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Investigation of Se-containing proteins in *Bertholletia excelsa* H.B.K. (Brazil nuts) by ICPMS, MALDI-MS and LC–ESI-MS methods

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ABSTRACT

Owing to recent studies which showed that Se supplementation in the diet can reduce the risk of several forms of cancer, efforts have been directed to identify anticarcinogenic activity in Se compounds from natural sources with the objective of using those as food supplements. As a part of a continuing research project directed at identifying Se-containing species in food stuffs, the distribution of selenium-containing proteins in water-soluble protein fraction of seeds of *Bertholletia excelsa* H.B.K., which typically have a high Se content, was studied under non-denaturing conditions and the effectiveness of protein fractional precipitation by ammonium sulfate was investigated with the objectives to preconcentrate the protein(s) of interest and to reduce the matrix complexity. By SEC–ICPMS, Se-containing proteins (selenoproteome with some additional proteins) were found demonstrating the usefulness of ICPMS as an "on-line assay" in sub-proteomics investigations. Fractions with Se-containing proteins, collected from SEC, were tryptically digested and tryptic digests were analyzed by MALDI-TOF-reflectron MS and capLC–ESI-QTOF-MS. Isotope patterns which are different from the typical isotope patterns of peptides containing C, H, O, N and S were used to identify selenium-containing peptides in the digests. Observed isotope patterns were slightly different from the predicted isotope patterns and partial deamidation of selenium-containing peptides is suggested to explain the modified isotope pattern. To our knowledge, this is the first report of the effect of partial deamidation of selenium-containing peptides on the observed selenium isotope pattern, although the evidence is indirect. According to the SEC–ICPMS study, Se-containing proteins in water-soluble protein fraction of *Bertholletia excelsa* H.B.K. seeds can be divided into two main sub-groups-high molecular weight Se-containing proteins and low molecular weight Se-containing proteins. LC–ESI-MS and MS/MS analysis of tryptic digest of low-molecular weight Se-containing proteins identified 2S albumins, which are rich in methionines; therefore, with a high probability of non-specific selenomethionine incorporation—as the proteins present in that fraction.

1. Introduction

Se, discovered in 1817, was considered a toxic element until it was reported in 1957 to be an essential trace element for mammals [1,2]. Now it is well established that Se is beneficial at lower concentrations, but toxic at higher concentrations, and the range from deficiency, essentiality, and toxicity is narrow. Regular consumption of food, which has more than 1 mg kg^{-1} Se, results in toxicity while food with less than 0.1 mg kg^{-1} Se results in deficiency [3]. Even though the effects of Se were described in terms of the total elemental concentration in earlier studies, now it has been shown that the chemical form of Se, as well as the dose, determine its biological activities as an essential element or a toxicant. In environmental and in biological systems, Se has been detected as inorganic species as well as in organoselenium forms, which range from small molecules, such as methyl selenol (MeSeH) to very complex selenoproteins. Most of these are analogous to their sulfur counterparts. In selenoproteins, Se is incorporated in the amino acid chain in the form of selenocysteine (now recognized as the 21st amino acid) or as selenomethionine.

In 1973, Se was identified as a stoichiometric, covalently bound component of glutathione peroxidase (GPx) [4,5]. Se is present in this enzyme as a selenocysteine residue integrated to the amino acid chain. In the majority of mammalian selenoproteins, selenium occurs in the form of selenocysteine.

Studies carried out in 1969 generated interest in the research on Se for cancer chemoprevention and in 1977 Se was reported to be a potential human cancer protective agent [6,7]. As far as

Abbreviations: SEC, size exclusion chromatography; ICPMS, inductively-coupled plasma mass spectrometry; MALDI-TOF-reflectron MS, matrix-assisted laser desorption ionization time-of-flight reflectron mass spectrometry; CapLC–ESI-MS, capillary liquid chromatography electrospray ionization mass spectrometry.

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human nutrition is concerned, the most recognized role of Se is its ability to serve as an antioxidant and possible cancer-preventive agent [8,9]. Owing to the high prevalence of this disease, searching for naturally occurring agents, which may serve as cancer chemopreventives, has been an important objective of various research efforts. Since recent studies have shown that Se supplementation in the diet can reduce the risk of several forms of cancer [8], efforts have been directed to identify anticarcinogenic activity in Se compounds from natural sources, with the objective of using those as food supplements. The Se content of food is highly dependent on the amount of Se in the soil that varies, not only from country to country, but also from region to region within a country. Moreover, it is also dependent on the ability of plants to take up and accumulate various Se forms. When considering selenoproteins, Se content is dependent on the developmental stage of the plant as well, since different proteins are expressed in different levels at different times. The majority of biological and biomedical effects appear to be mediated by proteins that contain selenium. The amount of selenium in proteins from plants, yeast and yogurt has been shown to be dependent on the extent of nonspecific incorporation of selenomethionine and selenocysteine [10]. It is known that selenomethionine can be nonspecifically inserted into proteins by the usual methionine incorporation process [11,12]. This type of substitution is very common in biological systems, and in humans and other animals, selenomethionine from ingested foods can be incorporated randomly into cellular proteins. It has been shown in studies with bacteria, that several proteins, especially rich in methionine, were radio-labeled because of nonspecific ^{75}Se selenomethionine substitution throughout the polypeptide chain, even when a relatively low level of labeled substrate was provided [13].

The proteins which contain selenium are classified into three groups: (A) the proteins which contain selenium in the form of genetically encoded selenocysteine. These are named selenoproteins. (B) In a second category, selenium is incorporated nonspecifically into proteins in place of methionine by replacing the sulfur and these are termed selenium-containing proteins; therefore, these are mainly found in methionine-rich proteins. (C) The third group comprises the specific proteins in which selenium is only attached to the molecule. There are few examples reported so far for this category [14–16]. Any peptide fragment containing selenocysteine and/or selenomethionine are termed as Se-containing peptides.

According to previous research, Brazil nuts, the seeds of *Bertholletia excelsa* H.B.K., which is a large tropical tree of the *Lecythidacea* family, widespread in Latin American high and middle basins, are rich in lipids (65–70%) and contain 15–17% protein by fresh weight and around 50% protein in its defatted flour [17]. As early as 1892, it was discovered that Brazil nuts have unusually high content of the sulfur-containing amino acids, cysteine and methionine, 8.3% by weight [18,19]. The protein content of the Brazil nut has been fractionated into three size classes of proteins, namely the 11S, 7S and 2S proteins [20]. While the level of methionine in all fractions is higher than that of many other plant seed storage proteins [21], the 2S fraction, albumin, is exceptionally rich in sulfur containing amino acids, about 30% methionine and cysteine, and comprises about 30% of the total protein fraction [22,23]. It is expected that there will be similar chemical or biochemical behaviors for S and Se because of their relationship in the periodic table.

It has been indicated that soil in some parts of Brazil is rich in Se and previous studies have shown that Brazil nuts from trees growing in those areas are rich in Se [24]. Previous research carried out by our group has shown that Se in Brazil nuts is bound to proteins and according to these results, Se bound to protein exists in two different forms, namely firmly bound Se and weakly bound Se. The term weakly bound has been ascribed to selenodisulfides (RS-Se-SR′) or methylselenylsulfides (RS-SeCH_3) [25]. In an attempt to characterize the Se-containing species in a Proteinase-K digest of Brazil nut proteins, a tentative structure was suggested but unconfirmed, based on electrospray-MS data [26]. Another study has shown that Se in the protein fraction of Brazil nut is covalently linked to the two amino acids, selenomethionine and selenocysteine [27].

Several studies have been carried out in the last few years to characterize Se-containing peptides by experimental procedures used in proteomics research, namely single or multidimensional chromatography and mass spectrometric techniques, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS). In these studies, Se-containing peptides were sequenced by high performance liquid chromatography ESI-MS/MS (LC–ESI-MS/MS). In LC–ESI-MS/MS experiment, ions with intensities which are below the pre-determined intensity value are not subjected to collision-induced dissociation. In such a case, m/z value of the ion of interest must be selected by the operator. When compared with the intensities of other peptides, the intensity of Se-containing peptides are very low (except in Se-enriched samples) and as a result those are not selected for collision-induced dissociation. Therefore, it is necessary to determine the m/z value of Se-containing peptide in order to input that value in setting up the ESI-MS/MS experiment. As Se has a unique isotope distribution, Se-containing peptides can be identified by MALDI spectrum through the characteristic isotope pattern (though modified to some extent) and m/z value of Se-containing peptides can be obtained. In some of the studies carried out to characterize Se-containing peptides, purified selenoproteins have been used [28–30] and in all other studies, a peptide mixture containing selenopeptides has been examined [31–40]. For studies in which a peptide mixture was analyzed, the sample investigated was selenium-enriched yeast (*Saccharomyces cereviseae*) except in a very recent study in which Se-containing peptides from Brazil nuts were investigated [41]. In that study, tryptic digests of the selenium-containing proteins were fractionated by size-exclusion chromatography, and then concentrated fractions were analyzed by reversed phase nanoLC–ICPMS (RP-nanoLC–ICPMS) and RP-nanoLC-nanospray-quadrupole time of flight (QTOF) mass spectrometry under identical elution conditions. Selection of Se-containing peptides for an ESI-MS/MS study has been carried out by examining the LC–ESI spectra for characteristic Se-isotope patterns at elution times when a Se signal is observed in nano-RP-LC–ICPMS. Their justification for this approach is that it is necessary because of the low-level of Se-containing peptides in Brazil nuts [41]. In studies in which purified selenoproteins were used (as in Ref. [28]), conventional or standard proteomics technologies have been used and expected results have been obtained-that is, sequencing Se-containing peptides by LC–ESI-MS/MS methods. Also, in such cases, necessities such as sample enrichment and minimizing or removing signal suppression do not arise. In studies in which a peptide mixture was analyzed, the sample investigated was selenium-enriched yeast (*S. cereviseae*) (except in one study, Ref. [41]). Therefore, in those studies too, expected results have been obtained by following standard proteomics methodologies as Se-containing proteins are expressed in considerable quantities in Se-enriched yeast. That is, such a sample is also not an analytical challenge. Even though Brazil nut is a natural sample, it has a higher content of Se (when compared with the content of Se in normal plants or, in general, other living specimens) owing to the elemental composition of the soil in which Brazil nut plants grow, but less than Se-enriched yeast. Therefore, standard or conventional proteomics methodologies may not generate polypeptides mixtures (by enzymatic digestion) with a considerable amount of Se-containing peptides (when compared with the quantity of other peptides) and free of signal suppression.