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Extracellular Antigens from *Listeria monocytogenes*

I. Purification and Resolution of Hemolytic and Lipolytic Antigens from Culture Filtrates of *Listeria monocytogenes*

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Two antigens were purified from culture filtrates of *Listeria monocytogenes* 7973 by the following procedure: (i) acid precipitation with 4 N HCl at *p*H 3.7, (ii) Sephadex G-75 column fractionation, (iii) diethylaminoethyl-Sephadex A50 batchwise adsorption, and (iv) rechromatography on Sephadex G-75. This procedure resulted in the resolution of two distinct antigens. One antigen, designated a hemolytic antigen because of its ability to lyse erythrocytes from a variety of species, had a specific activity of 25,000 units/mg of protein and an estimated molecular weight of at least 171,000. The other antigen, designated a lipolytic antigen because of its ability to hydrolyze egg yolk saline substrate, had a specific activity of 400 units/mg of protein and an estimated molecular weight of 52,500.

Listeria monocytogenes produces a soluble hemolysin on solid and in liquid media. Previous studies established that the hemolysin was a protein, migrating electrophoretically as gamma globulin and highly antigenic in rabbits (6, 16). In addition to investigations on the hemolytic nature of the hemolysin, several workers reported that the hemolytic character of listerial strains correlated well with the ability of the same strains to produce opacity in egg yolk-agar (5, 6, 8, 9, 13, 19). Since attempts to separate the seemingly dual biological activity expressed by the hemolysin were unsuccessful, the hemolytic and lipolytic activities were ascribed to the same protein.

In an effort to establish a relationship between hemolytic-lipolytic activity and the mechanism(s) of pathogenicity in listeriosis, further attempts were made to isolate these activities in relatively pure form. The dual biological activity that was earlier reported to be the expression of a single hemolytic antigen (10) is herein described as the result of two antigens with distinct properties. The purpose of this communication is to describe the purification and characterization of the hemolytic and lipolytic antigens from culture filtrates of *L. monocytogenes*.

MATERIALS AND METHODS

Measurement of biological activity. Hemolytic activity was measured by the method previously reported from this laboratory (16). Lipolytic activity was measured by the method of O'Leary and Weld (17) with egg yolk saline as a substrate. The detection and assay of an erythrocyte-sensitizing substance (ESS) followed the procedure outlined in detail by Moskowitz (15).

Chemical tests. Protein concentration was determined by the method of Lowry et al. (12) with bovine serum albumin as a standard. The nucleoprotein concentration was estimated at various stages in the purification procedures by the diphenylamine (2) and orcinol (3) procedures. Hexose was determined by the anthrone procedure (11) at 625 nm.

Specific activity of the hemolytic and lipolytic antigens. The determination of hemolytic antigen specific activity was by the method described by Maheswaran and associates (14). The number of hemolytic units contained in a 1.0-ml sample was divided by the milligrams of protein present in 1.0 ml of the same sample. The specific activity units of the hemolytic antigen were expressed as units per milligram of protein. The specific activity of the lipolytic antigen was determined by a modification of the turbidimetric method reported by O'Leary and Weld (17). Egg yolk supernatant fluid suspensions were made containing 4.0 mg of total lipids per ml in 3% saline solution. Reaction mixtures consisted of 1 ml of egg yolk supernatant fluid, lipolytic antigen, and 0.002 M sodium barbital buffer (*p*H 6.5) in a total volume of 3 ml. These mixtures were incubated in a water bath at 37 C with the increase in turbidity (due to insoluble free fatty acids released by substrate hydrolysis) followed in a Spectronic-20 spectrophotometer at 625 nm. One unit of lipolytic activity was defined as the amount required to increase the optical density under these conditions by 1.00 after 16 hr of incubation at 37 C. Specific activity units were determined by dividing the reciprocal of the highest dilution of lipolytic activity with an optical density of at least 1.00 at 625 nm by the milligrams of protein in the undiluted sample.

Procedure for the purification of hemolytic and lipolytic antigens. *L. monocytogenes* strain 7973 was used as the source of crude hemolysin. For routine production of the crude filtrate, 1.5 liters of Trypticase soy broth (BBL) was inoculated with 10 ml of an 18-hr broth culture of the organism and incubated in stationary culture for 21 hr at 37 C. The supernatant fluid was harvested in a Sorvall continuous-flow centrifuge at 4 C.

Step 1: acid precipitation. The culture filtrate was adjusted to *p*H 3.7 with 4 N HCl and allowed to stand at 4 C overnight. A black-brown precipitate was recovered by centrifugation at 7,500 × *g*. The precipitate was collected in 10 ml of demineralized water and adjusted to *p*H 10.5 with 4 N NaOH to dissolve the active component; the *p*H of the solution was finally adjusted to 6.8 with 4 N HCl.

Step 2: Sephadex G-75 column fractionation. Sephadex G-75 (particle size 40 to 120 μm; Pharmacia, Uppsala, Sweden) was suspended in water for 2 days. The fine particles were removed with the supernatant fluid, and the slurry was washed several times with 0.025 M phosphate buffer (*p*H 6.8). The washed gel was packed in a column (2.4 by 75 cm) by gravity at 4 C with the uniformity of packing determined by the use of 0.1% blue dextran 2000 (Pharmacia) in the same buffer. The void volume was 100 ml as determined by measuring the volume of liquid required to elute the blue dextran. The eluate containing the peak concentration of blue dextran, measured at 630 nm, was the last eluate fraction used in calculation. A 10-ml sample containing 1.6 mg of protein/ml was subjected to molecular sieving with 0.025 M phosphate buffer (*p*H 6.8) as the mobile phase at 4 C. Eluates were collected in 10-ml fractions and examined for hemolytic and lipolytic activity. The most active fractions were pooled.

Step 3: DEAE-Sephadex batchwise separation. Diethylaminoethyl (DEAE)-Sephadex A50 (Pharmacia) with an exchange capacity of 3.5 ± 0.5 meq/g was used. The DEAE-Sephadex was prepared by allowing the material to swell at room temperature in an excess of 0.025 M phosphate buffer (*p*H 6.8) for 1 day. The fines were removed with the supernatant fluid, and the slurry was washed several times in the same buffer. The pooled eluates, from step 2, were added to the slurry of DEAE-Sephadex A50. After constant stirring for 30 min at 4 C, the slurry was filtered as dry as possible by vacuum suction and rinsed with several portions of phosphate buffer. The recovered eluate was concentrated to approximately 0.1 of its original volume by Carbowax 4000 (Union Carbide Co.).

Step 4: rechromatography by Sephadex G-75 filtration. A 10-ml sample, from step 3, was rechromatographed on the same Sephadex G-75 column at 4 C. The flow rate was adjusted to 45 ml/hr, and 10-ml fractions (150 drops) were collected. Each fraction was used for hemolytic, lipolytic, and erythrocyte-sensitizing assays and for the determination of the protein, nucleoprotein, and carbohydrate content. The fractions were stored at −28 C for several months without loss of activity.

Preparation of antisera. Adult New Zealand white rabbits were immunized subcutaneously with crude acid-precipitated culture filtrate in incomplete Freund's adjuvant (Difco) by the method described by Freund (4). The recovered antiserum, hereafter referred to as anti-crude acid filtrate serum, was stored at −28 C. Four rabbits were immunized biweekly for 4 weeks with the hemolytic antigen (500 specific activity units/ml), and four rabbits were immunized with the lipolytic antigen (400 specific activity units/ml). The rabbits were given 1-ml injections of the antigens mixed with an equal volume of Freund's incomplete adjuvant. After the last injection, the rabbits were rested for 2 weeks and then bled via cardiac puncture. All antisera were heat-inactivated at 56 C for 30 min and adsorbed with sheep erythrocytes. Specific adsorption of antisera by acetone-dried *Listeria* whole cells (7973) was accomplished by adding 20 mg of cells to each milliliter of serum. The serum-cell mixture was incubated at 37 C for 2 hr and then overnight at 4 C.

Serological inhibition reaction. Hemolytic inhibition was assessed quantitatively by determining the highest dilution of antiserum that prevented the lysis of a 1% suspension of sheep erythrocytes in the presence of 500 specific activity units of hemolytic antigen. After mixing, the suspension was incubated at 37 C until the cells in the erythrocyte control tube (500 units of hemolytic antigen and undiluted normal serum) lysed. The lipolytic inhibition test employed a series of dilutions of serum ranging from 1:4 to 1:1,024 in 1-ml volumes of 0.002 M barbitol buffer (*p*H 6.5). One milliliter of 300 specific activity units of lipolytic antigen was added to each tube in the test series. The tubes were incubated in a water bath at 37 C for 30 min. After this period, 1.0 ml of substrate (egg yolk saline) was added to each tube. After the completion of the second incubation (37 C overnight), hydrolysis of the egg yolk saline was followed photometrically. Control tubes contained (i) buffered substrate, lipolytic antigen, and normal serum and (ii) buffered substrate alone.

Immunodiffusion. The method of Ouchterlony (18) was slightly modified by using barbitone acetate buffer (0.05 M, *p*H 8.6) and 1% Panagar obtained from Colab Laboratories, Chicago Heights, Ill.

Molecular weight determination by column chromatography. The molecular weights of the hemolytic and lipolytic antigens were determined by the gel filtration technique described by Andrews (1). A column (2.4 by 75 cm) of Sephadex G-75 was prepared and equilibrated in a manner previously mentioned. Samples (5 ml) of hemolytic antigen (treated with a 10^{-2} M concentration of cysteine) and lipolytic antigen (heat-treated at 60 C for 30 min) containing a specific activity of 25,600 and 400 units, respectively, were added to the top of the column and washed through with 0.025 M phosphate buffer (*p*H 6.8) at a flow rate of 45 ml/hr. Fractions of 150 drops (10-ml volume) were collected. The molecular weight was determined by comparing the elution volume of the antigens with those of several proteins of known molecular weight. Reference proteins, cytochrome *c* (molecular weight, 13,000), gamma globulin

TABLE 1. *Purification of hemolytic and lipolytic antigens*

Fractionation procedure	Protein (mg/ml)	Hemolytic activity			Lipolytic activity		
		Units/ml	Specific activity[a]	Purification (-fold)	Units/ml	Specific activity	Purification (-fold)
Cell-free supernatant	2.40	128	53	1	64	27	1
Acid-precipitated filtrate	1.60	4,096	2,560	48	128	80	3
First gel filtration							
Sephadex G75 fraction	0.27	1,024	3,793	72	64	237	9
Final gel filtration							
Hemolytic-active fraction	0.04	1,024	25,600	483			
Lipolytic-active fraction	0.02				8	400	15

[a] Units divided by protein (mg/ml).

(bovine fraction II; molecular weight, 171,000), and pepsin (three times crystallized; molecular weight, 35,500), were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

RESULTS

Purification and separation of hemolytic and lipolytic antigens. The *L. monocytogenes* (7973) culture filtrate was purified and resolved into two distinct antigens by (i) precipitation with 4 N HCl, (ii) Sephadex G-75 column fractionation, (iii) DEAE-Sephadex A50 batchwise separation, and (iv) rechromatography on the same Sephadex G-75 column.

The purification procedure is summarized in Table 1. Acid precipitation of the cell-free supernatant in the recovery of hemolytic and lipolytic antigens is indicated by an increase in specific activity of 48- and 3-fold, respectively. On Sephadex G-75, the hemolytic and lipolytic-active components moved with the solvent front and did not require gradient elution. Figure 1 illustrates a typical elution pattern of hemolytic activity and the protein and carbohydrate content when passed initially through a column of Sephadex G-75. One peak of hemolytic activity, several carbohydrate peaks, and two major protein peaks were observed. The hemolytic activity emerged from the column simultaneously with or immediately after a high-molecular-weight protein of the void volume. Lipolytic activity was closely associated with and parallel to the hemolytic activity. Ribonucleoprotein and the ESS were also observed in every tube within the first major protein peak. The second major protein peak tubes 25 to 32) did not contain hemolytic activity. Figure 2 represents the elution pattern after ion-exchange batch separation, concentration, and rechromatography through Sephadex G-75. The purification procedures employed were effective in the removal of the ESS and in the reduction of carbohydrate and ribonucleic acid (RNA) content. The cell-free filtrate, crude acid-precipitated

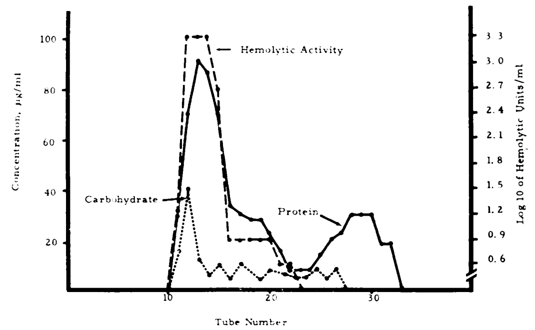

FIG. 1. *Fractionation of crude acid-precipitated filtrate of Listeria monocytogenes (7973) supernatant fluid on a Sephadex G75 column. Buffer, 0.025 M phosphate (pH 6.8); flow rate, 45 ml/hr; column size, 2.4 by 75 cm; tube volume, 10 ml; void volume, 100 ml.*

filtrate, and the hemolytic-active fraction recovered from the first gel filtration on Sephadex G-75 (Table 1) possessed titers of 1:512, 1:2,048, and 1:256, respectively. No ESS titer was detected after DEAE-Sephadex batchwise adsorption. These results are supported by previous work carried out in this laboratory (10). The employment of a DEAE-Sephadex column gave comparable results. Occasionally the hemolytic and lipolytic antigens separated slightly, but generally both antigens emerged from the column simultaneously.

The hemolytic activity was directly associated with the level or concentration of protein, possessed a specific activity of 25,600, and represented a 483-fold purification of the crude extract. A similar association between the protein concentration and lipolytic activity was not evident (Fig. 2). The lipolytic preparation had a specific activity of 400 and represented a 15-fold purification of the crude extract. Chemically the hemolytic and lipolytic activities differed in protein-carbohydrate-RNA content in that the lipolytic activity by comparison had a lower protein con-

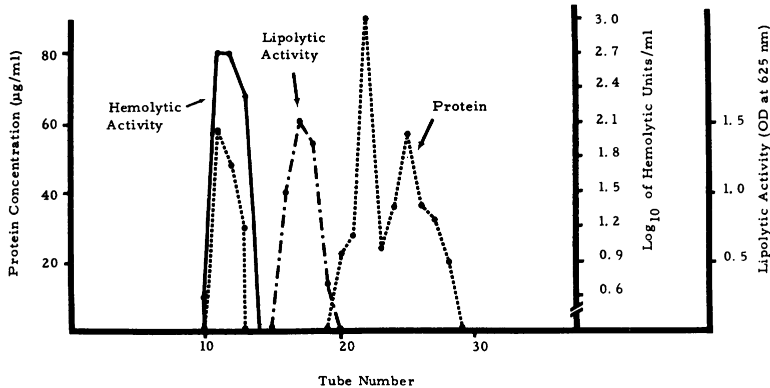

FIG. 2. *Resolution of hemolytic and lipolytic antigens by rechromatography on Sephadex G75 after ion-exchange batch adsorption of the hemolytic-active fraction in Fig. 1.*

TABLE 2. *Chemical analyses of the purified hemolytic and lipolytic antigens obtained from Listeria monocytogenes 7973*

Fraction	Protein (%)	Hexose (%)	Ribonucleic acid (%)
Hemolytic antigen....	91	4.5	4.5
Lipolytic antigen.....	25	43.7	31.3

centration but a higher concentration of total carbohydrate (Table 2). Deoxyribonucleic acid was not detected in any preparation.

Serological studies indicated that an imperfect separation of hemolytic from lipolytic antigens occurred and was most likely due to some overlap of the trailing and leading edges between antigens. Antisera prepared against the crude acid-precipitated filtrate and the resolved hemolytic and lipolytic antigens possessed both antihemolytic and antilipolytic activity (Table 3). The adsorption of these antisera with acetone-dried whole cells quantitatively removed antilipolytic antibodies with minimal or no loss of antihemolytic antibodies, indicating that the lipolytic antigen is predominately unassociated and distinct from the hemolytic antigen. To achieve the complete separation of the hemolytic and lipolytic antigens, investigations on the effect of heat and stability in the presence of cysteine added data to the accumulated evidence for nonidentity of these antigens. The serological and biological activities of the hemolytic antigen, irrespective of specific activity units, were destroyed when held at 60 C for 30 min. The lipolytic antigen remained unaffected under the same conditions. Treatment with a 10^{-2} M concentration of cysteine destroyed the serological and biological activities of the lipolytic antigen, whereas the biological activities of the hemolytic antigen, under these same conditions, were enhanced. This suggests that the

TABLE 3. *Effect of adsorption by Listeria whole cells on hemolytic- and lipolytic-inhibiting titers*

Serum tested	Hemolytic-inhibiting titer (log 10)		Lipolytic-inhibiting titer (log 10)	
	Unadsorbed	Adsorbed	Unadsorbed	Adsorbed
Anti-crude acid filtrate serum.......	2.5	2.5	1.6	<1.0
Antihemolytic serum.......	3.1	3.1	1.3	0
Antilipolytic serum.......	1.9	1.6	2.2	0

activities of the hemolytic antigen may parallel those of streptolysin o (7). As a final step, we are now treating the hemolytic antigen with 10^{-2} M cysteine which abolishes lipolytic activity. The lipolytic antigen is being subjected to heat treatment at 60 C for 30 min to destroy hemolytic activity.

Molecular weight. Since the hemolytic-active fraction emerged from the column simultaneously or immediately after the void volume in a single peak (Fig. 2) and was similar to the elution pattern of the gamma globulin standard, a molecular weight of at least 171,000 was assigned. Lipolytic activity was not observed until the 16 to 19 fraction, indicating a molecular weight of 52,000 (Fig. 3).

The results of immunodiffusion analyses may be summarized as follows. (i) A comparison of diffusion patterns before and after the initial Sephadex G-75 fractionation of crude acid-precipitated filtrate revealed a reduction from several precipitin lines to only one prominant band formed against the crude acid filtrate antiserum. This observation suggested that gel filtra-

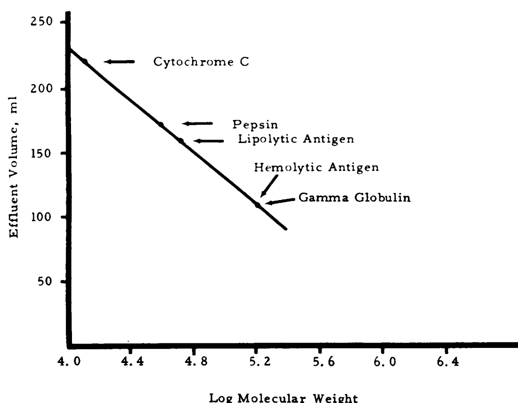

FIG. 3. *Determination of the molecular weights of the hemolytic and lipolytic antigens by gel filtration on a Sephadex G-75 column (2.4 by 75 cm) calibrated with chemicals of known molecular weight.*

tion was an effective means in the removal of extraneous antigenic impurities. (ii) After batchwise adsorption with DEAE-Sephadex A50 and rechromatography on Sephadex G-75, the resolved hemolytic and lipolytic antigens were placed in adjacent wells and allowed to diffuse toward a center well containing either hemolytic or lipolytic antiserum. No differences were observed with respect to the immunodiffusion patterns. A faint line of precipitation from each antigen well eventually joined in an apparent reaction of identity. (iii) Heat treatment (60 C for 30 min) of the lipolytic antigen and treatment of the hemolytic antigen with 10^{-2} M cysteine significantly affected the immunodiffusion patterns. A reaction of nonidentity was observed when either hemolytic or lipolytic antiserum was allowed to react against the treated antigens. This observation indicated that the determinant groups of the hemolytic and lipolytic antigens were clearly not identical and that the antisera must have contained both hemolytic and lipolytic antibodies formed against incompletely resolved antigens. (iv) When adsorbed hemolytic and lipolytic antisera were allowed to react against the heat-treated lipolytic antigen, no precipitin line was formed; however, a weak precipitin line was formed against the cysteine-treated hemolytic antigen.

DISCUSSION

The four-step procedure described herein resulted in the separation of two biologically active antigens in a more highly purified state than any method reported to date. A hemolytic antigen, tentatively designated because of its ability to lyse erythrocytes from a variety of species, had a specific activity of 25,600 units/mg of protein. The molecular weight of the hemolytic antigen as estimated by gel filtration on Sephadex G-75 was at least 171,000. The lipolytic antigen, tentatively designated because of its ability to hydrolyze the egg yolk saline substrate, had a specific activity of 400 units/mg of protein. The molecular weight was estimated to be approximately 52,500.

Rechromatography was necessary to resolve the hemolytic and lipolytic antigens. The reasons for separation by this means are not readily available. It is possible that the erythrocyte-sensitizing substance, hexose, and RNA constituents (before DEAE-Sephadex adsorption) represented interfering materials that initially masked antigen resolution and the subsequent removal of these materials enhanced separation Further, it is realized that the chromatographic separation presented is preliminary to more complete antigen resolution studies by techniques such as polyacrylamide gel electrophoresis.

An earlier communication from this laboratory (9) described an ammonium sulfate-calcium phosphate gel purification procedure; however, it was later found that this procedure yielded a final product that was heterogenous with respect to purity when subjected to immunoelectrophoresis and disc polyacrylamide gel electrophoresis (*unpublished data*). This was due largely to two factors: (i) the inability to separate the two antigens by the ammonium sulfate-calcium phosphate gel procedure and (ii) the reduced biological activity as a consequence of the prolonged dialyzation for the removal of ammonium sulfate ions in the initial purification steps.

Although there was an overlapping of activity as indicated by serological inhibition studies, the possession of distinct activity is without question. The adsorption of antisera by whole *Listeria* cells specifically removed lipolytic-inhibiting antibodies and suggests that the lipolytic-active component is a surface antigen (Table 3). The serological cross-reaction also provided insight into the purification procedure. Data indicated the incomplete separation of the hemolytic and lipolytic antigens by gel filtration techniques. Additional purification procedures, i.e., cysteine treatment of the hemolytic preparation and heating of the lipolytic preparation at 60 C for 30 min, resulted in two relatively purified fractions. Heat treatment of a portion of the fraction recovered from the ion-exchange process did not alter the mobility of the lipolytic antigen through Sephadex G-75. No changes in the mobility of the hemolytic antigen treated with cysteine were observed. Hence, it is felt that the molecular weights of these antigens were not seriously affected by these treatments.

Earlier, we reported that, in general, only

virulent strains have consistently expressed hemolytic and lipolytic activity. Avirulent strains and most nonhemolysin-producing strains have shown either diminished or no lipolytic activity (8). Investigations are now underway to critically assess the role of these two antigens in the pathogenesis of listeriosis.

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