Escherichia coli Heat-Stable Enterotoxin: Rapid Method of Purification and Some Characteristics of the Toxin

R. LALLIER,¹* S. LARIVIERE,¹ AND S. ST-PIERRE²

Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec,¹ and Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec,² Canada

In this study we used *Escherichia coli* strain F11(P155) of porcine origin. The heat-stable enterotoxin (ST) was produced with a batch fermentor under agitation (500 rpm) and forced aeration (5 liters/min) in Casamino Acids yeast extract medium containing 0.2% glucose. The pH varied from 7.2 to 7.8. The maximum amount of ST was obtained after 7 h of growth. ST was purified by ammonium sulfate precipitation, ultrafiltration on Amicon membranes, and chromatography in Bio-Gel P-4. The enterotoxin, which was purified approximately 1,000 times, was active at nanogram levels. On 20% polyacrylamide gel electrophoresis, ST exhibited an R_f of 0.6. ST was recovered from the gel slices by eluting in buffer and testing the activity in suckling mice, since no band appeared on the gel after staining with Coomassie brilliant blue R, Schiff reagent, or red oil. ST was resistant at pH 2 to 10 and at 100°C for 15 min, but it was inactivated at 121°C; it did not lose biological activity after treatment with pronase, lipase, or amylase. In suckling mice antiserum obtained from rabbits or goats immunized with ST neutralized the enterotoxin activity of a cell-free supernatant of purified ST.

Enterotoxigenic *Escherichia coli* can cause diarrhea by producing enterotoxins (14, 16, 21). The heat-labile enterotoxin has a high molecular weight and appears to be a protein, whereas the heat-stable enterotoxin (ST) has a low molecular weight (19). Whereas only ST has been reported from bovine and ovine strains (20), both the heat-labile enterotoxin and the ST have been detected in *E. coli* strains of human and porcine origin (17, 20). Conditions for production and purification of the heat-labile toxin have been studied (6, 9, 18), and antigenic relationships between the *Vibrio cholerae* enterotoxin and *E. coli* heat-labile toxin have been demonstrated (11).

The purification of ST has been complicated by the laborious and expensive pig gut loop system used for its detection. Since the suckling mouse test (5) and 6-h rabbit gut loops (7) were reported to detect ST, some characteristics of the ST have been described. Precipitation of a crude supernatant with methanol allowed the separation of two fractions, namely, a methanol-soluble fraction active in the suckling mouse system but not in the rabbit system and a methanol-insoluble fraction inactive in the suckling mouse system but active in rabbit gut loops (3). Alderete and Robertson (2), starting with 30 liters of cell-free culture supernatant, succeeded in purifying an ST, as detected by the suckling mouse test. After a number of purification steps

they obtained 2 mg of a 5,100-dalton protein, which was poorly antigenic.

The present paper describes an easy and rapid procedure for producing large amounts of purified ST. We found that this preparation is antigenic and that antisera capable of neutralizing its activity could be obtained after immunization with relatively large amounts of antigen.

MATERIALS AND METHODS

Bacterial strain. *E. coli* strain F11(P155) was derived from non-enterotoxigenic avian strain F11 (serotype O18ab:K?:H14) by conjugation with porcine strain P155 from H. W. Smith (22) and now has the ability to produce both heat-labile enterotoxin and ST.

Culture medium composition. Enterotoxin production was tested in either a complex or a semisynthetic medium. Brain heart infusion (Difco Laboratories) was used as complex medium. The semisynthetic medium (CA) was prepared basically as described by Finkelstein (8) and contained the following (in grams per liter): Na₂HPO₄, 5.0; K₂HPO₄, 5.0; NH₄Cl, 1.18; Na₂SO₄, 0.089; MgCl₂·6H₂O, 0.042; MnCl₂·4H₂O, 0.004; FeCl₃·4H₂O, 0.005; Casamino Acids (Difco), 30.0. The pH of this medium was 7.3. CA-YE medium was CA medium containing 0.6% yeast extract (Difco). These media were prepared both with and without glucose.

Conditions for growth. *E. coli* F11(P155) was grown in brain heart infusion with agitation for 4 h at 37°C. Then, 300 ml of the same medium used for fermentation was inoculated with 0.3 ml of the 4-h

culture. After overnight incubation without agitation at 37°C, 250 ml was transferred into 5 liters of fresh medium in a model 19 fermentor (New Brunswick Scientific Co.). Except when otherwise noted, the cells were allowed to grow for 7 h at 37°C with vigorous agitation (500 rpm) and forced aeration (5 liters/min), and the pH was automatically controlled. Samples were withdrawn at hourly intervals for measurement of growth and enterotoxin production. Growth was evaluated by optical density at 540 nm and by plate counts. Cells were removed by centrifugation at $10,000 \times g$ for 20 min, and culture supernatants were stored at -20°C until assayed.

Test systems for enterotoxin detection. Swiss albino mice (1 to 3 days old) were separated from their mothers just before use and randomly divided into groups of three. Samples containing ST were assayed by making twofold serial dilutions in physiological saline (containing 1 drop of 2% Evan blue dye), and 0.1 ml of each sample was administered via the oroesophageal route. After the mice were maintained at room temperature for 4 h, they were killed, and their responses were determined by the method of Dean et al. (5). The last dilution that gave a response greater than 0.09 was considered the endpoint and was expressed as the number of units per 0.1 ml.

Rabbit gut loop test. Jejunal loops, as described by Larivière et al. (12), were used. Young adult rabbits weighing 1.5 kg were starved for 24 h before surgery. Samples (2 ml) were injected into 4- to 6-cm loops, and the rabbits were sacrificed after 6 h. Only results from rabbits with appropriate positive (response greater than 0.5 ml of fluid per cm of loop) and negative control loops were accepted.

Concentration methods. Toxin was precipitated by slowly adding 650 g of $(\text{NH}_4)_2\text{SO}_4$ per liter of cell-free supernatant with vigorous agitation at 4°C, and the precipitate was allowed to settle overnight at 4°C. After centrifugation at $12,000 \times g$ for 45 min, the precipitate was suspended in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 8.0).

Ultrafiltration. The resuspended precipitate was filtered through an Amicon PM-30 membrane, and the filtrate was concentrated 10 times by flash evaporation.

Gel filtration chromatography. The concentrated filtrates were applied in 10-ml volumes to columns (70 by 2.5 cm) of Bio-Gel P-4 equilibrated with 5% acetic acid. The eluate was collected in 10-ml fractions. The enterotoxigenicity of each fraction was determined, and enterotoxic fractions were pooled. The acetic acid was removed by three successive lyophilizations and resuspensions in distilled water.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel (20 or 7.5%) electrophoresis was done by the procedure of Weber and Osborn (23). The gels were stained with Coomassie brilliant blue, Schiff reagent, or red oil. After electrophoresis, 0.5-cm gel slices were placed in vials containing 2 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.0. Samples were incubated overnight at room temperature, and the presence of ST activity in each fraction was determined by the suckling mouse test.

Protein determination. Protein was determined by the method of Lowry et al. (13), using crystalline

bovine serum albumin as a standard.

Characterization of the toxin. (i) **Methanol fractionation of purified ST.** The methanol extraction of ST was performed as described Mullan et al. (15).

(ii) **Effect of heat on enterotoxin activity.** Portions of the test sample were heated at 100°C for 30 min or at 121°C for 15 min. After heating, the samples were reconstituted to their original volumes.

(iii) **Effect of pH on enterotoxin activity.** Samples were taken to the required pH by adding either 2 M HCl or 2 M NaOH and held at this pH for 24 h at room temperature. After incubation the solutions were neutralized and assayed for enterotoxin activity.

(iv) **Effect of selected enzymes on enterotoxin activity.** Samples were treated with the following enzymes: pronase, bacterial alpha-amylase, and crystallized pancreatic lipase (all from Calbiochem, San Diego, Calif.). The mixtures of ST with pronase and ST with amylase were prepared with 0.05 M phosphate buffer (pH 6.9), whereas 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 8.1) was used for the lipase treatment. All mixtures contained 100 µg of enzyme per ml. Before being tested for ST activity, the mixtures were incubated for 4 h at 37°C.

Antiserum production. Immune sera were obtained from rabbits and a goat immunized with purified ST. The ST solutions were mixed with Freund complete adjuvant for the first immunization and with Freund incomplete adjuvant for the subsequent immunizations. The rabbits received 1.5 mg of ST at each immunization at 10-day intervals and were bled 20 days after the third injection. The goat received two injections of 35 mg of ST at 10-day intervals and was bled 1 month after the last injection.

Neutralization of ST activity. For neutralization experiments we used strain F11(P155) cell-free supernatant or purified ST suspended at 60 U/ml in physiological saline; 0.5-ml amounts of previously prepared twofold serial dilutions of serum were added to tubes containing 0.5 ml of toxin. After mixing, the tubes were incubated at 37°C for 30 min. After incubation, the solutions were assayed immediately for neutralization of toxin activity in suckling mice.

RESULTS

Enterotoxin production. Preliminary experiments were done with brain heart infusion broth cultures. Forced aeration and agitation considerably increased ST production, and maximal yield of ST was obtained after 7 h of incubation. Approximately the same amount of ST was produced in CA medium without glucose as in brain heart infusion medium. However, a high variation in toxin production was observed with bacterial cultures grown in different batches of Casamino Acids, but the bacterial cell growth was unaffected by the batches. CA medium was chosen as a starting medium, but each new lot of Casamino Acids was carefully controlled for ST production, and only batches with which the production of toxin was high were retained.

The addition of 0.6% yeast extract to CA medium led to a slight increase in ST production. When CA-YE medium was supplemented with 0.2 or 1% glucose, the production of ST increased, but maximum concentration of ST was obtained with 0.2% glucose (Table 1).

The pH also influenced ST production. When the pH was maintained at 6.8 or 8.0, a low production of ST was observed. However, when the pH was maintained at 7.2 or varied between 7.2 at the start and 7.8 at the end, maximal ST production occurred (Table 2).

Enterotoxin level was not increased by adding 1% ribonucleic acid, substituting lactose for glucose, or increasing the inoculum. The highest yield of ST was obtained by using CA-YE medium containing 0.2% glucose and maintained between pH 7.2 and 7.8 in a fermentor under forced aeration and agitation for 7 h.

Purification of ST. Concentration by acetone precipitation was unsuccessful, giving at

best less than 20% recovery of enterotoxin activity. Precipitation of cell-free supernatant with 90% $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C permitted the recovery of 80 to 95% of the activity.

The filtrate obtained after passage through an Amicon membrane contained the same activity as the resuspended precipitate, but the specific activity increased 200-fold (Table 3). Further purification was achieved by Bio-Gel filtration (Fig. 1). Two fractions were usually observed, and most of the ST activity was in the second fraction. This fraction had a specific activity of 14 ng, with a purification activity of 800-fold.

After the gels were run in 7.5% polyacrylamide, no bands appeared by staining. The ST activity was detected in the fastest moving ion band (relative mobility, 1) only after elution. However, if the percentage of polyacrylamide was increased to 20%, more than 90% of the ST activity was recovered (relative mobility, 0.6).

The purified ST was stable at 100°C for 30 min and was destroyed at 121°C after 15 min. It retained its activity between pH 2.0 and 10.0 but was destroyed at pH's of more than 10. Toxin activity was not altered by pronase, amylase, or lipase. The purified ST was not precipitated by methanol; all of the ST activity was recovered in the methanol extract, as tested by the gut loop and suckling mouse test. The ST activity present in a cell-free supernatant or in the purified product was neutralized by homologous antiserum (Table 4).

DISCUSSION

Before starting the purification of the ST produced by *E. coli* F11(P155) (porcine type), we first established standard conditions for production of ST, under which reproducible, high amounts of toxin could be obtained with a minimum of cell product or medium contamination. Using a complex medium (such as brain heart infusion), we confirmed previous observations (10) that forced aeration and agitation were necessary for ST production. Under appropriate conditions a semisynthetic medium allows for the production of ST in equivalent or higher amounts than a complex medium. With a suitable lot of Casamino Acids complemented with yeast extract, ST is produced in the same amount as with complex medium. Alderete and Robertson (1) found that repression of ST synthesis by glucose was not due to a decrease in pH, as is the case for the enterotoxin of *V. cholerae* (4). We also found that a high concentration of glucose (1%) slightly decreased ST production. The pH could slightly influence the production of ST. Best results were obtained when the pH was not allowed to decrease below 7.2 or increase above 8.0.

TABLE 1. Effect of additives to basal medium (Casamino Acids) on the production of ST by *E. coli* F11(P155)

Additive(s) ^a	Enterotoxin activity ^b	
	U/ml ^c	U/10 ⁹ cells
None	200	30
0.6% Yeast extract	300	40
1% Glucose	200	15
0.6% Yeast extract + 1% glucose	400	50
0.6% Yeast extract + 0.2% glucose	400	90

^a Basal medium (3% Casamino Acids) was complemented with the different additives. Cells were grown for 7 h in a fermentor with agitation (400 rpm) and forced aeration (5 liters/min), and pH was maintained at 7.2.

^b The enterotoxin activity was determined in the suckling mouse test.

^c The supernatant was twofold serially diluted in sterile phosphate-buffered saline; the last dilution was 1:10, which gave a gut-to-body weight ratio of ≥ 0.09 .

TABLE 2. Effect of pH on the production of ST by *E. coli* F11(P155)^a

pH	Enterotoxin activity	
	U/ml	U/10 ⁹ cells
6.8	200	30
7.2	400	80
7.2 at start to 7.8 at end	400	90
8.0	200	30

^a Cells were grown in medium containing 3% Casamino Acids, 6% yeast extract, and 0.2% glucose for 7 h in fermentor with agitation (400 rpm) and forced aeration (5 liters/min).

The purification of ST from *E. coli* F11(P155) was based on $(\text{NH}_4)_2\text{SO}_4$ precipitation, ultrafiltration, and gel filtration. The toxin obtain from Bio-Gel filtration appeared to be homogeneous, as indicated by one peak of activity in sodium dodecyl sulfate-gel electrophoresis and by the appearance of a symmetrical peak during Bio-Gel filtration in the presence or absence 6.0 M urea and 1.1 M KCl (data not shown). The urea and KCl were added to prevent the aggregation of the toxin by hydrogen bonding and electrostatic interaction, respectively. A batchwise purification step was desirable in order to decrease the volume of crude material down to manageable amounts. Ammonium sulfate precipitation was advantageous since the procedure does not increase the original volume (like acetone fractionation) and more than 90% of the enterotoxigenic activity could be recovered from the precipitate. Alderete and Robertson (2) suggested the solu-

TABLE 3. Purification summary for ST of *E. coli* F11(P155)

Step	Total amt of protein (mg)	Effective dose (ng) ^a	Recovery (%)	-Fold purification
Supernatant (5 liters)	8,600	11,000	100	
Ammonium sulfate precipitate	NT ^b	NT	90	NT
PM-30 ultrafiltrate	125	50	90	220
Bio-Gel P-4 (peak B elution volume, 290 to 340 ml) ^c	10.5	14	80	785

^a The effective dose was the amount of protein necessary in the suckling mouse assay to give a gut-to-body weight ratio of 0.09.

^b NT, Not tested.

^c See Fig. 1.

TABLE 4. Seroneutralization of ST activity

Serum	Toxin	Titer ^a
Undiluted normal rabbit serum	<i>E. coli</i> F11(P155) supernatant	0
Undiluted normal goat serum	<i>E. coli</i> F11(P155) supernatant	0
Rabbit serum 3 anti-ST	<i>E. coli</i> F11(P155) supernatant	1:4
Rabbit serum 3 anti-ST	Purified ST ^b	1:8
Goat serum anti-ST	<i>E. coli</i> F11(P155) supernatant	1
Goat serum anti-ST	Purified ST	1:8

^a The last dilution of serum which could neutralize the ST activity.

^b ST was suspended at 60 U/ml in physiological saline and mixed with an equal volume of serum.

bility of ST in acetone and a lack of precipitation by $(\text{NH}_4)_2\text{SO}_4$, whereas both acetone and ammonium sulfate were used to precipitate ST (7, 23). However we observed in preliminary experiments that ST produced by bovine strains is not precipitated by ammonium sulfate or 5 volumes of acetone. The ST produced by porcine strain 1362 was not precipitable by ammonium sulfate (2); all of these data suggested that differences could exist between the STs of different strains. Amicon ultrafiltration with membranes retaining molecular species larger than 1,000 daltons (UM₂ membrane) was eliminated since these membranes had a low flow rate and only 10% of the activity was recovered in the ultrafiltrate (data not shown). The use of an Amicon PM-30 membrane (cut-off point, 30,000 daltons) was advantageous since all activity was recovered in the filtrate, along with only 10% of the protein. An amicon PM-10 membrane (cut-

FIG. 1. Concentrated ultrafiltrates were applied in 10-ml volumes to columns (70 by 2.5 cm) of Bio-Gel P-4 equilibrated with 5% acetic acid. The ST activity in each fraction was determined by the suckling mouse test. OD, optical density.

off point, 10,000 daltons) does not increase the specific activity but shows a tendency to plugging. The ultrafiltrate was concentrated by flash evaporation and further purified by gel filtration. In our early experiments we observed elution at about 1 column volume and a low recovery of biological activity when ST from *E. coli* F11(P155) was applied to Sephadex G-25. On Bio-Gel P-4 the ST activity was eluted in 1 column volume, with a recovery of more than 90% of the activity. Further purification with different ion-exchange columns was useless. With both anionic and cationic Bio-Gel ion-exchange columns the toxin was recovered in the void volume, and the specific activity was not increased. Since known techniques for protein, carbohydrate, and lipid staining were unable to localize ST after polyacrylamide gel electrophoresis, we had to detect the activity after elution of gel slices. It is important to note that in order to separate ST from the migration front, it was necessary to increase the polyacrylamide concentration to 20%. Under these conditions, the ST activity was eluted at a relative mobility of 0.6, and no band was observed after staining, although 10^6 U of ST was applied on the gel.

Purified ST was methanol soluble, and the extract was active in the suckling mouse test and rabbit gut loops. We were unable to confirm the results of Mullan et al. (15), who found that a methanol extract elicited a response in the mouse test but not in the rabbit system. Purified ST retained the properties of ST in a cell-free supernatant; these included resistance at 100°C but destruction at 121°C, resistance at pH 2 but inactivation at pH 12, and resistance to pronase, lipase, and amylase.

ST has been reported as apparently non-immunogenic (3). An anti-ST activity of serum from immunized rabbits has been reported (2). In this study we found that ST activity could be neutralized by serum from rabbits or a goat immunized with ST. The sera could neutralize the ST activity present in a cell-free supernatant or the ST activity of the purified preparation. From a comparison of our neutralization titer and the titers of Alderete and Robertson (2), it appears that ST could be immunogenic if a sufficient amount of product in combination with adjuvant is used. Whereas at the end we obtained an ST which was apparently less pure than the ST described by Alderete and Robertson (2), our procedure has advantages. In fact, with this method milligram amounts of ST could be obtained in 2 days. Starting with 5 liters of supernatant, we obtained 10 mg of ST which was active at nanogram levels and was immunogenic, at least in rabbits and goats. Work is in

progress in our laboratory to further purify ST by high-pressure liquid chromatography in order to elucidate the structure of the molecule.

ACKNOWLEDGMENTS

This work was supported by grant MMV75-603 from Conseil des Recherches et Services agricoles du Québec and by Conseil des Recherches en Santé du Québec, subvention d'établissement.

It is a pleasure to acknowledge the valuable assistance provided by Francine Bernard.

LITERATURE CITED

1. Alderete, J. F., and D. C. Robertson. 1977. Repression of heat-stable enterotoxin synthesis in enterotoxigenic *Escherichia coli*. Infect. Immun. 17:629-633.
2. Alderete, J. F., and D. C. Robertson. 1978. Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. Infect. Immun. 19:1021-1030.
3. Burgess, M. N., R. J. Bywater, C. M. Cowley, N. A. Mullan, and P. M. Newsome. 1978. Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits, and calves. Infect. Immun. 21:526-531.
4. Callahan, L. T., III, and S. H. Richardson. 1973. Biochemistry of *Vibrio cholerae* virulence. Nutritional requirements for toxin production and the effects of pH on toxin elaboration in chemically defined medium. Infect. Immun. 7:567-572.
5. Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harder. 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhoea in children in Honolulu. J. Infect. Dis. 125:407-411.
6. Dörner, F. 1975. *Escherichia coli* enterotoxin. Purification and partial characterization. J. Biol. Chem. 250: 8712-8719.
7. Evans, D. G., D. J. Evans, Jr., and N. F. Pierce. 1973. Differences in the response of rabbit small intestine to heat-labile and heat-stable enterotoxins of *Escherichia coli*. Infect. Immun. 7:873-880.
8. Finkelstein, R. A., P. Athasampunna, M. Chulasamaya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biologic activities of purified pro-cholerae. J. Immunol. 96:440-449.
9. 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:S120-S137.
10. Gianella, R. A. 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect. Immun. 14:95-99.
11. Gyles, C. L. 1974. Relationships among heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. 129:277-283.
12. Larivière, S., C. L. Gyles, and D. A. Barnum. 1972. A comparative study of the rabbit and pig gut loop systems for the assay of *Escherichia coli* enterotoxin. Can. J. Comp. Med. 36:319-328.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
14. Moon, H. W. 1974. Pathogenesis of enteric diseases caused by *Escherichia coli*. Adv. Vet. Sci. 18:179-211.
15. Mullan, N. A., M. N. Burgess, and P. N. Newsome. 1978. Characterization of a partially purified, methanol-soluble heat-stable *Escherichia coli* enterotoxin in infant mice. Infect. Immun. 19:779-784.

16. Sack, R. B. 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. 29:333-351.
17. Sack, R. B., S. L. Gorbach, J. G. Banwell, B. Jacobs, B. D. Chattejee, and R. C. Mitra. 1971. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. J. Infect. Dis. 123:378-385.
18. Schenkein, I., R. F. Green, D. S. Santos, and W. K. Maas. 1976. Partial purification and characterization of a heat-labile enterotoxin of *Escherichia coli*. Infect. Immun. 13:1710-1720.
19. Smith, H. W., and C. L. Gyles. 1970. The relationship between two apparently different enterotoxins produced by enteropathogenic strains of *E. coli* of porcine origin. J. Med. Microbiol. 3:387-401.
20. Smith, H. W., and C. L. Gyles. 1970. The effect of cell-free fluids prepared from cultures of human and animal enteropathogenic strains of *Escherichia coli* on ligated intestinal segments of rabbits and pigs. J. Med. Microbiol. 4:403-409.
21. Smith, H. W., and S. Halls. 1967. Studies on *Escherichia coli* enterotoxin. J. Pathol. Bacteriol. 93:531-543.
22. Smith, H. W., and S. Halls. 1968. The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. J. Gen. Microbiol. 52: 319-334.
23. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.