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Glycerophospholipid:Cholesterol Acyltransferase Complexed with Lipopolysaccharide (LPS) Is a Major Lethal Exotoxin and Cytolysin of *Aeromonas salmonicida*: LPS Stabilizes and Enhances Toxicity of the Enzyme

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An extracellular lethal toxin produced by *Aeromonas salmonicida* was purified by fast-protein liquid ion-exchange chromatography. The toxin is composed of glycerophospholipid:cholesterol acyltransferase (GCAT) (molecular mass, 25 kilodaltons) aggregated with lipopolysaccharide (LPS), the GCAT/LPS complex having a molecular mass of about 2,000 kilodaltons, estimated by gel filtration chromatography. The toxin is lethal for Atlantic salmon (*Salmo salar* L.) at a concentration of 0.045 μg of protein per g of body weight. The toxin is a hemolysin (T-lysin, active on fish erythrocytes), leukocytolysin, and cytotoxin. Antiserum to the purified toxin neutralized the lethal toxicity of the crude extracellular toxins, indicating this toxin to be the major lethal factor produced by *A. salmonicida*. In the crude extracellular products, small amounts of free GCAT were also present. This has been purified, and its activities and properties have been compared with those of the GCAT/LPS complex. The presence of LPS did not influence the GCAT activity of the enzyme with egg yolk or phosphatidylcholine (lecithin) as a substrate, but the specific hemolytic activity and lethal toxicity was about eightfold higher in the complexed form. Furthermore, the free GCAT was more susceptible to proteolytic and heat inactivation than was the GCAT/LPS complex. Recombination of LPS (phenol extracted from extracellular products of *A. salmonicida*) with free GCAT enhanced the hemolytic activity, lethal toxicity, and heat stability of the latter but did not influence its lecithinase activity. In native polyacrylamide gel electrophoresis, the GCAT/LPS complex and the recombined GCAT-LPS both showed a high-molecular-mass band which did not enter the gel, while the free GCAT produced a single band with low molecular mass. In isoelectric focusing gels, the GCAT/LPS and recombined GCAT-LPS produced a nonfocusing smear with pIs from pI 5.0 to 5.8, while the free GCAT produced a single band with pI 4.3. These data show that free GCAT can combine with LPS to produce a high-molecular-mass complex with enhanced toxicity and heat stability compared with those of free GCAT, similar to the preexisting GCAT/LPS complex, and indicate that the LPS moiety of the toxin plays an active role in toxicity.

The extracellular products (ECP) of *Aeromonas salmonicida* are pathogenic and lethal to fish (10). The ECP contain a variety of enzymes, including proteases (27), glycerophospholipid:cholesterol acyltransferase (GCAT) (5), and a variety of partially characterized toxic activities. These include hemolysins (13, 31), a leukocytolysin (12), a cytotoxin (7), and a lethal toxin (24). However, the molecular natures and identities of these toxins are not precisely known.

The major lethal toxin has been suggested by several workers to be a protease, but recent work has discounted this (9). A potent lethal toxin has recently been isolated and shown also to be a hemolysin. It was characterized as a glycoprotein with a high molecular mass of at least 200 kilodaltons (kDa) (24). However, the hemolysin, which is specific for fish erythrocytes, was claimed by other workers to be a protein of 56-kDa molecular mass (13), and although it was shown to contribute to the pathogenesis of this disease, it was not implicated as a lethal toxin (14). The leukocytolysin was claimed to be a glycoprotein with molecular mass of over 100 kDa, but it was not considered to be toxic to fish (12). The GCAT has a molecular mass of 24 kDa (5), but its effect on fish has not been studied.

The aim of the present study was to identify and characterize the molecular nature of the lethal toxin for fish.

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MATERIALS AND METHODS

Bacteria and ECP. *A. salmonicida* MT004 (URL 2862; Unilever Research Ltd.) was isolated during an epizootic of furunculosis in Atlantic salmon in Scotland. This strain, which was autoaggregating when first isolated, is now nonaggregating. Stock cultures were grown on tryptone soy agar (Oxoid Ltd.) for 28 h at 22°C. Two swabs of these bacteria suspended in 5 ml of phosphate-buffered saline (pH 7.2) (Dulbecco Ca^{2+} and Mg^{2+} free; GIBCO Laboratories) (PBS) were spread onto cellophane overlaying tryptone soy agar and grown for 48 h at 22°C. The ECP were harvested as previously described (23).

Total protein was measured by the method of Bradford (3) with bovine serum albumin as a standard. Protease activity was measured by Hide powder azure digestion. Enzyme solution (0.1 ml) was incubated and shaken with 25 mg Hide powder azure in 2.4 ml of PBS at 37°C for 15 min. On the addition of 2.5 ml of 10% trichloroacetic acid and centrifugation, the A_{600} of the supernatant was measured. Blanks were prepared by addition of trichloroacetic acid to the substrate prior to addition of the enzyme. One unit of protease activity was an increase in absorbance of 0.01 U. Hemolytic activity was measured by a standard microtitration method with rainbow trout or rabbit erythrocytes as described previously (15).

The inhibition of the protease was performed by incubat-

ing with 10% (vol/vol) 25 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co.) (in isopropanol) overnight at 4°C.

Fish and toxicity tests. Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) weighing approximately 20 and 200 g, respectively, were held in 200-liter tanks supplied with fresh running water at 13 to 15°C. Atlantic salmon were used for the lethal toxin test, and rainbow trout were used to obtain erythrocytes and leukocytes. The fish were anesthetized with benzocaine (ethyl *p*-aminobenzoate; Sigma) during the procedures of injection and bleeding. Lethal toxin tests were conducted by intraperitoneal (i.p.) injection. The minimum lethal dose, with batches of 10 fish per dose, was defined as causing at least 50% mortality. Mortalities were monitored for 48 h.

Purification of the lethal toxin. The ECP was desalted by passing through a Sephadex G-25 column (PD10; Pharmacia, Inc.), and 5 mg of ECP protein was fractionated by fast-protein liquid chromatography (FPLC; Pharmacia) with an anion-exchange column (Mono Q HR 5/5) equilibrated with 20 mM Tris(hydroxymethyl)methylamine, pH 7.7. Fractions were eluted with a sodium chloride gradient at a rate of 1 ml/min. Fractions (1 ml) were collected and stored at −20°C prior to being tested.

Molecular weight of native toxin. The purified toxin from the FPLC ion-exchange column was concentrated 10 times by vacuum dialysis, and 200 μl was applied to an FPLC Superose 12 gel filtration column (Pharmacia) which had been calibrated by using standard markers (Pharmacia). The sample was eluted with PBS at 0.5 ml/min.

Carbohydrate and total lipid content of the purified toxin. Carbohydrate was measured by the method of Dubois et al. (8) with glucose as a standard. Total lipids were measured by a colorimetric method with a total-lipid kit purchased from Boehringer GmbH.

Quantitative GCAT assay. Five milliliters of 2.5% (wt/vol) egg yolk (Sigma) in 0.1 M Tris hydrochloride buffer (pH 7.5) was emulsified by shaking with 5×10^5 dpm of [^{14}C]cholesterol (55 mCi/mmol; Amersham Corp.). Protein ECP (150 μg); 12.0, 9.2, 7.6, and 3.8 μg of protein GCAT/LPS; or 9.6, 7.7, 4.7, and 3.0 μg of free GCAT were incubated with the substrate at 37°C for 1 h, and the whole was extracted with 10 ml of chloroform-methanol (2:1). Samples of the lower phase were separated in thin-layer chromatography (TLC), and radiolabeled spots, denoting the esterified and nonesterified cholesterol, were visualized by using a radiochromatogram imaging system (AID Ltd.). Spots were excised from the TLC plate and extracted with chloroform-methanol (2:1) for 30 min. The extract was decanted and evaporated in a stream of nitrogen. ^{14}C activity was determined by liquid scintillation spectrometry. GCAT units are expressed as [(counts per minute in cholesteryl ester)/(counts per minute in cholesteryl ester + counts per minute in cholesterol)] × 1,000.

Assay of GCAT, lecithinase, leukocytotoxin, and rainbow trout gonad cytotoxin. The qualitative GCAT assay was modified from the method of Buckley et al. (5). One hundred milligrams of both lecithin (phosphatidylcholine) and cholesterol (Sigma) in 1 ml of buffer (0.1 M Tris hydrochloride, pH 7.5) was sonicated and used as a substrate for the detection of GCAT activity in ECP and purified lethal toxin. ECP (200 μl) or 50 μl of purified lethal toxin was incubated at 37°C for 3 h (shaking) with 0.5 ml of substrate. After incubation, the samples were extracted with 3 ml of chloroform-methanol (2:1). Lecithin, palmitic acid, cholesterol, and cholesteryl ester (all at 50 mg/ml of buffer), 100 μl of ECP, and 200 μl of substrate were also extracted with 3 ml of chloroform-methanol (2:1). Fifty microliters of the lower phase of each treatment was introduced onto a TLC plate (Merk & Co., Inc.). The TLC plate was developed in petroleum ether-ether-acetic acid (90:10:1), and the spots were visualized by exposure of the plate to iodine vapor.

Lecithinase activity was detected by placing samples in wells cut in agarose (1% in PBS) containing 1% lecithin and incubating overnight at 25°C. The appearance of transparent zones around the wells indicated the presence of lecithinase activity.

The method of Fuller et al. (12) was adopted for the leukocytotoxin assay but used rainbow trout leukocytes, and the cells were finally stained with trypan blue (0.4%). The purified lethal toxin was diluted to 1.63 μg of protein per ml with Hanks balanced salt solution prior to incubation with leukocytes.

Rainbow trout gonad type 2 tissue culture cells were grown in flat-base wells at 20°C overnight in minimal essential medium supplemented with fetal calf serum (GIBCO, 10% [vol/vol]), tryptose phosphate broth (GIBCO, 10% [vol/vol]), nonessential amino acids (GIBCO, 1% [vol/vol]), sodium bicarbonate (7.5% [vol/vol]), Tris hydrochloride buffer (4 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml). Cells were washed with PBS, and 100 μl of doubling dilutions of the purified toxin in PBS was added to wells and incubated at 20°C for 2 h. Control wells contained serial dilutions of 1 M NaCl in PBS.

Preparation of rabbit antitoxin (GCAT/LPS complex) antiserum. The purified toxin was treated with Formalin (40% [wt/vol] formaldehyde) to a concentration of 3% (vol/vol) and incubated for 48 h at 22°C. Rabbits were injected subcutaneously with 13 μg of protein of purified toxin per kg of body weight in complete Freund adjuvant. A similar booster injection was given 6 weeks later, and antiserum was collected after 2 weeks. Use of this antiserum to stain Western immunoblots of purified toxin and crude ECP demonstrated that the antiserum contained a mixture of antibodies to the GCAT protein and the LPS.

Crossed immunoelectrophoresis (CIE). CIE was performed by using the Multiphor system (LKB Instruments, Inc.) as described by Wallenborg and Andersson (33). Briefly, 40 μl of ECP (1.4 mg of protein per ml) was electrophoresed in the first dimension at 10 V/cm for 70 min. The second-dimension gels contained 3% (vol/vol) rabbit antitoxin antiserum. Electrophoresis was at 2 V/cm for 16 h in Tris-barbiturate buffer (pH 8.6). Gels were stained with Coomassie brilliant blue. Control CIE was performed with serum obtained before immunization.

Detection of hemolytic and phospholipase activity in CIE immune precipitates. (i) Hemolytic activity. CIE plates were washed in 20 volumes of PBS for 2 h and then overlaid with gels containing 1% agarose and 3% washed (three times) rainbow trout erythrocytes, in PBS for 2 to 4 h at 25°C. Following removal of the overlay, the CIE gel was washed again and stained with Coomassie brilliant blue.

(ii) Phospholipase activity. CIE gels were washed as described above, and the immune precipitates were excised, thoroughly minced, and incubated at 50°C for 10 min in 0.1 M Tris hydrochloride, pH 7.5. Lecithin (Sigma) (5 mg emulsified in 50 μl of 0.1 M Tris hydrochloride, pH 7.5) was added and incubated at 40°C for 2 h. Phospholipase A activity was measured by detecting the release of free fatty acids from lecithin with a kit (Wako Chemicals GmbH) by a colorimetric method for the quantitative determination of nonesterified fatty acids following the instructions of the

manufacturer. An equivalent amount of gel was excised from the CIE plate 1 cm above the immune precipitates and used as a control.

Electrophoresis and Western blotting. PAA4/30 gradient gels, Phastgel (12.5% polyacrylamide; Pharmacia), and Phastgel gradient (10 to 15%) polyacrylamide (Pharmacia) were employed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pharmacia low- and high-molecular-weight calibration kits were used as marker proteins. Electrophoresis was conducted according to the recommendations of the manufacturer for these gels. After electrophoresis in PAA4/30 gels, some samples were transferred onto a nitrocellulose membrane by Western blotting (Trans-Blot Cell; Bio-Rad Laboratories) according to the instructions of the manufacturer. The electroblotted nitrocellulose membranes were stained with either colloidal gold (Aurodye, Janssen) for total protein or immunostained with rabbit antitoxin antiserum (1:1,000). Controls used serum obtained prior to immunization. Staining was by goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (1:3,000) (Bio-Rad). Native PAGE was performed by using Phastgels (12.5% polyacrylamide). SDS and native PAGE gels were stained with Coomassie brilliant blue or silver as recommended by Pharmacia. LPS silver staining was performed by a modification of the methods of Tsai and Frasch (32) and Hitchcock and Brown (16) as published elsewhere (18).

Isoelectric focusing (IEF). IEF was conducted in the PhastSystem (Pharmacia) with Phastgel IEF3-9 (homogeneous polyacrylamide gels containing Pharmalyte carrier ampholytes). Broad-pI calibration kits (protein pI 3.50 to 9.30) (Pharmacia) were used as marker proteins in the pI value determination. After electrophoresis, IEF gels were stained with silver. The separation and development protocols followed the recommendation of the manufacturer.

Neutralization of ECP lethal toxicity by rabbit antitoxin antiserum. In relatively large doses, the extracellular protease of *A. salmonicida* is lethal for fish (19). Inhibition of the protease in ECP by PMSF increases the minimum lethal dose of ECP by only about 40% (9). Experiments were performed to investigate whether the lethal toxin purified as described above was the major lethal toxin in ECP. Various lethal doses of ECP (preincubated with PMSF to inactivate the protease, as described above) were incubated with rabbit antitoxin antiserum in a ratio of 10:1 (vol/vol) for 30 min at 25°C and then injected i.p. into Atlantic salmon (5 to 10 g). Mortalities were recorded for 48 h. Serum from rabbits injected only with complete Freund adjuvant was used as a control.

Rabbit antiserum to *A. salmonicida* extracellular protease. Fractions 15 and 16 from ion-exchange (Mono Q HR 5/5) FPLC separation of ECP yielded the protease contaminated with small quantities of hemolysin. The protease was purified from these fractions by slab electrophoresis in polyacrylamide gradient gels (PAA4/30, Pharmacia) under native conditions. Following this, an agarose gel (1%) containing 0.1% casein (wt/vol) (Sigma) in PBS was layered onto the PAGE gel and incubated at 22°C for 2 h. The band of protease in the PAGE gel, visualized by the clearing of the casein in the overlay, was excised, homogenized with complete Freund adjuvant, and injected subcutaneously into a New Zealand White rabbit. A similar booster injection, without adjuvant, was given after 6 weeks, and the rabbit was bled 2 weeks later. In SDS-gradient PAGE and Western blots of ECP, this antiserum stained a single band with a molecular mass of 64 kDa, characteristic of the protease (data not shown). In nongradient gels, this protease had a molecular mass of 70 kDa, in agreement with the results of others (13, 27).

Purification of free GCAT. Fraction 18 of FPLC Mono Q separation of ECP contained only two bands in SDS-PAGE (Phastsystem) with molecular masses of 70 and 25 kDa. Tests for protease and hemolysin activity of this fraction were both positive. Separation of the hemolysin and the protease was performed by using a column of protein A-agarose (4% cross-linked agarose; Sigma) bound with rabbit anti-*A. salmonicida* protease antibodies. Samples of fraction 18 were passed into the column, and the pure GCAT was eluted with PBS.

Extraction of LPS from *A. salmonicida* ECP. LPS was extracted by hot phenol (36) from lyophilized ECP, dialyzed against tap water for 48 h, and lyophilized.

Recombination of phenol-extracted LPS with free GCAT (GCAT-LPS). Extracted LPS was incubated in PBS with free GCAT at 22°C for 30 min in the same ratio of total lipids/protein as that measured in purified GCAT/LPS complex.

Thermostability of free GCAT, GCAT/LPS, and recombined GCAT-LPS. Heat stability was assessed by incubating ECP, GCAT/LPS complex, free GCAT, and recombined GCAT-LPS at 60°C for 5, 15, or 30 min.

The stability of the GCAT was assayed by using hemolysin titration and the lecithinase assay. Various concentrations of GCAT/LPS and free GCAT were used to calibrate a standard curve (log enzyme concentration against the radius of clear zones produced in lecithin-agarose plates). The radii of the clear zones of heated samples were measured, and their equivalence to the active enzyme concentration was read from the standard curve.

The heat stability of lethal toxicity of ECP and GCAT/LPS was assessed by incubation at 60°C for 30 min prior to i.p. injection into fish with doses higher than the minimal lethal doses.

Stability of free GCAT and GCAT/LPS complex to proteases. *A. salmonicida* protease was prepared from fraction 15 (FPLC ion-exchange Mono Q), which was stored at 4°C until hemolytic activity was completely destroyed. This preparation possessed 556 protease units/ml. Samples of free GCAT and GCAT/LPS were incubated with *A. salmonicida* protease at 22°C for 48 h or with proteinase K (BDH) (1 mg/ml of PBS) for 30 min at 37°C. Controls were incubated with PBS.

RESULTS

ECP possessed a hemolytic activity on trout erythrocytes of 5,851 HU (hemolytic units)/mg of protein and a minimum lethal dose of 1.2 μg/g of fish. ECP did not lyse rabbit erythrocytes. The hemolytic, lethal, and GCAT activities of the ECP were completely inactivated by heating at 100°C for 10 min or 60°C for 30 min.

Isolation of the lethal toxin. The FPLC profile of the ECP fractionation is shown in Fig. 1. To screen fractions for toxicity, two salmon were injected i.p. with 0.1 ml of each fraction from 14 to 32 and mortalities were monitored for 72 h. Fractions 15, 16, 27, 28, and 29 were lethal, all deaths occurring within 24 h. Fractions were tested for protease and hemolytic activity (selected data shown in Table 1). The protease eluted as a sharp peak in fractions 15 and 16. These fractions had no detectable carbohydrate content. When the protease activity of these fractions was inhibited by PMSF, they were no longer lethal on injection of 0.1 ml. Hemolytic activity for trout erythrocytes was detected in all fractions, but it peaked in fractions 27 and 28. Lethal fractions were

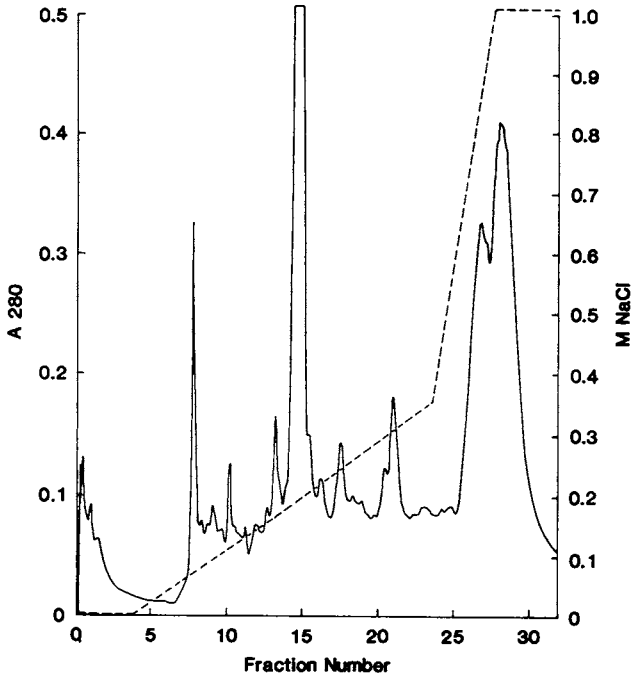

FIG. 1. Profile of extracellular products of *A. salmonicida* eluted from an FPLC anion-exchange column in a segmented NaCl gradient (dashed line).

analyzed by SDS-PAGE (Phastgels), and fractions 27 and 28 contained a single protein band with a molecular mass of 25 kDa (Fig. 2). With slab gradient SDS-PAGE (PAA4/30) for Western blotting, this protein always had a molecular mass of 27 kDa. Thus, the 25- and 27-kDa proteins reported here are the same protein. The other fractions containing hemolytic activity also possessed the 25-kDa band. Thus, the lethal toxin consisted of a single protein with a molecular mass of 25 kDa and possessed high hemolytic activity (157,538 HU/mg of protein). Hemolysis of trout erythrocytes caused by the purified toxin was incomplete (i.e., erythrocyte ghosts remained intact), in contrast to hemolysis by ECP, which resulted in complete solubilization of erythrocyte membranes.

The minimum lethal dose of the purified toxin was determined to be 0.045 μg of protein per g of fish. In terms of hemolytic units, the minimum lethal dose (0.1 ml) contained 512 HU/ml. GCAT activity of the purified toxin was demonstrated by the production of cholesteryl ester after incubation with lecithin-cholesterol liposomes, as detected in TLC plates (Fig. 3). The purified toxin also possessed leukocytolytic and cytotoxic activity. In assays performed in triplicate, 86 to 96% of rainbow trout leukocytes were lysed, as shown by their intense staining with trypan blue. Control leukocytes incubated in PBS contained about 10% dead cells. Rainbow trout gonad type 2 cells were lysed by the purified toxin to a dilution of 1:64 (0.41 μg of protein per ml). No lysis occurred in control cells incubated with dilutions of the eluting buffer (1 M NaCl, 20 mM Tris) in PBS under the same conditions.

TABLE 1. Lethal tests and related data of ECP and fractions of ion-exchange chromatography (FPLC)[a]

Fraction	Total protein (μg/ml)	No. killed/ total no.	HU/ml	Protease units/ml
ECP	1,400	5/5	8,192	2,010
14	42	0/2	4	20
15	520	2/2	256	2,180
15	—[b]	0/2	256	0
16	86	2/2	128	710
16	—	0/2	128	0
27	26	2/2	4,096	0
28	<20	2/2	1,024	0
29	<20	2/2	512	0

[a] Atlantic salmon, 20 g, were injected i.p. with 0.1 ml of ECP or fraction.
[b] —, Fraction inhibited by 10% PMSF.

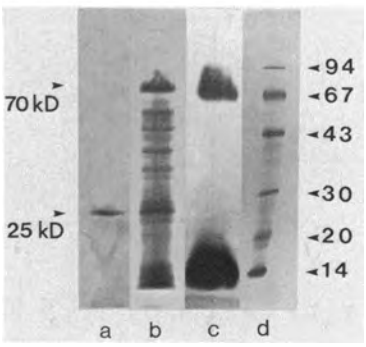

FIG. 2. SDS-PAGE of purified GCAT/LPS complex (64 ng of protein) (Phastgel protein silver stain) (lane a), ECP (Phastgel protein silver stain) (lane b), LPS silver stain of purified GCAT/LPS complex (2.56 μg of carbohydrate) (lane c), and molecular mass standards (Pharmacia) (phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa) (lane d). Sizes (in kilodaltons [kD]) are indicated at the sides.

Nature of the native toxin in ECP. The hemolytic, lethal, and GCAT activities of the purified toxin were inactivated by heating to 60°C for 30 min. In native-gradient Phastgels stained with Coomassie blue, the purified toxin showed a single band with very high molecular mass (data not shown). An estimation of the molecular mass of the pure toxin was made by passage through a FPLC Superose 12 gel filtration column with an exclusion limit of 2,000 kDa. Lecithinase activity, hemolytic activity, and lethal toxicity eluted as a single peak in the void volume (fraction 16) (Fig. 4). When

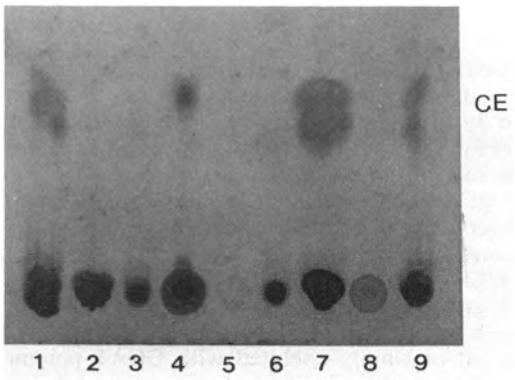

FIG. 3. TLC analysis of the GCAT activity of purified toxin and crude ECP, developed in petroleum ether-ether-acetic acid (90:10:1). Lanes: 1 and 9, Purified toxin-lecithin-cholesterol; 2, cholesterol; 3, lecithin-cholesterol (liposome); 4, ECP-lecithin-cholesterol; 5, palmitic acid; 6, lecithin; 7, cholesteryl palmitate; 8, ECP. CE, Cholesteryl ester.

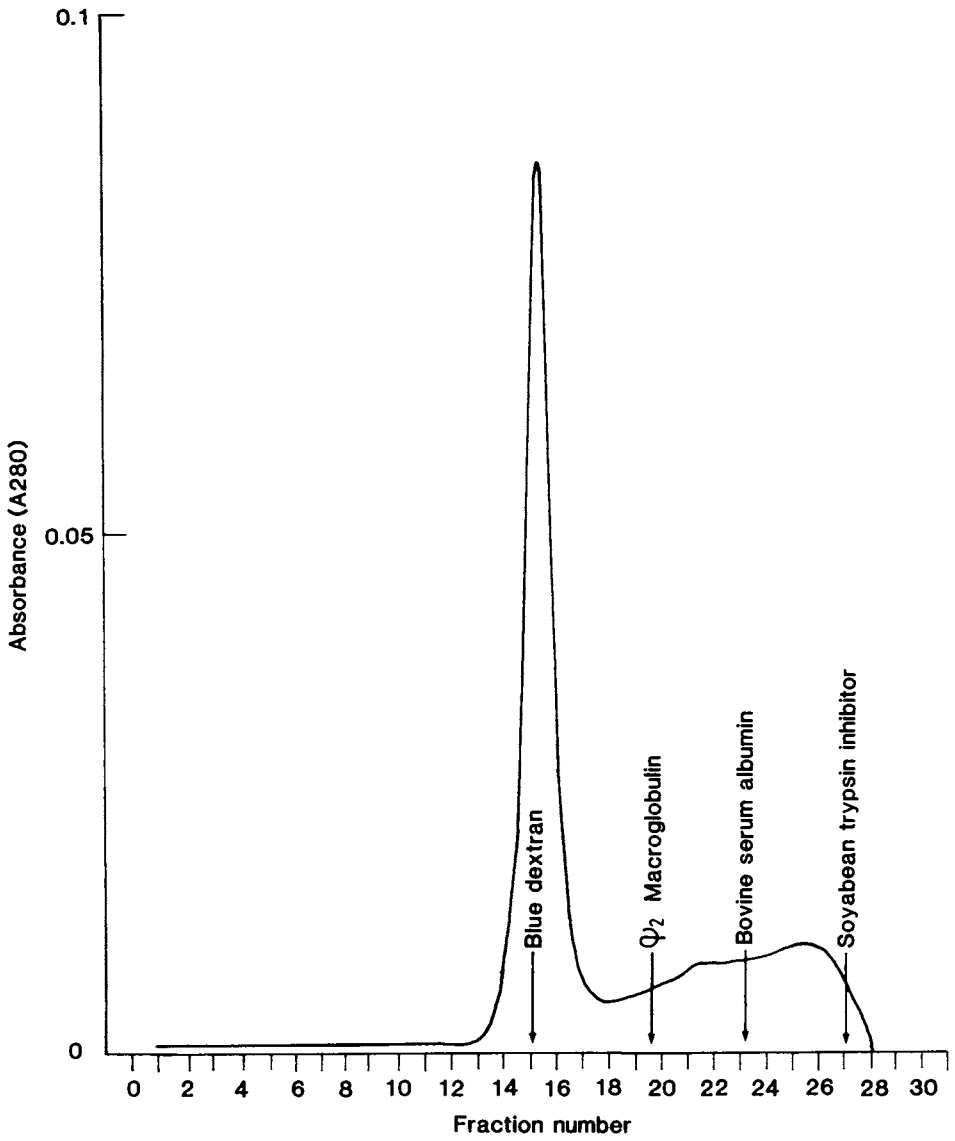

FIG. 4. Profile of purified toxin eluted from FPLC Superose 12 gel filtration column. Elution was with PBS, pH 7.2, at 0.5 ml/min. The column was calibrated with M_W standards (Sigma), and points of peak elution are indicated. The molecular masses of blue dextran, α_2-macroglobulin, bovine serum albumin, and soybean trypsin inhibitor are 2,000, 725, 67, and 20 kDa, respectively.

SDS-PAGE preparations of the purified toxin (from both the ion-exchange column and following gel filtration) were stained by the protocol of silver staining for LPS, a typical LPS profile with O-antigen bands (60- to 70-kDa equivalent) and the lipid A-core oligosaccharide (16- to 20-kDa equivalent) (Fig. 2, lane c) similar to that described by others (6, 11) was observed. The presence of LPS in the purified toxin was also confirmed in Western blots with rabbit antitoxin (GCAT/LPS) antiserum (Fig. 5, lane a). With this procedure, a much-greater heterogeneity of O antigens was observed than with silver staining. To assess whether this LPS was complexed or simply coeluted with GCAT polymers, the nature of the toxin in ECP was further investigated by CIE.

The rabbit antitoxin (GCAT/LPS) antiserum produced two precipitin arcs against native ECP in CIE (Fig. 6). Both arcs possessed hemolytic activity in erythrocyte-agarose overlays, though activity was detected earlier in arc 1. Phospholipase activity in excised immune precipitates was very high, and as with hemolytic activity, levels were higher in arc 1. Control parts of the CIE gels contained no detectable phospholipase activity. To investigate whether LPS had coprecipitated with the phospholipase activity in the CIE gels, arcs were excised from Coomassie blue-stained CIE gels, rehydrated and homogenized in SDS sample buffer containing 1 mg of proteinase K per ml, and incubated for 1 h at 37°C. Samples were analyzed in SDS-PAGE Western blots and stained with colloidal gold or rabbit antitoxin antiserum. Blots stained for protein by colloidal gold confirmed digestion of proteins in the immune complex. The immunostained blot showed a typical LPS pattern in sample prepared from arc 1, but no LPS was detected in arc 2 (Fig. 7). Thus, the majority of the native toxin in the ECP occurs as a high-molecular-mass aggregate containing GCAT complexed with LPS.

The purified toxin was composed solely of the arc 1 form, as demonstrated in CIE with rabbit antiserum to whole ECP

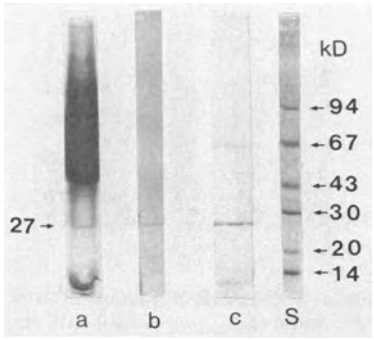

FIG. 5. SDS-PAGE and Western blotting of purified toxin (0.2 μg of protein) stained with rabbit antitoxin antiserum (lane a), purified toxin (0.2 μg of protein) stained with colloidal gold (lane b), and ECP (2.8 μg of protein) stained with rabbit antitoxin antiserum absorbed with boiled *A. salmonicida* MT004 cells (lane c). Lane S, Molecular mass standards (Pharmacia). Sizes (in kilodaltons [kD]) are indicated at the sides.

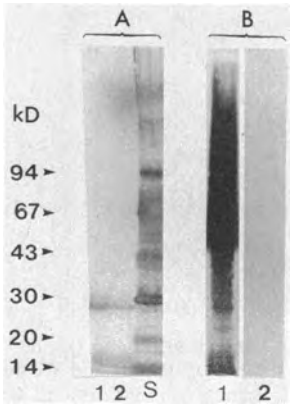

FIG. 7. SDS-PAGE and Western blotting of arcs 1 and 2 (Fig. 6) after digestion by proteinase K, stained with colloidal gold (A) or rabbit anti-toxin antiserum (B). Lanes 1, Arc 1 plus proteinase K; lanes 2, arc 2 plus proteinase K, lane S, molecular mass standards. kD, Kilodaltons.

(Fig. 8a). Determination of the carbohydrate and total lipid content of the purified toxin showed it to contain 65 mg of carbohydrate per mg of protein and 2.5 mg of total lipids per mg of protein.

When the rabbit antitoxin (GCAT/LPS) antiserum was absorbed with boiled cells of *A. salmonicida* MT004 (which lacks A-layer) (9) and then used in the CIE against ECP, only arc 2 was obtained (Fig. 8b). This absorbed antiserum, when used to stain Western blots of ECP, produced only very weak staining of LPS plus the 27-kDa protein, indicating that antibodies to LPS had been virtually removed (Fig. 5, lane c). Thus, the precipitation of the major hemolytic arc (arc 1) in the CIE was executed by rabbit antibodies to the LPS component of the complex, while antibodies to the 27-kDa protein were effective in precipitating only the form present in arc 2, which contained little LPS.

Occurrence of dimeric GCAT. During the course of these experiments, it became evident that following concentration of the purified toxin fraction by ultrafiltration (Millipore Corp.), analysis with Pharmacia PAA4/30 SDS gradient gels resulted in three protein bands: >200, 54, and 27 kDa. Frequently, the 54-kDa band was dominant (Fig. 9, lane a). When an unconcentrated sample was not boiled, a band with a 30-kDa molecular mass was obtained (Fig. 9, lane c).

Neutralization of ECP lethal toxicity by rabbit antitoxin antiserum. Following inhibition of the extracellular protease with PMSF, the ECP was still highly toxic to fish. The lethal toxicity of doses of ECP-PMSF, considerably higher than the 50% lethal dose, was neutralized by rabbit antitoxin antiserum (Table 2). Control rabbit serum had no effect on the lethal toxicity, and all fish died within 18 h.

Purification of free GCAT. Fraction 18 of FPLC Mono Q separation of ECP contained both protease and hemolysin activity. In SDS-PAGE, this fraction contained two protein bands with molecular masses of 70 and 25 kDa (Fig. 10, lane a). Staining of such preparations for the presence of LPS by using the silver method demonstrated the absence of LPS (5 ng of LPS was detectable by this method). Furthermore, carbohydrates (sensitivity, 10 μg/ml) and total lipids (sensitivity, 25 μg/ml) were undetectable in fractions 15 to 18. The protease was separated from this mixture by adsorption onto a protein A-agarose column bound with rabbit antiprotease antibodies, and the hemolysin was eluted in pure form with a molecular mass of 25 kDa in SDS-PAGE (Fig. 10, lane b). This preparation had no detectable protease activity. In the native state, the purified GCAT eluted as single peak in fraction 26 from a calibrated FPLC Superose 12 gel filtration column (Fig. 4), and the molecular mass was calculated to be 30 kDa. GCAT activity of this protein was demonstrated by using [^{14}C]cholesterol and egg yolk substrate assayed by TLC (Fig. 11). The free GCAT was identical antigenically to the enzyme in the GCAT/LPS complex, as shown by its staining with rabbit antitoxin (GCAT/LPS) antiserum in Western blots (Fig. 5).

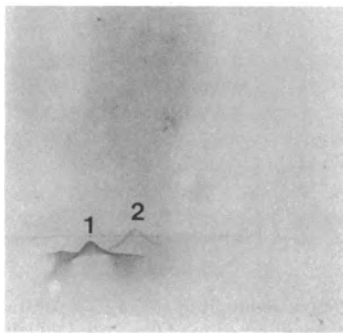

FIG. 6. CIE of *A. salmonicida* MT004 ECP against rabbit antitoxin antiserum showing two precipitation arcs (1 and 2).

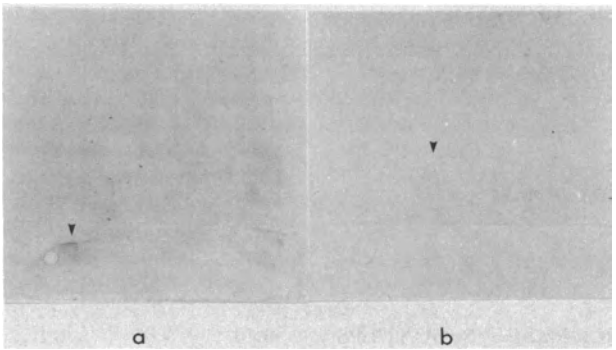

FIG. 8. (a) CIE of purified toxin against rabbit antitoxin antiserum. The arrowhead indicates arc 1. (b) CIE of *A. salmonicida* MT004 ECP against rabbit antitoxin antiserum absorbed with boiled whole cells. The arrowhead indicates arc 2.

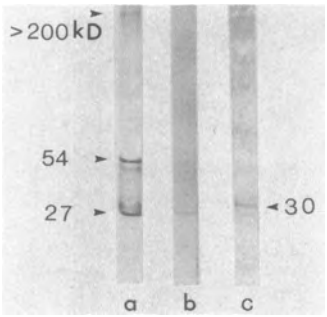

FIG. 9. Effect of concentration and boiling of GCAT/LPS complex prior to analysis in SDS-PAGE and Western blotting, stained with colloidal gold. Lanes: a, concentrated GCAT/LPS (2 μg of protein); b, GCAT/LPS (0.2 μg of protein); c, GCAT/LPS (0.2 μg of protein) not boiled in sample buffer. Sizes (in kilodaltons [kD]) are indicated at the sides.

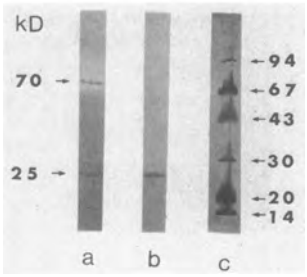

FIG. 10. Lane a, SDS-PAGE of fraction 18 from FPLC Mono Q ion-exchange separation of *A. salmonicida* ECP showing the protease (70 kDa) and GCAT (25 kDa); Phastsystem (Pharmacia) silver stain, 52 ng of protein applied (12.5% Phastgel). Lane b, SDS-PAGE of free GCAT obtained after affinity purification of fraction 18; Phastsystem silver stain, 16 ng of protein applied (12.5% Phastgel). Lane c, Molecular mass standards (Pharmacia): phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa. kD, Kilodaltons.

Recombination of phenol-extracted LPS with free GCAT. Phenol-extracted ECP LPS contained 0.46 mg of total lipids per mg of dry weight. Total lipids in GCAT/LPS were 2.5 mg per mg of protein. Therefore, a ratio of 2.5:1.0 (total lipids of ECP LPS/protein of free GCAT) was adopted to mimic the purified GCAT/LPS. Electrophoretic analysis of free GCAT and the mixture of GCAT and LPS demonstrated the latter to have combined into a stable GCAT-LPS complex. In native PAGE, the free GCAT produced a single fast-migrating band while the GCAT/LPS and recombined GCAT-LPS did not enter the gel (Fig. 12). In IEF gels, the GCAT/LPS and recombined GCAT-LPS produced a nonfocusing smear with pIs from 5.0 to 5.8, while the free GCAT produced a single band with pI 4.3 (Fig. 13).

Enzymic, hemolytic, and lethal toxic activities of GCAT. The GCAT activities of various concentrations of free GCAT and GCAT/LPS are shown in Fig. 14. This demonstrates that during the time of the assay, the conversion of up to 47% of the available cholesterol to cholesterol ester is proportional to the enzyme concentration. The specific GCAT activity of the enzyme preparations was calculated from assays which had conversion ratios of 35 to 46%, and the values for ECP, free GCAT, and GCAT/LPS complex are shown in Table 3.

There was no difference between the specific GCAT activities of the free enzyme and the GCAT/LPS with egg yolk as a substrate. Additionally, the lecithinase assay revealed that free GCAT, GCAT/LPS, and recombined GCAT-LPS possessed similar enzymic activities with phosphatidylcholine as a substrate. However, the hemolytic activity and lethal toxicity of the GCAT/LPS complex were about eight times higher than those of the free GCAT. The addition of phenol-extracted LPS to the free GCAT enhanced both the hemolytic activity and the lethal toxicity of the free GCAT about fourfold (Table 3).

In comparison with ECP, the GCAT/LPS complex was about six times more toxic. The free GCAT was about 0.8 times less toxic than ECP. In this preparation of ECP, the specific hemolytic activity of the ECP was greater than that reported in Table 1. This was reflected in the lower minimum lethal protein dose. On the basis of specific hemolytic units, the hemolysin content of ECP protein was 12.3%. However, as the specific hemolytic activities of free GCAT and GCAT/LPS are very different and ECP contains a mixture of the two, specific GCAT units provide a more-precise means of estimating the total GCAT content of ECP. Thus, on the

TABLE 2. Neutralization of ECP (0.4 mg of protein per ml) lethal toxicity for Atlantic salmon by incubation with rabbit antitoxin antiserum

Treatment[a]	Dose injected (ml)	No. killed (n = 4)	Mean wt of fish ± SD
ECP-PMSF + PBS	0.05	4	4.4 ± 1.0
	0.10	4	4.5 ± 0.8
	0.20	4	7.3 ± 0.4
	0.40	4	8.1 ± 0.8
PBS + PMSF	0.10	0	6.7 ± 0.8
	0.20	0	7.6 ± 0.9
ECP-PMSF + RαCFA	0.10	4	3.9 ± 1.1
	0.20	4	4.5 ± 1.3
ECP-PMSF + RαGCAT/LPS	0.10	0	6.1 ± 1.2
	0.20	0	7.9 ± 1.1

[a] ECP was incubated with 25 mM PMSF (10:1 [vol/vol] and then with serum or PBS (10:1 [vol/vol]). RαCFA, Rabbit anti-complete Freund adjuvant antiserum; RαGCAT/LPS, rabbit antitoxin antiserum.

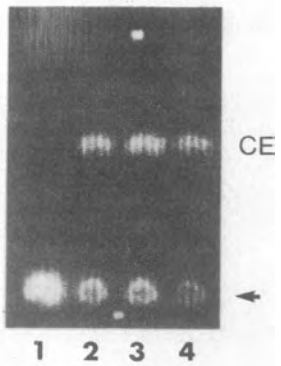

FIG. 11. Radiochromatogram of TLC separation of cholesteryl ester (CE) following incubation of egg yolk-[^{14}C]cholesterol with no enzyme (lane 1), ECP (lane 2), GCAT/LPS complex (lane 3), and free GCAT (lane 4). Arrow indicates sample origin.

FIG. 12. Native PAGE of free GCAT (96 ng of protein) (lane 1), recombined GCAT-LPS (96 ng of protein) (lane 2), GCAT/LPS complex (160 ng of protein) (lane 3), and ECP (6 μg of protein) (lane 4). Phastsystem native PAGE silver stain, 10 to 15% gradient Phastgel.

basis of specific GCAT units, the ECP protein contained 5% total GCAT.

Stability of free GCAT and GCAT/LPS to proteolytic inactivation. The effects of the *A. salmonicida* extracellular protease and proteinase K on the free GCAT and GCAT/LPS complex are shown in Tables 4 and 5. After 48 h of incubation with *A. salmonicida* protease, the hemolytic activity of the free GCAT had decreased by 75% compared with that of the control, in contrast to no decrease in GCAT/LPS activity. While the hemolytic activity of the free GCAT and GCAT/LPS resulted in partial lysis of erythrocytes (cell ghosts remained intact), mixing of the GCAT preparations with purified extracellular protease resulted in complete lysis (solubilization).

The effect of proteinase K on the hemolytic activity of free

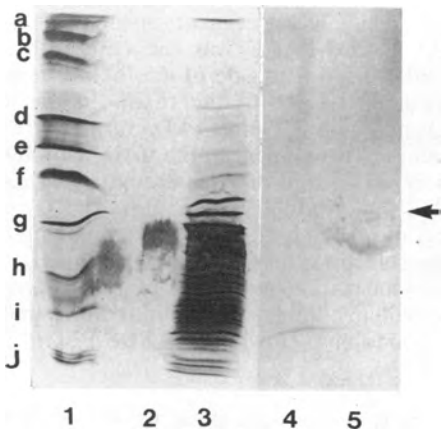

FIG. 13. IEF of pI marker proteins (lane 1), GCAT/LPS complex (160 ng of protein) (lane 2), ECP (6 μg of protein) (lane 3), free GCAT (96 ng of protein) (lane 4), and recombined GCAT-LPS (96 ng of protein) (lane 5). Phastgel IEF 3-9, Phastsystem IEF silver stain. Arrow indicates sample application point. a, Trypsinogen, pI 9.3; b, lentil lectin (basic), pI 8.65; c, lentil lectin (middle), pI 8.45; d, lentil lectin (acidic), pI 8.15; e, horse myoglobin (basic), pI 7.35; f, human carbonic anhydrase B, pI 6.55; g, bovine carbonic anhydrase B, pI 5.85; h, β-lactoglobulin A, pI 5.2; i, soybean trypsin inhibitor, pI 4.55; j, amyloglucosidase, pI 3.5.

TABLE 3. GCAT, hemolytic, and lethal toxic activity of *A. salmonicida* ECP, GCAT/LPS complex, free GCAT, and recombined GCAT-LPS

Sample	HU/μg of protein	GCAT units/μg of protein	Minimum lethal dose (μg of protein)/g of fish
ECP	21.8	2.4	0.260
GCAT/LPS	178.1	49.5	0.042
GCAT (free)	21.3	47.3	0.338
Recombined GCAT-LPS	85.3	NT[a]	0.079

[a] NT, Not tested.

GCAT was to reduce it by 87.5% in 30 min, with no effect on GCAT/LPS complex.

Heat stability of GCAT. The hemolytic activities of ECP, GCAT/LPS complex, free GCAT, and recombined GCAT-LPS following incubation at 60°C for various time periods are shown in Table 6. When hemolytic titers were determined after 2 h of incubation with erythrocytes, the free GCAT was completely inactivated after heating for 5 min while the recombined GCAT-LPS, GCAT/LPS, and ECP required 15, 30, and 30 min heating, respectively, for complete inactivation. When assays were prolonged for 20 h, heating the free GCAT for 15 min completely destroyed hemolytic activity, in contrast to the extremely high activities retained by GCAT/LPS, recombined GCAT-LPS, and ECP even after heating for 30 min. After heating at 60°C for 30 min, GCAT/LPS and ECP were not toxic in vivo with doses of 0.26 μg of protein per g of fish and 7.5 μg of protein per g of fish, which are 6- and 28-fold higher than their minimal lethal doses, respectively (Table 3).

Thermostability of the lecithinase activities of GCAT/LPS, free GCAT, and recombined GCAT-LPS following incubation at 60°C for various time periods is shown in Table 7. The lecithinase activity of free GCAT was completely inactivated after heating for 15 min, while recombined GCAT-LPS required 30 min. The lecithinase activity of GCAT/LPS was still not completely inactivated after heating for 30 min.

DISCUSSION

By ion-exchange FPLC, the extracellular lethal toxin of *A. salmonicida* was purified. The toxin was also extremely hemolytic for fish erythrocytes. Resolution was poor, but in one fraction of the eluted peak, the toxin was shown to contain a single protein which in SDS-PAGE (Phastgel) had a molecular mass of 25 kDa. This fraction, referred to hereafter as the toxin, was collected in a discontinuous and rapidly changing salt gradient of about 0.5 to 0.7 M. In a continuous salt gradient from 0 to 1 M, the 25-kDa protein eluted over the whole range without forming a peak (data not shown). In native PAGE, the toxin showed a single protein band with a very high molecular mass (>200 kDa), indicating that in ECP the toxin occurs as a high-molecular-mass complex. The toxin eluted in the void volume of an FPLC Superose 12 gel filtration column, which has an exclusion limit of 2,000 kDa, indicating an extremely high molecular mass for this toxin.

The toxin was identified as GCAT complexed with LPS (GCAT/LPS complex). The enzymatic characteristics of GCAT have been studied by others (5, 21, 22), who showed this enzyme to possess both phospholipase A_2 and acyltransferase activity. Initially, these workers considered the native

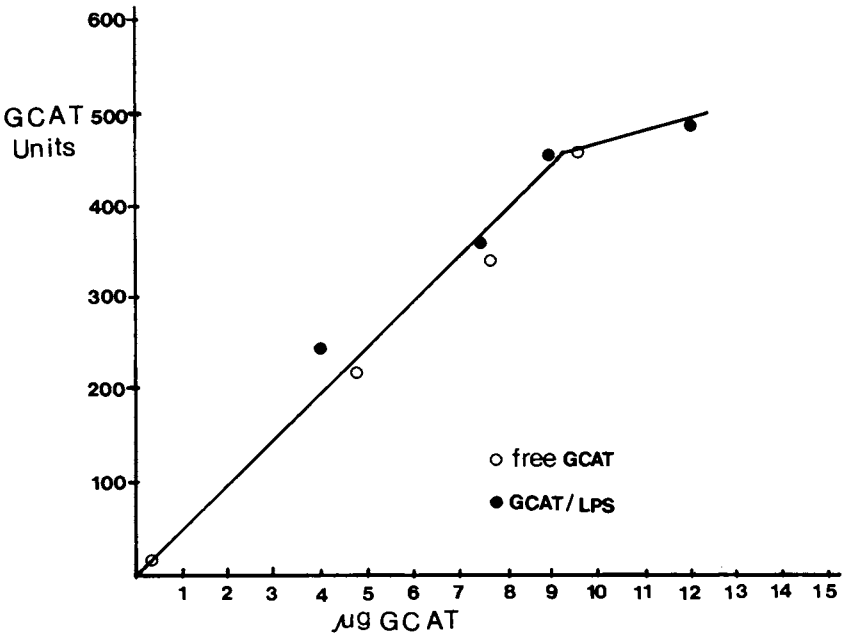

FIG. 14. GCAT activities of various concentrations of free GCAT and GCAT/LPS complex incubated with 5 ml of egg yolk and [^{14}C]cholesterol at 37°C for 1 h.

GCAT to be secreted in a form attached to membrane vesicles, but later (22) it was claimed that such complexes were artifacts resulting from the $(NH_4)_2SO_4$ precipitation step in concentrating the ECP. The present work used ECP produced by the cellophane overlay method without concentration. Assuming that this procedure had no effect on the native state of the GCAT, one may conclude that it is secreted in a very high M_W complex.

The toxin was shown by an LPS silver-staining method and Western blotting to contain large amounts of LPS. Evidence is presented which indicates that the toxin is a complex of GCAT with LPS and that this is the major form of the toxin in ECP, though some free GCAT is also present. In CIE analysis of ECP against rabbit antitoxin antiserum, two precipitin arcs were obtained. Both contained hemolytic and phospholipase activity, with a higher activity being in arc 1. In SDS-PAGE Western blot analysis of these arcs stained with rabbit antitoxin antiserum, the typical LPS pattern was obtained from arc 1 but not arc 2. The purified toxin had properties identical to those of arc 1 of ECP. Thus, the facts that GCAT and LPS present in the toxin had coeluted from both the ion-exchange and gel filtration columns and were coprecipitated in CIE by rabbit antitoxin antiserum indicate that these two moieties form a stable complex. This conclusion is further indicated by data from IEF (see below). Furthermore, when the rabbit antitoxin antiserum was depleted of antibodies to LPS by absorption with whole bacterial cells, leaving only antibodies to the GCAT protein, and used to develop CIE of ECP or purified toxin, neither the latter nor arc 1 of ECP was precipitated. Thus, the GCAT/LPS complex was precipitated only by the antibodies to LPS and not by anti-GCAT antibodies, which precipitated only arc 2 of the ECP. This arc, which lacked LPS, therefore indicates the presence of free GCAT in the ECP. The heterogeneous nature of the LPS may explain the failure to elute the GCAT in ion-exchange chromatography as a distinct peak in a continuous salt gradient and also to focus it in IEF.

A curious result in experiments using Western blotting from PAA4/30 SDS-PAGE was the ease with which the high-molecular-mass aggregate of the toxin was dissociated in concentrated samples of the toxin. In this case, two dominant bands with 27- and 54-kDa forms were obtained. Furthermore, after treatment of the toxin with SDS without boiling, there was a shift of the protein (GCAT) component from the 27-kDa form to a 30-kDa form. This is very similar to the effect of phenol extraction prior to boiling the GCAT in SDS reported by others (22). These workers also commented upon the marked tendency of the GCAT to aggregate with other proteins, lipids, and carbohydrates under a wide variety of conditions. However, in SDS-PAGE analysis of

TABLE 4. Hemolytic units of free GCAT and GCAT/LPS complex following incubation (48 h) with *A. salmonicida* protease

Sample treatment	HU at:	
	Time 0	48 h
Free GCAT + PBS	4,096	2,048
Free GCAT + protease	4,096	512
GCAT/LPS complex + protease	4,096	4,096
Protease	0	

TABLE 5. Hemolytic units of free GCAT and GCAT/LPS complex following incubation (30 min) with proteinase K

Sample treatment	HU at:	
	Time 0	30 min
Free GCAT + proteinase K	4,096	512
Free GCAT + PBS	4,096	4,096
GCAT/LPS + proteinase K	8,192	8,192
GCAT/LPS + PBS	8,192	8,192
Proteinase K	0	

TABLE 6. Thermostability of hemolytic activity of crude ECP, GCAT/LPS complex, free GCAT, and recombined GCAT-LPS incubated for various times at 60°C

Heating time (min)	Time (h) of endpoint determination[a]	HU in: ECP (1.5 mg/ml)	GCAT/LPS (92 μg/ml)	Free GCAT (24 μg/ml)	Recombined GCAT-LPS (24 μg of protein, 60 μg of lipids/ml)
0	2	2^{15}	2^{14}	2^{9}	2^{11}
5	2	2^{13}	2^{12}	0	2^{3}
	20	2^{17}	2^{15}	2^{4}	2^{12}
15	2	2^{8}	2^{7}	0	0
	20	2^{13}	2^{14}	0	2^{10}
30	2	0	0	0	0
	20	2^{11}	2^{14}	0	2^{9}

[a] Titers were determined after 2 and 20 h of incubation with erythrocytes.

the purified toxin with Phastgels, a single protein band with a 25-kDa molecular mass was consistently obtained.

The data reported here allow the unification of various activities of ECP reported by other workers. Cytotoxic activity on rainbow trout gonad type 2 cells has been previously reported and claimed to be the property of a glycoprotein (7). A leukocytolysin was purified and characterized as a glycoprotein with a molecular mass of over 100 kDa (12). Recently, a potent hemolytic toxin (salmolysin), with exactly the same 50% lethal dose as that reported here has been reported to be a glycoprotein (with 68% carbohydrates) with a molecular mass of over 200 kDa (24). All of these activities were expressed by the purified toxin obtained in the present work. The glycoprotein nature claimed by some authors may be explained by the aggregation of the GCAT with LPS. In the present study, the GCAT/LPS complex contained 65 mg of carbohydrate and 2.5 mg of total lipids per mg of protein. The carbohydrate content was much higher than that reported for the toxin by Nomura et al. (24), but this may be due to the different methods used in preparation of the ECP. The report of a high-molecular-mass hemolysin (24) contrasts with another claim that the hemolysin was a protein of 56 kDa (13). However, the latter is consistent with the present findings that in concentrated toxin, the dominant band in SDS-PAGE is a dimeric form of the GCAT (found here at 54 kDa).

The lethal toxicity of ECP and the toxin always correlated with the hemolytic activity. The specific hemolytic activity of the purified toxin was 26 times greater than that of the ECP from which it was purified. This is exactly the difference in minimum lethal doses, which was 1:26. On the basis of these data, the toxin constitutes 3.8% of the total ECP protein. However, this is variable among batches of ECP, with proportions of the toxin being up to 12% of total ECP protein, as based on specific hemolytic activities.

Previous workers have claimed that the extracellular protease of ECP is the major toxin (29) or pathogenic factor (28). Other work by the present authors has shown that when the protease in ECP is inhibited by PMSF, while there is a prolongation of the time to death, there is only a small increase in the minimum lethal dose (9). Thus, the protease is not a primary lethal toxin in ECP, but it does hasten the time to death. Further investigations have shown that purified protease is lethal in large doses, and studies with combinations of protease and GCAT/LPS complex have shown an additive relationship in lethality, with the GCAT/LPS being 55 times more lethal (in nanograms of protein per gram of fish) than the protease (19). Thus, the minimum lethal dose of ECP depends not only upon the absolute concentrations of the protease and GCAT/LPS but also upon their relative concentrations in the ECP. The present work has also demonstrated that the GCAT/LPS is the major lethal toxin in the ECP. Following the inhibition of protease activity by PMSF, the ECP was still highly toxic. However, this toxicity was specifically neutralized by rabbit antitoxin (GCAT/LPS) antiserum.

Hemolysis of trout erythrocytes by the purified GCAT/LPS complex was incomplete, i.e., the erythrocyte ghosts remained intact, in contrast to the complete solubilization of erythrocyte membranes by the ECP. This incomplete hemolysis is similar to the T_1 lysin activity described by others (31) which required the extracellular protease (present in the ECP) to cause complete lysis. In the present work, when the GCAT/LPS was mixed with purified *A. salmonicida* extracellular protease (70 kDa), complete lysis of the trout erythrocytes occurred, confirming similarity of GCAT/LPS with T_1 lysin activity.

The toxin possessed extremely high hemolytic activity for fish, but not rabbit, erythrocytes. The reason the GCAT/LPS was so selectively hemolytic is probably to be found in differences in the phospholipids of the erythrocyte membranes. The optimal substrate for the GCAT has been reported to be phosphatidylcholine substituted with unsaturated fatty acids (4). It is well established that fish tissues are much richer in polyunsaturated fatty acids than are those of mammals and that, furthermore, the proportion of phosphatidylcholine in the erythrocyte membranes of Atlantic salmon is 58.6% total phospholipids (20), compared with 29.5% in human erythrocytes (34). It would seem, therefore, that the enzymatic activity of the GCAT is well suited to digesting fish tissues. The LPS alone has no toxic effect in the fish, and heating the toxin to 60°C for 30 min destroyed its activity. Nevertheless, the role of the LPS in the high-molecular-mass GCAT/LPS complex was shown to be considerable.

While the major form of the toxin was shown to be GCAT/LPS complex of very high molecular-mass, the ECP also contained some GCAT in the form of a free monomeric protein. This was purified and shown to have a molecular mass of 30 kDa as determined by gel filtration and a molecular mass of 25 kDa in nongradient SDS-PAGE gels. Furthermore, experiments were performed to investigate the effect on various activities of free GCAT when the latter was recombined with LPS purified from ECP (recombined GCAT-LPS). In native Phastgel, GCAT/LPS and recombined GCAT-LPS did not migrate into the gel, while free GCAT produced a single fast-migrating protein band. These

TABLE 7. Thermostability of lecithinase activity[a] of GCAT/LPS, free GCAT, and recombined GCAT-LPS incubated for various times at 60°C

Heating time (min)	Lecithinase activity (%) of: GCAT/LPS	Free GCAT	Recombined GCAT-LPS
0	100.0	100.0	100.0
5	22.4	3.5	6.3
15	6.0	0.0	2.8
30	2.5	0.0	0.0

[a] The enzyme activity of each treatment was calculated from a standard curve described in Materials and Methods and expressed as a percentage of the unheated sample.

results indicate that a high-molecular-mass complex is formed when free GCAT and LPS are recombined. In IEF gels, the recombined GCAT-LPS focused heterogeneously, similar to GCAT/LPS, while free GCAT produced a single protein band focusing at pI 4.3. Furthermore, the heat stability of GCAT was shown to be greatly enhanced when recombined with LPS. These results indicate that free GCAT and LPS can recombine to form a complex with physicochemical properties similar to those of the GCAT/LPS in the ECP. Various other activities of free GCAT were compared with those of the GCAT/LPS and recombined GCAT-LPS complexes. The free GCAT was antigenically identical with the GCAT complexed with LPS, since the former was stained in Western blots by rabbit antitoxin (GCAT/LPS) antiserum. While the specific enzyme activity on egg yolk substrate or phosphatidylcholine was the same for free GCAT, GCAT/LPS, and recombined GCAT-LPS, the last two possessed eight and four times, respectively, the hemolytic and lethal activity of the other. The minimum lethal dose of the free GCAT was higher than that of the crude ECP, indicating that this form of the enzyme contributes very little to the toxicity. The reasons the recombined GCAT-LPS was slightly less hemolytic and toxic compared with GCAT/LPS complex may be some slight change in the LPS during phenol extraction or some feature in the conditions affecting recombination. Nevertheless, it is quite clear that recombination of GCAT with LPS results in enhanced hemolytic and lethal activities as well as protection against heat inactivation. The LPS component of the toxin is not intrinsically toxic or hemolytic, since heating to 60°C for 30 min destroyed these activities. However, hemolytic activity of the heated GCAT/LPS or ECP was still detected after prolonged (20-h) incubation with erythrocytes, indicating that not all of the activity of GCAT when complexed with LPS is destroyed by heating.

The lack of toxicity in salmonid fish of *A. salmonicida* LPS (up to 0.714 mg/g of fish) has been reported by others (25, 35). This is more than 2,900-fold higher than the LPS content in terms of total lipids of the minimum lethal dose of the GCAT/LPS. It is well known that LPS has an affinity for eucaryotic cell membranes and that this affinity is inhibited by phospholipids and cholesterol (17). Studies with phospholipid monolayers have shown that LPS can penetrate such layers most readily when they are composed of phospholipids with unsaturated fatty acids (17). Such compounds are common in the cell membranes of salmonids (20) and are the preferred substrates for the GCAT (4). Hence, the mechanism whereby the hemolytic activity of GCAT is enhanced by complexing with LPS may be to aid the enzyme to penetrate the cell membrane and target the GCAT into the host cell membranes exactly where the optimal substrates for the GCAT are present. Certain other bacterial hemolysins, e.g., the cell-bound hemolysin of *Serratia marcescens* (26) and the alpha-hemolysin of *Escherichia coli* (1, 2), or lipase of *Pseudomonas aeruginosa* (30) also exist as complexes with LPS, though the role, if any, of the LPS has not been demonstrated.

The hemolytic activity of the GCAT/LPS was resistant to inactivation by proteinase K, while the free GCAT was rapidly inactivated. The effect of the *A. salmonicida* extracellular protease was similar, but inactivation of free GCAT was much slower. This finding is similar to the reported protease resistance of salmolysin, which was resistant to papain and pepsin (24). This property may have significance in vivo during inflammatory responses by protecting the toxin from inactivation by host (e.g., leukocyte)-derived proteases and may, in part, explain the greater in vivo toxicity of the LPS-complexed GCAT. The mechanism of protection of the GCAT from proteolytic attack by the LPS may be simply by steric hindrance similar to the interference with binding of anti-GCAT antibodies. Thus, the GCAT/LPS seems to be well adapted to act as a toxin in salmonid fish.

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