# Purification and Characterization of PC-Like Cadmium-Binding Peptide from Root of *Rumex crispus*†

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**ABSTRACT** : This research investigated the process of removing cadmium and tested the detoxification mechanism of the cadmium-binding peptide (Cd-BP) from *Rumex crispus*. Phytochelatin-like cadmium-binding peptide (PC-Cd-BP) of *Rumex crispus* was purified and identified. *Rumex crispus* was exposed to 4.3 mg Cd/L for seven days. Heat-treated supernatant fraction taken by root tissues showed traces of PC-Cd-BP. An analysis of the material through Gel-filteration chromatography on the Sephadex G-75 column showed two symmetrical Cd-BP peaks. The major peak with the smaller molecular weight was further purified by $C_{18}$ reverse-phase HPLC to produce apparent homogeneity. The amino acid composition of Cd-BP from *Rumex crispus* included cysteine (22.6%), glutamate and glutamate acid (20%), and glycine (12%). It was similar the amino acid composition of most PC. The molecular weight of the purified peptide was determined at 568-706 Da by MALDI-TOF MS. Therefore, the Cd-BP of *Rumex crispus* was PC-Cd-BP consisting of isopeptides.

***Key words*** : Amino acid composition, MALDI-TOF MS, Phytochelatin-like cadmium-binding peptide (PC-Cd-BP), *Rumex crispus*

## INTRODUCTION

Plant is important connector delivering excessive heavy metals as a food chain and biological circulation (Wierzbicka and Antosiewicz 1993). If heavy metal flows internal system, it then hinders absorption of heavy metal or separately storages in cell organ or binds heavy metal binding material (Mitra *et al.* 1975, Tomsett and Thurmam 1988). And it maintains the consistency of the heavy metal inside the cell at a lower level.

A characteristic material in this plant is Phytochelatin (PC), which is composed of cysteine, glutamate and glutamic acid and glycine. Its general formula is ($\gamma$ Glu-Cys)$_n$Gly(n=2~11). PCs are small polypeptide compounds produced by plant enzymes in response to heavy metals ($Cd^{2+}$, $Pb^{4+}$) or oxy anions ($SeO_4^{2-}$) (Salisbury and Ross 1992). When synthesized and induced by heavy metal (like cadmium), the induced PC's sensibility to accumulation and detoxification increases (Rasuer 1995). Because of the implication of PC in heavy metal detoxification, chelation of heavy metals by the newly synthesized PC and activation of PC synthase have received a wide attention (Howe and Merchant 1992). This studies sought to identify heavy metal contaminated sites, suggest ways to clean them, and maximize their productivity (Anne 1995).

We conducted cadmium-binding peptide of *Rumex crispus* was isolated and purified, its cadmium tolerance tested on species (data not shown), and its characteristics analyzed.

## MATERIALS AND METHODS

### Cadmium exposure and extraction

*Rumex crispus* (64 individuals) were adapted for three days in hydroponics cultivations, were exposed to 4.3 mg Cd/L for seven days. Each pot was aerated continuously. The growth chamber was maintained for 18 hours a day/6 hours a night under 25℃ conditions. The plant was washed with distilled water to eliminate traces of exposure. The root part was ground in the bowl with liquid nitrogen and its weight measured and homogenized with the homogenate buffer (20 mM Tris-HCl (pH 8.0), 2 mM $\beta$ -Mercaptoethanol, 5 mM ascorbic acid, 5 mM Thiourea, and 1 mM KCN) of its 1.5 times volume using a homogenizer (SS Motor SSC811EA, National, Japan). Metal contents in the homogenate were analyzed by GF-AAS (HGA 800, Perkin Elmer, USA) after acid digestion.

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The homogenates were centrifuged at 20,000 × g for 30 minutes at low temperature. The supernatants were heated at 60℃ for 10 minutes and re-centrifuged at 20,000 × g for 30 minutes, then heavy metal in supernatant and pellet analyzed also the above.

### Purification of Cd-BP (cadmium-binding peptide)

The heat-treated 15 mL supernatant was applied to Sephadex G-75 by loading the 20 mM Tris-HCl (pH 8.0) and 2 mM $\beta$ -Mercaptoethanol buffer at 1.6 mL/min. Each fraction was collected 4mL and its cadmium concentration and $A_{254}$, $A_{280}$ measured by GF-AAS (HGA 800, Perkin Elmer, USA) and UV-VIS Spectrometer (Model 8453, Hewlett Packard, Germany). The Sephadex G-50 column was calibrated with the following molecular weight standards: bovine serum albumin (66 kDa), rabbit metallothionein (MT)-1 (6.5 kDa), and Vitamin $B_{12}$ (1.35 kDa). Eluant protein levels were monitored at $A_{254}$ and $A_{280}$, and cadmium concentrations were measured in the 3 3-ml fractions collected.

Then, the fractions with high cadmium content were collected and freeze-dried. The sample' s concentrates were repurified using HPLC (High-Performance Liquid Chromatography) system (Model 1100 Palo Alto, Hewlett Packard, Germany). The column, $\mu$-Bondapak $C_{18}$ reverse-phase column (3.9×300 mm; 10 $\mu$M particle size; 125 Å pore size, the Waters) was used and flowed solvent A (20 mM Tris-HCl (pH 8.0)) and solvent B (25% Acetonitrile in 20mM Tris-HCl (pH 8.0)) until they becomed 0~100% linear gradient by solvent B for 60 minutes. The flow rate stayed at 0.8 ml/min (Chemestation software). The DAD (Diode-Array Detector) at 214 nm, 254 nm has shown absorbance and cadmium content was measured by GF-AAS. High cadmium content peak was also collected and removed unnecessary salinity by the dialysis membrane (Spectra/Por® Ce (Cellulose Exter, MWCO: 500, US/ Canada)) using distilled water.

### Amino acid composition

Aliquots of the purified peptides were desalted using aby dialysis membrane (Spectra/Por® CE, MWCO 500, Spectrum) with several buffer changes of distilled water [xxx this is not very clear - distilled water is not a buffer]. Peroxidized samples were hydrolyzed in HCl and analyzed by theusing a reverse-phase HPLC system (Hewlett-Packard 1100) using with an Eclipse WDB $C_{18}$ column (Hewlett-Packard) for the detection of phenylisothiocyanatyl derivatives.

### Mass determination

One fraction of the exchanged sample was prepared with matrix solvent (10 mg/mg CHCA[alpha-cyano-4-hydroxy cinnamic acid]) at a ratio of 1:1 by weight. The molecular weights were determined by MALDI-TOF MS (Matrix-assisted laser de-absorption ionization, time of flight mass spectrometry, Voyager-DE™ STR Biospectrometry workstation, Perseptive Biosystems, USA).

## RESULTS AND DISCUSSION

### Cadmium partitioning of *Rumex crispus*

To determine the cadmium distribution of the *Rumex crispus* root that was exposed to cadmium, the cadmium partitioning of cytosolic (soluble) and pellet (insoluble) fractions was investigated. In the control group, total cadmium level was 297.88 $\mu$g/g (Table 1).

However, in the 4.3 mg Cd/L exposed group, total cadmium level was 2129.25 $\mu$g/g (7-fold of control). Cytosolic (soluble) cadmium level was 729.75 $\mu$g/g (34.3%), pellet(insoluble) cadmium level was 1398.5 $\mu$g/g (65.7%) in the 4.3 mg Cd/L exposed group. The cytosolic (soluble) and pellet (insoluble) cadmium content showed both high. When the maize was exposed to Cd, the cell wall (insoluble) and cytosolic (soluble) fraction all showed a high Cd content that it showed a similar tendency above result (Lozano-Rodriguez *et al.* 1997). Similarly, Nishizono *et al.* (1988) reported that 90% of Cu existed in the cell wall when *Athyrium yokoscense* was exposed to Cu. It was that the pellet (insoluble) contributed to Cu-tolerance and the Cu-tolerance mechanism will exist in the cytoplasm (soluble) of the root-cell, and the rest of Cu will move into the cytoplasm (soluble) fraction.

### Isolation of cadmium-binding peptide

In order to isolate Cd-binding ligand, which was cytosolic (soluble) fraction conducted Gel-filtration chromatography (Sephadex G-75) (Fig. 1). In the control group, the cadmium-binding fraction did not exist. In the 4.3 mg Cd/L exposed group, there were two separated symmetrical Cd peaks seen in the chromatogram. The small peak was measured at about 60 kDa (fraction 17~25) with High Molecular Weight Ligand (HMWL). The large peak was at about 730 Da (fraction 51~67) with Low Molecular Weight Ligand (LMWL). Under this condition when leaves of *Brassica oleracea*

Table 1. Partitioning of cadmium ($\mu$g/g of wet weight) between cytosolic and pellet fraction of *Rumex crispus* exposed to control, 4.3 mg Cd/L for 7days

| Cd exposure | Total homogenate | Cytosol | | Pellet | |
|---|---|---|---|---|---|
| mg/L | $\mu$g/g | $\mu$g/g | % | $\mu$g/g | % |
| control | 297.88 | 37 | 12.4 | 260.88 | 87.6 |
| 4.3 | 2128.25 | 729.75 | 34.3 | 1398.5 | 65.7 |

Values are expressed in $\mu$g/g of wet weight. About 64 individuals corresponding 1 g of wet weight.

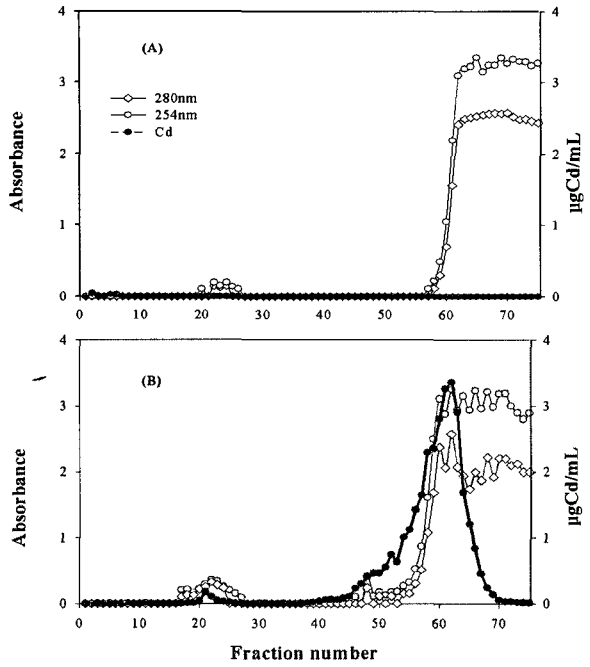


Fig. 1. Elution profile of Gel-filteration chromatography on Sephadex G-75 of untreated(A), cadmium treated(B) from *Rumex crispus* roots. Root extract from *Rumex crispus* treated with 0 mg Cd/L, 4.3 mg Cd/L for 7days.

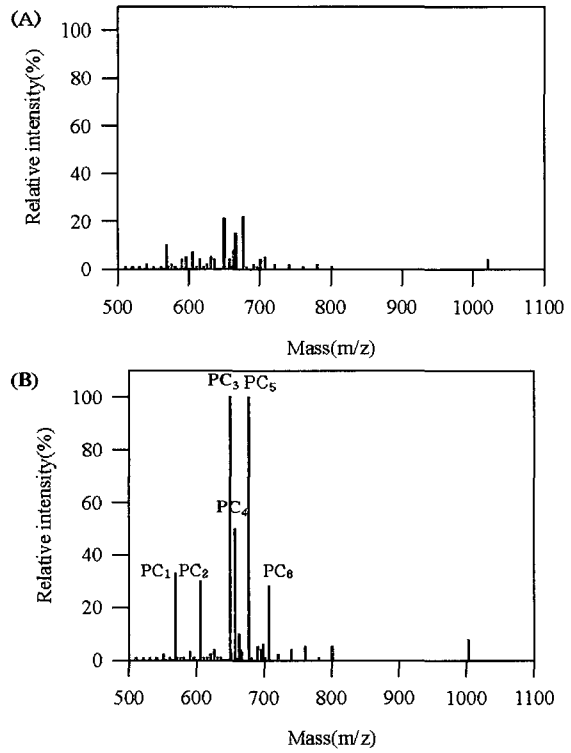


Fig. 2. MALDI-TOF MS obtained for plant culture extracts after their purification by reversed-phase HPLC and lyophilization. (A): not treated Cd, (B): treated 4.3 mg Cd/L.

are exposed to Cd, a Sephadex G-50 is used to isolated cadmium binding ligand. The pattern was similar to the peak of 20 kDa and 1 kDa (Grill *et al.* 1987). When Soybeans exposed to Cd were isolated with Sephadex G-75, The LMWL was measured at 2.2 kDa, showing a very small peptide like *Rumex crispus*.

The LMWL, which indicated high Cd content peak, collected, concentrated, and repurified by HPLC (data not shown) was that of *Rumex crispus* containing Cd-binding peptides. After the 24-hour exchanged buffer dialysis, the presence of PC (Phytochelatin) was confirmed in an amino acid composition analysis. The rest confirmed the molecular weights using MALDI-TOF MS again.

## Characterization of cadmium-binding peptide

The result that led to induction of PC was obtained through an amino acid composition analysis (Table 2). The cystein as an impor-

Table 2. A comparison of the amino acid composition of PC from *Rumex crispus* and other species

(residue % of each amino acid)

| Amino acid residue \ Species | *Rumex crispus* | *Athyrium yokoscense* | *Agrostis gigantea* |
|---|---|---|---|
| Cys | 22.6 | 27 | 29 |
| Asp/Asn | 1.8 | 8 | 2.5 |
| Glu/Gln | 19.5 | 20 | 42 |
| Ser | 2.7 | 5 | 9 |
| Gly | 12.0 | 15 | 6 |
| His | 0.4 | 0 | 4 |
| Arg | 5.1 | 1 | 0 |
| Thr | 0 | 5 | 1 |
| Ala | 3.9 | 6 | 2 |
| Pro | 1.1 | 3 | 0 |
| Tyr | 7.2 | 0 | 0 |
| Val | 3.2 | 5 | 1 |
| Met | 3.3 | 1 | 0 |
| Ile | 5.5 | 1 | 0 |
| Leu | 1.9 | 4 | 1 |
| Phe | 1.9 | 0 | 0 |
| Trp | 0 | 0 | 1 |
| Lys | 8.0 | 2 | 2 |
| Total | 100 | 100 | 100 |

The *Athyrium yokoscense* data are from Nishizono *et al.* (1988), *Agrostis gigantea* data are from Rauser (1984).

tant composition of PC was 22.6%. The glutamate and glutamate acid was 20% and glysine was 12%. The PC general composition ratio was as follows: Cys: Gln/Glu: Gly = 2 : 2 : 1 (Grill *et al.* 1985) and they were almost exactly alike at 1.9: 1.6: 1 and similar to the ratio of 1.8: 1.3 :1 of *Athyrium yokoscense*. Thus, the Cd -BP of *Rumex crispus* root induced PC-Cd-BP.

The molecular weight of *Rumex crispus* by MALDI-TOF MS was observed at six peaks. According to molecular weight, each was classified as $PC_1$, $PC_2$, $PC_3$, $PC_4$, $PC_5$ and $PC_6$. Each level was at 568 Da, 604 Da, 649 Da, 656 Da, 676 Da, and 706 Da. When molecular weights of *Silene cucubalus, Agrostis tennuis* and *Rauvolfia serpentina* that were exposed to 300 $\mu$M $Cd^{2+}$ measured using the ESI MS-MS (Electrospray ionization tandem Mass spectrometry), the major peak of *Silene cucubalus* was at 777.2 Da, *Agrostis tennuis* was at 727, 743, 772 Da and *Rauvolfia serpentin* was at 770, 879 Da (Véronique *et al.* 1999). Compared with the above, the molecular weight of *Rumex crispus* was found to be similar to the three species known PC-BP.

Therefore, considered as a whole Cd-BP of *Rumex crispus* was PC-Cd-BP consisting of isopeptides.

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