Physiochemical Properties of a Heat-Stable Enterotoxin Produced by *Escherichia coli* of Human Origin

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Concentrated cell-free filtrates, prepared from a human strain of enterotoxigenic *Escherichia coli*, were subjected to isoelectric focusing, molecular sieve chromatography, and polyacrylamide disc gel electrophoresis. Isoelectric focusing in a pH 3 to 5 gradient resulted in two biologically active peaks, I and II, that electrofocused at pI 1.5 and 3.8, respectively. Molecular sieve chromatography of the major enterotoxigenic peak (II) at pH 3.8 indicated a molecular weight of 2,500. Polyacrylamide disc gel electrophoresis of ST revealed a single protein band containing enterotoxigenic activity.

Few laboratories have reported on the physiochemical properties of *Escherichia coli* heat-stable enterotoxin. Several have used *E. coli* strains isolated from swine, and a variety of molecular weights have been obtained (3, 4, 7). The present study investigates the physiochemical properties of ST enterotoxin produced by a strain of *E. coli* of human origin.

Enterotoxigenic *E. coli* strain PB-122-B1 was isolated from a case of travelers' diarrhea in Mexico and has maintained stability of the ST plasmid in vitro (6). Heat-stable enterotoxin was routinely produced by shake culture in the defined medium of Alderete and Robertson (2) for 18 h at 37°C. The cultures were harvested by centrifugation at $16,000 \times g$ for 1 h, and the supernatants were sterilized by pressure filtration (0.45 μ m; Millipore Corp., Bedford, Mass.). The sterile filtrates were subjected to Amicon PM-10 ultrafiltration (Amicon Corp., Lexington, Mass.), and the resulting filtrate was concentrated 100-fold and washed on an Amicon UM-2 ultrafilter.

The infant mouse model of Dean et al. (5) was used to assay for toxic activity. One toxic unit was defined as the reciprocal of the highest dilution yielding a ratio of intestine to body weight of 0.100. Aliquots (0.1 ml) from pooled fractions of isoelectric focusing or column chromatography were serially diluted twofold in 0.134 M K-phosphate buffer (pH 7.0) unless noted otherwise.

Isoelectric focusing was conducted with concentrated UM-2 cell-free filtrates. A 110-ml isoelectric focusing column (LKB Products, Rockville, Md.) was used for all experiments; 4 mg of protein sample was applied to each column. The final column contained a 5 to 50% sucrose gradient; focusing was achieved at 800 V (Voltage and Current DC Power Supply, Buchler, Fort Lee, N.J.). Fractions of 2 ml each were collected

in an automatic fraction collector, and the pH and optical density at 280 nm were immediately determined.

Isoelectric focusing in a restricted pH range (pH 3 to 5; BioLyte, Bio-Rad Laboratories, Richmond, Calif.) resulted in two distinct toxicity peaks (I and II; Fig. 1). Peak I had a pI of 1.5 and corresponded to a lower yellow band in the column. Peak II focused at a pI of 3.8 and contained the major portion of enterotoxigenic activity. This low pI indicates that ST enterotoxin contains many acidic amino acids. Abdurushid et al. (1) have found that glutamic acid is the most common amino acid in ST enterotoxin. Alderete and Robertson (3) have shown that highly purified ST enterotoxin from a porcine strain of *E. coli* contained four aspartic acid and five glutamic acid residues per molecule.

Toxic fractions from electrofocusing peak II were pooled, dialyzed (Spectrapor 3; Spectrum Medical Industries Inc., Los Angeles, Calif.), lyophilized, rehydrated in 1.0 ml of column buffer, and applied to a Sephadex G-25 gel column (90 by 1.5 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated in 0.05 M Sorensen citrate buffer (pH 3.8). As shown in Fig. 2, two separate protein peaks were obtained; ST enterotoxin eluted in a single peak. All protein standards eluted in several fractions, each as a symmetrical peak. Initially, bioactivity (by the pooling method) was associated with fractions 40 through 49. However, further analysis of each fraction, with a dilution of 1:10, indicated that fraction 45 contained the major portion of ST activity. Absorbance at 280 nm was not observed in fractions 46 through 49. This observation may reflect the finding of Alderete and Robertson (3) that ST enterotoxin absorbs maximally at 270 nm.

The molecular weight of ST enterotoxin, when determined by comparison with known molecu-

FIG. 1. Isoelectric focusing (pH 3 to 5) of concentrated *E. coli* cell-free filtrate prepared from defined medium. Symbols represent pH (---), ratio of intestine to body weight (○), and absorption at 280 nm (▼). Each fraction represents 2.0 ml.

FIG. 2. Molecular sieve chromatography (Sephadex G-25) at pH 3.8 of pooled and concentrated electrofocusing peak II (pH 3 to 5). Symbols represent ratio of intestine to body weight (○) at a dilution of 1:10, and absorption at 280 nm (▼). Each fraction represents 2.0 ml. Inset represents standard curve generated by elution of proteins with known molecular weight (see text for details).

lar weight markers, was 2,500 (Fig. 2, inset). Protein markers included cytochrome *c*, 12,700 daltons (Worthington Biochemicals Corp., Free-

hold, N.J.); insulin, 5,700 daltons (E.R. Squibb & Sons, New York, N.Y.); insulin A chain, 2,300 daltons (D. Babin, Creighton University School

of Medicine); glucagon, 3,460 daltons (Sigma Chemical Co., St. Louis, Mo.), and hemoglobin fraction, 1,680 daltons (D. Babin).

Other studies have shown variable molecular weights for ST enterotoxin. Recently, enterotoxic activity during slab-gel electrophoresis was associated with a rapidly migrating band having a molecular weight of 2,000 to 4,000 (G. Ruiz-Palacios, D. G. Evans, D. J. Evans, Jr., and H. L. DuPont, Clin. Res. 25:383A, 1977). Contrary to these results, Alderete and Robertson (3), using an *E. coli* porcine isolate, have obtained a molecular weight of 5,100 when calculated by amino acid analysis. Results of the present study are comparable to those of Giannella (Gastroenterology 74:1124, 1978) who found a molecular weight of 2,500 to 3,500.

The specific activity of ST enterotoxin was one toxin unit per 25 ng of protein (8). This value substantially agrees with those of other recent studies. However, the definition of an effective dose was higher (0.100) in the present study than in other investigations (1, 3, 7).

Polyacrylamide disc gel electrophoresis, in the presence and absence of sodium dodecyl sulfate and urea (8 M), was performed by the method of Neville (9). The final gel concentration was 15%, with a 1:10 cross-linkage. Protein bands were detected by labeling with fluorescamine (10) and thus could not be readily photographed. In the absence of dissociants, electrophoresis of aliquots from the single toxic peak obtained from Sephadex G-25 gel chromatography resulted in

FIG. 3. Polyacrylamide disc gel electrophoresis (15%) of the toxic peak from Sephadex G-25 chromatography (see Fig. 2). A and B represent electrophoresis in the absence and presence of 1% sodium dodecyl sulfate and 8 M urea, respectively; a and b represent single protein bands.

a single band approximately 2 cm above the fluorescent marker front. Gel sectioning, followed by elution with K-phosphate buffer (pH 7.0), indicated that ST enterotoxin activity was associated with this band. In the presence of sodium dodecyl sulfate and urea, a single band, located 0.5 cm below the tracer front, was observed (Fig. 3). These observations reflect the low molecular weight and homogeneity of the *E. coli* ST enterotoxin sample.

These studies indicate that, because of the low pI of ST enterotoxin (pH 3.8), electrofocusing provides a simple yet powerful procedure for the isolation of this protein; subsequent molecular sieve chromatography at the pI yields an apparently homogeneous toxic protein with an estimated molecular weight of 2,500.

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