Characterization of envelope proteins from *Pasteurella haemolytica* and *Pasteurella multocida*

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A method was devised for the reproducible isolation of envelopes from *Pasteurella haemolytica* serotype A2. It was also possible to prepare envelopes from other serotypes of *P. haemolytica* and *Pasteurella multocida* using this methodology. Examination of these preparations by SDS-PAGE showed major differences between strains of *P. haemolytica* and strains of *P. multocida* which allowed the clear distinction of isolates of these species. Amongst the *P. haemolytica* serotypes it was possible to distinguish envelope preparations made from A biotype and T biotype organisms easily, but it was not possible to identify individual serotypes from each other. Envelope profiles were sufficiently different between the individual *P. multocida* serotypes examined to allow each to be identified by its polypeptide profile. Experiments using radiolabelling, antibody absorption, and susceptibility to protease digestion, together with heat modifiability and detergent solubility characteristics indicated that 13 of the envelope proteins were probably surface-located. A high molecular mass immunogenic envelope protein was shown, by immunoblotting, to be present in all strains of *P. haemolytica* and *P. multocida* examined.

Introduction

Pasteurella haemolytica is responsible for respiratory and septicaemic infections of cattle, sheep and goats. To date, 16 serotypes (Biberstein, 1978; Fodor *et al.*, 1988) have been identified, belonging to two biotypes; A (majority arabinose-fermenting) and T (majority trehalose-fermenting). Serotyping depends on the presence of surface capsular polysaccharides, and to date five of these have been purified and characterized (Adlam *et al.*, 1984, 1985a, 1985b, 1986, 1987).

Prevention of pneumonic pasteurellosis is at present effected by vaccination using formalin-killed whole-cell vaccines, extracts of whole cells or live attenuated vaccines. Protection with current vaccines is variable, with little or no protection being provided by extract vaccines against heterologous serotype challenge (Gilmour *et al.*, 1983). Extract vaccines prepared from serotype A2 (the serotype of most importance in ovine pasteurellosis) have not proved useful, probably because of the poor immune response to the serotype-specific capsular polysaccharide, which is composed of sialic

acid, a common constituent of host membranes (Adlam *et al.*, 1987). Development of a better vaccine giving protection against all serotypes might be facilitated by the identification of common antigens.

In the present study we have isolated and analysed the envelopes from several serotypes of *P. haemolytica* with a view to determining whether such common protein antigens exist. In addition, envelopes have been compared with those isolated from the related pathogen *P. multocida*. The putative surface location of envelope polypeptides has been inferred by their fractionation in sucrose density gradients, susceptibility to protease degradation, ability to be radiolabelled on intact organisms, heat modifiability, and reaction with immune antisera.

Methods

Reagents. All chemicals were of AnalaR grade and obtained from BDH unless otherwise stated.

Bacteria. Strains and serotypes of *P. haemolytica* and *P. multocida* used are given in Table 1. All bacteria were maintained freeze-dried in the Wellcome Culture Collection. In all cases serology was confirmed by taking single colonies of each strain and submitting them to passive haemagglutination (Biberstein, 1978).

Abbreviations: CBB, Coomassie Brilliant Blue; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-octulosonate); PMSF, phenylmethylsulphonyl fluoride; TLCK, *N*- α -tosyl-L-lysine chloromethyl ketone.

Table 1. Strains of *P. haemolytica* and *P. multocida* used

Serotype*	Wellcome culture number
<i>P. haemolytica</i>	
A1	5492, 7766, 7770, 7827
A2	358, 832, 4610, 4676, 7776, 8439
T3	5494, 6950, 7800, 8605
T4	5495, 7801, 7785
A5	5496, 7837
A6	5497, 7775, 7777, 7778
A7	5498, 7318, 7768, 7802
A8	5499, 7803, 8284
A9	5500, 7773, 7774, 7767
T10	4596, 4611, 7804
A11	7806, 7842
A12	5503, 7807, 8332
A13	7844
A14	7845
T15	4598, 8263
<i>P. multocida</i>	
A	5517, 7826, 8333
B	4110, 5523, 5835, 6864
D	5518, 5837, 8146, 8151
E	3780, 3781, 5522

* Serotyping was carried out by passive haemagglutination (Biberstein, 1978).

Growth of bacteria. Organisms were cultured for 15 h at 37 °C in one litre of Pasteurella 7 broth (Wellcome) in two-litre conical flasks with orbital shaking (140 r.p.m.).

Radiolabelling of *P. haemolytica* cells. Cells were washed in 100 ml 0.02 M-Tris/HCl (pH 7.8) at 4 °C and centrifuged at 5000 g for 20 min. The pellets were resuspended in ~5 ml 0.02 M-Tris/HCl (pH 7.8) and kept on ice prior to labelling with ¹²⁵I as follows. Fifty Iodobeads (Pierce and Warriner) were incubated at room temperature with 5 µl Na¹²⁵I (Amersham) (250 µCi; 9.25 MBq) and 995 µl 0.02 M-Tris/HCl (pH 7.8) for 5 min with occasional shaking. The bacterial suspension (5 ml) was added to the Iodobeads and maintained on ice for 15 min with mixing. The cell suspension was removed by pipette and centrifuged at 6000 g for 10 min. Cells were washed three times with 5 ml 0.02 M-Tris/HCl (pH 7.8) to remove excess ¹²⁵I, prior to resuspension in 2 ml of this buffer.

Protease treatment of *P. haemolytica* cells. Cells were resuspended in 10 ml portions of 10 mM-Tris/HCl (pH 7.8) and incubated at 37 °C with protease (proteinase K, 40 µg ml⁻¹, or trypsin, 200 µg ml⁻¹). After 1 h the reaction was terminated by addition of phenylmethylsulphonyl fluoride (PMSF, Sigma; for proteinase K treated cells) or *N*-α-p-tosyl-L-lysine chloromethyl ketone (TLCK; for trypsin treated cells) to a final concentration of 0.02 M. The mixture was centrifuged for 20 min at 20000 g at 4 °C and the pellet resuspended in 0.2 M-Tris/HCl buffer (pH 7.8) containing 0.01 M-EDTA and 0.005 M each of PMSF and TLCK, prior to preparation of envelopes from the cells by the standard method.

Protease treatment of envelope preparations. Envelope preparations suspended in 0.01 M-Tris/HCl (pH 7.8) containing 0.005 M-EDTA were incubated at 37 °C with protease (proteinase K, 40 µg ml⁻¹ or trypsin, 2% of total protein). After 1 h the reaction was terminated by adjusting to 0.02 M with PMSF or TLCK. The mixture was centrifuged for 1 h at 150000 g at 4 °C, the pellet resuspended in 0.01 M-Tris/HCl (pH 7.8) containing 0.005 M-EDTA and 0.002 M of PMSF and TLCK and stored at -40 °C.

Isolation of bacterial envelopes. Envelopes were prepared from bacteria grown in Pasteurella 7 broth using a modification of the Witholt method (Witholt *et al.*, 1976). Cells were harvested by centrifugation for 20 min at 4 °C and 6000 g. The pellet was resuspended in 200 mM-Tris/HCl (pH 7.8), 10 mM-EDTA, 4 mM-PMSF containing lysozyme (200 mg ml⁻¹) and DNAase I and RNAase (each at 10 µg ml⁻¹) and incubated at 37 °C for 90 min. The suspension was then diluted with three vols cold distilled water and centrifuged twice for 20 min at 4 °C and 6000 g. The resulting supernatant was recentrifuged for 1 h at 4 °C and 150000 g. The pellet was then washed twice in 10 mM-Tris/HCl (pH 7.8), 10 mM-EDTA, 2 mM-PMSF by centrifugation for 1 h at 4 °C and 150000 g. The washed envelope pellet was finally resuspended in a minimal amount of 0.01 M-Tris/HCl (pH 7.8) containing 5 mM-EDTA and 0.001 M-PMSF and either used immediately or stored at -20 °C.

Fractionation of envelope preparations. (i) *Sucrose density gradient centrifugation.* Envelopes (~3 mg protein) were applied to the top of a continuous sucrose density gradient (55–30%, w/w, sucrose, total gradient volume 19 ml) and centrifuged for 18 h at 100000 g at 4 °C. Fractions (1.5 ml) were collected from the base of the tube using a peristaltic pump. Sucrose concentrations were estimated by refractometry.

(ii) *Extraction with Triton X-100.* Each envelope sample was extracted once with 0.01 M-Tris/HCl (pH 7.2) containing 2% (v/v) Triton X-100 and 0.01 M-MgCl₂ (Schnaitman, 1973). Treated samples were allowed to stand for 15 min at room temperature before centrifugation for 20 min at 50000 g. The pellets were resuspended in 0.01 M-Tris/HCl (pH 7.2) containing 0.005 M-EDTA and 0.002 M-PMSF.

Biochemical assays. Protein was estimated by the method of Lowry or by measuring A₂₈₀, using a standard of bovine serum albumin (Sigma).

Protein in sucrose density gradient fractions was precipitated prior to assay, by addition of 10% (w/v) trichloroacetic acid (Sigma). The precipitate was resuspended to the original fraction volume in distilled water. Fractions were assayed for 2-keto-3-deoxyoctonate (KDO) by the method of Osborn (1963) and for reduced NADH dehydrogenase activity by the method of Osborn *et al.* (1972).

SDS-PAGE. This was done by the method of Laemmli (1970) using 12.5% (w/v) acrylamide gels. Gels were stained overnight using 0.05% (w/v) Coomassie Brilliant Blue R (CBB) dissolved in a mixture of methanol/distilled water/glacial acetic acid (9:9:2, by vol.). Destaining was performed in the same solvent (without CBB).

Autoradiographs. Where appropriate, gels were dried using a heated (80 °C) slab-gel drier (Bio-Rad) attached to a vacuum line. For autoradiography, dried gels were exposed to X-OMAT S X-Ray film (Kodak) for 20 h at -70 °C. Autoradiographs were developed for 5 min in Phenisol (Ilford) developer (diluted 1:4) and fixed for 1 min in Kodafix (Kodak).

Studies of heat modifiability of envelope polypeptides. For routine analysis, samples of envelope preparations were heated in Laemmli sample buffer for 10 min at various temperatures (30, 40, 50, 60, 70, 80 and 100 °C) before SDS-PAGE.

For more detailed studies, envelopes were heated at 30 °C in Laemmli sample buffer and polypeptides were resolved by SDS-PAGE and stained with CBB. Bands thought to be heat modifiable were excised and extracted by electroelution. This was achieved by placing the excised bands in a dialysis sac positioned between the electrodes of an electroblotting apparatus. Electroelution in buffer containing 0.025 M-Tris, 0.192 M-glycine and 0.1% (w/v) SDS was performed at 20 V for 24 h. Resulting samples were dialysed with distilled water for 24 h and lyophilized. The lyophilized samples were resuspended in Laemmli buffer, reheated for 10 min at 100 °C and re-examined by SDS-PAGE.

Western blotting. The method of Towbin *et al.* (1979) was used to transfer polypeptides from SDS-PAGE gels to nitrocellulose filters (pore size 0.45 µm; Sartorius). Transfers were performed at 0.11 A for 18 h at 20 °C. Immunoblots were maintained in phosphate-buffered saline (PBS; pH 7.2) composed of 0.15 M-NaCl, 0.0032 M-Na₂HPO₄ and 0.0015 M-KH₂PO₄, containing 0.5% (v/v) Tween 20 (Sigma) to prevent non-specific binding of protein to the nitrocellulose.

Blots were incubated at room temperature for 2 h in primary antibody diluted 1:100 in PBS/Tween 20. Immunoblots were washed three times for 10 min in PBS/Tween 20. Bound antibody was detected following incubation for 2 h at room temperature in a solution of protein A conjugated to horseradish peroxidase (Sigma), diluted 1:300 in PBS/Tween 20. After washing in PBS/Tween 20, reacting antigens were visualized by incubation for 3 min at room temperature in freshly prepared 'developing' reagent consisting of 60 mg 4-chloro-1-naphthol (dissolved in 10 ml methanol), 90 ml PBS/Tween 20 and 50 µl (30%, w/v) hydrogen peroxide. Immunoblots were photographed immediately following development.

Production of antisera. Antisera were raised in rabbits following multiple intradermal injections (50 µl) of antigen. Injections were repeated at weekly intervals for four occasions. The antigen (2 mg ml⁻¹) was dissolved in 0.15 M-NaCl and emulsified with an equal volume of incomplete Freund's adjuvant (Wellcome). Three weeks after the final vaccination, rabbits were anaesthetized and exsanguinated.

Purification of immunoglobulins. All sera were processed as described by Owen (1985). Briefly, immunoglobulins were precipitated by the addition of ammonium sulphate (25 g per 100 ml serum). Precipitates were collected and dialysed against PBS (pH 7.2). The final volume of the immunoglobulin solution was adjusted to be one-tenth of the original serum volume.

Absorption of antisera. Sera were absorbed for 1 h at 20 °C using various amounts of live bacteria ($1-8 \times 10^{12}$ cells) or envelopes isolated by the standard procedure. These were then removed by centrifugation for 20 min at 6000 g for cells and 1 h at 50000 g for envelopes.

Results

Isolation of envelopes

A suitable method for the preparation of envelopes from *P. haemolytica* (see Methods) was developed after optimization of spheroplast formation by adjustment of EDTA and lysozyme concentrations. Experiments (not shown) to optimize spheroplast lysis and involving the use of the French pressure cell, sonication and osmotic shock showed that the osmotic shock procedure was the most convenient and reproducible method. Thus, when a French pressure cell was used, poor separation was achieved, with an apparent loss of putative inner-membrane material. Sonication was more successful but results varied between experiments. SDS-PAGE analysis of typical A2 serotype envelopes prepared by osmotic shock as described in Methods is shown in Fig. 1. The polypeptide bands were numbered and their respective molecular masses calculated with reference to a panel of protein standards (Bio-Rad; see Table 2).

Fig. 1. CBB-stained SDS-polyacrylamide gel of a typical envelope preparation isolated from *P. haemolytica* A2 serotype (CN 8439). Track contains 70 µg protein. Labels on the right-hand side show polypeptides separable by this technique (see Table 2). Positions of molecular mass standards are indicated on the left-hand side.

The same isolation techniques gave reproducible envelope preparations from other serotypes of *P. haemolytica* and *P. multocida*.

Comparison of envelope profiles from *P. haemolytica* and *P. multocida*

Envelopes isolated from different serotypes of *P. haemolytica* and *P. multocida* were compared by SDS-PAGE (Fig. 2). Different isolates of the same serotype

Table 2. Summary of SDS-PAGE polypeptide profile of a typical A2 serotype envelope preparation

Band no.	~ Mol. mass (kDa)	Sucrose density gradient*	Triton X-100/Mg ²⁺ solubility†	¹²⁵ I-radiolabelling‡	Proteinase K treatment§	Trypsin treatment§	Antibody absorption
1	154.5	+	I	—	d	d	—
2	141.1	+	I	—	d	d	—
3	98.4	+	I	—	r	r	—
4	92.3	+	I	*	r	d	+
5	89.2	+	I	—	r	d	—
6	84.0	+	I	—	r	d	—
7	80.1	+	I	*	r	p	+
8	77.3	—	I	—	r	d	—
9	72.8	+	I	*	r	d	+
10	69.4	+	I	—	r	d	—
11	66.9	+	I	—	r	d	—
12	63.8	+	I	—	r	d	—
13	61.5	+	I	—	r	d	+
14	58.7	—/+	i/S	—	p	p	+
15	56.6	+	I	*	r	p	+
16	53.7	—/+	I/s	**	r	d	—
17	52.0	+	I	—	r	r	—
18	49.6	+	I	—	r	r	—
19	47.2	—	i/S	*	r	r	—
20	45.0	+/-	I/s	—	r	r	—
21	44.0	+/-	i/S	—	r	r	—
22	43.5	+	I	**	r	r	—
23	42.4	+	I	**	r	r	+
24	41.4	—	i/S	—	r	r	—
25	40.9	+	I	—	r	p	—
26	40.0	+	I	—	p	p	—
27	39.5	—/+	S	**	p	p	—
28	38.5	+	i/S	—	r	r	—
29	38.1	—/+	i/S	—	r	r	+
30	37.6	—/+	S	**	r	d	+
31	36.7	—/+	I	—	d	d	+
32	35.9	—	I	—	r	d	—
33	33.8	+	I	**	d	r	+
34	32.6	+	I	**	d	r	+
35	31.8	+	S	*	d	r	+
36	30.7	—	I	—	d	p	+
37	28.2	—	S	—	d	r	+
38	26.9	—	S	—	r	d	+
39	25.9	—	S	—	r	r	—
40	24.4	—	S	—	r	r	+
41	20.4	+	I	**	r	r	—
42	19.4	+	I	**	r	r	—
43	18.0	+	I	**	r	r	—
44 in LPS staining area	< 18.0	+	I	**	r	r	—

* Presence in fraction 4 of sucrose density gradient [this fraction was taken as a typical outer-membrane protein fraction (sp. gr. = 1.21 g cm⁻³)]. +, Polypeptide present; —, polypeptide absent; +/-, polypeptides of similar molecular mass but present in greater amounts in other sucrose fractions.

† I, Triton X-100/Mg²⁺-insoluble (putative outer-membrane protein); S, Triton X-100/Mg²⁺-soluble (putative inner-membrane protein); i/S, I/s, polypeptides of similar molecular mass found in both fractions but with the greater amount of polypeptide in the fraction represented with a capital letter.

‡ **, Strongly ¹²⁵I-radiolabelled polypeptide; *, weakly ¹²⁵I-radiolabelled polypeptide; —, no ¹²⁵I-radiolabelling.

§ Envelopes produced from cells treated with protease (proteinase K or trypsin) prior to envelope isolation. r, Resistant to protease; d, degraded by protease; p, partially degraded by protease.

|| Absorption of specific antibody from hyperimmune A2 envelope serum by live A2 serotype bacteria. +, Antibody absorbed from serum by A2 serotype bacteria; —, no antibody absorption.

had similar polypeptide profiles with the single exception of an A7 isolate (CN 5498; Fig. 2, track 7). This isolate produced a different envelope polypeptide profile in the 30–70 kDa region to three other A7 isolates

(CN 7318, CN 7768 and CN 7802), which produced profiles similar to each other. The envelope profile of one of the typical A7 strains (CN 7768) is shown in Fig. 2, track 6.

Fig. 2. SDS-PAGE of envelopes isolated from representative isolates of serotypes of *P. haemolytica* and *P. multocida*. Tracks 1 and 18 contain molecular mass standards. Remaining tracks are as follows. Tracks 2-17, *P. haemolytica*: 2, serotype A1; 3, A2; 4, A5; 5, A6; 6, A7 (CN 7768); 7, A7 (CN 5498); 8, A8; 9, A9; 10, A11; 11, A12; 12, A13; 13, A14; 14, T3; 15, T4; 16, T10; 17, T15. Tracks 19-22, *P. multocida*: 19, serotype A; 20, B; 21, D; 22, E. All tracks were loaded with 60 μ g of protein. Arrow indicates polypeptide of ~38.5 kDa found in envelopes of *P. multocida* serotype B.

Fig. 3. Comparison by SDS-PAGE of Triton X-100/ Mg^{2+} -insoluble fractions of envelopes from various *P. haemolytica* and *P. multocida* serotypes. Track 1 contains molecular mass standards. Remaining tracks are as follows. Tracks 2-17, *P. haemolytica*: 2, serotype A1; 3, A2; 4, A5; 5, A6; 6, A7 (CN 5498); 8, A8; 9, A9; 10, A11; 11, A12; 12, A13; 13, A14; 14, T3; 15, T4; 16, T10; 17, T15. Tracks 18-21, *P. multocida*: 18, serotype A; 19, B; 20, D; 21, E. All tracks were loaded with 50 μ g protein. Arrow indicates Triton X-100/ Mg^{2+} -insoluble polypeptide of ~89.2 kDa which is common to all *P. haemolytica* and *P. multocida* serotypes.

Considerable qualitative similarities were noted within the A biotype isolates (Fig. 2, tracks 2–13) and within the T biotype isolates (Fig. 2, tracks 14–17) but major differences were apparent between the two sets of biotype isolates. Likewise, many similarities were found between *P. multocida* serotypes (Fig. 2, tracks 19–22), but distinct differences were observed when they were compared with polypeptide profiles of *P. haemolytica* A and T serotypes (Fig. 2, tracks 2–17).

The SDS-PAGE polypeptide profiles of envelope preparations were sufficiently different to distinguish between A biotypes and T biotypes of *P. haemolytica* and also sufficiently different to differentiate between *P. haemolytica* and *P. multocida* species. Sufficient differences existed between *P. multocida* serotypes to identify each serotype by its polypeptide profile. In contrast to the other *P. multocida* serotypes, envelopes from serotype B possessed a polypeptide of molecular mass ~38.5 kDa (Fig. 2, track 20, arrow).

Polypeptides in the 200–40 kDa range from T serotypes were frequently separated by SDS-PAGE as diffuse or 'seagull' shaped bands (Fig. 2, tracks 14–17).

Comparison of Triton X-100/Mg²⁺-insoluble fractions of envelope preparations of *P. haemolytica* and *P. multocida*

Triton X-100/Mg²⁺ extraction of envelopes simplified SDS-PAGE profiles since the procedure separated envelope proteins into detergent insoluble and soluble fractions. In an attempt to detect the presence of any common or serotype-specific polypeptides, comparison was made between the SDS-PAGE polypeptide profiles of untreated envelopes (Fig. 2), and the Triton X-100/Mg²⁺-insoluble envelope fractions (Fig. 3) from various serotypes.

These results confirmed that there were distinct differences between biotypes A and T and between species *P. haemolytica* and *P. multocida*.

One major Triton X-100/Mg²⁺-insoluble polypeptide of ~89.2 kDa (Fig. 3, arrow) appeared to be common to all serotypes of *P. haemolytica* and *P. multocida* examined. Five major polypeptides of ~58.7, ~56.5, ~52.3, ~27.8, and ~27.1 kDa were Triton X-100/Mg²⁺-soluble (data not shown) and also appeared to be common to all serotypes of *P. haemolytica* and *P. multocida* examined,

Fig. 4. SDS-PAGE of fractions obtained following isopycnic sucrose density gradient centrifugation of *P. haemolytica* serotype A2⁺ (CN 8439) envelopes. Track S contains molecular mass standards. Track 1 contains fraction 1 (54.7% sucrose); 2, fraction 2 (52.7%); 3, fraction 3 (48.4%); 4, fraction 4 (45.0%); 5, fraction 5 (38.5%); 6, fraction 6 (38.5%); 7, fraction 7 (37.4%); 8, fraction 8 (35.0%); 9, fraction 9 (32.7%); 10, fraction 10 (31.0%); 11, fraction 11 (26.5%); 12, fraction 12 (22.5%); 13, fraction 13 (22.0%). Each track was loaded with 60 µl of the respective fraction. Track E contains 70 µl of envelope protein. Arrows indicate two major polypeptides of ~58.7 and ~53.7 kDa found in all *P. haemolytica* A serotypes.

although there was quantitative variation of these bands between preparations.

Fractionation of envelopes on sucrose density gradients

Separation of envelope components by isopycnic sucrose density centrifugation into clearly defined fractions has been used as preliminary evidence for the probable location of envelope proteins (Osborn *et al.*, 1973). The profile obtained when envelopes from A2 serotype organisms were fractionated in this way is shown in Fig. 4. Envelope proteins were clearly separable into three subfractions: putative outer-membrane proteins in fractions 1–5, putative inner-membrane proteins in fractions 9–13, and intermediate material in fractions 6–8. The ~89.2 kDa polypeptide seemed to be found mainly in fractions 1–7 but could be detected in all fractions.

Sucrose density gradient fractionation of ^{125}I -radiolabelled envelope preparations (data not shown) showed that, as expected, most of the radiolabelled polypeptides were found in that region of the gradient characteristic for outer-membrane proteins. For all *P. haemolytica* A serotypes examined, two major bands (Fig. 4, track 7, arrows) of ~58.7 and ~53.7 kDa were found predominantly in fractions 6–8 and 7–10 (specific gravity 1.170–1.158 and 1.158–1.143 g cm $^{-3}$), respectively, of the sucrose density gradients. The position of these two bands in the gradient might suggest an inner- rather than an outer-membrane location; this is further supported by their Triton X-100/Mg $^{2+}$ solubility.

Characterization of sucrose density gradient fractions

Fractions obtained from the sucrose density gradient were characterized for their content of sucrose, protein, NADH dehydrogenase activity and KDO. Results of a typical experiment performed on A2 serotype envelopes are shown in Fig. 5. These results confirmed that a satisfactory separation of inner and outer membranes had been achieved, as gradient fractions with high KDO (an outer-membrane marker) content were fractions 1–7 inclusive. Those gradient fractions with highest NADH dehydrogenase (an inner-membrane marker) activity were fractions 12–14 inclusive.

Similar separations (data not shown) were obtained from isopycnic centrifugations of envelopes from all of the other serotypes of *P. haemolytica*.

Studies of heat modifiability of envelope proteins

Analysis by SDS-PAGE of an A2 envelope preparation heated at various temperatures in Laemmli buffer showed that the migration of several polypeptides was

Fig. 5. Results of assays on sucrose density gradient fractions obtained following isopycnic centrifugation of envelopes from *P. haemolytica* serotype A2. ■, A_{280} ; ▲, amount of protein (mg ml $^{-1}$); ●, 100 × concn of KDO (mg ml $^{-1}$); ○, concn of NADH [mM (mg protein) $^{-1}$].

temperature dependent (Fig. 6). Several new bands (arrowheads) appeared in the 100 °C-heated profile compared with the profile from envelopes incubated at 30 °C.

A major polypeptide of molecular mass ~160 kDa (track 2, arrow) was only present in samples heated below 40 °C. To ascertain the apparent molecular mass of this component when heated to 100 °C, the band was excised, heated to 100 °C and reexamined by SDS-PAGE. The excised polypeptide now migrated as a major polypeptide with an apparent molecular mass of ~53.7 kDa (Fig. 6; track 8, arrow). Minor polypeptides with molecular masses of ~92.3 and ~43.5 kDa were also observed in this electroeluted material.

Another polypeptide of ~43.5 kDa was prominent at temperatures >60 °C (Fig. 6, track 7, arrow). An increase in staining of some other bands with increasing temperature was also noted (arrowheads). In part, this might reflect the different solubilities of proteins in Laemmli buffer at the different temperatures.

Triton/Mg $^{2+}$ -extraction of radiolabelled envelopes

Experiments were done to assess the solubility of envelope polypeptides in a detergent, Triton X-100. Envelopes prepared from ^{125}I -radiolabelled cells of *P. haemolytica* serotype A2 were extracted with Triton X-100/Mg $^{2+}$ buffer. The envelope preparation prior to detergent treatment (Fig. 7, tracks 1 and 4), the Triton X-100/Mg $^{2+}$ -insoluble residue (track 2) and the Triton X-100/Mg $^{2+}$ -soluble fraction (track 3) were compared by

Fig. 6. SDS-PAGE of A2 envelopes heated at various temperatures prior to electrophoresis. Track S contains molecular mass standards. Tracks 1–7, A2 envelopes heated for 10 min at 30 °C; various temperatures: track 1, 2, 40 °C; 3, 50 °C; 4, 60 °C; 5, 70 °C; 6, 80 °C; 7, 100 °C; 8, excised polypeptide (apparent molecular mass 160 kDa) heated at 100 °C. Tracks 1–7 were loaded with 60 µg of protein and track 8 was loaded with 10 µg of protein. See text for explanation of arrows.

SDS-PAGE, in conjunction with CBB staining (Fig. 7a) and autoradiography (Fig. 7b). Sixteen bands with varying levels of ^{125}I incorporation were observed. Of these bands, 13 appeared insoluble in Triton X-100/ Mg^{2+} . Two major and one minor radiolabelled polypeptides were found in the Triton X-100/ Mg^{2+} -soluble fraction. Summary data are given in Table 2.

The supernatant produced after centrifugation of the cell-free cell lysate at 150 000 g showed little radiolabelling apart from two bands (~ 42.4 and ~ 28.2 kDa) which seemed to correspond to ^{125}I -labelled envelope polypeptides also found in the Triton X-100/ Mg^{2+} -insoluble fraction. This would argue against significant cell lysis occurring during the labelling procedure.

Effect on envelope polypeptide profile of treatment of A2 serotype cells with proteolytic enzymes

Treatment of cells with proteases followed by isolation of envelopes by the standard method was used as an alternative procedure to assess surface expression of polypeptides. This method yielded simpler envelope profiles (Fig. 8, tracks 2 and 3) than that produced from untreated cells (Fig. 8, track 1). Control experiments (data not shown) demonstrated that isolated envelope proteins were completely degraded with the exception of bands of 43.5 and 19.4 kDa.

Cells treated with proteinase K showed a different profile of degradation than those treated with trypsin.

Fig. 7. SDS-PAGE examination of the solubility in Triton X-100/ Mg^{2+} of envelope polypeptides prepared from ^{125}I -radiolabelled cells. (a) CBB-stained polyacrylamide gel, (b) direct autoradiograph of (a). Track S contains molecular mass standards. Track 1, envelopes isolated from ^{125}I -radiolabelled cells; 2, Triton X-100/ Mg^{2+} -insoluble fraction of envelopes isolated from ^{125}I -radiolabelled cells; 3, Triton X-100/ Mg^{2+} -soluble fraction of envelopes isolated from ^{125}I -radiolabelled cells; 4, envelopes isolated from ^{125}I -radiolabelled cells; 5, post-lysis supernatant following centrifugation at $150\,000\text{ g}$ for 1 h. All tracks were loaded with $60\text{ }\mu\text{g}$ of protein except tracks 2 and 3, loaded with material derived from $60\text{ }\mu\text{g}$ envelope protein.

Polypeptides that were completely removed by proteinase K action were those having molecular masses of ~ 154 , 141 , 98.4 and 36.7 kDa and those in the 32.6 – 28.2 kDa range (summarized in Table 2). Some reduction also occurred in the intensity of a band of $\sim 58.7\text{ kDa}$. New bands which appeared in the profile at ~ 70 , 36 , 29 and 27 kDa were presumed to result from proteolytic action.

Similar experiments conducted with trypsin demonstrated that certain envelope polypeptides in the 90 – 60 kDa region were completely removed. The heavily stained band seen in track 3 of Fig. 8 was probably derived from proteolytic digestion and represents a reinforcement of the weaker staining band of 43.5 kDa seen in the untreated profile (track 1). Polypeptides of ~ 52 – 41.4 kDa and ~ 38.5 , 38.1 , 33.8 , 31.8 kDa , and low molecular mass bands at 28 – 18 kDa were largely unaffected by trypsin action, and, in contrast, polypeptides in the 37 – 35 kDa region were almost completely removed.

The combined results of these experiments indicated that bands of ~ 43.5 , 42.4 , 19.4 and 18.0 kDa probably corresponded to proteins which are surface-located and resistant to proteolytic action, whilst bands of 154 , 141 , 98.4 and 36.7 kDa , as well as several minor bands of molecular mass 70 – 80 kDa , are probably surface-located and susceptible to protease degradation.

Reaction of envelope proteins with immune serum previously absorbed with A2 serotype cells

Immunoblotting experiments were conducted using A2 envelopes and both control anti-A2 envelope serum (Fig. 9, tracks 1 and 6) and identical serum which had been absorbed with differing concentrations of bacteria (tracks 2–5).

Progressive absorption of A2 envelope antiserum with increasing numbers of live A2 serotype bacteria resulted in the reduction or removal of immunostaining of 17 polypeptide bands (Fig. 9, arrows). These bands thus corresponded to proteins which were surface-located and accessible to antibodies. Two of these bands (asterisks, Fig. 9) were not reduced in intensity in similar experiments conducted with sera absorbed with heterologous (A1, A6, A7, A9, T3) serotype envelope preparations (data not shown). This suggests that these polypeptides may be specific to the A2 serotype and are probably surface-located.

Discussion

Envelopes from *P. haemolytica* and *P. multocida* were isolated successfully and reproducibly by a modification of the method of Witholt *et al.* (1976) which employed osmotic lysis. This obviated the need to use more damaging physical methods.

Envelopes isolated from *P. haemolytica* biotype A, *P. haemolytica* biotype T and *P. multocida* could be differentiated by their SDS-PAGE profiles. Furthermore, different isolates of the same *P. haemolytica* A strain always generated very similar SDS-PAGE profiles. *P. haemolytica* T envelopes gave simpler SDS-PAGE profiles which were typified by distorted bands in the high-molecular-mass region. The reason for this is unknown. These results confirm those of Thompson & Mould (1975), who analysed phenol/acetic acid cell extracts of *P. haemolytica* by SDS-PAGE and showed differences in the profiles of extracts derived from A strains and T strains. Donachie *et al.* (1984) also showed similarities in A strain extracts produced using sodium salicylate.

Fig. 8

Fig. 8. SDS-PAGE examination of A2 serotype envelopes prepared from cells subjected to proteolytic digestion prior to envelope isolation. Track S contains molecular mass standards. Track 1, A2 envelopes prepared from untreated cells; 2, A2 envelopes produced following treatment of cells with proteinase K; 3, A2 envelopes produced following treatment of cells with trypsin. All tracks were loaded with 60 µg of protein.

Fig. 9

Fig. 9. Immunoblotted A2 serotype envelopes probed with anti-A2 serotype envelope serum absorbed with A2 cells. Tracks S contains molecular mass standards. A2 serotypes were probed with the following: tracks 1 and 6, anti-A2 serotype envelope antiserum (AES); 2, AES absorbed with $\times 1$ concentration A2 serotype cells; 3, AES absorbed with $\times 2$ concentration A2 serotype cells; 4, AES absorbed with $\times 4$ concentration A2 serotype cells; 5, AES absorbed with $\times 8$ concentration A2 serotype cells. See text for explanation of arrows.

P. multocida serotype B could be distinguished from the other *P. multocida* serotypes by the presence of a major band at ~ 33 kDa. Profiles of *P. multocida* D strains were broadly similar to those reported from atrophic rhinitis-producing strains by Lugtenberg *et al.* (1984, 1986).

Sucrose density gradient centrifugation of membranes gave effective separation of proteins into putative inner-

and outer-membrane fractions. The separation achieved was broadly similar to that achieved by Squire *et al.* (1984) and confirmed the differences between *P. haemolytica* biotypes and *P. multocida* serotypes seen using SDS-PAGE of unfractionated membranes.

The criteria used for assigning putative outer-membrane proteins were surface labelling (^{125}I), insolubility in Triton X-100/ Mg^{2+} , specific gravity of $\sim 1.22 \text{ g cm}^{-3}$

following isopycnic sucrose density gradient separation, absorption of specific antibody by homologous serotype cells and susceptibility to protease digestion in intact cells. The behaviour of several polypeptides did not always meet the above criteria in all types of experiment.

With reference to the standard A2 profile given in Fig. 1 and based on the characteristics of individual polypeptides listed in Table 2, the following polypeptides appear to be located in the outer membrane: bands 4, 7, 9, 15, 16, 22, 23, 30, 33, 41, 42, 43 and 44. The following bands were probably not surface-located: bands 8, 24, 28, 29, 37, 38, 39 and 40.

Blotting experiments (data not shown) in which envelopes from selected serotypes of *P. haemolytica* and *P. multocida* were probed with an A2 serotype envelope antiserum detected several common proteins. One noticeable polypeptide band corresponded to a molecular mass of ~89.2 kDa (arrow in Fig. 3). This protein was always found in sucrose density gradient fractions associated with putative outer-membrane proteins. However, it did not appear to radiolabel with ^{125}I and antibodies to it were not absorbed from specific antisera using living whole cells. The polypeptide had unusual detergent solubilities in being soluble in Sarkosyl (data not shown) but insoluble in Triton X-100/ Mg^{2+} . It could, therefore, represent an outer-membrane protein which is not surface-expressed and with somewhat unusual detergent solubility properties.

The purification and characterization of this and other common envelope proteins may lead to an improved understanding of their role in pasteurellosis and of their vaccine potential.

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