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Analytical Methods

Extraction of Se species in buckwheat sprouts grown from seeds soaked in various Se solutions

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ABSTRACT

The transformation of selenium (Se) in buckwheat sprouts grown from seeds soaked in various Se solutions (Se-methionine (10 mg Se L^{-1}), selenate or selenite (5, 10, 20 mg Se L^{-1})) was investigated. The extraction procedure was optimised by (a) using optimal extraction media (water, phosphate buffer, 0.1, 0.2, 0.3 M HCl, the enzyme protease alone or in combination with cellulase, amylase or lipase), and by (b) optimising the ratio between sample and enzyme. For Se speciation analysis extracts with the highest percentage of soluble Se were analysed, and additionally the stability of the extracts was investigated. The results showed that uptake of Se by sprouts was dependent on the form and concentration of Se in the solution used for soaking. Optimal extraction efficiencies were obtained by hydrolysis with 0.3 M HCl and protease. Selenate (23.7–29.7% from Se(VI) sprouts and in trace amounts from Se(IV) and SeMet sprouts), Se-methionine (2.4–7.9%) and selenite (traces) were detected in all supernatants, regardless of soaking solution.

1. Introduction

Se is an essential nutrient for animals and humans, and forms the active centre of Se-containing enzymes (selenoenzymes), such as glutathione peroxidase, thioredoxin reductase, and iodothyronine deiodinase. As Se plays an important role in human nutrition, it is important that the diet is sufficiently supplied with it. The Se content of food varies depending on the Se content of the soil where the animal was raised or the plant was grown: organ meats and seafood, 0.4–1.5 μg g^{-1}; muscle meats, 0.1–0.4 μg g^{-1}; cereals and grains, less than 0.1 to greater than 0.8 μg g^{-1}; dairy products, less than 0.1–0.3 μg g^{-1}; and fruits and vegetables, less than 0.1 μg g^{-1} (WHO, 1987). The Se supply in almost all European countries is below the recommended daily intake. In these countries, Se fortification of foods and the use of Se supplements are quite popular to compensate for the low Se intake from diet (Lintschinger, Fuchs, Moser, Kuehnelt, & Goessler, 2000). Moreover, field treatment with Se or direct supplementation of food and fodder is not enough. The chemical form in which Se is present in the food used is of principal importance to improve the Se nutrition of livestock and people.

Nowadays, common buckwheat is becoming an increasingly important alternative crop in Europe. It is used as food (grain, sprouts) or herb (plant or leaves for herbal tea) (Smrkolj, Stibilj, Kreft, & Germ, 2006). Since the nutritional characteristics of this plant, like the high content of proteins and essential amino acids, suggest that Se is preserved as selenoamino acid derivatives, in particular, selenomethionine in proteins, similar to selenized yeast. Buckwheat sprouts are prized for their subtle, nutty flavour and high nutritional value. They are widely available and consumed in Japan and Korea. Therefore buckwheat is expected and known to be (Lintschinger et al., 2000) a good nutritional source of Se.

Smrkolj et al. (2006) reported that SeMet is one of the main selenocompounds in Se-enriched buckwheat seeds. In addition, Kitaguchi, Ogra, Iwashita, and Suzuki (2008) confirmed that the main Se species present in Se-enriched seeds of buckwheat, treated with barium selenate, was SeMet. Lintschinger et al. (2000) treated sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*) and alfalfa (*Medicago sativa*) seeds with selenate solution. The sunflower sprouts obtained were resistant and had the highest uptake rates, but almost 100% of the Se was extracted with water and found to be nonmetabolized selenate. The metabolism of selenate by wheat, alfalfa was inversely related to the uptake rates. At low Se enrichment <20% of the total Se content within the sprouts remained as inorganic Se, indicating a high metabolic rate (Lintschinger et al., 2000). Comparable results were reported by Chan, Afton, and Caruso (2010). In the selenite-enriched soybean root and leaf, inorganic Se utilised over 90% of the peak areas, and only a small portion of Se was converted to organoselenium compounds. In contrary, SeMet and $SeCys_2$ were the predominant Se compounds found in the bean (Chan et al., 2010). Sprouts of several edible plants (10 families and 28 species) were cultivated hydroponically in a high Se environment (10 μg mL^{-1} of Se as selenite), and the

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chemical species of Sein these Se-enriched sprouts were identified by Sugihara et al. (2004). They reported that by performing speciation analysis of the HCl extract, SeMeSeCys was found to be the main Se species in all sprouts. In addition SeMet, Se(IV), γ-glutamyl-Se-methylselenocysteine and an unknown Se compound were detected in several high Se sprouts.

Recently, the speciation of Se-containing molecules, such as selenoproteins and selenometabolits, has been primarily carried out by means of extraction procedures, mainly using the non-specific enzyme protease, followed by separation and detection by a hyphenated technique, namely high performance liquid chromatography (HPLC) in conjunction with inductively coupled plasma mass spectroscopy (ICP-MS), hydride generation atomic absorption spectrometry (HG-AAS) or hydride generation atomic fluorescence spectrometry (HG-AFS) (Dumont, Vanhaecke, & Cornelis, 2006; Mazej, Falnoga, Veber, & Stibilj, 2006; Polatajko, Jakubowski, & Szpunar, 2006).

The present study investigates the metabolism of Se in buckwheat sprouts from seeds soaked in various Se solutions in order to investigate the uptake of Se and to identify the Se species present in the sprouts. Therefore, the extraction procedure was optimised by (a) using optimal extraction media (water, phosphate buffer, hydrochloric acid, the non-specific enzyme protease alone or in combination with the specific enzymes cellulase, amylase or lipase), and by (b) optimising the ratio between sample and enzyme. In addition, extract stability was studied. The accuracy of the results obtained by HPLC-UV-HG-AFS was checked by HPLC-ICP-MS.

2. Materials and methods

2.1. Reagents and standards

The following chemicals were used: 96% H_2SO_4 (Merck, Suprapur), 65% HNO_3 (Merck, Suprapur), 30% HCl (Merck, Suprapur), 36% HCl (Merck, p.a.), 30% H_2O_2 (Merck, p.a.), V_2O_5 (Merck, p.a.), NaOH (Merck, puriss p.a.) and $NaBH_4$ (Fluka, Purum p.a.), $(NH_4)_2HPO_4$ (Fluka Chemie, puriss p.a.), pyridine (Fluka Chemie, puriss p.a.), diammoniumhydrogen citrate (Fluka Chemie, puriss p.a.), citric acid (Fluka Chemie, puriss p.a.), MeOH (Primar, Fisher Scientific UK, trace analysis grade), protease XIV from *Streptomyces griseus* (type XIV: bacterial, 4.4 units/mg solid; Sigma P-5147), α-amylase from porcine pancreas (type VI-B, 19.6 units/mg solid; Sigma A-3176), cellulase from *Aspergillus niger* (0.57 units/mg solid; Sigma C-1184), lipase from porcine pancreas (type II, 30–90 units/mg protein, Sigma L-3126).

For preparation of Se solutions for seed soaking and Se analysis, Na_2SeO_3 (Se(IV), Sigma–Aldrich, >98%), Na_2SeO_4 (Se(VI), Sigma–Aldrich, SigmaUltra), selenomethionine (SeMet, Fluka Chemie, >99%), selenocystine ($SeCys_2$, Fluka Chemie, >98%) and selenomethylselenocysteine (SeMeSeCys, Fluka Chemie, >98%) were used. Stock solutions of Se species containing about 1 mg Se g^{-1} in water were prepared and kept at 4 °C. For preparation of solutions and sample treatment, ultra-pure water (Milli Q, Millipore Corporation, Bedford, MA, USA) was used. For Se speciation analysis standards were prepared at concentrations of approximately 100 ng Se per g for each species in supernatants of the control group of buckwheat sprouts, to check for the possible different retention times of Se species caused by matrix interactions in the measurement system.

2.2. Germination and sample preparation

Common buckwheat (*Fagopyrum esculentum*) seeds, cultivar Darja, were bought in a Seedman's Shop in Ljubljana, Slovenia. About 500 mL (346 ± 30 g) of seeds (average seed mass was 0.026 ± 0.001 g) were soaked in 500 mL of MilliQ water, or a solution of sodium selenate (5, 10, 20 mg Se L^{-1}), or of sodium selenite (5, 10, 20 mg Se L^{-1}), or of SeMet (10 mg Se L^{-1}). Seeds were soaked for 4 h, than were separated from the solution, weighed to obtain the mass of water and Se absorbed, and distributed equally in plastic bowls, which were covered with filter paper.

During germination seeds were treated with tap water (Se content below detection limit), as needed. Buckwheat sprouts whose seeds were soaked in (i) Se(VI) solution, were grown in April for 22 days, when the average day temperature was 13 °C (13 h of daylight) and the average night temperature was 8 °C. Buckwheat sprouts, whose seeds were soaked in (ii) Se(IV) solution were treated in May for 8 days. The average day temperature was 25 °C (15 h of daylight) and the average night temperature was 18 °C. Seeds soaked in (iii) solutions of SeMet or Se(VI) or Se(IV) at a concentration 10 mg Se L^{-1} were treated in July for 11 days. Sprouts were treated at 22 °C in a bright air-conditioned room (16 h of daylight). For every treatment a control group (seeds soaked in water) was included. Sprout sampling (harvest) was done when the buckwheat sprouts developed two extended cotyledon leafs. The whole sprouts were taken, including the cotyledons and roots. Six replicates of a hundred randomly chosen sprouts were weighed to obtain the average sprout mass. Buckwheat sprouts whose seeds were soaked in Se(VI), Se(IV) and SeMet solution are named Se(VI), Se(IV) and SeMet sprouts in the following text.

For analysis, buckwheat sprouts were lyophilised at −50 °C and 0.050 mbar (CHRIST ALPHA 1–4, LOC-1, freeze-dryer), milled and homogenised in a planetary micro mill (FRITSCH, Pulverisette 7, Idar-Oberstein, Germany; speed 6, time 8 min with additional 2 min at speed 7). Finally, samples were sieved through a 0.25 mm nylon sieve.

2.3. Determination of total Se concentration

Digestion was carried out on 0.2 g of homogenised sample. This was weighed in a Teflon tube and mineralisation performed using HNO_3 (1.5 mL) and H_2SO_4 (0.5 mL) by heating the closed tube in an aluminium block, kept at 80 °C overnight and then for 1 h at 130 °C. After cooling, 2 mL of hydrogen peroxide was added and the tubes were heated for 15 min at 115 °C. This step was repeated. After the solution had cooled to room temperature, 0.1 mL V_2O_5 in H_2SO_4 was added and the tube reheated at 115 °C until the solution became blue in colour. To reduce selenate to selenite 2.5 mL of HCl was added to the solution and heated at 100 °C for 10 min. Samples were diluted with Milli Q water. Sensitive detection was achieved by HG-AFS with the chemical and instrumental operating conditions according to Smrkolj and Stibilj (2004). Working standard solutions of Se(IV) were prepared daily by dilution of a stock standard solution with a solution containing appropriate amounts of H_2SO_4 and HCl to obtain the same acid media as in the samples. To check the accuracy and precision of the method a standard reference material representing a similar matrix (NIST SRM 1570a, Trace Elements in Spinach Leaves) was analysed simultaneously.

2.4. Extraction and speciation

Extraction of the sprouts was performed in triplicate by adding separately (a) 12 g of water, 25 mM phosphate buffer (pH 7.5), HCl (0.1, 0.2, 0.3 M), a solution of 25 mM phosphate buffer (pH 7.5) containing protease (1, 10, 50, 90, 150 mg) separately or in combination with α-amylase (150 mg) or cellulase (150 mg) or lipase (150 mg) to 900 mg of dry sample, or (b) the sample in 25 mM phosphate buffer (pH 7.5) was frozen in liquid nitrogen and unfrozen in hot water three times. Additionally, 150 mg protease was added. In all cases the duration of incubation was 24 h at 37 °C. After the extraction procedure extracts were centrifuged at