

Purification of Protease from *Vibrio cholerae* O1 and its Partial Characterization

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Abstract: A protease produced by a clinical isolate of *Vibrio cholerae* O1 was purified to apparent homogeneity by ammonium sulfate fractionation, ultracentrifuge and successive column chromatography on a Bio-gel A5m and a TSK gel G-3000SW HPLC column. The molecular weight of the purified protease was estimated to be 32,000 on the basis of its mobility on SDS-PAGE and was identical to HA/protease biochemically and physicochemically as previously reported (Booth *et al.*, 1983; Honda *et al.*, 1987). The hemagglutinin activity of purified protease, however, was not detected as previously described. The purified protease seemed to be identical biochemically and physicochemically but did not show the character of a bifunctional protein molecule.

Key word: *Vibrio cholerae*, Protease, Hemagglutinin

A number of investigators have reported the purification and characterization of proteases or protease/HA purified from *Vibrio cholerae* O1 and non-O1, because extracellular proteases have been implicated as important virulence factors in diarrheal diseases caused by these organisms. Young and Broadbent (1982) reported the purification of three types of protease from *V. cholerae* O1: phenylmethylsulfonyl fluoride inhibitable type I, metalloprotease and serine protease inhibitor resistant type II and EDTA inhibitable type III. Booth *et al.* (1983) reported that soluble HA/protease is a metalloenzyme which can be inhibited by a variety of chelating agents and inhibitors of zinc metalloprotease and that it nicks cholera enterotoxin (1984). It has also been reported by Finkelstein *et al.* (1983) that a soluble zinc- and calcium dependent protease from *V. cholerae* O1 causes hemagglutination, hydrolyzes mucin, cleaves lactoferrin, and nicks the A subunit of heat labile enterotoxin of *Escherichia coli*. Honda *et al.*, furthermore, reported that the purified HA/proteases from *V. cholerae* O1 and non-O1 are identical physicochemically and immunologically (1987, 1989). In these papers, protease was described as a bifunctional protein molecule showing the character of both a proteolytic enzyme and a hemagglutinin. In this study, therefore, we describe the purification of protease and discuss the fact that we could not detect any hemagglutinin activity.

Purification of the protease from *V. cholerae* O1

Protease activity was screened among 67 strains of *Vibrio cholerae* isolated from cholera patients in Kenya in 1983 and in Thailand in 1988 by a single-diffusion technique in agar gel (0.75%) containing skim milk (1.5%) as a substrate as previously described by Honda *et al.* (1987). The strain used for the purification of protease was *V. cholerae* O1, serotype Ogawa, biotype El Tor (K23), isolated from a diarrheal patient in Kenya. The bacteria were cultured in a 5-liter flask containing 1 liter of heart infusion broth with shaking (72 cycles/min) at 37°C for 36 hr. Culture supernatant was obtained by centrifugation (40,000 × *g*) 1 hr and equilibrated with 1/20 volume of 0.5 M phosphate buffer (pH 7.0) (PB). The resultant supernatant was fractionated with ammonium sulfate. The 20-60% ammonium sulfate-insoluble materials were suspended in 25 mM PB (pH 7.0) and dialyzed against the same buffer. Dialysates were centrifuged at 100,000 × *g* for 1 hr to remove insoluble substance, and the supernatant was applied to a Bio-gel A5m column equilibrated with the same buffer (Fig. 1).

The fractions of the second protein peak, showing protease activity, were concentrated to about 2 ml on a membrane (PM-10; Amicon Corp., mass. U.S.A.) and applied to a TSK gel G-3000SW HPLC column equilibrated with 25 mM PB (pH 7.0) containing 0.2 M sodium chloride. The fractions containing protease activity were again applied to the same TSK gel G-3000SW HPLC column. Fractions with protease activity, which showed a single protein peak, were used as purified protease (Fig. 2).

For the quantitative assay of protease, a test tube method using azocasein (azocasein assay; Sigma Chemical Co., St. Louis, Mo.) as a substrate was performed by the procedure previously described (Hingley *et al.*, 1986). One protease unit (PU) was defined as the smallest dose needed to digest 50% of 0.5 mg of the substrate in 1 hr of the incubation. Specific activity of the purified protease was 0.62 PU/μg and final recovery was about 3%. To test inhibitory effects, various amounts of the following compounds were added to 5 PU of purified protease in the protease assay system: EDTA, *o*-phenanthroline, α₂-macroglobulin, and phenylmethylsulfonyl fluoride (Sigma). After incubation with these compounds for 30 min at 37°C, the residual activity was measured in the protease assay system. The protease acti-

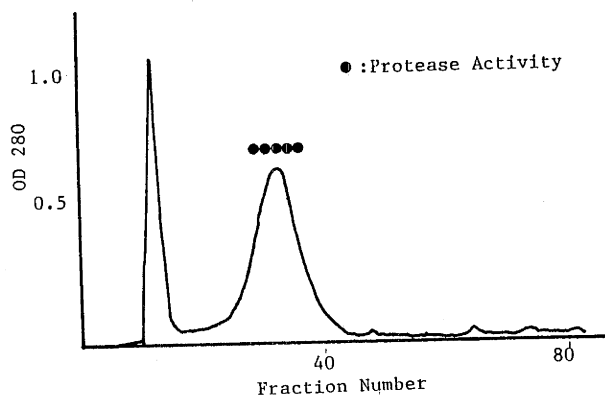


Fig. 1. Gel filtration profile of crude protease from Bio-gel A5m.

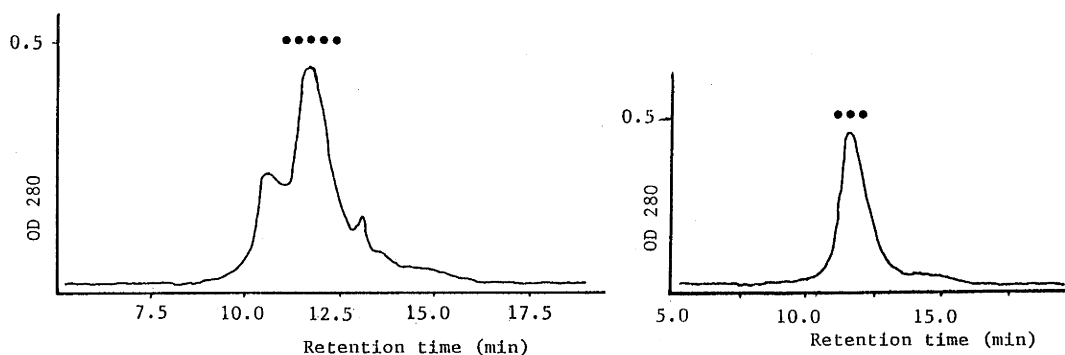


Fig. 2. Typical elution profiles of protease from TSK gel G-3000 SW HPLC column, 1st (left) and 2nd (right).

vity of purified protease was inhibited by EDTA (final concentration 5 mM), *o*-phenanthroline (0.8 mM), and α_2 -macroglobulin (100 μ g/ml) but not by phenylmethylsulfonyl fluoride (10 mM) or trypsin inhibitor (500 μ g/ml).

Sodium dodecyl sulfate-PAGE (SDS-PAGE)

SDS-PAGE in 0.1% SDS was carried out as described previously (Laemmli, 1970) in 12% polyacrylamide gel. The purified protease exhibited two protein bands on SDS-PAGE (Fig. 3).

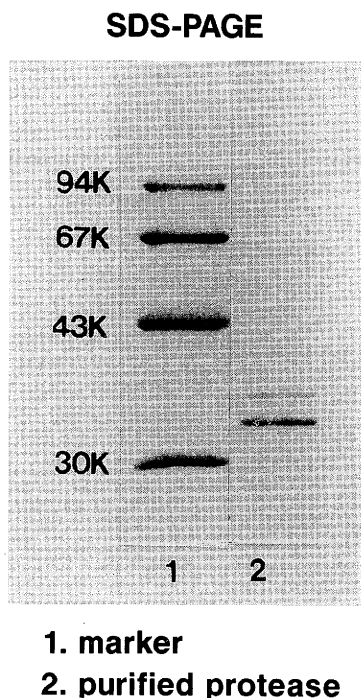


Fig. 3. SDS-polyacrylamide electrophoresis. Marker: molecular marker.

The molecular weight of the major protein band was estimated to be about 32,000 on the basis of its mobility on SDS-PAGE and that of the faint band was estimated to be about 34,000.

Hemagglutination assay

Microtiter quantitation of hemagglutinin activity (HA) on chicken erythrocytes was performed as previously described by Hanne and Finkelstein (1982). The titer is defined as the reciprocal of the highest dilution in which hemagglutinin was visible to the naked eye. HA preparations were diluted in two-fold series in round-bottomed microtiterplates in 25 μ l of Krebs-Ringer buffer (KRT). Red blood cells (RBCs) suspended to 1.5% concentration (vol/vol) were added in 25 μ l and incubated for 30 min at room temperature, and HA reactions were examined after 30 min. Five lots of chicken RBCs were used for the HA test. The hemagglutination titer of the bacterial culture supernatant was $\times 2^2$, but hemagglutination of the purified protease was not observed.

Purification of a protease produced by *V. cholerae* O1 to apparent homogeneity was achieved in this study. The molecular weight of the purified protease was estimated to be about 32,000. The faint band at the position of M.W. 34,000 can be considered to be a prototype of protease, because the protein band showing protease activity on conventional PAGE migrated at the position of M.W. 32,000 and 34,000 when the conventional disc gel was loaded on SDS-PAGE dimensionally, and the protein band at the position of M.W. 34,000 was shifted to the position of M.W. 32,000 on SDS-PAGE after storage at 4°C for several weeks (data not shown). The optimal pH for protease activity was about 7 to 9 and its protease activity was completely inactivated at 80°C for 15 min (data not shown). The results regarding the inhibitory effects of EDTA, *o*-phenanthroline, α_2 -macroglobulin, phenylmethylsulfonyl fluoride and trypsin inhibitor are the same as those of HA/protease as previously described (Booth *et al.*, 1983; Honda *et al.*, 1989). Five lots of chicken RBCs were used for the hemagglutination test, but the bacterial supernatant exhibited hemagglutination activity even though some were non-responder RBC. The hemagglutinin activity of purified protease, however, was not observed as described before. The purified protease seems to be identical physicochemically but does not show the character of a bifunctional protein molecule. Several possible explanations come from these results: 1) the active site of the hemagglutination of purified protease was degraded with other proteases contained in the culture supernatant or 2) autodigested during the course of purification, resulting in the loss of hemagglutination activity. In fact, it seems that the hemagglutination activity of purified HA/protease lowered during storage at 4°C, protease activity was well-maintained under these conditions (personal communication). 3) It might also be suggested that other components such as a soluble hemagglutinin were associated with or comigrated with the protease molecule at an undetectable level on SDS-PAGE. 4) Furthermore, the phenomenon of hemagglutination might be an autoagglutination of RBCs in which the hydrophobic regions were exposed by the digestion with protease, and this function might be lost in the course of maturation. It will be interesting to determine whether or not HA/protease really possesses a

bifunctional domain and whether it plays a significant role in the pathogenesis and immunity of cholera.

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