Se-Hg Dual-element Labeling Strategy for Selectively Recognizing Selenoprotein and Selenopeptide

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Abstract: An endogenous element-label plus exogenous element-tag strategy was proposed for inductively coupled plasma-mass spectrometry (ICP-MS) to screen and discriminate a family of ultratrace but biologically important biomolecules. The feasibility of this novel idea was demonstrated by setting seleno (SeCys) and Se-containing (SeMet) proteins (peptides) as an example. Se-label naturally occurring in the biomolecules acted as an identifier for picking up them out of a large amount of various coexisting proteins (peptides), and CH₃Hg-tag that could bind to SeCys rather than SeMet helped discriminating seleno and Se-containing ones simply based on the Se and Hg signals on ICP-MS. This strategy has been applied to screen and discriminate Seleno and Se-containing proteins (peptides) in water-soluble extracts of Se-enriched yeast, and seven selenoproteins (peptides) were detected with both ²⁰²Hg and ⁸²Se signals out of fifteen Se-containing species using RPLC/ICP-MS, providing valuable information for further identification using a high-resolution structure-selective mass spectrometer. This endogenous element-label plus exogenous element-tag dual-element approach implies that ICP-MS is not only able to quantify targeted proteins (peptides) but also helpful to discover unknown ones during a discovery-based proteomic study.

Key Words: Selenium; Mercury; Selenoprotein selenopeptide; Inductively coupled plasma mass spectrometry

1 Introduction

A very successful example of an endogenous element-label, nitrogen, for crude protein analysis with chemical back-titration developed by Sir Johan Kjeldahl nearly 130 years ago is still the universally accepted standard method today^[1]. In the last two decades, strategies based on such a traditional idea have been developed further and more successful to discover, track, identify and quantify proteins and/or peptides, and are far on the way to be extended for ever more applications, since the physicochemical properties of the elements are really attractive^[2]. Inductively coupled plasma mass spectrometry (ICP-MS), the element-specific analytical technique with the advantages of high sensitivity and selectivity, was successfully used in determining

low-abundance proteins (peptides) in complex biological matrix in combination of new element labeling strategy^[3–5]. Except the endogenous (naturally occurring, e.g. S, P, Se and Fe) element-label strategy, exogenous element-tag (chemically or biologically selective modification, e.g. Hg, I, Au and lanthanides) method attracted more and more attention due to its ability of expanding the application area and addressing the shortcoming of endogenous element-label strategy^[6–14]. Up to now, either an endogenous element-label or an exogenous element-tag has been used to study proteins (peptides). There are rare reports on combination of two or more element-labels and/or tags to study trace but biologically important proteins (peptides) in a complex biological specimen. The difficulty lies in how to build the relationship between the endogenous element-label and exogenous

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element-tag focusing on a targeted class of proteins (peptides), and to make the best use of their individual advantages for specific recognition and highly sensitive determination. In general, an ideal endogenous element-label plus exogenous element-tag dual-element labeling strategy should meet the following requirements. First, there should be a covalently-bonded endogenous element-label which could uniquely represent the target proteins (peptides) as an identifier. Second, an exogenous element-tag should be highly reactive for labeling the targeted proteins (peptides). Third, the endogenous element-label and exogenous element-tag must be simultaneously determined with an appropriate technique such as ICP-MS.

In our previous works, quantifications of the selenoproteins and Se-containing species *via* determination of the endogenous Se-label with HPLC/ICP-MS and the model proteins (peptides) through determination of the exogenous Hg-tag, which was chemically labeled through the specific interaction between the –SHs in the molecules and monofunctional organic mercurials, were accomplished^[14–19]. Se and Hg are an interesting pair, and one typical example of the best known biological antagonism interactions existing universally in organisms^[20–22]. Se mainly exists in the –SeH group in selenoprotein/peptides, which can be regarded as an ideal labeling site for exogenous Hg-tag. It is theoretically feasible to use Se and Hg as endogenous and exogenous elemental-label respectively to detect selenoproteins(peptides). In this work, the labeling of selenoproteins by exogenous Hg tag was investigated, and the recognition and detection of selenoproteins by Se-Hg dual-element labeling strategy were demonstrated.

2 Experimental

2.1 Instruments and reagents

Esquire-LC ESI ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), and the measurements were performed in positive ion mode with scan range of m/z 50–2000. Bruker microflex LRF matrix assisted laser desorption/ionization time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used for ESI-MS, and a SERIES 200 HPLC system (PerkinElmer, SCIEX, Canada) for ICP-MS equipped with an auto-sampler, a vacuum degasser and a 200- μ L loop. ELAN DRC II ICP-MS (PerkinElmer, SCIEX, Canada) equipped with an integral peristaltic pump and a PC³ Low Temperature Peltier Cooled Cyclonic Chamber (Elemental Scientific Inc. Omaha, US).

Oxygen (O₂, 99.999%) was purchased from the Beijing AP Beifen Gases Industry Co. (Beijing, China). S determination was achieved *via* monitoring ³²S¹⁶O⁺ by introducing O₂ (0.6

mL min^{−1}; RPQ 0.45) into the dynamic reaction cell (DRC), thus eliminating the interference of ¹⁶O₂⁺ in ³²S⁺ detection. Se and Hg were determined by ICP-DRC-qMS *via* monitoring the isotopes of ⁸²Se⁺ and ²⁰²Hg⁺.

Selenocystine (seleno-DL-cystine, (SeCys)₂), dithiothreitol (DTT), thiosalicylic acid (HOOC₆H₄SH, 97%), 2,5-dihydroxybenzoic acid (DHB) and glutathione peroxidase 1 (GPx1, from bovine erythrocytes, 84.5 kDa, lyophilized powder, ≥ 300 units per mg protein) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Methylmercuric chloride (CH₃HgCl, 98.5%) was obtained from Dr. Ehrenstorfer GmbH (Germany) and Commercial Se-enriched yeast (Sel-Plex) from Alltech, Nicholasville, KY, USA). Molecular weight markers used for size exclusion chromatography calibration, including pyruvate kinase (237.0 kDa), bovine serum albumin (66.5 kDa), ovalbumin (44.3 kDa), lysozyme (14.3 kDa) and cobalamin (1.4 kDa), were purchased from Sigma-Aldrich. Other reagents used were of at least analytical-reagent grade. All of the solutions were prepared with ultra-pure water (18.2 M Ω cm, Pen-Tung Sah Micro-Electro-Mechanical Systems Research Center of Xiamen University, China).

2.2 Experimental methods

2.2.1 Labelling SeCys, SeMet and GPx1 with CH₃Hg-THI

Briefly, equal molar concentrations of CH₃HgCl and HOOC₆H₄SH were mixed under pH 7.4 to obtain CH₃Hg-THI. The prepared stock solution (4 mM) was kept at 4 °C before use. The (SeCys)₂ (10 μ M, 0.2 mL) was reduced with different amounts (10 and 100 times molar excess) of DTT to obtain SeCys. The synthesized CH₃Hg-THI (4 mM, 0.2 mL) was used to label the SeCys in a buffer solution (20 mM NH₄COOCH₃, pH 7.4) at 37 °C for 1 h, and then CH₃Hg-tagged SeCys was identified by ESI-IT-MS. Meanwhile, SeMet (10 μ M, 0.2 mL) was prepared and analyzed under the same conditions as described above.

A stock solution (5 mg mL^{−1}) of GPx1 was prepared in buffer solution (20 mM NH₄COOCH₃, pH 7.4) and stored at −20 °C. The labeling processes of GPx1 with CH₃HgCl and CH₃Hg-THI was carried out at 37 °C for 1 h by addition of CH₃HgCl or CH₃Hg-THI solutions (5 μ L, 4 mM) into GPx1 solutions (1 mL, 25 μ g mL^{−1}) respectively. The GPx1 and its CH₃Hg-tagged products were analyzed with SEC/ICP-MS (size exclusion chromatography column Superdex G-75, 10/300 GL (GE Healthcare, USA); mobile phase: 5 mM Tris-100 mM NH₃HCO₃, pH 7.4; flow rate: 0.75 mL min^{−1}) and RPLC/ESI-MS (VP-ODS C₁₈ reversed-phase column (Shimadzu, Japan); mobile phase A: 0.05% TFA aqueous solution; mobile phase B: 0.05% TFA in acetonitrile; flow rate: 150 μ L min^{−1}. gradient programme: 0–5 min, 1% B; 5–10 min, 1%–5% B; 10–20 min, 5%–10% B; 20–25 min, 10%–30% B;

25–40 min, 30% B). The columns were washed with 1% DTT for 30 min after each analysis, and equilibrated with mobile phase before next injection. The same GPx1 samples were also analyzed by MALDI-TOF-MS.

2.2.2 Preparation and analysis of the Se-enriched yeast water-soluble extracts

0.5 g of Se-enriched yeast powder was extracted with 5 mL PBS buffer solution (1.4 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4 , 0.18 mM KH_2PO_4 , pH 7.4) for 5 min under ultrasonication at 4 °C. After centrifugation (10000 rpm, 30 min $\times$ 2 at 4 °C), the supernatant, *eg*, the Se-enriched yeast water-soluble extracts was collected. The supernatant (50 μL) was added with $\text{CH}_3\text{Hg-THI}$ (5 μL , 4 mM) to label the possible selenoproteins (peptides) and any other Cys-containing species coexisting in the fraction. After desalting on PD minitrapp G-10 column (GE Healthcare, UK), the products were analyzed with SEC/ICP-DRC-MS and RP-HPLC/ICP-DRC-MS under the conditions as described above in the case of GPx1. Before ICP-MS measurement (^{82}Se , ^{202}Hg and $^{32}\text{S}^{16}\text{O}$), the eluent of the chromatographic column was detected by a tandem UV detector (at 214 nm).

3 Results and discussion

3.1 Dual-element labeling principle

As shown in Fig. 1a, in selenoprotein (peptide) molecules,

Fig.1 Endogenous and exogenous Se-Hg dual-labeling strategy for recognizing selenoprotein/peptide

the endogenous Se exists as genetically encoded selenocysteine (SeCys) by the UGA codon, but not as selenomethionine (SeMet) randomly in non-specific Se-containing proteins (peptides)^[23]. SeCys and SeMet have the same structures respectively as Cys and Met with the replacement of S by Se. The SeCys-containing proteins (peptides) and Met-containing proteins (peptides), which are named as selenoproteins(peptides) and Se-containing proteins (peptides) respectively, exhibit different physiological functions. The $-\text{SeH}$ of SeCys residue in selenoprotein (peptide) can be labeled by Hg through reaction with CH_3Hg^+ which is dynamically dissociated from $\text{CH}_3\text{Hg-THI}$, whereas the $-\text{SeCH}_3$ of SeMet in Se-containing proteins (peptides) does not react with exogenous Hg. Since the pK_a of $-\text{SeH}$ in SeCys ($\text{pK}_a = 5.2$) is lower than that of cysteine $-\text{SH}$ ($\text{pK}_a = 8.5$), $-\text{SeH}$ is much more reactive than $-\text{SH}$, and thus is apt to react with CH_3Hg^+ ^[24,25]. Se-label would act as an identifier of selenoproteins (peptides) and Se-containing proteins (peptides), and Hg as a discriminator of them because only Se in the form of $-\text{SeH}$ (SeCys) is expected to be tagged with Hg. In this way it is possible to screen selenoproteins (peptides) and Se-containing ones out of large amount of coexisting biomolecules that may contain $-\text{SH}$ (Cys), and subsequently recognize selenoproteins (peptides) based simply on the ICP-MS signals of both Se and Hg, in addition to their specific isotope distribution patterns (Fig.1,b–d). The molecules which exhibited ICP-MS signals of both Se and Hg, and also had specific isotope distribution patterns of both Se and Hg on ESI-IT-MS were identified as selenoproteins (peptide), while

those which gave either Se or Hg signal only might be Se-containing proteins (peptides) or other proteins/ peptides containing Cys. Based on this principle, specifically recognizing selenoproteins (peptides) in complex biological matrix were accomplished.

3.2 Labeling –SeH group with CH₃Hg-THI

To test the feasibility of selectively labeling –SeH group with CH₃Hg-THI, selenocystine ((SeCys)₂) was firstly reduced with DTT to obtain SeCys, which was then labeled by CH₃Hg-THI (Fig.2a). The experimental results showed that by varying the molar ratio of (SeCys)₂ to DTT, SeCys with *m/z* 167.6 was successfully synthesized (Fig.2b). After reacting with CH₃Hg-THI, no MS signal of SeCys was detected, whereas MS peak of product CH₃Hg-Cys (*m/z* 404.6) was observed. Furthermore, the isotopic distribution of the product (*m/z* 404.6 (23.0%), 402.6 (16.3%), 403.5 (14.9%), 401.6 (12.8%), 406.6 (11.3%), 400.5 (8.4%), 405.5 (6.6%), 408.5 (2.9%), 399.5 (2.1%), 397.7 (0.9%) and 398.6 (0.8%)) was in well accordance with the theoretical value of H₂NCOOHCHCH₂SeHgCH₃ (Fig.2c), demonstrating the successfully labeling of –SeH in SeCys with Hg. In comparison, the MS signal of SeMet (*m/z* 197.9 and 394.8) remained unchanged after labeling reaction, indicating that –SeCH could be tagged with CH₃Hg-THI.

3.3 Labeling GPx1 with CH₃Hg-THI

GPx1, as a typical selenoprotein, belongs to the

selenoproteins family and can effectively eliminate the superoxide radical in living organisms^[26]. It consists of four monomers, each of which contains one –SeH (SeCys52) and two free –SH group^[27]. In this study, GPx1 was used as a model molecule to investigate whether the –SeH group in GPx1 could be labeled by CH₃Hg-THI. In a comparative experiment, GPx1 was labeled by both CH₃Hg-THI and CH₃Hg⁺ simultaneously. The experiment results showed that white protein precipitate appeared when GPx1 was labeled with CH₃Hg⁺ under physiological conditions (Fig. 3a), indicating the destroying of molecular structure and subsequent denaturation. The analytical results by SEC/ICP-MS also demonstrated the dramatic decrease of signal intensities of Se and Hg of residual CH₃Hg⁺-labeled GPx1 in reaction solution (Fig.3b). Compared with labeling with CH₃Hg⁺ directly, denaturation of GPx1 protein would not occur in the labeling reaction by using CH₃Hg-THI which indirectly and dynamically released CH₃Hg⁺^[17] to label GPx1(Fig.3a). In this case, very strong SEC/ICP-MS signals of Se and Hg could be detected at the same time, proving the successful labeling of GPx1 by CH₃Hg-THI and the tagged GPx1 was very stable in physiological solution.

Subsequently, the tagged GPx1 was analyzed by SEC/ESI-IT-MS and MALDI-TOF-MS (Fig.3c). The molecular weights of M (GPx1 monomer) before labeling and labeling product M' (M-(HgCH₃)) were 22651.5 and 23326.9 Da respectively, with a mass difference of 675.4 Da, proving the successful Hg-labeling of –SeH group and two –SH groups in GPx1 monomer. The analytical results by MALDI-TOF-MS showed that besides GPx1 monomer, the dimer, trimer and tetramer

Fig.2 (a) Reduction of (SeCys)₂ to SeCys by DTT and labeling with CH₃Hg-THI, (b) ESI-IT-MS of the reduction of (SeCys)₂ to SeCys by DTT and (c) labelling of the SeCys and SeMet with CH₃Hg⁺ dynamically released from CH₃Hg-THI

Fig.3 (a) Labeling of GPx1+CH₃Hg⁺ and GPx1+CH₃Hg-THI under the same experimental conditions. (b) Typical chromatograms of SEC-ICP-MS of native and labeled GPx1. Analysis of the native and CH₃Hg-tagged GPx1 by (c) ESI-MS and (d) MALDI-TOF-MS

of GPx1 could also be tagged by CH₃Hg-THI, again proving that the dynamically labeling by CH₃Hg-THI was in favor of the stability of GPx1. Further quantitative analysis by SEC/ICP-MS showed that based on the detection limit of ²⁰²Hg (45 pM), the detection limit of GPx1 was down to 10 fmol, more than one order of magnitude lower than that through determination of Se (⁸²Se) (100 fmol). The results demonstrated that labeling by exogenous Hg tag could not only help discrimination of the selenoproteins/peptides and se-containing proteins/peptides, but also improve the detection ability of selenoproteins/peptides, which is in favor of the detection of low-abundance biomolecules.

3.4 Potential selenoprotein (peptide) in se-enriched yeast water-soluble extracts

There are various Se species including selenoproteins (peptides) in yeast, especially in Se-enriched yeast. In fact, the Se-containing biomolecules involving in metabolic process of Se-enriched yeast were studied and many new Se species were discovered^[28–33]. Thus the Se-enriched yeast was used to demonstrate the feasibility of the proposed strategy. It should be noted that the water-soluble extracts of the Se-enriched yeast was not reduced with any reductant before Hg-tagging, thus avoiding the production of large amount of –SH group resulting from the breakage of the –S–S– (and possible –Se–Se–) bond ubiquitous in proteins. After labeling the water-soluble extracts of Se-enriched yeast with CH₃Hg-THI, the samples were analyzed by SEC/ICP-MS and RPLC/ICP-

MS. As shown in Fig.4a, the analysis of SEC/ICP-MS revealed five Se-containing fractions (9.6, 12.8, 21.2, 24.6 and 31.4 min) according to the ⁸²Se signals on SEC/ICP-MS. Among them, three fractions of 9.6, 12.8 and 21.2 min were supposed to contain potential selenoproteins (peptides) because both ²⁰²Hg and ⁸²Se signals were simultaneously determined. Compared with the retention time of the five molecular weight markers used, the possible selenoproteins in the fraction of 9.6 min had molecular weight of greater than 44.3 kDa, with Se content (0.084 μmol Se g^{–1}) accounting for 2.6% of the total Se determined in the soluble extracts. The fraction of 12.8 min (0.036 μmol Se g^{–1} corresponding to 1.1% of the total Se) might contain GPx1 according to the retention time of standard GPx1 under the same conditions, and those in the fraction of 21.2 min (1.9 μmol Se g^{–1}, 58.8% of the total Se in the soluble extracts) were the possible selenopeptides with molecular weights of 1.4–14 kDa. In addition, those in the fractions of 24.6 min and 31.4 min were inferred as small molecular-weight (< 1.4 kDa) Se-containing species (1.1 μmol Se g^{–1}, 33.4% of the total Se) because only ⁸²Se signal was detected. In RP-HPLC/ICP-MS analysis, seven peaks (* marked) with both ²⁰²Hg and ⁸²Se signals were detected out of fifteen Se-containing species (Fig.4b), which were identified as selenoproteins (peptides), and GPx1 was found within retention time of 26.5–30.0 min. As shown in Fig.4b, fractions with high-concentration ³²S¹⁶O⁺ were detected before 10 min, which were high-concentration cystein-containing proteins in water-soluble extracts of Se-enriched yeast. Anyway, the ability of recognizing selenoproteins (peptides), Se-containing

Fig.4 Typical chromatograms of the water-soluble extract of the Se-enriched yeast analyzed using (a) SEC-ICP-MS and (b) RP-ICP-MS. The marked (*) peaks indicated possible selenoproteins (peptides) in the extract. 1, 2, 3, 4 and 5 in (a) represent the elution time of the five molecular weight markers used (237.0, 66.5, 44.3, 14.3 and 1.4 kDa)

proteins (peptides) and other high-abundance proteins (peptides) under high background suggested that the Se-label plus Hg-tag strategy was effective for screening and discriminating selenoproteins (peptides). The combination of high-resolution multidimensional separation techniques together with high-resolution structure-selective molecular mass spectrometer such as FT-ICR-MS may facilitate further structure and composition analyses of the recognized selenoproteins (peptides).

4 Conclusions

An endogenous element-label plus exogenous element-tag strategy was developed in this study. The feasibility for screening and discrimination of a specific class of ultratrace but biologically important proteins (peptides) in a complex biological specimen was preliminarily demonstrated taking selenoproteins (peptides) and Se-containing species as an example. This novel approach allows ICP-MS being able to discriminate biomolecules with similar chemical composition but dissimilar function such as seleno- and Se-containing proteins (peptides). This strategy may find more applications, not limited in Se-label and Hg-tag, when more “label-tag”, “label-label” and “tag-tag” strategies are developed towards more targeted and unknown proteins.

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