Supplementary Material (ESI) for Lab on a Chip

Rapid in-gel processing and digestion of proteins using surface acoustic waves

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EXPERIMENTAL PROCEDURE

Materials

Triethylammonium bicarbonate buffer, iodoacetamide, BSA and trypsin were purchased from Aldrich Chemical Co. Inc., Wisconsin, USA. Reducing agent tris- (2-carboxyethyl)phosphine (TCEP) was purchased from Invitrogen Australia Pty Limited. High purity water was obtained from Millipore *MilliQ Academic* system.

SAW device operation

All reactions were conducted on a 19.65 MHz lithium niobate SAW device. The SAWs were generated by applying a sinusoidal oscillating electrical signal output to an interdigital transducer (250 nm thick Ti-Al electrodes) from an RF amplifier (10W1000C, Amplifier Research, Souderton PA USA) connected to a Agilent 33220A 20 MHz Function/Arbitrary waveform generator.

SDS PAGE separation of BSA and unknown proteins

Bovine Serum Albumin was separated on a 12% Bis-Tris polyacrylamide gel in triplicate and visualized using Coomassie Blue stain. One of the triplicates was used for SAW assisted digestion, one as a control without SAW and the last was processed using standard protocol with overnight digestion. In addition to the BSA standards, few proteins previously run on gel in the laboratory were excised to test the generality of the technique and its robustness in identifying "real-world" protein samples.

SAW-assisted in-gel digestion of proteins

Destaining, reduction and alkylation

Gel bands were excised and transferred to a reaction chamber (glass cuvette 1 cm in diameter, 1 cm high and 1 mm in thickness) followed by addition of 200 μL of destaining solution (50% v/v acetonitrile in 100 mM triethylammonium bicarbonate buffer) and 4 μL of reducing agent (50 mM Tris(2-carboxyethyl) phosphine hydrochloride in MilliQ water). A 40 μL drop of water was pipetted onto the SAW substrate (water acts as a coupler for SAWs to efficiently travel through the cuvette from the SAW substrate) and the cuvette containing the gel band placed on the drop of water. Destaining and reduction were achieved through exposing the gel bands to SAW (gain 6 on the amplifier, amplitude of 230 mVrms and 19.65 MHz sine wave on the signal generator) for 10 minutes by discarding the solutions, replenishing the cuvette with fresh solutions and replacing the cuvette on a topped up 40 μL drop of

water on the LN substrate after every 5 minutes until the gel piece appeared transparent. Remaining destaining solution was dispensed with and replaced by 100 µL of alkylating reagent (100 mM iodoacetamide in MilliQ water). A fresh 40 µL drop of water was laid onto the SAW substrate and the cuvette replaced on the water drop. Alkylation was accomplished by applying SAW to the cuvette (by reducing the gain to 3 on the amplifier) for 5 minutes. After removing the iodoacetamide solution, the gel piece was dehydrated with 2 X 100 µL acetonitrile for 5 minutes. This was performed by placing the cuvette on the SAW substrate at the second wash and applying SAW for 3 minutes to effect the acceleration of drying process and further air drying the gel piece for 2 minutes until the gel shrunk and became opaque-white in colour.

Trypsin digestion

LN substrate of the SAW device was washed with 70% ethanol/water and the dried gel band transferred onto the SAW substrate from the cuvette. Subsequently, tryptic digestion was performed by rehydrating the gel with 10 µL of trypsin stock solution (1 µg of trypsin in 40 µL of 50 mM triethylammonium bicarbonate buffer) and operating the SAW device for 1 minute. The rehydrated gel piece was overlaid with 40 µL of 50 mM triethylammonium bicarbonate buffer and the SAW device operated for an additional 8 minutes. The digested peptides were collected directly in glass vials. Digestion was stopped by acidifying the digested gel band with 10 µL of 1% formic acid and employing SAWs for 1 minute to wash the gel piece in order to maximize recovery of the digested peptides and minimize the loss of peptides by limiting their adsorption on the LN substrate. This 1% formic acid solution was pooled with the digested peptide solution for LC- MS/MS analysis.

Mass Spectrometry

Digested peptides were collected in glass vials (Waters) and separated by Agilent 1100 series nanoLC on a Zorbax 300SB-C18 5 µm (150 mm x 75µm) chip column (8 µL loading volume) using ChipCube interfaced at the front end of a LC/MSD Trap XCTplus 3D iontrap (Agilent Technologies). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 5% aqueous 0.1% formic acid in 95% acetonitrile. The elution gradient for the chip column was from 15% to 50% buffer B over 19 minutes. Peptides from the digests of unknown protein gel bands were separated on a C18 (100 mm x 75 µm) nanocolumn using Eksigent Tempo

nanoLC (15 µL loading volume) and detected on QSTAR ELITE (Applied Biosystems) QqTOF mass spectrometer. The elution gradient for the nano-column on the QSTAR ELITE was from 15% to 50% buffer B over 25 minutes. Data was acquired using the 6300 Series ion Trap LC/MS Software 6.1 (3-D Ion Trap) and Analyst 2.0 (QSTAR) respectively.

Database Search

MS/MS peaklists (.mgf) were generated using default parameters in DataAnalysis version 3.4 (build 175, Agilent Technologies). Peaklists for QSTAR Elite data were generated with default settings using the Mascot Script version 1.6b25 (Matrix Science).

MS/MS spectra were searched using Mascot search engine (provided by Australian Proteomics Computational Facility, APCF) against SwissProt database (April 2009, 428650 sequences; 154416236 residues) with the following search criteria:

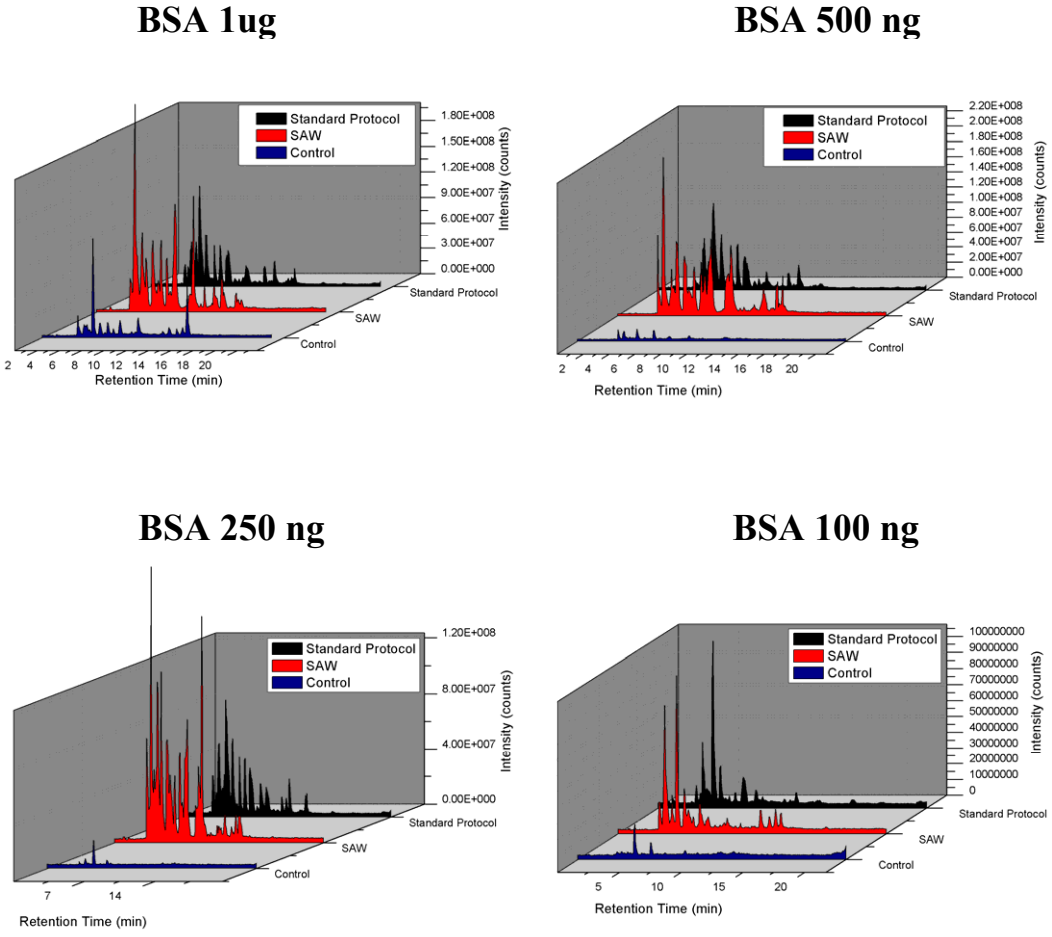
Modifications Fixed: Carbamidomethyl (C) Variable: oxidation (M). Peptide and Fragment mass tolerance 0.8 Da, Enzyme: Trypsin, with up to 2 missed cleavages allowed with no species restriction, significance threshold was set to $p< 0.01$ and expect value cut off to 0.8 to remove false positives.

Results

Comparison of the base peak chromatograms

Base Peak Chromatograms (BPCs) from the LC trace (Figure S1) showed a dramatic improvement in the number of peptides detected (Table S1) for SAW assisted in-gel digestion (BPCs shown in red) of Bovine Serum Albumin (BSA) compared to the control (BPCs shown in blue).

Figure S1



Base Peak Chromatograms (BPC overlay) comparing SAW assisted in-gel digestion of BSA with control and standard overnight digest protocol

Table S1 Peptides identified after tryptic digest for different gel loading of BSA

Peptides identified	BSA 1µg	BSA 500 ng	BSA 250 ng	BSA 100 ng
SAW	16	11	17	7
Control	6	1	1	1
Overnight	13	12	12	7

The number of peptides detected (Table S1) as perceived from the LC trace (Figure S1) using the SAW in-gel digest protocol (BPCs shown in red) was comparable to the gels processed using the standard overnight digest (BPCs shown in black).

Table S2 Protein sequence coverage for different gel loading of BSA

Sequence coverage	BSA 1µg	BSA 500 ng	BSA 250 ng	BSA 100 ng
SAW	23%	18%	26%	9%
Control	10%	1%	2%	2%
Overnight	27%	23%	18%	13%

Figure S2

MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
STQTALA

Protein sequence coverage information (depicting 23% coverage) for BSA (1 µg)

Table S3 & Table S4 display the detailed peptide fragments of BSA (1 µg & 250 ng) produced in 10 minutes of SAW-based in-gel digestion. 16 matched peptides corresponding to 23 % sequence coverage (Figure S2) were detected for 1 µg of BSA protein sample and 17 matched peptides corresponding to 26 % sequence coverage were detected for 250 ng of BSA, both results comparable to those obtained by the conventional overnight in-gel digestion (Table S2).

Table S3 Peptides identified using SAW assisted in-gel digestion of 1 µg BSA using Mascot Bovine Serum Albumin (*Bos Taurus*)

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
GACLLPK	0.066	757.5454	0.1298	0
LSQKFPK	0.1	846.8654	0.3691	1
LCVLHEK	0.01	897.5254	0.0512	0
AEFVEVTK	0.12	921.6654	0.1847	0
YLYEIAR	0.075	926.5454	0.0593	0
YLYEIAR	0.021	926.6854	0.1993	0
KQTALVELLK	0.0019	1142.4654	0.7584	1
KQTALVELLK	0.00018	1142.4654	0.7584	1
SLHTLFGDELCK	0.00018	1418.9054	0.2190	0
ETYGDMADCCEK	0.0031	1477.4254	-0.0905	0
VPQVSTPTLVEVSR	0.021	1510.7054	-0.1301	0
LKECCDKPLLEK	0.0013	1531.6282	-0.1456	1
DAFLGSFLYEYSR	7.3e-006	1567.3654	0.6300	0
KVPQVSTPTLVEVSR	0.00012	1639.0454	0.1150	1
RPCFSALTPDETYVPK	0.027	1880.6854	0.7716	0
CCAADDKEACFAVEGPK	3e-010	1927.5254	0.7344	1

Table S4 Peptides identified using SAW assisted in-gel digestion of 250 ng BSA using Mascot Bovine Serum Albumin (*Bos Taurus*)

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
GACLLPK	0.093	757.7254	0.3098	0
YLYEIAR	0.022	926.8454	0.3593	0
LVVSTQTALA	0.00085	1002.0254	0.4497	0
KQTALVELLK	0.001	1142.2054	0.4984	1
KQTALVELLK	0.0055	1142.2454	0.5384	1
LVNELTEFAK	6.5e-006	1163.0054	0.3821	0
HLVDEPQNLIK	0.0036	1305.3454	0.6366	0
TVMENFVAFVDK + Oxidation (M)	1.1e-005	1415.3654	0.6852	0
SLHTLFGDELCK	6.8e-005	1419.1054	0.4190	0
ETYGDMADCCEK	4.4e-006	1478.2054	0.6895	0
LGEYGFQNALIVR	2.5e-006	1479.1854	0.3973	0
ETYGDMADCCEK + Oxidation (M)	0.0052	1493.9454	0.4346	0
EYEATLEECCAK	0.00099	1501.9054	0.2990	0
KVPQVSTPTLVEVSR	0.0025	1638.9054	-0.0250	1
MPCTEDYLSLILNR + Oxidation (M)	5.4e-007	1739.9654	0.1432	0
MPCTEDYLSLILNR + Oxidation (M)	0.077	1740.4054	0.5832	0
YNGVFQECCQAEDK	0.0025	1746.8254	0.1277	0

To evaluate nonspecific cleavage of the proteins, a "no enzyme" search was performed on the BSA protein digest (restricting the search to mammalian BSA) which failed to identify any non-tryptic peptide matches. Thus all the results can confidently be ascribed to the trypsin's action on BSA thereby validating the digest protocol.

To demonstrate the generality of the SAW technology five protein extracts from disparate sources (Table S5) were also digested.

Table S5

MASCOT Score	Coverage %	No. of peptides identified	Protein mass (*M*r)	Protein name
724	51%	12	34228	Mitochondrial import receptor subunit TOM40 homolog 1
962	48%	21	41181	HLA class I histocompatibility antigen, A-2 alpha chain
205	37%	4	13820	Beta-2-microglobulin
586	27%	13	41095	H-2 class I histocompatibility antigen, D-B alpha chain
454	25%	6	39309	Outer membrane protein F

MASCOT search results from SAW assisted tryptic digest of random protein gel bands

After a database query with MASCOT high sequence coverages were obtained for the arbitrarily digested proteins. Sequence coverage of 51% (Figure S3) with 12 matched peptides (Table S6) was obtained for the first protein identified as mitochondrial import receptor subunit TOM40 homolog 1.

Figure S3

MADLLPPLTA AQVDAKTKVD EKVDYSNLPS PVPYEELHRE ALMSLKSDNF
EGLRFDFTRA LNQKFSLSHS VMMGPTEVPA QSPETTIKIP TAHYEFGANY
YDPKLLLIGR VMTDGRLNAR LKADLTDKLV VKANALITNE EHMSQAMFNF
DYMGSDYRAQ LQLGQSALIG ATYIQSVTNH LSLGGEIFWA GVPRKSGIGY
AARYETDKMV ASGQVASTGA VVMNYVQKIS DKVSLATDFM YNYFSRDVTA
SVGYDYMLRQ ARVRGKIDSN GVASALLEER LSMGLNFLLS AELDHKKKDY
KFGFGLTVG

Protein sequence coverage for mitochondrial import receptor subunit TOM40 homolog 1

Table S6 Peptides identified for SAW assisted in-gel digestion of mitochondrial import receptor subunit TOM40 homolog 1 using Mascot

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
LKADLTDK	0.00021	902.4838	-0.0235	1
SDNFEGLR	0.077	936.3980	-0.0321	0
LKADLTDKLVVK	0.006	1341.7747	-0.0484	2
DVTASVGYDYMLR + Oxidation (M)	0.0013	1504.6530	-0.0338	0
ADLLPPLTAAQVDAK	1.4e-005	1521.7807	-0.0596	0
VSLATDFMYNYFSR + Oxidation (M)	0.082	1728.7727	-0.0090	0
LSMGLNFLLSAELDHK + Oxidation (M)	0.00049	1802.8601	-0.0635	0
IPTAHYEFGANYYDPK	0.0026	1884.8066	-0.0617	0
VDYSNLPSPVPYEELHR	0.0068	2013.9058	-0.0738	0
MVASGQVASTGAVVMNYVQK + 2 Oxidation (M)	9.1e-005	2070.9291	-0.0787	0
FSLSHSVMMGPTEVPAQSPETTIK + 2 Oxidation (M)	0.026	2605.1483	-0.0921	0
YETDKMVASGQVASTGAVVMNYVQK + 2 Oxidation (M)	6.3e-008	2707.2161	-0.0672	1

Second protein with 21 matched peptides (Table S7) was identified as HLA class I histocompatibility antigen, A-2 alpha chain with 48% sequence coverage (Figure S4).

Figure S4

MAVMAPRTLV LLLSGALALT QTWAGSHSMR YFFTSVSRPG RGEPRFIAVG
YVDDTQFVRF DSDAASQRME PRAPWIEQEG PEYWDGETRK VKAHSQTHRV
DLGTLRGYYN QSEAGSHTVQ RMYGCDVGSD WRFLRGYHQY AYDGKDYIAL
KEDLRSWTAA DMAAQTTKHK WEAAHVAEQL RAYLEGTCVE WLRRYLENGK
ETLQRTDAPK THMTHHAVSD HEATLRCWAL SFYPAEITLT WQRDGEDQTQ
DTELVETRPA GDGTFQKWAA VVVPSGQEQR YTCHVQHEGL PKPLTLRWEP
SSQPTIPIVG IIAGLVLFGA VITGAVVAAV MWRRKSSDRK GGSYSQAASS
DSAQGSDVSL TACKV

Protein sequence coverage for HLA class I histocompatibility antigen, A-2 alpha chain

Table S7 Peptides identified for SAW assisted in-gel digestion of HLA class I histocompatibility antigen, A-2 alpha chain using Mascot

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
FDSDAASQR	0.0022	995.3988	-0.0320	0
FDSDAASQR	0.0027	995.3988	-0.0320	0
FDSDAASQR	0.0036	995.4031	-0.0278	0
FDSDAASQR	0.0026	995.4031	-0.0278	0
FDSDAASQR	0.0026	995.4031	-0.0278	0
GYHQYAYDGK	0.00078	1200.5002	-0.0197	0
WEAAHVAEQLR	0.025	1308.6180	-0.0395	0
MYGCDVGSDWR	0.019	1344.5085	-0.0142	0
YLENGKETLQR	0.062	1349.6478	-0.0461	1
MYGCDVGSDWR + Oxidation (M)	9.9e-005	1360.4807	-0.0370	0
SWTAADMAAQTTK + Oxidation (M)	6.2e-009	1396.5835	-0.0458	0
WAAVVVPSGQEQR	0.00031	1425.6904	-0.0461	0
AYLEGTCVEWLR	6.6e-007	1495.6749	-0.0381	0
AYLEGTCVEWLR	0.005	1495.6904	-0.0225	0
HKWEAAHVAEQLR	0.014	1573.7627	-0.0487	1
FIAVGYVDDTQFVR	2.5e-006	1628.7728	-0.0471	0
FIAVGYVDDTQFVR	0.00023	1628.7782	-0.0417	0
GYYNQSEAGSHTVQR	0.00013	1695.7059	-0.0542	0
THMTHHAVSDHEATLR + Oxidation (M)	0.0042	1857.7731	-0.0810	0
APWIEQEGPEYWDGETR	1.8e-006	2061.8626	-0.0442	0
DGEDQTQDTELVETRPAGDGTFQK	9.7e-005	2636.1178	-0.0660	0

Third protein with 4 matched peptides (Table S8) identified as beta-2-microglobulin had 37% sequence coverage (Figure S5).

Figure S5

MSRSVALAVL ALLSLSGLEA IQRTPKIQVY SRHPAENGKS NFLNCYVSGF
HPSDIEVDLL KNGERIEKVE HSDLSFSKDW SFYLLYYTEF TPTEKDEYAC
RVNHVTLSQP KIVKWDRDM

Protein sequence coverage for beta-2-microglobulin

Table S8 Peptides identified for SAW assisted in-gel digestion of beta-2-microglobulin using Mascot

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
VNHVTLSQPK	0.0002	1121.5803	-0.0390	0
VEHSDLSFSK	0.031	1147.6211	0.0702	0
IEKVEHSDLSFSK	0.0041	1517.7328	-0.0397	1
SNFLNCYVSGFHPSDIEVDLLK	0.083	2553.1707	-0.0503	0

Fourth protein with 13 matched peptides (Table S9) was found to be H-2 class I histocompatibility antigen, D-B alpha chain had 27% sequence coverage (Figure S6).

Figure S6

MGAMAPRTLL LLLAAALAPT QTRAGPHSMR YFETAVSRPG LEEPRYISVG
YVDNKEFVRF DSDAENPRYE PRAPWMEQEG PEYWERETQK AKGQEQWFRV
SLRNLLGYYN QSAGGSHTLQ QMSGCDLGSD WRLLRGYLQF AYEGRDYIAL
NEDLKTWTAA DMAAQITRRK WEQSGAAEHY KAYLEGECVE WLHRYLKNGN
ATLLRTDSPK AHVTHHPRSK GEVTLRCWAL GFYPADITLT WQLNGEELTQ
DMELVETRPA GDGTFQKWAS VVVPLGKEQN YTCRVYHEGL PEPLTLRWEP
PPSTDSYMVI VAVLGVLGAM AIIGAVVAFV MKRRRNTGGK GGDYALAPGS
QSSEMSLRDC KA

Protein sequence coverage for H-2 class I histocompatibility antigen, D-B alpha chain

Table S9 Peptides identified for SAW assisted in-gel digestion of H-2 class I histocompatibility antigen, D-B alpha chain using Mascot

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
NGNATLLR	0.029	857.4470	-0.0248	0
FDSDAENPR	0.047	1049.4007	-0.0407	0
FDSDAENPR	0.053	1049.4051	-0.0363	0
FDSDAENPR	0.0019	1049.4094	-0.0320	0
FDSDAENPR	0.016	1049.4094	-0.0320	0
FDSDAENPR	0.0031	1049.4311	-0.0103	0
WASVVVPLGK	0.0016	1054.5838	-0.0337	0
YISVGYVDNK	0.00011	1156.5362	-0.0402	0
DYIALNEDLK	1.5e-005	1192.5548	-0.0427	0
GYLQFAYEGR	0.00028	1202.5430	-0.0290	0
TWTAADMAAQITR + Oxidation (M)	2.3e-008	1450.6256	-0.0619	0
YFETAVSRPGLEEPR	0.00064	1749.7790	-0.0895	0
APWMEQEGPEYWER + Oxidation (M)	0.074	1822.7106	-0.0514	0

Final protein with 6 matched peptides (Table S10) was identified as an outer membrane protein F with 25% sequence coverage (Figure S7).

Figure S7

MMKRNILAVI VPALLVAGTA NAAEIYNKDG NKVDLYGKAV GLHYFSKGNG
ENSYGGNGDM TYARLGFKGE TQINSDLTGY GQWEYNFQGN NSEGADAQTG
NKTRLAFAGL KYADVGSFDY GRNYGVVYDA LGYTDMLPEF GGDTAYSDDF
FVGRVGGVAT YRNSNFFGLV DGLNFAVQYL GKNERDTARR SNGDGVGGSI
SYEYEGFGIV GAYGAADRTN LQEAQPLGNG KKAEQWATGL KYDANNIYLA
ANYGETRNAT PITNKFTNTS GFANKTQDVL LVAQYQFDFG LRPSIAYTKS
KAKDVEGIGD VDLVNYFEVG ATYYFNKNMS TYVDYIINQI DSDNKLGVGS
DDTVAVGIVY QF

Protein sequence coverage for outer membrane protein F

Table S10 Peptides identified for SAW assisted in-gel digestion of outer membrane protein F using Mascot

Sequence	Expect Value	Experimental Mass	Mass delta	Missed Cleavages
AEQWATGLK	0.045	1002.6654	0.1520	0
DGNKVDLYGK	0.015	1108.2454	0.6894	1
YADVGSFDYGR	2.1e-005	1248.9654	0.4243	0
TNLQEAQPLGNGK	1.9e-005	1369.4654	0.7657	0
YDANNIYLAANYGETR	3.3e-006	1847.5454	0.6969	0
NYGVVYDALGYTDMLPEFGGDTAYSDD FFVGR + Oxidation (M)	2e-008	3570.2482	0.6872	0