Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

Ni^{2+}-affinity chromatography of cadmium response protein in microcrustacean *Moina macrocopa* *

Chanya Phromchaloem **
Amonrujee Nakphlaiphan ***
Thanit Pewnim ****

Abstract

Induction of sulfhydryl-rich protein synthesis was studied in microcrustaceans *Moina macrocopa* exposed to $CdCl_2$ at concentrations of 0 and 160 µg L^{-1} for 48 h. The results implicated that sulfhydryl-rich protein increased when *Moina macrocopa* were exposed to cadmium. This study was successful in employing a simple one-step method to purify sulfhydryl-rich protein from microcrustaceans *Moina macrocopa* after exposed to cadmium by using a metal-chelating affinity chromatography. As the results, a high molecular weight sulfhydryl-rich protein of approximately 60 kDa could be purified and separated from other proteins using Ni-Sepharose column. The purified sulfhydryl-rich protein was evaluated by SDS-PAGE and further identified by MALDI-TOF-MS analysis. A striking result is the strong induction of a Spätzle protein, which is well-known for the cysteine-knot domain in its structure. This research demonstrated for the first time the existence of a Spätzle protein in the microcrustaceans *Moina macrocopa* in response to cadmium exposure.

Keywords: cadmium, moina, affinity chromatography, sulfhydryl-rich protein

* This manuscript is a part of the thesis for the degree of Doctor of Philosophy Program.

** A Ph.D. student in Bioinorganic Chemistry, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: chanya.phromchaloem@gmail.com

*** Assistant researcher, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: amonrujee.nakphlaiphan@gmail.com

**** A thesis advisor, Assoc. Prof. Dr. Thanit Pewnim, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: thanit.pewnim@gmail.com

Introduction

Contamination of ecosystems and exposure to toxic metals are major concerns all over the world. Metals can enter the environment via a variety of inorganic species which can be bioconverted by the biota. Some of these metals such as cadmium (Cd) are the most abundant, ubiquitously distributed toxic elements in aquatic systems and are a concern to human health. Cadmium output to the environment is dramatically increasing due to its industrial uses, such as in Ni-Cd batteries, pigments, plastic stabilizers, smelters, alloys, and electroplating. Cadmium can be absorbed and accumulated in important organs, mainly the kidney and the liver which can cause damages to protein, DNA and lipid. Other effects are enzyme inhibition, cell signaling impairment, calcium homeostasis and changes in gene regulation (Ruangsomboon and Wongrat, 2006; Alhama, Romero-Ruiz et al., 2011; Wang, Pan et al., 2011; Thaenghin et al., 2017).

Living organisms use several mechanisms to counter cadmium toxicity. In higher eukaryotes, cadmium is sequestered by metallothioneins (MTs) or metallothioneins-like proteins (MTLPs) through their high cysteine content (Amiard et al., 2006). The sulfhydryl group (-SH) of cysteine can be a nucleophile, form disulfide bond, and importantly, has a strong affinity to binding toxic metal ions including cadmium. Interestingly, not only MTs, but also cadmium can be detoxified by chelating to glutathione (GSH) and other high sulfhydryl group proteins (Vido, Spector et al., 2001). It has been reported that the sulfur amino acid synthesis is strongly increased in response to cadmium treatments. These results demonstrated that high sulfhydryl group proteins function as a first response of defense against cadmium toxicity by chelation and sequestration of the toxic cadmium.

It is well known that sulfhydryl groups have a unique property to chelate transition metals. Based on this property, the aim of this study is therefore to purify sulfhydryl-rich protein from *Moina macrocopa* after exposure to cadmium using an affinity chromatography through a metal-chelating column. The advantage of affinity chromatography is that it is a simple one-step purification of proteins to achieve high purity, especially for proteins having structures or domains containing metal-chelating amino acids, such as cysteine in this case (Park et al., 2012; Honda et al., 2005; Nimsanor et al., 2016).

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

Materials and methods

The 1-mL HisTrap™ FF column was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). DL-dithio-threitol (DTT), Phenylmethylsulfonyl fluoride (PMSF) and cadmium chloride were from Sigma (Spain). Protein molecular weight markers for SDS-PAGE and protein assay dye reagents were from Bio-Rad (USA). All other reagents utilized were of analytical grade.

Microcrustacean culture and cadmium exposure

Microcrustacean *Moina macrocopa* were purchased from the aquaculture farm in Bangkok (Thailand). They were maintained in EDTA-free filtered water, pH 7.5-8.0 at 22°±1 °C under a light: dark cycle of 12:12 with continuous aeration. They were fed daily using live unicellular green algae *Chlorella vulgaris* at a concentration of 10^5 cells per mL. The exposure was performed for 48 h using 6-7 days old *Moina macrocopa* (1000 cladocera) in 2-L glass beakers containing $CdCl_2$ at concentration of 0 µg L^- and 160 µg L^{-1} for control group and cadmium exposure group, respectively. At the end of the exposure period, live moina were sieved and blotted to dryness before transfer to a microcentrifuge tube and flash freezing in liquid N_2, they were then kept at -80 °C until further analysis.

Protein extraction

Moina macrocopa samples (1 g) of both treatments were homogenized with a hand-held ceramic-grinder under liquid N_2 in three volumes of 20 mM Tris-HCl buffer, pH 8.6 containing 0.5 mM PMSF, 0.5 mM DTT, 0.5 M sucrose and 0.01% β-mercaptoethanol as a reducing agent. The homogenate was centrifuged at 30,000 x g for 30 min at 4 °C. To 1 mL supernatant, 1.05 mL of cold (-20 °C) absolute ethanol and 80 µL chloroform were added and the sample was spun at 6,000 x g for 10 min at 4 °C. To the supernatant, three volumes of cold absolute ethanol were added to a final concentration of 87% (v/v). The mixture was maintained at -20 °C and centrifuged to harvest the protein pellet at 6,000 x g for 10 min at 4 °C. The protein pellet was washed with 87% (v/v) ethanol and 1 % (v/v) chloroform in homogenizing buffer, centrifuged and dried under a nitrogen stream (Viarengo et al., 1997). The protein pellet was resuspended in 50 mM Tris–HCl, pH 7.4 containing 0.15 M NaCl. After being centrifuged at 6,000 × g for 2 min, the supernatant was immediately applied to a HiTrap™ FF column as described below.

Metal-chelating affinity chromatography

Affinity chromatography was performed using a 1-mL HiTrap™ FF column (Ni-Sepharose column) attached to a syringe. The supernatants from the protein pellet resuspension in 50 µL of 150 mM Tris-HCl, pH 7.4 as described above were applied to the column. Before sample loading, the column was equilibrated with 10 volumes of binding buffer (20 mM sodium phosphate containing 0.5 M NaCl, pH 7.4). After applying the supernatants, 10 volumes of binding buffer were passed through the column until the absorbance at 280 nm reached a steady baseline. The elution was performed by 10 mL of 500 mM imidazole in 20 mM sodium phosphate containing 0.5 M NaCl, pH 7.4. Fractions of 1 mL each were collected at a flow rate of 1 mL/min through the entire procedure. For each fraction, the protein absorbance at 280 nm was measured and cadmium concentration analyzed by ICP-OES.The presence of sulfhydryl groups in each protein fraction was investigated by Ellman's reagent (Ellman, 1959). The sulfhydryl group concentration was estimated using reduced glutathione (GSH) as a reference standard. The purification was performed at room temperature. Once the elution was completed, the column was washed with 10 volumes of binding buffer, followed by 10 volumes of distilled water to recover the column per the manufacturer's protocol. To characterize the proteins in the peak obtained from the elution, sulfhydryl-containing protein-positive fractions were pooled and concentrated by 500 Viva spin columns prior to separation by loading 20 µg in the 15 % SDS-PAGE gels and then stained with Coomassie Brilliant Blue R250. The purified protein bands on SDS-PAGE were cut and subjected to sequencing by MALDI-TOF-MS analysis. After sequencing, the protein sequence fragments of *Moina macrocopa* were determined using the protein BLAST (blastp) program.

Protein determination

Protein concentration was determined by the protein dye-binding method, using bovine serum albumin as standard (Bradford, 1976).

Results and discussion

It has been generally accepted that organisms respond to exposure to cadmium by the induction of cysteine-rich proteins such as metallothioneins, glutathione or phytochelatins to assist them in cadmium detoxification (Baykan et al., 2007; Knapen et al., 2007; Vido, Spector et al., 2001). In this study, we performed the purification of a cysteine-rich protein from microcrustacean *Moina macrocopa* using metal ion affinity chromatography. Purifications

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

of cysteine-rich proteins from *Moina macrocopa* control group and cadmium- exposed group at 160 ppb were carried out using commercial Ni-Sepharose (HiTrap™ FF) column according to the protocol provided by the manufacturer.

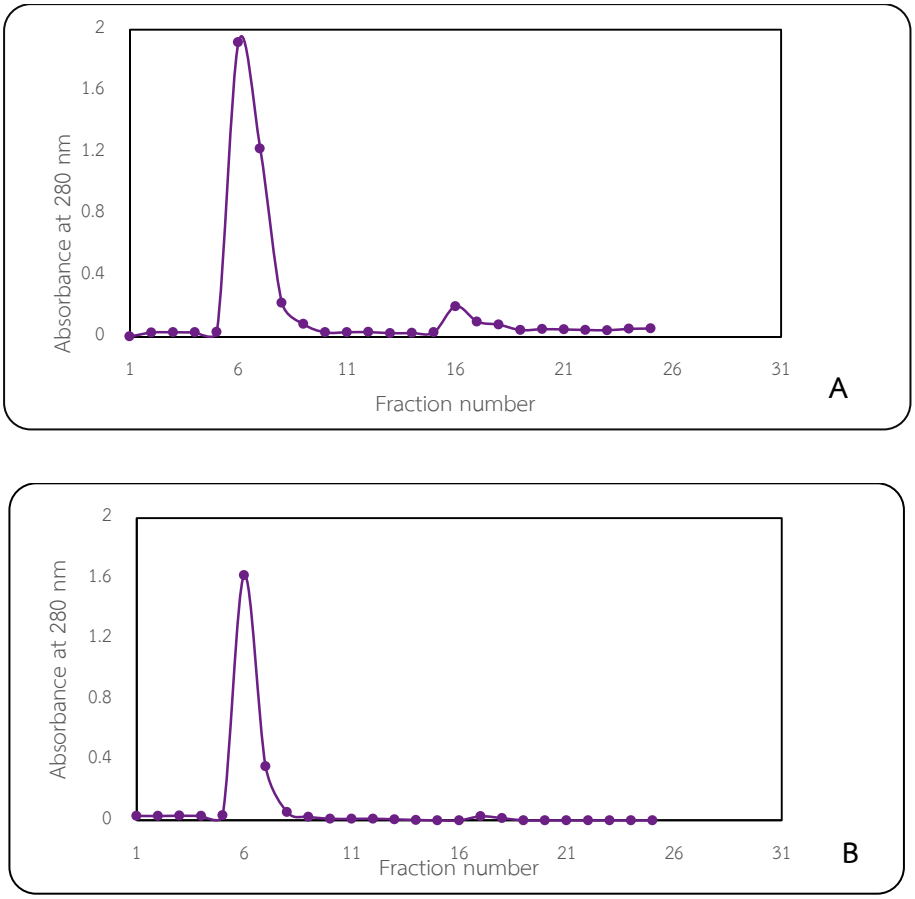

Fig. 1. (A) Elution profile on the purification of the *Moina macrocopa* protein extract from cadmium-exposed group using Ni-Sepharose column. (B) Elution profile was obtained during the purification of *Moina macrocopa* protein extract from control group using the same Ni-Sepharose column.

As shown in Fig. 1A, most of the proteins passed through the column in the binding step. For elution step, adsorbed proteins started to be eluted at fraction 15 through to fractions 16–19 using 500 mM imidazole at a flow rate of 1 mL/min. This result suggests that a small amount of the adsorbed protein exists in the flow-through fractions (fraction number 15-19) may be the metal-binding proteins. These fractions were combined and considered the pure protein as visualized on the 15% SDS-PAGE. The protein was subsequently utilized for amino acids sequence analysis. For sulfhydryl group analysis, the sulfhydryl-containing proteins in the eluted fractions were evaluated by Ellman's reagent. The sulfhydryl-containing protein

was detected in the eluted fractions (fraction number 15-19) of cadmium-exposed group (Fig. 2.). In contrast, the adsorbed protein and sulfhydryl-containing protein was not present in the eluted fractions of the control group (Fig. 1B). This result implicated that sulfhydryl-containing proteins increased when *Moina macrocopa* were exposed to cadmium. Moreover, the proteins adsorbed on the column were completely eluted with 500 mM imidazole, suggesting that this may be the cadmium-binding proteins in cadmium-exposed group.

Cadmium concentration was also determined in eluted fractions in order to determine the identity of the protein eluted from those fractions. Peak of cadmium was found to be at very low levels in the same fractions corresponding with sulfhydryl-containing protein eluted fraction 15-19 (Fig. 2). This is probably due to the metal exchange for Cd to Ni with those structurally attributed to cysteine-rich proteins in the binding step. This may explain why cadmium had low concentration in this eluted cadmium-binding protein. In the control group, cadmium was not present in any eluted fractions, when compared to the adsorbed protein and sulfhydryl contents of cadmium-exposed group mentioned above.

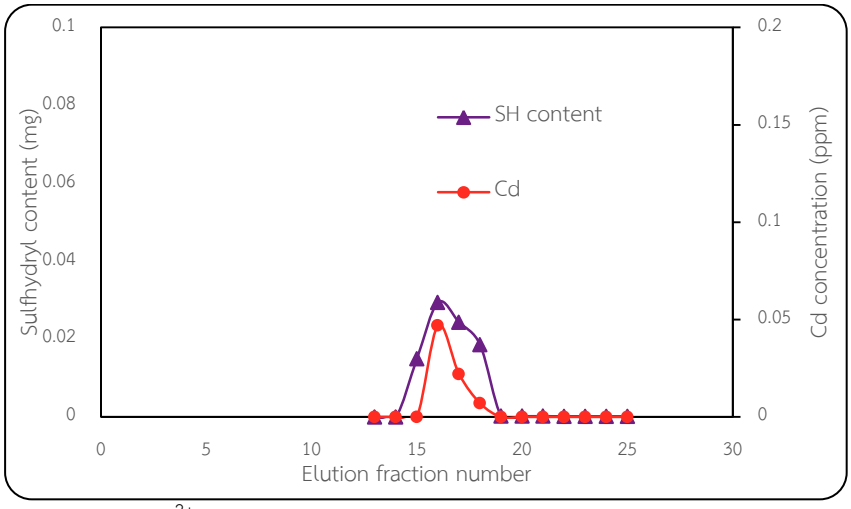

Fig. 2. Elution profile of Ni^{2+}-affinity column showing sulfhydryl and cadmium levels in eluted fractions of cadmium-exposed *Moina macrocopa* group. Sulfhydryl levels were determined by using Ellman's reagent while cadmium was quantified by ICP-OES.

To assess the purity of the proteins, sulfhydryl-containing fractions from the imidazole elution step were pooled and concentrated by ultrafiltration and were then separated by 15% SDS-PAGE. The proteins were stained with Coomassie Brilliant blue R250. Only one intense band was identified as shown in Fig. 3. The molecular mass of the intense sulfhydryl-binding protein band was estimated to be approximately 60 kDa based on SDS-PAGE

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

mobility. However, a very slightly blur bands at molecular weight of 50, 35 and ~10 kDa appeared in the gel. The protein with a molecular weight of ~10 kDa could be assumed to be a metallothionein due to its low molecular weight. These results suggest that among those cadmium-induced sulfhydryl-containing proteins, the high molecular-weight protein (MW of 60 kDa) has a special domain containing cysteine residues, which is essential for a strong binding to Ni^{2+} column. To understand the effect of cadmium stress on the expression of high molecular-weight protein that contain sulfhydryl groups, the amino acid sequencing of this purified protein band was analyzed by MALDI-TOF-MS analysis.

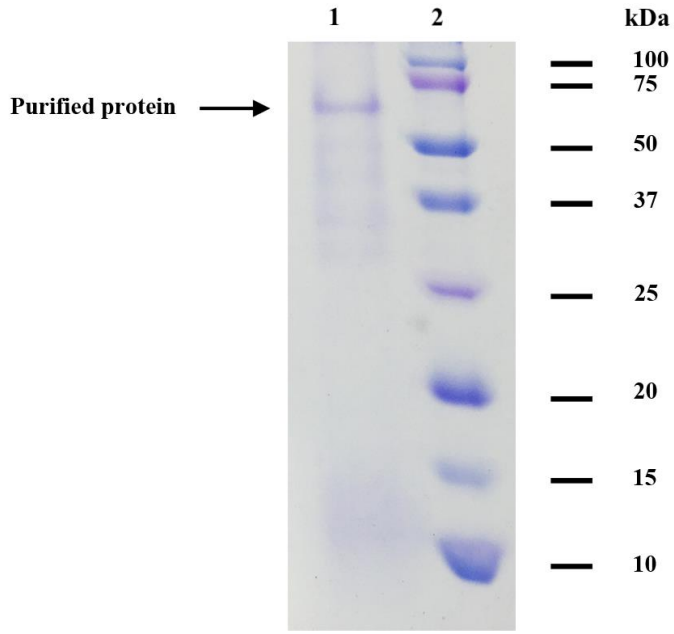

Fig. 3. A typical SDS–PAGE of sulfhydryl-containing proteins from *Moina macrocopa* after the purification using Ni-Sepharose affinity chromatography. Lane 1 refers to the pooled sample obtained after the imidazole elution step. Lane 2 is standard protein markers.

Protein sequence alignment using BLAST

Protein sequences identified by MALDI-TOF-MS were compared to sequences available in the NCBI database. Protein sequence comparisons using the Basic Local Alignment Search Tool Program (BLAST) was used to find similar proteins and the families to which the proteins could be related. The bioinformatics tool found that four different hypothetical proteins from *Daphnia pulex* were of very high identity to the type of protein purified in this study (MW of 60 kDa). The results of these analyses are summarized in Table 1 and Table 2.

The best protein hit with four different hypothetical proteins from *Daphnia pulex* is protein Spätzle. From this result, it is initially hypothesized that high molecular weight sulfhydryl-containing protein purified from *Moina macrocopa* cadmium-exposed group by Ni-affinity column is a protein Spätzle.

Table 1. Blast results of the purified protein using Ni Sepharose column.

Protein	organism	Accession number	MW (Da)	Protein score
Hypothetical protein DAPPUDRAFT_313415	*Daphnia pulex*	gi|321475101	32226	967
Hypothetical protein DAPPUDRAFT_308972	*Daphnia pulex*	gi|321474770	29805	566
Hypothetical protein DAPPUDRAFT_309050	*Daphnia pulex*	gi|321475069	29391	301
Hypothetical protein DAPPUDRAFT_313555	*Daphnia pulex*	gi|321475029	19827	256

*The protein score probability limit is 42 where $p < 0.05$

Table 2. Best hit results and protein domains.

Accession number	Best hit protein	Families and domains	Functions
gi|321475101 gi|321474770 gi|321475069 gi|321475029	Protein Spätzle Protein Spätzle Protein Spätzle Protein Spätzle,	Nerve growth factor-like ligands that form a cysteine knot structure.	(a) Controlling dorsal-ventral axis formation in *D. melanogaster.* (b) Immune response against bacteria and fungi

* Best hit results were obtained using a blastp search.

The Spätzle protein is the identified endogenous Toll receptor ligand which plays a critical role in initiating innate immune responses and controlling dorsal-ventral axis formation in *Drosophila melanogaster*. Spätzle has been shown to participate in immune responses against bacteria and fungi. As an implication from this study, Spätzle may act in the initiation of

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

"immune response" to cadmium toxicity in *Moina macrocopa*. Protein Spätzle contains a signal peptide, a Spätzle processing enzyme cleavage site, and a nerve growth factor NGF-like domain that adopts a cysteine rich domain or cysteine knot fold to bind the Toll receptor (Yu et al., 2015). Base on the property of sulfhydryl groups of cysteine, it can be inferred that cysteine knot in Spätzle protein could be a ligand to bind cadmium or nickel in this case of *Moina macrocopa*.

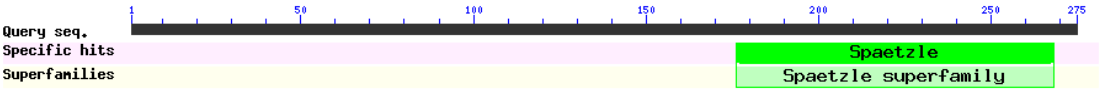

Fig. 4. The specific hits of purified sulfhydryl-containing protein from *Moina macrocopa* with Spätzle superfamily in *Daphnia pulex*. (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Conclusion

It is well known that the sulfhydryl groups of cysteine residues enable proteins to bind to heavy metal ions such as Cd^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+}. Based on this property, it is logical to use the single step method for purification of high sulfhydryl-containing proteins by Ni^{2+}-affinity chromatography. As expected, the sulfhydryl-containing proteins from cadmium-exposed microcrustaceans *Moina macrocopa* were purified at one step. The amino acid sequence of the 60 kDa protein was analyzed by MALDI-TOF-MS analysis. It was found that the purified sulfhydryl-containing protein has high identity to Spätzle proteins from *Daphnia pulex* (Fig. 4.). This observation suggested that the sulfhydryl-containing protein identified here may be referred to as a Spätzle protein. To date, Spätzle has been identified in insects and crustaceans, including the fruit fly, mosquito and brine shrimp. However, a Spätzle protein in water flea *Moina macrocopa* has not yet been described. Thus, this study demonstrated, for the first time, the existence of the Spätzle protein that was expressed in the microcrustaceans *Moina macrocopa* in response to cadmium.

Acknowledgements

We thank Professor Shireen A. Davies and Mr. Pablo Cabrero from University of Glasgow for supporting our research on protein sequencing and alignment. This research was supported by Ministry of Science and Technology (Thailand) through a Ph.D. scholarship to Ms. Chanya Phromchaloem.

References

Alhama, Jose, Antonio Romero-Ruiz, Jamel Jebali, and Juan López-Barea. (2011). "Total Metallothionein Quantification by Reversed-Phase High-Performance Liquid Chromatography Coupled to Fluorescence Detection after Monobromobimane Derivatization." **In:Environmental Research Journal** 5(5): 1-17.

Amiard, J. C., C. Amiard-Triquet, S. Barka, J. Pellerin and P. S. Rainbow. (2006). "Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers." **Aquatic Toxicology** 76, 2: 160-202.

Baykan, Umut., Guluzar Atli and Mustafa Canli. (2007). "The effects of temperature and metal exposures on the profiles of metallothionein-like proteins in *Oreochromis niloticus*." **Environmental Toxicology and Pharmacology** 23, 1: 33-38.

Bradford, Marion M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." **Analytical Biochemistry** 72, 1: 248-254.

Ellman, George L. (1959). "Tissue sulfhydryl groups." **Archives of Biochemistry and Biophysics** 82, 1: 70-77.

Honda, Rubens T., Roziete M. Araújo, Bruno B. Horta, Adalberto L. Val, and Marilene Demasi. (2005). "One-step purification of metallothionein extracted from two different sources." **Journal of Chromatography B** 820, 2: 205-210.

Knapen, Dries, Hans Reynders, Lieven Bervoets, Erik Verheyen and Ronny Blust. (2007). "Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations." **Aquatic Toxicology** 82, 3: 163-172.

Nimsanor, Supanee, Siriruk Chimpalee, Amporn Rungrod, Chutchanun Trakulnaleamsai, Boonsri Jongsareejit, and Boonhiang Promdonkoy. (2016). "Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated *Bacillus thuringiensis*." **Veridian E-Journal, Science and Technology Silpakorn University** 3, 5: 228-236.

Park, Nayoung, Ryu Jihye, Sohye Jang, and Hyun Soo Lee. (2012). "Metal ion affinity purificationof proteins by genetically incorporating metal-chelating amino acid." **Tetrahedron** 68: 4649-4654.

Ruangsomboon, Suneerat, and Ladda Wongrat. (2006). "Bioaccumulation of cadmium in an experimental aquatic food chain involving phytoplankton (*Chlorella vulgaris*), zooplankton (*Moina macrocopa*), and the predatory catfish *Clarias macrocephalus* × *C. gariepinus*." **Aquatic Toxicology** 78, 1: 15-20.

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

Thaenghin, Panathira, Thanit Pewnim, and Amonrujee Nakphayphan. (2017). "Effect of Cadmium to growth rates, cytotoxicity and pigment contents in Riceberry (*Oryza sativa L.*). " **Veridian E-Journal, Science and Technology Silpakorn University** 4, 3: 10-20.

Viarengo, Aldo, Enrica Ponzano, Francesco Dondero, and Rita Fabbri. (1997). "A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs." **Marine Environmental Research** 44, 1: 69-84.

Vido, Karin, Daniel Spector, Gilles Lagniel, Sebastien Lopez, Michel B. Toledano, and Jean Labarre. (2001). "A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*." **J Biol Chem** 276, 11: 8469-8474.

Wang, Lin, Luqing Pan, Na Liu, Dong Liu, Chaoqun Xu, and Jingjing Miao. (2011). "Biomarkers and bioaccumulation of clam *Ruditapes philippinarum* in response to combined cadmium and benzo[α]pyrene exposure." **Food and Chemical Toxicology** 49, 12: 3407-3417.

Yu, M., Y. Zhang, X. Tang, J. Ren and Y. Zhang. (2015). "The first mollusk spätzle homolog gene in the clam, *Paphia undulate*." **Fish & shellfish immunology** 47, 2: 712-716.