Production and Partial Purification of Heat-Stable Enterotoxin (A) Produced by Enterotoxigenic *Escherichia coli*

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Summary

A total of (25) stool samples were collected from children and adults (2- 4) years old suffering from diarrhea to isolate *E. coli* strains that produce heat-stable enterotoxin a (STa), and after performing microscopic examination, cultural characterization and biochemical identification only (11) isolates showed positive *E. coli*. STa activity was estimated by using suckling mouse assay (SMA) and from these (11) isolates only (5) showed STa activity and the one with the highest STa activity was selected for large scale production of STa, which was followed by partial purification using ion-exchange chromatography (normal phase) using DEAE sephadex A-50 column. After purification and determination of protein concentration by using the standard curve of bovine serum albumin, the concentration of toxin-protein was estimated as (1.08) mg/ml. The specific activity varied from (350) U/mg protein at the first step of purification to (2366.6) U/mg protein at the final step, while the final purification of the toxin was about (6.76) fold and with a yield of (18.25) %.

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انتاج وتنقية جزئية للسم المعوي الثابت حراريا (أ) المنتج من قبل بكتريا ايشيريشيا القولون السمية المعوية

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الخلاصة

جمعت (25) عينة خروج من مرضى يعانون من الاسهال لغرض عزل عزلات ايشيريشيا القولون المنتجة لـ (STa) , وبعد اجراء الفحص المجهري و التشخيص الزرعي و الفحوصات البايوكيميائية, شخصت (11) عزلة ايجابيا على انها ايشيريشيا القولون.وقدرت فعالية الـ (STa) عن طريق استخدام فحص الفئران الرضع (suckling mouse assay), وبعد استخدام هذا الفحص فقط (5) عزلات اظهرت فعالية سمية و من هذه العزلات تم انتخاب العزلة التي اظهرت اعلى فعالية من اجل استخدامها للانتاج المعياري الواسع لـ (STa) والذي اتبع بالتنقية الجزئية عن طريق استخدام كروماتوغرافيا التبادل الايوني (ion-exchange chromatography). في نهاية التنقية وبعد حساب تركيز البروتين, تم الحصول على 0.108 ملغم/ مل بروتين, وبفعالية نوعية قد تراوحت من 350 وحدة/ملغم بروتين في بداية التنقية الى 2366.6 وحدة/ملغم بروتين في الخطوة النهائية لعملية التنقية , اما عدد مرات التنقية للسم فكانت 6.76 مع حصيلة بروتينية مقدارها 18.25%.

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Introduction

Like most mucosal pathogens, *E. coli* is said to follow a requisite strategy of infection: colonization of mucosal site, invasion of host defense, multiplication, and host damage. The most highly conserved feature of diarrheagenic *E. coli* strains is there ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut (1, 2).

Six major groups of *E. coli* are associated with diarrheal diseases; First, *E. coli* strains that produce enterotoxins which called (Enterotoxigenic *Escherichia coli* / ETEC), there are numerous types of these toxins some of them are cytotoxic, damaging the mucosal cells, whereas other are merely cytotonic, including only the secretion of water and electrolytes. The second group of *E. coli* strains has invasion factors and cause tissues destruction and inflammation resembling the effect of *Shigella*, called (Enteroinvassive *Escherichia coli* / EIEC). The third group called (Enteropathogenic *Escherichia coli* / EPEC) includes strains associated with outbreaks of diarrhea in newborn nurseries but not producing recognizable toxins or invasion factors (2; 5). ETEC strains produce two types of enterotoxins; heat liable-toxin (LT) and heat-stable toxin (ST). LT is a large oligomeric toxin that is closely related to cholera toxins. In contrast, ST is a small monomeric toxin that is closely related to *Shigella* toxins (4).

The heat-stable enterotoxin (ST) is one of the two major types of enterotoxins produced by enterotoxigenic *Escherichia coli*, which causes diarrhea in neonatal animals, human infants, and travelers to undeveloped countries (5). The heat-stable enterotoxins are a family of closely related peptides. They are classified into two structurally, functionally, and immunogenically unrelated types, namely STa and STb (6), which, respectively, are also known as STI and STII. STa is methanol-soluble and infant mice-active peptide toxin, while STb is methanol-insoluble and active in pigs, but inactive in infant mice. The toxic activity of STa is resistance to protease (7), while that of STb is inactivated by treatment with trypsin (8).

The biochemical, physiological and immunological properties and amino acid composition of STa enterotoxins characterized so for are remarkably similar (9), they have a common, highly conserved region with ten amino-acids, including six cysteine residues, located in the same relative positions and linked intramolecularly by three disulphide bonds, which suggests that these enterotoxins have similar tertiary structures and are responsible for the toxic feature of the toxin (10).
The aim of the study is obtaining STa and large scale production of it due to its medical importance in cancer treatment especially in colon cancer.

Materials and Methods

All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England).
Collection of samples: Stool samples were taken from patients (2-40) years old, during (15/2 – 13/4/ 2006) suffering from yellow watery diarrhea in AL-Yarmuk Teaching hospital / Baghdad. Each stool sample was collected from the patient into a sterile tube containing 5ml peptone broth, and incubated at 37C° for (18 to 24) hr before being plated on a screening medium, macConky agar (11).

Isolates were identified depending on their gram staining and microscopic characteristics. Six biochemical tests were used for further identification of E. coli. These are Indole, Methyl red, Voges- Proskaur, Simmon's strate, Urease and Triple sugar iron (TSI) tests.
Production of STa: The production of STa was done according to the method of (12). Suspected isolates that were collected from stool and which has been identified as *E. coli* by biochemical tests, were used for the experiment regarding production of STa, and they were

considered as stock cultures. The starter culture was loop-inoculated from the stock cultures into a tube containing 10ml brain heart infusion broth (for bacterial activation), then incubated at 37C° for 18hr. After that 0.1ml from the starter culture ($1X10^6$ cell/ml) was inoculated into 10ml CA-YE medium in a 100ml flask capacity, then incubated at 37C° for 18hr in a shaking water bath at 140 shakes per min. The culture was then centrifuged at 6,000rpm for 30min, and the respective supernatant was carefully separated and filtered through millipore filter (0.45μm) before testing the STa activity. The *E. coli* isolate that showed the highest STa activity were used for the lab-scale production of STa by inoculating 2.5ml from the starter culture into 250ml CA-YE medium in 2000ml flask capacity, then incubated at 37C° for 18hr in a shaking water bath at 140 shakes per min. The culture was then centrifuged at 6,000rpm for 30min, and the respective supernatant was carefully separated and filtered through millipore filter (0.45μm) and kept at 4C° to be ready for the purification.

Detection and determination of STa activity: Suckling mouse assay (SMA) has been used for the detection of STa activity, and 4 mice were used for each test. Newborn Swiss albino suckling mice (3-5) days old were separated from their mothers immediately before use. Each mouse was inoculated (intragastric, percutaneous injection) with 0.1ml of crude culture filtrate containing 2 drops of 2% Evan's blue stain per ml. After 3-4 hr the mice were killed by cervical dislocation. The abdomen was opened, and the entire intestine (excluding the stomach) was removed with forceps. The intestines and carcasses of the four animals were pooled, and weighed, and the ratio of the intestine weight to remaining carcass weight was calculated and referred to as intestine to body-weight (IW / BW) ratio. The assay was considered as positive if the (IW/BW) ratio is above 0.085. The activity was determined by using the bovine serum albumin (BSA) standard curve by plotting the (IW/BW) ratio against the corresponding concentrations of BSA. Animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded (13).

Determination of protein concentration: the concentration of unknown protein was determined by using the standard curve of bovine serum albumin ,which was carried out by using different concentrations (0, 20, 40, 60, 100, 120, 140μg/ml) from BSA stock solution. Then to test tubes containing 0.1ml of each concentration, 0.4ml Tris-HCl buffer and 2.5ml of Coomassie brilliant blue G-250 stain was added, mixed well, and left to stand for 5min. The optical density at 595nm for each tube was measured using UV-Vis spectrophotometer and the relationship between the absorbance and BSA concentrations was plotted to determine the standard curve of BSA (14).

Preparation of crude STa: After collecting culture supernatant and detecting STa activity and estimating its concentration, crude toxin was prepared, and this was done depending on the method used by (15). Crystalline ammonium sulfate (50mg/100ml) was added to 250ml supernatant of crude culture filtrate and kept at 4C° for 2hr. Then the resulting precipitate was discarded after centrifugation at 6,000 rpm at 4C° for 15min. Then the supernatant was filtered through millipore filter (0.45μm) and the filtrate was concentrated in the oven at 45C° until its volume became (5ml).

After that, the concentrated filtrate was filtered through 0.45μm and then 0.22μm millipore filters. Then the concentrated material was dialyzed against 0.01M phosphate buffer using membrane tubing which cuts off molecules with molecular weight of less than 1,000 dl .The dialyzed material were used as a crude toxin, and stored at ˉ20C° until use. Both the activity and the concentration of the toxin were determined.

Preparation of DEAE-Sephadex A-50 column: DEAE-Sephadex A-50 column was prepared by dissolving 3gm of the resin in 250ml D.W., then it was kept in a shaker water-bath at 85-90C°for (1-2) hr. After cooling, it was washed several times with phosphate buffer (0.01M, pH .7.0) until the pH become near (7.0). Gas bubbles were degassed using vacuum pump.

Then it was poured accurately on the inner side of the column (2 x 10 cm) and equilibrated with the phosphate buffer (0.01 M, pH 7.0) until the pH was about (7.0) (16).
Purification procedure: Crude toxin 5ml was added gently to DEAE-Sephadex A-50 column that has been previously equilibrated with the phosphate buffer (pH 7.0). The fractions have been collected at a flow rate of 30 ml/hr and with a fraction size of 3 ml/tube. Then 50ml of (0.1) M phosphate buffer was added to the column until the absorbency at 280nm reached the base line, and that represent the washing part. After that proteins were eluted using 200ml of (0.01) M phosphate buffer (pH 7.0) that contains gradient of NaCl (0.1-1.0) M and that represent the elution part. Protein contents were traced in accordance to absorbency at 280nm and plotted as absorbency versus the elution volume. STa activity and concentration were also determined (15).

Results and Discussion

Isolation of *E. coli*: Isolates were first identified depending on their gram staining and microscopic characteristics. *E. coli* isolates were found to be gram negative, short bacillus, non-spore forming, occur singly under the light microscope as were also described by (18). Accordingly, (21) isolates were suspected to belong to *E. coli*. Further identification of *E. coli* was also achieved by the six biochemical tests used for this purpose. *E. coli* showed positive reaction for both indole and methyl red tests, but negative reactions for voges-proskauer, citrate and urease tests. In triple sugar iron (TSI) test, *E. coli* isolates turned the color of both the slant and butt to yellow with bubbles formation (19). Results showed that only (11) isolates were identified as *E. coli.*
Production of STa: Synthetic media have been effectively employed for the production of STa from human strains of *E. coli*. However the yield of toxin was shown to vary from medium to medium (20). More than three media were used by authors for the production of STa, including; the trypticase soy (TS) broth (21), the casamino acids-yeast extract (CA-YE) medium (22), and the brain heart infusion (BHI) broth (23).

In this study two media were used for the production of STa, including; BHI broth and CA-YE medium. The results showed that, CA-YE medium is more effective for the production than BHI broth. These results came in agreement with those obtained by (24, 25). They had compared between TS broth, BHI broth and CA-YE medium for the production of STa, and they showed that CA-YE medium was shown to be highly recommended for the production. It yield more toxin with less protein contamination than did other media, beside each of its components was tested for its contribution to the growth and toxin production. The trace salts in this medium (Mg^{+2}, Mn^{+2}, Fe^{+3}) were shown to play an important role to the growth STa producing *E. coli* strains, since elimination of these salts resulted in poor growth. Also STa that had been detected when using CA-YE medium, was not detected when other media were used especially when these media contain vitamins or glucose, which shown to reduce STa production (24). But at the same time, these results disagreed with those obtained by some other authors. Merson (21) and Naline (26) used TS broth for the production of STa. While, Whipp (23), used BHI broth for the production. Those authors showed that, these two media were more effective than CA-YE.
Detection of STa activity: Eleven *E. coli* isolates were tested for STa production by using suckling mouse assay (SMA), and from which only five isolates were shown to produce STa. Different methods were used to detect STa activity, including; dog loop assay (26), intestinal loop assay in adult mouse (15), and rabbit ligated intestinal loop assay (27), in addition to suckling mouse assay.

In this study, SMA was used and it was shown to be effective for detecting STa activity, although comparing with other assays was not done, but it was shown to be also more

effective model by many other investigators comparing with previous assays (3, 20, 24), since dog loop assay requires concentrated culture supernatant, adult mice do not response effectively as suckling mice, and rabbits are not easy to handle comparing with suckling mice assay which require unconcentrated supernatant, response more effectively and mice are more easy to handle.

Partial purification of STa: Purification of crude STa was done from the *E. coli* isolate that showed the highest STa activity by SMA. However, after extraction of STa; protein concentration, STa activity then specific activity were determined after each step of the purification. Results showed that bacterial supernatant had (0.16) mg/ml of crude STa with an activity of (56) U/ml which had a specific activity of (350) U/mg after one fold of purification for (250) ml of bacterial supernatant as shown in table 1.

The purification step was started with precipitation by saturated ammonium sulfate then by concentration in the oven until the volume of the culture supernatant became (5) ml. Protein concentration, STa activity and specific activity in (5) ml were recorded to be (0.41) mg/ml, (594) U/ml and (1448.7) U/mg respectively, with a purification fold of (4and13) and a yield of (21.21%).Unlike many other proteins, STa was collected from the supernatant and not from the precipitate, after precipitation by ammonium sulfate, from which most of the proteins were usually collected (15). Concentration of STa was done by authors usually by using an Amicon-filter in the ultrafiltration-cell. In this study, concentration by oven was used, since it was not easy to obtain an Amicon-filter, depending on the heat-stable nature of the STa (STa can withstand boiling at $100C^o$ for up to 30min), STa activity was detected and it was shown to be not affected by concentration in the oven.

Partial purification of STa was done by ion exchange chromatography using DEAE-Sephadex A-50 column. The concentrated sample from the previous step was passed through the DEAE- Sephadex column. Results shown in figure (1) indicated that washing with (50) ml of (0.01) M phosphate buffer (pH 7.0) allowed the presence of one peak which was represented by fractions (6-13). Then after elution with (200) ml of (0.01) M phosphate buffer (pH 7.0) containing gradient of NaCl (0.1-1.0) M, three peaks were obtained which were represented by fractions (21-31), (31-35) and (36-41). Each peak was tested for STa activity. Accordingly, only one peak in the elution part (21-31) was able to reflect STa activity.

Table (1): Purification steps results of STa using ion-exchange chromatography (DEAE-Sephadex A-50) column (2x10 cm)

Steps	Volume (ml)	Activity (U/ml)	Protein concentration (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Supernatant	250	56	0.16	40	14,000	350	1	100
Crude STa	5	594	0.41	2.05	2,970	1448.7	4.13	21.21
DEAE-Sephadex A-50 column chromatography	27	95	0.04	1.08	2,556	2366.6	6.76	18.25

After that, the fractions of this peak were tested for STa activity, and only nine fractions (21-30) were showed STa activity. Then, these fractions were pooled and collected. Protein concentration, STa activity and specific activity were determined, and results shown in the table (1) indicated a protein concentration of (0.04) mg/ml with STa activity of (95) U/ml and a specific activity of (2366.6) U/mg with a purification fold of (6.76) and a yield of (18.25) in (27) ml.

Partial purification of STa by ion-exchange chromatography was applied by many authors, and all of them indicated the presence of STa activity in the elution part only, and no activity was detected in the washing part (15and 28). In addition, they declared the presence of STa activity in the first peak only of the elution part, except with (28) who indicated STa activity in two peaks.
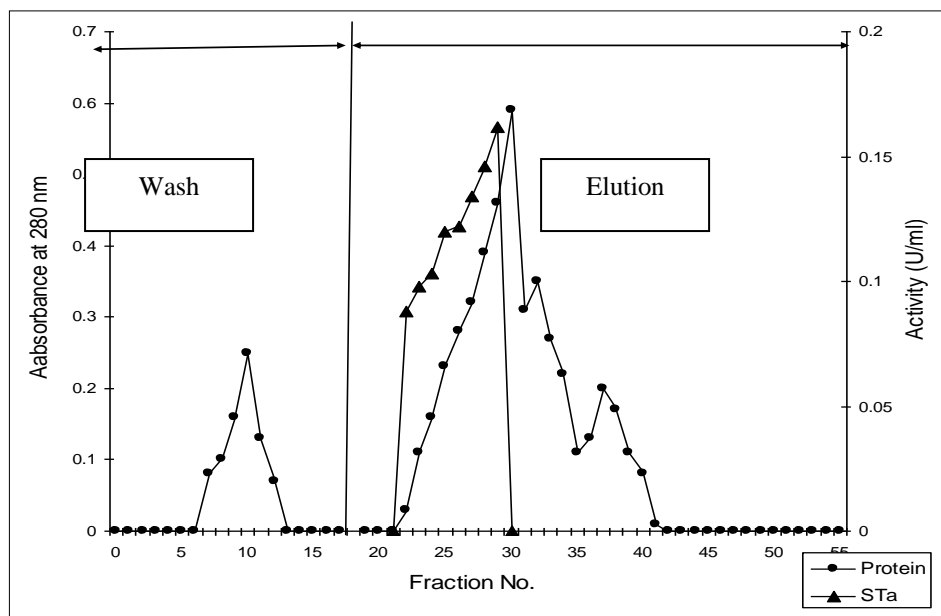

Figure (1): Purification of STa using ion-exchange chromatography (DEAE- Sephadex A-50) column (2x10 cm).

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