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PURIFICATION OF THE SOLUBLE HEMOLYSINS OF *LISTERIA MONOCYTOGENES*

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Abstract

Jenkins, E. M. (Tuskegee Institute, Tuskegee, Ala.), A. N. Njoku-Obi, and E. W. Adams. Purification of the soluble hemolysins of *Listeria monocytogenes*. J. Bacteriol. **88**:418–424. 1964.—A method is described for obtaining relatively purified hemolysin preparations from both virulent and avirulent strains of *Listeria monocytogenes*. These hemolysins are protein in nature as shown by heat lability, nondialyzable properties, precipitation with trichloroacetic acid, and electrophoretic mobility. The hemolysins are antigenic in rabbits as shown by serum neutralization tests. The potency of the purified hemolysin was markedly increased by cysteine, sodium hydrosulfite, and a number of reducing agents. Many of the actions of the purified hemolysin seemed to parallel that of streptolysin O, and certain of these activities could be explained by the "thioldisulfide hypothesis."

It has been known for a long time that *Listeria monocytogenes* produces clear hemolysis on blood-agar plates and that this hemolysin is soluble and filterable. Apart from papers by Njoku-Obi and Jenkins (1962), Girard, Sbarra, and Bardawil (1963), and Njoku-Obi et al. (1963), very little information is available regarding the nature, identity, and role of this hemolysin in listeria infections. In our search for toxins formed by this organism, 157 strains were studied. These strains were obtained from all over the world, including Japan, Eastern and Western Europe, Australia, and South America. Of these strains, 10% produced no detectable hemolysins, whereas 6% produced very high-titered hemolysins in the range of 1:1,024 to 1:4,096 (Njoku-Obi and Jenkins, 1962).

We sought to obtain a suitable method of purification of these hemolysins recovered from certain high-producing strains, and attempted a partial chemical characterization of this hemolysin fraction. Herbert and Todd (1941) successfully purified and characterized the hemolysin of group A streptococci (streptolysin O), and suggested that all streptolysins were similar if not identical both chemically and immunologically.

The approach selected to characterize the hemolysin of *L. monocytogenes* was guided by the belief that this hemolysin might conceivably share some of the chemical and immunological properties of streptolysin O.

Materials and Methods

The *L. monocytogenes* cultures used in this study were both virulent and avirulent strains which consistently produced hemolysins of high titers. Virulent strain 9-125 was obtained from K. F. Girard, Boston, Mass.; virulent strain 1122-3 was received through the courtesy of M. L. Gray, Bozeman, Mont. The avirulent strains 7647 and 7648 were provided by J. W. Osebald, Davis, Calif.

The strains of streptococcus group A were obtained from the American Type Culture Collection. These strains proved to be mediocre hemolysin producers. The purified hemolysin recovered from these strains was in the range of 256 to 512 hemolytic units (HU)/ml.

The soluble hemolysins from *L. monocytogenes* were obtained from either Brain Heart Infusion broth culture supernatant fluids or saline extracts of Brain Heart Infusion Agar cultures. However, in this study the organism was grown in Trypticase Soy Broth (BBL). Trypticase Soy Broth was dispensed in 50-ml portions in screw-capped tubes (60 ml capacity) and sterilized by autoclaving. The standard inoculum used was 2.5 ml of a culture grown at 37 C for 24 hr. After incubation the crude hemolysin was obtained by centrifugation (Servall super-speed centrifuge, type SS-34) of the cultures at 7,500 × *g* for 45 min at 0 C. The supernatant fluid was collected and stored at 10 C.

The method of purification was a modification

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of a procedure described by Herbert and Todd (1941) for the purification of streptolysin O. The procedure involved fractionation with $(NH_4)_2SO_4$ followed by adsorption and elution from calcium phosphate gel. Details of a typical preparation are described below.

Purification of hemolysin. The starting material was 1,000 ml of the crude hemolysin recovered by centrifugation of cultures. The titer of this crude preparation was usually in the range of 512 to 8,196 HU/ml. Sterile, filtered hemolysin (1,000 ml) was treated with crystalline ammonium sulfate to 60% saturation and stored overnight at 10 C. After 12 to 15 hr, a heavy brown precipitate was observed. This precipitate was recovered by centrifugation in the cold at 7,500 × *g* for 30 min. The supernatant fluid, which showed very little hemolytic activity, was discarded. The brown precipitate was placed in 0.15 M sodium phosphate buffer (pH 7.0), and was eluted five times with 10-ml portions of this same buffer. The elutions were carried out under centrifugation until immediate hemolysis at room temperature was no longer observed. The final eluate was discarded along with the pale-gray precipitate. The above solution was then dialyzed against six changes of distilled water containing Merthiolate at a concentration of $1{:}10^6$. When the solution was free of ammonium sulfate, as determined by the addition of barium chloride, 12 ml of a 0.2 M phosphate buffer (pH 7.2) were added to the ammonium sulfate-free solution. To the ammonium sulfate-free solution, 4 ml of a 1 M solution of calcium acetate were added. After standing at room temperature for 30 min, this mixture was centrifuged at 7,500 × *g* for 30 min. The supernatant fluid was discarded, and the precipitate was eluted three times with 10-ml portions of a 25% solution of saturated ammonium sulfate. This mixture was then centrifuged at 7,500 × *g* for 20 min, and the supernatant fluid was collected and dialyzed against several changes of distilled water to which Merthiolate ($1{:}10^6$) had been added. To the dialysate were added 6 ml of a 0.2 M phosphate buffer solution (pH 7.2) and 3 ml of a 1 M solution of calcium acetate. After standing for 30 min at room temperature, this preparation was eluted three times with 5-ml portions of a 25% solution of saturated ammonium sulfate. Finally, the eluted solution was exhaustively dialyzed against several changes of the distilled water-Merthiolate solution until $BaCl_2$ tests were negative for ammonium sulfate. The final yield was usually 12 to 14 ml of the purified hemolysin having a titer of 32 to 128 HU/ml, depending upon the *Listeria* strain used. The hemolysin preparation was either stored at −28 C or lyophilized immediately until ready for use.

Effect of oxidizing and reducing agents. The effects of various reducing and oxidizing agents on the hemolysin preparations were determined as follows. Lyophilized preparations were reconstituted with streptolysin O buffer (Cappell Laboratories, Westchester, Pa.), and the frozen preparations were thawed slowly at 10 C. Equal volumes of the hemolysin and the reducing or oxidizing agent were permitted to react for 10 min at room temperature; then this mixture was titrated in 0.5 ml of streptolysin O buffer in twofold dilutions. Sheep red cells were washed and stored in streptolysin buffer. To perform the test, 0.5 ml of these cells (1%, v/v) was added to the hemolysin dilutions to make a final volume of 1 ml. All solutions of oxidizing and reducing agents were brought to a neutral pH before use. Initial readings were made after 2 hr of incubation at 37 C, and the final readings were made after overnight incubation at 4 C. The reciprocal of the tube showing complete hemolysis was used as the end point of hemolytic activity.

Production of antihemolysin sera. Antihemolysin serum was prepared in New Zealand white rabbits by using the purified hemolysin from strains 1122-3 and 9-125 in an alum-precipitated vaccine. The frozen or lyophilized hemolysin preparations, or both, were passed through a 45-mμ Millipore filter membrane (type HA) by using positive pressure. A porcelain glass-type filter was used. The only impediment to flow was the filter membrane; no prefilter or filter-paper base was employed. The filtrate was tested for sterility in Trypticase Soy or thioglycolate broth and divided into two equal volumes. One portion was adsorbed with 60% aluminum hydroxide, permitted to stand at room temperature for 1 hr, and then centrifuged at 2,000 × *g* for 30 min. The alum gel was reconstituted to a 25% suspension with sterile saline and stored at 4 C until ready for use. The second portion of the hemolysin filtrate was also stored at 4 C.

Normal sera were obtained before immunization procedures were begun. The rabbits were inoculated subcutaneously with 2 ml of the alum preparation five times on alternate days; 6 days

after the fifth injection the rabbits were trial-bled and the antibody titers were found to be 1:1,000. The immunization procedures were continued, but with daily intravenous injections of 2 ml of the purified hemolysin for a period of 5 days. At the end of the second immunization procedure, the antibody titers were 1:10,000. Antibody titers were determined in the following manner. The antigen was diluted in tenfold dilutions but the serum was undiluted. To perform the tests, 0.1 ml of serum was placed in capillary tubes and overlaid with 0.1 ml of diluted antigen. A positive reaction was indicated by ring formation at the serum-antigen interphase; 6 days after the final immunization injection the rabbits were bled by cardiac puncture, and the sera were preserved in Merthiolate and stored at −28 C until ready for use.

RESULTS

In the inactivated state, the purified hemolysins from both the virulent and avirulent strains of *L. monocytogenes* showed a slightly higher titer than did streptolysin O. However, like the streptolysin O, the hemolytic activity was markedly increased in the presence of reducing agents. Cysteine hydrochloride yielded much higher titers than did thiolactic acid or sodium hydrosulfite.

Properties of the purified hemolysin. The precipitated fraction on drying was tan in color and readily soluble in distilled water or dilute salt solutions. The exact solubility has not been determined but 10% solutions were obtained. Concentrated solutions were usually clear, but occasionally the color was a dull white or light tan. The hemolysin was easily precipitated with trichloroacetic acid, concentrated solutions of ammonium sulfate, alcohol, and acetone. These solutions were nondialyzable.

The hemolysin coagulated when boiled or when placed in 0.5% formalin solution. Concentrated solutions were stable for 15 weeks at 0 C, after which time an appreciable part of the activity was lost. Weak solutions were less stable. Lyophilized preparations showed only a slight change in titer after 16 months, and frozen preparations stored at −28 C showed a slight drop in titer after 6 months.

Filtration through Seitz, Selas, and sintered-glass filters, and treatment with formalin or Merthiolate, inactivated the purified hemolysin; however, approximately one-half of the original titer could be restored by activation with cysteine.

On paper electrophoresis (Beckman Spinco, Durrum Cell, model H), the purified hemolysin behaved with the relative mobility of gamma-type globulins.

Effect of oxidizing and reducing agents on the crude hemolysin. In contrast to streptolysin O, the crude hemolysins of *L. monocytogenes* were extremely active; in fact, cysteine and other oxidizing and reducing agents had no effect on the crude hemolysins. The titer of the crude hemolysins was comparable to that of the purified hemolysins after activation of the latter with cysteine. Other reducing agents were not as potent as cysteine. The usual titer range of the crude hemolysins was 512 to 16,284 HU/ml. After concentrating the crude hemolysin 20 times by pervaporation, it was easily activated with cysteine. Filtered Merthiolate or formalin-inactivated crude hemolysins could also be activated to approximately one-half the original titer with cysteine. This action suggests that these hemolysins not only are auto-oxidizable but also can be altered chemically.

Effect of oxidizing and reducing agents on the purified hemolysin. The purified listeria hemolysin, like streptolysin O, showed very little activity when tested in the absence of cysteine and other activating agents. The hemolytic activity after activation was usually 30 to 50 times that of the unactivated hemolysin. The effect of a large number of oxidizing and reducing agents on the purified hemolysin from three high-producing strains is shown in Table 1. In each experiment cysteine was included, and the relative degree of activation is expressed as a percentage of the activation with cysteine, which was given an arbitrary value of 100%. Controls were carried out on all compounds tested by adding 0.5 ml of a 0.2 M solution of the test substance to a 1% suspension of sheep red cells. There was no observable hemolysis except in the case of ascorbic acid and hydrogen peroxide, which was not considered significant because hemolysis did not extend beyond a 1:4 dilution of the activating agent.

The substances that were tested could be divided into three groups. (i) The —SH containing compound, cysteine, and sodium thiosulfate and sodium sulphite, which had similar maximal activating effects (30 to 50 times that of the

control). (ii) The —S—S— compound, cystine, which had no activating effect and ascorbic acid which had only a slight activating effect. The slight activating effect of ascorbic acid was attributed to its hemolytic effect on red cells that was observed in the control solution. (iii) KCN and sodium thiosulphite occupied an intermediate position, the activating effect being only three to five times that of the control, much less than that observed in group 1.

Activation of the listeriolysin by reducing agents was completely reversible (Table 2); this phenomenon was shown in the foollwing way. When cysteine was added to the purified hemolysin preparation, the titer was greatly increased. However, when the hemolysin-cysteine solution was dialyzed until all of the cysteine was removed, the hemolytic activity fell again to its original low level. The addition of cysteine once more to the hemolysin resulted in an increase of hemolytic activity as much as before.

TABLE 1. *Activation of the purified listeria hemolysin by various oxidizing and reducing agents*

Strain	Oxidizing or reducing agent	Hemolytic activity	Relative activity (cysteine — 100)
		HU/ml	
9-125	Cysteine	4,096	100
	Thiolacetic acid	1,024	25
	$Na_2S_2O_3$	256	6.2
	$Na_2SO_3O_4$	1,024	25
	KCN	128	3.1
	Na_2SO_3	512	12.4
	Cystine	32	0.78
	Ascorbic acid	64	1.5
	H_2O_2	64	1.5
	None	32	0.78
1112-3	Cysteine	8,192	100
	$Na_2S_2O_4$	2,024	25
	Thiolacetic acid	2,024	25
	Na_2SO_3	1,024	12.1
	Na_2SO_3	512	6.2
	KCN	128	1.5
	H_2O_2	64	0.78
	Cystine	64	0.78
	Ascorbic acid	64	0.78
	None	64	0.78
7648	Cysteine	1,024	100
	Thiolacetic acid	512	50
	Na_2SO_3	512	11.5
	Na_2SO_4	512	50
	Na_2SO_3	512	50
	KCN	64	6.2
	H_2O_2	16	1.6
	Cystine	16	1.6
	Ascorbic acid	16	1.6
	None	16	1.6

TABLE 2. *Activation of hemolysin by cysteine*

Treatment	Hemolytic activity
	HU/ml
Unactivated listeria hemolysin	16
Unactivated listeria hemolysin after adding cysteine to a 0.2 M concentration	1,024
Listeria hemolysin-cysteine solution after 24 hr of dialysis (Sullivan's test for cysteine was negative)	32
Dialyzed solution after adding 0.2 M cysteine	512 to 1,024

Another portion of the hemolysin-cysteine solution was not dialyzed but kept at room temperature for 24 hr; this solution showed no decrease in hemolytic activity, and Sullivan's test for cysteine was positive. No attempt was made in any of these experiments to titrate the exact amount of cysteine or other reducing agents required for activation of any given amount of hemolysin. Instead, the aim was to keep equal volumes of the hemolysin solution containing 16 to 64 HU/ml and 0.2 M of the reducing agent together for a period of 10 to 25 min. The solution was then diluted with streptolysin O buffer until it contained less than 1 HU/ml. The final concentration of the reducing agent was thereby considered to be very small. Activation of the hemolysin by the reducing agents tested appeared to be rapid at room temperature.

Thiolacetic acid in 0.2 M concentration was found to be effective in increasing the activity of the hemolysin preparation, but it was not as active as cysteine.

Effect of pH on the purified hemolysin. Streptolysin O buffer was used routinely, because the hemolysin was found to be more active in this buffer than in 0.85% physiological saline. When the pH of the sodium phosphate buffer was below 6.4 or above 9.0, significant hemolysis was observed in the absence of the listeriolysin. Hemolytic activity of the hemolysin was greatest in the pH range of 6.8 to 7.2.

Effect of temperature on the purified hemolysin. The hemolysin appeared to be more active when incubated at 37 C. Temperatures of 45, 25, and 0 C greatly reduced its activity. The activity at 45 C was 50 to 75% that of the titer at 37 C. At 25 C, the activity was 50%, and at 0 C it was 5%. The purified hemolysin did not exhibit the so-called hot-cold lysis of the staphylococcus β-hemolysin.

Effect of iodoacetic acid and iodoacetamide. Because many enzymes are known to be activated by —SH compounds, the effect of iodoacetic acid and iodoacetamide was considered to be interesting. The enzymes papain, cathepsin, and succinic dehydrogenase are strongly and irreversibly inhibited by these compounds. However, iodoacetic acid and its amide had little or no effect on the listeriolysin. This action closely paralleled that described for streptolysin O.

Table 3 shows the effect of iodoacetic acid and its amide on the purified listeriolysin. Iodoacetic acid (0.02 M) had no effect on the hemolysin when kept with it for 20 min at room temperature. There was only partial inhibition at 37 C with 0.02 M iodoacetamide, which was not considered significant.

To test the hypothesis that the hemolysin

TABLE 3. *Effect of iodoacetic acid and iodoacetamide on the purified hemolysin*

Hemolysin no.	Iodoacetamide at 37 C for 20 min	Iodoacetic acid at room temperature for 30 min	Control
7648	128*	256	256
9-125	1,024	2,048	2,048
1122-3	1,024	1,024	2,048

* Indicates hemolytic activity expressed as hemolytic units per ml.

TABLE 4. *Effect of iodoacetic acid on cysteine-activated hemolysin*

Treatment	Hemolytic activity
	HU/ml
Unactivated hemolysin (strain 9-125)	64
Hemolysin + cysteine	2,048
Hemolysin + cysteine + iodoacetic acid (0.04 M for 30 min)	128
Hemolysin + 0.2 M cysteine + 0.04 M iodoacetic acid for 30 min	512

TABLE 5. *Effect of iodoacetic acid on thiolacetic acid-activated hemolysin*

Treatment	Hemolytic activity
	HU/ml
Unactivated hemolysin (strain 9-125)	32
Hemolysin + thiolacetic acid	512
Hemolysin + thiolacetic acid + iodoacetic acid (0.04 M for 30 min)	16
Hemolysin + thiolacetic acid + iodoacetic acid (0.04 M for 30 min)	256

activity depends upon a thioldisulfide system, an excess of iodoacetic acid was allowed to act upon the hemolysin activated by cysteine and thiolacetic acid, and presumably in the —SH state. A 2-ml portion of 0.04 M iodoacetic acid was kept with 2 ml of listeria hemolysin for 30 min. On testing again, it was found that the hemolytic activity had disappeared. The activation was partly reversible; however, on adding a further excess of thiolacetic acid or cysteine, the hemolysin activity was restored to the extent of 50% of its original titer. The results of typical experiments are shown in Table 4.

The hemolysin after activation by cysteine and thiolacetic acid was not completely inhibited by 0.04 M concentration of iodoacetic acid (Tables 4 and 5). It is also considered important that the partially inactivated hemolysin could be restored to one-half the initial activated titer by the addition of the reducing agents. These results compared favorably with those reported for streptolysin O (Herbert and Todd, 1941), and seemed to indicate that the hemolysin, like streptolysin O, is not dependent upon reducing —SH groups, or that the —SH groups of these hemolytic agents react slowly with iodoacetic acid.

Serological properties. Herbert and Todd (1941) showed that both the purified and the crude preparations of streptolysin O were neutralized to the same extent with antistreptolysin O serum. The effect of antihemolysin serum on the purified and crude hemolysins and streptolysin O are shown in Table 6. Neutralization tests were performed in the following manner. To avoid nonspecific reactions, the antihemolysin serum was inactivated by heating (56 C for 30 min). The purified hemolysin preparation was activated with 0.5 M cysteine hydrochloride at a neutral pH and then titrated. Amounts corresponding to exactly 16 HU (0.5 ml) were placed in a series of tubes con-

taining 0.5 ml of the antiserum diluted twofold in streptolysin O buffer. After standing at room temperature for 15 min, 1 ml of a 1% suspension of sheep red cells was added, and the tubes were incubated for 30 min at 37 C. The reciprocal of the tube showing complete inhibition of lysis was taken as the end point. Antisera for the hemolysin of *L. monocytogenes* neutralized both crude and purified hemolysins from *L. monocytogenes* as well as streptolysin O (Table 6). The crude hemolysin was neutralized to a lesser degree than was the purified hemolysin, which seemed to indicate that during the purification process the hemolysin was modified or the crude hemolysin contained two or more hemolysins which were lost on concentration. The antihemolysin serum completely abolished the lytic effect of streptolysin O, and the normal control rabbit serum showed protection only in the undiluted and 1:4 dilutions. There was no inhibition of lysis by the normal serum against the purified hemolysins.

Effect of various substances on the purified hemolysin. The α-hemolysin of group A *Clostridium perfringens* is activated by calcium and inhibited by substances which combine with calcium (i.e., phosphates, citrates, fluorides). These substances have no effect on streptolysin O (Herbert and Todd, 1941). Experiments were conducted to determine whether this was true for listeria hemolysins. After activation of the purified hemolysins with cysteine, the dilutions were made up in 0.9% saline which contained the various substances to be tested, and the pH was adjusted to 6.5. The red cells were suspended in 0.9% saline. A typical experiment is shown in Table 7.

TABLE 6. *Highest dilution of antiserum causing complete inhibition of listeria hemolysin and streptolysin O*

Listeria hemolysin	9-125 ALHS*	1122-3 ALHS	Normal serum
Crude hemolysins			
7648	1:1,024†	1:20,480	1:4
1122-3	1:2,048	1:81,920	1:4
9-125	1:163,840	1:20,480	Undiluted
Streptolysin O	1:40,960	1:81,920	Undiluted
Purified hemolysins			
7648	1:163,840	1:40,960	0
1122-3	1:81,920	1:81,920	0
9-125	1:163,840	1:163,840	0
Streptolysin O	1:81,920	1:81,920	0

* ALHS, antilisteria hemolysin serum.

† Figures indicate dilutions.

TABLE 7. *Effect of certain chemicals on the purified hemolysin**

Substance added	Hemolytic activity
	HU/ml
None	2,048
0.001 M $CaCl_2$	512
0.01 M Potassium oxalate	512
0.01 M Sodium citrate	1,024
0.02 M NaF	1,024
0.01 M $MgCl_2$	512

* These results show that there is not a significant decrease in the hemolytic activity by the addition of the above substances.

DISCUSSION

The results of this study clearly show that hemolysins of high titer are produced by certain virulent and avirulent strains of *L. monocytogenes.* Relatively highly purified preparations were obtained by fractionation with ammonium sulfate followed by adsorption and elution from calcium phosphate gel.

The listeriolysin, like streptolysin O, is a protein that contains the —S—S— linkage which can be reversibly reduced to the —SH groups. The interpretation of this action is that the —S—S— form of the protein is hemolytically inactive, whereas the —SH form is active. The action of cysteine and other reducing agents apparently is to reduce the protein —S—S— groups to —SH groups. In this behavior, the listeriolysin and streptolysin O resemble many enzymes (e.g., urease, papain, cathepsin, and succinic dehydrogenase). All of these enzymes are strongly inhibited by iodoacetic acid. However, the resistance of the listeriolysin and streptolysin O to iodoacetic acid, along with their rather feeble activation by KCN, would indicate that the hemolytic activity was not solely dependent upon reduced —SH groups or that the reducing agents reacted slowly with the available —SH groups. Therefore, it was concluded that, based upon available data, the "—SH theory" only partly explained the activity of the hemolysin. Fruton and Bergman (1940) found the —SH theory unacceptable as an explanation for the hemolytic activity of certain enzymes. They suggested that reducing agents

act as coenzymes and form a dissociable complex with a particular enzyme.

The manner in which listeriolysin acts upon red cells is considered important. Preliminary studies indicate that this hemolysin may well share the lecithinase activity of the alpha toxin of *Clostridium perfringens*. This toxin produces a flocculent precipitate when added to a solution of lecithovitellin prepared from egg yolk (MacFarlane and Knight, 1941). The hemolysin preparations produced this flocculent precipitate when added to a solution of lecithovitellin, and also produced opalescence in normal human and rabbit serum. This apparent lecithinase activity seems to be directly related to the hemolytic titer of the hemolysin preparation. Any procedure that reduced the hemolytic activity also reduced the capacity of the hemolysin to produce opalescence in serum and lecithovitellin. The strict parallelism between hemolysis by the listeriolysin and the free lipoid from lecithovitellin, a known lipoprotein, makes it tempting to suggest that this action is a manifestation of the same enzyme reaction. Hemolysis may well follow the breakdown of the lipoprotein complex in the red blood cell.

An explanation for the high hemolytic activity of the crude hemolysin preparation is not readily available. It was felt that the presence of traces of activating compounds derived from the broth or the growth of organisms might be responsible for this increased hemolytic activity. Because the —SH groups of the inactive hemolysin must be assumed to be, like most —SH groups, auto-oxidizable in air, the small residual activity of the purified hemolysin may indicate more complete oxidation due to the purification process. In the purification process, there was a decrease in hemolytic activity during the stage of adsorption and elution from the calcium phosphate precipitate. In addition, the hemolytic activity of the purified hemolysin was completely abolished by antilisteria hemolysin serum. This seems to suggest that the high hemolytic activity of the crude hemolysin is due to a different protein. Agar-gel diffusion studies are under way to more clearly define the antigenic structure of the crude and purified hemolysins.

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