Protocol no. 89-008

Purification of a cytotoxic enterotoxin from a cholera toxin gene-negative *Vibrio cholerae* 01 strain

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INTRODUCTION

A number of cholera toxin gene-negative (CT^-) strains of *Vibrio cholerae* 01, isolated from intestinal and extraintestinal infections and from genetically engineered laboratory strains have been used as candidates for live oral vaccines against cholera (12,16,18). These strains, when fed to human voluteers, caused diarrhea in about one-third of them (16). In 1983, Sanyal *et al.* (28) demonstrated that these candidate live oral vaccine strains of CT^- *V. cholerae* 01 produce another enterotoxin, which subsequently was named "new cholera toxin" (NCT), caused intestinal secretory responses in animal models. The immunobiological studies of this enterotoxin showed that cholera toxin gene-positive (CT^+) *V. cholerae* 01 strains, including hypertoxigenic strain 569B, also produce this toxin, which is identical to that produced by CT^- *V. cholerae* 01 strains (23,25,26,29).

Cholera toxin (CT), the most important pathogenic factor of *V. cholerae* 01, has been isolated in pure state and subjected to extensive physical and chemical characterization (9). On the other hand, the toxin of CT^- *V. cholerae* strain is as yet poorly characterized, although it is known to differ from CT in its antigenicity, receptor site, mode of action and genetic homology (23,27,29). Further, unlike CT, our present observation shows the toxin to have cytotoxic activity in addition to its enterotoxic properties. It has been suggested, without any

experimental data, that this toxin is a Shiga-like toxin (SLT) (19). We, however, have not find any immunological relationship of this toxin with SLT (Seterunnahar Saha, unpublished data) and the strain did not hybridize with SLT1 and SLT2 gene probes (P. Echeverria, personal communication). In view of these findings and recent reports of isolation of CT^- *V. cholerae* 01 strains from diarrheal patients (1,10), it seems evident that further characterization of this *V. cholerae* cytotoxic-enterotoxin (VCCE) is of paramount importance for complete evaluation of its role in disease.

This paper describes the purification and partial characterization of recently observed VCCE (formerly called NCT) from a CT^- strain of *V. cholerae* 01. In addition, attempts were made to determine the immunobiological activities of the purified toxin.

MATERIALS AND METHODS

Bacterial strain. The CT^- *V. cholerae* 01 strain X-392 was provided by Dr. J.B. Kaper, Center for Vaccine Development, University of Malryland School of Medicine, Baltimore, MD, USA.

Purification of VCCE. Purification of the toxin involved culture filtrate preparation, followed by differential ammonium sulfate precipitation, anion and cation exchange chromatography and Sephadex G-200 gel filtration. All chromatographic procedures were performed at 4^oC.

Culture filtrate preparation. Culture filtrates from the strain X-392 were prepared in syncase medium (7) by the method of Saha and Sanyal (24). Five ml of a 4 h culture, seeded with 5-6 smooth colonies from an overnight growth on a nutrient agar plate, was inoculated into 400 ml of broth in 2-liter conical flasks. Cultures were grown at 37°C for 18 h with constant shaking (120 oscillation/min) and centrifuged at 4°C. The supernatant was filtered through a membrane of 0.45 µm average pore diameter.

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the culture filtrate to 30% saturation with constant stirring in the cold and the mixture was kept at 4°C for 2 h. The precipitate was removed following centrifugation at 25,000 X *g* for 30 min at 4°C, the supernatant was further adjusted to 80% saturation with ammonium sulfate and kept at 4°C for 2 h. The second precipitate was collected by centrifugation (as above) and dissolved in phosphate buffered saline (PBS) at pH 7.2 and dialyzed against the same buffer with 6-8 changes to remove the ammonium sulfate completely. This dialysate constituted the enterotoxin preparation, the protein content of which was estimated by the method of Bradford (2) with bovine serum albumin as standard.

Anion-exchange chromatography. The dialyzed pooled material from ammonium sulfate precipitation, i.e. crude VCCE preparation, was applied to a DEAE-Sephadex A_{50} column (45 cm x 2 cm) equilibrated

in phosphate buffer (0.02m, pH 7.2). The column was washed with 30 ml of the same buffer at an elution rate of 0.3 ml/min with 450 ml of 0.0, 0.1, 0.5 and 1.0 M KCl gradient in 10 mM phosphate buffer (pH 7.2). Elution of protein was monitored at 280 nm. Five ml volumes were collected and assayed for cytotoxic and enterotoxic activities. Fractions with peak levels of toxin activities were pooled and concentrated against polyethylene glycol (PEG 15,000-20,000).

Cation-exchange chromatography. The pooled and concentrated material from anion-exchange chromatography was applied to a CM-Sephadex column (15.00 cm x 1.25 cm) equilibrated in phosphate buffer (pH 7.2). The column was washed with 10 ml of buffer at an elution rate of 0.2 ml/min. Fractions of 3 ml were collected with 200 ml of 0.0, 0.25, 0.5 and 1.0 M KCl in buffer. Elution of protein was monitored at 280 nm and assayed for toxin activities. Fractions containing peak levels of activities were pooled and subjected to Sephadex G-200 column chromatography.

Sephadex G-200 column chromatography. Pooled fractions from the cation-exchange step was applied to a Sephadex G-200 column equilibrated in phosphate buffer (0.02M, pH 7.2) at 0.2 ml/min. Fractions of 3 ml were collected with phosphate buffer. The protein containing eluates were pooled, ultracentrifuged at 100,000 X *g* for 3 h and the supernatant was assayed for biological activity. This material was the purified VCCE and used in further study.

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)-PAGE (15) was carried out using 13.5% resolving gel and 4.5% stacking gel concentration. Protein samples and low molecular weight markers were individually mixed with 25 µl of dissociating buffer [0.5 M Tris-HCl (pH 6.8), 2.0% SDS, 5.0% 2-mercaptoethanol, 10.0% glycerol] and heated at 100^{o}C for 5 min. Each sample was loaded onto individual lanes of the stacking gel. Five µl of 0.1% bromophenol blue was mixed with an equal volume of dissociating buffer and loaded onto a lane of stacking gel as a marker dye.

Polyacrylamide gel electrophoresis under non-denaturing condition (4) was carried out in 7.5% separating and 4.5% stacking gels. Electrophoresis was carried out overnight at a constant current of 7 mA until the dye-front reached the bottom of the resolving gel. Protein bands were detected by Coomassie brilliant blue staining.

Antiserum preparation. Albino rabbits (New Zealand) weighing 2.5 kg were immunized with purified VCCE, following our previously described method (23). Thus, 0.5 ml of toxin (100 µg of protein) in Freund's incomplete adjuvant was injected subcutaneously (SC); a further six doses (50 µg) without adjuvant were given SC at 5 days intervals, and the rabbits were bled 5 days after the last injection. The unabsorbed serum was used as the anti-toxin serum. Serum collected before immunization served as the negative control.

Western blotting. Purified toxin was first subjected to SDS-PAGE on 13.5% gels. It was electrophoretically transferred (2 h at constant current of 400 mA) at room temperature from the gel to a nitrocellulose (NC) membrane (30) in the TransBlot system of Bio-Rad. After transfer, NC paper was incubated in 0.01 M Tris-HCl (pH 7.4), containing 1% BSA, 0.15 M NaCl, 0.05% Tween-20 (TBS-T) at room temperature for 1 h to coat residual binding sites. Next the NC paper was incubated with antibodies against VCCE and CT (1:200 in TBS) at 4^{o}C overnight with constant shaking and then washed twice with TBS-T. The strip was incubated with a 1/1000 dilution of horseradish peroxidase conjugated anti rabbit immunoglobulin in TBS-T at 37^{o}C. Finally, the sheet was washed with Tris-HCl and developed with a solution of 0.3% 4-chloro-1-naphthol, (w/v) in methanol, 10 mM Tris-HCl buffer (pH 7.3), 30% H_2O_2 (ratio 1:5:0.002 v/v).

Cytotoxicity. Doubling dilutions of the toxin were made in Eagle's minimal essential medium (with Earle's salt supplemented with 10% fetal bovine serum) and 50 µl of each dilution was pipetted into each well of a confluent HeLa cell monolayer in microtiter plates. The plates were incubated at 37^{o}C in 5% CO_2 for 24 h. The monolayers were checked under an inverted microscope for cytotoxic effects. Rounding and detachment of the cells were considered as a positive effect. The cytotoxin titer, i.e. cytotoxic dose 50 (CD_{50}) was expressed as the highest dilution of the sample that killed 50% of the cell monolayer

after 24 h. Each sample was tested in duplicate.

Enterotoxicity. The enterotoxic property of the toxin was assayed in rabbit ileal loops (RIL) by the method described previously (24). Graded doses of purified toxin were used as the inocula and injected into RIL. Experiments were done in duplicate for each dose of toxin, and results were expressed as the average volume of fluid accumulated per cm of gut in 18 h. The amount of toxin (as protein) required to give a 50% reaction, i.e. effective dose 50 (ED_{50}), equivalent to one unit of toxin, was calculated by interpolation of the dose response curve of the toxin plotted as the volume of fluid accumulation per cm of gut against $\log_{10}$ toxin concentration. Culture filtrate of *V. cholerae* strain 569B (hypertoxigenic) served as positive control and PBS was the negative control.

Neutralization study. Attempts were made to neutralize the enterotoxic activity of purified toxin with homologous antiserum in ligated ileal loops of rabbit (24). Aliquots (0.5 ml) of the toxin (containing 2 ED_{50} protein) were added to each of a series of doubling dilutions of antitoxin (to 1 in 512) in PBS. The mixtures were incubated in a water bath at 37°C for 30 min and tested thereafter in RIL to obtain the highest dilution of antitoxin giving complete neutralization of enterotoxic activity of VCCE. An aliquot (0.5 ml) of the toxin and pre-immunization rabbit serum served as positive and negative control, respectively.

Effect of heat. Aliquots of 1.0 ml of pure toxin (containing 4 ED_{50}) were heated at 56°C for 10 min, 65°C for 30 min and 100°C for 10 min in a water bath. The aliquots were then cooled to room temperature and tested in RIL for enterotoxic activity.

RESULTS

Purification of VCCE. Differential ammonium sulfate precipitation was used as the initial purification and concentration step of the toxin from culture filtrate and tested for both enterotoxic and cytotoxic activities. The precipitate from 80% saturation contained 51.56% of the enterotoxic activity and gave 17-fold purification. The same preparation gave 2.12-fold purification with 60% cytotoxic activity (Table).

Next, the toxin preparation was subjected to anion-exchange chromatography using 0.1, 0.5 and 1.0 M KCl gradient. The biologically active peak was eluted with 1.0 M KCl (Fig. 1) and resulted in 2.75- and 8.25-fold purification of enterotoxic and cytotoxic activity with the yield of 13.9 and 39.5%, respectively.

During cation-exchange chromatography, the active peak was eluted only with phosphate buffer (Fig. 2). Pooled material was purified 25-fold with 37.5% cytotoxic activity and 4.23-fold with 5.07% enterotoxic activity (Table).

The final purification step, Sephadex G-200 gel filtration

(Fig. 3), resulted in a preparation that was purified 5-fold with a recovery of 1.6% enterotoxic and 62.5-fold with 20.0% cytotoxic activity (Table). This purification scheme produced 0.8 mg of purified protein from 5 liter of culture filtrate.

When analyzed by PAGE (Fig. 4a), the ammonium sulfate fraction showed several bands (lane a) and the toxin after purification from the columns gave a single homogenous band (lane b). SDS-PAGE analysis of the biologically active fraction at different steps of purification are shown in Fig. 4b. Fractions of anion-exchange chromatography were more impure (lane 4) than cation-exchange chromatography (lane 3). Pooled fractions of Sephadex gel filtration gave a single protein band (lane 2) which was considered as the purified toxin. The band migrated as a protein with molecular weight (MW) of approximately 61,000, calculated from the migration values of MW-marker proteins (lane 1).

Western-blotting. Western-blot analysis of the purified toxin with homologous anti-toxin showed a single band of MW 61,000 (Fig. 5). No band was demonstrated with anti-CT.

Cytotoxicity. At each step of purification, fractions were tested for cytotoxic activity on HeLa cells (Fig. 6a, 6b). Detachment and rounding of cells were seen at 18-24 h after exposure to toxin . The CD_{50} of purified toxin was 2.0 ng and the total CD_{50}/mg of toxin was calculated to be 5.0×10^5 (Table).

Enterotoxicity. The toxin when tested in RIL for its enterotoxic activity, the ED_{50} showed a value of 11 µg for the purified toxin. This was calculated to be equivalent to 5.5×10^2 cytotoxic units.

The neutralization effect of antitoxin on its homologous toxin (VCCE) was tested in the RIL. The highest dilution of antitoxin serum, that completely neutralized the enterotoxic activity, was 1:512; no neutralization was observed when the toxin was mixed with pre-immunization serum. There was proportionately less neutralization of enterotoxic activity (i.e. more fluid accumulation with higher serial dilutions of antitoxin).

Effect of heat. The pure toxin (4 ED_{50}/aliquot) after exposure at different temperature was tested in RIL to determine the enterotoxic activity. The toxin remained active after heating at 56°C for 10 min. The activity was lost completely after exposure at 65°C for 30 min and 100°C for 10 min.

DISCUSSION

In this study, a cytotoxic enterotoxin was purified from a CT^- *V. cholerae* 01 strain. The production of the toxin was extracellular and thus the culture filtrate preparation in a synthetic medium was used as the starting material of purification with sequential steps of ammonium sulfate

precipitation, ion-exchange chromatography and Sephadex gel filtration. The toxin showed enterotoxic activity in rabbit ileal loops and cytotoxic property on HeLa cells. Therefore, at each step of purification the toxin was screened on the basis of its enterotoxic and cytotoxic activities.

The purified toxin after ion-exchange chromatography and Sephadex gel filtration showed a single band of protein in PAGE under non-denaturing condition and after Coomassie brilliant blue staining, indicating the electrophoretic homogeneity of the toxin protein. In SDS-PAGE, the toxin molecule with molecular size of approximately 61,000 remained undissociated after treatment with 2-mercaptoethanol and heating, unlike the well-studied enterotoxins, such as, CT, *Escherichia coli* LT, Shiga, Shiga-like toxins, etc. which dissociated into sub units. This considerable difference between the toxins are not readily explainable. Several types of enterotoxins/cytotoxins with various characteristics have been isolated from a number of enteropathogens using different purification methods. A homogeneous LT of 102,000 MW has been isolated from *E. coli* strain P-263 with no indication of subunit structure (6). Dafni *et al.* (3) purified a homogenous fraction with a MW of 50,000 using the same *E. coli* strain while Finkelstein *et al.* (8) isolated single polypeptide chains of varying MWs. An undissociated cytotoxic enterotoxin from a strain of *Aeromonas sobria* has also reported by Potomski and coworkers (22).

There are several other reports in agreement with our

results where the toxins showed both cytotoxic and enterotoxic properties. Several authors (13,20,21) reported cytotoxic, as well as enterotoxic properties of Shiga and SLTs isolated from *Shigella dysenteriae* and *E. coli* strains, respectively. Hostaska and coworkers (21) also found cytotoxic enterotoxin from *Aeromonas* sp. in a fraction with MW of 60,000.

Western-blot analysis of purified toxin using polyvalent anti-toxin revealed the presence of a protein band with MW of 61,000, which correspond to the band of the toxin, i.e. VCCE demonstrated by SDS-PAGE. This result confirms that the toxin moiety remains undissociated in SDS-PAGE. However, no band of VCCE was demonstrated in the same set of experiments when anti-CT was used as the antibody. This observation further demonstrates that the toxin is different from CT in its antigenicity and established its difference in molecular characteristics as well.

The biological activities of toxin were demonstrated at each step of purification and the enterotoxic activity could be completely neutralized by antibody raised against purified toxin. After purification, the cytotoxic activity showed an increase in specific activity of 62.5-fold and the unit obtained in HeLa cell cytotoxicity was 5.0×10^5/mg of protein which is comparable with Shiga and Shiga-like toxins (14.17,31). The same fraction of toxin was found to be enterotoxic in RIL and the dose required was comparable with CT and LT (8,9). The enterotoxic activity however increased in specific activity with the gradual steps of purification with 5-fold and yeild of 1.6% only (Table). All

the purification steps were done at 4°C, therefore, the loss of some activity might be due to some ions or factors in the purification process .

In the study VCCE was found to be antigenic and elicited an antibody response when inoculated in rabbit. It is possible that local antibody may be produced *in vivo* that confer immunity to the secretory response of the toxin, as has been shown for other enterotoxins. This aspect of the immune response needs to be studied.

In summary we report the purification to homogeneity of a cytotoxic and enterotoxic protein "VCCE" from a CT^- *V. cholerae* 01 strain which has no similarity with CT, LT (23,26,27) and SLT (unpublished data). The recovery and overall yield of the protein using the scheme is however low and the procedure needs to be optimised using other methods including affinity chromatography. Further studies on the toxin need to be conducted using monoclonal antibodies. Studies also need to be carried out on the receptors on host cell surfaces for the toxin. The presence of antibody to VCCE in the serum of patients who have recovered from cholera also needs to be examined.

ACKNOWLEDGEMENTS

This research was supported by the Government of Australia and the ICDDR,B. The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is supported by countries and international agencies which share its concern for the health problems of developing countries. Current donors include: The aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, the Netherlands, Norway, Sweden, Switzerland, the United Kingdom and the Unites States; international organizations including the United Nations Capital Development Fund, the United Nations Development Programme, the United Nations Children's Fund and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank Manzurul Haque of ICDDR,B for his secretarial assistance.

REFERENCES

1. Batchelor, R.A., and F.S. Wignall. 1988. Non-toxigenic 01 *Vibrio cholerae* in Peru: A report of two cases associated with diarrhea. Giagn. Microbiol. Infect. Dis. 10:135-138.

2. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

3. Dafni, Z., R.B. Sack, and J.P. Craig. 1978. Purification of heat-labile enterotoxin from our *Escherichia coli* strains by affinity immunoadsorbent: evidence for similar subunit structure. Infect. Immun. 22:852-860.

4. Davis, B.J. 1969. Disc electrophoresis II. Method and application to human serum proteins. Am. NY Acad. Sci. 121:404-427.

5. Donohue-Rolfe, A., G.T. Keusch, C. Edson, D. Thorby-Lawson, and M. Jacewicz. 1984. Pathogenesis of Shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. J. Exp. Med. 160:1767-1781.

6. Dornar, F. 1975. *Escherichia coli* enterotoxin, purification and partial characterization. J. Biol. Chem. 250:248-254.

7. Finkelstein, R.A., P. Atthasampunna, M. Chulasamya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biologic activities of purified procholeragen A. J. Immunol. 96:440-449.

8. Finkelstein, R.A., M.K. LaRue, D.W. Johnston, M.L. Vasil, G.J. Cho, and J.R. Jones. 1976. Isolation and properties of heat-labile enterotoxin(s) from enterotoxigenic *Escherichia coli*. J. Infect. Dis. 133(Suppl):S120-S137.

9. Finkelstein, R.A., and J.J. Lospalluto. 1970. Production, purification and assay of cholera toxin. J. Infect. Dis. 121(Suppl):S63-S72.

10. Honda, S.L., K. Shimoirisa, A. Adachi, K. Saito, N. Asano, T. Taniguchi, T. Honda, and T. Miwatani. 1988. Clinical isolates of *Vibrio cholerae* 01 not producing cholera toxin. Lancet 2:1486.

11. Hostaska, A., I, Ciznar, B. Korych, and J. Karoleck. 1982. Toxic factors of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Zent. Bacteriol. Mikrobiol. Hyg. I. Abt. Origin. A. 252:525-534.

12. Kaper, J.B., and M.M. Levine. 1981. Cloned cholera enterotoxin genes in the study and prevention of cholera. Lancet 2:1162-1163.

13. Keusch, G.T., and M. Jacewiz. 1975. Pathogenesis of *Shigella diarrhea*. V. Relationship of Shiga enterotoxin, neurotoxin and cytotoxin. J. Infect. Dis. 131:533-539.

14. Kongmuang, U., T. Honda, and T. Miwatani. 1987. A simple method for purification of Shiga or Shiga-like toxin from *Shigella dysenteriae* and *Eschercihia coli* 0157:H7 by immunoaffinity column chromatography. FEMS Microbiol. Lett. 48:379-383.

15. Laemelli, U.K. 1970. Cleavage of the structural bacteriophage T_4. Nature (London). 227:680-685.

16. Levine, M.M. 1986. Prospects for vaccine. The Magazine of the World Health Organization, April, p.24-26.

17. Mac Leod, D.L., and C.L. Gyles. 1990. Purification and characterization of an *Escherichia coli* Shiga-like toxin II variant. Infect. Immun. 58:1232-1239.

18. Mekalanos, J.J., S.L. Moseley, J.J. Murphy, and S. Falkow. 1982. Isolation of enterotoxin structural gene deletion mutations in *Vibrio cholerae* induced by two mutagenic Vibriophages. Proc. Natl. Acad. Sci. USA. 79:151-152.

19. O'Brien, A.D., M.E. Chen, J.B. Kaper, and M.M. Levine. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. Lancet i:77-78.

20. O'Brien, A.D., and R.H. Holmes. 1987. Shiga and Shiga-like toxins. Microbiol. Rev. 51:206-220.

21. O'Brien, A.D., and G.D. Laveck. 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. 40:675-683.

22. Potomski, I., V. Barke, J., Robinson, D. Fumarola, and E. Miragliotta. 1987. Aeromonas cytotonic enterotoxin cross-reactive with cholera toxin. J. Med. Microbiol. 23:179-186.

23. Saha, S., and S.C. Sanyal. 1988. Cholera toxin gene-positive *Vibrio cholerae* 01 Ogawa and Inaba strains produce the new cholera toxin. FEMS Microbiol. Lett. 50:113-116.

24. Saha, S., and S.C. Sanyal. 1989. Immunobiological relationships among new cholera toxins produced by CT gene-negative strains of *Vibrio cholerae* 01. J. Med. Microbiol. 28:33-37.

25. Saha, S., and S.C. Sanyal. 1989. Neutralization of the new cholera toxin by antiserum against crude enterotoxin of cholera toxin gene-positive *Vibrio cholerae* 01 in rabbit ileal loop model. Indian J. Med. Res. 89:117-120.

26. Saha, S., and S.C. Sanyal. 1990. Immunobiological relationships of the enterotoxins produced by cholera toxin gene-positive (CT^+) and negative (CT^-) strains of *Vibrio cholerae* 01. J. Med. Microbiol. 32:33-37.

27. Sanyal, S.C., K. Alam, P.K.B. Neogi, M.I. Huq, and K.A. Al-Mahmud. 1984. A new enterotoxin produced by *Vibrio cholerae* 01. J. Diarr. Dis. Res. 2:3-12.

28. Sanyal, S.C., P.K.B. Neogi, K. Alam, M.I. Huq, and K.A. Al-Mahmud. 1983. A new cholera toxin. Lancet 1:1337.

29. Sanyal, S.C., S. Saha, S.K. Saha, and C.R. Ahsan. 1987. Immunologic and genetic interrelationship between enterotoxins. P.669-679. *In*: Gopalakrishnakone, P., and C.K. Tan (eds), Progress in Venom and Toxin Research. National University of Singapore, Singapore.

30. Towbin, H., T. Staehelim, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

31. Yutsudo, T., T. Honda, T. Miwatani, and Y. Takeda. 1986. Characterization of purified Shiga toxin from *Shigella dysenteriae*. Microbiol. Immunol. 30:1115-1127.

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TABLE. Purification of VCCE from CT⁻ *V. cholerae* strain X392

Purification step	Total protein (mg)	Specific activity units/mg of protein		% yield		Fold purification	
		Entero-toxicity assay (ED_{50})	Cyto-toxicity assay (CD_{50})	Entero-toxicity assay	Cyto-toxicity assay	Entero-toxicity assay	Cyto-toxicity assay
Culture filtrate	250.0	18.18	8.0×10^3	100.00	100.00	0.00	0.00
Ammonium sulphate precipitation	75.0	31.25	1.6×10^4	51.56	60.00	1.70	2.12
Anion-exhcnage chromatography	12.0	50.00	6.6×10^4	13.19	39.50	2.75	8.25
Cation-exchange chromatography	3.0	76.90	2.0×10^5	5.07	30.00	4.23	25.00
Sephadex gel filtration	0.8	90.90	5.0×10^5	2.39	20.00	5.00	62.50

LEGEND TO FIGURES

Fig. 1. Representative protein elution profile from anion-exchange chromatography of ammonium sulfate precipitated VCCE of *V. cholerae* strain X392.

Fig. 2. Representative protein elution profile from cation exchange chromatography of pooled anion-exchange fractions which contained VCCE activities.

Fig. 3. Representative protein elution profile from Sephadex G-200 gel filtration of pooled cation-exchange fractions which contained VCCE activities.

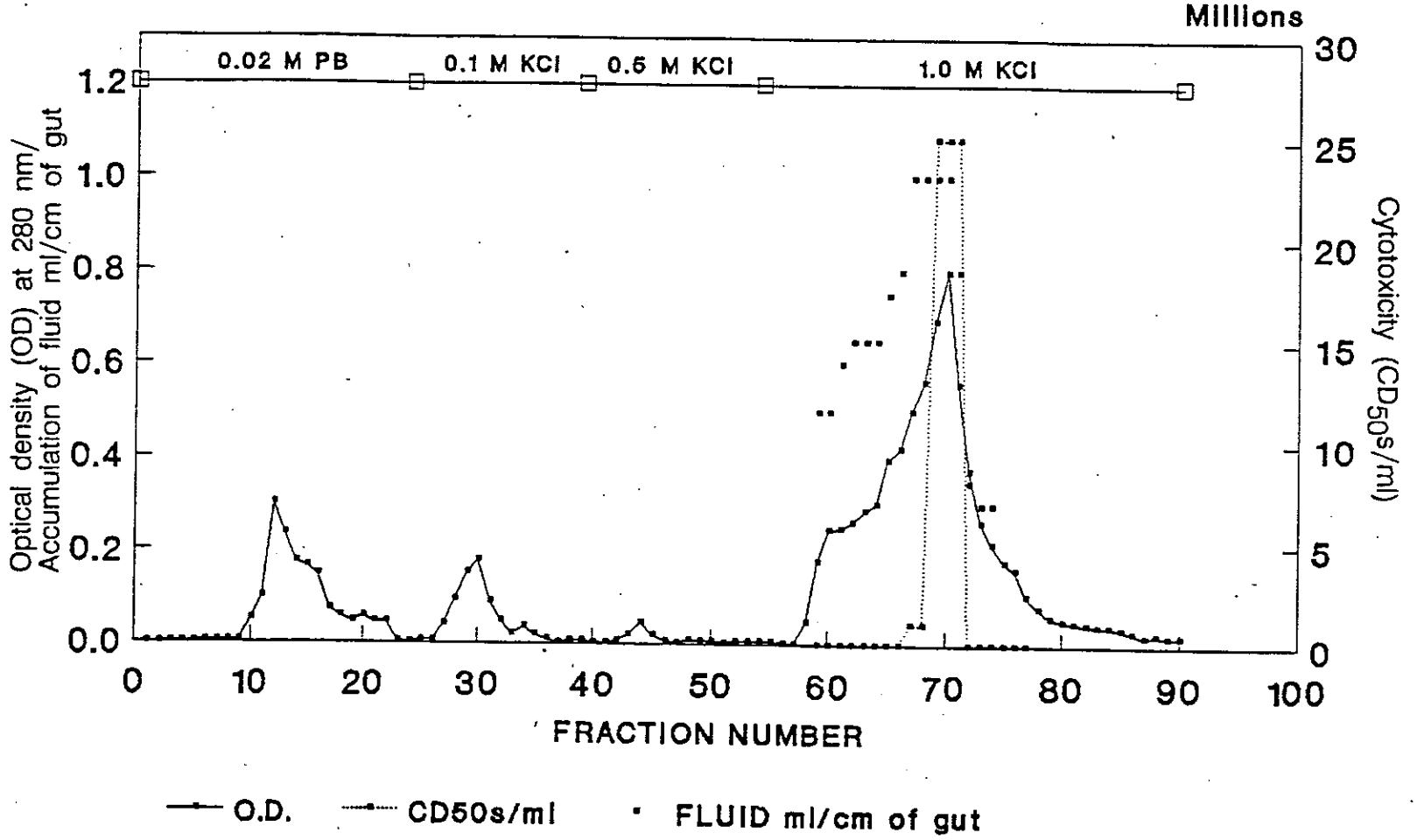
Fig. 4a. PAGE showing different protein bands of ammonium sulfate precipitated (Lane a) and purified VCCE (Lane b).

Fig. 4b. SDS-PAGE showing different protein bands of each step of purification. Lane 1 contains standard protein MW markers (K), Lane 4 and 3 contain anion and cation exchanged active fractions, respectively, and Lane 2 contains the purified VCCE.

Fig. 5. Western-blot of purified VCCE (MW 61,000 K) immunostained with rabbit polyclonal antiserum of VCCE.

Fig. 6a. HeLa cells prior to any treatment.

Fig. 6b. HeLa cells after treatment with purified VCCE.


Millions
0.02 M PB
0.1 M KCl
0.5 M KCl
1.0 M KCl
Optical density (OD) at 280 nm/
Accumulation of fluid ml/cm of gut
Cytotoxicity (CD50s/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER
O.D.
CD50s/ml
FLUID ml/cm of gut

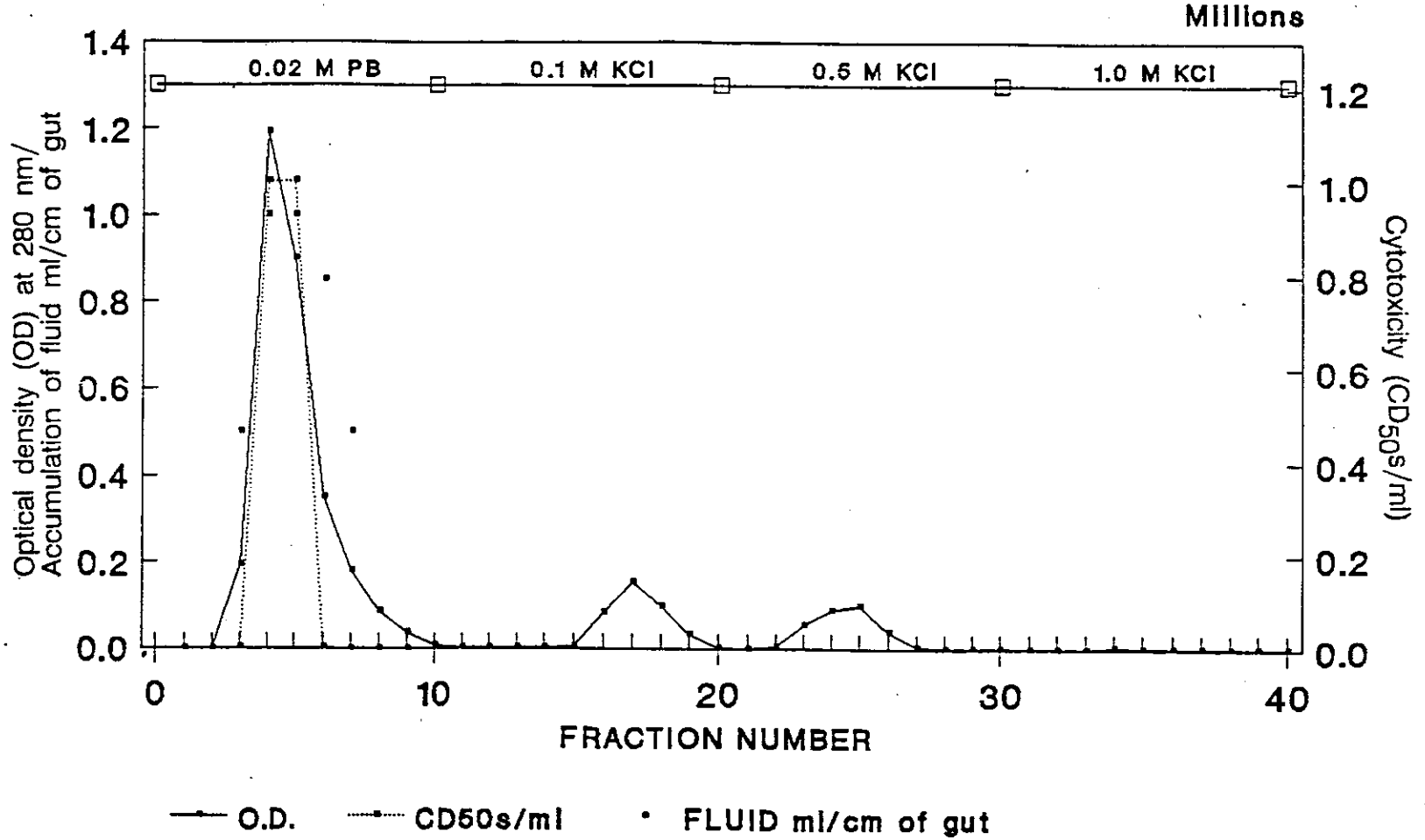
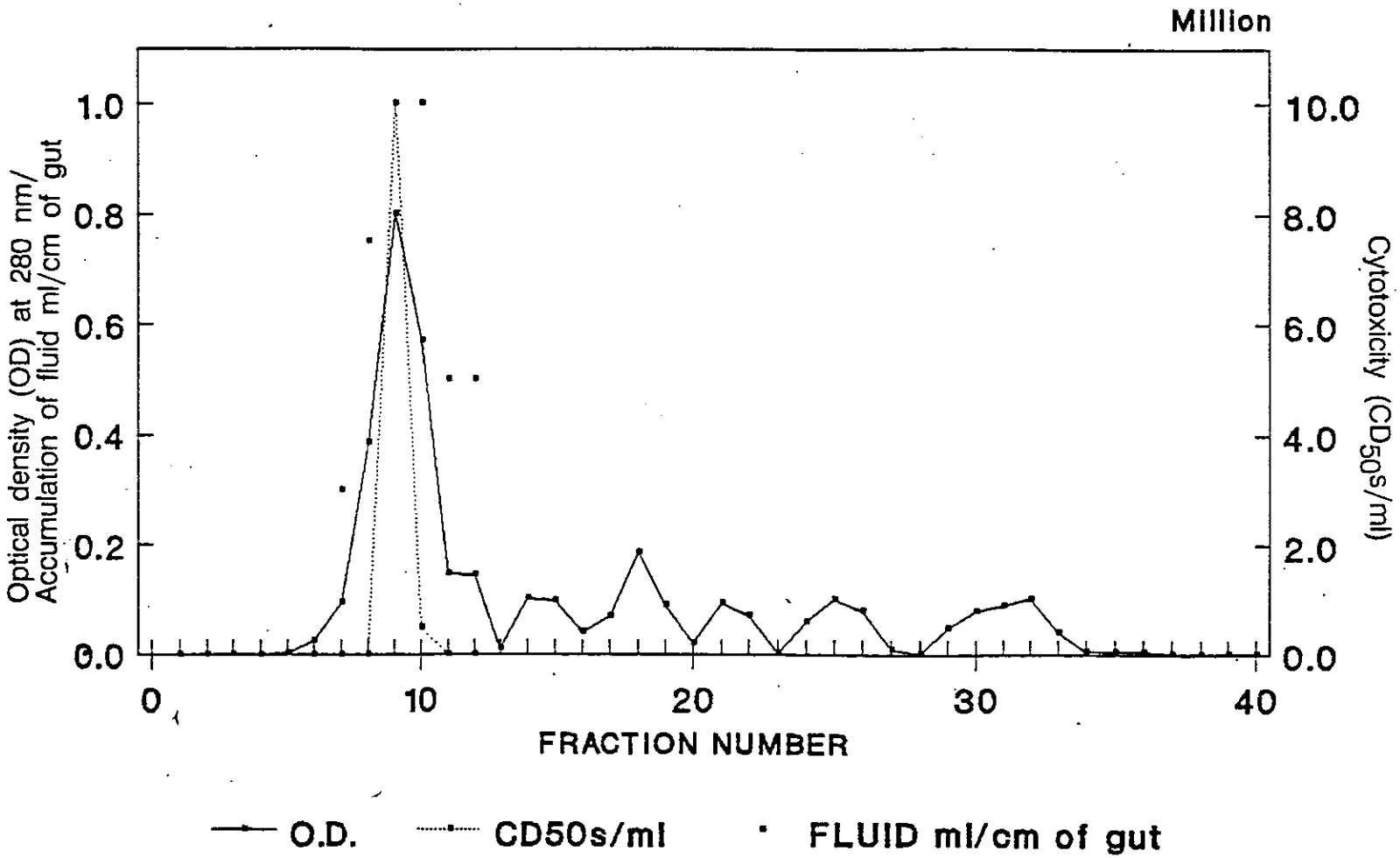

Millions
0.02 M PB
0.1 M KCl
0.5 M KCl
1.0 M KCl
Optical density (OD) at 280 nm/
Accumulation of fluid ml/cm of gut
Cytotoxicity (CD50s/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
FRACTION NUMBER
O.D.
CD50s/ml
FLUID ml/cm of gut

Fig. 2


Million
Optical density (OD) at 280 nm/
Accumulation of fluid ml/cm of gut
Cytotoxicity ($CD_{50}s$/ml)
1.0
0.8
0.6
0.4
0.2
0.0
10.0
8.0
6.0
4.0
2.0
0.0
0
10
20
30
40
FRACTION NUMBER
O.D.
CD50s/ml
FLUID ml/cm of gut

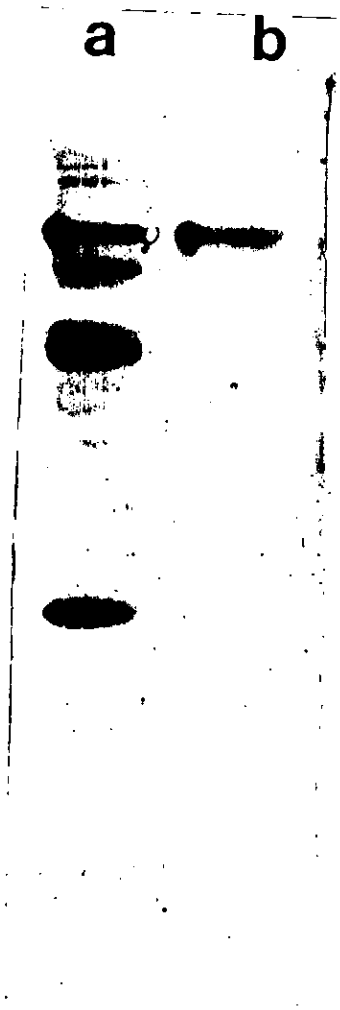


Fig 4a

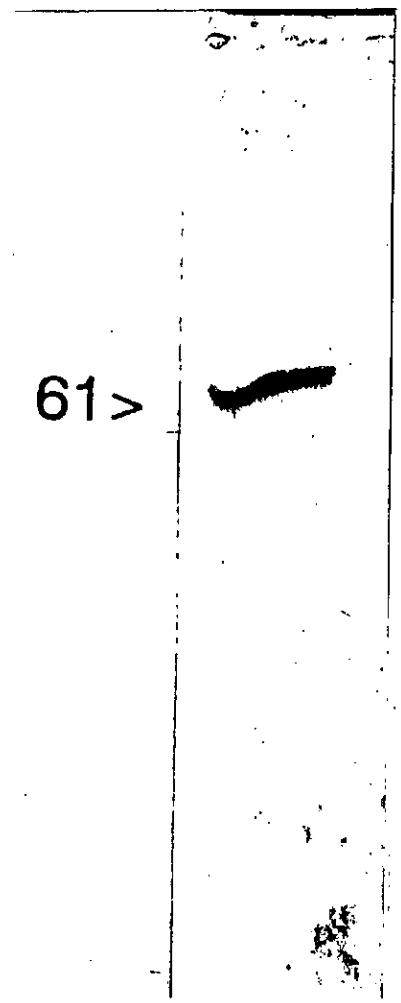


Fig 5

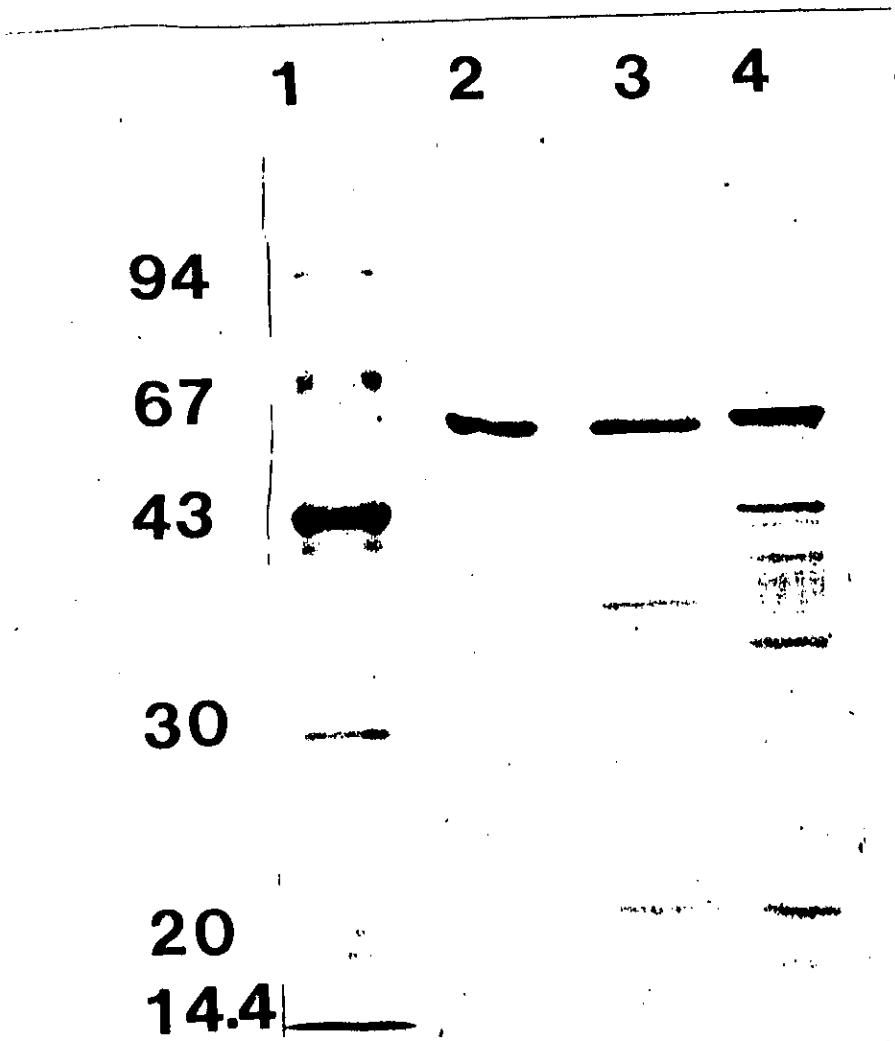


Fig 1A