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Purification of Dermonecrotic Toxin from a Sonic Extract of *Pasteurella multocida* SP-72 Serotype D

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A procedure was developed to purify dermonecrotic toxin (DNT) from a sonic extract of a serotype D strain of *Pasteurella multocida*. Sonic extract containing DNT was applied to a DEAE-Sephacel column and eluted by a linear gradient of NaCl. Upon rechromatographing, fractions with dermonecrotic activity for guinea pigs were applied on a second Sephacel column, and a pooled fraction with the toxic activity was filtered through a Sephadex G-200 column. Pooled fractions with the toxic activity were subjected to polyacrylamide disc gel electrophoresis (PAGE), and the toxic substance was eluted from each sliced gel. Eluted fractions with the toxic activity were rechromatographed on a second Sephadex G-200 column, and a pooled fraction with high dermonecrotic activity was referred to as a purified DNT. The activity of purified DNT was increased by 1,000 times, and the average yield was about 1.8%. The purified DNT was homogeneous as determined by Ouchterlony double immunodiffusion, crossed immunoelectrophoresis, and thin-layer isoelectric focusing in polyacrylamide gels and gave a single band on PAGE and sodium dodecyl sulfate-PAGE. The molecular weight of the toxin was ca. 160,000 as determined by sodium dodecyl sulfate-PAGE. The isoelectric point of the toxin was ca. 4.7 to 4.8. Amino acid analysis of the purified DNT revealed that the toxin was composed of characteristically high proportions of glutamic acid, aspartic acid, glycine, proline, alanine, and leucine. The minimal necrotizing dose of the toxin was about 1 ng of protein, and the 50% lethal dose per mouse was 0.2 μg. The purified DNT was heat labile and sensitive to inactivation by trypsin, Formalin, and glutaraldehyde.

Coinoculation of *Bordetella bronchiseptica* and *Pasteurella multocida* causes severe necrosis of the epithelia of the upper respiratory tract and deformities and atrophy of the turbinates and snout, a condition known as atrophic rhinitis (AR) in specific-pathogen-free or in gnotobiotic piglets (21, 23, 24). Phase I organisms of *B. bronchiseptica* cause marked loss of cilia accompanied by morphological changes in the nasal mucosa after intranasal inoculation into gnotobiotic piglets (30). Injection of pure cultures of *B. bronchiseptica* alone often results in typical nasal turbinate atrophy in gnotobiotic piglets (13, 28). Intranasal administration of *P. multocida* alone, on the other hand, fails to produce gross lesions typical of AR (24), and the nasal mucosa of pigs experimentally inoculated with *P. multocida* shows almost normal morphology, although the bacteria had been recovered from nasal turbinates (14). Therefore, *B. bronchiseptica* has been considered as a primary causative agent in AR. Some evidence, however, indicates that *P. multocida* enhances pathogenicity of *B. bronchiseptica* (21, 23, 24).

Some strains of *P. multocida* produce a factor causing erythema and necrosis in the skin of guinea pigs and mortality or splenotoxicity in mice (26; T. Nakai, A. Sawata, M. Tsuji, and K. Kume, Am. J. Vet. Res., in press). This factor, designated dermonecrotic toxin (DNT), is a heat-labile protein (24, 26; Nakai et al., in press) and is produced only by those strains of *P. multocida* belonging to a capsular (3) serotype D (23, 26). Intranasal inoculation of toxigenic strains of *P. multocida* alone into gnotobiotic piglets results in formation of mild-to-moderate lesions of the snout, but not the gross lesions typical of AR (24). No correlation has been found between incidences of AR and isolation of toxigenic *P. multocida* in an epidemiological survey study with field pigs (26). Hence, a primary role of toxigenic *P. multocida* in the pathogenicity of AR is not well defined.

The DNT of *B. bronchiseptica* has been suggested as a factor responsible for nasal turbinate atrophy, a typical AR lesion, in piglets (9). Sonicated extracts of *B. bronchiseptica* containing DNT have produced AR in specific-pathogen-free piglets by intranasal instillation. Similar macroscopic lesions have been experimentally produced by live phase I organisms of *B. bronchiseptica* in young mice (25) and newborn rabbits (12). Our interest in analyzing the possible role of *P. multocida* DNT in the occurrence of swine AR, therefore, prompted us to develop a purification scheme for obtaining a *P. multocida* DNT preparation of biologically active material. This paper presents the results of our studies concerning the extraction and purification of DNT from the serotype D strain SP-72 of *P. multocida*. Some biological 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 examined (16). Titers of samples were the reciprocal of the highest sample dilution showing a positive necrotic lesion more than 5 mm in diameter at 48 h after injection (Nakai et al., in press).

Four-week-old ddY mice were injected intraperitoneally with 0.2-ml portions as described previously (Nakai et al., in press). The 50% lethal dose (LD_{50}) for the mice was estimated by the method of Reed and Muench (22).

Amounts of protein were estimated by the method of

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Lowry et al. (11) with bovine serum albumin (Sigma Chemical Co.) as the standard.

Purification of DNT. Unless otherwise noted, all of the following steps were done at ca. 4°C.

(i) Stage 1: preparation of crude DNT. *P. multocida* serotype D (3) strain SP-72 (Nakai et al., in press) was grown from lyophilized cultures on yeast extract-Proteose Peptone (Difco Laboratories)-cystine agar medium (7) for 6 h at 37°C, 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, Perkin Elmer Co.). The suspension was treated by sonication, centrifuged, and filtered through a sterile 0.22-nm-pore membrane filter (Millipore Corp.) (Nakai et al., in press). The filtrate was used as the crude DNT of *P. multocida*.

(ii) Stage 2: DEAE-Sephacel column chromatography. The crude DNT (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 500 ml of the same buffer followed by a linear gradient of 0 to 0.5 M NaCl in the same buffer (pH 7.2). Fractions (10 ml) with dermonecrotic activity were pooled, concentrated by ultrafiltration, and dialyzed against 0.05 M sodium phosphate buffer (pH 7.2). Upon rechromatography, 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) with the activity were pooled, concentrated, and dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 0.1 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) 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. Disc polyacrylamide gel electrophoresis (PAGE) was carried out by the methods described by Davis (5). The Sephadex eluate I containing 200 μg of protein was mixed with the sample polyacrylamide gel containing 3% acrylamide and 0.27% *N,N*-methylenebisacrylamide (BIS) and placed on the top of the gel containing 10% acrylamide and 0.27% BIS. Electrophoresis was carried out at 4°C for 5 h at a constant current of 2 mA per tube. Gels were cut into strips 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 (disc eluate).

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

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) or purified (stage 5) DNT preparation adjusted to 10 mg of protein per ml as follows. The DNT diluted twofold with distilled water was inactivated with 1% Formalin at 37°C for 1 h, and the formalinized sample was injected subcutaneously into rabbits twice per week, beginning with 0.5 ml given with 0.5 ml of Freund complete adjuvant (Difco Laboratories). Subsequent 1-ml injections of the sample were given subcutaneously 20 times at 3-day intervals. One week after the final injection, blood samples were withdrawn. In each immunization group, the sera with neutralization antibody titers of 1:64 (Nakai et al., in press) against purified DNT in guinea pigs were pooled and stored at −20°C.

Immunological procedures. The plate method of Ouchterlony (20) was employed with 1% agarose in a phosphate-buffered solution (pH 7.0) containing 0.85% NaCl. Crossed immunoelectrophoresis (IEP) was performed with the LKB 2117 Multiphor apparatus and IEP kit. The general methodology presented in the LKB instruction manual and application note 249 and in the quantitative IEP manual of Axelsen

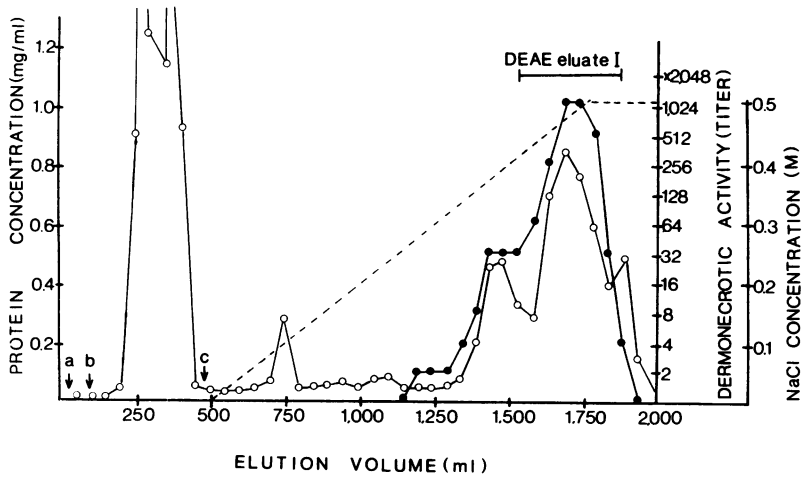

FIG. 1. DEAE-Sephacel chromatography of crude DNT of *P. multocida* SP-72. The crude DNT (stage 1) was applied (40 ml) to the column (2.5 by 30 cm) and eluted at a flow rate of 30 ml/h by a linear gradient of NaCl (– – – –) from 0 to 0.5 M at 4°C. The protein concentration (○) was determined by the method of Lowry et al. (11). Dermonecrotic activity (titer) (●) was the reciprocal of the highest sample dilution showing positive dermonecrotic lesions on the skin of guinea pigs 2 days after intradermal injection. a, Sample application; b, elution with 0.05 M sodium phosphate buffer (pH 7.2); c, elution with an NaCl gradient.

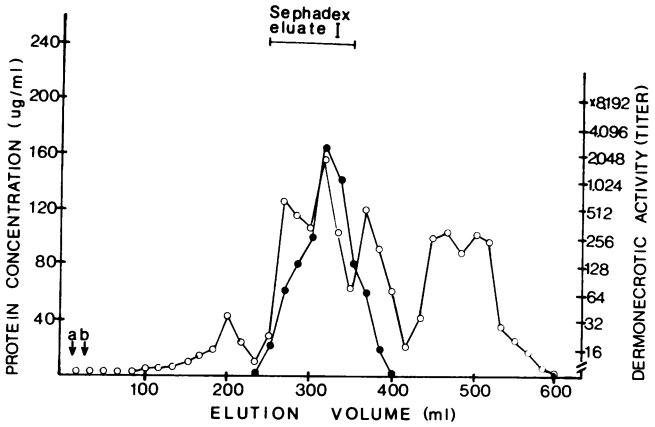

FIG. 2. An elution profile of gel filtration with a Sephadex G-200 column (3.5 by 85 cm) of DEAE eluate II (stage 2). A 4-ml portion was applied to the column and eluted at a flow rate of 6 ml/h in 0.01 M Tris buffer containing 0.1 M NaCl (pH 7.2). The protein concentration (○) was determined by the method of Lowry et al. (11). Dermonecrotic activity (titer) (●) was as described in the legend to Fig. 1. a, Sample application; b, elution with Tris buffer.

et al. (1) was followed. Crossed IEP 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, and destained. The location of DNT activity in the monomer concentration gels 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 the location of Coomassie blue-staining bands in gels subjected to PAGE under identical conditions.

Analytical isoelectric focusing. Analytical thin-layer isoelectric focusing in polyacrylamide gels was performed with the LKB 2117 Multiphor electrophoresis apparatus and the gel plates (a linear pH gradient of 3.5 to 9.5) as recommended by the manufacturer, except that samples (20 μl) were applied directly to the gel surface (2 cm from the cathode) and were electrofocused at a fixed maximum of 25 W and a maximum of 2,000 V and 50 mA for ca. 1 h at 4°C. Gels were fixed, stained with Coomassie blue R-250, and destained as recommended by the manufacturer, except that the fixed gels were soaked for 15 min in destaining solution before staining.

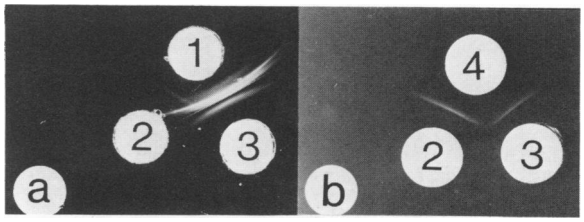

FIG. 3. Gel diffusion analysis of crude (stage 1) and purified (stage 5) DNTs of *P. multocida* SP-72 with rabbit anti-crude DNT (a) or anti-purified DNT (b). Each antiserum had a neutralization antibody titer of 1:64 against purified DNT. Well 1 (4 mm in diameter) contained rabbit anti-crude DNT antiserum (18 μl), wells 2 and 3 contained purified (20 μg of protein) and crude (200 μg of protein) DNTs, respectively, and well 4 contained anti-purified DNT antiserum (18 μl).

SDS-PAGE. Polyacrylamide gels containing 10% 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. (29). A sample of about 50 μg of protein in 50 μl of Tris buffer (pH 6.8) in SDS was heated for 1 min at 100°C. β-Mercaptoethanol was added to 5% before heating. Finally, 1 drop of glycerol was added, and the samples were layered under the buffer directly on the gels. Electrophoresis was performed at room temperature for 15 h at a constant current of 2 mA per tube. Then the gels were stained with Coomassie blue R-250 and destained by diffusion in a solution of 10% methanol and 10% acetic acid.

Determination of the molecular weight of purified DNT by SDS-PAGE. The molecular weight of the purified DNT was estimated by PAGE in parallel with markers of known molecular weight in 10% monomer concentration gels containing 0.1% SDS. The markers ranged in molecular weight from 21,500 to 165,000 and 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 Corp.). All samples were reduced in the presence of 1% SDS and 5% β-mercaptoethanol. The gels were run as described above. The distance of migration was plotted against the log of the molecular weights, giving a straight line (29). The molecular weight of the purified DNT was determined by comparison with this plot. The molecular weight shown is the mean of 10 determinations.

Amino acid analysis of purified DNT. The amino acid composition of purified DNT was determined by use of a Nihon-Denshi (model JLC-5AH) amino acid analyzer. A

TABLE 1. Purification of DNT prepared from a sonic extract of the serotype D strain SP-72 of *P. multocida*

Fraction	Total vol (ml)	Total protein[a] (mg)	Total dermonecrotic activity[b]	Specific dermonecrotic activity[c]	Realtive dermonecrotic activity	Yield of dermonecrotic activity (%)
Crude DNT (stage 1)	40	21,886	$2,095 \times 10^3$	96	1	100
DEAE eluate I	20	162	$1,049 \times 10^3$	6,470	67.4	50.1
DEAE eluate II (stage 2)	4	53	$7,092 \times 10^2$	13,381	139.4	33.9
Sephadex eluate I (stage 3)	2	8.2	$2,048 \times 10^2$	24,976	260.1	9.8
Disc eluate (stage 4)	1	1.6	824×10^2	51,552	536.7	3.9
Sephadex eluate II[d] (stage 5)	1	0.38	372×10^2	97,895	1,019.7	1.8

[a] Protein concentration was determined by the method of Lowry et al. (11).
[b] Dermonecrotic activity (titer) was determined as described in the legend to Fig. 1. Total activity = total volume (milliliters) × dermonecrotic activity (titer)/0.1 ml × 10.
[c] Specific activity = total activity/total protein (milligrams).
[d] Purified DNT.

sample (500 μg of protein) was hydrolyzed by heating in 2 ml of 5.7 N HCl at 110°C for 24 h in a vacuum-sealed tube. Then the mixture was dried by evaporation and dissolved in 2 ml of 0.2 M citrate buffer (pH 2.2). A 0.8-ml sample was examined in the analyzer.

Inactivation studies with purified DNT. The purified DNT containing 0.2 μg of protein per 0.1 ml was treated by heating at various temperatures (37, 56, or 70°C) for 30 or 60 min. Treatment with trypsin (P-L Biochemicals Co.) 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.) was done at 37°C for 1 h (Nakai et al., in press).

RESULTS

Purification of DNT. Crude DNT (stage 1) prepared by sonication of a serotype D strain SP-72 of *P. multocida* was applied to a DEAE-Sephacel column, and a typical chromatographic profile on the column is shown in Fig. 1. Fractions with dermonecrotic activity were pooled, concentrated, and dialyzed (DEAE eluate I). Upon rechromatographing, the DEAE eluate I preparation was then applied on a second DEAE-Sephacel column (stage 2). A pooled fraction with the toxic activity (DEAE eluate II) was filtered through a Sephadex G-200 column (stage 3). Six protein peaks were obtained, and the toxic activity was found in three of the peaks (Fig. 2). Fractions with the toxic activity were pooled, lyophilized, dissolved in distilled water, dialyzed, and subjected to PAGE (stage 4) (Sephadex eluate I). After PAGE, the substance was eluted from each sliced gel. Eluted fractions with the toxic activity were pooled and rechromatographed on a second Sephadex G-200 column (stage 5). The pooled fraction having a high dermonecrotic activity (Sephadex eluate II) was referred to as purified DNT. Toxic activity was increased by 1,000 times, and the average yield was about 1.8% (Table 1).

Purity of DNT. The crude DNT (stage 1) gave at least five precipitation lines with anti-crude DNT antiserum, whereas the purified DNT (stage 5) gave one of these precipitation lines (Fig. 3a), and the two DNT preparations gave one line with anti-purified DNT antiserum corresponding to each other (Fig. 3b) by the agar gel diffusion tests. The higher-resolution techniques of crossed IEP (Fig. 4), PAGE, analytical thin-layer isoelectric focusing in polyacrylamide gels (Fig. 5), and SDS-PAGE (Fig. 6) revealed homogeneity of the final purified DNT preparation (stage 5).

The 280/260 nm absorbance ratio of the purified DNT was ca. 1.5, thus indicating the absence of contaminating nucleic acid. The isoelectric point (pI) of the purified DNT was determined to be ca. 4.7 to 4.8 (Fig. 5). The molecular weight of the purified DNT was determined to be about 160,000 by SDS-PAGE (Fig. 6).

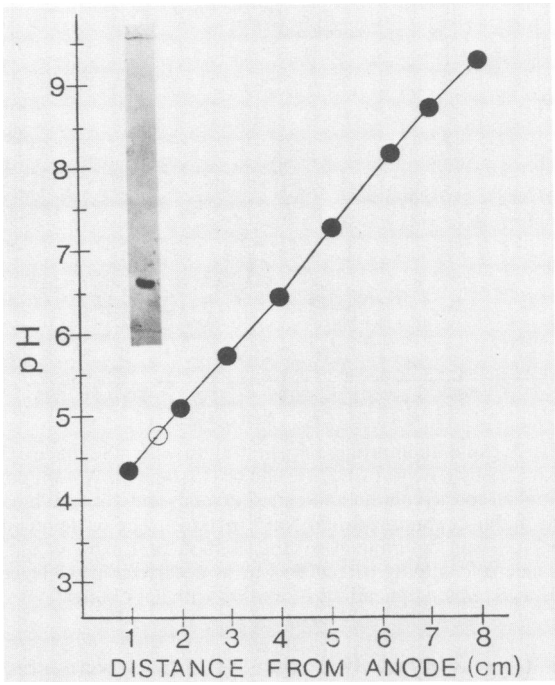

FIG. 5. Analytical thin-layer isoelectric focusing of purified DNT of *P. multocida* SP-72 (stage 5) stained for protein with Coomassie blue R-250 (cathode at top of gel). The sample (100 μg) was isoelectrically focused in the polyacrylamide gel containing carrier Ampholine, pH 3.5 to 9.5. Symbols: ●, pH at the indicated distance from the anode; ○, isoelectric point of the purified DNT (stage 5).

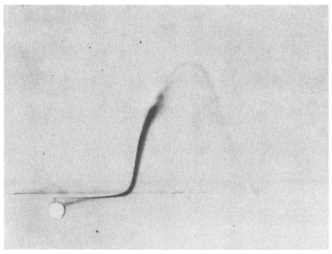

FIG. 4. Crossed IEP of purified DNT of *P. multocida* SP-72 (stage 5) stained for protein with Coomassie blue R-250. A sample (100 μg of protein in 18 μl) was placed into well (4 mm in diameter, indicated by the circle) that was cut into a gel composed of 1.2% (wt/vol) agarose (Boehringer Mannheim Corp.) in Veronal buffer (pH 8.6) and subjected to electrophoresis (anode to 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 1:32 against crude DNT. Electrophoresis (anode at top of gel) was performed at 2.5 V/cm for 3 h at 10°C.

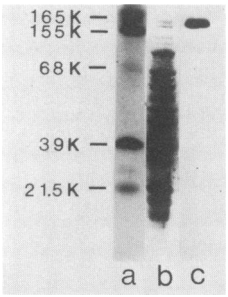

FIG. 6. SDS-PAGE of crude (stage 1) and purified (stage 5) DNTs of *P. multocida* SP-72 stained for proteins with Coomassie blue R-250. Polyacrylamide gels containing 10% acrylamide and 0.27% BIS in Tris buffer, (pH 8.8), 0.1% in SDS, were used. Lanes: a, marker (20 μg); b, crude DNT (100 μg); c, purified DNT (50 μg). The numbers on the left show the molecular weights of the markers in thousands (K): trypsin inhibitor (21.5K), RNA polymerase from *E. coli* α subunit (39K), bovine serum albumin (68K), RNA polymerase from *E. coli* β subunit (155K), and RNA polymerase from *E. coli* β′ subunit (165K). SDS-PAGE was carried out as described in the text.

Dermonecrotic and lethal activities of purified DNT. Both dermonecrotic activity for guinea pigs and lethal activity for mice of the purified DNT were examined as described above. The minimal necrotizing dose (MND) of purified DNT was 1 ng of protein, and the LD_{50} per mouse was 0.2 μg. Splenic atrophy was observed in all of the surviving mice injected with purified DNT (0.1 to 0.2 μg of protein per mouse).

Inactivation study and amino acid composition of purified DNT. Dermonecrotic activity of the purified DNT was completely inactivated by heating at 70°C for 30 min and partially at 56°C for 30 min (Table 2). The activity was reduced by treatments with trypsin, Formalin, or glutaraldehyde, depending on the concentration. The amino acid composition of purified DNT is shown in Table 3.

DISCUSSION

Organisms belonging to the genus *Bordetella*, such as *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*, are well known to produce DNTs with similar toxic activities for experimental animals (2, 7, 8, 10, 23; Nakai et al., in press). Crude DNTs extracted from *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis* are closely related serologically (6, 8). A previous study in our laboratory indicated that crude DNTs of *P. multocida* and *B. bronchiseptica* are serologically different, although they have similar biological properties and toxic activities for experimental animals (Nakai et al., in press). Onoue et al. (19) purified *B. pertussis* DNT by sequential calcium phosphate gel treatment, salt fractionation, potassium phosphate precipitation, and by column chromatography on DEAE-cellulose; however, the DNT still contained both agglutinogen and protective antigens. Nakase et al. (16) obtained purified *B. pertussis* DNT by preparative PAGE and reported that DNT is a protein with sugar. The entire molecule of their purified toxin has a sedimentation coefficient of 1.4. *B. bronchiseptica* DNT, however, has never been obtained in a purified state, probably because of its instability. The purified DNT available in the present study would clarify the relationship of DNT of *P. multocida* to other DNTs.

TABLE 2. Inactivation of purified DNT of *P. multocida* SP-72

Treatment	Dermonecrotic activity (titer)[a]
None (purified DNT)[b]	128
Heating	
37°C for 60 min	64
56°C for 30 min	16
70°C for 30 min	<2
Trypsin[c] (%)	
0.25	4
0.5	<2
Formalin[d] (%)	
0.5	8
1	<2
Glutaraldehyde[d] (mM)	
4	8
40	<2

[a] Determined as described in the legend to Fig. 1.
[b] Dose of DNT was 0.2 μg of protein per 0.1 ml).
[c] Incubation of DNT (0.2 μg of protein per 0.1 ml) with trypsin at 37°C for 2 h.
[d] Incubation of DNT (0.2 μg of protein per 0.1 ml) with Formalin or glutaraldehyde at 37°C for 1 h.

TABLE 3. Amino acid composition of purified DNT of *P. multocida*

Amino acid	mmol/100 g of DNT[a]	mol/100 mol of amino acids
Aspartic acid	15.52	9.41
Threonine[b]	9.20	5.58
Serine[b]	8.82	5.35
Glutamic acid	19.47	11.81
Proline	14.02	8.51
Glycine	15.04	9.12
Alanine	13.40	8.13
Valine	9.45	5.73
Methionine	3.08	1.87
Isoleucine	7.13	4.33
Leucine	13.11	7.95
Tyrosine	5.54	3.36
Phenylalanine	7.18	4.36
Lysine	9.16	5.56
Histidine	5.59	3.39
Arginine	7.33	4.44
Half-cystine	1.78	1.08
Tryptophan		

[a] When given as grams per 100 g, this refers to grams of amino acid residues per 100 g (dry weight).
[b] The value was corrected with factors of 1.13 for threonine and 1.11 for serine.

The purified DNT (stage 5) was confirmed to be homogeneous by Ouchterlony double immunodiffusion (Fig. 3), crossed IEP (Fig. 4), thin-layer isoelectric focusing in polyacrylamide gels (Fig. 5), and SDS-PAGE (Fig. 6). It was apparently free of the four antigens since antibodies against these antigens presented in the anti-crude DNT antiserum did not form by fusing precipitin lines with the purified DNT (Fig. 3a). Anti-purified DNT antiserum was also confirmed actively to be free from antibodies against capsular (3) and somatic (18) antigens present in the crude DNT (stage 1), when determined by indirect hemagglutination (3) and acid agglutination (18) tests (data not shown). The purified DNT, therefore, might be free from these two common antigens (3, 18) in *P. multocida*. Crude *B. bronchiseptica* DNT (Nakai et al., in press) does not form any precipitin lines against anti-purified and anti-crude DNTs of *P. multocida* antisera (data not shown). This observation may be supported by our previous conclusion that the DNTs from the two bacterial species are serologically distinct (Nakai et al., in press).

The chemical nature of the purified *P. multocida* DNT was presumably protein, having a molecular weight of 160,000, and, therefore, may be larger than the purified DNT of *B. pertussis*, which has a sedimentation coefficient of 1.4. (7). The pI of *P. multocida* DNT was ca. 4.7 to 4.8 (Fig. 5). The *P. multocida* DNT was composed of various amino acids (Table 3). The MND of the purified DNT for guinea pigs was about 1 ng of protein, and the LD_{50} per mouse was 0.2 μg. The toxic activity of the purified *P. multocida* DNT was similar to that of a purified *B. pertussis* DNT, i.e., an MND of 2.5 to 3.5 ng and an LD_{50} of 0.4 to 1.5 μg per mouse (27).

The DNTs of *B. pertussis* and *B. bronchiseptica* have been found both in the cells and in the supernatant fluids of liquid cultures. These toxins can be extracted from the cells by various procedures that disrupt the cells, such as sonication or freeze-thaw cycles. Recently, Cowell et al. (4) concluded that the DNT in *B. pertussis* cells is an intracellular cytoplasmic component that is not secreted by actively growing cells.

Generally, the crude DNT of *B. pertussis* extracted from the cells grown on an agar medium or in a liquid medium is used for further purification (2, 18, 19). A previous study in our laboratory showed that the crude DNTs with high toxic activities can be extracted from *P. multocida* or *B. bronchiseptica* grown on agar media (Nakai et al., in press). When concentrations of the suspended cells were adjusted before sonication, dermonecrotic activities of the crude DNTs of *P. multocida* and *B. bronchiseptica* was equal in titers (Nakai et al., in press) and similar to that of *B. pertussis* crude DNT (27). Further study is necessary to clarify the location of DNT in *P. multocida* cells in relation to the virulence of DNT. It is also of interest to speculate whether or not the DNT of *P. multocida* is a true exotoxin.

P. multocida is well known as an extracellular pathogen that does not fuse with the nasal epithelial cell membranes or invade the cytoplasm. In spite of a lack of direct evidence that the DNT of *P. multocida* causes deformity of nasal turbinates in piglets, however, a toxigenic strain of *P. multocida* has been emphasized as a causative agent for swine AR (21, 23, 24). If the DNT of *P. multocida* alone is responsible for the production of AR in piglets, it may result in an invasion of DNT into bone cells after being released from the cells colonizing the nasal mucosa. A homogeneous *P. multocida* DNT preparation of biologically active material obtained in the present study and resulting anti-purified DNT antiserum can be useful tools to analyze the role of DNT in production of nasal turbinate atrophy in piglets both in vivo and in vitro. The purification procedure preliminarily described herein yielded only 1.8% of the total DNT activity; however, the procedure resulted in a ca. 1,000-fold increase in specific activity (Table 1). The purification scheme presented herein would be helpful for obtaining a *B. bronchiseptica* DNT preparation.

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