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Influence of reducing agents on the integrity of selenocompounds. Exploratory work for selenoproteome analysis†

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Three selenium species (selenomethionine, selenocystine and selenocystamine) were studied for their behaviour in the presence of the reducing agents dithiothreitol and tributylphosphine, and of a derivatising agent, iodoacetic acid. These chemicals are commonly used in proteome analysis by means of gel electrophoresis. To study the effect of these chemicals on the selenospecies, two separation techniques were used, capillary electrophoresis and liquid chromatography, both with ICP-MS as an element-specific detector. Electrospray-mass spectrometry provided molecular information. Tributylphosphine (TBT) was shown to partly reduce selenocystine to selenocysteine, and iodoacetic acid to derivatise all the species, with conservation of the species information. Dithiothreitol (DTT), on the contrary, partly sequestered selenium from the species to form a DTT–Se molecule.

Introduction

Selenium speciation is more and more entangled with proteomics and can be performed either with gel electrophoresis[1,2] or capillary HPLC.[3] One of the bottlenecks of such an analysis is the stability of the side-chain of the aminoacids. Selenocysteine, for example, is not stable during separation. Protein seleno-amino-acids are related to their sulfur counterparts, methionine and cysteine, and methods developed for classical proteomics could be applied to selenoproteomics. For gel electrophoresis, various protocols have been optimised to prevent oxidation of the sulfur-containing side-chains. The most recent procedures for the reduction of sulfur-bridges are based on the use of DTT[4] or, even more recently, on TBP[5] as the reducing agent. Iodoacetamide or iodoacetic acid can be used to derivatise the sulfur-side-chain and prevent re-oxidation. It has already been demonstrated that iodoacetamide is not reactive enough to fulfil this role for selenium.[1,6] The goal of this work is to determine which chemical among the remaining three best preserves the two selenoamino-acids encountered in proteins, selenomethionine and selenocysteine. Secondly, the separation methods used in the context of this work were evaluated for their relevance for selenium-speciation after gel electrophoresis.

Materials and methods

Ultrapure water (resistivity >18 MΩ cm) was produced with a Milli-Q system (Millipore, Bedford, MA, USA). The standards, selenocystine, selenocystamine and selenomethionine, were from Sigma–Aldrich (Bornem, Belgium) (respectively noted $SeCys_2$, SeCa and SeMet). The three additives, tributylphosphine, dithiothreitol and iodoacetic acid were also from Aldrich and will be denoted TBP, DTT and IAc. See Table S1† for the structures of these various compounds. Tetraethylammonium chloride (TEACl) and methanol, used for HPLC, were respectively from Sigma-Aldrich and Panreac (Barcelona, Spain). Na_2HPO_4 (Agilent, Diegem, Belgium) and trimethyl (tetradecyl)ammonium bromide (Sigma–Aldrich), denoted TTAB in the following, were used for the capillary electrophoresis (CE) measurements. Ammonia and formic acid (Vel, Louvain, Belgium) were used to set the pH of the standards. All these products were of analytical purity at least.

Selenocystine, the oxidised form of selenocysteine, had to be taken as model for the latter since the reduced form is hardly stable outside the active site of some enzymes. Nonetheless, after gel electrophoresis selenocysteine is expected to be present in the protein spots, if they stem from a true selenoenzyme. A chemical able to preserve this form would thus be of high interest. Selenocystamine, although not encountered in proteins, was also investigated since it possesses a selenium-bridge, as does selenocystine. First, incubation of the standards with the chemicals of interest was performed. The derivatisation with iodoacetic acid was performed as previously reported.[1] Second, semi-quantitative analysis of the resulting species was carried out and, finally, the species were tentatively identified by ES-MS(-MS). For the series of experiments in the positive mode, for both ES-MS and CZE, twelve standard solutions were prepared: all cross-combinations between the three selenocompounds and the three derivatives plus blank solutions.

The three $SeCys_2$ solutions contained 30 μM $SeCys_2$, the SeCa series 40 μM SeCa and the SeMet series 51 μM SeMet, all in 4 ml formic acid l^{-1} water. These solutions also contained either no additive, 5 mM TBP, 13 mM DTT or 13 mM DTT plus 65 mM IAc (in the following, this standard will be referred to as IAc, even if DTT was present). The derivatisation with IAc has already been described.[1] These samples were used without further dilution for ES-MS. For HPLC and CZE, these solutions were diluted 20 times with the respective eluent.

For the experiments in negative mode, the same cross-combinations were chosen. The solutions contained either 23 μM $SeCys_2$, 21 μM SeCa or 90 μM SeMet. Sensibly, the same amounts of additives as in the positive mode were added to these solutions. NH_4OH was then added to a concentration of 4 ml per l of water. For CZE, these solutions were diluted 50 times with the electrolyte.

All solutions were kept in the fridge and under an argon atmosphere until analysed.

HPLC-ICP-MS

The method optimised by Dumont *et al.*[7] was applied here. Briefly, it consisted of a separation on a C_{18} column (XTerra

† Electronic supplementary information (ESI) available: selenocompounds and additives, measurement parameters for CZE/ICP-DRC-MS and ES-ICP-MS, and chromatograms from CZE/ICP-DRC-MS in positive mode. See http://www.rsc.org/suppdata/ja/b4/b412754d/

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MS C_{18}) by ion-pairing/reverse phase chromatography, allowing the baseline separation of SeMet and $SeCys_2$. The mobile phase consisted of 0.1 g TEACl l^{-1} and 20 ml methanol l^{-1} with a pH brought to 4.5 with formic acid. The HPLC system was hyphenated to a quadrupole-based ICP-MS instrument (Elan 5000, PerkinElmer Sciex, Woodbridge, ON, Canada).[7]

CE-ICP-DRC-MS

A CE-system (Agilent 3D, Palo Alto, CA, USA) was coupled to an ICP-dynamic reaction cell-MS (Elan DRC plus, PerkinElmer Sciex) with an interface from CETAC (CEI-100 CETAC, Omaha, NE, USA).

Two capillary zone electrophoresis (CZE) methods were applied. First, a method adapted from Michalke *et al.*,[8] in positive mode. Second, a method adapted from Mounicou *et al.*,[9] in negative mode. The main change brought to these methods was the detection method, namely ICP-DRC-MS, as described in Chéry *et al.*[2] for maximum sensitivity. The different parameters are summarised in Table S2†.

ES-MS-MS

All measurements were performed in infusion mode on a Quattro Micro system, a triple quadrupole instrument (Micromass, Manchester, UK). Compromise conditions were chosen so that all species could be detected without adapting the measuring conditions, as summarised in Table S3†.

Results and discussion

Separation techniques

Species stability. Liquid chromatography of the standards with additives showed that the presence of the latter induces major alterations to the pattern of retention times, as can be seen in Fig. 1. The separation pattern of the original standards is as formerly reported,[7] even with the present addition of formic acid. DTT is at the origin of a new species at a retention time of 250 s for all standards (only $SeCys_2$ and SeMet shown). At this stage, it can be hypothesised that DTT sequesters Se from the selenocompounds to form a unique compound. TBP does not disturb the pattern of retention times, but it is noteworthy at this stage that *ca.* half of the original Se is no longer detected, suggesting a loss on the column (perhaps as TBP-SeCys, see below). As expected, IAc quantitatively derivatised the selenocompounds, even SeMet, with the conservation of the speciation information: the three compounds were not, after derivatisation, reduced into one single product, but maintained their specificity. Further, it is already known that IAc reacts with the amino-acids only at the Se site,[10] which is confirmed by the presence of only one peak for each derivatised species.

In order to confirm these findings, CZE was also applied to the separation of these species. First, the species were determined in positive mode, a mode where only positively charged species migrate towards the detector. Although this mode does not offer a baseline separation between SeMet and $SeCys_2$, it helps to confirm the results obtained with HPLC (Fig. S1†). DTT seriously disturbs the pattern of the pure standards and forms one specific new species at a migration time of *ca.* 500 s, as was noticed with HPLC. TBP does not seem to perturb the separation pattern, neither in terms of migration time nor in peak-area. For this reason, it can be concluded that TBP does not sequester any part of the molecules. IAc quantitatively derivatised the selenocompounds, as previously noted. However, the new species formed are not resolved from each other in this mode. Second, the same experiment was performed in negative mode (Fig. 2). The areas under the peaks of the selenocoumpounds in contact with DTT are lower, confirming a loss; the peaks of the species in contact with TBP are much broader, suggesting an interaction, but not necessarily a loss; iodoacetic acid totally changes the separation pattern.

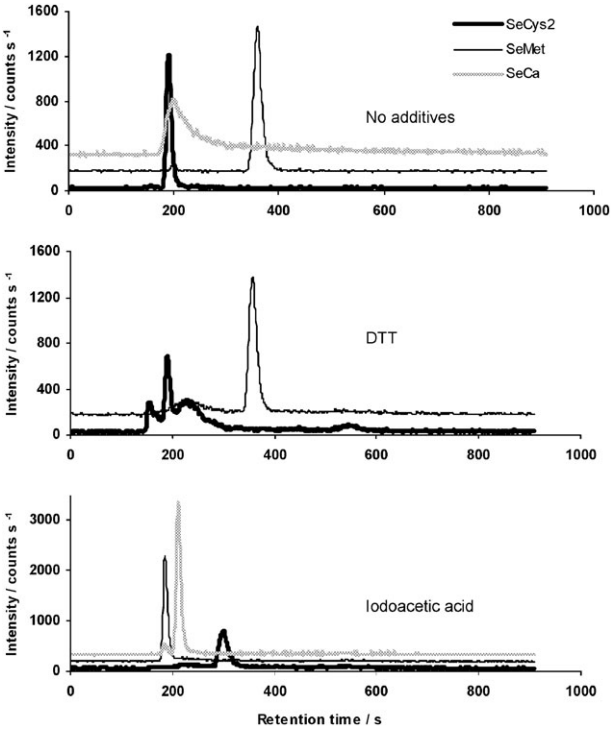

Fig. 1 HPLC-ICP-DRC-MS chromatograms. The concentration of the standard was held constant between the additives experiments. Offsets of 150 and 300 counts s^{-1} were used for, respectively, SeMet and SeCa (for the sake of clarity, the chromatograms or electropherograms that only differ in intensity from the original standards are not shown, like most results with TBP).

Limit of detection. Since the goal of this work is to apply some of these techniques to the identification of the selenoamino-acids after gel electrophoresis, the limit of detection was determined. CZE in negative mode was selected since it offered a baseline separation between SeMet and $SeCys_2$.

The limit of detection for both species (SeMet, $SeCys_2$) was 1 μg Se l^{-1} (3 standard deviations on the background of the blank, with a background 3 times as long as the full width at half maximum of the peak). This limit of detection is at least 15 times lower than previously reported with a similar coupling.[9] This improvement partially stems from the ability to measure the most abundant Se isotope, ^{80}Se, free of interference.

Confirmation by ES-MS(-MS)

The measurements, all with infusion, were optimised for the detection of the parent species, namely $SeCys_2$, SeMet or SeCa. With these parameters, the spectra obtained for the solutions without additive, with additives and blanks were compared with each other. The selection of the species of interest was based on two criteria: the presence of Se and a significant difference from the blank and the standard without additives. This selection was made easier by the isotopic pattern of selenium. The tentative identification of the species present after the addition of the reducing agents was based on mass and isotopic pattern.

First, it should be recognised that TBP partly reduces $SeCys_2$ to selenocysteine (SeCys), as detected in positive mode. Besides the reduced SeCys, an association between TBP and SeCys is readily detectable at mass 370 (see Fig. 3; note that the isotopic pattern perfectly corresponds to the proposed identity of the molecule if the background given by TBP is subtracted). This mass corresponds to $C_3H_7NO_2Se$, *i.e.*, SeCys, minus one hydrogen atom, plus TBP. This reduction is confirmed by the fact that SeCa is also partly reduced to C_2H_6NSe with TBP, at mass 326 (C_2H_6NSe + TBP, see Fig. 3). In order to confirm this association between TBP and reduced SeCa or SeCys, these molecules were further analysed with MS^2. The two main

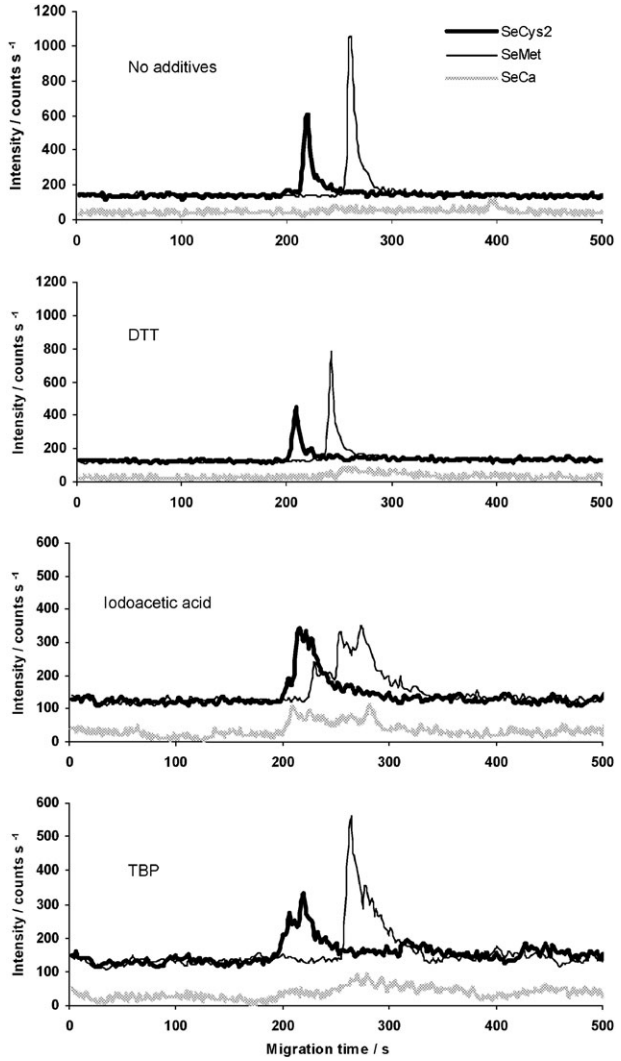

Fig. 2 CZE in negative mode. The concentrations of the standards are constant among the experiments. An offset of 100 counts s^{-1} was used for both SeMet and $SeCys_2$.

peaks obtained in the MS mode, from the molecules containing ^{76}Se and ^{80}Se, were selected, for SeCys at mass 368 and 370, respectively, and for SeCa at mass 324 and 326. At a collision energy of 40 eV, the molecules stemming from both SeCys and SeCa yielded fragments at m/z of 280 (from 368 and 324) and 282 (from 370 and 326). Since the mass difference is the same as between the parent ions, these molecules still contain Se. Their

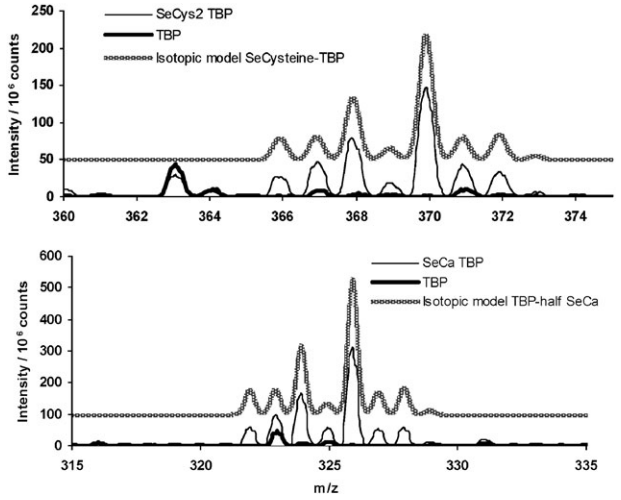

Fig. 3 Mass spectrum of SeCys and SeCa standards in presence of TBP.

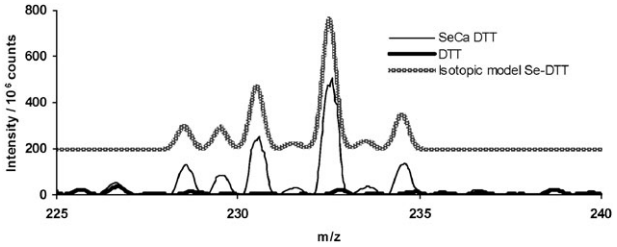

Fig. 4 Mass spectrum of SeCa (or $SeCys_2$) standard in presence of DTT.

masses correspond to TBP-Se. It is noteworthy that this compound was not originally present in the original selenocompound–TBP solution. This means that TBP does not split the compounds to form TBP–Se but, partly, forms a TBP–SeCys compound, preserving the species. Such an association was not detected for SeMet and TBP. In negative mode, although $SeCys_2$ could be detected, no TBP–SeCys species was to be seen, which should also be above its pI and thus negative. This shows that the negative charge of SeCys is bridged to TBP, perhaps through a covalent bonding.

DTT can sequester selenium to form DTT–Se, detected at mass 233 (=154 from (DTT–2H) + 80 from $Se + H^+$, probably a cyclic molecule) in positive mode from SeCa and $SeCys_2$ (see Fig. 4). In negative mode, the DTT–Se molecule could not be detected. This difference in pattern is probably due to the difficult ionisation in negative mode of a possibly cyclic DTT–Se molecule. A further interesting point is that a weak signal for reduced SeCys is also present in the solution with DTT, probably due to the increase in reducing power of DTT in alkaline solution.

Since DTT seems to degrade the original amino-acids into DTT-Se, with consequent loss of the speciation information, this reducing agent should be used cautiously. TBP can convert $SeCys_2$ into selenocysteine or TBP–selenocysteine, conserving the information.

The identification of the selenoamino-acids present in one single protein will now be the goal of this work, achieved by the combination of the high separation capacity of two-dimensional gel electrophoresis (2DE) and the low absolute LOD of CE/ICP-DRC-MS, allowing the detection of the species in the small amount of material obtained after 2DE. Indeed, if one assumes that, in the worst case, a protein contains only one selenoamino-acid, *ca.* 10 pg Se is obtained per spot revealed by silver-staining, which, after proteolysis, is reconstituted in approximately 1 μl, resulting in 10 μg Se l^{-1}, a concentration above the detection limits mentioned in this work. The next stage of this work will thus be to apply the most promising reducing agent, TBP, to gel electrophoresis and identify the amino-acids present (SeCys or SeMet) in the various Se-containing proteins.

References

1 C. C. Chéry, E. Dumont, R. Cornelis and L. Moens, *Fresenius' J. Anal. Chem.*, 2001, **371**, 775.
2 C. C. Chéry, D. Günther, R. Cornelis, F. Vanhaecke and L. Moens, *Electrophoresis*, 2003, **24**, 3305.
3 D. Schaumlöffel, J. R. Encinar and R. Lobinski, *Anal. Chem.*, 2003, **75**, 6837.
4 A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber and W. Weiss, *Electrophoresis*, 2000, **21**, 1037.
5 B. Herbert, M. Galvani, M. Hamdan, E. Olivieri, J. MacCarthy, S. Pedersen and P. G. Righetti, *Electrophoresis*, 2001, **22**, 2057.
6 H. E. Ganther, in *Advances in Nutritional Research*, ed. H. H. Draper, Plenum Press, New York, 1979, p. 107.
7 E. Dumont, F. Vanhaecke and R. Cornelis, *Anal. Bioanal. Chem.*, 2004, **379**, 504.
8 B. Michalke, O. Schramel and A. Kettrup, *Fresenius' J. Anal. Chem.*, 1999, **363**, 456.
9 S. Mounicou, S. McSheehy, J. Szpunar, M. Potin-Gautier and R. Lobinski, *J. Anal. At. Spectrom.*, 2002, **17**, 15.
10 T. C. Stadtman, *Method Enzymol.*, 1974, **107**, 576.