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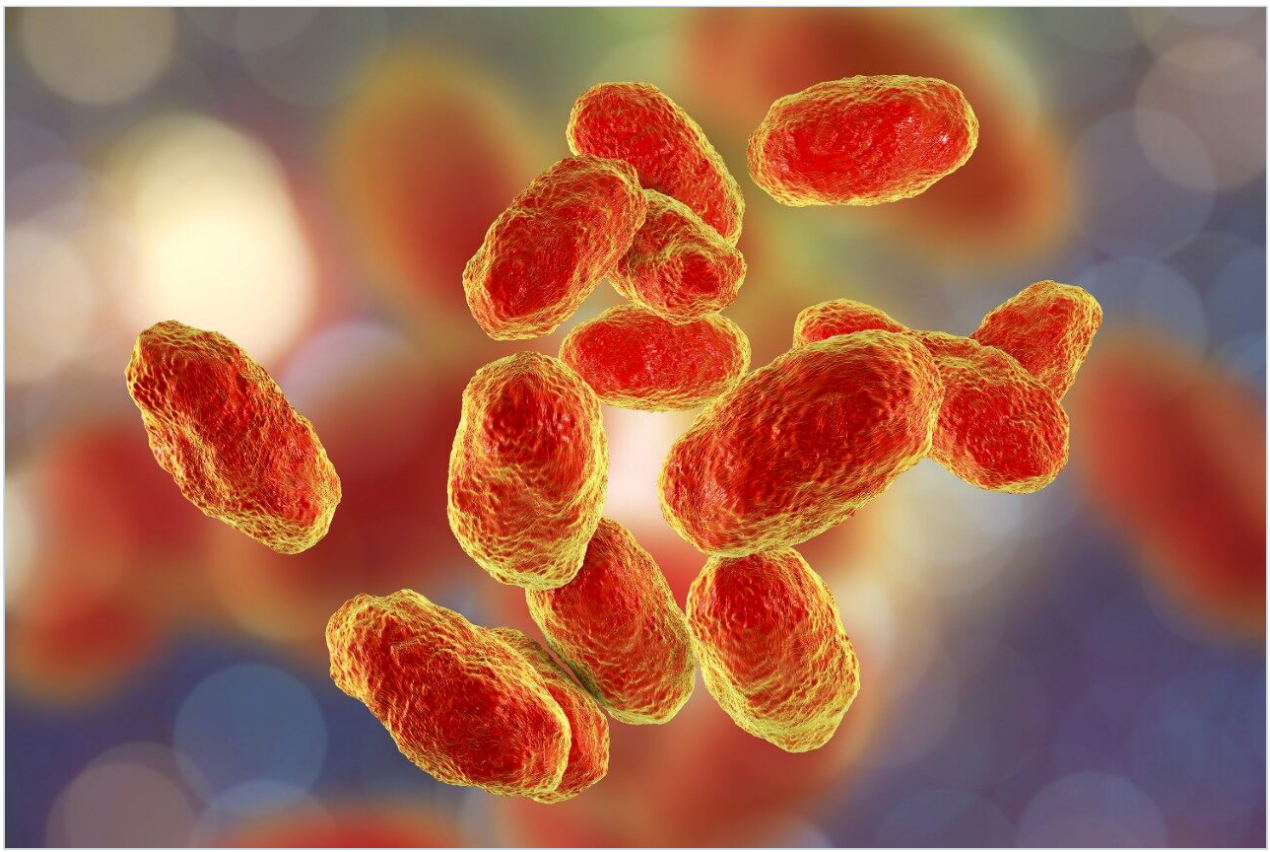
응용 자료

Monitoring Protein Expression in *Haemophilus Influenzae* using 2D Semi Preparative Electrophoresis and Mass Spectrometry

Kajsa Thorén, Elisabet Gustafsson, Thomas Larsson, Carol Nilsson

Waters Corporation, Göteborg University



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Abstract

In this paper we have used two dimensional preparative electrophoresis to separate proteins in an extract from *H. influenzae b*.

Introduction

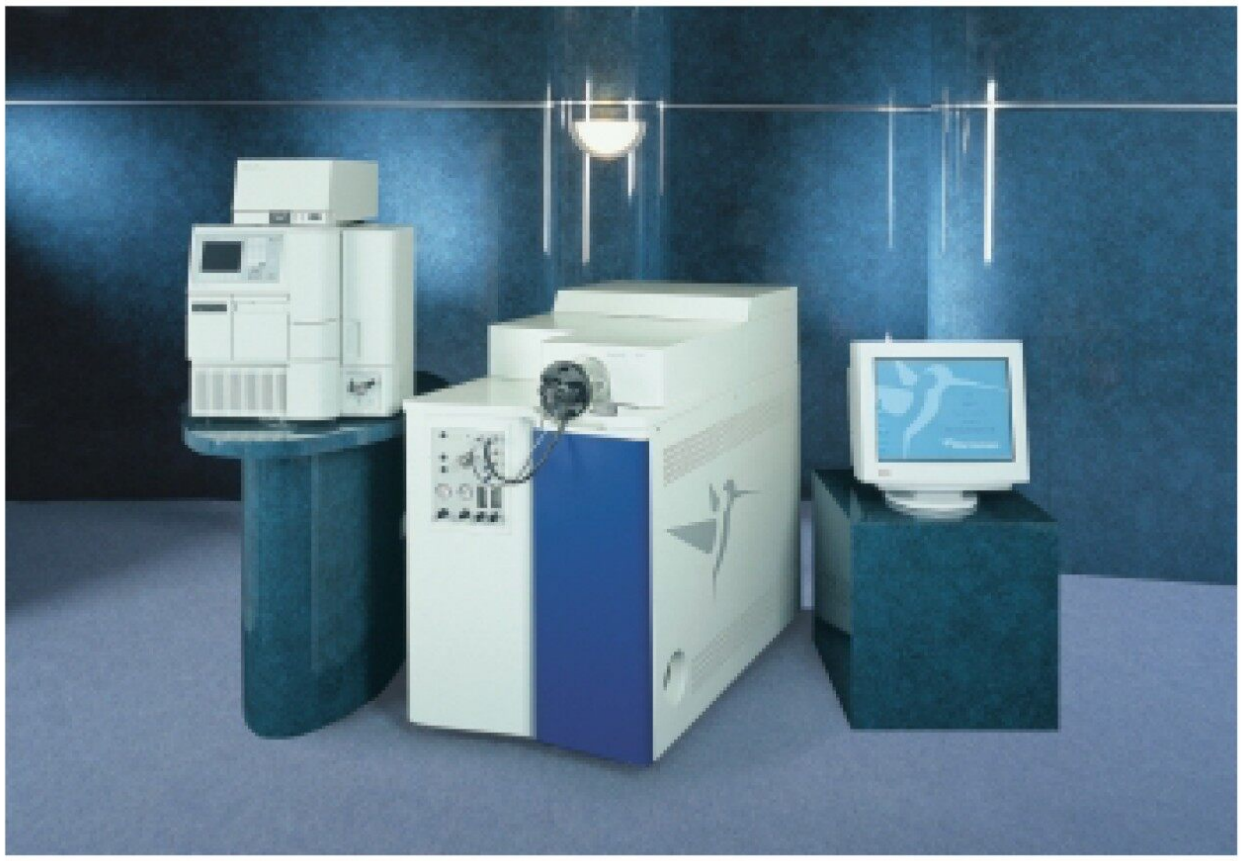
Haemophilus influenzae is a major pathogen responsible for a wide variety of human infections. This virulent bacterium causes serious diseases such as meningitis, epiglottitis, bacteremia, cellulitis, acute otitis media, and sinusitis. The physiology of this pleomorphic, Gram-negative coccobacillus bacterium has been well characterized over recent years and it was the first genome of a free-living organism to be completely sequenced (Fleischmann *et al.*, 1995).

The cell walls of *H. influenzae* contain lipooligosaccharide, which resembles the lipopolysaccharide of Gram-negative bacilli but has shorter side chains (hence the designation oligosaccharide rather than polysaccharide). *H. influenzae* does not make toxins or other extracellular products that account for their ability to produce infection. The mechanism of pathogenesis of *H. influenzae* infections is not completely understood. However, the presence of the type b polysaccharide capsule is a major factor in virulence.

Encapsulated organisms can penetrate the epithelium of the nasopharynx and invade blood capillaries directly.

The bacterium use pili and fibrils during the process of attachment and colonisation of the host. These cell surface macro structures contain proteins that may be potential vaccine candidates. However, identification of membrane associated proteins is difficult using traditional proteomics techniques such as 2D PAGE. This is because the membrane proteins contain hydrophobic transmembrane domains that span the phosopholipid membrane. The result of these hydrophobic domains is that the protein is difficult to solubilise and will not remain in solution during the isoelectric focusing on an IPG strip.

In this paper we have used two dimensional preparative electrophoresis to separate proteins in an extract from *H. influenzae b*.



Experimental

Material and Methods

Bacterial Cultivation and Protein Preparation

The *H. influenzae b* strain (D6) was cultivated in PDM antibiotic sensitivity medium (PDM, haemoglobin, isovitalex) at 37 °C in a low partial pressure of oxygen. The bacteria were harvested and the pellet was washed in PBS. Bacteria from a single culture dish were used.

Two Dimensional Preparative Electrophoresis Liquid Phase IEF

The samples were dissolved in 1 ml digitonin (0.1%) and ultra sonicated for 10 min. Another 14 ml of digitonin (0.1%) and servalyte (2%) was added to the sample. Liquid phase IEF was performed according to experimental procedures described in (1, 2). The sample was loaded into the (pre-cooled) Rotofor apparatus

(Bio-Rad Laboratories, Hercules, CA) for fractionation in a wide range pH gradient (pH 3–10). Constant power (10W) was applied to the system. The initial voltage applied was 880V and the system was run until a voltage plateau was reached, after about 2 hours. Twenty fractions were and pH was measured. The fractions were analysed with the NuPAGE System which includes 10% Bis-Tris gels, 3-(N-morpholino)- propane sulfonic acid (MOPS) sodium dodecyl sulphate (SDS) running buffer system. The proteins were detected using colloidal Coomassie blue (Gel Code Blue, Pierce).

SDS-PAGE and Electroelution

Rotofor fractions were concentrated by vacuum centrifugation, dissolved in 200 µL NuPAGE SDS sample buffer (Novex) and heated at 100 °C for 2–3 min. Samples were separated using the NuPAGE System run at 200V for 55 min. The fraction was then extracted using the mini whole gel eluter (Bio-Rad) following the manufacturer's instructions from the whole gel eluter and mini whole gel eluter, (Davidsson, ***et al***, 1999). The system was run at 50V.

Gel eluter fractions were dried to a volume of 200 µL. 600 µL of ice cold acetone was added and the samples were incubated in freezer for 2 hrs. The samples were centrifuged and dried after the supernatant was removed. The proteins were dissolved in digestion buffer (0.1 mM $CaCl_2$, 0.1 M NH_4HCO_3) and 10 µL of trypsin (10 ng/µL) was added followed by incubation at 37 °C for 4 hrs. The samples were dried and dissolved in 25 µL TFA (0.1%).

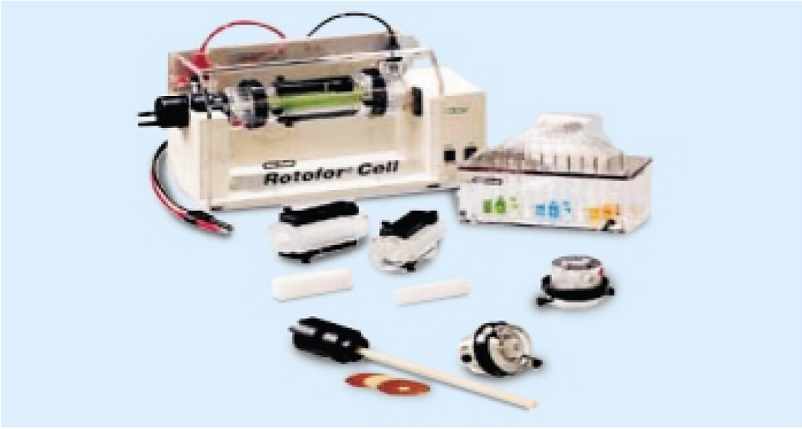


Figure 1. Rotofor apparatus with components were used to perform isolectric focusing in liquid phase.

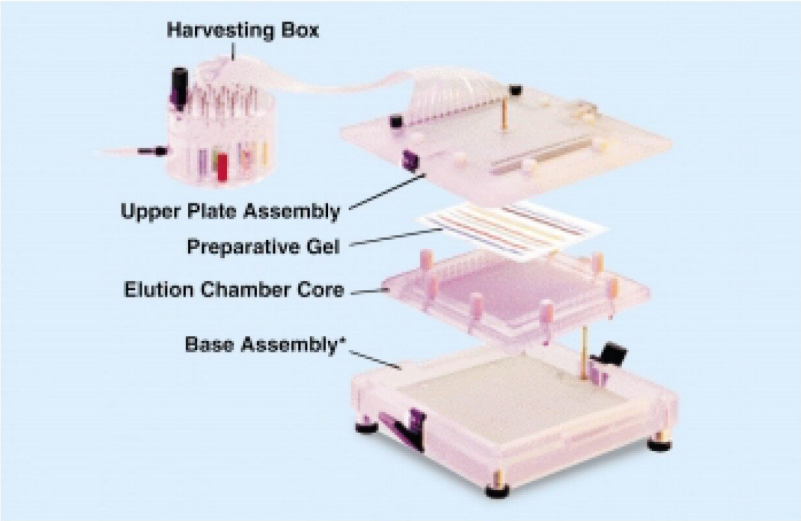


Figure 2. Mini Whole gel eluter apparatus with components were used to elute proteins into 14 liquid fractions.

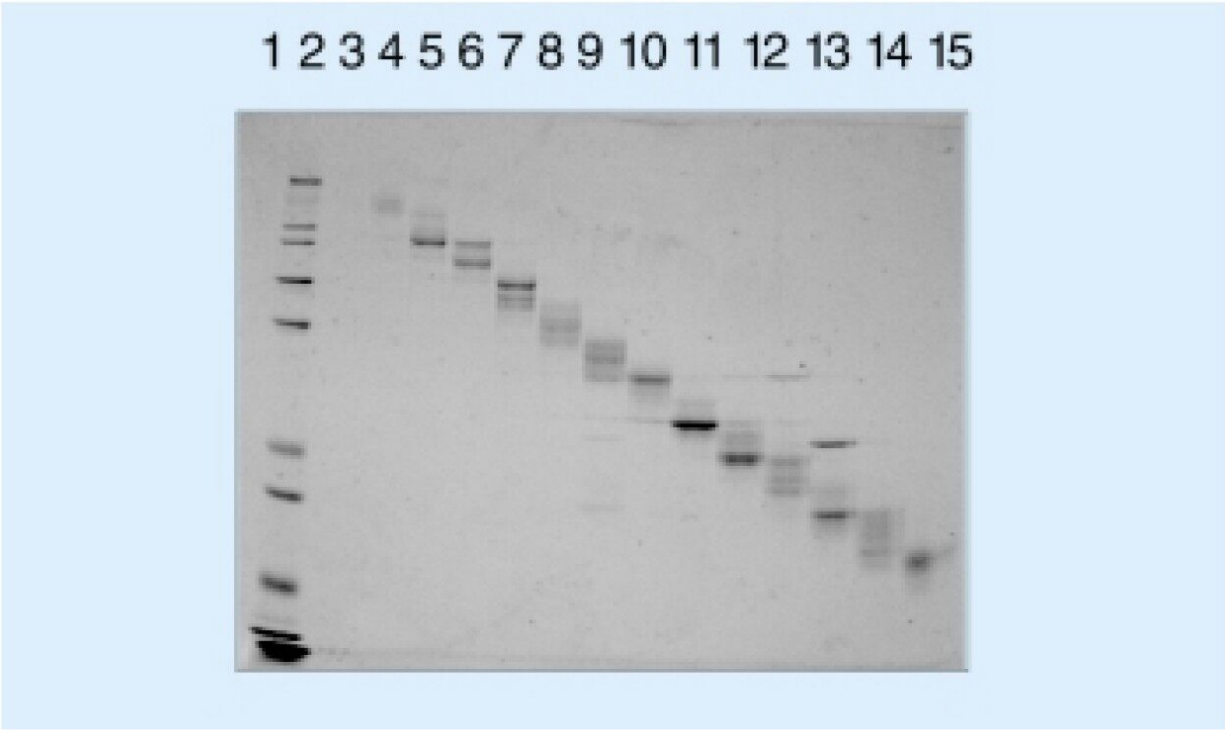


Figure 3. Proteins from Rotofor fraction 3, eleuted with the Mini Whole gel eluter were visualised by the NuPAGE system. Lane 1: Molecular weight standard, lanes 2-15 contains fractions 3:1-3:14.Fractions 3, 7, 15 (with pH 3, 5, 7) repectively, were analysed using MALDI TOFMS, ESI qTOF MS/MS and bioinformatics. Proteins identifications as listed in Table 1.

MALDI-TOF MS

MALDI-MS analysis was performed using a TofSpec 2E (Micromass). Dried tryptic digests were reconstituted in 25 µL 0.1% TFA and treated with C_{18} Zip Tips (Millipore) according to the manufacturer's instruction. 0.5 µl sample was mixed with 0.5 µl matrix solution (α-cyano-4- hydroxy cinnamic acid 10 mg/mL in acetonitrile: H_2O, 1:1) directly on the MALDI probe and allowed to dry under ambient conditions. Peptide spectra were acquired in reflectron mode at an accelerating voltage of 20 kV and 200 laser shots were summed. External calibration using angiotensin II and ACTH was used. MALDI-spectra were analysed using the MassLynx Software in a WindowsNT environment. Resulting values for monoisotopic peaks were searched against the NCBInr database.

ESI-QTOF MS/MS

Samples for which no confident identification could be made using peptide mass fingerprinting were analysed by nanoflow electrospray tandem mass spectrometry on a Q-Tof* (Micromass, UK). Samples were

enriched using Zip Tips, eluted with acetonitrile:H_2O containing 0.1 % formic acid, and sprayed from coated borosilicate capillaries (Micromass). Doubly and triply protonated peptides were fragmented using argon as a collision gas. Instrument calibration was performed using fragment ions from Glu-fibrinopeptide B and a fourth-order polynomial fit. Fragment ion spectra were post-processed in a standard text file format and searched in the NCBI nr database.

Table 1. Proteins identified from *H. influenzae b* using 2D preparative electrophoresis, MALDI-TOF MS, ESI-qTOF MS/MS and bioinformatics						
Function	ID	Accession no	MOWSE score	Mascot score	Mol.Weight	pI (calc.)
Translation	Elongation factor Tu (tufB)	1169492	1.21E+008		43354.6	5.26
Cell envelope	Outer membrane protein P5 *	585614		78	37571.0	9.54
	Iron ABC transporter * #	1073824	1.68E+004		32463.4	8.75
	periplasmic-binding protein					
	D-ribose periplasmic protein precursor *	1172866	6.4E+003		30315.0	7.81
	Major outer membrane porin P2 variant * #	2981129		132	39929.0	4.32
	Outer membrane protein P2 precursor *	129129		107	39677.0	9.09
	Outer membrane protein P1 *	881592		53	46461.0	9.14
	Outer membrane protein P4 precursor *	1170200		66	30412.0	8.98
	(lipoprotein E precursor)					
	Outer membrane protein 26 *	4574284		58	20691.0	
	28 KD membrane protein *	148838	2.07E+003		29814.1	5.4
Energy metabolism	Phosphoenolpyruvate carboxykinase	1172573		78	59404.6	5.38
	Fumarate reductase flavoprotein subunit	1169737		95	65892.0	5.72
	Glycerolphosphoryl diester	1169954		70	41875.0	6.47
	phosphodiesterase precursor					
	Dihydrolipoamide acyltransferase	1171889		73	59374.0	5.15
	component of pyruvate dehydrogenase					
	complex (E2)					
	Phosphoenol pyruvate carboxykinase (pcKA)	1172573	4.73E+004		59404.0	5.38
	Pyruvate dehydrogenase	1171888	2.83E+011		99131.5	5.44
	Aspartate ammonia-lyase	1168534	1.26E+003		51483.6	5.29
Cellular processes	DNAK protein	1169375		103	68239.0	4.72
	60 KD chaperonin (Gro E1)	1168916		82	57541.0	4.89
	Trigger factor (TF)	1174696		57	48302.0	4.96
	Periplasmic oxireductase	400828	1.68E+003		22964.4	6.83
Translation	30S ribosomal protein S1	2500383		84	59521.0	5.13
Transport protein	Heme-binding protein A precursor *	282095		88	60655.0	5.99
	D-galactose-binding periplasmic	1169276	2.92E+006		35517.9	5.62
	protein precursor					
Transport and binding proteins	Spermidine/putrescine-binding *	1075322		34	42157.0	5.29
	periplasmic protein					
ORF	Hypothetical protein precursor HI0146	1175165		57	36490.0	6.37
	Hypothetical protein HI1681	1176070		81	23381.0	8.95
Salvage of nucleotide	2´,3´-Cyclic-nucleotide 2´-	1168981		40	72718.0	6.67
	phosphodiesterase precursor					
* represents membrane and membrane-associated proteins						
# not earlier identified using 2D PAGE						

Table 1. Protein identifications from fractions 3, 7, 15 with pH 3, 5, 7, respectively. 28 proteins were identified, 9 by MALDI and 19 by ESI-MS-MS.

Results and Discussion

We have used a rapid 2D preparative electrophoresis technique to isolate 30 proteins from *H. influenzae b* and identify them using MALDI-TOF MS, ESI-QTOF MS/MS, and bioinformatics. Out of the 28 proteins 11 (39%) were identified as membrane or membrane- associated proteins. To the best of our knowledge two proteins not previously identified using 2D PAGE were identified using preparative electrophoresis as a separation technique. This method, which is rapid and requires no prefractionation, can be used as a complement to 2D PAGE in studies of protein expression.

References

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