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The Effect of Divalent Cations on the Prophenoloxidase Enzyme Cascade Activity in the Freshwater Crayfish Cambarus Latimanus

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by

Hans Skailand Eikaas

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I have submitted this thesis in partial fulfillment of the requirements for the degree of Master of Science.

12/6/99
Date

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Hans Skailand Eikaas

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ABSTRACT

The effect of divalent cations such as cadmium, calcium, copper, lead and magnesium upon the prophenoloxidase system (proPO) was studied in hemocytes of the crayfish *Cambarus latimanus*. It was demonstrated that cadmium, calcium, copper and lead increased proPO activity significantly, whereas magnesium had no statistically significant effect on the system. Also, the molecular weight of the proPO enzyme was estimated using SDS-PAGE and found to be approximately 76 kDa.

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I. INTRODUCTION

Almost any kind of habitat supports some type of invertebrate animal. They are abundant in fresh water, soils, forests, plants, deserts, wastelands, mountaintops, and as parasites in and on the bodies of plants and animals, including other invertebrates. Whether it be tiny dipterans such as chironomids, or larger crustaceans such as crayfish, an arthropod's dispersal and survival is largely dependent on successful defenses against various kinds of micro-organisms, parasites, and dissolved organic and inorganic chemical pollutants which are often found in their habitats. Vertebrates have developed an immune defense that includes an adaptive memory with immunoglobulin antibodies in order to defend themselves against pathogenic microorganisms (Söderhäll *et al*, 1995). Initially they respond non-specifically by the actions of phagocytic cells, natural killer cells and through the Complement system. Having an open circulatory system, invertebrates must have rapid and immediate defense and coagulation mechanisms to entrap parasites and to prevent blood loss after wounding. Invertebrate animals lack antibodies, but they do possess proteins with domains belonging to the immunoglobulin superfamily (Lanz, Mendoza and Faye, 1996). In arthropods, the immune processes are mainly carried out by hemocytes. Similar to humans, the hemocytes can react to and remove foreign particles that have succeeded in gaining entry into the body cavity of an arthropod. Thus, it appears these animals can differentiate non-self from self, and hence a system that can carry out this process ought to be present

(Söderhäll & Cerenius, 1993). This system must be efficient in recognizing and combating potentially harmful foreign substances in their environment.

A. Arthropod Immunity and Complement

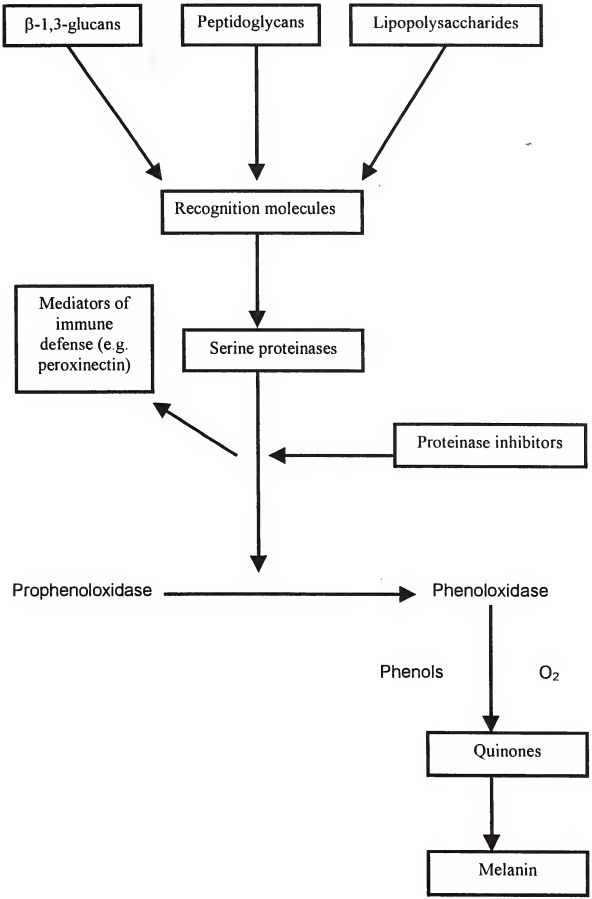
Söderhäll *et al.* (1995) has suggested that the most primitive complement system one can envision would constitute a recognition protein and a receptor on an immunologically active cell. In mammals there is a pathway for triggering an immune response without any interaction with antibodies, called complement. This is triggered by polysaccharides presented in a pattern typical for certain pathogens. The existence in invertebrates of pattern recognition molecules capable of mediating different immune-like responses in the presence of foreign particles is of significance because it acts as an innate immune system. Vertebrate complement components are capable of carrying out lysis of foreign cells, opsonizing and enhancing phagocytosis of foreign particles (Cárdenas & Dankert, 1997), releasing lymphokines and regulating the immune response (Söderhäll & Cerenius, 1995). Lacking true antibodies (Söderhäll & Cerenius, 1998) and hence also an adaptive immune response, invertebrates have to rely solely on innate immune mechanisms such as the production of antimicrobial peptides as a response to foreign body entry. Some of these peptides are similar to complement in that several of the components are present in a zymogenic form which will become biologically active upon a limited proteolysis (Aspàn *et al.*, 1995). Evidently, some sort of recognition of the foreign particle has to take place in order to transfer a message to the cells that will synthesize appropriate antimicrobial peptides. This non-self recognition of foreign material is believed to

occur through recognition molecules in the hemolymph of invertebrates, which induce activation of the prophenoloxidase (proPO) enzyme cascade system, causing a melanization reaction FIGURE 1.1. Additionally, its activation appears to elicit other defense reactions such as phagocytosis, nodule and capsule formation, and hemocyte locomotion (Arechiga *et. al.*, 1993).

B. Melanization

The melanization reaction, a common response to non-self particle entry in invertebrate animals, especially arthropods, is due to the activity of the oxidoreductase enzyme phenoloxidase (PO). This enzyme cascade, called the proPO enzyme cascade system, has been widely studied in a variety of arthropods and is believed to play a major role in mediating non-self recognition and host defenses (Hall and Söderhäll, 1983). Ashida *et. al.*(1983), and Söderhäll *et. al.* (1993) report that PO facilitates melanin formation by oxidizing phenols to quinones, which are then converted to melanin through a series of auto-catalytic nonenzymic reactions that are calcium ion dependent.

Figure 1.1 A scheme for prophenoloxidase activation in arthropods.


β-1,3-glucans
Peptidoglycans
Lipopolysaccharides
Recognition molecules
Mediators of immune defense (e.g. peroxinectin)
Serine proteinases
Proteinase inhibitors
Prophenoloxidase
Phenoloxidase
Phenols
O_2
Quinones
Melanin

Melanin deposits can readily be seen on animals that have been subjected to infection (FIGURE 1.2) or trauma (FIGURE 1.3). The PO enzyme occurs in arthropods as the inactive proenzyme proPO. Early studies of the proPO have dealt with the activated form of the enzyme because it is activated immediately upon removal of hemolymph from the study organism (Ohnishi, 1954; Ashida and Ohnishi, 1967). This renders the isolation of inactive proPO difficult. However, in an appropriate buffer solution, proPO can be prevented from being activated.

C. Phenoloxidase Enzyme Activators

The enzyme is proposed to be a non-self recognition system because conversion of proPO to active enzyme can be brought about by miniscule amounts of foreign objects such as bacterial agents (Söderhäll, 1982, and Ratcliffe *et al.*, 1982), parasites (Salt, 1956), lipopolysaccharides (Cerenius and Söderhäll, 1998), wounds (Söderhäll *et. al.* 1993) proteases (Schweiger and Karlson, 1962; Preston and Taylor, 1970; Thomson and Sin, 1970), lipids (Heynemann and Vercauteren, 1968), chloroform (Bodine and Allen, 1938), organic solvents (Preston and Taylor, 1979), detergents (Inaba et al., 1963), products of microbial origin (FIGURE 1.4), (Pye, 1974), heat (Ashida and Söderhäll, 1984), and aggregation of proPO itself (Mitchell and Weber, 1965; Munn and Bufton, 1973).

FIGURE 1.2 *Cambarus latimanus* with melanized areas on propodus (Circled) of chela and middle part of the merus (Arrow)

FIGURE 1.3 *C. latimanus* with melanized area around syringe penetration point (Arrow).

FIGURE 1.4 *Cambarus latimanus* with melanized area around a fungal infection on the branchiostegal portion of the carapace near the caudal flange (Circled)

D. Enzyme Efficiency and Biological Activity

The recognition of foreign material in invertebrates appears to be much more efficient than in vertebrates. Söderhäll and Cerenius (1988) report that the proPO non-self recognition system in invertebrates can recognize and respond to picograms per liter of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucans from fungi. These polysaccharides do not directly activate the catalytically inactive proPO into enzymatically active PO but do interact with a serine proteinase component of the proPO system. Subsequently upon activation of the system, the associated proteins gain biological activity and participate in the cellular defense reactions of the host organism (Söderhäll and Cerenius, 1998). Most proPOs are present as zymogens and are located in vesicles within the hemolymph cells. Proteins that are associated with the proPO system have been shown by Söderhäll (1993) to be directly involved in the communication between different blood cell types. In crayfish their release from the hemolymph vesicles has been shown to involve the engagement of specific receptors followed by a regulated exocytosis of the proPO components from the cells. During the cellular defense reaction the foreign particles are blackened in the host hemolymph by the deposition of melanin.

E. Prophenoloxidase and phenoloxidase

According to Söderhäll *et al.* (1993), the activity of the terminal component of the proPO system, PO, has been detected in several invertebrate groups, such as crustaceans, insects, millipedes, mollusks, bivalves, brachiopods, echinoderms, and ascidians, but the biochemical mechanism of proPO system

activation has been studied in greatest detail in the silkworm *Bombyx mori* and the freshwater crayfish *Pasifastacus leniusculus*. The PO is the enzyme involved in melanin formation. This enzyme catalyzes the oxidation of phenols to quinones which then will polymerize non-enzymatically to melanin. The intermediate compounds formed as well as melanin itself are toxic to micro-organisms (Söderhäll & Cerenius, 1998). ProPO in arthropods is activated by proteolytic cleavage by a native proteinase, serine proteinase, to yield an active PO. According to Kanost (1999), this ~30 kDa serine proteinase cleaves the proPO by hydrolysis specifically between an Arginine-Phenylalanine bond approximately 50 residues from the amino terminus. In all cases where the inactive proenzyme has been purified, the monomer has a mass of about 70-80 kDa and, after proteolytic activation, the active enzyme PO has a mass of 60-70 kDa, depending on the species.

F. Prophenoloxidase Enzyme Cascade Regulation

The proPO activating system must be controlled and regulated to avoid the deleterious effects of active components of the system, in particular active PO which can produce highly toxic intermediates (Söderhäll, 1998). Crustaceans have no equivalents to vertebrate red blood cells, but analogues of the white blood cells appear to exist. These cells, called hemocytes, can be grouped into three subpopulations: granular, semi-granular, and hyaline cells (Söderhäll and Smith, 1983) The granular and semigranular circulating secretory hemolymph cells of crayfish contain both proPO and the serine proteinase (Smith & Söderhäll, 1991). Thus compartmentalization of the system in these vesicles

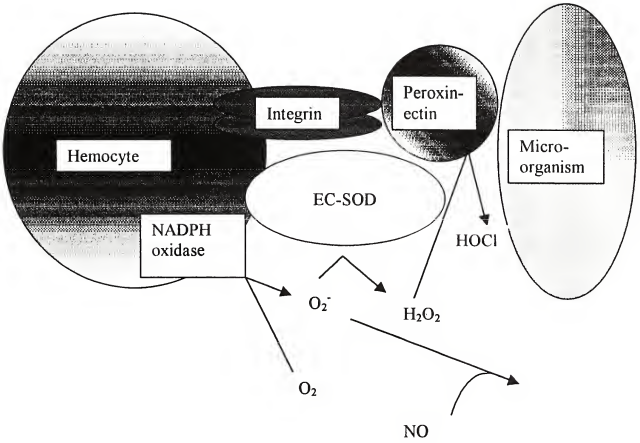
may be one important way to control and regulate the release of the proPO system at least in crustaceans. Normally, PO activation is regulated *in vivo* as a local reaction of brief duration (Aspán *et al.*, 1990). Pasifastin, a serine proteinase inhibitor, (Iwake *et al.*, 1996) and to a lesser degree, α-macroglobulin, inhibit proPO activation. Also, proPO factors are controlled by proteolytic degradation. Söderhäll reports that in crayfish this occurs via a 76 kDa cell-adhesion protein which is then degraded into a 30 kDa peptide with lower biological activity. This enzyme regulation prevents massive melanization throughout the entire circulatory system of the host organism.

G. Peroxinectin and Prophenoloxidase Interactions

The activation exerted by β-1,3-glucans is very specific (Söderhäll *et. al.* 1993). Almost all fungal cell walls contain β-1,3-glucans and thus it is not surprising to see that proteins capable of binding these molecules and induce activation of the proPO system are present in the plasma of both insects and crustaceans. Some organisms are capable of producing wall-less (protoplasts) cells without elicitor that are capable of evading the innate responses of invertebrate immune systems. Simultaneously with the activation of the proPO system, the cell adhesion protein peroxinectin is also activated, but the detailed mechanism for this is still unknown. The cell adhesion molecules are of vital significance in crustacean host defense in that the factor is secreted from the blood cells when they degranulate encountering non-self entities (Johansson *et. al.*, 1988). Melanin formed after the activation of PO is then deposited in and around the developing capsule. Holmblad and Söderhäll (1999) report that this

strengthens the capsule structure. Also, inside this walled-off pocket, reactive substances such as hypohalous acids and lysosymes will be formed by the participation of a peroxidase, peroxinectin. The mechanisms that destroys the invading entity includes an oxidative burst where the enzyme complex NADPH oxidase forms superoxide (O_2^-) from molecular oxygen (O_2). The superoxide ions are used by extracellular superoxide dismutase (EC-SOD) to produce hydrogen peroxide (H_2O_2) (FIGURE 1.5). The peroxinectin then uses the hydrogen peroxide to form toxic compounds such as hypochlorous acid (HOCl), or toxic peroxynitric acid ($ONOO^-$) may be formed if nitric oxide (NO) is present. Holmblad and colleagues (1999) attribute the destruction of the invading organism and complete successful defense on these toxic compounds.

Figure 1.5 Schematic drawing of proteins and their proposed actions in the contact between a hemocyte and a foreign microorganism (Adapted from Söderhäll, 1995)


Peroxin-
ectin
Integrin
Micro-
organism
Hemocyte
EC-SOD
NADPH
oxidase
HOCl
O_2^-
H_2O_2
O_2
NO

H. Metal Ion Regulation in Crayfish

Four crustacean epithelia are specialized for transport of various metal ions that are needed for the physiological activities of the crayfish. The gills are the site of passive calcium ion diffusion loss and uptake, the digestive epithelium effects calcium uptake from food, drink and storage deposits of the digestive system, the antennal gland may be involved in post-filtrational reabsorption of urinary calcium, and the cuticular hypodermis affects demineralization and mineralization at different stages of the molting cycle (Wheatly, 1999). The passive methods require no energy expenditure by the crayfish and work along a concentration gradient. Trace metals are transported by facilitated diffusion across permeable membranes and into the cells, where they bind with a series of metal-binding protein ligands of increasing affinity (Dallinger and Rainbow, 1992). Dallinger and Rainbow, 1992, reported that cadmium ions had a higher affinity for the calcium ion active transport mechanisms of many invertebrates by a factor of up to ten. Active transport mechanisms are also present, not only for calcium ions, but for other metal ions that work as cofactors in enzymatic reactions. Once the metal ions are inside the invertebrate, metallothioneins can function as storage proteins for the essential metals such as zinc and copper, or as chelating agents to bind toxic metals such as cadmium or mercury.

I. Literature Search Results

A literature search did not reveal anything related to studies of the effect of chemical pollutants, in particular metals such as calcium, cadmium, lead, magnesium and copper, upon the proPO enzyme cascade. In addition a protocol to isolate the inactive enzyme from arthropods had not been developed when this work was started.

J. Objectives

This research will address these issues: Will metals activate or inhibit the proPO enzyme cascade *in vitro,* and what implication(s) could that have *in vivo* for the organism? Are calcium ions truly necessary for the melanization process? What is required to isolate the inactivated form of the enzyme?

Thus, an objective of this research was to determine the effects of divalent heavy metals such as cadmium, calcium, copper, lead and magnesium upon the proPO enzyme cascade. The role of metal ion interactions with the apoenzyme was accomplished by exposing the purified apoenzyme to the "selected" metal and measuring the enzyme activity. The change in activity observed could possibly indicate heavy metal binding sites or a co-factorial role of the selected metal.

Also, a protocol for isolation of the proPO enzyme complex in its inactivated form was developed.

II. MATERIALS AND METHODS

A. Research Organism

a. Crayfish Background Information

The crayfish is a shellfish or crustacean, meaning that it has a hard exoskeleton composed of chitin. For the animal to grow, it is necessary that it periodically molt or shed its shell and grow a new one. Molting usually occurs in a burrow or some other cover required for protection during this period of vulnerability. Crayfish are also cannibalistic and will eat other crayfish that are molting. Crayfish were chosen as the research organism because many species are economically important. Crayfish also serve as indicators for environmental monitoring. Thus, this study can serve as a baseline study on the *in vitro* effect of divalent metals upon the proPO enzyme system that can later be compared to *in vivo* studies.

b. *Cambarus latimanus*

In the Chattahooche drainage, the range of *C. latimanus* extends from the lower Fall Line Hills to the upper reaches of the Piedmont. *Cambarus latimanus* typically inhabits the more sluggish areas of streams as well as lentic habitats. Given adequate cover, *C. latimanus* may remain concealed among rocks for most of the year (Hobbs, 1978). *Cambarus latimanus* was relatively abundant in the sampling area and had not previously been studied, and was therefore selected as the research organism.

B. Organism Handling and Care

The sample site for *Cambarus latimanus* that was used in this research project lies on both sides of Woodruff Farm Road near East Columbus Industrial Park in East Columbus, Ga. Background levels of metals have not been determined for this site. This area is a part of the Bull Creek drainage area and has proven to provide plentiful supplies of the target organism. Crayfish were collected using minnow traps baited with catfood, a method that worked well. Specimens were brought to Columbus State University, Department of Chemistry and Geology. Live-storage tanks with constant aeration and timed lighting provided a temporary home for the crayfish until they were released into the same drainage after the project was completed. Water in the storage tanks was changed once a week throughout the testing period. The organisms were fed shrimp pellets as their primary source of food. Throughout the testing period, the mortality rate was very low. Only four crayfish were lost over a period of twelve months.

C. Hemolymph Sampling Protocol:

a. Reagents:

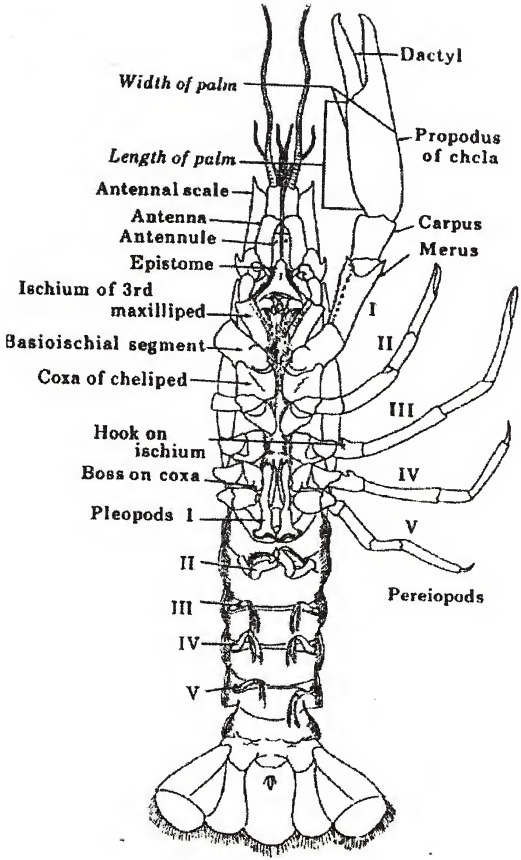
Cacodylate(CAC)-citrate buffer containing 0.01 M Na CAC, 0.45 M NaCl, and 0.1 M trisodium citrate was prepared and the pH adjusted to 7.0. This served as stock CAC-citrate buffer from which the remaining heavy metal containing buffers were made. A zymosan elicitor solution was made by suspending 500 micrograms of zymosan in the above CAC-citrate buffer, then centrifuged for 10 minutes at 20,000 rpm at 4 ^{o}C. An L-Dopa (DL-β-3,4-

dihydroxyphenylalanine) solution to serve as a substrate for melanin formation was prepared by dissolving 3 mg of L-Dopa in one liter of distilled water. L-Dopa solution has a limited shelf-life, and was made fresh every four weeks during the testing period. Solutions containing 0.01 M, 0.05 M. and 0.1 M of calcium, cadmium, copper, lead and magnesium respectively were made by dissolving the appropriate grams of the metal salts in CAC-citrate buffer and adjusting the pH with nitric acid until the salts were completely dissolved. All of the above solutions were stored in -4^{o}C cold storage.

b. Sampling Protocol:

Before withdrawing hemolymph from the crayfish, they were placed in an icebath for two minutes to ease handling. Hemocytes were then collected from the distal and proximal joints of the carpus (FIGURE 2.1) of the crayfish with a 1.2 mm hypodermic needle. Samples from individual crayfish were pooled and kept on ice. The hemolymph was then homogenized for eight minutes using a glass piston homogenizer in order to release the proPO enzyme from the granular and semi-granular hemocytes. Subsequently the homogenized hemolymph was centrifuged for 30 minutes in a Sorvall Superspeed centrifuge at 20,000 rpm at 4 ^{o}C. According to Söderhäll and Smith (1983) this should leave the proPO suspended above the hemocyte cell membranes that make up the pellet on the bottom of the tube. Aliquots of 1000 ml CAC-citrate buffer

Figure 2.1 Crayfish Anatomy, ventral side showing

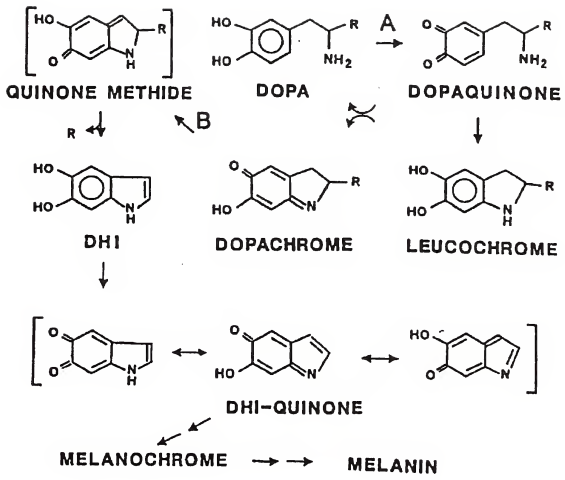

Dactyl
Width of palm
Propodus of chela
Length of palm
Antennal scale
Antenna
Carpus
Antennule
Merus
Epistome
Ischium of 3rd maxilliped
I
II
Basioischial segment
Coxa of cheliped
III
Hook on ischium
IV
Boss on coxa
Pleopods I
V
II
Pereiopods
III
IV
V

containing the various concentrations of heavy metals, 500 ml Zymosan elicitor, and 100 ml L-Dopa were put in a constant temperature bath (37 ^{o}C.) for twenty minutes. The centrifuged hemolymph was also incubated in the waterbath to bring all the solutions to the same temperature before mixing. After incubation, the aliquots were mixed with 1000 μl of purified hemolymph and the absorbance at 490 nm was immediately measured using a Varian UV-visible spectrophotometer. Melanization occurs as dihydroxyindole is oxidized to its quinone which further reacts to form dopachrome, or melanin (FIGURE 2.2). According to Söderäll, this is a general reaction mechanism that occurs in all arthropods. The initial absorbance was recorded as well as absorbance at 20 minutes, 60 minutes and 120 minutes in order to see if the response of the proPO enzyme cascade to the selected heavy metals was linear over time. The first 20 minute period was used for reporting enzyme activity. An increase in absorbance due to dopachrome production indicated enzyme activity .

D. Protein Complex Molecular Weight Determination Protocol:

In this study, SDS-PAGE was used as the analytical tool. The pore size of the gel can be controlled by choosing various concentrations of acrylamide and methylenebisacrylamide at the time of polymerization. SDS and the β-mercaptoethanol break the disulfide bonds of the protein and disrupts nearly all noncovalent interactions. Therefore, only the molecular weight of the substance determines the distance traveled

Figure 2.2 Mechanism of arthropod melanogenesis (Adapted from Söderhäll *et al.*, 1996).


QUINONE METHIDE
DOPA
A
DOPAQUINONE
B
DHI
DOPACHROME
LEUCOCHROME
DHI-QUINONE
MELANOCHROME
MELANIN

through the gel. Molecular weight markers ranging from 6500 daltons to 205.000 daltons were used to estimate the weights of the proteins in the purified hemolymph.

a. Reagents:

A 45% (w/v) solution of polyacrylamide was prepared from powdered acrylamide and stored in a brown glass bottle in a cold-room. 1.6% bisacrylamide (w/v) was prepared in warm water and then stored in a brown glass bottle in the cold-room until its use. Two Tris-HCl buffers, one of 1.5 M and pH 8.8 and another of 0.5 M and pH 6.8 were prepared, both stored at -4^{o}C. The pH of the buffers was adjusted with dilute nitric acid (which does not form insoluble lead chloride). A 4% solution of sodium dodecyl sulfate was prepared and stored at room temperature. Ammonium persulfate solution of 0.56% (w/v) was also prepared fresh daily. TEMED (Tetramethylethylene-diamine), a polymerizing agent, was used as supplied from the distributor. Tris-glycine reservoir buffer containing 25mM Tris, 192 mM glycine at pH 8.3, and 0.1% SDS (w/v) was stored in the cold-room as well. The gels were run in a Hoeffer SE 600 Series vertical gel apparatus.

b. Pouring the gels:

TABLE 2.1 gives the recipes for the preparation of a 7.5 % acrylamide 16X18 cm resolving slab gel which was topped by a 5% acrylamide stacking gel, both of which were 0.075 cm thick. Due to the high concentration of ammonium persulfate, the mixture needed to be degassed. The resolving gel mixture was poured into assembled gel plates, leaving sufficient space on top for the stacking

TABLE 2.1. Recipes for the preparation of a 7.5% Acrylamide 16X18 cm resolving slab SDS-PAGE vertical gels, 0.075 cm thick, and a 5% Acrylamide stacking gel.

Ingredients:	Volume (7.5% Gel)	Stacking Gel (5%)
Acrylamide (45%) - Bisacrylamide stock solution	7.5mL	0.9mL
Tris-HCl, pH 8.8 (1.5 M)	7.5mL	0.7mL (pH 6.8)
SDS (4%)	1.2mL	2.5mL
Water	7.1mL	0.5mL
Ammonium persulfate (0.56%)	1.5mL	0.7mL
TEMED	40 μL	15mL

gel. A 0.1% SDS solution was placed on top. After polymerization the overlay was removed, the gel was rinsed twice with water and a small amount of stacking gel mix. Stacking gel was then layered on top of the acrylamide gel and the comb for the sample wells inserted.

E. Assay of Phenoloxidase Activity -- Zymosan Elicitor Present

1000μL of crayfish hemolymph was incubated at 37°C for 5 minutes. A mixture of 500μL zymosan elicitor, 100μL L-Dopa, and 1000μL of CAC-Citrate buffer with varying concentrations of the selected metal ions were also incubated at 37°C for 5 minutes. The two solutions were then mixed together and the absorbance at 490nm was measured immediately. This first reading was designated time zero, and additional recordings of the absorbance were taken at 20 minute intervals. The effects on the proPO enzyme cascade by the selected metals in the presence of a zymosan elicitor was expressed as change in absorbance

over time for the first twenty minute interval. The control consisted of hemolymph, Zymosan elicitor, L-Dopa, and CAC-citrate buffer.

F. Assay of Phenoloxidase Activity -- No Zymosan Elicitor Present

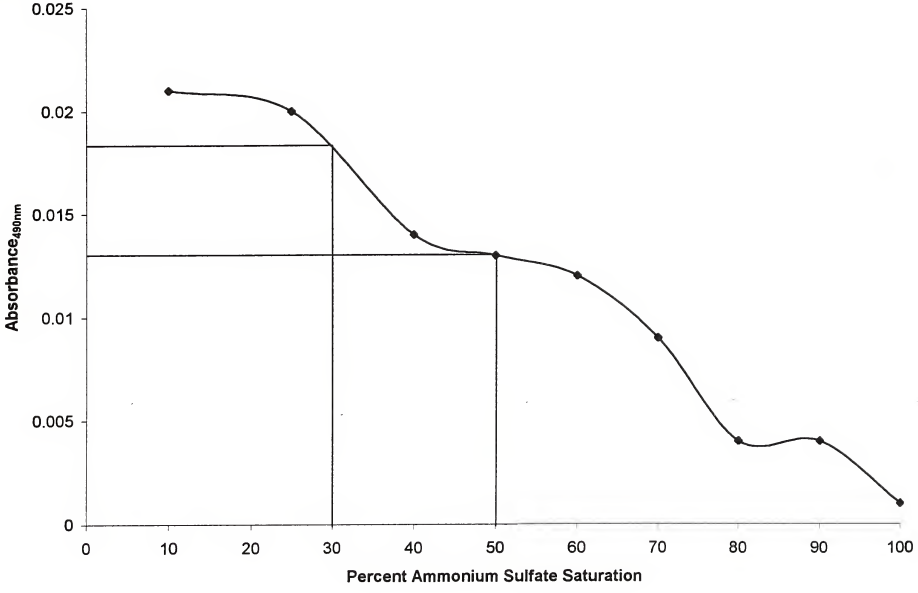
This was performed in the same fashion as the assay for PO activity in the presence of the zymosan elicitor, only the elicitor was replaced by 500μL of CAC-Citrate buffer without added metal ions. The control consisted of hemolymph, L-Dopa, and CAC-citrate buffer.

G. Preparation and Assay of Enzyme for Molecular Weight Determination

Hemolymph from 15 individuals was collected and pooled. The crude hemolymp was lysed using a glass piston homogenizer, then centrifuged at 20,000 rpm for 20 minutes. This extract was then separated in an SDS-PAGE gel under reducing conditions. Liquid ammonium sulfate solutions ranging from 10 to 100 percent saturation were prepared. Thus, by analyzing the various salt fractions, one can find the conditions where the proteins of interest, proPO and PO, are still enzymatically active as measured spectrophotometrically. 100μL of centrifuged hemolymph extract was put into 1000μL of the various concentrations of ammonium sulfate and centrifuged at 10,000 rpm for 10 minutes. 500μL of these centrifuged solutions were then incubated with 500μL Zymosan elicitor, 100μL L-DOPA, and 1000μL of CAC-citrate containing 3.85 mM Pb^{2+} ions. These solutions were then checked for enzyme activity and an activity curve was established to determine the point at which proPO precipitated out of solution (FIGURE 2.3).

Based on the activity curve obtained as outlined about, crude centrifuged extract was then saturated to 30 percent with ammonium sulfate and centrifuged. The precipitate was discarded and the supernatant further saturated to 50 percent ammonium sulfate concentration. The supernatant was discarded and the precipitate was re-suspended in CAC-citrate buffer and desalted by putting the sample in a dialysis membrane under continuous flow deionized water for four hours. Molecular weight was estimated by taking the log of the molecular weight standards plotted against the relative mobility of the marker proteins to

FIGURE 2.3 Optical Activity of proPO Extract Immediately following Precipitation with Ammonium Sulfate. Most of the Optical Activity is retained at 25% Saturation, but drops considerably around 40% Saturation and again around 70% Ammonium Sulfate Saturation.


0.025
0.02
0.015
0.01
0.005
0
Absorbance490nm
0
10
20
30
40
50
60
70
80
90
100
Percent Ammonium Sulfate Saturation

obtain a slope (FIGURE 5.9, APPENDIX B). Based on the slope obtained, the log of the crayfish proteins was calculated and the estimated protein weight in daltons (Da) calculated.

H. Project Funding:

Most of the chemicals needed for this research were already in stock with the department of chemistry. However, it was necessary to obtain some electrophoresis chemicals from Sigma Chemicals. The cost of these chemicals was covered by the Department of Chemistry and Geology. Syringes for hemolymph tissue withdrawal as well as paper for printing the work was provided free of charge by Dr. Jack H. Blalock, Jr., M.D., P.C.. All equipment necessary for the research was provided by the Department of Chemistry & Geology as well as the Department of Biology. The cost of feeding the research organisms was covered by the author.

III. Results:

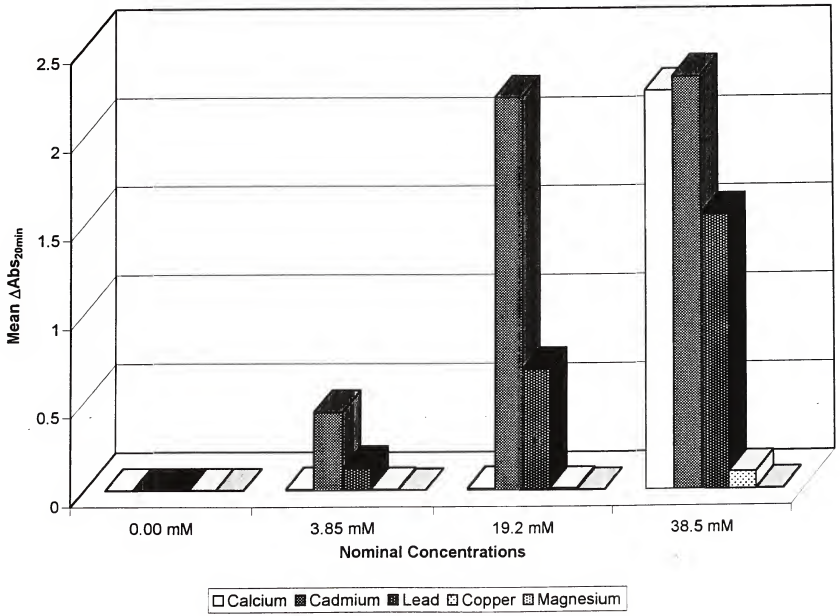
A. Testing for Prophenoloxidase Enzyme Activity to Determine if Zymosan Elicitor is Necessary for Enzyme Cascade Activation (Single-Factor ANOVA)

Summary tables for divalent cation testing on the proPO can be found in APPENDIX A, as well as in FIGURE 3.1 where the enzyme activity is graphed for comparison of activity among the metals tested. Single-Factor analysis of variance with replication failed to establish that the Zymosan elicitor was necessary for the proPO enzyme cascade to take place. Testing of the enzymatic response at 38.5 mM Ca^{2+} in the presence of the Zymosan elicitor and without the Zymosan elicitor gave a P-value of 0.08 which means that there was no significant difference in the change in absorbance observed over a twenty minutes period between the two groups tested at 95% confidence level (APPENDIX A, TABLE 5.7). It appears that calcium can by itself elicit the observed enzymatic response.

B. Enzyme Activity in the Presence of Zymosan Elicitor (Two-Factor ANOVA)

Two-Factor ANOVA (APPENDIX A, TABLE 5.8) showed that there was a significant change in absorbance observed with increased concentration of the various cations tested (P-value < 0.001), among the various divalent cations tested (P-value < 0.001), and among all the cations and concentrations tested (P-value < 0.001).

FIGURE 3.1 Mean prophenoloxidase enzyme activity over a 20 minute period for nominal concentrations of 0.00 mM, 3.85 mM, 19.2 mM, and 38.5 mM of the divalent cations Ca^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, and Mg^{2+} respectively.


2.5
2
1.5
1
0.5
0
Mean ΔAbs20min
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Nominal Concentrations
Calcium
Cadmium
Lead
Copper
Magnesium

Regression analysis to determine whether a correlation between divalent cation ionic radii, solvent interactions not considered, and mean enzyme activity at the three concentrations tested revealed no significant correlations at 95 percent confidence level (see TABLES 5.9 - 5.11 and FIGURES 5.1 - 5.3). The R squared values were 0.059, 0.069, 0.330 for 3.85 mM, 19.2 mM, and 38.5 mM respectively. An R squared value above 0.5 would have indicated a correlation between ionic radii and observed change in absorbance over a twenty minute period.

C. Enzyme Activity in the Presence of Zymosan Elicitor and Various Concentrations of Cadmium

Even at the lowest concentration tested (3.85 mM Cd^{2+}) the initial absorbance was somewhat higher than for most of the other metals tested (Mean absorbance = 0.207) (APPENDIX A, TABLE 5.1). At the two higher concentrations (19.2 mM and 38.5 mM Cd^{2+}) the initial absorbances were even higher and the solution appeared translucent grayish in color. The cadmium ions were probably interacting with some proteins in the hemolymph to give the translucent grayish color or some other reaction or cadmium binding must also have taken place to cause the initial high absorbances at the higher concentrations of cadmium tested. Single Factor Analysis of Variance revealed a significant change in absorbance over time (P-value <0.001) (APPENDIX A, TABLE 5.12) despite the high initial absorbances. The response of proPO to cadmium was not linear with increasing concentration (APPENDIX B, FIGURE 5.4). There appears to be a drastic change in slope when the nominal cadmium

ion concentration is increased from 3.85 mM to 19.2 mM, giving a sigmoid curve appearance.

D. Enzyme Activity in the Presence of Zymosan Elicitor and Various Concentrations of Calcium

Single Factor Analysis of Variance showed that there was a significant increase in change in absorbance over time with increasing calcium ion concentrations. The F-value was 40480.7, F-critical 3.49 for 3 degrees of freedom (APPENDIX A, TABLE 5.13). The initial absorbance at 38.5 mM Ca^{2+} was much higher compared to the lower concentrations (APPENDIX A, TABLE 5.2). Again, as with the cadmium testing, this could be due to a reaction other than proPO activation taking place simultaneously, causing light scattering. As with the cadmium testing, the enzymatic response to calcium is not linear. A threshold was reached when the concentration was increased to 38.5 mM (APPENDIX B, FIGURE 5.5).

E. Enzyme Activity in the Presence of Zymosan Elicitor and Various Concentrations of Divalent Copper

The initial absorbances were more similar to one another for the copper testing than for calcium or cadmium testing for the various concentrations tested (APPENDIX A, TABLE 5.3). However, this divalent cation also elicited a significant increase in enzymatic activity over time with increasing copper (II) ion concentration (APPENDIX A, TABLE 5.14). The Single Factorial Analysis of Variance gave an P-value < 0.001. For copper (II), an increase in enzyme activity

was observed when the concentration was raised from 0.0192 M and 38.5 mM copper (II) (APPENDIX B, FIGURE 5.6). A saturation level was not reached.

F. Enzyme Activity in the Presence of Zymosan Elicitor and Various Concentrations of Divalent Lead

Lead testing results can be found in TABLE 5.4 (APPENDIX A). Single Factorial Analysis of Variance showed that the change in absorbance over time changed significantly with increased lead (II) ion concentration (APPENDIX A, TABLE 5.15). The P-values was less than 0.001. Based on FIGURE 5.7 (APPENDIX B), an upper threshold was not reached for lead (II).

G. Enzyme Activity in the Presence of Zymosan Elicitor and Various Concentrations of Magnesium

Although the initial absorbances increased with increasing concentrations of magnesium ions (APPENDIX A, TABLE 5.5), the change in absorbance over time did not differ significantly (APPENDIX A, TABLE 5.16). The P-value was 0.119. Lower levels of magnesium actually lowered the enzymatic activity, below the level of controls. No upper threshold was reached (APPENDIX B, FIGURE 5.8).

H. Molecular Weight Determination

A total of six protein bands were observed in an extract of crayfish hemolymph purified as stated in the methods section. An aliquot of this extract was tested for enzyme activity to confirm the presence of proPO and PO. Duvic and Söderhäll (1990) reported a 76 kDa proPO protein complex in the hemolymph tissue of the crayfish *Pasifastacus leniusculus*, and protein

complexes of this size have also been observed in other species. According to my own results, I also observed a single band estimated to be 76.2 kDa in size (TABLE 5.17, APPENDIX A, and FIGURE 5.10, APPENDIX B). This experiment was duplicated with consistent results.

IV. DISCUSSION AND CONCLUSIONS

A. General:

I must conclude that the Zymosan elicitor, consisting of β-1,3-glucan and β-1,6-glucan from gram-negative bacteria, is not completely necessary for the activation of the proPO enzyme cascade, at least at the 38.5 mM Ca^{2+} concentration level tested. The Zymosan elicitor contains gram-negative bacterial cell membrane coating components, and it does make sense that the proPO is sensitive to this. However, Ca^{2+} in itself elicits a response of the proPO enzyme cascade that was not significantly different for the response seen in the presence of Ca^{2+} and the Zymosan elicitor. Also, the Zymosan by itself elicited a response significantly lower than calcium by itself or the combination of calcium and Zymosan.

It is interesting to note that calcium did not have a significant impact upon the proPO enzyme cascade until the highest concentration was tested. This contradicts results found by Ashida and Söderhäll (1984) who found calcium to elicit a biphasic response, and also contradicts the results by Gollas-Galvan *et al.*, (1997).

Cadmium, on the other hand, gave a significant increase in the observed absorbance compared to the control at all levels. However, reactions other than proPO and PO activity must have occurred simultaneously in the presence of cadmium as explained shortly. Preliminary *in vivo* studies of cadmium resulted in the death of the crayfish at all levels tested (Lovelette and Wright, 1996, unpublished).

Lead also increased the absorbance significantly at all levels compared to the control. Lead is suspected of being capable of utilizing the same transport mechanisms as calcium to enter an organism. If this is the case, considering lead elicits a significantly higher enzyme activity level than calcium, lead could put significant stress on the crayfish immune system.

Copper and magnesium, the two other elements tested that are ubiquitous in the environment, had almost no impact on the enzyme at any level.

Due to the small differences in ionic radii, one would not expect a significantly different enzymatic response due to size differences among the metal ions tested. Ionic radii regression analysis confirmed that there was no correlation between divalent cation ionic radii and enzymatic activity at any concentrations. Even though the lead ion is larger than the cadmium ion, the cadmium ions elicited a significantly larger change in absorbance than the lead ions. This is likely due to light scattering in addition to light absorbance, and the instrument used for measuring absorbance is not capable of distinguishing light scattering from light absorbance.

B. Cadmium

Even low levels of cadmium ions induced high levels of proPO enzyme activity in the presence of Zymosan elicitor. Cadmium is considered a highly toxic contaminant in the environment, and it is not surprising that even low levels elicits immediate activation of the proPO system, an innate immune response of arthropods. The translucent grayish color of the hemolymph solution observed with the higher concentrations of cadmium ions suggests that some events other

than proPO response had also taken place. Again, the observed "absorbance" might have been due to light scattering as well as absorbance. My protocol did not attempt to preclude enzymatic activity from enzymes other than proPO. Also, proPO activity in Zymosan free solution was not measured, which would have established whether cadmium could have activated the enzyme cascade in the absence of an elicitor.

C. Calcium

Naturally, in crayfish the gills are primarily responsible for calcium homeostasis (Wheatly, 1999). Calcium is necessary for crustaceans in hardening their exoskeleton, and crayfish actively regulate the calcium balance in the body through the above mentioned secretory organs.

Zymosan is capable of activating crayfish proPO system probably by inducing protease activity as demonstrated by Ashida and Söderhäll (1984). When crayfish hemolymph was incubated with Zymosan elicitor in the presence of various concentrations of calcium ions, an increased enzyme activity was recorded. As noted in the results section, calcium also induced proPO activation in the absence of Zymosan elicitor. This suggests that calcium alone is capable of inducing activation of the proPO system. In calcium free solution, minimal activity was observed. It is possible that under natural conditions, the activation of the proPO system occurs in multiple steps. The first must be degranulation of the semi-granular and granular hemocytes that contain the proPO enzyme, which can subsequently be transformed into its active form (PO) by higher than physiological levels of calcium ions unless the crayfish can actively transport out

the excess calcium ions. This threshold level effect suggests that calcium ions act as a co-factor in the enzyme cascade.

D. Copper (II)

The primary amino acid sequences of several arthropod proPOs have been determined and all have been found to contain two putative copper binding sites, designated CuA and CuB (Aspàn *et. al.*, 1995), and two copper atoms (Cerenius and Söderhäll, 1998). The amount of copper in purified PO has been determined by atomic absorption to be 0.21%, which corresponds to two atoms of copper per molecule (Cerenius and Söderhäll, 1998). These copper binding sites play a role in sclerotization of the cuticle and encapsulation of foreign particles (Hughes, 1999).

As noted in the results section, there was a statistically significant increase in the observed absorption over time with increased copper ion concentration. However, the increase in enzymatic activity was minimal compared to calcium, cadmium and lead. Copper binding sites could have bound the supplied copper ions prior to significant activation. Further research should be conducted to elucidate if this is the exact role of copper.

E. Lead (II):

Lead (II) is a common pollutant in the environment from industry and mining drainage. It is also considered to be toxic at small doses. Significant increase in enzyme activity indicates that this divalent metal is recognized by the proPO system by acting as a pseudo co-factor and triggers the immune response *in vitro*. *In vivo*, it is possible that lead is actively transported out of the organism

by the same mechanisms that transport calcium ions. Work by Lovelette and Wright (1996) found lead to exhibit a biphasic mode of action. Crayfish subjected to 5ppm lead in their water exhibited an increase in enzyme activity after the first week of exposure compared to their control, but decreased in the second and third weeks. This suggests that the crayfish have some means of sequestering the lead *in vivo*.

F. Magnesium:

Magnesium is ubiquitous in the environment and gave no significant increase in observed absorbance over time, as mentioned in the results section, for the various concentrations tested. Magnesium is a common cofactor in many enzyme cascades.

G. proPO Molecular Weight Determination

Based on the results of this research, the molecular weight for proPO in *C. latimanus* has been determined to be approximately 76kDa. This is consistent with the size determined for this enzyme in other species such as *P. leniusculus* and *Procambarus clarkii* (Duvik and Söderhäll, 1990). Further work with respect to the proPO enzyme *in C. latimanus* should be to raise specific monoclonal antibodies for the protein complex. This could aid in further purifying the hemolymph extract to eliminate the other proteins present in the solution. Also, the primary amino acid structure should be determined.

H. Environmental Significance

My *in vitro* experiments were performed in a controlled laboratory setting. The results can serve as a baseline for comparison with *in vivo* studies to be

undertaken soon, which upon completion can be compared to the *in vivo* study to see if a correlation exists. Crayfish serve an important role in the environment as keystone grazers and predators, in nutrient processing, and as an important source of food for fish in larger lakes and river systems. Both cadmium and lead has been reported to be taken up in moderate concentrations by crayfish (Phillips and Rainbow, 1993). Physiological indicators of contamination include deformities, sores, or lesions (Kennish, 1992). Crustaceans are also economically significant, and the more that is known about metal effect on their innate immune system, the better one can estimate the effects of real life applications. One could possibly adjust all the metal ion concentrations in growing ponds for crabs, lobsters, and crayfish for optimal immune system functioning.

Crayfish also serve as indicator species for environmental studies, indicating moderately clean water (McDonald, Borden, and Lathrop, 1990). If their presence or absence in an area is due to the metal ion concentrations in the habitat, the significance of my study is paramount.

Wheatly reported (1999) that very high levels of calcium interferes with the molting cycle. If high levels of calcium also compromises the immune system of the crayfish, one would not expect the presence of crayfish in a habitat extremely high in calcium ion concentration.

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V. APPENDIX - A Raw Data Tables for Cation Testing

TABLE **PAGE**

TABLE 5.1 Cadmium testing summary table. The table shows the dates of testing, the nominal concentrations of cadmium ions used, the absorbance measured at T_{ZERO} and T_{20}, and the change in absorbance over this time period.

Date	Concentration	Abs T_{zero} (A)	Abs T_{20} (A)	Δ Abs
07/10/1999	Control	0.133	0.135	0.002
07/17/1999	Control	0.135	0.136	0.001
07/18/1999	Control	0.136	0.137	0.001
07/24/1999	Control	0.135	0.137	0.002
07/10/1999	3.85 E-3 M	0.189	0.605	0.416
07/17/1999	3.85 E-3 M	0.234	0.680	0.446
07/18/1999	3.85 E-3 M	0.205	0.660	0.455
07/24/1999	3.85 E-3 M	0.200	0.648	0.448
07/10/1999	1.92 E-2 M	1.420	3.650	2.230
07/17/1999	1.92 E-2 M	1.205	3.401	2.196
07/18/1999	1.92 E-2 M	1.235	3.420	2.185
07/24/1999	1.92 E-2 M	1.410	3.650	2.240
07/10/1999	3.85 E-2 M	1.316	3.650	2.334
07/17/1999	3.85 E-2 M	1.291	3.623	2.332
07/18/1999	3.85 E-2 M	1.308	3.649	2.341
07/24/1999	3.85 E-2 M	1.299	3.600	2.301

TABLE 5.2. Calcium testing summary table. The table shows the dates of testing, the nominal concentrations of calcium ions used, the absorbance at T_{ZERO} and T_{20}, and the change in absorbance over this time period.

Date	Concentration	Abs T_{zero} (A)	Abs T_{20} (A)	Δ Abs
05/24/1999	Control	0.135	0.136	0.001
06/24/1999	Control	0.135	0.136	0.001
06/25/1999	Control	0.134	0.136	0.002
06/30/1999	Control	0.135	0.136	0.001
05/24/1999	3.85 E-3 M	0.157	0.165	0.008
06/24/1999	3.85 E-3 M	0.175	0.183	0.008
06/25/1999	3.85 E-3 M	0.138	0.145	0.007
06/30/1999	3.85 E-3 M	0.170	0.178	0.008
05/24/1999	1.92 E-2 M	0.257	0.267	0.010
06/24/1999	1.92 E-2 M	0.175	0.186	0.011
06/25/1999	1.92 E-2 M	0.136	0.145	0.009
06/30/1999	1.92 E-2 M	0.189	0.198	0.009
05/24/1999	3.85 E-2 M	1.141	3.390	2.249
06/24/1999	3.85 E-2 M	1.460	3.702	2.242
06/25/1999	3.85 E-2 M	1.230	3.430	2.200
06/30/1999	3.85 E-2 M	1.420	3.650	2.230
05/24/1999	3.85 E-2 M, no Zymosan	1.298	3.36	2.062
06/24/1999	3.85 E-2 M, no Zymosan	1.350	3.424	2.074
06/25/1999	3.85 E-2 M, no Zymosan	1.215	3.45	2.235
06/30/1999	3.85 E-2 M, no Zymosan	1.316	3.508	2.192

TABLE 5.3 Copper testing summary table. The table shows the dates of testing, the nominal concentrations of copper ions used, the absorbance at T_{ZERO} and T_{20}, and the change in absorbance over this time period. The reference solutions contained equal amounts of copper containing buffer as the solutions tested.

Date	Concentration	Abs T_{zero} (A)	Abs T_{20} (A)	Δ Abs
08/25/1999	Control	0.130	0.131	0.001
08/26/1999	Control	0.132	0.134	0.002
08/30/1999	Control	0.132	0.133	0.001
08/31/1999	Control	0.134	0.135	0.001
08/25/1999	3.85 E-3 M	0.119	0.124	0.005
08/26/1999	3.85 E-3 M	0.120	0.125	0.005
08/30/1999	3.85 E-3 M	0.110	0.115	0.005
08/31/1999	3.85 E-3 M	0.118	0.123	0.005
08/25/1999	1.92 E-2 M	0.127	0.135	0.008
08/26/1999	1.92 E-2 M	0.124	0.133	0.009
08/30/1999	1.92 E-2 M	0.126	0.133	0.007
08/31/1999	1.92 E-2 M	0.186	0.196	0.010
08/25/1999	3.85 E-2 M	0.139	0.230	0.091
08/26/1999	3.85 E-2 M	0.139	0.235	0.096
08/30/1999	3.85 E-2 M	0.139	0.236	0.097
08/31/1999	3.85 E-2 M	0.148	0.246	0.098

TABLE 5.4 Lead testing summary table. The table shows the dates of testing, the nominal concentrations of lead ions used, the absorbance measured at T_{ZERO} and T_{20}, and the change in absorbance over this time period.

Date	Concentration	Abs T_{zero} (A)	Abs T_{20} (A)	Δ Abs
08/06/1999	Control	0.137	0.139	0.002
08/13/1999	Control	0.135	0.136	0.001
08/18/1999	Control	0.135	0.136	0.001
08/19/1999	Control	0.137	0.138	0.001
08/06/1999	3.85 E-3 M	0.142	0.268	0.126
08/13/1999	3.85 E-3 M	0.143	0.269	0.126
08/18/1999	3.85 E-3 M	0.142	0.269	0.127
08/19/1999	3.85 E-3 M	0.144	0.267	0.123
08/06/1999	1.92 E-2 M	1.280	1.970	0.690
08/13/1999	1.92 E-2 M	1.300	1.980	0.680
08/18/1999	1.92 E-2 M	1.310	1.970	0.660
08/19/1999	1.92 E-2 M	1.280	1.970	0.690
08/06/1999	3.85 E-2 M	1.850	3.380	1.530
08/13/1999	3.85 E-2 M	1.830	3.410	1.580
08/18/1999	3.85 E-2 M	1.830	3.390	1.560
08/19/1999	3.85 E-2 M	1.890	3.400	1.510

TABLE 5.5 Magnesium testing summary table. The table shows the dates of testing, the nominal concentrations of magnesium ions used, the absorbance measured at T_{ZERO} and T_{20}, and the change in absorbance over this time period.

Date	Concentration	Abs T_{zero} (A)	Abs T_{20} (A)	Δ Abs
08/19/1999	Control	0.135	0.136	0.001
08/19/1999	Control	0.132	0.133	0.001
09/09/1999	Control	0.132	0.133	0.001
09/09/1999	Control	0.134	0.135	0.001
08/19/1999	3.85 E-3 M	0.164	0.164	0.000
08/19/1999	3.85 E-3 M	0.164	0.165	0.001
09/09/1999	3.85 E-3 M	0.164	0.165	0.001
09/09/1999	3.85 E-3 M	0.159	0.160	0.001
08/19/1999	1.92 E-2 M	0.170	0.170	0.000
08/19/1999	1.92 E-2 M	0.173	0.174	0.001
09/09/1999	1.92 E-2 M	0.170	0.171	0.001
09/09/1999	1.92 E-2 M	0.172	0.173	0.001
08/19/1999	3.85 E-2 M	0.187	0.188	0.001
08/19/1999	3.85 E-2 M	0.183	0.185	0.002
09/09/1999	3.85 E-2 M	0.183	0.184	0.001
09/09/1999	3.85 E-2 M	0.185	0.187	0.002

TABLE 5.6 Complete metal ion testing summary table. This table lists the change in absorbance over time for all the selected metals tested, at 0.00 mM concentration serving as control, 3.85 mM, 19.2 mM, and 38.5 mM concentrations respectively. This table was used for analysis of variance.

	Absorbance Change (Δ A) at Various Concentrations			
	0.00 mM	3.85 mM	19.2 mM	38.5 mM
Calcium	0.001	0.008	0.010	2.249
	0.001	0.008	0.011	2.242
	0.001	0.007	0.009	2.200
	0.001	0.008	0.009	2.300
Cadmium	0.002	0.416	2.230	2.334
	0.001	0.446	2.196	2.332
	0.001	0.455	2.185	2.341
	0.002	0.448	2.240	2.301
Lead	0.002	0.126	0.690	1.530
	0.001	0.126	0.680	1.580
	0.001	0.127	0.660	1.560
	0.001	0.123	0.690	1.510
Copper	0.001	0.005	0.008	0.091
	0.002	0.005	0.009	0.096
	0.001	0.005	0.007	0.097
	0.001	0.005	0.010	0.098
Magnesium	0.001	0.000	0.000	0.001
	0.001	0.001	0.001	0.002
	0.001	0.001	0.001	0.001
	0.001	0.001	0.001	0.002
Zymosan alone	0.008			
	0.007			
	0.006			
	0.007			

TABLE 5.7 Singe-Factor ANOVA table showing that there is no significant difference (F-value = 4.077, F-critical = 5.98) in the change in absorbance over time for prophenoloxidase enzyme solutions containing 38.5 mM Ca^{2+} ions (nominal) in the presence of Zymosan elicitor and no Zymosan elicitor.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
No Zymosan	4	8.563	2.14075	0.00738892
Zymosan	4	8.921	2.23025	0.00046825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0160205	1	0.0160205	4.0779331	0.0899765	5.98737415
Within Groups	0.0235715	6	0.00392858			
Total	0.039592	7				

TABLE 5.8 Two-Factor ANOVA table showing significant difference in absorption with increasing concentrations (F-value = 30485.41, F-critical = 2.75 for 3 degrees of freedom), change in cation tested (F-value = 19910.38, F-critical = 2.52 with 4 degrees of freedom), and interaction within (F-value = 7635.89, F-critical = 1.92 for 12 degrees of freedom).

SUMMARY	0.00 mM	3.85 mM	19.2 mM	38.5 mM	Total
Calcium					
Count	4	4	4	4	16
Sum	0.004	0.031	0.039	8.991	9.065
Average	0.001	0.00775	0.00975	2.24775	0.5665625
Variance	0	2.5E-07	9.16667E-07	0.001681583	1.005286929
Cadmium					
Count	4	4	4	4	16
Sum	0.006	1.765	8.851	9.308	19.93
Average	0.0015	0.44125	2.21275	2.327	1.245625
Variance	3.33333E-07	0.00029825	0.000696917	0.000315333	1.14681385
Lead					
Count	4	4	4	4	16
Sum	0.005	0.502	2.72	6.18	9.407
Average	0.00125	0.1255	0.68	1.545	0.5879375
Variance	0.00000025	3E-06	0.0002	0.000966667	0.395565929
Copper					
Count	4	4	4	4	16
Sum	0.005	0.02	0.034	0.382	0.441
Average	0.00125	0.005	0.0085	0.0955	0.0275625
Variance	0.00000025	0	1.66667E-06	9.66667E-06	0.001650396

Table 5.8 Continued

Magnesium					
Count	4	4	4	4	16
Sum	0.004	0.003	0.003	0.006	0.016
Average	0.001	0.00075	0.00075	0.0015	0.001
Variance	0	0.00000025	0.00000025	3.33333E-07	2.66667E-07

Total				
Count	20	20	20	20
Sum	0.024	2.321	11.647	24.867
Average	0.0012	0.11605	0.58235	1.24335
Variance	1.68421E-07	0.030194892	0.771336345	1.081282239

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	16.62882093	4	4.157205231	19910.38406	6.18845E-93	2.525212039
Columns	19.09512224	3	6.365040746	30484.51995	1.97798E-95	2.75807821
Interaction	19.13211058	12	1.594342548	7635.892549	9.65591E-91	1.91739602
Within	0.01252775	60	0.000208796			
Total	54.86858149	79				

TABLE 5.9 Ionic radii regression analysis table and regression statistics for a nominal concentration of 3.85 mM concentration of Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}. The regression analysis revealed no correlation (R square = 0.0597) between ionic radii and enzymatic activity.

3.85 mM	Radius (A)	Enzyme Activity
Mg	0.78	0.00075
Cu	0.96	0.005
Cd	1.03	0.44125
Ca	1.06	0.00775
Pb	1.32	0.1255

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.244
R Square	0.060
Adjusted R Square	-0.254
Standard Error	0.219
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.009095624	0.009095624	0.190	0.692
Residual	3	0.143304376	0.047768125		
Total	4	0.1524			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.000752365	0.118516311	8.444005333	0.00348595	0.623580214	1.377924516
Enzyme Activity	0.252026152	0.577561801	0.436362223	0.692069746	-1.586034993	2.090087296

TABLE 5.10 Ionic radii regression analysis table and regression statistics for a nominal concentration of 19.2 mM concentration of Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}. The regression analysis revealed no correlation (R square = 0.0693) between ionic radii and enzymatic activity.

19.2 mM	Radius (A)	Enzyme Activity
Mg	0.78	0.00075
Cu	0.96	0.0085
Cd	1.03	2.21275
Ca	1.06	0.00975
Pb	1.32	0.68

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.263
R Square	0.069
Adjusted R Square	-0.241
Standard Error	0.217
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.010563199	0.010563199	0.223	0.669
Residual	3	0.141836801	0.047278934		
Total	4	0.1524			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.998728183	0.117612948	8.491651616	0.003429464	0.624430939	1.373025428
Enzyme Activity	0.05369935	0.113607011	0.472676377	0.668704875	-0.3078492	0.4152479

TABLE 5.11 Ionic radii regression analysis table and regression statistics for a nominal concentration of 38.5 mM concentration of Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}. The regression analysis revealed no correlation (R square = 0.330) between ionic radii and enzymatic activity.

TABLE 5.12 Single Factor Analysis of Variance Table showing that increasing the concentration of Cadmium has a significant effect on the Change in Absorbance over time on the proPO system isolated from Crayfish hemolymph. The F-value of 17493.4 is higher than the F-critical value of 3.49, for 3 degrees of freedom.

SUMMARY Cadmium

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	0.006	0.0015	3.33333E-07
3.85 mM	4	1.765	0.44125	0.00029825
19.2 mM	4	8.851	2.21275	0.000696917
38.5 mM	4	9.308	2.327	0.000315333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.19827525	3	5.732758417	17493.48	4.185E-22	3.490
Within Groups	0.0039325	12	0.000327708			
Total	17.20220775	15				

TABLE 5.13 Single Factor Analysis of Variance Table showing that increasing the concentration of Calcium has a significant effect on the Change in Absorbance over time on the proPO system isolated from Crayfish hemolymph. The F-value of 40480.7 is higher than the F-critical value of 3.49, for 3 degrees of freedom.

SUMMARY Calcium

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	0.014	0.0035	0.000019
3.85 mM	4	0.031	0.00775	2.5E-07
19.2 mM	4	0.039	0.00975	9.16667E-07
38.5 mM	4	8.921	2.23025	0.00046825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.82860319	3	4.942867729	40480.7	2.728E-24	3.490
Within Groups	0.00146525	12	0.000122104			
Total	14.83006844	15				

TABLE 5.14 Single Factor Analysis of Variance Table showing that increasing the concentration of Copper has a significant effect on the Change in Absorbance over time on the proPO system isolated from Crayfish hemolymph. The F-value of 2845.6 is higher than the F-critical value of 3.49, for 3 degrees of freedom.

SUMMARY Copper

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	0.005	0.00125	0.00000025
3.85 mM	4	0.02	0.005	0
19.2 mM	4	0.034	0.0085	1.66667E-06
38.5 mM	4	0.382	0.0955	9.66667E-06

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.024721188	3	0.008240396	2845.6	2.242E-17	3.490
Within Groups	3.475E-05	12	2.89583E-06			
Total	0.024755938	15				

TABLE 5.15 Single Factor Analysis of Variance Table showing that increasing the concentration of Lead has a significant effect on the Change in Absorbance over time on the proPO system isolated from Crayfish hemolymph. The F-value of 6758.2 is higher than the F-critical value of 3.49, for 3 degrees of freedom.

SUMMARY Lead

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	0.005	0.00125	0.00000025
3.85 mM	4	0.502	0.1255	3E-06
19.2 mM	4	2.72	0.68	0.0002
38.5 mM	4	6.18	1.545	0.000966667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.929979188	3	1.976659729	6758.29	1.256E-19	3.490
Within Groups	0.00350975	12	0.000292479			
Total	5.933488938	15				

TABLE 5.16 Single Factor Analysis of Variance Table showing that increasing the concentration of Magnesium has no significant effect on the Change in Absorbance over time on the proPO system isolated from Crayfish hemolymph. The F-value of 2.4 is not higher than the F-critical value of 3.49, for 3 degrees of freedom.

SUMMARY Magnesium

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	0.004	0.001	0
3.85 mM	4	0.003	0.00075	0.00000025
19.2 mM	4	0.003	0.00075	0.00000025
38.5 mM	4	0.006	0.0015	3.33333E-07

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0000015	3	0.0000005	2.4	0.1187	3.490
Within Groups	0.0000025	12	2.08333E-07			
Total	0.000004	15				

TABLE 5.17 Protein Marker Calibration Table. The relative mobility (R_f) of the protein markers and the log10 of the molecular weight was used to establish the slope for the electrophoresis gel. The slope was then used to calculate the molecular weight of the purified proteins based on their relative mobility in the gel.

Protein	Molecular weight (Da)	Marker R_f	Log(MW)	Protein R_f	Log(MW)	Protein weight (Da)
α2-Macroglobulin	205000	0.06	5.31	0.03	5.26	184072
β-Galactosidase	116000	0.10	5.06	0.21	4.88	76227
Phosphorylase B	97000	0.18	4.99	0.41	4.46	28621
Catalase	58100	0.23	4.76	0.46	4.35	22404
Alcohol Dehydrogenase	39800	0.32	4.60	0.48	4.31	20314
Carbonic Anhydrase	29000	0.39	4.46	0.50	4.27	18418
Trypsin Inhibitor	20100	0.49	4.30			
Lysozyme	14300	0.55	4.16			
Aprotinin	6500	0.73	3.81			

APPENDIX - B Regression Analysis Charts

FIGURE **PAGE**

FIGURE 5.1 Ionic regression analysis chart for a nominal concentration of 3.85 mM for Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}, showing no correlation (R square = 0.0597) between ionic radii and enzyme activity.

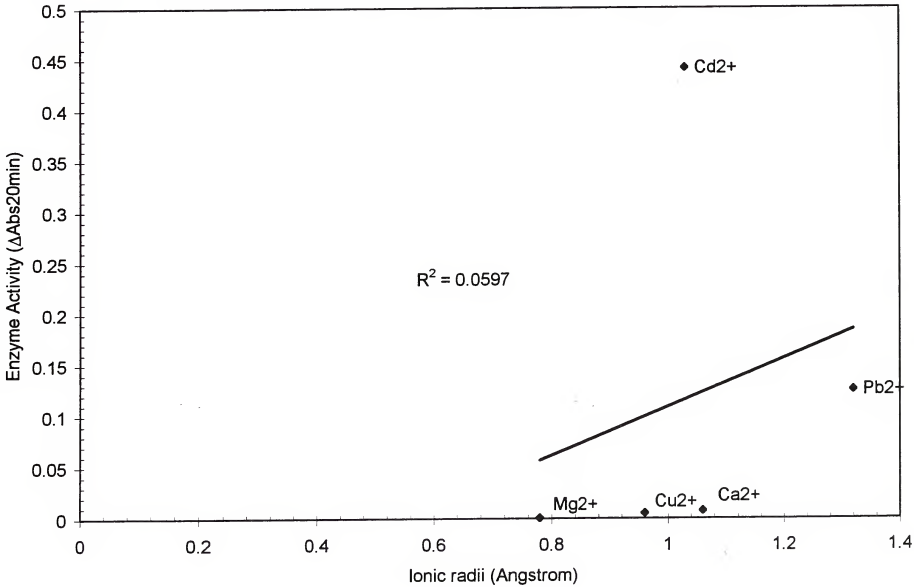
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Enzyme Activity (ΔAbs20min)
Cd2+
R2 = 0.0597
Pb2+
Mg2+
Cu2+
Ca2+
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Ionic radii (Angstrom)

FIGURE 5.2 Ionic regression analysis chart for a nominal concentration of 19.2 mM for Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}, showing no correlation (R square = 0.0693) between ionic radii and enzyme activity.

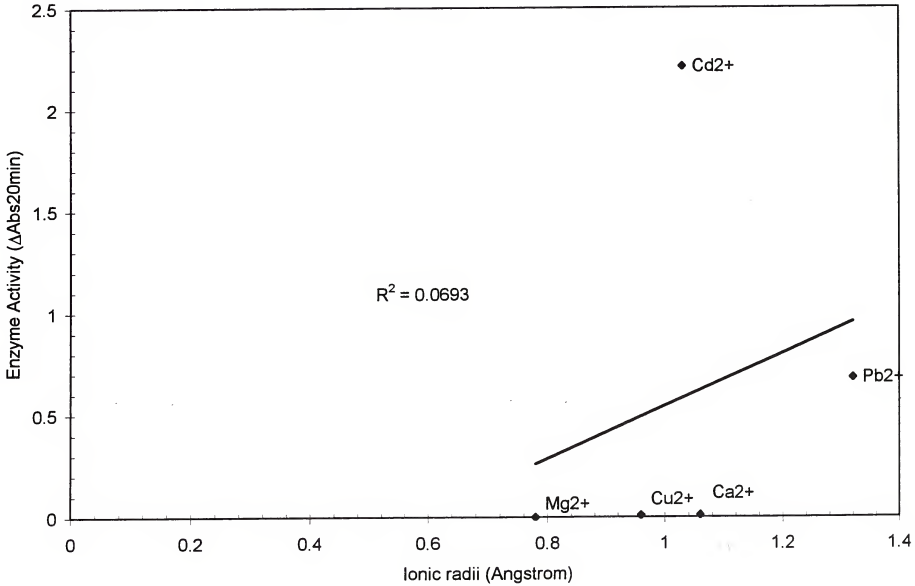
2.5
2
1.5
1
0.5
0
Enzyme Activity (ΔAbs20min)
Cd2+
R2 = 0.0693
Pb2+
Mg2+
Cu2+
Ca2+
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Ionic radii (Angstrom)

FIGURE 5.3 Ionic regression analysis chart for a nominal concentration of 38.5 mM for Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}, showing no correlation (R square = 0.330) between ionic radii and enzyme activity.

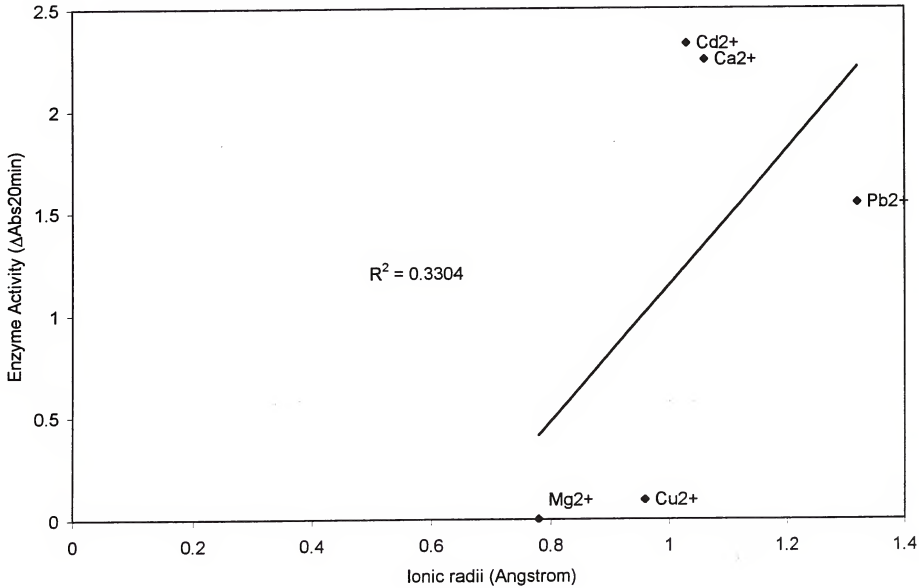
Cd2+
Ca2+
Pb2+
R^2 = 0.3304
Mg2+
Cu2+
Enzyme Activity (ΔAbs20min)
Ionic radii (Angstrom)
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4

FIGURE 5.4 Cadmium testing comparison chart. This chart shows that there is a marked increase in the rate of dopachrome formation when the concentration of cadmium is increased from 3.85 mM to 19.2 mM Cd^{2+}. ANOVA analysis indicated that there was a significant change in the rate of dopachrome formation with increasing cadmium ion concentration (F-value = 17493.5, F-critical = 3.49).

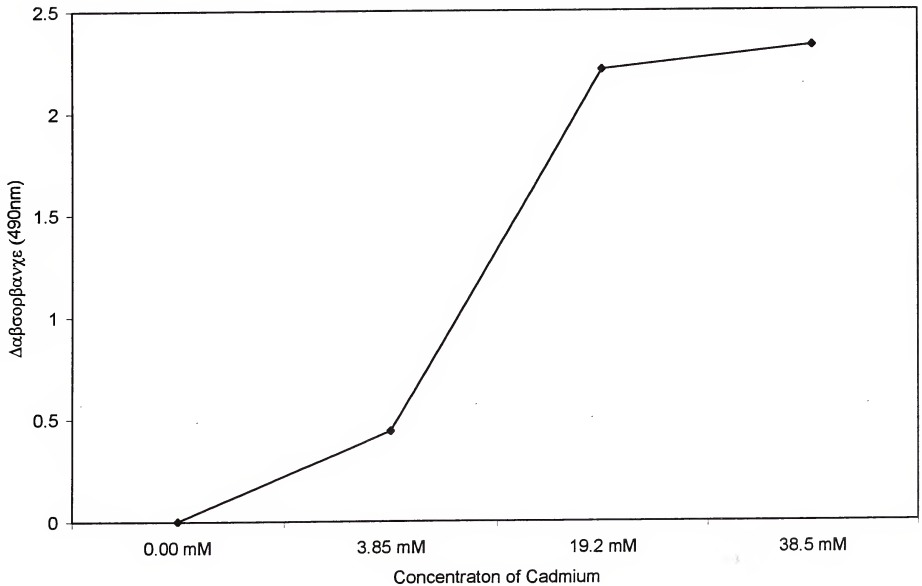
2.5
2
1.5
1
0.5
0
Δαβσορβανχε (490nm)
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Concentraton of Cadmium

FIGURE 5.5 Calcium testing comparison chart. This chart shows that there is an apparent threshold level at which the rate of dopachrome formation occurs more rapidly, as indicated by the change in slope above 19.2 mM Ca^{2+}. There appears to be a leveling off at the higher concentration of calcium. ANOVA analysis confirmed a significant change in dopachrome formation with increasing calcium ion concentration (F-value = 40480.7, F-critical = 3.49).

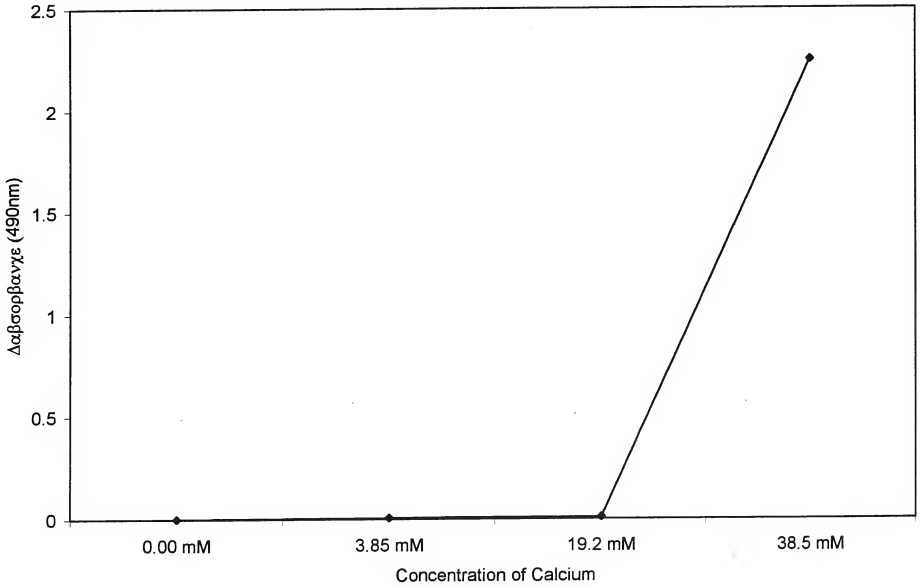
2.5
2
1.5
1
0.5
0
Δαβσορβανχε (490nm)
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Concentration of Calcium

FIGURE 5.6 Copper testing comparison chart. The rate of dopachrome formation increases linearly up to 19.2 mM Cu^{2+}, then a threshold is reached and the rate increases significantly as indicated by the change in slope. ANOVA analysis revealed significant change in dopachrome formation with increased copper ion concentration (F-value = 2845.6, F-critical = 3.49).

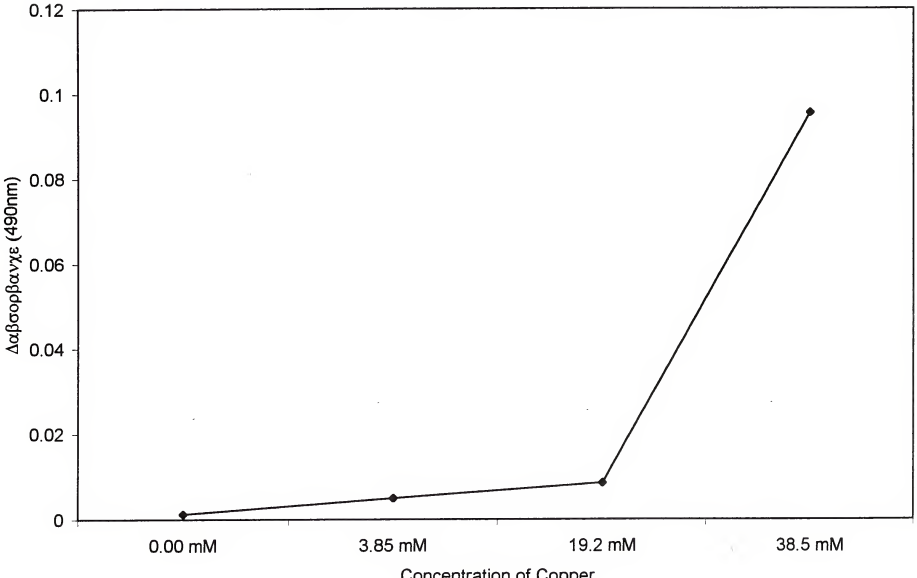
0.12
0.1
0.08
0.06
0.04
0.02
0
Δαβσορβανχε (490nm)
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Concentration of Copper

FIGURE 5.7 Lead testing comparison chart. The change in the rate of dopachrome formation appears to increase incrementally with increasing lead ion concentration. The upper threshold was not reached. ANOVA analysis revealed a significant change in dopachrome formation with increasing lead ion concentration (F-value = 6758.3, F-critical = 3.49).

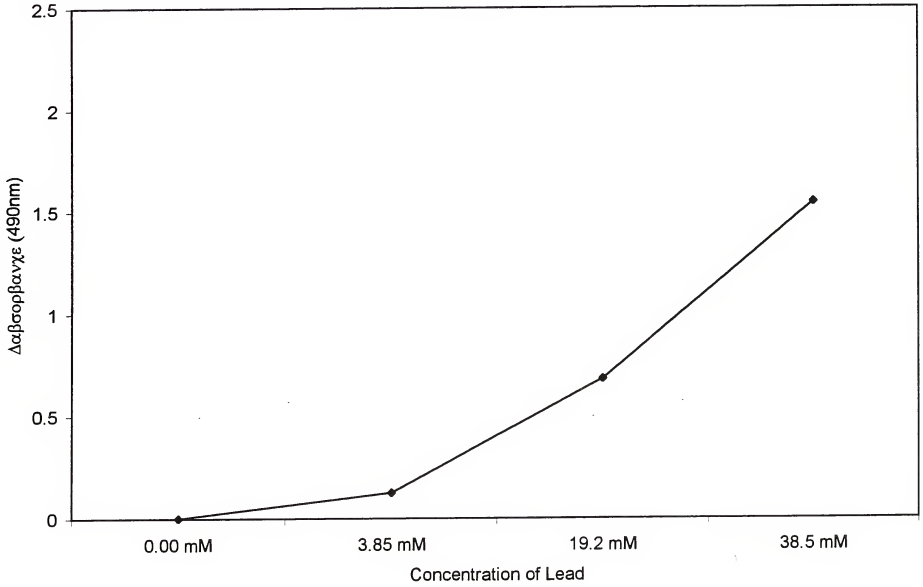
2.5
2
1.5
1
0.5
0
Δεβσορβανχε (490nm)
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Concentration of Lead

FIGURE 5.8 Magnesium testing comparison chart. The lower levels of magnesium tested appeared to suppress dopachrome formation slightly. At the highest concentration tested, a slight increase in dopachrome formation was observed. No upper threshold was reached. Previous ANOVA analysis revealed no correlation between magnesium ion concentration and rate of dopachrome formation (F-value = 2.4, F-critical = 3.49).

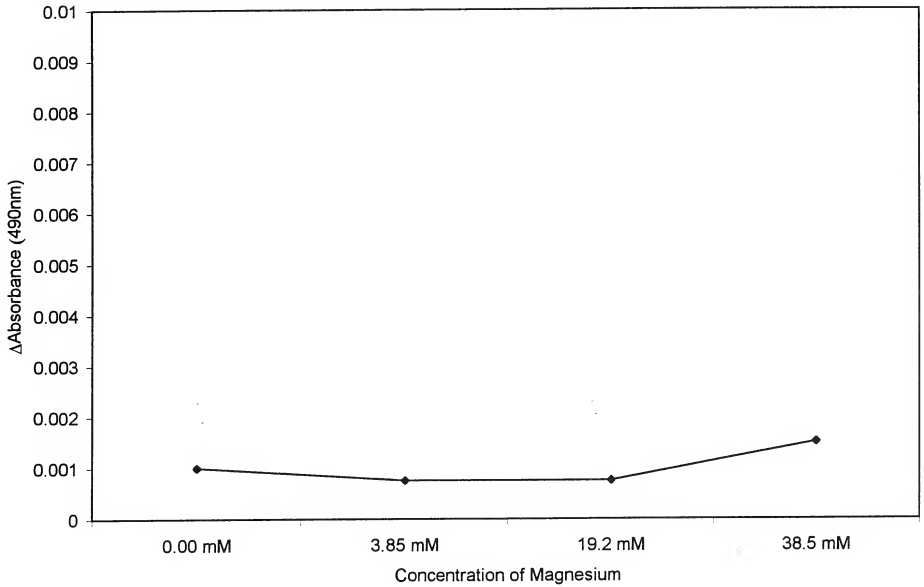
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
ΔAbsorbance (490nm)
0.00 mM
3.85 mM
19.2 mM
38.5 mM
Concentration of Magnesium

FIGURE 5.9 Calibration Curve for 7.5% SDS-PAGE. The relative mobility (R_f) of the protein markers were plotted against the log of their molecular weight. A least square fit line was then inserted to obtain the slope.

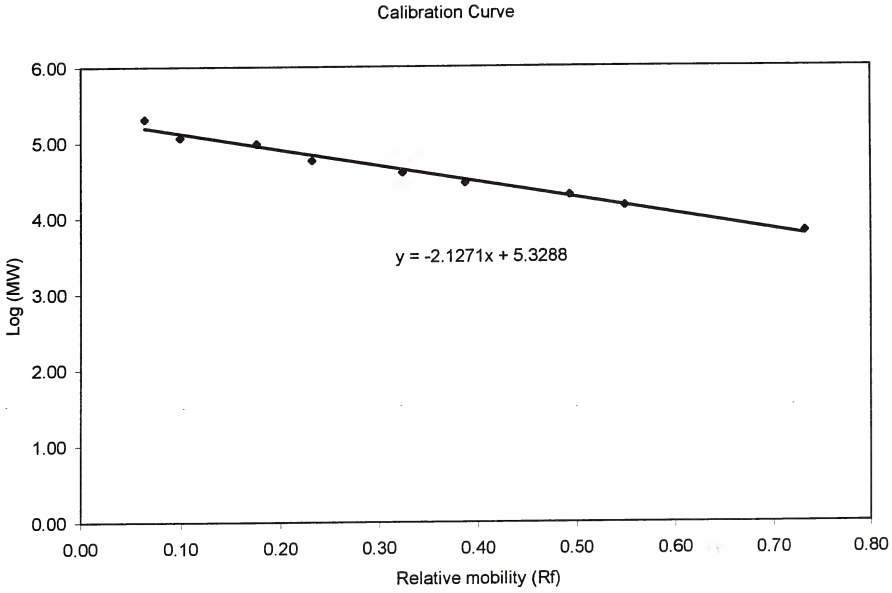
Calibration Curve
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Log (MW)
y = -2.1271x + 5.3288
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
Relative mobility (Rf)

FIGURE 5.10 7.5% SDS-PAGE showing three lanes. The first lane shows the protein markers, the second lane shows protein bands in crude hemolymph extract, and the third lane shows protein bands in purified hemolymph extract. The scanned image has been digitally enhanced to better show the protein bands in the marker. So, the band darkness is no indication of protein abundance.

38.5 mM	Radius (A)	Enzyme Activity
Mg	0.78	0.0015
Cu	0.96	0.0955
Cd	1.03	2.327
Ca	1.06	2.24775
Pb	1.32	1.545

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.575
R Square	0.330
Adjusted R Square	0.107
Standard Error	0.184
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.050350694	0.050350694	1.480	0.311
Residual	3	0.102049306	0.034016435		
Total	4	0.1524			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.906867102	0.130561824	6.945882584	0.006120619	0.491360717	1.322373487
Enzyme Activity	0.099033175	0.081399619	1.216629471	0.310731158	-0.160016984	0.358083333