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# Purification of a Protective Antigen from a Saline Extract of *Pasteurella multocida*

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It has been shown previously that soluble material extracted from *Pasteurella multocida* P-1059 by a 2.5% NaCl solution protects turkeys from generalized septicemia at a subsequent challenge exposure to the organism. In the present study, a protective antigen was purified from the crude soluble material by chromatographic methods. Four protein peaks were obtained by gel filtration with Sephadex G-200. The protective antigen was detected only in the first peak fraction, which contained a substantial amount of carbohydrate. The peak 1 fraction was adsorbed onto DEAE-cellulose and eluted by a linear gradient of NaCl. Fractions corresponding to a single protein peak were pooled and passed through an immunoadsorbent column to remove any possible serum component originating from the growth medium. The purified antigen had a carbohydrate/protein ratio of 1.5 and formed a single precipitin line with rabbit antiserum against the crude material in gel diffusion and immunoelectrophoresis analyses. The antigen produced antibodies in rabbits and turkeys which formed a single precipitin line against the crude material. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified antigen showed three protein bands, corresponding to molecular weights of 44,000, 31,000, and 25,000, and one carbohydrate band. The carbohydrate band did not correspond to any of the three protein bands. Upon isoelectric focusing gel analysis, the purified antigen showed two bands (pI = 3.5 to 4.0 and 4.5 to 5.5), but the two bands were antigenically identical by isoelectric focusing crossed immunoelectrophoresis. The 50% protective dose of the purified antigen was between 10 and 50 μg of protein in trials where two doses were given at 14-day intervals to 10- to 20-week-old turkeys.

Strains of *Pasteurella multocida* cause acute septicemic diseases, characterized by high morbidity and mortality, in some animal species (4). These diseases include fowl cholera, an acute to peracute septicemic disease of fowl which is prevalent all over the world and causes severe losses in domestic and wild fowl (12). Birds that recover naturally gain solid immunity against reinfection, and, therefore, many attempts have been made to develop an effective vaccine against the infection. Currently prevailing preventive practices in domestic poultry are exposure to a live strain of low pathogenicity (3) and immunization with inactivated whole cells of *P. multocida* (11). These vaccination methods are generally effective, but occurrence of the disease is not rare in a flock which has been properly vaccinated. Improvement in prevention has particularly been needed for domestic turkeys, which are one of the most susceptible species to the disease.

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Attempts have been made to identify subcellular materials of *P. multocida* that induce protective immunity in chickens and turkeys in the hope that a vaccine of higher potency than the types currently available would be developed. A lipopolysaccharide (LPS)-protein complex has been isolated with formalinized saline solution and found to be immunogenic in chickens (18). LPS has been further purified from the complex by two successive extractions with hot phenol followed by centrifugation in CsCl (19). The purified LPS containing 3.6% protein induces antibodies in adult chickens; these antibodies are protective upon subsequent transfer to 7-day-old chicks. Active immunity in highly susceptible hosts such as turkeys, however, has not yet been reported with purified LPS.

A ribosomal fraction purified from *P. multocida* induces active immunity in chickens at a dose of 1 mg (dry weight) (2). A factor capable of provoking active cross-type immunity in turkeys has been found in whole lysate of *P. multocida* grown in vivo, but the factor has not yet been purified (20). A soluble fraction extracted from

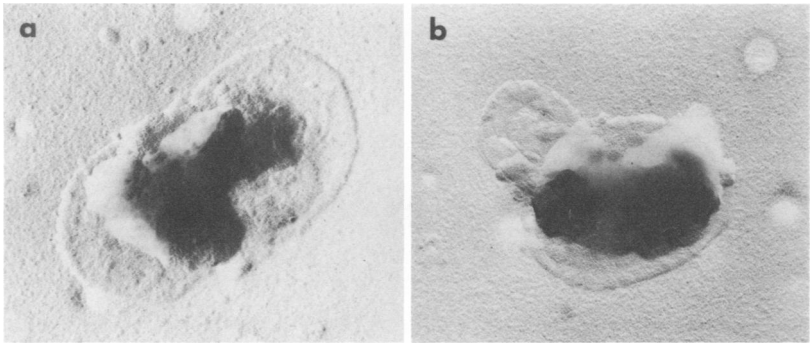


FIG. 1. Electron micrographs of *P. multocida* P-1059 before (a) and after (b) extraction for 60 min at 56°C in a 2.5% NaCl solution.

*P. multocida* with potassium thiocyanate is immunogenic in chickens (9).

We have previously reported that a soluble fraction extracted from *P. multocida* with a 2.5% NaCl solution induces protective immunity in turkeys (14). The polysaccharide antigen purified from the extract, however, was not immunogenic. In the present study, a protein-carbohydrate antigen with protective capacity was purified from the crude extract by chromatographic methods. The antigen contained three protein components, and the carbohydrate/protein (C/P) ratio was 1.5.

## MATERIALS AND METHODS

**Bacterial strain.** *P. multocida* P-1059 (11) was supplied by K. R. Rhoades, National Animal Disease Center, Ames, Iowa. After four passages in turkeys, the organism from a single colony was propagated on sheep blood agar, harvested in broth, and stored frozen at −70°C. The frozen culture was used to produce antigens and to infect turkeys.

**Preparation of CE.** An agar medium was prepared in plastic petri dishes (150 by 15 mm) containing the following materials in 900 ml of distilled water: 37 g of dehydrated brain heart infusion broth (Difco Laboratories, Detroit, Mich.); 5 g of Casamino Acids (Difco); 5 g of polypeptone (Daigo Nutritional Chemical Co., Osaka, Japan); 5 g of NaCl; 12 g of agar (Difco); and 10 mg of hemin (type 1; Sigma Chemical Co., St. Louis, Mo.). To the autoclaved medium, 50 ml of fresh yeast extract (10) and 50 ml of heat-inactivated (56°C for 30 min) turkey serum were added. The frozen culture was recovered on the agar medium, and 8- to 10-h growth at 37°C was harvested in 100 ml of brain heart infusion broth. The bacterial suspension containing approximately $10^8$ colony-forming-units (CFU) was spread onto the agar plates (0.5 ml per plate). The plates were incubated at 37°C for 18 to 20 h. The bacterial growth was harvested in a 2.5% NaCl solution (7 ml per plate) and agitated for 1 h at 56°C as described by Maheswaran et al. (16). The suspension was centrifuged at

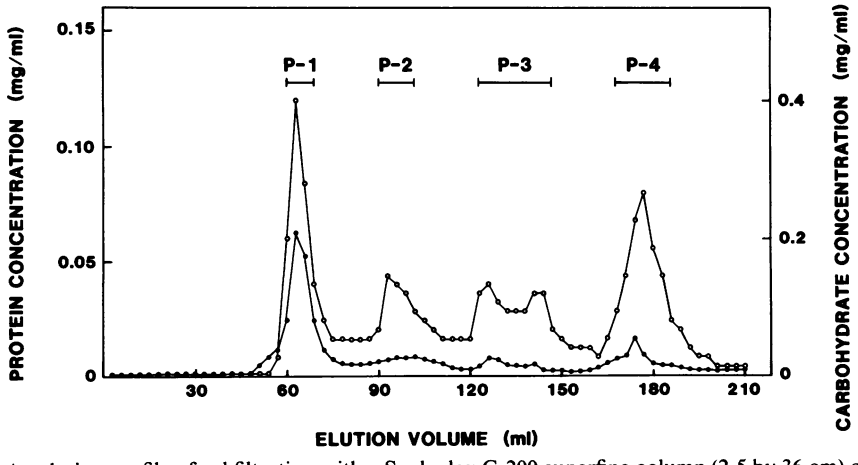


FIG. 2. An elution profile of gel filtration with a Sephadex G-200 superfine column (2.5 by 36 cm) of CE of *P. multocida*. A 4-ml portion was applied to the column and eluted at a flow rate of 7.5 ml/h in 0.01 M phosphate buffer (pH 6.8). The protein concentration (○) was determined by the method of Lowry et al. (15), and the carbohydrate content (●) was determined by the phenol-sulfuric acid method (6).

TABLE 1. Protein and carbohydrate contents of fractions obtained from CE of *P. multocida* by gel filtration with Sephadex G-200[a]

| Sample | Protein[b] | | Carbohydrate[c] | | C/P ratio |
|---|---|---|---|---|---|
| | Amt (mg/ml)[d] | Recovery (%) | Amt (mg/ml)[d] | Recovery (%) | |
| CE | 1.054 | 100 | 0.656 | 100 | 0.62 |
| P-1 | 0.220 | 20.9 | 0.318 | 48.5 | 1.44 |
| P-2 | 0.121 | 11.5 | 0.057 | 8.8 | 0.47 |
| P-3 | 0.187 | 17.7 | 0.046 | 5.7 | 0.21 |
| P-4 | 0.251 | 23.8 | 0.082 | 12.6 | 0.33 |

[a] See legend to Fig. 2 for details.
[b] Protein concentrations were estimated by the method of Lowry et al. (15) with bovine serum albumin as the standard.
[c] Carbohydrate concentrations were estimated by the phenol-sulfuric acid method with glucose as the standard (6).
[d] The volume of each fraction was adjusted to that of the original crude extract.

17,000 × *g* for 20 min, and the supernatant was recentrifuged, dialyzed for 48 h in a 0.85% NaCl solution containing 0.01% thimerosal, and then filtered through 0.22-μm-pore-size membrane filters (GS filter; Millipore Corp., Bedford, Mass.). The filtrate is referred to as crude extract (CE).

**Antiserum.** Adult New Zealand White rabbits were subcutaneously injected with 0.5 ml of CE or other antigens in 100-μg amounts of protein emulsified in an equal amount of complete Freund adjuvant (Difco). The injection was repeated twice at 30-day intervals with the antigen emulsified in incomplete Freund adjuvant (Difco). At day 14 after the third injection, the animals were exsanguinated.

**Electron microscopy.** The bacterial suspension was poured onto dried peptone agar in a petri dish. The cells were picked on a collodion membrane which was subsequently placed on a grid and shadowed with platinum-paladium alloy at an angle of 10 to 13 degrees as described previously (13). The specimens were examined with a Phillips 300 electron microscope.

**Chromatography.** A 4-ml portion of CE was applied to a Sephadex G-200 superfine column (2.5 by 36 cm) in 0.01 M sodium phosphate buffer (pH 6.8). The material was eluted at 7.5 ml/h at 4°C, and 3-ml fractions were collected. The first protein peaks of Sephadex G-200 effluent from several runs were pooled, and this fraction, containing 3.5 mg of protein, was adsorbed onto a DEAE-cellulose column (1.5 by 30 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). The material was eluted by a linear gradient of NaCl from 0 to 0.5 M with a flow rate of 30 ml/h at 4°C. For further purification an immunoadsorbent was used to remove turkey serum components. Whole turkey serum containing 30 mg of protein in 0.5 ml was coupled to 15 ml of Sepharose 4B by the method of Avrameas and Ternynck (1). Rabbit antiserum against whole turkey serum (30 ml) was passed through the adsorbent column, and antibodies were eluted with 3 M KSCN at 0°C. The eluted antibodies, containing 15 mg of protein, were in turn coupled to 15 ml of Sepharose 4B. The pooled fraction from the DEAE-cellulose column containing the specific antigen was passed through a second immunoadsorbent column to remove any residual turkey serum components.

**Electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (23) to assay the purity and determine the molecular weights of protein components by using the following markers: human immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.); bovine serum albumin (Sigma); ovalbumin (Miles); myosin (Sigma); and cytochrome *c* (Sigma). Disc electrophoresis was done by the method of Davis (5). Protein bands were stained with Coomassie brilliant blue R (Sigma), and carbohydrate was stained with periodic acid-Schiff reagent by the method of Felgenhauer (7). The stained gels were scanned with a densitometer (model 2950; Transydine General Corp., Ann Arbor, Mich.). In one instance 44,000 (44K) band was extracted from the gel by an isotonic saline solution containing 0.1% SDS followed by the removal of SDS with 1 M urea. For isoelectric focusing (IEF) in an agar gel column, 0.15 ml of sample was mixed at 56°C with 0.09 ml of ampholyte (IsoGel Ampholyte; FMC Corp., Rockland, Maine) and 1.5 ml of 1% agarose (IsoGel; FMC) and then gelated in glass columns (0.5 by 9.5 cm) at 4°C. Electrophoresis was done at 65 mW at 20°C for 4 h. The gels were stained and destained according to the method described in

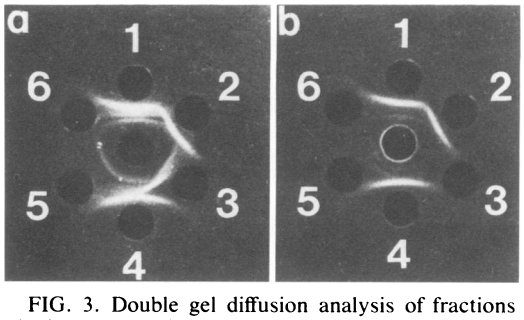


FIG. 3. Double gel diffusion analysis of fractions obtained by gel filtration with rabbit anti-CE serum. Wells 1 and 4 contained CE from *P. multocida* P-1059; wells 2, 3, 5, and 6 contained fractions P-1, P-2, P-3, and P-4, respectively. The center well contained rabbit anti-CE serum only (a) or the antiserum mixed with normal turkey serum (1:128) (b).

TABLE 2. Immunological characteristics of the fractions obtained from CE by gel filtration[a]

| Fraction | Precipitin line(s)[b] with | | | Amt of protein (mg) per dose[c] | Antibody response[d] (no. positive/no. inoculated) | Protection in turkeys (no. survived/no. inoculated) |
|---|---|---|---|---|---|---|
| | RACE | RACE + TS | TACE | | | |
| Original CE | + | + | + | 0.527 | 6/6 | 5/6 ($P < 0.01$) |
| P-1 | + | + | + | 0.110 | 4/5 | 4/5 ($P < 0.01$) |
| P-2 | + | − | − | 0.061 | 3/6 | 0/6 |
| P-3 | + | + | − | 0.094 | 0/6 | 0/6 |
| P-4 | − | − | − | 0.126 | 0/5 | 0/5 |
| P-1a[e] | − | − | − | 0.150 | 0/6 | 1/6 |
| Unimmunized control | | | | 0 | 0/7 | 0/7 |

[a] See legend to Fig. 2 for details.

[b] Precipitin lines observed (+) or not observed (−) in a gel diffusion test with rabbit antiserum against CE (RACE) with or without a 1:128 dilution of normal turkey serum (TS) or with turkey antiserum against CE (TACE).

[c] Fifteen-week-old turkeys were subcutaneously injected with the antigens emulsified in incomplete Freund adjuvant, followed by a second injection 14 days later with the same amounts. The turkeys were intramuscularly inoculated with 220 CFU of *P. multocida* P-1059 at day 14 after the second injection and were observed for 14 days.

[d] Serum samples obtained at the time of challenge exposure were assayed in the gel diffusion test against the P-1 fraction.

[e] The P-1 fraction was subjected to SDS-PAGE. A band corresponding to a molecular weight of 44K was isolated, and SDS was removed by treatment with urea.

the IsoGel manual. A blank gel was sliced at 3-mm intervals after electrophoresis and suspended in 0.85% NaCl for pH determination. In one instance, the electrofocused gel was sliced, and two protein components were extracted by electrophoresing the sliced gel at 200 V for 5 h in 0.05 M Tris-glycine buffer (pH 8.5). For IEF crossed immunoelectrophoresis, the focused agar gel column was placed on the cathode side of an agarose plate (105 by 80 by 1.5 mm) containing 8% rabbit anti-CE serum and electrophoresed perpendicularly at a constant voltage of 30 V for 16 h.

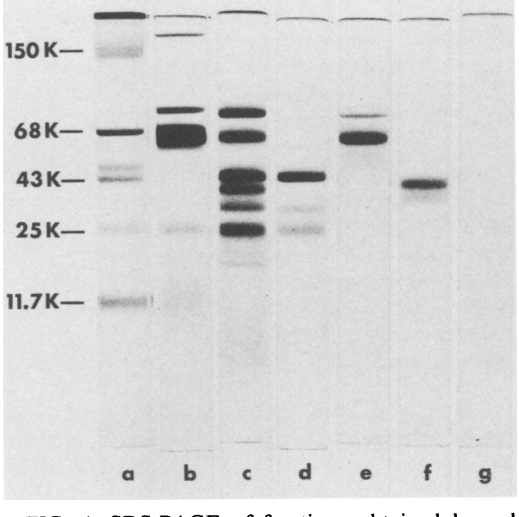


FIG. 4. SDS-PAGE of fractions obtained by gel filtration of CE of *P. multocida* P-1059. Lanes: a, markers; b, extract of growth medium; c, CE; d, fraction P-1; e, fraction P-2; f, fraction P-3; and g, fraction P-4. The numbers on the left represent the molecular weights of the markers (see Fig. 8).

**Other immunological tests.** Gel diffusion tests were done with a standard six-well Ouchterlony pattern in 1% agarose. Immunoelectrophoresis was done by the method of Scheidegger (21).

**Chemical analysis.** Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as the standard (15). Total carbohydrate content was determined by the phenol-sulfuric acid method with glucose as the standard (6).

**Protection test.** Test antigens emulsified in incomplete Freund adjuvant were inoculated subcutaneously to 10- to 20-week-old Nicholas Large White turkeys. Two inoculations were done, with a 14-day interval. At day 14 after the second inoculation, the animals were intramuscularly inoculated with diluted live cultures of *P. multocida* P-1059 (see Tables 2 and 3 for the exact numbers of CFU inoculated). The mortality was checked daily, and survivors were killed at day 14 after exposure to detect *P. multocida* from liver swab cultures. The organism was detected in none of the cultures taken from the surviving birds in all of the protection tests.

**Statistics.** The chi-square test was used to analyze the difference in mortality between immunized groups and nonimmunized control groups.

## RESULTS

**Morphological change by the extraction.** It has been shown previously that CE prepared from *P. multocida* by heat treatment in a 2.5% NaCl solution induces protective immunity in turkeys (14). To examine the effect of extraction on the morphology of *P. multocida*, the bacterial cells were examined before and after the extraction procedure under an electron microscope. The

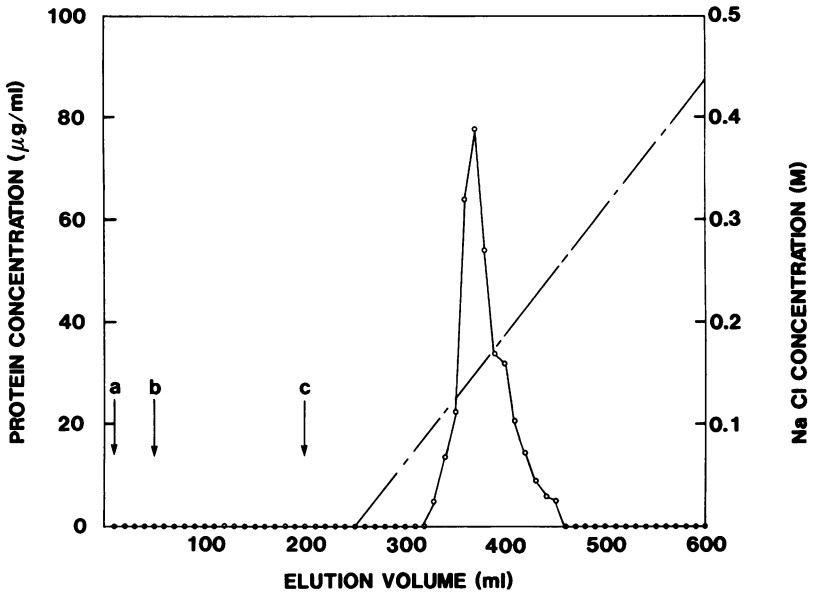


FIG. 5. DEAE-cellulose chromatography of fraction P-1. The fractions obtained from four separate gel filtration runs were pooled, applied to the column (1.5 by 30 cm), and eluted by a linear gradient of NaCl (— - —) from 0 to 0.5 M at 4°C. Protein concentrations (○) were determined by the method of Lowry et al. (15). Sample application (a), elution with 0.01 M sodium phosphate buffer (pH 6.8) (b), and elution with the NaCl gradient (c) are indicated.

treatment caused minor shrinkage of the capsular-like structure on the surface of the organism (Fig. 1). Neither lysis nor decrease in numbers of the organism was evident after the extraction.

**Gel filtration.** CE was fractionated on the Sephadex G-200 superfine column, and fractions corresponding to four protein peaks were pooled (Fig. 2). Fraction P-1 contained 21% of the protein and 49% of the carbohydrate of the original CE (Table 1). The remaining fractions did not contain substantial amounts of carbohydrate. The gel diffusion analysis showed that fraction P-1 contained a major bacterial antigen which was not detected by immunodiffusion in the other fractions (Fig. 3 and Table 2). The precipitin lines observed between fraction P-2 and rabbit anit-CE serum did not appear in the presence of turkey serum, indicating that the fraction contained only turkey serum components that originated in the growth medium. Fraction P-3, however, contained traces of two bacterial antigens (Fig. 3b). In a protection test in turkeys, only the P-1 fraction induced immunity against challenge exposure (Table 2). Fraction P-2, however, induced an antibody response against the P-1 antigen in some animals. The P-3 fraction induced neither protection nor antibodies against the P-1 antigen, indicating that the bacteria-specific antigens in the P-3 fraction are not related to the protective antigen found in the P-1 fraction. An analysis by SDS-PAGE revealed that the P-1 fraction contained four protein components with molecular weights of approximately 80K, 44K, 31K, and 25K (Fig. 4). The 80K and 25K bands corresponded to medium components. The P-2 fraction contained three bands, all of which corresponded to medium components. The two bands observed in the P-3 fraction seem to be bacteria-specific compo-

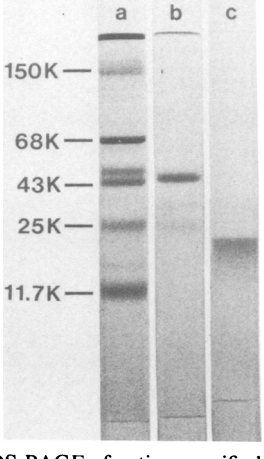


FIG. 6. SDS-PAGE of antigen purified by gel filtration, DEAE-cellulose chromatography, and immunoabsorption. Lanes: a, markers (see Fig. 8); b, antigen stained for proteins with Coomassie blue: c, antigen stained for carbohydrate with periodic acid-Schiff reagent.

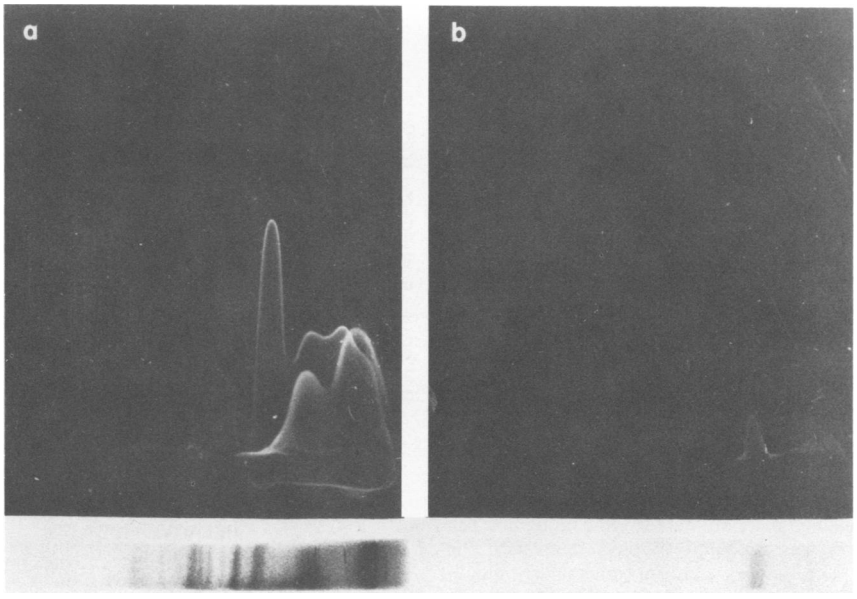


FIG. 7. IEF and subsequent crossed immunoelectrophoresis of the crude and the purified antigen of *P. multocida* P-1059. CE (a) and purified antigen (b) were isoelectrically focused on agarose gel columns with carrier ampholyte, pI 3.5 to 9.5, as shown in the bottom columns. Subsequently, the gels were placed on an agarose gel plate containing 8% rabbit anti-CE serum and electrophoresed perpendicularly.

nents, because no corresponding bands appeared in the growth medium sample. The two bands also did not correspond to any of the bands in the P-1 fraction. This observation supports the results obtained with the gel diffusion analysis. No band appeared in the P-4 fraction. The major 44K band of the P-1 fraction was extracted from the gel and treated with urea to remove SDS. The treated fraction, however, failed to induce an antibody response to the P-1 antigen and immunity against challenge exposure (Table 2). Furthermore, it did not form a precipitin line with antiserum against CE in the gel diffusion test, indicating a complete loss of the antigenicity.

**Further purification.** The P-1 fraction contained a major antigen and small amounts of other components, as determined by immunoelectrophoresis. To remove the minor components, the P-1 fraction was adsorbed onto DEAE-cellulose and eluted by a linear gradient of NaCl. The presence of pasteurella-specific antigen was detected by the gel diffusion analysis with rabbit anti-CE serum mixed with normal turkey serum. Those fractions containing the antigen were analyzed for their total protein contents. A single protein peak with a shoulder was obtained (Fig. 5). The pooled fraction from the entire peak was colorless, whereas the P-1 fraction was opaque with a slight yellow-brownish tinge. The pooled fraction was subsequently passed through an immunoadsorbent coated with antibodies against whole turkey serum to remove any possible serum component. The eluates were pooled and concentrated.

**Purity assay.** The purified fraction had a C/P ratio of 1.50 and formed a single precipitin line in gel diffusion and immunoelectrophoresis with rabbit anti-CE serum. Upon injection to rabbits and turkeys, the purified fraction induced antibodies which formed a single precipitin line against CE. This precipitin line also showed complete identity with those formed between the purified antigen and anti-CE sera. Upon SDS-PAGE analysis, three protein bands and one periodic acid-Schiff reagent-stained carbohydrate band appeared (Fig. 6). The latter band did not correspond to any of the three protein bands, suggesting that a bulk amount of carbohydrate may have been dissociated by the treatment with SDS in the electrophoretic procedure. In the IEF gel analysis the purified fraction showed two bands, a major band (pI = 3.5 to 4.0) and a minor band (pI = 4.5 to 5.5) (Fig. 7). When the two bands were subjected to crossed immunoelectrophoresis, a common antigenicity was detected between them. The two components were separated, electrophoretically extracted from the gel, and tested for immunogenicity in turkeys. Both components failed to induce protection in turkeys in the amount of 100 μg of protein per dose (Table 3).

**Molecular size.** In disc electrophoresis the purified antigen did not migrate in an acrylamide

TABLE 3. Induction of protective immunity in turkeys with various amounts of antigen purified from a 2.5% extract of *P. multocida* and with fractions obtained by IEF of the purified antigen[a]

| Sample (μg of protein per dose) | No. survived/no. inoculated | |
|---|---|---|
| | Expt 1 | Expt 2 |
| Purified antigen | | |
| 250 | 5/6 ($P < 0.01$) | ND |
| 50 | 5/6 ($P < 0.01$) | 6/8 ($P < 0.01$) |
| 10 | ND | 3/7 ($P > 0.05$) |
| 2 | ND | 0/8 |
| Fractions from IEF[b] | | |
| 100[c] | ND | 2/7 |
| 100[d] | ND | 1/7 |
| Unimmunized control | 0/7 | 0/6 |

[a] Turkeys 20 weeks old (experiment 1) or 10 weeks old (experiment 2) were subcutaneously injected with antigen emulsified in incomplete Freund adjuvant. The immunization was repeated 14 days later with the same dose. The turkeys were intramuscularly inoculated with 220 CFU (experiment 1) or 87 CFU (experiment 2) of live *P. multocida* P-1059 at day 14 after the second immunization. The birds were observed for 2 weeks thereafter, and mortality was recorded. ND, Not done.

[b] The purified antigen was subjected to IEF in a column gel as described in the text. The two protein bands were separated, extracted electrophoretically, and dialyzed. Samples from several runs were pooled and injected twice subcutaneously into turkeys with 100 μg of protein per dose in incomplete Freund adjuvant.

[c] Band a, pI = 3.5 to 4.0.

[d] Band b, pI = 4.5 to 5.5.

gel with the concentration of acrylamide as low as 3.5%. The purified antigen was eluted in 37% of the total column volume (2.5 by 95 cm) of Sepharose 6B in a 0.85% NaCl solution when its void volume was 34%. The results indicate that the antigen had a molecular weight of approximately $4 \times 10^6$. The molecular weight of the three proteins of the purified antigen was determined by SDS-PAGE. The three components showed molecular weights of 44K, 31K, and 25K (Fig. 8). The ratio of the protein concentrations of these three components was 71:9:20, and their molar ratio was 10:2:5.

**Minimum protective dose.** The purified antigen was emulsified in incomplete Freund adjuvant and inoculated into turkeys. After subsequent exposure to the live *P. multocida*, protection was observed in animals that received as little as 10 μg of protein (Table 3). The 50% protective dose was between 10 and 50 μg of protein when the injection was done twice. Liver swab cultures did not yield *P. multocida* from any of the survivors in the two experiments.

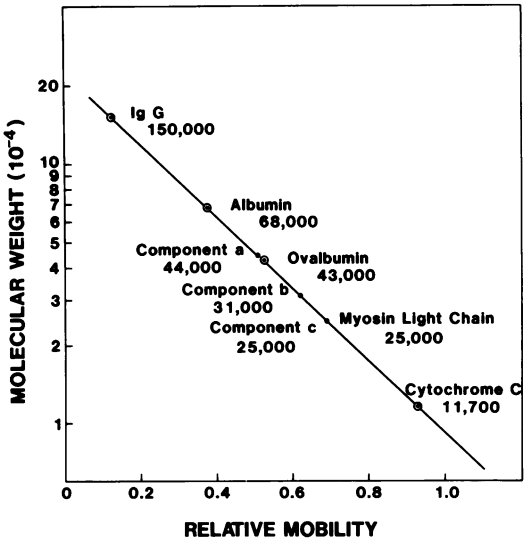


FIG. 8. Molecular weights of the three protein components of the purified antigen, determined by SDS-PAGE.

## DISCUSSION

Our previous study showed that CE obtained from *P. multocida* with a 2.5% NaCl solution is capable of inducing protective immunity in turkeys (14). In the present study, chromatographic methods were applied to purify the immunogenic fraction. Four protein peaks were obtained by gel filtration. The P-1 and P-3 fractions contained bacteria-specific antigens, and only the P-1 fraction induced immunity in turkeys. The P-1 fraction showed the presence of a single antigen in the gel diffusion test, but SDS-PAGE showed a minor contaminant with a molecular weight of 80K. The gel diffusion test and the SDS-PAGE analysis failed to detect bacteria-specific antigen in the P-2 fraction, but the P-2 fraction induced antibodies against the P-1 antigen in some turkeys (Table 2). The fraction may have contained a minor quantity of the P-1 antigen. Two antigens were detected in the P-3 fraction with rabbit antiserum against CE. With the gel diffusion test, the two antigens were shown to be different from the P-1 antigen with rabbit anti-CE serum, but no precipitin line was detected with the P-3 antigen against turkey anti-CE serum. The SDS-PAGE analysis also showed the presence of populations of proteins in the P-3 fraction that were different from those detected in the P-1 fraction (Fig. 4). These results together with the fact that the P-3 fraction failed to induce active immunity indicate that the P-3 fraction did not contain a significant protective antigen.

Anion exchange chromatography and immunoabsorption removed the minor contaminant in

the P-1 fraction. The purified fraction showed a single precipitin line against rabbit and turkey anti-CE sera. When injected into turkeys and rabbits, the purified fraction produced antibodies which formed a single precipitin line against CE, and it induced protection in turkeys in amounts greater than 10 μg of protein per dose. A protein band observed at 80K in SDS-PAGE of the P-1 fraction was removed by the purification, but a protein corresponding to a molecular weight of 25K remained in the purified fraction. Whereas the proteins in the purified fraction with bands at 44K and 31K are apparently bacteria specific, the 25K band corresponded to a band found in the growth medium sample (Fig. 4). The immunological tests mentioned above clearly showed the presence of a single antigenic substance in the purified fraction, but this does not exclude the possibility that a medium component with molecular weight of 25K, probably being nonantigenic by itself, is incorporated into the bacterial antigen. On the other hand, the 25K protein could be produced by the bacteria. Further study is needed to clarify the point. Since the purified antigen has definite capacity of inducing protection, it will be referred to as a protective antigen (PA) hereafter.

The purified PA had a C/P ratio of 1.5. The carbohydrate is not covalently linked to the proteins, because treatment of purified PA with SDS dissociated the carbohydrate moiety (Fig. 6). The immunogenicity of the carbohydrate moiety of purified PA was not tested. A similar carbohydrate antigen from *P. multocida* types B and E is immunogenic in cattle against hemorrhagic septicemia (17). The 50% protective dose, however, is as much as 3 mg. With the present PA, the protein components may be essential for immunogenicity when less than 200 μg of protein per dose of PA is used, because in our previous study, purified carbohydrate of CE retained neither antigenicity nor protective capacity (14). It is not known whether the protein component(s) alone is immunogenic. The major 45K protein component in PA was not immunogenic in turkeys when extracted after SDS-PAGE. The experiment to test the possibility that all three protein components are necessary for the immunogenicity or that the SDS treatment itself may destroy the antigenicity has not been done.

In the IEF analysis the purified PA separated into two bands. The two components had identical antigenicity, and neither fraction induced protection in turkeys. The fractions showed three identical protein bands upon SDS-PAGE (Fig. 6). Therefore, the difference between the two fractions detected in the IEF analysis is not significant in terms of antigenicity of PA. The reason why neither of the two components induced immunity is not known. It is possible that some carbohydrate which might be essential for immunogenicity may have dissociated from protein components in the carrier ampholyte.

The origin of the PA is not known. Antigens which are extracted by procedures similar to one employed in the present study have been referred to as "capsular" antigens (16), but an effort has not been made to elucidate exact topographical location of the antigens in pasteurella cells. Electron micrographs of the organism taken before and after the extraction showed few changes, but there was some shrinkage of capsule-like material on the bacterial surface (Fig. 1). Our previous study showed that when compared with extraction at 56°C for 60 min, extractions at 37 or 0°C for 60 min in a 2.5% NaCl solution yielded 24 and 4%, respectively, of the PA (unpublished data). This observation indicates that the linkage between PA and the cell surface is weak enough to be broken by high ionic strength and mild heat treatment. No direct evidence, however, has been provided in the present study to indicate that the antigen is clearly an extracellular product such as a capsule.

LPS-protein complex, obtained from a formalinized saline extract of *P. multocida*, has previously been shown to be immunogenic in mice, chickens, and turkeys (18). LPS purified from the complex by the Westphal method shows passive immunity only in chickens (19). The original LPS-protein complex contained another protein antigen in addition to LPS. Ganfield et al. (8) showed that the protective LPS-protein complex separates into three fractions by gel filtration with Sepharose 2B. The second peak (peak II) has immunogenicity in turkeys and is antigenically distinct from purified LPS. The purified LPS and the peak II fraction have C/P ratios of 5 to 8 (19) and 2.4 (8), respectively, both of which are considerably higher than the ratio of 1.5 obtained with PA. In another study, a protective activity in chickens was demonstrated in a soluble fraction which was prepared by extracting whole cells of *P. multocida* with potassium thiocyanate (9). Srivastava and Foster (22) have purified a protective antigen from a water extract of strain P-1059 by gel filtration and solvent fractionation. Two fractions were obtained and compared: a glycoprotein fraction was prepared by ether extraction and shows high immunogenicity in mice, and an LPS which is antigenically distinct from the glycoprotein antigen and showed low immunogenicity was prepared by phenol extraction. The glycoprotein antigen has a C/P ratio of 1.43, which is close to that of the PA investigated in the present study. A direct comparison of these antigens in terms of their chemical composition and antigenic

identity is possible only when they are purified from a single source and characterized in a uniform manner.

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