Partially Purification and Biological Properties of Shigella
dysenteriae Type 1 Shiga Toxin

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Abstract:
A toxin from Shigella dysenteriae type 1 (strain BB46) was partially purified. The steps used to purified the toxin included intermittent sonic oscillation of bacterial grown in modified synicase broth, ammonium sulphate precipitation, and chromatography on diethylaminoethyl sephadex and carboxymethyl sephadex. The partial purified preparation had a specific activity of $4.18 \times 10^5$ CD50/mg protein in Vero cells. The clinicopathological effects of intravenous administered partially purified toxin in rabbits and intraperitonial injection in mice were studied. The LD50 values were 0.2 and 40 µg/kg body weight in rabbits and mice, respectively. The clinical feature included diarrhea in some animals and a progressive flaccid paralysis, usually culminating in death. The histopathology of rabbits was characterized by edema and hemorrhage in the spinal cord and edema in the brain.

Introduction:
For over 100 years now, Shigella dysenteriae type 1 has been known to produce one of the most potent of the lethal microbial toxin. It was originally called Shiga toxin and classified as neurotoxin because it results in a delayed-onset limb paralysis terminating in death when administrated to sensitive animals (22, 23, 26). Shiga toxin is also cytotoxic to a restricted number of cultured cell lines, including HeLa and Vero (2, 6, 14), as well as enterotox (results in fluid secretion) when applied to intestinal mucosa (15).

Although the enterohemorrhagic Escherichia coli-produced Shiga like toxins, SLT1 and SLT2, are encoded by present in transforming phage, the Shiga toxin gene in Shigella, stx, is uniquely present on the chromosome of Shigella dysenteriae type 1 (26). The Shiga holotoxin contains a single A-subunit of approximately 32 kDa associated with a pentamer of 7.5 kDa B-subunit. The B-subunit binds to a membrane glycolipid, globotriasoylceramide (Gb3). The A-subunit cleaves a single adenine residue from the 28S rRNA component of eukaryotic ribosomes. The resulting inhibition of protein synthesis lead to death of the intoxicated cell (6, 10, 28).

Olitsky and Kligler first demonstrated Shiga toxin to be distinct from endotoxin in culture filtrates of Shigella (19). Early attempts at isolation of Shiga toxin involved either extraction at pH 11 from heat-killed bacteria (29) or enrichment from 24h culture filtrates by means of ultrafiltration and isoelectric focusing on polyacrylamide gels (12). Olnsnes and Eiklid described procedures using adsorption to acid–treated chitin columns (12).

In this investigation, the Shiga toxin from Shigella dysenteriae type 1, isolated from patient with bloody diarrhea, was partially purified using ion exchange chromatography, and its biological properties were studied.
Materials and Methods:

Bacterial Strains: A virulent strain of Shigella dysenteriae type 1, designated BB46 where isolated in Najaf city 2003, from stool of a patient with bloody diarrhea was used throughout the current study because it was known to produce Shiga toxin and had invasiveness property. (1)

Preparation of Cell Lysate: Bacteria were growing in a modified synancea broth [1% casamino acids (Difco, USA), 0.2% glucose, 0.004% tryptophan, and 0.002% nicotinic acid (BDH, England)] for 48h at 37°C with shaking (180 rpm), harvested by centrifugation (Segma, USA) at 4°C, suspended in buffer 1 (2.42 g tris-hydrochloride, and 5.84 g NaCl into 1 liter distilled water, pH 8), disrupted by 3 minutes of intermittent (15 sec. on, 10 sec. off) sonic oscillation (MES, UK), and the supernatant was collected by centrifugation. (16)

Cytotoxicity Assay: Cytotoxic activity was used to monitor Shiga toxin enrichment during purification steps. The extent of Vero cell detachment was employed as the indicator of cytotoxicity for Shiga toxin. (5) Vero cells (obtained from the Central Health Laboratory, Baghdad) were maintained at 36°C in growth medium consisting of Eagle minimum essential medium 199 with Hanks salts and glutamine (Flow Laboratories, UK) supplemented with 10% fetal calf serum (Flow Laboratories), 100U penicillin per ml, and 100 µg streptomycin per ml in 5% CO₂ atmosphere. To establish monolayers, fresh trypsinized cells were suspended at a concentration of 1.6x10⁶ cells per ml in growth medium, and 0.1 ml samples were dispensed into 96-well microtiter plates (Lab-Tek, USA). Cells were allowed to attach for 24h before experimental use. Serial dilutions (ten fold) were added (0.1 ml) and plates were incubated for an additional 24h. The end point of toxin activity was determined by fixing with 2% formalin in phosphate-buffered saline (PBS), and staining with 13% crystal violet in 5% ethanol-2% formalin-PBS for 20 minutes, stained cell monolayers were dissolved in 50% ethanol. The absorbance (595 nm) of extract was determined with spectrophotometer. A logarithmic plot of dye absorbance versus the dilution of toxin allowed determination of the dilution yielding 50% cell detachment. With this assay, a unit cytotoxic dose is defined as that amount of toxin which causes a 50% reduction of the dye retained in a microtiter well. (8)

Protein Determination: Protein concentrations were estimated by the method of Lowry et al. (11) with bovine serum albumin (Segma) as a standard.

Ammonium Sulphate Precipitation: Ammonium sulphate was added to the pooled supernatant at 4°C to 40% saturation. The precipitate removed by centrifugation at 10,000 rpm for 10 minutes and further ammonium sulphate added to the supernatant to 60%. The second precipitate, collected by centrifugation as outlined, was resuspended in 5 ml of buffer 1, and dialyzed for 40h at 4°C with this buffer.

Column Chromatography: The preparation was divided into equal volumes (2 ml), and each was applied to a 2.5x30 cm diethylaminoethyl-sephadex A-50 (Pharmacia, Sweden), equilibrated with buffer 1. The column was eluted with the same buffer. Fraction size was equal to 2 ml per tube. Every ten fractions were pooled and detected for cytotoxicity in the Vero cell microtiter plates. The fractions with high Shiga toxin concentration were pooled, dialyzed against sucrose saturated solution, and applied (equal volumes of 1 ml each) to a 1x20 cm column of carboxymethyl-sephadex C-50 (Pharmacia) equilibrated with buffer 2 (13.86 g NaH₂PO₄, 1.75 g Na₂HPO₄, and 2.92 g NaCl in 1 liter distilled water, pH 6). The
collected fraction size were equal to 0.25 ml per tube. Beginning with fraction number one, every five fractions were pooled and detected for cytotoxicity in the Vero cell microtiter plates. The toxin containing fractions were pooled and ammonium sulphate was added to 70% saturation. After 4h, the precipitate was collected and dialyzed with PBS. All further steps were at 4°C and sterilized by 0.22 Millipore filters.

Neutralization of Biological Activity: The cytotoxin-neutralization activity was determined according to the method of Karmali et al. (9), by measurement of the dilution of antiserum which completely neutralize the cytotoxicity of a constant amount of Shiga toxin. Serial dilutions (4-fold) of antiserum were prepared and mixed with an equal volume of toxin solution. After incubation for 1h at 37°C, 0.1 ml of each mixture was added to Vero cell monolayers. After overnight incubation, the monolayers were fixed and stained as described above. The end point was defined as the dilution of antibody which completely neutralized the toxin.

Animal Toxicity Test: A total of 57 animals were employed, comprising in 25 adult New Zealand White rabbits, and 25 adult Swiss White mice. Groups of 5 animals of each species, were inoculated with 5-fold dilution of a partial purified Shiga toxin. The toxin was administrated intravenously to rabbits and intraperitoneally to mice. All animals were observed daily for symptoms. Death between days 1 and 10 inclusive were tabulated. Lethal dose 50 (LD50) was calculated by using the method of Reed and Muench (25). At the time of rabbits death, specimens were taken from different organs for histopathological changes observation.

Results: Purification of Shiga Toxin: The use of ion exchange chromatography to purification of Shiga toxin from Shigella dysenteriae type 1 has documented (3). During partially purification steps, a stained microtiter plates and the curves were used for cytotoxicity assay. The curve was obtained by plotting absorbance value at 515 nm eluted dye versus the log of toxin dilution. The cytoxic dose 50 (CD50) value was obtained by extrapolating one half of this value to the log scale of toxin dilution. According to the above, the cytotoxicity (CD50/ml) of the cell lysates centrifugation was 316×10^5 CD50/ml, and in the step of ammonium sulphate precipitation at 28% and then 50% saturation was 501 × 10^5 CD50/ml (Table 1). The toxin of Shigella dysenteriae type 1 was then purified by using diethylaminoethyl sephadex column. A typical protein elution profile with cytotoxic activity to Vero cells monolayers below the protein profile is shown in Figure (1). Several peaks of protein appeared. The cytotoxicity was not detected in the first and second peaks, put retained slightly and eluted as a broad band after the second eluted protein. The cytotoxicity was assayed in the toxin containing fractions as 125 × 10^5 CD50/ml (Table 1).
The product was then purified with carboxymethyl sephadex column. The elution profile of protein with photography of the toxin activity to Vero cell monolayers are shown in Figure (2). Several peaks were also appeared. The cytotoxicity was clearly identified in fractions from 26-40. The toxin containing eluent was pooled and precipitated with ammonium sulphate to 70% saturation. The cytotoxicity was assayed as $79 \times 10^5$ CD50/ml (Table 1).

The protein concentration, specific activity (CD50/mg protein), purification fold, total activity, and yield of the *Shigella dysenteriae* type 1 protein-toxin for various steps of purification scheme are also shown in Table (1). From 6 liters of culture medium, 19 mg/ml protein was obtained and 0.19 mg/ml protein-toxin was prepared at the final step of purification. The specific activity of the toxin varied from $1.3 \times 10^5$ CD50/mg protein at the first step of purification to $41.8 \times 10^5$ CD50/mg protein at the final step of purification. The final purification of the toxin was about 32.15 fold as compared with the starting material. However, the cytotoxic activity of the partially purified toxin was neutralized by rabbit antiserum.
Biological Activity in Animals: The *Shigella dysenteriae* type 1 partially purified toxin was lethal to rabbits and mice. The 50\% lethal does (LD50) of the preparation being 0.2 and 40 µg/kg body weight in rabbits and mice, respectively. Mice displayed no symptoms until typically the second day, when some mice were found dead, and others remained asymptomatic or developed flaccid paralysis, usually beginning in the hind legs. Rabbits also developed flaccid paralysis prior to death, although this was predominantly in the front legs. Some animals developed symptoms of diarrhea on the second or third day.

Histopathological examination of the dead rabbits appeared that spinal cord tense and swollen, and visible foci of hemorrhage were noticed in the gray matter at the level of cross sections of the cord. Microscopical observation of the spinal cord reveal that the gray matter was the most affected area, with occasional extension to the adjacent white matter. This change included focal patchy hemorrhage, vesicular congestion, and rarely associated interstitial edema (Figure 3). More advanced lesions showed patched of cellular vacuolization and interstitial edema of neurophils without associated patches of hemorrhage (Figure 4). The most severe lesions showed extensive areas of vascular congestion, hemorrhage, and interstitial edema (Figure 5). However, identical pathological changes including marked interstitial and cellular edema were detected in brain tissue (Figure 6). No histopathological abnormalities were detected in sections of small intestine,
colon, kidney, liver, spleen, lung, and heart in test rabbits, and in all sections of control rabbits.

Figure (3): Marked vascular congestion of blood vessels and thrombus formation of intravenous Shiga toxin in rabbit spinal cord. Haematoxilin and eosin stain; X100.

Figure (4): Marked cellular swelling and vacuolization of neuron cells of rabbit spinal cord after intravenous Shiga toxin. Haematoxilin and eosin stain; X100.
Figure (5): Wide spread hemorrhage and edema in the spinal cord of a Shiga toxin-intoxicated rabbit. Haematoxilin and eosin stain; X200.

Figure (6): Marked hydropic degenerated intracellular edema of intravenous Shiga toxin in rabbit brain tissue. Haematoxilin and eosin stain; X100.
Discussion:

The purification process described above was used to produce a Shiga toxin preparation of high biological activity. As shown in Table (1), very high cytotoxic activity was observed by *Shigella dysenteriae* type 1, strain BB46 in the cell lysates centrifugation. This is partly due to the high association constant between toxin and cell surface receptors, and to the high number of binding sites (about $10^6$/cell) (20), which insures that most of the Shiga toxin added to the culture is actually bound to the cells. However, the concentration of toxin-protein at the final step of purification was 0.19 mg/ml. Other investigators noted that although the Shiga toxin is extremely potent, its purification of such toxin is difficult due to its presence in extremely low concentration in the culture of the organisms (22, 24). The yield of protein-toxin per 250 liters of culture as reported by Brown *et al.* (3) was 1.2 mg/ml. By contrast, Olsnes *et al.* (21), reported yields of 150 ng of purified toxin from 3 liters of culture. While, O`Brein and LaVaeck (17), reported yield of up to 100 µg of purified protein-toxin from 4 liters of culture.

The specific activity of partially purified toxin in the present study is $4.18 \times 10^5$ CD50/mg protein at the final step of purification. However, a range of specific activity values have been reported, for example, $8.20 \times 10^5$ CD50/mg (3), $3.44 \times 10^7$ CD50/mg (5), and $5 \times 10^8$ CD50/mg (17). These difference in the specific activity could be related to the *Shigella dysenteriae* type 1 strains, sensitivity of cell monolayers used to detect cytotoxin, variation among laboratories in the method used to measure cytotoxicity, or to the accuracy of toxin purity.

In this study, the partial purified toxin was lethal to rabbits and mice. The LD50 of toxin for mice was 40 µg/kg (1.3 µg/mouse), was higher than reported by O`Brein and LaVaeck (17), when they found it only 0.1 µg for purified Shiga toxin, and lower than that reported by O`Brein *et al.* (18), they estimated as 23 µg/mouse, but for crude Shiga toxin. Such difference in the lethality may reflect differences in sensitivity of the mice and depending on purity of Shiga toxin. Results also show that mice were less susceptible than rabbits (LD50: 0.7 µg/rabbit) to lethal effect of partial purified toxin. This finding concords with that observed by Cavanagh *et al.* (4).

In the present study, the clinical illness of rabbits was dominated by neurological involvement and frequently culminated in death. Watery diarrhea and paralysis predominantly in the hind legs were the cardinal signs of illness. Brown *et al.* (3) reported that the intravenous inoculation of Shiga toxin paralyzed and killed rabbits. However, sections of spinal cords of rabbits showed vascular congestion of blood vessels and thrombus formation surrounded by interstitial edema, brain tissues showed intracellular edema, and no histological abnormalities were detected in any of other tissues. It has been suggested that hemorrhage into the spinal cord, due to the toxic effect on the endothelial cells, is the reason for paralysis and for the lethal effect of Shiga toxin (23). However, Richardson *et al.* (27) showed that Shiga-like toxin 1 (is virtually identical to Shiga toxin) dose not act directly on neuron but that it can cause secondary neurological disorders by its action on the vascular system of the spinal cord and brain. It has been reported by Nataro and Kaper (13), that Shiga toxin is concentrated in central nervous system and gastrointestinal rabbits tissues. The target of these tissues is related to uptake of highly
specific toxin. However, the glycolipid (globotriosylceramide, Gb3) is the functional binding receptor for Shiga toxin (7, 10, 14).

The lack of histological abnormalities in the small intestine and colon of the rabbits in this study, may be due to the use of partially purified toxin which only causes functional abnormalities (diarrhea) in these tissues without visible pathological disturbance. This result agree with those found by Richardson et al. (27), who observed that small intestine and colon of rabbit tissues contain Gb3 concentrated significant quantities of Shiga like toxin 1, but these tissues lack histological abnormalities.

In conclusion, partially purified Shigella dysenteriæ type 1 (strain BB46) toxin was cytotoxic for Vero cells and was paralytic and lethal for rabbits and mice. Future studies may be needed for identifying other factors necessary for Shigella dysenteriæ type 1 to produce shigellosis.

References: