Use of an affinity proteomics approach for the identification of low-abundant bacterial adhesins as applied on the Lewis^b-binding adhesin of *Helicobacter pylori*

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Abstract Microbial attachment to host cell surfaces is considered to be the first essential step for colonization and infection. In most known cases, attachment is mediated by a specific protein–carbohydrate interaction. We have used a carbohydrate-containing crosslinking probe to select bacterial surface adhesins for trypsin digestion, MALDI-TOF mass spectrometry and identification against genome sequence. The present paper describes this functional proteomics approach for identification of the recently cloned low-abundant Lewis^b-binding adhesin of *Helicobacter pylori*. Protein identification was obtained through the enrichment of approximately 300 fmol of adhesin from solubilized cells.

Key words: Bacterial adhesin; Low-abundance protein; Proteomics; Protein–carbohydrate interaction; *Helicobacter pylori*

1. Introduction

The abundance, diversity and specificity of expression of glycoconjugates on mammalian cell surfaces may explain why many microbes use specific protein–carbohydrate bindings for their tropism of infections [1,2]. This first essential step of microbe–host interaction may be a rational target for prevention or treatment of infections. The microbial protein (hemagglutinin, adhesin, toxin) may be used for vaccination, and the host saccharide for design of soluble synthetic analogues [2,3].

Although complex, a receptor glycoconjugate may be detected by rational binding assays on separation surfaces, for instance using radiolabelled bacteria [2,4]. The detected glycoconjugate may be structurally identified after isolation by biophysical methods, such as nuclear magnetic resonance (NMR) and mass spectrometry [4,5]. Knowledge of a receptor saccharide should be possible to use for the functional identification of the microbial protein, provided that the genome sequence of the microbe is available, using a proteomics approach [6] combined with affinity tagging [7]. With the sensitivity that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) provides, identification can be performed on very low quantities of protein (femtomolar range).

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The recently discovered Gram-negative bacterium *Helicobacter pylori* is an urgent object for study, because of its common occurrence in human stomach and importance for gastric ulcer and cancer [8,9]. This bacterium is uniquely complex concerning carbohydrate-binding specificities [2]. To determine the relevance of these for pathogenicity, it is necessary to identify the corresponding bacterial adhesins for knock-out and other studies. So far, however, only one adhesin has been identified with certainty for *H. pylori*, namely the Lewis^b-binding (BabA) adhesin [10], which recently was genetically cloned [7]. Similar to the case for uropathogenic *Escherichia coli* and the PapG adhesin [11], the BabA adhesin is a low-abundant protein, with only about 500 copies per bacterial cell [7].

Complete genome sequences are now available for several strains of *H. pylori* [12,13], allowing identification of adhesins by proteomics approaches. In order to optimize conditions for analysis of *H. pylori* adhesins, we chose the known BabA adhesin as a model for identification. Earlier attempts to visualize adhesins using analytical two-dimensional (2D) polyacrylamide gel electrophoresis (PAG)E were unsuccessful. Therefore, a novel combination of analytical methods was employed in order to functionally enrich the BabA adhesin for identification using a mass spectrometric strategy. The BabA adhesin was selectively biotinylated using a method previously described [7]. In short, living bacterial cells were incubated with an albumin-based photoprobe to which the Lewis^b saccharide and a biotinylated crosslinker structure had been coupled. The tagged adhesin was then extracted by sodium dodecyl sulfate (SDS) solubilization of entire cells and enriched by streptavidin beads. Elution, one-dimensional (1D) gel electrophoresis, in situ trypsin digestion, MALDI-TOFMS for peptide masses and bioinformatics searches resulted in a reliable BabA adhesin identity. The sensitivity of MALDI-TOFMS allowed the identity to be obtained from approximately 300 fmol purified adhesin.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Two different strains of *H. pylori* were used, CCGU 17874 and CCGU 17875 from the Culture Collection, Göteborg University. The cells were stored at −80°C in soy broth containing 15% glycerol by volume, and were grown on agar (14 g/l) containing 10% heat-inactivated fetal calf serum, brucella broth (28 g/l; Difco Laboratories, Detroit, MI, USA) and 1 ml/l IsoVitale X enrichment vitamins (Becton Dickinson Europe, Merlyan, France) in a humid (98%) microaerophilic chamber (O_2: 5–7%, CO_2: 8–10%, N_2: 83–87% and H_2: less than 2%) at 37°C. After 48 h, the cells were scraped off and washed three times in phosphate-buffered saline (PBS).

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2.2. Labelling of receptor conjugate

The trifunctional reagent sulfosuccinimidyl[2-6-(biotinamido)-2-(*p*-azidobanzamido)-hexanoamido]ethyl-1,3′-dithiopropionate (Sulfo-SBED, Pierce, USA) was conjugated to Lewis^b hexasaccharide-conjugated human serum albumin (Le^b-HSA, IsoSep, Sweden), following the instruction of the manufacturer. Briefly, 5 μl Sulfo-SBED (10 μg/μl) in dimethylsulfoxide was added to 100 μl Le^b-HSA (1 μg/μl) in 0.1 M potassium phosphate buffer, pH 7.2. The mixture was incubated at room temperature in the dark for 1 h. The product was desalted on a HiTrap Desalt column (Amersham Pharmacia Biotech, Sweden) in a 1 ml fraction and stored in the dark to be further used the same day.

2.3. Tagging of H. pylori adhesin

The BabA adhesin was tagged using a procedure previously described for cloning [7]. In short, freshly harvested bacteria from approximately one-third of a Petri dish were incubated for 30 min at ambient temperatures with the labelled Le^b glycoconjugate and irradiated under an ultraviolet lamp. The disulfide bond on the linker was reduced by the addition of dithiothreitol to give a final concentration of 50 mM. The tagged bacteria were washed three times in PBS, pH 7.4, and frozen.

2.4. Enrichment of biotinylated bacterial proteins with streptavidin-coated magnetic beads

Tagged bacterial pellets were dissolved in 2% SDS containing 25 mM Tris, pH 8.0, followed by dilution to 0.5% SDS with 25 mM Tris. 200 μl Bio Mag streptavidin-coated beads (PerSeptive Biosystems, Framingham, MA, USA) were washed three times in PBS. Bacterial extracts were incubated with beads for 16 h at 4°C and non-bound material was washed away from the beads using 0.5% SDS. The beads were finally heated to 95°C in SDS–PAGE sample buffer for 15 min.

2.5. Electrophoresis and detection of biotinylated proteins

SDS–PAGE and Coomassie staining were carried out with Pharmacia PhastSystem™ (Pharmacia Biotech, Uppsala, Sweden) or Bio-Rad Mini-PROTEAN II (Bio-Rad, Hercules, CA, USA) according to the protocols of the manufacturers. Briefly, samples (4 μl or 25 μl, respectively) extracted from beads were applied on a homogeneous gel of 12.5%. After electrophoresis, the gel was either stained with GelCode® Blue Stain Reagent (Pierce, USA) or electroblotted to a PVDF (0.2 μ) membrane according to the manuals. The transfer buffer consisted of 20% methanol, 192 mM glycine and 25 mM Tris at pH 8.3.

The PVDF membrane was preincubated in blocking solution: 3% bovine serum albumin (BSA), 50 mM HEPES–NaOH, 100 mM NaCl, pH 7.3 for 1.5 h and washed with 0.05% Tween-20, 50 mM HEPES–NaOH, 100 mM NaCl, pH 7.3 (washing buffer). The membrane was then incubated with horseradish peroxidase-conjugated streptavidin (HRP-NeutrAvidin™, Pierce, USA) in washing buffer with 1% BSA added. After 1.5–2 h, the membrane was washed five times in washing buffer, and biotin-containing protein bands were developed with 0.1% H_2O_2 and 0.02% 3,3′-diaminobenzidine hydrochloride (DAB, Pierce, USA) in washing buffer. The membranes were then washed with water and dried at room temperature.

2.6. Enzymatic digestion

Each protein band from the Coomassie-stained gel was cut out, placed in separate siliconized tubes and gently macerated with a hand-hold mixer. The dye was removed by adding 85 μl 25 mM NH_4HCO_3 in 50% CH_3CN and the tube was vortexed for 30 min before the supernatant was removed. This procedure was repeated twice. Destained gel pieces were dried for 40 min in a vacuum concentrator (SpeedVac, Savant Instruments, USA). 15 μl trypsin (porcine, sequencing grade, modified, Promega, USA) in 25 mM NH_4HCO_3, pH 8, was added. The tubes were incubated for 16 h at 37°C. Peptides were extracted from the gel by adding 15 μl 5% CF_3COOH, either in 75% CH_3CN or in water, to the tube. The tubes were then vortexed for 30 min and centrifuged for 2 min at 5000×*g*.

2.7. Mass spectrometry

Mass spectra were obtained using a TofSpecE (Micromass, Manchester, UK) time-lag focusing MALDI-TOF mass spectrometer equipped with a reflectron. Samples were prepared using the dried-drop method, by mixing 0.5 μl tryptic digest with 0.5 μl matrix directly on the MALDI target. The matrix used was α-cyano-4-hydroxy-cinnamic acid (Aldrich Chemie, Steinheim, Germany), 10 mg/ml in CH_3CN:water 1:1. If unsatisfactory spectra were obtained, samples were redissolved in 2.5% CF_3COOH and reanalyzed. Sample purification and concentration were achieved by using ZipTips (Millipore, Bedford, MA, USA) containing C_{18} material according to the manufacturer's instruction. 1 μl of the eluate was added to 0.5 μl of matrix solution and prepared using the dried-drop method as above. Peptide masses obtained were used to identify known proteins through database searches using MS-Fit (http://prospector.ucsf.edu).

3. Results

Two strains of *H. pylori* were compared, one, 17875, being a positive binder and one, 17874, being a non-binder of fucosylated glycoconjugates [7,10]. The binding specificity was confirmed by overlay of radiolabelled bacteria on thin-layer chromatograms with Le^b hexaglycosylceramide, using conditions described earlier [14]. In early attempts to detect the adhesin using separation with analytical 2D PAGE, after labelling of the living cells with the biotinylated probe, it was possible to barely detect a biotinylated protein in strain 17875 (but not 17874) on blots, and detection with streptavidin-peroxidase (unpublished data). However, silver staining was unsuccessful, and detection of the adhesin by mass spectrometry after trypsin digestion could not be performed. Therefore, the streptavidin-coated beads were used to selectively enrich biotin-labelled products from solubilized cells, followed by elution, 1D electrophoresis, blotting and staining.

Six protein bands were identified (Table 1) from the Coomassie-stained gel (Fig. 1A), two of which were stainable with streptavidin-peroxidase (Fig. 1B). The major one of these was identified as the endogeneous biotin carboxyl carrier protein (Table 1). The minor band produced the mass spectrum reproduced in Fig. 2, where three peaks could be derived from BSA from the band just below, and two from autodigestion of trypsin. The remaining five masses were used in database searching (Table 1). The eight top-ranked proteins are outer

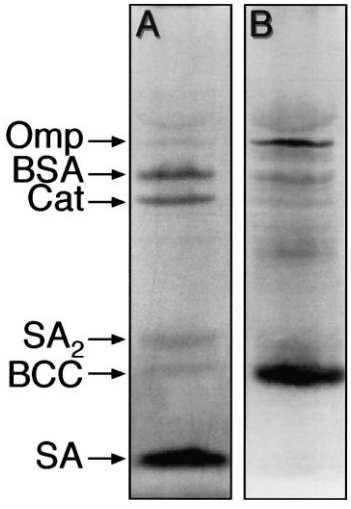

Fig. 1. Gel/blot. SDS–PAGE of proteins extracted from the magnetic beads after binding of biotin-tagged *H. pylori* proteins. A, gel stained with GelCode® Blue and B, stained with streptavidin-HRP/DAB. Bands indicated with arrows identified as: SA, streptavidin; BCC, *H. pylori* biotin carboxyl carrier protein; SA_2, streptavidin dimer; Cat, *H. pylori* catalase; BSA, bovine serum albumin; Omp, *H. pylori* outer membrane protein.

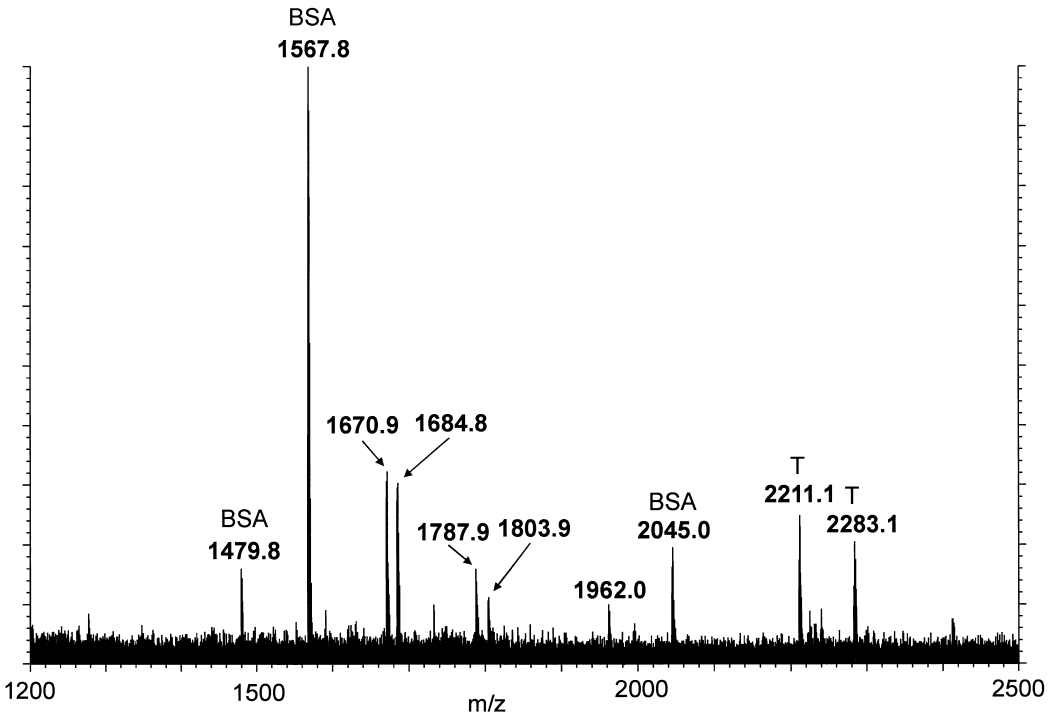

Fig. 2. MALDI-TOFMS spectrum of Omp (outer membrane protein) band (Fig. 1) after digestion with trypsin. BSA, tryptic peptide from bovine serum albumin. T, peptide from trypsin autolysis. Remaining peaks match peptides from the Le^b-binding adhesin.

membrane proteins that display a very high level of sequence homology; the second protein listed is the earlier reported BabA adhesin [7]. This protein was convincingly shown to be the Le^b-binding protein after inactivation studies [7]. The conclusion therefore is that the affinity proteomics approach used here is a reliable and sensitive method for identification of low-abundant bacterial adhesins.

4. Discussion

With the rapidly growing number of sequenced microbial genomes becoming accessible for public use, new ways to explain infection mechanisms will emerge, leading to improved therapeutics. A large number of carbohydrate-binding specificities have been detected for various microbes [1,15–17], and the general assumption is that these mediate the essential first contact with the host to colonize, infect or mediate a toxic effect. However, although specific protein–carbohydrate interactions have been proven to be important for simpler systems like bacterial toxins and viruses, in the case of bacteria and parasites, only a few cases have been critically studied in relevant infection models [2,15], and few adhesins have been identified. The functional proteomics approach described here will be a convenient way of identification of adhesins in cases where the saccharide receptor is known. Although the BabA adhesin is a low-abundant protein [7], only one-third of a Petri dish harvest of bacterial cells was required to obtain the correct protein identification. The amount of adhesin separated by 1D gel electrophoresis was in the order of 300 fmol and only a fraction of this was consumed during MALDI-TOFMS.

In case of an unknown host glycoconjugate receptor, the

Table 1
Proteins identified from stained bands cut from the gel in Fig. 1

		Accession #	Mw	Hits	MOWSE score	Coverage (%)
1	Streptavidin	494485	12 730	5/15	3.67×10^4	60
2	Biotin carboxyl carrier protein	2313469	17 133	6/10	7.44×10^4	41
3	Streptavidin (dimer)	494485	12 730×2	3/3	1.86×10^3	47
4	Catalase	4155380	58 527	7/10	1.21×10^5	20
5	Albumin, bovine	1351907	69 294	8/11	3.69×10^3	15
6	Omp-adhesin	4155400 2791666 4155775 2791668 2314032 2314407 2804778 2313410	80 608	5/5	2.86×10^3	9

Searches were made by searching against the NCBInr database (all species) with an error tolerance of 200 ppm. Table entries all received the highest scores in searches. For the adhesin, three masses could be derived from BSA and were not used in the search.

following experimental approach may be adopted. A receptor-active host glycoconjugate is detected by overlay assays [2,14] using a radiolabelled microbe and separation surfaces (thin-layer chromatograms for glycolipids, and membrane blots after electrophoresis for glycoproteins). Improved glycotechnology methods including NMR and mass spectrometry make identification of the saccharide-binding epitope accessible, provided that the necessary human saccharides are available [2]. Univalent biotinylated probes are easier to prepare, but the multivalency of saccharide as used here may be essential in case of low-affinity protein–saccharide specificities [2,15].

Final proof that a protein identified by this proteomics approach is the actual adhesin requires knock-out experiments and documentation that binding is lost [7]. If essential for infection, a microbe–host protein–carbohydrate interaction provides a rational basis for improved anti-infection treatments, including vaccination against the protein and use of soluble saccharide analogues designed from three-dimensional structures of protein–saccharide complexes.

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