Characterization and Inactivation of Verotoxin 1 Produced by *Escherichia coli* O157:H7

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Verotoxin 1 (VT-1) produced by *Escherichia coli* O157:H7 was purified to homogeneity by standard liquid chromatography and FPLC with a yield of 268 μg from 70 g of cells. Subunits A, A_1, A_2, and B were separated by SDS–PAGE with a 5–30% gradient and then transferred to PVDF membrane, stained with Coomassie Brilliant Blue R-250, and partially sequenced. The molecular weights of subunits A, A_1, A_2, and B were 32 000, 28 000, 3700, and 4700, respectively. N-Terminal amino acid sequencing of A, A_1, and A_2 revealed that the A and A_1 subunits have the same N-amino acid terminus and that the A_2 subunit was formed by nicking prior to amino acid 252. Subunit B was sequenced through 50 amino acids. Pretreatment of VT-1 with Ca^{2+} and EDTA increased verotoxicity, whereas Fe^{3+} and various divalent cations and lipids had no measurable effect on toxicity. VT-1 toxicity was stable for up to 60 min at 70 °C, but verotoxicity was reduced to 90% at 80 °C for 15 min and to 99% at 80 °C for 30 min. Verotoxicity was completely neutralized at 80 °C for 60 min and at 85 °C for times greater than 5 min. Treatment at pH 4.5 for 16 h reduced activity by 90%. Although the biological activity of ingested VT-1 is unknown, the thermal and pH tolerance of the toxin may have important practical implications for the food industry.

INTRODUCTION

Escherichia coli O157:H7, a food-borne pathogen responsible for recent outbreaks of hemorrhagic colitis (Riley et al., 1983), produces one or more verotoxins. These toxins are believed to be virulence factors involved in the organism's pathogenesis. Studies in mice have revealed that verotoxin injected intraperitoneally was lymphocytotoxic to spleen cells and caused necrosis of colonic epithelial cells, paralysis of lower extremities, and death (Padhye et al., 1987). Verotoxin 1 (VT-1), which is structurally and immunologically similar to Shiga toxin, is principally an intracellular toxin produced by many strains of *E. coli* O157:H7. Because of its similarity to Shiga toxin, it is often referred to as Shiga-like toxin I.

Purification of VT-1 from *E. coli* O157:H7 has been previously reported by others (Noda et al., 1987; Kongmuang et al., 1987). Additionally, VT-1 and VT-1-like toxins have been purified from other strains of *E. coli* (O'Brien and La Veck, 1983; Petric et al., 1987), and there is immunological evidence which indicates that VT-1-like proteins are produced by many other bacteria including *Salmonella* (Stockbine et al., 1985), *Campylobacter jejuni* (Moore et al., 1988), *Vibrio cholerae* (Stockbine et al., 1985), and *Vibrio parahaemolyticus* (Stockbine et al., 1985). These verotoxins have been shown to be immunologically similar to Shiga toxin by ELISA, Ouchterlony immunodiffusion, or cross-neutralization studies (Stockbine et al., 1985). However, reported physicochemical characteristics, such as isoelectric point and molecular weight, of some *E. coli* verotoxins differ from those of Shiga toxin.

There are many aspects of VT-1 that remain to be elucidated. For example, the mechanism of action of VT-1 is not fully understood at the molecular level, although the toxin has been shown to inhibit protein synthesis by inactivating the 60S ribosomal subunit (Igarashi et al., 1987). Furthermore, there have been no detailed studies of the stability of VT-1. The purpose of this study was to determine the effect of heat, pH, and various cations and lipids on the activity of VT-1. In addition, partial

N-terminal sequencing of VT-1 was done to allow for a more conclusive comparison of the toxin purified and characterized in this study with verotoxins purified by others.

MATERIALS AND METHODS

Production of Toxin. *E. coli* O157:H7 strain 932 (originally isolated from a patient with hemorrhagic colitis) was streaked on a fresh slant of Trypticase soy agar and grown for 18 h at 37 °C. A loopful of inoculum was transferred to each of nine 250-mL Erlenmeyer flasks containing 50 mL of Trypticase soy broth and incubated for 4 h at 37 °C with agitation (160 rpm). Cells were sedimented by centrifugation at 16000g for 10 min and resuspended in 80 mL of mineral basal medium (MBM) (Padhye et al., 1986). Two carboys each containing 12 L of MBM plus 350 mL of 40% glucose and 1400 mL of 10% casamino acids were prewarmed to 37 °C and then inoculated with 35 mL of resuspended cells. The media were aerated by sparging air through a sterile cotton-filled tube and stirring with a 10-cm stirbar over a magnetic stir-plate. The aeration rate was adjusted so as to minimize foaming. The culture was incubated for 16 h at 37 °C and then filtered and concentrated by using a Pellicon cassette system containing a 0.45-μm Durapore cassette (Millipore Corp, Bedford, MA). Cells were rinsed four times with 1 L of 10 mM Tris-HCl, pH 7.5, and 300 mM NaCl. Cells were sedimented by centrifugation (16000g, 15 min), washed, suspended in lysis buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.0), and adjusted spectrophotometrically to an OD_{640} of 100–300. Phenylmethanesulfonyl fluoride (PMSF) (10 μg/mL) was added to the cell suspension, which was then lysed by repeated sonication (140 W, 5 min, 5 times). Unlysed cells were sedimented by centrifugation and resonicated. Toxin was precipitated from the supernatant fluid between 30 and 60% saturated ammonium sulfate. Precipitated toxin was suspended in 50 mM Tris-HCl, pH 7.5, containing 300 mM NaCl and 5% glycerol.

Toxin Purification. *Gel Filtration.* Toxin was applied to a Sephacryl S-200 superfine column (111 × 5 cm; Pharmacia LKB, Piscataway, NJ) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 300 mM NaCl. The column flow rate was 0.5 mL/min, and fraction volume was 15 mL. Fractions were assayed by Vero cell cytotoxicity testing (Padhye et al., 1986). Toxic fractions from the major peak were pooled, concentrated with an Amicon stirred-cell concentrator using a PM-10 membrane, and

dialyzed in 50 mM Tris-HCl, pH 8.0 (12 000–14 000 molecular weight cutoff).

Ion Exchange. Sample was applied to a Q–Sepharose fast flow column (10 × 1.5 cm; Pharmacia LKB) equilibrated with dialysis buffer (50 mM Tris-HCl, pH 8.0). The column was washed with 200 mL of dialysis buffer, and the toxin was eluted with a linear NaCl gradient (250 mL of dialysis buffer × 250 mL of 200 mM NaCl–50 mM Tris-HCl, pH 8.0).

Chromatofocusing. The major toxic peak was concentrated and dialyzed in 20 mM Bis-Tris-HCl buffer, pH 7.2, and applied to a Mono-P HR 5/30 (Pharmacia LKB) on an FPLC system (Pharmacia LKB) equilibrated with Bis-Tris-HCl dialysis buffer. Toxin was eluted with 3 mM Buffalyte 3–10, pH 6.3 (Pierce Chemical Co., Rockford, IL). Fractions were assayed for verotoxicity, and major toxic peaks were analyzed by using SDS–PAGE.

Hydrophobic Interaction Chromatography. Fractions from the major toxic peaks were pooled and dialyzed with 1.2 M $(NH_4)_2SO_4$–50 mM Tris HCl, pH 6.0, and applied to a phenyl-Superose HR 5/5 column (Pharmacia LKB) on an FPLC system equilibrated with dialysis buffer. The column was washed with 8 mL of dialysis buffer, and the toxin was eluted with a nonlinear gradient progressing from dialysis buffer (0% B) to 20 mM Tris-HCl, pH 7.5 (100% B) (4 mL, 0–40% B; 36 mL, 40–100% B) (% B indicates the ratio of the mixture of the beginning buffer and the ending buffer). Flow rate was 0.5 mL/min, and fraction size was 1.2 mL. The major toxic peak, as determined by verotoxicity assay, were pooled and dialyzed in 20 mM $(NH_4)HCO_3$. If necessary, the toxin was further purified by chromatography on hydroxylapatite. A 2.5-mL hydroxylapatite column was equilibrated with 10 mM Tris-HCl, pH 7.0, and the toxin was eluted with a multiple-step gradient (15 mL/step) of 3, 15, 25, 35, 45, 55, 65, 75, 85, 95, 120, 200, and 400 mM $NaH_2PO_4 \cdot H_2O$–NaOH in 10 mM Tris-HCl, pH 7.0. Purity was confirmed by SDS–PAGE with silver staining and isoelectric focusing.

Molecular Weight Determination by Gel Filtration. A Superose 12 HR 10/30 column on an FPLC system, equilibrated with 50 mM Tris-HCl, pH 7.5, with 300 mM NaCl, was injected with a 100-μL sample of pure VT-1 at 440 μg/mL and eluted with a flow rate of 0.67 mL/min. Fractions of 1 mL were collected. The molecular weight of VT-1 was obtained by comparing the data obtained from VT-1 with the data obtained from molecular weight standards (Sigma Chemical Co., St. Louis, MO) (200 000, 150 000, 66 000, 29 500, 12 500, and 6500) run under the same conditions.

Polyacrylamide Gel Electrophoresis. Purity of the proteins was verified by SDS–PAGE gels [15% total acrylamide (T), 2.6% bis(acrylamide) (C)] on Mini-Protean II and Protean II systems (Bio-Rad) or with 8–25% Phastgels on a Phastsystem (Pharmacia LKB). Samples containing less than 100 ng of protein/band were silver stained (Merrill et al., 1981).

Subunit Separation and Sequencing. The procedure used as developed for minigels at the University of Wisconsin—Madison Biotechnology Center by Cynthia Wadsworth (personal communication) and was a modification of the method described by Matsudaira (1987). This procedure was expanded by us to large gels, and cooling was added to prevent loss of resolution. All SDS used in the procedure was recrystallized by the method of Hunkapillar (1983). All glassware was acid-washed. Two linear gradient SDS–PAGE gels (1 mm × 16 cm × 16 cm) were formed on a Protean II system. A gradient of 5% T and 2.6% C to 30% T and 5% C was used with a separating gel buffer of 0.75 M Tris-HCl and 0.1% SDS, pH 8.8. Pure toxin (10–15 μg) at 1 mg/mL was treated with an equal volume of sample buffer with reducing agent (125 mM Tris-HCl, pH 6.8, 4.6% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) and heated at 60 °C for 30 min. The toxin was then loaded onto the gel. Reservoir buffer of the gel system was composed of 50 mM Tris, 384 mM glycine, and 0.1% SDS, pH 8.3–8.8. The gel system was precooled for 30 min at 6 °C (LKB Multi-Temp II, Pharmacia LKB). Gels initially were run for 30 min at 15 mA/gel, constant current, and then for 6 h at 35 mA/gel with cooling continued at the prerun setting. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore Corp.) and sequenced (Matsudaira, 1987). Sequencing was done by the Biotechnology Center at UW—Madison.
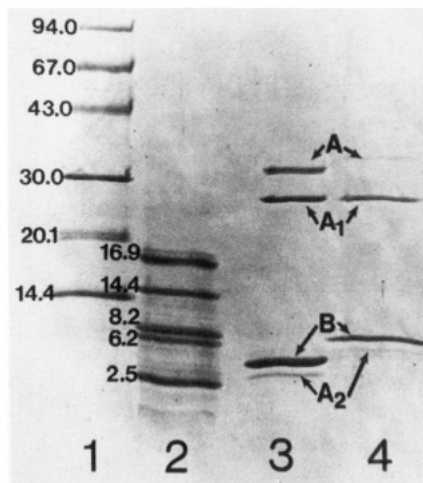

Figure 1. SDS–PAGE of VT-1 (lane 3) and VT-2 (lane 4).

Isoelectric Focusing (IEF). Pure toxin (20 μL at 50–100 μg/mL) and IEF standards (broad-range p*I* standards, Pharmacia LKB) were loaded onto an LKB Ampholine PAG plate 3.5–9.5 on an LKB Multiphor system with the coolant plate cooled to 4 °C before sample application. Electrode buffers were 1 M NaOH and 1 M H_3PO_4. Gels were run for 1 h at 750 V, 25 mA, and 15 W, and then the sample wick was removed. Focusing was continued for an additional 3 h at 1500 V, 50 mA, and 30 W. The gel was then fixed with 20% trichloroacetic acid and silver stained (Merrill et al., 1981).

Effect of Lipids on Toxicity. VT-1 (0.1 μg/mL) was incubated with cardiolipin, cholesterol, glycophorin, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, or phosphatidylethanolamine at 1, 10, and 100 μg/mL for 1 h at 37 °C. The toxin–lipid mixtures were serially diluted (1:3) and applied to Vero cells.

Effect of Cations on Toxicity. VT-1 (1 μg/mL) was serially diluted (1:3) in Hepes buffer (10 mM Hepes, 2 mM KCl, 137 mM NaCl, pH 7.2) containing 4.1 mM $FeCl_3$, 4.1 mM $FeCl_2$, 0.5 mM Na_2EDTA, 0.015 mM $ZnCl_2$, 0.14 mM $MnCl_2$, 0.14 mM $MgCl_2$, 22.2 mM $CaCl_2$, or 0.014 mM $Cu(C_2H_3O_2)_2$ and incubated for 1 h at 37 °C. The toxin was then transferred to Vero cells.

Effect of pH on Toxicity. VT-1 was diluted to 1 mL at 0.141 μg/mL, in a broad-range buffer composed of 20 mM sodium citrate, 20 mM sodium succinate, 20 mM sodium acetate, 20 mM NaH_2PO_4, 20 mM Tris, and 20 mM boric acid, pH 7.2. This preparation was dialyzed for 16 or 96 h against 100 volumes of the same buffer at various pH values ranging from 4 to 9 with two changes. NaOH or HCl was used to adjust the dialysis buffer to the desired pH value. Samples were then dialyzed twice with 100 volumes of buffer, pH 7.5, filter-sterilized, and tested for verotoxicity. The pH was determined between dialysis steps to ensure the desired pH was achieved. Dialysis was done at 4–6 °C.

Effect of Temperature on Toxicity. Forty 1.25-mL samples of purified VT-1 of equal concentration in Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) were treated in a water bath at 25, 37, 45, 55, 65, 70, 75, 80, or 85 °C for 15, 30, or 60 min or at 75, 80, or 85 °C for 5 min. Timing started when samples were immersed. Warm-up and cool-down times, which were measured with a thermocouple and a calibrated recorder, were minimal compared to the incubation times. Immediately after heating, samples were immersed in an ice bath. Samples were then tested for mouse toxicity and verotoxicity. Duplicate studies were done.

RESULTS

A total of 268 μg of highly purified VT-1 was obtained from 70 g (wet weight) of *E. coli* O157:H7 cells. SDS–PAGE of VT-1 revealed four subunits, i.e. A, A_1, A_2, and B with molecular weights of 32 000, 28 000, 3700, and 4700, respectively (Figure 1). This compares with molecular weights of 34 000, 28 000, 5600, and 7300 for subunits A,
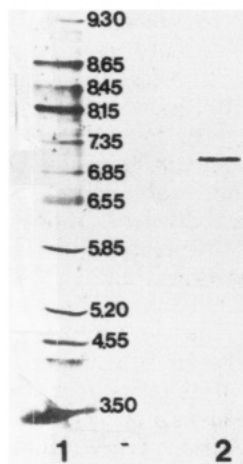
Figure 2. IEF of VT-1.

Table I. N-Terminal Amino Acid Sequences of VT-1 Subunits

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         1           5              10             15
A₁   Lys-Glu-Phe-Thr-Leu-Asp-Phe-Ser-Thr-Ala-Lys-Thr-Tyr-Val-Asp-Ser-Leu

A    Xᵃ-Glu-Phe-Thr-Leu-Asp-Phe-Ser-Thr-Ala-Lys-Thr-Tyr-Val-Asp-Ser-Leu-Asn


     252         255            260            265
A₂   X -Ala-Ser/Phe-Asp-gluᵇ-Phe/gly-Pro- X -Met- X -Pro-Ala-Asp-Gly-
                        270
     asnᶜ-Val- X -Gly-Ile-Thr



         1           5              10             15
B    Thr-Pro-Asp-Asp-Val-Thr-Gly-Lys-Val-Glu-Tyr-Thr-Lys-Tyr-Asn-

                        20             25             30
     -Asp-Asp-Asp-Thr-Phe-Thr-Val-Lys-Val-Gly-Asp-Lys-Glu-Leu-Phe-

                        35             40             45
     -Thr-Asn-Arg-Trp-Asn-Leu-Gln-Ser-Leu-Leu-Leu-Ser-Ala-Gln-Ile-

                        50
     -Thr- X -Met- X -Val
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ᵃ X, undetermined because of several peaks in cycle. ᵇ Amino acids that are not capitalized indicate there is less confidence in their identification. ᶜ Underscore indicates variance from previously reported sequences.

A_1, A_2, and B, respectively, of *E. coli* O157:H7 verotoxin 2 (VT-2) (Figure 1). An isoelectric point of 7.03 was determined for VT-1 by analytical isoelectric focusing (Figure 2). The molecular weight as measured by gel filtration on FPLC Superose-12 was 40 000.

N-Terminal amino acid sequences of the subunits are shown in Table I. Subunits A, A_1, A_2, and B were sequenced through 18, 20, 20, and 50 residues, respectively. The sequences of A and A_1 were identical. The A_2 subunit started at residue 252 as determined by comparison with deduced amino acid sequences reported previously (Jackson et al., 1987).

None of the lipids evaluated had a significant effect on verotoxicity of VT-1 (data not shown). Cardiolipin was toxic to Vero cells. Of the different cations evaluated, only calcium influenced verotoxicity (Figure 3). Calcium (22 mM) slightly increased verotoxicity as compared to the control ($P < 0.025$, Student's t-test). EDTA (0.5 mM) also slightly increased VT-1 verotoxicity; however, confidence in these results is less because EDTA above 1 mM was toxic to Vero cells.
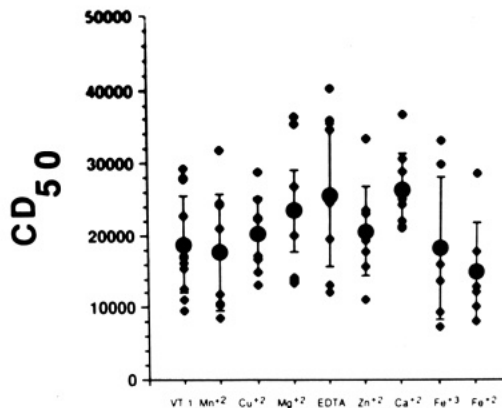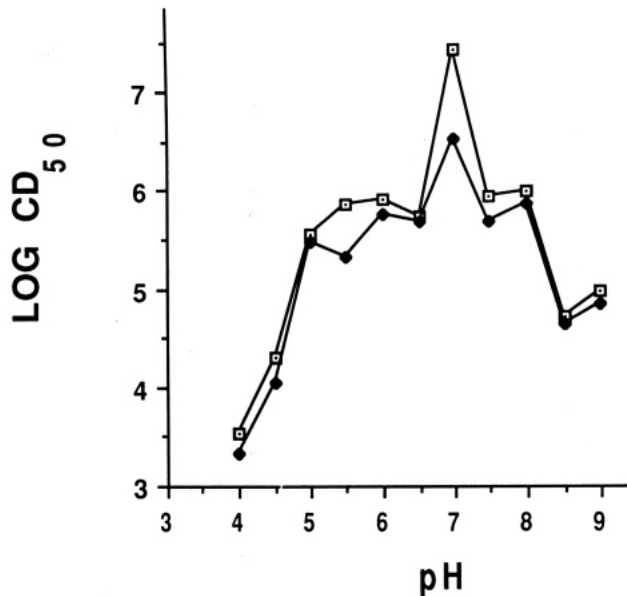

Figure 3. Cation or EDTA treatment of VT-1.

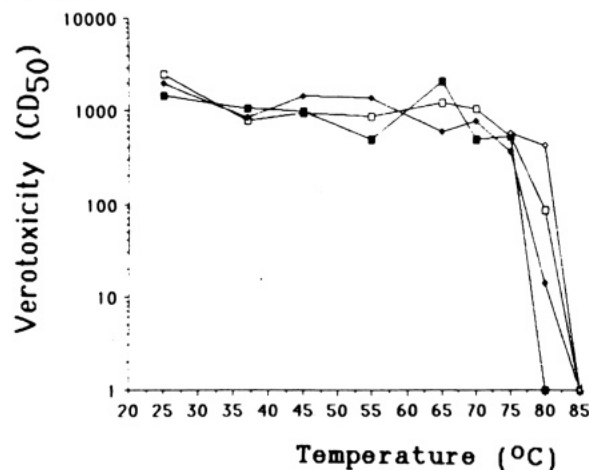**Figure 4.** Treatment of VT-1 at various pH values for 16 (□) or 96 h (◆).

Figure 5. Treatment of VT-1 at various temperatures for periods of 5 (O), 15 (□), 30 (◆), or 60 (□) min.

Treatment of VT-1 for 16 h at pH 4.5 reduced toxicity 10-fold, and at pH 4.0 toxicity was reduced by >99% (Figure 4). Results of a similar study done by exposing VT-1 to the same pH values for 96 h closely paralleled the samples treated for 16 h (Figure 4).

The activity of VT-1 treated at 45–70 °C for periods of 15, 30, or 60 min remained essentially the same (Figure 5). There was a time-dependent decrease in the activity of VT-1 heated above 70 °C, with heating at 80 °C for 60

min or at 85 °C for 5 min completely inactivating VT-1 with an initial activity of 1000–2000 Vero CD_{50}.

DISCUSSION

Previously published accounts of purification studies of VT-1 from *E. coli* O157:H7 have indicated yields of 208 μg from 180 g of cells (Noda et al., 1987) by use of standard liquid chromatography (LC) and 750 μg from 2 g of cell lysate by use of immunoaffinity chromatography (Kongmuang et al., 1987). The purification scheme used in this study provided a 28% better yield from 60% fewer cells than the previously reported procedure that used standard LC, and it bypasses the necessity for producing the antibodies needed for immunoaffinity chromatography to purify the toxin.

VT-1 and Shiga toxin have the same isoelectric point and are immunologically similar to each other and distinct from VT-2 by Ouchterlony immunodiffusion and verotoxicity neutralization assays (Yutsudo et al., 1987; Kongmuang et al., 1987). Recent studies revealed that there is immunological similarity between the A, A_1, and A_2 subunits of VT-1 and VT-2 as determined in Western blots and ELISA using monoclonal antibodies, even though these antibodies do not immunoprecipitate or completely neutralize the opposing toxin (Padhye et al., 1989). These data are consistent with reports of 56% homology of amino acids between VT-1 and VT-2 and the conservation of sequences (Jackson et al., 1987). Neutralization of VT-1 using monoclonal antibodies (anti-VT-1) only occurred with antibodies capable of reacting with the B subunit as seen on Western blots (Padhye et al., 1989).

The molecular weights reported here for subunits A, A_1, and B are consistent with previous values for VT-1 purified from *E. coli* O157:H7 (Noda et al., 1987; Kongmuang et al., 1987). Values reported previously for subunit B were 4000–5000 (Noda et al., 1987) or "near the dye front" (Kongmuang et al., 1987); our results indicate a molecular weight of 4700. The A_2 subunit has not been reported previously as being observed on SDS–PAGE. This may be due to the lack of resolution afforded by gel formulations used in previous studies or the lack of sensitivity of the staining method used.

The separation of subunits A, A_1, A_2, and B of VT-1 achieved by the SDS–PAGE procedure in this study allowed sequencing directly from blots of gels, small proteins which are very close in molecular weight. Hence, the amino acid sequence of a given protein band could be correlated with its molecular weight. The 32 800 A subunit and the 27 000 A_1 subunit were determined to have the same N-amino acid terminus. The N terminus of the A_2 subunit, which was formed by nicking of the holotoxin, apparently by endogenous proteases, was the 252nd residue of the A subunit. Takao et al. (1988) reported nicking to occur prior to residue 254. However, sequencing by Takao et al. (1988) was done on fragments prepared by tryptic digestion. This may account for the difference in the nicking site. Residue 266 was found to be asparagine as compared to arginine reported previously (Takao et al., 1988).

Up to 32 residues of subunit B of VT-1 were sequenced by Takao et al. (1988) and compared to the sequence of Shiga toxin (Seidah et al., 1986); complete identity was observed between the sequences of the two toxins. However, results of the study reported here indicate that residue 4 of VT-1 B subunit is aspartic acid and not cysteine as has been previously reported at this position. Therefore, the intramolecular disulfide bridge proposed by Seidah et al. (1986) may not be essential or even present for the binding activity of the B subunit.

Studies were done to determine if cofactors, inhibitors, or enhancers of verotoxicity could be identified by pretreating VT-1 with substances previously reported to affect enzyme or toxin activities. Inhibition, as measured by loss of cytotoxic activity, could be caused by blockage of binding, uptake of the toxin, or inactivation of RNA N-glycosidase activity (Endo et al., 1988). Studies with β-bungarotoxin revealed that divalent cations were required for binding the toxin to frog motor nerve terminals, whereas EDTA significantly reduced binding (Caratsch et al., 1985; Rehm and Betz, 1982). Other studies with divalent cations using Lettre cells and measuring membrane permeability revealed that several agents, including Sendai virus, polylysine, bee venom protein melittin, and Triton X-100, induced membrane leakage/permeability. This leakage was prevented by addition of Zn^{2+} (0.1 mM), Ca^{2+} (1 mM), or Mg^{2+} (5 mM) (Bashford et al., 1986).

Of the cations tested in our study, 22 mM Ca^{2+} had the most significant effect on VT-1 toxicity. This increase in toxicity may be due to indirect or direct effects of Ca^{2+} on the toxin. Because EDTA and Ca^{2+} both increased VT-1 activity, it is likely that Ca^{2+} and other divalent metal ions are not necessary for VT-1 activity. Perhaps they act to prevent degradation of toxin prior to uptake into the cell or increase the rate of toxin uptake into the cell, thereby decreasing the opportunity for degradation before cell uptake. Fe^{3+} has been shown to reduce Shiga toxin and VT-1 production in *Shigella dysenteriae* 1 and *E. coli*, respectively, but it was not determined if Fe^{3+} had any effect on the toxin itself (Weinstein et al., 1988; Chart et al., 1989). Our results indicate Fe^{3+} had no significant effect on VT-1 verotoxicity. In contrast, similar studies done by us with VT-2 revealed a >95% decrease in verotoxicity when the toxin was treated with 4 mM $FeCl_3$ before being applied to Vero cells (unpublished data).

Results of thermal inactivation studies revealed a significant reduction in activity of VT-1 when heated between 75 and 85 °C for ≤60 min. These results were similar to those reported in a previous but less comprehensive study (Petric et al., 1987). These investigators reported that VT-1 was inactivated by 50% after heating at 80 °C for 10 min and by 99% when heated at 80 °C for 30 min. Although the biological activity of ingested VT-1 is unknown, the thermal tolerance of the toxin may have important practical implications for the food-processing industry and the consumer because the times and temperatures often used for cooking meat are inadequate to inactivate VT-1. For example, meat changes color from red to gray near 60 °C and a "rare" steak may only reach an internal temperature of 40 °C for a brief period (Ingram and Simonsen, 1980). In addition, the stability of VT-1 from pH 5 to 10 also may have practical implications to the food industry because the pH value of many foods is the range 5–7.

The increased recognition of food-borne outbreaks attributed to *E. coli* O157:H7 raises questions about the significance of preformed verotoxins in human illness. Hence, studies of toxin stability and function have important practical significance. It is unknown if *E. coli* O157:H7 can produce VT-1 in foods or if ingestion of preformed VT-1 in foods will cause illness in humans. However, these studies have revealed that VT-1 is a relatively heat-stable toxin that may remain active in cooked foods that receive a mild heat treatment or in foods having a pH of 5–10. Additional studies in foods are needed to fully elucidate the significance of preformed verotoxins as health hazards.

E. coli Verotoxin 1

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