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Purification and Characterization of the Heat-Labile Toxin of *Bordetella pertussis*

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A procedure is described for purification of pertussis heat-labile toxin (PEHLT) from cells of *Bordetella pertussis*. The purification procedure, performed in the cold and in the presence of protease inhibitors, gives 1,350-fold purification with yields of about 60%. The toxin was shown to be a single-chain polypeptide of 140 kDa, pI 6.02. It was completely inactivated by heating at 56°C for 60 min. Rabbit antiserum prepared against PEHLT neutralized the toxin and gave a single precipitin line on immunodiffusion. In immunodiffusion assays, this anti-PEHLT serum did not react with pertussis toxin, filamentous hemagglutinin, or preparations of pertussis adenylate cyclase. Purified PEHLT elicited dermonecrosis and atrophy of the spleen. PEHLT is extraordinarily active; 0.4×10^{-12} g caused necrotic lesions in newborn mice, and with 18- to 20-g mice the 50% lethal dose was about 11×10^{-9} g.

Bordetella pertussis produces several protein toxins which may participate in pathogenesis and protective immunity against disease. In addition to pertussis toxin (33) and adenylate cyclase (13, 14), the organism expresses a toxic activity that causes necrotic lesions after intradermal injection. This activity was described in 1909 by Bordet and Gengou (5) shortly after the first isolation of *B. pertussis*. Inactivation of this toxic action by heating at 56°C led subsequent investigators to call this material heat-labile toxin (23). Others have referred to this material as dermonecrotic toxin (32). To differentiate this toxin from the heat-labile toxins produced by other bacteria (16, 17), the acronym PEHLT (for pertussis heat-labile toxin) is used in this the report.

The actions of PEHLT are distinct from those of pertussis toxin. Pertussis toxin is resistant to treatment at 56°C and acts through ADP-ribosylation of adenylate cyclase regulatory protein G_i (4, 15). The lethal action of PEHLT can be inhibited by giving animals corticosteroids at the time of toxin challenge (27, 28), but detailed information describing the biochemical basis for the action of PEHLT is unavailable.

PEHLT has been reported to be localized in the cytoplasm and is released only when the cells are disrupted (9). Physical characterization of PEHLT has been hindered by its instability in crude preparations. Only partially purified preparations that rapidly lose activity have been reported (2, 3, 12, 24–26, 29, 30, 36). PEHLT has been identified as a protein (9) on the basis of sensitivity to protease. Its sedimentation coefficient has been reported to range between 1.4S and 20S (25). More recently, the molecular size was reported as 89 and 102 kDa (19, 24).

Here, the purification and characterization of PEHLT is described. The use of low temperature and protease inhibitors during purification has overcome problems with instability. The toxin, isolated in high yield, was found to be a single polypeptide of 140 kDa. Identification of this polypeptide as PEHLT is supported by the ability of antibodies prepared against the purified protein to neutralize toxicity and give a single precipitin line on immunodiffusion. In an immunodiffusion assay, these antibodies did not react with pertussis toxin, filamentous hemagglutinin, or preparations of adenylate cyclase from *B. pertussis*.

MATERIALS AND METHODS

Bacteria. Frozen cell pastes of *B. pertussis* CS (7) were used for isolation of PEHLT. The bacteria were grown in Stainer-Scholte medium in a 100-liter New Brunswick fermentor (1). Cell pastes of bacteria were obtained by centrifugation and stored at −20°C prior to use.

Reagents. Affi-Gel blue (AGB), acrylamide, bisacrylamide, Coomassie blue, sodium dodecyl sulfate (SDS), and Dowex AG50WX-4 were obtained from Bio-Rad Laboratories, Richmond, Calif. Sephacryl S-200, DEAE-Sephacel, RNase, and bovine serum albumin were obtained from Pharmacia, Uppsala, Sweden. Hydroxylapatite was prepared by the method of Levin (18). Soybean trypsin inhibitor (STI), phenylmethylsulfonyl fluoride (PMSF), ATP, catalase, and alcohol dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo. Creatine phosphate and creatine kinase were obtained from Boehringer GmbH, Mannheim, Germany. [^{32}P]ATP and ^{3}H-labeled cyclic AMP were purchased from Dupont, NEN Research Products, Boston, Mass. Pertussis toxin was prepared as previously described (33). Filamentous hemagglutinin was kindly donated by J. Armand and F. Arminjon, Institut Merieux, Lyon, France. Partially purified adenylate cyclase was obtained after chromatography on DEAE-Sephacel (see purification step 5, below). Peak activity fractions contained predominantly (i.e., greater than 50%) a 43-kDa species.

Antibodies against PEHLT were raised by immunizing rabbits with 20 μg of the purified toxin emulsified in complete Freund's adjuvant (34). Rabbits were boosted twice with 20 μg of antigen in incomplete Freund's adjuvant at 4-week intervals. Antibody titers in serum were monitored by immunodiffusion.

Assays. PEHLT was assayed in the suckling mouse model

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(9). NIH mice, 3 to 4 days old, were injected subcutaneously in the nuchal region with 0.05 ml of toxin serially diluted in phosphate-buffered saline containing 0.1 mM EDTA sodium salt, 1 mM dithiothreitol, and 2% gelatin. The endpoint, determined after 12 h, is the dose giving a necrotic lesion about 5 mm in diameter. This amount of toxin is defined as 1 PEHLT U. Adenylate cyclase was determined by the method of Salomon et al. (31). Activity is expressed as ^{32}P recovered or nanomoles of cyclic AMP produced after correction for losses during chromatography. Protein was determined by using the method of Lowry et al. (20) as modified by Cabib and Polacheck (6). Endotoxin was assayed by using the *Limulus* amebocyte assay by the U.S. Pharmacopoeia procedure.

Characterization. The sedimentation behavior of PEHLT at 4°C was determined on 5 to 20% sucrose gradients for 15 h (22). The sedimentation coefficient and apparent molecular mass were estimated by using catalase (247 kDa) and alcohol dehydrogenase (83 kDa) as standards. The molecular size of PEHLT was estimated by chromatography on a column (1.5 by 100 cm) of Sephacryl S-200 equilibrated with 50 mM Tris-HCl (pH 7.5)–0.5 M NaCl–2 mM $CaCl_2$. RNase (13.7 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and aldolase (158 kDa) were used as standards (21). SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 7% gels as previously described (35).

Isoelectric focusing was performed in an LKB 8100 isoelectric focusing cell at 4°C by using pH 3.5 to 10 ampholines. The pH gradient was stabilized by sucrose, and equilibrium was obtained after about 20 h. Fractions of 2 ml were collected for determination of pH and toxin activity. Recovery ranged between 4.5 and 30.5%.

Purification. All steps were conducted at 4°C, and extreme care was taken to avoid warming of protein solutions above this temperature. *B. pertussis* is a human pathogen, and appropriate precautions should be taken to avoid exposure to aerosols. In addition, PEHLT is a potentially lethal toxin and should be handled appropriately.

Step 1: sonication and ammonium sulfate fractionation. *B. pertussis* cell paste (30 g) was suspended in a 250-ml solution containing 2.0 mM $CaCl_2$, 0.5 mM PMSF, 0.5 mM benzamidine, and 0.02 mg of STI per ml. The suspension, in a 300-ml Branson sonic power cup immersed in ice, was sonicated by using a Fisher 300 sonicator with 15 1-min pulses interspersed with 1-min intervals for cooling. The sonic extract was centrifuged at 7,500 × *g* for 30 min at 4°C. Ammonium sulfate (0.114 g/ml) was added to the supernatant, and after 1 h of stirring at 4°C the suspension was centrifuged for 30 min at 7,500 × *g*. PEHLT was precipitated from the resulting supernatant solution by adding ammonium sulfate (0.276 g/ml). The resulting PEHLT suspension can be stored at 4°C for several days prior to further processing.

Step 2: AGB. The suspension resulting from 500 g of cell paste was centrifuged at 7,500 × *g* for 20 min at 4°C. The pellet was dissolved in 270 ml of 50 mM Tris-HCl (pH 7.5) with 0.5 mM benzamidine–0.5 mM PMSF–2 mM $CaCl_2$–0.02 mg of STI per ml (buffer A). This solution was applied to a column (5.6 by 15 cm) of AGB (200/400 mesh) equilibrated with buffer A. The column was washed with 1.0 liter of 1.0 M potassium phosphate (pH 7.5) containing protease inhibitors and then washed with 700 ml of buffer A. PEHLT was eluted by washing the column with buffer A containing 2.0 M magnesium chloride. This procedure resulted in elution of PEHLT as a single protein peak with the final buffer (Fig. 1).

Step 3: Sephacryl S-200. The pool obtained from the preceding step was applied to a column (5 by 75 cm) of Sephacryl S-200 equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 110 ml/h. PEHLT eluted as a single peak of activity (Fig. 2). Fractions containing more than 2 × 10^6 PEHLT U/ml were pooled.

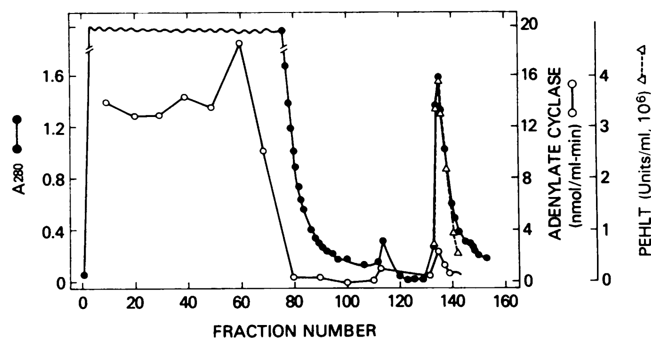

FIG. 1. Elution pattern of PEHLT and adenylate cyclase from a column (5.6 by 15 cm) of AGB. The ammonium sulfate precipitate obtained from 500 g of cells was dissolved in buffer A (270 ml) and applied to the column. The column was sequentially eluted with buffer A containing 1.0 M potassium phosphate (1,000 ml), no additions (700 ml), or 2.0 M magnesium chloride. Fractions of 13 ml were collected and monitored for PEHLT activity, adenylate cyclase activity, and A_{280} as indicated.

Step 4: DEAE-Sephacel. The protein solution obtained from the preceding step was diluted with 2 volumes of cold distilled water and applied to a column (2.3 by 13.5 cm) of DEAE-Sephacel equilibrated with 50 mM Tris-HCl (pH 7.5) containing 2.0 mM $CaCl_2$ and 0.02 mg of STI per ml (buffer B). The column was eluted at 70 ml/h with a linear gradient between 400 ml of buffer B and 400 ml of buffer B containing 0.3 M NaCl. Fractions from the DEAE-Sephacel column were monitored for protein and PEHLT activity (Fig. 3). The toxin eluted as a single peak, and fractions containing toxin at more than 8 × 10^6 PEHLT U/ml were pooled (94 ml).

Step 5: hydroxylapatite. The PEHLT pool obtained after DEAE-Sephacel chromatography was diluted with 2 volumes of cold deionized water and applied to a column (1.8 by 8.6 cm) of hydroxylapatite equilibrated with 10 mM potassium phosphate (pH 7.5) containing 0.02 mg of STI per ml.

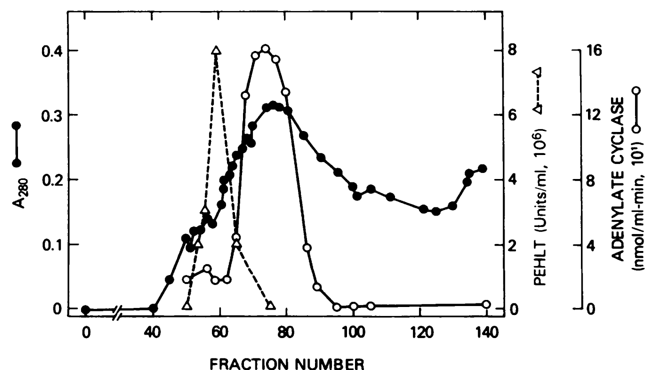

FIG. 2. Molecular exclusion chromatography on a column (5.0 by 75 cm) of Sephacryl S-200. The PEHLT pool (220 ml) obtained after purification step 3 was chromatographed in the presence of buffer A containing 0.1 M sodium chloride. Fractions of 11 ml were collected and monitored for adenylate cyclase, PEHLT, and protein as indicated.

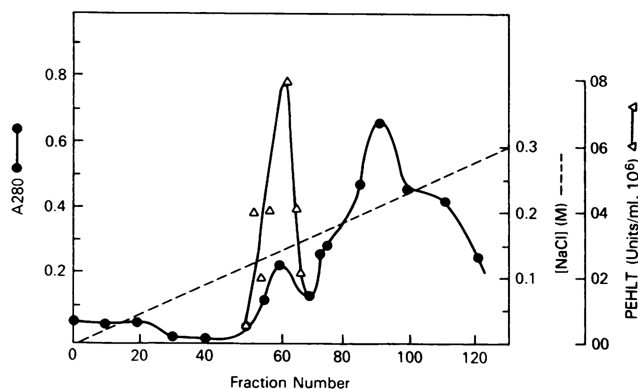

FIG. 3. Further purification of PEHLT by chromatography on a column (2.3 by 13.5 cm) of DEAE-Sephacel. The diluted PEHLT pool obtained after molecular exclusion chromatography was loaded onto the top of the column. The results shown were obtained after elution with a linear gradient (800 ml) established between buffer B and buffer B containing 0.3 M sodium chloride. Fractions of 6 ml were collected, and protein (A_{280}) and PEHLT activity were monitored as indicated.

The column was eluted at 14 ml/h with a linear gradient formed between 100 ml of starting buffer and 100 ml of 250 mM potassium phosphate (pH 7.5) containing STI. The results obtained by monitoring for PEHLT and protein are shown in Fig. 4. The toxin eluted as a single peak, and fractions containing more than 12.8×10^8 PEHLT U/ml were pooled (16.5 ml).

In some purifications, chromatography on a second column of hydroxylapatite was employed. In these instances, the pool obtained after the first hydroxylapatite column was diluted with 2 volumes of cold distilled water and applied to a column (1.8 by 8 cm) of hydroxylapatite equilibrated as described above but at pH 7.8. The toxin was eluted as a single peak with a gradient between 100 ml of starting buffer and 100 ml of 0.5 M potassium phosphate (pH 7.8) containing STI.

RESULTS

Purification. The purification procedure resulted in 1,350-fold purification of PEHLT and recovery of about 65% (Table 1). Estimation of recovery, however, can be variable because quantitation of toxin is achieved by endpoint titration in a biologic assay. The fractions of PEHLT obtained during purification were characterized by SDS-PAGE (Fig. 5). Progressive enrichment of PEHLT activity corresponded to the presence of a protein band at 140 kDa. When gels were overloaded with purified PEHLT, some contamination with low-molecular-weight materials was observed. Immunodiffusion of purified PEHLT against rabbit antibody resulted in a single precipitin line (Fig. 6).

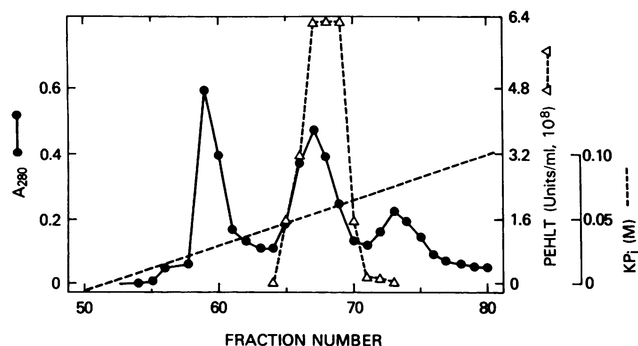

FIG. 4. Elution pattern of PEHLT from a column (1.8 by 8.6 cm) of hydroxylapatite. The PEHLT pool (94 ml) obtained in purification step 5 was diluted (2:1) with water and applied to the column. The results shown were obtained upon elution of the column with a linear, pH 7.5, gradient (200 ml) established between 0.01 and 0.25 M potassium phosphate in the presence of STI. Fractions of 2.8 ml were collected and monitored for protein (A_{280}) and PEHLT.

TABLE 1. Purification of PEHLT from 500 g of *B. pertussis* cells

Step	Vol (ml)	Amt of protein (mg)[a]	Activity: Total (U, 10^8)	Activity: Specific (U, 10^8/mg)[b]	Yield (%)
1. Centrifugation of sonic extract	4,030	16,500	320	0.02	100
2. Ammonium sulfate fractionation	270	15,700	430	0.03	134
3. AGB	220	724	280	0.39	88
4. Sephacryl S-200	210	370	340	0.92	106
5. DEAE-Sephacel	94	25.4	150	5.9	47
6. Hydroxylapatite	16.5	7.8	210	27	66

[a] Protein was determined by the method of Lowry et al. (6, 20).
[b] Dermonecrotic activity was monitored by use of the suckling mouse model (9).

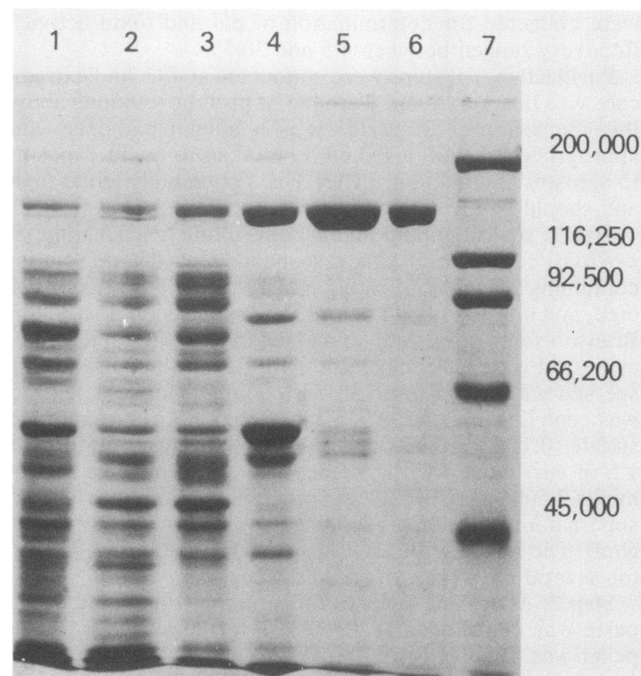

FIG. 5. Analysis of PEHLT fractions obtained at various stages of purification by SDS-PAGE. Protein samples were treated with SDS and β-mercaptoethanol as previously described (33). Protein samples (approximately 25 μg) were applied to the top of a 7% acrylamide gel. After electrophoresis, the gel was fixed and stained with Coomassie blue. Samples analyzed: 1, ammonium sulfate (60% saturation); 2, AGB; 3, Sephacryl S-200; 4, DEAE-Sephacel; 5, hydroxylapatite I; 6, hydroxylapatite II; 7, molecular weight standards (myosin [200 kDa], β-galactosidase [116 kDa], phosphorylase *b* [97 kDa], bovine serum albumin [66.2 kDa], and ovalbumin [45.0 kDa]).

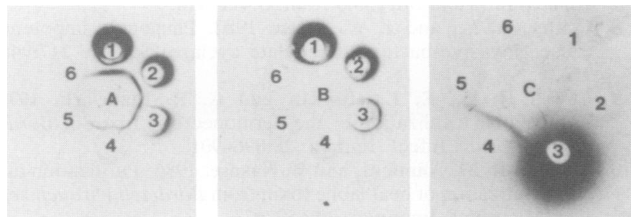

FIG. 6. Antigen specificity of rabbit antibody raised against purified PEHLT. Rabbits were immunized with purified PEHLT as described Materials and Methods. Serum thus obtained was tested for reactivity against specific antigens by immunodiffusion. Immune (A) and preimmune (B) sera were tested against purified PEHLT (20 μl at 0.1 mg/ml) applied to the center well. Wells 1 to 6 contained 10 μl of the respective serum serially diluted (1:2). The center well in panel C contained immune serum (20 μl, diluted 1:2) which was tested against partially purified adenylate cyclase (wells 1 to 3), PEHLT (10 μl at 0.1 mg/ml) (well 4), pertussis toxin (10 μl at 0.1 mg/ml) (well 5), and filamentous hemagglutinin (10 μl at 0.1 mg/ml) (well 6). After diffusion, gels were fixed and stained with Coomassie blue.

TABLE 2. Factors that affect PEHLT stability

Treatment	Activity (U/ml)[a]	Change (%)
Expt 1[b]		
4°C	3.2×10^6	100
37°C	1.6×10^6	50
45°C	0.08×10^6	2.5
56°C	$<0.004 \times 10^6$	<0.1
Expt 2[c]		
None	1.0×10^5	100
STI (0.02 mg/ml)	8.0×10^5	800
Benzamidine (0.5 mM)	4.0×10^5	400
PMSF (0.5 mM)	4.0×10^5	400

[a] Activity was determined as described in Materials and Methods by using the suckling mouse model.
[b] PEHLT from purification step 6 was diluted 1:20 into Tris-HCl buffer, pH 7.5. Samples were then incubated for 1 h at the indicated temperatures.
[c] Supernatant from purification step 1 was incubated for 21 h at 25°C in the presence of the specified protease inhibitors.

After chromatography of the *B. pertussis* sonic extract on AGB (Fig. 1), adenylate cyclase activity eluted with PEHLT. These two activities were resolved by subsequent chromatography on Sephacryl S-200 (Fig. 2). Similarly, adenylate cyclase was resolved from PEHLT by chromatography on DEAE-Sephacel and hydroxylapatite (data not shown). The relationship between PEHLT and adenylate cyclase may, however, be more complex. The amount of adenylate cyclase in partially purified PEHLT is variable. Storage for several days at 4°C can result in increased adenylate cyclase. In one instance, an increase of nearly 10-fold was observed.

To explore whether the presence of adenylate cyclase during purification might be related, purified PEHLT was tested for its ability to elevate cyclic AMP in human neutrophil preparations (8). Under conditions in which parallel experiments using supernatant from crude bacterial sonic extract gave a 100-fold increase in cyclic AMP, no effect was seen when purified PEHLT was present at a concentration of 45 μg/ml. Since reaction mixtures with pure protein contained eight times more bioactive PEHLT than those with the crude sonic extract, a relationship between the activity of PEHLT and adenylate cyclase is not apparent.

Use of rabbit antiserum prepared against pertussis toxin and filamentous hemagglutinin in an enzyme-linked immunosorbent assay and immunodiffusion assays demonstrated that PEHLT preparations are free from detectable levels of either antigen.

Physical characterization. SDS-PAGE of purified PEHLT gives an apparent molecular mass of 140 kDa (Fig. 5). Centrifugation in sucrose gradients yielded an $s_{20,w}$ of 6.41S. With an assumed partial specific volume of 0.725 cm^3/g, the calculated molecular weight was 114 kDa. Estimation of size by molecular exclusion chromatography on Sephacryl S-200 yielded a result of 130 kDa. The similarity of the molecular sizes determined under denaturing and nondenaturing conditions, in addition to the presence of only minor contamination at low molecular weight on SDS-PAGE, suggests that PEHLT is a single-chain polypeptide.

Preparative-level isoelectric focusing of material obtained after chromatography on hydroxylapatite results in recovery of less than 30% of the original activity. During isoelectric focusing, much of the protein which collected at the anode was inactive. Active PEHLT, however, was recovered with an apparent pI of 6.02.

Stability. In crude extracts, PEHLT activity was lost rapidly with storage at either 37 or 4°C. Addition of protease inhibitors such as PMSF, benzamidine, and STI was effective in preventing loss of PEHLT activity (Table 2). Accordingly, these protease inhibitors were included in buffers during purification.

Consistent with its designation as a heat-labile toxin, purified PEHLT was rapidly inactivated upon heating. When the toxin was heated at 56°C, activity was lost after 30 min. Milder treatment, 37°C for 75 min, resulted in about 50% inactivation. For these reasons, all protein solutions were maintained at 0 to 4°C throughout the purification.

Purified PEHLT was further stabilized by addition of glycerol to a 50% concentration. Toxin solutions thus prepared did not lose activity when stored for more than 6 months at −70°C.

Biologic activity and neutralization by specific antibody. Only a miniscule amount of PEHLT is necessary for a biologic effect. In the suckling mouse model, as little as 0.4×10^{-12} g was sufficient to induce a positive response. In 18- to 20-g mice, a dose of about 11×10^{-9} g caused 50% mortality. Pure PEHLT caused atrophy of the spleen (data not shown), thus conforming to the previously described action of partially purified preparations (19, 24, 25, 32).

Rabbits immunized with purified PEHLT produced antiserum that gave a single precipitin line on immunodiffusion. The antiserum did not react with filamentous hemagglutinin, pertussis toxin, and partially purified adenylate cyclase (Fig. 6) in immunodiffusion assays. Immune serum diluted to 1/320,000 was effective in neutralizing the action of PEHLT (data not shown). Together, these data support the identity of the 140-kDa polypeptide as active PEHLT.

DISCUSSION

Purification of PEHLT (19, 23–25) has been hindered by instability of the active protein. By conducting purification in the presence of protease inhibitors (i.e., PMSF, STI, and benzamidine) and in the cold, the purified toxin was obtained in a high overall yield. Although some contamination was observed on SDS gels at low molecular weight, it is likely

that this resulted in part from nicking of the intact toxin molecule by proteolytic enzymes.

PEHLT is a single-chain polypeptide with a molecular mass of about 140 kDa, as determined under both nondenaturing and denaturing conditions. Earlier reports of molecular size gave variable results, probably because of the extraordinary biologic activity of the toxin. Since only about 0.4×10^{-12} g of PEHLT is sufficient to induce a dermonecrotic lesion, quantities of active toxin nondetectable by standard detection methods for proteins may have been present in crude preparations of what was thought to be purified PEHLT. In earlier studies, purified preparations of PEHLT gave a minimal necrotizing dose of 10×10^{-6} g in guinea pigs. The potency of purified PEHLT seen here is comparable to the potency seen with bacterial toxins such as tetanus and diphtheria toxins.

Purification of the dermonecrotic–heat-labile toxin from *B. bronchiseptica* has been reported (10, 16, 17). This toxin is a single polypeptide of 145 (16) and 190 (17) kDa. It has a pI between 6.3 and 6.7 and is a necrotizing agent at nanogram or subnanogram levels. Since *B. bronchiseptica* and *B. pertussis* are closely related species, it would be informative to establish the similarities or differences between toxins isolated from the two sources. Preparations of heat-labile toxin from *B. parapertussis* were shown to interact with endotoxin in producing dermonecrotic lesions (11). While the possibility for this type of interaction has not been explored in depth with PEHLT, the presence of less than 30 ng of endotoxin per mg of pure protein suggests that endotoxin is not essential in PEHLT-mediated dermonecrosis in suckling mice.

The dermonecrotic activity present in extracts from *B. pertussis* was the first toxic activity attributed to the organism (5). However, the difficulty experienced in obtaining the purified toxin has limited progress in understanding not only the role of PEHLT in pathogenesis and immunity to disease but also its pharmacologic actions. With purified PEHLT and monospecific antitoxin, it will be possible to examine the role of the toxin in disease and gather insight into its biochemical mode of action.

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