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Physicochemical and Biological Properties of Purified *Escherichia coli* Shiga-Like Toxin II Variant

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Purified *Escherichia coli* Shiga-like toxin II variant (SLT-IIv) was characterized with regard to selected physical, chemical, and biological properties. N-terminal amino acid sequencing confirmed the identities of 33,000-, 27,500-, and 7,500-molecular-weight (MW) bands seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified SLT-IIv as the A subunit, A_1 fragment, and B subunit, respectively. The arginine-serine bond between amino acids 247 and 248 in the A subunit was determined to be the site for proteolytic cleavage into A_1 and A_2 fragments. As with other SLTs, gel filtration chromatography of SLT-IIv gave estimates of the MW of holotoxin that were variable and less than predicted for a 1-A-subunit–5-B-subunit configuration. The MWs were estimated to be 40,000 and 43,000 by Sephacryl S-100 and Sephadex G-100 and <2,000 by Bio-Sil Sec-250 gel filtration chromatography. The isoelectric point of SLT-IIv holotoxin was 9.0. Cytotoxicity of SLT-IIv was destroyed by heating at 65°C for 30 min and by incubation with 2-mercaptoethanol and dithiothreitol, but it increased 30-fold by incubation with trypsin, chymotrypsin, or pepsin and 2-fold by incubation with thermolysin. SLT-IIv cytotoxic activity was stable at neutral and alkaline pH values but was lost at pHs 3, 4, and 5. SLT-IIv was stable in fluid from the anterior and posterior small intestines of pigs but was not enterotoxic in pig intestinal loops. The smallest doses of SLT-IIv that inhibited protein synthesis in porcine endothelial cells and Vero cells were 0.1 ng and 0.1 fg, respectively.

Shiga-like toxins (SLTs) or verotoxins (VTs) are a family of cytotoxins produced by certain strains of *Escherichia coli* (7, 17, 19, 25). Three antigenically distinct SLTs produced by *E. coli* of human origin have been characterized (13, 19, 36). SLT-I (VT1) is almost identical to Shiga toxin, differing in only one amino acid in the A subunit (3, 28). Shiga toxin and SLT-I are cross neutralized by antibodies to each other (28, 36), and production of both toxins is regulated by iron (4). SLT-II is less closely related to Shiga toxin and shares 58% overall DNA sequence homology with SLT-I (3, 16). SLT-II is not iron regulated (37), and its activity is not neutralized by antibodies to Shiga toxin or SLT-I (36). VT2 was once considered to be identical to SLT-II, but recent neutralization studies suggest that they are distinct toxins (13). SLT-II variant (SLT-IIv) is produced by edema disease strains of *E. coli* (7, 10, 20, 25, 35) and shares 91% overall DNA sequence homology with SLT-II (11, 40). SLT-IIv is not regulated by iron (21, 40) and is neutralized by antibodies to SLT-II and VT2 but not by antibodies to SLT-I (10, 22, 25). SLT-IIv was termed a variant of SLT-II because, unlike SLT-II, it was less active on HeLa cells than on Vero cells (10, 22, 25).

SLT-producing *E. coli* strains have been implicated in diarrhea, hemolytic uremic syndrome, and hemorrhagic colitis in humans (17, 19, 33, 35, 36) and in edema disease of pigs (7, 20, 25). The pathogenesis of these diseases appears to involve colonization of the intestine by certain strains of *E. coli* (2, 24), production of SLT which is absorbed from the gut, and damage to vascular endothelium (23, 29) resulting in pathological lesions in target organs (23, 33).

SLT-I, SLT-II, and VT2 were the first of the SLTs to be purified to homogeneity (9, 14, 28, 32). They consist of an enzymatically active A subunit and multiple B subunits that are responsible for binding the toxin to specific receptors (6) on target cell surfaces. Enzymatic cleavage of the A subunit yields a larger, enzymatically active A_1 fragment, and a smaller A_2 fragment (9, 14, 28, 32). The A_1 fragment exhibits *N*-glycosidase activity which removes the adenine base at position 4324 of the 28S ribosomal RNA (34) and inhibits elongation factor 1-dependent binding of aminoacyl tRNA to the A site of the 60S ribosomal subunit, thereby preventing peptide chain elongation (15, 30).

SLT-IIv has recently been purified to homogeneity (22) and has been used to reproduce the clinical signs and pathological lesions seen in naturally occurring edema disease in pigs (23). Impure SLT-IIv preparations have also been shown to impair protein synthesis and to possess *N*-glycosidase activity in *Xenopus* oocytes (34). However, purified SLT-IIv has not yet been well characterized with regard to its physical, chemical, and biological characteristics. The purposes of this study were to further characterize purified SLT-IIv and to demonstrate the effect of SLT-IIv on protein synthesis in porcine endothelial cells, which appear to be the target cells for this toxin in edema disease.

MATERIALS AND METHODS

Bacteria and culture conditions. *E. coli* TB1(pCG6) was used as the source of toxin (11) and was stored as previously described (11, 22, 23). A single bacterial colony from a tryptic soy agar culture was inoculated into a 500-ml Erlenmyer flask containing 100 ml of glucose-syncase broth (21) and incubated at 37°C for 18 h in a Gyrotory (200 rpm) water bath shaker (model G76; New Brunswick Scientific, Edison, N.J.). Two 12-liter fermenter flasks containing 7.5 liters of glucose-syncase broth supplemented with 100 μg of ampicillin per ml were each inoculated with 50 ml of the 18-h culture and incubated for 24 h at 37°C with aeration (22). The bacterial pellet was harvested and washed twice (22).

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Purification of SLT-IIv. Toxin purification has been described previously (22). Purified toxin was assessed for purity on silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels and assayed for endotoxin (sensitivity, 0.1 ng of endotoxin per ml) by the *Limulus* amoebocyte lysate assay (Sigma Chemical Co.). Protein content was assayed as previously described (21). Purified SLT-IIv was aliquoted and stored at −20°C until needed.

Vero cell assay for SLT-IIv. SLT-IIv activity was measured in a microtiter Vero cell assay as described previously (22) except that toxin samples were serially diluted twofold rather than fivefold with Eagle modified essential medium in 96-well tissue culture plates (GIBCO BRL Canada, Burlington, Ontario, Canada). The toxin titer was expressed as the highest dilution of the sample that killed 50% of the Vero cell monolayer after 72 h (CD_{50}). One milligram of purified SLT-IIv had 4.4×10^9 CD_{50}s of activity.

N-terminal amino acid sequencing. Purified SLT-IIv was separated into its subunits on 15% SDS-polyacrylamide gels run under reducing or nonreducing conditions and electrophoretically transferred to Immobilon-P transfer membranes (Millipore Corp., Mississauga, Ontario, Canada) as previously described (22). After transfer, the membranes were stained with Coomassie brilliant blue (R-250; Bio-Rad Laboratories, Mississauga, Ontario, Canada) for 5 min and destained with a solution of 40% methanol and 10% acetic acid. Bands corresponding to the A subunit, A_1 fragment, and B subunit were cut from the membranes and stored at −20°C. The first 15 amino acids at the N terminus of each protein band were determined with a gas-phase sequentor (model 470A; Applied Biosystems, Mississauga, Ontario, Canada) by standard methods (26).

Determination of MW. The molecular weight (MW) of SLT-IIv holotoxin was determined by gel filtration chromatography using Sephacryl S-100 and Sephadex G-100 (Pharmacia LKB Biotechnology, Baie d'Urfé, Quebec, Canada) columns (2.5 by 90 cm) equilibrated in phosphate-buffered saline (PBS). A 1-ml volume of purified SLT-IIv (10^8 CD_{50}s/ml) was loaded onto the columns and chromatographed at 0.25 ml/min at room temperature. One-milliliter volumes were collected and assayed for SLT-IIv activity. Blue dextran 2000 (>100,000 MW) (Pharmacia LKB Biotechnology), transferrin (76,000 MW), ovalbumin (45,000 MW), chymotrypsin (25,000 MW), and cytochrome *c* (12,000 MW) (Sigma Chemical Co.) were used as MW markers. Elution of protein was measured at 280 nm.

The MW of SLT-IIv holotoxin was also determined by high-pressure liquid chromatography with a Bio-Sil Sec-250 (Bio-Rad Laboratories) gel filtration column (0.75 by 30 cm) equilibrated with PBS. A 100-μl volume of purified SLT-IIv (10^8 CD_{50}s/ml) was chromatographed at 0.5 ml/min. Elution of protein was measured at 280 nm, and 100-μl volumes were collected and assayed for SLT-IIv activity. Thyroglobulin (670,000 MW), gamma globulin (158,000 MW), ovalbumin (44,000 MW), myoglobin (17,000 MW), and cyanocobalamin (1,350 MW) (Bio-Rad Laboratories) were used as MW markers.

Isoelectric point. The isoelectric point (pI) of the SLT-IIv holotoxin was determined by agarose gel isoelectric focusing (Isolab Inc., Akron, Ohio). An anode buffer of 0.5 M acetic acid and a cathode buffer of 0.5 M NaOH were used for a pH range of 3 to 10. Purified SLT-IIv and pI markers (Pharmacia LKB Biotechnology) were (5 μl each) applied to individual lanes of the gel and electrophoresed at 4°C under the following conditions: 5 W for 5 min, 10 W for 20 min, and 20 W for 5 min. After electrofocusing, one lane containing SLT-IIv holotoxin was cut into 2-mm sections, incubated at 4°C overnight in PBS, and assayed for SLT-IIv activity. The remaining two lanes, which contained pI markers or SLT-IIv holotoxin, were fixed and silver stained (Isolab Inc.).

Temperature treatment of SLT-IIv. One-milliliter samples of purified SLT-IIv (5×10^6 CD_{50}s/ml) were heated in a model H2025-1 block heater (Canlab, Mississauga, Ontario, Canada) at 55, 60, 65, 70, and 75°C. Samples were held at each temperature for 5, 10, 15, 20, 25, and 30 min, cooled to room temperature, and assayed for SLT-IIv activity.

Effect of reducing agents on SLT-IIv activity. One-milliliter samples of purified SLT-IIv (10^6 CD_{50}s/ml) were incubated at 37°C for 1 h with 1 mM dithiothreitol (Sigma Chemical Co.) or 0.1 M 2-mercaptoethanol (Bio-Rad Laboratories) and assayed for SLT-IIv activity.

Effect of proteolytic enzymes on SLT-IIv activity. One-milliliter samples of purified SLT-IIv (5×10^6 CD_{50}s/ml) were incubated at 37°C for 1 h with 10, 50, 100, or 250 U of trypsin, chymotrypsin, pepsin, or thermolysin (Sigma Chemical Co.), respectively. Proteolytic activity was stopped by the addition of 1.5 times the corresponding number of units of trypsin inhibitor, chymotrypsin inhibitor, or pepstatin (Sigma Chemical Co.), and samples were assayed for SLT-IIv activity. In the case of treatment with thermolysin, no inhibitor was used. Mixtures of proteolytic enzymes plus PBS and of proteolytic enzymes, inhibitors, and PBS were tested on Vero cells at the highest concentrations of enzymes and inhibitors used in the tests.

Susceptibility of SLT-IIv to pH. One-milliliter volumes of purified SLT-IIv (5×10^6 CD_{50}s/ml) were adjusted to pH values ranging from 3 to 11, at 1-pH-unit intervals, with 1 M HCl or 1 M NaOH and incubated at 37°C for 1 h. Samples were readjusted to pH 7.4 by the addition of 1 M HCl or 1 M NaOH and assayed for SLT-IIv activity.

Effect of intestinal fluid on SLT-IIv activity. Intestinal fluid was collected from two pigs that were anesthetized following 24 h of food deprivation. An anterior and a posterior segment of small intestine were each injected with 20 ml of distilled water and left for 5 min. Intestinal fluid was removed from each segment with a needle and syringe, centrifuged at $10,000 \times g$ for 10 min, and filter sterilized (0.22-μm-pore-diameter filter; Millipore Corp.). One-milliliter samples of purified SLT-IIv (5×10^6 CD_{50}s/ml) were incubated at 37°C for 1 h with an equal volume of fluid from the anterior or posterior intestine and assayed for SLT-IIv activity. Controls consisted of samples of intestinal fluid and PBS.

Enterotoxicity. Purified SLT-IIv was tested for enterotoxicity in pig intestinal loops. Three weaned pigs (4 to 6 weeks old, 10 to 20 kg) (Arkell Swine Research Facility, University of Guelph, Guelph, Ontario, Canada) were fasted for 24 h and anesthetized. Twelve segments, approximately 10 cm long with 2 cm of intestine between loops, were constructed in the small intestine of each pig. One-milliliter volumes of doubling dilutions of purified SLT-IIv or PBS (negative control) were injected in duplicate in each animal. Toxin doses ranged from 1×10^8 to 1.6×10^9 CD_{50}s/ml. Pigs were euthanized at 16 h postinoculation by overdose with barbiturate (Euthanyl Forte; M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada), and the volumes of fluid and lengths of the intestinal loops were measured.

Inhibition of protein synthesis. Protein synthesis was determined by measuring incorporation of [^{3}H]L-leucine (27, 29). Porcine aortic endothelial (PEC-15 [39]) and African green monkey kidney cells (Vero cells; ATCC CCL81; American Type Culture Collection, Rockville, Md.) were cultured in Eagle modified essential medium without L-leu-

cine (EMEM-L), supplemented with 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 1% nonessential amino acids, 1% vitamins, and 1% antibiotic-antimycotic solution (GIBCO BRL Canada). Confluent monolayers were harvested with 0.05% trypsin–0.02% EDTA (GIBCO BRL Canada), filtered through a sterile 25-μm-pore-size nylon mesh to obtain a suspension of single cells, and adjusted to 3.4×10^5 viable cells per ml in EMEM-L. A 100-μl volume of cells was pipetted into each well of 96-well tissue culture plates and incubated in 5% CO_2 at 37°C for 18 h.

Immediately prior to the addition of toxin to the cell monolayers, the culture medium in each well was replaced with 100 μl of EMEM-L supplemented with 2 μCi of L-[4,5-^{3}H]leucine (60 Ci/mmol; ICN Biomedicals, Inc., St. Laurent, Quebec, Canada). Serial dilutions of purified SLT-IIv (100 μl per well) were then added to the monolayers in quadruplicate. Doubling dilutions were made for endothelial cells, and 10-fold dilutions were made for Vero cells. Control wells received 100 μl of EMEM-L. Plate cultures were incubated for various times in 5% CO_2 at 37°C. Protein was extracted from the cells and monitored for radioactivity as described previously (29). Inhibition of protein synthesis was expressed as a percentage of that of the PBS control, and values were then adjusted so that the control value was 0%.

Monolayers from replicate plates were harvested with 0.05% trypsin–0.02% EDTA solution containing 0.04% (wt/vol) trypan blue to determine cell death (26, 27). Cell death was expressed as a percentage of that of the PBS control, and values were then adjusted so that the control value was 0%.

Effects of SLT-IIv antiserum. To determine whether SLT-IIv antiserum prepared in pigs (22) could neutralize inhibition of incorporation of ^{3}H-leucine by SLT-IIv, antiserum which neutralized 4×10^4 CD_{50}s of SLT-IIv in the Vero cell assay was incubated with purified SLT-IIv for 1 h at 37°C and then overnight at 4°C. A 10-CD_{50} dose of toxin as determined on the cell line under examination was used. The toxin-antitoxin mixture was then assayed for its effects on the incorporation of ^{3}H-leucine and for lethality on PEC-15 cells and Vero cells, and these effects were compared with the effects of a toxin-PBS mixture. To determine the rapidity of binding of toxin to PEC-15 cells and Vero cells, the same amount of antiserum as that indicated above was added to cell monolayers at 5-min intervals after the addition of 10 CD_{50}s of toxin. Controls consisted of toxin alone, antiserum alone, and PBS alone added to the monolayers. Plates were incubated for 72 h at 37°C in 5% CO_2. Monolayers were harvested and measured for the inhibition of incorporation of ^{3}H-leucine and for the percentage of dead cells.

Replications. All experiments, with the exception of N-terminal amino acid sequencing, were performed three times.

RESULTS

N-terminal amino acid sequencing. The sequences of the first 15 amino acids at the N terminus of the 33,000-, 27,500-, and 7,500-MW bands seen after SDS-polyacrylamide gel electrophoresis (PAGE) of purified SLT-IIv are shown in Fig. 1. Analysis of the 33,000-MW band seen under nonreducing electrophoresis conditions revealed the presence of two peptides, which were identified as the A subunit (Fig. 1, line A) and A_2 fragment (Fig. 1, line B). The 27,500-MW band observed under reducing electrophoresis conditions had an N-terminal sequence identical to that of the A subunit and was identified as the A_1 fragment (Fig. 1, line D). The

	1	5	10	15
A	Gln-Glu-Phe-Thr-Ile-Asp-Phe-Ser-Thr-Gln-Gln-Ser-Tyr-Ser-Ser			
B	Ser-Val-Arg-Ala-Val-Asn-Glu-Glu-Ser-Gln-Pro-Glu-Cys-Gln-Ile			
C	Ala-Asp-Cys-Ala-Lys-Gly-Lys-Ile-Glu-Phe-Ser-Lys-Tyr-Asn-Glu			
D	Gln-Glu-Phe-Thr-Ile-Asp-Phe-Ser-Thr-Gln-Gln-Ser-Tyr-Ser-Ser			

FIG. 1. N-terminal amino acid sequences of the 33,000-MW (lines A and B), 7,500-MW (line C), and 27,500-MW (line D) peptides derived from SDS-PAGE of purified SLT-IIv.

7,500-MW band, which was identified as the B subunit (Fig. 1, line C), had identical N-terminal amino acid sequences under both reducing and nonreducing conditions.

Determination of MW. The MW of the SLT-IIv holotoxin was estimated to be 40,000, 43,000, and <2,000 by gel filtration chromatography on Sephacryl S-100, Sephadex G-100, and Bio-Sil Sec-250 columns, respectively. A single sharp peak which coincided with SLT-IIv activity was seen on chromatography with the Bio-Sil Sec-250 column (data not shown). No protein peaks were associated with toxin recovered from Sephacryl S-100 or Sephadex G-100, because of the larger column volumes and less sensitive systems for monitoring absorbance.

Isoelectric focusing. Isoelectric focusing of purified SLT-IIv demonstrated one protein band with a pI of 9.0 (Fig. 2). Cytotoxic activity was detected in gel sections which corresponded to the stained SLT-IIv holotoxin band.

Temperature treatment and reducing agents. The heat lability of purified SLT-IIv is illustrated in Fig. 3. Cytotoxicity was reduced by 75% by heating at 50°C for 30 min and was completely inactivated by heating at 65°C for 30 min. SLT-IIv activity was completely destroyed by incubation with 2-mercaptoethanol or dithiothreitol.

Effect of proteolytic enzymes. SLT-IIv cytotoxicity was increased 30-fold by incubation with as little as 10 U of

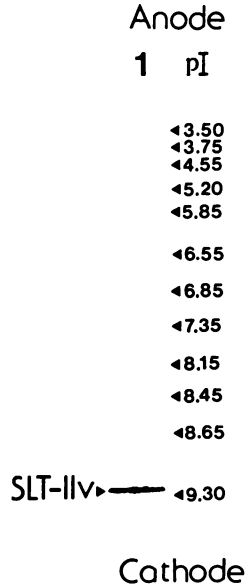

FIG. 2. Silver stained agarose isoelectric focusing gel of purified SLT-IIv. Lane 1, Purified SLT-IIv (1 μg of SLT-IIv); pI, isoelectric focusing markers.

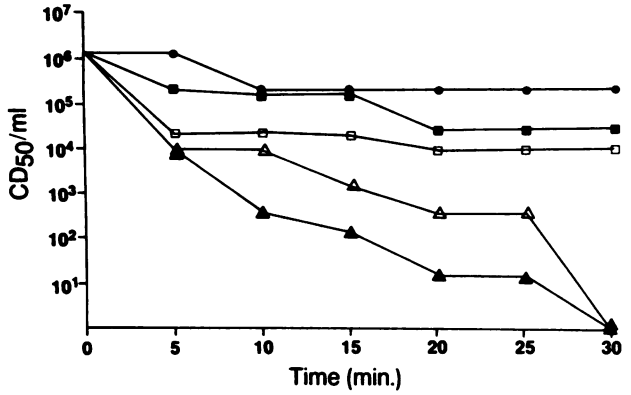

FIG. 3. Inactivation of purified SLT-IIv (5×10^6 CD_{50}s) heated at 50°C (●), 55°C (■), 60°C (□), 65°C (△), and 70°C (▲) for the indicated times.

trypsin, chymotrypsin, or pepsin and 2-fold by incubation with 100 U of protease. Incubation with higher concentrations of trypsin, chymotrypsin, or pepsin did not further increase the activity of SLT-IIv. Proteolytic enzymes and mixtures of the enzymes with inhibitors were not toxic for Vero cells.

Effect of pH and intestinal fluids on SLT-IIv activity. Purified SLT-IIv was stable at pH values ranging from 6 to 11, but 96% of the activity was destroyed by incubation at pH 3, 4, or 5. Cytotoxicity was not altered by incubation with intestinal fluids from the anterior or posterior intestine.

Enterotoxicity. SLT-IIv was not enterotoxic in pig intestinal loops at the doses tested.

Inhibition of protein synthesis. Inhibition of incorporation of ^{3}H-leucine was time dependent and proportional to concentration of toxin for both PEC-15 and Vero cells (Fig. 4A and 5A). In PEC-15 cells, inhibition was observed with 0.1 ng of SLT-IIv and, at higher doses, was evident at 6 h of incubation (Fig. 4A). In Vero cells, inhibition occurred with 0.1 fg of toxin and was detected 1 h after exposure to toxin (Fig. 5A). The inhibitory effect in PEC-15 cells increased over the 72-h period but reached a maximum at 12 to 24 h for Vero cells. For both cell lines, dead cells were first detected at 18 h of incubation and the percentages gradually increased to maximum values at 72 h (Fig. 4B and 5B).

Effect of SLT-IIv antiserum. Preincubation of toxin with SLT-IIv antiserum completely neutralized the cytotoxicity of SLT-IIv in the Vero cell assay and lethality in both PEC-15 cells and Vero cells (Table 1), but residual toxicity was detected by the inhibition of protein synthesis in both cell lines (Table 1). Antiserum alone had no effect (Table 1). Antiserum added to monolayers at 5-min intervals from 5 to 60 min after the addition of toxin did not neutralize cytotoxicity or prevent inhibition of the incorporation of ^{3}H-leucine in either cell line (data not shown).

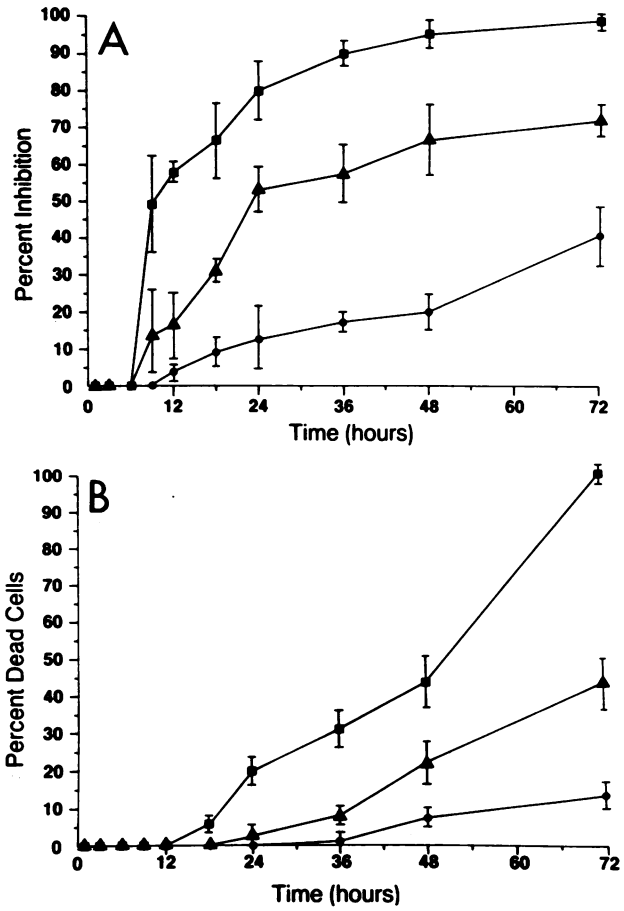

FIG. 4. Effects of purified SLT-IIv on the inhibition of incorporation of ^{3}H-leucine (A) and on cell viability (B) in porcine vascular endothelial cells. Cell monolayers were incubated with 0.4 ng (■), 0.2 ng (▲), and 0.1 ng (●) of purified SLT-IIv for various times. The incorporation of ^{3}H-leucine was measured, and cell death was determined by trypan blue dye exclusion. The inhibition of protein synthesis and the cell death results were expressed as percentages of those of the PBS controls, and values were then adjusted so that the control values were 0%.

DISCUSSION

N-terminal amino acid sequencing confirmed the identities of the protein bands seen on SDS-PAGE analysis of purified SLT-IIv (22). Two N-terminal sequences were identified in the 33,000-MW material which represents the A subunit. We conclude that these represent the N termini of the A subunit and its disulfide-bound A_2 fragment and that the site for proteolytic cleavage of the A subunit into A_1 and A_2 fragments is the arginine-serine bond between amino acids 247 and 248 in the A subunit (11, 40). The N-terminal amino acid sequences of the A and B subunits were identical to those predicted from the nucleotide sequence data (11, 40), thus confirming the identities of these subunits. The cleavage site in the A subunit is consistent with the data from SDS-PAGE analysis of purified SLT-IIv (22). The site for proteolytic cleavage of the A subunit of Shiga toxin and SLT-I is between alanine and serine at amino acid positions 253 and 254 (38). On the basis of the nucleotide sequences of the SLT-IIv genes, the MWs of the A and B subunits were predicted to be 33,069 and 7,583, respectively (11, 40). The A_2 N terminus is identical to a 15-amino-acid sequence within the A subunit, predicted from the nucleotide sequence data. On the basis of these data, the MWs of the A_1 and A_2 fragments were calculated to be 26,610 and 6,459, respectively. Contaminating proteins were not detected in any of the samples, confirming the purity of the toxin preparations.

On the basis of the observation that the MW of the largest cross-linked complex was 66,000, Donohue-Rolfe et al. (8) suggested that Shiga toxin was composed of one A subunit

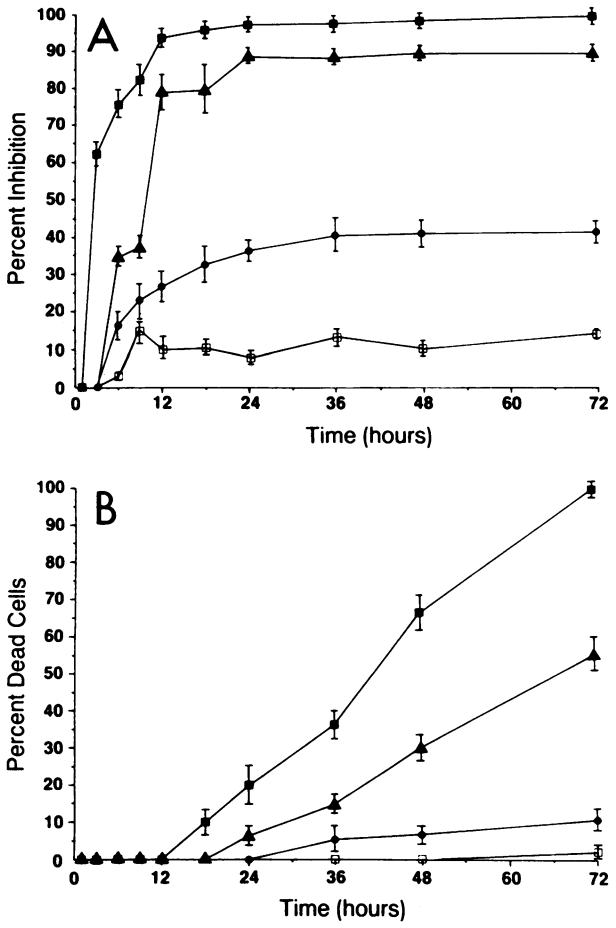

FIG. 5. Effects of purified SLT-IIv on the inhibition of incorporation of ^{3}H-leucine (A) and on cell viability (B) in Vero cells. Cell monolayers were incubated with 0.1 ng (■), 1.0 pg (▲), 10 fg (●), and 0.1 fg (□) of purified SLT-IIv for various times. The incorporation of ^{3}H-leucine was measured, and cell death was determined by trypan blue dye exclusion. The inhibition of protein synthesis and the cell death results were expressed as percentages of those of the PBS controls, and values were then adjusted so that the control values were 0%.

TABLE 1. Neutralization of SLT-IIv activity on PEC-15 cells and Vero cells

Prepn tested	% Inhibition[a]		% Dead cells[b]	
	Vero	PEC-15	Vero	PEC-15
Toxin–SLT-IIv antiserum[c]	22.0 ± 2.3	32.0 ± 4.7	0.0 ± 0.0	0.0 ± 0.0
Toxin-PBS[d]	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Serum alone[e]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Mean ± standard error of the mean from three experiments. The inhibition of protein synthesis was determined by measuring the incorporation of ^{3}[H]leucine and was expressed as a percentage of the PBS control, and values were then adjusted so that the control value was 0%.

[b] Mean ± standard error of the mean from three experiments. The percentage of dead cells was measured by trypan blue exclusion and expressed as a percentage of the PBS control, and values were then adjusted so that the control value was 0%.

[c] Antiserum which neutralized 4×10^4 CD_{50}s of SLT-IIv (22) was incubated with 10 CD_{50}s of toxin for 1 h at 37°C then overnight at 4°C.

[d] A 10-CD_{50} dose of toxin determined on Vero cells and on PEC cells was used on Vero cells and PEC cells, respectively.

[e] Same as antiserum described in footnote *c*.

with a MW of 32,000 and five B subunits with MWs of 6,500. However, MWs of 32,000 and 45,000 (28), 25,000 to 39,000 (28, 32), and 42,000 (14) have been reported for Shiga toxin, SLT-I, and VT2 holotoxins, respectively, by gel filtration chromatography. In this study, the estimated MW of SLT-IIv holotoxin was also much lower than expected from a 1-A-subunit–5-B-subunit toxin molecule (MW, 70,984). Binding of toxin to the gel matrix could be an explanation for the MW values determined by gel filtration. The severe retardation of SLT-IIv on the Bio-Sil Sec-250 could be useful in purification of the toxin.

The pI of SLT-IIv was 9.0, a value which is quite different from those reported for Shiga toxin (7.02 to 7.1) (8, 28), SLT-I (6.72 to 7.02) (28, 32), SLT-II (5.2) (9), and VT2 (6.1) (14). The pIs of the SLT-IIv A and B subunits predicted from the nucleotide sequence of the SLT-IIv genes were 8.7 and 10.2, respectively (11, 40). The pI value may be helpful in identifying the SLTs. For example, an SLT from *E. coli* B2F1 was referred to as an SLT-II variant (31), but the toxin had a pI of 6.1, indicating that it is different from SLT-IIv produced by edema disease strains of *E. coli*. Indeed, the pI and other characteristics suggest that the toxin described by Oku et al. (31) is more like VT2 than like SLT-IIv (14, 31).

Purified SLT-IIv was more heat-labile than purified Shiga toxin, SLT-I, and VT2 (14, 28). O'Brien and LaVeck (28) reported no loss of activity of Shiga toxin after incubation at 65°C for 30 min but complete inactivation after boiling for 2 min. Petric et al. (32) reported that VT1 required heating at 80°C for 20 min for a noticeable reduction in cytotoxicity. Head et al. (14) reported that VT2 was more heat labile than VT1 (SLT-I) and was reduced in cytotoxicity by 50% at 60°C for 30 min. Heat stability studies with crude toxin preparations indicate that SLT-II is more heat stable than SLT-IIv (10).

Treatment of holotoxin with reducing agents completely inactivated cytotoxicity, indicating that disulfide linkages within the intact molecule are necessary for biological activity. Two cysteine residues in the A subunit (11, 40) are involved in forming the disulfide bond which joins the A_1 and A_2 fragments. Two cysteine residues in the B subunit (11, 40) are similar in their location to their counterparts in the B subunit of cholera toxin (5). As in cholera toxin, these cysteine residues are presumed to form a disulfide bridge in the B subunit of SLT-IIv.

Several bacterial protein toxins which act in the gut, including *E. coli* heat-labile enterotoxin and Shiga toxin, exhibit increased biological activity after treatment with proteolytic enzymes such as trypsin (12, 28, 38). The exact mechanism by which trypsin, chymotrypsin, and pepsin increased SLT-IIv cytotoxicity is not known. A conformational rearrangement involving conversion of the A subunit to A_1 and A_2 fragments (28, 38) has been proposed to be necessary for a fully active toxin molecule. Since SLT-IIv appears to be partially nicked (22), increased activity could be due to the completion of the nicking of toxin molecules or to proteolytic cleavage at other sites.

SLT-IIv was stable at neutral and alkaline pHs but was inactivated at pHs 3, 4, and 5. Other bacterial protein toxins which act in the gut have similar pH stabilities (1). The tests with intestinal fluids suggest that SLT-IIv produced in the intestine during naturally occurring disease would be stable in that environment.

Diarrhea has been associated with edema disease in pigs (2, 20, 35) and with SLT-associated diseases in humans (33).

However, a previous study had demonstrated that SLT-IIv was only weakly enterotoxic in rabbit ileal loops (22), and in this study, purified SLT-IIv injected intraintestinally did not cause fluid accumulation in the pig intestine. It is therefore likely that the diarrhea observed in association with naturally occurring and experimentally reproduced edema disease was the result of enterotoxins produced by the infecting strains of *E. coli* (20, 35).

Previously, the clinical signs and pathologic lesions of naturally occurring edema disease were reproduced by injecting pigs intravenously with purified SLT-IIv (23). Microscopic lesions were related to vascular injury and included perivascular edema, hemorrhage, and vasculitis. The cerebellar folia, submucosa and mucosa of the stomach, cecum, and colon were apparently specific targets (23). This study demonstrates that purified SLT-IIv has a direct dose-related cytotoxic effect on porcine vascular endothelial cells, similar to that demonstrated for Shiga toxin on human umbilical vein endothelial cells (29). A steep dose-response curve was observed with PEC-15 cells and was similar to that observed in pigs injected intravenously with purified SLT-IIv (23). Vero cells were approximately 1,000,000-fold more sensitive to the inhibitory effects of SLT-IIv than were PEC-15 cells. This could be due to differences in the numbers of SLT-IIv receptors (6) or rates of metabolism of the cells. Inhibition of protein synthesis in Vero cells proved to be a more sensitive and rapid indicator of cytotoxicity due to SLT-IIv than was the classical Vero cell system (21, 22). A similar finding has been reported for Shiga toxin (for a review, see reference 18). As with Shiga toxin, SLT-IIv appeared to bind rapidly to PEC-15 and Vero cells, and it was not possible to neutralize the toxin 5 min after addition to the monolayers.

In this study we characterized purified SLT-IIv with regard to certain chemical, physical, and biological properties. The site for cleavage of the A subunit into A_1 and A_2 fragments was shown to be similar to that for Shiga toxin and SLT-I. Like Shiga toxin and other SLTs, SLT-IIv had a lower MW determined by gel filtration chromatography than would be expected from a 1-A-subunit–5-B-subunit arrangement. SLT-IIv differed from Shiga toxin and other SLTs with regard to pI and heat lability but had the molecular organization typical of Shiga toxin and SLTs. SLT-IIv was stable in the gut environment but, unlike other SLTs, did not appear to be enterotoxic. SLT-IIv was cytotoxic for porcine vascular endothelial cells and for Vero cells and appeared to bind rapidly to both cell lines. However, the relative insensitivity of porcine aortic endothelial cells to SLT-IIv compared with the sensitivity of Vero cells is worth further investigation. Perhaps porcine endothelial cells from tissues targeted in edema disease would show greater susceptibility.

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