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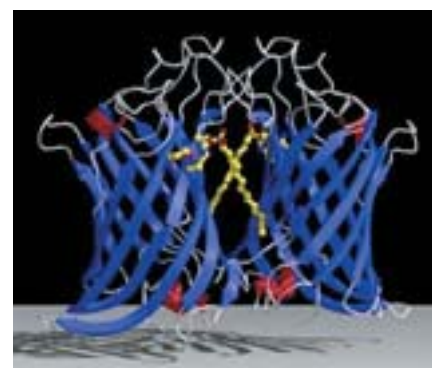
Whole Protein Separation on C8-silica with 1000Å pores

AOHUPO membrane protein standard

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Introduction

The Asia Oceania Human Proteome Organization (AOHUPO) started an initiative to analyze the membrane-associated part of the proteome. In the initial phase of the Membrane Proteomics Initiative (MPI), a protein standard was developed in the laboratories of Bill Jordan at the Victoria University in Wellington, NZ, and distributed to participating laboratories in order to develop and optimize a separation protocol that would be applicable to other proteomics samples. The sample was prepared from the large lobe of the liver of male C57BL/6J mice.



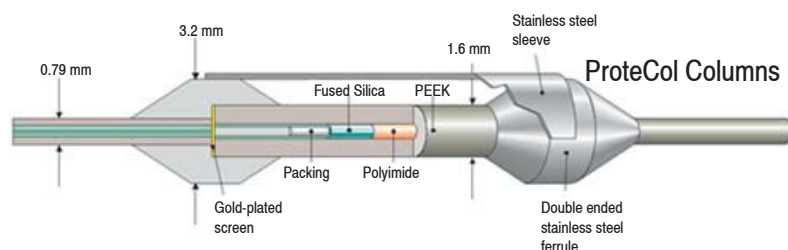
Membrane associated proteins perform very important functions in disease markers and cell recognition, signaling and trafficking. They also form a particularly challenging sample for RPLC separation due to their nature. Membrane proteins usually contain

relatively hydrophilic sections forming the intra- or extracellular domains and very hydrophobic membrane spanning domains. However, because of their exposure to the cell surface they form interesting commercial targets for both diagnostic and therapeutic purposes.

The columns employed were specifically developed for protein separation, utilizing a 3µm C8-modified silica with 1000Å pore size (Figure 1).

Figure 1

Schematic cross-section of a ProteCol capillary column



Column Length - 50, 100 or 150mm

IDs - 75, 150, 300 or 530 µm

Frit design - Deactivated gold-plated woven stainless steel mesh 0.5µm porosity, 140µm thickness

Integrated connection tubing: PEEKsil (PEEK coated fused silica) with 25 or 50µm ID, 1/32" OD

Standard inlet length - 200mm

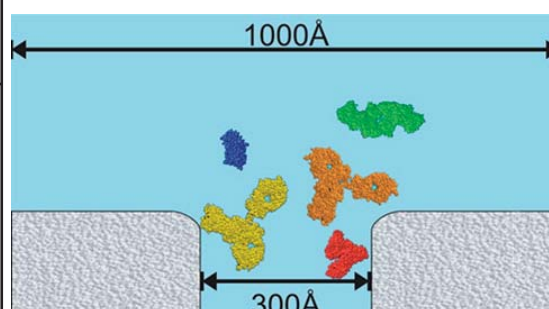
Standard outlet length - 100m

Encapsulated: stainless steel tube for added protection

Capillary ends: precision cut and polished to allow true zero-volume butt connections

The pore size is of importance for the protein separation. While most proteins would theoretically fit into a 300Å pore, a large and late-eluting protein that coincidentally gets adsorbed at the pore entrance can effectively block the pore for most of the analysis time and, thus, prevent all sample components trapped inside the pore from eluting (Figure 2).

Figure 2 - Relative sizes of proteins and pores



The 5 most abundant proteins in serum:

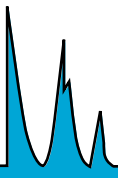
Serum albumin - shown in red

IgG - shown in orange

IgA - shown in yellow

Transferrin - shown in green

Antitrypsin - shown in blue



Experimental Outline

- Perform analytical separation on a 300 μ m ID capillary column
- Collect fractions from 3 separations on a 2mm ID glass lined column
- Freeze-dry fractions and perform a tryptic digest on all fractions
- Analyze digested fractions on a capillary C18 column with 300 Å pore size

Sample Preparation

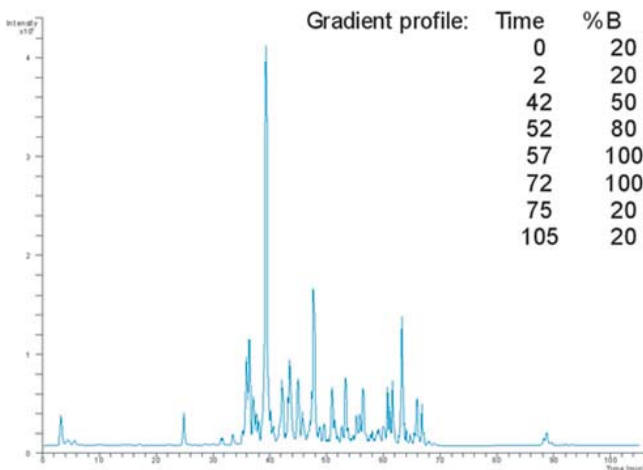
1. Dilute the MPI mouse liver microsomal membrane protein (11.3mg/ml) to 5mg/ml with Milli-Q. i.e. Aliquot 110 μ L Milli-Q to the bottom of Eppendorf tube (1.7mL) and 90 μ L of liver microsomal protein is added to the tube and the solution is mixed by pipetting up-and-down several times.
2. Aliquot 100 μ L of the diluted protein solution to the bottom of a separate Eppendorf tube (1.7mL). So, each tube will have 100 μ L protein solution.
3. Dry samples in a centrifugal vacuum concentrator (Speed-Vac).
4. Add 200 μ L of 80% formic acid/Milli-Q to each dried sample and sonicate in a water bath for 30 seconds.
5. Dry samples in a centrifugal vacuum concentrator (Speed-Vac).
6. Add 500 μ L of 80% formic acid/Milli-Q to each dried sample and sonicate in a water bath for 30 seconds or until a clear solution results.
7. Centrifuge the sample in a bench top centrifuge at 13000rpm for 5min.

Note: The final sample protein concentration would be approximately 1.0 mg/mL in 80% formic acid/Milli-Q.

Chromatographic Conditions for Analytical Separation

System: Agilent 1100 CapLC with Agilent MSD-iontrap MS
 Column: ProteCol-C8 3 μ m; 1000 Å 150mm x 300 μ m ID
 Sample: 3 μ L AOHUPO-MPI standard
 Flow rate: 5.0 μ L/min
 Temperature: 80 $^{\circ}$ C
 Mobile Phase A: 0.1% formic acid in water
 Mobile Phase B: 0.09% formic acid in acetonitrile

Base peak chromatogram of membrane proteins on a capillary column

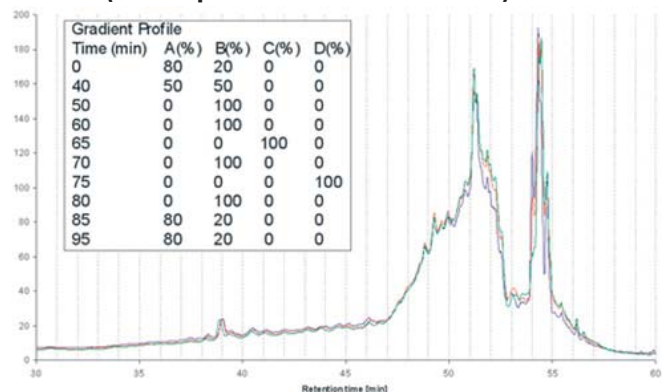


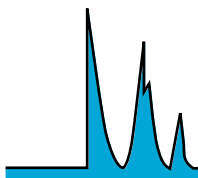
Chromatographic Conditions for Preparative Separation

HPLC separation of proteins is performed on an automated Agilent 1200 LC system equipped with a quaternary pump, an autosampler, a multiple wavelength detector and a fraction collector.

Column: C8 silica, 2.0 mm id x 150mm, 1000 Å , 3.0 μ m.
 Flow rate: 0.2 mL/min
 Temperature: 80 $^{\circ}$ C
 UV detection: 214 & 280nm
 Fraction size: 1min (0.2mL/fraction)
 Injection volume: 200 μ L
 Sample: 0.8mg/mL in 80% formic acid/Milli-Q.
 Solvent A: 0.1% TFA in Milli-Q
 Solvent B: 0.08% TFA in acetonitrile (ACN)
 Solvent C: 20% Formic acid in acetonitrile
 Solvent D: 100% isopropanol

UV chromatogram of membrane proteins on a 2mm ID column (lines represent collected fractions)





Analysis of Protein Fractions

Sample preparation

The dried fractions were reconstituted in 100mM NH₄HCO₃ and digested with trypsin overnight, acidified with 1% formic acid (FA), concentrated and rediluted to 10μL with 1% FA.

Chromatographic conditions

System: TSP4000 pump, Surveyor autosampler, LCQ Deca ion trap
 Column: SGE ProteCol-C18 3μm 300Å, 150μm ID x 100mm
 Sample volume: 10μL
 Mobile Phase A: 0.1% FA in 5% acetonitrile
 Mobile Phase B: 0.1% FA in 90% acetonitrile
 MS: 400-1500 mass range; top 3 ions fragmented with 39% collision energy

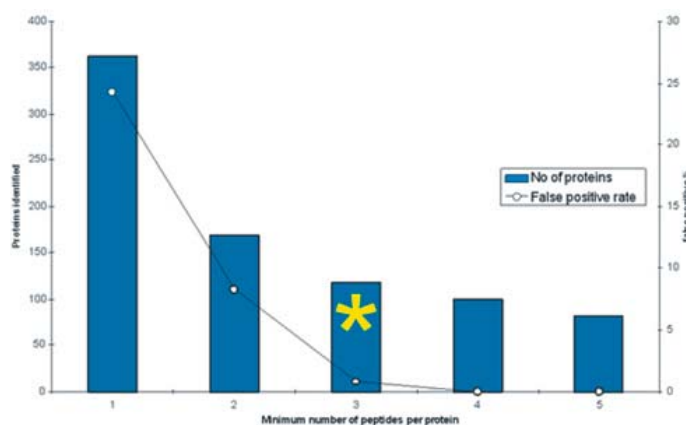
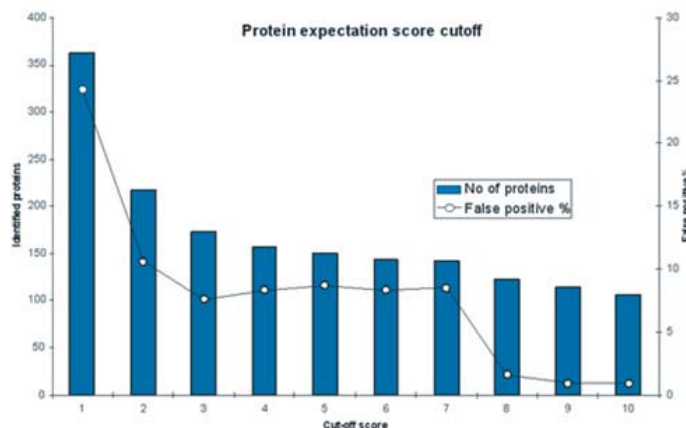
Data analysis

Raw data files were converted to mzXML and searched against the ENSEMBL mouse database using Xtandem algorithm (GPM-XE software). Parent ion mass accuracy 4Da, fragment ion mass accuracy 0.4Da.

Having a protein expectation cutoff value of 10-8, 122 proteins were identified with 1.6% false positive - 111 proteins when contaminants are excluded (Figure on right).

Having a minimum of 3 peptides per protein 118 proteins were identified with 0.8% false positive (109 proteins after the exclusion of contaminants). The full results are listed in Figure 6 (see last two pages)

Number of protein and false positive portion in relation to filtering criteria

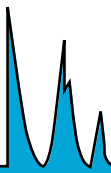


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Figure 6 – List of identified proteins with at least 3 positive peptides per protein

Identifier	No. of peptides/protein	Mr (kDa)	Description
ENSMUSP00000025549	155	15.2	cytochrome b-5
ENSMUSP00000003100	139	55.9	cytochrome P450, family 2, subfamily f, polypeptide 2
ENSMUSP00000072803	101	60	UDP glucuronosyltransferase 1 family, polypeptide A9
ENSMUSP00000031183	97	60.1	UDP glucuronosyltransferase 2 family, polypeptide B1
ENSMUSP00000008684	82	17.5	microsomal glutathione S-transferase 1
ENSMUSP00000006094	79	56.7	cytochrome P450, family 2, subfamily d, polypeptide 26
ENSMUSP00000073444	63	60	UDP glucuronosyltransferase 1 family, polypeptide A9
ENSMUSP00000058683	63	59.7	UDP glucuronosyltransferase 1 family, polypeptide A9
ENSMUSP00000072555	62	57.2	cytochrome P450, family 2, subfamily d, polypeptide 10
ENSMUSP00000020637	49	67.2	calnexin
ENSMUSP000000097037	45	69.8	solute carrier family 27 (fatty acid transporter), member 2
ENSMUSP00000047551	42	52.5	epoxide hydrolase 1, microsomal
ENSMUSP000000068282	40	60.8	UDP glucuronosyltransferase 2 family, polypeptide B5
ENSMUSP00000079065	36	55.7	cytochrome P450, family 2, subfamily c, polypeptide 50
ENSMUSP00000031195	34	61.1	UDP glucuronosyltransferase 2 family, polypeptide A3
ENSMUSP00000073061	34	21.7	progesterone receptor membrane component 1
ENSMUSP00000029729	33	60	flavin containing monoxygenase 5
ENSMUSP00000031186	32	60.4	UDP glucuronosyltransferase 2 family, polypeptide B35
ENSMUSP00000044004	29	504.4	low density lipoprotein receptor-related protein 1
ENSMUSP000000097943	27	34.1	cytochrome b5 reductase 3
ENSMUSP000000086530	26	56.9	cytochrome P450, family 2, subfamily d, polypeptide 9
ENSMUSP00000039252	26	35.6	retinol dehydrogenase 7
ENSMUSP00000046585	24	76.2	acyl-CoA synthetase long-chain family member 5
ENSMUSP00000006524	24	38.5	RIKEN cDNA1300013D18 gene
ENSMUSP00000034860	22	58.1	cytochrome P450, family 1, subfamily a, polypeptide 2
ENSMUSP0000005651	21	76.8	P450 (cytochrome) oxidoreductase
ENSMUSP00000028222	21	72.4	heat shock 70kD protein 5 (glucose-regulated protein)
ENSMUSP00000074990	21	56.1	cytochrome P450, family 2, subfamily a, polypeptide 12
ENSMUSP00000034400	21	16.3	cytochrome b5 type B
ENSMUSP00000092233	20	61	UDP glucuronosyltransferase 2 family, polypeptide B36
ENSMUSP00000071889	19	70.9	heat shock protein 8
ENSMUSP00000020329	19	134.8	epidermal growth factor receptor
ENSMUSP00000026552	19	56.8	cytochrome P450, family 2, subfamily e, polypeptide 1
ENSMUSP00000034046	18	77.9	acyl-CoA synthetase long-chain family member 1
ENSMUSP00000031181	16	60.8	UDP glucuronosyltransferase 2 family, polypeptide B34
ENSMUSP00000008927	16	99.6	ubiquitin C
ENSMUSP000000003137	14	55.7	cytochrome P450, family 2, subfamily c, polypeptide 29
ENSMUSP00000037583	13	24.9	transmembrane emp24-like trafficking protein 10 (yeast)
ENSMUSP00000023786	12	59.5	keratin 6B
ENSMUSP000000060912	12	50.3	gulonolactone (L-) oxidase
ENSMUSP00000018699	12	32.6	asialoglycoprotein receptor 1
ENSMUSP00000003066	11	35.8	apolipoprotein E
ENSMUSP00000023709	11	61.7	keratin 5
ENSMUSP00000017270	11	50.1	keratin 42
ENSMUSP0000007272	11	52.8	keratin 14
ENSMUSP00000026122	11	57	prolyl 4-hydroxylase, beta polypeptide
ENSMUSP00000037259	11	59.9	flavin containing monoxygenase 1
ENSMUSP0000002090	10	18.9	signal sequence receptor, delta
ENSMUSP00000070751	10	42.4	basigin
ENSMUSP00000023790	9	65.6	keratin 1
ENSMUSP00000014625	8	56.9	cytochrome P450, family 2, subfamily d, polypeptide 13
ENSMUSP00000007280	8	51.7	keratin 16
ENSMUSP00000004608	7	26	transmembrane emp24 protein transport domain containing 4
ENSMUSP00000044687	7	26.4	camello-like 2
ENSMUSP00000019896	7	32.8	iodotyrosine deiodinase
ENSMUSP00000000335	7	29.5	catechol-O-methyltransferase
ENSMUSP00000044050	7	87.4	dipeptidylpeptidase 4
ENSMUSP00000043660	7	25.3	
ENSMUSP00000029476	7	24.7	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)
ENSMUSP00000006961	6	56.9	keratin 10
ENSMUSP00000025668	6	60.5	RIKEN cDNA5730596K20 gene
ENSMUSP00000075345	6	31.1	transmembrane emp24 protein transport domain containing 9
ENSMUSP000000092002	6	20.8	ferritin light chain 1
ENSMUSP00000031314	6	68.6	albumin 1
ENSMUSP00000026398	6	28	methyltransferase like 7B
ENSMUSP00000072236	6	59.6	UDP glycosyltransferases 3 family, polypeptide A2
ENSMUSP00000034304	5	42	hydroxysteroid (17-beta) dehydrogenase 2
ENSMUSP00000081370	5	58.3	cytochrome P450, family 4, subfamily a, polypeptide 12 B
ENSMUSP00000025181	5	41.8	histocompatibility 2, K1, K region
ENSMUSP000000097754	5	62.8	keratin 76
ENSMUSP00000016338	5	32.3	hydroxysteroid 11-beta dehydrogenase 1
ENSMUSP00000002091	5	27.9	B-cell receptor-associated protein 31
ENSMUSP00000030299	5	57.7	cytochrome P450, family 2, subfamily j, polypeptide 5
ENSMUSP00000006749	5	103.1	solute carrier family 4 (anion exchanger), member 1
ENSMUSP00000026565	5	14.9	interferon induced transmembrane protein 3
ENSMUSP00000032916	4	48.7	MYC-associated zinc finger protein (purine-binding transcription factor)
ENSMUSP00000073094	4	158.2	deleted in colorectal carcinoma
ENSMUSP00000023952	4	54.5	keratin 8
ENSMUSP00000028004	4	55.9	aldehyde dehydrogenase 9, subfamily A1
ENSMUSP00000026211	4	56.4	cytochrome P450, family 2, subfamily c, polypeptide 44



Identifier	No. of peptides/ protein	M _r (kDa)	Description
ENSMUSP00000047790	4	23.6	glutathione S-transferase, pi 1
ENSMUSP00000019861	4	120	zinc finger protein 451
ENSMUSP00000026743	4	52.8	ubiquinol-cytochrome c reductase core protein 1
ENSMUSP00000015256	4	73.9	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13
ENSMUSP00000004375	4	33.3	prohibitin 2
ENSMUSP00000032539	4	76.2	solute carrier family 27 (fatty acid transporter), member 5
ENSMUSP00000046772	4	33.4	hydroxysteroid (17-beta) dehydrogenase 13
ENSMUSP00000026270	4	67.1	SAC1 (suppressor of actin mutations 1, homolog)-like (S. cerevisiae)
ENSMUSP00000085494	4	25.4	reticulon 3
ENSMUSP00000037665	4	57.8	cytochrome P450, family 3, subfamily a, polypeptide 11
ENSMUSP00000059501	4	11.3	vesicle-associated membrane protein 8
ENSMUSP00000028045	4	164.9	mannose receptor, C type 1
ENSMUSP00000095794	4	5.6	hemoglobin, beta adult major chain
ENSMUSP00000065271	3	28.1	methyltransferase like 7A
ENSMUSP00000021940	3	40.4	lectin, mannose-binding 2
ENSMUSP00000068013	3	28.9	ATP synthase, H ⁺ -transporting, mitochondrial F0 complex, subunit b, isoform 1
ENSMUSP00000033176	3	48.2	ubiquinol cytochrome c reductase core protein 2
ENSMUSP00000007602	3	31.2	mannose-6-phosphate receptor, cation dependent
ENSMUSP00000043722	3	22.3	receptor accessory protein 6
ENSMUSP00000048832	3	21.2	RAB3 D, member RAS oncogene family
ENSMUSP00000031251	3	32.9	hydroxysteroid (17-beta) dehydrogenase 11
ENSMUSP00000080806	3	52.5	
ENSMUSP00000035468	3	183.2	ATP-binding cassette, sub-family A(ABC1), member 6
ENSMUSP00000056227	3	268.7	jumonji domain containing 1 C
ENSMUSP00000036128	3	43.3	pelota homolog (Drosophila)
ENSMUSP00000027675	3	84.9	polymeric immunoglobulin receptor
ENSMUSP00000015011	3	30.4	surfeit gene 4
ENSMUSP00000032143	3	68.5	ribophorin 1
ENSMUSP00000018702	3	35.2	asialoglycoprotein receptor 2