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CHARACTERIZATION OF A TYPE II METALLOTHIONEIN
FROM *Helianthus annuus*
USING RECOMBINANT DNA TECHNIQUES

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ABSTRACT

Metallothioneins (MTs) are ubiquitous low molecular weight, cysteine rich proteins with a pronounced affinity for metal ions with d^{10} configuration such as Cu, Cd and Zn. These heavy metals form metal-thiolate clusters with cysteine side chains. In contrast to the vertebrate forms, knowledge about the properties of members of the plant metallothionein family is still scarce. We describe here a method of isolation and purification of metallothionein from the plant *Helianthus annuus*, performed by affinity chromatography using glutathione-agarose column. In this study, alignment of gene sequence of the isolated *H. annuus* metallothionein cDNA with known MTs showed that it belongs to the type 2 of the plant MTs. The gene sequence encoding MTs was cloned into a suitable vector and the protein was overexpressed in *Escherichia coli*. The purified metallothionein was evaluated by SDS-PAGE and characterized by UV spectra of the apo-and metal bound protein. Detection of metal bound protein was also carried out by using chemiluminescence assay. The metal binding ability of metallothionein was evaluated by 5, 5 dithio (2-nitrobenzoic acid) (DTNB) analysis. The affinity of metal ions for metallothionein was in the order of $Cu > Cd > Zn$.

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ABBREVIATIONS

MT – Metallothionein

PC – Phytochelatin

GSH - Glutathione

GST – Glutathione s-transferase

DTNB – 5, 5 dithio (2-nitro benzoic acid)

SDS – Sodium dodecyl sulphate

PAGE – Polyacrylamide gel electrophoresis

BSA – Bovine serum albumin

PBS – Phosphate buffer saline

IPTG – Isopropyl β -D-thiogalactoside

TEMED - (N, N, N', N' - tetramethylethylenediamine)

TBS – Tris buffer saline

(-SH) –Sulfhydryl

CHAPTER I

OBJECTIVES

Certain plants take up higher than normal quantities of toxic metals from the soil (1). They are called hyperaccumulators and *Helianthus annuus* (dwarf sunflower) is one such a plant. So this study was mainly based on the hypothesis that hyperaccumulation of metal ions in *H. annuus* depends largely on the availability of metal binding proteins, membrane transporters and enzymes to uptake, translocate and sequester metals. They are induced when the plants are exposed to toxic metals. The long-term goal of this study is to examine and understand the molecular mechanisms underlying the hyperaccumulation in *H. annuus*. In this project the study was focused on the isolation and cloning of a metallothionein (MT) gene from *H. annuus* followed by purification and characterization of the expressed MT. The specific aims of this project are:

- (i) To isolate and purify MT from the plant *H. annuus*.
- (ii) To evaluate the purified MT by SDS-PAGE and characterization of the protein by UV Spectroscopy.
- iii) To study the metal binding ability of MT by DTNB analysis.
- iv) To detect the MT using chemiluminescence assay.

CHAPTER II

BACKGROUND

2.0. Phytoremediation of toxic metals

Metals, radionuclides and other inorganic contaminants are among the most prevalent forms of environmental contaminants and their remediation in soils, sediments and water is rather a difficult task (2). While environmental pollution by these substances has become the major environmental problem, the most remediation approaches available are costly and complicated. So the technique of using plants is emerging as an innovative approach with great potential for achieving sustainable development (2). This novel technique of using terrestrial plants for environmental remediation is called phytoremediation (21).

This approach has a great potential for application in the remediation of toxic metals in the environment since is a low-cost effective and environmentally friendly. A phytoremediation system capitalizes on the synergistic relationships among plants, microorganisms, water, and soil that have evolved in nature over many years. Various biological processes for instance, participate in phytoremediation, which include plant-microbe interactions and other rhizosphere processes, plant uptake, translocation and tolerance in plant (compartmentation, degradation) and the chelators involved in storage and transport (4).

Phytoremediation includes (i) phytoextraction in which metal accumulating plants are used to transport and concentrate metals from the soil into the harvestable roots and above-ground shoots, (ii) rhizofiltration in which roots absorb, precipitate and concentrate toxic metals from polluted effluents and (iii) phytostabilization in which heavy metal-tolerant plants are used to reduce the mobility of heavy metals, thereby reducing the risk of further environmental degradation by leaching into the ground water or by airborne spread (3).

2.0.1. Toxicity of heavy metals

Plants possess some characteristic features which enable them to absorb heavy metals from soil and water that are essential for their growth and development (3). These metals include iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo) and nickel (Ni). Plants also accumulate toxic metals with no biological function, such as silver (Ag), cadmium (Cd), chromium (Cr), cobalt (Co), mercury (Hg), lead (Pb), selenium (Se). However, excessive accumulation of these metals can be toxic to plants (22). They inhibit root and shoot growth by affecting nutrient uptake and homeostasis. Heavy metals also interact with membrane components altering its permeability, potential and enzymatic activity (23). Based on the chemical and physical properties of heavy metals, three different molecular mechanisms of heavy metal toxicity can be distinguished: (i) production of reactive oxygen species by autoxidation and Fenton reaction; these reactions are typical for transition metals such as Fe or Cu, which upon oxidation give rise to free radicals that cause damage to the cell (6); (ii) blocking of essential functional groups in biomolecules, which is typical for non-redox reactive heavy metals such as Cd,

Hg (6) and (iii) displacement of essential metal ions from pigments or enzymes, disrupting the function of these biomolecules (6). Some transition metals initiate hydroxyl radical production, which can not be controlled by antioxidants. Exposure of plants to non-redox reactive metals also results in lipid peroxidation, H_2O_2 accumulation and an oxidative burst. Cadmium and some other metals cause a transient depletion of glutathione (GSH) and inhibition of antioxidative enzymes, especially of glutathione reductase. So, cadmium when not removed rapidly may trigger, the disturbance of the redox control of the cell, a sequence of reactions leading to growth inhibition, lignification and finally cell death (6).

2.0.2. Defense mechanisms against toxic metals

The sensitivity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms such as (i) uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents; (ii) efflux of heavy metals from cytoplasm to extraplasmatic compartments including vacuoles; (iii) complexation of heavy metal ions inside the cell by various substances, for example organic acids, amino acids, ferritins, phytochelatins, and metallothioneins. (iv) biochemical stress defense responses such as the induction of oxidative enzymes (10). Other types of metal-triggered defense mechanisms include accumulation of pathogenesis-related proteins (PRs), activity of heat shock proteins and formation of reactive oxygen species during biotic stress (24). The potential chelators may also be involved in the process of detoxification and tolerance of toxic metals; include amino acids, organic acids, phytosiderophores and nicotianamine (4).

2.1. Binding and sequestration of metal ions

Plant properties important for metal accumulation are the metal tolerance and accumulation, which are determined by metal uptake, root-shoot translocation, intracellular sequestration, chemical modification and general stress resistance (4). The pathway of transport and translocation of metal ions taken up by plants across the root cell membrane is symplast → xylem apoplast → shoot apoplast and involves specific membrane transporter proteins and organic acid chelators. Once inside the shoot cells, these metal ions are translocated to a final destination where they can do the least harm to essential cellular processes through a process that involves membrane metal transporters, sequestration by chelators or formation of conjugates (4). Of these chelation of metals by high-affinity ligands is potentially a very important mechanism of heavy metal detoxification and tolerance (26). Detoxification of inorganic pollutants (metals) in plants generally involves conjugation followed by active sequestration of toxic metal into the vacuole and apoplast.

The potential ligands mainly involved in this process of detoxification through sequestration include: phytosiderophores facilitate uptake of iron and other metals in grass. These are biosynthesized from nicotianamine, which is composed of three methionines coupled via nonpeptide bonds (18). Nicotianamine also chelates metals and may facilitate their transport (19). Organic acids (e.g. citrate, malate, histidine) not only can facilitate uptake of metals into roots but also play a role in transport, sequestration and tolerance of metals (21). Metals are also bound by the thiol-rich peptides glutathione (GSH) and phytochelatins (PCs), or by the cysteine rich metallothioneins (7). After chelation of metal ions by GSH or PCs, an ABC-type transporter actively transports the

metal-chelate complex to the vacuole, where it is further complexed by sulfide (4). On the other hand, detoxification of organic pollutants typically comprises three phases: chemical modification, conjugation and finally sequestration. Conjugation to GSH also plays a role in sequestration and tolerance of organic pollutants. The glutathione-S-conjugates are actively transported to the vacuole or the apoplast by ATP-dependent membrane pumps (4).

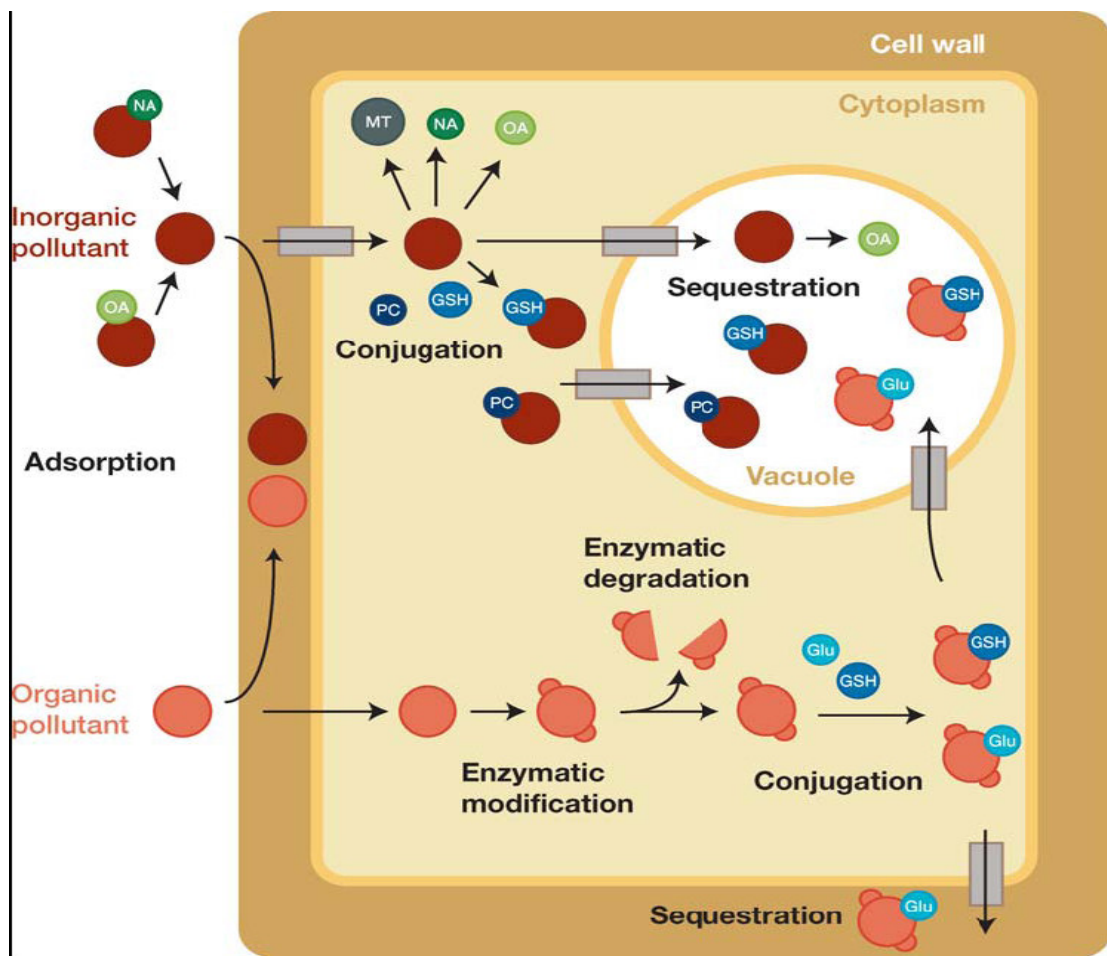


Figure 1. A model showing the mode of binding and sequestration of organic pollutants and inorganic pollutants (metal ions) by various chelating agents namely, GSH: glutathione, Glu: glucose, MT: metallothioneins, NA: nicotianamine, OA: organic acids, PC: phytochelatins.

2.2. Protein induction upon heavy metal stress

Certain types of plants take up higher than normal quantities of toxic metals (e.g. Pb, Cd, and Cu) from the soil (1). This is called hyperaccumulation. These plants therefore evoke different defense mechanisms to resist the cytotoxic burden of the accumulated metals and scavenging mechanism for the efficient uptake of these potentially toxic elements from the soil (10). These metals are first taken up by the root system and are subsequently distributed to stems and leaves. At any point along the pathway of transporting these metals, they could be converted to a less toxic form through chemical conversion or by complexation (3). For instance, one way to reduce toxic properties of metals would be to induce certain types of proteins that would affect the metal uptake processes or sequester them (26). Some of the strategies developed by the plants to accumulate metals may include the synthesis or induction of metal binding proteins. Thiol-rich peptides glutathione (GSH), phytochelatins (PCs) and metallothioneins have been studied extensively (4). These peptide chelators use thiol groups as ligands, so the sulfur biosynthetic pathways are critical for sequestering heavy metals (1). These contribute to metal detoxification by buffering cytosolic metal ions. Another group of proteins that are not directly related to metal binding are heat shock proteins (HSPs); they were shown to be induced in plants in response to heavy metal stress (26).

2.3. Metal binding proteins

The proteins described below are believed to be involved in binding toxic metals in plants. They are phytochelatins, metallothioneins and metallothioneins.

2.3.1. Phytochelatins

Phytochelatins (PCs) are a group of small cysteine rich metal binding peptides present in both plants and fungi (8). They are induced in plants when the plants encounter heavy metal stress (27). The structure of phytochelatins consists mainly of three types of amino acids namely cysteine (Cys), glycine (Gly) and glutamic acid (Glu). These are arranged generally in a conformation $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where $n = 2$ to 8. They are structurally related to glutathione [GSH: $(\gamma\text{-Glu-Cys-Gly})$]. Phytochelatins are not gene-encoded, but enzymatically synthesized from glutathione (7). They are synthesized from glutathione by the enzyme phytochelatin synthase, which is strongly activated by metal ions such as Cd, Pd and Hg (9). The catalytic mechanism of action and the function of phytochelatin synthase in the biosynthesis of phytochelatins is as follows: the C-terminal domain of the enzyme acts as a local sensor of heavy metal ions, such as Cd. The cysteine residues bind Cd ions, bringing them into closer proximity and transferring them to the activation site in the N-terminal, catalytic domain. The activated N-terminal domain catalyzes the transfer of the $\gamma\text{-Glu-Cys}$ moiety of a molecule of GSH onto a second molecule of GSH or an existing PC_n molecule to form a PC_{n+1} product (27). Phytochelatins are thought to be involved in the accumulation, detoxification and metabolism of heavy metal ions such as cadmium, lead, copper and mercury in plant cells (7). They act by binding to metal ions and transport the metal ions to vacuoles for storage, where metals are less accessible to cells (27).

2.3.2. Metallothioneins

These are a group of low molecular weight cysteine rich intracellular metal binding proteins found in plants, animals and eukaryotes (11). In contrast to the vertebrate forms, knowledge about the properties of members of the plant metallothionein family is still scarce (29). In general the molecular mass of metallothioneins range from 3500-14000 Daltons. They are gene encoded unlike PCs that are enzymatically synthesized (7).

Metallothioneins (MT) are classified based on the arrangement of cysteine residues. Class I MTs contain 20 highly conserved cysteine residues and are wide spread in vertebrates. Those without this strict arrangement of cysteines are referred to as class II MTs and include all those from plants, fungi and non vertebrate animals. The class II proteins in the plants can be further classified based on amino acid sequence as type I and type II MTs. Type I MTs contain a total of 6 Cys-Xaa-6Cys motifs (Xaa is another amino acid) that are distributed equally among two domains that are separated by 40 amino acids that include aromatic residues. This is a common feature of plant MTs. Type II MTs have either a configuration of 6Cys-6Cys or 6Cys-Xaa-Xaa-6 Cys. The biosynthesis of metallothioneins is regulated at the transcriptional level and is induced by several factors such as hormones, cytotoxic agents and metals (8). Some of the functions of MTs include heavy metal detoxification, homeostasis and providing defense against oxidative stress.

Apo-metallothioneins have a non-rigid three dimensional structure with two different cysteine clusters, one at the N-terminal (β -domain) and the other at the C-terminal (α -domain). Each cluster is able to bind four and three metal ions, respectively (16). These two metal binding domains give them a dumbbell conformation (28). The

randomly coiled polypeptide form of MT adopts its tertiary structure or holoprotein form upon the chelation of metal ions (32). The chelation of metal ions is mediated through the cysteine residues which are highly conserved between species and they are arranged to present their sulphur atoms to the metal ions in a way that meets the coordination geometry of the ions without placing a strain on the protein (28). Thus the large number of cysteine residues in MT bind a variety of metals by mercaptide bonds to form stable metal thiolate clusters with some of the toxic metals like Cd, Zn, Cu and Hg (32). MTs in animals as well as plants are not only involved in homeostasis of essential metals and metal detoxification, but are also implicated in a range of physiological processes, including scavenging reactive oxidant species, regulating cell growth, proliferation and response to stress conditions (33).

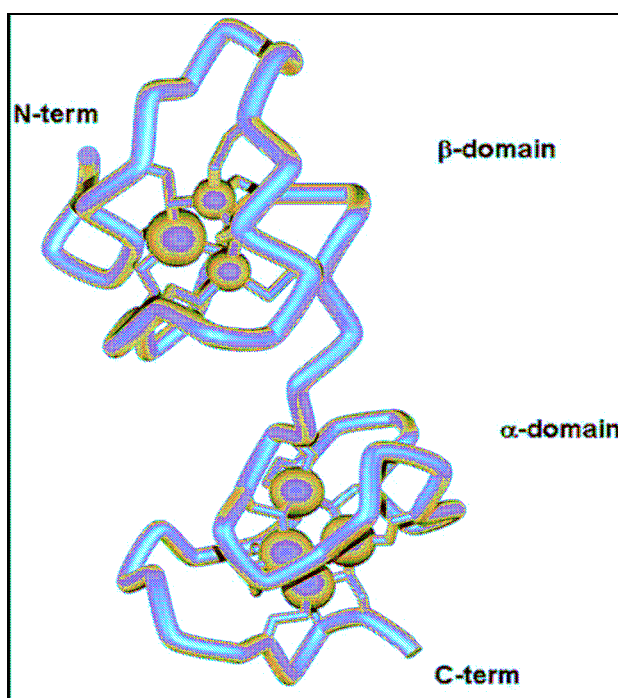


Figure.2. Structure of type 2 metallothionein showing the two metal binding cysteine rich domains, β -domain (N-terminal) and α -domain (C-terminal).

2.3.3. Metallothioneins

Two small multimeric histidine-rich proteins, AgNt84 and Ag164, encoded by two nodule-specific cDNAs isolated from nodule cDNA libraries of the actinorhizal host plant *Alnus glutinosa*, represent a new class of plant metal binding proteins (15). AgNt84 and Ag164 are not related to the metal binding metallothioneins or phytochelatins. They represent a new class of plant metal binding proteins. They were also found to play a biological role in symbiosis and bioremediation (15).

2.4. Proteomics

Metallothioneins are difficult to identify in plants and it was necessary to employ genomic techniques to produce milligram amount of MTs.

2.4.1. Fusion proteins

Fusion proteins, also known as chimeric proteins, are proteins created through the joining of two or more genes which originally coded for separate proteins. Translation of this fusion gene results in a single polypeptide with function properties derived from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology for use in biological research or therapeutics. This is achieved by removing the stop codon from the DNA sequence of the first protein and then appending the DNA sequence of the second protein in frame. This DNA sequence will be then expressed by cells as a single protein. This technique is often used for identification and purification of proteins, by using a GST protein or a hexa histidine peptide (6x histidine tag) which can be isolated by using affinity chromatography.

2.4.2. Glutathione S-transferase (GST) gene fusion system

The glutathione S-transferase gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in bacterial, yeast, mammalian and insect cells. The sequence encoding the GST protein is incorporated into an expression vector such as pGEX-4T-1. The sequence encoding the protein of interest is then cloned into this vector. Induction of the vector results in expression of a fusion protein - the protein of interest fused to the GST protein. The fusion protein can then be released from the cells and purified.

Purification of the fusion protein is facilitated by the affinity of the GST protein for glutathione residues. Glutathione residues are coupled to an agarose resin and the expressed protein product is brought into contact with the resin. The fusion protein binds to the glutathione-resin complex and all other non-specific proteins are washed off. The fusion protein can then be released from the resin using glutathione elution buffer at low pH. The fusion protein having the GST can be separated from the protein of interest by using a number of different enzymes (thrombin, factor X), which cleave specific sites between the GST and the protein of interest.

CHAPTER III

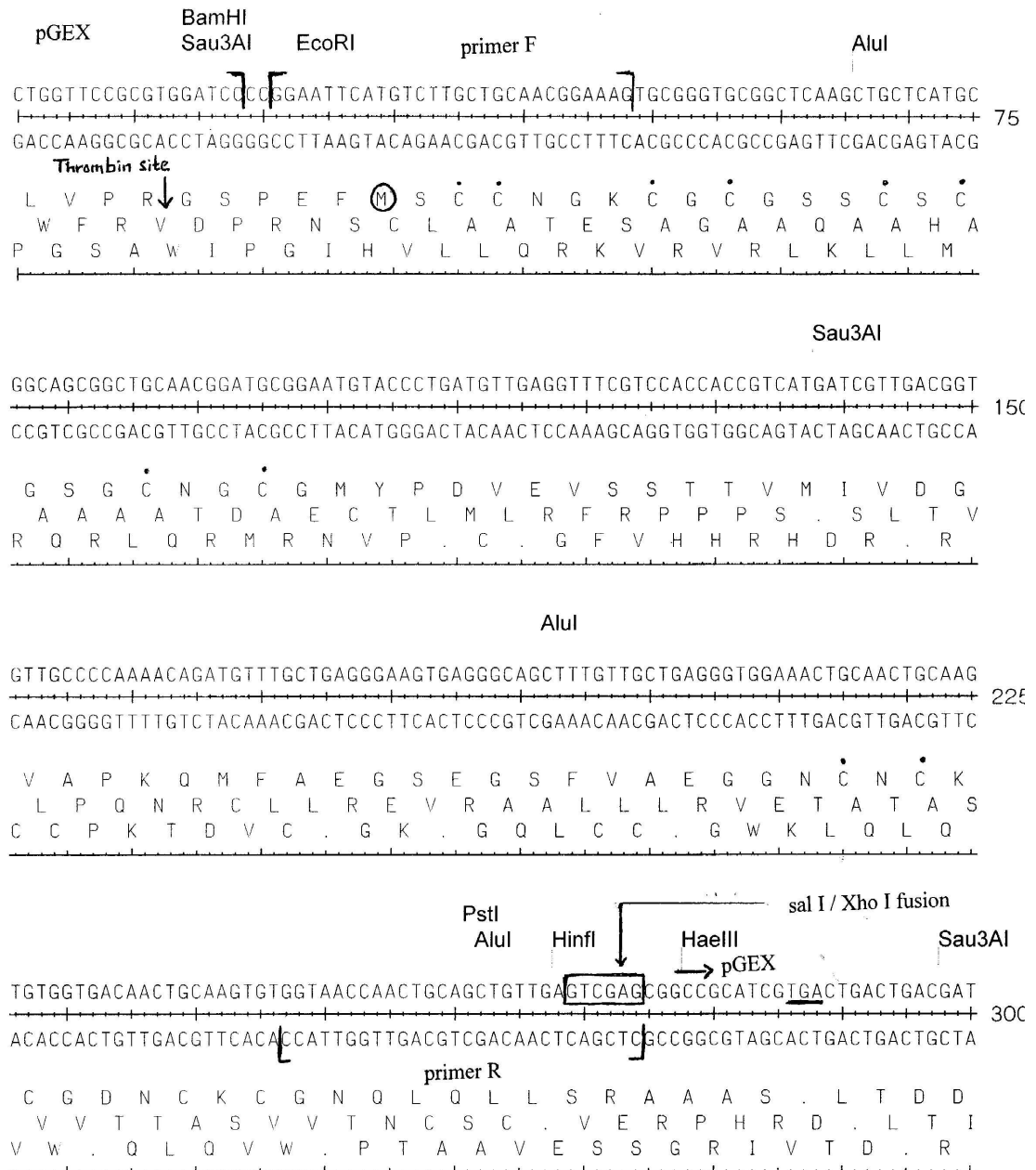
EXPERIMENTAL METHODS

3.1. Isolation and cloning of the Metallothionein Gene

The following experimental methods described below were based on the methodology described in the literature (14). Metallothionein (MT) used in these experiments was the translated product of specific mRNA. The expression of MT genes can be detected primarily through the examination of steady state levels of MT RNA. To overcome certain drawbacks associated with the direct isolation of proteins from plant material such as plant growth, low yields and proteolytic degradation, isolation of the protein was done by cloning the plant MT gene into suitable vectors for recombinant protein expression in *E.coli*. This provided highly pure MT in sufficient yield for the present investigations.

MT cDNA used in this project was obtained from the plant *Helianthus annuus*. Alignment of gene sequence of the isolated *Helianthus annuus* MT cDNA (clone 4) with known MTs showed that it belongs to the type 2 of the plant MTs (Fig. 3). In this experiment the expression and purification of Glutathione-S-Transferase (GST) fusion proteins was performed following the protocol shown in Figure 3. The gene sequence encoding the MT cDNA of *H. annuus* was cloned in frame with the Glutathione-S-transferase (GST) gene of the expression vector pGEX-4T-1. The recombinant plasmid

Figure 3. Clone 4 metallothionein cDNA in pGEX-4T-1



was then used to produce a fusion protein, where in N -terminus is GST and C-terminus is the plant MT (13). Then *E.coli* was transformed with the recombinant plasmid DNA containing the MT cDNA to produce pGST-MT cells as described.

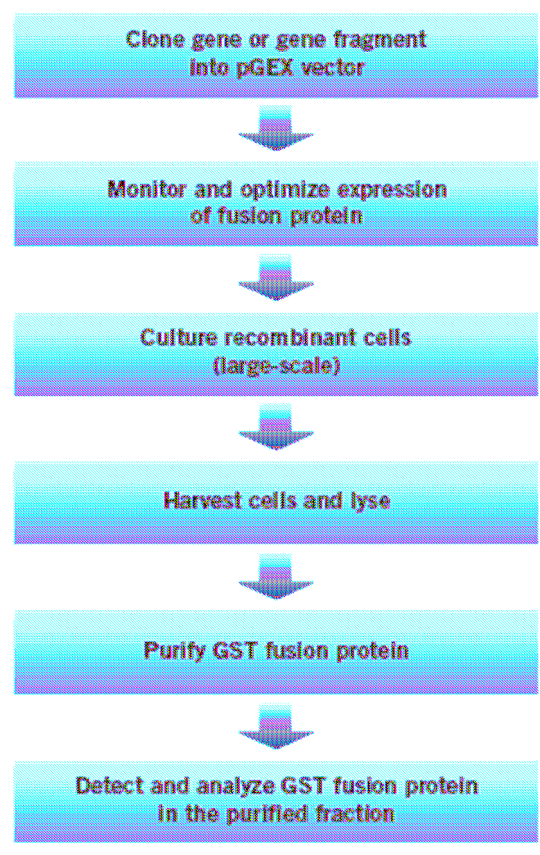


Figure 4. A typical protocol for expression and purification of GST fusion proteins.

3.2. Growth of *E.coli* cells and expression of the recombinant fusion protein (GST-MT)

Transformed *E.coli* cells were grown in Luria-Bertani (LB) broth containing 100µg/mL ampicillin at 37 °C overnight. In the following day the overnight culture was diluted to 100 mL using fresh LB broth containing ampicillin. Incubation continued under similar

conditions for one hour. Isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM to induce protein synthesis. Following incubation for 5-6 h at 37 °C, cells were pelleted by centrifugation at 5000 rpm for 10 min.

3.3. Purification of the expressed fusion protein

The pelleted cells were resuspended in 500 -1000 μ L of Bacterial Protein Extraction reagent (B-PER) to lyse the cells. Cell lysis was further aided by the addition of 10 μ L of lysozyme solution, 6 μ L of 1M $MgCl_2$ followed by the addition of 10 μ L of endonuclease enzyme. The suspension was then transferred into a micro centrifuge tube and was gently mixed at room temperature to facilitate solubilization of proteins.

The suspension was centrifuged at 14000 rpm for 10 min and the supernatant was collected into another centrifuge tube. The supernatant containing the recombinant fusion protein (GST-MT) was purified by affinity chromatography using a Glutathione-Agarose column (Pierce) that was pre-equilibrated with 1X phosphate buffered saline (PBS). The supernatant was passed through the column 4-5 times to make sure all the fusion protein (GST-MT) was bound to the column. This was followed by 3-4 column volumes of PBS buffer until all other non-specifically bound proteins were eluted off the column. This was further confirmed by checking with coomassie blue based protein assay reagent whose color turns from pink to purple in the presence of protein. The fusion protein (GST-MT) bound to the column was eluted with 50 mM Tris-Glutathione elution buffer. Then the first ten fractions, each of 400-500 μ L were collected in centrifuge tubes and checked for the presence of fusion protein using coomassie blue based protein assay reagent. The fractions containing the fusion protein were pooled and the purity of these

pooled fractions was further analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). From this analysis, fractions containing the fusion protein were pooled and dialyzed overnight at 4°C in 2 L of Tris buffer saline (TBS) to remove glutathione.

3.4. Thrombin cleavage of the GST-MT

Thrombin is an endolytic serine protease that selectively cleaves any fusion protein containing a thrombin cleavage site. Cleavage of fusion protein can be done while it is bound to the column or in solution after elution from the column. On-column cleavage is generally recommended since many potential contaminants can be washed out and the target protein eluted with a higher level of purity. Off-column cleavage is suggested if optimization of cleavage conditions is necessary. In this experiment cleavage of the fusion protein was carried out off the column to optimize the cleavage conditions. The optimal pH range for cleavage of fusion protein was between 8 and 9. The cleavage of 1 mg of fusion protein in a final volume of 1 mL was carried out using thrombin agarose resin as described. The dialyzed samples containing the fusion protein (GST-MT) were subjected to digestion with thrombin to cleave the GST-MT protein into GST and MT. Before digestion, 100 µL of thrombin agarose resin was thoroughly resuspended into homogenous slurry using 500 µL of thrombin resuspending buffer. Then it was centrifuged at 2500 rpm and the supernatant was removed. This step was repeated 2-3 times to thoroughly wash the resin. After these washing steps, 100 µL of the 10X thrombin resuspension buffer was added to the centrifuged resin to resuspend it. The fusion protein was then added to the resuspension containing the thrombin agarose resin.

This resuspension was incubated overnight at room temperature with gentle agitation to keep the resin resuspended. In the following day the suspension was gently centrifuged for 5 min at 2500 rpm to remove the resin. The supernatant collected was then passed through glutathione - agarose column where GST binds to the column and the flow through collected contains only the metallothionein (MT). Once again the column was washed with PBS buffer to make sure the entire MT was eluted. Cleavage of fusion protein was also carried out by directly using thrombin solution. One unit of thrombin can cleave more than 90 % of the 0.1 mg of fusion protein.

3.5. Protein concentration determination using Bio-Rad protein assay

In this assay, concentrations of the purified GST-MT and MT were determined using Bio-Rad protein assay. The Bio-Rad Protein Assay is a dye binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. This dye binds to primarily basic and aromatic amino acid residues, especially arginine. Thus by applying Beer's law, protein quantitation can be calculated by using an appropriate ratio of dye volume to sample concentration.

A standard procedure of determining protein concentration using the Bio-Rad assay kit was as follows: dye reagent of 50 mL was prepared by diluting 10 mL of dye reagent with 40 mL of deionized water. Several dilutions of a protein standard, bovine serum albumin (BSA) were prepared. The concentrations of these dilutions were in the range 0.2 to 0.9 mg/mL. Then 100 μ L each standard and sample solution were pipetted

into a clean dry test tube. Protein solutions were normally assayed in duplicate. Five mL of diluted dye reagent was added to the protein solutions and mixed well. These mixtures were incubated at room temperature for 5-60 min and the absorbance was measured at 595 nm. A calibration curve was constructed by plotting the absorbance at 595 nm against protein concentration of the standards and protein concentration of the sample was determined.

3.6. Analysis of purified protein by SDS-PAGE

The purpose of SDS-PAGE is to separate proteins according to their size. The other function includes estimation of sample purity. The main principle involved in this method is that sodium dodecyl sulphate (SDS) is an anionic detergent, which when dissolved confers a net negative charge on the protein over a wide pH range. A polypeptide chain binds SDS in proportion to its relative molecular mass. The negative charges on SDS perturb the conformation of proteins thus allowing the protein to interact with detergent, with the generation of a net negative charge. Thus all proteins are attracted towards a positively charged anode in an electric field. The polyacrylamide gel used is a cross-linked matrix that functions as a sort of sieve to hold the molecules as they are transported by the electric current. In this experiment an SDS/PAGE gel was run to analyze whether the pooled fractions contain the fusion protein and also to see if the eluted sample after cleavage contains the metallothionein. Before this process, polyacrylamide gel needed for the experiment was made as described below.

3.6.1. Materials

The materials needed for the preparation of gel include acryl/bis acryl solution (50 % acrylamide and 0.235 % bisacrylamide) (Bio-Rad), 1 M Tris (pH 8.6), 1 M Tris (pH 6.8), 10 % SDS, freshly prepared 10 % ammonium persulfate, distilled H₂O, TEMED (N, N, N', N'-tetramethylethylenediamine) and isobutanol. A stock solution of Tris-glycine running buffer (4X) was prepared by mixing Tris (0.025 M), glycine (0.192 M) and SDS (0.1 %) and pH was adjusted to 8.3. Working solution (1X) of 800 mL was prepared by diluting the stock solution. Sample buffer/SDS reducing buffer was prepared by mixing 0.0625 M Tris-HCl (pH 6.8), 2 % SDS, 5 % β-mercaptoethanol and 0.001 % pyronin Y.

3.6.2. Gel preparation

Electrophoresis gels required for SDS/PAGE were made up of separating gel and stacking gel. The separating gel was first made on which then a stacking gel preparation was poured and polymerized.

3.6.3. Separating gel

After assembling the gel plates, separating part of the gel was prepared by mixing 11.2 mL of 1 M Tris (pH 8.6), 9.6 mL of polyacrylamide (50 % acrylamide and 0.235 % bisacrylamide), 0.3 mL 10 % SDS and 8.7 mL of milliQ water in a 50 mL beaker. Before pouring the above mixture in between the gel plates, 0.35 mL of 10 % ammonium persulfate and 35 µl of TEMED were added to the beaker. This solution was mixed uniformly and quickly poured into the gel assembly up to three quarters and was overlaid with isobutanol to get rid off any air bubbles present on the surface of the gel. Then it was allowed to polymerize for at least 30 min.

3.6.4. Stacking gel

After polymerization of separating gel, overlaid isobutanol layer was poured off and then washed with milliQ water to completely remove isobutanol. Stacking part of the gel was prepared by thoroughly mixing 0.9 mL of 1 M Tris (pH 6.8), 0.75 mL polyacrylamide (50 % acrylamide and 1.33 % bisacrylamide), 80 μ l of 10 % SDS and 5.6 mL of milliQ water. To the above mixture, 75 μ l of 10 % ammonium persulfate and 7.5 μ l of TEMED were added, thoroughly mixed and poured above the separating part of the gel. Then the comb with 10 wells was placed properly without introducing air bubbles and the gel was allowed to polymerize for at least 45 min.

3.6.5. Gel electrophoresis

Once the gel was polymerized, comb was removed from gel without disturbing the wells and the gel with the 10 well slots was placed into the gel chamber. Tris-glycine running buffer was prepared from 4X stock buffer by dilution. The gel chamber was filled with running buffer. The purified fusion protein fractions and the metallothionein samples were prepared for gel electrophoresis by mixing with a sample buffer /SDS reducing buffer. Fifteen μ L of each protein sample was transferred to a centrifuge tube and 5 μ L of the 4X sample buffer was added to it and mixed well. These samples were then heated at 95 °C for 5 min. The heated samples of 20 μ L each were then loaded in the order after loading protein marker in the first slot. After the samples were loaded, covering lid was placed properly on the gel chamber by connecting corresponding electrodes (black to black and red to red). Other ends of electrodes were connected to a voltage supply unit. An initial voltage of 50 V was applied until all samples were stacked in the stacking part

of the gel and after this the voltage was increased to as high as 150 V to 200 V. The gel was allowed to run for sufficient time until all the samples and the marker reach the bottom. Then the electrophoresis apparatus was disconnected from the voltage source. The gel was taken out carefully and the stacking part of the gel was removed. Then the gel was placed carefully in a tray and to have better staining of the protein bands, the protein bands on the gel were initially fixed with 50 % methanol and 10 % acetic acid for 30 min. The gel was washed with water for an hour and then it was subjected to different staining methods.

3.7. Staining Methods

3.7.1. Coomassie blue staining

After the protein bands on the gel were fixed, the gel was then stained with Coomassie blue dye for an hour. This was followed by destaining the gel with 28 % methanol and 9 % acetic acid overnight.

3.7.2. Silver staining

Silver staining method is much more sensitive than the coomassie blue staining technique, which is generally weak for small proteins with low abundance of hydrophobic and aromatic residues such as MTs. Consequently, this is the method of choice when low molecular weight proteins like metallothioneins and very low amounts of protein have to be detected on electrophoresis gels.

3.8. Tricine-SDS-PAGE

In a regular SDS/PAGE system, proteins with in the molecular mass range of 20-100 kDa can be separated using Tris-Glycine as running buffer. But using the same system to separate proteins like MT whose molecular mass is less than 20 kDa can be partly achieved or not at all. So a new system namely Tricine-SDS-PAGE was tried to analyze proteins with molecular mass less than 20 kDa. The principle involved in this is as same as the previously discussed SDS/PAGE except that it differs in the composition of separating gel and the running buffer.

3.8.1. Gel Preparation

The materials needed for preparation of gel are acryl/Bis acryl solution (50 % acrylamide and 0.235 % bisacrylamide), 2 M Tris (pH 8.45), 0.1 M Tricine, 10 % SDS, milliQ water, 10 % ammonium persulfate, TEMED. Cathode buffer (1X) was prepared by mixing 0.1 M Tris, 0.1 M Tricine and 0.1 % SDS and the pH was adjusted to 8.25. Anode buffer was prepared by making 0.2 M Tris and the pH was adjusted to 8.9.

Electrophoresis gels required for Tricine-SDS-PAGE were made up of separating gel and stacking gel. Separating gel was prepared by mixing 9.6 mL of polyacrylamide (50 % acrylamide and 0.235 % bis acrylamide), 15 mL of 2 M Tris HCl (pH 8.45), 0.3 mL of 10 % SDS, 5.1 mL of MilliQ water, 0.3 mL of 10 % ammonium persulfate and 35 μ L of TEMED. Ammonium persulfate and TEMED were then added at the end and the solution was mixed properly. This was applied in between the gel plates and overlaid with isobutanol. After the separating gel was polymerized, stacking part of the gel prepared as discussed in SDS-PAGE was applied above the separating part of the gel and was allowed to polymerize for at least 45 min.

3.8.2. Gel Electrophoresis

When the gel is polymerized, comb was carefully removed from the gel without disturbing the wells and the gel was assembled in the electrophoresis apparatus as discussed in the SDS-PAGE. Cathode buffer was placed in cathode chamber and anode buffer was put in the anode chamber. Samples were loaded into the gel slots and the gel chamber was covered with lid. Initially 50 V of voltage applied until all the samples were stacked and after it was increased to 150-200 V. The gel was allowed to run for sufficient time until all the samples and the marker reach the bottom. Then the electrophoresis apparatus was disconnected from the voltage source. The gel was taken out carefully and the stacking part of the gel was removed. Then the gel was placed carefully in a tray and to have better staining of the protein bands, the protein bands on the gel were initially fixed with 50 % methanol and 10 % acetic acid for 30 min. The gel was washed with water for an hour and then it was subjected to different staining methods such as coomassie blue staining and silver staining methods as previously discussed.

3.9. Spectroscopic Characterization of the protein

Metallothioneins are the proteins with low abundance of aromatic amino acids and histidine (17). Because of this unusual amino acid composition, it is not detected by standard spectrophotometric assays (30). The binding of group-2B metal ions to the apoprotein intensifies the far UV absorption and introduces characteristic absorption shoulders whose positions differ as a function of the metal (31). These metal ions are bound to the proteins through mercaptide bonds forming thiolate complexes. The position and intensity of these so called ligand-to-metal charge transfer bands is indicative for the

sort of metal ions bound (17). Therefore, UV-Visible spectroscopy is the method of choice for the initial characterization of a new plant MT. As described above, in this experiment metallothionein isolated from the plant *H. annuus* was characterized by studying the UV spectra of the apoprotein and of the metal-protein complex at 260nm, following the methodology described in the literature (10).

3.10. Reaction of the protein with Ellman's reagent

DTNB [5,5 dithio (2-nitrobenzoic acid)] is commonly called Ellman's reagent and is a symmetrical aryl disulfide which readily undergoes the thiol-disulfide interchange reaction in the presence of thiol rich peptides like the metallothionein (35).

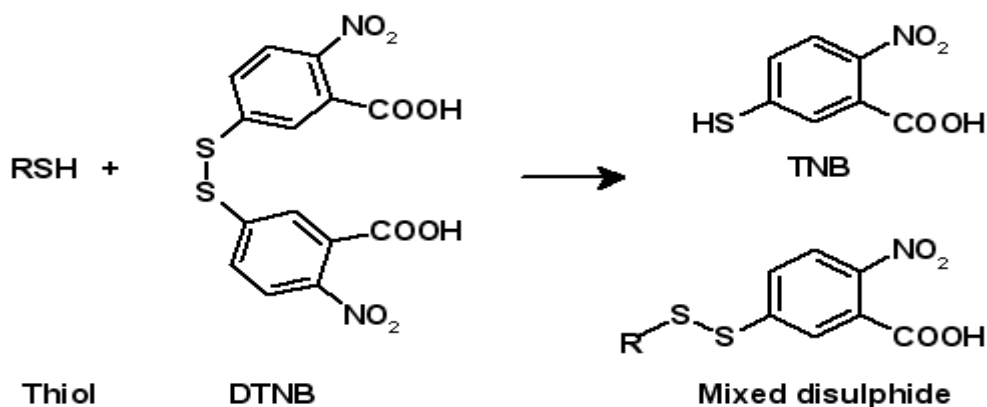


Figure 5. Generalized reaction of DTNB with thiol compounds.

DTNB is one of the favorite reagents for the spectrophotometric measurement of protein sulfhydryls or the thiol group rich peptides like the metallothionein. The main principle involved in this process is that the colorless DTNB on reaction with the thiol group is converted into a yellow colored compound namely 2-nitro-5-thiobenzoate (TNB)

which has maximum absorbance at 412 nm. The competitive reaction with DTNB is used to assess the accessibility of DTNB to protein sulfhydryls, which relates to protein structure and metal binding affinity. In this experiment the competitive reaction of apo-metallothionein (apo-MT) and different metal bound MTs with DTNB was studied by measuring the change in absorbance at 412 nm during various time intervals. DTNB is also used for the determination of total thiol (-SH) content in the protein samples such as metallothioneins. The free thiol content in a peptide was calculated using the formula,

$$\frac{(\text{Total volume} / \text{Protein sample volume}) \times \text{Absorbance at 412 nm}}{13600}$$

13600 = Extinction coefficient of DTNB

3.11. Detection of metalloproteins using chemiluminescence assay

Metalloproteins such as metallothioneins bind transition metals and identification of the metal cofactor in a protein can greatly facilitate its functional assignment. Existing methods for the detection of metalloproteins such as atomic absorption spectrometry consume large amounts of protein, require expensive equipment and are not much sensitive (34). The method described here is a chemiluminescence assay that is sensitive, consumes minimal amounts of protein, is inexpensive and is fast and adaptable to a large number of samples. This method was mainly used for the detection of proteins such as metallothioneins that contain the most common transition metals such as Cu, Ni, and Zn (34).

Chemiluminescence assay was used for the detection of metal bound metallothionein based on the fact that luminol, in the presence of certain catalysts will produce chemiluminescence when mixed with a base and an oxidant (Fig. 5). Transition metals such as Fe, Cu, Mn, Co, Ni and Zn mainly act as catalysts for this reaction. In this experiment, protein samples to be analyzed by chemiluminescence assay were prepared by mixing 50 μL of apo-MT with different concentrations of metals such as Cu, Cd and Zn. To these solutions, 50 μL of luminol solution (11 mM luminol + 500 mM Na_2CO_3 + 230 mM H_2O_2) was added and the final volume was made 1000 μL using phosphate buffer (pH: 7.2). The change in intensity of light emitted was measured for the apo-MT and metal bound MTs with a luminometer (Turner Biosystems). The intensity of light emitted was measured as arbitrary emission units at different time intervals. A graph was constructed by plotting the arbitrary emission units against the time in minutes.

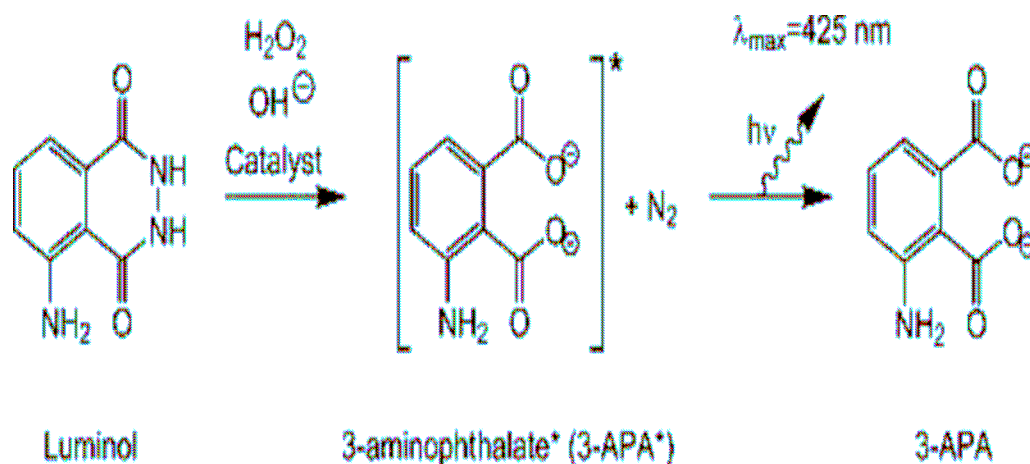


Figure 6. Light producing luminol reaction. Certain transition metals can act as the catalyst for this reaction.

CHAPTER IV

RESULTS AND DISCUSSION

4.0.1. Analysis of fusion protein fractions by SDS- PAGE

As discussed in the section 3.3, the supernatant collected after cell lysis and centrifugation usually with a volume of 2 mL containing the fusion protein glutathione-s-transferase-metallothionein (GST-MT) was applied to a glutathione-agarose column. The supernatant was passed through the column repeatedly to make sure all the fusion protein was bound to the column. The fusion protein bound to the column was eluted with 50 mM Tris-Glutathione elution buffer and ten fractions, each of volume 500 μ L were collected in centrifuge tubes. Out of the ten fractions collected, fractions from 2 to 8 were subjected to SDS-PAGE analysis using Tris-glycine as the running buffer to check the presence of fusion protein and purity of the samples. The gel was stained with Coomassie Blue, destained overnight and a picture of the gel was taken to analyze the protein bands.

Lanes 3 and 4 contain the fractions 3 and 4 show two distinct bands (figure 6). Out of these bands, one of them appears in the mass range of about 35 kDa, which is likely the fusion protein (GST-MT) band. Another distinct band appears with in a molecular mass range of about 26 kDa and is the GST protein. The fractions other than 3 and 4 did not show any GST-MT band, which shows that these fractions do not have any fusion protein. So from this analysis fractions 3 and 4 containing the fusion protein were pooled

and they were subjected to overnight cleavage with 10 μ L of thrombin at room temperature. In the following day these fractions containing the cleaved GST-MT were passed through glutathione-agarose column so that only GST would bind to the column and the fractions containing MT would not be retained. The eluant from this column treatment was further analyzed by Tricine SDS-PAGE gel.

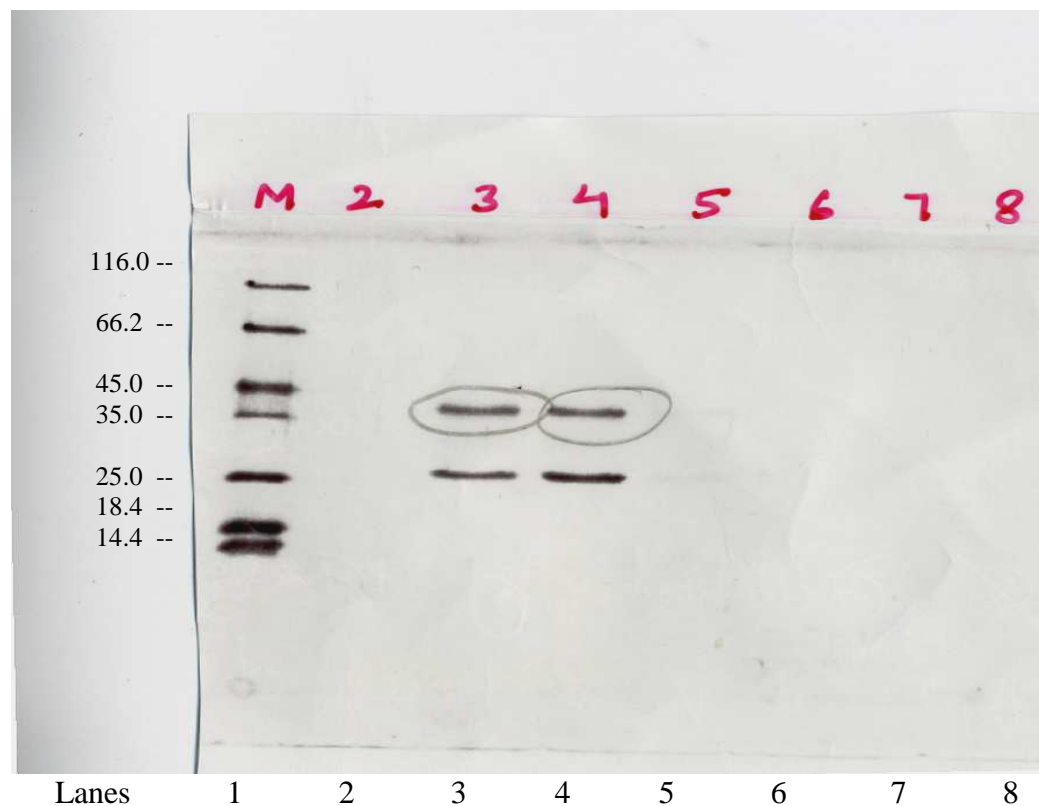


Figure 7. SDS-PAGE analysis of fusion protein (GST-MT) using Tris-glycine running buffer; lane 1: Molecular Marker, lanes 2-8: fusion protein fractions.

4.0.2. Analysis of protein samples by Tricine SDS/PAGE:

To ascertain cleavage of the GST-MT and to check the purity of the sample, the eluted samples were analyzed on the Tricine SDS-PAGE gel. Tricine [N-tris (hydroxymethyl) methylglycine] was used instead of Tris-glycine buffer as the cathode buffer, because Tricine SDS-PAGE is useful for analyzing proteins with molecular mass below 20 kDa, such as metallothioneins and other small peptides. The electrophoresis procedure for using Tricine as running buffer was described in the sections 3.8.1 and 3.8.2. The gel was initially stained with Coomassie Blue, destained overnight and a picture of the gel was taken to analyze the protein bands.

Lane 2 shows two distinct bands (Fig.7). Out of these bands, one appears in a mass range of about 35 kDa and is judged to be the GST-MT prior to cleavage by thrombin. Another protein band had mass range of about 26 kDa, therefore it is likely to be the GST. Lane 3 is the supernatant fraction following cleavage but before passing through the glutathione-agarose column. But this lane showed no visible protein bands, which may be due to not loading enough sample into the well. Lanes 4 and 5 were from the eluted MT fractions. Lane 4 shows no visible protein band, which could be due to the incomplete elution of protein from the column. A faint band with a mass range of 14 kDa is seen in the lane 5, which could be due to the presence of MT. It may be that the light staining is attributed to the absence of aminoacids which react with the dye. There are reports which indicate that MT is not properly stained by Coomassie Blue (17). So to test whether the faint band observed was due to the poor reaction of the Coomassie Blue with MT, the same gel was restained with silver stain.

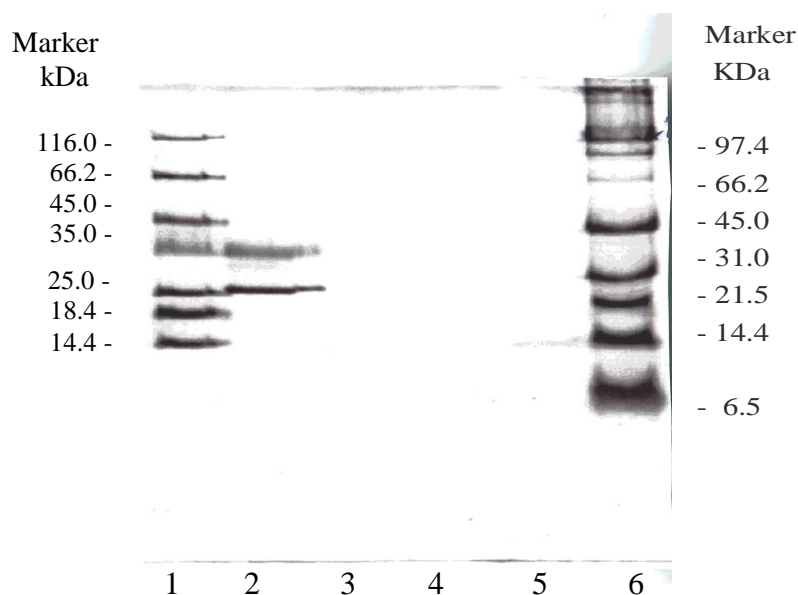


Figure 8. Coomassie Blue stained Tricine SDS/PAGE analysis of MT samples. Lanes 1 and 6 are protein markers, lane 2: GST-MT before cleavage, lane 3: GST-MT after cleavage but before column treatment, lane 4, 5: eluted MT fractions.

Silver staining of the gel resulted in appearance of a distinct MT band at 14 kDa (Fig. 8, lane 5), which was barely visible in the Coomassie Blue stained gel (Fig. 7, lane 5). This indicated that the metallothionein migrated in SDS-PAGE at an apparent molecular mass of 14 kDa, while the mass of MT is around 7 kDa. Lane 3 is the supernatant fraction following cleavage but before passing through the glutathione-

agarose column and three visible protein bands are seen (Fig. 8). Out of these bands, one light band corresponded to a mass range of about 35 kDa, indicating that it contains GST-MT that was not completely cleaved by thrombin. Another band had a mass range of 26 kDa, which is the cleaved GST from GST-MT. A distinct band corresponding to 14 kDa band was seen and that is the cleaved MT. But the same lane did not show any visible protein bands in the Coomassie Blue stained gel (Fig.7)

The results in the figures 7 and 8 showed that the metallothioneins are not easily detectable by Coomassie dye. This could be mostly due to the low level of aromatic aminoacids in the MT, which react with the dye. Coomassie Blue staining was found to be weak for small proteins with low abundance of hydrophobic and aromatic residues such as MTs (17). Alternatively the reasons for not seeing the MT band in lane 4 (figure 7 and 8) could be due to the fact that the protein was not completely eluted from the column. Furthermore, the MT band appeared to be 14 kDa in lane 5 (figure 7 and 8). This means that the MT migrated in SDS-PAGE at an apparent molecular mass of 14 kDa, while the calculated mass of MT is around 7 kDa. The nucleotide sequence of isolated MT cDNA of *H.annuus* showed that it has a molecular mass of 7 kDa. So, these discrepancies or unpredicted migrations have been described frequently for proteins containing disulfide bridges such as MTs, which preclude sufficient binding of the SDS owing to their compact structure, leading to a lower negative charge and consequently to a higher apparent molecular mass in the experiment (29). Increased apparent masses have been described frequently for metallothioneins and were thought to be associated with the oxidative formation of multimeric species (17). The metallothionein (MT) could have been dimerized and therefore, appeared at 14 kDa, instead of at 7 kDa.

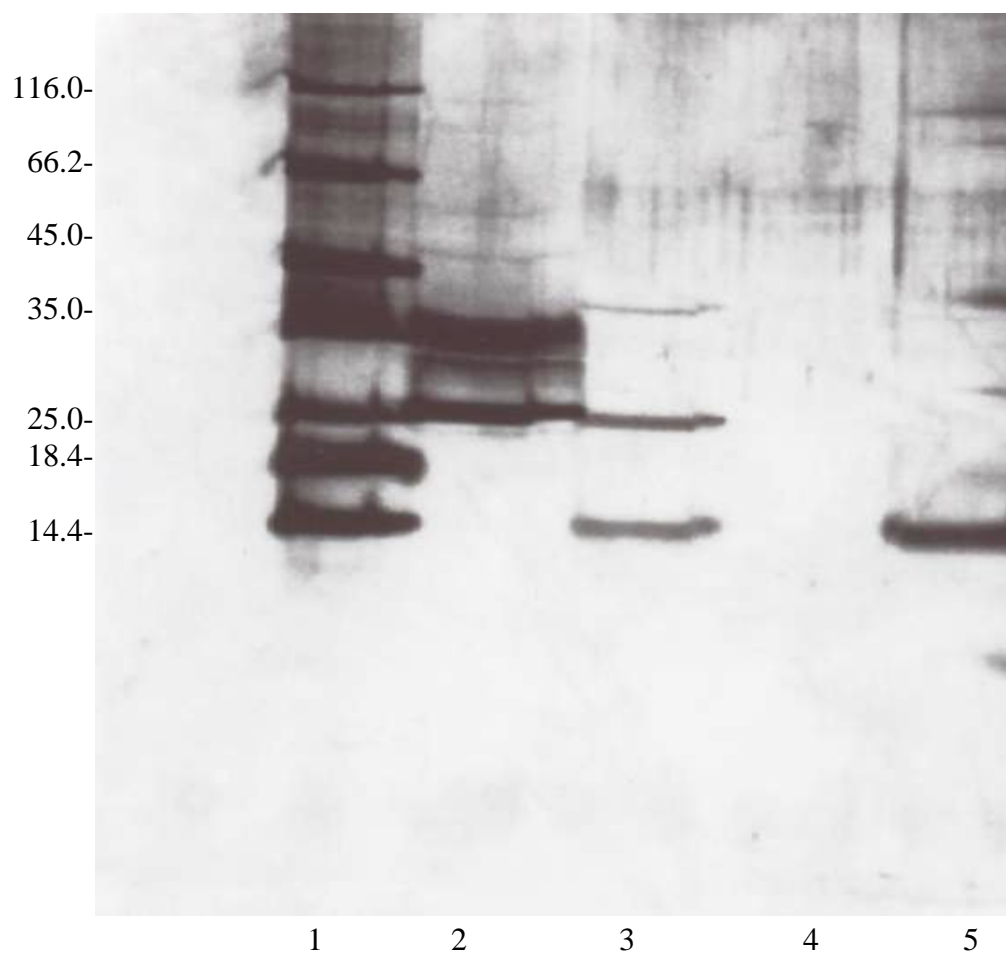


Figure 9. Silver stained Tricine SDS/PAGE analysis of metallothionein samples. left, Lane 1: molecular marker, Lane 2: GST-MT before cleavage, Lane 3: fusion protein fraction following cleavage but before the column treatment, Lane 4 and 5: MT fractions.

4.0.3. Spectroscopic Characterization of the protein

The results in the figures 7 and 8 showed that the MTs were not easily detectable by Coomassie Blue which mainly reacts with proteins abundant in arginine, aromatic amino acids and histidine (17). This could be due to the low level of aromatic aminoacids and histidine in the metallothioneins (31). Because of this unusual amino acid composition with low levels of aromatic aminoacids which show an appreciable absorption above 250 nm, metallothioneins are not readily detected by standard spectrophotometric assays which measure absorbance in long UV and Visible range. However, the binding of group-2B metal ions such as Cu, Cd and Zn to the apoprotein intensifies the far UV absorption and introduces characteristic absorption shoulders above 250 nm whose positions differ as a function of the metal (31). These metal ions are bound to the proteins through mercaptide bonds forming thiolate complexes (32). Therefore, UV-Visible spectroscopy is the method of choice for the initial characterization of a new plant MT and metal ion binding to MTs can be observed by UV spectroscopy due to the characteristic absorption bands originating from the metal-thiolate cluster formation.

In this study metallothionein isolated from *H. annuus* was investigated by measuring the UV spectra of the apo- and of the Cu^{2+} - protein complex at 260 nm, following methodology described in the literature (16). Absorbance at 260 nm of apo-MT sample was very low and the absorbance increased as Cu^{2+} concentration increased from 0 to 50 μM to the apo-MT samples (Fig.9). Thus the change in absorbance was proportional to the increase in Cu^{2+} content within the indicate range and these results were similar to the results reported in the literature (16). This change in absorbance could be mostly due to the formation of metal-thiolate clusters. In this study the concentration

of metal was confined to the indicated range (0 to 50 μM). However it could be that with increase in concentration of Cu^{2+} beyond the indicated range, the absorbance is expected to increase proportionally until saturation with Cu^{2+} is reached. Similar experiments were conducted with copper itself and it showed little absorbance at this wavelength. Similar studies were also done using other metals namely Cd and Zn ions. But the change in absorbance was not proportional to the metal concentration and did not agree with the above results. This could be due to the interaction of the metals with some nonspecific sites on MT.

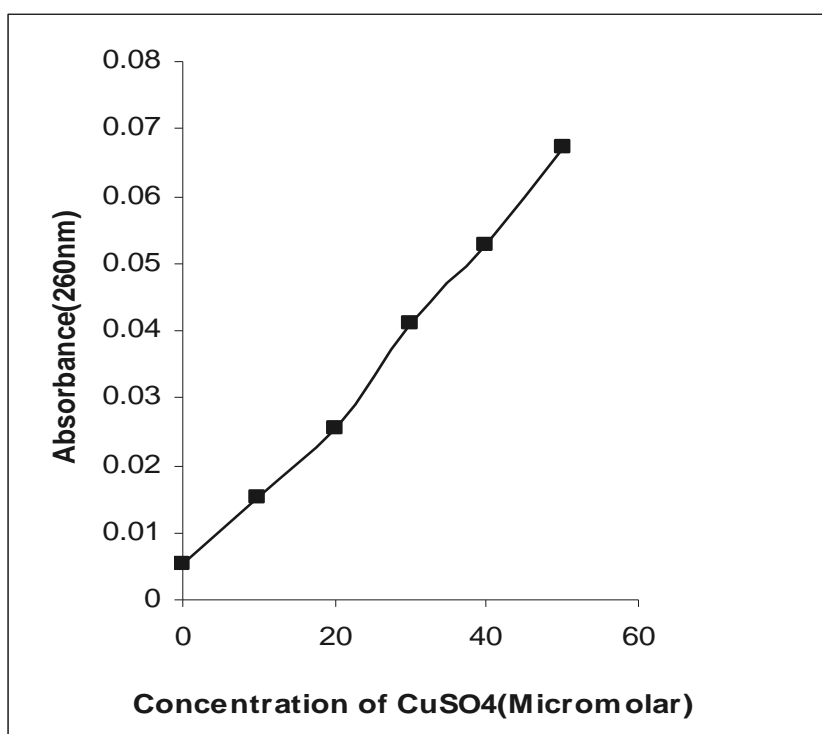


Figure 10. Absorbance change obtained as a function of the Cu^{2+} concentration.

4.0.4. Reaction of MT with DTNB reagent

Metal binding ability of metallothionein was also evaluated by use of the Ellman's reagent (5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB). DTNB is a symmetrical aryl disulfide which readily undergoes the thiol-disulfide interchange reaction in the presence of thiol peptides like metallothionein (35). It absorbs little visible radiation but the absorbance increases when it forms 2-nitro-5-thiobenzoate (TNB) which has a relatively intense absorbance at 412 nm (35). DTNB has been used for the determination of thiol content in the protein (37). When a small amount of the sample containing MT and DTNB were mixed together, the solution turned yellow, indicating the presence of thiol groups on the MT. Therefore the reaction of MTs with DTNB make thiol group unavailable for binding metal ions.

In this experiment the metal binding ability of MT towards metals Cu, Cd and Zn was studied by reaction with DTNB following the procedure described (36). This study showed that the competitive reaction of apo-MT and metal bound MTs with DTNB therefore affect the change in absorbance at 412 nm differently. The results relating the absorbance changes at 412 nm with time are shown (Fig.10). It showed that the affinity of metal ions namely Cu, Cd and Zn for MT was in the order of $Cu > Cd > Zn$. Study was also conducted relating different concentrations of Cu, Cd to the change in thiol content of MT. Thiol content of MT was calculated following the method described in method 4.0. A decrease in titratable thiol content and absorbance was observed with the increase in metal content. This could be due to the competitive reaction of metal ions with DTNB for thiol groups in the MT, which resulted in decrease in titratable thiol content available for DTNB to react with and caused the decrease in absorbance.

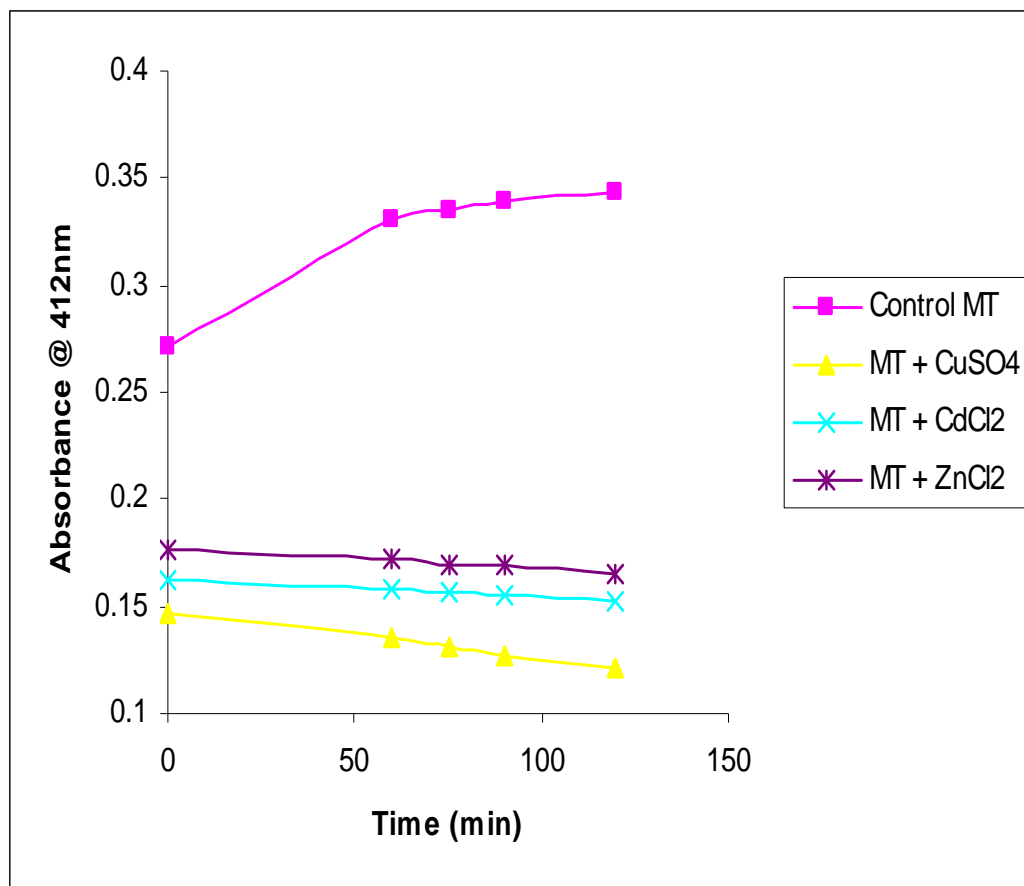


Figure 11. Change of absorbance of MT at 412 nm in the presence of Cu, Cd and Zn ions.

4.0.5. Chemiluminescence Assay

Proteins such as metallothioneins and peptides such as phytochelatins bind transition metals and the identification of a metal bound to them would help to understand the role and functional assignment of the protein (34). However the existing methods for the detection of metalloproteins consume large amounts of protein and are not sensitive. So another method was tested, namely chemiluminescence assay. It is less expensive and potentially more sensitive. This method was used for the detection of metalloproteins such as metallothioneins that contain the most common transition metals such as Cu, Ni, and Zn (34). The method is based on the principle that luminol, in the presence of certain catalysts will produce chemiluminescence when mixed with a base and an oxidant. Transition metals such as Fe, Cu, Mn, Co, Ni and Zn can act as catalysts for this reaction. So, in this experiment metal-bound MT was detected by using a chemiluminescence assay.

In the experiment the MT was first equilibrated with Cu, Cd or Zn at a concentration of 10 μ M (see method 4.1). These metal-bound MT samples were then mixed with 50 μ L of a luminol solution (11 mM luminol + 500 mM Na₂CO₃ + 230 mM H₂O₂). The intensity of light emitted was measured before and after the addition of luminol by luminometry. A change in intensity of light emitted by Cu-MT, Zn-MT and Cd-MT was compared to the apo-MT. Theoretically, the metal-MT complex should show an increase of luminescence. The results (Fig. 11) show an increase in luminescence of Cu-MT and Zn-MT as compared to apo-MT. However a similar change was not obtained with Cd-MT and the readings were even lower than that of apo-MT. The reason could be that the Cd may not be acting as a catalyst for the luminol reaction.

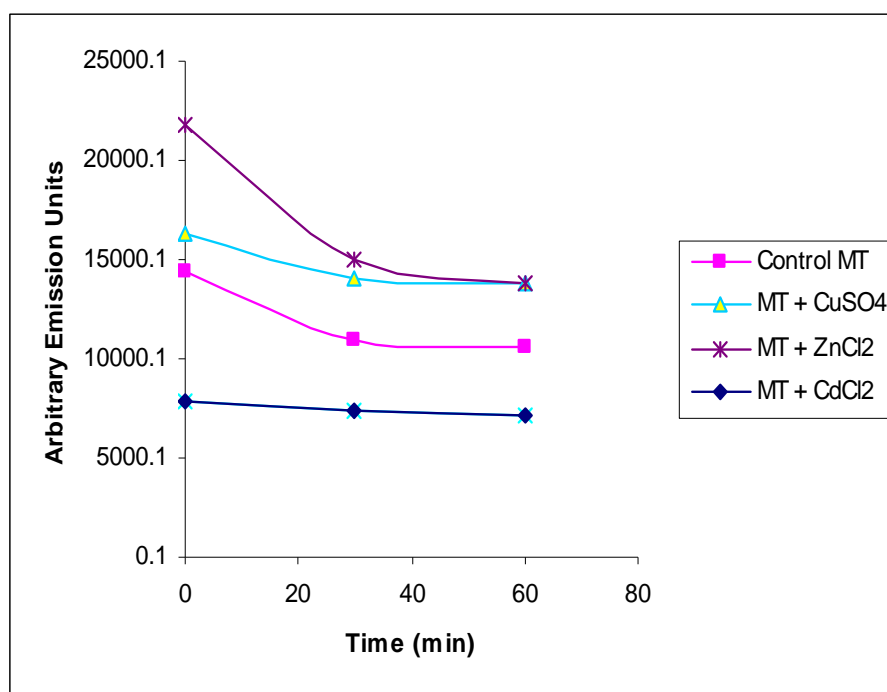


Figure 12. Reactivity of apo- and metal bound MTs with luminol solution.

CHAPTER V

FUTURE STUDIES

Much remains to be done and the following are to be examined in future studies:

1. Procedures for preparing metallothioneins in pure form have to be improved and the conditions in terms of MT yield, conditions for purifying MT on the glutathione-agarose column need to be optimized.
2. Metal binding properties such as the metal specificity and capacity of MT need to be determined and examined.
3. The methods described in this study for the detection of metal bound MTs need to be repeated and expanded with other metals.
4. The purified MT need to be further characterized with respect to its affinity, capacity and selectivity in complexation with Cu, Cd, Ni and Zn to examine if there is expression of metal specific metallothionein.
5. Determine the molecular weight and amino acid sequence of the MT by mass spectrometry.

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