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Shotgun Proteomic Analysis of Cerebrospinal Fluid Using Off-Gel Electrophoresis as the First Dimension Separation

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Abstract

Shotgun proteomic analysis usually employs multidimensional separations with the first dimension most commonly being strong cation exchange (SCX) liquid chromatography (LC). SCX-LC is necessarily a serial process for preparation of multiple samples. Here we apply a newly available tool, off-gel electrophoresis (OGE), for first dimension separation of peptide mixtures from digests of cerebrospinal fluid (CSF), a complex and low total protein-containing sample. OGE first dimension fractionation enabled identification of a total of 156 unique proteins compared to 115 identified in previous work using first dimension SCX fractionation. OGE can be used to process multiple samples unattended with easy retrieval of the separated fractions. Thus shotgun analysis using OGE as the first dimension separation offers a significant advantage both in terms of sample throughput as well as increased numbers of identified proteins.

Keywords

OGE; cerebrospinal fluid; shotgun proteomics

Introduction

Shotgun analysis is an increasingly applied tool in proteomics.^{1,2} This method entails digestion of a mixture of intact proteins prior to any form of fractionation, followed by multidimensional separation of the resulting peptide mixture and mass spectrometry (MS) analysis. A variety of separation techniques have been used for shotgun analysis¹ including isoelectric focusing (IEF)^{3,4}, strong cation exchange (SCX) liquid chromatography (LC), capillary electrophoresis (CE) and reversed phase (RP) LC. In the original shotgun proteomic method (dubbed “MudPIT” for “multidimensional protein identification technology”), the multidimensional separation was tandem SCX and RPLC with online electrospray ionization (ESI) MS analysis.⁵

The online 2D LC-MS/MS approach, although providing an all-in-one setup^{1,6}, results in inefficient use of instrument time. The off-line approach, where peptides are separated in the first dimension before being subjected to second dimension RP-MS analysis, makes more

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efficient use of mass spectrometer time. On the other hand, it is a tedious process requiring fraction collection of the eluted peptides, and the increased sample handling can increase sample losses. An offline first dimensional separation comparable to SCX, but without some of the disadvantages, would provide a significant improvement in efficiency of both time and sample recovery. We report here the use of Off-Gel electrophoresis (OGE) as the first dimension separation in shotgun analysis of cerebrospinal fluid (CSF).

Another liquid phase IEF separation device used in 2D separations of proteins is the Rotofor, a commercial product available from BioRad. This instrument fractionates proteins in chambers separated by plastic mesh using carrier ampholytes.^{7,8,9} The ZOOM IEF fractionator, sold by Invitrogen, is a miniaturized system using carrier ampholytes, but with the chambers separated by isoelectric membranes.¹⁰ Further miniaturization of this approach in a chip device has also been reported.¹¹ In all of these devices, the separated fractions contain carrier ampholytes, which need to be removed for subsequent steps. A recently available fractionation tool known as the digital Proteome Chip™ (Protein Forest) uses an electric field to fractionate proteins and peptides into a series of immobilized pH gel plugs.¹² The OGE system has the advantage of using immobilized pH gradient strips to yield separated fractions without carrier ampholytes. Another advantage of this technique is the commercial availability of IPG strips of various pI ranges.

OGE is a method of isoelectric focusing where sample fractions can be recovered in solution with the option of not using carrier ampholytes¹³, which may interfere with downstream mass spectrometry analysis. The original design consisted of a protein solution being introduced into a flow chamber containing a specified pH gradient gel strip. Further development of the OGE system included a sample-focusing buffer combination used in a multiwell device that provided a narrow pI range in each well, thereby giving better separation of proteins.^{8,14} In addition to the separation, OGE has the added advantage of concentrating the focused components. The OGE technique has been used to separate mixtures at the intact protein level, yielding fractions that, in turn, could undergo digestion with the resulting fragments separated at the peptide level using an OGE peptide format.^{15,16,17} A variety of samples have been analyzed with this OGE approach, including *E. coli* lysate¹⁷, murine macrophage-infected cells¹⁸, human plasma¹⁵ and recently, extraocular muscle.¹⁹

OGE has been commercialized by the Agilent corporation as the model 3100 OFFGEL Fractionator, which can be used for separation of either protein or peptide mixtures.¹⁵ Previous analytical studies show that the OFFGEL device provides a 6-7 mm well width structure that allows the recovery of 90% of peptides in the maximum of two well compartments.^{17,20} The work reported here applies peptide OGE as the first dimension separation in shotgun analysis of CSF.

Cerebrospinal fluid is a complex biological sample that has been studied in efforts to find protein biomarkers of CNS diseases.^{21,22} CSF is a clear fluid produced in the choroid plexus of the ventricles that surround the brain and spinal cord containing peptides, proteins, sugars, and salts.^{23,24,25} Normal human CSF protein concentration is in the range of 0.18-0.8 mg/mL, which is more than two orders of magnitude lower than serum or plasma

protein concentration levels.^{24,25} The low overall concentration and large dynamic range of protein concentration in CSF, as much as twelve orders of magnitude, complicates proteomic analysis.²⁶ Because of this complexity and low total protein concentration, there is a need for not only efficient first dimension separation, but also for concentration of the peptide fragments. In this work, OGE was incorporated into a protocol previously established in our laboratory for shotgun analysis of CSF ^{27,28}, with replacement of the first dimension SCX fractionation with OGE fractionation prior to RP-LC-ESI-MS analysis. This approach yielded an improved method for shotgun proteomics analysis of CSF as reflected by an increased number of identified proteins compared to the conventional method using SCX.

2. Experimental Section

2.1 Materials

Urea, dithiothreitol, iodoacetamide, and proteomics grade trypsin were purchased from Sigma -Aldrich corporation (St. Louis, MO). C-18 Sep-Pak (2.0 mL) vacuum manifold solid-phase extraction (SPE) cartridges were purchased from Waters corporation (Millford, MA). The 3100 OFFGEL Low Resolution Kit, pH 3-10, containing urea, thiourea, dithiothreitol, a glycerol solution, ampholytes, mineral oil, electrode pads, frames, cover seals, and IPG strips, was purchased from Agilent Technologies (Wilmington, DE). Amicon Ultra 5 kDa molecular weight cutoff filters were purchased from the Millipore Corporation (Billerica, MA).

2.2 Human CSF

A pool of human CSF was obtained from excess clinical specimens under IRB approval as previously described.^{27,28} The total protein concentration of this CSF pool was determined to be 454 µg/mL. The CSF pool was stored at -80° C.

2.3 Buffer Exchange and Removal of Biological Salts

CSF was centrifuged, and the supernatant (2.5 mL, 1.135 mg total) was loaded onto a 15.0 mL capacity 5 kDa Amicon Ultra molecular weight cutoff filter. The volume was then increased to 15.0 mL total by adding 0.2 M NH₄HCO₃ and cartridges were centrifuged at 3000 × g until the volume was reduced to 200 µL. This buffer exchange process was repeated two more times resulting in the substantial reduction of biological salt concentration. The retentates containing CSF proteins were then removed and aliquotted into 5 equal fractions (40 µL amounts containing 227 µg each), which were dried by vacuum centrifugation.

2.4 Reduction, Alkylation, and Digestion of CSF Proteins

This protocol was as previously described with slight modification.^{27,28} CSF proteins in each sample were denatured in 9.0 M urea. Disulfide bonds were reduced by 10 mM dithiothreitol for 1 hr at 37° C in the dark. Alkylation was performed via addition of 50 mM iodoacetamide followed by incubation for 30 min at 37° C in the dark. The sample was diluted to 1.85 M urea concentration by addition of 0.2 M NH₄HCO₃, yielding a pH of 8.5 for protein digestion. Proteomics grade trypsin was added (20 µg, Sigma Aldrich), and the

proteins were digested overnight at 37° C in the dark. Tryptic peptides were then isolated via solid-phase extraction with a C-18 Sep-Pak Vac cartridge (Waters Corporation) according to manufacturer's instructions. Peptides were eluted with 70% acetonitrile/0.1% formic acid and were dried using vacuum centrifugation.

2.5 Off-Gel Electrophoresis

The 3100 OFFGEL Fractionator and the OFFGEL Kit pH 3-10 (Agilent Technologies) were used according to the manufacturer's protocol. This experiment utilized the 12-well tray. The corresponding 13-cm long IPG gel strip with a linear pH gradient ranging from 3 to 10 was rehydrated with the addition of 20 μ L of rehydration solution per well. Next, electrode pads were wetted and placed on the anodic and cathodic ends. The strip was allowed to rehydrate for 15 min. The CSF tryptic digest was diluted to 1.8 mL with the addition of OFFGEL peptide fractionation buffer and 150 μ L was placed into each well. The cover seal was placed over the well apparatus followed by addition of cover fluid on anodic and cathodic ends of the tray. Electrodes were placed on each side and set to run until 20 kVh was reached (approximately 13 h). Afterwards, the solution was removed from each well, yielding recovery amounts ranging from 100-200 μ L per well to give a total of 12 peptide containing fractions. The fractions were then dried via vacuum centrifugation.

The OGE analyses used the same pool of CSF as used for previous experiments employing strong cation exchange (SCX) chromatography as a first-dimension separation.^{27,28} Offline SCX chromatography was performed using a PolySULFO-ETHYL A column, 200 mm \times 2.1 mm (PolyLC, Inc.). The column was equilibrated with buffer A (10 mM KH_2PO_4 and 25% acetonitrile, pH < 3.0) for 30 min, after which the CSF peptides solubilized in 2.0 mL of buffer A were loaded onto the column. The peptides were eluted with a linear gradient of 0-50% buffer B (10 mM KH_2PO_4 , 1.0 M KCl, and 25% acetonitrile, pH < 3.0) over 60 min. A UV absorption cell ($\lambda = 214$ nm) was used to monitor the elution of CSF peptides. A total of 8 peptide-containing fractions were collected in 5 min intervals. Collected fractions were then dried via vacuum centrifugation.

2.6 Reversed-Phase LC/MS/MS

Fractions were analyzed using slight modification of a previously reported procedure regarding SCX fractions.²⁷ OGE fractions were solubilized with 200 μ L of 2% acetonitrile/0.1% formic acid (solvent A) and 20 μ L was loaded onto a 0.3 mm \times 5.0 mm C18 trap column (LC Packings, Dionex), which was then eluted onto an analytical RPC18 column, 15 cm \times 75 μ m (Microtech Scientific) and separated with a 100 min linear gradient of 0-50% solvent B (95% acetonitrile/0.1% formic acid) followed by a 20 min linear gradient (50-70% solvent B) at a flow rate of 180 nL/min using a Dionex ULTIMATE system. Eluted peptides were analyzed online with a LTQ mass spectrometer (Thermo Electron) using the Thermo nanospray ion source. The mass spectrometer was set to collect MS/MS spectra on the 5 most intense ions observed in the MS spectrum collected over m/z 400-2000. Other parameters included a nanospray voltage of 2.0 kV, a normalized collision energy of 35%, a default charge state of +5, and an isolation mass window of 2.5 amu. Dynamic exclusion was enabled for all experiments with a duration of 3.0 min, a repeat count of 2, a repeat duration of 0.5 min, and a rejection mass window of 2.0 amu.

The fractions from the SCX separation were analyzed by RP-LC-MS/MS using the same conditions as for the OGE fractions, except that the loading of each SCX fraction on the C18 trap column was followed by a wash for 30 min prior to sample flow onto the RP-C18 analytical column. The wash was unnecessary for the OGE fractions.

2.7 Data Analysis

MS/MS spectra were searched against an indexed *Homo sapiens* database, which was extracted from the NCBI nonredundant *H. sapiens* database, using the Turbo SEQUEST algorithm, a component of the Bioworks 3.2 software suite (Thermo Electron). Peptides with up to two missed cleavages were allowed. Dynamic chemical modifications of +16 and +57 mass units corresponding to M-oxidized and C-carboxyamidomethyl modifications, respectively, were included as search parameters. A precursor ion accuracy of 2.0 amu was used. Resulting protein identifications were filtered using two protein and two peptide filters, protein probability $P < 0.001$, minimum of two unique peptides for a protein identification, peptide probability $P < 0.001$, and Xcorr (cross correlation) versus charge state of at least 1.5, 2.0, and 2.5 for +1, +2, and +3 ions, respectively. Using Bioworks 3.2, multi-consensus reports were generated for each set of twelve reversed-phase experiments (i.e. LC/MS runs for each of the twelve first-dimension separation fractions) for each sample. All protein identifications resulting from only two unique peptides were further examined. Each peptide sequence was searched using the BLAST algorithm, and when both of the two peptide sequences were found in more than two proteins in the NCBI nonredundant *H. sapiens* database (e.g. multiple protein variants), the protein identification was deemed inconclusive and eliminated from the identification list. Thus, the identified proteins listed were only those for which at least two peptides were identified that matched to only one protein.

3. Results and Discussion

3.1 LC/MS/MS Analysis of OGE Separated Peptides

The dried CSF protein samples were reduced, alkylated, and digested prior to fractionation using the peptide OFFGEL electrophoresis format. After the separation time was complete, the wells were inspected to make sure that none had gone to dryness (due to osmotic pumping). All wells contained fluid, and a range of ~100-200 μ l of separated peptide solution was recovered from the wells. Each well fraction was analyzed and the proteins identified are shown in Table 1. They are given in the order of relative abundance, which was calculated using the total spectrum count (TSC) method described previously.²⁹ Briefly, this method entails normalizing the TSC (number of peptide MS² spectra matched to a particular protein) of each protein by dividing by its molecular weight. The LC-MS/MS analyses were repeated two more times. In the repeated analyses additional proteins were identified with the total identification tending toward a plateau after three analyses.²⁹ The same pool of CSF was used as in prior SCX-RP analyses.²⁶ The first run using OGE yielded 97 protein identifications, the second yielded 135, and the third yielded 99, for a total of 156 proteins identified (Table 1). As expected, there were overlapping and unique proteins seen during each run (Fig. 2). Shotgun analysis of the same CSF pool using SCX as the first dimension yielded 86, 94, and 83 proteins in first, second, and third runs, for a total of 115

proteins identified.²⁶ Of these, 101 proteins were observed using either SCX or OGE fractionation (Figure 3). There were a total of 14 unique proteins observed with SCX fractions, while 55 unique proteins were observed in OGE fractions (Table 2). Some of the proteins identified using the OGE separation were ones that were previously seen only after abundant protein depletion of cerebrospinal fluid.²⁸

3.2 Advantages of OGE-LC/MS/MS Method

Because OGE does not require addition of salts or detergents, peptides do not have to undergo an extensive or time consuming cleaning step prior to the second dimension separation and MS analysis. A 30 min wash step in the SCX method is eliminated by use of OGE, significantly reducing the instrument time required for the analysis. Also, OGE provides an automated environment for sample separation and stability of the separation in that after separation, the peptides remain focused until the fractions are removed from the wells. Because OGE concentrates as well as fractionates peptides into their respective pI range, lower abundance proteins can be identified than when the first-dimensional separation only fractionates. OGE also offers the opportunity for increased throughput in that as many as twelve samples can undergo parallel first dimension separation. Table 3 shows a comparison between SCX and OGE workflows. Even though sample run times are longer for OGE analysis, samples can be fractionated in parallel overnight unattended. SCX separation is necessarily a serial process and entails collection of fractions every 5 min for a minimum of 2.5 hrs of instrument time per sample, not including blank gradients normally run between samples to insure no cross contamination.

4. Conclusions

This work demonstrates the utility of OGE as the first dimension peptide separation for proteomic analysis of CSF. Use of OGE enabled observation of more proteins than with the conventional SCX first dimension separation in CSF that had not been depleted of abundant proteins. OGE also offers increased analytical throughput in that multiple samples can be run in parallel. As such, shotgun analysis using OGE as the first dimension separation offers a significant advance in proteomic analysis technology.

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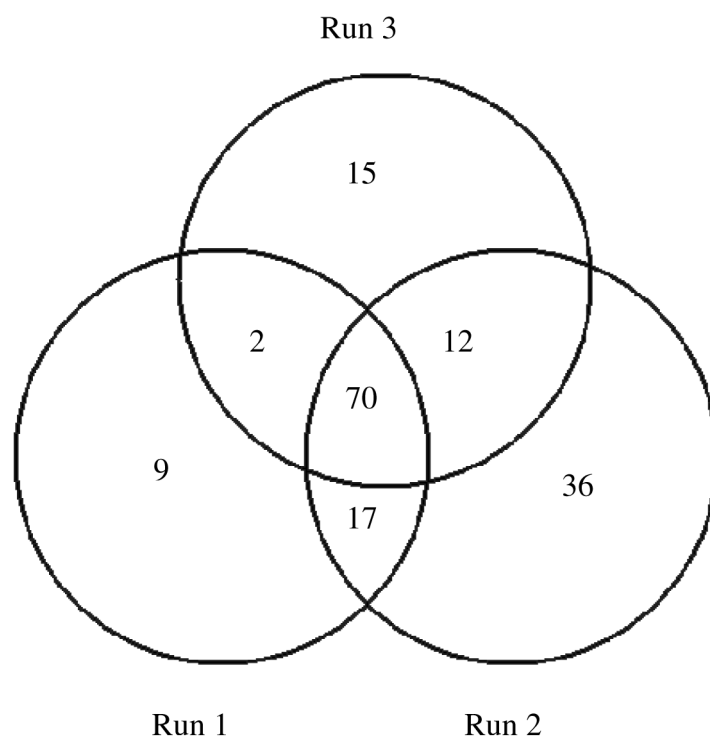


Figure 1. Venn diagram showing the number of proteins identified in three runs RP-LC-MS/MS analyses of the OGE separated peptides. Each number with no overlap of circles shows the number of proteins uniquely seen in that run, while overlapping circles show the numbers of identified proteins common to 2 or to 3 of the analyses.

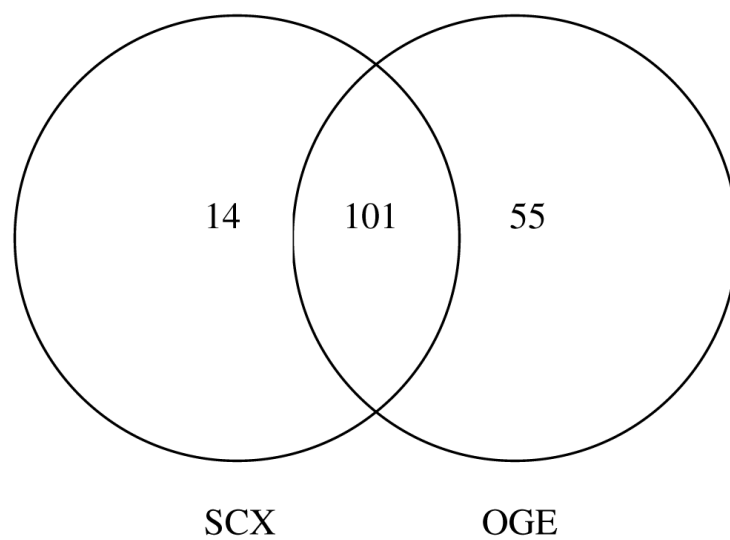


Figure 2. Venn diagram showing the numbers of proteins identified using SCX and using OGE as the first dimension separation in shotgun analysis of undepleted CSF. A total of 101 identified proteins were observed in both of the methods, while 14 additional proteins were unique to the SCX method, and 55 were unique to OGE method.

Table 1

List of proteins identified using the OGE method in order of abundance as indicated by the total spectrum count (TSC). The percent composition (% comp) was calculated as the percent of the summed normalized TSC's (see text). Proteins were considered identified if at least two identified peptides were matched to only that protein.

Protein ID	MW	Accession	TSC	%Comp.
Albumin	69321	P02768	1911	22.63
Transthyretin	15877	P02766	191	9.87
Hypothetical protein LOC651928	26229	946295	176	5.51
Hypothetical protein LOC649897	22059	Q49AS2	102	3.80
Cystatin C	15789	P01034	68	3.54
Transferrin	77000	P02787	303	3.23
Anti-RhD monoclonal T125 gamma1 heavy chain	52253	Q5EFE5	196	3.08
Alpha-1 antitrypsin	46707	P01009	163	2.86
Prostaglandin D2-synthase	21015	Q5SQ09	64	2.50
PREDICTED: similar to Ig gamma-3 chain C region	18069	947052	51	2.32
Apolipoprotein A-II preproprotein	11167	P02652	27	1.98
Apolipoprotein A-I preproprotein	30758	P02647	70	1.87
Apolipoprotein E	36131	P02649	71	1.61
PREDICTED: similar to Ig alpha-1 chain C region isoform 1	28490	Q8NCL6	54	1.56
Alpha 2 globin	15247	Q86YQ5	26	1.40
Beta globin	15988	P68871	25	1.28
Vitamin D-binding protein	52883	P02774	80	1.24
Apolipoprotein D	21261	P05090	29	1.12
Haptoglobin	45176	P00738	56	1.02
Syntaxin binding protein 2	66396	15833	81	1.00
Haptoglobin-related protein	39004	P00739	44	0.93
Hemopexin	51643	P02790	57	0.91
Orosomuroid 1	23496	P02763	24	0.84
Clusterin isoform 1	57795	Q96AJ1	59	0.84
Delta globin	16045	P02042	15	0.77
Serpin peptidase inhibitor, clade A, member 3	47620	P36955	44	0.76
PREDICTED: similar to Ig gamma-4 chain C region .	47414	P01861	39	0.68
PREDICTED: similar to Ig kappa chain V-III region HAH	16578	642113	13	0.64
Beta-2-microglobulin	13705	Q9UM88	10	0.60
Apolipoprotein A-IV precursor	45344	P06727	32	0.58
Complement component 4A preproprotein	192663	Q5INX2	117	0.50
Complement component 3	187045	Q6LDJ0	110	0.48
Alpha-2-glycoprotein 1, zinc	34237	P02765	20	0.48
Orosomuroid 2	23587	Q5T538	13	0.45
Angiotensinogen preproprotein	53120	P01019 ACS	29	0.45
Alpha 1B-glycoprotein	54219	P04217	28	0.42

Protein ID	MW	Accession	TSC	%Comp.
PREDICTED: similar to Ig gamma-1 chain C region	64280	652050	32	0.41
Serine (or cysteine) proteinase inhibitor, clade F	46283	Q4R6H4	23	0.41
Alpha-2-macroglobulin	163188	P01023	81	0.41
A-gamma globin	16118	P69891	8	0.41
Apolipoprotein H	38286	P02749	19	0.41
Insulin-like growth factor binding protein 6	25306	P24592	12	0.39
Kininogen 1	47852	P01042	22	0.38
CD14 antigen precursor	40050	P08571	18	0.37
Retinol-binding protein 4, plasma	22995	P02753	10	0.36
Ceruloplasmin (ferroxidase)	122127	P00450	53	0.36
PREDICTED: similar to Ig kappa chain V-II region Cum	13912	P01614	6	0.35
C-type lectin domain family 3, member B	22552	P05452	9	0.33
Gelsolin isoform B	80590	P06396	32	0.33
Pancreatic ribonuclease	17632	P07998	7	0.33
Alpha-2-HS-glycoprotein	39299	P02765	15	0.31
Family with sequence similarity 3, member C	24664	Q5HY75	9	0.30
Actin, gamma 1 propeptide	41765	P63261	15	0.29
PREDICTED: similar to Ig gamma-2 chain C region	39968	P01859	14	0.29
Kallikrein 6 isoform B	15045	Q92876	5	0.27
EGF-containing fibulin-like extracellular matrix 1 For	54604	Q12805	18	0.27
Complement component 1 inhibitor precursor	55119	O60860	18	0.27
Synovial sarcoma, X breakpoint 4B isoform a	21844	O60224	7	0.26
Complement factor B preproprotein	85478	P00751	27	0.26
Dickkopf homolog 3	38365	Q4R417	12	0.26
Lysozyme	16526	Q8N1E2	5	0.25
Proprotein convertase subtilisin/kexin type 1 inhibitor	27355	P29120	8	0.24
Galectin 3 binding protein	65289	P17931	19	0.24
Serine (or cysteine) proteinase inhibitor, clade C	52568	P32661	15	0.23
Secreted phosphoprotein 1 isoform A	35401	Q4W597	10	0.23
Alpha-1-microglobulin/bikunin	38973	Q5TBD7	11	0.23
PREDICTED: hypothetical protein XP_939253	28963	647006	8	0.23
Chitotriosidase	51648	QHVG6	14	0.22
Insulin-like growth factor binding protein 7	29111	Q16270	7	0.20
Complement factor H isoform b	50974	P08603	11	0.18
Amyloid precursor-like protein 1 isoform 2	72131	P51693	15	0.17
Serine (or cysteine) proteinase inhibitor, clade F	46283	Q4R6H4	9	0.16
Secretogranin III	52972	Q8WXD2	10	0.15
PREDICTED: similar to FXVD domain-containing ion	10640	A8K0R4	2	0.15
Complement component 1, q subcomponent, B chain	26704	Q5T960	5	0.15
PREDICTED: similar to Ig heavy chain V-III region	16286	646057 -	3	0.15
VGF nerve growth factor inducible	67217	Q9UDW8	12	0.15
Osteoglycin preproprotein	33900	P20774	6	0.15

Protein ID	MW	Accession	TSC	%Comp.
Autotaxin isoform 2 preproprotein	98929	I035181	17	0.14
PREDICTED: similar to peptidylprolyl isomerase A	18660	P62937	3	0.13
Complement component 7	93457	Q8TCS7	14	0.12
SPARC-like 1	75201	Q14515 ACS	11	0.12
Phospholipid transfer protein isoform a	54704	Q53H91	8	0.12
Afamin	69024	P43652	10	0.12
PREDICTED: similar to Ig heavy chain V region 102	13898	P01743	2	0.12
Insulin-like growth factor binding protein 2	35114	P18065	5	0.12
Carnosinase 1	56656	Q96KN2	8	0.12
Fibrinogen, gamma chain isoform gamma-B	51478	068656	7	0.11
Complement factor H isoform A	138978*	P08603	18	0.11
Vitronectin	54271	P04004	7	0.11
Peroxiredoxin 2 isoform b	15979	Q6P390	2	0.10
Cell adhesion molecule with homology to L1CAM	136612	Q59FY0	17	0.10
Superoxide dismutase 3, extracellular	25864	O14618	3	0.10
Complement component 9	63132	Q9UG14	7	0.09
Plasminogen For	90510	P06733	10	0.09
PREDICTED: similar to actin-like protein	46174	O96019	5	0.09
Fibulin 1 isoform B	65427	Q59G97	7	0.09
PREDICTED: similar to Ubiquitin carboxyl-terminal	113359	Q9H9C5	12	0.09
Leucine-rich alpha-2-glycoprotein 1	38154	P02750	4	0.09
DJ-1 protein	19878	Q99497	2	0.08
Hypothetical protein LOC79441	69606	Q725Q5	7	0.08
Complement component 1, r subcomponent	80147	Q53HT9	8	0.08
Hypothetical protein LOC160518	140512	Q6NUJ0	14	0.08
PREDICTED: similar to Prostate, ovary, testis alpha-N-acetylglucosaminidase	121366	P54802	12	0.08
Fibronectin 1 isoform 3 preproprotein	259061	P02751	25	0.08
Alpha 1 actin	42023	P68133	4	0.08
Chitinase 3-like 1	42586	P36222	4	0.08
Alpha-2-plasmin inhibitor	54561	P08697	5	0.08
Fibrinogen, beta chain preprotein	55892	P02765	5	0.07
Tissue inhibitor of metalloproteinase 1	23155	P01033	2	0.07
Coagulation factor II	69992	Q53H04	6	0.07
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 4	7088	Q43505	4	0.07
Complement component 1, s subcomponent	76634	Q53HU9	6	0.06
Fructose-bisphosphate aldolase C	39431	P09972	3	0.06
Kallikrein 6 isoform A preproprotein	26838	Q6T774	2	0.06
Peptidoglycan recognition protein L	67927	Q96PD5	5	0.06
Heparin cofactor II	57034	P05546	4	0.06
Microfibrillar-associated protein 4	28629	A8KAJ1	2	0.06
Inter-alpha (globulin) inhibitor H4	103293	Q59FS1	7	0.06
Histidine-rich glycoprotein	59540	P04196	4	0.06

Protein ID	MW	Accession	TSC	%Comp.
Inter-alpha globulin inhibitor H2 polypeptide	106396	A2RTY6	7	0.05
Nel-like 2	91284	Q99435	6	0.05
PREDICTED: similar to Fc fragment of IgG binding	30792	945689	2	0.05
PREDICTED: similar to Ceruloplasmin (Ferroxidase)	32078	877964	2	0.05
Neuronal cell adhesion molecule isoform B	130964	A4D0S3	8	0.05
Limbic system-associated membrane protein	37370	Q13449	2	0.04
Fibulin 1 isoform D	77190	Q96K89	4	0.04
Intercellular adhesion molecule 5	97185	Q9UMF0	5	0.04
Aldolase A	39395	P04075	2	0.04
UNC13 (C. elegans)-like	180529	O14795 ACS	9	0.04
ATP-binding cassette, sub-family E, member 1	67271	P61221	3	0.04
Neogenin homolog 1	159859	Q59FP8	7	0.04
Chromogranin A	50657	P10645	2	0.03
Inter-alpha (globulin) inhibitor H1	101338	P19827	4	0.03
Hypothetical protein LOC440307	53187	A6NNM8	2	0.03
Hypothetical protein LOC51244	53924	Q6P1I3	2	0.03
Hypothetical protein LOC9816	170460	Q14146	6	0.03
Amyloid beta A4 protein precursor, isoform A	86888	P05067	3	0.03
MutS homolog 5 isoform c	92816	O46196	3	0.03
Complement component 4 binding protein, alpha chain	66989	Q5VVQ8	2	0.02
Coagulation factor XII	67774	P00748	2	0.02
Integrator complex subunit 8	113015	Q75QN2	3	0.02
Chromogranin B	78199	P05060	2	0.02
Carbamoylphosphate synthetase 2	242827	P27708	6	0.02
Complement component 2	83214	A2AAQ4	2	0.02
Apolipoprotein B	515209	P04114	12	0.02
Tuberous sclerosis 2 isoform 3	195776	P49815	4	0.02
Trinucleotide repeat containing 15	149977	Q6Y7W6	3	0.02
Calsyntenin 1 isoform 1	109723	Q94985	2	0.01
Ephrin receptor EphA4	109789	P54764	2	0.01
Contactin 1 isoform 1 For	113249	Q12860	2	0.01
Calcium channel, voltage-dependent, alpha 2/delta	123105	Q17R45	2	0.01
Fc fragment of IgG binding protein	571719	P01876	9	0.01
Myosin VIIA	254242	Q13402	3	0.01
Complement component 4B preproprotein	192629	Q6U2E9	2	0.01
Thyroid hormone receptor interactor 11	227498	Q15643	2	0.01

Table 2

Lists of uniquely identified proteins observed using the SCX and using the OGE methods (i.e. observed using one method but not the other). Proteins denoted by an asterisk were observed in previous SCX fractionation analysis only after abundant protein depletion.

Unique Protein Identifications Observed in SCX Fractions	Unique Proteins Identifications Observed in OGE Fractions
Antitrypsin	Actin, gamma 1 propeptide*
Brevican isoform 1	Aldolase A*
CD59 antigen p18-20	Alpha 1 actin
Complement component 1, q subcomponent	Amyloid beta A4 protein, isoform a
Epididymal secretory protein E1	Apolipoprotein B*
Fibrinogen, alpha polypeptide isoform alpha	ATP-binding cassette, sub-family E, member 1*
Fibulin 1 isoform C	Calcium channel, voltage-dependent, alpha 2/delta*
Gelsolin isoform A	Carbamoylphosphate synthetase 2
Insulin receptor substrate 1	Coagulation factor XII*
Neural cell adhesion molecule 1 isoform 1	Complement component 2*
Prion protein preproprotein For	Complement component 4 binding protein, alpha chain*
Prosaposin	Contactin isoform 1*
Thy-1 cell surface antigen	DJ-1 protein
Thymosin-like 3	Ephrin receptor EphA4
	Fructose-bisphosphate aldolase C*
	Heparin cofactor II*
	Hypothetical protein LOC160518
	Hypothetical protein LOC440307
	Hypothetical protein LOC51244
	Hypothetical protein LOC 79441
	Hypothetical protein LOC9816
	Insulin-like growth factor binding protein 6
	Integrator complex subunit 8
	Inter-alpha (globulin) inhibitor H1*
	Intercellular adhesion molecule 5
	Kallikrein 6 isoform A preproprotein
	Leucine-rich alpha-2-glycoprotein 1*
	Microfibrillar-associated protein 4*
	MutS homolog 5 isoform c
	Myosin VIa
	Nel-like 2*
	Neogenin homolog 1*
	Peroxiredoxin 2 isoform b
	Phospholipid transfer protein isoform a*
	PREDICTED: hypothetical protein XP_939253
	PREDICTED: similar to actin-like protein
	PREDICTED: similar to ceruloplasmin*

Unique Protein Identifications Observed in SCX Fractions

Unique Proteins Identifications Observed in OGE Fractions

- PREDICTED: similar to Fc fragment of IgG binding*
 - PREDICTED: similar to FXYD domain-containing ion
 - PREDICTED: similar to Ig heavy chain V region 102
 - PREDICTED: similar to Ig heavy chain V-III region
 - PREDICTED: similar to Ig kappa chain V-II region C
 - PREDICTED: similar to peptidylprolyl isomerase A
 - PREDICTED: similar to prostrate, ovary, testis alpha-N-acetylglucosaminidase
 - PREDICTED: similar to ubiquitin carboxyl-terminal
 - Serine (or cysteine) proteinase inhibitor, clad
 - Serine (or cysteine) proteinase inhibitor, clade
 - Superoxide dismutase 3, extracellular
 - Synovial sarcoma, X breakpoint 4B isoform a
 - Syntaxin binding protein 2
 - Thyroid hormone receptor interactor 11
 - Tissue inhibitor of metalloproteinase 1
 - Trinucleotide repeat containing 15
 - Tuberous sclerosis 2 isoform 3
 - UNC13 (C. elegans)-like
-

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Table 3

Comparison of workflows and times for OGE and SCX fractionation. Note the shorter time for instrument setup as well as for the LC-MS/MS runs for the OGE method. A 30 min difference in LC-MS/MS times is due to the absence of a desalting step in the OGE workflow that is normally needed with SCX fractions. The OGE instrument allows the analysis of up to twelve samples at a time, giving a further advantage of parallel sample processing.

	OGE	SCX
Instrument Setup Time	25 min	3 hrs (includes equilibration time for SCX column)
Separation and Fraction Collection Time	13 hr (fraction collection from wells-5 min)	3.5 hr (fraction collection every 5 min over a 150 min gradient followed by a 1 hr wash)
Sample number	up to 12 samples	1 sample
LC-MS/MS	180 min per fraction	210 min per fraction