BACTERIAL ECOLOGY AND PATHOGENICITY: MODELS OF INFECTION

A collagen-binding protein produced by *Aeromonas hydrophila*

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The gastrointestinal pathogen *Aeromonas hydrophila* strain A186 produces a collagen-binding protein (CNBP) which is found extracellularly and loosely associated with the cell surface. The cell-associated CNBP was purified by sequential ammonium sulphate precipitation, size-exclusion chromatography and ion-exchange chromatography, or by sequential ammonium sulphate precipitation and affinity chromatography with collagen-Sepharose. The purified CNBP was homogeneous in SDS-PAGE, and had a mol. wt of c. 98 kDa. Cyanogen bromide cleavage of the CNBP destroyed collagen-binding activity; however, enzymic digestion with *Staphylococcus aureus* V8 protease generated >10 polypeptide fragments, from which a 30-kDa polypeptide contained the strongest collagen-binding activity. Binding of collagen by the CNBP was restricted to the $\alpha 1$ (I) chain of the collagen molecule and binding seemed to involve both the carbohydrate moieties and certain peptide sequences on the collagen. Collagen-saccharides generated by alkaline hydrolysis inhibited collagen binding by *A. hydrophila*. Also, glycosidase digestion and chemical alteration of the carbohydrate residues of collagen reduced its ability to be bound by the CNBP. Collagen-homologous synthetic peptides inhibited binding of ^{125}I -collagen by the bacteria.

Introduction

Collagens, a family of proteins characterised by a triple-helix structure, the repeating Gly-X-Y triplets, and a high content of hydroxyproline and hydroxylysine in its peptide chains [1], display a complex pattern of interactions with different types of cells, with other extracellular matrix components such as fibronectin, laminin and heparan sulphate and with cell receptors called integrins [2, 3]. Recent studies have shown that several bacterial pathogens bind to various collagens [4–9]. Also, bacterial receptors for collagens and other extracellular matrix components have been proposed as potential virulence factors because of their ability to enhance the adherence of bacterial pathogens to epithelial and endothelial tissues [6, 10, 11].

Previous studies on the binding of soluble and latex bead-immobilised collagens by the human gastrointestinal pathogen *Aeromonas hydrophila* have shown that the bacteria recognise fibre-forming collagen type I and basement membrane collagen type IV [12, 13].

Because of the potential use of adhesins as vaccine candidates against infections produced by *A. hydrophila* and related species in mammals and fish, the current work was carried out to purify and define some biochemical properties of a collagen-binding protein produced by *A. hydrophila*, a pathogen which has become increasingly implicated as a causative agent of human gastroenteritis and is thought to have an aetiological role in travellers' diarrhoea [14].

Materials and methods

Chemicals and materials

Collagen type I (from rat skin), and endo- β -galactosidase (from *Bacteroides fragilis*) were purchased from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). Iodobeads (N-chloro-benzenesulphonamide-derivatised polystyrene beads) were obtained from Pierce Chemical Co. (Rockford, IL, USA), and ^{125}I from Radiochemical Centre, Amersham, UK. Chromatography media and HPLC columns were purchased from Pharmacia-LKB (Uppsala, Sweden). Peroxidase-conjugated immunoglobulins and 1,2-phenylenediamine (OPD) tablets were from Dakopatts A/S (Glostrup, Denmark). Endoglycosidase F (from *Flavobacterium meningosepticum*), endoglycosidase H (from *Strepto-*

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myces griseus), *Vibrio cholerae* neuraminidase and the polypeptide mixture (collagen hydrolysates) Polypep^R were purchased from Sigma. Arginine-glycine-aspartic acid (RGD)-containing synthetic peptides were purchased from Telios Pharmaceuticals Inc. (San Diego, CA, USA). The collagen-derived octapeptide H-Lys-Pro-Gly-Glu-Pro-Gly-Pro-Lys-OH was obtained from Nova Biochem (Läufelfingen, Switzerland). Proline-rich protein (PRP-1) was a gift from Dr D. I. Hay (Forsyth Dental Centre, Department of Biochemistry, Boston, MA, USA) and collagen-analogous synthetic peptides were provided by Dr H. Weingarten (Department of Chemistry, Louisiana State University, USA). Culture media and individual culture media ingredients were purchased from Difco (Detroit, MI, USA), except Blood Agar Base No. 1, which was obtained from LabM (London). Immobilon-PVDF membranes were from Millipore (Bedford, MA, USA).

Bacteria and growth conditions

A. hydrophila strain A186, which was isolated from an infected human patient, is a high binder for collagen type I [13]. Bacterial suspensions were prepared for binding assays by growing them overnight at 32°C on blood agar base supplemented with citrated horse blood 4% (blood agar). Cells were harvested by centrifugation (6000 *g* at 4°C for 30 min), washed once with 0.02 M potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS) and suspended in PBS at a density of 10¹⁰ cells/ml. For the extraction of cell-associated collagen-binding protein (CNBP), the bacteria were grown in a marine bacteria broth (CIB-4), adjusted to 0.15 M NaCl, as described previously [15].

Assays for collagen binding and inhibition of collagen binding

Collagen type I (Cn-I) from rat skin was labelled with 0.2 mCi of ¹²⁵I, with Iodobeads to catalyse the reaction [16]. The binding assay was run as described previously [13]. Briefly, 50 µl of ¹²⁵I-labelled protein (*c.* 25 000 cpm, specificity 1.5 × 10⁷ cpm/µg) solution in PBS containing bovine albumin 0.1% w/v were incubated with 100 µl of an *A. hydrophila* cell suspension (10⁹ cells) in a polystyrene centrifuge tube for 1 h at 20°C. After adding 2 ml of ice-cold PBS containing Tween 20 0.1% v/v, the mixtures were centrifuged (4500 *g* at 4°C for 10 min) and the radioactivity of the pellets was measured in a gamma counter. Incubation mixtures without bacteria were used as background cpm controls and this value was subtracted from the value of the other tubes before the percentage of binding was calculated. The amount of ¹²⁵I-labelled Cn-I bound to the bacterial cells was expressed as a percentage of the amount of radio-labelled protein incubated with the 10⁹ bacteria. Triplicate determinations were made and the average was determined in all cases.

Binding-inhibition assays were made by incubating

bacterial suspensions (100 µl containing 10⁹ cells) at 20°C for 1 h with 100 µl of PBS (control), 50 µg of unlabelled fragments and 50 µg of synthetic peptides, before incubating the bacteria with ¹²⁵I-Cn-I. The treated cells were washed with 2 ml of PBS, suspended in 100 µl of buffer and used in the binding assay described above. To measure CNBP in fractions or extracts, 100 µl of sample were incubated with ¹²⁵I-Cn-I (25 000 cpm in 50 µl of PBS) at 22°C for 1 h before incubation with the bacteria. Inhibition values were calculated as the relative percentage of ¹²⁵I-Cn-I binding by bacterial cells suspended in PBS without inhibitor and the values extrapolated to calculate the µg of protein required to produce 50% inhibition of ¹²⁵I-Cn-I binding.

Extraction of cell-associated collagen-binding protein

A. hydrophila grown in CIB-4 broth and washed with PBS was suspended in water or in various extraction buffers (0.2 M glycine buffer, pH 2.2; 3 M, 4.5 M and 6 M urea; 1 M lithium chloride; sucrose 30% w/v in 50 mM Tris buffer, pH 10; n-octyl-β-D-glucopyranoside 1 mg/ml; 10 mM EDTA; PBS, pH 7.2 containing 1 mM phenylmethylsulphonyl fluoride (PMSF), at a final concentration of 4 *g* of cells (wet weight)/100 ml of extraction buffer. Cell suspensions were incubated with gentle stirring at 4°C for 30 min and the bacteria were removed by centrifugation (12 000 *g* for 30 min at 4°C). The supernatant fluids were filtered through a nitrocellulose membrane (0.45 µm), dialysed against 0.01 M ammonium bicarbonate and assayed for CNBP activity by determining their ability to inhibit binding of ¹²⁵I-Cn-I (as described above).

Purification of the CNBP

A. hydrophila PBS extracts were precipitated with ammonium sulphate (60% saturation at 4°C). The precipitate was collected by centrifugation (18 000 *g* at 4°C for 30 min), dissolved in distilled water and dialysed exhaustively against 0.01 M ammonium bicarbonate. The solution was lyophilised and kept at 4°C until used. The lyophilised preparation was reconstituted in PBS (30 mg/ml) and filtered (0.22 µm) to remove insoluble particles. Five ml of solution were applied to a Sephacryl S-400 (100 × 2.5 cm) chromatographic column (Pharmacia-LKB) equilibrated with the same buffer. The fractions in the major peaks absorbing at 280 nm were pooled, and the pools (pools 1–3) were assayed for the ability to inhibit binding of ¹²⁵I-Cn-I as described above. The active fractions (pools 2 and 3) were lyophilised.

The CNBP preparation purified by ammonium sulphate precipitation and chromatography with Sephacryl S-400 was reconstituted in 20 mM piperazine buffer, pH 5 (1 mg/ml) and 200 µl of the solution were loaded on to a Mono Q H5/5 column (Pharmacia-LKB) equilibrated with the same buffer.

The column was first washed with two bed volumes of equilibrating buffer and then washed with a linear gradient (three bed volumes) of 0–1 M NaCl in 20 mM piperazine buffer at a flow rate of 1.5 ml/min. The major peak absorbing at 280 nm, which eluted at *c.* 0.45 M NaCl, contained the CNBP. The fractions constituting this peak were pooled and lyophilised.

An alternative purification procedure used to purify the CNBP was affinity chromatography with (Cn-I)-AH-Sepharose, with 0.01 M sodium acetate buffer (pH 5) as equilibrating buffer and CNBP eluted with a 0–1 M linear gradient of NaCl. CNBP activity was quantified by the Cn-I binding-inhibition assay as described above.

Immunisation

Adult New Zealand White rabbits were inoculated subcutaneously with highly purified CNBP (30 µg of protein) emulsified in Freund's complete adjuvant (Difco Laboratories). Booster doses of 20–30 µg of protein in Freund's incomplete adjuvant were injected subcutaneously 15 and 30 days later. The rabbits were bled on day 40 and the pooled sera were stored at –80°C. Western blot analysis of extracellular proteins and whole-cell lysates of *A. hydrophila* strain A186 showed that the antiserum was specific for the CNBP.

Electrophoresis

SDS-PAGE was run by the general method of Laemmli [17] with a stacking gel containing acrylamide 4.5% w/v and a separating gel containing acrylamide 12.5% w/v. Electrophoresis of the denatured and reduced samples, and of denatured and reduced mol.wt standards (BioRad Laboratories, Richmond, CA, USA) to calibrate the gel was done in a Protean II xi apparatus (BioRad) at 20 mA (constant current) initially and at 30 mA after the tracking dye entered the separating gel. The gel was stained with Coomassie Blue R-250.

Western blot analysis

The separated proteins (under denaturing conditions) were transferred electrophoretically to Immobilon-PVDF membranes, in a semi-dry trans-blot cell, for 2 h at 200 mA in 20 mM Tris containing methanol 20% v/v as anodic buffer and 25 mM Tris, 40 mM 6-amino-1-hexanoic acid and methanol 20% v/v as the cationic buffer. Additional binding sites were blocked by incubating the membranes with bovine albumin 3% w/v in 10 mM Tris-HCl, 0.15 M NaCl (pH 7.2) for 1 h at 22°C and the membranes were washed three times with 10 mM Tris-HCl, 0.15 M NaCl (pH 7.2) containing Tween-20 (TNT) 0.05% w/v. The membranes were probed at 22°C for 2 h with horseradish peroxidase (POD)-labelled Cn-I in 10 mM Tris-HCl, 0.15 M NaCl (pH 7.2) and after washing three times with TNT, the reactive bands were visualised with 3,3'-diaminobenzidine.

Partial digestion of CNBP

Peptide mapping of the CNBP was done following the general protocol of Cleveland *et al.* [18]. Polyacrylamide gel slices, containing protein bands excised from the primary gels and equilibrated in 0.125 M Tris-HCl buffer (pH 6.8) containing SDS 0.1% w/v and 1 mM EDTA (sample buffer), were loaded into the wells of polyacrylamide slab gels containing acrylamide 12.5% w/v. The number of slices per well was adjusted to provide a protein of 20–50 µg. Wells were filled with sample buffer containing glycerol 20% v/v, and 25 µl of *Staphylococcus aureus* V8 protease solution (25 µg/ml of sample buffer containing glycerol 10% v/v) was layered on top of the wells containing the gel slices. Electrophoresis and Western blotting were run as described above.

Preparation of collagen α -chains, peptides and disaccharides

Denatured collagen was obtained by heating a Cn-I solution [19]. Cn-I (2–5 mg) dissolved in acetic acid 0.05% v/v was heated at 56°C for 60 min, and the $\alpha 1(I)$ and $\alpha 2(I)$ chains were separated by ion-exchange chromatography with CM-cellulose. Cyanogen bromide (CNBr) peptides of Cn-I or of the $\alpha 1(I)$ -chain were obtained by established procedures [20]. Briefly, collagen preparations (1–5 mg/ml) were dissolved in formic acid 70% v/v, the solutions were flushed with nitrogen and, after addition of a 10-fold excess of CNBr (w/w), they were incubated in the dark for 24 h at 22°C. Reaction mixtures were then diluted with water to <5% formic acid and freeze-dried. O-Glycosidic disaccharides (2-O- α -D-glucopyranosyl-O- β -D-galactopyranosyl hydroxylysine) were isolated from an alkaline hydrolysate of Cn-I as described previously [21].

Binding of glycosidase treated-collagen by *A. hydrophila*

Glycosidase digestion of collagen. Microwell flat-bottom strips (Costar, Cambridge, MA, USA) were coated with Cn-I 10 µg/well by incubation at 4°C for 16 h with a solution (100 µl/well) of Cn-I in 0.1 M sodium bicarbonate buffer (pH 8.1) containing 0.15 M NaCl. The wells were washed with 10 mM Tris-HCl, 0.15 M NaCl (pH 7.2) containing TNT 0.05% v/v, and the immobilised collagen was treated with several glycosidases.

α -Glucosidase (0.5 mg/ml) digestion was done in 0.2 M sodium acetate buffer (pH 5) at 37°C for 16 h [21]. β -galactosidase (1 U/ml) digestion was done in 0.1 M sodium citrate buffer (pH 4.3) and the reaction was run at 37°C for 16 h. Neuraminidase (5 U/ml) treatment was done at 37°C for 16 h in 0.5 M sodium acetate buffer (pH 5.5) containing 4 mM CaCl₂. Endo- β -galactosidase (6 mU/ml) digestion was done at 37°C

for 24 h in 0.05 M sodium acetate buffer (pH 5.8) containing bovine albumin (0.2 mg/ml). Endo- β -N-acetylglucosaminidase H (7 mU/ml) digestion was performed at 37°C for 24 h in 0.025 M sodium acetate buffer (pH 5.5). Endo- β -N-acetylglucosaminidase F (1 mU/ml) digestion was performed at 37°C for 18 h in 0.25 M sodium acetate buffer (pH 6.5) containing 20 mM EDTA and 10 mM 2- β -mercaptoethanol. Cn-I immobilised on the ELISA plates was also treated with NaIO₄ (0.075 M), Na(BH₄) (0.075 M) and H₂SO₄ (0.025 M) for 1 h at 37°C; plates were washed with PBS and blocked with BSA 3% w/v as described above. All of the enzymic and chemical partial digestions were accompanied by controls containing the enzyme or the substrate separately. At the end of the incubations, plates were washed four times with TNT buffer and collagen binding was assayed as described below.

ELISA procedure for assay of soluble CNBP binding to treated collagen. One hundred μ l of PBS containing 10 μ g of purified CNBP were added to each glycosidase-treated collagen-coated well and the plates were incubated for 1 h at 37°C. The wells were washed four times with TNT buffer and 100 μ l of rabbit polyclonal antibodies raised against purified CNBP (diluted 1 in 500) were added to each well. After 2 h, the wells were washed four times with TNT buffer and 100 μ l of POD-labelled porcine IgG against rabbit IgG (diluted 1 in 1000) were added to each well and incubated at 37°C for 90 min. The wells were washed four times with TNT buffer, then the substrate solution (1 mg of OPD/ml of 0.05 M sodium citrate buffer, pH 5) was added to each well (100 μ l/well), the plates were incubated at 22°C for 30 min and the A₄₉₂ of each well was measured in an ELISA plate reader. The results are expressed as a relative percentage compared to the binding of CNBP to untreated collagen-coated wells.

Radiolabelling procedure for assay of soluble CNBP and cell-associated CNBP binding. One hundred μ l of a solution of ¹²⁵I-labelled CNBP (25 000 cpm; specific

activity 10⁶ cpm/ μ g), or 25 000 cpm of ¹²⁵I-labelled *A. hydrophila* cells were added to each glycosidase-treated collagen-coated well. After incubation for 1 h at 37°C, the wells were washed five times with TNT buffer and the bound radioactivity was counted in a gamma counter. Binding was expressed as the percentage of radioactivity bound to bacteria or CNBP immobilised on to the collagen-coated wells, and where the 100% end-point was 25 000 cpm. Background values were corrected for CNBP binding to glycosidase-treated collagen-coated wells (<10%).

Results

Isolation and purification of CNBP

A. hydrophila cell-associated proteins were solubilised by treatment of bacterial cells with water and various extraction solutions. Water, PBS, LiCl, sucrose and n-octyl- β -D-glucopyranoside yielded extracts with the largest amounts of CNBP activity as revealed by a specific collagen-binding-inhibition assay with ¹²⁵I-Cn-I as a probe (Table 1).

Based on both the amount of protein extracted from each bacterial treatment, the inhibitory activity on ¹²⁵I-Cn-I binding by *A. hydrophila* cells and the protein purity (extracts composed of a relatively low number of protein bands as revealed by electrophoresis and Western blot analysis), the PBS extraction procedure was used for further isolation and purification of CNBP.

Fractionation of the PBS extracts with ammonium sulphate precipitation gave two fractions, of which the 60% fraction was found to be most active. Further purification was achieved by sequential size exclusion-chromatography with Sephacryl S-400 (Fig. 1a) and ion-exchange chromatography with a Mono Q HR 5/5 column (Fig. 1b), or by affinity chromatography with Cn-I-AH Sepharose (data not shown). Although both purification procedures yielded similar purification

Table 1. Extraction of cell-associated collagen-binding proteins from *A. hydrophila* strain A186, revealed as inhibition of ¹²⁵I-Cn-I binding to *A. hydrophila* cells

Extraction medium	Protein yield after extraction (μ g/ml)	Protein (μ g) producing 50% inhibition of collagen binding
0.2 M Glycine buffer (pH 2.2)	127	56
1 M LiCl (pH 5)	50	6
6 M Guanidine-HCl	400	32
Sucrose 30% in 50 mM TRIS buffer (pH 10)	38	6
n-Octyl- β -D-glucopyranoside (1 mg/ml)	58	8
3 M Urea	400	No inhibition
4.5 M Urea	380	104
6 M Urea	670	174
10 mM EDTA	76	69
De-ionised water	58	7
PBS (pH 7.2)	62	11
Culture supernates	39	10

The data represent the means of triplicate measurements and the SD for each mean was <6%.

Fig. 1. Chromatographic purification of CNBP produced by *A. hydrophila* strain A186. **a**, size exclusion chromatography with sephacryl S-400; **b**, ion-exchange HPLC chromatography with Mono Q HR 5/5 column. **c**, affinity chromatography with (CnI)-AH Sepharose. Shaded peaks indicate fractions containing CNBP detected by its ability to inhibit binding of ^{125}I -Cn-I by *A. hydrophila*. Experimental conditions are described in the *Materials and methods* section.

results, affinity chromatography was not used further because of its inability to process large amounts of material, its yield of only small amounts of purified CNBP, higher losses of the biological activity of the CNBP and the short life of the chromatographic column. A summary of the purification data is given in Table 2.

The final purified CNBP preparation was homoge-

neous by SDS-PAGE and Western blot analysis, with POD-labelled collagen as a probe (Fig. 3). The analysis indicated that the protein had a mol.wt of c. 98 kDa under reducing conditions.

Partial digestion of the CNBP

Because one of the goals of these studies with *A. hydrophila* adhesins and lectins concerns their application as potential vaccine candidates, the CNBP was cleaved with CNBr and *S. aureus* V8 protease to define whether the collagen-binding site(s) are restricted to certain domains in the CNBP molecule, and to determine whether the generation of synthetic peptides homologous to the collagen-binding domains of the CNBP could be an alternative strategy for a vaccine candidate. CNBr cleavage of the CNBP gave one main polypeptide of 27 kDa and three minor bands (Fig. 2), but no collagen-binding activity was detected in the Western blot analysis (data not shown). However, proteolytic cleavage of the CNBP with *S. aureus* V8 protease gave >10 polypeptide bands. The band corresponding to a mol.wt of 30 kDa showed the strongest collagen-binding activity, as revealed by Western blot analysis (Fig. 3).

Location of the A. hydrophila CNBP binding sites on the Cn-I molecule

As both the microbial adhesin or the host cell receptor can be used as blocking agents for inhibiting bacterial adherence on to host tissues in anti-adhesive therapies during microbial colonisation and infection, this study evaluated whether the collagen molecule had specific binding sites recognised by the CNBP. Purified $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ chains, collagen oligosaccharides, collagen peptides generated by CNBr cleavage and collagen-homologous synthetic peptides (10 μg in 100 μl) were evaluated for their ability to inhibit ^{125}I -collagen-binding by the *A. hydrophila* cells. The $\alpha 1(\text{I})$ chain, but not the $\alpha 2(\text{I})$ chain, significantly inhibited collagen binding (Table 3). In addition, preparations of the carbohydrate moieties of the Cn-I also inhibited collagen binding (Table 3). To define better whether the glycosidic residues of the collagen molecule were involved in binding to the *A. hydrophila* CNBP, Cn-I was treated with glycosidases, sodium periodate, sodium borohydride and sulphuric acid. Although some differences were observed in collagen binding depending on the assay method, removal or alteration of the oligosaccharide units of the collagen molecule decreased collagen binding by the *A. hydrophila* CNBP (Table 4).

Studies with peptides generated by CNBr-cleavage and *S. aureus* V8 protease cleavage of Cn-I, blotted on to PVDF membranes and assayed for binding of purified ^{125}I -labelled CNBP, were unsuccessful in identifying the binding domains for the CNBP (data not shown). However, collagen-homologous peptides, proline-rich polypeptides and arginine-glycine-aspartic acid sequences (RGD) were studied for their ability to

Table 2. Purification of CNBP produced by *A. hydrophila* strain A186

Purification stage	Total protein (mg)	Collagen-binding inhibition (%)	Specific activity	x-Fold purification
1. PBS extract	100	6	28	1
2. (NH ₄) ₂ SO ₄ precipitation (60% saturation)	12.2	87	36	0.6
3. Size exclusion chromatography (Sephacryl S-400)				
Pool 2	2.7	90	2.0	11.5
Pool 3	1.2	90	1.7	13.5
4. Ion-exchange chromatography	1	48	13	8

*Specific activity is defined as the amount of protein (μ g) required to produce 50% inhibition of collagen binding.

Fig. 2. SDS-PAGE of *A. hydrophila* strain A186 CNBP and its CNBr-cleavage-generated fragments. Lane 1, CNBP; 2, CNBr-cleaved CNBP. Mol. wt standards (MW) were lysozyme, 14 400; soybean trypsin inhibitor, 21 500; bovine carbonic anhydrase, 31 000; ovalbumin, 45 000; bovine albumin, 66 200; rabbit muscle phosphor-ylase B, 97 400.

inhibit collagen binding. Neither proline-rich polypeptides nor the RGD sequences interfered in the collagen-binding process significantly, but some collagen-homologous peptides, containing a Gly-X-Y collagen triplet, inhibited collagen binding by *A. hydrophila* cells (Table 5).

Discussion

It is now generally accepted that, during the course of an infectious disease, bacteria colonise body sites by sequentially meshing their surface-bound adhesins with cognate receptors available on epithelial cells, endothelial cells, or with extracellular matrix components [22]. The production of collagen-binding surface proteins by gram-positive and gram-negative bacterial pathogens

has been addressed in a number of publications [4–9]. The present studies indicate that the human gastro-intestinal pathogen *A. hydrophila*, previously found to have a high affinity for collagen types I and IV [3], expresses a collagen-binding protein (CNBP) loosely associated with cells. Cell-surface extracts and culture supernates of *A. hydrophila* strain A186 contain CNBP(s) that competitively inhibits binding of collagen by the bacteria. Western blot analysis was used to determine whether the 98-kDa protein purified from the supernatant fluids was the same as the cell-associated CNBP, or represented another protein of similar mol. wt. Peroxidase-labelled collagen and rabbit anti-sera specific for the 98-kDa protein chromatographically purified from the bacterial cell surface recognised the 98-kDa CNBP (data not shown).

In the chromatographic procedures used to purify the *A. hydrophila* CNBP, an increase in the specific activity of the CNBP preparation was achieved, but some of its biological activity was lost as revealed by the x-fold purification (Table 2). A possible explanation could be that the ionic strength and pH of the buffers employed in the chromatographic procedures affected the stereochemical conformation of the CNBP, especially the affinity chromatographic procedure. For this reason, this last purification stage was omitted from further studies.

Although the receptor chosen necessarily restricts the possible fate of the bound pathogen, according to Hoepelman and Tuomanen [22] virtually every pathogen has more than one adhesin, thereby conferring the capability to interact with more than one cell receptor. In that regard, *A. hydrophila* has been reported to produce several adhesins and haemagglutinins [23–26]. Several lines of evidence indicate that the chromatographically purified 98-kDa CNBP binds to Cn-I in more than one manner, i.e., through some polypeptide sequences of Cn-I, and by the interaction of lectin-like CNBP domains with glycosidic residues in Cn-I. Removal or alteration of the carbohydrate residues in Cn-I, by enzymic or chemical methods, decreased collagen binding. Single monosaccharide precursors of the collagen disaccharide moieties (galactose and glucose) did not inhibit collagen binding [13]. However, the O-glycosidic branches of the collagen molecule, isolated by alkaline hydrolysis,

Fig. 3. Enzymic cleavage of CNBP from *A. hydrophila*. **a**, SDS-PAGE stained with Coomassie Blue R-250; **b**, Western blot analysis with peroxidase-labelled Cn-I. Lane 1, purified CNBP; 2, CNBP partially digested with *S. aureus* V8 protease. Mol. wt standards (MW) were as described for Fig. 2.

Table 3. Inhibition of ^{125}I -Cn-I binding to *A. hydrophila* A186 by Cn-I polypeptides and Cn-I saccharides

Collagen preparation	Collagen-binding inhibition (%)
Whole Cn-I molecule	70
$\alpha 1(1)$ chain	45
$\alpha 2(1)$ chain	0
CNBr-cleaved Cn-I	40
CNBr-cleaved $\alpha 1(1)$ chain	10
Saccharide residues (2-O- α -D-glucopyranosyl-O- β -D-galactopyranosylhydroxylysine)	10
Polypep ^R	19

The data represent the means of triplicate measurements and the SD for each mean was <6%.

inhibited binding of collagen by the bacterium. Also, collagen-homologous peptide(s), but not RGD-containing sequences or proline-rich polypeptides, inhibited collagen binding.

Inhibition of collagen binding by collagen-homologous synthetic peptides has also been observed in studies with *S. aureus* and *Escherichia coli* [6, 11]. The importance of the collagen glycosidic moieties has not been addressed in the interactions of these pathogens with collagen, except in the case of binding of type IV collagen by uropathogenic *E. coli* strains. In that case, it was shown that binding does not involve lectin-like interactions [10].

Although CNBr-cleaved collagen inhibits collagen binding by *A. hydrophila* and *S. aureus*, the CNBP

of *A. hydrophila* differs from the *S. aureus* CNBP [6] and *E. coli* CNBP [11] in being strongly inhibited only by the $\alpha 1(I)$ collagen chain. Attempts to define which CNBr-generated polypeptide is recognised by the *A. hydrophila* CNBP by Western blot analysis with ^{125}I -labelled CNBP were unsuccessful. The iodination procedure used appears to have destroyed the biological activity of the CNBP (data not shown).

Treatment of the purified CNBP with *S. aureus* V8 protease generated several polypeptides of different size, as revealed by electrophoretic analysis, but the collagen-binding domain was restricted to a polypeptide chain of *c.* 30 kDa, as revealed by Western blot analysis with peroxidase-labelled collagen as a probe. Conversely, CNBr cleavage of the CNBP generated a major polypeptide of *c.* 45 kDa that did not recognise

Table 4. Effect of glycosidase digestion of collagen type I for binding by *A. hydrophila* strain A186 and by its soluble CNBP

Collagen treatment	Percentage binding of glycosidase-treated collagen to		
	¹²⁵ I-labelled bacteria	¹²⁵ I-labelled CNBP	unlabelled CNBP
Control (PBS)	0	0	0
Endo-glycosidase			
Endo-β-galactosidase	10	34	14
Endo-glycosidase F	20	36	ND
Endo-glycosidase H	28	27	ND
Exo-glycosidase			
β-galactosidase	33	52	23
α-glucosidase	34	66	0
Neuraminidase	38	50	0
Chemical agent			
NaIO ₄	47	54	0
Na(BH ₄)	29	60	6
H ₂ SO ₄	43	9	0

ND, not done.
The data represent the means of triplicate measurements and the SD for each mean was < 6%.

Table 5. Inhibition of binding of ¹²⁵I-Cn-I to *A. hydrophila* A186 by synthetic peptides

Peptide sequence	Collagen-binding inhibition (%)
Collagen-homologous peptides	
Pz-Pro-Leu-Gly-Pro-D-Arg-OH	0
Ac-Gly-Pro-Leu-Gly-Ile-Ala-OC ₂ H ₅	22
Ac-Leu-Leu-Gly-Ile-Leu-OH	0
Ac-Pro-Leu-Gly-Leu-Leu-Gly-OC ₂ H ₅	70
Ac-Ala-Leu-Gly-Leu-Ala-Gly-OH	0
H-Lys-Pro-Gly-Glu-Pro-Gly-Pro-Lys-OH	0
RGD sequences	
H-Gly-Arg-Gly-Asp-OH	7
H-Gly-Arg-Gly-Asp-Ser-OH	2
n-Me-Gly-Arg-Gly-Asp-Ser-Pro	7
Gd-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys	0
Proline-rich polypeptides	
PRP-1	0
Poly-L-proline (mol. wt 7600)	0

The data represent the means of triplicate measurements and the SD for each mean was < 6%.

peroxidase-labelled collagen, thus indicating that the biological activity of the CNBP was destroyed by CNBr cleavage.

Recently, Kostrzynska *et al.* [27] reported on the antigenic diversity of two structural domains in the S-layer protein of *A. hydrophila* TF7. Further studies will be needed to determine if this antigenic diversity is also reflected in the *A. hydrophila* CNBP epitope that has the collagen-binding domain and might be useful for vaccine development.

As adhesion factors are most important in the early stages of an infection process, for the adhesion, colonisation and establishment of the pathogen in a host, the possibility of using adhesins and lectins as vaccine candidates is attractive. Ongoing studies in this laboratory are focused on the use of *A. hydrophila* antigen as immunogenic and immunoprotective agents

against infections produced by *A. hydrophila* and related species in marine fish. Antigens from *A. hydrophila* with affinity for mucosal constituents and collagenous proteins have recently been found to stimulate the mucosal immune system of rabbit [28] and fish (unpublished results), and are considered as vaccine candidates for *A. hydrophila* infections in fish farming.

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