THE PROTEASE FROM VIBRIO CHOLERAЕ NICKS ARGinine 
AT POSITION 192 FROM THE N-TERMINUS OF THE 
HEAT-LABILE ENTEROTOXIN A SUBUNIT FROM 
ENTEROTOXIGENIC ESCHERICHIA COLI

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It was examined where a protease purified from Vibrio cholerae might nick the heat-labile enterotoxin (LT) A subunit from enterotoxigenic Escherichia coli.

LT was digested by the protease and contained a fragment which had the same mobility on SDS-PAGE as that of the A1 fragment of LT digested by trypsin. The biological activity of LT by this protease was also identical to that of LT by trypsin. The amino acid sequence of the N-terminus of the A2-like fragment was Thr-Ser-Thr-Gly, which corresponded to the sequence from 193 to 196 of the A subunit.

These data suggest that this protease, like trypsin, nicks arginine at position 192 from the N-terminus of the A subunit and that the biological activation of LT by this protease is similar to that by trypsin.

INTRODUCTION

Extracellular proteases have been thought to play an important role in the pathogenesis of diarrhea caused by Vibrio cholerae. Protease-deficient mutants of V. cholerae are known to be less virulent (16). It has been reported that pathogenic V. cholerae produces a hemagglutinin/protease with the ability to activate the A subunit of cholera enterotoxin and cleaves other physiologically important substrates, including mucin, fibronectin and lactoferrin (1, 5, 17).

Cholera toxin (CT) produced by V. cholerae is composed of two subunits, A and B, similar to heat-labile enterotoxin (LT) from enterotoxigenic Escherichia coli. The B subunit recognizes the membrane component, GM1 ganglioside, on the cell membrane as a receptor and binds the holotoxin to the target cells, facilitating internalization of the A subunit (9). The A subunit is composed of A1 and A2 fragments, which are joined by a cysteine bridge (nicked state) in CT (6, 10) and by a polypeptide band (unnicked state) in LT (2). The A1 fragment activates adenylate cyclase in the target cell to increase the intracellular cAMP level. The nicking of the A subunit is necessary for activation of adenylate cyclase (7). In V. cholerae the nicking of the A subunit of CT occurs at position 192 and 194 or 195 from the N-terminus of the A subunit of CT (11, 13, 14), and increases its biological activity. However, it is not known which protease nicks the toxin in V. cholerae. In this study, we examined whether a protease purified from V. cholerae would nick LT at the same position as trypsin and increase its biological activity.
MATERIALS AND METHODS

Purification of the protease from *V. cholerae*

Protease was purified from *V. cholerae* 01, serotype Ogawa, biotype E1 Tor (K23) isolated in Kenya in 1983. Bacteria were cultured in 1 liter of heart infusion broth with shaking at 37°C for 36 h. Culture supernatants were mixed with 1/20 volume of 0.5 M phosphate buffer (pH 7.0) (PB) and 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 h, and the supernatant was applied to a Bio-gel A 5m column equilibrated with the same buffer. Fractions with protease activity were concentrated to about 2 ml on a membrane (PM-10; Amicon Corp., Lexington, Mass.) and applied to a TSK gel G-3000 SW HPLC column equilibrated with 25 mM PB (pH 7.0) containing 0.2 M sodium chloride. Fractions with protease activity, which showed a single peak with HPLC, were used as purified protease. Protease was assayed by a single agar-diffusion technique in agar gel containing 1.5% skim milk as the substrate, as previously described (5). In this paper this protease is named protease VC-I.

Purification of LT

LT was purified as described by Clements and Finkelstein (2). Detailed procedures of culture, isolation and purification of LT by successive column chromatographies on Bio-gel A 5m and Sephadex G-75 were performed as described previously (18).

SDS-polyacrylamide gel electrophoresis

SDS-PAGE in 0.1% SDS was carried out as described previously (14) in 15% polyacrylamide gel. Gels were stained with Coomassie brilliant blue then destained as described previously (12).

Digestion of LT by protease VC-I and by trypsin

The LT sample in 50 mM Tris-HCl containing 0.14M NaCl was mixed with purified protease VC-I and incubated at 37°C for various times. After digestion, the samples were analyzed with SDS-PAGE. LT was digested by trypsin as described previously (15).

Rabbit skin permeability test

The PF test was performed as described previously (3). The samples were diluted with borate-buffer (3) and 0.1 ml of each sample was injected subcutaneously.

Sequence determination

LT was digested with protease VC-I or trypsin. Then samples containing A1 and A2 fragments and the B subunit were analyzed by an automatic protein sequencer (model 477A/120A; Applied Biosystems, Foster City, Calif.). Each amino acid detected by this analyzer was compared with sequences already reported (4, 13, 19, 20), and the sequence of the 3 N-terminal amino residues were considered.

RESULTS

SDS-polyacrylamide gel electrophoresis of purified protease

The protease was purified as described in Materials and Methods. From 1 liter cultured medium we obtained 0.1 mg of protein. As shown in figure 1, the purified protease showed two bands. However, the protein contained in the upper band was modified and

![Figure 1. - SDS-polyacrylamide gel electrophoresis of purified protease](image-url)