Protocols for Selenium Biochemistry

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ABSTRACT

Selenium, an essential trace element for mammals, birds, fishes, amphibia and some bacteria, shows various remarkable biological effects, and it is definitely required in various metabolic systems such as antioxidative defence systems, hormone regulated biosynthesis, constituents of muscle and anaerobic redox catalysis. The biological significance of selenium may be related to the unique functions of various selenoproteins which contain a selenocysteine residue as an integral part of their active site. Glutathione peroxidase is one of the most extensively studied selenoenzymes, and its catalysis is closely related to the antioxidative effects of selenium. However, glutathione peroxidase and other known selenoproteins such as iodothyronine 5′-deiodinase cannot explain all the biological effects of selenium. Discovery of a new selenoprotein would give us a significant insight for further understanding the physiological roles of selenium in human health. This article introduces protocol and skills frequently used in selenium biochemistry; the use of selenium-75 in detecting and identifying selenoenzymes, chemical identification of the selenocysteine residue, and precautions in selenoprotein purification.

INTRODUCTION

Selenium is an essential trace element for mammals, birds, fishes, amphibia and some bacteria[1]. Its remarkable biological effects as a micronutrient for mammals may be related to unique functions of various selenoproteins[2]. Well-examined cases are the glutathione peroxidases in antioxidative systems[3], and type I iodothyronine 5′-deiodinase in thyroid hormone biosynthesis[4]. White muscle disease in mammals, observed in selenium deficiency, may be related to selenoprotein W[5]. Selenoprotein P, a heavily glycosylated monomeric protein whose cDNA sequence indicates the presence of 10 selenocysteine (Sec) residues in its polypeptide chain[6], is suggested to be involved in antioxidative defence system, but its catalytic and biochemical functions are not known[7]. The recent discovery[8,9] that mammalian thioredoxin

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reductase is a selenocysteine-containing protein provides evidence of an essential role of selenium in an important redox catalyst.

Although some of the significant biological effects of selenium may be closely related to these known selenoproteins, there still remain other important metabolic systems which require selenium but the responsible selenoproteins are not identified yet. In fact, the biological effects such as prevention of tumorigenesis induced by chemical carcinogens and viruses[10], activation of the immune system[11], and effective compensation of vitamin E deficiency[12] indicate the presence of undiscovered enzymes whose catalysis and function depend on selenium as an integral part of the molecule. Thus, discovery of additional selenoproteins may provide significant insight into the undiscovered biochemical roles of selenium in human health.

The present article describes skills and techniques for investigation of selenoproteins. The radioisotope ^{75}Se is a useful tool for the detection and identification of selenoproteins on SDS-PAGE gels, and the ^{75}Se-labeled Sec residue can be chemically identified by amino acid analysis following chemical derivatization. Technical precautions in purification of selenoproteins are also described. Most of the skills described here have been employed during the course of isolation and characterization of a thioredoxin-reducing selenoprotein (thioredoxin reductase) from a human lung adenocarcinoma cell line[8].

1. Labeling Selenoproteins with Selenium-75

The radioisotope selenium-75 (^{75}Se) is a powerful tool in selenium biochemistry. It emits γ-ray with 0.265 eV (59%) and 0.280 eV (25%), and decays with a half life of 120.4 days. ^{75}Se-Selenite can be purchased from the Research Reactor Facility, University of Missouri, Columbia. The radioisotope is delivered in the form of selenious acid (H_2SeO_3) in a very small volume of 7-30% nitric acid. Its specific activity is high enough to allow us to ignore the original content of selenium. It is strongly recommended to add a small amount of "cold" sodium selenite before use in order to avoid radio-colloid formation. The presence of ^{75}Se is readily detected using a portable Geiger-Muller counter, and the radioactivity is determined in a γ-ray scintillation counter such as the Beckmann γ 5500 counter or the Wallac 1470 Wizard Automatic γ counter.

Since selenite is one of the most effective chemical forms in labeling a selenocysteine residue with ^{75}Se, the sodium ^{75}Se-selenite is directly added to the culture broth and incubated for the desired period of time. In most cases the small amount of nitrate added with the selenite has no effect on the growth of cells. In the experiments with the human adenocarcinoma cell line NCI-H441, 0.1 μM sodium selenite containing ^{75}Se (368 μCi/L) was added to the culture media and incubated for 4 days. Cells were collected at 6, 24, 48, 72, and 96 hours, and they were directly analyzed by SDS-PAGE/autoradiography procedure.

2. SDS-PAGE Analysis

A convenient and sensitive method for detecting proteins that contain a ^{75}Se-labeled Sec residue is by autoradiography of SDS-PAGE gels. A cell suspension (5 mg wet weight in 30 μL PBS buffer) was mixed with 30 μL of SDS-PAGE sampling buffer, and boiled for 10 min. Then 5-20 μL portions of the heated sample were loaded on a SDS-PAGE gel. Selenoproteins can be developed on SDS-PAGE without any obvious degradation unless ammonium peroxodisulfate, a radical polymerization initiator, remains in the polyacrylamide gel. Oxidizing reagents and radical species are harmful for Sec residues, and ^{75}Se may be totally eliminated from selenoproteins during the course of electrophoresis. This can be avoided by running 10 μL of buffer containing 10% thioglycolate through the gel before the sample is loaded, or alternatively by including 5 mM 2-mercaptoethanol or DTT in the running buffer.

Recently, autoradiography is more conveniently and better performed using an imaging plate technique. Authors have noted that ^{75}Se radioactivity of 3000 cpm is enough for producing a clear image when the gel is attached on an imaging plate only for 2 hours. For the routine analysis, the exposure is usually performed overnight (Fig. 1).

3. Identification of Sec Residue

Identification of ^{75}Se-labeled Sec is the critical evidence for characterization of a selenocysteine-dependent selenoprotein[6,13]. Some proteins can bind elemental ^{75}Se or ^{75}Se-selenite with high affinity, others may contain significant amount of ^{75}Se-selenomethionine[14], and the resulting radioactive proteins may be confused with specific selenoproteins[15,16]. Sec can be identified on an amino acid analyzer but it requires chemical derivatization before the ^{75}Se-labeled protein is subjected to acid-hydrolysis. Sec can be almost completely decomposed when heated at 110°C in 6N HCl solution in the presence of trace amounts of oxygen (survival rate; 6%). Iodoacetate is frequently used for the protective derivatization of Sec residues, and the resulting *Se*-carboxymethyl-Sec (CM-Sec) can survive the entire procedure. A homologous derivatization can be carried out with 3-bromopropionate, which yields *Se*-carboxyethyl-Sec (CE-Sec). A combination of CM-Sec and CE-Sec can illustrate that the selenium moiety of the ^{75}Se-labeled protein is in the form of Sec as described in the following procedure.

Purified ^{75}Se-labeled protein (40 μg) was washed three times with distilled water and concentrated to 40 μL using a Centricon 10 microconcentrator (Amicon Inc., MA). The solution was mixed with 60 μL of 100 mM $NaBH_4$ in 20 mM NaOH aqueous solution, and incubated under argon at room temperature for 30 min. Sodium iodoacetate (or sodium 3-bromopropionate) was added to the mixture to the final concentration of 50 mM, and the mixture was incubated under argon at room temperature for 40 min. Then, 2-mercaptoethanol was added (10 μmol) to quench the alkylation reaction. The protein was washed three times with distilled water by ultrafiltration, taken to dryness, then hydrolyzed in 6N HCl at 155°C under argon. The hydrolysate was dried, treated with 100 μg of $NaBH_4$, mixed with authentic CM-Sec

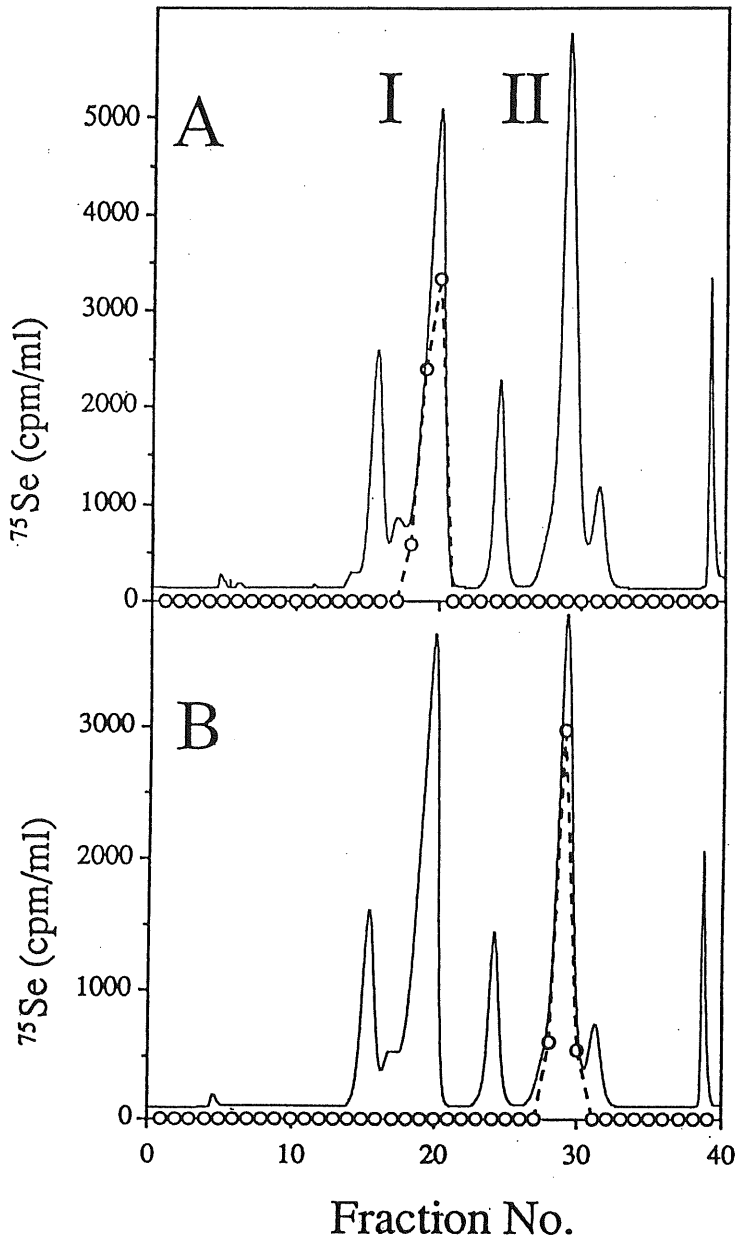

Fig. 1. Cells were grown in 50 mL of RPMI-1640 medium containing 10% fetal bovine serum and 680 kBq of ^{75}Se-labeled selenite for (1) 6, (2) 24, (3) 48, (4) 72, (5) 96 hours. The five samples were boiled in SDS-PAGE sampling buffer, and subjected to SDS-PAGE (left; Coomassie Brilliant Bule staining). The gel was dried, and subjected to autoradiography (right).

and CE-Sec (1.3 μmoles each), and chromatographed on an amino acid analyzer. The eluate from the analyzer column was collected in one minute fractions, and the radioactivity contained in these fractions was determined with a Beckman 5500 γ counter. On the amino acid analyzer, CM-Sec and CE-Sec are eluted at 19 and 29 min, respectively. When the labeled protein was alkylated with iodoacetate, the radioactive elution profile of the hydrolysate coincided exactly with CM-Sec. When 3-bromopropionate was used for the alkylation, ^{75}Se in the hydrolysate coincided with that of CE-Sec (Fig. 2). Throughout the procedure of alkylation, hydrolysis, and chromatography, the recoveries of ^{75}Se were 47% and 65% for CM-Sec and CE-Sec formation, respectively. ^{75}Se-Labeled selenomethionine was not detected in either experiment. The results clearly show that the selenium moiety of the ^{75}Se-labeled protein is Sec.

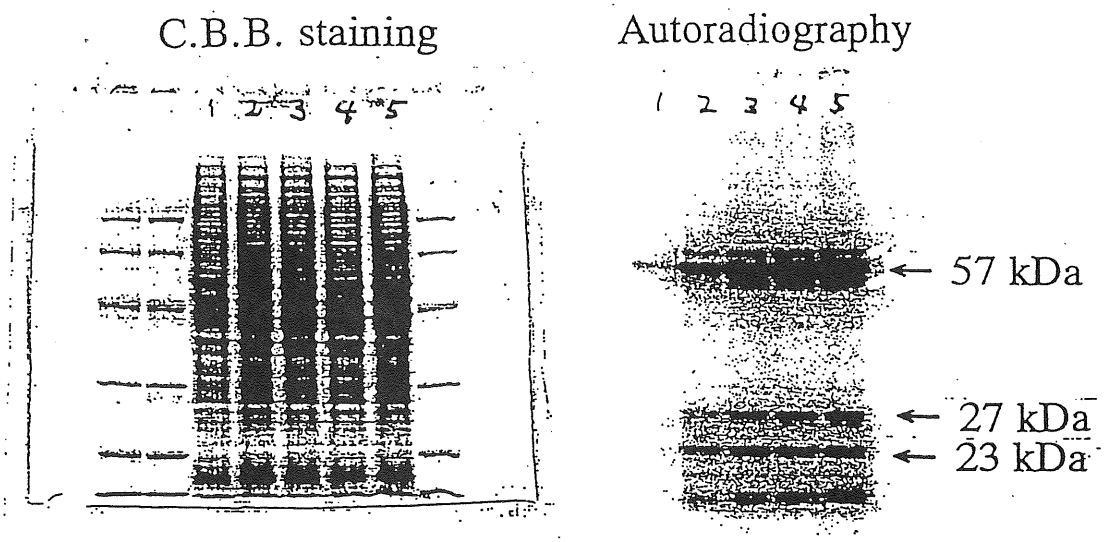

Fig. 2. Amino acid analyzer chromatogram of ^{75}Se-labeled compounds from acid hydrolysate of carboxymethylated (A) and carboxyethylated (B) selenoprotein. The hydrolysate was mixed with CM-Sec (I) and CE-Sec (II) before the chromatography. Solid lines represent amino acid elution and open circles represent ^{75}Se radioactivity.

4. Purification of Sec-containing Proteins

Purification of Sec-containing proteins can be carried out by conventional protein purification methods, if certain precautions are taken. First, Sec has a lower redox potential than cysteine, and it requires some reducing reagent to maintain the selenol state. In the absence of a reducing agent, it may undergo oxidation to seleninate ($-SeO_2H$) state, and consequently to dehydroalanine residue through α, β-elimination. Dithiothreitol (2 mM) is a favorite reagent for the present authors and it was always accompanied by EDTA-Mg,K complex (0.1 mM) to prevent metal-catalyzed thiol radical formation. Secondly, Sec residue is also a reactive residue toward various nucleophiles. Sodium azide is recommended to be kept out of the

buffer for gel filtration column chromatography.

^{75}Se-Labeled protein can be located in the column if one holds a Geiger-Muller counter close to the column tube. This physico-chemical property is particularly useful in estimating the elution of selenoprotein during gel filtration column chromatography.

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