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Investigation of a Hemolysin Produced by Enteropathogenic *Treponema hyodysenteriae*

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A hemolysin produced by *Treponema hyodysenteriae*, the etiological agent of swine dysentery, was investigated. A virulent isolate (B204) was inoculated into a standard culture medium consisting of Trypticase soy broth without dextrose (BBL Microbiology Systems) supplemented with 10% fetal calf serum in an atmosphere of 70:30 deoxygenated H_2–CO_2. Sterile cell-free filtrates were prepared at 2-h intervals and assayed for hemolytic activity by using washed sheep erythrocytes. The maximum hemolytic titer was obtained during the early log phase of growth (4 h). A loss of hemolytic activity was observed when cell-free filtrates were stored at 23 and 4°C. Storage at −20 or −80°C after lyophilization resulted in retention of the hemolytic titer for periods of up to 30 days. Enzymatic inactivation of the hemolysin was accomplished with pronase, but not with deoxyribonuclease, ribonuclease, lipase, or trypsin. Addition of exogenous ribonucleic acid-core to the standard culture medium resulted in a dose-dependent increase in the amount of hemolysin produced. The hemolysin could be purified by acid and ammonium sulfate precipitation followed by ion exchange and molecular sieve chromatography. The molecular weight of the hemolysin was 68,000 when determined by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis.

Recent studies on the etiology of swine dysentery indicate that *Treponema hyodysenteriae* is the primary pathogen (1, 9, 25, 34). However, other anaerobic microflora of the intestinal tract may act in a cooperative manner to initiate and induce this malady (3, 8, 20, 23, 34). The disease is characterized as a mucohemorrhagic diarrhea of swine with acute inflammation of the large intestine (9).

Both pathogenic and nonpathogenic *T. hyodysenteriae* have been cultured from swine and shown to be associated with the colonic mucosa (14, 29, 33). Although Hunter and Wood (12) have shown that in vitro biochemical reactions provide a means to separate pathogenic from nonpathogenic biotypes, differentiation by a more practical means, namely, hemolytic pattern, has been accepted (21, 31). Biotypes that show a complete hemolytic pattern on blood agar plates appear to remain virulent when used to orally infect specific-pathogen-free swine; biotypes that show an incomplete hemolytic pattern are avirulent (1, 6, 11, 14, 21-23, 25). Kinyon et al. (16) have indicated a significant role for hemolytic isolates of *T. hyodysenteriae* in the pathogenesis of swine dysentery in specific-pathogen-free swine. Recently, Kinyon and Harris (15) have suggested, based on enteropathogenicity (16, 30), hemolytic activity (16, 30), and DNA homology, that the nonpathogenic isolates be regarded as a new species of intestinal bacteria, *Treponema innocens*.

Although a correlation between hemolytic pattern and enteropathogenicity has been observed (16, 21-23, 25, 31), investigations on the precise nature and pathogenic role, if any, of the hemolysin remain unclear.

The present investigation describes the production, characterization, and purification of a hemolysin produced by an enteropathogenic isolate of *T. hyodysenteriae*.

MATERIALS AND METHODS

Microorganism. *T. hyodysenteriae* isolate B204 was kindly supplied by J. M. Kinyon and D. L. Harris (College of Veterinary Medicine, Ames, Iowa). The virulence of this isolate for swine has been determined (13, 16, 33). All stock cultures were maintained as previously described (17).

Growth conditions and hemolysin production. The growth of *T. hyodysenteriae* isolate B204 in a standard culture medium consisting of Trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) was carried out as previously described (17). All tubes were overlaid with 70:30 deoxygenated H_2–CO_2 2 days before inoculation. One milliliter of an 18-h broth culture of *T. hyodysenteriae* was inoculated into 50-ml test tubes (Corning no. 8424; Fisher Scientific Co., St. Louis, Mo.) containing 30 ml of standard culture medium, resulting in an initial optical density at 540 nm

of 0.05. The tubes were overlaid with gas mixture and incubated at 37°C. At 2-h intervals the optical densities at 540 nm of duplicate cultures were determined. Each culture was then centrifuged at 2,500 × *g* for 30 min, the supernatant was filter sterilized (0.2 μm; Millipore Corp., Bedford, Ma.), and the pH was determined. The sterile cell-free filtrate (CFF) was stored at −20°C unless stated otherwise.

The increased production of hemolytic activity by *Streptococcus pyogenes* has been observed when yeast ribonucleic acid (RNA)-core is added to the medium (2, 18). To determine the effect of RNA-core (Sigma Chemical Co., St. Louis, Mo.) on the production of hemolytic activity by *T. hyodysenteriae*, various concentrations were added to the standard culture medium at the time of inoculation. Culture conditions and collection of CFF were as described above.

Hemolysin assay. The hemolysin produced by *T. hyodysenteriae* was assayed by determining the effect of CFF on washed sheep erythrocytes. The CFF (1 ml) was serially diluted twofold with Veronal-buffered saline (pH 7.35) containing 0.1% gelatin (VBSG). Sheep erythrocytes were washed three times with VBSG and adjusted to 0.25% concentration, and a 2.5-ml volume was added to each dilution tube; the final reaction volume was 3.5 ml. The tubes were incubated at 37°C in a water bath for 1 h. After incubation, the tubes were centrifuged at 1,200 × *g* for 15 min (4°C) to remove unlysed erythrocytes. The supernatant was checked for optical density at 541 nm to determine hemoglobin release. The titer (hemolytic units per milliliter) was expressed as the reciprocal of the highest dilution of CFF yielding 50% hemolysis. All values were interpolated from a standard curve (32).

Ultrafiltration. Ultrafiltration procedures were carried out in an Amicon model 202 flow cell (Amicon Corp., Lexington, Mass.). A volume of 400 ml of CFF was placed above a Diaflo XM-50 membrane (nominal molecular weight cutoff, 50,000) and concentrated 10-fold at a constant pressure of 50 lb/in^2 dry nitrogen. The XM-50 filtrate was concentrated 10-fold on a Diaflo PM-10 (nominal molecular weight cutoff, 10,000) or UM-2 (nominal molecular weight cutoff, 1,000) membrane. The samples were twice washed with a 25-ml volume of 0.067 M potassium phosphate buffer (pH 7.35) and assayed for hemolytic activity as described above.

Enzymatic studies. The ability for deoxyribonuclease (Sigma), ribonuclease (Sigma), lipase (Worthington Biochemical Corp., Freehold, N.J.), trypsin (Sigma), and pronase (Calbiochem, La Jolla, Calif.) to inactivate hemolytic activity in CFF was determined. One milliliter of each enzyme was added to tubes containing 1 ml of CFF (108 hemolytic units per ml); enzyme concentrations were 1 mg/ml. The tubes were then incubated in a water bath at 37°C for 1 h. After incubation, tubes containing trypsin and pronase received 1 ml of soybean trypsin inhibitor (1 mg/ml of VBSG); all other tubes received 1 ml of VBSG. Duplicate tubes without CFF were used to assess the effect of enzyme on the nonspecific lysis of sheep erythrocytes. One milliliter from each tube was assayed for hemolytic activity.

The presence of protease in CFF was determined by a revised method of Sato et al. (28). To 1.0 ml of CFF, 1.0 ml of 2.5% casein yellow (Calbiochem) in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5) was added, and the mixture was incubated at 37°C for 2 h. After incubation, 1.0 ml of 8% trichloroacetic acid was added, and the sample was centrifuged at 2,000 × *g* for 30 min. To 0.5 ml of the superantant fluid, 0.5 ml of 0.5 N NaOH was added, and absorbance at 440 nm was measured. A reaction mixture without substrate was incubated and treated with 8% trichloroacetic acid and then with substrate to serve as a blank; trypsin was used as a positive control. One proteolytic unit was defined as the amount of enzyme that caused an absorption increase at 440 nm of 0.01 under the specified conditions.

pH effect on hemolysin. The effect of pH on hemolytic activity in CFF was determined. The CFF was adjusted to various pH values with 0.1 N HCl or 0.1 N NaOH and incubated at 4°C for 60 min. The samples were then readjusted to pH 7.25 with HCl or NaOH, and hemolytic activity was assayed after appropriate dilution.

Purification of hemolysin. The hemolysin was purified by a revised method of S. A. Saheb, N. Daigneault-Sylvestre, and B. Picard (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B21, p. 20). Culture supernatants (CFF) were adjusted to pH 4.0 with glacial acetic acid and stored overnight at 4°C. The precipitate was collected by centrifugation at 12,100 × *g* for 60 min and suspended in 0.067 M potassium phosphate buffer (pH 7.5) containing 0.85% NaCl. Ammonium sulfate (430 g/liter) was then added, and the precipitate was removed by centrifugation (12,100 × *g*; 60 min) and discarded. The supernatant was dilayzed against 15 liters of phosphate-buffered saline. After dialysis the sample was concentrated 10-fold on a PM-10 Diaflo membrane (Amicon Corp.) and applied to a diethylaminoethyl (DEAE)-Sephacel column (1.6 by 30 cm) equilibrated with phosphate-buffered saline. Material was eluted with about 200 ml of phosphate-buffered saline and then with 100 ml of a linear gradient of 0 to 1 M NaCl in the same buffer. Fractions from the single absorption peak were collected, concentrated on a PM-10 Diaflo membrane, and applied to a Sephadex G-100 column (1.6 by 70 cm) equilibrated with phosphate-buffered saline; material was eluted with the same buffer. Fractions containing hemolytic activity were dialyzed against 15 liters of distilled water, concentrated by lyophilization, and stored at −80°C until needed.

Polyacrylamide slab gel electrophoresis. The discontinuous buffer system of Ornstein (26) and Davis (4) was used, but modified for the inclusion of sodium dodecyl sulfate by the method of Dimmock and Watson (5). All samples were electrophoresed on a polyacrylamide gel slab 0.75 mm thick by using sample slots 4 mm wide formed in the stacking gel mixture. Immediately before electrophoresis, the samples were dissociated by boiling for 3 min in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.7)–1% sodium dodecyl sulfate–1% mercaptoethanol. The gels were run under a constant current of 15 mA per gel slab (0.75 by 120 by 140 mm) and stained with Coomassie blue to detect protein bands.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (19),

with bovine serum albumin (fraction V; Sigma) as a standard unless stated otherwise.

RESULTS

Growth conditions and toxin production. The growth of *T. hyodysenteriae* isolate B204 in the standard culture medium is shown in Fig. 1. After 6 h of growth the pH decreased to 7.16, reaching a pH of 6.70 at 24 h. Further incubation of the culture did not result in a pH change. However, a decrease in pH during culture was associated with a concomitant decrease in hemolytic titer (Fig. 1); further studies on the effect of pH on hemolytic activity were conducted. A decrease in hemolytic units contained in the CFF was not observed over the pH range of 3.0 to 9.0.

The time interval for optimal production of hemolytic activity by *T. hyodysenteriae* during culture was determined. Hemolytic activity increased between 2 and 4 h of culture; the levels at 4 h were well in excess of possible carry-over with the seed inoculum (Fig. 1). After 14 h of culture the hemolytic titer was minimal. The addition of RNA-core to the standard culture medium resulted in a 400-fold increase of hemolytic activity after an incubation period of 8 h (Fig. 2). As indicated, an increase in RNA-core concentration resulted in a dose-dependent increase of hemolytic activity. However, dose dependency was not observed at concentrations above 1,200 μg/ml of culture medium.

Ultrafiltration. The hemolytic activity of CFF after ultrafiltration was determined. Ultrafiltration of the CFF on an XM-50 Diaflo membrane resulted in recovery of 94% of the hemolytic activity. The slight loss (6%) of hemolytic activity could not be recovered from the XM-50 filtrate after subsequent concentration on a PM-10 or UM-2 membrane.

Temperature effects on hemolytic activity. The effect of storage temperature on hemolytic activity was investigated (Fig. 3). As shown, storage at 23 or 4°C resulted in a loss of hemolytic titer. The storage of CFF at −20 or −80°C or at −80°C after lyophilization allowed hemolytic activity to be retained for periods of up to 30 days (Fig. 3).

Enzymatic studies. The action of proteolytic, lipolytic, and nucleolytic enzymes on the hemolytic activity contained in CFF was determined. The treatment of CFF with deoxyribonuclease, ribonuclease, or trypsin for 1 h (37°C) did not cause a decrease in hemolytic activity upon subsequent assay (data not shown). However, the treatment of CFF with pronase (1 h, 37°C) resulted in an almost complete (98.3%) loss of hemolytic activity. These observations suggest that the hemolysin may be a protein. Further studies to detect proteolytic activity in CFF were unsuccessful, suggesting that the rapid loss of hemolytic activity in CFF (see above) was not associated with a protease produced by the organism.

Purification of hemolysin. Crude hemolysin, prepared as described earlier, was applied to a DEAE-Sephacel column; a typical chromatographic profile is shown in Fig. 4. Fractions from

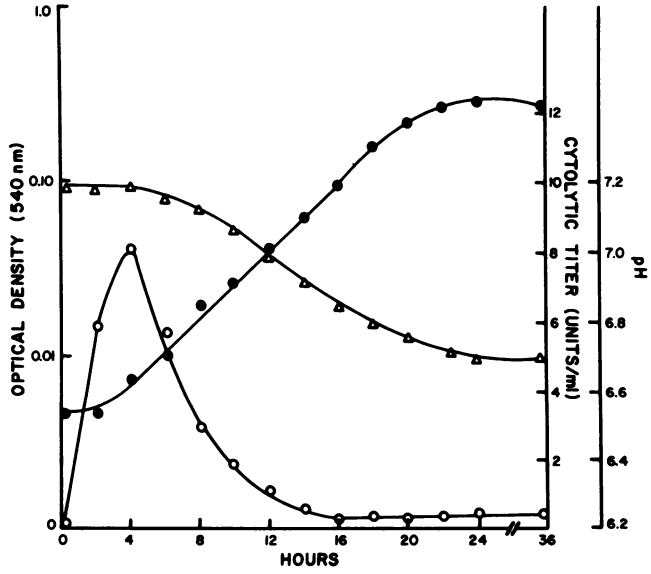

FIG. 1. *Growth of T. hyodysenteriae isolate B204 in the standard culture medium. Data shown represent optical density (●), hemolytic titer (○), and pH (△).*

the absorbance peak were collected, concentrated on a PM-10 Diaflo membrane, and chromatographed on a Sephadex G-100 column (Fig. 5). Selected fractions containing toxin (fractions 56 to 66) were dialyzed against water and lyophilized. The sample was then rehydrated and subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. This procedure resulted in a single homogeneous protein band (Fig. 5, insert). On occasion, an anomalous second protein band was observed; this band appeared to be diffuse and associated with the primary band and may be a result of sample overload.

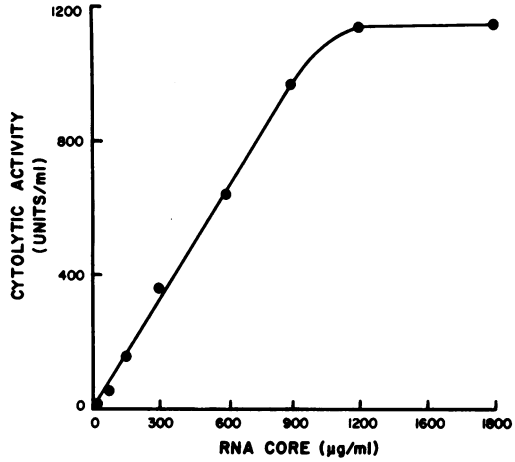

FIG. 2. *Hemolytic titer produced by T. hyodysenteriae cultured in the standard medium containing various concentrations of exogenous RNA-core.*

The specific activity of hemolysin increased from 0.46 in the CFF to 89,400 hemolytic units per mg of protein in the final material eluted

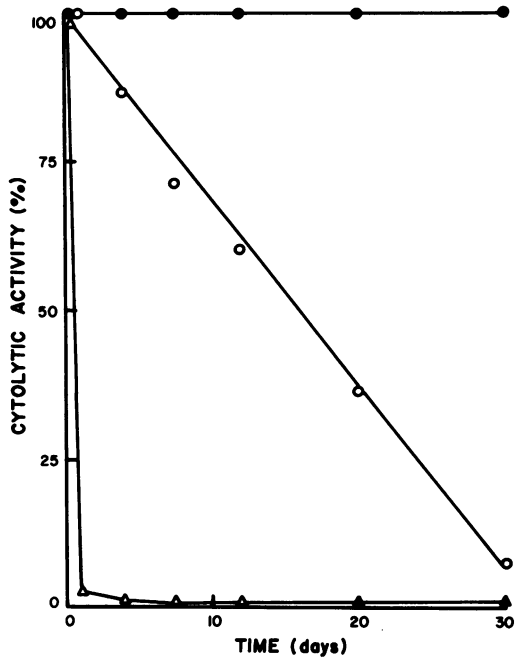

FIG. 3. *The effect of storage conditions on the hemolytic activity produced by T. hyodysenteriae isolate B204. Hemolytic activity is expressed as the percentage of total remaining after the designated time period. Symbols represent storage at 23°C (△), 4°C (○), and −20 or −80°C or −80°C after lyophilization (●).*

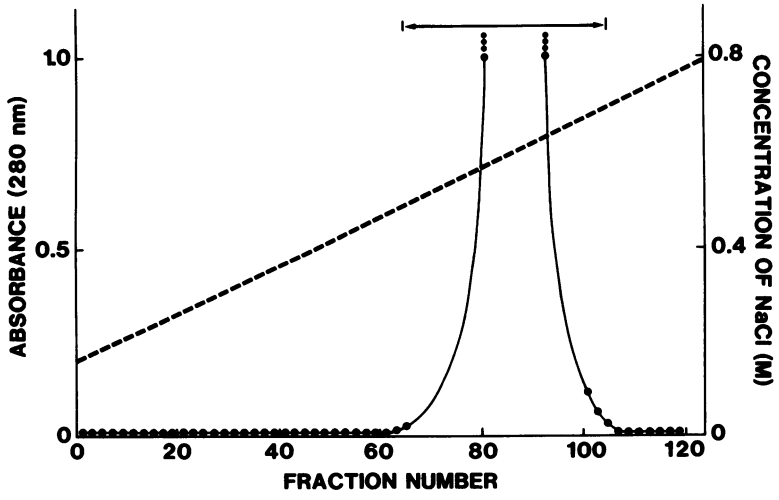

FIG. 4. *DEAE-Sephacel column chromatogram of crude hemolysin. After acid and ammonium sulfate precipitation, CFF was chromatographed on a DEAE-Sephacel column as described in the text. Symbols: ●, absorbance at 280 nm; broken line indicates concentration of NaCl; arrows (fractions 65 to 105) indicate fractions pooled and concentrated for further chromatography.*

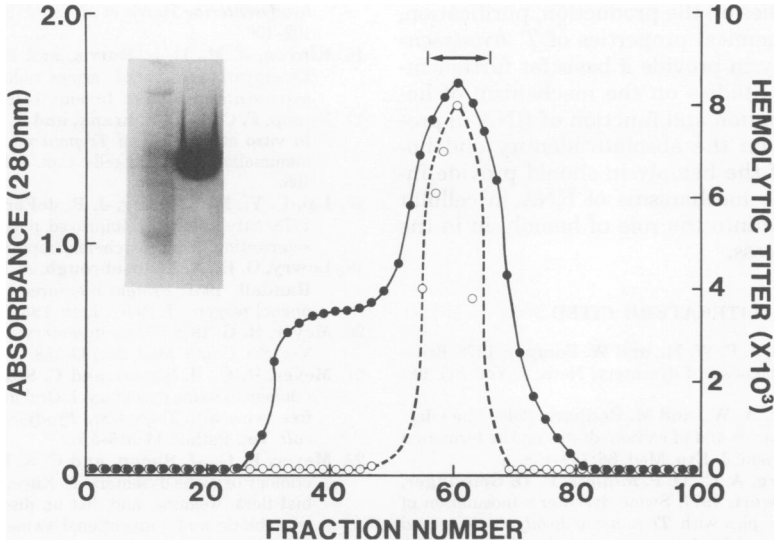

FIG. 5. *Sephadex G-100 column chromatogram of partially purified hemolysin. Pooled fractions from DEAE-Sephacel chromatography were chromatographed on a Sephadex G-100 column as described in the text. Symbols: ●, absorbance at 280 nm; ○, hemolytic activity (see text for details); arrows (fractions 56 to 66) indicate hemolytic fractions concentrated for further analysis by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (see insert). Insert: left lane represents sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of protein standards with known molecular weights of (top to bottom) 2×10^5, 1.3×10^5, 9.4×10^4, 6.8×10^4, and 4.5×10^4; right lane represents 60 μg of hemolysin; the anode is at the bottom.*

from the Sephadex G-100 column; the recovery was about 30%.

DISCUSSION

The induction of a mucohemorrhagic dysentery by oral inoculation of swine with pure cultures of hemolytic (complete) *T. hyodysenteriae* is well documented (1, 6, 7, 10, 16, 21, 22, 25). Kinyon et al. (16) have shown that the hemolytic pattern of pathogenic isolates appears unaltered with increased number of in vitro passages, and that hemolytic isolates play a significant role in the etiology of swine dysentery. To the contrary, nonpathogenic isolates produce an incomplete pattern of hemolysis when cultured on blood agar and remain avirulent for swine (30).

Although pathogenic isolates produce a zone of complete hemolysis when cultured on sheep blood agar plates, studies on the production and characterization of this hemolytic factor in a liquid culture medium have not been well documented. In the present study, hemolytic activity was produced optimally after 4 h of growth in a liquid medium consisting of Trypticase soy broth without dextrose supplemented with 10% fetal calf serum in an atmosphere of 70:30 deoxygenated H_2–CO_2.

The storage of CFF in the frozen (−20 and −80°C) or lyophilized state did not effect a loss of hemolytic activity. Storage of CFF at 23 and 4°C resulted in a loss of hemolytic activity. Thermal inactivation may account for the slight loss (6%) of hemolytic activity after XM-50 ultrafiltration. The concentration of hemolytic activity by ultrafiltration on an XM-50 Diaflo membrane suggested a molecular weight in excess of 50,000. Further studies with sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis indicate a molecular weight of approximately 68,000; subunits were not detected. Enzymatic studies suggest that the hemolysin may be a protein.

A 400-fold increase in hemolytic activity was observed when the organism was cultured in the standard culture medium containing RNA-core. This increase was dose dependent, reaching an optimal concentration of 1,200 μg/ml of culture medium. Similar observations on the ability of nucleic acids to increase hemolysin titer have been reported (2, 18, 24, 27). Lai et al. (18) and Okamoto (24) have shown that yeast RNA will induce the formation of a potent hemolysin in cultures of *Streptococcus pyogenes*. In addition to yeast RNA, Bernheimer and Rodbart (2) have shown that RNA from streptococci, wheat germ, and mammalian liver will induce the formation of streptococcal hemolysin. More recently, Picard et al. (27) have indicated the increased production of hemolysin by *T. hyodysenteriae* after culture in the presence of RNA-core.

These studies on the production, purification, and physiochemical properties of *T. hyodysenteriae* hemolysin provide a basis for further investigations. Studies on the mechanism of hemolysin formation and function of RNA in production and on the absolute identity and homogeneity of the hemolysin should provide insight into the mechanisms of RNA in cellular processes and into the role of hemolysin in the infective process.

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