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Selenium accumulation in protein fractions during germination of Se-enriched brown rice and molecular weights distribution of Se-containing proteins

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ABSTRACT

This study investigated the accumulation of selenium (Se) in protein fractions of albumin, globulin, prolamin and glutelin extracted from Se-enriched brown rice and the molecular weight distribution of Se-containing proteins. Results showed that the amount of total Se (T-Se) and protein-bound Se (PB-Se) in brown rice was significantly ($P < 0.05$) increased after germination with 10–60 μmol/l sodium selenite. Except prolamin, the amount of all the other three protein fractions decreased significantly ($P < 0.05$) with the increase of germination time. Low Se concentrations had promoting effects on degradation of albumin and globulin, while no significant effects were observed on prolamin and glutelin. The accumulation of T-Se and PB-Se were in the order of albumin > glutelin > globulin > prolamin. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed that Se was distributed in all the proteins of which molecular weights varied from 13.6 to 121.4 kDa; however, 84.34% of Se was observed in the proteins whose molecular weights less than 36.3 kDa.

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1. Introduction

Selenium (Se) is an essential micronutrient for human beings and animals that has received considerable attention. As the component of selenocysteine, the 21st amino acid, Se was related to the functions of some bio-enzymes such as glutathione peroxidases, thioredoxin reductase, iodothyronine deiodinases, and selenophosphate synthetase. (Allan, Lacourciere, & Stadtman, 1999; Letavayov, Vlckov, & Brozmanov, 2006). Recently, Amaral, Cantor, Silverman, and Malats (2009) reported that Se had a protective effect for bladder cancer development. Se deficiency was found to be associated with disease conditions and general impairment of the immune system. In China, Keshan disease, an endemic cardiomyopathy, and Kaschin-Beck disease, an osteoarthropathy condition, were found in some areas where Se levels, in the soils, are extremely low (Ge & Yang, 1993). Unfortunately, Se levels in foodstuffs such as cereals, grains, fruits and vegetables are relatively low and cannot meet people's daily dietary requirement. With the purpose of increasing people's dietary Se, a wide variety of Se-enriched foods such as green tea (Hu, Xu, & Pang, 2003), mushroom (Zhao et al., 2004), buckwheat and pumpkin (Stibilj, Kreft, Smrkolj, & Osvald, 2004) have been studied.

Rice is one of the most consumed cereals throughout the world. However, Se content in rice is usually low, and more importantly, a considerable proportion of Se is lost during the milling process of turning brown rice into white rice (Liu, Cao, Bai, Wen, & Gu, 2009). Some research has been done in order to increase Se contents in rice grain by foliar spraying methods (Fang et al., 2008; Hu, Chen, Xu, Zhang, & Pan, 2002). However, these methods were associated with high costs and potential environmental problems (Zhang, Shi, & Wang, 2006a, 2006b). Therefore, finding an alterative is essential.

Plant seeds are able to absorb Se in the environment and assimilate it from different organic and/or inorganic selenocompounds during germination. Lintschinger, Fuchs, Moser, Kuehnelt, and Goessler (2000) reported that significant accumulation of Se is possible in wheat, alfalfa, and sunflower seeds during sprouting using a Se-containing solution, and these sprouts might serve as an excellent Se food source, used directly for food or for supplementation of various diets. Our previous study provided an effective alternative to increase Se level in brown rice by supplying selenite during germination, and a considerable amount of inorganic Se could be transformed to Se-containing proteins (Liu & Gu, 2009). However, the distribution of Se in proteins is still not clear. Rice storage proteins consist of four fractions identified, according to their solubility in various extractants, which are albumins, globulins, prolamins and gluteins. Some research has been done with the purpose of understanding Se accumulation in different protein fractions of Se-enriched Brazil nuts (Chunhieng et al., 2004) and Se-enriched bacteria cells (Zhang et al., 2009). However, there is no information available on the accumulation of Se in different

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protein fractions of Se-enriched brown rice and the molecular weight distribution of Se-containing proteins.

The objectives of this work were to investigate the accumulation kinetics of total Se (T-Se) and protein-bound Se (PB-Se) in brown rice during the germination process, and to study the Se accumulation capacity in various protein fractions and the effects of both germination time and external Se concentrations, as well as to determine the molecular weights of Se-containing proteins.

2. Materials and methods

2.1. Grain materials

Rough rice grain of Zhendao 8 (Z8) was obtained from the Jiangsu Academy of Agricultural Science (JAAS). This cultivar was chosen because it is widely cultivated in the studied region, and showed relatively high capacity of Se accumulation based on our previous study (Liu & Gu, 2009). The brown rice was obtained after the removal of the husk using a hulling machine (JGMJ8098, Shanghai Jiading Grain and Oil Instrument Co. Ltd., China).

2.2. Preparation of Se-enriched brown rice

Brown rice seeds (10 g) were sterilised with 1% (v/v) sodium hypochlorite for 30 min and then washed three times with distilled water. Thereafter, they were well-distributed in a 15-cm Petri dish on top of two layers of filter paper. Fifteen millilitres of solution containing 10, 20, 30, or 60 μmol/l sodium selenite was added into the Petri dish. These Se concentrations were chosen because, based on a previous study (Liu & Gu, 2009), they had no influence on seeds germination and growth of sprouts. Germination was performed in darkness at 25 °C for 4 d. During germination process, an additional 1 ml of the corresponding Se solutions was added every 12 h. The control was cultivated with distilled water under the same conditions. During the entire germination period, the germinated seeds were collected every 24 h from different Petri dishes. After three times washing with distilled water, samples were stored at −20 °C until further use.

2.3. Protein extraction and determination

The procedure of total protein extraction was according to Zhang et al. (2009) with some modifications. Briefly, the frozen germinated rice seeds were freeze-dried, ground and then sieved through a 0.15 mm square aperture sieve-mesh. The flour sample (3 g) was defatted with hexane, homogenised and extracted with 0.25 mol/l NaOH at 25 °C for 2 h under continuous stirring. The supernatant was obtained by centrifugation (6000*g* for 20 min) and the residue was extracted twice with 0.25 mol/l NaOH. Then the supernatant was mixed and 95% saturated ammonium sulphate was added at 4 °C. The precipitate was collected after centrifugation (6000*g* for 20 min) and dissolved in Tris–HCl buffer (50 mmol/l, pH 8.5). This solution, containing total proteins, was filtered (0.45 μm filter) and dialysed (molecular weight cutoff of 3500 Da) against the same buffer, then it was freeze-dried and stored at −20 °C until use.

Protein fractions were prepared according to the method described by Guo and Yao (2006) with some modifications. The frozen germinated rice seeds were freeze-dried, ground, sieved through a 0.15 mm square aperture sieve-mesh and then defatted with hexane. The defatted flour (defatted flour/solvent ratio 1:10 w/v) was sequentially extracted with each of distilled water (albumin), 1 mol/l NaCl (globulin), 70% (v/v) aqueous ethanol (prolamin), and 0.1 mol/l NaOH (glutelin) for 2 h each at 25 °C under continuous stirring. Following each extraction procedure, the mixture was centrifuged at 6000*g* at 4 °C for 30 min. In order to extract most of the protein each extraction step was performed twice. The supernatants containing desired protein fractions were freeze-concentrated and stored at −20 °C. Protein contents of each extraction step were determined spectrophotometrically by the Bradford's (1976) method using bovine serum albumin as standard.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the discontinuous system (12.5% separating/4% stacking gel) according to the method of Laemmli (1970). Freeze-dried total protein samples were dissolved in a sample buffer containing Tris–HCl (pH 6.8), 20% glycerol (v/v), 0.05% bromophenol blue (w/v) and 2% SDS (w/v). Reduction of protein disulphide bonds was performed by adding to the samples 0.5% (v/v) 2-mercaptoethanol at 95–100 °C for 5 min. Samples were, then, centrifuged at 6000*g* for 10 min and the supernatants were subjected to SDS–PAGE. Gels were run at 4 °C and at a constant current of 30 mA. The gels were fixed using 10% acetic acid in 50% ethanol for 1 h, stained with Coomassie Brillant Blue R-250 for 2 h and then washed in destaining solution (8% acetic acid in 25% ethanol) overnight. A series of molecular weight markers (14.4–94.0 kDa) was also applied.

2.5. Determination of total Se and protein-bound Se

The contents of total Se (T-Se) and protein-bound Se (PB-Se) were determined by the method described by Liu and Gu (2009). Briefly, the frozen germinated rice seeds were freeze-dried, ground and then sieved through a 0.15 mm square aperture sieve-mesh. The sample was digested with 5 ml of a mixture of HNO_3 and $HClO_4$ (v/v, 4:1) at 130 °C for 1 h. After cooling, 5 ml of concentrated HCl was added and incubated at 115 °C for 20 min. Subsequently, a clear solution was obtained and made up to 50 ml with distilled water for the T-Se determination by hydride generation of atomic fluorescence spectrometer (HG-AFS) method. For the PB-Se determination, the semipermeable membrane device (SPMD) technique and the enzymatic hydrolysis (protease XIV, Sigma) were used (Liu & Gu, 2009).

2.6. Statistical analysis

The analysis of the variance was performed with the Statistical Analysis System software 8.2 (SAS, USA). Differences amongst means were evaluated using the Duncan's multiple range tests. The significance was established at $P < 0.05$.

3. Results and discussion

3.1. Accumulation of T-Se and PB-Se in brown rice

As shown in Fig. 1A, the amount of T-Se accumulated in brown rice increased significantly ($P < 0.05$) as the external selenite concentration increased from 10 to 60 μmol/l. A high correlation ($r = 0.987$ for 4 d germination) was found between T-Se contents in brown rice and the Se concentrations supplied in the germination solutions. After 4 d germination, the T-Se contents in brown rice reached 1.39, 2.81, 5.43, and 8.43 μg/g DW, respectively, when 10, 20, 30, and 60 μmol/l Se were supplied in the germination solutions. The results were consistent with those of previous studies (Lintschinger et al., 2000; Zhang et al., 2006b). No inhibitory effects on the brown rice germination rate and growth of sprouts were observed during the entire germination period (data not shown). The results were consistent with our previous study (Liu & Gu, 2009),

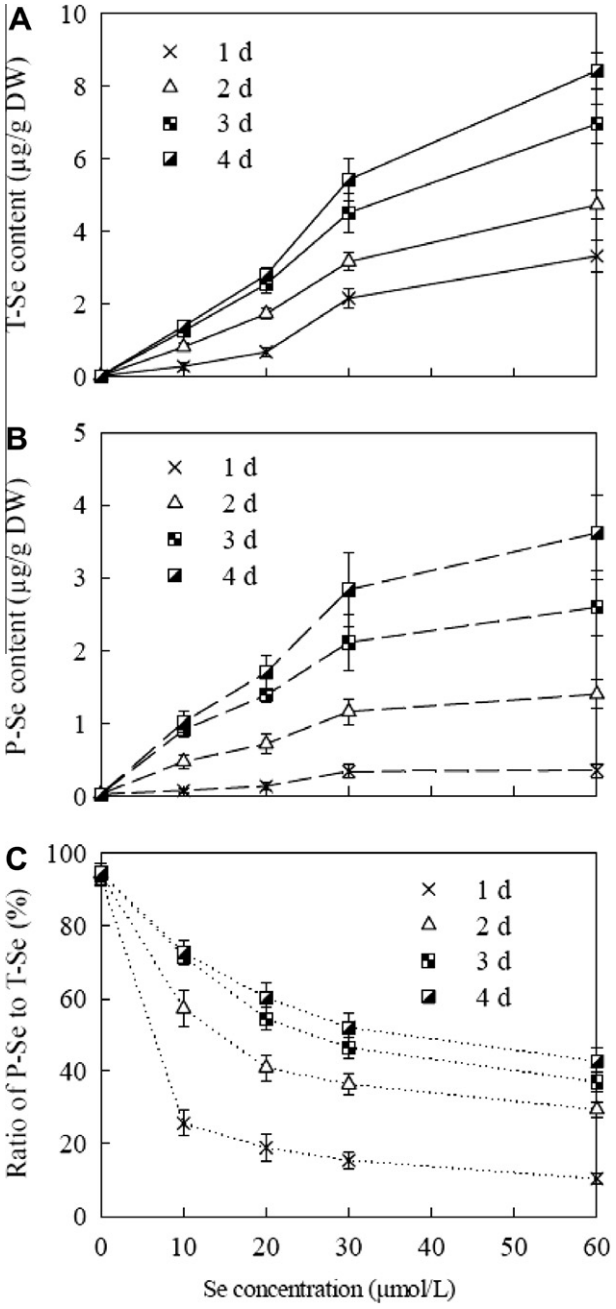

Fig. 1. T-Se (A) and PB-Se (B) contents in Se-enriched brown rice and the ratio of PB-Se to T-Se (C). The values shown are the mean of three replications ± SD. Se-enriched brown rice was obtained by germination at 25 °C with various selenite concentrations for 1–4 d, respectively. T-Se, total selenium; PB-Se, protein-bound selenium.

where an excess of Se showed inhibition on rice germination and sprout length, whereas, no significant effects were observed with an Se concentrations less than 90 μmol/l. In addition, it was evident that the T-Se content in brown rice increased significantly ($P < 0.05$) as the germination time increased. When 60 μmol/l selenite was supplied, the T-Se content in 4 d germinating brown rice was approximately 2.5-fold higher than that in 1 d germinating brown rice. Similar results have been reported previously (Lintschinger et al., 2000; Zhang et al., 2006b).

Similar to the trend of T-Se change, PB-Se accumulation in brown rice increased significantly ($P < 0.05$) as well with increasing Se supplied concentration and germination time (Fig. 1B). However, the ratio of PB-Se to T-Se decreased gradually as Se supplied concentration increased from 10 to 60 μmol/l (Fig. 1C). This indicates that brown rice has a certain capacity for transforming inorganic Se to PB-Se. As expected, the ratio of PB-Se to T-Se was significantly increased ($P < 0.05$) with increasing germination time. When 60 μmol/l selenite was supplied in the germination solution, the ratio of PB-Se to T-Se was 42.77% after 4 d germination, increasing by approximately 3-fold compared to that after 1 d germination. This may be due to Se accumulation at various germination stages through different processes. At the initial stage of germination, the driving force of selenite uptake is the water potential gradient between seeds and their surroundings. At this step, selenite may pass with the imbibing water through the aleurone layer into seeds and, consequently, into cells (Lintschinger et al., 2000). The biosynthesis of Se-containing proteins requires the presence of enzymes, such as cysteine synthase, which catalyses selenide to synthesise selenocysteine (Ng & Anderson, 1979). During the germination process, seeds firstly absorb water from their surroundings and swell, and then restore their metabolic activity (Yang et al., 2007; Yuan, Shan, Huai, Wen, & Zhu, 2001). Thus, it was concluded that only a small amount of sodium selenite is transformed to PB-Se at the early stages of germination because some correlative enzymes are not activated.

3.2. Changes in protein fractions during germination

3.2.1. Time-dependent kinetics of protein fractions

Changes in the protein fraction contents of brown rice at various stages of germination (ranging form 0 to 4 d) with distilled water are shown in Fig. 2. It is clear that the most abundant storage proteins in brown rice are glutelin (more than 60% of total), followed by globulin and albumin, while the quantitative ratio of prolamin is less than 2.4% at each germination stage. These results are similar to those from previous studies (Cao, Wen, Li, & Gu, 2009; Chandi & Sogi, 2007). After germinating for 1 d, no significant differences ($P > 0.05$) were observed in the contents of the four protein fractions compared to controls. This is because proteolytic enzymes, such as endopeptidases, carboxypeptidases, and aminopeptidases have few effects on the mobilisation of endosperm storage proteins during the early germination period (Yuan et al., 2001). Yang et al. (2007) also reported that the degradation of storage proteins in rice seeds mainly occurs after soaking for 48 h.

As shown in Fig. 2, albumin and globulin contents decreased gradually after 2 d of germination, in contrast to glutelin, which starts to decrease after 3 d of germination. This may be because

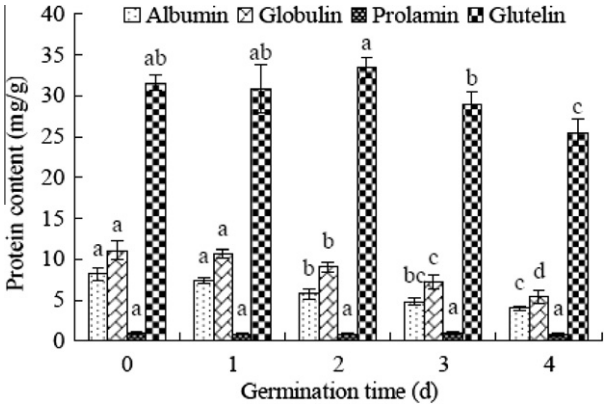

Fig. 2. Changes in protein fractions as a function of germination time. The values shown are the mean of three replications ± SD. Values of each protein fraction followed by the same letter are not significantly different ($P > 0.05$). Brown rice was obtained by germination at 25 °C with distilled water for 1–4 d, respectively.

albumin and globulin are dissolved more easily than glutelin, and are the main nutrient sources of embryos growing at the early germination stage. In addition, it must be noted that the prolamin contents showed no significant change ($P > 0.05$) during the entire germination period. Yuan et al. (2001) also reported that no change in the molecular weight distribution of prolamin during 14 d germination period for rice seeds. This is because prolamin mainly deposits in protein bodies-I and it is difficult to dissolve and digest (Ogawa et al., 1987, 1989).

3.2.2. Se concentration-dependent kinetics of protein fractions

Se treatments showed various effects with respect to protein fraction contents. At a range of 10–60 μmol/l (Fig. 3), Se had no significant effects ($P > 0.05$) on prolamin and glutelin contents after germinating for 4 d. However, albumin and globulin contents varied significantly ($P < 0.05$) amongst different Se concentration treatments. Compared to the controls, the albumin and globulin contents decreased by 10.28% and 14.80%, respectively, after germination with 20 μmol/l selenite for 4 d. Interestingly, when 60 μmol/l selenite was supplied in the germination solution, no significant effects on albumin and globulin contents were observed. This may be because low Se concentrations stimulate the activities of proteases, which hydrolyse albumin and globulin to amino acids or small peptides, while high Se concentrations have inhibitory effects on these proteases. Previous studies have shown that a low Se dosage has promoting effects on the growth of plants, while excess Se addition reduces plant yields (Valkama, Kivimaenpaa, Hartikainen, & Wulff, 2003; Xue, Hartikainen, & Piironen, 2001).

3.3. Se accumulation in protein fractions of Se-enriched brown rice

In order to understand Se accumulation in each protein fraction of Se-enriched brown rice, brown rice was germinated at 25 °C for 4 d with 60 μmol/l selenite. Protein fractions were extracted and their T-Se and PB-Se accumulations were analysed (Fig. 4). Substantial variations were found amongst the protein fractions with respect to both T-Se and PB-Se contents. Accumulations of T-Se and PB-Se in albumin were 2.78 and 0.98 μg/g DW, respectively, while only 0.24 and 0.17 μg/g DW of T-Se and PB-Se were accumulated in prolamin. Amongst the four protein fractions, the amount of T-Se and PB-Se accumulated was in the order of albumin > glutelin > globulin > prolamin. However, the ratio of PB-Se to T-Se showed a different trend. Albumin had the highest T-Se and PB-Se content amongst the four protein fractions; however, it showed the lowest ratio of PB-Se to T-Se. This indicates that albumin extraction included a considerable amount of inorganic Se, which was soluble in water. It is interesting that prolamin had the highest ratio of PB-Se to T-Se but the lowest amount of T-Se and PB-Se. One of the reasons for this may be the presence of polypeptide subunits of rice prolamin with a high content of sulphur-containing amino acids (Hibino et al., 1989; Ogawa et al., 1987). Since the chemical similarity between Se and sulphur, Se could be incorporated into sulphur-containing amino acids to replace sulphur (Brown, 1982; Pilon-Smits et al., 1999).

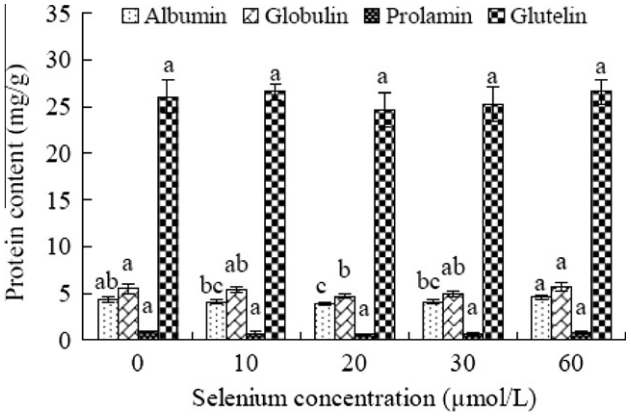

Fig. 3. Changes in protein fractions as a function of selenium concentrations. The values shown are the mean of three replications ± SD. Values of each protein fraction followed by the same letter are not significantly different ($P > 0.05$). Brown rice was obtained by germination at 25 °C with various selenium concentrations for 4 d.

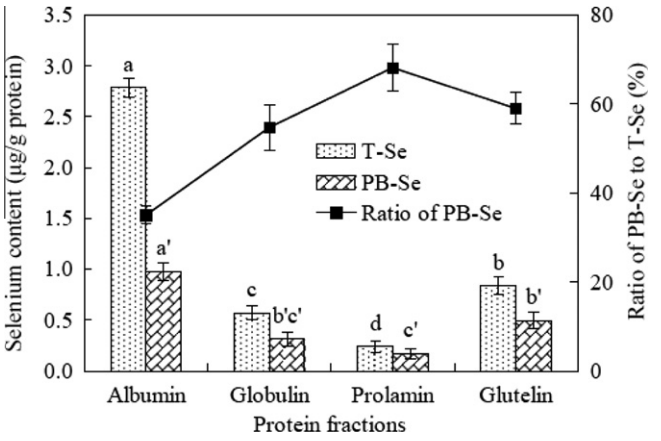

Fig. 4. T-Se and PB-Se accumulation in protein fractions of Se-enriched brown rice and the ratio of PB-Se to T-Se. The values shown are the mean of three replications ± SD. Values of T-Se and PB-Se followed by the same letter are not significantly different ($P > 0.05$). Brown rice was obtained by germination at 25 °C with 60 μmol/l selenite for 4 d. T-Se, total selenium; PB-Se, protein-bound selenium.

3.4. Molecular weights distribution of Se-containing proteins in Se-enriched brown rice

The total proteins extracted from Se-enriched brown rice were analysed by SDS–PAGE (Fig. 5). A total of eighteen proteins or their subunit bands were detected by the Quantity One software (version 4.6, Bio-Rad, USA). These bands were classified into seven groups (I–VII in Fig. 5) according to their molecular weights. It is clear that the proteins or their subunits extracted from Se-enriched brown rice had a large distribution of molecular weights, ranging from 13.6 to 121.4 kDa. Proteins with molecular weights of 13.6–15.8 kDa were predominant amongst the 18 bands, accounting for 37.8% of all proteins analysed by the Quantity One software.

In order to investigate the molecular weight distribution of Se-containing proteins, seven protein sections, representing the seven groups in Fig. 5, were cut out from the gel and the Se content of each section was determined. Fig. 6 shows the Se content and its relative quantity in each protein section. Results indicate that Se could be incorporated into all the proteins, which has molecular weights ranging from 13.6 to 121.4 kDa. This verifies the above results that external inorganic Se could transform to PB-Se (Fig. 1) and accumulate in all the four protein fractions (Fig. 4). Previous research also showed that Se could be incorporated into proteins with molecular weights ranging from 8.71 to 142.53 kDa in Se-enriched mushrooms (Zhao et al., 2004) and proteins with molecular weights ranging from 14.4 to 97.4 kDa in Se-enriched bacteria (Zhang et al., 2009). It was worth noting that a significantly non-uniform distribution of Se was observed in proteins with different molecular weights (Fig. 6). A negative correlation between proteins molecular weights and their Se contents was observed. Proteins with molecular weights of 13.6–15.8 kDa had

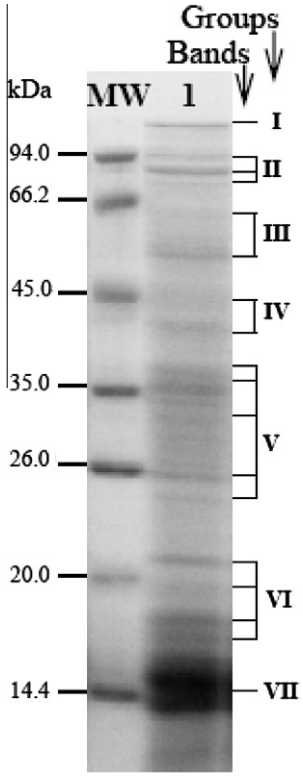

Fig. 5. SDS–PAGE analysis of total proteins extracted from Se-enriched brown rice. MW: molecular weight markers. Lane 1: the total protein extracted form Se-enriched brown rice, obtained by germination at 25 °C with 60 μmol/l of selenite for 4 d. Group molecular weights – I: 121.4 kDa; II: 74.2–94.9 kDa; III: 52.5–62.7 kDa; IV: 40.7–43.6 kDa; V: 23.8–36.3 kDa; VI: 16.8–20.6 kDa; VII: 13.6–15.8 kDa.

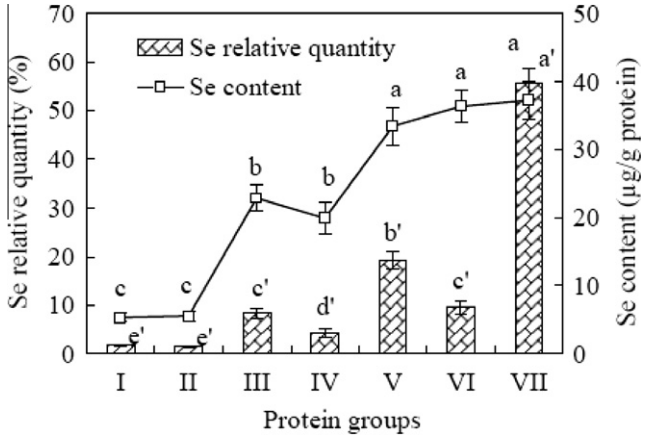

Fig. 6. Selenium contents in Se-containing proteins extracted from Se-enriched brown rice. The values shown are the mean of three replications ± SD. Values of T-Se and PB-Se followed by the same letter are not significantly different ($P > 0.05$). Brown rice was obtained by germination at 25 °C with 60 μmol/l selenite for 4 d. I–VII: Protein groups, see details in Fig. 5.

the highest Se content (37.31 μg/g protein), while those with a molecular weight of 121.4 kDa (5.32 μg/g protein) had the lowest Se content. About 84.34% of the Se available was observed in proteins with molecular weights less than 36.3 kDa, indicating that Se tends to incorporate into low-molecular-weight proteins. These results were in agreement with those of previous studies, where low-molecular-weight proteins accounted for the major amount of Se in Se-enriched mushroom (Zhao et al., 2004) and bacteria (Zhang et al., 2009). This may be due to the hypothesis that storage proteins are hydrolysed by proteases during germination. It is also possible that the disulphide bridges of proteins, which hold tertiary protein structures together, were reduced by β-mercaptoethanol during electrophoresis. This causes Se-containing proteins to fully denature and dissociate into the corresponding subunits.

4. Conclusions

This study shows that both T-Se and PB-Se contents in Se-enriched brown rice increased significantly with increasing external selenite concentration and germination time. Prolonged germination time could promote the transformation of selenite to PB-Se. Protein fractions of albumin, globulin, prolamin, and glutelin showed various changes affected by germination time and Se concentration. Se could accumulate in all the four protein fractions, and albumin was the major fraction to accumulate T-Se and PB-Se. Proteins with molecular weights from 13.6 to 121.4 kDa incorporated various levels of Se. However, a very non-uniform distribution of Se in Se-containing proteins was observed, 84.34% of the Se available was distributed in proteins with molecular weights less than 36.3 kDa. The structures and precise functions of these Se-containing proteins warrant further investigation.

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