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Purification of Heat-Labile Enterotoxin from an Enterotoxigenic *Escherichia coli* of Human Origin by Monoclonal Immunoaffinity Chromatography

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Heat-labile enterotoxin (LT) was purified from an enterotoxigenic Escherichia coli 015H11 of human origin. The purification steps included French pressure cell disruption of the bacteria, salting-out, DEAE-Sephacel on chromatography. Application of this procedure resulted in a 95.1-fold purification of LT with a yield of 19.9% as determined by rabbit ileal loop assay. The final LT preparation showed only one protein-staining band on polyacrylamide gel electrophoresis, indicating that the purified LT was homogeneous.

Key Words: *Purification of heat-labile enterotoxin, enterotoxigenic* E. coli

INTRODUCTION

Enterotoxigenic *E. coli* cause a diarrheal diasease in human and young animals by producing heat-labile (LT) and/or heat-stable (ST) enterotoxins (Donta et al., 1974; Sack, 1975). ST is clearly distinguishable from LT in physicochemical properties, antigenicity, and mechanisms for causing diarrhea (Smith & Gyles, 1970).

Numerous attempts to isolate highly purified LT have been made for a better understanding of pathogenesis and for the development of specific immunological assay. Thus, several investigators purified LT employing various techniques which include ion exchange chromatography (Moss & Richardson, 1978), gel filtration (Lariviere et al., 1973), hydrophobic chromatography (Kunkel & Robertson, 1979), isoelectric focusing (Konowalchuk et al., 1978), agarose affinity chromatography (Clement & Finkelstein, 1979) and immunoaffinity chromatography (Dafni et al., 1978).

Conventional antisera used in immunoaffinity chromatography require an elaborate procedure of immunological adsorption since they usually show cross-reactivity with antigenically related substances.

The recent hybridoma techniques has made to possible to obtain monoclonal antibodies with desired specificities (Kohler and Milstein, 1975). When a specific monoclonal antibody is used in immunoaffinity chromatography, there is no need to resort to immunological adsorption which renders conventional antisera specific.

We have recently reported a monoclonal antibody specific for LT (Kim et al., 1986). This paper describes the use of the monoclonal antibody in immunoaffinity chromatography to purify LT from an enterotoxigenic

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E. coli, 015H11 (LT+ ST–) of human origin.

METHODS AND MATERIALS

Production and purification of monoclonal antibody to LT

Hybridomas(1G8-1D1), producing a monoclonal antibody to LT (Kim et al., 1986), were expanded and injected intraperitoneally into BALB/c mice previously treated with pristane (2, 6, 10, 14-tetramethylpentadecane, Aldrich Chemical Co.). Ascitic fluid collected was brought to 50% saturation with ammonium sulfate and centrifuged at 48,000*g* for 30 min. The dialyzed solution was applied to a Sephadex G-200 column (2.6 x 900cm, LKB) previously equilibrated with the buffer and then eluted with the buffer. Unless otherwise stated, absorbance of elutes from all columns was measured at 280nm with a UV-visible spectrophotometer. Anti-LT antibody in the elutes was detected by GM1 enzyme-linked immunosorbent assay described below. Anti-LT antibody containing fractions were pooled and used for coupling to CNBr-activated Sepharose 4B.

Preparation of immunoaffinity column

The monoclonal anti-LT antibody was coupled to CNBr-activated Sepharose 4B according to the instruction of the manufacturer (Pharmacia). The coupled gel was suspended in 0.02M Tris-buffered saline PH 8.0 (TBS) and packed into a column (1.6 x 5cm).

Preparation of extract of whole-cell lysate

E. coli, 015H11 (LT+, ST–) was obtained from WHO Collaboration Center for Pnage Typing & Resistance of Enteric Bacteria, Division of Entric Pathogens, Central Public Health Laboratory, London, U.K. The bacteria were cultured in CYES-2 medium (Evans et al., 1973) with shaking at 37°C for 18 hrs.

This was harvested by centrifugation at 5,000*g* for 20 min. and disintegrated in a French pressure at 20,000 psi. The whole cell lysate was clarified by centrifuge at 20,000*g* for 20 min. The supernatant was brought to 60% saturation by the addition of solid ammonium sulfate and centrifuged at 10,000*g* for 30 min. The pellet was dissolved in 0.02M Tris buffer, pH 8.0 containing 0.5% (v/v) Triton X-100 and dialyzed against the same buffer. The dialyzed extract of whole-cell lysate was clarified by centrifugation.

DEAE-Sephacel Chromatography

The extract of whole-cell lysate was applied to a DEAE-Sephacel column (2.6 x 45cm) previously equilibrated with 0.02M Tris buffer, pH 8.0 containing 0.5% (v/v) Triton X-100 and eluted with a linear gradient of 0 to 0.5M NaCl in the equilibrating buffer. Fractions collected from the column were assayed for LT activity by double sandwich ELISA described below. LT activity-containing fractions were pooled and concentrated by ultrafiltration through Amicon PM10 membrane, followed by dialysis against the equilibrating buffer. The dialyzed fractions were again subjected to DEAE-Sephacel chromatography as described above.

Immunoaffinity chromatography

The DEAE-Sephacel column eluate was directly loaded onto the immunoaffinity column prepared as above and then washed with TBS. When the absorbance of the eluent at 280nm reached base line, 3M guanidine hydrochloride was applied. The eluted protein was dialyzed against TBS and kept frozen at −20°C before use.

GM1-ELISA

Eluates from the Sephadex G-200 column were tested for the presence of anti-LT antibody by GM1-enzyme-linked immunosorbent assay (GM1-ELISA) as described by Kim et al. (1986).

Wells of polyvinylchloride microtiter plates were coated overnight at 4°C with 50μl of 1.5 mM monosialoganglioside (GM1) in 10mM phosphate-buffered saline, pH 7.3 (PBS). Remaining binding sites were blocked by incubating with 150μl of 3% bovine serum albumin in PBS for 30 min. at 37°C. Partially purified LT (50μl) was added to each well and incubated for 2 hrs at 37°C, followed by 50μl of 1:100 dilutions of the eluates from the column.

Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Cappel Lab.) at a dilution of 1:1000 was then added and allowed to stand for 1hr. at 37°C. Subsequently, 50μl of the enzyme substrate solution (4mg of orthophenylenediamine and 20ul of H_2O_2 in 10ml of 0.1M phosphate-citrate buffer, pH 5.0) was added and incubated for 30 min. at 37°C. The enzyme reaction was stopped by the addition of an equal volume of 2N H_2SO_4. The absorbance was measured at 492nm with a Titertek Multiscan.

Double Sandwich ELISA

Fractions collected from column in purification steps were assayed for LT activity by double sandwich ELISA.

Wells of polystyrene microtiter plate were coated

100μl of monoclonal antibody adjusted to 100μg/ml in 0.04M borate buffer (pH 8.0) for 18hrs at 4°C. Remaining binding sites were blocked as GM1-ELISA.

The wells were added by 100μl of 1:100 dilutions of eluates from column and incubated for 1hr at 37°C. The wells were washed, followed by 100μl of 1:250 dilutions of rabbit anti-LT sera and incubation for 1 hr. at 37°C. Peroxidase-conjugated goat anti-rabbit Ig A, Ig M (Cappel Lab. Lot No. 14216) at a dilution of 1:4000 was added and allowed to stand for 1hr. at 37°C.

The next procedures were carried out as GM1-ELISA.

Protein determination

Protein was determined by the method of Lowry et al., (1951).

Rabbit ileal loop assay

Rabbit ileal loop assay was performed by a modified method of Kasai & Burrows (1966). Rabbits, weighing 1.5kg, were anesthetized with pentobarbital and lidocaine after 3 days of fasting. Six to ten ligated ileal segments, 8 to 10cm long, were made in each rabbit and 1 ml of toxin preparation in PBS were injected into each segment. The animals were sacrificed after 18hr, and the weight of accumulated fluid in grams and the length in centimeters were measured in each loop. LT activity was expressed as the ratio of accumulated fluid to length of the loop.

Polyacrylamide gel electrophoressis

Polyacrylamide disc gel electrophoresis was carried out in 11% polyacrylamide gel according to the method of Davis (1964). Electrophoresis was performed at a constant current of 3 mA per tube at 4°C until the bromophenol blue tracking dye reached the bottom of the gel. Gels were stained with Coomassie brilliant blue and then destained.

RESULTS

Production and purification of monoclonal anti-LT antibody

Monoclonal antibody specific to LT was prepared for use in immunoaffinity chromatography. Hybrid cells producing anti-LT antibody were expanded and injected intraperitoneally into BALB/c mice previously treated with pristane. Ascitic fluid collected from the mice was subjected to salting-out, followed by gel filtration. As expected from its isotype of Ig M, the monoclonal antibody appeared at the void volume (Fig. 1). This monoclonal antibody was coupled

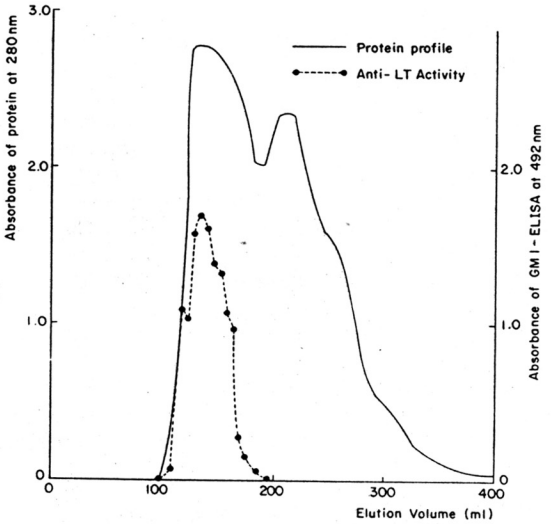

Fig. 1. Gel filtration of monoclonal anti-LT antibody (1G8-1D1, IgM) on Sephadex G-200.
Ascitic fluids were subjected to salting-out, followed by gel filtration on Sephadex G-200. The column (2.6 x 90cm) was eluted with 0.1M carbonate buffer, pH 8.3 containing 0.5M sodium chloride. Anti-LT antibody was determined by GM1-ELISA.

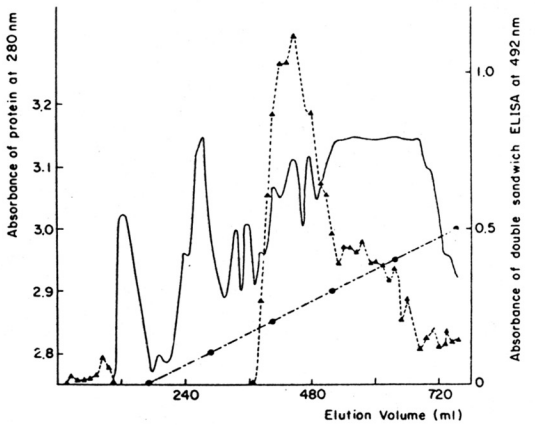

Fig. 2. Elution pattern of the first DEAE-Sephacel chromatography.
Whole-cell lysate of an enterotoxigenic *E. coli* was subjected to salting-out, followed by dialysis. The dialyzed sample was applied to DEAE-Sephacel column (2.6 x 45cm) equilibrated with 0.02M Tris buffer, pH 8.0 containing 0.5% (v/v) Triton X-100 and eluted with a linear gradient of 0 to 0.5M NaCl in the equilibrium buffer. LT activity was determined by double sandwich ELISA.
——— Absorbance of protein at 280nm
▲- - -▲ Absorbance of double sandwich ELISA at 492nm
●-·-·-● a linear gradient of 0 to 0.5M NaCl.

Table 1. Purification of heat-lablile enterotoxin (LT) from *E. coli* 015H11 (LT^+, ST^-)

LT preparation	Total ileal loop activity (ILU*)	Total protein (mg)	Specific activity (ILU/mg of protein)	Purification fold	Recovery of ileal loop activity (%)
Extract of whole-cell lysate	247,000	1710	144	1.00	100
1st DEAE-Sephacel column eluate	241,000	1130	213	1.48	97.6
2nd DEAE-Sephacel column eluate	106,000	203	522	3.63	42.9
Immunoaffinity column eluate	49,100	3.58	13,700	95.1	19.9

*One ileal loop unit (ILU) is defined as the amount of toxin required to produce a fluid accumulation(g) to length(cm) ratio of 0.5

to CNBr-activated Sepharose 4B for use in immunoaffinity chromatography.

Purification of LT

The purification of LT proceeded from whole cells of *E. coli*, 015H11. The whole cells harvested from culture were disintegrated and subjected to salting-out. The precipitate was harvested and applied to a DEAE-Sephacel column. The column was eluted with a linear gradient ranging from 0 to 0.5M NaCl in the equilibrium buffer. As shown in Fig. 2, LT activity was detected in the fractions containing 0.2 to 0.3M NaCl by double sandwich ELISA. As illustrated in Table 1, this purification step resulted in a 1.48-fold purification of LT with a good recovery of 97.6%. LT

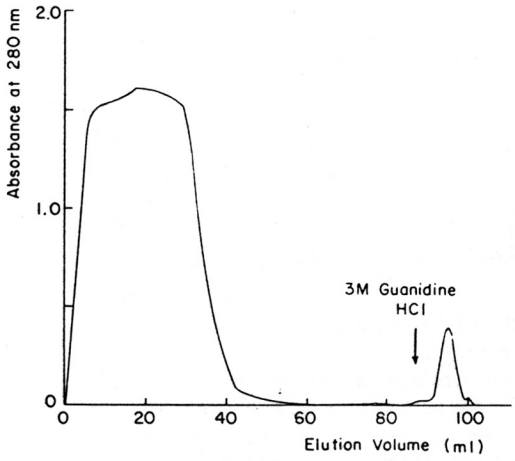

Fig. 3. Elution pattern of affinity chromatography.
The DEAE-Sephacel eluate was loaded onto a monoclonal immunoaffinity column (1.6 x 5cm) and then washed with 0.02M Tris-buffered saline, pH 8.0. LT activity was eluted by shifting the washing buffer to 3M guanidine hydrochloride.

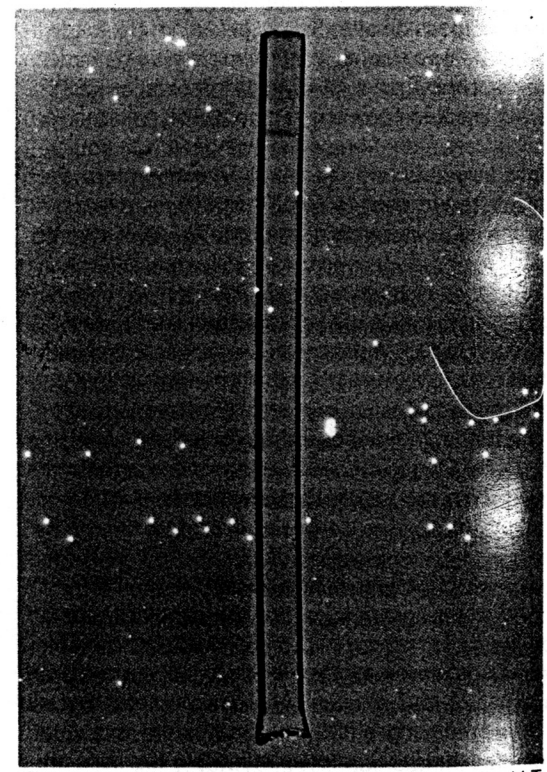

Fig. 4. Polyacrylamide gel electrophoresis of the final LT preparation.
Electrophoresis was performed in 11% polyacrylamide gel at a constant current of 3 mA/gel until the bromophenol blue tracking dye reached the bottom of the gel. Gels were stained with Commassiee brilliant blue.

activity-containing fractions were pooled and concentrated by ultrafiltration through Amicon PM10 membrane. The concentrated sample was run through the DEAE-Sephacel column again as before. The rechromatography resulted in a 3.63–fold purification with respect to the extract of whole-cell lysate but was accompanied by a decrease in LT recovery to 42.9% (Table 1).

LT-containing fractions from the DEAE-Sephacel column were directly loaded onto a monoclonal immonoaffinity column and washed with 0.02M Tris buffer, pH 8.0 containing 0.15M NaCl. As shown in Fig. 3, LT was eluted from the column by replacing the Tris-buffered saline with 3M guanidine hydrochloride solution. The immunoaffinity chromatography eliminated the bulk of foreign proteins, resulting in a 95.1–fold purification with a recovery of 19.9% with respect to the extract of the whole-cell lyzate (Table 1).

Polyacrylamide gel electrophoresis

The final LT preparation was subjected to electrophoresis on 11% polyacrylamide gel (Fig. 4). The result showed that there was a single protein-staining band in the final preparation, indicating that the purified LT was homogeneous.

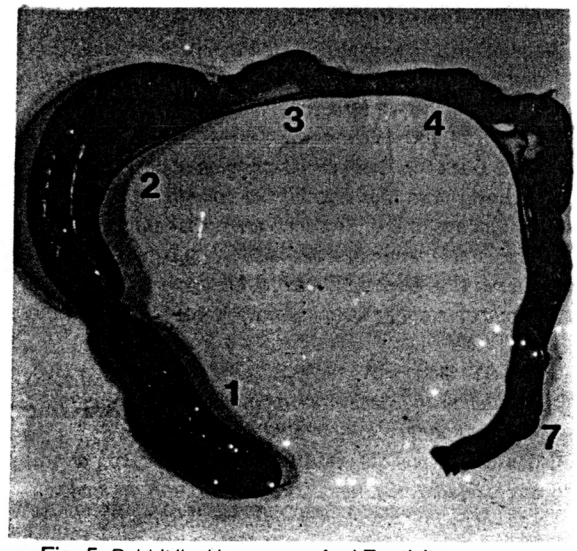

Fig. 5. Rabbit ileal loop assay for LT activity
Two-fold serial dilutions of a LT preparation were injected in volumes of 1ml in 0.02M phosphate-buffered saline PH 7.3. LT activity was expressed as the ratio of fluid accumulation in grams to length of the loop in centimeters

DISCUSSION

Clements and Finkelstein (1979) reported a purification of LT from cell-free supernatant, NaCl extract and whole-cell lysates of a porcine enterotoxigenic *E. coli*, using agarose-affinity chromatography. In their report whole-cell lysate was shown to be the richest source of LT. We therefore attempted to purify LT from a whole-cell lysate of an enterotoxigenic *E coli* of human not poreine origin using an agarose-affinity column. The column was washed before application of 0.1M *D*-galactose as described by Clements and Finkelstein (1979). Unexpectedly LT activity was detected in all washing-out fractions (Data were not shown), indicating that the column did not only have a strong affinity for LT but also was unable to separate LT on its molecular size. Whether the deficiency of the affinity was due to the different source of LT or to other unknown factors was not clear. On the other hand, the most likely explanation for the inefficient separation of LT on its molecular size was the association of LT with it's own or other hydrophobic proteins. Another evidence supporting this view was that LT could not be separated on a DEAE-Sephacel column by an elution buffer which did not contain detergents (Data were not shown). To prevent LT from the their molecular association, 0.5% Triton X-100 (v/v) was included in the LT preparation and in the elution buffer for the DEAE-Sephacel column.

Since polyclonal antibodies usually show cross-reactivity with antigenically related substances, they require immunological absorption which renders them speicific. But monoclonal antibody can be selected for desired specificities without immunological absorption. The monoclonal antibody to LT which we prepared was so specific for LT that it proved to be of great use in immunoaffinity chromatography. As shown in Table 1, the conventional procedure for purification of LT resulted in a few times increase in the specific activity which was accompanied by a great loss of activity. On the contrary the monoclonal immunoaffinity chromatography increased the specific activity of LT to about 100 times that of the starting material with a good recovery.

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