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The Analyst

Identification of Selenocysteine and Selenomethionine in Protein Hydrolysates by High-performance Liquid Chromatography of Their *o*-Phthaldialdehyde Derivatives

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A method for the identification of selenocysteine and selenomethionine in protein hydrolysates was developed. The proteins were subjected to acid hydrolysis after they had been carboxymethylated to prevent decomposition of selenocysteine during this process. After precolumn derivatization of the amino acids with *o*-phthaldialdehyde, the hydrolysate was chromatographed on C_{18} columns. The selenoamino acids were detected either by the fluorescence of their *o*-phthaldialdehyde derivatives (detection limit 30 pmol for selenomethionine and 170 pmol for selenocysteine) or by selenium determination in the eluate using atomic absorption spectrometry (detection limit 0.3 pmol) or, with ^{75}Se-labelled compounds, the measurement of the tracer activity. With the latter procedure the detection limit, which depends on the specific activity of the Se tracer, could be decreased to the femtomole range. The method was successfully applied to the identification of selenocysteine in several newly found mammalian selenium-containing proteins.

Keywords: *Selenocysteine; selenomethionine; selenoproteins; high-performance liquid chromatography; o-phthaldialdehyde; amino acid analysis*

Selenium is present in the biosphere in chemical forms analogous to those of sulfur, with selenocysteine (SeC) and selenomethionine (SeM) being the predominant forms in Se-containing proteins. With regard to the essential effects of the element, great differences have been found between these two amino acids. The insertion of SeC into proteins is genetically encoded and accomplished by a cotranslational mechanism.[1] In mammals, for instance, all proteins in which Se was found to be responsible for their biological functions contain the element in this form.[2]

In addition to the genetically encoded insertion of SeC, non-specific incorporation of selenoamino acids is known in organisms fed high doses of these compounds. This is especially the case with SeM, which is randomly incorporated into methionine-containing proteins in the place of methionine.[3] Of the Se-containing proteins, only those with genetically encoded selenocysteine are usually referred to as selenoproteins.

As the metabolic behaviour, the bioavailability and the effects of Se are thus dependent to a large extent on its chemical form, there is a demand for the determination of the selenoamino acids in foodstuffs and tissues in several fields of Se research. This analysis is of particular interest in the investigation of newly found Se-containing proteins, where the determination of SeC is an important step in the identification of a biologically active Se compound.

Three different techniques have so far been used in the separation and measurement of SeC: gas chromatography coupled to atomic emission spectrometry[4] or to mass spectrometry,[5,6] HPLC with reversed-phase columns[7] or ion-exchange columns, the latter as part of an amino acid analyser,[8,9] and thin-layer or paper chromatography.[10] In the separation of SeM similar techniques have been applied, as has previously been reviewed.[11]

In this study, a method was to be developed for the chromatographic identification of SeC and SeM in Se-containing proteins. The main problem with the determination of protein-bound SeC is the fact that the selenoamino acid is very easily oxidized and thus may decompose during acid hydrolysis of the proteins.[9,12] With cysteine and cystine, decomposition can be prevented by the formation of cysteic acid using treatment with performic acid[13] or by carboxymethylation to carboxymethylcysteine (CMC).[14] We therefore investigated the analogous derivatizations of SeC in order to protect the selenoamino acid during protein hydrolysis. This step was then included in the procedure for the separation of SeC and SeM which was to be achieved by adaptation of the widely used derivatization with *o*-phthaldialdehyde (OPA).[14]

Experimental

Materials

The amino acids were obtained from Sigma (St. Louis, MO, USA) and amino acid standards from Sigma and Pharmacia (Freiburg, Germany). Standards not commercially available were prepared from the amino acids in question. OPA was purchased from Amresco (Solon, OH, USA) and HPLC-grade methanol used in the chromatography from Promochem (Munich, Germany). Suprapur hydrochloric acid and acetic acid and all other analytical-reagent grade chemicals were purchased from Merck (Darmstadt, Germany).

Selenium-containing proteins were labelled *in vivo* by administration of [^{75}Se]selenite with a specific activity of 5.2 MBq μg^{-1} Se to either Se-deficient or Se-adequate rats as described elsewhere.[15] ^{75}Se-labelled glutathione peroxidase, which was used as a test substance, was prepared according to the procedure of Awasthi *et al.*[16]

Methods

Performic acid oxidation

For the oxidation by treatment with performic acid, the procedure described by Fontana and Gross[13] was used.

Carboxymethylation

Carboxymethylation of the proteins was carried out in a total volume of 500 μl, with β-mercaptoethanol as the reducing agent and either iodoacetic acid or iodoacetamide as the alkylating agent, as reviewed by Jones.[14] Carboxymethylselenocysteine (CMSeC) was prepared from selenocystine using $NaBH_4$ as the reducing agent.[17] The carboxymethylated proteins were desalted by means of a gel filtration column (P10DG column; Bio-Rad, Hercules, CA, USA) and lyophilized in a centrifugal drier (RC 10.22; Jouan, St. Nazaire, France).

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Gas-phase hydrolysis

An adaptation of the method of Swadesh *et al.*[18] was used. The proteins in question were taken up in water and redried in test-tubes of 6 × 50 mm. The reaction was carried out in a gas-phase hydrolysis vessel with PTFE stopcocks (Waters, Milford, MA, USA) using 250 μl of the hydrolysis acid. For one series of experiments the widely used hydrolysis acid composition of 6 M HCl with 0.5% phenol and 1% β-mercaptoethanol[19] was chosen (hydrolysis acid A). With the hydrolysis acid B used in other experiments, 10 mg ml^{-1} of sodium sulfite were added to 6 M HCl which had been purged with helium for 30 min.[18] The hydrolysis vessels were evacuated and refilled with nitrogen three times to purge off the remaining traces of oxygen. As the oxidation of CMSeC was found to be lower when using shorter reaction times at elevated temperatures, the hydrolysis was performed at 160 °C for 10–45 min.

Precolumn derivatization

The dried samples were taken up in 10–15 μl of the starting buffer. An equal volume of OPA-reagent [0.4 M sodium borate buffer (pH 10.9) containing 10% v/v methanol and 5 mg ml^{-1} OPA] was added to the samples. After an incubation period of 1.5 min, 20 μl of the reaction mixture were injected onto the HPLC column. Amino acid standards, which had been diluted to prevent overloading of the column, were treated in the same way.

Reversed-phase HPLC

HPLC was carried out using an LKB2249 gradient system equipped with a fluorescence detector (LKB, Bromma, Sweden) and an integrator for data acquisition and handling (Merck–Hitachi, Darmstadt, Germany). C_{18} columns (Vydac 218TP54, 5 μm, 250 × 4 mm id from MZ-Analysentechnik, Darmstadt, Germany, or Knaur-Vertex-OPA-Special, 5 μm, 250 x 4 mm id from Knaur, Berlin, Germany) with appropriate guard columns were used. A gradient for the elution of the OPA derivatives was started immediately after sample injection. The conditions were as follows. Buffer A was 100 mM sodium acetate (pH 7.2) containing 0.1% of acetonitrile and buffer B was methanol. The gradient breakpoints for the Vydac column were as follows (min/%B): (0/13), (6/13), (12/32), (20/32), (38/39), (49/63) and (51/100) at a flow rate of 2 ml min^{-1}. The Knaur column was eluted at a flow rate of 1 ml min^{-1} by means of a gradient with the following breakpoints (min/%B): (0/10), (2/17), (15/35), (25/35), (37/60) and (45/100). As the retention times of some of the amino acids, especially those of Met, Val, SeM and Phe, were found to be affected by changes in the temperature, all chromatographic runs were carried out slightly above room temperature at 32 °C. For the fluorescence measurements an excitation wavelength of 340 nm and an emission wavelength of 435 nm were chosen. Fractions of the hydrolysate were collected at 1 min intervals. The Se distribution among the fractions was determined by measuring the ^{75}Se activity or by elemental analysis using atomic absorption spectrometry.

Measurement of ^{75}Se activity

The tracer distribution was determined by means of a liquid scintillation counter (TriCarb, Canberra Packard, Meridea, CT, USA) using the cocktail Ultima Gold (Packard, Meridea, CT, USA). As the scintillation processes are quenched considerably by methanol, the 1 + 1 sample–cocktail mixture proposed by the manufacturer was changed to 1 + 4.5. The counting time was 20 min per sample and the counting efficiency 23.5%. Under these conditions the detection limit for ^{75}Se was about 1 Bq.

Atomic absorption spectrometry

Selenium was determined by electrothermal atomization using the transverse Zeeman effect for background correction. The method has been described in detail elsewhere.[20] A 10 μl volume of the sample was mixed with 10 μl of the chemical modifier (2.5 mg ml^{-1} $PdCl_2$) and injected into a pyrolytic-graphite furnace with a L'vov-platform of a Zeeman 5000 atomic absorption spectrometer (Perkin-Elmer, Überlingen, Germany). After thermal pretreatment for 30 s at 1150 °C, atomization was carried out for 5 s at 2200 °C. The Se concentrations were calculated from the absorption at 196.0 nm by comparing the peak areas of the samples with those of a commercial Se standard (Merck).

Results

Derivatization of Se *Compounds*

Selenoamino acids

CMSeC kept at 4 °C was found to be stable for several weeks, whereas free SeC, prepared by reduction of selenocystine with $NaBH_4$, decomposed within a few hours into Ser, Ala and a red precipitate which was probably elemental Se. Performic acid oxidation of SeC also led to complete decomposition into Ser and Ala. Amido-CMSeC, prepared by carboxymethylation of SeC with iodoacetamide, was stable for only a few days.

Selenoproteins

As with SeC, oxidation with performic acid resulted in complete decomposition of the selenoamino acid residue. After carboxymethylation, however, the ^{75}Se-labelled proteins remained intact (Fig. 1). The standard carboxymethylation method[14] was thus found to be suitable for the treatment of selenoproteins. The losses of ^{75}Se activity during carboxymethylation and chromatographic desalting were below 9% (data not shown). An experiment with an SeM standard showed that this selenoamino acid is partially degraded during carboxymethylation. However,

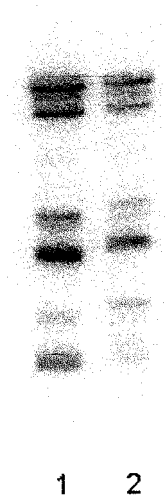

Fig. 1 Autoradiogram of ^{75}Se-labelled proteins from rat testis cytosol separated by sodium dodecylsulfate polyacrylamide gel electrophoresis. Lane 1, untreated proteins; lane 2, proteins after carboxymethylation. The Se-containing proteins remained intact after the treatment. The slight band shifts may be due to the carboxymethylation of all the selenol and thiol groups present in the proteins in question.

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in the investigation of Se-rich plants it was found that qualitative analysis of SeM in proteins is still possible under these conditions.

Optimization of Protein Hydrolysis

In the experiments carried out to optimize the hydrolysis conditions, SeC-containing ^{75}Se-labelled proteins from rat testis were used. After treatment with hydrolysis acid A, a ^{75}Se peak was eluted between 1 and 2 min and thus before the most polar amino acids (Fig. 2). No fluorescence signal was detected at this elution position. It was therefore most likely that this peak contained an inorganic oxidation product of SeC produced during acid hydrolysis as described elsewhere.[12] This was confirmed by the finding that with 6 M HCl without additives and without nitrogen purging of the hydrolysis vessel all of the Se was contained in this peak (data not shown). By careful purging of the vessel with inert gas and exact control of the hydrolysis time and temperature this oxidation peak could be lowered to about 25% of the total ^{75}Se activity.

The oxidative decomposition of SeC could be diminished more effectively by using hydrolysis acid B. In tests in which the hydrolysis time ranged from 10 to 45 min, optimum results with regard to the SeC oxidation were found after treatment of the proteins for 15–20 min. In some experiments the oxidation peak then disappeared completely (Fig. 3). Prolonged hydrolysis led to a rise in the oxidation peak, and shorter treatment of the selenoproteins carboxymethylated with iodoacetamide often resulted in the formation of unhydrolysed amido-CMSeC in addition to CMSeC.

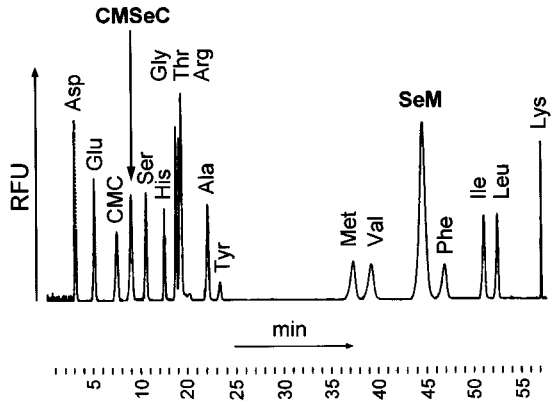

Fig. 2 Fluorescence chromatogram of the OPA derivatives of carboxymethylated selenocysteine, selenomethionine and some standard amino acids separated by reversed-phase HPLC.

HPLC

The OPA derivatives of CMSeC and SeM eluted as single fluorescence peaks. By chromatography of these selenoamino acid standards together with other amino acids, the elution gradient described above was developed which allowed the complete separation of CMSeC and SeM from all other amino acids (Fig. 2). The amino acids were identified from their retention times and the elution sequence of the peaks. Prolonged use of the columns may result in incomplete separation of SeM from Phe. In this case, SeM could be completely resolved by raising the elution gradient in the first 6 min and flattening it between 10 and 25 min. Under these conditions, a baseline separation of the CMSeC peak was still achieved but CMSeC then eluted closer to the CMC peak (data not shown). With new columns the gradient parameters have to be adjusted slightly.

As mentioned above, in hydrolysates of selenoproteins carboxymethylated with iodoacetamide, incomplete hydrolysis may result in the formation of amido-CMSeC, which is eluted about 2 min after CMSeC between His and Gly.

With the experimental parameters described above, for the amino acids investigated the following order of elution was found: cysteic acid, Asp, Glu, CMC, CMSeC, Ser, Gln, His, amido-CMSeC, Gly, Thr, Arg, taurine, Ala, Tyr, Met, Val, SeM, Phe, cystathionine, Ile, Leu and Lys.

Detection Limits

The selenoamino acids in the eluate were detected by the fluorescence of their OPA derivatives (detection limit 30 pmol for SeM and 170 pmol for SeC), by Se determination using atomic absorption spectrometry (detection limit 0.3 pmol) or, with ^{75}Se-labelled compounds, by the measurement of the tracer activity (detection limit 3 fmol). In the first case the values were calculated from the selenoamino acid standards because with the relatively small amounts of isolated selenoproteins the SeC fluorescence peaks of the hydrolysates were below the limit of detection. In the last two procedures the detection limits were calculated for Se-containing proteins which had been isolated from rat tissues by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the investigation of a cytosolic 63 kDa Se-containing protein present in the rat testis, it was found that with the losses during the different steps of protein enrichment, such as electrophoresis, electroelution, carboxymethylation, desalting and hydrolysis, and with four subsamples used in the hydrolysis and selenoamino determination, the original amount of the protein present in the tissue sample should exceed the detection limit by at least a factor of 50.

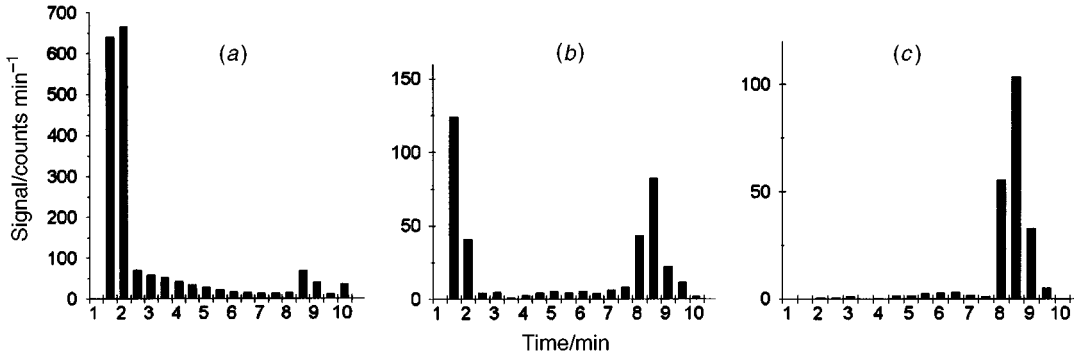

Fig. 3 Oxidation of selenocysteine during acid hydrolysis of ^{75}Se- labelled proteins from rat testis cytosol investigated by reversed- phase HPLC of the Se-containing compounds and measurement of the tracer distribution. Carboxymethylated selenocysteine eluted after 8–9 min and its oxidation product after 1–2 min. (*a*) Protein hydrolysis with 6 M HCl without any additives led to the complete decomposition of selenocysteine. (*b*) The oxidation was reduced by adding 0.5% phenol and 1% β-mercaptoethanol. (*c*) The addition of sodium sulfite to the hydrolysis acid further reduced the oxidative processes.

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Applications

The complete procedure was tested by analysing ^{75}Se-labelled glutathione peroxidase which had been enriched from rat kidney. With the identification of SeC in this well known selenoenzyme the reliability of the method was investigated (data not shown).

The applicability of the procedure was demonstrated in studies on several ^{75}Se-labelled Se-containing proteins which had been detected in tissues of the rat. By determining Se *via* the ^{75}Se activity we found that a 15 kDa protein present in the prostatic acini contained the element in the form of SeC (Fig. 4).[21] We also found SeC as a constituent of a lysosomal 18 kDa protein,[22] of a 34 kDa protein from rat testis[23] and of 63 and 20 kDa proteins from rat testis cytosol,[24] and thus identified these proteins as selenoproteins. Using atomic absorption spectrometry for the determination of Se, we were able to show that in Se-rich proteins obtained from *Lecythis ollaria*, in addition to some other Se compounds as yet unidentified, part of the element was present in the form of SeM.[25]

Discussion

A procedure has been developed that allows the identification of SeC and SeM in tissues and proteins after separation of the amino acids by reversed-phase HPLC. Other techniques applied so far in the simultaneous analysis of SeC and SeM include GC–MS which, however, was used only for the free selenoamino acids,[26] anion-exchange separation with electrochemical detection, which provided limits of detection similar to the fluorescence measurements,[27] and measurement with an amino acid analyser where SeC was determined as the mixed dimer of SeC and Cys.[9] Reversed-phase HPLC has so far been used in the determination of either SeC[7] or SeM[28] and OPA derivatization only in the separation of the enantiomers of SeM[28] or, combined with neutron activation analysis, in the determination of the total amount of free selenoamino acids.[29]

The main problem in the optimization of the method was caused by oxidative decomposition of SeC during the acid protein hydrolysis, also observed in other studies. This was avoided by repeatedly flushing the samples with nitrogen many times.[9] In the present study this difficulty was mainly overcome by carboxymethylation and the addition of sodium sulfite to the hydrolysis acid. As the C_{18} columns used in the reversed-phase HPLC are relatively cheap, the procedure can be carried out in any laboratory with access to standard HPLC equipment without great additional expense.

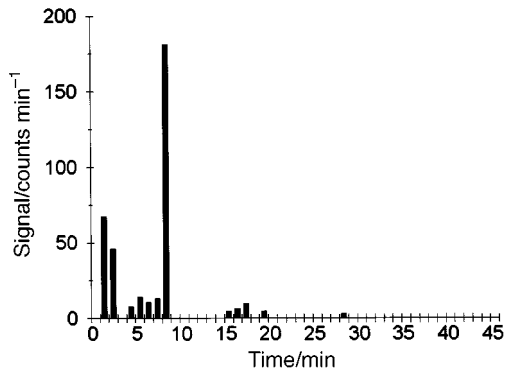

Fig. 4 Determination of the chemical form of Se in a Se-containing 15 kDa protein found in the rat prostatic epithelium.[21] After carboxymethylation and acid hydrolysis of the labelled protein the OPA derivatives were separated by reversed-phase HPLC. Peak 2 consisted of carboxymethylated SeC. Peak 1 was an oxidation product, while peak 3 resulted from incomplete hydrolysis and disappeared after prolonged treatment.

With the well known elution sequence of the OPA derivatives of the amino acids and the elution pattern of the selenoamino acids reported here, the procedure should be easily adaptable to any established OPA method using methanol as an eluent. In the adaptation of OPA techniques in which acetonitrile is used as the gradient eluent, it has to be taken into consideration that the elution sequence of Met, Val and Phe is known to be changed[19] and that SeM, which elutes between Val and Phe, might likewise be affected.

In the determination of the two selenoamino acids *via* atomic absorption spectrometry, much lower detection limits were reached than in the measurement of the fluorescence of the OPA derivatives. With a maximum of 10 mg of protein to be processed in the hydrolysis step and a yield of 10% after HPLC, atomic absorption spectrometry allows the determination of selenoamino acid concentrations exceeding 0.1 pmol mg^{-1} protein in whole sample hydrolysates and thus the direct determination of the seleno compounds in most of the tissues and foodstuffs. When ^{75}Se-labelled proteins are available, extremely small amounts can be detected. This was shown in an experiment in which, after administration of ^{75}Se with a very high specific activity of 37 MBq μg^{-1} Se to Se-deficient rats and measurement of the tracer activity in the labelled Se compounds by means of a low-level counting device, the detection limit could be lowered to amounts in the attomole range.[30] With the possibility of combining HPLC with different techniques of selenoamino acid determination in non-labelled and labelled samples, the procedure represents a versatile tool for the identification of SeC and SeM in biological materials. It may be used in various fields of the life sciences where information on the differences in the bioavailability and the metabolic behaviour of the two selenoamino acids is needed. With the low limits of detection to be achieved, it is of particular advantage in the investigation and characterization of newly found Se-containing proteins which are present in the tissues in only very low concentrations.

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