Chin.J.Geochem.(2014)33:357–364
DOI: 10.1007/s11631-014-0698-7

Accumulation and speciation of selenium in *Cardamine* sp. in Yutangba Se Mining Field, Enshi, China

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Received October 20, 2013; accepted December 29, 2013

Abstract A naturally selenium-accumulating *Cardamine* sp. is growing in Yutangba Selenium Mining Field, Enshi area, Hubei Province, China, where the geochemical environment is selenium-enriched and endemic selenosis ever occurred in humans. The present study investigated the characteristics of accumulation, speciation and quantity of selenium in *Cardamine* sp. with HPLC-ICP-MS. Results show that *Cardamine* sp. can accumulate Se at most 1427 mg/kg in seedling leaves. Even after the biomass incensement of growing up, the plant still could accumulate Se up to several hundred of mg/kg in concentration. Moreover, the biomass enrichment coefficient (BEC) of Se is exceedingly high, in the seedling leaves mostly, higher than 50 mg/kg; in the range of 43.7–68 mg/kg; and the lowest value is higher than 3 mg/kg in mature fronds. Se is present in the plant predominantly in form of $SeCys_2$ with the highest concentration in seeds; up to 1081 mg/kg as Se. In contrast, $SeCys_2$ levels are low during early growth period; they are 136.1 mg/kg as Se in seedling fronds and 39.4 mg/kg as Se in mature fronds, respectively. SeMet concentration is comparatively low; 10.6 mg/kg as Se in seedling frond and 5.3 mg/kg as Se in half mature fronds, respectively. This indicates that *Cardamine* sp. is extremely efficient in extracting Se from soil and translocating it into its above-ground biomass. Therefore, *Cardamine* sp., found in Yutangba Se Mining Field may be a new Se hyperaccumulator. It is still uncertain whether the Se-accumulation or detoxification of *Cardamine* sp. happens through the pathway of SeCys methylated to form Se-methylselenoCys or through the formation of Se-carboxymethyl- selenohomocysteine. Indeed, further study should be carried out on the determination of more Se species to explain the high Se hyperaccumulation in *Cardamine* sp.

Key words Enshi; Se hyperaccumulator; *Cardamine* sp.; Se speciation; $SeCys_2$

1 Introduction

There has been much scientific interest in recent years in using metal hyperaccumulating plants to clean up hazardous material-contaminated sites, a process called phytoremediation. Selenium (Se) is an important element from environmental and biological point of view. It is an essential element, but the margin between minimum necessary and already toxic levels is small (Ellis and Salt, 2003; Sager, 2006). It has been recognized as an integral component of different enzymes such as gluthathione peroxidease and thioredoxin reductase, which participate in the antioxidant protection of cells (Birringer et al., 2002). Moreover, Se is known to be a potent anti-carcinogen and has been applied in cancer prevention (Clark et al., 1996; Combs et al., 1996). Se-accumulating plants not only can be used in remediating Se polluted environment but also have a broad potential as a way to improve dietary habits or even to create anti-

carcinogen functional foods (Salt et al., 2001).

There are some wild plants that accumulate and transform selenium into bioactive compounds naturally and have important implications for human nutrition, health and the environment (Ellis and Salt, 2003). Some Se hyperaccumulator, such as *Astragalus bisulcatus* growing on Se-enriched soil in the western United States, can accumulated Se up to the level of to 0.65% (w/w) dry weight in the shoots (Byers, 1936). Using micro X-ray absorption spectroscopy and liquid chromatography-mass spectrometry, it was found that Se in the trichomes of young leaves of *A. bisulcatus* was presented in 30% as inorganic Se (selenate and selenite) in addition to 70% as $MeSeCys_2$. Inside the leaf edges of young *S. pinnata* leaves, with the use of liquid chromatography-mass spectrometry both $MeSeCys_2$ (88%) and selenocystathionine (12%) were detected (Freeman et al., 2006). Some selenoproteins play a role in free-radical scavenging, and consequently, Se has been shown to have anticarcinogenic activity, especially when consumed as methylselenocysteine ($MeSeCys_2$) and γ-glutamyl-$MeSeCys_2$ (Ellis and Salt, 2003). Therefore, As Ellis and Salt (2003) reported, "Se hyperaccumulators provide rich genetic resources that we are beginning to exploit to develop food crops that are enriched in anticarcinogenic Se compounds for improved public health. These genetic resources are also being used to develop plants that are ideally suited for the phytoremediation of Se-contaminated soil and water".

In China, there is a typical seleniferous area namely Enshi, situated in southwestern Hubei Province, central China, and it is a prospective place to find Se hyperaccumulators. Enshi is well-known for endemic selenosis. There were about 477 cases of human Se poisoning reported during the sixty five years from 1923 to 1987 and more than 10000 cases of swine Se poisoning had been recorred over the past 4 decades in Enshi. In order to investigate the truth behind the selenosis incidence, numerous scientists have carried out a great number of experiments (Yang et al., 1983; Yang and Xia, 1995; Mao et al., 1997; Zheng et al., 1993; Zhu et al., 2008, 2001, 2012; Fordyce et al., 2000). Previous studies primarily focused on the environmental geochemistry and intake of Se in local inhabitants. According to their researches, in an environment of high Se-concentration where rocks, soils, water, and crops were all seleniferous and residents took in excess selenium from local Se-enriched foods (corn and vegetable suffered selenosis) that resulted in selenosis. However, few local Se-enriched plants have been investigated. Investigated a few regional vegetables and forages, and found that two Se-accumulating species, cabbage and plantain contained Se of 224.30 and 235.71 mg/kg, respectively. Shao et al. (2013) studied the Se-species of a naturally Se-enriched string bean (*Phaseolus Vulgaris*) in Jianshi County, Enshi, China, and identified and quantified Se-methylselenocysteine (2.6 mg/kg as Se) and γ-glutamyl-Se-methylselenocysteine (1.2 mg/kg as Se) using HPLC-ESI-TOF-MS and orbitrap MS. We performed extensive investigations on Se-level in various species of wild plants in Ehshi, China, and found a native species of Se-caccumulator *Cardamine* sp. (then identified as *Thlaspi* L.). In this plant the Se-concentration reached 1427 mg/kg (Shao et al., 2007). Later Yuan (2013) also discovered a novel Se secondary accumulator (*Cardamine* hupingshanesis) in the same area.

The objective of this study was to characterize the accumulation and speciation of Se in *Cardamine* sp. from Yutangba Se Mining Field. This study could provide biogeochemical information on Se tolerance and accumulation mechanism, help the development of phytoremediation technology for the clean-up of Se contaminated environment and promote the development of Se supplementing products for the amelioration of human health.

2 Materials and methods

2.1 Sampling area

Sampling area was situated in Yutangba Se Mining Field (109°46′39″E and 30°20′16″N), in Enshi area, western Hubei Province, China (Fig. 1). It is a small, closed, intermountain basin with hot climate and heavy rains. There is an abundance of seleniferous rocks and ores exposed in the hillside, which have been exploited for many years since they had been discovered in 1980s. Of exposed rock strata, the Upper Permian stratum bears selenium ores. The exposed Permian Se-rich strata comprised carbon-siliceous rocks and carbonaceous shale (known locally as 'stone coal'). The Upper Permian Mokou and Wujiaping Formations contained the highest level of Se (up to 8500 mg/kg) (Zhu et al., 2008). Natural strong weathering of Se-enriched rocks and intense human activities, such as stone coal mining and agricultural soil amendment with coal ash, resulted in stress contamination of Se, V, Mo and other heavy metals in soils and water. The environmental contamination of Se led to serious selenosis in humans and livestock in Yutangba Village in the 1960s, and all local residents had to leave the area (Yang et al., 1983).

2.2 Sampling and treatment

From the sampling area mentioned above, sampling sites were selected at primarily Se-rich places including stone coal outcrops, waste ore, rock heaps, seleniferous farm land, and drainage areas of rivulet.

At the above mentioned sampling sites, various species of wild plants and their root soils were sampled. Of the collected wild plants, Se accumulator *Cardamine* sp. is in the focus of our research. In various seasons (spring, summer and autumn), seedling plants, half mature plants and mature seeds were sampled. Various organs of *Cardamine* sp, including roots, stems, leaves and seeds, were collected. Soil samples were taken from places where plants grow, and from near the surface (0–30 cm).

Plant samples were washed firstly with tap water and then with deionized water to exclude contamination from the surface and were dried with oven at low temperature about 40–50℃. After minimizing the water content, samples were milled into powder with a plant grinder and separated through 250 μm mesh for complete homogenization. Last, samples were sealed with paper bags and stored in the refrigerator at 4℃. Soil samples were air-dried at the room temperature. Clods were crushed using an agate-mortar, and then passed through a 2-mm sieve to remove rocks, pebbles and larger pieces of plant material. Sieved soils were transferred into plastic bags for storage.

2.3 Samples analysis

2.3.1 Determination of total Se content

Samples were digested with the help of a microwave system (Mars-5, CEM Corporation, Matthews, North Carolin, USA). A set of 0.05 g subsamples were placed in PTFE-coated digestion tubes and 6.0 mL of concentrated HNO_3 and 3.0 mL of H_2O_2 were added to each. The samples were left incubating overnight in atmospheric pre-digestion, then were digested according to the following ramp-to pressure protocol, 0–15 min for the pressure rising to 250 psi, 15–35 min for keeping at 250 psi, and 35–50 min decreasing to 70 psi. After digestion the sample solutions were transferred into PP vials. All dilutions were carried out based on weight.

Total Se concentration was determined with a thermo XⅡ ICP-MS instrument (Thermo Fisher Scientific., USA) using Rh as internal standard (Merck). Standard addition was executed for calibration, using a Merck Certi PUP grade 1000±2 mg/kg of Se standard.

2.3.2 Determination of Se species sample preparation

To liberate Se-species from the sample matrix an enzymatic digestion with nonspecific proteolytic enzymes was used. A set of 0.1 g subsamples was dissolved in 3 mL hyperpure water (10 mg protease E enzyme was added). The sample was treated with ultrasound for 30 min at 37℃. After the extraction, the samples were centrifuged at 14000 $r \cdot min^{-1}$ for 15 min at 25℃ and the supernatant was filtered through a 0.22 μm filter and used for selenium speciation analysis with HPLC-ICP-MS.

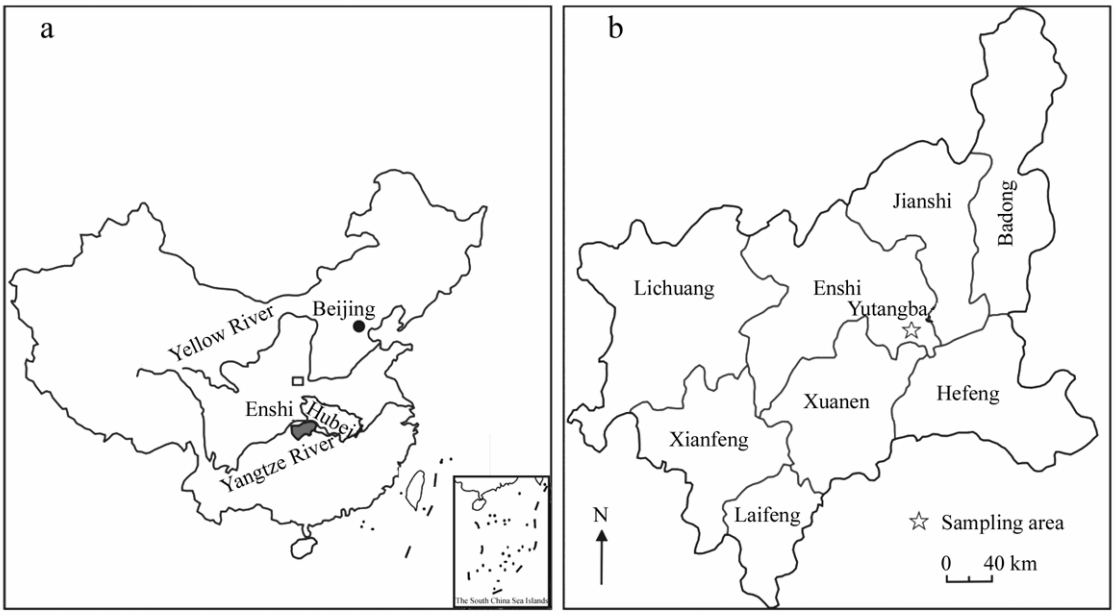

Fig. 1. Location of sampling area in Yutangba Mining Field. a. Enshi location in China; b. Sampling site in Enshi area. Shaded part denotes to Enshi area, and asterisk denotes to the location of Yutangba Mining Field in Enshi.

2.3.3 HPLC-ICP-MS set up

For the identification and quantification of Se-species extracted, a hyphenated HPLC-ICP-MS was used. A Hamilton PRPAX-100 anion exchange column (150 mm×4.6 mm×5 μm; Hamilton; Reno, Nevada, USA) was applied. Mobile phase was 5 mmol/L Citric acid (pH=4.69). The flow rate was 1.2 mL/min, and the column was kept at ambient temperature. The injection volume was 50 μl.

Quality control of quantification was executed with a standard addition of selenate (Se VI), selenite (Se VI), selenomethionine (SeMet), selenocysteine ($SeCys_2$), and Se-methylselenocysteine ($MeSeCys_2$) (PharmaSe, Lubbock, TX, USA). Concentrations of five Se species in the mixed standard solution all are 100 μg/L (as Se).

3 Results and discussion

3.1 Total Se contents in *Cardamine* sp.

We measured 5 sets of *Cardamine* sp. samples during three growth periods. Concentrations of total Se in *Cardamine* sp. and its root soils are presented in Table 1 and Fig. 2.

Cardamine sp. grew in seleniferous soils with Se concentration of 20–50 mg/kg and accumulated from several hundred to more than one thousand mg/kg of total Se; the highest level was 1427 mg/kg, found in a leaf sample. There are obvious differences in Se accumulation during three growth periods in various tissues (root, stem and leaf). During the seedling period, the mean value of Se concentrations are 1328 (1224–1427) mg/kg in leaf, 600 (512–700) mg/kg in stem, and 1060 (841–1168) mg/kg in root (Fig. 2a). During half mature period, the mean value of Se concentrations are 543 (439–717) mg/kg in leaf, 299 (241–331) mg/kg in stem, and 396 (348–452) mg/kg in root (Fig. 2b). During mature period, the mean value of Se concentrations dropped to 236 (185–326) mg/kg in leaf, 188 (142–319) mg/kg in stem, and 140 (129–152) mg/kg in root (Fig. 2c). In addition, for specialization of Se in *Cardamine* sp., the frond (stem and leaf together) and seeds of the plant were selected, measured during different growth periods (seedling, half mature, and mature), and the results of total Se content were listed in Table 2. Total Se concentrations were 868.3±17.8 mg/kg in seedling fronds, 343.7±5.1 mg/kg in half mature fronds, and 1220.8±12.3 mg/kg in mature seeds.

Table 1 Concentrations of total Se and BEC of Cardamine sp.

Growth period	Sample No.	Total Se (mg/kg, DW)				BEC[a] of plant vs. soil		
		Soil	Leaf	Stem	Root	Leaf	stem	Root
Seedling	1	28	1226	512	841	43.8	18.4	30.0
	2	21	1427	512	1121	68.0	24.4	53.4
	3	23	1396	648	1145	60.7	28.2	49.8
	4	24	1224	627	1027	51.0	26.1	42.8
	5	32	1365	700	1168	42.7	21.9	36.5
Half mature	1	38	463		365	12.2		9.6
	2	46	717	331	348	15.6	7.1	9.6
	3	49	600	331	348	12.2	6.8	7.1
	4	49	479			10.1		
	5	48	439	241	418	9.2	5.1	8.8
Mature	1	46	326	319		7.1	6.9	
	2	37	229	143	152	6.3	3.9	4.2
	3	39	251	158		6.4	4.0	
	4	38	188	177		4.9	4.6	
	5	48	185	142	129	3.8	3.0	2.7

Note: [a] BEC denotes to total Se content in plant/total Se content in soil.

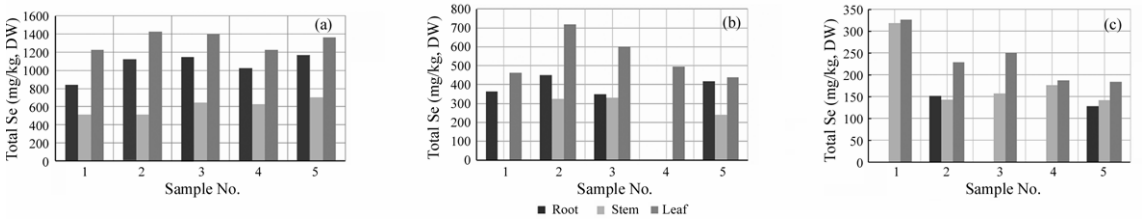

Fig. 2. Concentrations of total Se in tissues of *Cardamine* sp. during three growth periods. (a) seedling period; (b) half mature period; (c) mature period.

3.2 The biomass enrichment coefficient (BEC) of Se from soil to plant

To characterize the Se-translocation from soil to plant, BEC of Se was calculated (Table 1 and Fig. 3). The BEC of the seedling period is the highest, higher than 20; the mean BEC are 53.2 (42.7–68.0) for leaf vs. soil, 23.8 (18.3–28.2) for stem vs. soil, and 42.5 (30.0–53.4) for root vs. soil (Fig. 3a). The BEC of the half mature period are comparatively higher, in the range of 5–15, with the mean BEC of 11.9 (9.2–15.6) for leaf vs. soil, 6.3 (5.1–7.1) for stem vs. soil, and 8.8 (7.1–9.6) for root vs. soil (Fig. 3b). The BEC of the mature period are comparatively lower but almost all of them are higher than 3. The mean BEC are 5.7 (3.8–7.1) for leaf vs. soil, 4.5 (3.0–6.9) for stem vs. soil, and 3.4(2.7–4.2) for root vs. soil (Fig. 3c). Above BEC data show that seedling *Cardamine* sp. can concentrate Se in the leaves up to 50 times higher than the concentration in soil; mature *Cardamine* sp. accumulate Se is the least, but they still have BEC higher than 3. Thus, *Cardamine* sp. is extremely efficient in extracting Se from soil and translocating it into its above-ground biomass.

3.3 Se accumulation of *Cardamine* sp.

A plant is considered to be a Se-hyperaccumulator if it can accumulate Se higher than 1000 mg/kg dry weight when growing on Se-enriched soil. An accumulator is a plant, growing on Se-enriched soil, can concentrate Se up to several hundreds of mg/kg; while an ordinary plant (non-accumulator), even if growing on Se-enriched soil, accumulates lower than 100 mg/kg in Se (Baker and Brooks et al., 1989; Maryland et al., 1989). In Yutangba, the Se mining area, *Cardamine* sp. can accumulate Se up to 1427 mg/kg in seedling leaves. Even after the biomass incensement of growing up, the plant still could accumulate Se up to several hundred of mg/kg concentration. The typical Se-hyperaccumulators, *Astragalus bisulcatus* and *Stanleya pinnata* mostly accumulate Se in their young leaves and reproductive tissues with Se level up to 1% of plant dry weight. (Freeman et al., 2006). *Cardamine* sp. is similar to *Astragalus bisulcatus* and *Stanleya pinnata* and mostly stores Se in young leaves and the concentration can be higher than 1000 mg/kg of plant dry weight. Thus, *Cardamine* sp., growing in Yutangba Mining Field, Enshi, China, possesses the ability of hyperaccumulting Se. Moreover, its BEC of Se is very high eapecially for seedling leaves in the range of 43.7–68.0, indicating that *Cardamine sp.* is extremely efficient in extracting Se from soil and translocating it into its above-ground biomass. Therefore, *Cardamine* sp. in Yutangba Se mining area may be considered as a Se-hyperaccumulator. Of course, this needs further research for confirmation.

3.4 Speciation of Se in *Cardamine* sp.

Table 2 lists the concentration of total Se and Se species in *Cardamine* sp. and Fig. 4 shows the chromatograms acquired from the enzymaticaly digested *Cardamine* sp. samples separated with anion exchange chromatography. Fig. 4a shows a typical chromatogram of a mixture of Se species with standard addition containing 100 μg/L of Se. Fig. 4b, c presents $SeCys_2$, Se (IV), SeMet and Se (VI) in both the seedling and half mature fronds. Fig. 4d shows a huge $SeCys_2$ spike added to the mature seeds. The use of enzymatic digestion yields low extraction efficiency (39.2%) of Se from the half mature samples, but high extraction efficiency from the seedlings (62.2%) and seed (88.6%) (Table 2). Based on standard addition, concentrations of Se in *Cardamine* sp. were found to be 136.1 mg/kg of $SeCys_2$, 6.6 mg/kg of Se (IV), 10.6 mg/kg of SeMet and 386.7 mg/kg of Se (VI) as Se in seedling frond, 39.4 mg/kg of $SeCys_2$, 3.7 mg/kg of Se (IV), 5.3 mg/kg of SeMet and 87.2 mg/kg of Se (VI) as Se in half mature frond, and 1081 mg/kg of $SeCys_2$ as Se in mature seeds (Table 2).

3.5 Se Species distribution in *Cardamine* sp.

Data acquired from HPLC-ICP-MS measurements reveal that there are abundant organic Se compounds in *Cardamine* sp. including $SeCys_2$ and SeMet; the largest amount of organic Se is in the form of $SeCys_2$. Different growth periods have distinctly different organic Se species distributions. $SeCys_2$ and SeMet were detected in both seedling frond (136.1 and 10.6 mg/kg as Se, respectively) and half mature frond (39.4 and 5.3 mg/kg as Se, respectively).

Table 2 Contents of the total Se and Se speciation of *Cardamine* sp. during three growth periods in Yutangba Mining Field, Enshi, China (mg/kg, DW)

Sample	Total Se	$SeCys_2$	Se(IV)	SeMet	Se(VI)	EF[b]
Seedling frond	868.3±17.8	136.1	6.6	10.6	386.7	62.2
Half mature frond	343.7±5.1	39.3	3.7	5.3	87.2	39.4
Mature seed	1220.8±12.3	1081.4	-[a]	-	-	88.6

Note: [a] denotes to undetected, [b] denotes to extraction efficiency.

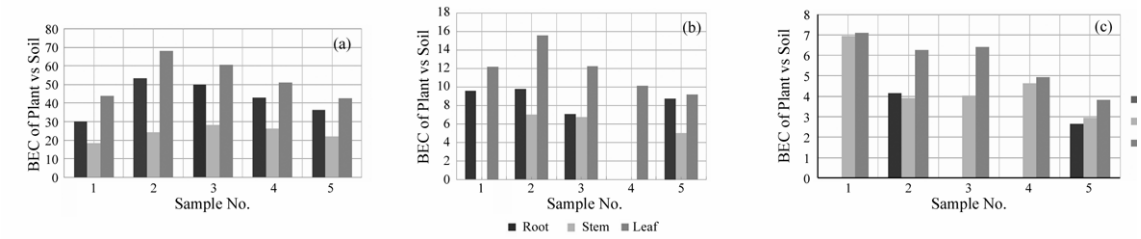

Fig. 3. BEC of *Cardamine* sp. tissues vs. soil during three growth periods. (a) seedling period; (b) half mature period; (c) mature period.

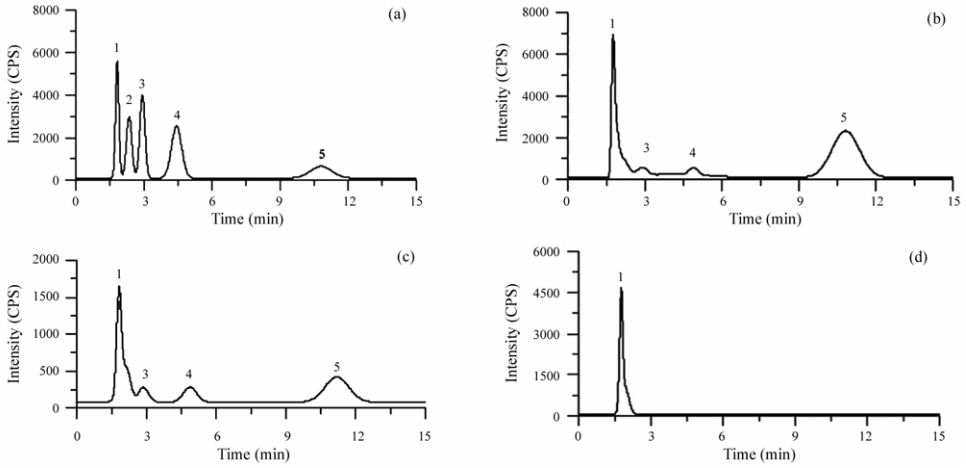

Fig. 4. Anion-exchange chromatogram of the Se species separation. (a) mixture of standard solution containing 100 μg Se /L separately; (b) frond of *Cardamine* sp. during Seedling period; (c) front of *Cardamine* sp. during half mature period; (d) seed of *Cardamine* sp. during mature period. 1. $SeCys_2$; 2. $MeSeCys_2$; 3. Se(IV); 4. SeMet; 5. Se(VI).

Only $SeCys_2$ was detected in mature seeds, up to 1081 mg/kg as Se. A research of Yuan et al. (2013) also indicated that over 70% of the total accumulated Se was in the form of selenocystine ($SeCys_2$) in Se-enriched *Cardamine hupingshanesis* (Brassicaceae). While inorganic Se is dominant during early growth period, with concentrations of 6.6 mg/kg of Se (IV) and 386.7 mg/kg of Se (VI) in seedling frond, and 3.7 mg/kg of Se (IV) and 87.2 mg/kg of Se (VI) in half mature frond. The accumulation of inorganic Se during early growth period of *Cardamine* sp. is regarded to be the consequence of insufficient rate of selenium metabolism; this phenomenon has also been observed in several mushroom and plant species (Dernovics et al., 2002; Ximénez et al., 2004).

In hyperaccumulating plants, accumulated Se is predominantly present as Se-methylseleno Cys ($MeSeCys_2$). For example in the young leaves of *Astragalus bisulcatus* (Brassicaceae), there was found 70% $MeSeCys_2$ in addition to 30% inorganic Se; *Stanleya pinnata* (Fabaceae) leaves containe 88% $MeSeCys_2$ and 12% selenocystathinoine (Freeman et al., 2006). In Se-accumulating plants the mechanism of selenium tolerance is a key step, when Seleno-Cys is methylated by selen-Cys methyl transferase to form Se-methylselenoCys (Neuhierl and Bock, 1996). In the present study, the accumulated Se in *Cardamine* sp. was predominantly present in the form of $SeCys_2$. For our experiment, the extraction efficiency is not high (Table 2); it is possible that there are some other

Se species as $MeSeCys_2$ instead of $SeCys_2$ in the plant. This assumption was proved to be true as a novel Se-species (Se-carboxymethyl-selenohomocysteine) in the plant using HPLC-ESI-TOFMS and HPLC-ESI-Orbitrap MS (Dernovics, pers, commun). It is still uncertain whether the Se-accumulation or detoxification of *Cardamine* sp. happens through the pathway of SeCys methylated to form Se-methylselenoCys or through the formation of Se-carboxymethyl-selenohomocysteine. Indeed, further study should be carried out on the determination of more Se species to explain the high Se hyperaccumulation in *Cardamine* sp.

4 Conclusions

Cardamine sp., growing in Yutangba mining field, Enshi, China, possesses the ability to hyperaccumulte Se in young leaves. It could accumulate at most 1427 mg/kg Se in seedling leaf. Even after the biomass incensement of growing up, the plant still could accumulate Se up to several hundred of mg/kg. Moreover, BEC of Se is exceedingly high, in the seedling leaves are mostly higher than 50; and the lowest value is still higher than 3 in mature fronds. This indicates that *Cardamine* sp. is extremely efficient in extracting Se from soil and translocating it into its above-ground biomass. Therefore, *Cardamine* sp. found in Yutangba Se Mining Field may be a new Se-hyperaccumulator.

Data acquired by HPLC-ICP-MS reveal that there are abundant organic Se compounds in *Cardamine* sp. The dominant Se-species in the plant is $SeCys_2$ with the highest content up to 1081 mg/kg as Se (in mature seeds). In contrast, the $SeCys_2$ level is lower during early growth period; its concentration is 136.1 mg/kg as Se in seedling fronds and 39.4 mg/kg as Se in mature fronds. SeMet concentration is comparatively low, 10.6 mg/kg as Se in seedling frond and 5.3 mg/kg as Se in half mature fronds, and no SeMet was detected in mature seeds. It is possible that there are some other Se species including $MeSeCys_2$ in the plant. It is still uncertain whether the Se accumulation or detoxification of *Cardamine* sp. happens through the pathway of SeCys methylated to form Se-methylselenoCys or through the formation of Se-carboxymethyl- selenohomocysteine. Indeed, further study should be carried out on the determination of more Se species to explain the high Se hyperaccumulation in *Cardamine* sp.

Acknowledgements This project was supported by the National Science Foundation of China (Grant No.40971287), the "Strategic Priority Research Program-Climate Change: Carbon Budget and Related Issues" of the Chinese Academy of Sciences (Grant No. XDA05010105), and the 12th Five-year Plan Project of State Key Laboratory of Ore-Deposit Geochemistry, Chinese Academy of Sciences (SKLODG-ZY125-08). The authors acknowledge the help of Dr. Orsolya Egressy-Molnar and Dr. Dernovics Mihaly in Corvinus University of Budapest, Hungary in the correction of the manuscript.

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