BACTERIAL PATHOGENICITY

Isolation and characterisation of putative adhesins from *Helicobacter pylori* with affinity for heparan sulphate proteoglycan

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A pool of heparan sulphate-binding proteins (HSBP) from *Helicobacter pylori* culture supernates was obtained by sequential ammonium sulphate precipitation and affinity chromatography on heparin-Sepharose. The chromatographic procedure yielded one major fraction that contained proteins with heparan sulphate affinity as revealed by inhibition studies of heparan sulphate binding to *H. pylori* cells. Preparative iso-electric focusing, SDS-PAGE and blotting experiments, with peroxidase (POD)-labelled heparan sulphate as a probe, indicated the presence of two major extracellular proteins with POD-heparan sulphate affinity. One protein had a molecular mass of 66.2 kDa and a pI of 5.4, whilst the second protein had a molecular mass of 71.5 kDa and a pI of 5.0. The N-terminal amino acid sequence of the 71.5-kDa HSBP did not show homology to any other heparin-binding protein, nor to known proteins of *H. pylori*, whereas the 66.2-kDa HSBP showed a high homology to an *Escherichia coli* chaperon protein and equine haemoglobin. A third HSBP was isolated from an outer-membrane protein (OMP) fraction of *H. pylori* cells with a molecular mass of 47.2 kDa. The amino acid sequence of an internal peptide of the OMP-HSBP did not show homology to the extracellular HSBP of *H. pylori*, or to another microbial HSBP.

Introduction

*Helicobacter pylori* is a micro-aerophilic gram-negative spiral-shaped bacterium that is considered as a prevalent human- and non-human-primate-specific pathogen [1–3]. It is a causative agent in chronic active gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma [4]. *H. pylori* is known by its characteristic association with gastric mucus-secreting cells in vivo [5] and it has been found within and beneath the mucus layer and attached to the gastric epithelial cells [6].

Several studies have indicated that this micro-organism has unique cellular characteristics that allow it to colonise the human stomach mucosa. These characteristics include the high motility of the bacteria [7, 8], the profuse production of urease [9–11] and the expression of surface lectins that recognise specific stomach mucosa cell receptors [4, 12]. A cytotoxin has been described that causes lethal effects on various mammalian cells and gastric epithelial lesions [8, 12–16]. *H. pylori* has an SOS regulon important in the repair of DNA damage caused by exposure to a low pH or reactive oxygen species produced by immune cells during bacterial infections [17].

Recently it was found that *H. pylori* binds with high affinity to the glycosaminoglycans such as heparin and heparan sulphate, and to heparin-dependent growth factors [18, 19]. Proteoglycans are important in the normal development of cellular function, blood coagulation, extracellular matrix organisation, cell adhesion and cytokine action [20, 21]. Yet, the distribution of various proteoglycans during human wound healing is poorly understood [22, 23].

The heparan sulphate (HS) proteoglycan participates as an adhesion molecule, binding extracellular matrix proteins (collagen, fibronectin and vitronectin) in interactions that can affect cell adhesion [24, 25]. Many organisms have been shown to use the extracellular matrix as a means of adhesion once the tissue is damaged.
It has been reported that some pathogens, such as *Streptococcus pyogenes*, *S. mutans* and *Staphylococcus aureus*, bind heparan sulphate, which also acts as the cell receptor for members of the herpes virus group [18, 26].

D. O. et al. suggested that *H. pylori* adhesion may be a multi-step process that begins with non-specific hydrophobic and electrostatic interactions, followed by a second step involving a much closer adherence process that may include adhesins and lectin interactions [27]. The adhesion process is further enhanced once an ulceration induced by the bacteria exposes the base membrane heparan sulphate, providing the pathogen with a more firm attachment.

It may be worthwhile to find out if bacterial proteins with affinity for proteoglycans, such as heparan sulphate, may be useful for the development of vaccine protocols. This study reports the purification and a partial biochemical characterisation of cell-associated and extracellular proteins from *H. pylori* with affinity for heparan sulphate proteoglycan.

**Materials and methods**

**Chemicals**

All chemicals of analytical grade were purchased from Sigma, unless otherwise stated. Heparin Hi-Trap column was purchased from Upjohn-Pharmacia, Uppsala, Sweden. Heparan sulphate (HS) purchased from Sigma (from swine gut mucosal tissue) was coupled to hors eradish peroxidase (POD-HS) as follows. POD was incubated with 0.1 mM sodium periodate for 20 min at room temperature and dialysed overnight against 1 mM sodium acetate (pH 4.4). HS (1 mg/ml) was mixed with the activated POD for 4 h at room temperature and the reaction was stopped by the addition of a sodium borohydride solution (4 mg/ml). Finally, glycerol was added at 50% and the mixture was stored at −20°C in the dark until used.

**Bacterial strain and growth conditions**

*H. pylori* strain 25 (clinical isolate from adenocarcinoma) was provided by Professor T. Wadström (Lund University, Sweden). This strain, which has previously been examined for haemagglutinin [18] and was also found to bind HS with high affinity [28], was selected for characterisation of HSBPs.

*H. pylori* was grown on solid GAB-CAMP (GC) agar (BBL, Becton Dickinson, USA) supplemented with lysed human blood (80°C, 20 min) 8.5%, inactivated horse serum (56°C, 30 min) 10%, cysteine hydrochloride 0.05% and the following antibiotics: vancomycin 6 μg/ml, nalidixic acid (20 μg/ml) and ketoconazole 3 μg/ml at 37°C for 3 days in micro-aerophilic conditions. The cells were then harvested and inoculated in two separate broth systems: (A) Brucella broth containing fetal calf serum 10% (BBFCS) and supplemented with antibiotics [29–32], and (B) a serum-free Brucella broth containing cyclodextrins (BBCD) and antibiotics [30, 33]. The culture media were then incubated as above; cells were harvested and stored at −20°C until used [9, 34]. The culture supernates were used for further protein isolation.

**Protein fractionation**

The proteins were precipitated from the culture supernates with ammonium sulphate at 0–40, 40–60, 60–80 and 80–100% saturation. The precipitated proteins in each fraction were centrifuged (18,000 g, 30 min, 5°C) and then resuspended in distilled water. The fractions were dialysed against four changes of 0.01 M ammonium bicarbonate. The protein concentration in each fraction was determined with the BioRad (Richmond, CA, USA), protein dye reagent with bovine serum albumin (BSA) for the construction of a standard curve. All the protein fractions were stored at −20°C until used.

**Affinity chromatography**

The protein fractions obtained from 60–80% ammonium sulphate precipitation of culture supernates of *H. pylori* cells were subjected to a modified heparin affinity-chromatography procedure [35]. Briefly, protein samples were filtered through a 0.45-μm filter and diluted 1:1 with 0.1 M sodium acetate (pH 5.0). One ml of diluted sample was applied to a 5-ml Heparin Hi-Trap column, previously equilibrated with 0.1 M sodium acetate buffer (pH 5.0). Proteins lacking affinity for heparin were washed through with 0.1 M sodium acetate (pH 5.0) at a flow rate of 1 ml/min. Absorbed proteins were eluted with an NaCl gradient (0–2 M) over 30 min at the same flow rate and 1-ml fractions were collected. Finally, the column was washed with 0.01 M NaOH and then regenerated with distilled water and 0.1 M sodium acetate buffer. The fractions collected were dialysed extensively against 10 mM ammonium bicarbonate and stored at −20°C until used.

**Outer-membrane protein (OMP) extraction**

OMPs were obtained as described previously [36, 37]. Briefly, bacterial cells were harvested, washed and resuspended in 1 mM TRIS-EDTA (pH 7.0) at a final concentration of 107 cells/ml. Cells were disrupted by sonication (10 × 30 s at 25 W on ice) and centrifuged (7500 g, 20 min) to remove cell debris. Total membranes were collected by centrifugation (20,000 g, 90 min) at 4°C. The pellet was resuspended in 0.5 ml of distilled water. Sodium lauryl/sarcosine was added to give a final concentration of 2% at pH 7.5 and the suspension was incubated at 37°C for 30 min with gentle shaking and then centrifuged (20,000 g, 90 min) at 4°C. The pellet was washed twice in 0.05 M Tris,
pH 7.5, and suspended in distilled water. OMP extracts were then stored at −20°C until used.

**SDS-PAGE**

The protein samples were electrophoresed according to the method of Laemmli [38] with the BioRad Mini-Protein II system or the Protean apparatus (BioRad). Protein samples were denatured before electrophoresis for 7 min at 100°C in sample buffer (0.6 M Tris-HCl, pH 6.8, glycerol 10%, SDS 10%, β-mercaptoethanol 5% and bromophenol blue 0.05%). Samples containing 5–10 μg of total protein and molecular mass standard were loaded on to a discontinuous acrylamide gel (stacking gel 4%, separating gel 12%), electrophoresed at 80 V for 2 h and stained with Coomassie Blue.

**Blotting**

Proteins were electrophoresed in the BioRad SDS-PAGE Mini-Protein II system and then transferred to Immobilon membranes (Millipore, Bedford, USA) by a semi-dry electroblotter at 190 mA for 2 h. The lane of the molecular mass markers was cut and stained with Coomassie Blue. The membrane was blocked with BSA 3% in saline phosphate buffer (PBS), washed with three 15-min changes of PBS-Tween 20 0.05% (PBS-T), followed by a wash with 0.1 M sodium acetate, pH 5.0. POD-HS was added to the membrane and incubated for 90 min, washed with three 20-min changes of sodium acetate buffer-Tween 20 and two 5-min changes of sodium acetate buffer. The membrane was developed with dianisobenzidine 2.5 mg and H2O2 2.5 μl in 10 ml of 0.1 M sodium acetate, pH 5.0. The reaction was stopped with 0.1 M sodium metabisulphite.

**Preparative iso-electric focusing**

Protein samples were adjusted to a concentration of 5–10 μg/ml in sample buffer (9.5 M urea, Triton X-100 2%, ampholine 3-10 2%, 50 mM dithiothreitol) before iso-electric focusing (IEF). The gel was prepared with acrylamide 5% and a pH range of 3–10 and placed in a horizontal electrophoretic chamber. The samples and pl standards (Pharmacia) were loaded on to the gel and electrophoresed at 500 V and 250 mA for 3 h. The gel was stained with Coomassie Blue R-250 and pl measurements were calculated from a pl standard curve according to the migration distance of the pl standards. A second electofocusing run was transferred to Immobilon membranes (Millipore) and developed with POD-HS as described above.

**NH2-terminal amino acid sequencing**

The proteins were electrophoresed with the Protean apparatus (BioRad) according to the protocol described above, transferred to Immobolon membranes (Millipore) and stained with Coomassie Blue. The HSBS bands were cut off, de-stained with methanol:acetic acid:distilled water (5:1:4) and thoroughly washed with distilled water. The amino acid analysis was performed on tryptophan hydrolysates of these proteins with a yield of 10 amino acids from the N-terminal chain. The analysis was done at the University of Arizona, Division of Biotechnology, under the supervision of Dr Wallace Clark.

**Results**

**Isolation of extracellular HSBS**

Electrophoretic profiles of culture supernates from both BBFCS and BBBCD revealed that the 0–40% and 40–60% ammonium sulphate precipitation fractions contained the highest composition and concentration of proteins (Fig. 1a). In both culture media, two bands were evident in the 40–60% fractions, one band at 66.2 kDa and the second at 54.4 kDa, but 44.3, 30.0- and 11.3-kDa protein bands could also be detected (the latter only in BBFCS).

In the 60–80% fraction of both culture media, two high molecular mass and one low molecular mass proteins were present at 71.5, 66.2 and 11.3 kDa. A few minor protein bands at 47.2 and 14.4 kDa were also present in both culture media. At 80–100% saturation, no protein bands could be detected by SDS-PAGE in either medium. Blotting studies with POD-HS as a probe showed the presence of two main protein bands with HS binding activity at 66.2 and 54.4 kDa (Fig. 1b) in the 0–40 and 40–60% fractions of both culture media. Also, a smear was observed in the range 20–31 kDa in BBFCS. In BBBCD, two proteins with POD-HS affinity were detected at 47.2 and 30.0 kDa, and at 60–80% ammonium sulphate precipitation, three HSBSs were evident, again at 71.5, 66.2 and 54.4 kDa. A comparative SDS-PAGE and blotting analysis of the BBFCS and BBBCD cultures indicated that the proteins produced and secreted into the BBBCD culture supernates were present in lower concentrations. However, both BBFCS and the BBBCD supernates showed common protein bands (Fig. 1a), mainly in the molecular mass range 40–65 kDa. Some bands that were present in BBFCS were less evident in BBBCD (Fig. 1a). These could be caused by the presence of fetal calf serum in the medium with proteins with similar molecular mass to those secreted by *H. pylori*. In either case, the three major HSBSs (71.5, 66.2 and 54.4 kDa) were present in both culture media (Fig. 1b).

**Affinity chromatography**

The ammonium sulphate fractions were subjected to affinity chromatography (Fig. 2). SDS-PAGE analysis of the eluted proteins obtained from the Hi-Trap
column (LKB-Pharmacia, Uppsala, Sweden) in different elution conditions revealed that all the HSBPs co-purified in the same fraction (Fig. 2, shaded zone), where the 71.5-, 66.2- and 54.4-kDa protein bands were detected, correlating with the electrophoretic profile of the 60–80% ammonium sulphate precipitation that was put on the chromatographic column.

**Cell-associated HSBP**

Proteins obtained from the bacterial cell surface by distilled water and 3 M urea extraction of cells harvested from solid GAB-CAMP, BBFCS and BBCD cultures were analysed by SDS-PAGE and blotting analysis (Fig. 3a). Similar protein bands were observed in both extraction procedures, especially the 66.2-kDa
protein. Also 53.0-, 44.3- and 40.1-kDa protein bands were present in both extracts.

Blotting analysis revealed that the 66.2-, 44.3- and 40.1-kDa proteins exhibited POD-HS affinity (Fig. 3b). Nevertheless, in the distilled water extract, the 44.3- and 40.1-kDa HSBP bands were the predominant proteins, whereas in the urea extract a 23.8-kDa HSBP was predominant.

**OMP HSBP**

SDS-PAGE and blotting analysis of the OMP fractions of *H. pylori* cells cultured on GAB-CAMP medium or in BBFCS and BBCD revealed the presence of a large number (seven proteins) of HSBP (Fig. 4). In this cell compartment, the 66.2-kDa HSBP was also evident in all the extracts, but the predominant protein band was detected at 47.2 kDa, also with HS-POD affinity.

Minor protein bands at 51.2, 44.3, 34.2, 20.4 and 29.8 kDa with affinity for POD-HS were also present in the three bacterial OMP preparations. Although the starting concentration of bacterial cells was 10⁶ cells/ml, some difference in the protein proportion was observed among the extracts, mainly in the BBCD harvest, where low molecular mass proteins were detected at 30.4 and 29.8 kDa, both of which had enhanced HS-POD affinity.

**Iso-electric focusing**

Electrofocusing of the 60–80% ammonium sulphate protein fraction from BBCD culture showed that most
of the proteins focused at pH 4–6 (Fig. 5A). Blotting analysis revealed two main protein bands with HS-binding activity at pl 5.4 and 5.0, and a weaker band with a pl of 5.2 (Fig. 5B).

To determine the correspondence between the molecular mass data and the pl measurements, electrophoresed proteins were cut out of the SDS-polyacrylamide gel with a stained gel as reference, and the protein was eluted from the gel. The eluted proteins were then loaded on to an iso-electric focusing gel. The results indicated that the 66.2-kDa protein had a pl of 5.4 and the 71.5-kDa protein had a pl of 5.0.

NH2-terminal amino acid sequence

On the basis of previous studies [28], in which high molecular mass proteins showed the highest affinity for HS, two HS·BPs (71.5 and 66.2 kDa) from the 60–80% fraction obtained from BBCCD culture (Fig. 1) were analysed by amino acid sequencing. One HS·BP band was designated as HS·BP54 (molecular mass 66.2 kDa, pl 5.4) and the second HS·BP band was designated as HS·BP50 (molecular mass 71.5 kDa, pl 5.0). Amino acid sequencing of these two proteins after 10 cycles (10 amino acids) provided the sequence information shown in Table 1. The major OMP-HS·BP was also sequenced; however, the protein was first hydrolysed with trypsin and a major internal polypeptide fragment was subjected to Edman degradation and NH2-terminal amino acid sequencing (Table 1).

![Fig. 5](image-url)  
**Fig. 5.** Iso-electric focusing (IEF) of proteins secreted by *H. pylori* in BBCCD precipitated by 60–89% (F80) ammonium sulphate saturation. (A) IEF, (B) blotting analysis. Right margin arrows indicate the presence of the HS·BP; M, marker.

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<tr>
<th>Table 1. Amino acid sequences of three <em>H. pylori</em> HS·BPs</th>
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<tr>
<td><strong>HS·BP label</strong></td>
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<td>HS·BP50&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>HS·BP54&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>OMP-HS·BP&lt;sup&gt;2&lt;/sup&gt;</td>
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<sup>1</sup>The single-letter amino acid code is used.
<sup>2</sup>Amino-terminal sequence.
<sup>3</sup>Internal sequence.

**Discussion**

It is important to establish the conditions that make the adherence and, therefore, the colonisation of the gut mucosal surfaces by bacteria possible. Several candidate receptors for attachment of *H. pylori* to target cells have been proposed, including sulphogalactosylceramide [39, 40], and extracellular matrix (ECM) proteins [10, 22, 41]. Furthermore, Slomiany et al. demonstrated that there is specificity of the bacterium for lactosylceramide sulphate and GM1 gangliosides [42]. Borén et al. reported that soluble glycoproteins that possessed the Le<sup>b</sup> antigen inhibited bacterial adhesion *in situ*. It has also been found that *H. pylori* has strong binding affinity for HS proteoglycan [12]. Some of the adhesive molecules from *H. pylori* responsible for binding to host receptors have been isolated and characterised [12, 43, 44].

Kamisago et al. addressed the role of sulphatides in attachment of *H. pylori* to a gastric cancer cell line, KATO III, and found that the adhesion process can be significantly inhibited by heparin [40]. Based on the assumption that *H. pylori* has adhesive molecules that enable the bacterium to interact with host-sulphated glycoconjugates as an important step during the adhesion and colonisation process, the present study isolated and characterised a group of extracellular, cell-associated and OMP proteins from *H. pylori* with affinity for HS proteoglycan, which may enable the pathogen to target sulphated glycoconjugates exposed on mucosal epithelial cells.

Studies have shown the presence of HS·BP in *H. pylori* [18, 19, 28, 45], where the interaction of these bacterial proteins with host components was exploited. It could be determined that these proteins recognised heparin, gastrointestinal cell surface HS, in an association inhabitable by the presence of sulphated carbohydrate polymers in the media. Furthermore, *H. pylori* HS·BP were shown to interact with heparin-dependent growth factors, interfering with the tissue regeneration induced by these factors [19], and Chmiela et al. observed that the binding of different *H. pylori* strains to cell lines was decreased by pretreatment of bacterial cells with heparin [46].

Furthermore, recent work in this laboratory has shown that oral immunisation of BALB/c mice with vaccine composed of *H. pylori* HS·BP prevented bacterial...
colonisation of the gut mucosa by a mouse-adapted *H. pylori* strain, as evidenced by histopathological examination, culture, rapid urease test and PCR assays [47]. This vaccine administration resulted in a reduction of the adhesion of the bacteria to the gastrointestinal tract, from 100% in unvaccinated mice to 6.6% in the HSBP-CTB-immunised group, providing additional evidence on the role that the *H. pylori* HSBP play in the disease development.

A chromatography procedure allowed the purification of a set of extracellular proteins, with different molecular masses, showing high reactivity towards POD-HS. However, these proteins appear to differ from the *H. pylori* heat-shock protein described above. For example, their NH₂-terminal amino acid sequences do not have any homology with the 62-kDa heat shock protein described by Evans et al. in *H. pylori* [35] and they do not react with mouse polyclonal antibodies which recognise the 62-kDa heat-shock protein (data not shown).

The findings in the present study indicate that *H. pylori* yields mainly two extracellular proteins (71.5 kDa, pl 5.0 and 66.2 kDa, pl 5.4) and one OMP (45 kDa) that exhibit strong affinity for POD-HS proteoglycan, shown by blotting analysis. None of them show any similarity with a known heparin-related lectin. The NH₂-terminal amino acid signature of these three HSBPs did not show homology either to other adhesins previously described in *H. pylori*, or to each other. An amino acid sequence analysis of the OMP with the BLAST-system protein bank revealed that this protein differs from that found in the extracellular space of *H. pylori* and from other bacterial HSBPs.

An interesting observation is that the 66.2-kDa extracellular HSBP has a remarkable homology to the haemoglobin α-chain, human tumour necrosis factor receptor-2 precursor and to an Excherichia coli chaperon protein. However, when equine haemoglobin was used for inhibition experiments of binding of HS by *H. pylori* cells, or binding of HS to HSBP immobilised on Immobilon-P membranes, no inhibitory activity was seen (data not shown).

To rule out the possibility that the extracellular HSBPs were heparin-binding proteins in the fetal calf serum used in the culture media, the study also evaluated whether the HSBPs were present in the serum-free broth media, and if the HSBPs were also associated with the bacterial cell. For this assessment, bacterial cells were grown in BBCD instead of BBFCS. SDS-PAGE and blotting analysis showed the presence of HSBPs in the culture supernates of the serum-free broth media. Also, four proteins of molecular mass similar to those HSBPs from the culture supernates of BBFCS were detected in the urea extracts, which suggests that the HSBPs are associated with the bacterial cell.

The results of the present study may suggest that growth of *H. pylori* in broth media allows the secretion of the proteins into the culture medium, although this can also be caused by the absence of an anchored pedestal for these antigens [4, 12]. The concentrations found in cell-associated proteins extracted from *H. pylori* cells grown in BBFCS and BBCD were similar on the basis of protein concentration obtained from the same bacterial mass (wet mass) and with the same extraction volumes. The presence of these HSBPs in *H. pylori* may suggest a potential vaccine candidate for the development of alternative immunoprophylactic strategies against *H. pylori*-associated gastritis and duodenal ulcers. Work is being carried out in this laboratory to elucidate the immunostimulant and immunoprotective properties of the extracellular HSBPs.

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