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Subcellular and molecular localization of rare earth elements and structural characterization of yttrium bound chlorophyll *a* in naturally grown fern *Dicranopteris dichotoma*

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Abstract

A rare earth element (REE) hyperaccumulator, *Dicranopteris dichotoma*, sampled from an REE mining area in South-Jiangxi region, was chosen for analysis of 15 REEs at subcellular and molecular levels by inductively coupled plasma-mass spectrometry (ICP-MS). The sum of the concentrations of 15 REEs ($\sum$REEs) of *D. dichotoma* leaf was about 0.1% dry mass. Results indicated that the $\sum$REEs of different subcellular fractions of *D. dichotoma* leaf were cell walls>organelles>the 'soluble' fraction (i.e., cytosol and vacuoles)>cell membranes. ICP-MS results also showed that REEs existed in chlorophylls and lutein, though REE concentrations in carotene and pheophytin were both lower than the procedural blank levels. The $\sum$REEs of crude lipopolysaccharide and Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) obtained from *D. dichotoma* leaf were 1200 and 343 mg/kg, respectively. The extended X-ray absorption fine structure (EXAFS) study of *D. dichotoma* chlorophyll *a* indicated that yttrium was bound to the porphyrin ring of chlorophyll *a*, and one yttrium atom was surrounded by four nitrogen atoms with the average yttrium–nitrogen bond length being 0.236 nm. These data might be useful for understanding of the physiological role of REEs in hyperaccumulator *D. dichotoma*.

Keywords: Rare earth elements; Subcellular localization; *Dicranopteris dichotoma*; ICP-MS; EXAFS

1. Introduction

In recent years, rare earth elements (REEs) have been widely used in functional materials, catalysts, and additive, due to their specific physical and chemical properties [1,2]. Moreover, discoveries that yield and quality of the agricultural products can be significantly improved by applying REE micro-fertilizers have led to a large-scale usage of REEs in agricultural fields in China since 1970s [3–5]. More and more REEs are getting into the environment as a result of their usage. Although REEs possess less toxicity than other heavy metals such as copper and cadmium, the long-term hazardous effect of REEs on human health is still serious. For example, it was reported that the mean intelligence quotient and memory of children in REE polluted areas were significantly lower than those in the control areas [6]. REEs have already been classified as main environmental pollutants in China since 1990s [7]. Therefore, remediation of REE contaminated soils is drawing more and more attentions.

Phytoremediation is the application of hyperaccumulator, plant that can hyperaccumulate metals, to make soil

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contaminants nontoxic, and is often also referred to as bioremediation, or green remediation. This method was firstly introduced in 1983 [8], and subsequently gained public exposure in 1990s due to its low cost and high efficiency. A natural perennial fern, *Dicranopteris dichotoma*, grown in southern China can hyperaccumulate REEs to about 0.1% in its dry leaves [9]. The understanding of uptake, translocation, and storage of REEs in *D. dichotoma* is the key to apply this plant in phytoremediation of REE contaminated soils. However, up to now, although there have been many studies on the uptake and translocation of heavy metals in their hyperaccumulators [10–12], details of subcellular and molecular localization of REEs in *D. dichotoma* are less studied, and the structures of REE bound biological molecules in *D. dichotoma* are obscure.

The few studies of subcellular and molecular localization of REEs are mainly because of the very low concentrations of REEs in plant, especially for some heavy REEs [13]. Conventional analytical methods, which cannot achieve the required sensitivity and accuracy for REE determination, are not applicable for these studies. Even with using the relatively sensitive techniques, such as inductively coupled plasma-atomic emission spectrometry (ICP-AES) and neutron activation analysis (NAA), satisfactory results cannot be obtained. On the one hand, the concentration data obtained by these techniques are not always precise or sensitive enough because the abundances of REEs in plant are only trace. On the other hand, these analyses do not cover all the members of REEs [14–16]. To measure the REEs in plants, an analytical method with high sensitivity and accuracy is required. Recently, the inductively coupled plasma-mass spectrometry (ICP-MS) technique has been applied to determine REEs in biological and environmental samples, as a result of its inherent, low detection limits, simple spectra, wide dynamic range, and multielement determination capability [17]. The detection limits of ICP-MS are about 0.01 to 0.001 of those of ICP-AES or NAA, so the REE determination obtained by ICP-MS is much more accurate.

REE bound biological molecules prepared in vivo are always poorly crystallized, so, studies of their structures present particular analytical difficulties. Recently, the method of extended X-ray absorption fine structure (EXAFS) has received increased attention as a result of the development of synchrotron radiation. EXAFS can be used to determine the local structure of an atom, such as bond length and coordination number. It is not necessary that a sample used for EXAFS must be well crystallized, so that both solid and liquid samples can be used in the measurement. Furthermore, one can quantitatively obtain the structural parameters of one selected element in the milligrams per kilogram concentration range in a complex material by EXAFS. Therefore, EXAFS is particularly suitable for the studies of bound forms of REE ions in biological molecules [18–20].

In the present work, ICP-MS will be used to investigate the localization of REEs at subcellular and molecular levels, and EXAFS will be used to characterize the structure of yttrium bound chlorophyll *a*, which would be very useful for elucidating the physiological role of REEs in hyperaccumulator *D. dichotoma*.

2. Experimental

2.1. Reagents and materials

Stock solutions of REEs, 1 mg/ml, were prepared by National Research Center for Geoanalysis (Beijing, China). Analytical standard solutions of REEs, 20 ng/ml, were prepared from stock solutions by serial dilution. Microcrystal cellulose was purchased from the Second Reagent of Shanghai, analytical reagent (A.R.) grade. Before use, it was ground to pass a 100-mesh sieve and then activated at 70 °C for 4 h. All reagents used were of A.R. grade, and all organic solvents used were dried free of water and redistilled before use. Petroleum ether with boil point of 60–90 °C and water (18 mΩ) prepared with a Milli-Q system were used throughout.

D. dichotoma samples were collected at a heavy REE-enriched mining area in South-Jiangxi region in September. The fresh leaves of *D. dichotoma* were thoroughly washed with water, blotted with paper tissue, and then stored in liquid nitrogen immediately before use.

2.2. Leaf tissue fractionation

The subcellular distribution of REEs within the leaves was determined using the protocol described by Carrier et al. [21] and Ni and Wei [22] with some modifications. Twenty-five grams of leaves was homogenized in 50 ml of chilled extraction buffer [50 mM Tris–HCl (pH 7.5), 250 mM sucrose, 1 mM dithioerythritol]. The homogenate was sieved through a nylon cloth and centrifuged at 500×*g* for 5 min in order to pellet the cell debris. The pellet was designated as crude cell wall fraction. The supernatant was centrifuged for 30 min at 10,000×*g* to isolate cell organelles. The supernatant was then centrifuged at 100,000×*g* for 30 min to separate the membrane-containing fraction from the soluble fraction. All steps were performed at 4 °C. All of the fractions were then dried for ICP-MS analysis.

Intact chloroplasts were isolated using the protocol described by Baryla et al. [23] with some modifications. Twenty grams of leaves was homogenized in 45 ml of chilled extraction buffer [50 mM Tris–HCl (pH 7.6), 10 mM NaCl]. The homogenate was sieved through six layers of cheesecloth and centrifuged at 500×*g* for 5 min at 4 °C in order to pellet the cell debris. The supernatant was centrifuged at 3000×*g* for another 30 min also at 4 °C to