Arch. Inst. Razi, 1982, 33, 33-42

SOME PROPERTIES OF BETA TOXIN PRODUCED BY CLOSTRIDIUM HAEMOLYTICUM STRAIN IRP - 135 (*)

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Abstract - Six culture media were evaluated for the optimization of ß-toxin production by *Clostridium haemolyticum* (strain IRP - 135) using both batch and dialysis culture techniques. The lethal component of ß-toxin remained active for 13 days when maintained at 37°C but was inactivated by heating at 60° for 20 min. A 1: 10,000 dilution of trypsin inactivated the toxin in 15 min. Preliminary data from electrophoresis in SDS acrylamide gel indicate the molecular weight of the ß-toxin to be approximately 32,000.

Key words: Clostridium haemolyticum, ß-toxin of Clostridium haemolyticum, culture media

INTRODUCTION

Clostridium haemolyticum is the obligate anaerobic bacterium causing bacillary hemoglobinuria or red water disease of cattle and sheep, which has been described by Records and Vawter [1 - 3] in Nevada and Montana [4]. *Clostridium haemolyticum* has also been reported as the causative agent of bacillary hemoglobinuria in many other parts of the world [5 - 11]. Records and Vawter [1, 3] demonstrated that *Cl. haemolyticum* forms two toxic fractions; one is hemolytic and unstable, and the other is a necrotizing fraction that is stable at incubator temperature. Oakley, Warrack and Clark [12, 13] demonstrated a relationship between the ß-toxin derived from *Cl. haemolyticum* and that isolated from *Cl. oedematien* type B.

In recent years the ß-toxin of *Cl. haemolyticum* has received little attention. In 1948, Bard and Mc Clung [14] demonstrated that lecithinase B reduced the hemolytic activity of *Cl. novyi type B and Cl. haemolyticum* toxin. More recently, Lozano and Smith [15] demonstrated that twelve distinct fractions are present in the toxic culture fluid of *Cl. haemolyticum.* Seven of these fractions were present in the uninoculated medium. Four of the five remaining metabolic fractions of *Cl. haemolyticum* were identified as either proteinase, lipase or phospholipase.

(*) Reprinted from: Comp. Immun. Microbiol. infect Dis., vol. 4. NDS. 314 PP. 307 - 316. 1981.

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To obtain more data on the physical and chemical characteristics of *Cl. haemolyticum* ß-toxin, two different culture techniques using six different media were studied for optimization of toxin production. The *Cl. haemolyticum* toxin was concentrated on and the effect of some physical and chemical agents in its activity were examined.

MATERIALS AND METHODS

Organism

Clostridum haemolyticum IRP – 135 was obtained from the National Animal Disease Center, Ames, IO, U. S. A.

Media

Cooked meat medium was used for maintaining the organism. Several media were tested for toxin production: (1) brain and heart infusion broth (BHIB)*; (2) trypticase peptone^{+} 3%, yeast extract $^{+}$ 0. 5%, glucose 0. 5% (TYG); and (3) chopped meat glucose (CMG). The CMG was prepared in two steps. First, 500g of fat-free ground beef was added to a solution containing 1000 ml distilled water, 25 ml 0. 1 N Na OH, boiled for 20 min and then filtered. The filtrate was adjusted to its original volume by the addition of a solution containing 3% proteose peptone, 0. 5% Na_2HPO_4 and 0.5% glucose.

The remaining media were prepared as follows: (4) 5% trypticase peptone$_{+}$,
0. 5% proteose peptone*, 0. 5% yeast extract $_{+}$, 0. 5% glucose, 0. 05% cysteine hydrochloride and 0. 1% sodium thioglycollate, **(TPYG)**; (5) fluid thioglycolate* (FT); and (6) cooked meat medium* (CMM). Each of the above media was adjusted to a pH of 7. 6 by the addition of buffer before use.

Solid medium was prepared from the suitable broth medium by adding 2% agar and dispensed into plates. Culture plates were incubated anaerobically at 37°C using the Gas Pak system $_{+}$.

The optical density of each culture was measured spectrophotometrically at 620 um wavelength after 18 hr incubation. The supernatant of each culture was inoculated into mice and the minimum lethal dose of toxin was determined by titration. Lecithovithelline was prepared as described by Jasmin [16]. A 1% suspension of sheep red blood cells in physiological saline was prepared for the hemolysis test.

Kinetics of toxin production

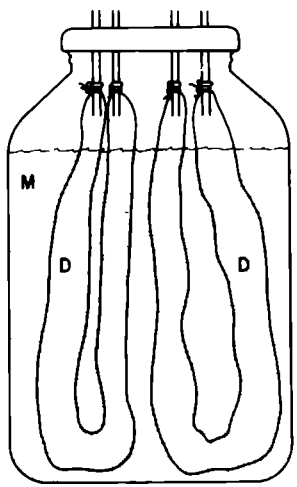
Clostridium haemolyticum was initially grown 24hr in cooked meat medium and then a 5% (v/v) incoculum transferred into fluid TPYG medium and incubated at 37°C. One milliliter aliquots were collected every 2 hr and centrifuged at 6000 **g** for 10 min. The supernatant fluid was collected and frozen at – 70°C until tested for toxic activity.

Assay of toxin

The toxin neutralization test described by Sterne and Batty [17] was used to identify the toxin in the supernatant fluid. Pairs of mice were inoculated i. v. with 0. 5 ml of a mixture consisting of 0. 3 ml of the TPYG containing the toxin, 0. 1 ml of *Cl. haemolyticum* antitoxin and 0. 1 ml of diluent (0. 85% of NaCl solution). Control mice were inoculated with a mixture of 0. 3 ml of the toxic fluid and 0. 2 ml of saline diluent. Each mouse was observed for 3 days and the results recorded.

Dialysis culture

Greater toxin production was obtained from dialysis culthre. The dialysis culture was made in a 41 wide mouth jar shown in Fig. 1. There were 4 glass tubes inserted through the cap of the jar. A 2 foot long dialysis bag was tied to the internal end of 2 glass tubes forming a U shape. Two dialysis bags were used in each flask. The dialysis bags were filled with 500 ml of distilled water. Three liters of TPYG medium was added to the jar and the container and contents were autoclaved at 121°C for 30 min. The dialysis bags were inoculated with washed cells of a 24 hr culture of *Cl. haemolyticum IRP – 135 in cooked meat medium. Two milliliters of inoculum containing* 8×10^6 colony forming units per ml (CFU/ml) of the organism were used to inoculate each bag. The container was incubated at 37°C for 18 hr. After incubation, the contents of each diaylsis bag were poured into tubes and centrifuged in a Sorval RCB-2 model refrigerated centrifuge at 6000**g** at 4° for 30 min. The supernatant was used for purification of toxin.



I - INOCULATION & GAS OUTLET TUBES
D - 3" DIALYSIS SAC
M - MEDIUM

Fig. 1. Dialysis culture.

Batch culture

Batch culture of *Cl. haemolyticum* was performed by inoculating a 41 wide mouth jar containing 31 of sterile TPYG medium with 2 ml of culture with 8×10^6 CFU/ML and incubating at 37°C for 18 hr, After the 18 hr incubation period, ten-fold dilutions of the supernatants from both the dialysis culture and batch culture were inoculated into mice.

Concentration of toxin

The toxin-containing supernatant recovered from the dialysis culture was precipitated and collected by centrifugation at 600g at 4°C for 20 min. The toxin precipitate was resuspended in sterile distiled water and poured into a dialysis bag dialysed against 3 – 4 changes of distilled water over a 24 hr period at 4°C. The toxin dialysate was concentrated by contact with polyethylene glycol for 24 hr at 4°C and stored at 4°C.

Purification of toxin by Sephadex gel filtration

Sephadex G 100[a] was swollen in 0.05 M Tris (hydroxy methylene) ammonium methane buffer at pH 7. 6 (containing 0. 1 M KCL and 0. 02% sodium azide) and poured into a 7×93 cm column. The concentrated toxin was loaded on the column and eluted at 4°C. The effluent was collected in fractions of 240 drops per tube at a speed of 20 drops per min. The fractions were assayed by their ultra violer absorption at 280 um wavelength. The fractions with greatest protein concentration were examined for their biological activity (lecithinase, hemolytic and lethal toxins).

Hemolytic activity was identified by setting a test aliquot to a suspension of washed 1% RBC. Hemolytic activity was inhibited by adding antitoxin of *Cl. haemolyticum* to demonstrate specificity.

The lethal activity of the toxin was determined by the intravenous (i.v.) inoculation of healthy test mice and observation for death within 3 days. The lethal effect was neutralized by prior incubation of the toxin with *Cl. haemolyticum* antitoxin.

The lecithinase activity was determined by adding an aliquot of the test sample to egg yolk solution. Lecithinase activity was inhibited by adding *Cl. haemolyticum* antitoxin. The fractions with the greatest protein concentration were pooled and dialysed against disilled water at 4°C. The dialysed material was concentrated by immersion of the dialysis bag in polyethylene glycol at 4°C. The concentrated toxin was stored at 4°C for further evaluation.

Toxin molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to determine the molecular weight and purity of the toxin. A 10 cm long acrylamide gel was prepared in a 15 cm glass tube with an inside diameter of 6 mm. Gels were polymerized chemically by TEMED and ammonium

persulphate. The stacking gel of 4. 3% acrylamide (1 cm) was placed on top of the resolving gel. The surface of the gel was rinsed with upper chamber buffer before filling the upper chamber for electrophoresis. The sample was dissociated in boiling water for 2 – 3 min, then applied to the gel. Bromphenol blue tracking dye was added to the sample. Electrophoresis was performed at a constant current of 100 mA for the first 30 min then continued at 150 mA for 16 hr until the marker reached the botton of the gel. The protein was fixed in the gel with 50% trichloracetic acid overnight. The gel was stained for 1 hr at 37°C and then destained in 5% acetic acid.

Protein detection

Proteins were quantitated by the Lowry method [18] with crystallized bovine serum albumin* as the standard.

Stability of toxin at 37°C

The toxin was poured into several tubes and incubated at 37°C. At 24 hr intervals one tube was removed and the contents tested for toxicity.

Stability of toxin at high temperatures

The toxin was divided into several tubes and placed in a water bath at temperatures of 50, 60 70 and 94°C. Samples were tested for toxicity at 5 min intervals for toxin held at 50 and 60° and at 2 min intervals for toxin held at 70 and 94°C.

Effect of trypsin on toxin

Trypsin was used in different concentrations for this experiment (a stock trypsin solution). Trypsin dilutions of 1: 2500, 1: 10,000, 1: 25,000, 1: 50,000 and 1: 100,000 were added to toxin and samples removed at 5 min intervals. Each sample was immediately inoculated i.v. into pairs of mice and observations recorded over a 3 day period.

Immunodiffusion

The template microimmunodiffusion procedure [19] was used to measure immunodiffusion characteristics.

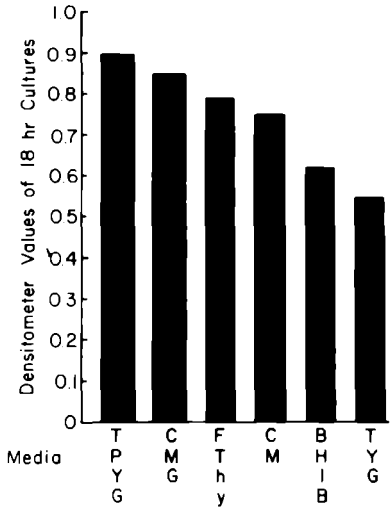
RESULTS

Media

Maximun production by *Cl. haemolyticum* was obtained in TPYG broth and CMG medium (figs. 2 and 3).

Kinetics of toxin production

Toxin production in TPYG medium was detectable 2 hr after inoculation and maximum production of the toxin occurred between 12 and 18 hr following inoculation (Fig. 4). Following the period of peak production, no additional toxin was produced.



TPYG = TRYPTICASE PEPTONE, PROTEOSE PEPTONE, YEAST EXTRACT, GLUCOSE
CMG = CHOPPED MEAT GLUCOSE
F.Thy = FLUID THIOGLYCOLATE
CM = COOKED MEAT
BHIB = BRAIN HEART INFUSION BROTH
TYG = TRYPTICASE PEPTONE, YEAST EXTRACT, GLUCOSE

Fig. 2. Growth evaluation of *Clostridium haemolyticum* in various media.

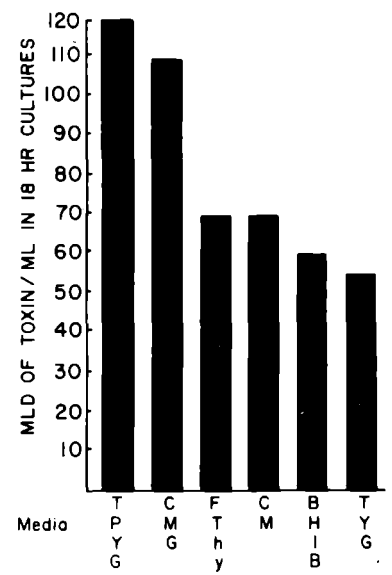


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Fig. 3. Toxin production by *Clostridium haemolyticum* in various media.

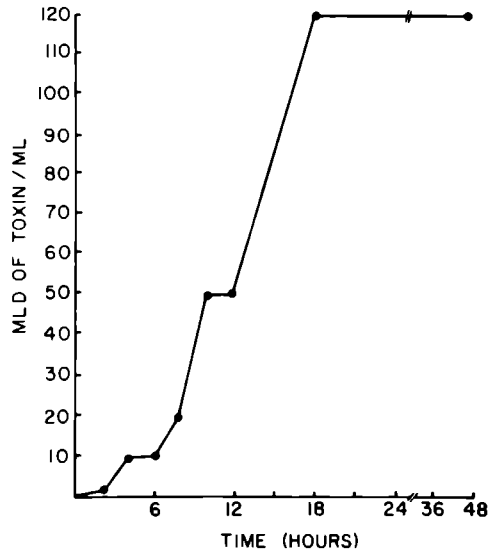

Fig. 4. Kinetics of toxin production by *Clostridium haemolyticum* in TPYG medium.

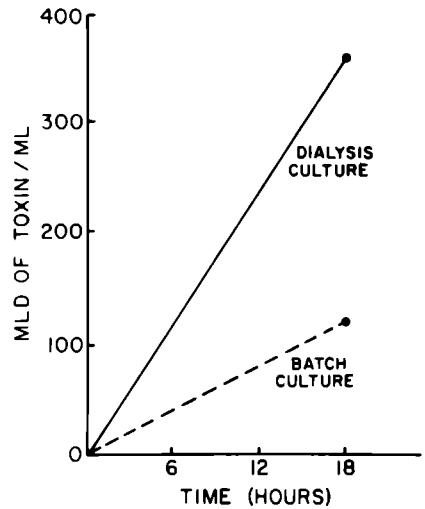

Fig. 5. Kinetics of toxin production by *Clostridium haemolyticum* comparing batch and dialysis culture.

Dialysis and batch culture

The toxicity of the supernatants from both the dialysis and batch culture were titrated in mice. The supernatants from the dialysis culture was demonstrated to be 3 times more toxic than the supernatant from the batch culture (Fig. 5).

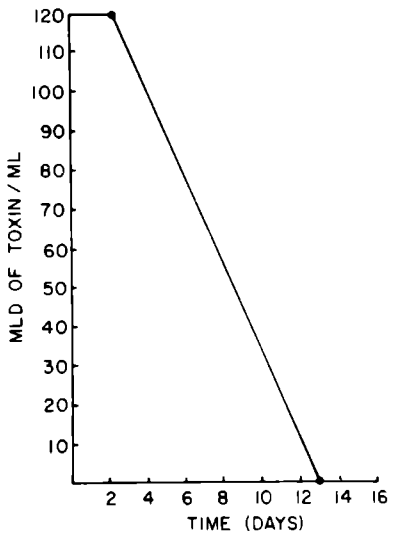

Fig. 6. Toxin stability at 37°C temperature.

Stability of toxin

Active toxin was detectable for 13 days when the sample was incubated at 37°C (Fig. 6). The MLD of the toxin began to decrease after 2 days of incubation at 37°C.

Stability of high temperature

The toxin was exposed to various temperatures and demonstrated to be stable at 50°C for 60 min, but the toxin inactivated after exposure to 60°C for 20min. The toxin was inactivated after only brief exposure to 70 and 94°C.

Effect of trypsin on toxin

Toxin was rapidly inactivated in trypsin diluted 1: 2500 and 1: 10,000. Toxic activity was detectable for 15 min in trypsin diluted 1: 25,000. Trypsin diluted 1: 50,000 and 1: 100,000 did not inactivate the toxin.

Determination of molecular weight

The molecular weight of the concentrated partially purified toxin was determined to be approximately 32,000 by comparison with other compounds of known molecular weight by SDS acrylamide gel.

DISCUSSION

Clostridium haemolyticum is a fastidious anaerobic bacterium and is one of the organisms most sensitive to presence of oxygen. Many media and procedures have been proposed for the isolation and quantitation of *Cl. perfringens* types, an anaerobic bacterium that is not as fastidious as the *C.*

novyi types [20]. Difficulty in obtaining consistent and reliable growth of *C. novyi* types on solid media has been reported [21].

Both densitometry and the MLD tests showed that the TPGY broth and CMG medium were quite suitable for high yield toxin production by *Cl. haemolyticum* IRP - 135, but because preparation of the TPYG broth is easier than CMG medium, so it is preferable to use TPYG broth medium.

Dialysis culture was found to be superior to batch culture for the production of toxin. The toxin produced in dialysis culture was confined to the bag and present in a 3-fold higher concentration than that found in the batch culture.

The immunodiffusion test revealed a cross-reaction between ß-toxin from *Cl. haemolyticum* and antitoxin of *Cl. novyi type* B. This confirms a similarity between the ß-toxin of *Cl. haemolyticum* and that of *Cl. novyi* type B.

Although *Cl. haemolyticum* ß-toxin was found to be highly unstable at temperatures above 60°C, it can be stored safely for at least 2 days at 37°C without significant decrease in activity.

The kinetics of ß-toxin production in TPYG broth revealed an initial lag phase lasting about 6 hr followed by a period of rapid production which increased and peaked at about 18 hr following inoculation. After 18hr, there was no futher increase in the rate of toxin production.

Of considerable interest is the high degree of sensitivity of *Cl. haemolyticum* ß-toxin to trypsin. Other workers have reported that trypsin administered i.v. to guinea pigs did not affect the specific activity of *Cl. tetani* toxin [22]. The mice subjected to experimental intoxication with *Cl. haemolyticum* toxin in this study survived when the toxin was incubated with trypsin dilutions prior to intoxication.

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