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Determination of arsenic speciation and the possible source of methylated arsenic in *Panax Notoginseng*

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HIGHLIGHTS

- Arsenic species in PN and soil were investigated using field and pot experiments.
- Inorganic As and methylated As were present in considerable amounts in PN.
- As(V) was the major species in soil, although methylated As was detected.
- Methylated As level in PN is positively correlated with ArsM gene abundance in soil.
- The methylated arsenic in PN was most likely from the planting soil.

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ABSTRACT

Arsenic species and a possible source of methylated arsenic in a *Panax Notoginseng* (PN) medicinal plant were explored to further understand the change of inorganic arsenic to the less toxic methylated form to minimize the health risks associated with its medicinal use. Arsenic speciation in PN from major planting areas was determined using high-performance liquid chromatography coupled with hydride generator-atomic fluorescence (HPLC-HG-AFS). Pot experiments were performed to explore the source of methylated arsenic in PN, and the arsenite methyltransferase (arsM) gene abundance was determined using quantitative reverse transcription PCR (q-RTPCR). Methylated arsenic (monomethylarsonic acid (MMA) + dimethylarsinic acid (DMA)) accounted for 43% ± 30% of the total arsenic in PN from planting areas, while the primary species in soil was As(V) (94% ± 0.12%). In the pot experiments, methylated arsenic accounted for 37%–49% of the total arsenic in PN, and As (V) was the primary species in soil (>98%). The four detected arsenic species in PN increased as the amount of As added to soil increased. The methylated arsenic contents in the PN root were significantly positively correlated with the ArsM gene abundance in soil, suggesting that methylated arsenic in PN is likely from the planting soil.

1. Introduction

Arsenic is a ubiquitous element in the environment. Thousands of people all over the world are suffering from the toxic effects of arsenic contamination, which exerts a variety of adverse health effects, such as dermal changes and carcinogenic effects, among others, in humans after acute and chronic exposures (Duker et al., 2005; Hopenhayn, 2006). However, it is well known that arsenic toxicity depends on the total concentration and the chemical species. In general, inorganic arsenic species are more toxic than organic forms. Of the inorganic forms, arsine is highly toxic, and arsenite [As(III)] is accepted as being more toxic than arsenate [As(V)] (Chen and Wang, 1990). The methylated organic species monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are less toxic, and organoarsenicals, arsenobetaine (AsB) and arsenocholine (AsC) are generally considered non-toxic to humans (Grund et al., 2008). However, in recent studies, As methylation was considered to be a bioactivation process, and the metabolites

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MMA(III) and DMA(III) were more toxic than inorganic arsenic (Styblo et al., 2000; Kligerman et al., 2003). The arsenic level in soil can be elevated due to natural and anthropogenic causes (Sierra-Alvarez et al., 2006). The major arsenic species in terrestrial plants, such as rice, are the more toxic inorganic species and the methylated species (Williams et al., 2005), while the non-toxic forms AsB and AsC are the major species in seafood and seaweed (Borak and Hosgood, 2007; Súñer et al., 2002). Therefore, to understand the As toxicity in soil, foodstuffs and other environmental samples, it is essential to monitor both the arsenic content and its chemical species.

The inorganic chemical species of As can be methylated to form methylated organic species, as has been found in fungi. Although limited information is available about the transformation pathway in higher plants, it can be inferred from the Challenger pathway (Zhao et al., 2010) in fungi. In this pathway, As(V) is the initial species, and it can be reduced to As(III) by arsenate reductase (AR), simultaneously converting glutathione (GSH) to oxidized glutathione (GSSG). To convert As(III) to MMA (V), the methylation reaction occurs using the methyl donor *S*-adenosyl-L-methionine (SAM) and the arsenic methyltransferase (ArsM) enzyme, generating *S*-adenosylmethionine (SAH). The product MMA (V) is reduced to MMA (III) by AR. Methylation and reduction steps continue to produce di-(DMA) and trimethyl (TMA) compounds (Fig. S1). The arsM genes encoding As methyltransferases have been identified in bacteria, fungi (Liu et al., 2011; Qin et al., 2006), and some algae (Marapakala et al., 2012; Zhang et al., 2013). For higher plants, no arsM genes have been identified, and no direct evidence exists for As methylation, although a few indirect proofs suggest that higher plants may be able to perform As methylation (Nissen and Benson, 1982; Quaghebeur et al., 2003). Lomax et al. (2012) found that methylated species in rice are due to microorganisms in the soil. Therefore, the search for additional evidence for As methylation and the determination of the source of methylated As in higher plants are required.

Panax Notoginseng (PN) is a highly valued Chinese medicine prepared from the root of the herb *Panax notoginseng* (Burk.) FH Chen (Araliaceae) and is widely used in China, Korea, Japan and other countries. PN is traditionally used as a hemostatic to control external bleeding but has also been used for the treatment of cardiovascular diseases, inflammation, trauma, and various body pains (Dong et al., 2003; Ng, 2006); currently, it is also used as an anticarcinogen (Sun et al., 2010) and in health products (Zhu et al., 2015). The primary cultivation areas of PN are distributed in the southeast part of Yunnan Province, China. Mining and smelting have widely elevated the arsenic level in the soil and plants. In addition, arsenic-containing pesticides are occasionally used during PN plantation, causing the As levels in some PN samples to exceed the standard (2 mg kg^{-1}) set by China and other organizations. Several authors have demonstrated As contamination in PN, but the level of PN contamination by As was not serious (approximately less than 20%) (Feng et al., 2003). The cultivation area and management practices can influence the pollution degree. PN in geo-authentic habitats and good agriculture practice (GAP) planting areas showed less As contamination (Li, 2004). However, few studies on the As species in PN have been conducted, and there is certainly a lack of information about the source of methylated As in PN.

The growth environment of PN plants is under shady, moist conditions, similar to rice-growing conditions. Because a considerable amount of methylated arsenic was detected in rice, we inferred that methylated arsenic also existed in PN plants. Therefore, the objectives of our study were as follows: 1) to determine the As chemical species in PN and the attached soil using a field study and a pot experiment and 2) to explore the possible source of methylated As in PN by examining the correlation between As speciation in PN and the abundance of ArsM genes in soil via a pot experiment.

2. Materials and methods

2.1. Field survey

PN plants and the attached soil (0–20 cm depth) were simultaneously collected from 20 plantations in the southeast area of Yunnan Province in October 2011, as described in a previous study by our group (Zhu et al., 2016). A map of the sampling sites is shown in Fig. 2S.

2.2. Pot experiment

The pot experiment was performed at Wenshan Experimental Station, Yunnan, China (24.7197°N, 104.3927°E, 1468 m above sea level) beginning in January 2012. Disodium hydrogen arsenate ($Na_2HAsO_4 \cdot 7H_2O$) was added to the soil to attain As treatments of 0 (control (CK)), 10 and 30 mg kg^{-1}; then, the soils were placed in 10 L plastic pots. Five replicates were performed for each treatment. The soil used was from the surface (0–20 cm) of a field near the station, with a pH of 4.94, an organic content (OC) of 27.40 g kg^{-1}, a cation exchange capacity (CEC) of 19.27 mmol kg^{-1}, a total P of 0.21 mg g^{-1}, a rapidly available P value of 117.95 mg kg^{-1}, and an As concentration of 35.12 mg kg^{-1}. After the added As had stabilized in the soil for one week, PN plants were transplanted into each pot. The pots were arranged in a randomized block design in the greenhouse. PN and soils were sampled for laboratory analysis after 22 months in October 2013.

2.3. Sample preparation and As speciation determination

The samples were immediately transferred to the laboratory. The PN roots were washed thoroughly to remove the soil and were cut into small pieces with a ceramic knife. Then, the roots and soils were freeze-dried in a freeze dryer (GHRIST, Germany) for approximately 36 h and then ground using a grinding mill (ZM200 pulverisette 14, Germany). The lyophilized roots (0.50 g) were weighed (Mettler Toledo AG 204, Switzerland) into a 15 ml centrifuge tube and extracted using a methanol-water mixture (4 ml, 1 + 1 v/v) by the microwave (MCR-3, China) method (15 min, 60 °C, 100 W). The extracted solution was centrifuged (SC -3614, China) at 6000 rpm for 20 min. The supernatant was transferred and subjected to a two-fold dilution. For the soil samples, the extraction solution was 0.3 M phosphoric acid (H_3PO_4) (4 ml); the extraction process was the same as that for the roots, and the supernatant was five-fold diluted. The final solution was filtered through a 0.22 μm cellulose-nitrate ester filter before being injected into the analytical instrument. The water used in the experiment was ultrapure water (Mill-Q, USA).

Four As chemical species (As(III), As(V), DMA and MMA) were detected using the high-performance liquid chromatography coupled with hydride generator-atomic fluorescence (HPLC-HG-AFS) (PS Analytical, Orpington, UK) method. After proper optimization, the experimental conditions were determined. The arsenic species were separated using a PRP-X100 SAX column (4 × 250 mm id, 10 μm) from Hamilton (Reno, USA). The mobile phase consisted of 50 mM phosphate buffer (pH 6.0), and the mobile phase flow rate was 1.0 ml min^{-1}. The acid carrier was 0.1% (m/v) $Na_2S_2O_8$ in 10% (v/v) HCl, and the reductant was 0.8% (m/v) $NaBH_4$ in 0.4% (m/v) NaOH. The gas carrier was 0.3 MP highest purity Ar. Arsenic compounds were quantified by external calibration with standard solutions (0, 1, 5, 20, and 50 ng mL^{-1}) of As(III), As(V), DMA and MMA