INFECTION AND IMMUNITY, July 1983, p. 313-320
0019-9567/83/070313-08$02.00/0

Vol. 41, No. 1

Separation and Purification of the Hemagglutinins from *Bordetella pertussis*

YUJI SATO,[1] JAMES L. COWELL,[2]* HIROKO SATO,[3] DON G. BURSTYN,[2] AND CHARLES R. MANCLARK[2]

First[1] and Second[3] Departments of Bacteriology, National Institute of Health, Tokyo 141, Japan, and Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20205[2]

Received 9 February 1983/Accepted 23 April 1983

The role of the filamentous hemagglutinin (FHA) and the lymphocytosis-promoting factor hemagglutinin (LPF) in pertussis pathogenesis and immunity is the subject of active investigation. To be certain of their role as protective antigens, the hemagglutinins must be pure and free of each other. This report describes procedures to separate and purify FHA and LPF from the culture supernatant of stationary cultures of *Bordetella pertussis* Tohama, using hydroxylapatite, haptoglobin-Sepharose, and Sepharose CL-6B filtration chromatography. Purified FHA contained less than 0.002% active LPF assayed by histamine-sensitizing activity, and both hemagglutinins contained less than 0.01% of each other based on antigenic activity measured by an enzyme-linked immunosorbent assay, using affinity chromatography-purified antibody to each hemagglutinin. LPF and FHA were also shown to be antigenically distinct by immunodiffusion and were judged to be highly purified proteins by polyacrylamide.gel electrophoresis. In addition, the purification procedures yielded milligram amounts of each hemagglutinin with very good recovery of starting activities.

Phase I strains of *Bordetella pertussis* produce two distinct hemagglutinins (3, 15, 16). The lymphocytosis-promoting factor hemagglutinin (LPF) appears as spherical structures 6 nm in diameter by electron microscopy (4, 15, 22), has a relatively low hemagglutinating activity, and induces a variety of biological responses, including leukocytosis and lymphocytosis, histamine sensitization, lethality, mitogenicity, adjuvant effects, and potentiation of insulin secretion (3, 15, 16, 22, 24). Munoz and Bergman refer to LPF as pertussigen (26), and it is now clear, based on biological activities (24), antigenic properties (7, 21), and chemical and physical properties (15, 16, 22) that LPF is also identical to the islet-activating protein also isolated from *B. pertussis* (32, 33). The filamentous hemagglutinin (FHA) appears as fine filaments about 2 nm in diameter and 40 to 100 nm in length (3, 22) and has a hemagglutinating activity (HA) per milligram of protein five to seven times greater than that of LPF (3, 16).

There is much interest in these hemagglutinins regarding their role in the pathogenesis of and immunity to *B. pertussis*. Active immunization against intracerebral infection of mice (23, 25, 27, 29) and passive immunization against aerosol-induced respiratory infection of mice (30) indicate that both hemagglutinins are protective antigens. Munoz et al. (25), however, have presented data suggesting that their FHA preparations which protected mice from intracerebral challenge did so because of contamination with LPF.

This communication describes the separation and purification of FHA and LPF. In the past, it was difficult to obtain FHA totally free of LPF. The procedures of Arai and Munoz (1) and Arai and Sato (3) resulted in FHA preparations which still contained LPF (25). The FHA preparations obtained by using the procedures of Morse and Morse (22) and Irons and MacLennan (15, 16, 27) have not been rigorously tested for LPF contamination, but these preparations protect mice from intracerebral challenge (16, 23, 27), which could reflect contamination with LPF (25).

To better define FHA as a protective antigen, a procedure is needed to obtain FHA free of LPF. In this paper, we report such a procedure. A preliminary report of part of this work was presented at the International Symposium on Bacterial Vaccines, National Institutes of Health, Bethesda, Md., September 15 to 18, 1980 (7).

MATERIALS AND METHODS

Organism and growth. *B. pertussis* Tohama, phase I, was grown from freeze-dried cultures as described previously (28). For production of FHA and LPF, two

growth procedures were used. Method 1 was done at the National Institute of Health, Tokyo, Japan, and method 2 was done at the National Center for Drugs and Biologics, Bethesda, Md. In method 1, cells from 24-h growth on Bordet-Gengou agar slants were transferred to 500-ml Erlenmeyer flasks containing 200 ml of modified Morse and Bray medium (28). After 20 h of incubation with shaking (150 rpm), the growth was used to inoculate Roux bottles, containing 100 ml of modified Stainer-Scholte medium (14, 31), to a final absorbance at 650 nm of 0.1. In method 2, the procedures described above were followed, except that the inoculum was grown in Cohen-Wheeler medium (5) and used to inoculate 150 ml of modified Stainer-Scholte medium in 650-ml polystyrene tissue culture flasks (Costar). In both methods, the final growth was for 4 to 5 days at 35°C under stationary conditions.

Biological assays. For hemagglutination assays, two-fold serial dilutions of each sample were made in 0.05-ml volumes with 0.01 M sodium phosphate-buffered saline (PBS) (pH 7.4) by using rigid polystyrene V-bottom plates (Dynatech Laboratories). An equal volume of a 0.7% (vol/vol) suspension of washed goose erythrocytes or a 0.7% (vol/vol) suspensions of glutaraldehyde-fixed chicken erythrocytes was added, and the mixture was incubated at 23°C for 1 h. Units of HA in the undiluted sample were expressed as the reciprocal of that dilution of hemagglutinin which, after the addition of the erythrocytes, caused complete agglutination.

Erythrocytes from adult ganders and 1-day-old chickens in Alsever solution were obtained commercially (Truslow Farms, Chestertown, Md.). The chicken cells were fixed with glutaraldehyde after being washed at 4°C twice with Alsever solution and twice in PBS. The washed pellet was resuspended to 10% (vol/vol) in PBS, and 25% glutaraldehyde (Sigma Chemical Co.) was slowly added to a final concentration of 1.8%. The mixture was stirred at 23°C while the glutaraldehyde was added and then stirred at 4°C for 1 h. The fixed cells were washed at 4°C three times in PBS containing 10 mM glycine and once with PBS and resuspended to 0.7% (vol/vol) in PBS containing 0.1% sodium azide, 0.01% gelatin, and 0.1% bovine serum albumin. Unfixed chicken or goose cells were washed three times with PBS and used fresh (within 3 days) in PBS.

Leukocytosis-promoting (LP) and histamine-sensitizing (HS) activities were determined as described previously (3) by using 14- to 16-g female mice, strains N:NIH(S) and HSFS/N (19). One unit of LP activity is that amount of sample giving an increase of 10,000 leukocytes per mm^3.

ELISA. The enzyme-linked immunosorbent assay (ELISA) for detection of LPF or FHA was essentially that described by Maiolini and Masseyeff (18). Concentrations of antigens and conjugates used were determined by checkerboard titrations. The wells of flat-bottomed polystyrene microtiter plates (Dynatech) were sensitized with 50 μl of affinity chromatography-purified goat anti-LPF or anti-FHA at 5 μg/ml in 0.1 M carbonate buffer (pH 9.6). After overnight incubation at 28°C, wells were washed with 0.85% NaCl plus 0.1% Brij 35 (Sigma) in a Multiwash (Flow Laboratories). Samples were diluted in 0.01 M PBS (pH 7.2) containing 0.1% Brij 35 and added to wells in 50-μl volumes. Plates were incubated for 3 h at 28°C and then washed as previously described. Affinity chromatography-purified goat anti-LPF or anti-FHA conjugated with alkaline phosphatase (Sigma; type III) as described previously (9) was added in PBS containing 0.1% Brij 35. Plates were incubated overnight at 28°C and washed, and the enzyme reaction was initiated by the addition of a 1 mg/ml solution of *p*-nitrophenylphosphate (Calbiochem) in 1 M Tris-hydrochloride buffer (pH 9.8) plus 0.3 mM $MgCl_2$. Substrate was added in 100-μl volumes. Enzymatic reactions were measured with a MR580 microelisa reader (Dynatech) at 405 nm when the optical density of the most reactive well was about 1.0. The elapsed time was noted, and all values were extrapolated to 100 min.

Preparation of antibody to FHA and LPF. Yearling goats, 15 to 16 months of age, were bled before immunization as a source of normal gamma globulins. For the preparation of anti-FHA, 500 μg of FHA protein in 50 mM TRIS-hydrochloride (pH 8.0) containing 1 M NaCl was emulsified with 2 volumes of incomplete Freund adjuvant (Difco Laboratories) and injected intramuscularly. After 6 weeks, a booster dose of 150 μg of soluble protein was injected intramuscularly, and the animals were bled 2 weeks later. Anti-LPF was prepared as described above, except that the protein was treated with Formalin (28) and the primary and booster doses were 150 and 100 μg, respectively. The gamma globulin fractions of the sera were prepared by the methods of Harboe and Ingild (12), except that the DEAE-Sephadex chromatography step was omitted.

Preparation of affinity chromatography-purified antibody. Purified FHA or LPF was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described previously (15) with 5 to 10 mg of protein per 3 to 4 g of dry gel. The gamma globulin fraction of the goat anti-FHA or anti-LPF was applied to the appropriate columns (1.5 by 15 cm) equilibrated with 0.1 M TRIS-hydrochloride (pH 7.5) containing 0.5 M NaCl (TNB). The antibodies were applied in TNB containing 1 mM EDTA and eluted with TNB containing 3 M KSCN,

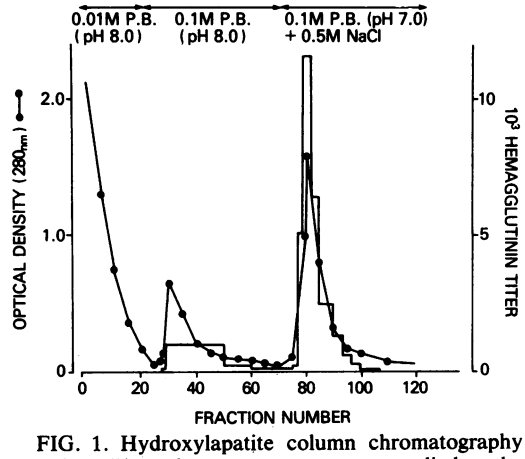

FIG. 1. Hydroxylapatite column chromatography (pH 8.0). The culture supernatant was applied to the column (3 by 35 cm) at a flow rate of 500 ml/h. The column was then washed with PB at 50 ml/h. Each fraction contained 10 ml. Hemagglutination was assayed with fixed chicken erythrocytes.

both at 23°C. The elution buffer was replaced with PBS by dialysis, and antibody activity was measured by ELISA, using a modification of the methods of Engvall and Perlman (9).

Preparation of haptoglobin-Sepharose affinity adsorbent. Haptoglobin was purified from a 10 to 20% polyethylene glycol fraction of pooled human plasma (6, 11) and coupled to CNBr-activated Sepharose 4B as described above.

Chemical assays. Protein was measured by the method of Lowry et al. (17) with bovine serum albumin as the standard. Carbohydrate was determined by the phenol-sulfuric acid method (8), and lipid was determined by the method of Marsh and Weinstein (20) after extraction of the material into chloroform. Amino acid analyses were performed after hydrolysis of samples, sealed under vacuum, in 6 N HCl at 110°C for 22 h by using an amino acid autoanalyzer (Hitachi T835).

Electrophoretic analysis. Analytical polyacrylamide gel electrophoresis was done with the pH 3.8 gel system III as described by Gabriel (10). Stacking gels were not used. Gels were polymerized with either riboflavin or ammonium persulfate. Electrophoresis was conducted at 4 mA per gel at 13 or 23°C. Gels were fixed and stained with 0.01% Coomassie blue G250 in 12.5% trichloroacetic acid for 16 h at 23°C. For final color development, gels were placed in 5% acetic acid.

RESULTS

Separation and purification of FHA and LPF. All purification steps were conducted at 8 to 10°C unless indicated otherwise. After growth, cultures were centrifuged (16,300 × *g* for 20 min), and the culture supernatant (8 to 15 liters) was adjusted to pH 8.7 with 1 N sodium hydroxide. Supernatants were applied at 500 to 1,000 ml/h to a column (3.0 by 35 or 5.0 by 23 cm) of hydroxylapatite (Spheroidal; Gallard-Schlessenger) equilibrated with 0.01 M sodium phosphate buffer (PB) (pH 8.0). It was desirable to obtain the culture supernatant relatively free of bacterial cells, since they bound to the hydroxylapatite and impeded flow through the column. Under these conditions, all the FHA was bound, whereas 90% of the LPF passed through the column. The column was then washed as shown in Fig. 1. The material eluted with 0.1 M PB (pH 8.0) contained less than 1% of the HA applied to the column and about 1% of the original LP activity. FHA was recovered from the column in the cold by a wash with 0.5 M sodium chloride in 0.1 M PB (pH 7.0) at 50 to 100 ml/h. This step was begun with the buffer at room temperature, since salt crystals tended to form in the buffer below 10°C. The fractions containing the bulk of FHA activity were pooled and directly applied at 45 ml/h to a column (2.0 by 6.0 or 1.5 by 13.5 cm) of haptoglobin-Sepharose 4B equilibrated with 0.5 M sodium chloride in 0.1 M PB (pH 7.0). Under these conditions, LPF binds to the column, but FHA passes through. Pooled fractions with FHA activity were precipitated over 16 h with 4.7 g of ammonium sulfate per 10 ml. The pellet, after centrifugation (27,000 × *g* for 20 min), was redissolved in 0.05 M Tris-hydrochloride (pH 8.0)–0.5 M NaCl by dialysis into the same buffer and separated on Sepharose CL-6B (2 by 80 or 2.5 by 92 cm). The single peak of FHA activity (Fig. 2) was pooled and stored at −70°C at 1 mg/ml. FHA has been stored for at least 1 year in this manner without loss of HA. A summary of results of a typical purification of FHA is shown in Table 1.

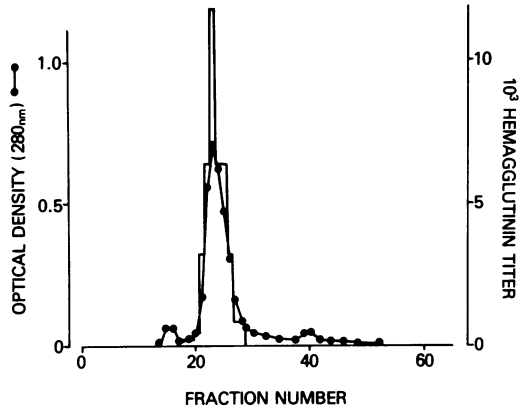

FIG. 2. Sepharose CL6B column chromatography. A concentrated FHA fraction (5 ml; 6 mg of protein per ml) from the haptoglobin-Sepharose 4B column was applied to the column (2 by 80 cm) at 10 ml/h. The elution buffer was 0.05 M Tris-hydrochloride (pH 8.0) containing 0.5 M NaCl. Fractions of 3.3 ml were collected. Hemagglutination was assayed with fixed chicken cells.

TABLE 1. Purification of FHA

Fraction	Vol (ml)	Total protein (mg)	LP activity		HA[a]	
			Total	U/mg	Total (× 10⁶)	U/mg (× 10⁴)
Culture supernatant	14,088	4,212	0.92 × 10⁶	217	72	1.71
Hydroxylapatite eluate	154	188	None[b]		98	52.1
Haptoglobin-Sepharose pass through (concentrated)	14	183	None		67	36.6
Sepharose CL-6B	78	100	None		50	50

[a] With fixed chicken erythrocytes.
[b] With this fraction, HS activity was detected when 61 μg of protein was injected per mouse.

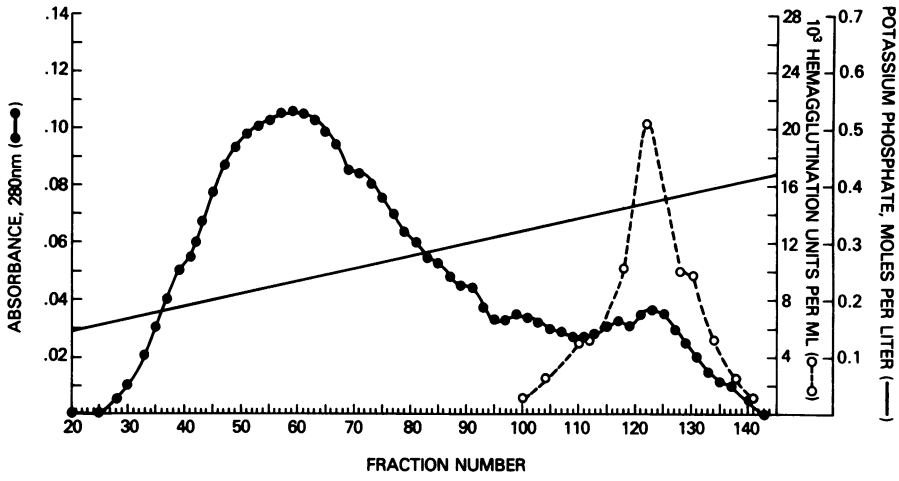

FIG. 3. Hydroxylapatite column chromatography (pH 6.0). The material that passed through the pH 8.0 hydroxylapatite column was applied at pH 6.0 to the column (5 by 16 cm) at a flow rate of 1 liter/h. The column was then washed with a linear gradient (10 to 700 mM) of potassium phosphate buffer (pH 6.0) at 100 ml/h. Each fraction contained 16.5 ml. Hemagglutination was assayed with goose erythrocytes.

LPF was further purified as follows. The solution obtained after passage through the pH 8.0 hydroxylapatite column was adjusted to pH 6.0 with 1 N HCl and applied at 250 to 1,000 ml/h to a column (2.0 by 40 or 5.0 by 16 cm) of hydroxylapatite equilibrated in 0.01 M PB (pH 6.0). Under these conditions, all of the LPF was bound. The column was then washed with a linear gradient (10 to 700 mM) of potassium phosphate buffer (pH 6.0) at about 100 ml/h. The bulk of the protein was eluted between 0.2 to 0.3 M, whereas the LPF was eluted between 0.35 and 0.4 M (Fig. 3). Pooled fractions with LPF activity were applied at 20 to 35 ml/h to a column (2.0 by 6 or 1.5 by 13 cm) of haptoglobin-Sepharose 4B at 23°C in 0.1 M PB (pH 7.0) containing 0.5 M NaCl. The column was washed as shown in Fig. 4. A small amount of material lacking LPF activity was sometimes eluted with 0.1 M PB (pH 8.0) containing 0.5 M NaCl. LPF was eluted with the same buffer containing 3 M potassium thiocyanate. The fractions containing LPF were pooled and dialyzed for 16 h against 1,000 volumes of 0.5 M NaCl in 0.1 M PB (pH 7.0). Some of the LPF precipitated during this dialysis; however, the precipitated LPF could be solubilized in an active form by using the buffer described above containing 4 M urea. The majority of the LPF did not precipitate during dialysis and was further dialyzed against the buffer described above containing 50% glycerol. This LPF was stored at −70°C. LPF has been stored under these conditions for at least 1 year without loss of activity. When stored in 4 M urea, LPF loses about 70% LP activity after 6 weeks at 8°C and about 40% of this activity after thawing from storage at −70°C. A summary of results of a purification of LPF is shown in Table 2.

Electrophoretic analyses. The LPF migrated as a single component (Fig. 5A) when as much as 30 μg of protein was analyzed. The molecular weight of this component, estimated by the procedure of Hedrick and Smith (13) as described by Arai and Sato (3), was 107,000 (data not shown). Based on electrophoretic patterns, two different preparations of FHA were obtained, FHA I was obtained at the Japan National

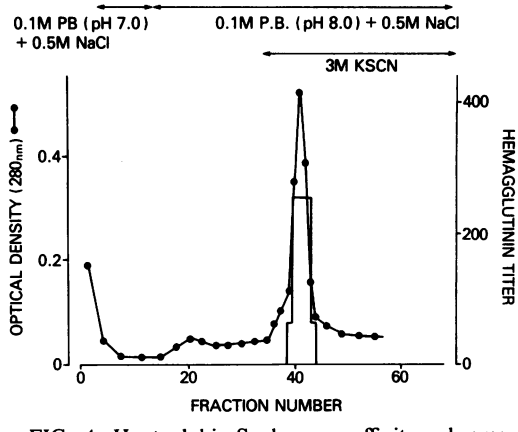

FIG. 4. Haptoglobin-Sepharose affinity chromatography. LPF (100 ml; 70 μg of protein per ml) was applied to the column (2 by 6 cm) at 20 ml/h. The column was washed with PB as shown. Fractions of 2 ml were collected. Hemagglutination was assayed with goose erythrocytes.

TABLE 2. Purification of LPF

Fraction	Vol (ml)	Total protein (mg)	LP activity		HA[a]	
			Total ($\times 10^6$)	U/mg	Total ($\times 10^6$)	U/mg
Culture supernatant	14,088	4,212	0.92	217	72	17,100
Hydroxylapatite pass through (pH 6.2)	15,000	4,200	0.83	196	2.4	571
Hydroxylapatite eluate	285	54	0.79	14,600	1.8	33,200
Haptoglobin-Sepharose	22	22.5	0.89	39,600	2.2	98,000

[a] With goose erythrocytes.

Institute of Health, and FHA II was obtained at the National Center for Drugs and Biologics. FHA I (Fig. 5B) migrated as a single component, whereas FHA II (Fig. 5C) had one major band in addition to a few more slowly moving components. Additional data (B. An der Lan, J. L. Cowell, D. G. Burstyn, C. R. Manclark, and A. Chrambach, manuscript in preparation) indicate that the more slowly moving components in FHA II are oligomeric forms of the monomer (major band). The molecular weight of FHA I was 130,000 by gel electrophoresis (13), which is identical to the molecular weight of the major band in FHA II. Also, both preparations of FHA were antigenically identical as determined by double immunodiffusion against goat anti-FHA II (data not shown). So far, the only major difference observed between FHA I and FHA II is the presence of apparent aggregated forms in FHA II.

Immunodiffusion. To evaluate further the separation and purity of the preparations of FHA and LPF, each was reacted in an immunodiffusion assay against rabbit antibody prepared against a mixture of *B. pertussis* antigens (crude antigen). When reacted with the crude antigen, the antibody detected several antigens but gave only a single precipitin line diffused against purified FHA and LPF (Fig. 6). In addition, the precipitin lines that developed with FHA and LPF showed reactions of antigenic nonidentity.

Additional criteria for separation of FHA and LPF. The following biological and antigenic assays were used to further judge the separation and distinct nature of these two proteins. When injected into the tail vein of mice, 0.025 μg of LPF caused an increase in the peripheral leukocyte count of 10,000/mm^3, and 0.002 μg sensitized 50% of HSFS/N mice to the lethal effects of histamine. LP and HS activities were not

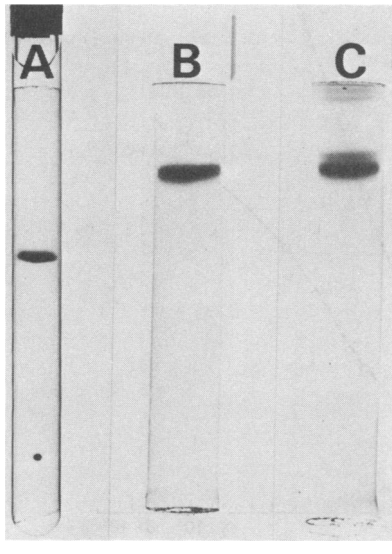

FIG. 5. Electrophoretic analyses. (A) 30 μg of LPF. (B) 25 μg of FHA I. (C) 25 μg of FHA II. The gel composition was 7.5% acrylamide–0.05% bisacrylamide. Electrophoresis was from the top (anode) to the bottom (cathode).

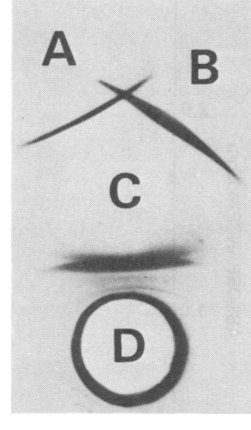

FIG. 6. Immunodiffusion. (A) LPF (200 μg of protein per ml). (B) FHA I (200 μg of protein per ml). (C) Rabbit anti-crude antigen. (D) Crude antigen (20 mg of protein per ml). Identical results were obtained with FHA II. Immunodiffusion was done with 1% agarose in 0.05 M PB (pH 7.2), containing 1 M NaCl. Diffusion was for 24 h at 23°C. After diffusion, the gels were washed, dried, and stained with Coomassie blue R250 by standard procedures. The crude antigen and antibody to the crude antigen were prepared as described previously (30).

TABLE 3. Effect of cholesterol on the HA of purified FHA and LPF

Hemagglutinin	Cholesterol (μM)	HA[a]	
		Goose cells	Chicken cells
LPF	0	3.1×10^4 (100)[b]	0.39×10^4
	125	4.1×10^4 (132)	1.7×10^4
FHA	0	5.3×10^5 (100)	5.3×10^5
	125	0.29×10^5 (5.5)	0.29×10^5

[a] Expressed as units per milligram of protein. The assay was done as described in the text, except that after the dilution of the hemagglutinins in 0.05-ml volumes, an equal volume of cholesterol (Sigma; grade I) or PBS was added. After 15 min at 23°C, 0.05 ml of a 0.7% (vol/vol) suspension of washed goose or unfixed chicken erythrocytes was added, and the mixture was incubated at 23°C for 1 h. A uniform suspension of cholesterol was made by first dissolving it in 99% ethanol and then diluting the sample to 250 μM in PBS at 50°C while vortexing.

[b] Values in parentheses show the percent activity relative to that obtained in the absence of cholesterol.

detected in purified FHA when as much as 140 μg was injected per mouse. The more sensitive HS assay indicated that FHA contained <0.002% of active LPF.

When FHA or LPF was incubated with 125 μM cholesterol, the ability of FHA to agglutinate fresh chicken or goose erythrocytes was inhibited 95%, whereas the ability of LPF to hemagglutinate was not inhibited, but stimulated, especially when chicken cells were used (Table 3). The distinct nature of the purified hemagglutinins was also evidenced by the fact that the HA per milligram of protein of FHA was similar with chicken and goose erythrocytes, whereas the HA of LPF was 10 times greater with goose erythrocytes compared to chicken cells (Table 3).

To test the possibility that each hemagglutinin could contain the other in a biologically inactive form, the ability of each to react with affinity chromatography-purified goat anti-LPF and anti-FHA was measured by using an ELISA (Fig. 7). Anti-FHA could detect FHA at a protein concentration as low as 10 ng/ml but did not react with LPF at a protein concentration of 100 μg/ml. Similarly, anti-LPF could detect LPF at 10 ng of protein per ml but did not react with FHA at 100 μg/ml. These data indicate that cross contamination of LPF and FHA in the respective preparations is less than 0.01%.

Chemical analyses. The carbohydrate content of LPF was about 1%, and lipid was not detected at a limit of less than 0.5%. The carbohydrate content of FHA was 0.4%, and the lipid content

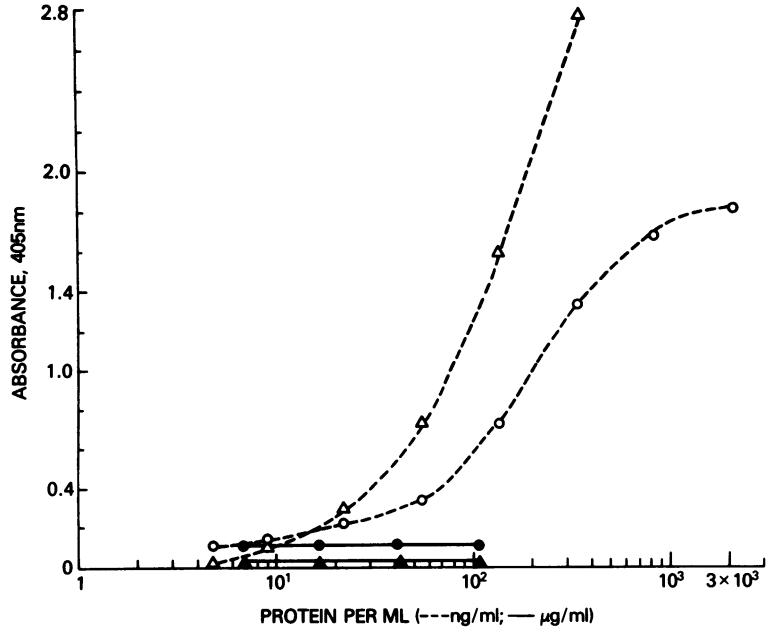

FIG. 7. ELISA for the detection of FHA and LPF. The circles show the reaction of anti-FHA with purified FHA (nanograms of protein per milliliter) (○) and LPF (micrograms of protein per milliliter) (●). The triangles show the reaction of anti-LPF with purified LPF (nanograms of protein per milliliter) (△) and FHA (micrograms of protein per milliliter) (▲).

TABLE 4. Amino acid composition of LPF and FHA

Amino acid	Residue (% of total)	
	LPF	FHA
Aspartic acid	7.5	10.5
Threonine	7.4	6.1
Serine	8.0	7.6
Glutamic acid	9.5	9.0
Proline	6.0	2.2
Glycine	9.7	13.7
Alanine	9.9	15.3
Cysteine	1.9	0.2
Valine	5.7	8.3
Methionine	3.0	1.1
Isoleucine	3.1	3.3
Leucine	8.5	8.6
Tyrosine	5.0	1.3
Phenylalanine	3.2	1.4
Lysine	4.1	4.6
Histidine	1.8	1.7
Arginine	5.7	5.1

was 0.7%. Representative amino acid analyses of LPF and FHA are shown in Table 4. The amino acid composition of LPF is very similar to what is now recognized as the same protein purified by other workers by different procedures (22, 32). The amino acid composition of LPF and FHA are distinctly different.

DISCUSSION

Sato and colleagues (3, 28) have shown that when FHA and LPF are coprecipitated from supernatants of stationary cultures, they copurify when a variety of fractionation methods are used. Possibly, FHA and LPF form a nonspecific complex. These considerations led us to develop a method of separating FHA and LPF directly from stationary culture supernatants in which the protein concentration was low and protein-protein interactions minimal. Spheroidal hydroxylapatite chromatography of the culture supernatant gave a satisfactory separation of the two hemagglutinins and also allowed the handling of large volumes of supernatants. Flow rates through spheroidal hydroxylapatite of 1 to 2 liters/h could be used. At a high pH (8.5 to 9.0), FHA was bound to the column, whereas most of the LPF was not. The LPF which passed through the column was recovered in a purified and concentrated form by applying the material at pH 6.0 to another hydroxylapatite column.

Irons and MacLennan (15, 16) have shown that LPF, but not FHA, binds to an affinity column of haptoglobin coupled to Sepharose 4B. We used this procedure to further purify LPF and to remove trace amounts of LPF still present in the FHA eluted from hydroxylapatite. Thus, FHA was made free of LPF by hydroxylapatite and haptoglobin-Sepharose chromatography. Recently, Askelöf and Gillenius (4) used fetuin instead of haptoglobin as the ligand coupled to Sepharose 4B to purify LPF. We have also found that fetuin-Sepharose 4B can replace the haptoglobin-Sepharose 4B in our purification procedures.

A major goal of this research was to obtain FHA free of LPF in sufficient quantity for further study. FHA contained less than 0.002% of active LPF assayed by HS activity, and both hemagglutinins contained less than 0.01% of each other based on antigenic activity measured by ELISA. Thus, both hemagglutinins were obtained free of detectable contamination by the other. In addition, the purification procedure yielded milligram amounts of each hemagglutinin with very good recovery of starting activities (Tables 1 and 2). The hemagglutination caused by FHA and LPF was also distinct. As previously reported (2, 16), the hemagglutination produced by LPF was of a granular type, and only the HA of FHA was inhibited by micromolar concentrations of cholesterol (Table 3). Also, the HA per milligram of protein of FHA was similar when fresh chicken or goose erythrocytes were used, whereas the HA of LPF was 10 times greater with goose erythrocytes compared to chicken cells.

Besides separating the two hemagglutinins in high yield, our purification procedures gave highly purified and distinct proteins based on gel electrophoresis, immunodiffusion, and chemical analyses. We are now using these proteins in immunization studies with mouse animal models to help determine their roles as protective antigens against *B. pertussis* infection. We have found (M. Oda, J. L. Cowell, D. G. Burstyn and C. R. Manclark, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B51, p. 26) that the purified, detoxified LPF protected mice from both intracerebral and respiratory infection with *B. pertussis*, but the purified FHA protected only from respiratory infection. This is consistent with the data of Munoz et al. (25).

A major concern in pertussis vaccine research is to produce a vaccine which contains the necessary protective antigens with a minimum content of toxic components. The above purification procedures could prove useful in the development and production of such a vaccine.

ACKNOWLEDGMENTS

We thank Dave Hussong for technical assistance, Masaaki Oda and Ronald Sekura for kindly supplying affinity chromatography-purified goat antibody to FHA and LPF, and Birgit An der Lan for helpful discussions concerning electrophoretic analysis of FHA.

LITERATURE CITED

1. **Arai, H., and J. J. Munoz.** 1979. Purification and crystallization of fimbrial hemagglutinin from *Bordetella pertussis*.

Infect. Immun. 25:460–462.
2. **Arai, H., and J. J. Munoz.** 1981. Crystallization of pertussigen from *Bordetella pertussis*. Infect. Immun. **31**:494–499.
3. **Arai, H., and Y. Sato.** 1976. Separation and characterization of two distinct hemagglutinins contained in purified leukocytosis-promoting factor from *Bordetella pertussis*. Biochim. Biophys. Acta **444**:765–782.
4. **Askelöf, P., and P. Gillenius.** 1982. Effect of lymphocytosis-promoting factor from *Bordetella pertussis* on cerebellar cyclic GMP levels. Infect. Immun. **36**:958–961.
5. **Cohen, S. M., and M. W. Wheeler.** 1946. Pertussis vaccine prepared with phase-I cultures grown in fluid medium. Am. J. Public Health **36**:371–376.
6. **Connell, G. E., and R. W. Shaw.** 1961. The purification of haptoglobin. Can. J. Biochem. Physiol. **39**:1013–1019.
7. **Cowell, J. L., Y. Sato, H. Sato, B. An der Lan, and C. R. Manclark.** 1982. Separation, purification and properties of the filamentous hemagglutinin and the leukocytosis promoting factor-hemagglutinin from *Bordetella pertussis*. Semin. Infect. Dis. **4**:371–379.
8. **Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. **Engval, E., and P. Perlman.** 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
10. **Gabriel, O.** 1971. Analytical disc gel electrophoresis. Methods Enzymol. **22**:565–578.
11. **Hao, Y. L., and M. Wickerhauser.** 1978. An integrated plasma fractionation system based on use of polyethylene glycol, p. 372–379. *In* H. E. Sandberg (ed.), Proceedings of the international workshop on technology for protein plasma fractionation. DHEW publication no. (NIH) 78-1422. U.S. Government Printing Office, Washington, D.C.
12. **Harboe, N., and A. Ingild.** 1973. Immunization, isolation of immunoglobulins, and estimation of antibody titer. Scand. J. Immunol. **2**(Suppl. 1):15–169.
13. **Hedrick, J. L., and A. J. Smith.** 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**:155–164.
14. **Hewlett, E. L., and J. Wolff.** 1976. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*. J. Bacteriol. **127**:890–898.
15. **Irons, L. I., and A. P. MacLennan.** 1979. Isolation of the lymphocytosis promoting factor-hemagglutinin of *Bordetella pertussis* by affinity chromatography. Biochim. Biophys. Acta **580**:175–185.
16. **Irons, L. I., and A. P. MacLennan.** 1979. Substrate specificity and purification by affinity combination methods of the two *Bordetella pertussis* hemagglutinins, p. 338–349. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. DHEW Publication no. 79-1830. U.S. Government Printing Office, Washington, D.C.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Maiolini, R., and R. Masseyeff.** 1975. A sandwich method of enzymeimmunoassay. I. Application to rat and human alpha-feto protein. J. Immunol. Methods **8**:223–234.
19. **Manclark, C. R., C. T. Hansen, P. E. Treadwell, and M. Pittman.** 1975. Selective breeding to establish a standard mouse for pertussis vaccine bioassay. II. Bioresponses of mice susceptible and resistant to sensitization by pertussis vaccine HSF. J. Biol. Stand. **3**:353–363.
20. **Marsh, J. B., and D. B. Weinstein.** 1966. Simple charring method for determination of lipids. J. Lipid Res. **7**:574–576.
21. **Mizushima, Y., A. Imaizumi, T. Ogita, and T. Nakamura.** 1982. Immunologic, adjuvant and islet cell activating protein of *Bordetella pertussis*. Semin. Infect. Dis. **4**:390–394.
22. **Morse, S. I., and J. H. Morse.** 1976. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J. Exp. Med. **143**:1483–1502.
23. **Morse, S. I.** 1976. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. **20**:9–26.
24. **Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski.** 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect. Immun. **33**:820–826.
25. **Munoz, J. J., H. Arai, and R. L. Cole.** 1981. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from *Bordetella pertussis*. Infect. Immun. **32**:243–250.
26. **Munoz, J. J., and R. K. Bergman.** 1977. *Bordetella pertussis*. Immunological and other biological activities, p. 1–235. *In* N. Rose (ed.), Immunological series, vol. 4. Marcel Dekker, Inc., New York.
27. **Robinson, A., D. C. Hawkins, and L. I. Irons.** 1981. The preparation of distinct protective antigens from *Bordetella pertussis*. FEMS Microbiol. Lett. **10**:241–244.
28. **Sato, Y., H. Arai, and K. Suzuki.** 1974. Leukocytosis-promoting factor of *Bordetella pertussis*. III. Its identity with protective antigens. Infect. Immun. **9**:801–810.
29. **Sato, Y., K. Izumiya, M. A. Oda, and H. Sato.** 1979. Biological significance of *Bordetella pertussis* fimbriae or hemagglutinin: a possible role of the fimbriae or hemagglutinin for pathogenesis and antibacterial immunity, p. 51–57. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. DHEW Publication no. 79-1830. U.S. Government Printing Office, Washington, D.C.
30. **Sato, Y., K. Izumiya, H. Sato, J. L. Cowell, and C. R. Manclark.** 1981. Role of antibody to leukocytosis-promoting factor hemagglutinin and to filamentous hemagglutinin in immunity to pertussis. Infect. Immun. **31**:1223–1231.
31. **Stainer, D. W., and M. J. Scholte.** 1962. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63**:211–220.
32. **Yajima, M., K. Hosoda, Y. Kanbayashi, T. Nakamura, K. Nogimori, Y. Mizushima, Y. Nakase, and M. Ui.** 1978. Islet-activating protein (IAP) in *Bordetella pertussis* that potentiates insulin secretory responses of rats. Purification and characterization. J. Biochem. (Tokyo) **83**:295–303.
33. **Yajima, M., K. Hosoda, Y. Kanbayashi, T. Nakamura, I. Takahashi, and M. Ui.** 1978. Biological properties of islets-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. J. Biochem. (Tokyo) **83**:305–312.