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Analytical Methods

Accumulation and species distribution of selenium in Se-enriched bacterial cells of the *Bifidobacterium animalis* 01

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ABSTRACT

Bifidobacterium animalis 01 (*B. animalis* 01) could absorb 16.7–39.6% of inorganic selenium in the medium and transform most of it into organic selenium. Most of the organic selenium (50.7–63.0%) was found in the protein fraction, 9.62–18.7% in the polysaccharide fraction, 0.273–0.754% in the nucleic acid fraction, and 20.8–30.9% in other components. Furthermore, the selenium content of different protein extractions was in the following order: Alkaline-soluble protein-bound Se (46.5–53.4%) > Water-soluble protein-bound Se (27.4–30.8%) > Salt-soluble protein-bound Se (7.79–11.9%) > Alcohol-soluble protein-bound Se (not detected). Additionally, the molecular mass of most proteins or protein subunits containing selenium was about 10–20 kDa. Analysis by LC–MS showed that selenomethionine (SeMet) is the major selenocompound in protein.

1. Introduction

Selenium (Se), an essential micronutrient with antioxidant properties, has received considerable attention for potential roles in cancer prevention for both human beings and animals (Berry, 2005). However, it was regarded as a toxic element before 1973 (Dubois & Belleville, 1988). Evidences indicated that there is a narrow gap between toxic and essential levels of Se in human beings (Suhajda, Hegoczki, Janzso, Pais, & Vereczkey, 2000). Thus, there is a special emphasis on selenium in relation to disease and a variety of degeneration diseases, such as Keshan, Kashin-Beck, and cancer (Tan et al., 2002). Moreover, severe selenium deficiency occurs in some remote areas where people eat primarily plant-derived foods grown locally in selenium-deficient soil, especially in some regions of China (Burk, 2000). It is therefore suggested to find proper sources of dietary selenium supplement. Some authors suggest that organic Se is an ideal additive because animals and humans absorb and retain it more than inorganic Se. The main source of organic Se is seafood and fish, whereas foods like cereals, meat, nuts, mushrooms and eggs can also increase the dietary selenium intake (Muniz-Naveiro et al., 2005). Recently, more researches showed that Se-enriched yeast and some plant resources such as Se-enriched onion, garlic, fungi and tea were considered as effective organic selenium supplement (Dumont, Vanhaecke, & Cornelis, 2006; Ip, Lisk, & Stoewsand, 1992; Whanger, Ip, Polan, Uden, & Welbaum, 2000).

It is noteworthy that as well as discussing foods as a selenium source, the impact of selenium on nutritional bioavailability also should be discussed (Kohrle, 2004). Recent studies showed that selenium was found in the active site of many selenoenzymes in which selenium is present as selenocysteine, including glutathione peroxidase, iodothyronine deiodinase, and thioredixin reductase (Brigelius-Flohe, 1999). Most researchers found selenium could also form selenomethionine (SeMet) by replacing sulphur in methionine and could then be incorporated nonspecifically into proteins instead of methionine (Behne & Kyriakopoulos, 2001). In addition, selenium can be incorporated into other biological macromolecules, such as seleno-tRNA-s and selenosugar, influencing the synthesis of selenoproteins, respectively (Kobayashi et al., 2002; Suzuki, Somekawa, & Suzuki, 2006).

Several selenium supplements, such as Brazil nut, Se-enriched yeast and Se-enriched *Ganoderma lucidum*, had already been investigated on Se-accumulation (total selenium content) and also on species distribution (Chunhieng et al., 2004; Chassaigne, Chery,

Abbreviation: Se, selenium; LC, liquid chromatography; MS, mass spectrometry; SeMet, selenomethionine; Se-$(Cys)_2$, selenocystine; MRM, multiple reactions monitoring.

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Bordin, & Rodriguez, 2002; Zhao et al., 2004; Ponce De Leon, Bayon, Paquin, & Caruso, 2002). However, there is no information available on Se-enriched *Bifidobacterium* from these two aspects. *Bifidobacterium* is one of the major bacteria in human intestinal flora and is well known for their beneficial functions on health, such as control of gastrointestinal infections, stimulation of the immune system, anti-ulcer activity, anti-tumour activity and antioxidative effects (Jack, Tagg, & Ray, 1995; Picard et al., 2005). Therefore, it is of interest to know whether bifidobacterum could also be used as an effective carrier for Se enrichment. If bifidobacterum could accumulate inorganic selenium from culture medium, Se-enriched *Bifidobacterium* could be explored as a dietary Se supplement source.

The objective of this study is to evaluate the capability of *Bifidobacterium. animalis* 01 to accumulation Se and to characterise the species distribution of selenium in the Se-enriched *B. animalis* 01. To accomplish this study, Se-enriched samples were first obtained, and then nucleic acid, polysaccharide, and proteins were separated respectively. The cell proteins were also separated on the basis of their affinity for different solvents. Then the selenium content was analysed. Finally, a liquid chromatography method was coupled to electrospray tandem mass spectrometry (LC–ESI–MS) to confirm the presence of selenocystine (Se-$(Cys)_2$) and selenomethionine (SeMet) in proteins.

2. Materials and methods

2.1. Materials

All solvents/chemicals used were of analytical reagent grade. selenomethionine (SeMet) and selenocysteine (SeCys) were obtained from Sigma Chemical Co. Nitric acid (70.0% v/v), hydrochloric acid (37% v/v) and perchloric acid (GR, Beijing Chem. Co.) were used to perform the acid digestion of the samples. A stock standard solution of selenium, 1000 g L^{-1} SeO_2 in 0.5 N nitric acid was used to prepare the calibration standards. Glassware and plasticware were soaked in 10% nitric acid for at least 24 h and then rinsed three times with double-distilled water and kept dry ready for use.

2.2. Instrumentation

HPLC were carried out with an Agilent 1100 equipped with a binary pump, an autosampler, a vacant de-gasser system and a thermostated column compartment. The chromatographic column used was an Alltima C_{18} (150 mm × 3.9 mm i.d. 5 μm, Alltech, USA). The HPLC conditions were as follows: 0.1% heptafluorobutyric acid – methanol (70:30, v/v) as a mobile phase; elution at a flow rate 0.50 ml/min; injection volume was 10 μL.

LC–ESI–MS was carried out using an API 4000 mass spectrometer triple-quadrupole with Turboionspray heated at 450 °C as ionisation source. The mass spectrometer was operated in positive ion mode. Nitrogen was used as curtain and collision gas. The Multiple reactions monitoring (MRM) mode was used to monitor the parent and product ions of each amino acid.

2.3. Bacteria and culture conditions

B. animalis 01 (China General Microbiological Collection Center 1353), identified by the method based on 16S rRNA sequences analysis, was isolated from the intestines faeces of the healthy centenarian volunteers who reside in Bama longevity villages, the fifth macrobian district in the world in Guangxi Province of China. It was cultured and sub-cultured in MRS broth at 37 °C for 24 h under anaerobic conditions (High pure N_2) (Pan, Li, & Liu, 2006).

2.4. Preparation of Se-enriched samples

The cultivation method of Se-enriched *B. animalis* 01 was basically the same as that of *B. animalis* 01. The only difference was that a series of 2.5, 5.0, 8.0, and 10.0 μg ml^{-1} selenium in the form of sodium selenite was added to the culture medium for obtaining Se-enriched *B. animalis* 01 sample of Se 2.5, Se 5.0, Se 8.0, and Se 10.0, respectively.

To ensure all the preparations started with the same amount of cells, aliquots containing 1×10^6 cells ml^{-1} bacteria were used for inoculation (Ponce De Leon et al., 2002). Then, the bifidobacteria were harvested by centrifugation at 6000 rpm for 10 min. To remove the inorganic selenium adsorbed to the bacteria cells, the pellet was resuspended twice in distilled water and centrifuged at 6000 rpm for 20 min. Finally, all samples were collected, lyophilised, and stored at −20 °C for use.

For determination of selenium incorporation into bifidobacteria cells the biomass was treated with ultrasonic disruptions. Sonication was performed in an ice-water bath for 80 cycles of 5 s, with 5 s intervals.

2.5. Analysis of organic selenium in Se-enriched B. animalis 01

One gram of freeze-dried and powdered Se-enriched *B. animalis* 01 was dialysed (8000–12000 Da, 48 h) against double-distilled water for 96 h by changing the water every 12 h until no selenium was detected in the dialysing water. Thus, Se compounds left in the sample were considered as organic selenium (Zhao et al., 2004).

2.6. Fractionation of nucleic acid from Se-enriched B. animalis 01

Three grams of freeze-dried and powdered Se-enriched *B. animalis* 01 was stirred into 100 ml of 12% NaCl at 90–95 °C (Rivas et al., 2001; Zhao et al., 2004). After 2 h, the supernatant was obtained by filtration and the residue was repeated twice with 50 ml of 12% NaCl. Then the supernatant was mixed and the protein in the supernatant was removed using the Sevag method (1:4 butanol and chloroform) (Qin, Huang, & Xu, 2002). Finally, by adjusting the pH value of the supernatant to 2.5, the nucleic acids were precipitated at 4 °C after 12 h. The precipitate was collected, lyophilised, and stored at −20 °C to use.

2.7. Fractionation of polysaccharide from Se-enriched B. animalis 01

Three grams of freeze-dried and powdered Se-enriched *B. animalis* 01 were stirred into 100 ml of 1.0 M NaOH at 60 °C. After 4 h, the supernatant was obtained by filtration and the residue was washed and re-precipitated twice with 50 ml of 1.0 M NaOH. Then the supernatant was mixed and the protein in the supernatant was removed using the Sevag method (Qin et al., 2002). Ethanol was added to the supernatant until 75% (v/v) ethanol was obtained. After 12 h, the resultant precipitate was collected by centrifugation and then dissolved in 10.0 ml of double-distilled water. This solution was filtered and dialysed against distilled water at 4 °C three times to remove any other small molecules. The solution left was lyophilised and stored at −20 °C to use (Liu et al., 2003).

2.8. Fractionation of total proteins from Se-enriched B. animalis 01

Total protein extraction was carried out on 3 g of freeze-dried and powdered Se-enriched *B. animalis* 01, which was stirred with 150 ml 0.25 M NaOH at 50 °C for 4 h. The supernatant was obtained by filtration and the residue was repeated twice with 50 ml of 0.25 M NaOH. Then the supernatant was mixed and was made 95% saturated with ammonium sulphate at 4 °C. The resulting precipitate was collected by centrifugation and then dissolved

in 10.0 ml of 50 mM Tris–HCl (pH = 8.0). This solution was filtered and dialysed against 1.0 L of 50 mM Tris–HCl (pH = 8.0) at 4 °C three times to remove any other small molecules. Finally, the solution left was lyophilised and stored at −20 °C to use (Chunhieng et al., 2004; Zhao et al., 2004).

Fractionation of proteins was carried out four times to obtain four different fractions. They were each analysed independently. Fist of all, a water extraction, followed by an extraction with 0.5 M NaCl, and then an extraction with 75% (v/v) alcohol, finally, an extraction with 0.1 M NaOH was done (Chunhieng et al., 2004; Zhao et al., 2004).

2.9. Extraction of water-soluble protein

Three grams of freeze-dried and powdered Se-enriched *B. animalis* 01 stirred into 100 ml of double-distilled water. Four hours later, the supernatant was obtained by centrifugation. After adjustment to pH 4.5 by the addition of 1 M HCl, the supernatant was made 100% saturated with ammonium sulphate at 4 °C. Other procedures were the same as those used for the total protein extraction.

2.10. Extraction of salt-soluble protein by NaCl

Extraction was carried out with the residue of the preceding operation, which was stirred into 100 ml of 0.5 M NaCl. Four hours later, the supernatant was obtained by centrifugation. Other procedures were the same as those used for the water-soluble protein extraction.

2.11. Extraction of alcohol-soluble protein by 75% alcohol

Extraction was carried out with the residue of the preceding operation, which was stirred into 100 ml of 75% (v/v) alcohol. Four hours later, the supernatant was obtained by centrifugation and added to 100 ml of distilled water at 4 °C. The resultant precipitate was collected by centrifugation and dissolved in 75% (v/v) alcohol. This solution was filtered and dialysed against 1.0 L of distilled water at 4 °C three times to remove any other small molecules. Finally, the solution left was lyophilised and stored at −20 °C for use.

2.12. Extraction of alkaline-soluble protein by NaOH

Extraction was carried out with the residue of the preceding operation, which was stirred into 100 ml of 0.1 M NaOH. Other procedures were the same as those used for the salt-soluble protein extraction.

2.13. Protein separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to Laemmli, 1970. In a 120 × 100 × 1.5 mm cell with 12 lanes, 6 lanes were loaded with blanks and others were loaded with test samples. A 0.3 g sample of freeze-dried and powdered Se-enriched *B. animalis* 01 was suspended in 6 ml SDS reducing buffer (60 mM Tris–HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; and 0.05% bromophenol blue). Then the solution was boiled for 5 min and the supernatant was isolated by centrifugation at 6000 rpm for 20 min. Proteins in the supernatant were concentrated in 4% polyacrylamide gel and electrophoretically separated in 12% gel at constant voltage of 120 V for 4 h. To visualise the bands, the gel was then stained with Coomassie Brilliant Blue R-250 overnight and destained in a mixture with 9% acetic acid and 45% methanol for two days. The gel between the first visualised protein band and the last one was cut into 12 segments every 1 cm, which corresponded to twelve different molecular mass ranges of proteins. After deducting the parallel blank, the Se content of each segment was analysed by HG-AAS.

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).

2.14. Determination of total selenium content by HG-AAS

The selenium concentration of tested samples was determined by hydride generation-atomic absorption spectrometry (HG-AAS, haiguang analytical Co., China) as reported recently (Muniz-Naveiro et al., 2005). The HG-AAS conditions were as follows: wavelength, 196.0 nm; slit width, 0.7 nm; sample loop 500 μl; Ar flow rate 125 ml ml^{-1}, quartz cell temperature 850 °C; measurement mode, peak height; carrier solution HCl, 4 M; reducing solution, $NaBH_4$, 0.2% (m/v). The sample pre-treatment was carried out in three steps. In the first step, samples were digested in a 5 ml mixture of nitric acid and perchloric acid (4:1 v/v). The samples were left for 10 h at room temperature and then heated to 80 °C for 1 h. Then the temperature was elevated to 150 °C for 2 h and to 180 °C until white smoke appeared where the samples were left for another 30 min. In the second step, after cooling the vessels down to 50 °C, 5 ml of concentrated hydrochloric acid was added to reduce Se(VI) to Se(IV). Afterwards, the samples were heated at 120 °C for 30 min. Finally, the samples were transferred to volumetric flasks and made up to a final volume of 25 ml with double-distilled water. After those processes, the total selenium determination of samples was carried out by HG-AAS.

2.15. Determination of protein content and amino acid composition

According to the national standard method of China (GB-2905, National Standard Bureau), total raw protein content was determined with a model KN-01 Kjeldahl nitrogen analyser (Mitsubishi Chemical Industries, Inc., Japan). Amino acid composition was determined with a Beckman model 121 amino acid analyser after the tested samples were hydrolysed with 10 ml 6 M HCl at 110 °C for 22 h in airtight ampoules (Suhajda et al., 2000; Wang, Xie, & Peng, 1996; Zhao et al., 2004).

2.16. Selenocystine and selenomethionine identification by LC–MS/MS

Determination of selenocystine (Se-$(Cys)_2$) and selenomethionine (SeMet) in Se-enriched *B. animalis* 01 hydrolysate was investigated by ion-pair reversed-phase liquid chromatography coupled with electrospray (ESI) tandem mass spectrometry (LC–ESI–MS/MS) under conditions previously described and optimised for the analysis of the two seleno-amino acids (SeCys and SeMet) (Zhang et al., 2006). Samples were hydrolysed with 10 ml 4 M HCl at 50 °C for 50 h in airtight ampoules. Se species eluting directly from the column were then measured by multiple reaction monitoring (MRM). SeMet was measured on its two product ions resulting from the transitions MRM 198.1/152.0, MRM 198.1/181.0 and Se-$(Cys)_2$ was measured via MRM 337.0/248.0, MRM 337.0/88.2, respectively.

2.17. Statistics analysis

The data were analysed using the Statistical Analysis System (SAS 1998) package software for the analysis of variance, Duncan's test, and Student's test. All experiments were carried out in triplicate. The significance was established at $p < 0.05$.

3. Results and discussion

3.1. The capability of selenium accumulation of B. animalis 01

The total Se concentration was measured by HG-AAS. Selenium was found to be concentrated in *B. animalis* 01, when sodium selenite was added to the culture medium (Table 1). A high correlation was found between the bacterial Se content and the Se concentration in the medium. The Se concentration in biomass were (mean ± s.d.) 528 ± 48, 641 ± 24, 898 ± 74 and 1017 ± 48 μg g^{-1} dry weight respectively for *B. animalis* 01 when 2.5, 5.0, 8.0 and 10.0 μg ml^{-1} Se^{4+} were present in the medium. And the accumulative rate were 39.6 ± 1.9, 33.4 ± 3.8, 31.6 ± 2.4 and 16.7 ± 1.8%, respectively. However, the presence of 10.0 μg ml^{-1} Se^{4+} in the medium, or higher, lowered the growth of *B. animalis* 01 compared to unsupplemented culture.

The Se content in sample Se 5.0, cultivated in the medium of 5.0 μg ml^{-1}selenium, was 641 ± 24 μg g^{-1}. With the same selenium concentration in the medium, the Se content in Se-enriched *Lactobacilli delbrueckii* ssp. *Bulgaricus* (ATCC 11842) was over 1000 μg g^{-1}. According to the report by Calomme, Van Den Branden, and Vanden Berghe (1995), the selenium concentrations in lyophilised cells, cultured in the presence of 1 mg L^{-1} Se^{4+}, were respectively 375 ± 33, 253 ± 50 and 407 ± 108 μg g^{-1} for *Lactobacilli Plantarum*, *L.. delbrueckii* ssp. *Bulgaricus* and *Lactobacilli casei ssp. csei*. This content was equivalent to that found in Se-enriched *B. animalis* 01. It means that Se-enriched *Bifidobacterium* can also be explored as a dietary Se supplement source.

Otherwise, Se concentration of 10.0 μg ml^{-1} in the medium, or higher, yielded a pink or reddish biomass. It should be understood that selenium can accumulate in bacterial cells in organic and/or inorganic form and in certain circumstance elemental selenium is formed, which gives a reddish colour to the bacterial cells (Suhajda et al., 2000).

3.2. Distribution of organic Se in Se-enriched B. animalis 01

Since the bioavailability of selenium is closely correlated with its distribution and chemical forms, information on speciation in natural products is vital (Chassaigne et al., 2002). Percentage of organic Se in total Se of Se-enriched *B. animalis* 01 for various Se concentrations in medium (2.5, 5.0, 8.0 and 10.0 μg ml^{-1}) is 83.3%, 81.6%, 86.6%, and 77.4%, respectively. It was indicated that most of selenium (over 77%) was incorporated in bacterial cells into the organic fraction and for all the treatment, the percentage of inorganic-selenium remained lower than 22.6%. This is supported by previous findings about selenium speciation in selenite-treated plants (Wang et al., 1996), Se-enriched yeast (Chassaigne et al., 2002) and Se-enriched *G. lucidum* (Zhao et al., 2004).

The results above indicate that Se-enriched *B. animalis* 01 could absorb 16.7–39.6% of inorganic selenium in the medium and transform most of it into organic selenium, which accounted for 77.4–86.6% of absorbed selenium.

Table 2 shows the percentage distribution of organic selenium in the Se-enriched *B. animalis* 01 sample of Se 2.5, Se 5.0, Se 8.0 and Se 10.0. There was no significant interrelation between the percentage distribution of organic selenium and the selenium concentration in the medium. Most of the organic selenium (50.7–63.0%) was found in the protein fraction, 9.62–18.7% in the polysaccharide fraction, and 0.273–0.754% in the nucleic acid fraction with 20.8–30.9% remaining in other components, which possibly included lipids and other low molecular weight selenocompounds.

Table 1
Enrichment of selenium by *B. animalis* 01.[A]

Item	CK	Se 2.5	Se 5.0	Se 8.0	Se 10.0[B]
Se content in 100 ml medium (μg)	0	250	350	800	1000
Biomass in 100 ml medium (mg)	179 ± 6 b	187 ± 4 a	182 ± 5 b	176 ± 7 b	164 ± 7 c
Organic Se content in bacteria (μg g^{-1})	*7.17* ± 0.11 c	*528* ± 48 b	*641* ± 24 b	*898* ± 74 b	*1017* ± 48 a
Accumulating rate (%)[C]	–	39.6 ± 1.9 a	33.4 ± 3.8 b	31.6 ± 2.4 b	16.7 ± 1.8 c

[A] Values are the means ± standard deviations ($n = 3$). Values followed by different letters in the same row are significantly different ($p < 0.05$).
[B] The colour of the product is pink.
[C] Accumulating rate of Se-enriched *B. animalis* 01 was calculated according to the following equation: accumulating rate (%) = (organic Se content in bacteria × biomass in 100 ml medium/Se content in 100 ml medium) × 100.

Table 2
Distribution of organic Se in Se-enriched *B. animalis* 01.[A]

Item	Protein-bound Se	Nucleic acid-bound Se	Polysaccharide-bound Se	Other organic Se
Se 2.5				
Se content (μg/g)	299 ± 9 a	3.98 ± 0.12 d	99.1 ± 1.5 c	127 ± 11 b
Percentage in organic Se (%)	56.5 ± 1.9	0.754 ± 0.011	18.7 ± 0.4	24.0 ± 0.2
Percentage in total Se (%)	47.0 ± 0.6	0.627 ± 0.004	15.6 ± 0.1	20.0 ± 0. 8
Se 5.0				
Se content (μg/g)	325 ± 9 a	2.79 ± 0.08 d	115 ± 8 c	198 ± 15 b
Percentage in organic Se (%)	50.7 ± 1.1	0.435 ± 0.017	18.0 ± 1.3	30.9 ± 2.7
Percentage in total Se (%)	41.3 ± 0.4	0.355 ± 0.011	14.7 ± 1.2	25.2 ± 4.1
Se 8.0				
Se content (μg/g)	498 ± 3 a	2.81 ± 0. 39 d	132 ± 2 c	166 ± 12 b
Percentage in organic Se (%)	62.3 ± 3.7	0.352 ± 0.044	16.5 ± 2.7	20.8 ± 0.19
Percentage in total Se (%)	54.0 ± 1.7	0.305 ± 0.021	14.3 ± 0.7	18.0 ± 0. 12
Se 10.0				
Se content (μg/g)	641 ± 15 a	2.77 ± 0.09 d	97.9 ± 2.6 c	276 ± 12 b
Percentage in organic Se (%)	63.0 ± 3.2	0.273 ± 0.019	9.62 ± 0.54	27.1 ± 1.6
Percentage in total Se (%)	48.8 ± 0.7	0.211 ± 0.006	7.44 ± 0.04	21.0 ± 2.1

Values followed by different letters in the same row are significantly different ($p < 0.05$).
[A] Values are the means ± standard deviations ($n = 3$).

Thus, proteins in Se-enriched *B. animalis* 01 showed the strongest ability to incorporate Se, whereas the nucleic acid fractions hardly bound Se. The present results indicated that, similar to other bacteria such as yeast, *L.. delbrueckii* ssp. *Bulgaricus* and *Flammulina velutipes* (Calomme et al., 1995; Chassaigne et al., 2002; Suhajda et al., 2000), as well as *G. lucidum* (Zhao et al., 2004), *B. animalis* 01 can also be used to accumulate selenium, and selenium tends to be incorporated into its protein components.

3.3. Se Distribution in proteins of Se-enriched B. animalis 01

The protein extracts of different solubilities were obtained and analysed for their selenium content. The results showed that selenium was unequally distributed in the four protein fractions (Table 3). The alkaline-soluble protein extraction had the highest Se content (>46% of organic selenium), whereas the salt-soluble protein had the least (<12% of organic selenium). There was an absence of proteins extracted with 75% ethanol. This was also reported for the Brazil nut: *Bertholletia excelsa* (Chunhieng et al., 2004). Additionally, in all the treatments, the selenium content of the different protein extractions was in the following order: Alkaline-soluble protein-bound Se (46.5–53.4%) > Water-soluble protein-bound Se (27.4–30.8%) > Salt-soluble protein-bound Se (7.79–11.9%) > Alcohol-soluble protein-bound Se (not extracted). Therefore, the alkaline-soluble protein and water-soluble protein were the major proteins to bind selenium as the selenium incorporated with them accounted for over 73% of total organic Se and over 32% of the total Se in the Se-enriched *B. animalis* 01.

3.4. Investigation of molecular mass distribution of selenium-containing proteins in Se-enriched B. animalis 01 by SDS–PAGE

SDS–PAGE aimed at the separation of the selenium-containing proteins in Se-enriched *B. animalis* 01. Typical scans of the silver stained gel of the sample CK, Se 2.5, Se 5.0, Se 8.0 and Se 10.0 proteins after SDS–PAGE are shown in Fig. 1(A). A large distribution of proteins is observed in the mass range investigated (using high-molecular-mass protein markers between 14.4 kDa and 97.4 kDa). More than 16 bands can be visually distinguished in all samples. As it can be seen, the attenuation of bands of molecular

Table 3
Distribution of Se in protein fractions of Se-enriched *B. animalis* 01.[A]

Item	Water-soluble protein	Salt-soluble protein	Alkaline-soluble protein	Unextracted protein
Se 2.5				
Se content (μg/g)	81.9 ± 0.3 b	24.2 ± 0.2 d	139 ± 0.7 a	53.8 ± 0. 8 c
Percentage in protein-bound Se (%)	27.4 ± 0.8	8.09 ± 0.12	46.5 ± 0.3	18.0 ± 0.3
Percentage in total Se (%)	12.9 ± 0.1	3.87 ± 0.11	21.9 ± 0.2	8.47 ± 0.61
Se 5.0				
Se content (μg/g)	99.1 ± 0.38 b	27.5 ± 0.7 d	164 ± 6 a	34.5 ± 0.072 c
Percentage in protein-bound Se (%)	30.5 ± 0.3	8.45 ± 0.17	50.5 ± 1.6	10.6 ± 0.8
Percentage in total Se (%)	12.6 ± 0.9	3.49 ± 0.24	20.1 ± 2.7	4.39 ± 0.15
Se 8.0				
Se content (μg/g)	149 ± 11 b	59.4 ± 4.9 d	248 ± 21 a	41.2 ± 0.9 c
Percentage in protein-bound Se (%)	29.9 ± 1.7	11.9 ± 0.5	49.8 ± 1.5	8.28 ± 0.97
Percentage in total Se (%)	16.2 ± 1.9	6.44 ± 0.07	26.9 ± 0.7	4.47 ± 0.27
Se 10.0				
Se content (μg/g)	198 ± 18 b	49.9 ± 2.1 d	343 ± 16 a	50.9 ± 3.3 c
Percentage in protein-bound Se (%)	30.8 ± 2.8	7.79 ± 0.93	53.4 ± 1.5	7.94 ± 0.11
Percentage in total Se (%)	15.0 ± 0.4	3.80 ± 0.17	26.1 ± 0.9	3.87 ± 0.67

Values followed by different letters in the same row are significantly different ($p < 0.05$).
[A] Values are the means ± standard deviations ($n = 3$).

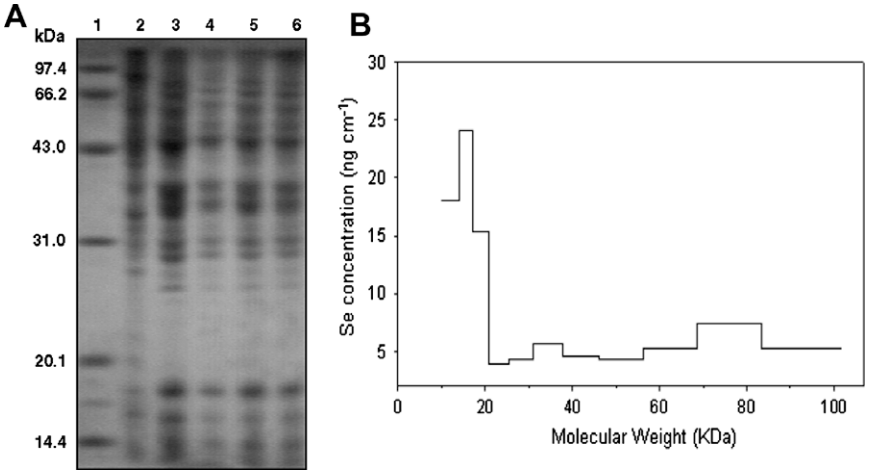

Fig. 1. (A) SDS–PAGE analysis of regular *B. animalis* 01 and Se-enriched *B. animalis* 01 sample (Se 2.5, Se 5.0, Se 8.0 and Se 10.0) with a series of 2.5, 5.0, 8.0, and 10.0 μg/ml Se in the form of sodium selenite was added to the culture medium respectively. Lane 1, molecular weight markers; Lane 2, regular *B. animalis* 01 (CK); Lane 3, Se 2.5; Lane 4, Se 5.0; Lane 5, Se 8.0; Lane 6, Se 10.0. (B) Molecular mass distribution of Selenoproteins or protein subunits in one-dimensional gel for protein fractions of the sample of Se 2.5 (Fig. 2(A), Line 3).

weight of about 38, 35 and 16 kDa was increased in Se-enriched samples of Se 2.5, Se 5.0, Se 8.0 and Se 10.0. Additionally, there was a new protein band appearing at the molecular weight of about 28 kDa in the Se-enriched samples. The present results indicated that selenium could not only influence the synthesis of proteins but alter the protein composition of all Se-enriched samples.

Table 4
Distribution of amino acids in *B. animalis* 01 and Se-enriched *B. animalis* 01.

Amino acid	Content for given samples (%, w/w $n = 3$)				
	B. animalis 01	Se-enriched *B. animalis* 01			
	CK	Se 2.5	Se 5.0	Se 8.0	Se 10.0
Asp	3.31	5.08	5.39	6.40	6.38
Thr	1.19	1.64	1.68	2.15	2.17
Ser	0.98	1.41	1.46	1.98	2.02
Glu	4.13	5.70	6.09	7.48	7.64
Pro	1.07	1.36	1.42	1.83	1.89
Gly	1.50	2.11	2.45	2.79	2.75
Ala	3.43	4.78	5.23	6.05	5.92
Cys	***0.22***	***0.18***	***0.24***	***0.26***	***0.30***
Val	1.65	2.21	2.28	2.80	2.83
Met	***0.74***	***1.00***	***1.08***	***1.09***	***1.12***
Ile	1.30	1.93	1.98	2.46	2.45
Leu	2.01	2.70	2.74	3.45	3.55
Tyr	0.92	1.17	1.17	1.52	1.53
Phe	1.07	1.40	1.41	1.79	1.81
Lys	2.38	3.83	4.05	4.68	4.77
His	1.24	2.11	2.22	2.56	2.72
Arg	1.67	1.83	1.87	2.37	2.46
Trp	0.43	0.40	0.34	0.47	0.42
Total amino acids	**29.24**	**40.90**	**43.06**	**52.13**	**52.73**

For investigation of molecular mass distribution of selenium-containing proteins, the resultant gel with the Se 2.5 sample was cut into 11 segments (about 1.0 cm/segment) and, at the same time, analysed for their Se content. The results (Fig. 1B) shows that selenium is distributed in all proteins or protein subunits. The protein bands of molecular weight between 10 and 20 kDa had the highest Se content (up to 24 ng/cm gel). It was indicated that the molecular mass of most proteins or protein subunits containing selenium was about 10–20 kDa. It should be understood that selenium tends to be incorporated into proteins with lower molecular weight, and/or the ionic interactions, hydrogen bonds, and weak interactions between the protein and mineral would be completely disrupted by the SDS–PAGE conditions used in these experiments (salt concentration of 60 mM, 5% β-mercaptoethanol, and the presence of 2% SDS) (Wang et al., 1996). This should be further confirmed by the association of selenium with amino acids.

3.5. Amino acid content of Se-enriched B. animalis 01 and control B. animalis 01

To discover the form of organically incorporated selenium in the bacterial cells the amino acid content of Se-enriched *B. animalis* 01 was determined and compared with regular *B. animalis* 01. It was expected that the amount of sulphur-containing amino acids

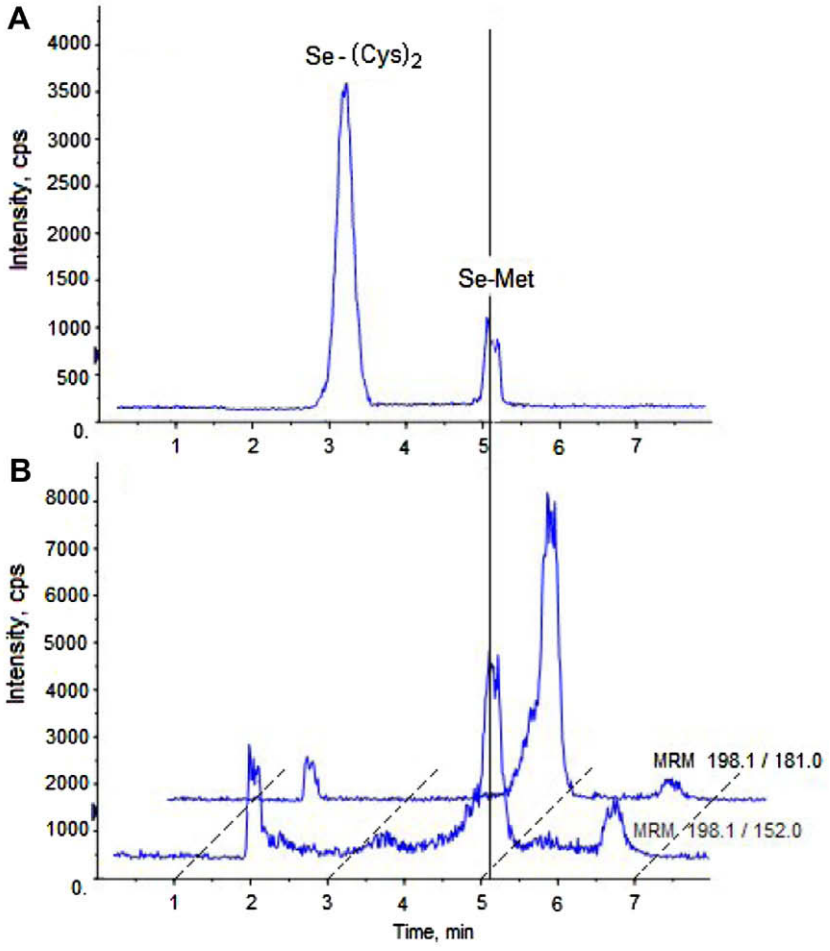

Fig. 2. (A) Total ion chromatogram of a mixed standard solution of Se-Cys and Se-Met obtained by LC–ESI–MS–MS in MRM scan mode; (B) LC–ESI–MS–MS chromatogram obtained from a 4 M HCl extract of Se-enriched *B. animalis* 01: detection of Se-Met.

(Met, Cys) would change because of the chemical similarity of sulphur and selenium (Chassaigne et al., 2002; Suhajda et al., 2000). According to the data shown in Table 4 not only the two amino acids mentioned above, but the entire amino acid content of the *B. animalis* 01 were increased when the Se level in culture medium was raised. It was indicated that selenium accumulation could enhance nutritive value in the organism by elevating the contents of total amino acids (Shu, Chen, & Jun, 2007).

The increased amino acid content of the Se-enriched *B. animalis* 01 is not only accounted for selenium incorporated into the proteins, since not only sulphur-containing amino acids (Met, Cys) increased, but also sulphur may be replaced by selenium. Selenium can also be incorporated into other biological macromolecules, such as seleno-tRNA-s and selenosugar (Kobayashi et al., 2002; Suzuki et al., 2006).

3.6. Identification of selenocystine and selenomethionine in Se-enriched *B. animalis* 01

Selenium is known to be incorporated into proteins as selenocysteine and selenomethionine. In the present study, regular *B. animalis* 01 sample and Se-enriched *B. animalis* 01 sample of Se 2.5, Se 5.0, Se 8.0 and Se 10.0 were analysed for the non-protein organic seleno-amino acids Se-$(Cys)_2$ and SeMet by using LC–ESI–MS–MS. Because multiple reaction monitoring (MRM) is the most sensitive mode of measurement with ESI–MS–MS, the limits of detection of some commonly detected organic Se species were determined for the separation method applied here, measuring in MRM mode. The results are showed in Fig. 2. Of the two seleno-amino acids, none of them was detected in regular *B. animalis* 01 and only SeMet was detected in Se-enriched samples according to the conditions used in this experiment. All the Se-enriched *B. animalis* 01 samples (Se 2.5, Se 5.0, Se 8.0 and Se 10.0) with different Se content did contain a certain amount of SeMet, which is 681.9 ± 11, 780.1 ± 24, 1494.3 ± 39, 1490.3 ± 21 μg/g, respectively.

Additionally, SeMet measured is equivalent to 52.39 ± 0.43%, 49.04 ± 0.86%, and 67.55 ± 1.0%, 59.47 ± 0.99% of the total selenium in Se-enriched samples of Se 2.5, Se 5.0, Se 8.0 and Se 10.0. It is a direct result indicating that selenium replaced sulphur in methionine to form selenomethionine. Moreover, the study by Calomme et al. showed that selenomethionine, the major selenocompound in commercialised Se-yeast and tends to accumulate in tissues, was not detected in Se-enriched *lactobacilli* (Se-Lb) (Calomme et al., 1995). In Se-enriched Mushroom species of the *Genus ganoderma*, SeMet and SeCys were both detected but the content of SeMet decreased with the increase of Se content in proteins (Zhao et al., 2004). SeMet and SeCys were also identified in the *B. excelsa*, however, the content of SeCys was very weak and not easily quantifiable (Chunhieng et al., 2004). In present study, only SeMet was detected and identified in Se-enriched *B. animalis* 01, whereas SeCys was not. Therefore, SeMet might be the major selenocompound in proteins.

4. Conclusions

In this work, total selenium content, selenium distribution and speciation in Se-enriched *B. animalis* 01, was investigated. *B. animalis* 01 could absorb 16.7–39.6% of inorganic selenium in the medium and transform most of it into organic selenium. The content of Se-enriched *B. animalis* 01 can be up to 1017 ± 48 μg g^{-1} dry weights. And a large portion (50.7–63.0%) of organic selenium was incorporated with protein extracts. The alkaline-soluble protein and water-soluble protein were the major proteins to incorporate selenium. Using LC–ESI–MS, the seleno amino acid, SeMet, was identified in Se-enriched *B. animalis* 01, confirming that selenium is non-specifically incorporated in bacterial cells by replacing sulphur in methionine to form selenomethionine. The molecular mass of most proteins or protein subunits containing selenium was about 10–20 kDa. Furthermore, selenium could not only influence the synthesis of proteins but alter the protein composition of all Se-enriched samples. This preliminary work indicates that *B. animalis* 01 could not only be used as an effective carrier for Se enrichment, but the extracted protein fractions were also very rich in selenium and could constitute a health food that could be interesting to consume with moderation.

However, questions remain to be answered more accurately in identifying the other selenium species, which may assist in understanding their role, if any, in cancer chemopreventive effects of Se-enriched *B. animalis* 01. Nowadays, the researches of Se-enriched microorganisms relate to many aspects such as enrich condition and the form of organic Se, the report on loss of organic Se is rare, which might be caused by some free amino acids with Se when dialysis for instance. Whether the organic Se lost from intracellular to extracellular was also concerned when we study the species distribution of selenium in Se-enriched bacterial cells of the *B. animalis* 01, and found that it is difficult to isolate and identify the organic Se *in vitro*, especially the trace of organic Se in a small weight. Nevertheless, we consider this as a very interesting issue which should be study further. Additionally, the more sensitive techniques, e.g. HPLC–ICP–MS or laser ablation with online ICP–MS detection should also be applied to analysis of total concentration of selenium and some of the selenium species present.

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