Proteolysis of *Clostridium perfringens* Type A Enterotoxin during Purification

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Received 21 November 1989/ Accepted 9 March 1990

The small satellite bands of enterotoxin frequently seen in polyacrylamide gels following purification of *Clostridium perfringens* enterotoxin were found to be due to endogenous protease activity and were not present if phenylmethylsulfonyl fluoride (PMSF; 1 mM) and EDTA (10 mM) were used in the purification protocol. The use of PMSF was avoided by passing gel filtration-purified enterotoxin material through DEAE-Sephacel. This modified protocol resulted in an 11.4-fold purification of enterotoxin and a 26.8% yield. Contrary to previous reports (B. R. Dasgupta and M. W. Pariza, Infect. Immun. 38:592–597, 1982), if PMSF and EDTA were included during purification, we were unable to detect the novel enterotoxin ET-1 produced by strain NCTC 10240. *C. perfringens* proteases cleaved homogeneous enterotoxin into two additional fragments, suggesting that ET-1 was a product of endogenous protease action during purification.

The enterotoxin produced by *Clostridium perfringens* is the etiological agent of *C. perfringens* food poisoning in humans (3). The toxin is produced during sporulation in the small intestine following ingestion of large numbers of vegetative cells. Since its identification, numerous methods for purification have been described (1, 4, 5, 7, 14, 15). However, regardless of the method of purification, minor enterotoxin bands immediately anodal or cathodal to the principal enterotoxin band frequently appear following electrophoresis (1, 4, 6, 14; R. G. Labbé, unpublished data). This effect has been ascribed to the action of proteolytic enzymes from exogenous sources (6). The purification of a second distinct enterotoxin, ET-1, has been reported by Dasgupta and Pariza (2). Our previous experience with *C. perfringens* intracellular proteases (9) led us to suspect that these previous results were caused by the action of endogenous proteases.

Enterotoxin was purified from *C. perfringens* NCTC 8798 or NCTC 10240 by (i) the method of Granum and Whitaker (5), (ii) the method of Reynolds et al. (12), (iii) the method of Dasgupta and Pariza (2), both with and without 1 mM phenylmethylsulfonyl fluoride (PMSF) plus 10 mM EDTA (9), or (iv) our method. In the last case, cells were grown for 7 to 8 h in a defined medium (13), harvested by centrifugation (10,000 × g, 15 min), and washed twice with ice-cold 0.02 M sodium phosphate buffer (pH 6.8) containing 1 M NaCl. Washed cells were suspended in about 20 ml of the same buffer without NaCl but containing 1 mM PMSF plus 10 mM EDTA and broken with a previously chilled French pressure cell (American Instrument Co.) at 82.8 MPa. After centrifugation (10,000 × g, 20 min), pellets containing unbroken cells were suspended in the same buffer and passed through the French pressure cell again. Cell extracts were pooled and cell debris was removed by centrifuging the cells twice at 10,000 × g for 20 min. Ice-cold, 80%–saturated ammonium sulfate was slowly added with constant stirring to an equal volume of the chilled crude extract. After 30 min at 4°C, precipitated enterotoxin was collected by centrifugation (10,000 × g, 20 min), suspended in about 20 ml of the buffer described above containing 0.2 mM PMSF plus 1 mM EDTA, and dialyzed overnight against the same buffer. The dialyzed enterotoxin was then treated with an equal volume of 30%–saturated ammonium sulfate solution. After 30 min of stirring at 4°C, precipitated enterotoxin was collected by centrifugation (10,000 × g, 20 min), suspended in 7 ml of the same buffer, and mixed with 0.25 g of sucrose. The crude enterotoxin was applied to a Sephacryl S-200 high-resolution (HR) column (2.5 by 95 cm) and eluted with 0.02 M phosphate buffer (pH 6.8) containing 0.2 mM PMSF plus 1 mM EDTA at a flow rate of 40 ml/h. Enterotoxin fractions (5 ml per tube) eluted in the second peak and were pooled. In one series of experiments, toxin was purified by the same method, but in the absence of PMSF and EDTA. In this case, the pooled enterotoxin fractions were dialyzed against 0.01 M potassium phosphate buffer (pH 8.0), applied to a DEAE-Sephacel column (2.5 by 25 cm), and eluted with a linear (0.01 to 0.06 M) gradient of potassium phosphate (pH 8.0). Flow rate was 40 ml/h with fraction volumes of 5 ml per tube. All chromatography was conducted at 4 to 6°C.

Slab polyacrylamide gel (9%) electrophoresis under non-denaturing conditions was performed as described by Hames (7). Resolved proteins were electrophotographically transferred to 0.2-µm-pore-size nitrocellulose membranes (Micron Separation, Inc.) with a Trans-Blot cell (Bio-Rad Laboratories) and stained with colloidal gold as previously described (11). For dot blotting, grids (1 by 1 cm) were drawn on nitrocellulose membranes, which were then wetted in 20 mM Tris (pH 7.5) containing 500 mM NaCl (TBS) and air dried on filter paper. Sample (5 µl) was applied to each square, and the membranes were allowed to air dry completely. Nitrocellulose membranes were immunostained with horseradish peroxidase according to the protocol of the manufacturer (Bio-Rad), except that the blocking solution was composed of 5% nonfat dry milk. The first antibody solution consisted of 100 µl of antienterotoxin serum in 100 ml of TBS plus 0.3% Tween 20.

Enterotoxin concentrations were determined by dot blotting on nitrocellulose membranes followed by immunostaining. Titers were the highest serial double dilutions which gave positive staining. The toxin concentrations of unknown samples were determined by multiplying the concentration of standard enterotoxin by the ratio of the titer of the

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unknown sample to that of the standard. Antitenterotoxin serum was prepared with electrophoretically pure enterotoxin as previously described (10), except that equal volumes of Maalox (William H. Rorer, Inc.) were used as an adjuvant.

The method of Granum and Whitaker (5) for purification of enterotoxin employs one chromatography step in which enterotoxin characteristically elutes as the second peak. However, immuno-, gold, or Coomassie blue staining of Western blotted (immunoblotted) gels of such purified enterotoxin by us and by others frequently reveals a second minor band. We also detected a faint but visible minor band by immunoblot analysis by using the purification methods of Reynolds et al. (12). The minor band was not visible when protease inhibitors (PMSF and EDTA) were included during preparation of cell extracts and in the elution buffer (method iv) (Fig. 1).

By an alternative method, which avoided the use of the toxic PMSF, enterotoxin from the gel permeation step was further purified with DEAE-Sephacel (Fig. 2). A small enterotoxin shoulder appeared prior to the main enterotoxin peak, and a later protein peak of unknown composition also appeared. Immunostaining (as well as colloidal-gold staining [not shown]) of polyacrylamide gels of enterotoxin obtained by this method revealed one band (not shown). By this protocol, fold purification and yield were 11.4 and 26.8%, respectively, which are similar to the results of the method of Granum and Whitaker (5).

When protease inhibitors were included during cell disruption, Sephacryl S-200 chromatography, and dialysis, we failed to detect the novel enterotoxin (ET-1) from strain NCTC 10240 which was reported by Dasgupta and Pariza (2) (Fig. 3). In another experiment, we removed protease inhibitors by dialysis from Sephacryl S-200-purified enterotoxin and treated the toxin with a mixture of purified intracellular and extracellular proteases (Park and Labbé, unpublished data). This treated toxin was then separated on a DEAE-Sephacel column. Three distinct peaks of enterotoxin (ET-1, ET-2, and ET-3) were eluted at potassium phosphate molarities of 0.02, 0.03, and 0.04, respectively (Fig. 4). As determined by its mobility on gradient (5 to 15%) gel electrophoresis, ET-2 corresponded to standard enterotoxin. ET-1 and ET-3 were more cationic and anionic, respectively, than was standard enterotoxin (not shown).

Our results suggest that ET-1 is an artifact of proteolysis...
by endogenous proteases which act during preparation of cell extracts and purification procedures. Thus, the toxin can be cleaved by its own protease, resulting in relatively minor (satellite band on polyacrylamide gel electrophoresis) or major (ET-1) cleavage products.

Granum and Whitaker (5) claimed that during enterotoxin purification, the first peak to elute during gel filtration of ammonium sulfate-precipitated cell extracts was composed of nucleic acids, while more recently, others (12) have stated that this peak is an aggregated form of enterotoxin which lacks immunological reactivity. When we treated the first peak with DNase or RNase, a rapid drop in absorbance was observed (not shown), indicating that the peak observed of nucleic acids. However, fractions from this first peak did show a small degree of reactivity against antienterotoxin serum (determined by dot blotting), implying some interaction of nucleic acids with enterotoxin.

This study was supported in part by U.S. Department of Agriculture Hatch grant 643.

LITERATURE CITED


