

PARTIAL ISOLATION AND SOME PROPERTIES OF ENTEROTOXIN PRODUCED BY *BACILLUS CEREUS* STRAINS

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Extracellular proteins produced by Bacillus cereus AL-42 and AL-15 were fractionated by chromatography on QAE-Sephadex and Sephadex G75. This last chromatographic process resulted in three peaks. The major peak showed vascular permeability activity to rabbits, lethality to mice, and cytotoxicity to Vero and Hela cells. The analysis by SDS-PAGE after ultrafiltration confirm recent findings that the enterotoxin is a compound with molecular mass > 30.000.

Key words: *Bacillus cereus* – enterotoxin – toxic factor – purification

Some extracellular proteins produced by *Bacillus cereus* strains seem to be involved in a diarrhea-type syndrome whose predominant symptoms are diarrhea and abdominal cramps (8-16 hr), and an emetic-type syndrome characterized by vomiting after a short incubation time of 1-5 hr (Gilbert 1979; Turnbull, 1981).

The diarrhea caused by *B. cereus* is attributed to a enterotoxin (ET) (Molnar 1962; Goepfert et al., 1972; Gilbert et al., 1981), similar to that of *Vibrio cholerae* (De et al., 1960) or *Escherich coli* (Evans et al., 1973; Moon et al., 1970), and cell extracts of *Clostridium perfringens* (Nilo, 1971). Although several attempts have been made to isolate the ET (Spira & Goepfert 1972; Ezepechuk et al., 1979; Turnbull, 1981), only recently were homogeneous ET preparations obtained.

Two different proteins, with molecular weights of 33-34 KDa and 45 KDa, were isolated from fluid cultures of *B. cereus* using two chromatographic steps DEAE-Sephadex and Sephadex G-75 (Shinagawa et al., 1991a). The 33-34 KDa protein was lethal to mice whereas the 45 KDa protein exhibited in addition vascular permeability activity (Schinagawa et al., 1991a, b). This last toxin seems to be responsible for the diarrheagenic effects of the emetic syndrome (Schinagawa et al., 1991b).

We have used two chromatographic processes, QAE-Sephadex and G 75 Sephadex to isolate a major fraction from *B. cereus* strains with several biological activities.

MATERIALS AND METHODS

Bacterial strains and toxin production – *B. cereus* strains AL-42 and AL-15 isolated from food in Rio de Janeiro (Rabinovitch et al., 1985) were used for ET production. Sporulated stock cultures were maintained on extract-soil-agar slants at 4 °C (Rabinovitch et al., 1985). For ET production, a fresh vegetative culture in nutrient broth incubated overnight at 33-35 °C, was used to inoculate 100 ml of medium (brain heart infusion broth; BHI-Difco Laboratories) in two 250 ml Erlenmeyer flasks. After shaking (120 rpm) for 12 hr at 33-35 °C both cultures were combined and a 5 ml volume was transferred to each of 500 ml volumes of BHI contained in 2 L Erlenmeyer flasks. These cultures were incubated with shaking (120 rpm) for a further 12 hr as above, and bacterial cells were removed by centrifugation at 6.000xg for 30 min at 4 °C. The supernatant fluid was used as the starting material for purification of ET.

Isolation of ET – The procedure for isolating ET is shown in Fig. 1. Briefly, the supernatant from 10 L of culture was concentrated with Aquacide III (Calbiochem-Hoechst) and extensively dialyzed (60 l, three days at 4 °C) against 20 mM phosphate buffer pH 7.0 containing 0.02% NaN₃. After dialysis, any in-

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soluble substances formed were removed by centrifugation. The clear supernatant fluid was added to 400 ml of QAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with phosphate buffer. The mixture was gently rotated overnight at 4 °C. The resin was allowed to settle and the supernatant was decanted. The QAE gel was then packed into a R25 column (Pharmacia Fine Chemicals, Uppsala, Sweden), washed with 2 L of phosphate buffer (0.02 M, pH 7.0), and the adsorbed proteins were eluted with 2 L liters of phosphate buffer containing 0.05 M, 0.1 M and 0.5 M NaCl. The eluates were concentrated to 15 ml by Aquacide III and dialysed extensively against phosphate buffer (0.02 M, pH 7.0). After dialysis, the samples corresponding to both peak 1 and 2 were subjected separately to a column (4 cm x 100 cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) in phosphate buffer (0.02 M, pH 8.0) containing 0.15 M NaCl. The columns were developed at a flow rate of 25 ml/h at 4 °C and the fractions containing ET composing the major peak were collected and tested for biological activity.

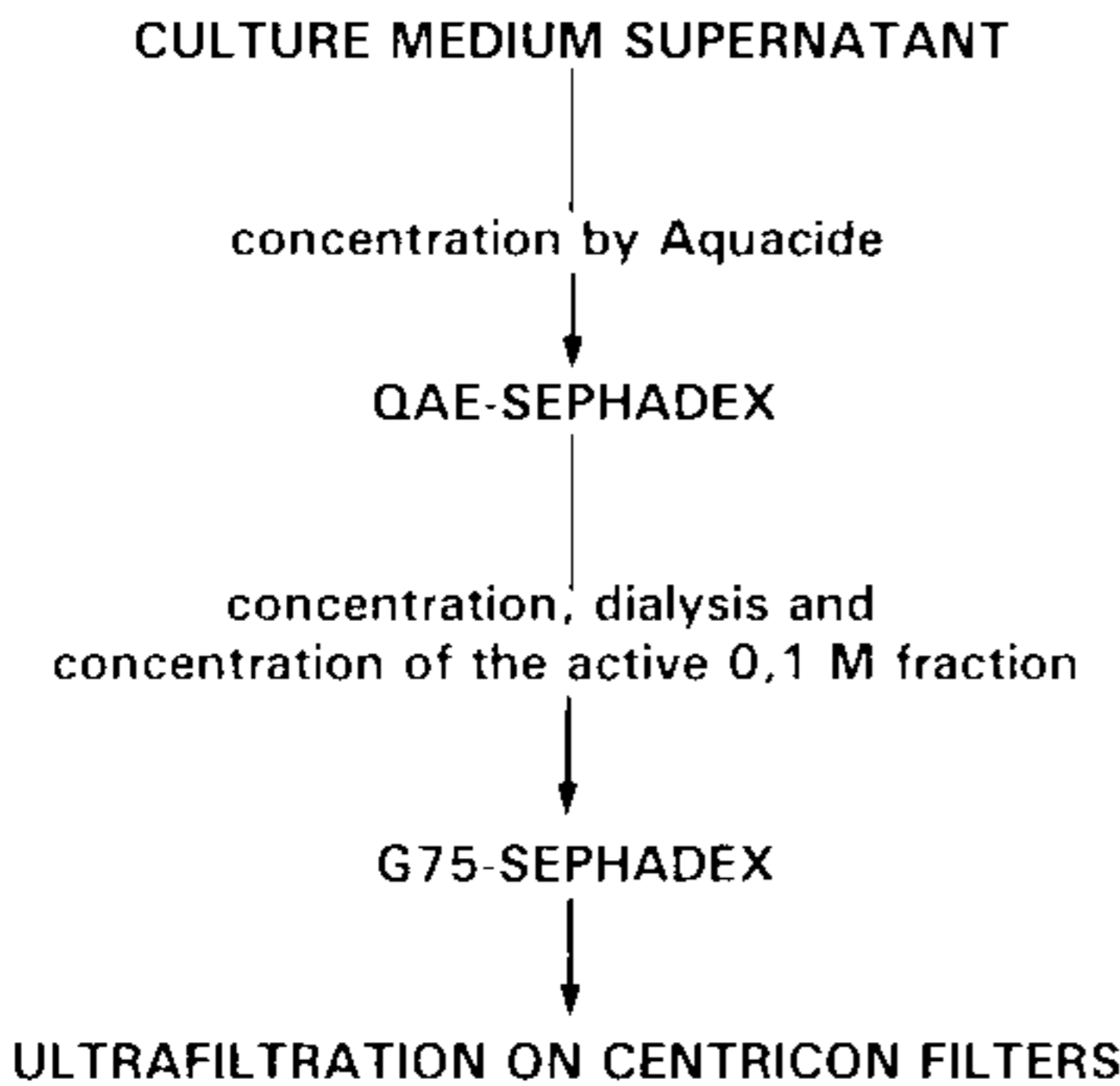


Fig. 1: schematic representation of the procedure used to isolate toxic fraction of *Bacillus cereus* from culture supernatant.

Biological assays – The vascular permeability (VP) and necrosis were assayed in rabbits by the methods described previously (Glatz & Goepfert, 1973). Mouse lethal toxicity (MLT) was assayed by the intraperitoneal injection of 0.5 ml of the fractions 1 and 2. Death within 15 min was considered to be a positive response. *In vitro* cytotoxic activity

was determined by the use of Vero and Hela cells. The cells were maintained in Eagle medium with 10% and 5% fetal calf serum. Test fractions were dialyzed against phosphate buffer (0.02 M, pH 7.0) and 100 µl was added to each plate with cellular monolayer. Phosphate buffer controls were run concurrently with the tests. Cells were incubated in 5% CO₂ at 37 °C and observed at intervals up to 1 h for disruption of the cell monolayer.

Polyacrylamide gel electrophoresis – SDS-PAGE was performed by the method described by Laemmli (1970).

RESULTS AND DISCUSSION

ET activity from the supernatant fluid obtained from isolated strains was found to adsorb to QAE-Sephadex at pH 7.0, although the SDS-PAGE pattern showed a dissimilarity in some bands (Fig. 2). The eluates obtained by increasing the ionic strength (0.05 and 0.1 M

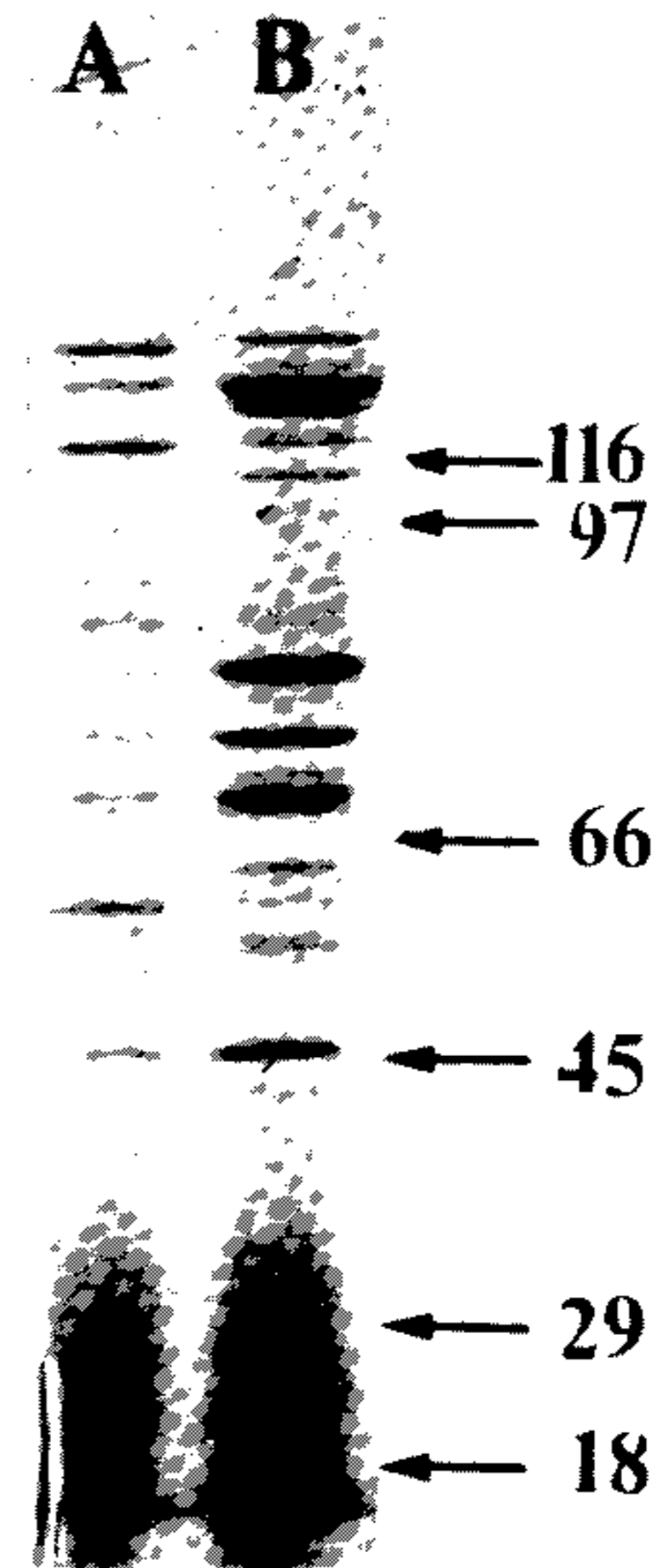


Fig. 2: SDS-PAGE of total supernatant fluid obtained from AL-42 (A) and AL-15 (B) isolated *Bacillus cereus* strains.

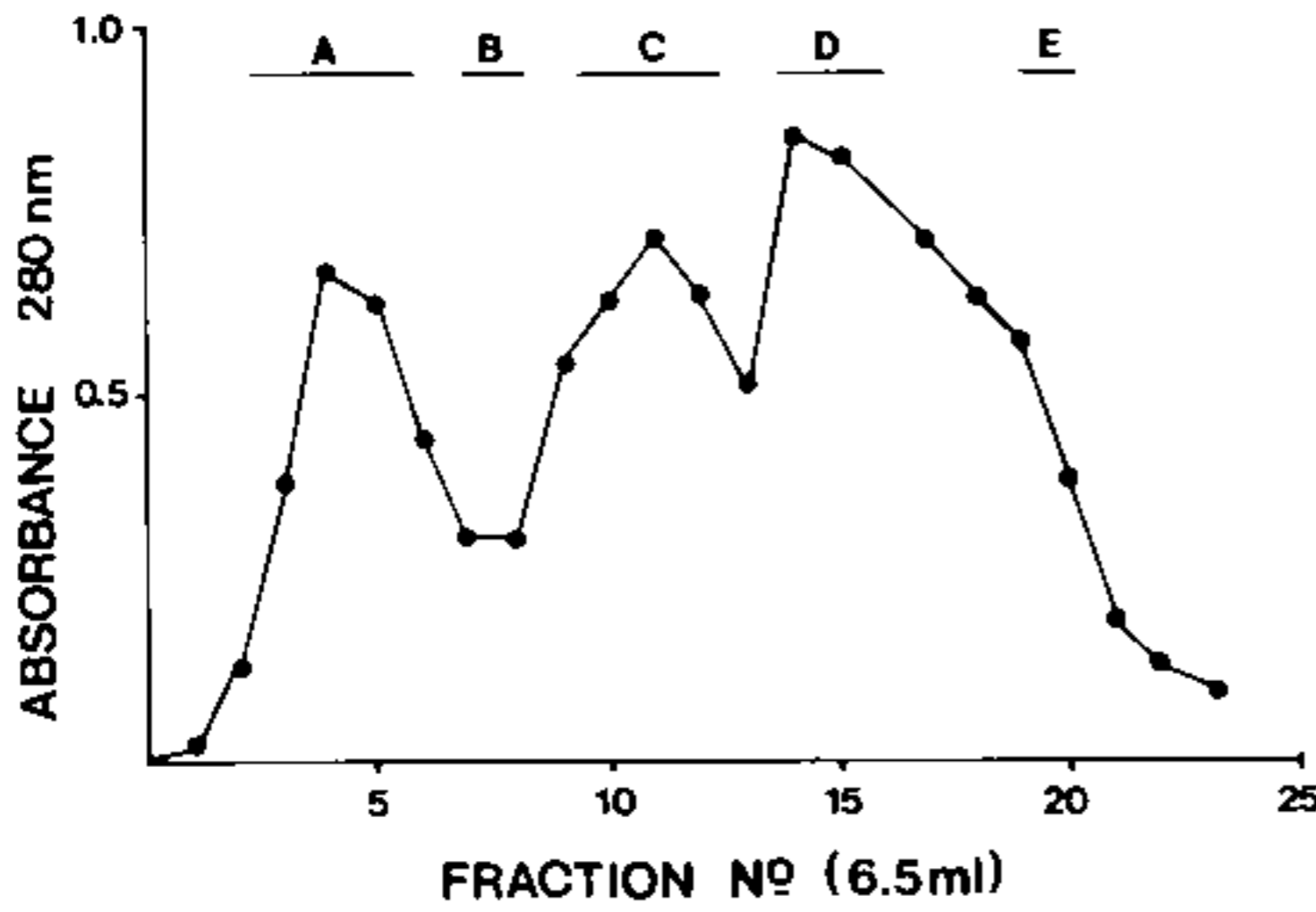


Fig. 3: elution pattern by gel filtration on Sephadex G75 (1.5 x 85 cm) of the 0.1 M fraction obtained from QAE-Sephadex. The letter A-E represent the pooled tubes analyzed for biological activities. Fractions of 6.5 ml were collected from the column at flow rate of 5 ml h⁻¹ using as eluting buffer 0.075 M phosphate pH 7.2.



Fig. 4: SDS-PAGE analysis of the active gel filtration fraction D, after ultrafiltration on Amicon membranes. Retained fraction (A) and ultrafiltered material (B).

NaCl) after concentration showed VP activity, lysis to Vero and Hela cells and lethality to mice. The major activity was observed in the 0.1 M fraction. This fraction was concentrated

by Aquacide and further fractionated by gel filtration on Sephadex G75. Three major protein peaks appeared as shown in Fig. 3 but the ET activity was concentrated in peak 3 (fraction D). The corresponding tubes were concentrated by ultrafiltration on Amicon membranes using a cut-off of 30.000. The retained fraction showed VP activity, cytotoxicity to Vero and Hela cells and lethality to mice. The SDS-PAGE revealed bands with 40-70 KDa (Fig. 4A). Although no stability studies such as inactivation by heating or exposure to low pH values were carried out, the proteic nature of the ET is consistent with previous findings in the other isolated (Glatz et al., 1974; Spira & Goepfert, 1975; Turnbull, 1981) and partially purified vascular permeability factors (Shinagawa et al., 1991b). Thus, the ET preparation with VP activity obtained in this study appear to be responsible for the diarrhoeal-type food poisoning caused by *B. cereus* strains isolated in Rio de Janeiro (Rabinovitch et al., 1985).

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