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Determination of Nanoscale Elemental Selenium Content in Selenium-Enriched Proteoglycans by High-Performance Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry Postprint

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Abstract

This study aimed to establish an efficient, accurate, and rapid method for the determination of nano-elemental selenium content in selenium-enriched protein polysaccharides. A 25 mg sample of selenium-enriched protein polysaccharide produced by Enterobacter cloacae Z0206 was suspended in 3 mL of distilled water and sonicated for 30 min at 37 °C in the presence of 3 mg of type XIV protease. The precipitate (nano-elemental selenium) obtained by centrifugation of the enzymatic hydrolysate was digested at room temperature using a hydrogen peroxide-hydrochloric acid (H2O2-HCl) system, and the resulting solution was directly analyzed by high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) [using a PRP X100 anion-exchange column to separate SeO3ˆ2- in the sample, with a mobile phase consisting of 5 mmol/L aqueous citric acid solution (pH 4.5)], yielding a nano-elemental selenium content of 2,439 g/g in the sample. Using the national standard (GB/T 13883-2008) [where the sample is digested with a nitric acid (HNO3)-perchloric acid (HClO4) system and analyzed by hydride generation-atomic fluorescence spectrometry (HG-AFS)] as a control, the measured nano-elemental selenium content in the sample was 2,450 g/g. The results obtained by the two methods were highly consistent. Compared with the method described in GB/T 13883-2008, the oxidation system established in this experiment is milder, faster, and easier to control, making it suitable for rapid detection of nano-elemental selenium.

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Full Text

Measurement of Nano Elemental Selenium in Selenium-Enriched Polysaccharides Using High Performance Liquid Chromatography Coupled With Inductively Coupled Plasma Mass Spectrometry

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Abstract

This experiment was conducted to establish an efficient, accurate, and rapid method for determining nano elemental selenium (Nano-Se) content in selenium-enriched polysaccharides. A 25 mg sample of selenium-enriched polysaccharides produced by *Enterobacter cloacae* Z0206 was suspended in 3 mL distilled water and subjected to ultrasonic treatment for 30 min at 37 ℃ with 3 mg protease type XIV. The precipitate (nano elemental selenium) obtained after centrifugation of the enzymatic hydrolysate was digested at room temperature using a hydrogen peroxide-hydrochloric acid (H O -HCl) system. The resulting solution was directly analyzed by high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) using a PRP X100 anion exchange column with a mobile phase consisting of 5 mmol/L citric acid solution (pH 4.5). The nano elemental selenium content in the sample was determined to be 2,439 g/g. Using the national standard method (GB/T 13883-2008) as a control—where samples are digested with nitric acid (HNO)-perchloric acid (HClO) and analyzed by hydride generation-atomic fluorescence spectrometry (HG-AFS)—the nano elemental selenium content was measured at 2,450 g/g. The results from both methods showed excellent agreement. Compared with the method described in GB/T 13883-2008, the oxidation system established in this study is milder, faster, and easier to control, making it suitable for rapid detection of nano elemental selenium.

Keywords: high performance liquid chromatography; inductively coupled plasma mass spectrometry; selenium-enriched polysaccharides; nano elemental selenium

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Introduction

Selenium (Se) is an essential nutrient for many animals. China' s "Feed Additive Catalogue (2013)" lists sodium selenite and selenium yeast as mineral feed

additives for selenium supplementation in livestock. While sodium selenite is abundant and inexpensive, it suffers from low bioavailability, a narrow margin between toxic and required doses, high toxicity, and environmental pollution risks, leading to strict limitations on its usage. Several countries and regions, including the European Union, have restricted or banned sodium selenite as a nutritional supplement; for example, Sweden has limited its use in piglet feed, and Japan has prohibited its addition to animal feed altogether. Consequently, there is a growing trend toward replacing sodium selenite with other forms of selenium in feed applications.

Naturally occurring selenium exists primarily as selenides, elemental selenium, selenates, selenites, and organic selenium compounds. Selenites and selenates have a very narrow margin between toxic and required doses, whereas elemental selenium was historically considered both non-toxic and biologically inactive. However, red nano elemental selenium particles with diameters ranging from 20–350 nm have attracted increasing attention due to their remarkable safety profile and biological activity. Numerous studies have demonstrated that certain bacteria can effectively reduce sodium selenite to red nano elemental selenium, thereby mitigating toxicity. Furthermore, when nano elemental selenium is dispersed on bacterially secreted proteins or polysaccharides, it significantly enhances the antioxidant activity and other functional properties of selenium-enriched polysaccharide products.

In our previous research, a selenium-tolerant exopolysaccharide-producing bacterial strain Z0206 was obtained through adaptive evolution and identified as *Enterobacter cloacae* through morphological, physiological, biochemical, and 16S rDNA gene sequence analysis. *Enterobacter cloacae* Z0206 can tolerate high concentrations of sodium selenite and produces substantial amounts of extracellular protein polysaccharides, demonstrating strong antioxidant and immune-enhancing functions in mice and poultry. Recent studies have revealed that a portion of the selenium exists in the zero-valent state with nanoparticle dimensions, identified as nano elemental selenium. This research focuses on the quantitative analysis of nano elemental selenium.

Conventional techniques for determining zero-valent selenium typically involve open wet digestion or microwave-assisted closed digestion. Reported methods generally involve adding strong oxidants such as nitric acid (HNO) or perchloric acid (HClO) to samples, followed by repeated heating reflux or microwave digestion. For instance, the national standard method for selenium determination in feed (GB/T 13883-2008) requires overnight digestion in mixed acid (HNO -HClO) followed by repeated heating on a hot plate the next day. These methods operate on the principle of oxidizing zero-valent selenium to tetravalent selenium (Se) before detection. However, these procedures are time-consuming, require large amounts of reagents, demand extreme caution when handling high-concentration acid media, and necessitate additional acid removal steps to prevent instrument damage and signal suppression during subsequent HPLC-ICP-MS analysis. Therefore, this study improves upon traditional digestion methods

by replacing HNO and HClO heating digestion with a room-temperature reaction using a hydrogen peroxide (H O)-hydrochloric acid (HCl) system to oxidize nano elemental selenium, yielding a solution suitable for direct HPLC-ICP-MS detection. This approach enables rapid, accurate, and safe determination of nano elemental selenium in selenium-enriched polysaccharides, with the entire sample processing cycle controlled within 2 hours.

Materials and Methods

1.1.1 Reagents

Protease type XIV (produced by *Streptomyces griseus*), selenium powder (purity 99.95%), sodium selenite standard (purity 98%), and HPLC-grade methanol were purchased from Sigma-Aldrich (USA). Citric acid, HCl, 30% H O , potassium borohydride (KBH), and potassium hydroxide (KOH) were all analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd.

1.1.2 Instruments

The major instruments included: a Nexion 300 HPLC-ICP-MS system (PerkinElmer, USA); a KQ-500E ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); a Millipore ultrapure water system (EMD Millipore, Germany); a Mettler Toledo analytical balance (Switzerland); an ST 40R centrifuge (Thermo, USA); a Mettler Toledo pH meter (USA); and an AFS-8220 hydride atomic fluorescence spectrometer (Beijing Jitian Instrument Co.) equipped with a high-performance hollow cathode lamp (wavelength: 196.0 nm, General Research Institute for Nonferrous Metals, Beijing).

1.2 Preparation of Selenium-Enriched Polysaccharide Samples

Following previously reported methods from our research group, sodium selenite was added to *Enterobacter cloacae* Z0206 cultures during deep fermentation. The fermentation broth was first centrifuged to remove mycelia, then the supernatant was precipitated with 95% ethanol, and the precipitate was freeze-dried to obtain selenium-enriched polysaccharide samples, which served as the experimental material.

1.3 Sample Pretreatment

A 25 mg sample of selenium-enriched polysaccharide was weighed into a 5 mL centrifuge tube, and 3 mg protease type XIV was added along with 3 mL distilled water. The mixture was ultrasonicated for 30 min at 37 °C. The enzymatic hydrolysate was then centrifuged for 30 min at 10,000 r/min, and the precipitate was collected. The precipitate was washed by adding 3 mL distilled water, mixing, and centrifuging again at 10,000 r/min for 30 min. After discarding the supernatant, 100 L of 6 mol/L HCl was added to the precipitate, followed by

3 mL H O , and the mixture was reacted at room temperature for 30 min. The reaction solution was transferred to a volumetric flask and diluted to 500 mL with distilled water, then filtered through a 0.45 m membrane before HPLC-ICP-MS analysis.

1.4 Preparation of Standard Curve

Accurately weighed 25.0 mg sodium selenite was dissolved in 250 mL water and serially diluted to prepare standard working solutions at concentrations of 0.500, 0.300, 0.200, 0.050, and 0.025 g/mL for HPLC-ICP-MS analysis.

1.5.1 HPLC-ICP-MS Analysis Conditions

The analytical conditions were as follows: PRP-X100 anion exchange column (250 mm × 4.1 mm × 10 m, Hamilton, USA); injection volume 25 L; mobile phase 5 mmol/L citric acid solution (pH 4.5); flow rate 0.8 mL/min. ICP-MS parameters: RF power 1,100 W; plasma gas (argon) flow 16.0 L/min; auxiliary gas (argon) flow 1.3 L/min; nebulizer gas (argon) flow 0.91 L/min; collision gas (helium) flow 3 mL/min; mass-to-charge ratio 78.

1.5.2 HG-AFS Analysis Conditions

Instrument parameters: photomultiplier tube negative high voltage 290 V, atomizer height 8 mm, hollow cathode lamp current 80 mA, carrier gas flow 300 mL/min, shield gas flow 800 mL/min, carrier solution 5% HCl, injection volume 0.5 mL. The selenium standard working solution concentration was 10 ng/mL. For standard curve preparation, 5% HCl was used as diluent, and the instrument automatically diluted to series concentrations of 2, 4, 6, 8, and 10 ng/mL. All samples were measured in triplicate, and average values were calculated.

1.6 Data Processing and Analysis

Data were processed and calculated using Excel 2010 statistical analysis software, with results expressed as mean values.

Results

2.1 Sample Pretreatment of Selenium-Enriched Polysaccharides

After pretreatment, the precipitate obtained from final centrifugation is shown in [Figure 1: see original paper]-A. Upon addition of H O and HCl with shaking, the precipitate became well dispersed ([Figure 1: see original paper]-B). After standing at room temperature for 30 min, all samples appeared clear, colorless, and transparent ([Figure 1: see original paper]-C). This phenomenon indicates that red nano elemental selenium can be rapidly digested into a colorless solution state by the H O -HCl system at room temperature.

2.2 Linear Range and Accuracy

The chromatogram of sodium selenite standard working solution is shown in [Figure 2: see original paper], with a retention time of 5.008 min. Using sodium selenite concentration as the x-coordinate and the integrated area of Se as the y-coordinate, the standard curve equation and correlation coefficient for selenite (SeO ²) concentration were calculated as: y = 167,993.58x + 2,364.94 (R = 0.999).

To validate the digestion method, nano elemental selenium precipitates obtained by centrifugation were simultaneously digested using both H O -HCl and HNO -HClO systems, followed by HPLC-ICP-MS and HG-AFS analysis, respectively. The chromatogram of nano elemental selenium digested by the H O -HCl system is shown in [Figure 3: see original paper]. HPLC-ICP-MS analysis determined the nano elemental selenium content to be 2,439 g/g (), while the HNO -HClO digestion with HG-AFS analysis yielded a content of 2,450 g/g. The results from both methods were remarkably consistent.

Discussion

3.1 Effect of Enzymatic Hydrolysis on Quantitative Analysis of Nano Selenium

Selenium in selenium-enriched polysaccharides exists in various forms, including nano selenium, organic selenium, and possibly selenite. To effectively extract nano elemental selenium, this study employed enzymatic hydrolysis to break down macromolecular selenium-containing compounds into water-soluble small molecules, thereby separating them from nano selenium. Among the enzymes evaluated—including protease type XIV, proteinase K, and pronase—protease type XIV completely hydrolyzed the sample within 30 min, and ultrasonic treatment could effectively replace conventional shaking incubation (48 h). The addition of protease type XIV hydrolyzed proteins in the sample, allowing hydrophobic nano elemental selenium to precipitate under centrifugation while water-soluble selenoamino acids and any sodium selenite remained in the supernatant, achieving effective separation.

3.2 Comparison of H O -HCl and HNO -HClO Digestion Methods

The current national standard for selenium determination in feed (GB/T 13883-2008) requires overnight digestion in mixed acid (HNO -HClO) followed by repeated heating on a hot plate. Complete digestion is judged by perchloric acid fuming and color changes, requiring extreme caution to prevent sample dryness. Adapting this method for nano elemental selenium detection in selenium-enriched polysaccharides would be both time-consuming and cumbersome.

The oxidizing effect of H O in selenium determination has been confirmed in

previous studies. The reaction principle is as follows: Se + H O + HCl → H SeO + Cl + 2HCl + 3H O . Traditional selenium determination typically employs HG-AFS, where samples digested with HNO -HClO are reduced from Se to Se in HCl medium, then further reduced to selenium hydride (SeH) using KBH for detection. This process requires overnight standing, generally taking over 12 hours per sample. In contrast, our H O -HCl system required only 100 L of 6 mol/L HCl and 3 mL H O with 30 min at room temperature to completely oxidize nano elemental selenium to SeO ² . Using the H O -HCl digestion with HPLC-ICP-MS detection yielded a nano elemental selenium content of 2,439 g/g, while the HNO -HClO digestion with HG-AFS gave 2,450 g/g. The results were essentially identical. Compared with traditional HNO -HClO -based methods, our established method is rapid, simple, environmentally friendly, highly accurate, and safe, making it suitable for rapid detection of nano elemental selenium.

Conclusions

1. This study successfully established an ultrasonic-assisted enzymatic hydrolysis method using protease type XIV for selenium-enriched polysaccharides. By suspending the sample in water and ultrasonicating at 37 ℃ for 30 min with protease type XIV, complete enzymatic hydrolysis was achieved.

2. Nano elemental selenium obtained from centrifugation of the enzymatic hydrolysate can be completely digested to SeO ² by the H O -HCl system at room temperature. The precipitated nano elemental selenium reacted completely within 30 min at room temperature under the action of 100 L of 6 mol/L HCl and 3 mL H O , producing detectable SeO ² .

3. This study developed a direct HPLC-ICP-MS analytical method for quantitative detection of SeO ² in the reaction solution. Using a PRP X100 anion exchange column with 5 mmol/L citric acid solution (pH 4.5) as mobile phase at 0.8 mL/min flow rate, HPLC-ICP-MS can complete SeO ² analysis within 10 min.

4. The nano elemental selenium content in selenium-enriched polysaccharides determined by our established method showed high consistency with results from the classic HNO -HClO digestion HG-AFS method, demonstrating that our method is rapid, simple, environmentally friendly, highly accurate, and safe, making it suitable for rapid detection of nano elemental selenium.

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