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Selective fluorescence detection method for selenide and selenol using monochlorobimane

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ABSTRACT

The low redox potential of selenide and selenol is physiologically important, as it confers efficient catalytic abilities to selenoproteins. Quantitative determination of selenol and selenide provide important clues for understanding the metabolism and physiological function of selenium. However, selective detection of selenol and selenide is extremely difficult because of their chemical similarity to thiol and sulfide. In this study, we established a highly sensitive, selective, quantitative, and simple method for detection of selenol and selenide, using a reaction with monochlorobimane (MCB), followed by ethyl acetate extraction of the product *syn*-(methyl,methyl)bimane. We analyzed selenide production from selenite, catalyzed by human glutathione reductase, and also determined selenide and selenol concentrations in Hepa1-6 cells using the MCB method, to demonstrate its practical applications. This study provides a new tool for selenium detection in biology.

Introduction

The essential trace element selenium (Se) is required for normal embryonic development [1] and DNA biosynthesis [2]. Moderate selenium deficiency has been linked to various defects, such as male infertility [3], thyroid dysfunction [4], and several neurologic diseases [5]. Se exists in several valence states: +VI, +IV, +II, 0, and −II, in inorganic and organic compounds under different redox environments [6]. Among them, Se (−II), including selenol (R-SeH) and selenide (Se^{2-}), shows a wide biological distribution. Selenocysteine residues in selenoproteins are typical selenols. Selenoprotein biosynthesis requires selenide as an essential selenium source [7]. Recently, it was also reported that functions of many proteins are redox regulated by selenide and selenol [8,9]. The low redox potentials of selenide and selenol are physiologically important. Detection and quantification of selenol and selenide helps understand the metabolism and physiological function of selenium.

Determination of unstable seleno-compounds is challenging [10–14], in contrast to the determination of the stable seleno-compounds, Se (VI), Se (IV), selenomethionine, and selenocysteine [13,15]. Moreover, because of the similarity between chemical properties of selenium and sulfur, distinguishing selenol and selenide from thiols, using a chemical reaction, is difficult. Furthermore, intracellular concentration of glutathione (GSH), the most prevalent intracellular thiol in mammalian cells, is abundant (0.5–10 mM) [16], in comparison to the trace amounts of selenium. For example, level of GSH in 1 g rat liver is in the micromolar range [17], whereas selenium is in the nanomolar range [18]. Thus, under physiological conditions, a large excess of thiols, in the form of GSH, makes the measurement of selenol compounds difficult. Methods for highly selective and sensitive determination of selenide and selenol are yet to be exploited.

Various detection techniques [19–23] have been reported for the determination of seleno-compounds. However, these techniques require expensive special equipment, which might not be available in many laboratories [24]. In addition, these methods also require tedious multistep pretreatments of samples [19–23].

Monochlorobimane (MCB), a non-fluorescent molecule, forms the fluorescent bimane-glutathione conjugate (bimane-GS) from its reaction with glutathione (GSH). Thus, MCB has been used for

Abbreviations: MCB, monochlorobimane; bimane-GS, bimane-glutathione conjugate; GR, glutathione reductase; HBSS, Hank's balanced salt solution; ESI-MS, electrospray ionization mass spectrometry.

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quantitative analysis of intracellular GSH [25]. Furthermore, derivatization of several thiols with bimane has been used for high performance liquid chromatography (HPLC) analyses [26–28]. However, the reaction of MCB with selenide and selenol has not been explored. In this study, we demonstrated the formation of *syn*-(methyl,methyl)bimane, for the first time, from the reaction of MCB with selenocysteine or selenide. Thus, easy detection and selective separation of the fluorescent compound enables us to successfully establish a simple, quantitative method for the detection of selenide and selenol.

Materials and methods

Materials

Sodium selenite, ATP, NADPH, dithiothreitol, and GSH were purchased from Nacalai Tesque (Kyoto, Japan). MCB, *syn*-(methyl,-methyl)bimane, and Dulbecco's modified Eagle's medium was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). M-PER was purchased from Thermo Fisher Scientific Pierce Biotechnology (Rockford, IL, USA). Hank's balanced salt solution (HBSS) was purchased from Gibco (Carlsbad, CA, USA). Recombinant human glutathione reductase (GR) was purchased from Abfrontier (Seoul, Korea).

Purification of selenocystine

To purify a selenocystine reagent, selenocystine powder (10 mg) was rinsed with water, and the supernatant containing selenate and selenite was removed. The sample was dissolved in 2 mL of 12% ammonia solution, passed through a filter paper to remove Se^0 amorphous, and lyophilized.

Cell culture

The Hepa1-6 cell line used in this study was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 5% CO_2.

Production and purification of syn-(methyl, methyl)bimane

A solution of sodium selenite or selenocystine (each 100 mM, 3 mL) was added individually to 4 mL dithiothreitol and GSH (each 1 M) under Tris-HCl buffer (1.5 M, pH 8.8) with N_2 purge. Sodium selenite solution was connected with a vial containing 10 mL of 10 mM MCB in 10 mM Tris-HCl buffer (pH 7.4) (trap 1a) and 3 mL of 0.1 M $AgNO_3$ (trap 2) in tandem, and N_2 gas was introduced into the system at a flow rate of 200 μL/min for 10 min. Without interrupting the gas flow, 1 mL of 11 M HCl was injected rapidly into the sample vial, and N_2 purge was continued for another 10 min. Selenocystine-added GSH solution was directly incubated with 10 mM MCB under N_2 purge (trap 1b). After removing the trapping vials, contents were extracted by adding 250 mL chloroform to trap 1a and trap 1b vials, and the pooled extracts were concentrated by rotoevaporation. After inspissation, the samples were fractionated using flash column chromatography with silica gel column and chloroform with 5% methanol. The fluorescent fractions were subjected to ^{1}H-NMR, HPLC, and electrospray ionization mass spectrometry (ESI-MS) analyses.

NMR and ESI-MS analyses

^{1}H-NMR spectra were obtained from a JEOL JNM-AL-300 FT NMR system spectrometer. The instrument was calibrated using residual nondeuterated solvent and tetramethylsilane as internal references. ESI-MS analysis was carried out with an Applied Biosystems API3000 spectrometer. Samples (2 mg each) were diluted with 500 μL of acetonitrile/methanol (2:1 v/v) containing 0.1% (v/v) ammonium formate (pH 6.5), and subjected to ESI-MS analysis.

HPLC analyses

All HPLC analyses were performed at 25 °C, using a Capcell Pak C_{18} SG120 column (4.6 × 250 mm; Shiseido, Japan) and a Waters HPLC system (Waters, USA). The column was equilibrated with 5% 20 mM phosphate buffer (pH 7.0) in 50% acetonitrile solution at a flow rate of 1.0 mL/min. Eluents were monitored by fluorescence spectroscopy, using excitation and emission wavelengths of 380 nm and 480 nm, respectively. Under these conditions, *syn*-(methyl,-methyl)bimane was eluted as a major peak at 3.6 min.

Thin layer chromatography

Thin layer chromatography (TLC) was performed using TLC aluminum sheets (20 cm × 20 cm Silica gel 60 F_{254}, Merck KGaA, Germany). The sample (3 μL each) was spotted onto the plate. The plate was developed with *n*-butanol—acetate—water (4:1:2). Spots were detected by fluorescence under UV light (360 nm).

Extraction of the reaction products of MCB with selenocysteine, selenide, cysteine, and sulfide

Selenocystine, sodium selenite, cystine, and sodium sulfide (1 mM each) were mixed with 3 mM GSH in 50 mM Tris-HCl (pH 7.4) at 37 °C to produce selenocysteine, selenide, cysteine, and sulfide, respectively. After 1 min, samples were incubated with 100 μM MCB at 37 °C for 1 h. Then, the contents from 1 mL samples were extracted with 500 μL ethyl acetate. After dilution with 10 vol of water, the extracted compounds were subjected to HPLC analysis.

Detection of selenide produced via selenite reduction by GR and GR assay

NADPH (1 mM), EDTA (2 mM), sodium selenite (500 μM), GR (500 nM), and MCB (2 mM) were incubated in 50 mM Tris-HCl (pH 7.4) at 37 °C. After 3 h, 200 μL of the mixture was extracted with 200 μL ethyl acetate. After dilution with 40 vol of water, the reaction product was subjected to HPLC analysis. Measurements of the selenite reduction activity of GR were performed at 37 °C in the solution containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 200 μM NADPH, using a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan). The oxidation of NADPH in the presence of 0 mM–10 mM selenite was followed by measuring the absorbance at 340 nm (ε_{340} = 6.2 $mM^{-1}cm^{-1}$). The value obtained in the absence of selenite was subtracted from that obtained in the presence of selenite.

Quantification of selenocysteine and selenide using MCB

Selenocystine (0 μM–100 μM) and selenite (0 μM–10 μM) were reduced with 2 mM GSH in 50 mM Tris-HCl (pH 7.4) at 37 °C to yield 0 μM–200 μM selenocysteine and 0 μM–10 μM selenide solutions, respectively. After 1 min, samples were incubated with 400 μM MCB for 1 h. Then, the contents in 300 μL samples were extracted with 600 μL ethyl acetate. The extracted contents were analyzed with a fluorometer (Shimadzu, Japan) with excitation and emission wavelengths of 380 nm and 480 nm, respectively.