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Partial Purification and Properties of *Enterobacter cloacae* Heat-Stable Enterotoxin

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Cell-free preparations of the whole-cell lysate and ultrafiltration (UF) fractions of broth cultures of a strain of *Enterobacter cloacae*, isolated from a Puerto Rican with tropical sprue, were assayed for their ability to induce in vivo net water secretion in the rat jejunum. The whole-cell lysate and UM-10 retentate of broth cultures were inactive. The UM-2 retentate and filtrate were active at a concentration of 100 μg/ml or more; the toxigenic activity was entirely retained, and increased to 1 μg/ml, by a UM-05 membrane; washing this retentate yielded a fraction with an activity of 10 ng/ml. Stationary aerobic culture conditions yielded the most active UF fractions when ammonium sulfate was used as the precipitating agent, whereas anaerobic culture conditions produced the most active fractions in broth cultures precipitated by acetone. Passage of the active acetone-precipitated UF fractions through a Sephadex G-25 column yielded eluate pools with enhanced toxigenic activity in, or adjacent to, the void volume, but maximum activity of the ammonium sulfate-precipitated UM-05 retentate eluated at a K_{av} of 0.38 to 0.52. Neither of the most active gel filtration elution fractions of the UM-05 retentates contained detectable carbohydrate, suggesting that the toxin is not associated with endotoxin. Toxigenic activity was unaltered by exposure to a temperature of 100 C for 30 min, lowering the pH to 1, or incubation with either Pronase or trypsin. These observations indicate that the strain of *E. cloacae* under study elaborates a heat-stable enterotoxin that has approximately the same molecular weight and shares many of the characteristics of the heat-stable enterotoxin produced by some strains of *Escherichia coli* and *Klebsiella pneumoniae*.

It is now well established that transient contamination of the small intestine by *Enterobacteraciae* often occurs in persons with acute diarrhea in the tropics (9, 13, 28, 33), and chronic colonization occurs among malnourished children with diarrhea (3, 11, 15, 26) and persons with tropical sprue (1, 10, 20, 41; F. A. Klipstein, H. B. Short, R. F. Engert, L. Jean, and G. A. Weaver, Gastroenterology, in press). *Escherichia coli* is the most commonly encountered species of *Enterobactericiae* among most persons with acute diarrhea and malnourished children. *Klebsiella pneumoniae* is often found in association with *E. coli* in many of these individuals (9, 11, 13, 15) and, in some it is the predominant organism (3, 28). *K. pneumoniae* also is often the only coliform species isolated from the jejunum of persons with tropical sprue (20; Klipstein et al., in press). Enterotoxigenic strains of both *E. coli* (2, 6, 17, 33, 37) and *K. pneumoniae* (13, 18, 19; Klipstein et al., in press) elaborate, either singly or in combination, a small-molecular-weight, heat-stable (ST) or a large-molecular-weight, heat-labile (LT) form of toxin. In vivo marker perfusion studies in experimental animals have shown that both the ST and LT produced by either *E. coli* (2, 12, 29, 36) or *K. pneumoniae* (19, 21; Klipstein et al., in press) induce net secretion of water and electrolytes into the intestinal lumen.

Enterobacter cloacae has also been isolated from the small intestine of persons with acute diarrhea (9) or tropical sprue (20), but the actual prevalence of this species among persons with diarrhea in the tropics is uncertain since most investigators have not completely characterized the specific species of *Enterobacteraciae* cultured from intestinal aspirates or stools. We have reported that a crude cell-free broth filtrate of a strain of *E. cloacae*, isolated from a Puerto Rican with untreated tropical sprue, evokes fluid secretion in the rabbit ligated loop model (20, 22). In the present study, we have determined the enterotoxigenic activity of the whole-cell lysate and ultrafiltration fractions of

the cell-free broth filtrate of this strain, evaluated the optimum culture conditions for production of the heat-stable enterotoxin produced by it, additionally purified this toxin by gel filtration chromatography, and characterized some of its properties.

MATERIALS AND METHODS

Organism. A strain of *E. cloacae*, API biotype no. 3305763 (38), which was the predominant organism cultured from the midjejunal aspirate of a Puerto Rican with tropical sprue (20), and which we have examined in previous studies (22), was used.

Enterotoxin production. Whole-cell lysates were obtained by sonication (5, 37) of cells harvested after 18-h aerobic culture on Trypticase soy agar (BBL, Cockeysville, Md.). Aerobic broth cultures were prepared by 18-h stationary or agitated (at 200 shakes/min) incubation in 250 ml of Trypticase soy broth (BBL) in a 2-liter flask. For anaerobic cultures, bacteria were inoculated in 500 ml of broth in a 1-liter flask and incubated for 18 h in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The broth cultures and sonication material were centrifuged at 25,000 × *g* for 45 min, and the supernatant was filtered through a 0.45-μm membrane filter adapted with a type AP 25 microfiber glass prefilter (Millipore Corp., Bedford, Mass.). Material in the cell-free broth filtrates was then precipitated at 4 C with either 8 volumes of acetone (2, 21) or 90% ammonium sulfate (AMS) (6). The acetone precipitates were solubilized in distilled water, the AMS precipitates were dissolved in 0.02 M ammonium bicarbonate (pH 7.8), and both these solutions were then fractionated by ultrafiltration.

UF. The acetone and AMS precipitates were subjected to sequential ultrafiltration (UF) at 4 C through an Amicon UM-10 and then through either a UM-2 or a UM-05 membrane (Amicon Corp., Lexington, Mass.). The retentates of these membranes were taken to 10% of the original volume without washing and they and the filtrates were concentrated by lyophilization and stored at −20 C until assay or further purification procedures.

Gel filtration chromatography. A column (5 by 85 cm) was packed with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and equilibrated with 0.02 M ammonium bicarbonate (pH 7.8). Excluded and included volumes were determined using blue dextran 2000 (Pharmacia) and NaCl. A 200- to 500-mg amount of the UF fraction, dissolved in 10 ml of the above buffer, was eluted from the gel at 4 C at a flow rate of 24 ml/h. The eluate was collected in 20-ml fractions; each tube was monitored at 280 nm for protein content and every third tube was monitored at 620 nm for total hexose content, as determined by the anthrone reaction (39), on a model 139 Hitachi Perkin-Elmer spectrophotometer. The fractions were then separated into pools, which were concentrated by lyophilization.

Chemical analysis. Protein concentrations were determined by the method of Lowry et al. (24) using bovine serum albumin as the standard, and carbohydrate was determined by the anthrone reaction (39) using mannose as the standard. Polyethylene glycol was determined by the turbidimetric technique (25). Osmolalities were determined by measuring the freezing point depression with an advanced digimatic osmometer (Advanced Instruments, Inc., Needham Heights, Mass.).

Effect of proteolytic enzymes. One-milligram quantities of the UM-05 retentate of actone-precipitated, anaerobically grown broth filtrate were mixed with Pronase (Calbiochem, LaJolla, Calif.) in a substrate-to-enzyme ratio, based on protein concentration, of 10:1 and incubated in 0.1 M phosphate buffer, pH 7.4, at 37 C for 24 h (17). The same quantities of these fractions were also mixed in the same substrate-to-enzyme ratio with trypsin (Worthington Biochemicals, Freehold, N.J.) using tris-(hydroxymethyl)aminomethane-hydrochloride buffer with a final calcium concentration of 0.001 M and a pH of 8.1 and incubated at 37 C for 3 h (17). Tryptic activity was confirmed with TAME (16). After incubation the test fractions were perfused at a concentration of 10 μg/ml.

Assay for enterotoxic activity. Test fractions were assayed for their ability to induce net secretion of water in the rat jejunum by means of standard marker perfusion techniques for the in vivo evaluation of intestinal transport (21, 30). Single 20-cm jejunal segments were cannulated in 150-g Sprague-Dowley rats and perfused at a rate of 0.5 ml/min using a model 1201 Harvard peristaltic pump (Harvard Apparatus Co., Inc., Millis, Mass.). Test preparations were perfused during both a 30-min steady-state period and six subsequent 30-min test periods. The electrolyte solution described by Powell et al. (30), as modified in this laboratory (21) when necessary to maintain an iso-osmolality of 317 mosmol/kg, was used. The concentration of enterotoxin added is expressed as micrograms per milliliter; the total dosage of enterotoxin perfused equaled this concentration times the volume of 105 ml perfused during the total 210 min of perfusion time.

Net transport of water was calculated by changes in the polyethylene glycol concentrations by the usual water marker technique equation (30). Values are expressed in microliters per centimeter per 30 min. Net lumen to blood transport is termed absorption, which is signified by a plus sign, whereas a minus sign refers to net blood to lumen transport, or secretion. This laboratory (19, 21) and others (12, 40) have previously reported, and we so noted in the present study, that marginal concentrations of enterotoxin will induce net secretion during only one or two of the test periods. For this reason, the values reported are for that single 30-min test period during which there was minimal absorption or maximal secretion of water. The minimum effective dose of toxin is defined as that amount (in dry weight) of toxin that induces net secretion.

The effect of control preparations has been reported previously (21). Minimum water absorption during the six test periods among 20 rats perfused with the electrolyte solution alone was +41 ± 3, and that after perfusion of the whole-cell lysate and UM-2 retentate of broth filtrate of a nontoxigenic strain of *E. coli* (API biotype 5044572) was +34 ± 4

and +37 ± 7, respectively, when each preparation was tested in five rats at a concentration of 2,000 μg/ml.

RESULTS

Enterotoxigenic activity of fractions. The effect on water transport of the whole-cell lysate and UF fractions of broth cultures is shown in Table 1. The sonicated preparation was inactive when tested at a maximum dosage of 2,000 μg/ml. Due to the fact that previous studies indicated that anaerobic culture conditions enhance the production of *K. pneumoniae* ST (19), preliminary screening of the UF fractions was conducted, using broth cultures grown anaerobically. The UM-10 retentates of both the acetone- and AMS-precipitated broth filtrates were inactive at a dosage of 2,000 μg/ml. Both the UM-2 retentate and filtrate of the acetone precipitate and the UM-2 retentate of the AMS precipitate were active, with a minimum effective dose of 100 μg/ml. The UM-2 filtrate of the AMS precipitate induced net water secretion only when perfused at a concentration of 2,000 μg/ml.

Effect of culture conditions on ST production. The effect of varying degrees of aerobiosis during bacterial growth on the enterotoxigenic activity of UM-2 ultrafiltration fractions of broth filtrates precipitated with either AMS or acetone is shown in Table 2. All UF fractions of cultures grown under agitated aerobic conditions were inactive. Stationary aerobic culture conditions produced the most active UF fractions when AMS was used as the precipitating agent, whereas anaerobic culture conditions yielded the most active UF fractions in broth cultures precipitated by acetone.

Enterotoxigenic activity of UM-05 UF fractions (Table 3). The finding that enterotoxigenic activity was present in both the retentate and filtrate fractions of the UM-2 membrane (molecular weight cutoff, approximately 1,000) suggested that the molecular size or configuration of the active material is such that it might be completely retained by a membrane with a smaller pore size. Table 3 compares the enterotoxigenic activity of the UM-2 retentate versus the UM-05 (molecular weight cutoff, approximately 500) retentate and filtrate fractions of material prepared under the optimal conditions: acetone precipitates after anaerobic growth and AMS precipitates after stationary aerobic growth. Both of the UM-05 filtrates were inactive when perfused at a concentration of 100 μg/ml. The UM-05 retentate of the acetone precipitate had a minimum effective dose of 1 μg/ml, which represented a 100-fold increase in activity over that of the UM-2 retentate. On the other hand, both the UM-2 and the UM-05 retentates of the AMS-precipitated material were equally active, with an minimum effective dose of 1 μg/ml. Repeated washing of the acetone-precipitated UM-05 retentate with 0.02 M ammonium bicarbonate yielded a product with an minimum effective dose of 10 ng/ml, which represented a further 100-fold increase in activity.

TABLE 1. *Enterotoxigenic activity of cell lysates and UF fractions of broth filtrates*

Medium[a]	Ppt[b]	Fraction	Water transport		
			2,000 μg/ml[c]	100 μg/ml	10 μg/ml
Agar	Sonicate		+73		
BF	Acetone	UM-10 R	+51		
BF	Acetone	UM-2 R	−120	−5	33
BF	Acetone	UM-2 F		−21	+3
BF	AMS	UM-10 R	+52		
BF	AMS	UM-2 R	−106	−7	+68
BF	AMS	UM-2 F	−105	+17	

[a] Broth filtrates (BF) were grown anaerobically.
[b] Material used to precipitate the broth filtrates.
[c] Dosage.

TABLE 2. *Effect of culture condition on enterotoxigenic activity of the UM-2 UF fractions*[a]

Culture conditions	AMS[b]		Acetone[b]	
	R	F	R	F
Agitated	+10	+34	+35	+74
Aerobic	−112	−9	0	+21
Anaerobic	−7	+17	−5	−21

[a] Fractions were tested at a concentration of 100 μg/ml.
[b] Broth filtrates were precipitated with either AMS or acetone. R, Retentate; F, filtrate.

Fractionation by gel filtration. (i) UM-2 UF fractions. Eluates from Sephadex G-25 columns after passage of the retentate (Fig. 1) and the filtrate (Fig. 2) of acetone-precipitated, anaerobically grown broth cultures were divided into either three or four pools, each of which was screened for enterotoxigenic activity at an initial concentration of 10 μg/ml (Table 4). Maximum enterotoxigenic activity among the eluates of the retentate was in pool 2, which had a K_{av} of between 0.24 and 0.50. This pool contained a distinct protein, but no carbohydrate, peak. The most active elution pool of the UM-2 filtrate was in the void volume, which did not contain a distinct protein peak; no carbohydrate peaks were detected in the elution fractions, although analysis of the lyophilized pools showed carbohydrate in each (Table 4).

(ii) UM-05 UF fractions. Passage of the UM-05 retentate of acetone-precipitated, anaerobically grown broth culture yielded several dis-

TABLE 3. *Enterotoxigenic activity of UM-2 and UM-05 UF fractions prepared under optimal conditions*

Growth conditions	Ppt[a]	Fraction	Water transport			
			100 μg/ml[b]	10 μg/ml	1 μg/ml	0.1 μg/ml
Anaerobic	Acetone	UM-2 R	−5	+33	+42	
Anaerobic	Acetone	UM-05 R	−103	−67	−8	+23
Anaerobic	Acetone	UM-05 F	+26			
Aerobic	AMS	UM-2 R	−112	−39	−25	+17
Aerobic	AMS	UM-05 R	−74	−27	−18	+36
Aerobic	AMS	UM-05 F	+28			

[a] Material used to precipitate the broth filtrates.
[b] Dosage.

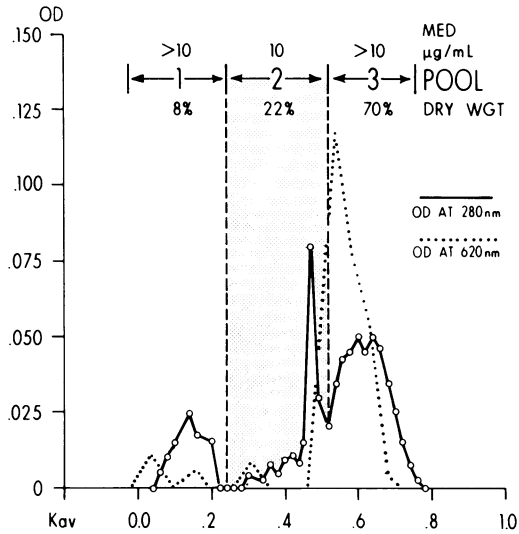

FIG. 1. *Gel filtration through a Sephadex G-25 column of the UM-2 retentate of acetone-precipitated broth culture. The minimum effective dose of the UF fraction was 100 μg/ml. The hatched area indicates the most active elution pool.*

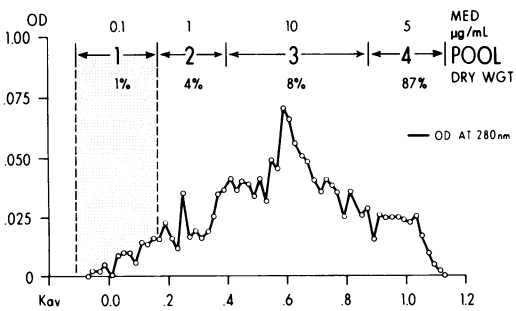

FIG. 2. *Gel filtration of the UM-2 filtrate of acetone-precipitated broth culture. The minimum effective dose of the UF fraction was 100 μg/ml. No carbohydrate was detected in the elution fractions.*

crete protein peaks and a carbohydrate peak at the end of the elution (Fig. 3). Pool 1, the void volume, was the most active enterotoxigenic fraction, although the adjacent pool, which had a K_{av} of from 0.14 to 0.40, also contained material that had a 10-fold increase in enterotoxigenic activity (Table 5).

The elution pattern of the AMS -precipitated broth filtrate after stationary aerobic growth (Fig. 4; Table 5) was quite different from the acetone-precipitated material. Increased enterotoxigenic activity was detected only in pool 3, which had a K_{av} from 0.38 to 0.52 and contained a discrete protein peak. No carbohydrate peaks were detected in any of the elution fractions or the lyophilized pools of this column.

Composition of the most active fractions (Table 6). Passage of all three UF retentate fractions through the G-25 column yielded, in each instance, an elution pool containing material whose enterotoxigenic activity was increased by 10- to 20-fold. The activity of the single filtrate so treated was enhanced 1,000-fold; it is probable that a significant proportion of this increase was due to desalting by the gel filtration procedure.

As would be expected, the protein concentration of both the UM-2 and UM-05 UF retentates of the acetone-precipitated material was appreciably higher than that of the filtrate; the protein content of both acetone retentates was also greater than the UM-05 retentate of the AMS-precipitated broth culture. After gel filtration, the most active elution pool of all three acetone-precipitated UF fractions contained, in each instance, the highest protein concentration of the elution fractions, but such was not the case for the AMS-precipitated UM-05 material (Table 5). All of the UF fractions of the acetone-, but not AMS-, precipitated material contained carbohydrate. No carbohydrate was detected in the most active gel filtration elution pools of either of the UM-05 retentates.

Effect of physical changes or treatment with proteolytic enzymes (Table 7). The effect of various physical treatments on the UM-05 retentate of acetone-precipitated, anaerobically grown broth filtrate is shown in Table 6. Neither heat treatment, 100 C for 30 min, nor acid treatment, consisting of lowering the pH to 1

TABLE 4. *Composition and enterotoxigenic activity of gel filtration eluates of UM-2 UF fractions*

UF fraction[a]	GF[b] pool	Protein (%)	CHO[c] (%)	Water transport				
				10 μg/ml[d]	5 μg/ml	1 μg/ml	0.1 μg/ml	0.05 μg/ml
Retentate	1	46.5	3.9	+27				
	2	50.0	5.4	−23	+7			
	3	18.7	9.1	+36				
Filtrate	1	12.0	5.0		−63	−26	−14	+28
	2	10.2	2.3		−93	−29	+23	
	3	9.8	9.3	−3	+30			
	4	9.6	0.7	−18	−2	+13		

[a] The minimum effective dose of both UF fractions before gel filtration was 100 μg/ml.
[b] GF, Gel filtration.
[c] CHO, Carbohydrate.
[d] Dosage.

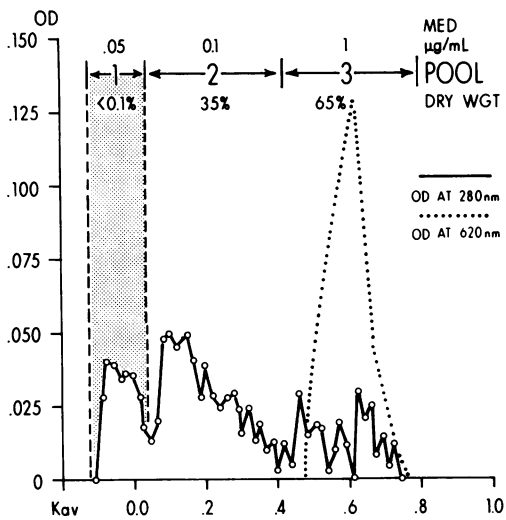

FIG. 3. *Gel filtration of the UM-05 retentate of acetone-precipitated broth culture. The minimum effective dose of the UF fraction was 1 μg/ml.*

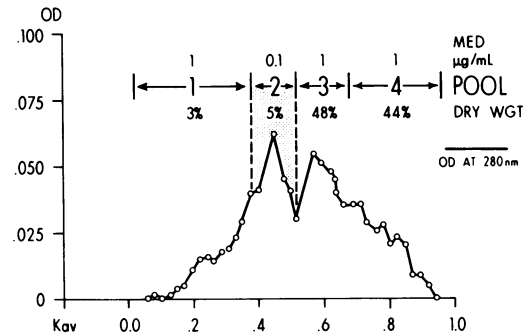

FIG. 4. *Gel filtration of the UM-05 retentate of AMS-precipitated broth culture. The minimum effective dose of the UF fraction was 1 μg/ml. No carbohydrate was detected in the elution fractions.*

for 4 h by the addition of concentrated HCl, affected enterotoxigenic activity. We have previously reported (19) that perfusion of a rat with 100 ml of electrolyte solution containing 32.7 μg of trypsin, an amount slightly less than that incubated with the toxin fraction in the present study, does not affect water transport at all, and that perfusion with 33.8 μg of Pronase alone does not alter water transport during the first two 30-min collection periods. After exposure to these proteolytic enzyme preparations, the enterotoxin preparation continued to induce striking net secretion during the first two test periods, indicating that its effect was not impaired.

DISCUSSION

Until recently *E. coli* was the only species of *Enterobacteriacae* recognized to be enterotoxigenic (33). Strains of *K. pneumoniae* have now been described that elaborate an enterotoxin that evokes fluid secretion in the ligated rabbit ileal loop (18, 20, 22), induces net water and electrolyte secretion when perfused through the rat small intestine (19, 21; Klipstein et al., in press) and yields a positive Chinese hamster ovary assay (13). Enterotoxigenic strains of both *E. coli* (5, 6, 13, 33, 37) and *K. pneumoniae* (13, 18, 19; Klipstein et al., in press) have been shown to elaborate ST and LT, either singly or in combination. The results of the present study indicate that the strain of *E. cloacae* we evaluated also produces a low-molecular-weight, heat-stable enterotoxin. No enterotoxigenic activity was detected in the cell-free, whole-cell lysate of bacterial growth, the procedure usually used to produce LT (5, 37). Whether other strains of *E. cloacae* produce LT, either alone or in addition to ST, remains to be determined; it would seem likely that such will prove to be the case.

Culture conditions for the optimal production of *E. cloacae* ST depended on the chemical

TABLE 5. *Composition and enterotoxigenic activity of gel filtration eluates of UM-05 retentates*

Ppt[a]	GF[b] pool	Protein (%)	CHO[c] (%)	Water transport			
				1 μg/ml[d]	0.1 μg/ml	0.05 μg/ml	0.01 μg/ml
Acetone	1	84.1	0		−18	−7	+38
	2	82.4	0	−28	−10	+12	
	3	36.3	9.9	−29	+8		
AMS	1	35.8	0	−89	+40		
	2	21.0	0	−98	−39	+33	
	3	10.0	0	−95	+10		
	4	10.0	0	−24	+33		

[a] Ppt, Precipitate. The minimum effective dose of both UM-05 retentates before gel filtration was 1 μg/ml.
[b] GF, Gel filtration.
[c] CHO, Carbohydrate.
[d] Dosage.

TABLE 6. *Composition of the most active fractions obtained during sequential purification procedures*

Culture	Ppt[a]	UF fraction	GF[b] pool	Carbohydrate (%)	Protein (%)	MED (μg/ml)[c]		% Initial dry wt[d]
						Dry wt	Protein	
Anaerobic	ACET	UM-2 R	–	5.5	59.9	100	59.9	28.9
			2	5.4	50.0	10	5.0	3.1
Anaerobic	ACET	UM-2 F	–	0.9	15.1	100	15.1	28.9
			1	5.0	12.0	0.1	0.01	0.2
Anaerobic	ACET	UM-05 R		8.5	48.1	1	0.48	34.2
			1	0.0	84.1	0.05	0.04	0.7
Aerobic	AMS	UM-05 R	–	0	13.1	1	0.13	14.2
			2	0	21.0	0.1	0.02	0.5

[a] Ppt, Precipitate; ACET, acetone.
[b] GF, Gel filtration.
[c] MED, Minimum effective dose.
[d] Based on the weight of the crude broth filtrate expressed as milligrams per milliliter taken as 100%.

TABLE 7. *Effect of heat, acid, and proteolytic enzymes on enterotoxigenic activity*

Treatment	Duration (h)	Water transport
None		−67
100 C	0.5	−81
pH 1	4.0	−58
Pronase	24.0	−86
Trypsin	3.0	−90

agent used to precipitate the toxin. When AMS was used, stationary aerobic conditions yielded the most active preparation, whereas anaerobic growth produced the most active preparation in acetone precipitates. In neither case was ST produced under agitated aerobic conditions. We have previously found that the same set of culture conditions pertains to ST elaborated by strains of *E. coli* (C. S. Lee and F. A. Klipstein, unpublished observations) and *K. pneumoniae* (19).

The UM-05 retentate of broth cultures precipitated by AMS differed from the same UF fraction precipitated by acetone in the following respects: (i) growth conditions of the culture that yielded maximum toxin production; (ii) the UM-2 retentate of AMS-precipitated material was equally active; (iii) the protein concentration was lower and there was no detectable carbohydrate; (iv) the AMS-precipitated UF eluted from the Sephadex G-25 column at a different K_{av}. Whether these differences reflect the fact that AMS precipitation yields a form of ST that has a different molecular structure than that precipitated by acetone or that AMS modifies certain characteristics of the same ST molecule is uncertain.

Most strains of coliform bacteria isolated from persons with acute diarrhea elaborate LT (13, 27, 34), but strains that produce just ST have been recovered from the stools of some of these individuals (13, 27, 31, 35) as well as from the jejunal aspirates of persons with tropical sprue (18; Klipstein et al., in press). The information presently available suggests that the ST forms of enterotoxin produced by *E. coli*, *K. pneumoniae*, and *E. cloacae* all have approximately the same molecular weight and share a number of other characteristics. Working with UF fractions of an acetone precipitate of an enterotoxigenic strain of *E. coli*, Bywater found toxigenic activity absent in the UM-10 reten-

tate but present in both the UM-2 retentate and filtrate (2). We have previously reported similar findings with *K. pneumoniae* ST (18, 19) and found such to be the case with the *E. cloacae* ST under study. These observations suggested that a UF membrane with a smaller pore size might be more effective in retaining the active fraction and, in the present study, passage through a UM-05 membrane yielded activity only in the retentate which, in the case of the acetone precipitate, was 100-fold greater than in the UM-2 retentate. Subsequent repeated washing of the UM-05 retentate resulted in a further 100-fold increase in activity.

Previous investigations using UF or gel filtration indicate that the apparent molecular weight of ST elaborated by both *E. coli* (2, 17) and *K. pneumoniae* (18, 19) is in the range of between 1,000 and 10,000. Our observations in the present study show that such is the case for *E. colacae* ST. After separation of the UF fractions of the acetone precipitate through a G-25 Sephadex column, the most active elution fractions of both the UM-2 filtrate and the UM-05 retentate appeared in the void volume, and that of the UM-2 retentate was in the fraction adjacent to the void volume.

The responses of *E. cloacae* ST to various physical, chemical, and enzymatic treatments are also similar to those of both *E. coli* and *K. pneumoniae* ST. Exposure of *E. coli* (5, 29, 37), *K. pneumoniae* (18, 19), and *E. cloacae* ST to a temperature of 100 C for 30 min does not abolish activity. Further, the activity of the ST form of toxin elaborated by all three coliform species is resistant to pH values as low as 1 (17, 19, 23) and to treatment with either Pronase (17, 19) or trypsin (14, 17, 19, 23). Previous studies have suggested that *K. pneumoniae* ST is not related to the endotoxic carbohydrate-lipid-protein complex (19), and the results of the present observations suggest that the same holds true for *E. cloacae* ST: neither of the most active eluate fractions of the UM-5 retentates after passage through a G-25 Sephadex column contained detectable carbohydrate.

It has been proposed that immunological protection against *E. coli* enterotoxins may represent a feasible approach towards irradication of the intestinal disorders induced by the toxigenic forms of these bacteria (8, 33). Whereas *E. coli* LT is antigenic and induces immunological protection (4, 6, 32), crude preparations of ST derived from strains of *E. coli* isolated from animals (37) and humans (6) are said not to be antigenic in that they did not arouse an immunological response protective against their ability to evoke fluid secretion in ligated animal loops. It is not known, however, whether more purified fractions may be able to induce a protective immunological response; such proved to be the case with preparations of cholera enterotoxin (7). In addition, the low molecular weight of ST suggests that it may he haptenic and not produce immunological complexes unless it is bound to a carrier molecule. As a preliminary step, it would seem to be important that the structure and composition of ST elaborated by the various species of enterotoxigenic coliform bacteria be precisely defined.

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