

**ASSESSMENT ON THE ACCUMULATION AND PHYTOCHELATIN
PEPTIDE PROFILING OF THE WATER SPINACH
[*Ipomoea aquatica* Forsk] GROWN UNDER
HEAVY METAL (LEAD) STRESS**

A THESIS PROPOSAL

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IRISH A. TEJANO

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CHAPTER 1

INTRODUCTION

Human evolution has led to immense scientific and technological progress. Global development, however, raises new challenges, especially in the field of environmental protection and conservation (Bennett *et al.*, 2003). Ironically, it is the economic, agricultural and industrial developments that are often linked to polluting the environment (Ikuoria and Okieimen, 2000). The world faces nowadays a deleterious environmental problem triggered by disasters stemming from ecologically destructive practices of man. Many ecosystems have been frayed to the point where they are no longer resilient and able to withstand natural disturbances.

One of the most serious ecological threats we face today is water pollution. Since the beginning of the industrial revolution, water pollution by toxic metals has accelerated dramatically. This affects plants and organisms living in bodies of water and, in almost all cases the effects were damaging either to individual species and populations, but also to the natural communities. According to Nriagu (1996) about 90% of the anthropogenic emissions of heavy metals have occurred since 1990 AD.

Man's exposure to heavy metals comes from industrial activities like mining, smelting, refining and manufacturing processes (Nriagu, 1996). Heavy metals and a number of chemicals from industries in the coastal areas have resulted in significant discharge of industrial effluents into the coastal water bodies. Human activities indeed have led to a substantial accumulation of heavy metals not only in our soil but also in our water. These toxic substances contribute to a variety of toxic effects on living organisms in the food chain (Dembitsky, 2003) by bioaccumulation and bio-magnification (Manohar *et al.*, 2006).

In response to environmental stresses, certain plants and organisms developed adaptive mechanisms enabling their growth and survival. Consequently, plants respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins. These mechanisms have been reviewed comprehensively by Sanita di Toppi and Gabbrielli (1999) for plants exposed to Cd, the heavy metal for which there have been arguably the greatest number and most wide-ranging studies over many decades. Understanding the molecular and genetic basis for these mechanisms will be an important aspect of

developing plants as agents for the phytoremediation of contaminated sites (Salt *et al.*, 1998). One recurrent general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex. A number of metal-binding ligands have now been recognized in plants. The roles of several ligands have been reviewed by Rauser (1999). Extracellular chelation by organic acids, such as citrate and malate, is important in mechanisms of aluminum tolerance. For example, malate efflux from root apices is stimulated by exposure to aluminum and is correlated with aluminum tolerance in wheat (Delhaize and Ryan, 1995). Some aluminum-resistant mutants of *Arabidopsis* also have increased organic acid efflux from roots (Larsen *et al.*, 1998).

Notably one of the most prolific aquatic plants in the Philippines is water spinach. Examining plant's ability to hyperaccumulate lead-contaminated growth medium and characterizing its metal-binding peptides, the phytochelatins, would be the main concerns of this study.

1.1 Statement of the Problem

Plants are exposed to all sorts of environmental stresses such as drought, cold, salinity, heavy metal toxification, mechanical wounding or infections by fungi and other organisms. Plants, like other organisms, have adaptive mechanisms whereby they are able to respond to both nutrient deficiencies and toxicities.

The water spinach, locally known as "**Kangkong**" being prolific in the tropical and subtropical countries, is typically exposed to abiotic stress (e.g. heavy metal or waste water pollutants). In relation to this view, this study opts to examine the plant's ability to accumulate amount of lead consequent to heavy metal stress in its growth medium and to perform protein profiling of the metal-binding peptides (phytochelatins) of the plant extract.

1.2 Objectives of the Study

This study aims to examine the ability of water spinach to hyperaccumulate heavy metal when treated with different level of lead and

to detect and quantify metal-binding peptides (phytochelatins) of the plant extract.

Specifically, this research aims to:

1. test and identify the plant organ (sorted roots, leaves and stems of water spinach) that accumulates the greatest amount of lead through Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) technique ;
2. isolate and analyze phytochelatins peptides chromatograms found in plant organ with greatest amount of lead using HPLC analysis; and
3. identify the amino acid sequence of some proteins distinctly expressed by the plant consequent to heavy metal stress condition employing the LC-MS/MS technique.

1.3 Significance of the Study

The results of this study could possibly provide a vital data for the utilization of water spinach as hyperaccumulator to clean up lead-polluted areas and could generate its applicability for phytoremediation. This study could also provide details on the amino acid sequence of the plant when grown under heavy metal stress condition. This could also give

information to the public about the health hazards of the water spinach specially when consumed.

1.4 Scope and Limitations of the Study

This study is limited to the isolation and characterization of phytochelatins present in water spinach upon treatment of lead gradient (0-1000 μM). The ability of the plant to accumulate heavy metal would be examined consequent to heavy metal stress. Chromatographic analysis and amino acid sequencing of metal-binding peptides would also be performed, but due to unavailability of the instruments, plant sample prior to analysis would be sent to other universities or analytical institutions here or outside the country.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Developing cost effective and environmentally friendly technologies for the remediation of soils and wastewaters polluted with toxic substances is a topic of global interest. A promising new technology, referred to as phytoremediation, offers promise for cleanup of polluted areas in a cost-effective and environment friendly manner (Salt *et al.*, 1995, 1998; Raskin *et al.*, 1997). This technology involves removal of toxic heavy metals from contaminated soils and waters, or rendering them harmless by accumulating, chelating, or transforming these contaminants into biologically inactive forms through green plants. One possible approach for phytoremediation is to use "hyperaccumulators," plant species that have evolved to accumulate high concentrations of heavy metals in their biomass.

The value of metal-accumulating plants to wetland remediation has been recently realized (Black, 1995). Several terrestrial plants that have been identified in the last two decades as highly effective in absorbing and accumulating various toxic trace elements are being evaluated for their role in the phytoremediation of soils polluted with trace elements (Baker *et*

al., 1994; Tang *et al.*, 2001). Raskin *et al.* (1994) defined rhizofiltration as the use of plant roots to absorb heavy metals from polluted effluents. *Lemna minor* L. (duckweed) and *Azolla pinnata* R. Br. (water velvet) have been shown to bioconcentrate metals such as Fe and Cu by up to 78 times the concentrations in the wastewater (Jain *et al.* 1989). Pinto *et al.* (1987) demonstrated that water hyacinth would remove silver from industrial wastewater for subsequent recovery with high efficiency in a fairly short time. The accumulation of some other heavy metals and trace elements in many species of wetland plants has also been demonstrated (Dunbabin and Bowmer, 1992; Delgado *et al.*, 1993; Fett *et al.*, 1994; Salt *et al.*, 1995, Zaranyika and Ndapwadza, 1995; Zayed *et al.*, 1998; Zhu *et al.*, 1999). Water hyacinth has been used successfully in wastewater treatment systems to improve the quality of water by reducing the levels of organic and inorganic nutrients (Brix, 1993; Delgado *et al.*, 1995) and readily reducing the level of heavy metals in acid mine drainage water (Falbo and Weak, 1990). Furthermore, the quantity of trace elements that can be accumulated by water hyacinth has been shown to correlate well with concentration of heavy metals in the water (Ismail *et al.*, 1996).

2.1 *Ipomoea aquatica* Forsk

Ipomoea aquatica Forsk locally known as kangkong is a member of the Convolvulaceae family. It is a semi-aquatic tropical plant grown as a leaf vegetable. It is known in english as water spinach, water morning glory, water convolvulus, or by the more ambiguous names "Chinese spinach" and "swamp cabbage" (Gothberg et al., 2004). The aquatic plant water spinach (*Ipomoea aquatica* Forsk) grows wild and is cultivated throughout Southeast Asia and is a widely consumed vegetable in the region. Many of the waters where *I. aquatica* grows serve as recipients for domestic and other types of waste water. Because these waters contain not only nutrients, but often also a wide variety of pollutants, such as heavy metals from various human activities, many people are in high risk of poisoning. Water spinach is also supposed to possess an insulin-like activity according to indigenous medicine in Sri Lanka (Malalavidhane et al., 2000). Only a very few scientific studies have been conducted on its medicinal aspect. These include the inhibition of prostaglandin synthesis (Tseng et al., 1992) effects on liver diseases (Badruzzaman and Husain, 1992), constipation (Samuelsson et al., 1992), and hypoglycemic effects (Malalavidhane et al., 2000).



Figure 1 Picture of *Ipomoea aquatica* (adapted from photobucket.com).

Evaluation on the effect of *Ipomoea aquatica* aqueous and dichloromethane/methanol extracts on the glucose absorption using a rat intestinal preparation *in situ* showed a significant inhibitory effect on glucose absorption tested orally at the dose of 160 mg/kg when compared with control animals. The most pronounced effect was observed with the aqueous extract. On the other hand both plant extracts inhibited the gastrointestinal motility suggesting that the inhibition of glucose absorption is not due to the acceleration of intestinal transit (Sokeng *et al.*, 2006).

In 2004, Huang and colleagues examined possible antioxidant and antiproliferative activities of 95% ethanol and or water extract from water spinach organs employing DPPH staining, total phenolic compounds, total flavonoid content, DPPH radical, reducing power method, FTC method, and inhibition of cancer cell proliferation. They found out that ethanol extract of stem had the highest content of the total phenolic compounds, as well as the highest reducing power and FTC activity. Ethanol extract of leaf had the highest amount of flavonoids. While extract of stem had the highest radical-scavenging activity, followed by ethanol extract of leaf. The antiproliferative activities of water spinach extracts were studied in vitro using human lymphoma NB4 cells, and the following results were found: water extract of stem had the highest antiproliferative activity with an EC₅₀ of 661.40 ± 3.36 µg dry matter/mL, followed by ethanol extract of stem and ethanol extract of leaf. The water extract of leaf had the lowest antiproliferative activity (EC₅₀>1000 µg dry matter/mL) under the experimental conditions.

Comparative study on oral hypoglycaemic activity of an aqueous extract of the green leafy vegetable *Ipomoea aquatica* with that of the known oral hypoglycaemic drug tolbutamide in glucose challenged Wistar rats, showed that the mean blood glucose level of the *Ipomoea aquatica*

treated group was 47.5% lower than that of the control group treated with distilled water while tolbutamide treated group showed a mean blood glucose level which was only 33.8% lower than that of the control group. However, statistical analysis indicated that the blood glucose levels of the *Ipomoea aquatica* treated group were not significantly different from that of the tolbutamide treated group. With these results, the researchers concluded that aqueous extract of *Ipomoea aquatica* is as effective as tolbutamide in reducing the blood glucose levels of glucose-challenged Wistar rats (Malalavidhane *et al.*, 2000).

2.2 Metal of Interest

2.2.1 Lead

As reviewed by Sengar *et al.* (2008), lead is a metallic pollutant emanating from various environmental sources including industrial wastes, combustion of fossil fuels, and use of agrochemicals. Lead may exist in the atmosphere as dusts, fumes, mists, and vapors, and in soil as a mineral. Soils along roadsides are rich in lead because vehicles burn leaded gasoline, which contributes to environmental lead pollution. Other important sources of lead pollution are geological weathering, industrial processing of ores and minerals, leaching of lead from solid wastes, and

animal and human excreta. Lead is nondegradable, readily enters the food chain, and can subsequently endanger human and animal health. Lead is one of the most important environment pollutants and deserves the increasing attention it has received in recent decades. The effort made by Sengar and colleagues was undertaken to review lead stress effects on the physiobiochemical activity of higher plants. Lead has gained considerable attention as a potent heavy metal pollutant because of growing anthropogenic pressure on the environment. Lead-contaminated soils show a sharp decline in crop productivity. Lead is absorbed by plants mainly through the root system and in minor amounts through the leaves. Within the plants, lead accumulates primarily in roots, but some is translocated to aerial plant parts. Soil pH, soil particle size, cation-exchange capacity, as well as root surface area, root exudation, and mycorrhizal transpiration rate affect the availability and uptake of lead by plants. Only a limited amount of lead is translocated from roots to other organs because there are natural plant barriers in the root endodermis. At lethal concentrations, this barrier is broken and lead may enter vascular tissues. Lead in plants may form deposits of various sizes, present mainly in intercellular spaces, cell walls, and vacuoles. Small deposits of this metal are also seen in the endoplasmic reticulum, dictyosome, and dictyosome-derived vesicles. After entering the cells, lead inhibits activities

of many enzymes, upsets mineral nutrition and water balance, changes the hormonal status, and affects membrane structure and permeability. Visual, nonspecific symptoms of lead toxicity are stunted growth, chlorosis, and blackening of the root system. In most cases, lead inhibition of enzyme activities results from the interaction of the metal with enzyme - SH groups. The activities of metalloenzymes may decline as a consequence of displacement of an essential metal by lead from the active sites of the enzymes. Lead decreases the photosynthetic rate of plants by distorting chloroplast ultrastructure, diminishing chlorophyll synthesis, obstructing electron transport, and inhibiting activities of Calvin cycle enzymes.

Lead is a nonessential element in metabolic processes and may become toxic or lethal to many organisms even when absorbed in small amounts. Boonyapookana *et al.*, (2005) showed that Pb caused phytotoxic effect including chlorosis, necrosis, stunt growth of root/shoot, and less biomass production on *Helianthus annuus*, *Nicotiana tabacum* and *Vetiveria zizanioides*.

2.3 Heavy-Metal-Binding Peptides of Plants

2.3.1 Phytochelatins (PCs)

A. Structure and Biosynthetic Pathway

Phytochelatins (PCs) are small, heavy metal-binding, cysteine-rich polypeptides with the general structure of $((\text{c-Glu-Cys})_n\text{Gly}$ ($n \geq 2-11$)), present in plants, fungi, and other organisms (Grill *et al.*, 1985; Gekeler *et al.*, 1988; Piechalak *et al.*, 2002). They are synthesized from glutathione (GSH) in the presence of heavy metals by the enzyme phytochelatin synthase (PCS) (Grill *et al.*, 1989; Tomaszewska *et al.*, 1996; Vatamaniuk *et al.*, 2000) and form complexes with some of those ions, then subsequently transported from the cytosol into the vacuole (Salt and Rauser, 1995). PCs form a family of structures with increasing repetitions of the ^o-GluCys dipeptide followed by a terminal Gly; $(\text{^o-GluCys})_n\text{-Gly}$, where n is generally in the range of 2 to 5. PCs have been identified in a wide variety of plant species and in some microorganisms. In addition, a number of structural variants, for example, $(\text{^o-GluCys})_n\text{-Ala}$, $(\text{^o-GluCys})_n\text{-Ser}$ and $(\text{^o-GluCys})_n\text{-Glu}$, have been identified in some plant species. PCs are structurally related to glutathione (GSH; ^o-GluCysGly), and various physiological, biochemical, and genetic studies have confirmed that GSH is the substrate for PC biosynthesis (Cobbett, 1994;

Rausser, 1995 and 1999; Zenk, 1996). According to Grill *et al.*, 1989; Chen *et al.*, 1997, their biosynthesis is due to the transpeptidation of γ -glutamylcysteinyl dipeptides from GSH by the action of a constitutively present PC synthase. Recently, the gene encoding PC synthase has been cloned by several laboratories (Clemens *et al.*, 1999; Ha *et. al.*, 1999; Vatamaniuk *et al.*, 1999). PC synthase is activated by heavy metal ions such as Cd^{2+} , Cu^{2+} , Ag^{1+} , Hg^{2+} , and Pb^{2+} , which are characterized as class B and borderline elements (Nieboer and Richardson, 1980). Subsequently, these ions are complexed by the induced PCs via thiolate coordination (Grill *et al.*, 1985; Grill, 1989; Strasdeit *et al.*, 1991; Mehra *et al.*, 1995, 1996a, 1996b; Mehra and Mulchandani, 1995; Salt *et al.*, 1995; Pickering *et al.*, 1999). In particular, genetic studies have confirmed that GSH-deficient mutants of *S. pombe* as well as *Arabidopsis* are PC deficient and hypersensitive to Cd. A list of mutants that identify a role for particular genes in PC biosynthesis or function is shown in Table 1. In addition, a schematic diagram including the PC biosynthetic pathway is illustrated in Figure 2.

TABLE 1 Mutants affected in phytochelatin biosynthesis and function

Organism ^a	Gene/locus	Activity/function	Reference
PC biosynthesis			
<i>Sp</i>	<i>Gsh1</i> °	γ-glutamylcysteine synthetase/ GSH biosynthesis	(11, 22)
<i>At</i>	<i>CAD2/RML1</i> °	γ -glutamylcysteine synthetase/ GSH biosynthesis	(7, 41)
<i>Sp</i>	<i>Gsh2</i>	glutathione synthetase/ GSH biosynthesis	(11, 22)
<i>At</i>	<i>CAD1</i>	PC synthase/PC biosynthesis	(14)
<i>Sp</i>	<i>Pcs1</i>	PC synthase/PC biosynthesis	(11, 13)
<i>Ce</i>	<i>Pcs1</i>	PC synthase/PC biosynthesis	(40)
PC function			
<i>Sp</i>	<i>Hmt1</i>	PC-Cd vacuolar membrane	(26, 27)
<i>Sp</i>	<i>Ade2, 6, 7, 8</i>	Metabolism of cysteine sulfinate to products involved in sulphide biosynthesis; also required for adenine biosynthesis	(14,33)
<i>Sp</i>	<i>Hmt2</i>	Mitochondrial sulfide: quinine oxidoreductase/ detoxification of sulphide	(38)
<i>Ca</i>	<i>Hem2</i>	Porphobilinogen synthase/ siroheme biosynthesis (cofactor for sulfite reductase)	(16)

^aAt, *A. thaliana*; Sp, *S. pombe*; Ca, *Candida albicans*; Ce, *C. elegans*.

Adapted from Cobbett and Goldsbrough, 2000.

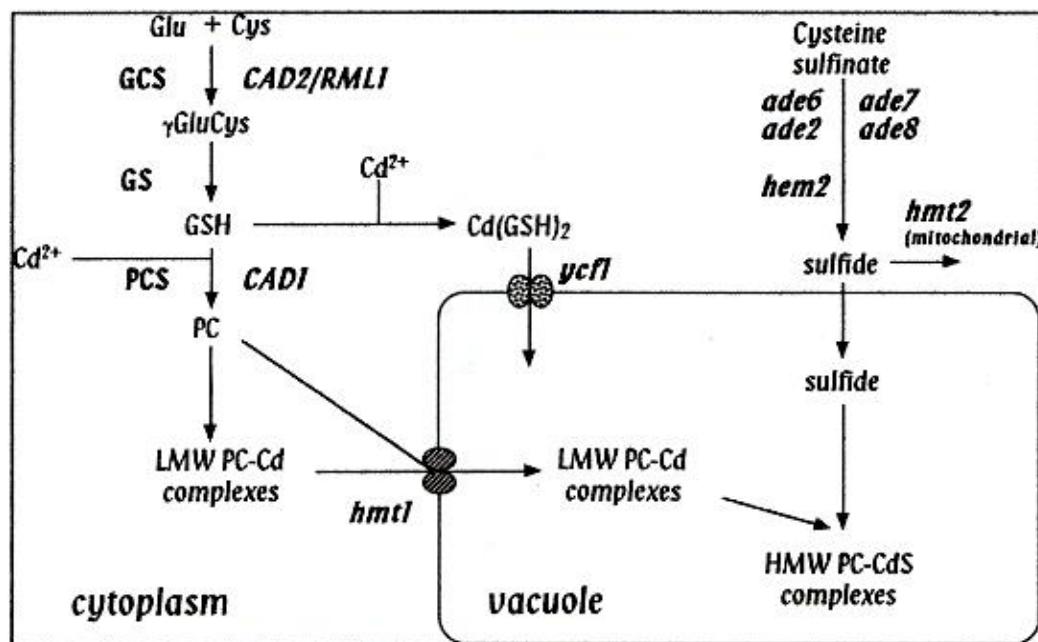


Figure 2 Genes and functions contributing to Cd detoxification in plants and fungi as a composite of various functions identified through the isolation of Cd-sensitive mutants of different organisms that express PCs. Gene loci are shown in italics: *CAD1* and *CAD2/RML1* are in *Arabidopsis*; *hmt1*, *hmt2*, *ade2*, *ade6*, *ade7*, and *ade8* are in *S. pombe*; *ycf1* is in *S. cerevisiae*; and *hem2* is in *C. glabrata*. Enzyme abbreviations: GCS, γ -glutamylcysteine synthetase; GS, glutathione synthetase; PCS, phytochelatin synthase (Adapted from Cobbett, 2000).

B. Identification of PC Synthase Genes

Phytochelatin synthase, the enzyme catalyzing the biosynthesis of PCs from GSH, was first characterized by Grill *et al.*, (1989). However, it was not until 1999 that the cloning of PC synthase genes was described. It was in *Arabidopsis* where PC synthase gene was first genetically

identified. Investigations on the expression of Arabidopsis and wheat cDNA libraries in *S. cerevisiae* isolated PC synthase genes *AtPCS1* (Vatamaniuk, 1996) and *TaPCS1* (Clemens et al., 1999), respectively conferred an increased Cd resistance. Another study had identified *AtPCS1* through the positional cloning of the *CAD1* gene of Arabidopsis (Ha et al., 1999). This combination of genetic, molecular, and biochemical data was a conclusive demonstration that these genes encode PC synthase. Full-length or partial cDNA clones encoding presumptive PC synthases have also been isolated from other plant species, including *Brassica juncea* and rice.

2.3 The Recent Phytoremediation Techniques

Jadia and Fulekar (2008), reported that the current remediation technique of heavy metal from contaminated soil-water are expensive, time consuming and environmentally destructive. Unlike organic compounds, metals cannot degrade, and therefore effective cleanup requires their immobilization to reduce or remove toxicity. In recent years, scientists and engineers have started to generate cost effective technologies that include use of microorganisms/biomass or live plants to clean polluted areas. Phytoremediation is an emerging technology for

cleaning up contaminated sites, which is cost effective, and has aesthetic advantages and long term applicability. It is best applied at sites with shallow contamination of organic, nutrient or metal pollutants that are amenable to one of the five applications; phytotransformation, rhizosphere bioremediation, phytostabilization, phytoextraction and rhizofiltration. The technology involves efficient use of plants to remove, detoxify or immobilize environmental contaminants in a growth matrix (soil, water or sediments) through the natural, biological, chemical or physical activities or processes of the plants. They added that for this clean-up method to be feasible, the plants must (1) extract large concentrations of heavy metals into their roots, (2) translocate the heavy metal into the surface biomass, and (3) produce a large quantity of plant biomass. In addition, remediative plants must have mechanisms to detoxify and/or tolerate high metal concentrations accumulated in their shoots. In the natural setting, certain plants have been identified which have the potential to uptake heavy metals. At least 45 families have been identified to hyperaccumulate heavy metal; some of the families are Brassicaceae, Fabaceae, Euphorbiaceae, Asteraceae, Lamiaceae and Scrophulariaceae. *Brassica juncea*, commonly called Indian mustard, has been found to have a good ability to transport lead from the roots to the shoots (United States Protection Agency, 2000). Indian mustard (*B. juncea*) is a high biomass,

rapidly growing plant that has an ability to accumulate Ni and Cd in its shoots. It is a promising plant for phytoremediation (Terry *et al.*, 1992). Aquatic plants such as the floating Eichhornia crassipes (water hyacinth), Lemna minor (duckweed), and Pistia have been investigated for use in rhizofiltration (Karkhanis *et al.*, 2005). Recently, a fern Pteris vitatta has been shown to accumulate as much as 14,500 mg kg⁻¹ arsenic in fronds without showing symptoms of toxicity (Ma *et al.*, 2001). Corn, sunflower and sorghum (Jadia and Fulekar, 2008) were found to be effective due to their fast growth rate and large amount of biomass (Pilon-Smits, 2005; Schmidt, 2003; Tang *et al.*, 2003). Gardea-Torresdey *et al.*, 2000 have shown that alfalfa is a potential source of biomaterials for the removal and recovery of heavy metal ions.

2.4 SDS-PAGE Analysis (adapted from www.molecularStation.com)

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as

well as higher order protein folding, posttranslational modifications and other factors).

The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

SDS bind in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform mass: charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

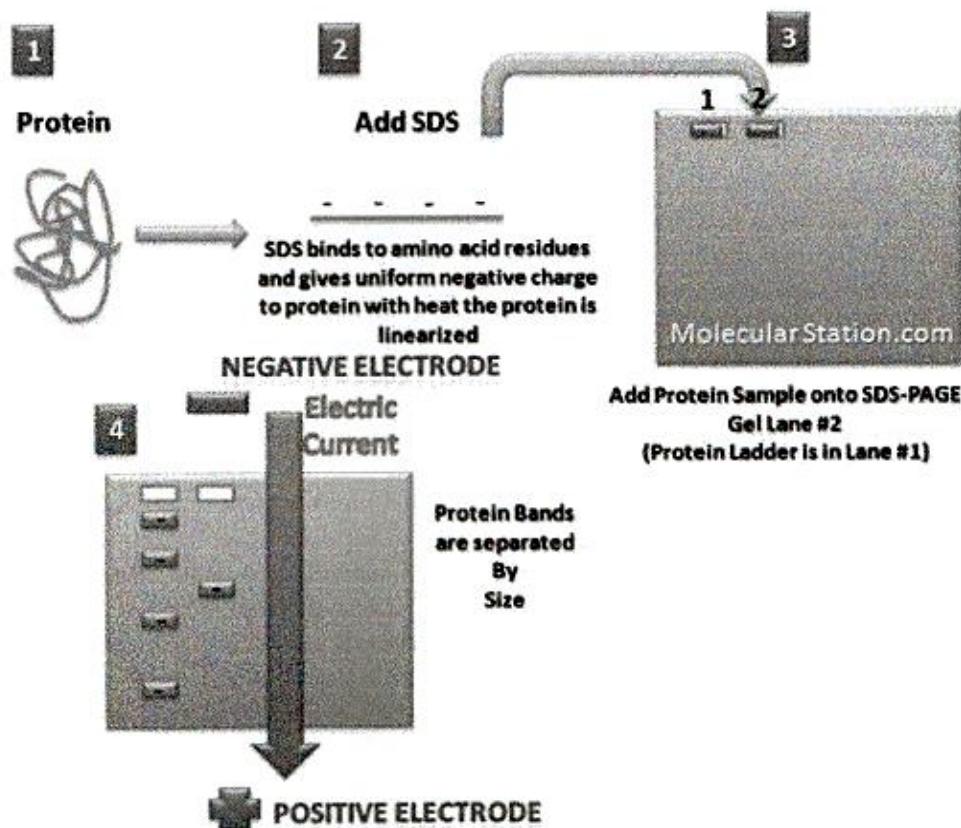


Figure 3 Overview of the Protein Gel Electrophoresis Method.
Adapted from www.molecularStation.com

Electrophoresis is a technique for separating, or resolving molecules in a mixture under the influence of an applied electric field, also called electrophoretic mobility. Dissolved molecules in an electric field move, or migrate at a speed determined by their charge: mass ratio. For example if two have the same mass and shape, the one with the greater net charge will move faster toward an electrode. The separation of small

molecules, such as amino acids and nucleotides, is one of the many uses of electrophoresis. In this case, a small drop of sample is deposited on a strip of filter paper or other porous substrate, which is soaked with a conducting solution. When an electric field is applied at the ends of the strip, small molecules dissolved in the conducting solution move along the strip at a rate corresponding to the magnitude of their charge.

2.4 Amino Acid Sequencing (adapted from www.epc.com)

Protein sequencing determines the order of amino acids in a protein. Among chemical methods N-terminal sequencing by Edman degradation is by far the most commonly used. By this method information about the order of amino acids from the amino-terminal end is obtained.

Sequence information can be used to identify a protein or homologous proteins through searches in databases; the minimal number of residues for a successful search is ten, but often more residues are required. The N-terminal sequence is often used for confirmation of the identity of a protein; in that case a few residues are enough. The sequence also gives information about post translational cleavage points. In addition, the sequence results give information about the purity of a

preparation; limits of detectable contamination depend on the sequences of the analysed proteins.

The most important prerequisite for a protein to be sequenced by Edman degradation is that the N-terminal residue is not blocked, but free to react in the first step of the sequencing procedure. Proteins can be blocked either naturally e.g. by acetylation, or during purification by cyclisation of glutamine. It is estimated that >50% of all proteins are blocked. In these cases no sequence is obtained.

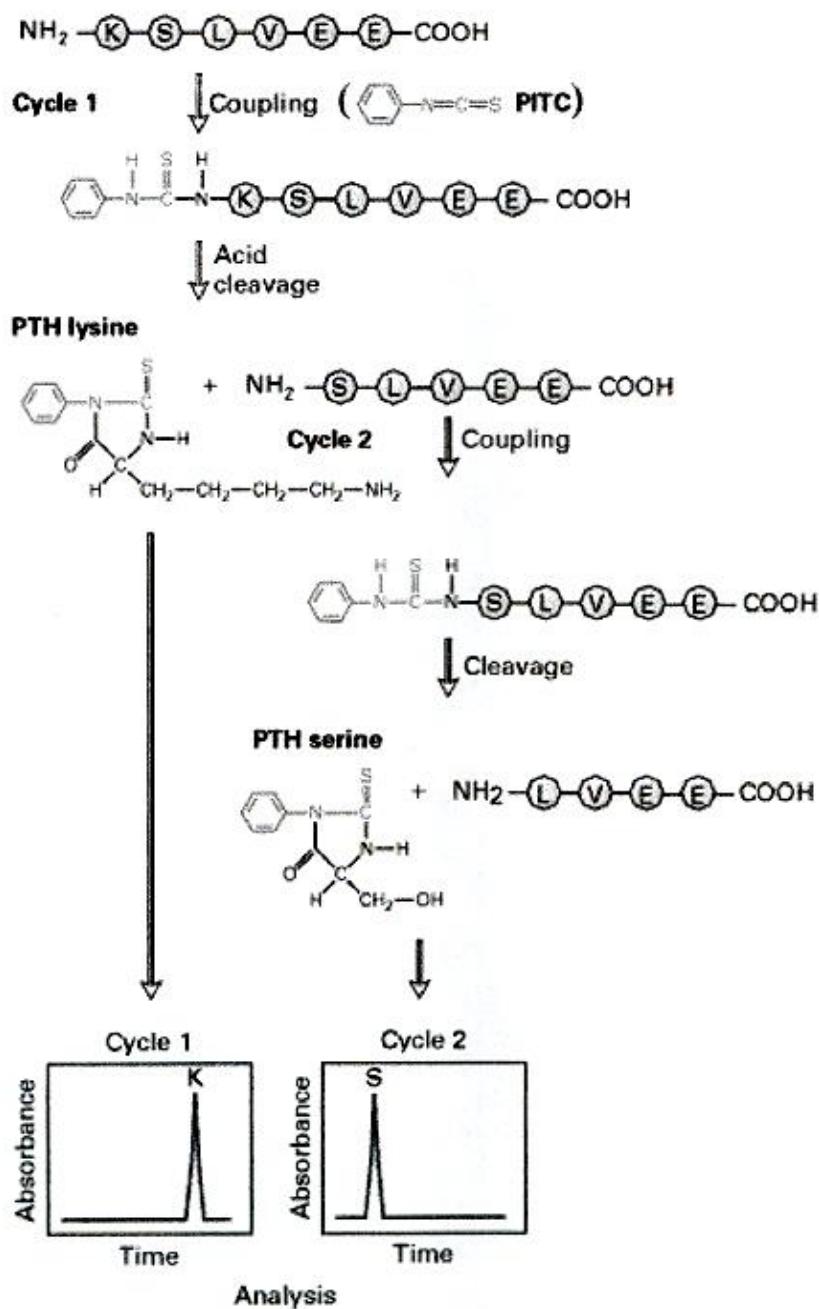


Figure 4 Overview of the Chemical determination of the sequence of a protein by Edman degradation.

In the first step, the polypeptide N-terminus is reacted with phenylisothiocyanate (PITC). In the second step, the N-terminal amino acid is cleaved from the polypeptide by acid hydrolysis, yielding the cyclic phenylthiohydantoin (PTH) derivative and a polypeptide that is shorter at its N-terminus by one residue. These two steps are then repeated with the shortened polypeptide. The PTH derivative formed in each cycle is identified by liquid chromatography (W. H. Freeman and Company, 2000).

CHAPTER 3

METHODOLOGY

3.1 Preparation of Plant Material

- **Materials Needed:**

- Nutrient Solution
- Lead nitrate
- Deionized water
- Greenhouse
- Hydroponic Tank

Stem cuttings will be employed for propagating the plant. Water spinach will be grown under hydroponic conditions, using Hoagland nutrient solutions (to be made with deionized water), with varying lead concentration and with sand to be used as a mechanical support. The plants will be grown under greenhouse at the vicinity of MSU-IIT, Iligan City. Three pots per treatment of heavy metal with three replicates (plants) per pot will be used. Seedlings will be grown for 5 weeks. Each pot would be spiked with 0, 50, 125, 375, 750 and 1,000 μM $\text{Pb}(\text{NO}_3)_2$ concentrations for once a week.

3.2 Analysis of Lead Content (Dry Ashing)

- **Materials needed:**

- crucible, 15 mL, porcelain, tall form
- muffle furnace
- water bath
- filter paper, Whatman no.541
- Hydrochloric acid; approx. 6 M
- Nitric acid, concentrated

After five weeks the water spinach will be harvested and sorted into different parts: roots, stems and leaves. Sorted plant parts will be dried in an oven at 70 °C. A 2.5 g of dried and ground plant material will be placed into an acid-washed porcelain crucible. The crucibles with samples will then be put in a muffle furnace with the temperature slowly raised over 2 h to reach 500 °C and ashed for at least 4 h. Thereafter, crucible will be removed and allowed to cool. Ten milliliter of 6 M HCl will be added then covered and will be heated on a steam bath for 15 min. One milliliter of concentrated nitric acid will then be added and evaporated to dryness. Heating will be continued for 1 h to dehydrate silica. One milliliter of 6 M HCl will be added. The solution will be consequently swirled and will be added also with 10 mL of deionized water. It will be heated again on a

steam bath to complete dissolution. Cool and filter through a Whatman filter paper into a 25 mL volumetric flask and make up to the mark with water. A blank will then be prepared by repeating procedure but omitting the plant sample.

3.3 Phytochelatin Analyses

3.3.1 Protein Extraction prior for HPLC Analysis

- **Materials needed:**

- plastic bag
- blender
- ice bath/ liquid nitrogen
- centrifuge instrument
- chelating agent EDTA (1 M)
- 96% acetic acid
- 60% Perchloric acid
- cryovials

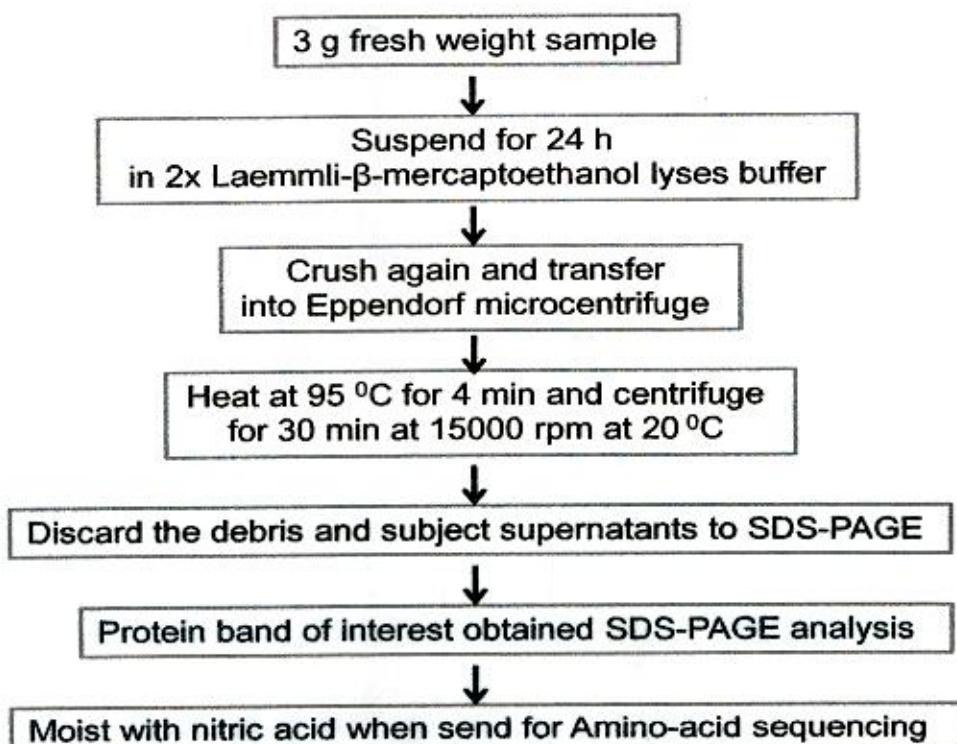
Plant's organ (roots, stems or leaves) of high concentration of lead level as detected by ICP-OES will undergo PC analysis. Plants that are mature and fully developed will be harvested. Each part will be weighed, cut into pieces and ground using a blender. Two grams of fresh weight will

be taken and will be added with 0.8 mL of chelating agent EDTA. Homogenates will then be vortexed for 10 min and added with 0.8 mL of 96% acetic acid. Vortex again for 5 min and add 2.4 mL of 60% perchloric acid. Homogenates will then be transferred to centrifuge tubes and centrifuge at 9000 g for 10 min at 4°C. Supernatants will be transferred in an eppendorf tube and ready for HPLC analysis. A synthetic PC will be purchased for further detection of PCs in the experimental sample and will also be subjected for HPLC analysis.

3.3.2 Protein extraction prior for SDS-PAGE and Amino-acid Sequencing Analyses

- **Materials needed:**

- Stirring rod
- Ice bath
- Laemmli- β -mercaptoethanol lyses buffer
- Eppendorf microcentrifuge tubes
- centrifuge



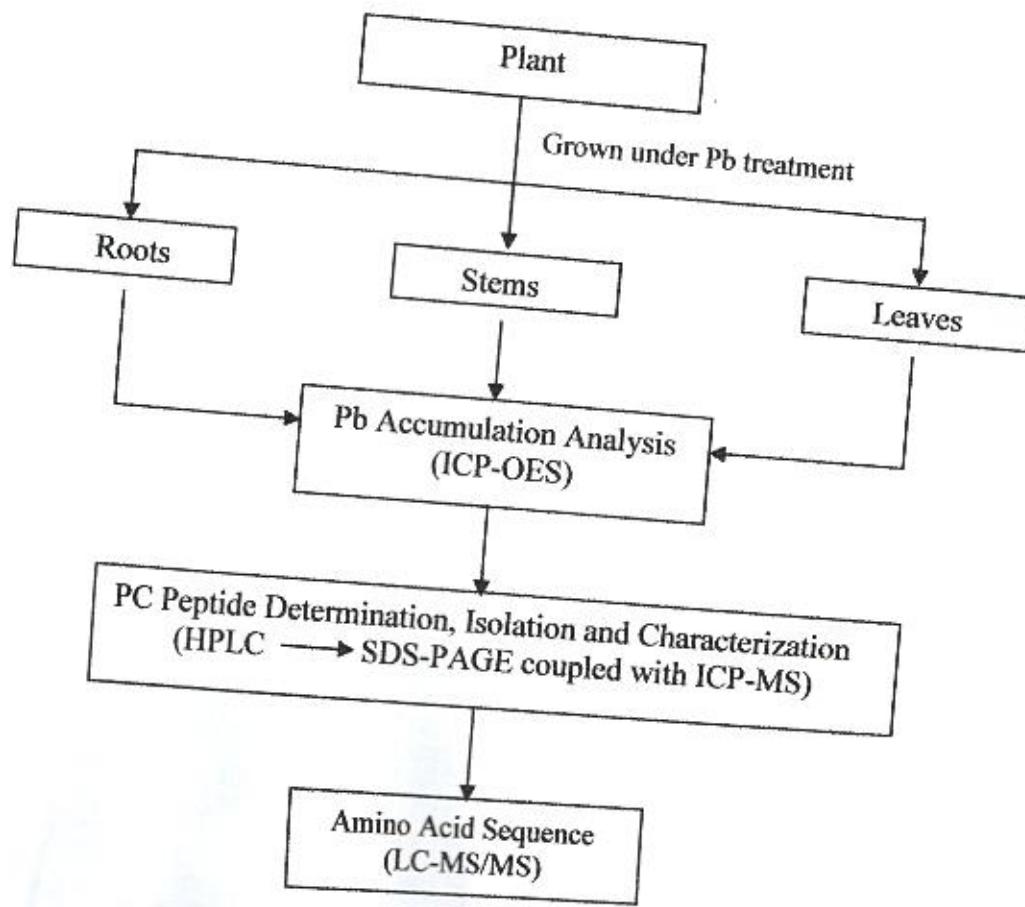


Figure 5 The experimental framework of the study.

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LINE ITEM BUDGET

ITEM	QUANTITY	PRICE
CHEMICALS:		
• Hydrochloric acid; approx. 6 M	4L	1,000
• Nitric acid, concentrated	4L	2,000
• chelating agent EDTA (1 M)	1L	3,000
• 96% acetic acid	4L	800
• 60% Perchloric acid		
• Laemmli-β- mercaptoethanol lyses buffer	1L	1,200
• Lead Nitrate	4L	3,000
• Nutrient Solution	50L	4,000
• Synthetic PCs	10 pcs.	5,000
		20,000
APPARATUS:		
• Hydroponic tank	8 pcs.	1,000
• Eppendorf Tubes & cryovials	30 pcs.	2,000
• Porcelein crucible	30 pcs.	3,000
• Greenhouse	1 unit	5,000
		11,000
ANALYSES FEE:		
• ICP-OES		10,000
• HPLC		5,000
• SDS-PAGE		10,000
• Amino acid Sequencing		15,000
		40,000
Manuscript Expenses/ Documentation:		10,000
TOTAL COST		81,000

TIME TABLE OF THE STUDY

Month	Scheduled Work	Duration
• last week of March	• Seed Propagation	• one week
• April	• Hydroponic cultivation of Plant • Lead treatment	• one mo.
• May	• Harvest • Preparation of sample for ICP-OES • Protein Extraction for HPLC • Protein Extraction for SDS-PAGE and LC-MS/MS Analysis	• one mo.
• June- July	• Waiting for the Results	• two mos.
• August	• Analysis and Interpretation of the Data and Results	• one mo.
• Sept.- Oct.	• Manuscript Writing	• two mos.