Talanta 99 (2012) 186–193

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Synthesis and application of a Sec_2-containing oligopeptide for method evaluation purposes in selenium speciation

Mihály Dernovics [a,*], Andrea Vass [a], Anikó Németh [a], Anna Magyar [b]

[a] Department of Applied Chemistry, Corvinus University of Budapest, 29-33. Villányi út, 1118 Budapest, Hungary
[b] Research Group of Peptide Chemistry, Eötvös Loránd University, Hungarian Academy of Sciences, 112 POB 32, 1518 Budapest, Hungary

ARTICLE INFO

Article history:
Received 14 February 2012
Received in revised form
17 May 2012
Accepted 19 May 2012
Available online 26 May 2012

Keywords:
Selenocysteine
Selenocystine
Oligopeptide
Selenium
Speciation

ABSTRACT

Sec_2-containing oligopeptide was synthesized directly from Sec_2 with the traditional liquid phase peptide synthesis without addressing the usually applied and complex solid phase (SPPS) protocol driving through a protected Sec residue and site-oriented oxidation into a diselenide bridge. Effective solubilization of Sec_2 in dimethylformamide and its pH-controlled access to pentachlorophenol-activated peptides to couple with were of crucial importance to achieve good yield ($>50\%$) of synthesis, monitored by HPLC-UV, SEC–ICP-MS and HPLC–ESI-MS techniques. To demonstrate the possible application of the new compound, (Boc-GGFG)–Sec_2–(Boc-GGFG) (m/z 1173.3, $[M+H]^+$), it was utilized to compare the effect of the two most addressed sample preparation techniques, i.e., methanesulphonic acid (MSA) based digestion and proteolytic digestion with protease XIV, on the Sec residue. The study revealed that the use of MSA resulted in the decomposition of Sec even after derivatization with iodoacetamide.

1. Introduction

Quantitative determination of selenocysteine (Sec or U) content from biological samples (including tissues and different kinds of food) has become a target in the field of selenium speciation [1–3]. Basically, the driving forces of this analysis originate in three facts. First, selenocysteine is the active residue of selenoenzymes [4] that have become the quantitative targets of bioanalytical studies [5–7]. Second, selenocysteine content of selenium-enriched food materials is considered to be an indication of biologically active incorporation of Se instead of inorganic Se accumulation and partly instead of selenomethionine (SeMet) enrichment [8]. Third, Sec-containing selenoglutathiones occurring, e.g., in selenium-enriched yeast, have been identified as important factors in peptide folding and in glutathione-peroxidase (GSHPx) related mechanisms [9].

The quantification of SeMet, the most often analyzed selenoamino acid, can be adequately kept under analytical quality control (QC) due to a relevant CRM SELM-1 (NRC, Canada), interlaboratory comparisons [10], synthetic [11] or isotopically enriched SeMet-peptides [12] and isotopically enriched SeMet standards [13,14]. As reviewed lately [15], quantification of the other natural selenoamino acid, Sec, may suffer from several analytical pitfalls arising from sample preparation steps, low quality HPLC separations, etc. The stability of Sec is a critical point during the whole analysis, because non-derivatized Sec residues may decompose even during the LC–ESI-MS^n analysis [16]. In order to achieve high recovery of Sec from real samples, several techniques have been proposed. These include the derivatization of Sec-containing proteins with N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid [17], with *o*-phthaldialdehyde [18], with iodoacetic acid [19], carbamidomethylation (CAM) with iodoacetamide [15] or a derivatization-free technique through an orthogonal LC-separation method intended for the purification of Sec-containing glutathione conjugates [20].

Ideally, the analytical QC of quantitative Sec analysis should monitor all the sample preparations steps that directly act on Sec, namely reduction, derivatization, proteolytic or acidic protein digestion, clean-up methods and final analytical determination. The need for the reduction step should be underlined as Sec can either be naturally bridged in selenoproteins with another Sec residue [21] or bridged with Cys residues in selenoglutathiones [20,22] and presumably in selenium containing proteins where Sec is non-specifically incorporated [23]. The traditional methods toward this end have used a commercially available selenoenzyme, bovine glutathione peroxidase (GSH-Px, EC 1.11.1.9) [24]. However, as Sec is present in this enzyme in reduced form, the complete analytical procedure including the reduction of Se–S and Se–Se bridges cannot be monitored.

Theoretically, a diselenide-containing synthetic oligopeptide could serve as a matching validation material. The presence of the dimer of Sec can be the result of either the oxidation of Sec-containing peptides into diselenide-bridged compounds or

* Corresponding author. Tel.: +36 14826161; fax: +36 14664272.
E-mail address: mihaly.dernovics@uni-corvinus.hu (M. Dernovics).

0039-9140/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.05.038

the direct incorporation of Sec_2 into peptides. The previous technique requires first the synthesis of selenocysteine peptides. There are doable protocols for this goal for laboratories equipped especially for solid phase peptide synthesis (SPPS) and the native chemical ligation technique as recently reviewed [25]. The second—oxidizing—step to form inter- or intrachain Sec_2 is a less frequently addressed and less straightforward process [26–28]. Therefore, alternative methods such as the direct modification of bridged Cys residues into bridged Sec (through an admittedly difficult) stepwise conversion technique [29], aryl substitution [30], or the substitution of Cys residues of a protein possessing a naturally occurring disulfide bridge with Sec residues during biological protein synthesis [31] have been proposed.

All of these techniques require either sophisticated organic chemistry or molecular biology background with the relevant instrumentation and practical experience. Interestingly, an alternative approach, i.e., the direct incorporation of Sec_2 into peptides with the classical liquid phase (homogeneous) synthesis has not been addressed. In our study, this direct coupling was investigated, aiming at an affordable and straightforward synthesis of a diselenide-containing oligopeptide for method validation purposes in selenium speciation. In order to assure the unambiguous assessment of the resulting structure, orthogonal chromatographic clean-up methods and the use of LC–ICP-MS and HPLC–ESI-MS/MS systems were chosen. The resulting oligopeptide was used as a substrate to compare the effect of the two most utilized sample preparation techniques, i.e., methanesulphonic acid (MSA) based digestion and proteolytic digestion with protease XIV, on the Sec residue.

2. Experimental

2.1. Reagents and standards

For the peptide synthesis, derivatization and hydrolysis, seleno-L-cystine (Sec_2; ≥98.0%), 4-methylmorpholine (NMM; ≥98.0%), N,N′-dicyclohexylcarbodiimide (DCC; ≥99.0%), pentachlorophenol (PCP; 98%), heptafluorobutyric acid (HFBA; ≥99.5%), Tris–HCl (≥99%), iodoacetamide (IAM; ≥99%), dithiothreitol (DTT; ≥99%), methanesulphonic acid (MSA; 99.5%), and protease XIV enzyme (4.8 U mg^{-1}) were obtained from the Sigma-Aldrich group (Schnelldorf, Germany). Hydrochloric acid (≥37%), diethyl ether (99%) and N,N-dimethylformamide (DMF; 99%) were purchased from Molar Chemicals (Budapest, Hungary). The peptide Boc–Gly–Gly–Phe–Gly-OH (referred from now on as "Boc-Pept") was purchased from Bachem (Boc-GGFG-OH; product no. A-4450, M_r: 436.47, $C_{20}H_{28}N_4O_7$; Weil am Rhein, Germany). For chromatographic purposes, methanol (HPLC gradient grade), formic acid (98–100%) and acetonitrile (≥99.0%) were bought from Scharlau (Barcelona, Spain), while trifluoroacetic acid (TFA; ≥99.0%), ethyl acetate (≥99.5%), glacial acetic acid (≥99.0%), pyridine (≥99%), and ammonium acetate (≥98.0%) were obtained from Reanal (Budapest, Hungary). Milli-Q water (18.2 MΩ cm, Millipore, Molsheim, France) was used throughout. For the determination of total selenium, HNO_3 (a.r., ≥65%), and the 1.000 g l^{-1} standards of Se and Rh were obtained from Merck (Darmstadt, Germany).

2.2. Solubilization of Sec_2

30 mg (~90 μmol) of Sec_2 was mixed with 1.0 ml of DMF in a V-bottomed borosilicate glass test tube equipped with a glass stopper and it was solubilized with the addition of the stoichiometric amount (70 μl of 6.0 M) of hydrochloric acid. The resulting clear yellow solution was placed at −18 °C overnight to freeze out water that forms ice on the bottom. Afterwards, the $Sec_2 \times 2$ HCl solution was decanted and used for the peptide synthesis.

2.3. Active ester formation of the Boc-Pept

Activation of the Boc-Pept was carried out with the PCP-DCC protocol as described by Bodánszky and Bodánszky [32]. Briefly, 110 mg of the peptide (~250 μmol) was dissolved in 3.0 ml of ice-cold DMF, and 57 mg of DCC (~275 μmol) and 73 mg of PCP (~275 μmol) were added. The mixture was vortex mixed continuously and kept at 0 °C for 3 h, then at ambient temperature overnight. The efficiency of the active ester formation was monitored on TLC plates covered with Kieselgel-60 (0.25 mm; Merck, Darmstadt, Germany) that were eluted with the 120:20:6:11 v:v:v:v mixture of ethyl acetate: pyridine: glacial acetic acid: water at regular intervals. Because of possible water-based deactivation of the reagents, additional amounts of 230 μmol DCC and PCP (47 mg and 61 mg, respectively) were required to obtain esterification yield that finally reached >95%, based on the determination of peak areas by HPLC-UV at 220 nm. The resulting PCP-activated peptide was cleaned up with repeated processes of filtration through disposable 0.22 μm PTFE syringe filters, vacuum evaporation and dissolution in diethyl ether.

2.4. Liquid phase coupling of PCP-activated peptide and $Sec_2 \times 2$ HCl

The PCP-activated peptide was dissolved in 2.0 ml of ice-cold DMF, and 1.0 ml of $Sec_2 \times 2$ HCl solution in DMF was added. The mixture was vortex mixed, kept at 0 °C and continuously purged with N_2 to prevent oxidation. The coupling was initiated and advanced by the additions of 2 μl NMM every 15 min for 3 h to liberate Sec_2 from the HCl salt. The pH of the solution was checked at each NMM addition in order not to exceed pH=9.0 where decomposition of Sec_2 into red elemental selenium rapidly occurs. The solution was finally kept at ambient temperature overnight with maintained N_2 purging and vortex mixing, then vacuum centrifuged and lyophilized (Scanvac; Scanlaf, Lynge, Denmark). The resulting light yellow powder was kept at −20 °C prior to the SEC-based clean-up process targeting the (Boc-Pept)–Sec_2–(Boc-Pept) molecule.

2.5. CAM-derivatization of (Boc-Pept)–Sec_2–(Boc-Pept)

All reagents were dissolved in Tris–HCl buffer (0.1 M, pH=7.5). 1.2 mg (Boc-Pept)–Sec_2–(Boc-Pept) was dissolved in 0.5 ml buffer in an Eppendorf tube, then 130 μL of 0.2 M DTT was added. After 30 min, 250 μL of 0.2 M IAM was added and the solution was kept in dark for 2 h. Finally, 240 μL of 0.2 M DTT was added and kept in dark for 1 h. To purify the derivatized peptide, Boc-Pept-CAM-Sec, with analytical scale SEC, the volume of the solution was increased to 1.5 ml with the Tris–HCl buffer.

2.6. Enzymatic and acidic hydrolysis of the Boc-Pept-CAM-Sec peptide

0.5 mg of the Boc-Pept-CAM-Sec peptide was dissolved in 2.5 ml Tris–HCl buffer (0.1 M, pH=7.5) and 5 mg of protease XIV was added. The solution was vortexed and kept at 37 °C for 24 h. Afterwards, the solution was filtered through a disposable 0.22 μm PTFE syringe filter and used for the ion-pairing HPLC–ICP-MS analyses after 1:10 dilution with the appropriate starting HPLC eluent.

For the acidic hydrolysis, 0.5 mg of the Boc-Pept-CAM-Sec peptide was dissolved in 5.0 ml of 4 M MSA and boiled under reflux according to the procedure of McSheehy et al. [11]. Following the completion of the reaction the solution was diluted to 50 ml with deionized water and further 1:10 diluted with the appropriate starting HPLC eluent before the ion-pairing HPLC–ICP-MS analysis.

2.7. HPLC-UV system

A WellChrom K-1001 HPLC pump (Knauer; Berlin, Germany) was used in gradient mode with UV detection at 220 nm. The column was a Synergi MAX-RP C_{12} (250 mm × 4.6 mm × 4 μm; Phenomenex, Torrance, CA, USA) eluted with solution A (water with 0.1% v/v TFA) and solution B (80:19.9:0.1% v/v acetonitrile: water: TFA). A 60-min linear gradient starting from 5% B to 95% B was applied. Injection volume was 20 μl.

2.8. SEC–ICP-MS and HPLC–ICP-MS systems

20 mg of the sample was dissolved in 0.5 ml of water-acetonitrile solution (80:20% v/v), then filtered through a low dead-volume disposable 0.22 μm PVDF syringe filter (Ø 4 mm, Isodisc; Sigma-Aldrich). The preparative scale SEC purification procedure of (Boc-Pept)–Sec_2–(Boc-Pept) was based on the use of a HiLoad 16/60 Superdex 30 pg column (GE Healthcare-Amersham Biosciences, Uppsala, Sweden). The column was eluted with 0.1 M ammonium acetate in a water-acetonitrile solution (80:20% v/v) at the flow rate of 0.86 ml min^{-1}. Fractions were collected at 1.5 min intervals. To set-up a selenium elution profile, 50 μl of each fraction was analyzed with an Agilent 7500ce type ICP-MS instrument (Agilent; Santa Clara, CA, USA) operated in normal (without addressing the collision cell) mode on the isotopes of ^{77}Se and ^{82}Se. The SEC fractions selected for further analyses were vacuum-centrifuged, frozen and lyophilized. For the determination of column recovery, a separate batch of 20 mg of the sample was dissolved into solution and filtered, and then digested as described in Section 2.10, along with the aliquots of selected fractions of the SEC run.

The analytical scale SEC–ICP-MS set-up for the purification of the Boc-Pept-CAM-Sec peptide included a Superdex Peptide 10/300 GL column (GE Healthcare), eluted with 0.1 M ammonium acetate (pH = 7.5) for 45 min at the flow rate of 0.8 ml min^{-1}. 30 independent runs were carried out with the individual injections of 50 μl. The selected SEC fraction from each run was pooled and lyophilized.

The HPLC–ICP-MS analysis of the Boc-Pept-CAM-Sec peptide and the purification of its hydrolytic end-products were carried out on a Waters XTerra C_{18} reversed phase column (250 mm × 4.6 mm × 5 μm; Waters, Milford, MA, USA) used in ion-pairing mode. For the chromatographic gradient, solution A was water with 0.05% v/v HFBA, and solution B was methanol with 0.05% v/v HFBA. The flow rate was 0.7 ml min^{-1}. Gradient elution was set as follows: 0–10 min 4% B; 10–12.5 min up to 10% B; 12.5–20 min 10% B, 20–21 min down to 4% B.

2.9. HPLC–ESI-MS and flow injection-ESI-MS analyses

The HPLC–ESI-MS couplings were carried out in all cases by using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). The samples were dissolved in water with 0.1% v/v formic acid and 10 μl was injected on a Zorbax Eclipse XDB-C_8 reversed phase column (150 mm × 4.6 mm × 5 μm; Agilent). For the chromatographic gradient, solution A was water with 0.1% v/v formic acid, and solution B was acetonitrile (without formic acid). Gradient elution was set as follows: 0–5 min 10% B; 5–25 min up to 90% B; 25–30 min 90% B.

For the analysis of (Boc-Pept)–Sec_2–(Boc-Pept), a QTRAP 3200 triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems/Sciex; Foster City, CA, USA) was used either in the Enhanced Q3 Single MS (EMS) mode for the full-scan experiments with an integration time of 1 s or in Enhanced Product Ion (EPI) mode for MS/MS analyses. The related instrumental parameters are described in the supplementary material (SM) SM-Table 4.

For the analysis of the Boc-Pept-CAM-Sec peptide and its hydrolytic end-products, a 6530 Accurate Mass QTOF LC/MS system (Agilent) was applied. The QTOF-MS instrument was operated with an Agilent 6220 instrument derived dual ESI ion source in positive ionization mode. The tuning parameters and other settings are described in the supplementary material (SM-Table 4).

2.10. Microwave digestion

For the determination of total selenium and column recovery, the digestion of samples was carried out on a CEM Mars-5 microwave digestion system (CEM; Matthews, NC, USA). A sample (or aliquots to analyze) was mixed with 5.0 ml of conc. HNO_3 in PTFE digestion tubes. The pressure was raised to 250 psi over 20 min and held for 20 min. The total Se concentration was determined with the Agilent 7500ce ICP-MS on the ^{77}Se and ^{82}Se isotopes by the method of standard addition using Rh as an internal standard.

3. Results and discussion

3.1. Oligopeptide formation with Sec_2

The usual peptide coupling with Sec_2 without reduction is hampered by the inadequate solubility of this dipeptide in general organic solvents. Esterification with methanol and HCl provides a soluble form of Sec_2; however, the removal of methyl groups with alkalic treatments results in the decomposition into black elemental selenium (results not presented).

Interestingly, providing the HCl salt of Sec_2 renders this dipeptide good solubility in DMF, which is not common among usual amino acids. On the other hand, when liberating Sec_2 from its HCl salt, the sensitivity to alkalic pH must be taken into consideration: one-step liberation of all the Sec_2 resulted in complete decomposition. Therefore, special care was taken to make Sec_2 accessible for coupling with the active ester in discrete steps with the limited stepwise addition of NMM.

In order to facilitate the complete procedure, a commercially available Boc-protected peptide without sidechain acidic residues, Boc-GGFG-OH, was chosen to limit the number of possible reaction products. As shown in the supplementary material

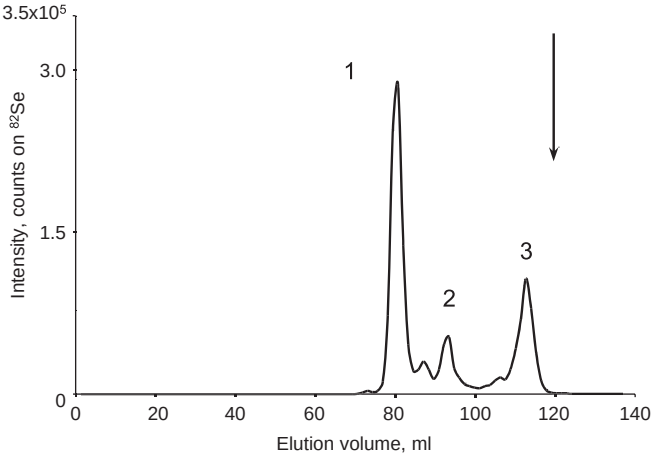

Fig. 1. SEC-ICP MS elution profile (recorded on the ^{82}Se isotope) of the reaction products obtained in the peptide coupling process. The arrow indicates the total volume (120 ml) of the SEC column. Peaks assigned with numbers 1–3 were collected for further characterization.

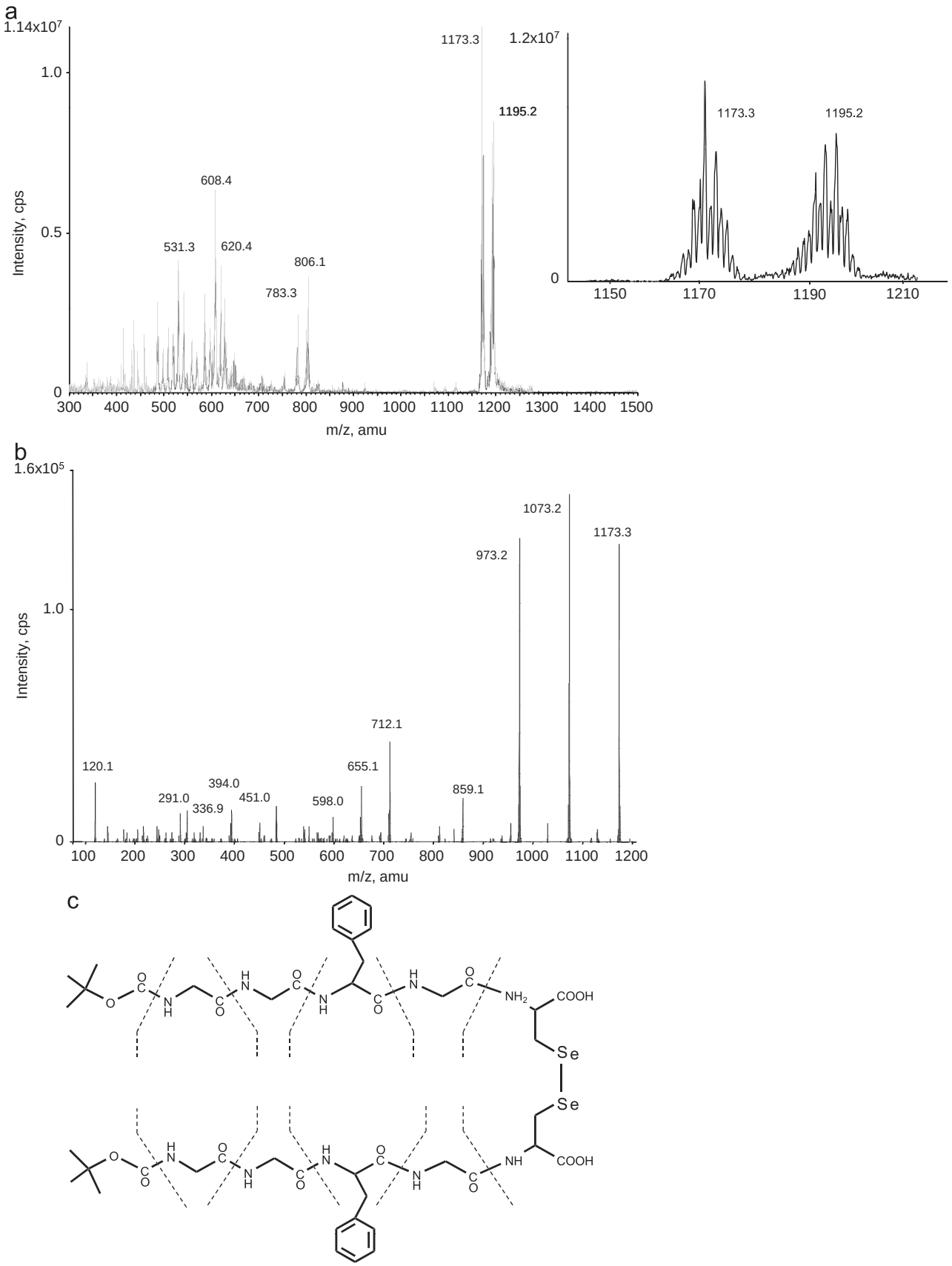

Fig. 2. (a) Flow-injection ESI-MS analysis the first eluting SEC fraction (cf. no. 1 in Fig. 1). The inset shows the characteristic doubly selenized isotope pattern of the Sec_2-containing oligopeptide and its Na-adduct. (b) ESI-MS/MS collision induced dissociation (CID)—fragmentation spectrum of the *m*/*z* 1173.3 (monoisotopic) ion. (c) Fragmentation pathways of the Sec_2-containing oligopeptide. For details, see SM-Table 1. Note that due to the symmetry of the molecule and the repeated sequence of G residues several fragmentation possibilities can result in the same fragment masses.

(SM), SM-Fig. 1, its active ester formation could be synthesized in a straightforward manner.

3.2. SEC–ICP-MS purification and ESI-MS based characterization of the reaction products

Fig. 1 presents the selenium elution profile of the compounds arising from the peptide coupling reaction from the SEC column by ICP-MS monitoring. Three main peaks were detected, and all of them were collected for total selenium determination and ESI-MS characterization.

The flow injection ESI-MS full scan spectrum of the most abundant peak (No. 1 in Fig. 1) eluting first in the SEC run is shown in Fig. 2a. The spectrum shows both the protonated and sodium adducts with the characteristic isotopic pattern of molecules having two selenium atoms. Considering the monoisotopic mass of the expected Sec_2–oligopeptide with the complete coupling of two Boc-Pept molecules, m/z 1173.29 ($[M+H]^+$; $C_{46}H_{65}N_{10}O_{16}Se_2^+$), the two intensive ions can be identified as the pseudomolecular ion (m/z 1173.3) and its sodium adduct (m/z 1195.2; see the inset). The MS/MS analysis of the monoisotopic ion is presented in Fig. 2b, along with the fragment information in Fig. 2c and in SM—Table 1. However the compound follows the general peptide fragmentation pathways, it is noteworthy that the energy provided by the collision cell (Q2) was not high enough to dissociate the diselenide bond to provide monoselenized fragments. According to Bean and Carr [33], this phenomenon shown in positive mode coincides with the fragmentation behavior of protonated peptide ions with disulfide bridges.

The flow injection ESI-MS full scan spectrum of the peak No. 2 eluting after the previous one (cf. Fig. 1) is shown in SM—Fig. 2a. The spectrum contains a doubly selenized molecule at m/z 755.11 ($[M+H]^+$; $C_{26}H_{39}N_6O_{10}Se_2^+$), referring to the oligopeptide formed by the coupling of one Boc-Pept and a Sec_2 molecule. Therefore, the fragments obtained in the MS/MS experiment (see SM—Fig. 2b, c and SM—Table 2) have been partly identified at the previous, doubly coupled oligopeptide (cf. Fig. 2b and c).

The last abundant peak of the SEC run eluted close to the total volume of the column, indicating a low molecular weight (between 0.4 and 0.1 kDa) species. Indeed, as reinforced by the flow injection ESI-MS acquisition shown in the supplementary material SM—Fig. 3, non-reacted Sec_2 was present in this fraction.

Aliquots of the three analyzed fractions were digested in order to determine their total selenium concentrations. Compared to

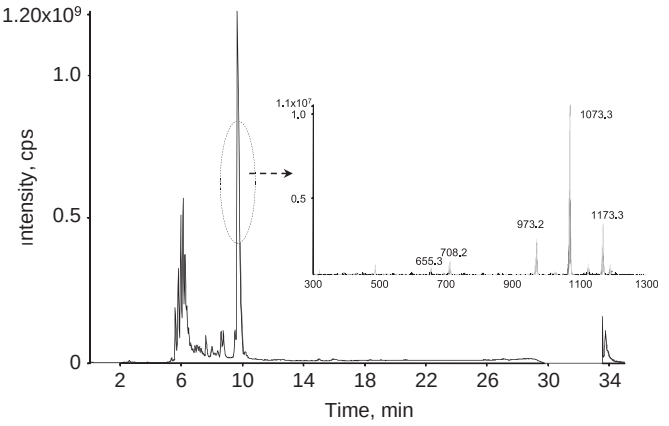

Fig. 3. TIC (total ion chromatogram) obtained with the HPLC–ESI-MS analysis of the doubly coupled Sec_2–oligopeptide (m/z 1173). The inset shows the full scan spectrum recorded at the apex of the most intense peak, t_R=9.7 min.

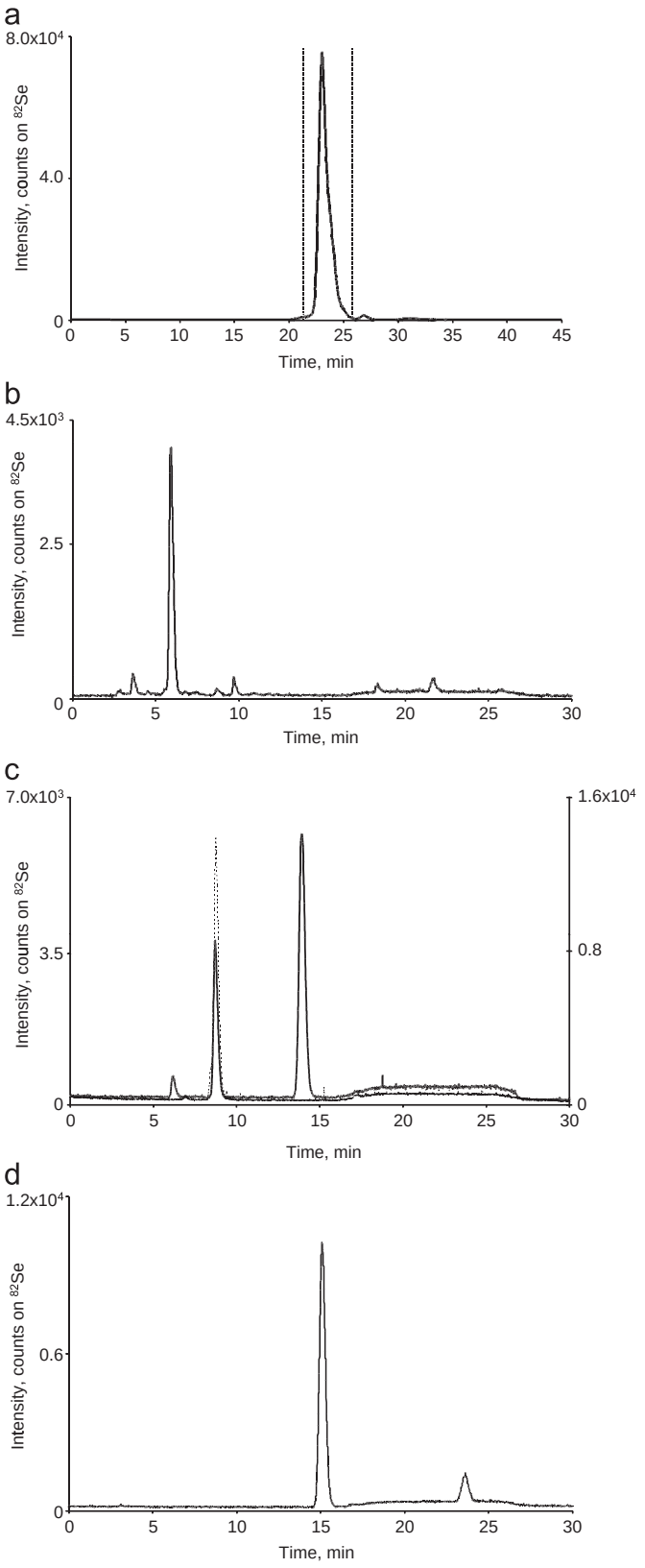

Fig. 4. (a) SEC–ICP-MS chromatogram (recorded on the ^{82}Se isotope) of the reaction products obtained after the derivatisation of (Boc-Pept)–Sec_2–(Boc-Pept). The fraction in-between the lines was collected for further analysis. (b) IP-RP C_{18} HPLC–ICP-MS chromatogram of the SEC-purified derivatization product, the Boc-Pept-CAM-Sec. (c) Effect of enzymatic hydrolysis/protease XIV/ on the Boc-Pept-CAM-Sec peptide, monitored with HPLC–ICP-MS. The dotted line indicates the elution of the CAM-Sec standard, scaled to the second y-axis. (d) Effect of acidic hydrolysis/MSA/ on the Boc-Pept-CAM-Sec peptide, monitored with HPLC–ICP-MS.

the injected amount of Se, the three fractions accounted for 93% of selenium, that is, taking into account the non-analyzed less abundant peaks, column recovery exceeded 95%. The ratio of total selenium in the fractions was 57:11:32, which denotes to a ca. 5:1 ratio of the completely (doubly) and mono-coupled Sec_2 oligopeptides, and an overall yield of ca. 53% (counting only the doubly coupled species).

Neither the SEC profile, nor the elution of the genuine Boc-Pept on an RP HPLC column provides reliable information on the retention characteristics of the final reaction product under usual RP conditions. Therefore, an aliquot of the SEC fraction containing the doubly coupled Sec_2–oligopeptide (m/z 1173.3) was dried in a vacuum centrifuge, dissolved in water with 0.1% v/v formic acid and injected on a C_8 column for the HPLC–ESI-MS analysis. Fig. 3 presents the related TIC (Total Ion Chromatogram), where the dominating peak contained the Sec_2–oligopeptide and its in-source fragments (see the inset). This elution indicates that this final product can be fit into the analysis of selenopeptides due to its adequate retention on RP columns [34,35]. On the other hand, as it is soluble in water with 0.1% v/v formic acid, other

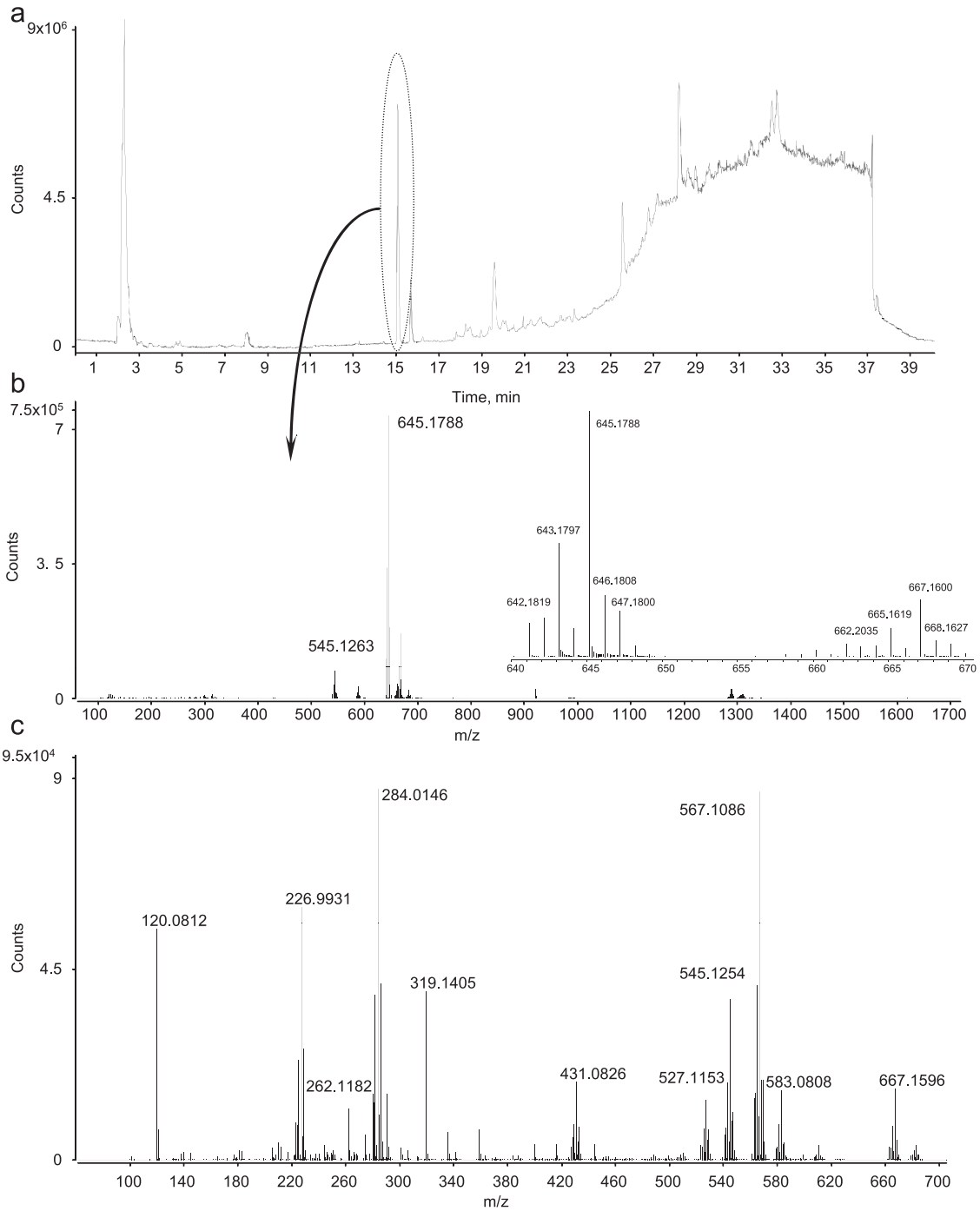

Fig. 5. (a) TIC (total ion chromatogram) obtained with the RP C_8 HPLC–ESI-QTOF-MS analysis of the Boc-Pept-CAM-Sec peptide. (b) Full scan spectrum of the chromatographic peak eluting at t_R=15.05. The inset shows the Boc-Pept-CAM-Sec peptide and its Na^+ adduct. (c) Targeted ESI-QTOF-MS/MS spectrum of the peptide with the molecular mass of m/z 645.1788. Note the selenium isotopic profile of the selenium containing fragments due to the relatively wide mass selection window. For the details on mass accuracy, elemental composition, fragment and structure information, see SM—Table 3 and SM—Fig. 5.

chromatographic systems generally not applying organic solvents (i.e., SEC) can be also addressed to exploit this selenized oligopeptide.

3.3. Application of the oligopeptide for the evaluation of sample preparation techniques

In order to determine whether the synthesized oligopeptide can be used to follow the general sample preparation methodology of selenium speciation targeting the quantification of Sec, the oligopeptide was first reduced with DTT and derivatized with IAM [13]. The resulting derivatized peptide was purified with SEC chromatography (see Fig. 4a) and its purity was checked with HPLC–ICP-MS and HPLC–ESI-QTOF-MS set-ups. As presented in Fig. 4b, the fraction collected from SEC contained one abundant compound on the HPLC–ICP-MS chromatogram (t_R=6.1 min) and some less abundant by-products detected by the ICP-MS. According to the QTOF-MS and MS/MS results Fig. 5a–c, SM—Table 3 and SM—Fig. 5, the most abundant selenium-containing compound was the Boc-Pept-CAM-Sec peptide/$C_{25}H_{37}N_6O_9Se^+$, [M+H$^+$]=645.1788, Δ=0.93 ppm/. The less abundant by-products included the Boc-free peptide/$C_{20}H_{29}N_6O_7Se^+$, [M+H$^+$]=545.1253, Δ=−0.73 ppm/ and other artifact compounds arising from the sample preparation such as the *o*-methylated form/$C_{26}H_{39}N_6O_9Se^+$, [M+H$^+$]=659.1929, Δ=−1.37 ppm/ (see SM—Fig. 4).

The derivatized and purified peptide was subjected to two separate analyses. First, enzymatic digestion with protease XIV was carried out, which is the most widespread technique suitable for SeMet and Sec quantification [36]. The HPLC–ICP-MS chromatogram of the hydrolytic products (see Fig. 4c) indicated incomplete digestion: however the derivatized peptide eluting at t_R=6.1 min was almost completely digested, there were two abundant peaks, with the first one co-eluting with the standard of the CAM-Sec residue (t_R=8.7 min), prepared according to the study of Dernovics and Lobinski [15]. The two abundant compounds were purified from the ion-pairing chromatographic set-up and were analyzed with the HPLC–ESI-QTOFMS set-up for the unambiguous identification. As shown in Fig. 4c and Fig. 6 a and b, the first purified compound matches not only the ion-pairing retention time of the CAM-Sec standard but its mass and isotopic profile also fit $C_5H_{11}N_2O_3Se^+$, [M+H$^+$]=226.9939, Δ=4.40 ppm/.

The incomplete digestion can be a result of the relatively short peptide sequence (only five amino acid residues), which also occurred at the enzymatic digestion of the tripeptide selenoglutathione [15]. On the other hand, this has been the first artificial peptide that was engineered to be a substrate for Sec quantification. The identification of the other abundant peak eluting at t_R=13.9 min was not successful (see Fig. 4c).

The second digestion was carried out with MSA, the acidic reagent widely used for SeMet quantification [10]. Fig. 4d shows that MSA digestion resulted in a complete hydrolysis of the peptide without arriving at a detectable amount of CAM-Sec. Indeed, only one abundant compound was detected at t_R=15.1 min, mismatching the retention time of the CAM-Sec standard. However this compound was purified from the ion-pairing HPLC system, its identification by the QTOFMS was unsuccessful.

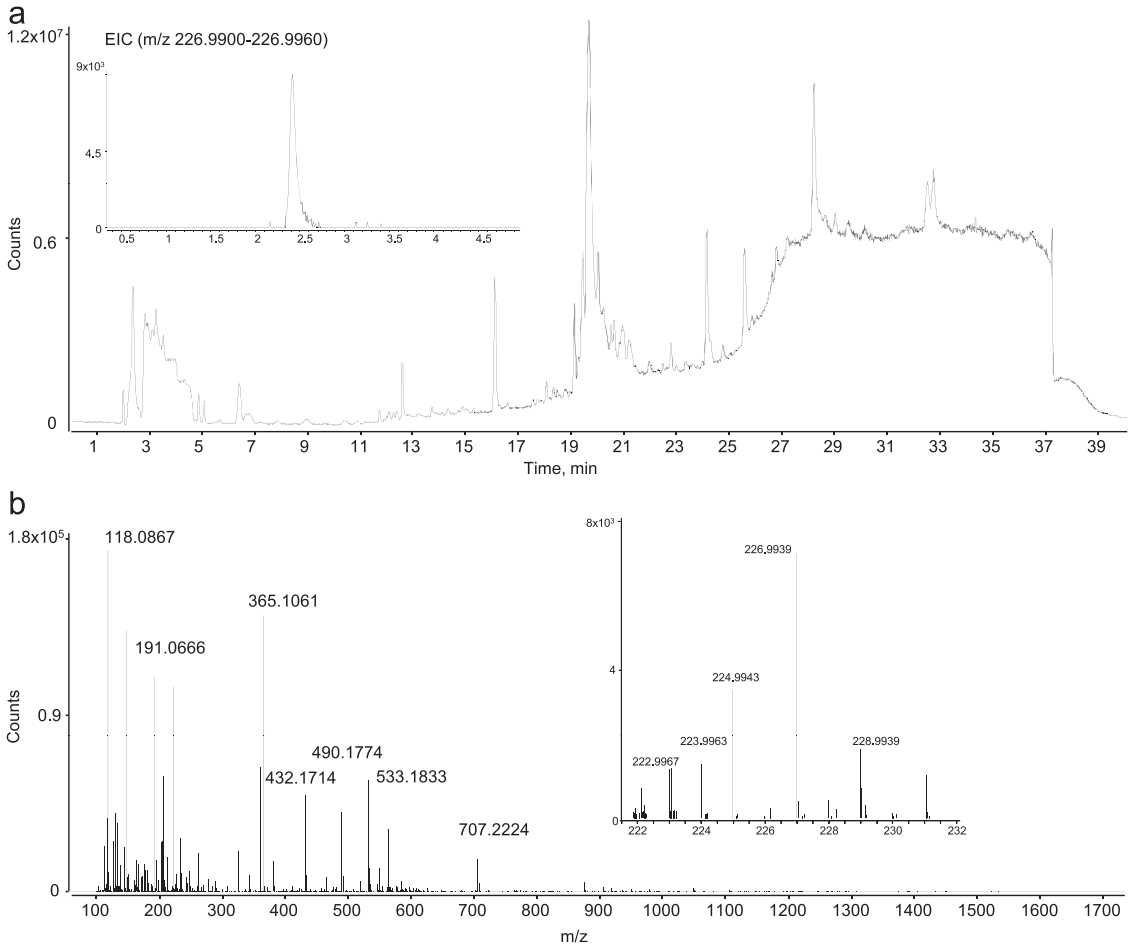

Fig. 6. (a) TIC (total ion chromatogram) obtained with the HPLC–ESI-QTOF-MS analysis of the peak supposed to contain CAM-Sec and fractionated from the HPLC–ICP-MS analysis (see Fig. 4c) after enzymatic hydrolysis. The inset shows the extracted ion chromatogram (EIC) for the mass range *m*/*z* 226.9900–229.9960. (b) Full scan spectrum of the chromatogram at the elution time t_R=2.42 min. The inset shows the detected CAM-Sec with its selenium-containing isotopic profile.

The use of MSA has recently been reported to coincide with the alteration of the genuine enantiomeric distribution of SeMet residues [37]. The actual study also questions its applicability for Sec analysis because of the total decomposition of this analyte.

4. Conclusion

The coupling of Sec_2 via the traditional liquid phase peptide synthesis instead of applying the demanding protocol of reduction of Sec_2-derivatization (i.e., protection of selenol groups)-oxidation to re-form Sec_2 offers a straightforward method to arrive at a selenocystine-peptide. The present strategy is suitable for the synthesis of non-internal Sec_2-containing oligopeptides of symmetrical construction (X–Sec–Sec–X, where X=peptide) with all types of amino acids at the C-terminal of the peptides. The developed technique was successfully used for the synthesis of an oligopeptide that can be considered a conjugate of two Sec-containing peptides, thus forming an interchain Se–Se bridge. The oligopeptide could be used to follow the usual method of sample preparation aiming at selenocysteine quantification including reduction, CAM derivatization and hydrolysis. The comparison of enzymatic and acidic digestion schemes revealed that the MSA based hydrolytic approach decomposes the derivatized Sec residue and it is therefore not recommended for selenium speciation studies targeting this analyte.

Acknowledgment

Mihaly Dernovics acknowledges the Marie Curie Grant no. MERG-CT-2006-044951. The authors thank Zsanett Miklán (ELU) for help with the HPLC-UV experiments and also acknowledge the financial help of the TÁMOP 4.2.1./B-09/1/KMR-2010-0005 grant for the HPLC–ESI-QTOF-MS experiments.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.038.

References

[1] Z. Pedrero, Y. Madrid, Anal. Chim. Acta 634 (2009) 135.
[2] H. Maeda, K. Katayama, H. Matsuno, T. Uno, Angew. Chem. Int. Ed. 45 (2006) 1810.
[3] E. Lipiec, G. Siara, K. Bierla, L. Ouerdane, J. Szpunar, Anal. Bioanal. Chem. 397 (2010) 731.
[4] T.C. Stadtman, PLoS Biol. 3 (2005) 2077.
[5] M. Roman, M.L. Fernández Sánchez, A. Sanz-Medel, H. González Iglesias, P. Cescon, C. Barbante, J. Anal. At. Spectrom. 26 (2011) 100.
[6] G. Ballihaut, L.E. Kilpatrick, W.C. Davis, Anal. Chem. 83 (2011) 8667.
[7] J. Duan, B. Hu, Talanta 79 (2009) 734.
[8] M.P. Rayman, H.G. Infante, M. Sargent, Br. J. Nutr. 100 (2008) 238.
[9] S. Yoshida, F. Kumakura, I. Komatsu, K. Arai, Y. Onuma, H. Hojo, B.G. Singh, K.I. Priyadarsini, M. Iwaoka, Angew. Chem. Int. Ed. 50 (2010) 2125.
[10] H. Goenaga-Infante, R. Sturgeon, J. Turner, R. Hearn, M. Sargent, P. Maxwell, L. Yang, A. Barzev, Z. Pedrero, C. Cámara, V. Díaz Huerta, M. Fernández Sánchez, A. Sanz-Medel, K. Emese, P. Fodor, W. Wolf, R. Goldschmidt, V. Vacchina, J. Szpunar, L. Valiente, R. Huertas, G. Labarraque, C. Davis, R. Zeisler, G. Turk, E. Rizzio, L. Mackay, R. Myors, D. Saxby, S. Askew, W. Chao, W. Jun, Anal. Bioanal. Chem. 390 (2008) 629.
[11] S. McSheehy, L. Yang, Z. Mester, Microchim. Acta 155 (2006) 373.
[12] A. Polatajko, J.R. Encinar, D. Schaumloffel, J. Szpunar, Chem. Anal. 50 (2005) 265.
[13] K. Bierla, M. Dernovics, V. Vacchina, J. Szpunar, G. Bertin, R. Lobinski, Anal. Bioanal. Chem. 390 (2008) 1789.
[14] Z. Pedrero, J.R. Encinar, Y. Madrid, C. Camara, J. Chromatogr. A 1139 (2007) 247.
[15] M. Dernovics, R. Lobinski, J. Anal. At. Spectrom. 23 (2008) 744.
[16] S. Ma, R.M. Caprioli, K.E. Hill, R.F. Burk, J. Am. Soc. Mass Spectrom. 14 (2003) 593.
[17] W.C. Hawkes, M.A. Kutnink, J. Chromatogr. B 576 (1992) 263.
[18] C. Hammel, A. Kyriakopoulos, U. Rosick, D. Behne, Analyst 122 (1997) 1359.
[19] C.C. Chery, E. Dumont, R. Cornelis, L. Moens, Fresenius J. Anal. Chem. 371 (2001) 775.
[20] M. Dernovics, R. Lobinski, J. Anal. At. Spectrom. 23 (2008) 72.
[21] V.A. Shchedrina, S.V. Novoselov, M.Y. Malinouski, V.N. Gladyshev, Proc. Natl. Acad. Sci. USA 104 (2007) 13919.
[22] H.G. Infante, G. O'Connor, M. Rayman, R. Hearn, K. Cook, J. Anal. At. Spectrom. 21 (2006) 1256.
[23] K. Bierla, J. Szpunar, R. Lobinski, Anal. Chim. Acta 624 (2008) 195.
[24] K. Bierla, V. Vacchina, J. Szpunar, G. Bertin, R. Lobinski, J. Anal. At. Spectrom. 23 (2008) 508.
[25] M. Muttenthaler, P.F. Alewood, J. Pept. Sci. 14 (2008) 1223.
[26] L. Moroder, J. Pept. Sci. 11 (2005) 187.
[27] S. Pegoraro, S. Fiori, S. Rudolph-Bohner, T.X. Watanabe, L. Moroder, J. Mol. Biol. 284 (1998) 779.
[28] T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, N. Fujii, Chem. Pharm. Bull. 41 (1993) 502.
[29] M. Iwaoka, C. Haraki, R. Ooka, M. Miyamoto, A. Sugiyama, Y. Kohara, N. Isozumi, Tetrahedron Lett. 47 (2006) 3861.
[30] P.P. Phadnis, G. Mugesh, Org. Biomol. Chem. 3 (2005) 2476.
[31] S. Müller, H. Senn, B. Gsell, W. Vetter, C. Baron, A. Böck, Biochemistry 33 (1994) 3404, US.
[32] M. Bodánszky, Á. Bodánszky, The Practice of Peptide Synthesis, Springer-Verlag, Berlin, 1984, pp. 120.
[33] M.F. Bean, S.A. Carr, Anal. Biochem. 201 (1992) 216.
[34] J.R. Encinar, L. Ouerdane, W. Buchmann, J. Tortajada, R. Lobinski, J. Szpunar, Anal. Chem. 75 (2003) 3765.
[35] L. Tastet, D. Schaumloffel, B. Bouyssiere, R. Lobinski, Talanta 75 (2008) 1140.
[36] B. Gammelgaard, M.I. Jackson, C. Gabel-Jensen, Anal. Bioanal. Chem. 399 (2011) 1743.
[37] O. Egressy-Molnár, A. Vass, A. Németh, J.F. García-Reyes, M. Dernovics, Anal. Bioanal. Chem. 401 (2011) 373.