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Separation and detection of selenium-containing proteins in human serum

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Abstract Selenium-containing proteins or their subunits in human serum were separated and detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the amount of selenium in each protein band was determined by HPLC with a fluorescence detector after derivatization with 2,4-diaminonaphthalene (DAN). This procedure provides a detection limit of 0.06 ng in a linear range of 0–1.5 ng. A protein is defined as a selenium-containing protein if its mean Se content exceeds twice the detection limit (0.12 ng) and twice the standard deviation of three replicates in sample determination. At least 4 selenium-containing bands with apparent molecular masses of 57–74, 46–56, 40–42 and 21–22 kDa could be detected from human serum collected from 4 volunteers.

Introduction

Selenium is an essential trace element to animals and humans. It has been related to a variety of human health problem [1]. Many papers have contributed to the biological role of selenium in mammalian cells and tissues. While about 30 selenium-containing proteins or subunits in tissues of mammals have been reported [2–6], only a few of them, glutathione peroxidases (GSH-Px) family, type-1 iodothyronine 5'-deiodinase and selenoprotein P, have been identified and sequenced in human tissues. Therefore, the search for new Se-containing proteins and the study of their characteristics and biological functions has been continuing.

Techniques used in separation and detection of selenoproteins in mammalian tissues include gel filtration chromatography [7,8], affinity chromatography [9–11], electrophoresis [7] and immunoassay [12]. By combining affin-

ity chromatography with electrothermal atomic absorption spectroscopy, selenium distribution among albumin, selenoprotein P and plasma GSH-Px in human plasma or serum was investigated rapidly and conveniently [13, 14]. But affinity chromatography as well as gel filtration can be used to separate only a group of proteins from a mixture, and they suffer from low resolution for protein separation. In addition, a large volume of effluents used during separation dilutes the sample, making the subsequent steps more difficult. Electrophoresis, such as SDS-PAGE, is a high-resolution technique for separating numerous proteins from a biological sample simultaneously, but the amount of protein in each band is so small that a very sensitive method for Se determination is required. In previous work, identification of Se-containing proteins in mammalian tissues has been successfully performed using electrophoresis after in-vivo labeling with ^{75}Se -selenite [5, 6]. However, the ^{75}Se -isotope technique can hardly be applied to studies in human tissues.

In this paper, an approach to the separation and determination of the Se-containing proteins in human serum has been proposed. The approach includes protein separation by SDS-PAGE and sensitive determination of selenium in the protein bands. The method for the determination of selenium consists of a pre-column derivatization with 2,4-diaminonaphthalene (DAN), followed by separation using high performance liquid chromatography (HPLC), with fluorimetric detection (FLD).

Materials and methods

Sample collection and pretreatment. Human blood samples were donated by 4 volunteers in the laboratory. Each 20 mL blood sample was put in a glass tube with neither anticoagulant nor heparin. The samples were left at room temperature for 20 min and were centrifuged at 2500 g for 15 min. The supernatant was transferred into a clean tube and combined with 6 volumes of Laemmli's buffer solution [15], which contained 50 mM dithiothreitol (Sigma) as a reducing agent, 20 μM leupeptin (Sigma), 0.5 mM phenylmethyl sulfonyl fluoride (PMSF, Sigma), as well as 50 μM chymostatin (Sigma) as protease inhibitors. After mixing, the sample was boiled for 10 min and the insoluble material was removed by centrifuga-

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tion at 10000 g for 5 min to obtain the test solution. This test solution was used for immediate protein separation by SDS-PAGE.

Protein separation by SDS-PAGE. SDS-PAGE was carried out according to Laemmli [15]. In a $200 \times 10.5 \times 3$ mm cell with 12 lanes, 6 lanes were loaded with blanks, the solution used for homogenizing the sample, and the others with test solution. Proteins in test solution were concentrated in 4% acryl amide gel and electrophoretically separated in 12.5% gel at constant voltage of 120 V for 8 h. To visualize the bands, the gel was then stained with Coomassie Brilliant Blue R-250 (Fluka) for 6 h (with 7% acetic acid and 30% methanol, GR, Beijing Chem. Co.) and destained in a mixture with 10% acetic acid and 30% methanol for two days. Visualized protein bands and the parallel blanks of the same width were separately sliced for Se determination.

The relative molecular weights of the proteins in the gel were calibrated by comparison with the following marker proteins (Shanghai Promega): phosphorylase B (rabbit muscle, MW = 97400), serum albumin (bovine, MW = 66200), ovalbumin (chicken egg, MW = 42700), carbonic anhydrase (bovine erythrocytes, MW = 31000) and lysozyme (chicken egg white, MW = 14400).

Selenium determination. Sliced gel bands from SDS-PAGE were digested in a mixture of nitric acid and perchloric acid (5:2 v/v, GR, Shanghai Chem. Co.). The samples were left for 10 h at room temperature and then heated to 80 °C until the sliced bands in the mixture disappeared. Then the temperature was elevated to 150 °C for 5 h and to 200 °C until white smoke appeared where the samples were left for another 30 min. After digestion, the reduction of selenate to selenite was achieved by adding an equal volume of concentrated hydrochloric acid and heating the tubes in a water bath (100 °C) until the fumes of nitrogen dioxide disappeared. After cooling the sample was derivatized by adding of 0.5 mL of 0.1 M EDTA and 1 mL of 2% hydroxylammonium chloride (GR, Beijing Chem. Co.). The pH of the solution was adjusted to 1.5–2.0 using 5 M $\text{NH}_3 \cdot \text{H}_2\text{O}$ or 5 M HCl (GR, Beijing Chem. Co.). After further adding of 0.5 mL of 1% 2,4-diaminonaphthalene (DAN, AG, Sigma) in 0.1 M HCl, which was purified by 7 times extraction with cyclohexane, the mixture was heated at 50 °C for 20 min and the fluorescent derivative, the naphtho-selenodiazol, was extracted into 0.5 mL of cyclohexane.

Fig. 1 Typical HPLC chromatograms, (a) DAN reagent before purification, (b) DAN reagent after extraction (7 times) with cyclohexane and (c) Se-containing protein with purified DAN

The mixture was centrifuged at 4000 g and 100 μL of the organic phase was injected into normal phase HPLC (Waters 201 HPLC W/510 with Shimadzu RF-535 fluorescence detector (366/520 nm) and μ -Porasil C_{18} - NH_2 column (mobile phase: cyclohexane/tetrahydrofuran (THF) 9:1, v/v). The signal for naphtho-selenodiazol in the chromatogram was characterized by its retention time that was determined using a naphtho-selenodiazol standard prepared in our laboratory. The relative fluorescent intensity was recorded as peak height for calculating the selenium content according to the calibration curve.

The stock solution of selenite (100 mg/L) was prepared by dissolving known amount of selenium dioxide (GR, Beijing Chem. Co.) in 0.1 M hydrochloric acid and diluted to a final concentration of 1 ng/mL before use. The calibration curve was obtained by adding 0–1.5 ng Se to distilled water, followed by the same procedure for selenium determination. Calibration was carried out together with sample measurements. The validity of the method for quantitative analysis were performed by using a certified reference material (rice flour with Se content of 70 ng/g, Japan Environmental Agency, NIES 10) and by the addition of selenite standard to human serum.

Detection of selenium-containing bands. Three replicates from each sample were subjected to the separation and Se determination. The mean Se content and the standard deviation for each protein band was calculated ($n = 3$) after subtracting the parallel blank bands of the same width. The protein bands with mean Se content exceeding twice the detection limits (i.e. 0.12 ng) and twice the standard deviations were defined as to be selenium-containing bands.

Results and discussion

Calibration of Se determination

For a sensitive determination of selenium, the blank has to be considered. Typical HPLC chromatograms for blanks are shown in Fig. 1, in which only DAN reagent was subjected to the thermal derivatization procedure before (Fig. 1a) and after extraction (7 times) with cyclohexane (Fig. 1b). The peak denoted with asterisk in the chromatogram arise from naphtho-selenodiazol and other peaks from impurities in commercial DAN reagent and its thermal decomposition

products when heated to 50°C. The interfering peaks of the reagent blanks can be reduced to a minimum by repeated extraction with cyclohexane but not totally eliminated. The purity of DAN is of utmost importance to the accuracy and precision of the method. Therefore purified DAN was used in all procedure for selenium determination. A chromatogram of sample determination with purified DAN was shown in Fig. 1c.

In conventional fluorimetric determination of Se with DAN, the detection limit can be as low as 0.4 ng/mL sample with a precision better than 7% [16]. The conventional fluorimetric method cannot discriminate the naphtho-selenodiazol peak from other fluorescent impurities, which result either from DAN reagent or its degraded products during thermal derivatization and cannot be eliminated simply by reagent purification. The illustrated HPLC separation after thermal derivatization of DAN allows identification of naphtho-selenodiazol peak from other fluorescent signals. For example, the total area in chromatogram Fig. 1b corresponds to 0.25 ng Se, which is equal to the fluorescent intensity obtained by conventional fluorometry, but the peak area of naphtho-selenodiazol (denoted with asterisk) corresponds only to 0.5 pg Se. Therefore, the interference could be removed from fluorescent signals by HPLC separation.

Interference may also be introduced during wet digestion of samples. To assess the interference from the whole procedure, 10 replicate determinations were performed on 2 mL of distilled water. The result was 0.08 ± 0.02 ng Se per sample, which corresponds to a detection limit of 0.06 ng Se (3 times the standard deviation of 10 duplicated determinations for 2 mL of distilled water). Obviously, the proposed method is one of the most sensitive

methods for selenium determination reported. To obtain this sensitivity, careful operation is required and EDTA and hydroxylammonium chloride should be repetitively purified. We found that no commercial sulfuric acid can be used in wet digestion procedure for this sensitive analytical method. It is also concluded that interference was mainly deduced by replacing wet digestion by a close-vessel microwave digestion using only purified nitric acid. We are still working in this direction.

Under optimal conditions, a calibration curve can be obtained in a range of 0–1.5 ng Se (Fig. 2) and a linear relationship between dosage of Se-selenite and fluorescent intensity can be observed ($r^2 = 0.9974$).

To validate the analytical procedure, a reference material, rice flour from Japan Environmental Agency (NIES 10), was used and a recovery of $95.2 \pm 1.5\%$ was obtained ($N = 3$). The results are shown in Table 1.

To test the recovery of Se from human serum, 0.60 ng of Se in form of selenite was spiked to a serum sample containing a measured amount of 0.54 ng Se. The results showed a recovery of $108.6 \pm 2.6\%$ ($N = 3$), as shown in Table 1.

Separation and detection of selenium-containing proteins in human serum

Figure 3 shows a typical photo of the stained gel from SDS-PAGE separation of human serum protein. A total of 29 bands in the gel could be visually distinguished. After the selenium content in each protein band was determined, only bands with a mean Se content exceeding twice the detection limit (or 0.12 ng Se) and twice the standard deviations for 3 replicates were considered to be selenium-containing bands. In this way, four bands were defined as selenium-containing proteins and the results are illustrated in Table 2.

Selenium is known to be incorporated into at least three human serum proteins [17, 18], i.e., albumin, selenoprotein-P (Se-P), as well as plasma glutathione peroxidase (p-GSH-Px). The widest band in Fig. 3 (MW = 57–74 kDa) is albumin, whose reported molecular weight is 69 kDa. This protein is the most abundant protein in human serum (about 60% of total in serum proteins). Albumin has so far not being reported as a specific selenoprotein. It was reported that Se can incorporate to albumin in form of selenomethionine which replaces the methionine in

Fig. 2 Calibration of selenium determination at ng levels

Table 1 Recoveries of spiked Se from certified material (rice flour) and human serum

^a 14 mg of rice flour with Se content of 70 ng/g from Japan Environmental Agency (NIES 10)

^b 0.01 mL of human serum (volunteer 1) and 0.6 ng Se-selenite

Rice flour ^a			Human serum ^b			
Sample ng	Found ng	Recovery %	Sample ng	Se-selenite added (ng)	Found ng	Recovery %
0.98	0.93	94.9	0.56	0.60	1.29	111.2
0.98	0.92	93.9	0.56	0.60	1.23	106.0
0.98	0.95	96.9	0.56	0.60	1.26	108.6
Mean $\pm$ SD	0.93 $\pm$ 0.02	95.2 $\pm$ 1.5			1.26 $\pm$ 0.03	108.6 $\pm$ 2.6

Fig. 3 Typical stained gel after SDS-PAGE separation of human serum. Right side: molecular weight of standard proteins, left side: selenium-containing protein bands identified

a non-specific and unregulated manner [14, 19, 20], or in form of selenide which binds to one of its 17 disulfide bonds [21, 22]. Since selenide would be lost in the process of SDS-PAGE operation, it is suggested that the Se binding should be in form of selenomethionine in albumin.

Selenoprotein P, the major selenoprotein in serum, is a glycoprotein with molecular weight of 57 kDa. It is the only selenoprotein found containing multiple selenocysteine residues [23]. It takes from 39 to 69% of total selenium in human plasma and the percentage distribution depends on human Se nutritional state [13, 14, 19]. Accordingly, selenoprotein P could also be present in the band of 57–74 kDa in Fig. 3. The protein band for albumin is so wide that the protein band representing selenoprotein P cannot be separated from the band in our study.

Recent studies have revealed that there exists at least two isoforms of the protein in rat plasma [24–26]. One isoform, migrating at 45 kDa on SDS-PAGE, is a truncated protein with termination of protein synthesis occurring at the second selenocysteine codon. The selenium-containing protein present in 46–56 kDa band could be probably an isoform of selenoprotein P similar to that in rat plasma.

The band at MW 21–22 kDa could be the subunit of plasma GSH-Px, which has been purified from human plasma [27, 28]. GSH-Px is a tetramer composed of four identical 22 kDa subunits and each subunit contains one selenocysteine residue at its active site.

Among the four selenium-containing protein bands detected, the 40–42 kDa protein band has not been reported so far. Since the protease inhibitors were added to the serum immediately and the separation of proteins by SDS-PAGE was completed in about 10 h after sampling, it is unlikely the fragment of large selenoprotein produced by accidental proteolytic decomposition. Incomplete dissociation of multimeric protein into their subunits in the process of SDS-PAGE might result in some more selenium-containing bands originating from the protein, but it is possibly a selenium-containing protein, which has not been reported so far. Since this work is only a preliminary screening of selenium-containing proteins in human serum, further studies to confirm the protein bands are necessary.

Selenium distribution in human serum

The distribution of selenium in serum proteins depends to a large extent on its chemical forms and their bioavailability in the diet [19]. For example, more Se in form of glutathione peroxidase was found in human plasma when supplemented with selenate than that supplemented with selenomethionine [20]. The distribution of Se in human serum is affected also by Se intake or Se status in human body. Previous research has shown that the lower the blood Se concentration, the greater the percentage of total erythrocyte selenium associated with glutathione peroxidase [29]. The incorporation of selenium into selenoprotein-P is in preference to that into the plasma glutathione peroxidase at a low selenium status in plasma [30, 31]. Other factors, such as species and the physiological state, may also influence speciation of selenium in biological materials [32]. The Se distribution among 4 serum protein bands, as

Table 2 Se content in protein bands of different molecular weight in serum of four healthy adults

Volunteer	Serum (ng Se/mL)	Loading ^a (ng Se)	Protein band with molecular weight of ^b				Recovery (%)
			57–74 kDa	46–56 kDa	40–42 kDa	21–22 kDa	
1	56 ± 4	1.85	0.68 ± 0.05 (43.6)	0.19 ± 0.06 (12.2)	0.36 ± 0.04 (24.0)	0.33 ± 0.06 (21.2)	84.3
2	72 ± 8	2.38	0.95 ± 0.26 (42.4)	0.46 ± 0.05 (20.5)	0.32 ± 0.13 (14.3)	0.51 ± 0.13 (22.8)	94.1
3	115 ± 5	3.80	1.03 ± 0.07 (35.6)	0.78 ± 0.25 (27.0)	0.36 ± 0.12 (12.5)	0.72 ± 0.18 (24.9)	76.0
4	131 ± 5	4.32	1.02 ± 0.20 (30.4)	1.10 ± 0.02 (32.8)	0.45 ± 0.17 (13.4)	0.78 ± 0.04 (23.4)	77.5

^a 33 µL serum in SDS-PAGE for a single run

^b Data in parenthesis show the percentile distribution of Se in each Se-containing protein band with respect to the total recovered Se

listed in Table 2, was only informative, because of the limited sample volume in this study. Nevertheless, one can find from the 4 samples that the serum albumin and/or selenoprotein P (MW = 57–74 kDa) were the dominant forms in human serum, which takes about 30–43% of the total recovered Se. Combined with Se present in 46–56 kDa band that may be an isoform of selenoprotein P, albumin and selenoprotein P can take about 58–63% of total recovered Se. The result is similar to that reported by Harrison [13] and Plecko [14]. (In conventional Sephadex gel chromatography, these two bands can hardly be separated.) Se in glutathione peroxidase (MW = 21–22) was obviously dose-dependent and was saturated when serum Se exceeds 0.1 µg/L. Similar results have been reported previously by measuring the enzymatic activity of glutathione peroxidase [1].

In the last column of Table 2, we also give the recoveries of selenium after SDS-PAGE separation. The recoveries for serum samples vary from 76 to 94.1%. This is an indicator for the existence of selenium-containing proteins that cannot be detected by the present method or were neglected according to our criteria of selection. Also, selenium in serum may associate with serum proteins nonspecifically or weakly, or may be present as small molecules, such as selenoamino acids, selenide, etc., which would be lost from the gel during the separation procedure. The technique applied in the present study can distinguish specific selenoproteins; whereas other procedures, such as Sephadex chromatography, can hardly distinguish specific bounded from loosely bounded selenium to proteins, thus causing error in investigation of Se distribution. While the proposed approach has been adapted for the speciation of selenium in human serum, it was still constrained by its detection limit. As discussed before, the interference attributed by Se in DAN thermal derivation procedure is only 0.5 pg, while the interference attributed to Se contained in the chemicals used and caused by contamination in wet digestion is 0.08 ng or 80 pg. There is potential to further reduce interference by clean digestion technology, so that more selenoproteins can be identified on the gels. On the other hand, preparative SDS-PAGE technique can be used to separate more amounts of proteins, which can improve the accuracy of selenium determination.

Conclusions

By combining SDS-PAGE and HPLC-FLD, a method for the separation and detection of selenium-containing proteins in human serum has been proposed. The procedure provides a detection limit of 0.06 ng and selenium recoveries of 76–94%. The selenium distribution was determined as 30.4–45.6% of recovered Se present in band of 57–

74 kDa, 12.2–32.8% in band of 46–56 kDa, 12.5–24% in band of 40–42 kDa and 21.2–24.9% in band of 21–22 kDa. The procedure can also be used to investigate selenium distribution in human tissues.

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