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Isolation and Characterization of a Protective Antigen-Containing Particle from Culture Supernatant Fluids of *Erysipelothrix rhusiopathiae*

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The mouse-protective activity of *Erysipelothrix rhusiopathiae* culture supernatant fluids exists in a polydisperse form, ranging in density from aggregates which sediment at 10,000 × *g* for 3 hr to soluble units which will not sediment at 198,000 × *g* for 12 hr. A partially purified protective antigen has been isolated from the aggregates sedimented from a concentrate of the culture supernatant fluid at 20,000 × *g* for 3 hr. These aggregates contained the major protective antigen or antigens of *E. rhusiopathiae*, since, in addition to inducing active immunity, they adsorbed essentially all of the passively protecting antibody from rabbit antiserum produced by immunization with whole culture. The protective activity in these aggregates was destroyed by trypsin and greatly diminished by muramidase and heating at 64 C, but was not affected by lipase or ribonuclease.

Erysipelothrix rhusiopathiae, having been one of the earliest infectious agents identified, has been the subject of much research in terms of its pathogenicity and control. Although effective vaccines have been available since the time of Pasteur (13), there is little known about the particular antigen or antigens responsible for specific protective effects.

Early vaccines consisted of either viable virulent cultures given with immune serum or avirulent cultures alone (19). In 1947, Traub (16) reported that certain strains of *E. rhusiopathiae* grown in the presence of horse serum were capable of producing enough protective antigen in vitro to be useful as killed vaccines. Later studies (4) indicated that these strains, which belonged to Dedié's serotype B, were protective against both serotypes A and B. Traub's finding (16) that part of the protective activity of these vaccine cultures was the result of soluble protective antigen dissociated from the bacterial cell was confirmed by Gledhill (7).

It is unknown whether the protective activity is caused by a single protective antigen or whether more than one antigen is present. There is some evidence of type specificity at low levels of immunity (17, 18).

Various chemical and physical characteristics of the protective antigen have been reported. Bergmann (3) noted that protective activity could be destroyed by proteolytic enzymes, indicating that a protein or peptide moiety was essential for protection. Nani (12) found that, although most of the protective activity of whole cells was extracted in the protein fraction when using the Boivin method, a minor portion of the activity was associated with a glycolipid fraction. The protective antigen is destroyed by heating to 64 C (7), is sensitive to freezing (11), and is resistant to ethanol at room temperature (7).

An investigation was undertaken to characterize the protective antigen of *E. rhusiopathiae* further. Soluble antigen in culture supernatant fluids was used as the source of protective antigen in these studies.

MATERIALS AND METHODS

Bacterial strains. The strain used for the production of protective antigen was *E. rhusiopathiae* 829-2, an avirulent strain of serotype B. Strain 2646, of unknown serotype, was used as the challenge organism. All cultures were stored in the lyophilized state in a 10% milk-1% lactose medium. Fresh cultures were reconstituted with distilled water for each batch of antigen and for each challenge.

Cultural procedures. The agar medium used for slants and plates consisted of Difco Brain Liver Heart (Semisolid) broth with 1.5% agar and 7.5% horse serum added. The broth medium was prepared by the

following formula. To each liter of distilled water heated to 70 C were added peptone (Difco), 10 g; sodium phosphate, dibasic, 8 g; potassium phosphate, monobasic, 1 g; sodium citrate, 1 g; magnesium sulfate, 0.2 g; and thioglycolic acid, 0.3 g. This mixture was heated to boiling and then allowed to cool to room temperature. The *p*H was adjusted to 8.3, and the preparation was boiled for 10 min. It was cooled to 80 C, and 8 g yeast extract (Difco) and 1 g of oxgall (Difco) were added. It was then filtered through high porosity filter paper, distributed in 2-liter volumes into 4-liter aspirator bottles, and autoclaved at 121 C for 20 min. Upon cooling, sterile horse serum and 50% dextrose were added to provide a concentration of 7.5% horse serum and 2% dextrose.

For production of protective antigen, lyophilized cultures of strain 829-2 were reconstituted and subcultured three times through broth tubes at 12-hr intervals. Three-liter Fernbach flasks containing 2 liters of broth medium were inoculated with 10 ml of the last subculture and incubated at 37 C for 48 hr. The flasks were then incubated at room temperature with continuous stirring on a magnetic stirrer for 48 hr. The culture was centrifuged at 7,500 × *g* for 20 min at 4 C, and the supernatant fluids were pooled as the source of antigen.

Concentration, dialysis, and buffer adjustment of the culture supernatant fluid were performed at 4 C by using a modification of the negative pressure dialysis method described by Jesting (8). During this procedure, the nondialyzable components of the culture supernatant fluid were reduced to 10% of their initial volume.

Animal studies. For immunization, a stock preparation of adjuvant was prepared by diluting compressed aluminum hydroxide gel (Reheis F-5000) to a concentration of 2% (based on Al_2O_3) in 0.05 M sodium phosphate buffer, *p*H 6.0. This adjuvant was added to the sample to be tested in a volume equal to 20% of the sample. The *p*H was adjusted to 6.0, and the preparation was shaken at room temperature for 30 min to adsorb the protective antigen. Essentially all of the protective antigen was adsorbed to the aluminum hydroxide gel under these conditions. Fourfold dilutions of this preparation were made in 0.05 M phosphate buffer (*p*H 6.8) containing 10% adjuvant. A 0.5-ml amount was injected subcutaneously into the backs of 20- to 25-g white female mice. Groups of six to nine mice were used for each dilution. Fourteen days later, the mice were challenged and the 50% protective dose (PD_{50}) was determined by the method of Reed and Muench (14).

For challenge tests, a lyophilized culture of strain 2646 was seeded to a slant and incubated for 18 to 24 hr. It was then transferred to a second slant and allowed to grow for 12 hr. The cells were washed from the slant with 1% peptone water and diluted to a turbidity of 70 Klett units by using a green filter (500 to 570 nm), and 10-fold dilutions made in peptone water. One hundred LD_{50} units, approximately 200 cells, in 0.5 ml of peptone water was injected subcutaneously in the backs of the mice to be challenged. Deaths were recorded for 10 days.

To prepare antisera for passive protection tests, 4- to 5-lb (1.8 to 2.3 kg) male albino rabbits were injected intravenously with the antigen to be tested on 5 successive days, followed by a repetition of the same protocol after 2 weeks of rest. The successive injections of whole culture were 1, 1, 2, 3, and 3 ml, and those of the fractions were 1.5 mg in 2 ml of phosphate buffer. The rabbits were bled by cardiac puncture 1 week after the final injection.

Ammonium sulfate precipitation. The desired concentrations of powdered ammonium sulfate were slowly added to the antigen preparation in 0.05 M sodium phosphate buffer (*p*H 6.8) at 4 C with continuous stirring. The *p*H was maintained at 6.8 by addition of 5 N NaOH. The preparations were allowed to stand overnight at 4 C, and the precipitates were removed by centrifugation at 2,500 × *g* for 20 min at 4 C. The precipitates were dissolved in phosphate buffer, and the fractions were dialyzed to remove the ammonium sulfate.

Ion-exchange chromatography. Various ion-exchange celluloses (Serva) were used for ion-exchange chromatography. Both stepwise and gradient elution methods were employed in attempts to fractionate samples. Fractions were collected with a refrigerated Beckman fraction collector (model 132).

Molecular sieve chromatography. Molecular sieve chromatography was carried out by using a column (6 by 45 cm) of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, *p*H 6.8. The flow rate of the column was 50 ml/hr and 10-ml fractions were collected. The elution pattern was determined on the basis of the absorbance of the fractions at 280 nm as measured in a Beckman model DU spectrophotometer.

Preparative ultracentrifugation. Preparative ultracentrifugation was carried out at 4 C in a Spinco model L ultracentrifuge with a type 40 rotor. For forces greater than 144,000 × *g*, the Spinco model L-HV was used with a type 50 rotor.

Analytical methods. Dry weight determinations were performed on preparations dialyzed against distilled water and represent the total nondialyzable solids of the fraction under study. Hexose was determined by the anthrone procedure (9) and pentose was determined by the Bial method (2) by using glucose and ribose as the standards, respectively. Protein was determined by the method of Lowry et al. (cited in reference 9) by using bovine serum albumin as the standard. Lipid content was estimated by a modification of the method of Foch et al. (5). The samples were hydrolyzed in 6 N HCl for 2 hours in a boiling-water bath, the HCl was removed by lyophilization, and the dried material was extracted with a chloroform-methanol mixture (2:1, v/v). The dry weight of the material extracted in the chloroform-methanol was considered to indicate the amount of lipid in the sample.

Enzyme analysis. The sensitivity of the protective antigen to various enzymes was determined by incubating samples containing 1 mg of nondialyzable solids per ml with equal volumes of the following enzyme (Worthington) systems for 2 hr at 37 C: 18 units of trypsin per ml in 0.05 M tris(hydroxymethyl)-aminomethane buffer, *p*H 7.5; 0.1 units of wheat germ

lipase per ml in 0.5 M phosphate buffer, *p*H 7.5; 250 units of ribonuclease per ml in 0.05 M phosphate buffer, *p*H 7.0; 500 units of muramidase per ml in 0.05 M phosphate buffer, *p*H 6.5. The protective activity remaining after enzyme treatments was determined by using the routine animal protection test.

RESULTS

Although it has been reported (16) that the protective antigen of *E. rhusiopathiae* can be found free in the culture supernatant fluids of serotype B cells, the proportion of the total protective activity of the whole culture that this soluble antigen constitutes was unknown. To quantitate the part that this soluble antigen played in the overall immunizing activity of killed cultures, the cells obtained after centrifugation were resuspended to their original volume in sterile broth, and samples of whole culture, cells, and culture supernatant fluid were tested for protective activity. About 20% of the total activity of the whole culture was found in the culture supernatant fluid (Table 1). This was found to occur repeatedly with the strain used for protective antigen production.

Attempts to fractionate culture superntant fluid on columns of diethylaminoethyl (DEAE)-cellulose were unsuccessful because the antigen bound irreversibly to the column material and could not be eluted by increasing ionic concentration or by changes in *p*H from 4.0 to 9.0. The antigenic integrity of the adsorbed antigen was demonstrated by means of injections of DEAE suspensions obtained from the upper portions of the columns. These suspensions protected mice even in the absence of aluminum hydroxide. Subsequent experiments described below also demonstrate the strong binding properties of the protective antigen.

All of the protective activity could be precipitated at 50% ammonium sulfate saturation, but dialysis and restoration to original volume did not solubilize all of the precipitate. The turbid material remaining in suspension could not be sedimented at 5,000 × *g* for 30 min, but could be sedimented at 15,000 × *g* for 30 min. This sediment was resuspended in 0.05 M sodium phosphate buffer (*p*H 6.8), and the protective activity of this suspension and that of the centrifugal supernatant fluid were compared to that of the original 50% ammonium sulfate fraction.

About one-half of the protective activity of the ammonium sulfate preparation occurred in each of the centrifugal fractions (Table 2). Thus, a major portion of the protective activity appeared to reside in a large particle. Attempts to dissolve this sediment in acid and alkaline buffers were unsuccessful.

TABLE 1. *Protective activity of different fractions of whole culture*

Fraction	Dilutions				PD_{50}/ml
	1:1	1:4	1:16	1:64	
Whole culture	7/7[a]	6/7	4/7	4/7	38
Cells	7/7	7/7	4/7	2/7	32
Culture supernatant fluid	6/7	5/7	3/7	0/7	8
Filtered supernatant fluid	7/7	5/7	2/7	0/7	8

[a] Number of mice surviving over number challenged.

TABLE 2. *Protective activity of centrifugation fractions derived from ammonium sulfate fraction*

Fraction	Dilution		PD_{50}/ml
	1:1	1:4	
Ammonium sulfate fraction	6/6[a]	5/6	>4.0
15,000 × *g* Precipitate[b]	5/6	3/6	3.2
15,000 × *g* Supernatant fluid	5/6	3/6	3.2

[a] Number of mice surviving over number challenged.

[b] Suspension was centrifuged at 15,000 × *g* for 30 min.

Further studies were carried out on the culture supernatant fluid concentrated by negative pressure. This procedure not only concentrated the protective activity but also served as a means of partial purification of the protective antigen (Table 3). This was due to a decrease in the total nondialyzable solids. The experiment illustrated here showed a decrease of about 55%. This is probably the result of stretching of the dialysis tubing under negative pressure, causing an increase in the pore size of the tubing. Smaller molecules which were usually retained by dialysis tubing under normal conditions of dialysis would then escape. This smaller molecular weight material, which ranged from 30 to 60% of the total nondialyzable solids in the various preparations tested, did not appear to include any of the protective antigen. In addition, as shown in Table 3, this procedure brought the protective activity up to a level which was more suitable for further fractionation.

The size of the protective antigen-containing unit was investigated by means of molecular sieve chromatography. This was done with Sephadex

TABLE 3. *Effect of concentration by negative pressure dialysis on nondialyzable solids and protective activity of the culture supernatant fluid*

Preparation	Nondialyzable solids (μg/ml)	PD_{50}	
		Dilution	Amt (μg)
Culture supernatant fluid	3,595	1:16	225
Concentrate[a]	16,352	1:220	74

[a] Volume reduced to 10% of the original culture supernatant fluid.

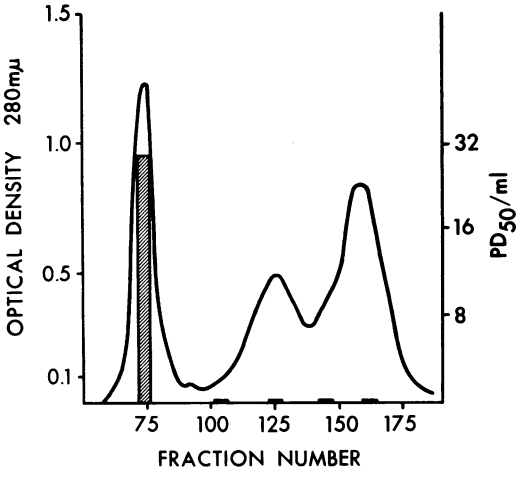

FIG. 1. *Sephadex G-200 fractionation of the concentrated culture supernatant fluid. Bar indicates location of protective activity.*

G-200 by using 25 ml of the concentrated culture supernatant fluid as the sample. Pooled fractions representing the three peaks of the elution profile shown in Fig. 1 were tested for protective activity. Protection tests showed that the first peak, which represented the material excluded from the gel, contained all of the protective activity. This indicated that the protective activity resided in a molecule or particle of 200,000 or greater molecular weight.

This molecular sieve chromatography revealed only a possible minimal molecular size. To approximate more closely the probable size of the protective antigen-bearing unit, differential ultracentrifugation of the concentrated culture supernatant fluid was undertaken, by using a method of sequential centrifugation. A sample of the concentrated culture supernatant fluid was centrifuged at 10,000 × *g* for 180 min, the precipitate was removed, and the supernatant fluid

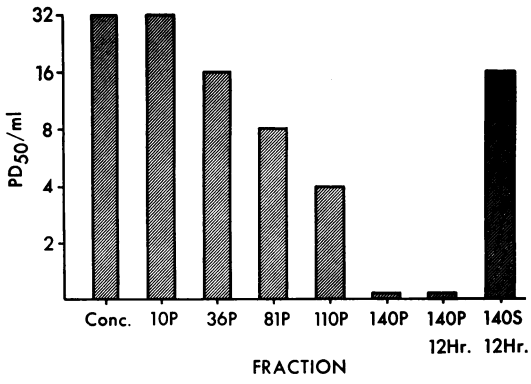

FIG. 2. *Protective activity of the fractions obtained by ultracentrifugation in the Spinco model L ultracentrifuge. The numerals indicate the centrifugal force in 1,000 × g, and the letters indicate the precipitate (P) and the supernatant fluid (S). The precipitate of each centrifugation was resuspended to its initial volume in sodium phosphate buffer, and the supernatant fluid was subjected to the next higher speed. Except as indicated, all were run for 180 min.*

was centrifuged at a higher speed. This process was repeated through speeds of 36,000, 81,000, 110,000, and 140,000 × *g* for 180 min. After a final centrifugation of 140,000 × *g* for 12 hr, the sediments were each resuspended to a volume equal to that of the supernatant fluid from which they were removed, and these, along with the final supernatant fluid, were tested for protective activity. The sediments were designated the 10*P* 36*P*, 81*P*, 110*P*, 140*P*, and 140*P*-12-hr fractions, and the final supernatant fluid was designated the 140*S* fraction by using the system suggested by Alexander (1).

Most of the activity was sedimented at relatively low speeds, indicating that the protective activity occurred in a particle of considerable size, one much greater than 200,000 molecular weight (Fig. 2). The amount of protective activity sedimented gradually decreased, until at 140,000 × *g* there was none present in the sediment. However, an appreciable amount of protective activity still remained in the supernatant fluid. A certain portion of the protective activity was not sedimented even at 140,000 × *g* for 12 hr. In subsequent experiments, this residual activity was found to be only partially sedimented at speeds of 198,000 × *g* for 12 hr. Thus, it appears that the protective antigen occurs in a variety of sizes ranging from particles which approach the borderline of solubility to molecules which are in solution, and which are probably of relatively small size or low density.

The relationship of the faster sedimenting protective material and that which remained in the

TABLE 4. *Chemical composition of the 20P fraction based on total nondialyzable solids*

Analysis	Per cent
Protein	64.0
Lipid	12.8
Hexose	2.4
Pentose	0.75

TABLE 5. *Protective activity remaining in the 20P fraction after enzyme treatment*

Enzyme treatment	PD_{50}/ml
Lipase	32
Ribonuclease	32
Muramidase	8
Trypsin	<1
Control	32

supernatant fluid was unknown. They might represent different antigens, or the latter might be the unit of which the heavier is composed. However, because the heavier material could be obtained relatively free from most of the soluble culture components by simple washing procedures, this form of the protective antigen was selected for further study and characterization. The material used for further studies was obtained from culture supernatant fluids centrifuged at 7,500 × *g* for 20 min, concentrated 10-fold by negative pressure dialysis, and sedimented at 20,000 × *g* for 180 min. This sediment, designated the 20*P* fraction, was washed twice in 0.05 M sodium phosphate buffer (*p*H 6.8) and resuspended in appropriate buffers for further studies. When resuspended, different preparations of this 20*P* fraction displayed a turbidity of about 60 to 100 Klett units at a concentration of 1 mg per ml of total nondialyzable solids. The 20*P* fraction yielded about 20% of the total activity present in the concentrated culture supernatant fluid, but the specific activity based on total nondialyzable solids was increased 28-fold. The average PD_{50} of four preparations chosen for analysis was 4.5 μg. When used to immunize rabbits, the 20*P* fraction induced the formation of antibody that was passively protective for mice challenged with *E. rhusiopathiae*.

The results of chemical analysis of the 20*P* fraction (Table 4) showed that it was predominantly protein and lipid. Although Bergmann (3) had reported that the protective antigen was sensitive to proteolytic enzymes and thus presumably protein in nature, the presence of these other chemical moieties in the 20*P* fraction made it necessary to attempt to determine the possible contribution of these components to the protective activity. Therefore, enzymatic degradation studies with trypsin, lipase, ribonuclease, and muramidase were undertaken. Neither lipase nor ribonuclease appeared to have any effect on the protective activity of the 20*P* fraction (Table 5). However, treatment with trypsin resulted in the loss of all activity, suggesting the presence of an essential protein or peptide moiety in the protective antigen. In addition, muramidase, which acts upon B-1, 4-glucosidic linkages in glycosamino peptides, removed approximately 75% of the protective activity of the 20*P* sample. This result with muramidase has been repeated several times on different 20*P* preparations, indicating the probable presence and essentiality of this B-1, 4-glucoside linkage in the protective antigen.

To compare further the protective antigen present in the 20*P* fraction to that which has been previously reported (7), the heat stability of the protective activity of the 20*P* fraction was studied. Samples (20 ml), containing 8 mg of the 20*P* fraction in 0.05 M sodium phosphate buffer (*p*H 6.8) were subjected to 1 hr of exposure in water baths at 50, 64, 75, 85, and 95 C. Results of protection tests (Fig. 3) indicated that there was no loss of activity at 50 C. At 64 C, the point at which Gledhill (7) reported that all activity was lost, there was still about 25% of the protective activity present. The protective activity remained at this level through treatment at 85 C but at 95 C again decreased.

To determine the relationship of the protective activity of the 20*P* fraction to that of the whole culture and that remaining in the high-speed supernatant fluid, absorption tests of protective anti-whole culture rabbit serum were carried out by using the 20*P* fraction as the absorbing agent. An 8-ml amount of sodium phosphate buffer containing 1 mg of sample based on nondialyzable solids was mixed with an equal volume of antiserum and incubated for 2 hr in a water bath at 37 C. It was then allowed to stand overnight at 4 C, and the precipitate was removed by centrifugation. The antiserum was then tested for protective activity.

The 20*P* fraction absorbed all of the protective activity of the antiserum prepared against whole culture (Table 6). This indicated that the 20*P* fraction contained the major protective antigen responsible for inducing the production of passively protecting antibody. In addition, if it is assumed that the soluble antigen in culture supernatant fluids is antigenic in the rabbit, these absorption results also indicate that the protective antigen in the 20*P* fraction is antigenically similar to that found in the high-speed supernatant fluid.

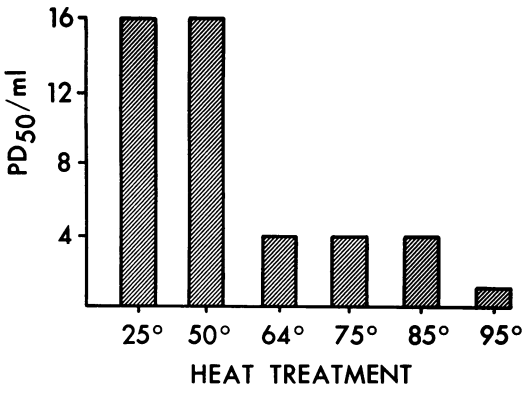

FIG. 3. *Effect of heating for 1 hr on the protective activity of the 20P fraction.*

TABLE 6. *Protective activity of unabsorbed and 20P-absorbed anti-whole culture rabbit serum*

Determination	Serum dilutions					PD_{50}
	1:2	1:5	1:10	1:20	1:40	
						ml
Untreated..	7/7[a]	6/7	8/8	8/8	4/8	0.025
Absorbed with 20*P*..	1/7	0/8	0/8	0/8	0/8	<0.5
Normal serum....	0/8	0/8				

[a] Number of mice surviving over number challenged.

DISCUSSION

The finding that the protective antigen in supernatant fluids of *E. rhusiopathiae* cultures can exist as a relatively large particle is not without precedent. In early work with culture supernatant fluids of *E. rhusiopathiae*, Gledhill (6) found that the protective activity could be filtered out by passage through 0.2-μm filters. This may have been due to the presence of antigen-containing units described above.

The size and lipid content of the 20*P* fraction suggest the possibility of cell membrane origin. However, the cell walls of *E. rhusiopathiae*, unlike the typical cell walls of gram-positive bacteria, have been reported (18) to contain relatively large amounts of lipid. In addition, an isolated cell wall antigen of *E. rhusiopathiae* has been reported (18) to contain muramic acid, a compound whose presence in the 20*P* fraction is suggested by the results of enzymatic analysis. Thus, the particles present in the 20*P* fraction might be portions of the cell walls containing the protective antigen. However, the protective antigen present in the high-speed supernatant fluids appears to be smaller units of protective antigen released into the supernatant fluid, and the particles found in the 20*P* fraction may be formed by spontaneous aggregation of these small units. This possibility is supported by the increased aggregative properties observed during ammonium sulfate precipitation.

It is significant that although an appreciable amount of the total protective activity of the concentrated culture supernatant fluid is not sedimented at 144,000 × *g* for 12 hr, this activity is excluded from Sephadex G-200 gel. This is indicative of a large-size, low-density molecule and is compatible with the lipoprotein nature of the particulate 20*P* fraction.

Although the chemical analyses are useful in characterizing the whole fraction, they do not necessarily indicate the composition of the protective antigen which may be only a portion of the 20*P* fraction. Assuming that the loss of activity was the result of enzymatic degradation and not simply blockage of antigenic determinants by nonspecific adsorption, the enzymatic studies produced more relevant results. The destructive effects of trypsin confirm Bergmann's (3) studies in demonstrating the presence of an essential protein or peptide moiety. The loss of protective activity as a result of muramidase treatment is highly suggestive of the presence of muramic acid as an essential part of the protective antigen. This is of some general interest, for, although it is ubiquitous in the bacterial world (15), muramic acid has not been previously reported to be a component of any protective antigen of bacterial origin. Based on the results of trypsin and muramidase treatments, it is probable that this protective antigen is structurally similar to the glycopeptide compounds found in many bacterial cell walls. The lack of activity by lipase on the protective antigen could indicate that the lipid present in the antigen is unnecessary for protective antigenicity, or that the lipid is within the antigen molecule and not exposed to the enzyme. Further studies are necessary to distinguish between these two possibilities.

Although both active immunization studies and serum absorption indicate that the antigen in the 20*P* fraction is responsible for the major protective activity of *E. rhusiopathiae* cultures, some suggestions remain that this antigen may have some structural diversity or that multiple protective antigens are present. Thus, the protective activity (about 25%) remaining after muramidase and heat treatment could be interpreted as being the result of the partial destruction of a single antigen or the presence of a second resistant antigen of lesser activity or smaller amount. The latter might

be the type-specific protective antigen described by Watts (17) and White (18).

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