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Properties of Dermonecrotic Toxin Prepared from Sonic Extracts of *Bordetella bronchiseptica*

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A toxin with dermonecrotic activity (DNT) was purified from sonic extracts of *Bordetella bronchiseptica* L3 of pig origin at phase I by chromatographic and electrophoretic methods. The purification procedure was one developed for obtaining the *Pasteurella multocida* DNT from sonic extracts with some modifications. Dermonecrotizing activity of *B. bronchiseptica*-purified DNT was increased by 600-fold compared with that of the crude extract, and the average yield was about 3%. The toxin was homogeneous, as determined by Ouchterlony double immunodiffusion, crossed immunoelectrophoresis, and disk isoelectric focusing in polyacrylamide gels. The toxin gave a single band on polyacrylamide disk gel electrophoresis (PAGE) and sodium dodecyl sulfate-SDS PAGE. The molecular weight of the toxin was ca. 190,000 ± 5,000, as determined by SDS-PAGE. The isoelectric point of the toxin was ca. 6.5 to 6.6. The minimal necrotizing dose of the toxin for guinea pigs was about 2 ng of protein per 0.1 ml, the 50% lethal dose per mouse was about 0.3 μg, and the minimal cytotoxic dose for embryonic bovine lung cells was about 2 ng/ml. The toxin was heat labile and sensitive to inactivation by trypsin, Formalin, and glutaraldehyde. The mildly trypsinized *B. bronchiseptica* DNT preparation dissociated into two polypeptide chains, with molecular weights of ca. 75,000 ± 4,000 (fragment 1) and ca. 118,000 ± 5,000 (fragment 2), after treatment with dithiothreitol-SDS or urea. Upon removal of dithiothreitol and urea from the dissociated DNT preparation, the fragments reassociated, and the DNT that was formed was indistinguishable from the native toxin.

The organisms belonging to the genus *Bordetella*, such as *Bordetella bronchiseptica*, *Bordetella pertussis*, and *Bordetella parapertussis*, produce dermonecrotic toxin (DNT) with similar biological properties and toxic activities in experimental animals (2, 6, 9, 12, 13, 15, 21, 24). The crude DNT preparations extracted from the three species of the bacteria were reported to be serologically related (10, 12). Purification of *B. pertussis* DNT has been described previously (2, 25, 26). Onoue et al. (26) purified *B. pertussis* DNT by sequential calcium phosphate gel treatment, salt fractionation, potassium phosphate precipitation, and column chromatography on DEAE-cellulose; however, the DNT preparation still contained both agglutinogen and protective antigens. Nakase et al. (25) obtained purified *B. pertussis* DNT by preparative polyacrylamide gel electrophoresis (PAGE) and reported that DNT is a protein with sugars.

Swine atrophic rhinitis (AR) is a disease characterized by severe necrosis of epithelia of the upper respiratory tract and by deformity and reduction both in volume and size of the nasal turbinates and snouts (5, 8, 18, 19, 23, 30, 36, 39). *B. bronchiseptica* causes marked loss of cilia accompanied by the characteristic morphological changes of the nasal mucosa, if the organisms at phase I (24) are intranasally inoculated into gnotobiotic or specific pathogen-free neonatal pigs (5, 8, 18, 19, 20, 30, 36, 39). Injection of pure cultures of *B. bronchiseptica* also results in severe nasal turbinate atrophy; therefore, *B. bronchiseptica* has been considered as a primary causative agent for swine AR. *B. bronchiseptica* DNT extracted from the phase I organisms has been considered as a virulence factor for the production of the turbinate atrophy in neonatal pigs (14) or in young mice (34).

In this report we describe the purification of DNT from sonicates of *B. bronchiseptica*. Some biological and physicochemical properties of the purified DNT were investigated.

MATERIALS AND METHODS

Assays. Dermonecrotic activity in guinea pigs was assayed as follows. Guinea pigs weighing about 300 g were depilated and injected intradermally with portions (0.1 ml) of twofold serial dilutions (in distilled water) of the preparations (25). Titers of samples were the reciprocal of the highest dilution showing a positive necrotic lesion more than 5 mm in diameter observed at 48 h after injection (22). One toxic unit (TU) was defined as the highest dilution causing necrotic lesion of ≥5 mm in diameter. The magnitude of toxicity was expressed as TU per milligram of DNT.

Four-week-old specific pathogen-free ddY mice were injected intraperitoneally with 0.2-ml portions as described previously (22). The 50% lethal dose for the mice was estimated by the method of Reed and Muench (29).

Cytotoxic activity for embryonic bovine lung cells was assayed by the method of Rutter and Luther (32), using the cells provided by P. D. Luther (Agricultural Research Council Institute for Research On Animal Disease, Berkshire, England).

Amounts of protein were estimated by the method of Lowry et al. (17) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Purification of DNT. The purification procedure was essentially that developed for obtaining the *Pasteurella*

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multocida DNT (23). Unless otherwise noted, all of the following steps were done at ca. 4°C.

(i) Stage 1: Preparation of crude DNT. *B. bronchiseptica* L3 (14) of pig origin was grown on the agar medium described by Bordet and Gengou (4) supplemented with 20% horse blood for 18 h at 37°C (about 60 petri dishes; diameter, 9 cm), harvested, and suspended in distilled water to a concentration of 10^{12} cells per ml by spectrophotometry (10^{10} cells per ml = optical density at 650 nm of 0.386) with a model 6/20 spectrophotometer (Coleman Instruments Division, The Perkin-Elmer Corp., Norwalk, Conn.). The suspension (40 ml) was disrupted by sonication, centrifuged, and filtered through a sterile 0.22-nm-pore membrane filter (pore size, 0.22 nm; Millipore Corp., Bedford, Mass.) (22). The filtrate was used as the crude DNT preparation.

(ii) Stage 2: DEAE-Sephacel column chromatography. The crude DNT preparation (40 ml) was applied to a DEAE-Sephacel column (2.5 by 30 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.2). The column was eluted at a flow rate of 30 ml/h with 50 ml of the same buffer, followed by a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions (10 ml each) with dermonecrotic activity were pooled (total volume, 500 ml), concentrated by ultrafiltration, and dialyzed against 0.05 M sodium phosphate buffer (pH 7.2). After the repeated chromatography, the preparation (referred to as DEAE eluate I [20 ml]) was applied to a second DEAE-Sephacel column and eluted in the same manner. Fractions (10 ml each) with the activity were pooled, concentrated, and dialyzed against 0.01 M Tris hydrochloride buffer (pH 7.2) containing 0.01 M NaCl (DEAE eluate II).

(iii) Stage 3: Sephadex G-200 gel filtration. DEAE eluate II (4 ml) was applied to a Sephadex G-200 column (3.5 by 85 cm) equilibrated with 0.01 M Tris buffer (pH 7.2) containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 6 ml/h. Fractions (3 ml each) with dermonecrotic activity were pooled and concentrated by lyophilization. The lyophilized sample was dissolved in 2 ml of distilled water and then dialyzed against 0.01 M phosphate buffer (pH 7.0) (Sephadex eluate I).

(iv) Stage 4: PAGE and acid precipitation. Disk PAGE was carried out by the method described by Davis (7). The Sephadex eluate I containing 200 μg of protein mixed with a sample gel was placed on the top of the gel containing 7.5% acrylamide and 0.27% *N,N*-methylenebisacrylamide (BIS). Electrophoresis was carried out at 4°C for 5 h at a constant current of 2 mA per tube (23). Gels were cut into strips that were 2 mm wide, and the DNT was extracted from each piece with 2 ml of distilled water to assay the dermonecrotic activity. Fractions with the activity were pooled and adjusted to pH 6.5 by the addition of acetic acid to precipitate the DNT in the eluates. The acid precipitate was dissolved in 1 ml of 0.01 M Tris buffer (pH 7.2) containing 0.1 M NaCl (disk eluate).

(v) Stage 5: Rechromatography on a Sephadex G-200 column. The disk eluate (1 ml) was again applied on a Sephadex G-200 column (1 by 76 cm) equilibrated with 0.01 M Tris buffer (pH 7.2) containing 0.1 M NaCl and eluted at a flow rate of 2 ml/h. Fractions (1 ml each) with the activity were pooled, concentrated by lyophilization, and referred to as Sephadex eluate II or purified DNT preparation.

Preparation of anti-DNT antisera. Anti-DNT antisera were prepared by injecting New Zealand White rabbits weighing about 2 kg with a crude (stage 1; 1 mg of protein per ml) or purified (stage 5; 0.1 mg of protein per ml) DNT preparations, by a previously described procedure (22). Sera with anti-DNT (neutralization) antibody titers of 64 were pooled, stored at −20°C, and used for immunological analysis. The neutralization antibody titer was estimated by a skin test in guinea pigs and expressed as the reciprocal of the highest serum dilution completely inhibiting necrotizing lesion formation by the purified DNT preparation (22).

Immunological procedures. The plate method of Ouchterlony (27) was employed with 1% agarose in a phosphate-buffered solution (pH 7.0) containing 0.85% NaCl. Crossed immunoelectrophoresis was performed with the LKB 2112 Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.) and immunoelectrophoresis kit. The general methodology presented in the LKB instruction manual and application note 249 and in the quantitative immunoelectrophoresis manual of Axelsen et al. (1) was followed. Crossed immunoelectrophoresis gels were pressed, washed, stained, and destained as described by Axelsen et al. (1).

Analytical PAGE. After PAGE was performed as described above, the gels were fixed, stained with Coomassie blue R-250 (Sigma), and destained. The location of DNT activity in 7.5% acrylamide gels, which were used to analyze the final purified DNT preparation (stage 5), was determined by cutting the gels into 1-mm sections, and the DNT was extracted from each section with 1 ml of distilled water to assay the dermonecrotic activity. The location of the activity was compared with that of the stained band, which had been subjected to PAGE under identical conditions.

Analytical isoelectric focusing. Analytical disk isoelectric focusing in polyacrylamide gels (a linear pH gradient of 3.5 to 9.5) was performed with the CD-12 disk electrophoresis apparatus (Toyo Kagaku Sangyo Corp., Tokyo, Japan). Samples (20 μl) were applied on top of the gels and electrofocused at a fixed maximum of 25 W and a maximum of 2,000 V and 50 mA for ca. 2 h at 4°C. Gels were fixed, stained with Coomassie blue R-250, and destained as described previously (23).

SDS-PAGE. The gels containing 7.5% acrylamide and 0.27% BIS in Tris buffer (pH 8.8)–0.1% in sodium dodecyl sulfate (SDS), were prepared by the method of Weber et al. (38). Methods for the preparation of sample gel and application of the heated sample in gels were described in a previous report (23). Electrophoresis was carried out at 4°C for 5 h at a constant current of 2 mA per tube. Then, the gels were fixed, stained with Coomassie blue R-250, and destained by diffusion in a solution of 10% acetic acid.

Determination of molecular weight by SDS-PAGE. The molecular weight was estimated by SDS-PAGE in parallel with markers of known molecular weights in 7.5% acrylamide gels containing 0.1% SDS (38). The markers included trypsin inhibitor (21,500), RNA polymerase from *Escherichia coli* α subunit (39,000), bovine serum albumin (68,000), RNA polymerase from *E. coli* β subunit (155,000), and RNA polymerase from *E. coli* β′ subunit (165,000) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). All the samples were reduced in the presence of 1% SDS and 5% β-mercaptoethanol. The gels were electrophoresed as described above. The molecular weight shown is the mean of 10 determinations.

Amino acid analysis of purified DNT. The amino acid composition of purified DNT (600 to 900 μg) was determined by use of a Nihon-Denshi (model JLC-5AH) amino acid analyzer, according to the procedure described previously (23).

Inactivation studies with purified DNT. The purified DNT containing 0.4 μg of protein per 0.1 ml was treated by heating at various temperatures (37, 56, and 70°C) for 30 or 60 min.

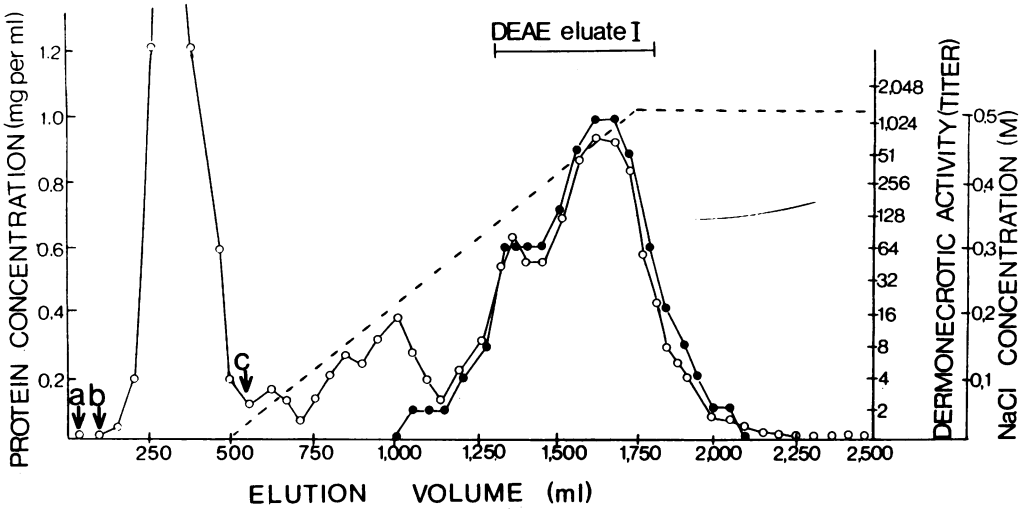

FIG. 1. DEAE-Sephacel chromatography of crude DNT of *B. bronchiseptica* L3. The crude DNT (stage 1) was applied (40 ml) to the column (2.5 by 30 cm) and eluted at a flow rate of 30 ml/h by a linear gradient of NaCl (-----) from 0 to 0.5 M at 4°C. The protein concentration (○) was determined by the method of Lowry et al. (17). Dermonecrotic activity (titer) (●) was the reciprocal of the highest sample dilution showing positive dermonecrotic lesions on the skin of guinea pigs 2 days after itradermal injection. a, Sample application; b, elution with 0.05 M sodium phosphate buffer (pH 7.2); c, elution with a NaCl gradient.

Treatment with trypsin (P-L Biochemicals, Inc., Milwaukee, Wis.) was done by incubating DNT with 0.25 or 0.5% crystal trypsin at 37°C for 2 h. Treatment with Formalin (0.5 or 1%) or glutaraldehyde (4 or 40 mM) (Eastman Kodak Co., Rochester, N.Y.) was done at 37°C for 1 h (22).

Trypsin treatment and thiol reduction of purified DNT. Treatment of the purified DNT preparation with trypsin (TT-DNT preparation) was carried out by the following procedures. The purified DNT (1 mg/ml) in 50 mM Tris buffer containing 1 mM EDTA (pH 8.2) was incubated with L-1-toluenesulfonylamide-2-phenylethyl-ketone-treated trypsin (1.5 μg/ml; Worthington Diagnostics, Freehold, N.J.) at 25°C for 60 min. At the end of the incubation, soybean trypsin inhibitor (Sigma) was added to 1.5 μg/ml (16). Thiol reduction of the purified DNT or TT-DNT preparations (1 mg/ml) in Tris buffer containing 1 mM EDTA (pH 8.2) was performed with 100 mM dithiothreitol (Wako Pure Chemical Inc., Tokyo, Japan) at 25°C for 60 min (thiol-reduced DNT or thiol-reduced TT-DNT preparations) (16).

Urea treatment of the thiol-reduced or thiol-nontreated TT-DNT. Urea treatment of the thiol-reduced or thiol-nontreated TT-DNT preparation was performed by adding

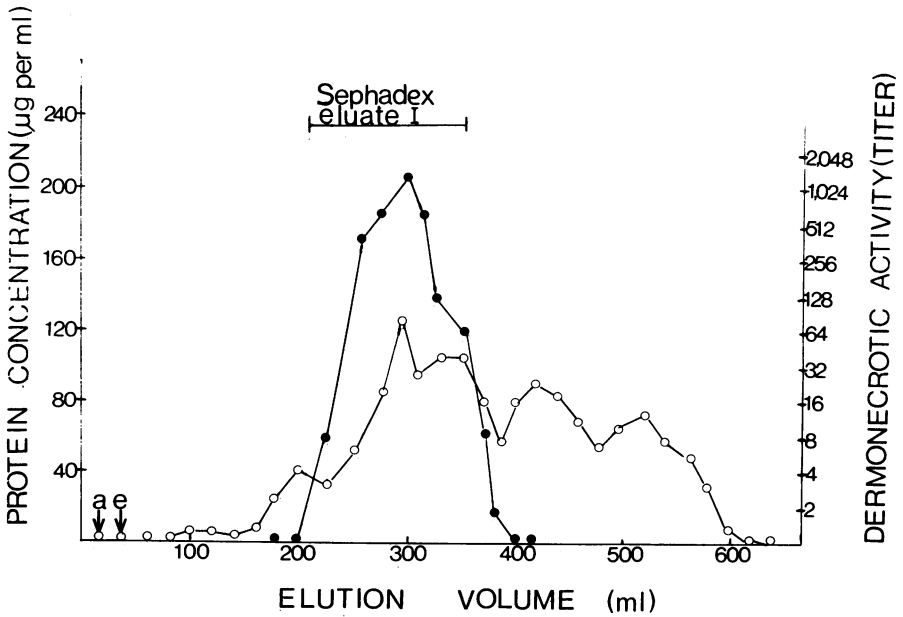

FIG. 2. An elution profile of gel filtration with a Sephadex G-200 column (3 by 76 cm) of DEAE eluate II (stage 3). A 4-ml portion was applied to the column and eluted at a flow rate of 6 ml/h in 0.01 M Tris buffer (pH 7.2) containing 0.1 M NaCl. The protein concentration (○) was determined by the method of Lowry et al. (17). Dermonecrotic activity (●) was expressed as described in the legend to Fig. 1. a, Sample application; e, elution with Tris buffer.

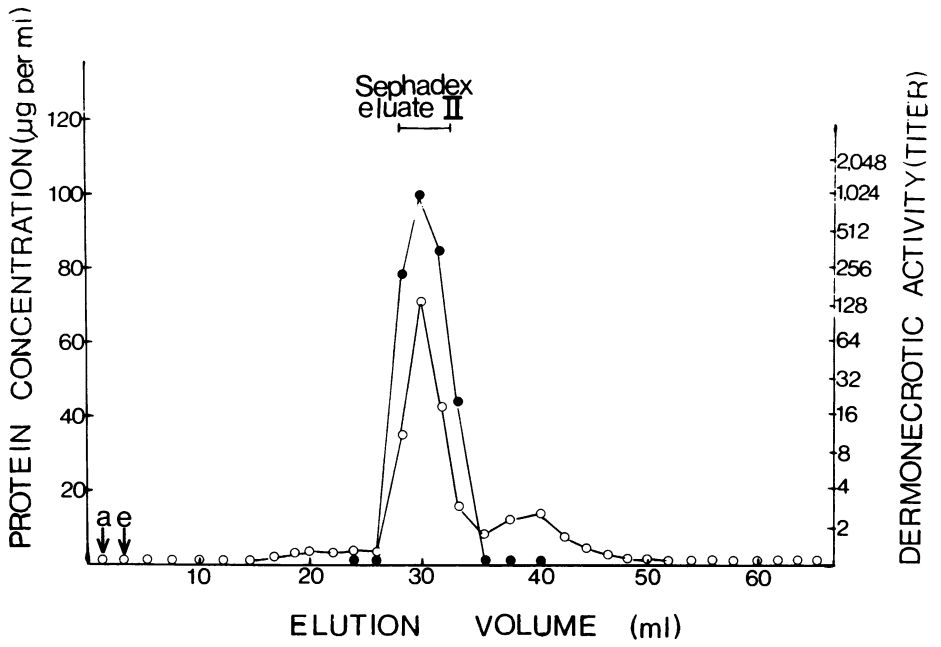

FIG. 3. An elution profile of gel filtration with a second Sephadex G-200 column (1 by 76 cm) of disk eluate (stage 4). A 1-ml portion was applied to the column and eluted at a flow rate of 2 ml/h in 0.01 M Tris buffer (pH 7.2) containing 0.1 M NaCl. The protein concentration (○) was determined by the method of Lowry et al. (17). Dermonecrotic activity (titer) (●) was determined as described in the legend to Fig. 1. a, Sample application; e, elution with Tris buffer.

100 μl of 0.25 M Tris buffer (pH 6.8) containing 7.2 M urea (Wako Pure Chemical) to each 20 μl of the DNT preparations for conventional or SDS-PAGE. For reversible dissociation of the thiol-reduced TT-DNT preparation, urea was added to the sample to a final concentration of 6 M (dissociated DNT preparation). The dissociated DNT preparation was dialyzed against 0.1 M sodium potassium phosphate buffer (pH 7.5) at 4°C for 48 h to remove urea and dithiothreitol from the sample (dialyzed DNT preparation).

RESULTS

Purification of DNT. Crude DNT (stage 1) was applied to a DEAE-Sephacel column, and a typical chromatographic profile of the column is shown in Fig. 1. The DEAE eluate I preparation was applied on a second DEAE-Sephacel column (stage 2), and the DEAE eluate II was applied to a Sephadex G-200 column (stage 3). Five protein peaks were obtained, with toxic activity in two of the peaks (Fig. 2). The Sephadex eluate I was subjected to PAGE (stage 4). The disk eluate was rechromatographed on a second Sephadex G-200 column (stage 5). The acid precipitation did not influence the yield of DNT. The Sephadex eluate II was referred to as the purified DNT preparation (Fig. 3). Toxic activity of the purified DNT preparation (stage 5) was increased by 600-fold compared with that of the crude extract (stage 1), and the average yield was about 3% (Table 1).

Purity of DNT. The crude DNT preparation (stage 1) gave at least three precipitation lines with anti-crude DNT antiserum, whereas the purified DNT preparation (stage 5) gave one of these lines by the immunodiffusion test. The two DNT preparations gave one fused precipitin line with anti-purified DNT antiserum by the immunodiffusion test, indicating the purity of the DNT tested (data not shown). The higher resolution techniques of crossed immunoelectrophoresis (Fig. 4), PAGE, analytical disk isoelectric focusing in polyacrylamide gels, and SDS-PAGE (Fig. 5, lane a) revealed homogeneity of the final purified DNT preparation (stage 5).

TABLE 1. Purification of DNT prepared from a sonic extract of *B. bronchiseptica* L3

Fraction	Total vol (ml)	Total protein (mg)[a]	Total dermonecrotic activity[b]	Specific dermonecrotic activity[c]	Relative dermonecrotic activity	Yield of dermonecrotic activity (%)
Crude DNT (stage 1)	40	18,253	$1,438 \times 10^3$	79	1	100
DEAE eluate I	20	148	$6,835 \times 10^2$	4,618	58.5	47.5
DEAE eluate II (stage 2)	4	61	$4,050 \times 10^2$	6,639	84.0	28.2
Sephadex eluate I (stage 3)	2	9.8	$1,018 \times 10^2$	10,388	131.5	7.1
Disk eluate (stage 4)	1	3.1	507×10^2	16,355	207.0	3.5
Sephadex eluate II[d] (stage 5)	1	0.91	431×10^2	47,363	599.6	3.0

[a] The protein concentration was determined by the method of Lowry et al. (17).
[b] Dermonecrotic activity (titer) was determined as described in the legend to Fig. 1. Total activity = total volume (milliliters) × dermonecrotic activity (titer) per 0.1 ml × 10.
[c] Specific activity = total activity per total protein (milligrams).
[d] Purified DNT.

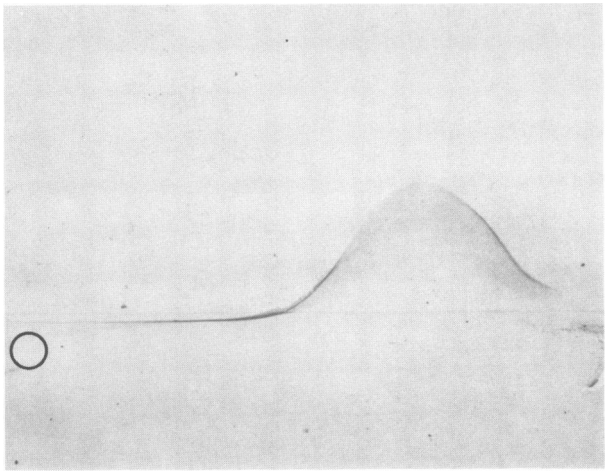

FIG. 4. Crossed immunoelectrophoresis of the purified DNT preparation of *B. bronchiseptica* L3 (stage 5) stained for protein with Coomassie blue R-250. A sample (100 μg of protein in 18 μl) was placed into a well (4 mm in diameter, indicated by the circle) that was cut into a gel composed of 1.2% (wt/vol) agarose (Boehringer Mannheim) in Veronal buffer (pH 8.6) and subjected to electrophoresis (anode to the right) at 2.5 V/cm for 90 min at 10°C. For the second dimension, the upper part of the gel (55 cm^2) was composed of 1.2% agarose (10 ml) containing 0.5 ml of anti-crude DNT antiserum with a neutralization antibody titer of 32 against crude DNT. Electrophoresis (anode at top of gel) was performed at 2.5 V/cm for 3 h at 10°C.

The 280/260 nm absorbance ratio of the purified DNT was ca. 1.9, thus indicating the absence of contaminating nucleic acid. The (pI) of the purified DNT was determined to be ca. 6.5 to 6.6 (data not shown). The molecular weight of the purified DNT was determined to be ca. 190,000 ± 5,000 by SDS-PAGE (Fig. 5, lane a).

Toxic activities of purified DNT. The minimal necrotizing dose of the purified DNT preparation for guinea pigs was ca. 2 ng of protein. The 50% lethal dose for mice was ca. 0.3 μg; splenic atrophy was observed in all of the surviving mice injected with the preparation (0.2 to 0.3 μg of protein per mouse). The minimal cytotoxic dose for embryonic bovine lung cells was ca. 2 ng/ml.

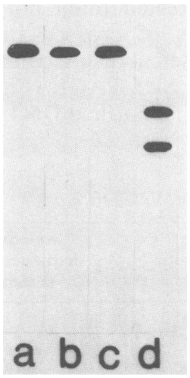

FIG. 5. SDS-PAGE of the purified DNT (stage 5) and trypsin-treated (1.5 μg/ml) or thiol-reduced (final concentration, 100 mM) DNT preparations or both of *B. bronchiseptica* L3 stained for proteins with Coomassie blue R-250. Polyacrylamide gels containing 7.5% acrylamide and 0.27% BIS in Tris buffer (pH 8.8)–0.1% in SDS were used. Approximately 50 μg of protein was applied on each gel. Lanes: a, purified DNT; b, TT-DNT; c, thiol-reduced DNT; d, thiol-reduced TT-DNT. Migration was from top to bottom.

TABLE 2. Amino acid composition of the purified DNT preparation of *B. bronchiseptica* L3

Amino acid	mol/100 mol of amino acids
Aspartic acid	11.24
Threonine	6.15[a]
Serine	5.29[a]
Glutamic acid	12.01
Proline	5.34
Glycine	8.64
Alanine	11.69
Valine	7.73
Methionine	1.96
Isoleucine	4.64
Leucine	7.87
Tyrosine	3.11
Phenylalanine	3.32
Lysine	5.02
Histidine	1.19
Arginine	4.47
Half-cystine	0.31
Tryptophan	ND[b]

[a] The value was corrected by factors of 1.13 for threonine and 1.11 for serine (37).
[b] ND, Not determined due to destruction.

Inactivation study and amino acid composition of purified DNT. Dermonecrotic activity of the purified DNT (5×10^5 TU per mg of protein) was completely inactivated (<1 TU) by heating at 70°C for 30 min and partially inactivated (2×10^3 TU) by heating at 56°C for 30 min. The activity was reduced by treatments with trypsin (0.25% = 5×10^2 TU; 0.5% = <1 TU), Formalin (0.5% = 5×10^2 TU; 1% = <1 TU), or glutaraldehyde (4 mM = 10^3 TU; 40 mM = <1 TU), depending on the concentration. The amino acid composition of the purified DNT preparation is shown in Table 2.

Dissociation of purified DNT. The purified DNT, TT-DNT, or thiol-reduced DNT preparation migrated as a single protein band on conventional PAGE. On SDS-PAGE, each sample moved as a single component with a molecular weight of ca. 190,000 (Fig. 5, lanes a through c). However, when the thiol-reduced TT-DNT preparation was subjected to SDS-PAGE, the preparation dissociated into two protein bands (Fig. 5, lane d). The faster moving component was designated as fragment 1 and the slower one as fragment 2. The molecular weights of fragments 1 and 2 were ca. 75,000 ± 4,000 and ca. 118,000 ± 5,000, respectively. The dissociation was not observed on conventional PAGE. Treatment of samples with SDS resulted in irreversible loss of toxicity and antigenicity.

Reversible dissociation of purified DNT. Treatment of the TT-DNT preparation individually with either urea (Fig. 6, lanes a and d) or dithiothreitol (Fig. 5, lane c) caused no changes in electrophoretic migration. However, when the thiol-reduced TT-DNT preparation was treated with 6 M urea and subjected to conventional PAGE, the preparation dissociated into two protein bands (Fig. 6, lane b) (dissociated DNT preparation). On SDS-PAGE, the dissociated DNT preparation gave two protein components (Fig. 6, lane e), corresponding to fragments 1 and 2, respectively.

Mild treatment of purified DNT with trypsin (TT-DNT) and treatment of the TT-DNT preparation with dithiothreitol (thiol-reduced TT-DNT) or urea (urea-treated TT-DNT)

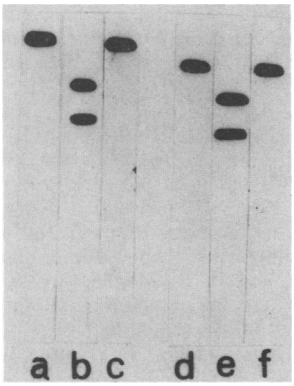

FIG. 6. Conventional (lanes a, b, and c) and SDS (lanes d, e, and f) PAGE of the urea-treated but thiol-nontreated TT-DNT (lanes a and d), the dissociated DNT (lanes b and e) preparations, and the dialyzed DNT preparation after removal of dithiothreitol and urea from the dissociated DNT preparation by dialysis (lanes c and f) of *B. bronchiseptica* L3 stained for proteins with Coomassie blue R-250. The method for the dissociation of the purified DNT preparation is described in the text. Polyacrylamide gels containing 7.5% acrylamide and 0.27% BIS in Tris buffer (pH 8.8), with or without 0.1% in SDS, were used. Approximately 50 μg of protein was applied on each lane of the slab gel. Electrophoresis was carried out as described in the text. Migration was from top to bottom.

alone had no effect on dermonecrotic activity (Table 3). After treatment of the TT-DNT preparation with dithiothreitol and urea, the thiol-reduced and urea-treated TT-DNT (dissociated DNT preparation) did not show dermonecrotizing activity. However, the dithiothreitol and urea were removed from the dissociated DNT preparation by dialysis (dialyzed DNT preparation), and the original dermonecrotic activity was restored. On conventional PAGE, the dialyzed DNT preparation migrated as a single protein band (Fig. 6, lane c) at a position corresponding to that of the purified DNT preparation (Fig. 5, lane a). The dialyzed DNT preparation was electrophoresed, and proteins were eluted from each of the sliced gels. The toxic activity was detected only at the single protein band of the purified DNT preparation (Fig. 5, lane a). The dialyzed DNT preparation formed a single precipitin line by the immunodiffusion test, which fused completely with that formed between the anti-DNT antiserum and the purified DNT preparation (Fig. 7).

DISCUSSION

The purified *B. bronchiseptica* DNT (stage 5) was shown to be homogeneous by Ouchterlony double immunodiffusion, crossed immunoelectrophoresis (Fig. 4), disk isoelectric focusing in polyacrylamide gels, and SDS-PAGE (Fig. 5, lane a). Apparently, it was free of at least two other antigens that were presented in the crude extract, because antibodies against these antigens presented in anti-crude DNT antiserum did not form precipitin lines with the purified DNT. Anti-purified DNT antiserum was also shown to be free of antibodies against K and O antigens (24) in studies reported elsewhere, and the DNT preparation was found to be free from histamine-sensitizing factor (31) and hemagglutinin (3) (data not shown).

The purified *B. bronchiseptica* DNT was presumably protein, having a molecular weight of ca. 190,000. Some biological properties, amino acid composition, and toxic activities of the *B. bronchiseptica*-purified DNT were similar to those of the *P. multocida*-purified DNT (23); however, the molecular weight of the *B. bronchiseptica* DNT (ca. 190,000) was larger than that of *P. multocida* (ca. 160,000), as determined by SDS-PAGE (23). The pI of *P. multocida* DNT was ca. 4.7 to 4.8 (23), whereas that of *B. bronchiseptica* DNT was ca. 6.5 to 6.6. Cross-neutralization tests between the *P. multocida*- and *B. bronchiseptica*-purified DNT preparations revealed that the toxins were serologically and immunologically distinct from each other (data not shown). Previously, we reported a similar observation with the crude DNT preparations (22).

In previous reports (16; T. Nakai and K. Kume, submitted for publication), we described the following on *P. multocida* DNT. (i) The purified DNT preparation consists of a single polypeptide chain (intact toxin) with a molecular weight of ca. 160,000, which can be cleaved with trypsin. (ii) The toxin molecular weight may be composed of three polypeptide chains (fragments a, b, and c), which have distinct antigenicities, are linked by at least two disulfide bridges and also are associated by noncovalent bonds that are dissociable with agents such as SDS or urea. The purified DNT preparation of *B. bronchiseptica*, consisting of a single polypeptide chain with a molecular weight of ca. 190,000, was reversibly dissociated into two polypeptide chains, with molecular weights of ca. 75,000 (fragment 1) and ca. 118,000 (fragment 2), by treatment with dithiothreitol and urea under similar conditions employed for the reversible dissociation of *P. multocida* DNT (Nakai and Kume, submitted). It seems justified to conclude that trypsin cleaves to produce fragments 1 and 2, and these must be linked by one or more disulfide bonds.

Elling and Pedersen (11, 28) reported that the toxigenic *P. multocida* strains induced swine AR which was character-

TABLE 3. Change in toxicity on treatment[a]

Determinants	Dermonecrotizing activity (10^5 TU/mg of DNT)[b]
Purified DNT	4.3–4.7
TT-DNT	3.8–4.5
Thiol-reduced TT-DNT	3.7–4.6
Urea-treated TT-DNT	3.9–4.4
Thiol-reduced and urea-treated TT-DNT[c]	<0.00001
Thiol-reduced and urea-treated TT-DNT[d]	3.8–4.4

[a] This method for the dissociation of the toxin is described in the text.
[b] Data are expressed as ranges obtained in two tests conducted at different times by using two lots of samples.
[c] Before dialysis.
[d] After dialysis.

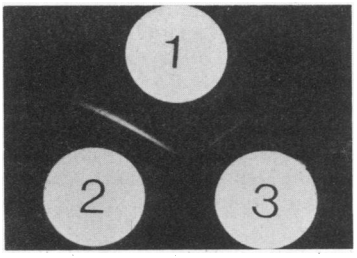

FIG. 7. Gel diffusion analysis of the purified DNT and the dialyzed urea- and thiol-treated TT-DNT preparations of *B. bronchiseptica* L3 with rabbit anti-purified DNT antiserum. The antiserum had a neutralization antibody titer of 32 against the purified DNT. Well 1 (4 mm in diameter) contained anti-purified DNT antiserum (18 μl); wells 2 and 3 contained purified DNT and dialyzed DNT preparations (20 μg of protein), respectively.

ized by the impaired osteogenesis in an early stage of the infection, although the infected nasal mucosa were reported to have normal structures. They concluded that the toxigenic *P. multocida* strains are able to induce the nasal turbinate atrophy in neonatal pigs. *B. bronchiseptica* DNT or *P. multocida* DNT was reported to be an intracellular component that was not secreted by the actively growing bacterial cells, although a low amount of cell-free DNT was detected in the culture supernatant after autolysis of the cells (21). It has been shown that *B. bronchiseptica* colonizes on the swine nasal mucosa and cause severe chronic catarrhal inflammatory reactions, whereas *P. multocida* is established poorly on the normal swine nasal mucosa but does not cause much damage to the mucosa (20, 39; T. Oyamada, T. Yoshikawa, H. Yoshikawa, M. Shimizu, T. Nakai, and K. Kume, Jpn. J. Vet. Sci., in press). Thus, a chemical irritation of acetic acid or *B. bronchiseptica* infection prior to the exposure to the toxigenic strains of *P. multocida* was employed for the colonization of *P. multocida* on the swine nasal mucosa (28, 33).

The nasal tissue fragments removed from the turbinate cross sections of neonatal pigs which were incubated in the medium containing the purified DNT preparations of *P. multocida* and *B. bronchiseptica* were equally injured by the toxins (T. Nakai, unpublished data). Colonization of *B. bronchiseptica* or *P. multocida* on the swine nasal mucosa and production of DNT after multiplication of the organisms on the nasal mucosa, therefore, are essential for nasal mucosal damage. A homogeneous *B. bronchiseptica* DNT preparation of biologically active material and the resulting anti-*B. bronchiseptica* DNT antiserum available in the present study would be useful tools to clarify the hypothesis that *B. bronchiseptica* DNT is responsible for the pathogenesis of swine AR.

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