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Arsenic complexes in the arsenic hyperaccumulator *Pteris vittata* (Chinese brake fern)

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Abstract

Pteris vittata (Chinese brake fern), the first reported arsenic (As) hyperaccumulating plant, can be potentially applied in the phytoremediation of As-contaminated sites. Understanding the mechanisms of As tolerance and detoxification in this plant is critical to further enhance its capability of As hyperaccumulation. In this study, an unknown As species, other than arsenite (As^{III}) or arsenate (As^{V}) was found in leaflets by using anion-exchange chromatography–hydride generation–atomic fluorescence spectroscopy and size-exclusion chromatography–atomic fluorescence spectrometry. The chromatographic behavior of this unknown As species and its stability suggest that it is likely an As complex. Although phytochelatin with two subunits (PC_2) was the only major thiol in *P. vittata* under As exposure, this unknown As complex was unlikely to be an As^{III}–PC_2 complex by comparison of their chromatographic behaviors, stability at different pHs and charge states. The complex is sensitive to temperature and metal ions, but relatively insensitive to pH. In buffer solution of pH 5.9, it is present in a neutral form.

Keywords: *Pteris vittata*; Arsenic; Metal complexes; Phytochelatins

1. Introduction

Arsenic (As) is a toxic element widely encountered in the environment and organisms [1]. Usually, plants contain trace levels of As. However, recently found *Pteris vittata* (Chinese brake fern) can accumulate up to 2.3% of As in their fronds without significant toxicity symptoms [2]. This fern, along with other recently identified other As hyperaccumulating ferns from genus Pteris (order Pterdales) [3], i.e., Pityrogramma calomelanos [4], Pteris cretica, Pteris longifolia and Pteris umbrosa [5–7], can be potentially used for phytoremediation of As-contaminated sites. In these As hyperaccumulating ferns, As is mainly accumulated in their fronds, and only inorganic forms of As, i.e., arsenite (As^{III}) and arsenate (As^{V}), are present [2,5,8]. It is unclear why *P. vittata* accumulates such high levels of As and how it tolerates As. Uncovering As tolerance mechanism in this hyperaccumulating fern is essential to understand As hyperaccumulation and the evolution of this unique capacity.

One proposed mechanism of As tolerance in *P. vittata* is chelation followed by sequestration. According to this hypothesis, As^{V} is first reduced to As^{III} in cytoplasm, and then As^{III} is chelated by ligands to avoid the consequences of cellular toxicity. Arsenic complexes are eventually sequestrated into vacuoles to be stored. This hypothesis is supported by energy dispersive X-ray microanalyses (EDXA), which reveals that As is primarily located in the upper and lower epidermal cells, probably in the vacuoles [9]. Thiol-containing compounds, e.g. glutathione (GSH) and phytochelatins (PCs) are considered to be the ligands of As^{III}. It has been confirmed that As^{III} can be chelated by these thiol-containing compounds to form As^{III}-tris-thiolate complexes through thiolate bonds by using size-exclusion chromatography (SEC) or electrospray ionization mass spectrometry (ESI-MS) [10,11]. GSH may be involved in As detoxification in Indian mustard [12]. PCs, a group of thiol-rich peptides with the general structure $(\gamma\text{-GluCys})_n$–Gly ($n = 2$–11), are synthesized from

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GSH by phytochelatin synthase [13,14]. PCs are induced by As and play an essential role in As detoxification in *Holcus lanatus* [15] and *Cytisus striatus* [16]. PC synthesis in *P. vittata* is also induced under As exposure [17,18]. The major compound induced by As was purified and characterized as PC_2 [17]. However, PC_2 seems to play a limited role in As tolerance in *P. vittata* [18,19]. Therefore, other kinds of As complexes, which may play a major role in As tolerance, may exist in the plant. Determining the presence of As complex is critical to understand the mechanisms of As hypertolerance and hyperaccumulation in *P. vittata*. Up to date, there has been no direct evidence to suggest the presence of As complexes in *P. vittata*. As speciation analyses using anion exchange chromatography (AEC) or SEC have shown that only inorganic As^{III} and As^{V} are present in *P. vittata*, and As^{III} is predominate species in its leaflets [8,20]. The lack of evidence for As complexes in the plant is probably due to the instability of these complexes and/or improperly selected analytical conditions [8]. In an effort to identify possible As complexes in *P. vittata*, we improved extraction methods and employed two separation methods, i.e., anion-exchange chromatography (AEC) and SEC, to determine whether As complexes might be overlooked in previous research.

2. Experimental

2.1. Experimental plants

P. vittata was collected from Central Florida where they were first discovered [2]. The ferns were returned to Miami where they were grown in 30 cm pots containing peat moss (Lamber, Canada), in a greenhouse environment. High levels of thiols were induced in the plants by treating them with sodium arsenate (500 ml of 13.3 mM solution), which was slowly spiked to the soil at two week intervals for a total of five times. After harvesting, the leaflets were immediately washed with deionized water (dH_2O), blotted dry with paper towel, frozen in liquid nitrogen, and ground to fine powder with a mortar and pestle. The powder was immediately extracted separately using the following ice cold solutions: dH_2O; 50% dH_2O–methanol; 0.015 M EDTA solutions; sodium acetate buffer (pH 4.0); 0.015 M potassium phosphate buffer (pH 5.9); 0.015 M potassium phosphate buffer (pH 7.0); or 0.015 M Tris–HCl buffer (pH 8.0). Extracts were centrifuged at 4 °C and 12,000 rpm for 10 min, and supernatants were analyzed by HPLC to determine As species.

2.2. As speciation

Arsenic speciation was determined by HPLC coupled with hydride generation atomic fluorescence spectrometry (HPLC–HG–AFS). A Millennium Excalibur atomic fluorescence system (P.S Analytical, Kent, UK) coupled with a Spectra-Physics HPLC System (Fremont, CA, USA) was used for these analyses. The Millennium Excalibur system is an integrated atomic fluorescence system incorporating vapor generation, gas–liquid separation, moisture removal and atomic fluorescence stages. The detailed experimental conditions of the HG–AFS system can be found in our previous report [8]. Data were acquired by a real-time chromatographic control and data acquisition system. The HPLC system consisted of a P4000 pump and an AS 3000 autosampler with a 100 μl injection loop. Both anion exchange column (PRP X-100, 250 mm × 4.6 mm, 10 μm particle size, Hamilton) and size exclusion column (OH-PAK SB-8025 HQ, 300 mm × 8.0 mm, Shodex) were used for As speciation. HPLC pump flow rate was 1 ml/min for both columns. Potassium phosphate (0.015 M) at pH 5.9 was used as mobile phase for the anion-exchange column. Sodium acetate (0.015 M containing 0.1 M NaCl at pH 4.0), potassium phosphate (0.015 M containing 0.1 M NaCl at pH 5.9 and 7.0), and Tris–HCl (0.015 M, containing 0.1 M NaCl at pH 8.0) were used as mobile phases for SEC.

2.3. Preliminary separation of the potential As complex

Fresh leaflets (200 g) were collected from the plants exposed to As and rinsed with dH_2O. The leaflets were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. An ice-cold EDTA solution (0.015 M; 300 ml) was added to the powder and the slurry was filtered through cheesecloth. Debris was extracted again by the ice-cold EDTA solution (0.015 M; 100 ml). Extracts were combined and centrifuged (12,000 rpm for 10 min; 4 °C). Supernatant was filtered through two layers of filter paper (Whatman No. 4) using a Buchner funnel. The filtrate was lyophilized using a freeze-dryer (Freezone, 6 L, Labconco). The lyophilized filtrate was dissolved in 5 ml dH_2O. The As complex was eluted from a Sephadex G-25 column (Superfine, Pharmacia, 80 cm × 2.6 cm) with ice-cold EDTA (0.015 M; 1.5 ml/min flow rate). Fractions (15 ml each) were collected using a fraction collector (FRAC-100, Pharmacia) and directly analyzed with AEC and SEC.

2.4. Analysis of the reconstituted As^{III}–PC_2 complex

PC_2 was purified from As-exposed leaflets by covalent chromatography combining with preparative reversed-phase HPLC [17]. As^{III} and the purified PC_2 with a stoichiometry of one As to three thiol groups was used to reconstitute As^{III}–PC_2 complex in vitro. All buffer solutions were degassed with He to prevent thiol oxidation before use in reconstitution in vitro. Purified PC_2 (10 μl, 1.86 mM) and As^{III} (10 μl, 1.24 mM) were added to 80 μl of 0.015 M different SEC mobile phase (pH 4.0, 5.9, 7.0 and 8.0) under He protection [11]. An aliquot of 20 μl of the reconstituted As^{III}–PC_2 complex was subject to HPLC.

As^{III}–PC_2 complex was analyzed by SEC or AEC with post-column derivatization device for on-line detection of

thiols at 412 nm. Mobile phases and flow rates for As complex analysis were the same as those described above for As speciation. A homemade device consisting of a reaction coil (Teflon tubing; 3 m × 0.5 mm i.d.) and an isocratic pump (Acuflow Series I, Fisher) was used for post-column derivatization [17]. Derivatization reagent was made of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; 1.8 mM) in 0.3 M phosphate buffer (pH 8.0) containing 15 mM EDTA. The solution was pumped at 0.5 ml/min [17].

3. Results and discussion

3.1. As speciation analysis with AEC–HG–AFS

In our previous study, only As^{III} and As^{V} were detected in lyophilized fronds extracted by methanol–water (1:1) at room temperature [8]. The absence of evidence for As complexes may have resulted from the use of harsh extraction conditions that may have decomposed the unstable complexes, and/or improper selection of the chromatographic condition that resulted in poor separation of the small quantity of As complexes. To minimize these possible shortfalls, different extraction solutions were used to extract fresh leaflets at low temperature and the extracts were analyzed with both AEC and SEC in the present work. Improved extraction procedures resulted in detection of an unknown As species in addition to As^{III} and As^{V}. When As species were analyzed in fresh leaflet extract by AEC–HG–AFS, a small peak appeared right after As^{III}. The level of the small peak was much lower than As^{III}, and its retention time is close to that of As^{III}. Most of the small peak was overlapped by a much larger As^{III} peak. In order to remove most of As^{III} interference from the extract, a Sephadex G-25 column was used. Fractions containing the small peak were collected and analyzed using AEC chromatography. AEC chromatogram clearly shows the presence of a small peak in addition to As^{III} and As^{V} when the concentrations of these ions are reduced (Fig. 1b). Compared to the chromatogram of the four As standards (Fig. 1a), the small peak elutes slightly ahead of dimethylarsinic acid (DMA). Since the small peak is much smaller than As^{III} peak and their retention times are close, the small peak is easily overlooked. This is especially true when fresh leaflet extracts were directly analyzed without first cleaning up the As^{III} interference with a Sephadex G-25 column. Overlook of the small peak seemed to happen in a previous As speciation study in *P. vittata* by Wang et al. [20]. In their study, the small peak was also present on the chromatogram of AEC. Unfortunately, the large As^{III} peak overlapped most part of the small unknown As species peak, causing it ignored. However, we cannot exclude the possibility that the small peak is actually DMA from AEC, since their retention times are close. Low levels of DMA have been reported to be present in some terrestrial plants [21,22].

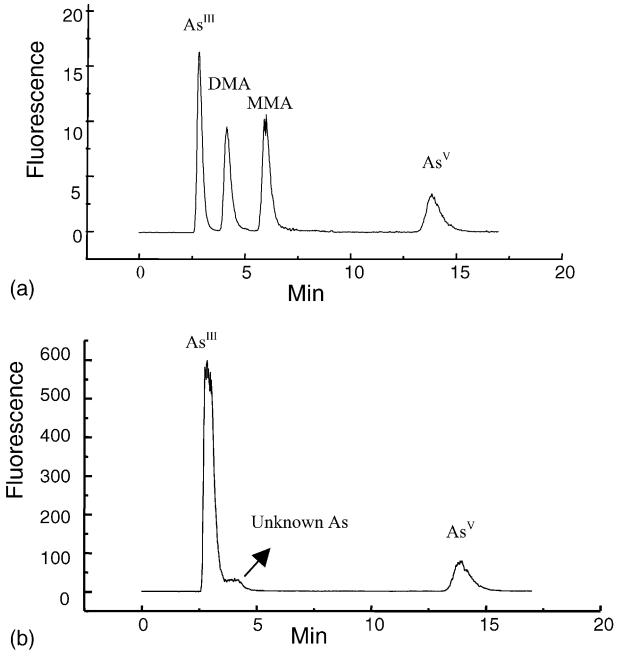

Fig. 1. Arsenic speciation in leaflets exposed to As with AEC–HG–AFS. Mobile phase: 0.015 M potassium phosphate buffer at pH 5.9. (a) Standard chromatogram of As^{III}, MMA, DMA and As^{V}. (b) As species in leaflets extracted with 0.015 M EDTA followed by preliminary separation by a Sephadex G-25 column.

3.2. As speciation analysis with SEC–HG–AFS

To further characterize the small peak, SEC was used to separate As species. SEC is a widely used method to study the formation of metal/metalloid complexes. Usually, complexes have an earlier retention time than metals on size exclusion column due to their larger molecular mass. Coupled with element specific detectors, e.g., atomic absorption spectrometry (AAS), AFS, and inductively coupled plasma (ICP)/MS, SEC is especially useful to probe the weak interaction of metal ions and their ligands [10,23,24]. On a size exclusion column, the four As standards produced only two peaks, one for As^{III} and the other consisting of unresolved DMA, monomethylarsonic acid (MMA) and As^{V}, using phosphate buffer (0.015 M, pH 5.9 with 0.1 M NaCl) as a mobile phase (Fig. 2a). Sample chromatogram of fresh leaflet extract clearly showed three peaks, As^{V}, As^{III}, and an unknown As species (Fig. 2b). The overlapped peak of DMA, MMA and As^{V} in the standard chromatogram (Fig. 2a) was replaced by a single As^{V} peak in the sample chromatogram (Fig. 2b), because DMA and MMA are not present in *P. vittata* [8,20]. Since the levels of As^{V} were much less than that of As^{III} in leaflets [8,20] and the retention time of the unknown As species was close to that of As^{V}, there was no interference from As^{III} to the separation on SEC and pre-separation of sample using Sephadex G-25 was not required (Fig. 2b). Chromatograms of SEC suggest that the unknown As species is not DMA. Several other experiments were further conducted to examine the proper-

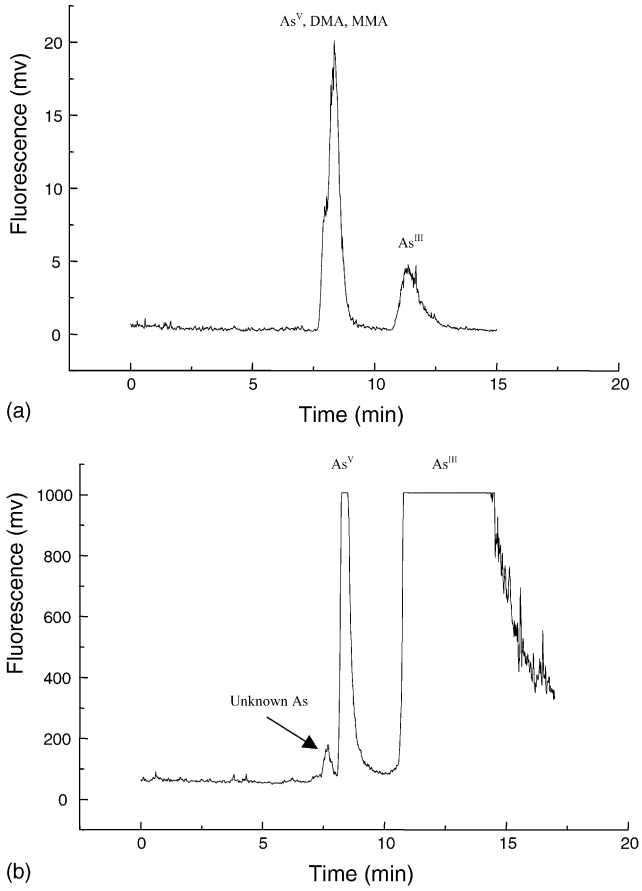

Fig. 2. Arsenic speciation in leaflets exposed to As with SEC–HG–AFS. Mobile phase: 0.015 M potassium phosphate buffer at pH 5.9 with 0.1 M NaCl. (a) Standard chromatogram of As^{III}, MMA, DMA and As^{V}. (b) As species in leaflets extracted with 0.015 M EDTA.

ties of the unknown As species. The unknown As species was not thermally stable. Extraction of leaflets with dH_2O at room temperature resulted in the complete decomposition of the unknown As species after 4 h, but at 4 °C, 25% of the peak remained after 24 h. DMA is relatively stable at room temperature, whereas the unknown As species is temperature sensitive and decomposed rapidly at room temperature, supporting the conclusion that the two species are different. Except for DMA, no other known As species have a similar chromatographic behavior to that of the unknown As on AEC. Therefore, this small peak is most likely an As complex based on its chromatographic behavior and stability.

It has been reported that thiol-containing peptide compounds, e.g. glutathione (GSH) and phytochelatins (PCs), can chelate As to form As^{III}–tris–thiolate complexes [10,11]. The formation of As^{III}–PC complexes has been confirmed by ESI-MS [11]. Considering PC_2 is induced under As exposure in *P. vittata* [17,18], we initially thought that the unknown As could be a As^{III}–PC_2 complex. Attempt to utilize reversed phase LC-ESI/MS for characterization of this unstable As complexes was not successful due to the poor separation obtained with a C_{18} column. Further purification of the sample extract could not be done because the unknown As was not stable enough. Therefore, an alternative method was developed to determine whether the unknown As was actually the As^{III}–PC_2 complex by comparing their stability at different pHs. As^{III}–PC_2 complex is relatively stable only at weak acid solution. At pH 4.0, in vitro reconstituted As^{III}–PC_2 complex shows the maximum stability and can be detectable on SEC or ESI-MS [11]. We tested the stability of the unknown As in SEC mobile phases with different pHs. When different pH buffer solutions (4.0, 7.0 and 8.0) were used as SEC mobile phase, elution profiles were similar to the profile at pH 5.9, indicating that this unknown As species is not sensitive to pH change. The stability of the unknown As species was also investigated in different extraction solvents. The unknown As was extracted with dH_2O, dH_2O–methanol (1:1), EDTA (0.015 M), acetate buffer (0.015 M, pH 4.0), potassium phosphate buffers (0.015 M, pH 5.9 and 7), and Tris–HCl buffer (0.015 M, pH 8.0). However, maximum stability was achieved with 0.015 M EDTA extraction, less than 25% of the unknown As species extracted with EDTA was decomposed after 48 h even at room temperature. The appearance of the unknown As on SEC in neutral and weak basic solutions suggests that it is unlikely the As^{III}–PC_2 complex. Extractions in a stainless steel homogenizer caused no detection of the unknown As, suggesting that the unknown As complex is sensitive to metal ions.

3.3. Reconstitution and analysis of As^{III}–PC_2 complex

To further confirm the presence of the unknown As complex, As^{III} and PC_2 were mixed together in SEC mobile phases at varying pH (4.0, 5.9, 7.0, and 8.0) to reconstitute As^{III}–PC_2 complex in vitro. The structures of PC_2 and As^{III}–PC_2 complex are shown in Fig. 3. As^{III}–PC_2 complex was only detected with mobile phase at pH 4.0 on a size-exclusion column (Fig. 4), indicating that at the pHs

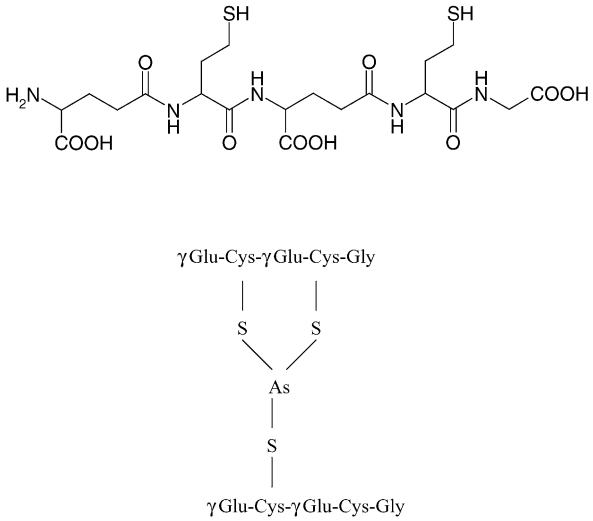

Fig. 3. The structures of PC_2 and As^{III}–PC_2 complex.

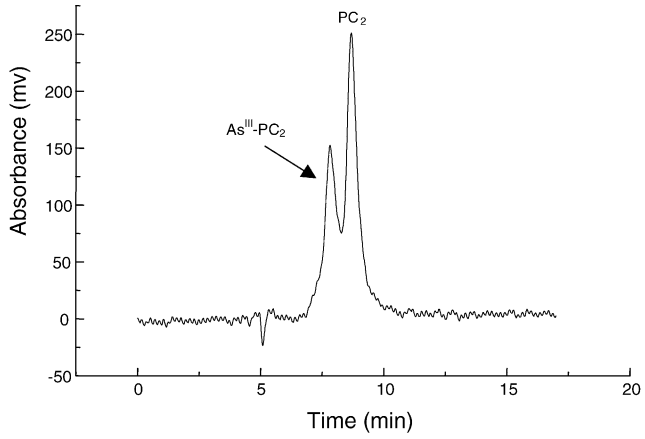

Fig. 4. Analysis of reconsitituted As^{III}–PC_2 complex with SEC-post column derivatization. As^{III}–PC_2 complex was reconstituted in sodium acetate buffer (0.015 M containing 0.1 M NaCl at pH 4.0). Thiols in PC_2 were spectrometrically monitored at 412 nm.

studied, As^{III}–PC_2 was most stable at pH 4.0. Our result was consistent with the report by Schmöger et al. [11], and further confirmed that the unknown As was not an As^{III}–PC_2 complex. On an anion-exchange column, however, neither PC_2 nor As^{III}–PC_2 complex could be eluted within 17 min using phosphate buffer (0.015 M, pH 5.9) as the mobile phase (data not shown). Different chromatographic behaviors of the unknown As complex (detectable on AEC) and the reconstituted As^{III}–PC_2 complex (not detectable on AEC) also indicate that the unknown As is not an As^{III}–PC_2 complex. The absence of As^{III}–PC_2 complex on AEC is probably due to its degradation in the mobile phase of pH 5.9. It is interesting to note that PC_2 was not detected on AEC either. This can be explained from its chemical structure. PC_2 is an acidic peptide with two γ-glutamic acids (pK_a = 2.39, 3.18, 4.01) [25] (Fig. 3). At pH 5.9, PC_2 is present as a trivalent anion. This trivalent anion was strongly adsorbed on the anion exchange column so that it could not be eluted with the mobile phase within 17 min. When anionic PC_2 chelates neutral As^{III}, only sulfhydryl groups from cysteine are involved in this process. Hence, the As^{III}–PC_2 complex likely has pKa similar to PC_2 and is still a trivalent anion at pH 5.9. Even if the As^{III}–PC_2 complex were stable enough at pH 5.9, it would not be easily eluted on AEC. At the same pH, the unknown As complex is eluted between neutral As^{III} (pK_a = 9.2, 12.1, 13.4) and DMA (pK_a = 6.2) on AEC, suggesting that the unknown As complex is also in a neutral form. Different charge states of the unknown As complex and As^{III}–PC_2 complex again indicate that they are not a same compound.

3.4. Possible role of the unknown As complex in As hypertolerance and hyperaccumulation

Arsenic detoxification mechanisms have been studied in a variety of As nonhyperaccumulating plants. PCs are essential for As detoxification in these plants [11,15,16,26]. Results from these studies suggest that As^{III} is chelated by PCs in cytoplasm and the As complexes are further transported into vacuoles. However, in this As hyperaccumulator, PCs were shown to play a limited role in As detoxification [18,19]. A PC-independent sequestration of As into vacuoles was suggested to play a major role in As tolerance in *P. vittata* [18,19]. The unknown As complex found in this study is probably related to the PC-independent sequestration and responsible for both As hypertolerance and hyperaccumulation in *P. vittata*. The peak of the unknown As species in plant leaflets seemed very small compared to that of As^{III} on the chromatogram. However, the concentration of the unknown As was estimated to be at several hundreds μmol/kg in the leaflets when As^{V} was used as the standard. The unknown As species is relatively stable to pH from weakly acidic to weakly basic environments, whereas As^{III}–PC complexes are only stable in weakly acidic environment. Therefore, when both the unknown ligand and PC_2 are present in the weakly basic cytoplasm, formation of the unknown As complex is more likely than As^{III}–PC_2 complex. The unknown As complex is perhaps formed in cytoplasm, and transported to vacuoles where it is degraded and ligand is further decomposed or reused. The unknown ligand probably functions as a shuttle to transport As^{III} from cytoplasm into vacuoles, and most of As^{III} is stored in the vacuoles. This may explain why the concentrations of the unknown As complex is much less than those of As^{III}. Further research is needed to figure out the detailed roles of this unknown As complex in As hyperaccumulation and hypertolerance in *P. vittata*.

4. Conclusions

An unknown As complex was found in the leaflets of *P. vittata* by using AEC–HG–AFS and SEC–HG–AFS. Its chromatographic behavior, stability at different pHs, and charge state suggest that the unknown As complex was not an As^{III}–PC_2 complex. The unknown As complex is sensitive to temperature and metal ions, but relatively insensitive to pH. At pH 5.9, the chromatographic behavior of the unknown As complex on AEC reveals that it is a neutral species. To our best knowledge, this is the first report to show the presence of an As complex in plants that is not an As^{III}–PC complex. This finding is useful for understanding the mechanisms of As hypertolerance and hyperaccumulation in *P. vittata*.

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