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Original Paper

CADMIUM-PEPTIDES COMPLEXES IN *DUNALIELLA SALINA* CELLS

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ABSTRACT

Dunaliella salina responds to cadmium present in cells by synthesizing phytochelatin. Reverse Phase (RP) and common HPLC analysis describe the formation of complexes between Cd and γ-glutamyl peptides in cells. The unique peptides chain chelate Cd by thiolate bonds formation. Two classes of Cd-PC_n complexes were identified as specific Cd-peptides complexes binding in D. salina.

Keywords : Cadmium, metal binding peptides, *D. salina*

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INTRODUCTION

Dunaliella salina has developed several defense mechanisms to counter the present of potential toxic compounds in the media (Hall, 2002; Muhaemin, 2004). The important metal detoxification mechanism involves intracellular thiol containing compounds such as methionine (Muhaemin, 2006), glutathione (GSH) (Muhaemin, 2008), and phytochelatin (PC_n). Phytochelatin are cysteine rich peptides with general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$. Phytochelatin are able to bind metal ions via thiolate coordination. The metal phytochelatin formation complexes in cell can reduce intracellular free metal ion concentration effectively (Morelli, *et al*., 2002). Phytochelatin synthesized by the PC synthase which is activated by the presence of metal ions; and even uses glutathione as substrate (Cobbett, 2000).

Two main groups of phytochelatin cadmium complexes consist of high molecular weight (HMW) and low molecular weight (LMW). The acid labile sulfide present has been demonstrated in HMW form with different chain length in which Cd binding. Only a number of LMW complexes have been described as distinct cadmium phytochelatin complexes in several marine diatoms (Morelli, *et al*, 2002; Scarano and Morelli, 2002). Only a few researches have demonstrated the characterization of metal phytochelatin complexes in marine diatom (Gekeler, 1988; Hu, *et al*, 2001; Scarano and Morelli, 2002).

This work investigated the cellular Cd speciation complexes in *Dunaliella salina* cells during the growth cycle. The dominant Cd species were determined to find their

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specific peptides binding compounds in *D. salina* cells.

MATERIALS AND METHODS

The research was conducted on June – August 2009 in Aquaculture Laboratory University of Lampung. The pure *D. salina* grown in cultured condition, at 22-23 ^{0}C, by using continuous illumination under fluorescent daylight (110 µmol photons.m^{-2}.s^{-1}).The growth medium was natural seawater enriched with one sixth the normal trace metal concentration without Cu. The medium sterilized by filtration on 0.2 µm sterile membrane filters.

The culture medium spiked with 1 µM Cd. The growth was monitored by Sedgwick-Rafter counting cell under a microscope. Algae sample (500 ml) was harvested daily by filtration onto 0.2 µm membrane filters. The algae placed in 2 ml of 50 mM N-[2-hydroxyethil]piperazine-N'-[2-etbanesulfonic acid]/50 mM NaCl buffer (pH 7.5), containing in 1 mM Tris (2-carboxyethyl) phosphine as antioxidant. All samples were carried out in an ice bath. The cellular homogenate was centrifuged at 5500 rpm for 15 min at 4^{0}C to remove cellular debris, and then filtered through 0.2 µm membrane filter.

Size Exclusion Chromatography (SEC) performed by Hi-Load grade column by using 50 mM HEPES/50 mM NaCl pH 7.5 as elution buffer at flow rate of 1 ml. min^{-1}. The system consists of biotech pump, injection valve, and UV detector set at 254 nm. Injection volume was 1 ml and the eluate was collected in 1 ml fractions. Prior to each injection, the column was cleaned by loading 5 ml of 0,2 M EDTA.

γ-Glutamyl peptides were determined by RP-HPLC after derivatization with the fluorescent tag monobromobimane (mBrB). Having been acidified (10 µl, 1.2 MHcl/50 mM DTPA, 25 min), the sample was spiked with 150 µl of 0.7 mM TCEP in 200 mM HEPPS/5 mM DTPA, pH 8.2 to protect the thiol groups from oxidation and subjected to derivatization (20 µl of 10 mM mBrB). The reaction allowed to proceed for 20 min in the dark at 45 ^{0}C; 20 µl of 100 mM Cysteine were added and 20 min later the reaction was stopped by adding 20 µl of 1 M MSA. The derivative samples stored in the dark at ± 5^{0}C until HPLC analysis. Blank samples were used to identify the reagent peaks. Cd concentration analyzed by using AAS (Muhaemin, 2004).

RESULTS AND DISCUSSION

Fig. 1 showed that *D. salina* growth rate (µ) was 2.1 doubling per day. The microalgae harvested daily, from early exponential until stationary phase. It showed that low concentration of Cd and γ-Glu peptides were detected in microalgae cells for the first 2 day of exposure (DOE). Low concentrations of Cd were detected in cells. Cd and γ-Glu peptides concentration increased rapidly in 3 and 4 DOE. In the earlier stationary growth phase, Cd sharply increased and γ-Glu peptides began to accumulate.

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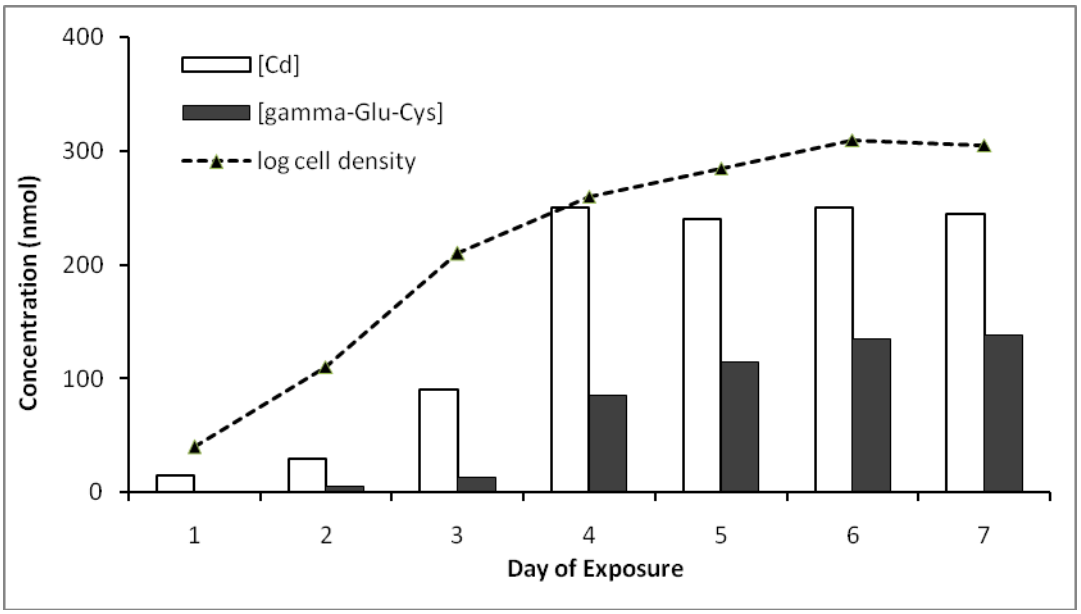

Fig 1. Concentration of Cd and total PC γ-Glu-Cys accumulation in *D. salina* cells.

The very low cellular Cd content assayed at the end of the 2nd DOE could be attributed to the dilution by cell division, that is higher during the early exponential phase. After the 4th DOE, depletion of the total dissolved Cd occurred and its concentration in the cells reached nearly constant value. The total γ-Glu peptides synthesis was almost linear from the 4th to 6th DOE. The stagnant concentration of γ-Glu-Cys in the *D. salina* cells indicated degradation of Cd-peptides complexes began during the 6th and 7th DOE.

Fig. 2 showed briefly a significant fraction of the Cd in 4th and 5th DOE cellular extracts eluted at the LMW column zone (fraction number 90-105). RP-Phase analysis revealed that PC_2, GSH, and γ-Glu-Cys peptide eluted in this fractions. The PC_2 peak maximum was coincident with a shoulder on the leading edge of the Cd peak which was indicating that a fraction of LMW Cd was bound to the oligomer. GSH co-eluted with the tailing edge of the Cd peak, suggesting that Cd-GSH complexes might be present.

(**Fig. 2**) showed that the kinetic analyses of the different Cd species accumulated in the cells correspond to the period in which the highest rate of metal uptake occurred. The dominant Cd species in *D. salina* cells were LMW-Cd compounds. Cd-PC_2 complexes were formed to a minor extent. The total cellular Cd was not tend to increase from 4th-7th DOE. At the 7th DOE, about 72 % of Cd still eluting in the LMW zone of the column as was identified as Cd-PC_2 complexes. The decrease of LMW-Cd suggests that the major fraction of Cd eluting as LMW-Cd was progressively transferred to different form of Cd-PC_n complexes. At the end of experiment, 81 % of total Cd eluted from SE column was bound to PC_n. the remaining Cd eluting at the exclusion volume of column. By the extended of exposure time, the Cd peaks related to both Cd-PC_n and CdS-containing PC_n complexes were slightly shifted toward shorter elution time. It indicates an increase of their apparent molecular weight.

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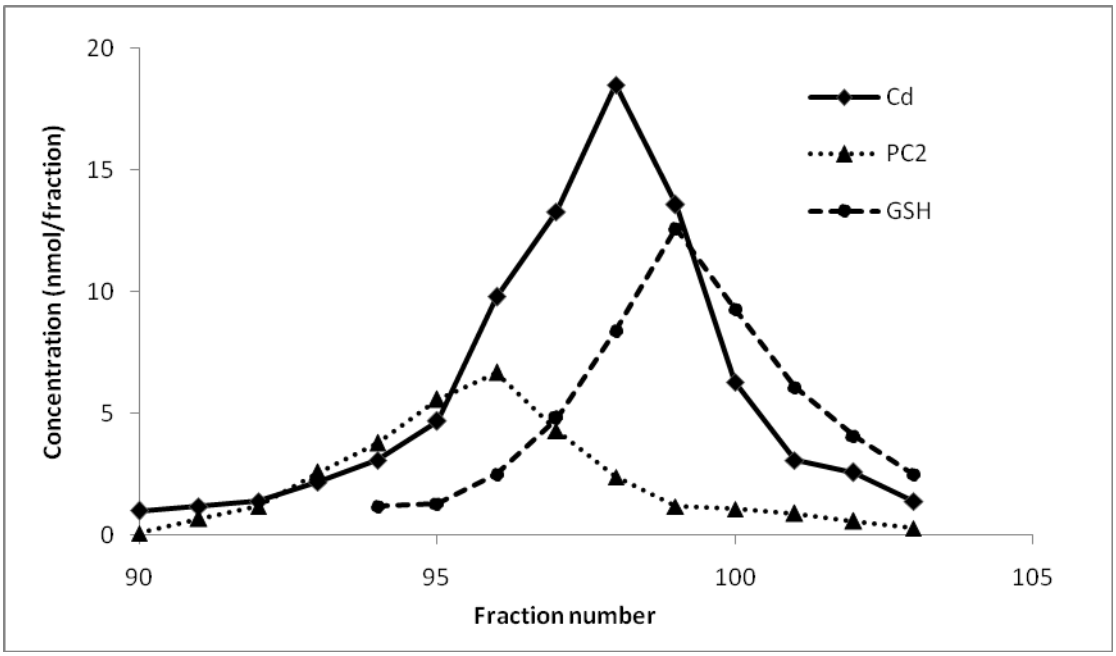

Fig 2. Concentration of Cd, PC_2, and GSH in the fraction eluting in the LMW zone

The result indicated that *D. salina* was able to sequent Cd in two classes of Cd-PC_n complexes. ***The first*** class was characterized by Cd-peptide complexes in which the peptides chelate Cd by formation of thiolate bonds. Morelli, *et al.,* (2002) noticed that these complexes have an apparent molecular weight according to the polymerization degree of the oligo-peptide involved. ***The second class*** of Cd-PC_n complexes was characterized by the higher apparent molecular weight and differs from the former. In these complexes, the metal ion might bind to a heterogeneous mixture of peptide with different chain length and to an acid labile sulfide to form CdS-containing PC complexes.

The kinetic of different species of Cd accumulated in the cells was consistent with the involvement of the GSH in the Cd sequestration mechanism through the initial formation of Cd-GSH complexes. The metal ion bound to GSH should be transferred to the newly synthesized $(\gamma\text{-Glu-Cys})_n$-Gly oligomers to form more stable Cd-PC_ncomplexes, or Cd-GSH complexes should be converted to Cd-PC_n complexes by the action of PC-synthase on this substrate (Cobbett, 2000). The early study of importance of sulfide association into PCn in Cd-tolerance is supported by several studies, such as metal tolerant of marine micro algae *P. tricornutum* incorporate into metal-PCn complexes a higher amount of sulfide than the sensitive one (Morelli, *et al.*, 2002) and Pb-methionine bio-sorption in *D. salina* (Muhaemin, 2006). Their capability to incorporate inorganic sulfide into Cd-peptide clusters could describe an important role for the defense of this marine micro algae to heavy metal stresses.

CONCLUSION

D. salina was able to absorb Cd along cultured phase. *D. salina* was able to sequent Cd in two classes of Cd-PC_n complexes; which was characterized by Cd-peptide complexes and noticed by a higher apparent molecular weight. These defense mechanisms might

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describe the important role of marine micro algae to combat heavy metal stresses in aquatic ecosystems.

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