

## Comparison of the *Pasteurella haemolytica* A1 Envelope Proteins Obtained by Two Cell Disruption Methods†

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**The French pressure cell and sonication methods of bacterial cell disruption were compared for the evaluation of surface proteins from *Pasteurella haemolytica* A1. Several protein bands were quantitatively different when compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and densitometry. With densitometry, sonicated preparations had higher concentrations of the 102-, 83-, 50-, 38-, and 30-kilodalton (kDa) proteins; French pressure cell preparations had higher concentrations of the 96-, 65-, and 42-kDa proteins. Qualitative differences between these two disruption methods were evident at the 102-, 96-, 91-, 50-, 38-, and 30-kDa protein bands. However, significant differences ( $P < 0.05$ ) were detected between the two methods at only the 102-, 96-, 91-, and 50-kDa bands.**

Bovine pneumonic pasteurellosis (shipping fever) is most frequently associated with *Pasteurella haemolytica* biotype A serotype 1 infection of feedlot cattle (10). Despite numerous attempts, *P. haemolytica* vaccines have been largely unsuccessful in preventing the natural disease (11). Several experimental vaccines, including live *P. haemolytica* (4, 13), bacterins (4, 16), and various other bacterial components (6, 17), have been studied. Many uncertainties still exist concerning the potential efficacy of these vaccines when tested in the field.

The major surface antigens of *P. haemolytica* have excellent potential as immunizing agents but have not been examined thoroughly (14). The initial step in isolating surface antigens is to obtain bacterial envelopes by cell disruption. There are two commonly used methods of bacterial cell disruption. These are pressure shearing with a French pressure cell (FPC) and lysis by ultrasonic treatment (3). The only published information on *P. haemolytica* A1 surface antigens was obtained by the FPC method (14). Ultrasonication is a more rapid and less labor-intensive technique, but information on its effect on *P. haemolytica* is not available.

The purpose of this study was to obtain cell envelopes by sonication and FPC shearing of *P. haemolytica* A1 and to analyze the surface antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and densitometry.

### MATERIALS AND METHODS

**Bacterial growth and harvesting.** *P. haemolytica* A1 was originally isolated from a feedlot steer and maintained as described previously (12). The culture was grown (6 h at 37°C in a 10% CO<sub>2</sub> incubator) on supplemented (5% bovine blood) brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Bacteria were harvested in phosphate-buffered saline (0.01 M, pH 7.4), centrifuged (13,000 × g, 30 min, 4°C), washed once in phosphate-buffered saline, and stored at -20°C.

**Sonication.** Frozen whole-cell suspensions were thawed at

4°C overnight. Ten milliliters was removed and centrifuged at 20,000 × g for 20 min (14). The pellet was suspended in 30 ml of 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.4; ICN Pharmaceuticals Inc., Cleveland, Ohio). Lysozyme (0.1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated at room temperature for 15 min. The suspension was sonicated on ice five times for 30 s each time at the maximum setting for the microtip, with 30-s rest periods between each sonication (Cell Disruptor 200; Branson Sonic Power Co., Danbury, Conn.). This preparation was centrifuged twice at 5,000 × g for 20 min each time to remove whole cells (3). The supernatant was centrifuged at 226,000 × g (50 Ti rotor, L8-60MR Ultracentrifuge; Beckman Instruments, Inc., Fullerton, Calif.) for 90 min. The pellet containing the cell envelopes was washed in cold, sterile distilled water three times (14). The remaining pellet was suspended in 3 ml of cold, sterile distilled water and stored at -20°C.

**FPC method.** A frozen whole-cell suspension was thawed and centrifuged as described above. Briefly, bacteria were suspended in 40 ml of cold 20% sucrose (wt/vol) in 0.01 M HEPES buffer (pH 7.4). One milligram each of DNase and RNase (Sigma) was added. Bacteria were passed once through an FPC (20K; SLM Instruments, Inc., Urbana, Ill.) at 15,000 to 20,000 lb/in<sup>2</sup> (14). The suspension of cell envelopes was incubated at 37°C for 40 min and centrifuged at 6,000 × g for 15 min. Two to three milliliters of sucrose-HEPES buffer and 0.1 mg of lysozyme per ml was added to the supernatant fluid, and the mixture was centrifuged at 226,000 × g (50 Ti rotor, L8-60MR Ultracentrifuge) for 90 min (14). The pellet containing the cell envelopes was washed in cold, sterile distilled water three times. The remaining pellet was suspended in 3 ml of cold, sterile distilled water and stored at -20°C.

**SDS-PAGE.** The protein content of each sample was determined (Bio-Rad Laboratories, Richmond, Calif.) and adjusted to a concentration of 1 mg/ml. Cell preparations were analyzed by discontinuous SDS-PAGE with a 10% acrylamide (Sigma) resolving gel and a 2% acrylamide stacking gel (9). After electrophoresis, the gels were stained with Coomassie brilliant blue R (Sigma). The molecular weights of the proteins were determined from their positions in the gel relative to those of known protein standards (1).

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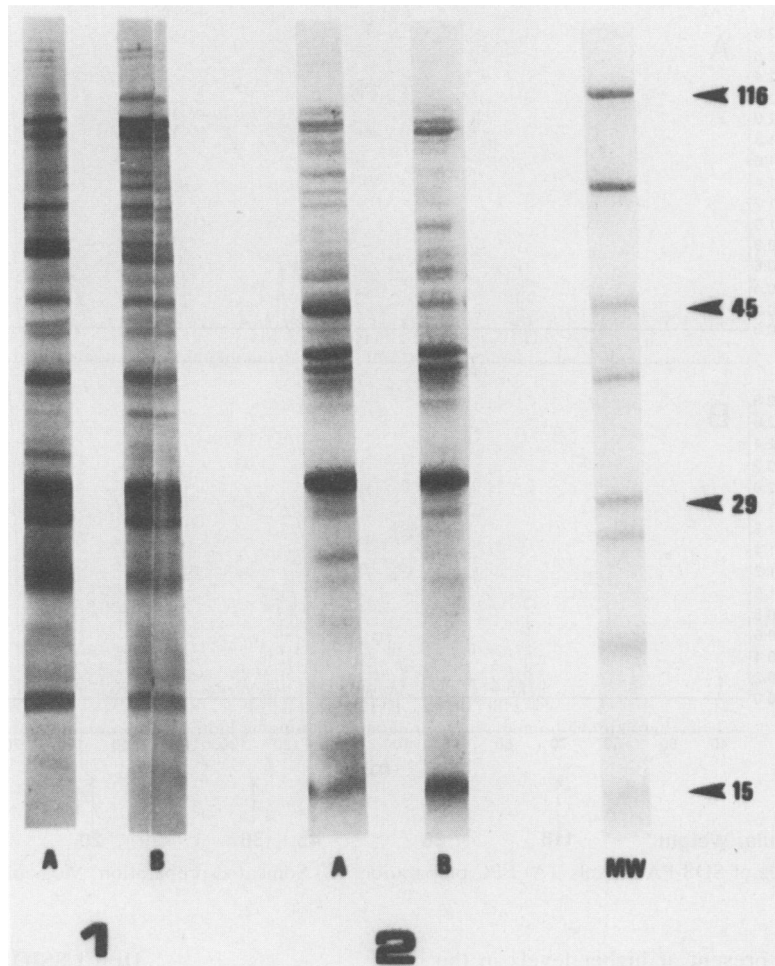


FIG. 1. SDS-PAGE (panel 2) and immunoblotting (panel 1) banding patterns of FPC-treated (lanes B) and sonicated (lanes A) *P. haemolytica* A1. MW, Molecular weight in thousands.

**Immunoblotting.** After SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) by the method of Towbin et al. (15). The membrane strip containing the molecular weight protein standards was removed, and the marker proteins were demonstrated with hyperimmune rabbit serum, biotinylated protein A, and streptavidin-horseradish peroxidase.

The remainder of the membrane containing the *P. haemolytica* protein was treated with bovine hyperimmune antiserum against *P. haemolytica* A1 diluted 1:25 in phosphate-buffered saline-Tween 20 buffer containing 1% gelatin for 1 h. After two washes, the membrane was incubated for 1 h in a 1:400 dilution of biotinylated, affinity-purified goat anti-bovine immunoglobulin G (Kirkegaard and Perry). After two additional washes, the membrane was incubated for 30 min with a streptavidin-horseradish peroxidase complex (Amersham Corp., Arlington Heights, Ill.), and the color was developed with 4-chloro-2-naphthol and hydrogen peroxide.

**Densitometry.** Dried gels (model 443 Slab Dryer; Bio-Rad) and nitrocellulose membranes were analyzed by densitometry in the reflectance mode (model 620 Video Densitometer; Bio-Rad). Analyses were performed with 1-D Gel Analyst software (Bio-Rad). Data are expressed as percentages of the total peak area optical density for each band relative to

the total peak area optical density for all bands. Data are presented as percent total peak area optical density.

**Statistics.** Differences in the same band from the two disruption methods were detected by Student's *t* tests (2).

## RESULTS

The data presented are the results of five separate analyses. Numerous protein bands were detected by SDS-PAGE for the FPC and sonicated samples (Fig. 1). Densitometric scans of the SDS-PAGE gels revealed 21 detectable protein bands for the FPC samples and 28 bands for the sonicated samples (Fig. 2). There were quantitative and statistical differences in the percentages of membrane surface proteins detected densitometrically for the two preparations (Table 1). The sonicated preparations had higher levels of the 102- and 83-kilodalton (kDa) proteins and lower levels of the 96-, 65-, and 42-kDa proteins than did the FPC preparations, as indicated by the mean percentages of the optical densities for each band. The levels of the 50-, 38-, and 30-kDa proteins were markedly different between the two preparations, being higher in the sonicated preparations.

Densitometric examination of the immunoblot revealed banding patterns similar to those in the SDS-PAGE gels, with 22 and 29 detectable bands for the FPC and sonicated preparations, respectively. As with SDS-PAGE, the 96- and

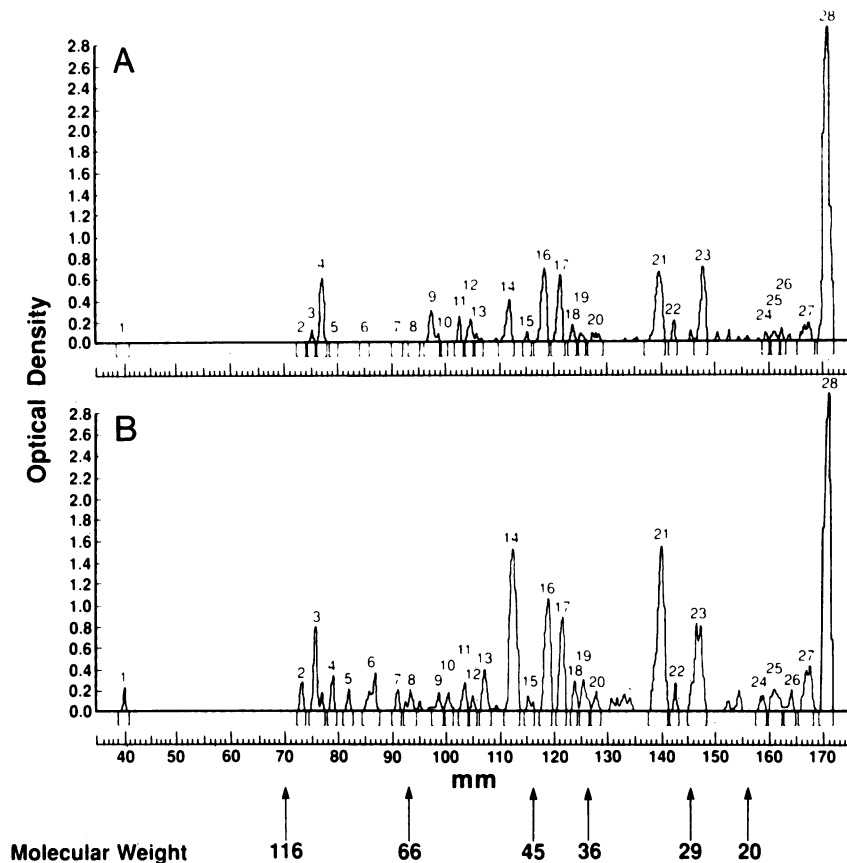


FIG. 2. Densitometric analyses of SDS-PAGE gels. (A) FPC preparation. (B) Sonicated preparation. Molecular weights are in thousands.

42-kDa protein bands were present at higher levels in the FPC preparations than in the sonicated preparations. The levels of the 50- and 38-kDa proteins were also noticeably different between the two preparations, both being higher in the sonicated preparations. The data for the 102-, 96-, 91-, and 50-kDa protein bands were statistically significant ( $P < 0.05$ ), with data for the 96-kDa protein band being highly significant ( $P = 0.001$ ).

TABLE 1. Comparison of densitometric areas of selected protein bands from SDS-PAGE of FPC-treated and sonicated *P. haemolytica* A1

Peak no. <sup>a</sup>	Mean % of total peak area $\pm$ SD for:		MW ( $10^3$ )	$R_f$ <sup>b</sup>	$P^c$
	FPC prepn	Sonicated prepn			
3	2.39 $\pm$ 1.08	4.91 $\pm$ 1.57	102	0.37	0.021
4	5.42 $\pm$ 1.08	1.40 $\pm$ 0.71	96	0.38	0.001
5	0.62 $\pm$ 0.58	1.74 $\pm$ 0.81	91	0.40	0.035
6	2.01 $\pm$ 1.64	2.65 $\pm$ 1.82	83	0.43	0.358
9	2.55 $\pm$ 1.23	1.69 $\pm$ 0.54	65	0.50	0.311
14	5.06 $\pm$ 1.08	10.36 $\pm$ 2.29	50	0.58	0.017
16	7.42 $\pm$ 0.87	7.33 $\pm$ 1.75	44	0.62	0.910
17	6.27 $\pm$ 2.31	4.92 $\pm$ 2.03	42	0.64	0.159
19	2.11 $\pm$ 1.30	2.61 $\pm$ 0.45	38	0.66	0.280
21	11.30 $\pm$ 0.77	13.45 $\pm$ 2.20	30	0.74	0.136
23	7.02 $\pm$ 2.49	7.21 $\pm$ 2.29	26	0.79	0.814
27	4.94 $\pm$ 2.27	4.62 $\pm$ 1.15	15	0.90	0.785

<sup>a</sup> Numbers correspond to those in Fig. 2.

<sup>b</sup>  $R_f$ , Relative mobility.

<sup>c</sup> Significant at  $<0.05$ .

## DISCUSSION

The present study indicates that there is a quantifiable variation in bacterial envelopes obtained by the two cell disruption procedures. Recent articles have focused on outer membrane proteins (OMPs) from a variety of organisms (1, 7, 8). Dunn et al. (5) have shown that there is little difference between the two methods for OMP yield with *Campylobacter jejuni*.

In the present study, the reason for quantitative differences in the envelope preparations obtained by the two techniques is unknown. Both disruption method samples were obtained from the same serotype, strain, and bacterial preparation. One possible reason may be that the FPC dissociates some OMP from the envelope because of shearing forces. Modifications that would enhance the yield of both techniques were not studied. Extension of the sonication time and multiple passages through the FPC would be examples of modifications. These modifications would help determine if the differences seen in protein yield with SDS-PAGE, immunoblotting, and densitometry were true differences.

Further studies will be conducted with the sonication disruption technique. This disruption technique yields higher levels of the proteins that we feel may be important antigenically. The effectiveness of OMPs as components in vaccines for shipping fever is yet to be determined. However, experimental vaccine work can be optimized by using this high-yield technique for OMPs.

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