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The Enterotoxin of *Bacteroides fragilis* Is a Metalloprotease

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During the past decade, strains of *Bacteroides fragilis* that produce an enterotoxin have been implicated in diarrheal disease in animals and humans. The extracellular enterotoxin has been purified and characterized as a single polypeptide (M_r, ~20,000). Single specific primer-PCR was used to clone a portion of the *B. fragilis* enterotoxin gene. The recombinant protein expressed by the cloned gene fragment reacted with monospecific antibodies to *B. fragilis* enterotoxin by enzyme-linked immunosorbent assay and immunoblot analysis. The deduced amino acid sequence revealed a signature zinc-binding consensus motif (HEXXHXXGXXH/Met-turn) characteristic of metalloproteases termed metzincins. Sequence comparisons showed close identity to matrix metalloproteases (e.g., human fibroblast collagenase) within the zinc-binding and Met-turn region. Purified enterotoxin contained 1 g-atom of Zn^{2+} per molecule and hydrolyzed gelatin, azocoll, actin, tropomyosin, and fibrinogen. The enterotoxin also underwent autodigestion. The N-terminal amino acid sequences of two autodigestion products were identical to the deduced amino acid sequence of the recombinant enterotoxin and revealed cleavage at Cys-Leu and Ser-Leu peptide bonds. Gelatinase (type IV collagenase) activity comigrated with the toxin when analyzed by gel fractionation and zymography, indicating that protease activity is due to the enterotoxin and not to a contaminating protease(s). Optimal proteolytic activity occurred at 37°C and pH 6.5. Primary proteolytic cleavage sites in actin were identified, revealing cleavage at Gly-Met and Thr-Leu peptide bonds. Enzymatic activity was inhibited by metal chelators but not by inhibitors of other classes of proteases. Additionally, cytotoxic activity of the enterotoxin on human carcinoma HT-29 cells was inhibited by acetoxymethyl ester EDTA. The metalloprotease activity of the enterotoxin suggests a possible mechanism for enterotoxicity and may have additional implications in the study of disease caused by *B. fragilis*.

Bacteroides fragilis, which makes up about 1 to 2% of the normal human colonic microflora (21), is the anaerobic bacterial species most commonly isolated from human clinical specimens and causes abscesses, soft-tissue infections, and bacteremias (37). Capsular polysaccharides have been implicated as virulence factors in the formation of abscesses by *B. fragilis* (4, 38). Little else is known about the pathogenic mechanisms of *B. fragilis* infections, although others have suggested a role for proteases produced by the organism (12). In 1984, Myers et al. (24) reported the presence of enterotoxicity associated with some strains of *B. fragilis* assayed in ligated lamb ileal loops. Since these initial reports, enterotoxigenic *B. fragilis* organisms were implicated as the cause of diarrhea in calves, piglets, foals, and infant rabbits (3, 8, 25–29). Enterotoxigenic strains of *B. fragilis* were first associated with diarrhea in humans in 1987 (30). In this study, enterotoxigenic strains were isolated from 12% of patients with diarrhea of unknown etiology compared with 6% of controls. More recently, larger controlled studies have implicated enterotoxigenic *B. fragilis* as an etiologic agent of acute diarrhea in children older than 1 year (32, 33). Furthermore, enterotoxigenic strains may be carried as a part of the normal colonic flora, since 22 of 237 (9%) isolates from a municipal sewage plant produced enterotoxin in culture (36). Other investigators recently found that a high percentage (52%) of *B. fragilis* strains isolated from human blood culture were enterotoxigenic (17).

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The enterotoxin was recently purified to homogeneity and characterized by Van Tassell and coworkers (40). It is released into culture supernatants as a protein with a molecular weight of approximately 20,000. It is cytotoxic for certain human intestinal carcinoma cell lines, particularly HT-29 (43), and causes fluid accumulation in ligated lamb ileal loops. Amino acid analysis of the N terminus revealed that it begins with an alanine, indicating that the enterotoxin is processed before release into culture supernatants.

We report here the cloning of a portion of the *B. fragilis* enterotoxin gene encoding the native (extracellular) enterotoxin by the technique of single specific primer (SSP)-PCR (34), employing a degenerate oligonucleotide primer based on the N-terminal sequence of the enterotoxin. Analysis of the deduced amino acid sequence of the cloned gene fragment revealed a zinc-binding consensus motif characteristic of metalloproteases. In this report, we present the sequence and characterization data that confirm that the *B. fragilis* enterotoxin is a zinc metalloprotease.

MATERIALS AND METHODS

Chemicals and reagents. Tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) was obtained from Boehringer Mannheim (Indianapolis, Ind.). Nitrocellulose transfer membranes BA-S85 were obtained from Schleicher & Schuell (Keene, N.H.). Immobilon polyvinylidene difluoride membranes were obtained from Millipore (Bedford, Mass.). OPTI-Mist horseradish peroxidase substrate was obtained from Transgenic Sciences Incorporated (Boston, Mass.). Immulon 96-well plates, type 2, were obtained from Dynatech Labs, Inc. (Alexandria, Va.). Oligonucleotide primers used in the PCR were purchased from National Biosciences Inc. (Plymouth, Minn.). High-fidelity Ultma polymerase was obtained from Perkin-Elmer (Branchburg, N.J.). Restriction endonucleases were purchased from New England BioLabs (Beverly, Mass.) and Bethesda Research Laboratories (Gaithersburg, Md.). T4 DNA ligase was purchased from Promega (Madison, Wis.). Shrimp alkaline phosphatase was purchased from United States

Biochemicals (Cleveland, Ohio). Zincov was purchased from Calbiochem (La Jolla, Calif.). Acetoxymethyl ester EDTA was purchased from Molecular Probes (Eugene, Ore.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Highly purified *B. fragilis* enterotoxin was prepared from culture filtrate of VPI strain 13784 as previously described (39).

SSP-PCR. High-fidelity Ultma polymerase, restriction enzymes, and shrimp alkaline phosphatase were used according to the manufacturers' protocol. Genomic DNA (2 μg) isolated from *B. fragilis* VPI 13784 as described previously (15, 23) was digested with restriction endonucleases for 4 h. Approximately 250 ng of digested genomic DNA and an equal amount of pUC19 digested with the same endonuclease and dephosphorylated with shrimp alkaline phosphatase were ligated overnight at 15°C with T4 DNA ligase. PCRs were performed with 50 ng of DNA from the ligation reaction, a degenerate SSP (designated 53B) based on the N-terminal amino acid sequence of the *B. fragilis* enterotoxin, and pUC reverse primer (located at −40). The degenerate primer was designed to contain a *Bam*HI restriction site at the 5′ end to facilitate cloning of the PCR product.

Cloning and sequencing of the SSP-PCR product. The PCR product was digested with appropriate restriction endonucleases (*Bam*HI and *Pst*I) and electrophoresed through a 1% agarose gel. The digested PCR product was then excised from the gel and purified with the Sephaglas Band Prep Kit (Pharmacia, Piscataway, N.J.). Isolated fragments were cloned into pUC19 and transformed into competent *Escherichia coli* DH5α (GIBCO BRL, Gaithersburg, Md.). Plasmids were isolated by the alkaline lysis method (19) and sequenced in accordance with the Sequenase version 2.0 kit (United States Biochemicals).

Protein homology database search. The PIR-protein data library was searched using the Sequence Analysis Software Package developed by the Genetics Computer Group at the University of Wisconsin.

Expression and purification of recombinant protein. The cloned PCR product was subcloned into the prokaryotic gene fusion vector pGEX-4T-1 (Pharmacia/LKB). The recombinant plasmid p4T-BF107 contains the gene fragment from the SSP-PCR fused in frame with the glutathione *S*-transferase (GST) gene of pGEX-4T-1. *E. coli* DH5α harboring p4T-BF107 was grown in 1 liter of TB medium (19) with 0.1 mg of ampicillin per ml to an optical density at 600 nm of 0.6. Recombinant protein expression was induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 3 h, cells were pelleted by centrifugation and suspended in 20 ml of phosphate-buffered saline (PBS), pH 7.4. Cells were then disrupted by sonication, and cellular debris was removed by centrifugation. Lysates were filtered through 0.45-μm-pore-size membranes. The recombinant protein was purified by affinity chromatography with glutathione-Sepharose 4B as described by the manufacturer (Pharmacia/LKB).

Protein assays and metal analysis. Protein concentrations were determined with the Coomassie Protein Assay kit (Pierce Chemical Co., Rockford, Ill.) as recommended by the manufacturer. Bovine serum albumin was used to generate the standard curves for determining protein concentrations. Purified enterotoxin was analyzed for the presence of zinc by atomic absorption spectroscopy, using inductive coupled plasma 61 software.

ELISA. The enterotoxin-specific enzyme-linked immunosorbent assay (ELISA) was performed in Immulon type 2 plates (96 wells), using monospecific rabbit and goat anti-enterotoxin and rabbit anti-goat immunoglobulin G (IgG) conjugate to alkaline phosphatase as previously described (42). Reactions were read at 405 nm on an automated ELISA plate reader (model E111; Dynatech). ELISA results are expressed as ELISA absorbances or as ELISA titers, i.e., the highest dilutions that yield absorbances greater than twice background readings of 0.2 to 0.3.

PAGE. Protein samples were separated by polyacrylamide gel electrophoresis (PAGE), using the discontinuous system of Laemmli (18). Electrophoresis was performed at 10°C in 0.025 M Tris–0.192 M glycine buffer, pH 8.3 (running buffer). Samples were boiled for 5 min in loading buffer (0.05 M Tris, 0.01 M dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.19 M bromophenol blue, 10% glycerol, pH 6.8) for SDS-PAGE analysis, and SDS was added to a concentration of 0.1% in the gel and running buffer. Gels were fixed and stained with Coomassie blue, followed by destaining, or proteins were transferred to nitrocellulose for immunoblotting.

Gelatinase zymogram analysis was performed by electrophoresing enterotoxin in PAGE gels containing 1% gelatin (10). Native gels, run in tandem with the zymogram gels, were used to determine the position of the enterotoxin by Coomassie blue staining. After electrophoresis, zymogram gels were incubated in PBS for 18 h at 37°C, stained for 30 min with Coomassie blue, and destained for 1 to 2 h. Gelatinase activity was indicated by a clear zone of hydrolysis in a background of stained gelatin.

Immunoblot analysis. Protein samples were separated by SDS-PAGE as described above. Proteins were transferred to nitrocellulose membranes by electrophoresis at 15 V and 10°C for 15 h. Membranes were blocked with 3% gelatin in 1× Tris-buffered saline (TBS), pH 7.5, for 2 h. The membrane was incubated with a 1:2,500 dilution of primary antibody (monospecific goat anti-enterotoxin IgG in TBS containing 1% gelatin) and then washed three times for 10 min (each) with TBS. The membrane was incubated for 1 h with a 1:7,500 dilution of secondary antibody (rabbit anti-goat IgG horseradish peroxidase conjugate in TBS with 1% gelatin) and washed six times for 10 min (each) in TBS. Membranes were developed with Opti-Mist horseradish peroxidase reagent (TSI). Purification of monospecific goat anti-enterotoxin IgG was described previously (41).

Autodigestion. Autodigestion of purified enterotoxin was performed as described previously (42). For N-terminal amino acid sequencing studies, toxin (500 μg/ml) was incubated in PBS at 37°C for 48 h. For inhibition studies, toxin (100 μg/ml) was incubated with inhibitors at 37°C and samples were taken at 8, 24, and 72 h. Samples were frozen at −20°C until analyzed for autodigestion by SDS-PAGE.

N-terminal amino acid sequence analysis. Autodigestion products were separated by high-performance liquid chromatography and N-terminal sequenced at the Biomolecular Research Facility at the University of Virginia, using an Applied Biosystems 470A sequenator with a 120A PTH analyzer (Foster City, Calif.).

Actin cleavage products were sequenced at the Virginia Polytechnic Institute and State University Protein Sequencing Facility with a model 477A protein sequenator (Applied Biosystems). Actin hydrolysates were analyzed by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes in 3-[cyclohexylamino]-1-propanesulfonic acid (10 mM; pH 11) for 20 min at 0.5 A. Membranes were stained with 0.1% Coomassie blue in 50% methanol for 5 min and air dried, and the bands were cut out for N-terminal sequencing.

Protease assays. Enterotoxin was added to tubes containing 1 ml of 10% gelatin in PBS, pH 6.8, and the mixtures were incubated for 1 h at 37°C. The stoppered tubes were then chilled on ice, inverted, and incubated at 25°C. Gelatinase activity was determined by observing the presence or absence of liquification as the tubes warmed.

Proteolysis of azo-substrates was determined by incubating enterotoxin with azocoll, azocasein, and azoalbumin (5 mg/ml in sterile PBS). After enterotoxin (0.5 μg) was incubated with 1 ml of azo-substrate at 37°C for up to 24 h, the mixture was precipitated with 1 ml of 5% trichloroacetic acid and microcentrifuged (12,000 × *g*) for 5 min, and the supernatant was intensified by the addition of 1 ml of NaOH (0.5 M). Enzyme activities were determined by measuring dye release spectrophotometrically at 440 nm. Collagenase IV from *Clostridium histolyticum* was used as a positive control.

Other substrates, including actin, tropomyosin, fibronectin, fibinogen, human IgA, laminin, myosin, filamin, and bovine serum albumin, were tested for hydrolysis by the enterotoxin. Proteins were incubated with 0.5 μg of toxin per ml in PBS (pH 6.5) at 37°C for up to 24 h and frozen at −20°C until analyzed for hydrolysis. Hydrolysis was determined by SDS-PAGE as described above. Collagen I, collagen V, and synthetic peptides were tested for hydrolysis as described by Worthington (44). Elastin was tested for hydrolysis with elastin-congo red as a substrate. Assays were performed as described above for the azo-substrates, and dye release was measured spectrophotometrically at 495 nm.

Temperature and pH optima. The effects of pH on proteolytic activity of the toxin were determined by using the azocoll and actin digestion assays. Substrates were mixed with sodium acetate buffer (200 M; pH 4.0, 4.5, 5.0, and 5.5), sodium phosphate buffer (200 mM; pH 6.0 and 6.5), Tris-HCl (200 mM; pH 7.0, 7.5, 8.0, and 8.5), and 3-[cyclohexylamino]-1-propanesulfonic acid (200 mM; pH 9.0, 9.5, and 10). After incubation of the enterotoxin with azocoll (6 h) and actin (2 h), samples were analyzed spectrophotometrically and by SDS-PAGE, respectively.

The optimal temperature for proteolysis was determined by using the azocoll and actin digestion assays at 6 and 2 h, respectively. All proteolytic assays were performed at pH 6.5 and 37°C unless otherwise noted.

Inhibition studies. Inhibition of proteolysis of gelatin, azocoll, and actin was determined by incubating enterotoxin with each of the substrates (as described above) in the presence and absence of a variety of classes of protease inhibitors. Inhibitors were preincubated with toxin (0.5 μg/ml) for 1 h at 37°C (see Table 2). Inhibition of gelatin hydrolysis was determined by the "tube gel" method (2 h, 37°C) and zymography. Percent inhibition of dye release from azo-substrates was determined after 6 h; released dye was measured at 440 nm. Inhibition of hydrolysis of actin was determined after 2 h by SDS-PAGE. Relative hydrolysis of actin was ranked as follows (see Fig. 5 for correlation of ranking with the degree of hydrolysis): ++++, complete hydrolysis (Fig. 5, 6 h); +++, strong hydrolysis (Fig. 5, 2 h); ++, moderate hydrolysis (Fig. 5, 1 h); +, weak hydrolysis (Fig. 5, 15 min).

Inhibition of autodigestion was determined by incubating the enterotoxin in PBS, pH 6.5, at 37°C in the presence of inhibitors for 48 h. Samples were analyzed by SDS-PAGE and SDS-capillary electrophoresis. SDS-capillary electrophoresis was performed on a Beckman PACE 2200 system, using the eCAP SDS-200 kit according to the standard procedures of the manufacturer (Beckman Instruments, Fullerton, Calif.). Samples were injected by using high pressure (30 s) and run at 14.4 kV for 25 min. Peaks were monitored by UV absorption at 214 nm (0.02 absorbance units full scale [AUSF]).

PAGE gel fractionation. Purified enterotoxin (1.25 μg) was run on a native PAGE gel in duplicate. One-half of the gel was stained with Coomassie blue to determine the position of the enterotoxin. The other half of the gel was rinsed in deionized water and cut into 1-mm strips, and each gel strip was placed in a glass tube (13 by 100 mm). PBS (0.5 ml) was added to each of the gel slices, which were then crushed with a glass rod; the proteins were allowed to diffuse into the buffer for 4 h at 4°C, and the slices were frozen at −20°C. Samples were thawed and microcentrifuged (12,000 × *g*, 5 min) to remove gel debris. Samples were tested for proteolytic activity on gelatin, azocoll, and actin as described above.

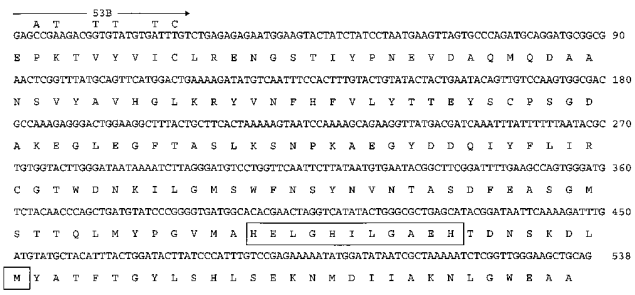

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the cloned SSP-PCR product rBF107. The position of the SSP used is indicated by the arrow. Nucleotides above the sequence are at positions of degeneracy in the primer. The zinc-binding signature motif and the methionine residue of the Met-turn are boxed.

Cytotoxicity inhibition assays. Cytotoxicity assays were performed on HT-29 cells (ATCC HTB 38), grown and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum, as described previously (40). Cytotoxic titers were expressed as the reciprocal of the highest dilution of toxin that caused greater than 50% cell rounding. To measure inhibition of cytotoxic activity by metal chelators, 0.5 μg of enterotoxin was treated with 10 mM acetoxymethyl ester EDTA for 30 min at room temperature. Equal amounts of enterotoxin were treated in the same manner without the chelator. Inhibition by acetoxymethyl ester EDTA was determined by comparing cytotoxic titers of treated and untreated enterotoxin.

RESULTS

Cloning and sequencing of an SSP-PCR product encoding a portion of the *B. fragilis* enterotoxin. The N-terminal amino acid sequence of *B. fragilis* enterotoxin was previously determined to be ALPSEPKTVYVICLRENGST. We developed a degenerate primer, designated 53B (Fig. 1), based on a portion of the N-terminal amino acid sequence for use in SSP-PCR. Ligation reactions of restriction endonuclease-digested pUC19 and genomic DNA from *B. fragilis* VPI 13784 were amplified by SSP-PCR. Ligation reactions were performed with DNA digested by a number of different restriction enzymes; however, only the *Pst*I-digested DNA generated a recombinant enterotoxin PCR product (designated rBF107). When cloned into pUC19 and sequenced, the rBF107 gene fragment was determined to be 538 nucleotides in length and encoded an open reading frame that was in frame with the deduced amino acid sequence of the 53B primer (Fig. 1).

Analysis of the deduced amino acid sequence. Protein homology searches revealed that rBF107 contained sequence identity to a number of metalloproteases, including *Pseudomonas aeruginosa* alkaline protease, *Serratia marcescens* protease, snake venom adamalysins and ruberlysin, and matrix metalloproteases. Furthermore, the sequence of rBFET contained the sequence HELGHILGAEH, which is consistent with the signature consensus sequence of the zinc-binding motif HEXX HXXGXXH found in the metalloprotease family termed metzincins (1). An additional feature found in the metzincins, a Met-turn region located downstream of the zinc-binding motif, is also present in the rBFET sequence. The zinc-binding and Met-turn region of rBF107 showed the closest identity to the matrix metalloprotease human fibroblast collagenase (1).

Immunoreactivity of recombinant protein with antibodies to the *B. fragilis* enterotoxin. The rBF107 gene fragment was subcloned to pGEX-4T-1 and expressed and purified as a GST fusion protein. The purified recombinant fusion protein, designated GST/BF107, was analyzed for reaction with monospecific goat anti-enterotoxin by ELISA and immunoblot analysis. The recombinant protein reacted strongly by immunoblot analysis (Fig. 2). GST alone showed no immunoreactivity with the specific enterotoxin antibody. When tested in the enterotoxin-specific ELISA, the GST/BF107 fusion protein had a titer of 10^2 to 10^3. This was similar to the ELISA response observed with an equal amount of purified enterotoxin (10^3). GST included as a negative control showed no ELISA reactivity. The purified recombinant protein did not have proteolytic or cytotoxic activity.

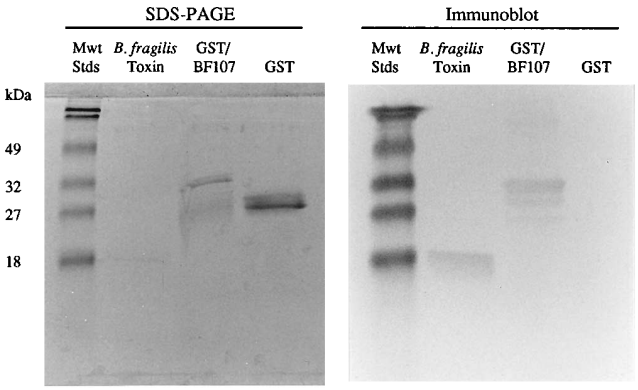

FIG. 2. SDS-PAGE and immunoblot of GST/BF107 fusion protein. GST was used as a control.

Characterization of autodigestion of the enterotoxin. We reported previously that incubation of purified enterotoxin at elevated temperature results in a breakdown of the enterotoxin into defined smaller peptides (42). Incubation of the enterotoxin with the zinc-specific metal chelators 1,10-phenanthroline and Zincov completely inhibited this breakdown, when analyzed by SDS-PAGE and SDS-capillary electrophoresis (data not shown). Two peptides generated by autodigestion were isolated by high-performance liquid chromatography and gave an unambiguous N-terminal sequence that was identical to the rBF107 deduced amino acid sequence. Table 1 lists these amino acid sequences and shows the predicted cleavage sites of autodigestion according to the deduced amino acid sequence of rBF107. The peptide bonds Cys-Leu and Ser-Leu were cleaved by autodigestion of the enterotoxin.

Zinc analysis. On the basis of a molecular weight of 20,000, the *B. fragilis* enterotoxin contained 1 g-atom of zinc per molecule when analyzed by atomic absorption spectroscopy.

Protease studies. We initially detected proteolytic activity of the *B. fragilis* enterotoxin in tube gelatinase assays. Tubes of gelatin incubated with toxin remained liquid after cooling and warming to 25°C. Gelatinase activity is considered to be equivalent to type IV collagenase activity (9). A time course of hydrolysis of azocoll by the *B. fragilis* enterotoxin is shown in Fig. 3, along with hydrolysis by *C. histolyticum* type IV collagenase included as a positive control.

TABLE 1. Cleavage sites of autodigestion and hydrolysis of actin by the *B. fragilis* enterotoxin

Substrate	Cleavage site[a]
B. fragilis enterotoxin	C ↓ LRENG
	S ↓ LKSNPKAEG
Actin	T ↓ LKYP
	G ↓ MGNK

[a] Cleavage sites deduced from the N-terminal sequence of the digestion products are indicated by arrows.

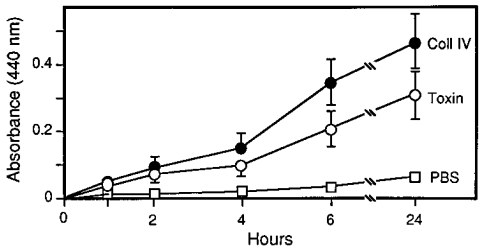

FIG. 3. Proteolysis of azocoll by the *B. fragilis* enterotoxin and collagenase type IV. Enzymes (0.5 μg/ml) were incubated with azocoll in PBS (pH 6.5) at 37°C. ○, *B. fragilis* toxin; ●, collagenase IV (coll IV); □, PBS.

To ensure that the observed proteolytic activity of the enterotoxin preparations was due to the enterotoxin and not a contaminating protease(s), we performed PAGE on enterotoxin preparations and tested gel slices for gelatinase activity. Gelatinase activity was detected only in gel fractions containing the enterotoxin (data not shown). To further ensure that the enzymatic activity was due solely to the enterotoxin, we performed gelatinase zymograms. As shown in Fig. 4, gelatinase activity comigrated with the enterotoxin, and activity was not observed anywhere else in the zymogram.

A number of other proteins, including actin, tropomyosin, fibronectin, fibrinogen, human IgA, laminin, and bovine serum albumin, were tested for hydrolysis. In addition to gelatin and azocoll (described above), the enterotoxin cleaved actin (Fig. 5). The cleavage products of actin hydrolysis were isolated and subjected to N-terminal sequencing. The cleavage sites predicted from the known amino acid sequence of rabbit skeletal muscle actin (39) are shown in Table 1. Initial cleavage occurred at the peptide bond Gly-Met, followed by cleavage at Thr-Leu. Enterotoxin also hydrolyzed tropomyosin and fibrinogen, although to a lesser degree than observed with actin (data not shown). None of the other proteins were hydrolyzed by the enterotoxin.

The optimum conditions for hydrolysis were determined by incubation of the enterotoxin with azocoll and actin at various temperatures and pH. The optimal temperature for hydrolysis

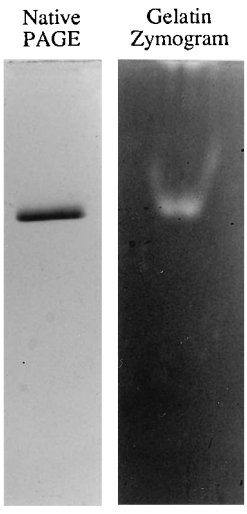

FIG. 4. Gelatin zymography of the *B. fragilis* enterotoxin. (Left) Native PAGE (12%) of purified toxin. (Right) Native PAGE gel (containing 1% gelatin) incubated for 18 h at 37°C in PBS prior to staining with Coomassie blue.

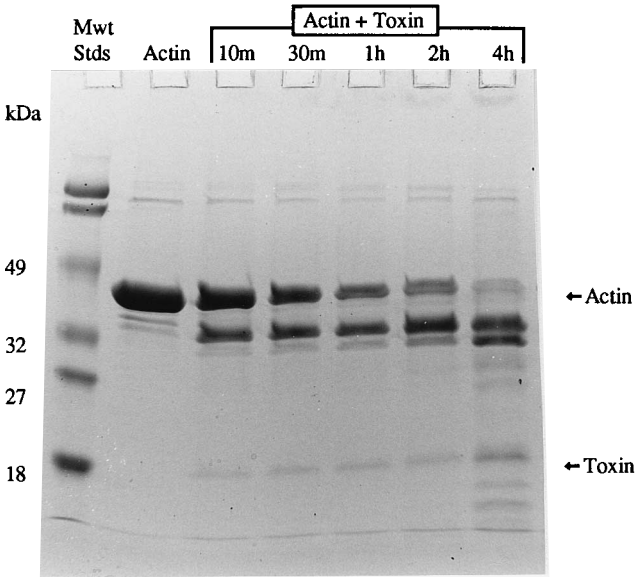

FIG. 5. Time course of hydrolysis of actin by the *B. fragilis* enterotoxin. Actin (100 μg/ml) was incubated with toxin (0.5 μg/ml) for 1 h at 37°C and analyzed by SDS-PAGE (12%).

of azocol and actin was ~37°C (Fig. 6a and b), and the optimal pH was ~6.5 (Fig. 6c and d).

Inhibition of proteolysis. The results of the inhibitor studies are summarized in Table 2. The specific zinc chelators 1,10-phenanthroline and Zincov, and other metal chelators (EDTA and 2,2′-dipyridyl), strongly inhibited enzymatic activity on all substrates tested, including enterotoxin autodigestion. The reducing agents thimerosal and dithiothreitol also inhibited proteolysis and autodigestion. α-Macroglobulin and diethylpyrocarbonate, known to inhibit matrix metalloproteases, were moderately inhibitory. Hydrolysis also was inhibited slightly by excess (10 mM) zinc. The serine protease inhibitors phenylmethylsulfonyl fluoride, 4-(2-aminoethyl)-benzenesulfonyl-fluoride, tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), and tosyl-lysine chloromethyl ketone (TLCK) did not inhibit proteolysis.

Inhibition of cytotoxicity. Cytotoxicity assays were performed with enterotoxin pretreated with 10 mM acetoxymethyl ester EDTA. The cytotoxic activity of enterotoxin decreased 1,000-fold (99.9% inhibition) when chelated with acetoxymethyl ester EDTA compared with enterotoxin treated in an identical manner without chelator.

DISCUSSION

Using SSP-PCR (35), we generated a PCR product (rBF107) that when cloned and sequenced contained an open reading frame in frame with the N-terminal amino acid sequence of the *B. fragilis* enterotoxin. The gene fragment was subcloned to an expression vector, and the expressed recombinant protein reacted with monospecific anti-enterotoxin by ELISA and immunoblot analysis. Analysis of the deduced amino acid sequence of the PCR product revealed sequence identity with a number of metalloproteases. The sequence similarities to all of these proteases was in the region of the enzymes containing the zinc-binding consensus motif HEXX HXXGXXH. In addition, the enterotoxin sequence included a Met-turn region similar to that of these and other metalloproteases that have recently been termed metzincins (1). This

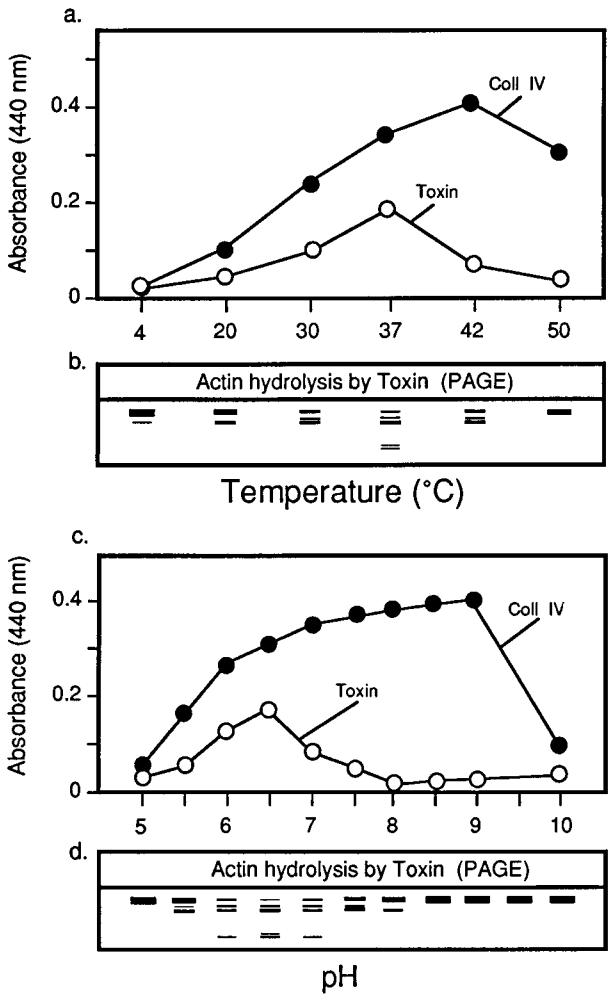

FIG. 6. Temperature and pH optima for proteolysis by the *B. fragilis* enterotoxin. (Graphs) Purified toxin (○) and collagenase IV (Coll IV; ●) were incubated with azocoll at (a) various temperatures for 6 h and (c) various pHs for 6 h at 37°C; dye release was measured spectrophotometrically (A_{440}). (Diagrams) Purified toxin was incubated with actin at (b) various temperatures for 6 h and (d) various pHs for 2 h at 37°C; hydrolysis was measured by SDS-PAGE analysis (see Fig. 5).

similarity and the presence of a threonine residue immediately following the zinc-binding motif suggest that the *B. fragilis* enterotoxin is most closely related to the subfamily of metzincins termed matrixins (matrix metalloproteases), which share the feature of a functionally equivalent serine at this position.

We recently reported that purified enterotoxin undergoes spontaneous breakdown upon incubation at 37°C (42). Upon discovering the sequence similarity shared by the enterotoxin and metalloproteases, we suspected that the breakdown occurred because of autodigestion. Incubation of purified enterotoxin with metal chelators known to specifically inhibit metalloproteases prevented the breakdown of the enterotoxin, suggesting to us that our previous observations were indeed due to the metalloprotease activity of the enterotoxin on itself. The N-terminal sequences of autodigestion products had 100% identity with the deduced amino acid sequence of the cloned SSP-PCR product, further evidence that the PCR product corresponds to the enterotoxin gene.

The sequence similarity of the *B. fragilis* enterotoxin and matrix metalloproteases suggested to us that the enterotoxin shares functional homology with these enzymes. Therefore, we wished to determine whether the enterotoxin has proteolytic activity. Purified enterotoxin tested in the tube gelatinase assay showed strong gelatinase (type IV collagenase) activity. Gelatinase activity in purified enterotoxin preparations comigrated with the enterotoxin band by gel fractionation and zymogram analysis. Therefore, the enzymatic activity was not due to a contaminating protease(s). In addition to gelatin and collagen, the enterotoxin hydrolyzed actin, tropomyosin, and fibrinogen. Cleavage sites identified in this study occurred between an uncharged polar amino acid (Cys, Ser, Gly, or Thr) and a hydrophobic amino acid (Leu or Met). In the case of actin cleavage, hydrolysis occurred at a faster rate at the Gly-Met site located near the N terminus of actin. Interestingly, this is the same region of actin that is readily cleaved by a number of other proteases, including the metalloprotease thermolysin (22).

The zinc-specific chelators 1,10-phenanthroline and zincov, as well as EDTA, strongly inhibited proteolysis of gelatin, actin, and enterotoxin (autodigestion). Addition of zinc to EDTA-chelated enterotoxin restored approximately 50% of the proteolytic activity on actin (data not shown). Proteolysis was moderately inhibited by excess zinc, as has been observed with other metalloproteases, presumably due to the formation of zinc complexes that reduce the accessibility of free zinc molecules. We observed moderate inhibition of actin hydrolysis by α-macroglobulin and diethylpyrocarbonate, both reported to inhibit other metalloproteases. Histidine residues are specifically blocked by diethylpyrocarbonate, thus interfering with zinc binding via the histidine residues of the zinc-binding motif. Taken together, these results confirm that the *B. fragilis* enterotoxin is a zinc-dependent metalloprotease.

Cytotoxic activity on HT-29 cells was inhibited by pretreatment of the enterotoxin with the metal chelator acetoxymethyl ester EDTA. These results support the idea that cytotoxic activity is due to proteolytic activity of the enterotoxin. The fact that it cleaves actin, tropomyosin, fibrinogen, and collagen, however, does not mean that cytotoxicity or enterotoxicity is due to cleavage of these substrates. Further studies to determine if the mechanism of action is due to cleavage of these or other proteins are ongoing.

Metalloproteases have emerged as important virulence factors in a number of diverse pathogenic organisms including bacteria and fungi (14). Metalloproteases participate in a wide variety of virulence functions, including adhesion, tissue invasion, activation of protoxins and other proenzymes, immunoavoidance mechanisms, and cytotoxicity. For example, *P. aeruginosa* and *Aspergillus fumigatus* produce elastases that contribute to their invasiveness and ability to establish infection in the lungs (11, 20). The hemagglutinating metalloprotease of *Vibrio cholerae* nicks and activates cholera toxin (2), while *Staphylococcus aureus* produces a metalloprotease required for activation of its V8 protease (7). *Streptococcus sanguis* and *Streptococcus pneumoniae* produce extracellular metalloproteases that hydrolyze IgA and also promote adherence of the organisms (13, 31). Recently, it was discovered that the neurotoxins produced by *Clostridium botulinum* and *Clostridium tetani* are zinc-dependent metalloproteases and cleave actin, although actin cleavage is not the mechanism of action (6, 35). Like *B. fragilis* enterotoxin, these toxins undergo autodigestion.

The ubiquity of metalloproteases in nature, including many endogenously produced by the colonic microflora in the human intestines, makes the discovery that the *B. fragilis* enterotoxin is a metalloprotease particularly intriguing, since the intestinal mucosa is continually exposed to metalloproteases.

TABLE 2. Inhibition of proteolysis by the *B. fragilis* enterotoxin

Inhibitor(s)[a]	Concn (mM)	Inhibition of:			
		Gelatin hydrolysis[b]	Azocol hydrolysis (%)[c]	Actin hydrolysis[d]	Autodigestion[e]
EDTA	1	+++[f]	84 ± 6	++++	+++
OPA	1	+++	92 ± 3	++++	++++
Zincov	1 mg/ml	+++	90 ± 5	++++	++++
Thimerosal	1% (wt/vol)	ND[g]	ND	+++	+++
$ZnCl_2$	10	−	60 ± 7	+++	+
DTT	50	+++	ND	+++	++++
2,2-Dipyridyl	1	ND	ND	+++	+
α-Macroglobulin	10 mg/ml	ND	ND	++	++
DEPC	2	ND	ND	++	ND
PMSF, AEBMF	10	−	0	−	−
TPCK, TLCK	10	−	0	−	−

[a] OPA, 1,10-phenanthroline; DTT, dithiothreitol; DEPC, diethyl-pyrocarbonate; PMSF, phenylmethylsulfonyl fluoride; AEBMF, 4-(2-aminoethyl)-benzenesulfonyl fluoride.
[b] Inhibition of gelatin hydrolysis was determined by the tube method after 2 h at 37°C.
[c] Inhibition of dye release from azocoll after 6 h at 37°C was determined at 440 nm (means of duplicate samples in two experiments ± standard deviations).
[d] Inhibition of hydrolysis of actin after 2 h at 37°C was determined by SDS-PAGE.
[e] Inhibition of autodigestion after 48 h at 37°C was determined by SDS-PAGE and capillary electrophoresis.
[f] Relative inhibition: ++++, complete; +++, strong; ++, moderate; +, weak; −, no inhibition.
[g] ND, not determined.

Researchers have shown fluid responses in ligated loop models with the metalloproteases from *P. aeruginosa* and *Vibrio mimicus* (5, 16). The finding that the enterotoxin is a metalloprotease also strengthens the possibility that the enterotoxin is involved in tissue destruction and invasiveness associated with *B. fragilis* soft-tissue infections. We now have DNA probes and an ELISA with which to analyze clinical samples. These recent advances make it possible for us to evaluate the involvement of the enterotoxin in the pathogenesis of *B. fragilis* infections. Further studies to determine if enterotoxicity associated with *B. fragilis* is attributable to proteolysis by the enterotoxin are ongoing.

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