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Preliminary study of the enrichment and fractionation of REEs in a newly discovered REE hyperaccumulator *Pronephrium simplex* by SEC-ICP-MS and MALDI-TOF/ESI-MS†

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Concentrations of rare earth elements (REEs) were determined in the laminae of 10 species of ferns and their acetone-extractable pigments, as well as their host soil and soil extract, by ICP-MS. A new REE hyperaccumulator, *Pronephrium simplex*, was discovered which could accumulate REEs up to 1.2 mg g^{-1} dry mass under natural growth conditions. Three typical species of ferns chosen were divided into lamina, petiole, stem and root for the study of REE translocation and fractionation. A hyphenated technique, size exclusion HPLC coupled with online UV/ICP-MS, was developed to provide reliable evidence of the existence of REE-binding proteins in the fern's lamina. A new REE-binding protein was discovered and separated from the lamina of natural grown *P. simplex*. Further characterization of the protein showed that its molecular mass is 5068.4 Da by MALDI-TOF-MS and ESI-MS. Amino acid composition analysis by RP-HPLC indicated that the protein has relatively high contents of proline and glycin.

Introduction

A metal-hyperaccumulator is a plant that has the ability to hyperaccumulate metals from its natural habitat, in other words, the hyperaccumulator plant's metal-concentration threshold in the shoot is exceeded under non-inhibitory growth conditions.[1] Phytoremediation, a technique using hyperaccumulator to remove contamination from soil and water, is a good choice as it is safe and cost-effective. Although the hyperaccumulators of heavy metals have been intensively studied,[2] less attention was paid to rare earth element (REE) hyperaccumulators. The most famous REE-hyperaccumulator, *Dicranopteris dichotoma*, found by Li *et al.* at a REE mining area in the south of the Jiang Xi province of China, can accumulate REEs up to 0.32% dry weight.[3] Guo *et al.*[4] and Wang *et al.*[5] recently separated out REE-binding proteins and/or peptides in *D. dichotoma*. However, detailed information about these REE-containing proteins and/or peptides had not been shown. In the present work, we established an integrated analysis system to seek for promising REE hyperaccumulators, and provide valuable information on REE distribution, fractionation and possible chemical existing form, which should be useful for further studies on the phytoremediation of REE contamination.

Experimental

Instrumentation

The HPLC system was composed of two Shimadzu LC-10ADvp pumps and a Shimadzu SPD-M10Avp PDA detector with an 8-μL flow cell. The system was controlled by a Shimadzu SCL-10Avp system controller and Shimadzu CLASS-VP 6.0 workstation. A micro flow cell of 2.5 μL was used in a micro-HPLC system for amino acid analysis.

A 10 mm id × 500 mm length Sephadex G-25 column was used to fractionate the extracted proteins in *P. simplex*. 50 mmol L^{-1} Tris-HCl (pH 8.0) was used as an eluent, and the flow rate was 1 mL min^{-1}. Size exclusion HPLC (SE-HPLC) was performed on a 7.9 mm id × 500 mm length column (Shimadzu, Shim-pack DIOL-300; particle size, 5 μm; molecular weight range from 500 to 1 000 000 Da) with a precolumn (Shim-pack DIOL, 4.0 mm id × 50 mm length; particle size, 10 μm). The standards (all Sigma) used for calibration consist of thyroglobulin (660 000 Da), transferrin (80 000 Da), metallothionein II (6918 Da) and Vitamin B12 (1355.4 Da). An eluent of 30 mmol L^{-1} Tris-HCl (pH 8.0) was used for separation; H_2O (pH = 8.0 adjusted by $NH_3 \cdot H_2O$) was used as an eluent for desalination, the flow rate being 0.75 mL min^{-1}. RP-HPLC analysis of amino acids was performed on a 2.0 mm id × 150 mm length C_{18} column (Shim-Pack VP-ODS; particle size, 4.6 μm) with a precolumn (Shim-pack GVP-ODS, 2.0 mm id × 5 mm length) through gradient elution. The flow rate was 0.20 mL min^{-1}. Eluent A: 0.13 mol L^{-1} NaAc + 6% acetonitrile (pH = 6.4); eluent B: 60% acetonitrile. Time programme: 0–5 min, B 0%; 5.01–12 min, B 8–10% (curve 6); 12–18 min B 10–12% (curve 6); 18–21 min B 12–37% (curve 10); 21–32 min B 37–41% (curve 6); 32–33 min B 41–100%.

A HP 4500 ICP-MS (Yokogawa, Japan) fitted with a Babington nebulizer kit was used for the determination of REEs. The working conditions were as follows: rf power, 1350 W; plasma gas flow rate, 16 L min^{-1}; carrier gas flow rate, 1.12 L min^{-1}; auxiliary gas flow rate, 1.0 L min^{-1}; sampling depth, 6.3 mm; sample uptake rate, 0.4 mL min^{-1}; acquisition mode, full quantification; integration time per point, 300 ms; and no. of replicates, 3. Detected element isotopes were: ^{89}Y, ^{139}La, ^{140}Ce, ^{141}Pr, ^{143}Nd, ^{147}Sm, ^{153}Eu, ^{155}Gd, ^{159}Tb, ^{161}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{172}Yb and ^{175}Lu. When it was coupled to SE-HPLC, the data acquisition mode was changed to time resolve. The accuracy of determinations was controlled by a Chinese standard reference material GBW 070603 (Geological Department, Academy of China). Good agreement ($P > 0.05$) was achieved between the obtained data and the certified values.

MALDI-TOF-MS was performed on a Bruker Reflex III instrument (Bremen, Germany) using sinapic acid as the matrix. ESI-MS was performed on Bruker ESQUIRE-LC™ ion trap MS

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(Bremen, Germany). A Hitachi F-4500 fluorescence spectrophotometer was used for the structural analysis of proteins.

Reagents

REE oxides (purity >99.9999%) were obtained from the Changchun Institute of Applied Chemistry of CAS. 2-Mercaptoethanol was purchased from Sigma, sodium dodecylsulfonate (SDS) and phenyl isothiocyanate (PITC) from TCI, phenylmethylsulfonyl fluoride (PMSF) from AMESCO, amino acids reference collection from BDH Chemicals Ltd., Poole, England and Sephadex G-25 from Pharmacia. All other reagents were of the highest available purity.

Sample collection, preparation and analysis

Ferns (*Alsophila spinulosa, Pronephrium lakhimpurense, Pteris fauriei, Gymnosphaera metteniana, Woodwardia japonica, Pronephrium simplex, Diplazium donianum, Arachniodes exilis, Lindsaea chienii, Microlepia hookeriana*) and their host soils were respectively collected from Nanjing Natural Reservation of tropical rain forest, Fujian province of China (North latitude: 24°56′20″, east longitude: 117°11′30″).

The ferns sampled were thoroughly washed with Milli-Q water, dried under 105 °C for 24 hours, and ground. Approximately 0.2 g of each was digested by HNO_3 and $HClO_4$ for the determination of REE content. Soil samples were digested with a mixture of HNO_3–HF–$HClO_4$ (5 : 1 : 1) after being dried and ground. Bioavailable REEs in the host soil were extracted by employing a 0.11 mol L^{-1} acetic acid solution.[6] Pigments in fern laminae were extracted with acetone. Suitable amounts of pigment extracts were dried by a current of N_2 and digested with HNO_3 before ICP-MS detection.

The crude proteins in *P. simplex* were extracted with buffer containing 0.2 mol L^{-1} Tris-HCl (pH 8.0), 1 mmol L^{-1} EDTA, 1 mmol L^{-1} SDS, 20 mmol L^{-1} 2-mercaptoethanol and 1 mmol L^{-1} PMSF.[7] The extract was applied to the Sephadex G-25 column. The eluate was collected every 5 mL, and analyzed by ICP-MS to find the fraction containing REEs, which was subjected to further separation and purification by SE-HPLC. One dominating REE binding protein from SE-HPLC was collected and characterized by MALDI-TOF-MS, ESI-MS and fluorimetry. The concentration of the protein in the fraction was determined by the G-250 method.[8]

The purified REE binding protein was hydrolyzed in 5.7 mol L^{-1} HCl containing 2% 2-mercaptoethanol, 1% phenol and 1% oxalate at 110 °C under vacuum for 22 h. Compositional analysis was conducted by the derivatization of amino acids with PITC followed by RP-HPLC. Losses of the unstable amino acids during hydrolysis were calibrated by the hydrolysis of standard amino acids under the same conditions.

Results and discussion

Method development

An integrated analysis system that was developed is shown in Table 1. The ICP-MS instrument was tuned for optimum signal-to-noise ratio on m/z 140 for the ^{140}Ce isotope. Typically, a sensitivity of approximately 35 000 counts per 0.1 s per ng Ce mL^{-1} in 2% (v/v) HNO_3 solution was achieved. A flow rate of 0.75 mL min^{-1} was chosen for the efficient separation and acceptable retention time of size-exclusion HPLC. A complex gradient elution strategy of RP-HPLC was optimized to gain both a good resolution between 18 amino acids and an acceptable retention time (<35 min). The calibration curve was constructed every day. The detection limit is below 10^{-12} mol for most amino acids by this method. ESI-MS and MALDI-MS were also tuned by the standard solutions for optimum sensitivity and accurate molecular mass.

REE accumulation and fractionation in the soil–fern system

The total amount (Σ_{REEs}, μg g^{-1}) of REEs except Pm in the laminae of the ferns are as follows: *A. spinulosa*, 2.11; *P. lakhimpurense*, 6.25; *P. fauriei*, 11.4; *G. metteniana*, 8.8; *W. japonica*, 152; *P. simplex*, 1234; *D. donianum*, 100; *A. exilis*, 294; *L. chienii*, 56; *M. hookeriana*, 46; Σ_{REEs} in the host soil, 34; and HAc extractable Σ_{REEs} in the soil, 2.24. Great differences were found among different fern species, indicating that the REE contents in the laminae of the ferns growing at the same site are even not of the same order of magnitude. Among all fern species studied, the Σ_{REEs} concentration in *P. simplex* is the highest, reaching 1.2 mg g^{-1}, which is 35 times higher than that, 34 μg g^{-1}, in its host soil. Obviously, it was recognized as a new REE-hyperaccumulator.

Three species of ferns (*P. simplex, D. donianum, L. chienii*) were chosen as examples for further studies, and each of them was divided into 4 parts: lamina, petiole, stem and root, respectively. Σ_{REEs} of the various parts are quite different among the different fern species. *P. Simplex* has much higher Σ_{REEs} in the lamina and petiole than that in its root, which is one of the hyperaccumulator characteristics. However, *D. Donianum* and *L. Chienii* have more than 50% REEs stored in the underground part.

It is known that uptake process of mineral substances in plants usually consists of 5 steps, *i.e.*, (1) migration from bulk soil to soil solution, (2) absorption from the solution by root, (3) translocation from root to stem, (4) stem to petiole, (5) petiole to lamina. The ratio of the sum of chondrite normalized light REEs (LREEs, La–Eu) value to that of heavy REEs (HREEs, Gd–Lu) ($\Sigma_{LREEn}/\Sigma_{HREEn}$) for all the parts of the three ferns was greater than 1, suggesting that fractionation may happen in every migration process, and LREEs accumulation was preferential, in most steps, to HREE accumulation. This might be due to the fact that LREEs have very similar ionic radii (9.5–10.6 nm) to the calcium ion (9.9 nm), resulting in competition between the LREE and Ca. In most cases, the LREE might be more capable of simulating Ca than the heavy REE; especially La^{3+}, which even has nickname "super-calcium". A delicate mechanism which retains the balance between Ca, LREE and HREE is very likely to exist; the fractionation between LREE and HREE in different migration processes might just be the homeostatic regulation by plant itself.

It has been verified that suitable amounts of REEs can enhance the photosynthesis of a plant, and our previous work has proved by ESI-MS that lanthanum could enter into chlorophyll-a and form a double-decker sandwich-like complex in spinach under the stress of La^{3+}.[9] Similarly, LREEs, such as La, Ce and Nd, were also found in the pigments of *P. simplex*.

Table 1 Analytical system developed

	Object	Method
Fern sampled from a natural reservation	REE concentration and distribution in laminae, pigments and proteins of fern species	Offline ICP-MS determination
	Seeking of REE's binding peptides or proteins	On line SE-HPLC/UV/ICP-MS
	Characterization of REE's binding peptides	MALDI-TOF-MS/ESI-MS/fluorimetry
	Amino acid composition of the REE's binding peptide	RP-HPLC

REE-accumulation behavior of *P. simplex*

In order to investigate the accumulation behavior of *P. simplex*, the plant was cultured with modified knop solution (Table S1†) containing 0, 15, 50, 100, 250, and 500 μg mL^{-1} La^{3+}, respectively, for 23 days. Phosphate was applied by spraying on the lamina surface to avoid precipitation with La^{3+} in the culture solution. The content of La in the laminae of *P. simplex* indicated that *P. simplex* has an incredible appetite for La^{3+}. It retains vigour under 100 μg mL^{-1} La^{3+}, with 12.7 mg g^{-1} La in its lamina; it survives even at a concentration as high as 500 μg mL^{-1} La^{3+}, but it was unbelievable that La content in the lamina was as high as 102 mg g^{-1}.

REE binding proteins in natural grown *P. simplex*

The proteins of naturally grown *P. simplex* were extracted and separated by size exclusion chromatography on a Sephadex G-25 column. The eluate was collected every 5 mL followed by UV and ICP-MS detection. Two fractions, which were found containing REEs, were collected and designated preliminarily purified REE-binding proteins. The Σ_{REEs} in fractions I and II were 3000 and 883 μg g^{-1}, respectively, much higher than that in their mother laminae, suggesting that REEs mainly associated with the proteins of *P. simplex*.

To depict the fractionation between different REE more clearly, the chondrite normalized REE patterns were shown (Fig. 1) in the form of a Masuda–Coryell diagram.[10] The distribution pattern of REEs in fractions I and II were quite similar, but clearly different from that in their mother laminae. In the proteins LREE contents decreased rapidly with the increase in the atomic number, whereas HREE only changed slightly, so that the whole pattern could be summarized as LREE-enrichment. However, in their mother laminae, HREE distribution was a declining line with the increase in their atomic number, which was quite different from that in the proteins. Furthermore, both fractions I and II showed a conspicuous positive Ce anomaly, while Ce was deficient in their mother laminae. It could be inferred that these proteins might have high affinity with Ce, and such affinity decreased with the increase in the atomic number of LREEs, while with HREEs, the selectivity was not so obvious.

The preliminarily purified REE-binding proteins were further purified by SE-HPLC, coupled with on-line UV and ICP-MS detection, to provide reliable evidence of the existence of REE-binding proteins. Chromatograms in Fig. 2 suggested that there was only one protein associated with REEs in fraction I; however, at least 3 REE-containing proteins were found in fraction II.

Since fraction I was abundant in REEs, further study was carried out to characterize this REE binding protein by MALDI-TOF-MS after desalination. The result showed that its molecular mass is 5068.4 Da, which was in agreement with

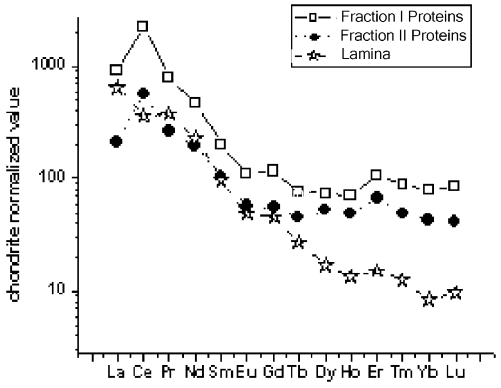

Fig. 1 Chondrite normalized REE patterns in the laminae and proteins of *P. simplex*. Chondrite normalized value = $[REE]_{sample}/[REE]_{chondrite}$.

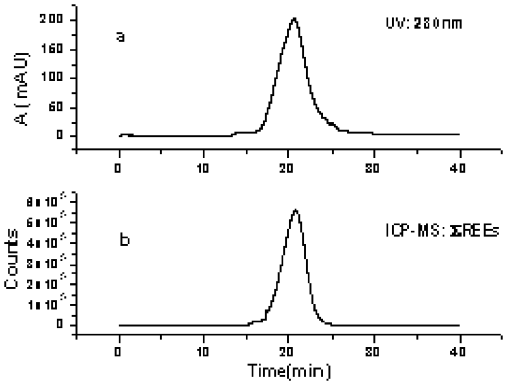

Fig. 2 SE-HPLC/UV/ICP-MS chromatogram of the fraction I proteins of *P. simplex*: a, chromatogram of the fraction I proteins by UV-Vis detector at 280 nm; b, chromatogram of the fraction I proteins by ICP-MS detector. Σ_{REEs} denotes the sum of the counts of all REEs except Pm.

the result (5073.6 Da) obtained by ESI-MS. In the ESI-MS spectrum, the strongest peak, 1692.2 (m/z), is of three positive charges. Moreover, this protein has natural emission fluorescence at 335.8 nm, which is mainly from tryptophan (Trp) residue. Compared with the emission range from 351 to 361 nm of free Trp, there is more than a 15.2 nm blue shift, indicating that Trp might locate in the hydrophobic kernel of the protein.[11] The existence of Trp was also confirmed by the results of amino acid composition analysis.

The amino acid composition of the purified protein I was analyzed by RP-HPLC: the protein has relatively higher contents of proline (Pro) and glycine (Gly), lower contents of phenylalanine and lysine. Such results are, to a certain degree, consistent with the stability order of the REE complexes with different amino acids.[12] The pyrrole ring of Pro can participate in binding with REE, Gly has minimum steric hindrance. Both of them can therefore form stable complexes with REE. However, the binding of REE to the protein might be more complicated: detailed coordination chemistry should have strong correlation with the conformation of the protein.

Further identification of REE-binding proteins from *P. simplex* is still ongoing.

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