a]

addition	activity (%)
none	100
EDTA	4 ± 1
EDTA + $CaCl_2$	2 ± 5
EDTA + $ZnCl_2$	0
EDTA + $CaCl_2$ + $ZnCl_2$	39 ± 2

[a] The purified protease was incubated with 5 mM EDTA to inactivate the protease, as described under Materials and Methods. The reaction was then made 10 mM with either $CaCl_2$ or $ZnCl_2$ or with a 5 mM sample of each combined. Samples were diluted to 1 mL in 150 mM NaCl/10 mM HEPES, pH 7.4, for the hide powder-azure assay. The values are the average of duplicate determinations (±SEM).

(*C*) *Metal Ion Reconstitution.* The identity of the metal ion(s) required for proteolytic activity was investigated by using a reconstitution assay. The protease was inhibited by using 5 mM EDTA, and reconstituted by using 10 mM $CaCl_2$, 10 mM $ZnCl_2$, or a mixture of 5 mM $CaCl_2$/5 mM $ZnCl_2$ (Table III). Interestingly, neither metal ion restored activity by itself. Partial activity was seen only when both $CaCl_2$ and $ZnCl_2$ were present, suggesting that both metal ions may be necessary for activity. This effect has also been seen in "calcium-stabilized, zinc-containing proteases" such as the *Vibrio cholerae* hemagglutinin/metalloprotease (Booth et al., 1983).

Discussion

Extracellular proteases are produced by many bacterial species and have been correlated with virulence in a number of cases (Schneider & Parker, 1978; Pavlovskis & Wretlind, 1979; Howe & Iglewski, 1984). *Vibrio* species in particular produce several well-studied proteases. A comparison of previously identified *Vibrio* proteases with the M_r 38 000 protease from *Vibrio anguillarum* reveals a few similar proteases, but most are distinctly different. *V. alginolyticus* produces five proteases, but these are all serine proteases, as shown by their sensitivity to phenylmethanesulfonyl fluoride (Hare et al., 1983). The endopeptidase from *Vibrio* B-30 (Merkel & Sipos, 1971) is larger (M_r 49 000) and is clearly a serine protease by virtue of its inhibition by diisopropyl fluorophosphate. The collagenase from *Vibrio* B-30 (Merkel & Dreisbach, 1978), while demonstrating sensitivity to EDTA and insensitivity to serine protease inhibitors, is a tetramer of M_r 105 000 composed of M_r 24 000 and 28 000 subunits. In this case, the subunits are much smaller than M_r 38 000; in addition, there is no evidence for a multimeric structure for the *V. anguillarum* protease since it eluted from the Sephacryl S-200 column with M_r 16 000.

Similarly, the *V. cholerae* hemagglutinin protease, although a metalloenzyme (Booth et al., 1983), is a multimer with subunits of M_r 32 000 (Finkelstein & Hanne, 1982). In contrast, the electrophoretic migration of the *V. cholerae* mucinase (Schneider & Parker, 1982; see also "type II protease", Young & Broadbent, 1982) is similar to the *V.*

anguillarum protease, but it is not inhibited by EDTA at concentrations as high as 25 mM. The type I protease from *V. cholerae* described by Young and Broadbent (1982) is a serine protease; however, the type III protease is a metalloprotease with a molecular weight of 43 000. The relationship between this type III protease from *V. cholerae* and the M_r 38 000 protease from *V. anguillarum* is not clear. In addition, the 32 000 molecular weight protease from *Vibrio cholerae* 01 and non-01 types may be related to the *Vibrio anguillarum* protease, as judged by their pH activity profiles, inhibition by EDTA and 1,10-phenanthroline, and lack of sensitivity to phenylmethanesulfonyl fluoride (Honda et al., 1989). Another protease which may be related is the 45 000–50 500 molecular weight protease from *V. vulnificus* (Kothary & Kreger, 1985; Miyoshi et al., 1987b). Although it is larger than the *V. anguillarum* protease, it is similarly resistant to phenylmethanesulfonyl fluoride, leupeptin, and pepstatin A, while showing significant inhibition by EDTA and 1,10-phenanthroline (Kothary & Kreger, 1987).

The role of the M_r 38 000 protease in vibriosis remains to be established. The extensive tissue damage seen in this disease suggests a role for extracellular proteases. Now that the major caseinolytic extracellular protease from *V. anguillarum* has been purified, it will be possible to further investigate its contribution to vibriosis. With purified M_r 38 000 protease, antiserum can be produced to test the effect of passive immunization on the outcome of the disease. A toxoid consisting of inactivated protease may also be useful in direct immunization against vibriosis. In addition, it should be possible to clone the gene, with a view toward creating specific mutants by marker exchange. Mutants of this kind would be especially useful to directly test the role of the M_r 38 000 protease in vibriosis.

Added in Proof

Genetic evidence for the role of a 36-kDa metalloprotease in vibriosis has now been reported (Norqvist et al., 1990).

Acknowledgments

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