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Purification and Characterization of Thiols in an Arsenic Hyperaccumulator under Arsenic Exposure

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***Pteris vittata* (Chinese brake fern) is the first reported arsenic hyperaccumulator. To investigate the arsenic tolerance mechanism in this plant, reversed-phase HPLC with postcolumn derivatization was used to analyze the thiols induced under arsenic exposure. A major thiol in the plant leaflets was found to be responsive to arsenic exposure. The arsenic-induced compound was purified on a large scale by combining covalent chromatography and preparative reversed-phase HPLC. About 2 mg of this compound was isolated from 1 kg of fresh leaflets. The purified arsenic-induced compound was characterized using electrospray ionization mass spectrometry. A molecular ion (M + 1) of 540 and fragments were obtained, which indicated that the arsenic-induced thiol was a phytochelatin with two subunits (PC_2). Compared to the classical methods for purification of phytochelatins, this new method is more specific, simple, and rapid and is suitable for purification of PCs in a large scale as well as sample preparation for mass spectrometry analysis.**

Heavy metals (e.g., Pb, Cd, Ni, Cu, Zn) and metalloids (e.g., As, Se) are nonessential or microessential elements for plants. Excessive amounts of these elements are phytotoxic. Toxicity may result from the binding of metals to sulfhydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacement of an essential element resulting in deficiency effects.[1] In response to excessive uptake of heavy metals, plants have evolved detoxification mechanisms. Complexation of heavy metals in cytoplasm and sequestration of heavy metals to vacuoles is considered one of detoxification mechanisms. Thiol-containing compounds, e.g., glutathione (GSH), phytochelatins (PCs) and metallothioneins (Mets), play an important role in the detoxification in plants through complexation of heavy metals.[2]

Pteris vittata (Chinese brake fern), the first reported As hyperaccumulator, is a plant with potential use for phytoremediation of As-contaminated soils.[3] This fern lacks a biochemical pathway to convert more toxic inorganic As to less toxic organic As, since only arsenate (As^V) and arsenite (As^{III}) were found.[4,5] Therefore, it is believed that some ligands are involved in the formation of As complexes to avoid acute toxicity of free As in *P. vittata*. Thiol-containing compounds, e.g., Mets, PCs, and GSH, are possible chelators of this kind. Mets, which are thiol-rich peptides encoded by a gene, were suggested to be involved in copper tolerance.[2] PCs, which possess the general structure (γ-Glu-Cys)$_n$-Gly ($n = 2-11$), are synthesized from GSH by phytochelatin synthase.[6,7] They are suggested to play roles in cellular heavy metal homeostasis and detoxification. PCs were induced in plants by Cd, Cu, Ag, Zn, Pb, Ni, and As.[8] It has been confirmed that As can be chelated by GSH and PCs to form As^{III}−tris−thiolate complexes through thiolate bonds by using size exclusion chromatography or mass spectrometry.[9,10] Moreover, in non-As hyperaccumulators, e.g., *Silene vulgaris*, *Rauvolfia serpentina*, *Holcus lanatus*, and *Cytisus striatus*, PCs were shown to play a constitutive detoxification role.[10−13] In Indian mustard, results from X-ray absorption spectrometric analysis indicated that GSH may be involved in As detoxification.[14] Our previous data showed that As induces thiol synthesis in *P. vittata*, and total thiols and acid-soluble thiols exhibit a strong and positive correlation in plant leaflets.[15] This result suggests that synthesis of thiols may be related to As detoxification in the plant.

To investigate the role of the thiols in the As detoxification and hyperaccumulation in *P. vittata*, methods for separation and identification of thiols in plant samples are needed. The most common method used for determining thiol-containing compounds is reversed-phase HPLC (RP-HPLC) coupled with postcolumn derivatization of thiol groups with Ellman's reagent (DTNB). The identification of thiols is based on their retention

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times in RP-HPLC. However, this can be done only with the presence of known standards. For unknown thiols without standards, this method is only suitable for quantitative analysis.[16,17] One of the ideal methods for the identification of unknown compounds in complex matrixes is on-line electrospray ionization mass spectrometry (ESI-MS) or tandem mass spectrometry (ESI-MS/MS) after HPLC. However, these methods are not feasible for compounds with low concentrations in complex matrixes. Proper sample isolation and concentration is required before samples are subject to ESI-MS. In classic purification methods, PCs are concentrated and preseparated from tissue extract by anion exchange chromatography. The preseparated low molecular weight PCs are isolated from the macromolecular proteins with ammonium sulfate fractionation and size exclusion chromatography. RP-HPLC is used to further purify the PCs.[6,18] The purification procedures are tedious and time-consuming. In this study, we report a novel purification method for PCs in plant samples. The method involves the preseparation of As-induced thiols using thiol-selective covalent chromatography (activated thiopropyl agarose) and a further separation and purification step with preparative RP-HPLC. Compared to the classic methods, the technique reported here is fast, specific, and simple for the purification of PCs and other thiols on a large scale.

EXPERIMENTAL SECTION

Plants. Experimental ferns were collected from Central Florida where they were first discovered.[3] After the spores were germinated in peat moss (Lamber, Canada), young ferns with three fronds were transplanted individually into 30-cm pots. After 3 months, sodium arsenate solution (500 mL, 500 ppm) was slowly added to the pots every 2 weeks for a total of five times. The plants were watered as needed, and no fertilizers were applied during the experimental period. Fresh leaflets of the ferns under As exposure were collected for thiol analysis and purification.

Analysis of Thiols by HPLC. Fresh leaflets were ground to powder under liquid nitrogen in a mortar and pestle. The powder was homogenized with 20 mM EDTA with a solid/liquid ratio of 1:5. After centrifuging at 12 000 rpm at 4 °C for 10 min, the supernatant was filtered using a cartridge with a 0.45-μm cellulose membrane. Dithiothreitol (DTT) (50 μL, 10 mg/mL) in 0.3 M phosphate buffer (pH 7.5) with 20 mM EDTA was added to 150 μL of extract. Reaction was allowed to develop at room temperature for 30 min. A 10-μL aliquot of sample was subject to HPLC for analysis.

Thiol analysis was performed using RP-HPLC with postcolumn derivatization as described by Rauser[19] with a modification to the chromatographic separation conditions in order to achieve better results. Briefly, a HPLC system (Thermo Separation Products, TSP) with an autosampler (AS3000) and UV−visible detector (1000) was used. A homemade postcolumn derivatization device consisting of a reaction coil and an isocratic pump (Acuflow Series I, Fisher) was used for postcolumn derivatization. The reaction coil was made of Teflon tubing (10 ft, 0.5-mm i.d.). A C_{18} column (Hypersil ODS, 5-μm, 4 × 250 mm, Agilent) was used for thiol separation. Mobile phases A and B were 0.1% trifluroacetic acid (TFA) and acetonitrile, respectively. The flow rate of the HPLC pump was 1 mL/min. A linear gradient of 0−20% acetonitrile was used for 40 min and followed by washing the column with 50% acetonitrile for additional 5 min. The postcolumn 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. The thiols were monitored by an UV−visible detector at 412 nm.

The As-induced thiols were also analyzed by HPLC−UV without postcolumn derivatization. Wavelengths of 220, 254, or 280 nm were used. The mobile-phase flow rate and gradient were the same as those for the method of using HPLC with postcolumn derivatization.

Synthesis of Activated Thiopropyl Agarose. Activated thiopropyl agarose beads were synthesized using a method described by Axen et al. with minor modification.[20] Briefly, Sepharose 6B gel (Sigma) was washed on a sintered glass funnel with distilled water and aspirated free of interstitial water. The gel (45 g) was suspended in 100 mL of NaOH (1.0 M) in an Erlemmeyer flask. Epichlorohydrin (45 mL) was added at room temperature, and the reaction was allowed to develop at 37 °C in an air bath shaker for 2 h. The epoxide-activated gel was washed with distilled water on a sintered glass funnel until reaching neutral pH and then allowed to react with 50 mL of sodium thiosulfate (2.0 M) under shaking using an air bath shaker for 6 h at room temperature. The synthesized *S*-alkyl thiosulfate agarose gel was washed free of sodium thiosulfate with distilled water. The *S*-alkyl thiosulfate agarose gel was then suspended in 30 mL of sodium bicarbonate solution (0.1 M) containing 1.0 mM EDTA. DTT (1 g), dissolved in 5 mL of EDTA (1.0 mM), was added to reduce the *S*-alkyl thiosulfate agarose gel to thiol agarose gel. The reduction was carried out at room temperature for 30 min. The thiol agarose gel was then washed on a sintered glass funnel with 300 mL of sodium bicarbonate solution (0.1 M) containing 1 M sodium chloride and 1 mM EDTA and followed by 1000 mL of EDTA (1 mM) solution. The gel was washed with 500 mL of acetone (60% in 0.05 M sodium bicarbonate solution containing 1 mM EDTA) and suspended in 30 mL of the same solvent. One gram of 2,2′-dipyridyl disulfide in 20 mL of the above solvent was immediately added to activate the gel. The reaction was allowed to develop at room temperature for 1 h. The product was washed with 500 mL of acetone (60% in water) and finally with 1000 mL of EDTA solution (1.0 mM). The activated gel was kept in the EDTA (1.0 mM) solution at 4 °C until use.

Purification of the As-Induced Thiol. Covalent chromatography and preparative RP-HPLC were used to purify the As-induced thiol. Fresh leaflets (1 kg) were collected and rinsed with deionized water. The leaflets were ground with liquid nitrogen in a mortar and pestle. Ice-cold EDTA solution (1500 mL, 10.0 mM) was added to the powder. After being homogenized for 10 min, the slurry was filtered using cheesecloth. The debris was extracted again with an EDTA solution (1500 mL, 10.0 mM). The crude extracts were combined and centrifuged at 12 000 rpm for 10 min. A Buchner funnel with two layers of filter paper was used to filter

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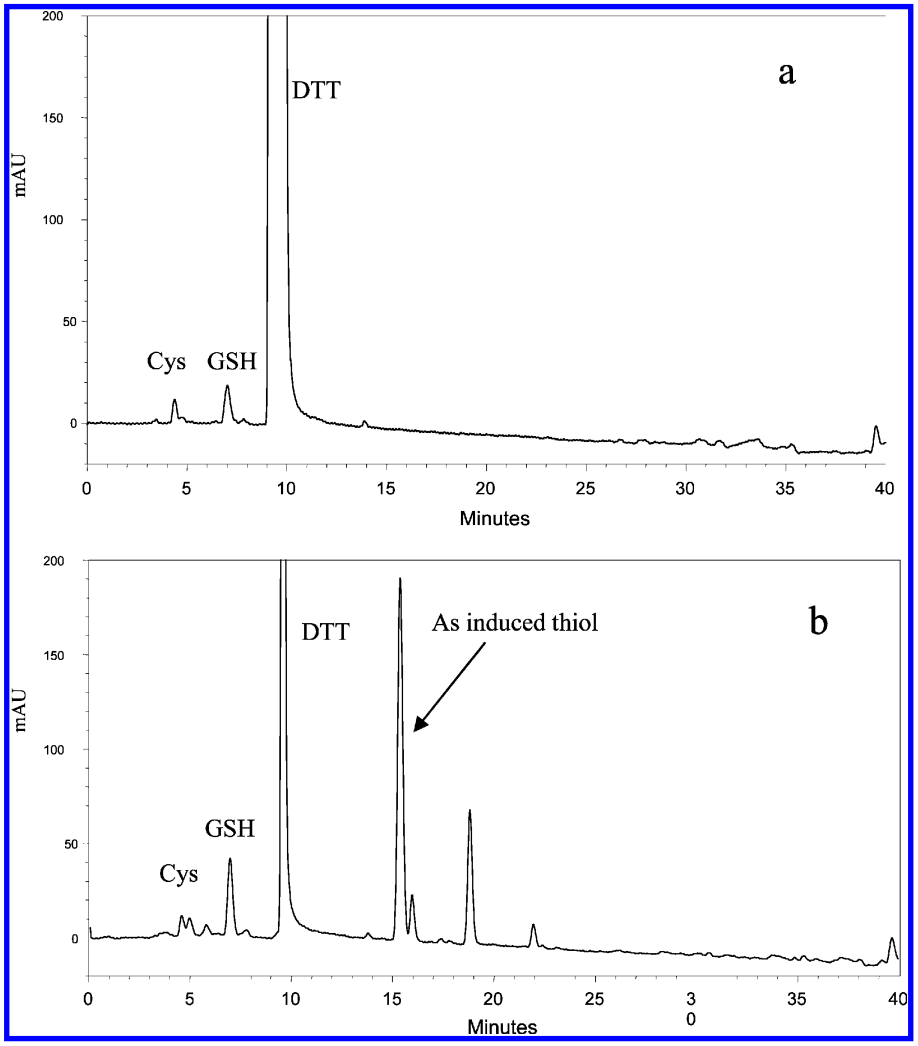

Figure 1. Chromatograms of thiol analysis by RP-HPLC with postcolumn derivatization: (a) control leaflets; (b) leaflets under As exposure.

the supernatant. The activated thiopropyl agarose (10 g) was then added to the filtrate. The pH of the mixture was adjusted to 7.8 with solid sodium bicarbonate. After the reaction was carried out at room temperature for 4 h, the gel was collected using a sintered glass funnel. The gel was washed with 200 mL of NaCl (0.1 M), and then with 200 mL of phosphate buffer (0.05 M, pH 7.4) containing 2.0 mM EDTA. The gel was slightly suctioned by vacuum and then transferred to a flask. Phosphate buffer (10 mL, pH 7.4) containing 100 mg of DTT was added to the gel to release thiols. The reaction was allowed to develop at room temperature for 2 h. The identity of the thiols separated using this method was confirmed using HPLC with the postcolumn derivatization technique. After analysis, the collected thiols was lyophilized and used in further purification by preparative HPLC.

A C_{18} column (5 μm, 21.2 × 250 mm, Supelco) was used in the preparative HPLC to separate thiols. Mobile phase was 5% acetonitrile in 0.1% TFA, and the flow rate was 10 mL/min. The thiols were monitored at 220 nm. The lyophilized crude thiols (~142 mg) were dissolved in 1.0 mL of deionized water. After centrifugation, a 0.5-mL aliquot was subject to HPLC. The As-induced thiol peak was collected, analyzed, and lyophilized. About 2 mg of pure As-induced thiol was obtained from 142 mg of thiols obtained using covalent chromatography.

Characterization of the As-Induced Thiol by LC/ESI-MS. Characterization of the purified thiol was carried out using LC/MS (Navigator, Thermo Finnigan) with a C_{18} column (2.1 × 10 mm, 3 μm, Mercury MS, Phenomenex). The mobile phase was 20% methanol in 0.05% formic acid solution. The flow rate of the HPLC was 0.2 mL/min. The experimental conditions of the ESI-MS are the following: cone voltage, 47 V; acquisition time, 4 s; mass range, 300−1400. The purified As-induced thiol (2 mg) was dissolved in 2 mL of deionized water, 5 μL of which was subject to LC−MS. The number of thiol functional groups in the thiol-containing compounds was determined by alkylation of the purified thiol with iodoacetic acid.[21] To do this, 100 μL of the thiol solution (~0.1 mg/mL) was reacted with 100 μL of iodoacetic acid (1 mg/mL) in 0.005 M Tris buffer (pH 8) containing 2.0 mM EDTA. The reaction was allowed to develop in the dark at room temperature for 1 h. The alkylated thiol was analyzed by LC/ESI-MS. The ESI-MS conditions were the same as those for the unalkylated thiols, except for the cone voltage being 10 V.

RESULTS AND DISSCUSSION

Analysis of As-Induced Thiols in Leaflet Extract with RP-HPLC. RP-HPLC with DTNB postcolumn derivatization is a

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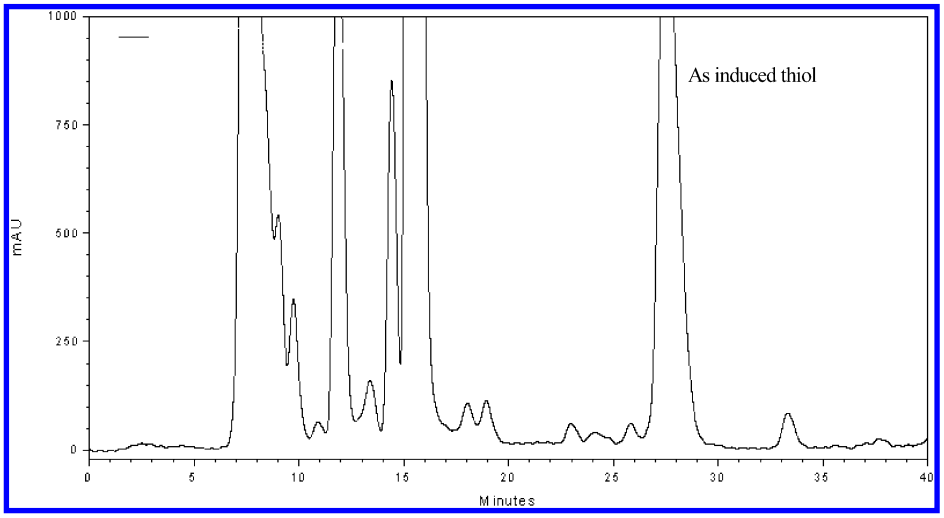

Figure 2. Preparative RP-HPLC chromatogram for the analysis of thiols after separation from As-treated plant by covalent chromatography.

traditional method for analyzing thiol-containing compounds in biological samples.[7,19] Through a thiol−disulfide exchange reaction, the aromatic disulfide (DTNB) is reduced by thiols in samples to release 5-mecapto-2-nitrobenzoate (MNB). MNB can be detected at 412 nm by UV−visible detector. Using this method, we analyzed the thiols in leaflets of *P. vittata* (Figure 1). In control leaflets, only cysteine (Cys) and GSH were found. In leaflets under As exposure, however, there was a major thiol-containing compound (referred as As-induced thiol in the following discussion) in addition to Cys and GSH. The presence of this thiol in the plant was clearly related to As exposure.[22] The peak at retention time of 18.8 min was from an unknown disulfide compound, since it only was present in the extract with DTT reduction (Figure 1b). The small peak appearing at 21.9 min was not identified.

Initially, efforts were made to directly collect the fractions containing the nonderivatized As-induced thiol for further characterization with LC/ESI-MS. This was done by using RP-HPLC with UV detection at three typical wavelengths: 220, 254, and 280 nm. However, the chromatograms of the control leaflets and As-exposed leaflets showed no obvious differences (date not shown), and the As-induced thiol could not be detected by UV detection. The results indicated that the As-induced thiol has a weak UV absorption or small content. Additionally, several other compounds were coeluted with the As-induced thiol. The fractions, which possibly contained the As-induced thiol, were collected and subject to LC/ESI-MS. No useful information was obtained from MS because of the low analyte signal and high interferences. Therefore, purification of the As-induced thiol before LC/ESI-MS characterization became required.

Purification of As-Induced Thiol with Covalent Chromatography and Preparative RP-HPLC. Because of the unknown properties of the As-induced thiol (e.g., pK_a), optimization of the separation condition for ion exchange chromatography would be time-consuming. Basing on the fact that the As-induced product contains thiol groups, we proposed a novel method, which utilized covalent chromatography coupled with preparative RP-HPLC to purify the As-induced thiol in the leaflets of *P. vittata*. Covalent

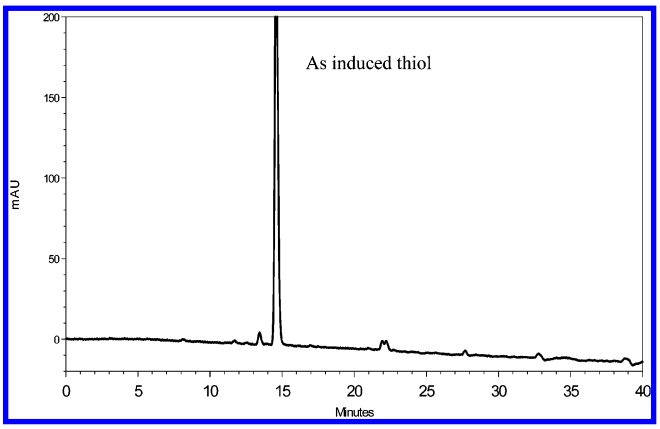

Figure 3. Chromatogram of the As-induced thiol after purification by covalent chromatography combined with preparative RP-HPLC.

chromatography, a special affinity chromatographic technique, has high selectivity and the capacity to absorb thiol-containing compounds.[23−25] Thiol groups react with activated thiopropyl agarose gel to form disufide bonds through a thiol−disulfide exchange reaction. After the impurities are washed off, the thiol-containing compounds are released from the gel by reduction of the disulfide bonds with DTT. Since the separation of thiols using this covalent chromatography is specific, it is not necessary to optimize separation conditions. In addition, covalent chromatography is especially useful to concentrate low levels of thiols from large volumes of matrixes. During the course of our experiment, covalent chromatography was demonstrated to be extremely efficacious to selectively concentrate trace levels of thiols on a large scale.

Thiols were concentrated from 3000 mL of extract obtained from 1 kg (fresh weight) of As-exposed leaflets using 10 g (wet weight) of the activated thiopropyl agarose gel. After 4 h of reaction, no thiols were found in the extract, indicating that all thiols formed covalent bonds with the activated gel. The absorbed thiols were easily released through the reduction of the disulfide

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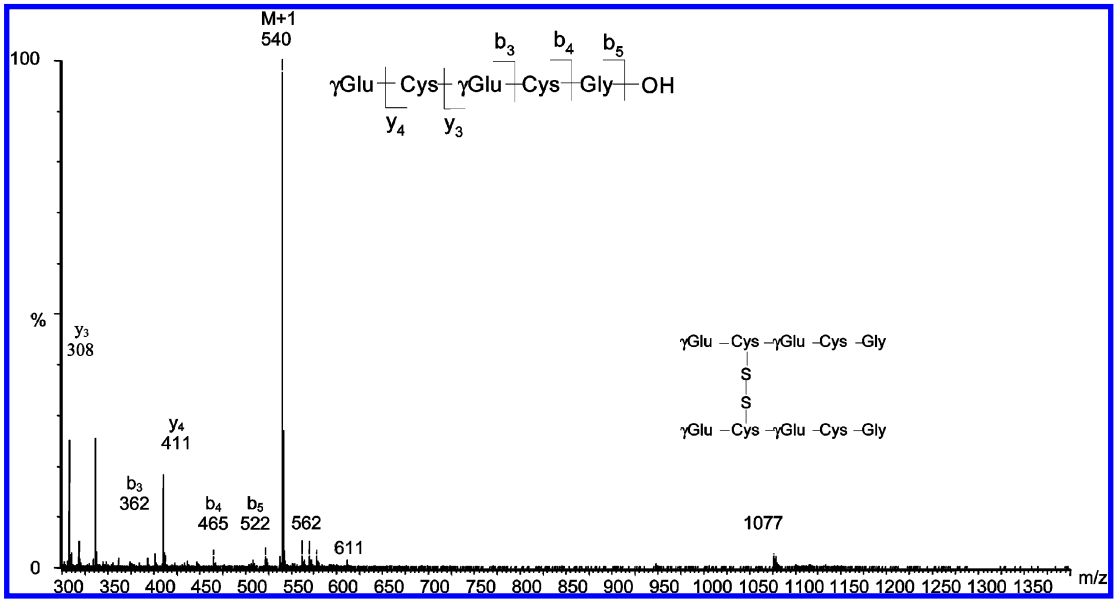

Figure 4. Electrospray ionization mass spectrum of the purified As-induced thiol (PC_2).

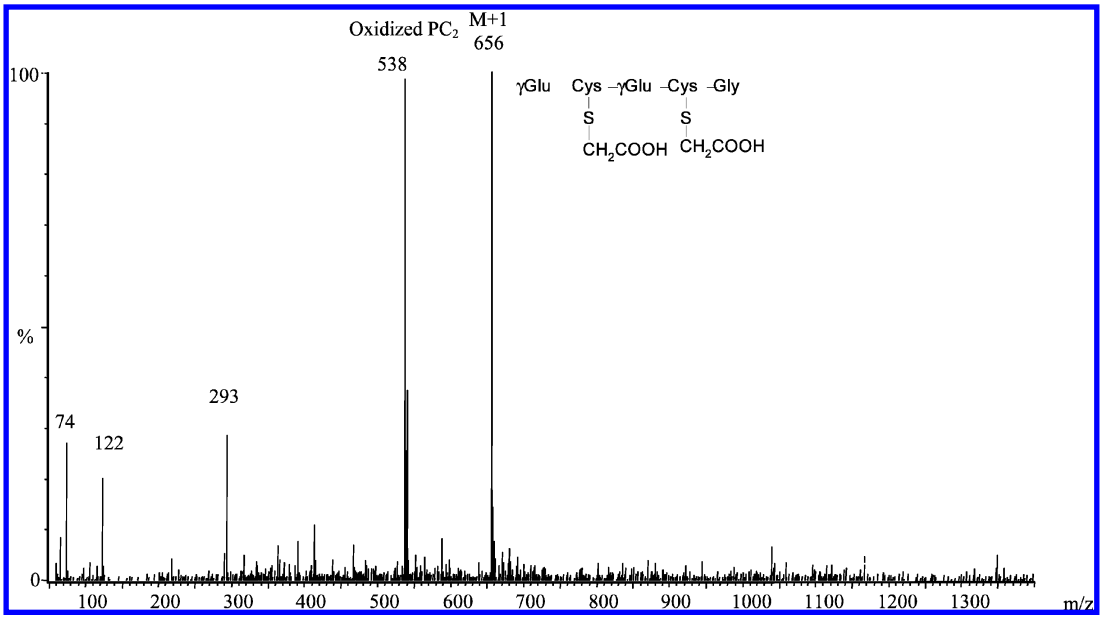

Figure 5. Electrospray ionization mass spectrum of alkylated As-induced thiol (PC_2). The peak at *m*/*z* 656 is the molecular ion of the alkylated PC_2, and *m*/*z* 538 is the molecular ion of the oxidized (formation an intramolecular disulfide bond) PC_2.

bonds with DTT. The enrichment factor of the covalent chromatographic adsorption for thiols is 300 if 10 mL of DTT solution is used to release thiols from the gel. However, this purified As-induced thiol using covalent chromatography was still not suitable for characterization by LC/ESI-MS due to high levels of salts and DTT present in the solution.

To further purify the As-induced thiol and remove salts, preparative RP-HPLC was used to separate the primarily purified thiols using covalent chromatography. Compared with a regular analytical column, the preparative column has a much higher sample loading capacity, which allows the separation of much more sample in one injection. However, optimal separation conditions on a preparative column are not as easy to develop as on an analytical column. We first optimized the separation conditions on an analytical column (C_{18}, 5 μm, 4.6 $\times$ 250 mm) with the same packing material as in the preparative column. These conditions were amplified on a 21.2 $\times$ 250 mm preparative column (C_{18}, 5 μm). The chromatogram obtained using preparative HPLC is shown in Figure 2. The separation was completed within 40 min, and a nice baseline separation of the As-induced thiol was achieved. Using this method, the As-induced thiol was rapidly purified within 2 h by injecting only twice (500 μL of each injection). About 2 mg of As-induced thiol was obtained from 1 kg of fresh leaflets by covalent chromatography combined with preparative RP-HPLC. HPLC analysis indicated that the As-induced thiol was successfully separated and collected with high purity using this method (Figure 3).

Characterization of the Purified As-Induced Thiol. To obtain structural information on the purified As-induced thiol, high cone voltage (47 V) was used for production of both molecular ion and fragments in ESI-MS. The mass spectrum of ESI-MS showed that the purified As-induced thiol has a molecular ion

(M + 1) 540 (Figure 4). The small signal at m/z 1077 indicated the presence of the dimer of the thiol. To further confirm the finding and structure of the As-induced thiol, derivatization of this compound was conducted using iodoacetic acid.[21] The alkylated compound showed molecular ion (M + 1) 656, which indicated that two thiol groups exist in this thiol compound (Figure 5). The results implied that the As-induced compound was PC_2 (γGlu-Cys-γGlu-Cys-Gly, M_r 539). Under our experimental conditions, the PC_2 fragmented primarily at the amine bonds. The charge can be retained on the amion terminus (type b-ion) or on the carboxy terminus (type y-ion). The interpretation of the fragments obtained are as follows. The peak at m/z 522 was attributed to desOH$-PC_2$; the peaks at m/z 465 and 362 were attributed to the type b-ions of desGly-PC_2 and desGlyCys-PC_2, respectively; and the peaks at m/z 411 and 308 were attributed to the type y-ions of desγGlu-PC_2 and desγGluCys-PC_2, respectively.

In summary, a novel method for purification of an As-induced PC in the leaflets of *P. vittata* on a large scale was developed successfully by using covalent chromatography coupled with preparative RP-HPLC. The purified thiol from *P. vittata* was characterized as PC_2 by using ESI-MS. The new method is rapid, simple, and specific. While the classical methods for PC purification require four-step separations, the new method only has two steps. This method can be used to purify and characterize other PCs. Purification and identification of the PCs synthesized in *P. vittata* is important for understanding the As tolerance mechanism in this hyperaccumulator.

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