Subcellular distribution of rare earth elements and characterization of their binding species in a newly discovered hyperaccumulator

Pronephrium simplex

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Received 31 October 2005; received in revised form 17 December 2005; accepted 22 December 2005

Abstract

Subcellular distribution of rare earth elements (REEs, including 14 lanthanides and yttrium) in a newly discovered REE hyperaccumulator, Pronephrium simplex (P. simplex), was determined by a chemical sequence extraction followed by ICP-MS analysis. Results showed that most REEs are associated with cell wall and proteins, and REEs concentration in the proteins, 2899.5 ± 9262 gg⁻¹, is much higher than those in the cell wall; in the chloroplast of P. simplex, REEs distribute almost equally in chloroplast membrane and thylakoid, while most REEs in the thylakoid are binding with photosystem II (PS II); a new REE-binding peptide in the lamina of P. simplex, which can accumulate REEs up to 3000 gg⁻¹ and has higher affinity with light REEs, was characterized, indicating that its molecular mass is 5073 Da, and may have β-sheet structure; isoelectrofocusing electrophoretic photograph indicated that it is acidic peptide with IP of 3.7. Such information should be useful for understanding of both the storage and physiological role of REEs in P. simplex and further studies on the phytoremediation of REEs contaminated environments.

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Keywords: Hyperaccumulator; Fern; Rare earth elements; Protein; Chloroplast; Photosystem II

1. Introduction

Rare earth elements (REEs) have been widely used in agricultural activities, and the resulted environmental contamination and accumulation in food chain would grow rapidly in the next few decades [1]. Phytoremediation, a technique using plant hyperaccumulator to remove contamination from soil and water, is a good choice as it is safe and cost-effective. Although the hyperaccumulators of other heavy metals were intensively studied [2], less attention was paid to REE-hyperaccumulator. REEs are very likely to exist in certain special forms in plants, and play important role in regular physiological activities. However, there always have been controversial opinions on whether REE can enter into plant cells and influence their physiological activities. Nagahashi et al. [3] have reported that the casparian strip could provide effective barrier to apoplastic movement of lanthanum. Moreover, in early stage many researchers believed that although REEs could bind to the surface of cells, they could not past the membrane and enter into the cell [4]. Recently, scientists discovered that low level of REE could easily pass across the membrane with the help of some carriers, such as protein, hormone, etc. [5–7]. Our previous study by electrospray ionization mass spectrometry (ESI-MS) showed that lanthanum could not only pass across cell wall but also enter into chlorophyll-a and form a double decker sandwich like complex in spinach under the stress of La⁺⁺ [8]. The results from Shan and co-workers [9,10] and Wei et al. [11] also showed that although most of REEs were deposited on cell wall, the rest of REEs could indeed enter into the interior, some even enter into the chloroplast of Dicranopteris dichotoma (D. dichotoma), a famous REE-hyperaccumulator. However, great difference was found between the results from different researchers concerning REEs distribution in the cell of D. dichotoma [9,11,12]. Different fractionation methods might be responsible for the great difference. Furthermore, researches on the mechanism of REE hyperaccumulation at molecular level is too few compared to those of other heavy metals, although the results obtained are somewhat inspiring [13–15]. Guo et al. [13] discovered two REE binding
proteins from \textit{D. dichotoma} by size exclusion chromatography (SEC) and neutron activation analysis (NAA), one of them has the molecular mass of about \(8.0 \times 10^5\) Da and the other less than \(12,400\) Da. Wang et al. \cite{14} found one REE binding peptide of \(2208\) Da from the same plant through ICP-MS and MALDI-MS analysis.

Most recently, a new REE-hyperaccumulator, \textit{Pronephrium simplex} (\textit{P. simplex}), which can accumulate REEs up to \(1.2\) mg g\(^{-1}\) dry lamina mass, was discovered in Nanjing national natural reservation of semi-tropical rain forest of China \cite{15}. In the present work, a chemical sequence extraction combined with ultracentrifuge fractionation and ICP-MS analysis was proposed to investigate the subcellular distribution of REEs in \textit{P. simplex}. A possible REE binding peptide in its laminas was recognized by size exclusion chromatography coupled with ICP-MS, and characterized by ESI-MS, isoelectrofocusing electrophoresis (IEF) and IR.

2. Experimental

2.1. Instrumentation

Beckman AvantiTM J-25 refrigerated centrifuge and Hitachi SCP 55H refrigerated ultracentrifuge were used for subcellular component fractionation. An ELAN DRC II ICP-MS (Perkin-Elmer SCIEX, USA) was used for the determination of REEs. The working conditions were shown in Table 1. ESI-MS was performed on Bruker ESQUIRE-LCTM ion trap MS (Bremen, Germany). IEF was performed on DYY-10C electrophoresis apparatus with DYCZ-27B electrophoresis tank (Beijing Liu Yi instrument plant, China). Avater 360 FT-IR spectrometer (Nicolet, USA) was used to obtain the IR spectrum of REE binding peptide.

2.2. Reagents and samples

REE oxides (purity \(\geq 99.9999\%\)) were obtained from Changchun Institute of Applied Chemistry of Chinese Academy of Science. 2-Mercaptoethanol was purchased from Sigma, sodium dodecylsulfonate (SDS) from TCI (Japan), phenylmethylsulfonyl fluoride (PMSF), Triton X-100 and 2-(N-morpholino) ethane sulfonic acid (MES, ultra pure) from AMESCO, dithioerythritol (DTT) (>99.0%), acrylamide (>99.9%), bis-acrylamide (>99.9%), nonidet P-40 (NP-40), urea (>99.5%), tetraethylethylene diamine (TEMED) (>99.0%) and ammonium persulphate (>98.0%) from BBI (Canada), isoelectric focusing calibration kit Broad PI (pH 3.0–10.0) and Ampholine\textsuperscript{TM} (pH 3.5–10) from Amersham (Sweden). All other reagents were at least of AR or BR grade.

\textit{P. simplex} is a short creeping fern that grows in forest or on stream banks at low altitudes distributing mainly in China, Viet Nam and Japan (Ryukyu Islands). \textit{P. simplex} and its host soil were collected from Nanjing natural reservation of semi-tropical rain forest, Fujian province of China (north latitude: 24° 56′ 20″; east longitude: 117° 11′ 30″). The photograph of this fern is shown in Fig. 1.

2.3. Cell fractionation of \textit{P. simplex} lamina

Cell fractionation method I adopted the protocol described by Shan et al. \cite{9}. The finally resulted precipitation by this method was designated as crude proteins and further subject to SEC-ICP-MS analysis using the conditions described in our previous work \cite{15}. The separated REE binding peptide was further characterized by ESI-MS, IEF and IR spectrum. Cell fractionation method II adopted the protocol described by Wei et al. \cite{11} with slight modifications.

Cell fractionation method III was established according to published references \cite{12,16}. Detailed procedures are: (1) 10 g Fern laminas were ground to powder under liquid N\(_2\), dried in shade, and extracted with ether for 8 h (soxlet extraction) to get crude lipids; (2) the resulted residue I was extracted with 150 mL boiling water for 3.5 h (repeat three times), combine
all the solutions and centrifuge at 800 × g for 10 min; (3) the supernatant obtained from step 2 was concentrated to 50 mL with rotary evaporator under reduced pressure, then 250 mL ethanol was added and placed overnight, the resulted precipitation is crude polysaccharides; (4) the residue obtained from step 2 was extracted with 100 mL 0.1 mol L⁻¹ NaOH at 80 °C for 2 h (repeat two times), combine all the solutions and centrifuge at 10,000 × g for 10 min. The resulted supernatant is crude proteins, and the residue is cellulose and pectin, which are the main components of cell wall. The crude polysaccharides obtained was further extracted with chloroform–normal butyl alcohol (4:1) for seven times to remove proteins and finally got purer polysaccharides. The chloroplast of *P. simplex* was extracted and fractionated by using the procedures previously proposed [17].

### 2.4. Determination of REEs by ICP-MS

All fractions obtained from lamina tissue or chloroplast fractionation were dried at 105 °C for 24 h, weighed, and digested with HNO₃–HClO₄ (5:1) for the determination of REEs content by ICP-MS. Proteins and all other soluble fractions were also digested and diluted to an appropriate volume for ICP-MS determination. Protein concentration was determined by G-250 method [18].

REEs content in the soil sample was determined by ICP-MS after dried and ground and digested with the mixture of HNO₃/HF/HClO₄ (5:1:1). Bioavailable REEs in the host soil were obtained by extracting with 0.11 mol L⁻¹ acetic acid [19].

### 2.5. Determination of the isoelectric point of REE binding peptide by IEF

The method proposed by He et al. [20] was modified to determine the IP of the REE binding peptide obtained. Ampholines™, of pH 3.5–10 was adopted for building the pH gradient. The migration conditions for isoelectric focusing were modified as follows: at beginning 200 V for 30 min, then gradually ramped to 1000 V and kept constant for 15 h; and then gradually ramped to 1100 V and kept constant for 15 h. Finally, the focusing gels were stained with Coomassie blue R-250.

### 3. Results and discussion

#### 3.1. Comparison of three cell fractionation methods and determination of REEs contents in different subcellular fractions of *P. simplex* lamina

The results shown in Table 2 indicated that large amount of REEs are binding with cell walls, which coincides with those in literatures [9,11,12]. However the exact values of REEs contents in the cell walls obtained from the three methods are quite different. As far as methods I and II are concerned, in order to retain the biological activity of biomolecules as much as possible, all the experiments are conducted under very mild conditions, resulting in low extraction efficiency. However, it is inapplicable to improve extraction efficiency only by increasing the solvent amount and extraction times. A relatively violent method should be therefore established to make extraction more efficient and quantificational. Based on such a consideration, a scheme of chemical sequence treatment was adopted, which extracts lipid by ether, gets total polysaccharides through boiling water, obtains total proteins by dilute base, and the finally resulted residue are the main components, cellulose and pectin of cell wall. The results obtained indicated that about 68% REEs are located in the cell wall of *P. simplex*. It is well known that pectin acid in cell wall is a kind of polygalacturonic acid, and most of its carboxyl groups have not been esterified, they can therefore provide many binding sites for metal ions. It has been proved that large amount of Ca is stored in cell wall as calcium pectinate to reinforce the cell wall. Since the ratio of charge to ionic radius of REE is higher than that of calcium, REEs may have higher intensity to bind with pectin acid [21,22]. It might be inferred that most REEs are deposited as REE–pectin acid complexes in cell wall when they translocate in the plant. However, about 30% REEs can still pass across the cell wall and bind with proteins. It is very likely that REE could regulate and control the physiologic function of the hyperaccumulator, *P. Simplex*, through the interactions with certain proteins. As for polysaccharide and lipid, they have much lower REE content, and cannot be the main reason of REE hyperaccumulation.

### Table 2. REEs of subcellular fractions obtained by methods I–III [μg g⁻¹ fresh lamina weight]

<table>
<thead>
<tr>
<th>Method</th>
<th>Lamina</th>
<th>Free amino acids and pigments</th>
<th>Organelle</th>
<th>Cell membrane</th>
<th>Soluble fraction</th>
<th>Cell wall</th>
<th>Cell debris</th>
<th>Lamina</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>430.5</td>
<td>308.1</td>
<td>4.3</td>
<td>1.8</td>
<td>15.3</td>
<td>394.2</td>
<td>1.53</td>
<td>415.6</td>
</tr>
<tr>
<td>II</td>
<td>415.6</td>
<td>40.1</td>
<td>4.3</td>
<td>1.8</td>
<td>15.3</td>
<td>394.2</td>
<td>1.53</td>
<td>415.6</td>
</tr>
<tr>
<td>III</td>
<td>456.2</td>
<td>11.7</td>
<td>4.3</td>
<td>1.8</td>
<td>15.3</td>
<td>394.2</td>
<td>1.53</td>
<td>415.6</td>
</tr>
</tbody>
</table>

* a Three measurements for each sample, R.S.D. < 5%.
* b ∑REE is the sum of the concentrations of 15 REEs.
* c % means the percentage of ∑REE in each fraction of lamina to that of the whole lamina.
ggesting selective uptake of light REEs by this fern. Furthermore, the amount of light REEs such as La, Ce, Pr and Nd account for 84.9% of total REEs in the protein fraction, indicating that light REEs may have much stronger intensity to bind with the proteins than heavy ones. REEs concentration in different subcellular components listed in Table 3 also indicated that proteins have much higher intensity to accumulate REEs than those of the others. If REEs deposition in cell wall is passive and uncontrollable to some extent, accumulation of REEs by the proteins in \textit{P. simplex} should be an initiative behavior.

### 3.2. REE distribution in the chloroplast of \textit{P. simplex laminas}

Chloroplast is the organelle where photosynthesis occurs in green plant cells. It is surrounded by double membranes. Inside the inner membrane is thylakoid, which contains photosystem I (PS I) and photosystem II (PS II) \cite{25,26}. The cooperation of these two systems makes the whole photosynthesis. It is well known that suitable amount of REEs can improve the photosynthesis of green plants; the binding sites of REEs in chloroplast are thus attracting more and more attention for understanding REE functions during plant growing. Karukstis et al. \cite{27} showed that REE ion could interact with the membrane of thylakoid by electrostatic attraction, since the mutual attraction exists between the negative charged membrane and the positive charged REE ion under physiological conditions \cite{28}. PS II is suggested to be another important existing place for REEs, the negatively charged carboxyl groups of aspartic acid and glutamic acid in PS II could provide possible binding sites for REEs under physiological pH condition \cite{29}. Ono \cite{30} reported that lanthanide could replace the functional calcium presenting in the photosynthetic oxygen-evolving center (OEC) of PS II. Kruk et al. \cite{31} proposed that the 33 kDa protein of PS II contained one lanthanide low-affinity binding site. We also observed that in lanthanum cultured spinach, lanthanum could not only partly replacing magnesium in the chlorophyll, but also share the common binding sites of PS II proteins together with the inorganic cofactors of calcium and manganese \cite{17}. In order to get further understanding of the influence of REEs on photosynthesis, the chloroplast of \textit{P. simplex} was fractionated and REEs concentration in each fraction was determined. Results were shown in Table 4.

In the chloroplast of \textit{P. simplex}, 46.6% REEs associate with chloroplast membrane and 53.4% REEs are located in thylakoid. Nearly half (47.1%) of the total amount of REEs in the chloroplast is associated with PS II in the thylakoid, while only 6.4% REEs is found in the fraction of PS I. All these results indicated that the main compartment of REE influencing photosynthesis of \textit{P. simplex} is PS II.

### Table 4

<table>
<thead>
<tr>
<th>REEs content (μg mg⁻¹ chl's chloroplast) in subcomponents of chloroplast</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast</td>
<td>10.4</td>
</tr>
<tr>
<td>Chloroplast membrane</td>
<td>4.9</td>
</tr>
<tr>
<td>Thylakoid</td>
<td>5.6</td>
</tr>
<tr>
<td>PS I</td>
<td>0.66</td>
</tr>
<tr>
<td>PS II</td>
<td>4.9</td>
</tr>
</tbody>
</table>

* REEs content in subcomponents of chloroplast containing 1 mg chlorophyll.

* The sum of the concentrations of 15 REEs.

* % means the percentage of \( \sum \text{REE} \) in each fraction of chloroplast to that of the whole chloroplast.

* Soluble fraction rich of PS I.

### Table 3

<table>
<thead>
<tr>
<th>REEs contents in biological molecules of \textit{P. simplex laminas} (μg g⁻¹ dry weight)</th>
<th>( \sum \text{REE} ) (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lipids</td>
<td>620.6</td>
</tr>
<tr>
<td>Polysaccharides (deproteinization)</td>
<td>32.5</td>
</tr>
<tr>
<td>Crude proteins</td>
<td>2899.5</td>
</tr>
<tr>
<td>Cellulose and pectin</td>
<td>672.9</td>
</tr>
</tbody>
</table>

* Three measurements for each sample, R.S.D. < 5%.

* \( \sum \text{REE} \) is the sum of the concentrations of 15 REEs.
3.3. Preliminary characterization of REE binding peptide in P. simplex

Since the protein fraction of P. simplex laminas has the highest REEs content, one dominating REE binding peptide (protein I in Fig. 3) was separated and determined by SEC-ICP-MS from the REEs content, one dominating REE binding peptide characterized in the lamina of P. simplex. The preference for light REEs of this peptide might partly explain light REEs enrichment in P. simplex. ESI-MS spectrum shown in Fig. 4 indicated that the calculated molecular mass of the peptide is 5073 Da that is in accordance with our previous result obtained by MALDI–TOF-MS. Furthermore, it could be calculated from IEF photograph that the isoelectric point of this peptide is about 3.7. It has been proved by amino acid analysis with HPLC that the peptide contains much more acidic amino acids such as glutamic acid and aspartic acid than basic amino acids such as arginine and lysine [15]. What worthy of note is that this acidic peptide may be negatively charged under physiological pH condition, which makes it possible to provide binding sites for REEs. IR spectrum showed that the amide I band of this peptide is around 1632 cm$^{-1}$, indicating that the peptide may contain β-sheet structure according to empirical rule [32].

4. Conclusion

The proposed chemical sequence extraction combined with ultracentrifuge fractionation and ICP-MS analysis could provide reliable subcellular distribution of REEs in P. simplex laminas. Cellulose and pectin, proteins are the main storage of REEs in P. simplex; PS II is found to be the main compartment of REEs in the chloroplast of P. simplex, giving the evidence that REEs participate in the photosynthetic process of the plant; the REE-binding peptide characterized in the lamina of P. simplex offers a clue to understand the hyperaccumulation of REEs by P. simplex. Moreover, this is also important for the phytoremediation of REE-contaminated environment by this fern and more possible transgenic hyperaccumulators of high biomass in the future.

Acknowledgements

This work was financially supported by National Basic Research Program of China (No. 2003CCA00500) and National Natural Science Foundation of China (Nos. 20535020, 20475046 and 20175019). We are also grateful to Dr. Zhenji Li of Department of Biology, Xiamen University, and Mr. Guozhong Lin of Nanjing National Natural Reservation of semi-tropical rain forest for helping us in sampling P. simplex. We appreciate the comments of anonymous referees on this paper.

References
