Partial characterisation of a soluble haemagglutinin from human diarrhoeal isolates of *Aeromonas*

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Summary. A soluble haemagglutinin has been identified in cell-free culture supernates of human diarrhoeal isolates of *Aeromonas sobria*, *A. hydrophila* and *A. caviae*. It was oligomeric; a major peak of haemagglutinating activity had an apparent mol. wt of 780 000 but there was haemagglutinating activity throughout the mol. wt range < 40 000–> 10⁶. Human group O, A and B, horse, rabbit, chicken and rat erythrocytes, but not those of sheep and cow, were agglutinated by the soluble haemagglutinin, in contrast to the cell-bound agglutinin. Agglutination was inhibited by fetuin, a complex glycoprotein, but not by simple sugars. The haemagglutinating activity was not affected by 0.5 M NaCl, dithiothreitol or the presence or absence of Ca⁺⁺. It was unrelated to the haemolytic, enterotoxigenic and proteolytic activities present in cell-free extracts of *A. sobria*. All *A. sobria*, 73% of *A. hydrophila* and 68% of *A. caviae* strains tested produced this soluble haemagglutinin. *A. caviae* does not appear to be an enteric pathogen, therefore this soluble haemagglutinin alone is unlikely to be a virulence factor in *Aeromonas* spp.

Introduction

Adherence of intestinal pathogens to the gut mucosa precedes diarrhoea, whether this is caused by enterotoxins or by invasion of intestinal epithelium. Haemagglutination has been widely used for characterisation of such adhesins. Cell-associated fimbrial haemagglutinins are important in the virulence of *Escherichia coli* (Satterwhite *et al.*, 1978). Several patterns of cell-associated haemagglutination have been detected with strains of *Aeromonas* spp. grown on solid media (Atkinson and Trust, 1980; Burke *et al.*, 1984a) but adhesins of *Aeromonas* spp. may be fimbrial (Atkinson and Trust, 1980) or non-fimbrial (Chang *et al.*, 1985). Their role as possible virulence factors is not known, but fucose-resistant haemagglutination correlates well with clinical isolates (Burke *et al.*, 1984b).

Soluble haemagglutinins produced by strains grown in liquid media have been reported in *Vibrio cholerae* (Finkelstein and Hanne, 1983) and may be important virulence factors (Finkelstein *et al.*, 1983). Soluble haemagglutinins of *V. parahaemolyticus* have also been described (Oishi *et al.*, 1979) but their significance is unknown. We have studied the production of soluble haemagglutinins by *Aeromonas*.

Materials and methods

Bacteria

Aeromonas spp. were identified according to the methods of Popoff (1984). Three strains were chosen for study: *A. sobria* isolated from an adult in Perth; *A. hydrophila* from a Thai adult; and *A. caviae* from an Indonesian child. All three patients had diarrhoea. We had previously shown that the strains of *A. sobria* and *A. hydrophila* were cytotoxic, haemolytic and enterotoxigenic (Burke *et al.*, 1981a) but the *A. caviae* strain did not produce cytotoxin, haemolysin or enterotoxin.

Growth conditions. Bacteria were stored at room temperature in maintenance medium. For the preparation of large batches of broth culture, bacteria were grown either on a blood-agar plate overnight at 37°C or in a 5-ml volume of Trypticase Soy Broth (Oxoid) with Yeast Extract L21 (Oxoid) 0.6% w/v (TSB-YE) in 50-ml Erlenmeyer flasks until growth was clearly visible (5–6 h at 37°C). These cultures were used to inoculate about 16 50-ml volumes of TSB-YE in 250-ml Erlenmeyer flasks, which were incubated for 24 h at 37°C and 300 rpm on an environmental shaker (New Brunswick Scientific, New Jersey, USA); each batch comprised *c.* 800 ml of broth culture. All cultures were checked for purity by subculture on to blood-agar plates which were incubated overnight at 37°C.

Cell-free supernates of culture were prepared by centrifuging the broth cultures at 5000 rpm for 30 min at 4°C (Sorvall, GSA rotor), and then filtering the supernate

under vacuum through a 0.45- μ m membrane filter (type HA, Millipore Corp., Bedford, USA). This cell-free preparation was extensively dialysed against 0.05 M NH_4HCO_3 at 4°C, and was then lyophilised and resuspended in a minimal volume of distilled water.

Toxin preparation

Crude toxins were precipitated from the cell-free preparation with cold saturated $(\text{NH}_4)_2\text{SO}_4$ 80% v/v. The precipitate was collected by centrifugation at 10 000 rpm (Sorvall, SM-24 rotor) for 15 min at 4°C and washed twice in a minimal amount of 80% saturated $(\text{NH}_4)_2\text{SO}_4$, dialysed extensively against 0.5 M NH_4HCO_3 at 4°C and lyophilised. This crude toxin preparation was stored at -20°C until required for further purification. Alternatively, the culture supernates were concentrated in dialysis tubing with Aquacide (Calbiochem Behring, Australia), dialysed against 0.05 M NH_4HCO_3 and lyophilised.

Gel filtration. The crude toxin preparation was filtered at 4°C on a Sephacryl S-300 (Pharmacia, South Seas Pty. Ltd) column (162 $\times$ 2.6 cm), poured according to the manufacturer's instructions and equilibrated with 0.1 M tris-HCl buffer, pH 8, containing 0.15 M NaCl. The lyophilised material was resuspended in column buffer and filtered through a membrane prefilter (Millipore), then applied to the top of the column and fractionation achieved at a flow rate of 30 ml/h. Fractions (c. 5 ml) were collected on an LKB Ultrarac fraction collector and the optical density at 280 nm determined by a flow-through spectrophotometer (Pharmacia, South Seas Pty. Ltd). Relevant fractions were pooled, extensively dialysed against 0.5 M NH_4HCO_3 , concentrated with Aquacide and stored at -20°C until required. Gel filtration on Fractogel HW 55F (Merck, W. Germany) was performed in 0.1 M tris-HCl buffer containing 0.3 M NaCl.

Assays

Enterotoxin assay. The suckling-mouse system was used to assay enterotoxigenic activity as described previously (Burke *et al.*, 1981b). Every second fraction from the gel-filtration run was assayed. Controls included the column buffer (negative control) and a fresh cell-free preparation of the *A. sobria* strain (positive control).

Haemolysin and haemagglutinin assays. The haemolytic and haemagglutinating activities of the gel-filtered fractions were assayed in microtitration trays with either human group O or rabbit erythrocytes (rbc) 1% v/v. To 100- μ l volumes of serial twofold dilutions of individual fractions in 0.01 M phosphate buffer, pH 7.3, containing 0.15 M NaCl (PBS) was added an equal volume of rbc. The trays were incubated at 37°C and the haemolytic or haemagglutinating titres were the highest dilutions that showed either haemolysis or agglutination after incubation for 1 h at 37°C.

Species specificity of haemagglutinin. Rbc obtained from cattle, sheep, horses, chickens and rats as well as rabbit and human O, A, and B cells were used to assay the

haemagglutinating activity of the fractions from the Sephacryl S-300 gel-filtration runs.

Effect of temperature on haemolytic and haemagglutinating activity. Pooled fractions from the Sephacryl S-300 fractionation of the cell-free supernate from *A. sobria* with either haemagglutinating or haemolytic activity were incubated at 56°C in a water bath and 100- μ l samples removed at 0, 0.5, 5, 10, 15, 30, 60 and 120 min for assay with rabbit rbc.

Inhibition of haemagglutinating activity. Pooled fractions from the Sephacryl S-300 fractionation of supernate from *A. sobria* with high haemagglutinating activity were diluted to give a haemagglutination titre of 10, and 50- μ l volumes of this dilution were preincubated with an equal volume of serial twofold dilutions of the sugars, amino acids and proteins listed in table II for 1 h at 37°C. Rabbit rbc 1% v/v (100 μ l) were then added and the haemagglutination recorded after incubation for 1 h at 37°C.

Effect of chemical reduction on the haemagglutinin. Pooled haemagglutinating fractions were incubated with 0.1 M dithiothreitol for 1 h at 37°C and haemagglutinating activity determined with rabbit rbc.

Protease activity. The proteolytic activity of the fractions from the Sephacryl S-300 gel filtration of the *A. sobria* supernates was determined with the general proteolytic enzyme substrate Azocoll (Calbiochem-Behring Pty. Ltd, Australia). The amount of red product released was measured with a spectrophotometer by absorption at 520 nm. Protease activity was also determined by the casein-agar-lysis method (Sokol *et al.*, 1979).

Physico-chemical studies

The mol. wts of the haemagglutinin and haemolysin from *A. sobria* were determined either by gel-filtration chromatography on Sephacryl S-300 or Fractogel HW 55 F or by gradient polyacrylamide gel electrophoresis (2.5–27%; Gradient Laboratories, Australia; G-PAGE). The mol.-wt markers used in gel filtration included thyroglobulin (mol. wt 669 000), albumin (67 000), ovalbumin (43 000), α -chymotrypsinogen (25 700) and ribonuclease (13 700). Markers used for G-PAGE included thyroglobulin, ferritin (440 000) catalase (232 000) lactase dehydrogenase (140 000) and albumin (67 000). After electrophoresis, the G-PAGE gels were cut into 0.5-cm wide sections and the gel disrupted in a teflon-coated pestle. The material contained therein was eluted with PBS and the eluate tested for haemagglutination and haemolysis.

Survey of soluble haemagglutinin among *Aeromonas* spp.

Assay for soluble haemagglutinin was performed with 70 strains of *A. sobria*, 55 of *A. hydrophila* and 75 of *A. caviae*. Cell-free preparations from haemolytic strains were heated to 56°C for 1 min; these conditions had been shown to abolish haemolytic activity and thus allow detection of haemagglutination.

Results

Fractionation of cell-free supernates

Fractions of the cell-free supernate from *A. sobria* separated on Sephacryl S-300 were assayed for total protein and haemolytic, haemagglutinating, proteolytic and enterotoxic activities.

Haemagglutination occurred in the high-mol.-wt region of the elution profile with the peak of activity eluting at a position corresponding to a protein with an apparent mol. wt of 780 000. In contrast, the major haemolysin peak eluted at a position corresponding to a mol. wt of 34 000; a smaller peak of haemolytic activity co-eluted with the high-mol.-wt haemagglutinin. Haemagglutinin and haemolysin each eluted as broadly based peaks, suggesting heterogeneity with regard to apparent mol. wt. A similar profile was also obtained by gel filtration on a Fractogel column; similar apparent mol. wts were obtained for the haemagglutinin but the apparent mol. wt of the haemolysin was 55 000.

Preparative G-PAGE studies in which electrophoresed material was eluted and tested for haemagglutinating activity also showed that the haemagglutinin was heterogeneous. Haemagglutinating activity was present throughout the gel (mol.-wt range < 40 000 to > 10⁶). Haemolytic activity was also detected in this and subsequent assays by a blood-agarose overlay technique. Two bands of haemolysis were observed corresponding to proteins with mol. wts of 127 000 and 110 000.

Enterotoxic activity measured by the suckling mouse assay corresponded in elution position and profile with haemolytic activity. Similarly, a small peak of enterotoxic activity co-eluted with the haemagglutinin and the high-mol.-wt haemolysin. Protease eluted at a position corresponding to a protein with an apparent mol. wt of 14 000. Protease activity was not detected in association with the haemagglutinating activity by use of either Azocoll or casein-agar substrates.

G-PAGE studies showed that haemagglutinin was also produced by *A. hydrophila* and by *A. caviae*. Gel filtration showed two haemagglutinin peaks in *A. caviae* supernates, the high-mol.-wt fraction eluting in the same position as the haemagglutinin of *A. sobria* and the low-mol.-wt fraction eluting in the same position as the haemolysin of *A. sobria*. There was no haemolytic activity in the *A. caviae* supernate. The haemagglutinins of *A. hydrophila* and *A. caviae* like that of *A. sobria* were heterogeneous with regard to apparent mol. wt as judged by preparative G-PAGE.

Characteristics of the haemagglutinin

Properties of the soluble haemagglutinin were compared with those of cell-bound haemagglutinins of *Aeromonas* spp. The soluble haemagglutinin agglutinated rbc from various species (table I), including horse, rabbit, chicken, rat and human (O, A and B). The highest haemagglutinin titres were found with rabbit and chicken rbc, but sheep or cow rbc were not agglutinated. In contrast, cell-bound haemagglutinin agglutinated all species of rbc tested, including those of sheep and cow. The soluble haemagglutinin was unaffected by the presence or absence of Ca⁺⁺ or by the chelating agent EDTA.

The inhibition profile of the haemagglutinin was determined with various compounds including sugars, amino acids and glycoproteins (table II). Of the sugars, only lactose inhibited haemagglutination but this activity was variable; haemagglutination-inhibition titres were in the range 0–32. Empirical observations suggested that the inhibitory capacity of lactose varied with the source of rabbit cells. None of the amino acids inhibited haemagglutination, and of the other compounds tested, only fetuin was inhibitory.

The thermal labilities of haemagglutinin and haemolysin were determined (table III). Both were labile at 56°C. However, the haemolysin was much more sensitive to heat than the haemagglutinin; at 56°C, haemolytic activity was abolished within 30 sec whereas haemagglutination was abolished only after 10 min. Haemagglutinating activity was detected in the haemolysin fraction after heating.

Table I. Reactivity of the soluble and cell-bound haemagglutinins from *A. sobria* with erythrocytes from various species

Erythrocytes	Soluble haemagglutinin titre*	Cell-bound agglutinin titre**
Human O	16	64
Human A	16	64
Human B	16	64
Horse	8	16
Rabbit	64	128
Chicken	32	64
Rat	16	64
Sheep	0	64
Cow	0	64

* The haemagglutinin used was the high-mol.-wt fraction from Sephacryl S-300 gel filtration.

** Agglutination of *A. sobria* cells by erythrocytes.

Table II. Inhibition of haemagglutination of rabbit rbc by the haemagglutinin from *A. sobria*

Inhibitor	Initial concentration of inhibitor (mg/ml)	Haemagglutination-inhibition titre*
L-Arabinose	50	0
Dulcitol	50	0
D-Fructose	50	0
N-Acetyl D-galactosamine	50	0
N-Acetyl D-glucosamine	50	0
L-Rhamnose	50	0
L-Sorbose	50	0
L-Fucose	50	0
D-galactose	50	0
D-glucose	50	0
D-mannose	50	0
α -Methyl-mannoside	50	0
Lactose	50	8†
Sialic acid	50	0
Ovalbumin	1	0
Bovine serum albumin	1	0
Fetuin	1	32
Creatine	13	0
Alanine	9	0
Arginine	17	0
Lysine	18	0

* Haemagglutination-inhibition titres are expressed as the reciprocal of the highest dilution of inhibitor solution that inhibited haemagglutination; the haemagglutinin was the high-mol.-wt fraction from Sephacryl S-300 gel filtration.

† The inhibitory activity of lactose in this system was highly variable; titres were in the range 0–32.

Table III. The influence of heat (56°C) upon the stability of haemagglutinin and haemolysin from *A. sobria*

Time of exposure to 56°C (min)	Titres in the HAG* fraction		Titres in the HL* fraction	
	HAG	HL	HL	HAG
0	32	0	16	ND
0.5	16	0	0	4
5	8–16	0	0	4
10	2	0	0	4
15	0	0	0	4
30	0	0	0	2
60	0	0	0	0

* The haemagglutinin (HAG) and haemolysin (HL) used were the major fractions from Sephacryl S-300 gel filtration. ND = not detected because of haemolysis.

This too was abolished by heat but only after incubation for 30 min. Neither haemolysin nor haemagglutinin were affected by reduction with dithiothreitol.

Previous studies of the fimbrial and heat-labile agglutinins of *Bordetella pertussis* (Askelöf *et al.*, 1982) indicated that a high salt concentration (0.5 M NaCl) inhibited haemagglutination by fimbrial but not by heat labile haemagglutinin. Thus, haemagglutination experiments with gel-filtration fractions from the strain of *A. sobria* were performed in the presence of 0.15 M NaCl or 0.5 M NaCl; no difference was found.

Soluble haemagglutinin in other strains of Aeromonas spp.

All strains of *A. sobria*, 40 of 55 strains of *A. hydrophila* and 51 of 75 strains of *A. caviae* tested produced soluble haemagglutinins.

Discussion

Although soluble haemagglutinins of *V. cholerae* (Finkelstein and Hanne, 1983) and *V. parahaemolyticus* (Oishi *et al.*, 1979) are recognised, production of haemagglutinins detectable in cell-free preparations from broth cultures by *Aeromonas* spp. has not been reported previously. The soluble haemagglutinins from strains of *A. sobria*, *A. hydrophila* and *A. caviae* demonstrated in this study had properties different from the cell-associated haemagglutinin of *Aeromonas* spp. previously described (Atkinson and Trust, 1980; Burke *et al.*, 1984a). Cell-bound haemagglutinin reacted with all species of rbc tested, but soluble haemagglutinin did not agglutinate rbc from sheep or cows. Furthermore, the soluble haemagglutinin could not be inhibited by simple sugars such as L-fucose, D-galactose or D-mannose, although lactose produced inconsistent inhibition. The glycoprotein fetuin was the only compound tested that consistently inhibited haemagglutination by the soluble haemagglutinin.

These properties are similar to those of the soluble haemagglutinins of *V. cholerae* (Finkelstein and Hanne, 1983) which are heat labile and unaffected by reduction with dithiothreitol and are not inhibited by simple sugars, a characteristic shared by some soluble haemagglutinins of *V. parahaemolyticus* (Oishi *et al.*, 1979).

The soluble haemagglutinins from *Aeromonas* spp. and *V. cholerae* exists in an oligomeric form. The mol. wt of the *V. cholerae* sub-unit is 32 000, similar to that of the low-mol.-wt haemagglutinin peak in cell-free supernates of *A. caviae* cultures. However, the relationship between the high-mol.-wt and the low-mol.-wt haemagglutinins in *Aeromonas* spp. is unknown.

Physico-chemical studies by gel-filtration and G-PAGE showed that the soluble haemagglutinin of *Aeromonas* spp. was a heterogeneous high-mol.-wt component comprising a major peak with an apparent mol. wt of 780 000. G-PAGE studies showed that the soluble haemagglutinins had apparent mol. wts in the range $< 40\,000 - > 10^6$. The haemagglutination profile did not parallel proteolytic, haemolytic or enterotoxigenic activities of *Aeromonas* spp. The *V. cholerae* haemagglutinin hydrolyzes several different protein substrates (Finkelstein *et al.*, 1983). We have been unable to demonstrate any correlation between haemagglutination and proteolytic activity in *Aeromonas* spp.

Gel-filtration studies showed that the apparent mol. wt of the haemolysin was 34 000 but the elution profile suggested that the haemolysin was heterogeneous with regard to mol. wt; previous studies have shown that the apparent mol. wt of isolated haemolysin in the presence of sodium dodecyl sulphate or urea is 50 000 (Bernheimer and Avigad, 1974; Buckley *et al.*, 1981). The G-PAGE studies described here indicated that the haemolysin comprised two components with apparent mol. wts of 110 000 and 127 000. Previous studies have suggested that the haemolysin is heterogeneous with regard to charge (Asao *et al.*, 1984). These data suggest that in normal aqueous systems the haemolysin aggregates or interacts in some way with the separating matrix. This conclusion is supported by

gel-filtration studies in the presence of 1M urea (Asao *et al.*, 1984) and by the gel-filtration studies on Fractogel described here in the presence of high salt concentrations. Gel filtration on Fractogel indicated that the haemolysin had an apparent mol. wt 55 000. The haemolysin profile was shown to parallel that of the enterotoxigenic activity, which suggests that the haemolysin had enterotoxigenic activity, as had been observed previously (Hošťacká *et al.*, 1982).

Although haemolytic and enterotoxic activities in *A. sobria* correlate with isolation of the strain from faeces rather than the environment and with the presence of diarrhoea (Burke *et al.*, 1984b), no such association has been found with the soluble haemagglutinin described here in *A. sobria*, *A. hydrophila* and *A. caviae*. This latter organism appears not to be an enteric pathogen (Burke *et al.*, 1984b). Therefore, the soluble haemagglutinin alone would not seem to be a virulence factor but it may facilitate colonisation and contribute to the virulence of those *Aeromonas* spp. capable of producing diarrhoea by enterotoxin production (Burke *et al.*, 1984) or by invasion of epithelial cells (Watson *et al.*, 1985).

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