两种不同镉积累类型水稻糙米中镉的存在形态*

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摘 要：采用组织化学及溶剂提取法研究了低镉积累水稻品种“广源占 No. 3”和高镉积累品种“珍桂矮”糙米中镉的存在形态。结果表明：镉在糙米中主要以配合物的形式存在，并且主要与蛋白质结合。在糙米主要蛋白中镉与谷蛋白和球蛋白结合量最多。与低镉积累品种“广源占 No. 3”相比，“珍桂矮”糙米中 0. 1mol/L EDTA、w=2. 5% NaCl 和 w=0. 2% NaOH 提取态镉以及球蛋白中镉的比例较高，初步证明了两个品种间的差异。凝胶层析出现了 3 个蛋白峰（F-I，F-II 和 F-III），镉的出峰位置与样品流份中可溶性蛋白的出峰位置大致相同。而两种水稻品种相比较镉在 3 个峰中的分布有所不同。“珍桂矮”糙米可溶部分中的镉大多与相对分子质量为 3 000的物质结合，属于植物螯合肽（PCs）或低相对分子质量物质。“广源占 No. 3”糙米中镉与 PCs 配合的组分（Cd- PCs）含量远小于“珍桂矮”，表明了镉在 PSC 和非 PSC 品种中分布的差异。

关键词：存在形态；镉；糙米
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Existing Forms of Cadmium in Brown Rice (*Oryza sativa* L.) of Two Cultivars Differing in Cd Accumulation

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Abstract: The existing forms of cadmium (Cd) in brown rice of two cultivars, a low-Cd accumulating cultivar Guangyuanzhan No. 3, and a high-Cd accumulating cultivar Zhenguiai, were studied through solvent extraction and biochemical methods. The results showed that Cd was mostly existed in brown rice in complex forms and mainly in the protein binding forms. Among the main proteins Cd was mainly bound with globular and glutelin. In comparison with Guangyuanzhan No. 3, Zhenguiai was higher in proportion of 0. 1 mol/L EDTA, 2. 5% NaCl and 0. 2% NaOH extractable-Cd and had higher Cd percentage in globulin, which preliminary proved the difference between the two cultivars. Three protein absorption peaks (F-I, F-II and F-III) were identified in Tris-HCl extraction of brown rice on Sephadex G 75. The distribution of Cd in the soluble fraction followed the same elution pattern as protein. While comparing the two rice cultivars Cd distribution in the three absorption peaks was different. In Zhenguiai the majority of soluble Cd was bounded to phytochelatins (PCs) or low-molecular-weight components with a molecular weight of ~3 kDa. However, the content of Cd-PCs in seed in the variety of Guangyuanzhan No. 3 was founded to be much lower than that in Zhenguiai, which possibly implying the difference of Cd allocation in PSC and non-PSC.

Key words: existing forms; cadmium (Cd); brown rice

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Cadmium (Cd) is one of the most mobile elements among all the toxic heavy metals. It can be readily taken up by plants and translocated to aerial organs where it can accumulate to high levels. Consequently, it can easily enter the food chain[1] and become detrimental to human and animal health. Clear evidence has linked human renal tubular dysfunction with soil Cd contamination in subsistence rice farm families in Asia[2]. In China more than 1.46×10^8 kg of agricultural products are polluted by Cd every year[3].

The toxicity of Cd in foods on animals and mankind depends upon the binding forms administered. Many studies have described the chemical forms and qualitative binding properties of Cd in polluted food products, such as bean (*Phaseolus vulgaris* L.) seeds[4], rice (*Oryza sativa* L.) and wheat (*Triticum aestivum*) seeds[5]. However up to date there is a gap of knowledge about the difference of chemical forms and binding properties of Cd in high-Cd and low-Cd accumulating cultivars. It is reported that different cultivars of rice accumulate quite different amounts of Cd in the seeds even when grown under the same conditions[6-9]. Moreover recently the concepts of pollution-safe cultivar (PSC)[9-11] have been proposed and applied in searching for crops with low levels of heavy metal uptake and accumulation, in order to cut down pollutant flow to the human food chain. While the form of Cd existing in brown rice of different cultivars is still unknown.

In this study, we filled this gap by examining the chemical forms of Cd and the patterns of Cd-binding protein in brown rice of two typical cultivars, Cd-PSC and non-Cd-PSC, which were screened and verified in our previous study[9]. It is one of the first attempts to reveal the binding forms of Cd in crops seeds of different cultivars differing in Cd accumulation. This knowledge might be helpful to identify the distribution and mechanism of Cd accumulation in rice to reduce the risk of Cd toxicity.

1 Materials and methods

1.1 Sample of brown rice

Brown rice of two cultivars, differing in Cd content when exposed to 75 mg · kg^{-1} Cd according to our previous result[9], were collected. One was cv. Guangyuanzhan No. 3, a typical Cd-PSC and Cd concentration in brown rice was 0.62 mg · kg^{-1}. Another was cv. Zhenguiai, a typical non-Cd-PSC and Cd concentration was 1.09 mg · kg^{-1}.

1.2 Extraction of chemical forms of Cd

Determination of Cd chemical forms in brown rice was carried out referring to the method of Yang et al.[12]. Cadmium in different chemical forms was extracted separately in the extracting solutions listed below. Brown rice were air-dried after grain chaffs were mechanically removed ground and diluted with the extracting solution at the ratio of 1:20 (w/v) under room temperature. After 17 ~ 18 h the extracting solution was collected in the flask bottle, then the same extracting solution was added and after 2 h was collected. The same performances were repeated 4 times. The solution in the flask bottle collected from the four-time extractions was the first extraction. The same performances were repeated by using the other extractive solutions, and then a total of 6 Cd extractions were obtained.

(1) Distilled water (F_W), extracting free Cd and water-soluble Cd of organic acids

(2) 0.1 mol/L EDTA (F_{EDTA}), strong chelator, extracting Cd combined with grain components

(3) 1% Hac (acetic acid, F_{Hac}), extracting weakly bound Cd and undissolved Cd phosphate

(4) 2.5% NaCl (F_{NaCl}), extracting protein integrated Cd mainly globulin Cd

(5) 0.2% NaOH (F_{NaOH}), extracting alkaline-dissolved protein integrated Cd mainly glutelin Cd

(6) 70% ethanol (F_E), extracting mellow-dissolved protein integrated Cd and inorganic Cd

The ratio of Cd extracted in different extracting solutions to the total Cd in brown rice was calculated as

$$R = \frac{C \times V}{C_0 \times W} \times 100\%$$

where R is extractive Cd ratio (%), C is the concentration of extracting solution (mg · L^{-1}), V is the volume of extracting solution (mL), C_0 is Cd concentration in brown rice (mg · kg^{-1}) and W is the weight of brown rice (g).

1.3 Extraction of main proteins

Albumin, globulin, prolamin and glutelin in brown rice were separated using extractive solutions in the order of distilled water, 5% NaCl, 70% ethanol and 0.2% NaOH[13]. The de-fated crushed brown rice was diluted with the extracting solution at the ratio of 1

: 10 (w/v), shaken for 1 h and placed in the refrigerator (4 ℃) for 10 h, centrifuged at 6 000 × g for 30 min and the supernatant was moved to a flask bottle. The same performances were sequentially repeated by using the second and other extractive solutions.

1.4 Cd-binding proteins separation by gel filtration chromatography

Cd-binding proteins separation in brown rice was carried out referring to the method of He et al.[14]. The whole protein in pulverized de-fated crushed brown rice was extracted with 0.05 mol/L Tris-hydrochloric acid (HCl) buffer (w/v 1: 10, pH 7.5) for 10 h. Then the extraction was separated and ultracentrifuged at 20 000 × g for 60 min and the supernatant solution was collected. The protein fractions were separated from the extracts by gel filtration on Sephadex G-75 in a 1.6 × 100 cm column equilibrated with 0.01 mol/L Tris-HCl buffer (pH 7.5). The column had been previously calibrated with molecular weight markers, including Ribonuclease (13 700), Chymotrypsinogen A (25 000), Ovalbumin (43 000), Albumin (67 000) and Blue Dextran (2 000 000). The sample was eluted with Tris-HCl at a flow rate of 18 mL · h^{-1}. Fractions of 4 ml per tube were collected by an automatic collector (BIO-RAD, California) and monitored by measuring conductivity and absorbance at 280 nm. After that, the samples were used for analysis of Cd concentration.

1.5 Cd analysis

The extraction of chemical forms and main proteins was dried at 70 ℃ to constant weight, and digested by $HNO_3 - H_2O_2$ in a microwave decomposition device (Microwave digester-7295, O. I. Corporation, College Station, TX). Cd concentrations in the digests and those in column effluents were analyzed with an Atomic Absorption Spectrophotometer (Perkin-Elmer AA 100, Norwalk, CT). A Certified Reference Material (CRM) of plant (GBW-07603, National Research Center for CRM, China, the certified Cd concentration is 0.057 mg · kg^{-1}) was used to ensure the precision of the analytical procedures, and the results averaged 0.059 mg · kg^{-1} with 0.099% relative standard deviation (RSD).

1.6 Data Analysis

Three replicates were performed for each treatment. The statistical software package SPSS 11.0 and EXCEL 2003 for Windows were used for one-way ANOVA and LSD tests.

2 Results and Discussion

2.1 Chemical forms of Cd

The extraction ratios of Cd by the six extracting solutions are shown in Table 1. The extraction ratio of Cd was different from distilled water, 0.1 mol/L EDTA, 1 % HAC, 2.5% NaCl, 0.2% NaOH and 70% ethanol . The largest proportion of Cd extracted by 0.1 M EDTA indicates that most Cd exists in complexes in brown rice. Followed by 2.5% NaCl, 0.2% NaOH and 1% Hac, quite a little Cd was combined with protein and other weakly binding states in the complex forms. Extractable-Cd by distilled water and 70% ethanol were the smallest ones, implied that little Cd was bounded with water-soluble components and mellow-dissolved protein in brown rice. The results agreed with the study of Yang et al[12] on polluted rice and wheat seeds.

Table 1 The extraction ratios (%) of Cd by the six extracting solutions

	GYZ	ZGA
F_{EDTA}	67.2 ± 6.8 b	74.1 ± 6.6 a
F_{NaCl}	42.2 ± 5.2 b	47.5 ± 3.9 a
F_{HAC}	33.1 ± 3.6 a	35.1 ± 5.2 a
F_{NaOH}	30.4 ± 4.1 b	34.5 ± 3.0 a
F_W	11.7 ± 2.4 a	9.5 ± 1.6 a
F_E	5.2 ± 0.7 a	4.8 ± 0.5 a

F_{EDTA}, F_{NaCl}, F_{HAC}, F_{NaOH}, F_W and F_E indicate the fractions extracted by 0.1 mol/L EDTA, 2.5% NaCl, 1% HAC, 0.2% NaOH, distilled water and 70% ethanol. GYZ and ZGA indicate the two tested cultivars cv. Guangyuanzhan No. 3 and cv. Zhenguiai, respectively. Different letters within the same row indicated significant difference between the two cultivars at 0.05 level. The same below

In comparison with PSC cv. Guangyuanzhan No. 3, non-PSC cv. Zhenguiai was significantly higher in ratio of the extractable-Cd by 0.1 mol/L EDTA. EDTA is a strong chelator and it can extract Cd combined with grain components. The result indicates that the capacity of binding Cd in non-PSC is higher than that in PSC. The extraction ratio of Cd by 2.5% NaCl and 0.2% NaOH in Guangyuanzhan No. 3 were significantly lower than that in Zhenguiai, indicating more Cd proportion bound with protein in the later. The difference between the two rice cultivars probably was the result caused by the different between PSC and non-

PSC. Because there is no report about this aspect up to date, the current study is preliminary and need further investigation in the future.

2.2 Cd integrated with proteins

According to the solubility in different solvents, seed protein is mainly classified into 4 categories: albumin, globulin, prolamin and glutelin[15]. Shown in Table 2 is the percent distribution of Cd in the main proteins extracted by the four extractants in the current study. The largest proportion of Cd was presented in globulin, which was extracted by 5% NaCl, then glutelin extracted by 0.2% NaOH, and prolamin extracted by 70% ethanol contained the least proportion of Cd. It is reported that nutrient substance binding with heavy metal mainly is protein in grain[16], and among proteins globulin and glutelin are the main ones to bound with heavy metal[13,17]. The result of the present study is consistent with the former observations.

Table 2 Percent distribution (%) of Cd in main proteins extracted by the four extractants

	GYZ	ZGA
Globulin	32.5 ±4.5 b	36.2 ±6.9 a
Glutelin	30.8 ±4.13 a	29.2 ±4.2 a
Albumin	26.9 ±4.3 a	24.7 ±3.6 a
Prolamin	9.8 ±1.1 a	9.9 ±2.1 a

Compared with PSC Guangyuanzhan No. 3, non-PSC Zhenguiai had higher Cd proportion in globulin and there were no significant difference in other three proteins. This result was basically accorded with the phenomena of the above experiment.

2.3 Cd distributions in Cd-binding fractions from gel filtration chromatography

The elution profiles of Tris-HCl buffer extractions of defatted brown rice on Sephadex G-75 are shown in Fig. 1 and Fig. 2. Three absorption peaks for rice were detected at 280nm, which is in agreement with the study of Lei et al.[18] and He et al.[14] in polluted flax (*Linum usitatissimum* L.) seed and brown rice. This represented the three major components (*F*-I, *F*-II and *F*-III) of the protein fraction. The first peak (*F*-I) eluting just after V_o (void volume) represents the high-molecular-weight components with a molecular weight ≥80 000. The second peak (*F*-II) eluted at the 35 elution fraction, corresponding to a molecular weight of 11 000. The third peak (*F*-III) eluted around V_t (total volume) with a molecular weight of ~3 000, probably represents low-molecular-weight components including peptides. The position of the protein peaks in the current study was slightly different from the former reports[14,18], probably due to the different crop species or cultivars[19].

The distributions of Cd in the elution fraction were basically the same as that of the absorption peaks, which is consistent with those observed by He et al.[14], Jiang et al.[20] and Wan et al.[21]. Comparing the two rice cultivars Cd distribution in the three absorption peaks was different. In the seed of Guangyuanzhan No. 3, *F*-I combined highest concentration of Cd, then *F*-II, and *F*-III was the lowest. While in Zhenguiai highest Cd concentration was present in *F*-III and *F*-II combined lowest Cd concentration.

Under Cd exposure, plants will synthesize phytochelatins (PCs) which can chelate Cd to reduce free Cd ion concentration[22]. Previous researchers observed the molecular weight of the PCs usually remained 2 000 ~4 000[22-23]. In this experiment Cd distributed in the *F*-III with molecular ~3 000 probably bound with PCs. In our former study more Cd in root/shoot of Zhenguiai was combined with low-molecular-weight components (probably PCs) than Guangyuanzhan No. 3[24], which may result in the high Cd concentration in the F-III in the seed of Zhenguiai. The distribution difference of Cd in protein in seed of the two rice cultivars further demonstrated the difference about Cd allocation in PSC and non-PSC. And the underlying mechanism for this genotypic difference is the subject of ongoing research.

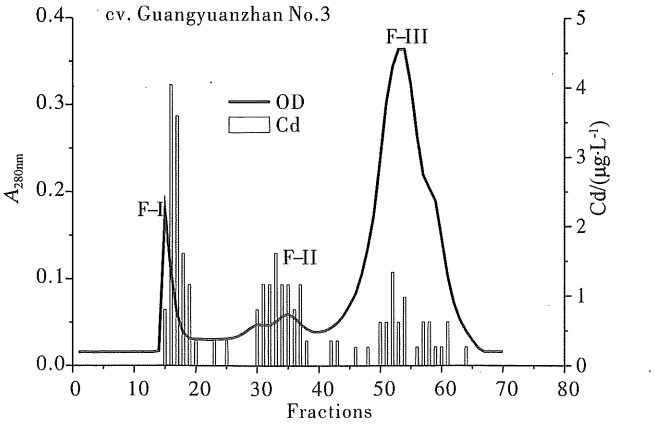

Fig. 1 Sephadex G-75 gel filtration profile of soluble protein fractions and concentration of Cd in each fraction in brown rice of cv. Guangyuanzhan No. 3

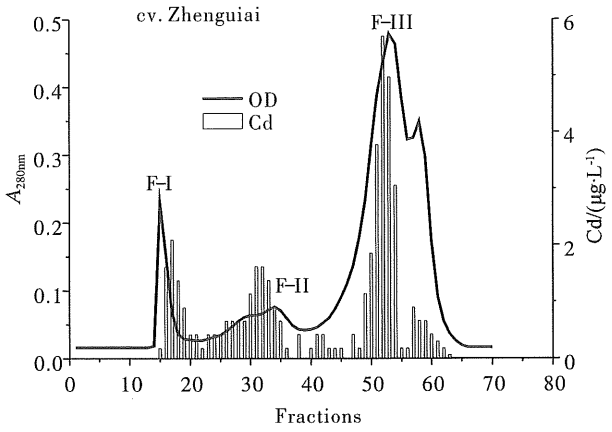

Fig. 2　Sephadex G-75 gel filtration profile of soluble protein fractions and concentration of Cd in each fraction in brown rice of cv. Zhenguiai

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