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Identification of selenium-containing proteins in HEK 293 kidney cells using multiple chromatographies, LC–ICPMS and nano-LC–ESIMS

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ABSTRACT

Our previous studies using HeLa and HEK 293 cells demonstrated that selenomethionine, SeMet, exerts more of an antagonistic effect on arsenic than other selenium species. These studies attributed the antagonistic effect of SeMet to decreased levels of reactive oxygen species, ROS, changes in protein phosphorylation and possible incorporation of SeMet into proteins. The present study employs a metallomics approach to identify the selenium-containing proteins in HEK 293 cells raised with SeMet. The proteins were screened and separated using two dimensional high performance liquid chromatography (HPLC)–inductively coupled plasma mass spectrometry (ICPMS), size exclusion chromatography (SEC) and reversed-phase chromatography (RPC). The Se-containing proteins were identified by peptide mapping using nano-HPLC–Chip–electrospray ionization mass spectrometry (ESIMS).

1. Introduction

Selenium (Se) is an essential micronutrient for humans and animals [1] and is considered to be associated with the function of numerous major cellular metabolic pathways [2–4]. Se is required for the activity of a number of selenium-dependent enzymes, such as glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases and selenophosphate synthetases [5,6]. The proteins containing selenium are termed as selenium-containing proteins; however, if the selenium incorporation is through selenocysteine, SeCys, these coded proteins are termed as selenoproteins. Though selenium-containing proteins are considered physiologically not as important as selenoproteins, they serve in synthesis of selenoproteins, thus having an indirect effect. It is thought that SeMet is incorporated into proteins through the sulfur metabolic pathway [7,8]. In other words, SeMet replaces methionines during the corresponding peptide synthesis. Selenium exerts its biological functions largely through its presence in selenoproteins; however, some low molecular weight selenium compounds, such as methyl selenic acid (MeSeA), methyl-selenocysteine (MeSeCys) and selenomethionine (SeMet) also have profound cellular effects. Recent studies have shown that Se-containing small molecules modulate tumor growth in animal studies and *in vitro* models [4,9–11]. Lack of dietary selenium, has been attributed to the increased risk of cancer, infections [12,13], cardiomyopathy [14], male infertility [15], Parkinson's disease [16], Alzheimer's disease [17], depleted immune response [18] and thyroid function [19]. Further, selenium deficiencies lead to decreased levels of selenoproteins, such as GPx, thus generating increased levels of ROS leading to numerous detrimental effects [2,20,21].

Selenium species can mitigate the toxicity of ingested and inhaled arsenic and mercury [22–24]. Our earlier studies using HeLa and HEK 293 cells demonstrated that SeMet exerts a greater antagonistic effect on arsenic than other selenium species [24,25]. Examination showed that SeMet decreased levels of reactive oxygen species (ROS) through its anti-oxidant function. Importantly, key changes in signaling cascades were noted via our phosphoproteomic analysis. It has been recently proposed that ROS is a key cellular signal transducer for cell growth and angiogenesis [26,27]. Thus, SeMet not only quenches the ROS induced from arsenic and mercury but also lowers the initiation capacity of these key cancer-signaling cascades. We hypothesize that this occurs through possible incorporation of SeMet into proteins. The first step in exploring this hypothesis is to demonstrate enhanced non-specific incorporation of SeMet into cellular proteins. To demonstrate the non-specific incorporation of SeMet into HEK 293 cell proteins, this study was undertaken.

Currently the most predominant method for identification of selenium-containing proteins is chromatographic separation followed by mass spectrometry [28,29]. Coupling High Performance Liquid Chromatography (HPLC) with Inductively Coupled Mass Spectrometry (ICPMS) or Electrospray Ionization Mass Spectrometry (ESIMS) provides a means of identifying selenoproteins and selenium-containing proteins, and this coupling technique

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was reviewed by Sanz-Medel [30,31] and Szpunar [32]. Lobinski et al. did extensive work on identifying selenoproteins and selenium-containing proteins in selenized yeast [28,33].

The goal of the study is to demonstrate the incorporation of selenium into the proteins via selenomethionine. This study was undertaken due to the fact that selenomethionine had been found to modulate arsenic toxicity on HEK 293 cells [34]. This antagonistic behavior of SeMet was found to be due to modulation of ROS, differential protein phosphorylation changes and a presumed selenium incorporation into proteins. Thus, this current study shows that SeMet has a role in incorporation of selenium in proteins, which builds on the earlier study [34] where differential protein phosphorylation between an arsenite inoculation and arsenite/SeMet inoculation of the HEK 293 cells. The secondary focus was to further verify our method for doing so, now using HEK 293 cells.

First, size exclusion chromatography (SEC) coupled to ICPMS was used to confirm the presence of selenium in various molecular weight regions, and then followed by fraction collection based on molecular weight ranges. To assess whether the selenium found through SEC–ICPMS is covalently bound to proteins or not, each fraction was further analyzed using capillary reversed-phase liquid chromatography (capRPLC) coupled to ICPMS. Any free selenium that is not covalently bound to the proteins would be found in this procedure, since denaturation of SEC protein fractions is done prior to capRPLC. To overcome the small capRPLC amounts, the developed method was transferred to normal bore reversed-phase liquid chromatography (RPLC, with peptide MW standards used for to assure the MW assignments for the fractions were correct). With the ICPMS offline, the fractions were isolated. The isolated fractions were tryptically digested and protein identification from their corresponding peptides was achieved through nanoLC–ESIMS^2 followed by database searching.

2. Experimental

2.1. Reagents

All the aqueous solutions were prepared in 18 MΩ cm^{-1} doubly deionized water (Sybron Barnstead, Boston, MA, USA). HEK 293 human kidney cell lines, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), M-Per protein extraction buffer, formic acid (FA), urea, ammonium acetate and ammonium bicarbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Trypsin-EDTA: 0.05% trypsin 0.53 mM EDTA ×4 Na, was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). L-selenomethionine (SeMet), Dithiothreitol (DTT), iodoacetamide (IAA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The HPLC grade solvents, water and acetonitrile (ACN), were of high purity and purchased from Burdick and Jackson (Muskegon, MI, USA). Sequence grade modified trypsin and acetic acid buffer were obtained from Promega (Madison, WI, USA).

2.2. Cell culture and cell lysate

Under humid conditions, human kidney cells (HEK293 cells) were cultured in 75 cm^2 tissue culture plates using DMEM medium containing 10% FBS at 37 °C with 5% CO_2. Fresh solutions of selenomethionine were made using the DMEM medium prior to dosing. After cells reached 80% confluence, they were raised for 24 hr with no SeMet dosing or with 100 μM of SeMet.

After incubation for 24 h with 100 μM SeMet, Trypsin–EDTA was used to isolate the cells, followed by washing with PBS to remove the traces of leftover media. M-Per protein extraction buffer was used for lyses and extraction. Cells were centrifuged at 10,000*g* for 10 min and supernatants were used for further analysis.

2.3. Size Exclusion Chromatography (SEC)

An Agilent 1100 series HPLC system equipped with a binary pump, vacuum membrane degasser, thermostated auto sampler, column oven, and diode array detector with a semi-micro-flow UV–vis cell was used for SEC chromatographic analysis. The entire system was controlled using Chemstation software (all from Agilent Technologies, Santa Clara, CA, USA). SEC was performed using TSK Gel 3000SW 7.5 × 300 mm (Tosoh Bioscience LLC, PA, USA). The size exclusion column was calibrated using a UV detector (wavelength, 280 nm) by using a gel filtration standard mixture (MW of thyroglobulin, 670 kDa; MW of γ-globulin, 158 kDa; MW of ovalbumin, 44 kDa; MW of myoglobin, 17 kDa; MW of vitamin B12, 1.3 kDa) purchased from Bio-Rad Laboratories (Life Science Research, CA, USA), and R=0.997 for the calibration. Mobile phases were: A—50 mM NH_4OAc, B—MeOH, Isocratic—5% B at flow rate of 0.5 ml/min. The fractions isolated based on the SEC–ICPMS signal were collected and pre-concentrated to dryness by lyophilization.

2.4. Inductively Coupled Plasma Mass Spectrometry (ICPMS)

An Agilent Technologies 7700 inductively coupled plasma mass spectrometer, equipped with a Micromist nebulizer, a Peltier-cooled spray chamber (2 °C), and an octopole collision/reaction cell with hydrogen gas pressurization (purity of 99.99%) was used. The entire system was controlled using Mass Hunter software (all from Agilent Technologies, Santa Clara, CA, USA). The ICPMS conditions were as follows: forward power, 1500 W; plasma gas flow rate 15 L min^{-1}, carrier gas flow, 0.91 L min^{-1}; make-up gas 0.12 L min^{-1}; collision gas, H_2, 4.0 ml min^{-1}; quadrupole bias, −16.0 V; octopole bias −18.0 V for a +2 V energy discrimination voltage. The isotope monitored was selenium ^{78}Se.

2.5. Capillary Reversed Phase Liquid Chromatography (capRPLC)

An Agilent 1200 HPLC system equipped with a binary pump, vacuum membrane degasser, thermostated auto sampler, column oven, and diode array detector with a semi-micro-flow UV–vis cell was used for chromatographic analysis. Reversed phase chromatography was performed using C-4 phase, Jupiter 150 × 0.50 mm, 5-μ-particle size (Phenomenex, CA, USA). The solvents used were 0.1% FA in H_2O (Solvent A) and 0.1% FA in 90% ACN (Solvent B). A flow rate of 10 μl/min was used. The following gradient was used in the analysis: 3 min 3% B; 3–60 min 65% B, 60–70 min 90% B, 70–75 min 90% B, 75–80 min 2% B and 80–90 min 3% B.

2.6. Tryptic digestion, ESI–MS and nano-LC-Chip

Tryptic digestions were performed as follows: the protein pellets obtained from the SEC fractions collected were re-suspended in 50 μL of 50 mM ammonium bicarbonate, 4 μL of 100 mM DTT were added as reducing buffer and the mixture was heated at 95 °C for 5 min; this step unfolds the proteins and reduces the disulfide bonds. After cooling the sample, an alkylation was carried out to protect the thiol groups of the cysteine residues by adding 6 μL of 100 mM iodoacetamide. The mixture was incubated in the dark for 20 min at room temperature. After the alkylation, 2 μL of modified sequence grade trypsin solution was added and incubated at 36 °C for 2 h. Then 2 μM of additional trypsin was added to complete the reaction, followed by incubation at 36 °C for 8 h. 2 μL of formic acid was added to stop the

reaction, and the solution was ultra-filtered through 5 kDa filters to eliminate the undigested proteins and the trypsin.

Electrospray experiments were performed using an Agilent 6300 Series nanoLC Chip/Ion Trap XCT system (Agilent Technologies, Santa Clara, CA). An Agilent 1200 LC, equipped with both capillary and nano-pumps, was used for loading and flushing the chip nano-column. This is equipped with both capillary and nano-pump and used for mass identification. The chip used for the analysis consists of a Zorbax 300SB C18 enrichment column (4 mm × 75 μm, 5 μm) and a Zorbax 300SB C18 analytical column (150 mm × 75 μm, 5 μm). Two microliters of sample were loaded via the capillary pump onto the on-chip enrichment column. Samples were loaded on to the enrichment column at a flow rate of 3 μL min^{-1} with a 97:3 ratio of solvent A (0.1% FA (v/v) in water) and B (90% ACN (acetonitrile), 0.1% FA (v/v) in water). After the enrichment column was loaded, the on-chip microfluidics switched to the analytical column at a flow rate of 0.3 μL min^{-1}. The following gradient conditions were used in the analysis: 0–5 min, 10% B; 5–85 min, 35% B; 85–90 min, 75% B; 90–95 min, 75% B; 95–98 min, 3% B; 98–105 min, 3% B. For MS/MS experiments, experimental conditions consisted of: m/z range: 100–2200, isolation width: 2 m/z units, 2 precursors, maximum accumulation time 150 ms and active exclusion after 3 spectra from 0.5 min, fragmentation energy: 30–200% fragmentation.

The MS/MS data obtained from the experiments were exported to the online MASCOT program (Matrix Science Inc.) and Spectrum Mill database search engines, and submitted with the following parameters: Taxonomy (Mammals), Enzyme (Trypsin), Missed Cleavages (Two), Fixed modifications (Carbamidomethyl), Peptide tolerance (2 Da), MS/MS tolerance (0.8 Da), Peptide charge (+1,+2,+3) and Instrument (ESI–TRAP). MASCOT then searched against Uniprot database and the reported hits were validated doing a blast analysis of the reported peptides.

3. Results and discussion

This study identifies the selenium containing proteins in HEK cells grown with and without100 μM SeMet (control vs. analytical sample). The strategy employed in the identification of selenium-containing proteins is shown in Fig. 1. Size Exclusion Chromatography was used for separation of proteins based on their molecular weight and the removal of low MW free selenium with ICPMS detection monitoring ^{78}Se. Insights into which fraction includes selenium containing proteins were therefore obtained. Fig. 2a shows the chromatograms (UV detection at 280 nm) of SEC fractions for cells grown with 100 μM SeMet and the control. Although slight differences are observed in the UV profiles, the differences obtained via the ICPMS ^{78}Se signal is considerable, as shown in Fig. 2b and it may be noted that element specific detection usually provides simpler to understand results, relative to universal detection, such as UV. Thus, the incorporation of SeMet into the higher molecular weight regions is extensive. Four fractions, as a function of molecular weight (hydrodynamic radii) were separated. For fraction collection, the ICPMS plasma is turned off and SEC fractions are collected multiple times to achieve an appropriate concentration prior to the capRPLC experiments. After fraction collection, the samples were lyophilized and denatured prior to the injection into capRPLC.

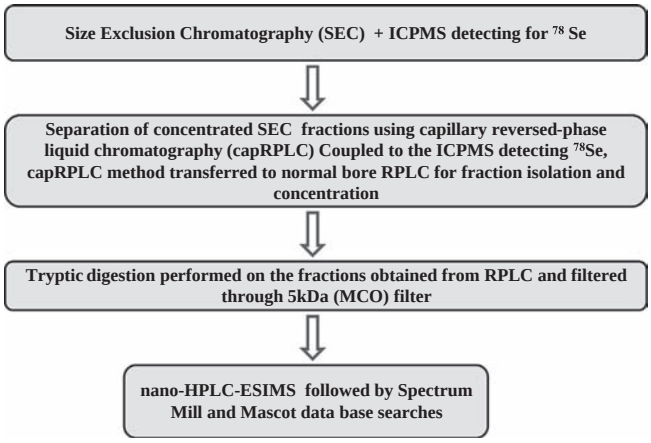

Fig. 1. Protein rich supernatant was obtained through lysis of HEK293 cells followed by extraction and centrifugation. Only a part of the sample supernatant was analyzed using SEC with in line monitoring of isotope ^{78}Se using ICPMS. With the plasma off, remaining supernatant is subjected to the SEC and fractions were collected. Fractions isolated were concentrated to the necessary level for further analysis using capRPLC–ICPMS. After monitoring the isotope ^{78}Se online using ICPMS, the capRPLC method was transferred to normal RPLC and fractions isolated. Isolated fractions were further concentrated. Tryptic digestions on the concentrated fractions were performed and subjected to nanoLC–ESIMS analysis.

3.1. capRPLC

SeMet incorporation into proteins is nonspecific and the incorporation of selenium in the selenium-containing proteins can be through covalent and non-covalent bonding. Though SEC separation based on hydrodynamic volume (an indication of molecular weight) provides purification of proteins from the free inorganic selenium, a confirmation of covalently bound selenium in these proteins is established by denaturing the protein fractions.

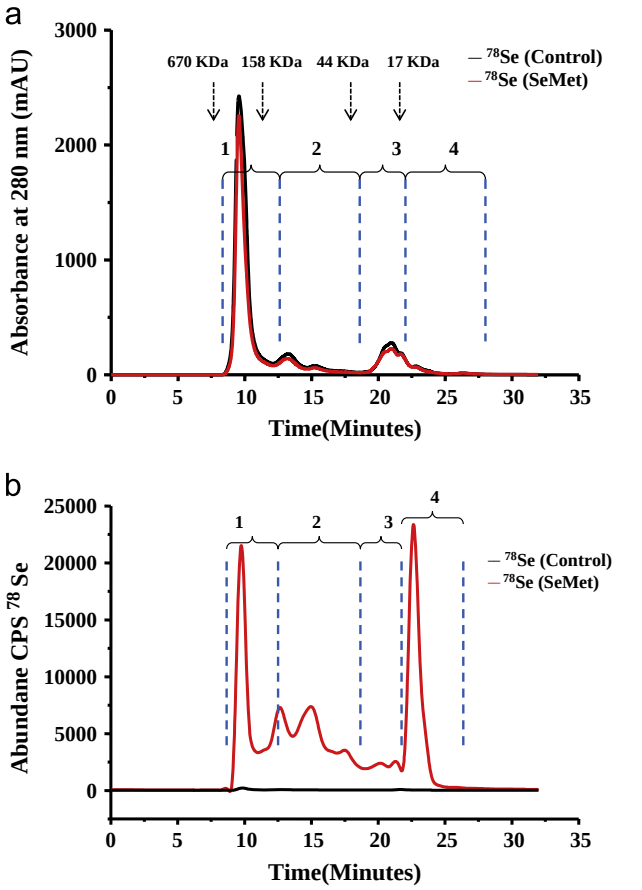

Fig. 2. (a) SEC chromatograms showing the UV signals monitored at 280 nm. (b) SEC–ICPMS chromatograms; counts of ^{78}Se isotope (*y*-axis) vs. Time (*x*-axis). The color traces, sample description, column, flow rate, solvent conditions are similar to Fig. 2a. It is apparent that the ICPMS chromatograms are simpler and generally more useful than with UV detection for screening selenium containing proteins.

Denaturing the fractions liberates free selenium not covalently associated. Further, these denatured protein fractions are separated using capRPLC and ^{78}Se signal is monitored by ICPMS.

Fig. 3a and b represents the ^{78}Se signal monitored over time using ICPMS for first and second SEC fractions, which contain various substances including proteins whose molecular weights are greater than 44 kDa. As can be seen from the figure there is no loss of selenium upon injection, suggesting all the selenium present is covalently bound. Fig. 3c and d represents the ^{78}Se chromatography for the third and fourth SEC fractions, whose

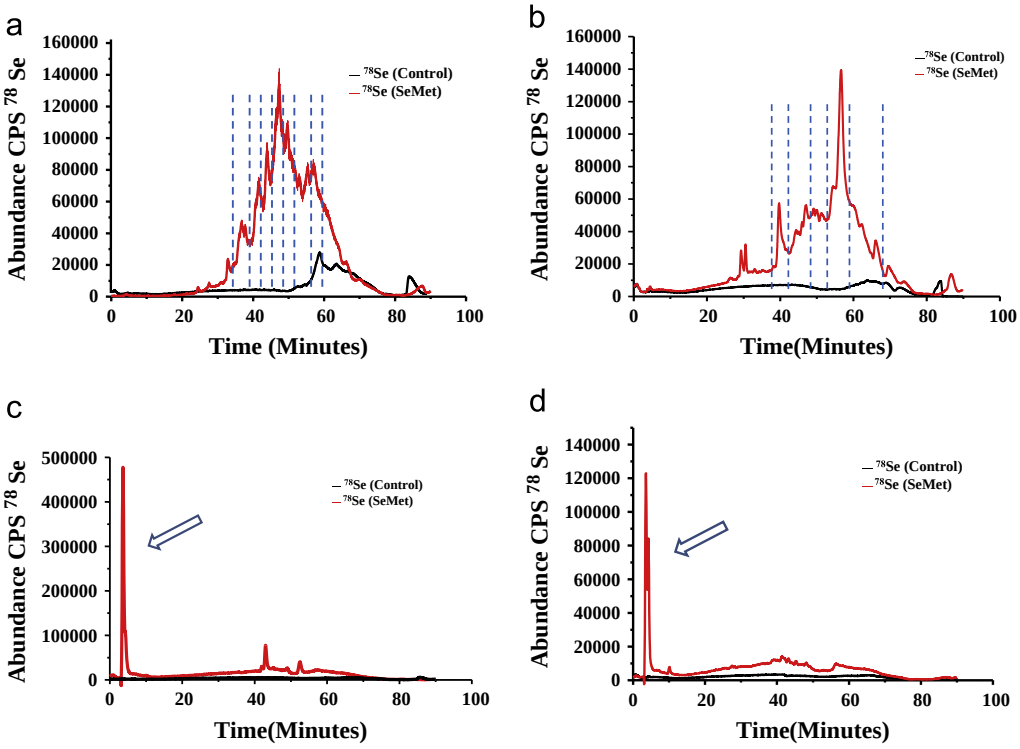

Fig. 3. All Fig. 3a–d represents counts of ^{78}Se isotope (*y*-axis) vs. Time (*x*-axis) and color traces, sample description, column, flow rate and solvent conditions are similar. (a&b) First and second fractions of SEC analyzed using capRPLC. (c&d) Third and fourth fractions of SEC analyzed using capRPLC, the arrow points the free selenium eluted upon injection.

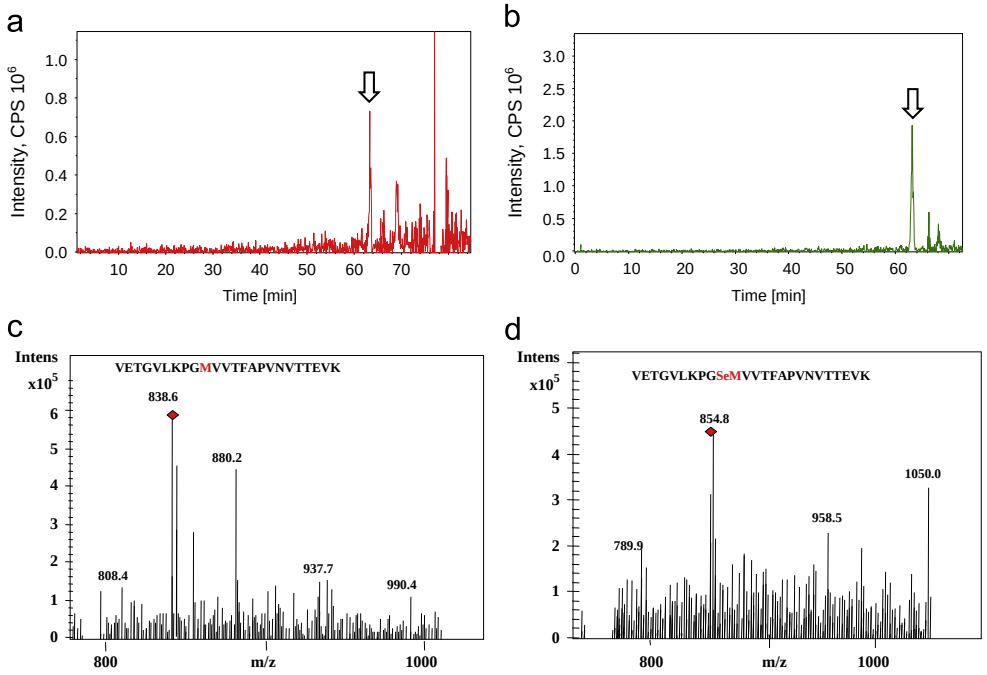

Fig. 4. (a) Extracted Ion chromatogram (EIC) of an S-peptide (VETGVLKPG**M**VVTFAPVNVTTEVK) belongs to Elongation factor 1-alpha and has *m*/*z* of 838 (b) EIC of Se-peptide (VETGVLKPG**SeM**VVTFAPV-NVTTEVK) belongs to Elongation factor 1-alpha and has *m*/*z* of 854; the retention time overlap is to be noted (c) MS/MS spectra of S-peptide (VETGVLKPG**M**VVTFAPVNVTTEVK) belong to Elongation factor 1-alpha and has *m*/*z* of 838 (d) MS/MS spectra of Se-peptide (VETGVLKPG**SeM**VVTFAPVNVTTEVK) belongs to Elongation factor 1-alpha and has *m*/*z* of 854.

molecular weights are less than 44 kDa. As can be seen from the figures there is high amount of selenium loss upon injection. This suggests that selenium is not covalently associated with proteins of <44 kDa.

Thus, coupling capRPLC with ICPMS provides greater insights about the covalent or non-covalent bonding of selenium in SEC separated fractions. As can be seen from Fig. 3a–d, fractions 1 and 2 (isolated by SEC) are important for further analysis, since the association of selenium in these protein fractions is through covalent bonding. It is not practical to collect fractions on capRPLC to the necessary levels to further perform ESIMS protein analysis. To overcome this, the gradient solvent conditions employed in the capRPLC were transferred to the normal bore RPLC to separate a much larger protein amount. These reversed phased fractions were further concentrated to necessary levels and tryptic digestion performed prior to nanoLC–ESIMS analysis.

3.2. Identification of Se-containing proteins

Based on the ^{78}Se signal provided by capRPLC–ICPMS, 12 fractions were collected from the scaled up normal bore experiment, digested with trypsin and analyzed by nano-LC–Chip–ESIMS. The resulting

Table 1
Shows the parent charge, score, peptides identified, accession number and protein name.

Parent charge	Score	Sequence	Matched parent mass of S-peptide	Matched parent mass of Se peptide	Accession number	Protein name
3	16.8	(R)VETGVLKPGMVVTFAPVNVTTEVK(S)	2515.3	2563.3	P68104	Elongation factor 1-alpha
3	16.51	(K)EKIRLDEAGVTDEVLDSAMQAFLLEIIVK(H)	3245.7	3293.7	O60507	Protein-tyrosine sulfotransferase
3	15.88	(K)FRLDASDKPLKVGLGMYSK(E)	2125.1	2173.1	Q9UFH2	Dynein heavy chain
3	15.83	(R)NTEELDMAGVQSLVPR(L)	1758.8	1806.8	Q68DX3	FERM and PDZ domain-containing protein
3	15.62	(K)LHLMFSLMDKVPNGIEPMLK(D)	2313.2	2361.2	Q93034	Cullin-5
3	14.16	(K)LEFAQDAHGQPDVSAFDFTSMMRAESSAR(V)	3201.4	3249.4	Q8TDZ2	NEDD9-interacting protein
3	14.1	(K)GGDIMLWNFGIKDKPTFIKGIGAGGSITGLK(F)	3191.7	3239.7	Q92466	DNA damage-binding protein
3	13.49	(K)EENLICSECGDEFTLQSQLAVHMEEHR(Q)	3261.4	3309.4	Q9UL36	Zinc finger protein
3	13.36	(K)EYSSEHQAADMAEIDARLKALQEYMNR(L)	3169.4	3217.4	Q9Y6R9	Coiled-coil domain-containing protein
3	13.27	(K)ELMGCQCCEEKPSIMVSNLHKEYDDK(K)	3200.3	3248.3	Q8WWZ7	ATP-binding cassette sub-family A member
3	13.15	(R)VHEKPFVAFGSGMESLVEATVGERVRIPAK(Y)	3240.7	3288.7	P35968	Vascular endothelial growth factor receptor
3	13.13	(K)GYLGAKGIQGMPGIPGLSGIPGLPGRPGHIKGVK(G)	3279.8	3327.8	P08572	Collagen alpha-2(IV) chain
3	13.05	(R)RQASSMPTGTTMGSPASPPGPNSKTGR(V)	2658.2	2706.2	Q9Y2E6	Protein deltex-4
3	12.96	(R)GVELMRFTTPGFPDSPYPAHARCQWALR(G)	3260.5	3308.5	Q9Y5Y6	Suppressor of tumorigenicity 14 protein
3	12.91	(R)VANIARTNATNNMNLSRSSSDNNTNTLGR(N)	3106.5	3154.5	Q9ULT8	E3 ubiquitin-protein ligase HECTD1
3	12.9	(K)LMSCPLAGLISKDAINLKAEALLPTQEPLK(A)	3234.7	3282.7	Q9NR48	Probable histone-lysine N-methyltransferase
3	12.73	(R)QGRVISVIAVSIGFLASVTGAMITSAAVAGIYR(V)	3278.8	3326.8	Q5T4T1	Transmembrane protein
3	12.63	(R)HGLPIPGSTPTPMVGSGRLGAPVGRSGGGASAR(S)	3054.6	3102.6	Q96S07	Proline-rich protein
3	12.45	(K)EMEEFVQSSGENGVVVFSLGSMVSNTSEER(A)	3264.4	3312.4	P06133	UDP-glucuronosyltransferase
3	12.44	(K)RCLTLMDRGFIFNLINDYISGFSPK(D)	2977.5	3025.5	Q5JSL3	Dedicator of cytokinesis protein
3	12.43	(R)YGGNLSLQSAMSVRFNSNGTQLLALRR(R)	2953.5	3001.5	Q96JK2	DDB1- and CUL4-associated factor
3	12.42	(K)SYLAWIGFSAEGTPCYVDSEGIVRMLNR(G)	3191.5	3239.5	O75717	WD repeat and HMG-box DNA-binding protein
3	12.4	(K)NKVNFIPTGSAFCPVKLLGPLLPASDLMLK(N)	3240.7	3288.7	Q86VQ1	Glucocorticoid-induced transcript 1 protein
3	12.23	(K)KAFATCSSHVAVVGLFYGAGIFTYMRPK(S)	3078.5	3126.5	Q8NG77	Olfactory receptor 2T12 OS=Homo sapiens
3	11.05	(R)GIVARLVQKCLPPEIIMEYGEEVLEEIK(N)	3255.7	3303.7	P51956	Serine/threonine-protein kinase
3	12.22	(R)SRYGESYLDQILNGMDEELGSLEELEK(K)	3117.4	3165.4	P16157	Ankyrin-1 OS=Homo sapiens
3	12.1	(K)KLPFIYGNLSQGMVSEPLEDVDPYYYKK(K)	3293.6	3341.6	Q01118	Sodium channel protein type 7 subunit alpha
3	11.98	(R)WSSYQNQTDNSVSNDPLLMTQYFK(K)	2866.2	2914.2	Q6PJP8	DNA cross-link repair 1A protein
3	11.95	(R)YTRLLLVKEHSNPMQVQQQLLDTGR(G)	2967.5	3015.5	P59046	NACHT, LRR and PYD domains-containing protein
3	11.71	(R)LGIPMSVLMGANIASEVADEKFCETTIGCK(D)	3241.5	3289.5	P21695	Glycerol-3-phosphate dehydrogenase [NAD+]
2	11.59	(R)MNNGDVDLTSDR(Y)	1336.5	1384.5	Q12860	Contactin-1
3	11.44	(K)AGMNIARLNFSHGSHEYHAESIANVR(E)	2881.3	2929.3	P30613	Pyruvate kinase isozymes R/L
3	11.38	(R)LTFPDREALAEHADLKSMVELIK(R)	2626.3	2674.3	Q8NE63	Homeodomain-interacting protein kinase
3	11.29	(K)TKQFAPIHAEAPEFMEMSVEQEILVTGIK(V)	3273.6	3321.6	P06576	ATP synthase subunit beta, mitochondrial
3	11.23	(K)QKDSLLQAPMHIDRNILMLILPLILLNK(C)	3253.8	3301.8	Q5PT55	Sodium/bile acid cotransporter

MS/MS spectra were database searched for peptide identification. Numerous proteins were found in the fractions isolated containing Se, though it would be presumptuous to declare these are Se-containing proteins. A generally accepted hypothesis is that SeMet is incorporated into proteins through non-specific pathways, thus SeMet and Met can be incorporated into the same type of proteins, further indicating the presence of both peptides, a S-peptide, the peptide containing Met, and a Se peptide, the peptide with the same sequence as the S-peptide except that the Met is replaced by SeMet. Calculations were made to predict the m/z of the Se-peptide based on the m/z of S-peptide using the formula shown below [35].

$$m/z_{(\text{S-peptide})} + n \times ({}^{80}\text{Se} - {}^{32}S)/z = m/z_{(\text{Se-peptide})}$$

In the above formula, $m/z_{(\text{S-peptide})}$ would be obtained from standard peptide mapping, $n = 1,2,3\ldots N$, where N is the number of Met in the S-peptide. The difference in atomic mass between ^{80}Se and ^{32}S is 48 and z is the charge of the S-peptide precursor ion (it is assumed that the charge is the same for the Se-peptide). Since a Se-peptide may contain more than one Met and SeMet may replace more of them, it is possible that multiple Se-peptides may be predicted from an S-peptide with multiple Met. It is expected that an S-peptide and its corresponding Se-peptide elute closely together (unless a carbamidomethylation is present on S-peptide and absent in Se-peptide) from the reversed-phase column during nano-LC-ESI-MS/MS analysis. The extracted ion chromatograms (EIC) of the m/z S-peptide and the predicted m/z Se-peptide were extracted from the total ion chromatogram (TIC). If an S-peptide and its corresponding Se-peptide are both present, a retention time match will be observed as shown in Fig. 4a and b, since they are structurally identical except the difference between S atoms (Met) and Se atoms (SeMet). A use of the formula is illustrated below.

All the selenium-containing proteins identified in 12 fractions isolated using normal bore LC are presented in Table 1. For a better understanding on how the selenium-containing proteins were isolated and identified, a discussion follows for one protein. The first peptide in the table is taken for the explanation. This peptide sequence confirms the presence of protein Elongation factor 1-alpha. The S-peptide (VETGVLKPG**M**VVTFAPVNVTTEVK) belongs to Elongation factor 1-alpha and has m/z of 838. The total ion chromatogram, extracted ion chromatogram for S-peptide and Se-peptide, MS/MS spectra of S-peptide and Se-peptide are shown in Fig. 4a–d. As it is expected that the S-peptide would elute at the same retention time compared to the Se-peptide (at least very close, since the change in net hydrophobicity would be small), the retention time matches should serve for identification. The Se-peptide (VETGVLKPG**SeM**VVTFAPVNVTTEVK) has an m/z of 854 and its EIC matches with EIC of S-peptide at the same retention time confirming the both peptides. The S-peptide mass is 2515.38 and its charge is +3, so the observed m/z is 838.46. The Se peptide mass is 2515.38 plus the difference between the two atomic weights of selenium and sulfur is 48. So, the net mass is 2515.38+48=2563.38, divided by its charge +3. Final observed m/z is 2563.38/3=854.46. A similar strategy was employed in identifying all the Se-containing proteins reported in Table 1 based on their S-peptide. Table 1 also shows the differences between the S and Se peptide masses for all proteins reported as obtained from the MS/MS spectra.

Though numerous proteins were found to contain the SeMet, only a few of those showing significance are discussed here. Further information can be found by following the accession numbers. WD repeat and HMG-box DNA-binding protein 1 has a role in DNA binding and acts as a replication initiation factor that brings together the mini-chromosome maintenance proteins (MCM2-7) helicase and the DNA polymerase alpha/primase complex in order to initiate DNA replication [36]. Suppressor of tumorigenicity 14 protein has a similar role to serine protease and is proposed to play a role in breast cancer invasion and metastasis [37]. Collagen alpha-2(IV) chain is the major structural component of glomerular basement membranes (GBM). Canstatin, a cleavage product corresponding to the collagen alpha 2(IV) NC1 domain, possesses both anti-angiogenic and anti-tumor cell activities [38].

4. Conclusion

A metallomics approach was used for identification of Se-containing proteins in HEK 293 cells cultured in the presence of SeMet. The use of SEC–ICPMS, resulted in preliminary peak screening and molecular weight fraction separation for selenium containing proteins by monitoring the ^{78}Se isotope. The fractions were isolated based on the MW with the ICPMS offline. Further use of cap-RPLC on SEC fractions clearly established the differences in fractions containing Se association by means of covalent or non-covalent bonding. Se incorporation in higher molecular weight fractions is through covalent bonding, while lower molecular weight fractions are through non-covalent bonding. The selected RPLC Se-containing fractions were analyzed by peptide mapping with HPLC–Chip–ESIMS and MASCOT database searches. Numerous selenium-containing proteins were identified based on the approach, thus providing evidence of the SeMet incorporation into the HEK proteins is non-specific.

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