PARTIAL PURIFICATION AND CHARACTERIZATION OF PHENOLOXIDASE IN COCONUT BEETLE LARVAE (*Oryctes rhinoceros L.*)

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Abstract

Coconut beetle larvae have a protein called phenoloxidase present in their hemolymph that is essential in arthropod immune response. Prior to characterization and isolation of an enzyme designing optimum assay conditions is crucial. This will stabilize the enzyme and ensure optimal activity. In this study, phenoloxidase activity was measured as a function of varying substrates, activators with their incubation periods and incubation temperatures, and buffer pH. The substrate 4-methylcatechol appeared as the best substrate for the enzyme. Among the activators tested, trypsin efficiently activated phenoloxidase within 20-60 minutes incubation periods at 30°C in sodium phosphate buffer with pH ranging from 6.0 to 8.0. The hemolymph was partially purified for pro-phenoloxidase through DEAE and SP ion-exchange chromatography. Fractions positive for enzyme activity were loaded on SDS-PAGE. Partially purified prophenoloxidase shows a molecular weight of 85-92 kDa while its active form (phenoloxidase) has a molecular weight of 62-67 kDa.

1.0 INTRODUCTION

1.1 Background of the Study

The coconut beetle (*Oryctes rhinoceros* L.) is the most destructive and widely distributed pest of coconut. It has an enzyme component called phenoloxidase that is important for defense by detecting, encapsulating and killing invading pathogen as well wound healing through melanin synthesis. It is the key enzyme in insect sclerotization and melanization, which also occupies several major roles in insect development and immunity. Phenoloxidase is universally present in insects due to the positive results of all tests from other insects. However, phenoloxidase has not been isolated and studied in a local insect like the coconut beetle.

This study focuses on purification and characterization of the activity of phenoloxidase enzyme in coconut beetle larvae. The wide range of immune activity triggered by phenoloxidase is due to the phenoloxidase cascade. From studies, it has been found out that phenoloxidase is present in other insects as an inactive zymogen (prophenoloxidase), thus it needs an activator before it could catalyze any reaction. Several variables such as substrate, activator (with its incubation period and temperature), and pH of the buffer were tested to determine the optimum activity of the phenoloxidase enzyme.

Purification involves isolation of the enzyme in order to observe the biological properties. This was done using centrifugation technique, SDS-PAGE and ion-exchange column chromatography.

Purification and characterization of the enzyme activity will lead to further understanding of the properties of phenoloxidase. This will be useful in determination of possible inhibitor for the enzyme activity. Inhibition of phenoloxidase activity entails a deficiency in the immune system of the coconut beetle. Detection of phenoloxidase inhibitors will therefore lead to possible biological pest control for the coconut beetle. Thus, this study aims to understand the phenoloxidase activity under different conditions and determine the molecular weight of the partially purified enzyme. However, further study is still needed to determine an effective inhibitor for the phenoloxidase enzyme in coconut beetle.

1.2 Statement of the Problem

1. What is the phenoloxidase activity of Philippine *Oryctes rhinoceros* larva in the presence of different substrates, activators (with its incubation period and temperature), and pH of the buffer?
2. What are the properties of partially purified phenoloxidase based on SDS-PAGE and column chromatography?

1.3 Research Objectives

1. To isolate and partially purify phenoloxidase in Philippine *Oryctes rhinoceros* larva hemolymph plasma

2. To measure phenoloxidase activity as a function of varying substrates, activators (with its incubation period and incubation temperature), and buffer pH
3. To determine the molecular weight of partially purified phenoloxidase

1.4 Significance of the Study

The Philippines remains as the world's leading supplier of traditional coconut products. The coconut is a crop that yearly provides benefits for 1.5 million farmers and farm workers. However, insect infestation causes damage to coconut trees. The great impact of insects on agriculture, food industry, and health leads to several researches about them. The study of phenoloxidase in coconut beetle larvae is a basic research that gives information regarding the immune response of the insect that is the primary pest in coconut trees. Through analysis of the enzymatic action of phenoloxidase with different substrates and with an activator, the specificity of the enzyme was determined.

This study may be the basis of other researches that aims to control pest infestation through the inhibition of the immune response of coconut beetle for an effective application in pest control. Through the obtained results, there will be additional knowledge about phenoloxidase thus providing broader background for the study of its inhibitor. Since phenoloxidase is universally present in arthropods, the study of

phenoloxidase activity in coconut beetle will also give additional information about the enzyme involved in insect immunity.

1.5 Scope and Limitation

Oryctes rhinoceros L. larvae were used as test animals. The larvae were gathered randomly from coconut fields of Quezon province. The hemolymph was tested for enzyme activity with different substrates in the presence of an activator. The incubation temperature and incubation period of the activator was varied to optimize the conditions needed for activation of the enzyme. The optimum pH of the buffer was also determined. Determination of the presence of phenoloxidase in *Oryctes rhinoceros* hemolymph (which includes the hemocytes and hemolymph plasma) was confirmed by enzyme assay and SDS-PAGE. Phenoloxidase was purified using column chromatography and its concentration was determined using calorimetric analysis or Bradford's Method.

Phenoloxidase activity was studied using different substrates that include L-dopa, 4-methylcatechol, catechol, hydroquinone and tyrosine by spectrophotometric assay. The wavelengths that were used in the spectrophotometric assay were predetermined from different journals stated in our methodology. These are the wavelengths wherein the toxic product of phenoloxidase and the substrate is absorbed. However, the products from the reactions were not identified but instead, the absorbance which implies the amount of products formed were emphasized. Activators with its varying

incubation period and temperature are trypsin, chymotrypsin, and propanol. Incubation period ranged from 0-60 minutes while incubation temperature ranged from $4^0C - 40^0C$. pH of the buffer were varied from 2.6 to 12.

Phenoloxidase in hemolymph was partially purified using anion (DEAE) and cation (SP) column chromatography. Presence of proteins in fractions was determined using UV-Vis spectrophotometric assay under the determined optimum conditions. Prophenoloxidase and phenoloxidase molecular weights was determined using SDS-PAGE on fractions with high absorbance in the assay. Protein bands in the gel having a molecular weight far from any proteins involved in phenoloxidase cascade were not identified. Molecular weights of light bands were not computed.

Study was conducted from July-December of 2001, which included sample collection and the actual experiment using the determined and established optimum conditions.

1.6 Definition of Terms

- Prophenoloxidase – the zymogen, or inactive form of the enzyme that needs an activator before it could catalyze any reaction (Söderhäll and Cerenius, 1998).
- Phenoloxidase Cascade – the reactions involve in enzyme immunity that involves phenoloxidase as the key enzyme; this starts from the recognition of pathogens and the activation of phenoloxidase up to the

production of metabolites for the insect's defense mechanism (Söderhäll and Cerenius, 1998).

- Enzyme Assay – indispensable biochemical tool that is used principally for determining the amount of an enzyme as well as the concentration of non-enzymic molecules such as substrates in a biological material.
- Enzyme Activity – behavior of the enzyme when catalyzing the reaction of substrates in the formation of products. This could be characterized by describing the spectrophotemetric absorbance of the enzyme with a substrate. At a specific wavelength, a specific product is absorbed. Therefore, a high absorbance implies a high production of enzyme-substrate products (Birch, 2001).
- SDS Polyacrylamide Gel Electrophoresis – characterize the number and size of protein chains or protein subunit chains in a given preparation regardless of its inherent solubility in aqueous solutions. Based from the Protein purification handbook (1999), separation of proteins by this technique led to observation that the mobilities of these proteins were a linear function of the logarithms of their molecular weights.
- Ion-exchange chromatography – based from the Protein Purification Handbook (1999), this is a special kind of adsorption chromatography defined as the reversible exchange of ions in solution with ions electrostatically bound to an adsorbent called ion-exchanger.

2.0 REVIEW OF RELATED LITERATURES

Phenoloxidase has been the most studied enzyme in insects. However, there has been no study of phenoloxidase characteristics in the coconut beetle in any of the four stages in its life cycle making our study novel. Phenoloxidase characteristics vary in species but related species has some similarities. Our review of related literatures include information of the species itself. An in-depth review of the mechanism of phenoloxidase and its characteristics in different species already studied will be included.

2.1 Coconut Beetle

The coconut rhinoceros beetle (*Oryctes rhinoceros* L.) is the most destructive and widespread pest involving the coconut. *Oryctes rhinoceros* is under Order Coleoptera and Family Scarabaeidae. These beetles grow to be between 1 and 2 ½ inches long. Since both sexes have horns, the only way to distinguish the two is the presence of less sensory hair at the end of the male abdomen. Their horns are used for digging, climbing, and mating. In males, the horns are also used for fighting over mate and breeding grounds. When they are stressed, they make a sound by rubbing their abdomens against the ends of their wing covers as a sign of defense. Outer shells are hard and tough to serve as protection (Corwin, 2001).

The coconut beetle is said to be the strongest creatures on earth, with it being able to carry 850 times their own weight. They eat rotting wood and compost material, with the larvae eating a great deal compared to the adult

(Corwin, 2001). Coconut beetle larvae help recycle plant matter back into the ecosystem through their feeding. Adults are the damaging stage of the insect wherein they feed by tunneling into the coconut leaves causing a V-shaped notch in the process. Rhinoceros coconut beetles are active during the night and hide during the day to prevent being eaten by predators. During the night, they are greatly attracted by light (Crop Protection Division, 1994).

2.1.1 Life Cycle

Coconut beetle have four stages of development in their life cycle namely eggs, larva, pupa, and adult. Eggs are laid in moist, dead and decaying coconut logs. They could also be found in sawdust, farmyard manure, sugarcane trashes, corn cubs, and rice straws but never in living tissues of plants. They are round and white in color having a diameter of 3 to 4mm. Incubation period of eggs is 7-18 days (Crop Protection Division, 1994). The eggs hatch into whitish grubs called curl grubs due to its form.

Larva is a white and curved creature with a brown head and three pairs of legs. Length of fully-grown larva is from 60 to 105 mm. Coconut beetle larvae have three instars before they change into a pupa. This means that they undergo three molts before going into the pupal stage. The entire larval period last about 80 to 130 days.

Pupal stage occurs in the deeper layers of the soil. Pupa is enclosed in a cocoon composed of soil and coconut fibers. The pupal period last about 14 to 29 days.

Adult is the destructive stage that last to about four months. They are brownish black with a median horn curved backward on its head. Their diet includes feeding on unopened leaves and bud. A female adult can lay as many as 70 to 140 eggs in its lifetime (Crop Protection Division, 1994).

2.1.2 Control Measure of Coconut Beetle

The beetle can be prevented and controlled by field sanitation, biological means and regular inspection (Crop Protection Division, 1994). Field sanitation involves elimination of potential breeding sites. Standing coconut logs, stumps and rubbish piles should be cleared away. These may be utilized for other purposes like lumber, fertilizers and firewood. Biological control involves the use of green muscardine virus (GMF) and/or baculovirus. The GMF infects the larvae, pupae and adult beetles by contact with the spores in the breeding site, thus the beetles die after 8-13 days of inoculation. The baculovirus is a microorganism that infects the coconut beetle in all its developmental stages except the egg. The infected beetles become inviable and diseased causing its death. Regular farm inspection is done by regularly scooping out beetles attacking young palms before heavy damage can occur (Crop Protection Division, 1994).

Researches are being conducted to study the enzymes in arthropods like the phenoloxidase, which is responsible for the immune response of coconut beetle against the invasion of microorganisms. Larvae are chosen to be the test animal when it is hard to obtain hemolymph from adult insects. The study of phenoloxidase will lead to analysis of phenoloxidase inhibitor

for possible application of pest control. Inhibition of phenoloxidase activity is a good method of eliminating insect pests because it is found in all arthropods and in all cases, it is the key enzyme for the insect's immune response. Before any biological pest application can be done concerning phenoloxidase, the enzyme's activity must be studied in the target insect along with the main pathway in which phenoloxidase triggers reaction.

2.2 Phenoloxidase Role in Invertebrate Immunity

The survival of invertebrates, including insects, depends on the extent of successful defenses against microorganisms and parasites. Since most invertebrates live in environments where microorganisms thrive, they must have efficient means of recognizing and combating harmful microorganisms (Söderhäll and Cerenius, 1998). However, invertebrate animals lack antibodies and adaptive immune response that is why they have efficient innate immune systems to defend themselves against invading foreign materials. The defense mechanism of invertebrates may be classified as cellular response and humoral immune response. The cellular response involves encapsulation, phagocytosis and nodule formation. The humoral immune responses are the clotting system, synthesis of potent antimicrobial proteins and the phenoloxidase activating system (Lee et al., 2000). These humoral responses are triggered by lipopolysaccharide (LPS), peptidoglycans or β-1,3 glucans.

The LPS, peptidoglycans and β-1,3 glucans bind specifically to a pattern recognition protein to activate the biological defense mechanism. The pattern recognition proteins serve as biosensors for detection of invading pathogens in the innate immune systems of vertebrate and invertebrate animals (Ma and Kanost, 1999). Upon binding to the foreign invaders, pattern recognition protein trigger defense pathways such as complement systems in vertebrates and the prophenoloxidase activation pathway in insects and other arthropods (Lee et al., 2000). Some pattern recognition proteins are β-1,3 glucan binding protein, peptidoglycan recognition proteins, lectins and hemolin.

2.2.1 Prophenoloxidase, the Inactive Form of Phenoloxidase

Since Ohnishi's paper was published in 1954, phenoloxidase has been accepted to be present in insect hemolymph in its inactive form (Ohnishi, 1954). Prophenoloxidase (proPO) is the precursor and inactive form of the enzyme phenoloxidase that is found in invertebrates both protostome and deuterostome. Most of them are located in vesicles within the blood cells and exist as zymogens. However, proPO may be synthesized in different parts. Crustacean and insect proPO are synthesized in the blood cells while crayfish hemocyanin are synthesized in the hepatopancreas. It is often studied in the hemolymph of arthropods, either the plasma or in the haemocytes (Söderhäll and Cerenius, 1998).

The monomer of the inactive proenzyme in arthropods has a mass of 70 to 80 kDa in cases where it has been isolated and purified (Söderhäll and

Cerenius, 1998). Several researches show that proPO can exist in polymeric form. Test done also indicates that proPOs is a carbohydrate-containing protein (Durrant et al., 1993). Presence of cupric copper was also detected in an arthropod (Ashida, 1971). Several arthropod proPOs have been determined to lack a signal peptide and contain two functional copper-binding sites, both of which are functional. Some species may have two or more forms of proPO wherein the different proPOs have different functions. Estimate of proPO molecular weights has been determined in many invertebrates including crayfish (Durrant et al., 1993), *Manduca* (Hall et al., 1995), *Bombyx* (Durrant et al., 1993), and many others as well as the coleopteran *Tenebrio* (Lee et al., 1999).

2.2.2 Proteins Involve in the Activation of Phenoloxidase

Activation of prophenoloxidase to phenoloxidase involves a Ca^{2+}-requiring enzyme cascade that involves serine proteinases with molecular masses of about 30 kDa. The proteinase activates the proPO through proteolytic cleavage (Söderhäll and Cerenius, 1998). So far, two serine proteases of insect proPO cascade have been purified. One, which was purified from the silkworm haemocyte, is referred to as BAEEase (BAEE: N^{α}-benzoyl-L-arginine ethyl ester) and is activated from the inactive zymogen pro-BAEEase through proteolysis. The other activating enzyme is named proPO-activating enzyme (PPAE). It was the first activating enzyme purified which was extracted from larval cuticles of silkworms (Satoh et al.,1999). Prophenoloxidase-activating system (proPO-AS) is activated when

the recognition protein in the organisms recognized the microbial cell wall constituents like the β-1,3 glucans, peptidoglycans, and lipopolysaccharide. In some species, endogenous lectins can also trigger the activation of proPO-AS (Söderhäll and Cerenius, 1998).

Research showed that β-1,3 glucan binding protein represents a suprisingly high percentage for a protein involved in the initial phase of prophenoloxidase activating system (Duvic and Söderhäll, 1990). It indicates that β-1,3 glucan binding protein may function in binding to foreign invaders carrying β-1,3 glucan in their cell wall and opsonic protein in the removal of the microorganisms from circulation (Duvic and Söderhäll, 1990). Other studies showed that β-1,3 glucan binding protein is expressed in body fat secreted into hemolymph. In the presence of laminarin (soluble glucan), β-1,3 glucan binding protein stimulated activation of prophenoloxidase in plasma of the insect *Manduca sexta* (Ma and Kanost, 2001).

Therefore, when β-1,3 glucan bind specifically to pattern recognition protein there will be an activation of coagulation cascade like the prophenoloxidase system. It will also result to an opsonic effect, degranulation of blood cells, and hemocyte nodule formation (Lee et al., 2000). Molecular weight of different enzymes involve in activating prophenoloxidase was determined in many invertebrates including the coleoptera *Holotrichia diomphalia* (Kwon et al., 2000)

2.2.3 Phenoloxidase Cascade

Phenoloxidase cascade is all the reactions involved in the activation of prophenoloxidase into phenoloxidase up to the formation of metabolic products involve in the immune response of invertebrates. In different invertebrates, phenoloxidase are usually found in the hemolymph and in the cuticle. Since insects is the usual test animal for the detection of this enzyme, phenoloxidase have been detected in accessory glands, egg cases, salivary glands, and mid-gut of different insects (Durrant *et al.*, 1993). After proteolytic activation of the inactive proPO, the active PO has a mass of 60 to 70kDa. The enzyme can exist as an oligomer in its native form. (Söderhäll and Cerenius, 1998).

Activation of the PO cascade will involve the catalyzation of several reactions, which includes the role of a tyrosinase, phenolase, o-phenol monooxygenase, o-diphenoloxidase, monoxidase, oxygen reductase, oxidase, catalyzing cresolase, and catecholase (Durrant *et al.*, 1993). Polyphenoloxidase, which has a similar catalyzing reactions like phenoloxidase has been found to posses two broad catalytic activities. These are 1) monophenolmonooxygenase or cresolase activity and 2) o-diphenoloxidase or catecholase activity. Cresolase activity involves hydroxylation of certain monophenols to o-dihydroxyphenols while catecholase activity coverts resultant diphenols to quinones (Sugumaran *et al.*, 1997). There are also some cases wherein high concentrations of calcium ions inhibit phenoloxidase activity (Brivio *et al.*, 1996). This is due to the

fact that PO is the key enzyme for the oxidation of phenolic substances like tyrosine, DOPA, and dopamine to melanin. These different reactions are part of the phenoloxidase role in sclerotization, melanin synthesis, and immune response that are interrelated. The PO responsible for the three results are referred to as laccase-type PO, granular PO, and injury PO respectively. Injury PO is found in both the hemolymph and cuticle. Present in the cuticle is the laccase-type PO, which is responsible for sclerotization of newly ecdysed cuticle. Granular PO, which is responsible for color pattern of the body and melanin synthesis, is also present in the cuticle and blood (Asano and Ashida, 2001).

In simple terms, the reaction involves the active enzyme catalyzing the oxygenation of monophenols to o-diphenols and oxidation of o-diphenols to quinones. The quinones in turn are polymerized nonenzymatically to melanin. Quinones, and other intermediate products of the PO cascade, are toxic to invading microorganisms and facilitate the formation of coagulum around the parasite and at the injured part (Satoh *et al.*, 1999). In support, melanin itself is synthesized for wound healing and encapsulation of pathogens.

PO has been implicated with the immune defense system of the invertebrates since they are concerned with the formation of highly toxic substances. Also, microbes and microbial parasites are melanized when they are encapsulated or nodulated (Durrant *et al*, 1993). Phenoloxidase cascade with concern to immunity is a nonself-recognizing cascade. It has been

discovered that PO activation is linked to blood coagulation activation implicating they have evolved from a common ancestral protease cascade (Nagai and Kawabata, 2000). Therefore, to prevent the production of products toxic to pathogens, inhibition of phenoloxidase should be executed. Inhibition of the phenoloxidase cascade will lead to higher mortality of the insect. But before inhibition can be done, the phenoloxidase activity with different molecules as well as its activation must be studied. Phenoloxidase activity in coconut beetle has not been previously studied. Some studies done in other animals include phenoloxidase activity under different pH in silkworm hemolymph (Ashida & Ohnishi, 1967) and activity of *Paraplerurus* phenoloxidase under different substrates (Nellaiappan & Ramalingam, 1980).

3.0. MATERIALS AND METHODS

3.1 Test Animal (Coconut Beetle Larvae)

The test animals that used were coconut beetle (*Oryctes rhinoceros* L.), also known as rhinoceros beetle, in their larval stage without specificity in instars. The larva has a prominent curled white body, brown head, and three pairs of anteriorly located legs. It is commonly found in rotting wood and compost material. One hundred larvae were collected from the fields of Tayabas, Quezon province. The larvae were raised in a container full of soil, compost, and coconut shavings in the National Institute of Molecular Biology and Biotechnology (NIMBB) where all the experiments are conducted.

3.2 Preparation of the Haemocyte-Lysate from the Specimen

Preparation of haemocyte-lysate was adapted from Durrant *et al.* (1993). Larvae were chilled at -4°C for 10-15 min in the freezer. Each larva was pre-injected with 100 µl ice-cold anticoagulant (10mM EDTA, 0.1M Glucose, 145 mM NaCl, 30mM trisodium citrate, 26 mM citric acid, pH 4.6).

The hemolymph was obtained by incision of the antero-ventral part of the larvae by cutting its anterior legs. The incised part of the larva was bled into a container until all the hemolymph was extracted. The hemolymph was centrifuged at 5000rpm for 2 min at 4°C using Sorvall RC 28S centrifuge. The supernatant obtained was dissolved in 50mM sodium phosphate buffer before analysis (Durrant *et al.*, 1993).

3.3 Determination of Enzyme Concentration Using Bradford's Method

The hemolymph's enzyme concentration was tested using a Bradford's Method. This method quantifies binding of Coomasie brilliant blue to an unknown protein and compared this binding to that of different amount of standard protein (Bovine Serum Albumin or BSA). This was done by the addition of duplicate aliquots of 0.5 mg/ml BSA (5, 10, 15, 20 µl) into microcentrifuge tube. The volume of each tube was brought to 100 µl with 0.15M NaCl. Two blank tubes containing only 100 µl of 0.15M NaCl were prepared. 1 ml of Coomassie blue solution will be added to each tube and mixed at vortex mixer for 2 min at room temperature. The absorbance at 595 nm was measured using a 1-cm pathlength microcuvette. A standard curve of absorbance and protein concentration were made. The absorbance of the unknown was determined at the same wavelength. The protein concentration of the unknown was determined using the BSA standard curve. If the unknown protein concentration is high, the protein was diluted and a smaller aliquot was assayed. A standard curve in a higher concentration range may also be prepared according from the method of Ausbel *et al.*, (1997).

3.4 Spectrophotometric Assay of Phenoloxidase Activity

The haemocyte-lysate supernatant was assayed using Beckman DU 650 UV-Vis spectrophotometer and a 3 ml glass cuvette. Prior to it, the phenoloxidase activity was optimized under different conditions using the following amount for blank: (1) 2.5 ml buffer, (2) 0.3 ml 5mM substrate, and

(3) 0.2 ml 1mg/ml activator. The protein was assayed with the following components: (1) 2.4 ml buffer, (2) 0.3 ml 5mM substrate, (3) 0.2 ml 1mg/ml activator, and (4) 0.1 ml crude extract.

A component (substrate, activator incubation period, activator incubation temperature, and sodium phosphate buffer with different pH) was varied alternately while the other components remain constant. The conditions varied for the substrates are (1) L-dopa (SIGMA) assayed at 490 nm, (2) 4-Methylcatechol (SIGMA) assayed at 520 nm, (3) Catechol (SIGMA) assayed at 520 nm, (4) Hydroquinone (SIGMA) assayed at 520 nm, and (5) Tyrosine (SIGMA) assayed at 520 nm. The activators namely Trypsin (GibcoBRL), Chymotrypsin (SIGMA), and Propanol were incubated at different incubation periods (0 min, 10 min, 20 min, 30 min and 60 min) and different incubation temperatures (4^0C, 10^0C, 25^0C, 30^0C, and 40^0C). Sodium phosphate buffer with pH 2.6, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were also used, and optimum pH was determined.

The results of optimization procedure were tabulated and graphed. The optimum conditions were used in the assay of fractions from the ion-exchange column chromatography. Spectrophotometric readings were taken every 30 seconds for 5 minutes.

3.5 Determination of Protein Molecular Weight using SDS-PAGE

The hemolymph plasma and the fractions obtained from the column chromatography were tested for the presence of phenoloxidase. Vertical slab

electrophoresis was performed in 10% polyacrylamide gels in the presence of SDS. A 20 µl portion of the samples from hemolymph were loaded in wells composed of 7.5% running gel and 4% acrylamide stacking gel solution. For determination of molecular mass, a Bio-Rad SDS-PAGE broad range molecular weight standard electrophoresis kit containing aprotinin (6.5 kDa), lysozyme (14.4 kDa), soybean trypsin (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116.25 kDa) and myosin (200 kDa) were used. Gels were stained with Coomasie Brilliant Blue (Hames, 1990). The procedure followed for SDS-polyacrylamide gel electrophoresis was adapted from Protein Electrophoresis Applications Guide (1994).

3.5.1 Preparation of Running Gel

The vertical slab gel unit were assembled and ensured that there were no leaks by putting agar sealant at the bottom of the gel. The solutions for running gel were mixed in a 50 ml conical tube by pipetting in and out. TEMED (SIGMA) and APS (SIGMA) were added last so that the solution will not instantly solidify. The solution was pipeted down the spacer into each sandwhich to a level about 4 cm from the top (big gel), or 2 cm (minigel). Approximately 0.3 ml (big gel) or 100µl (minigel) of n-butanol were applied on both corners of the slab. A very sharp liquid-gel interface became visible when the gel had polymerized. After polymerization, the casting stand was tilted to pour off overlay and rinse surface once with the running gel overlay. 1ml overlay was added while preparing the stacking gel.

3.5.2 Preparation of the Stacking Gel

The solutions for the stacking gel were added in a 50 ml conical tube. APS (SIGMA) and TEMED (SIGMA) were added last. The running gel overlay was poured off. The comb was inserted and the glass sandwhich was filled with the stacking gel solution. It must be taken into consideration not to trap air bubbles below the teeth of the comb. A very sharp liquid-gel interface became visible when the gel had polymerized.

3.5.3 Preparation of the Sample to be Loaded

In a 1.5 ml microcentrifuge tube, 7µl 2X treatment buffer was combined with 7.0µl protein sample. The tubes were placed in a boiling water bath for 3 minutes. The samples were placed in ice until ready for use. The treated samples were stored at -20^0C for 6 months for future runs.

3.5.4 Loading the Gels

The combs were removed from gel carefully to avoid disrupting the well dividers. The wells were rinsed well with tank buffers. The samples were loaded using a gel-loading tip. 5-15µl is recommended for each lane or 5-30µg of sample protein for each well.

3.5.5 Running the Gel

The buffer chamber was filled with tank buffer. The lid was placed on the gel electrophoresis unit and was connected to a power supply. The power was set to a constant 30mA and run for 4 hours. When the dye reached the bottom of the gel, the power supply was turned off and the power cables were

disconnected. The glass plates were separated and the gel was removed for staining or blotting.

3.5.6 Staining SDS-PAGE Separated with Coomasie Brilliant Blue

After removing the buffer and the glass plates, the gel will be placed in a container with rapid staining fixing solutions. It was shaken slowly for 10-15 minutes. The fixing solution was replaced with rapid Coomasie stain. It was shaken slowly for 2 hours or until the bands were visible. The Coomasie stain was replaced with destaining solution II until the background was clear. Folded tissue papers were added into the corners to facilitate destaining. It was stored in 7% acetic acid or distilled water.

3.5.7 Drying PAGE Gel using Hoefer Drygel Sr.

Two pieces of cellophane wider than the gel were cut and moistened by water. The gel was sandwiched by the cellophane. The sandwiched gel was placed onto the surface of the Hoefer Drygel unit and covered with rubber sheet attached to the unit. The vacuum pump and the drying apparatus were switched on. The unit was set at 50^{0}C and the vacuum timer at 1-5 hours. After drying, the dried gel was inserted between 2 heavy objects in order to be flattened.

3.6 Partial Purification of Phenoloxidase Using Ion-Exchange Chromatography

3.6.1 Anion Chromatography

Column fractionations were performed continuously at 4°C inside a cold cabinet and procedures are modified from Nakamura *et al.* (1985). Around 250 ml of DEAE(diethylaminoethyl)-Toyopearl 650c (TOSOH) was washed 3 times with ddH_2O (distilled water) in a beaker with a stirring rod. The gel was allowed to settle and the water was decanted afterwards. The hydrated gel was then washed with 50mM sodium phosphate buffer (pH 7.0) before packing the washed gel in a 20-cm column and allowing it to settle. Extreme caution, in watching the amount of buffer present, was practiced to prevent the gel from drying-up. Buffer five times the 100ml volume of the packed gel was allowed to flow through the column in order to stabilize the gel. The pH of elute was monitored until the pH of elute was the same as the pH of the buffer source. Then, the flow rate was calibrated to 1ml/min.

The crude haemocyte-lysate was then cautiously loaded into the column with a Pasteur pipette covered with a rubber tube on the tip to prevent abrasions. Loading was slow and carefully done to prevent bubble formation. The fraction collector was set to 3 minutes per tube in order to collect 3 ml per fraction.

Unbound protein was eluted using sodium phosphate buffer. Protein content of each fraction was determined by reading absorbance value of 1ml of the fraction in a quartz cuvette using Beckman DU 650 UV-Vis

spectrophotemetry at 280nm. Each fraction was also assayed for enzyme activity with trypsin incubated for 30min at room temperature (Method 3.4). Fractions were monitored until absorbance reading became zero or negligible. After, bound proteins were eluted using salt gradient of 0.5M NaCl with the set-up shown in Appendix B. Absorbance and enzyme activity of these fractions using a salt gradient were also determined using the same procedures done to the unbound proteins. Then, the remaining proteins that were still bounded after the gradient was used were eluted with pure 0.5 M NaCl. Reading of absorbance was done simultaneously with fraction collection until the reading is zero.

Molecular weight of the proteins fractions with high absorbance reading was determined using 8% SDS-PAGE minigel (refer to Method 3.5).

3.6.2 Cation Chromatography

SP(sulfopropyl)-Toyopearl 550c (TOSOH) was used instead of DEAE and the same procedure as anion chromatography was followed. The fractions from the anion chromatography column with high absorbance readings were combined and then loaded into the cation chromatography column. Same procedures in eluting and monitoring the protein in the fractions collected were done as in anion chromatography including. UV-Vis spectrophotometric assay of enzyme activity versus its absorbance at 280nm was also obtained (Method 3.6.1). Molecular weight of the proteins in fractions with relatively high absorbance reading was determined using 8% minigel SDS-PAGE (Method 3.5).

4.0 RESULTS

4.1 Determination of Enzyme Concentration through Bradford Method

The concentration of the unknown protein was determined through Bradford assay. The binding of Coomasie brilliant blue was compared to the standard protein used which is BSA or bovine serum albumin (Figure 1). Results showed that the unknown protein had a concentration of 17μg/μl.

4.2 Optimization of Phenoloxidase Activity Using Spectrophotometric Assay

The phenoloxidase in crude protein was measured as a function of varying substrates, activators with its incubation periods and incubation temperatures, as well as buffer pH. It was observed that the purified phenoloxidase ion in 2.4 ml sodium phosphate buffer pH 7 slightly turned to brownish solution in aged preparation. This result is supported by the observation of Ashida on silkworm phenoloxidase (1971). In such preparations, several percentages of total copper components of phenoloxidase were reduced to cuprous form due to prolonged standing. The partial oxidation of copper would be accompanied by the inactivation of enzyme.

4.2.1 Phenoloxidase Activity of Crude Protein with Different Substrates

The enzyme without the substrate showed almost no activity as indicated by its absorbance. The crude phenoloxidase with L-tyrosine and hydroquinone had an absorbance slightly higher than enzyme alone but still

showed no activity with succeeding minutes. This indicates that the two chemicals were not ideal substrates for phenoloxidase. Catechol, L-dopa, and 4-methylcatechol showed increasing activity with phenoloxidase within the span of time. Of the three, 4-methylcatechol showed the highest activity as indicated by its absorbance (Table 1; Figure 2).

4.2.2 Phenoloxidase Activity of Crude Protein with Different Activators

Trypsin, chymotrypsin, and propanol, all showed an increase in the absorbance compared to substrate and phenoloxidase alone. Although, trypsin and propanol showed the highest absorbance, trypsin showed better promises as an activator of phenoloxidase due to the consistency of its absorbance with phenoloxidase and the substrate 4-methylcatechol. Using propanol as an activator had produced precipitate upon addition in a solution of 4-methylcatechol, buffer, and phenoloxidase that affected the absorbance of the spectrophotometric assay (Table 2; Figure 3).

4.2.3 Phenoloxidase Activity of Crude Protein with Different Incubation Period of Activator

There was an increase in absorbance as shown starting at around 20-60 minutes incubation period of trypsin with the crude hemolymph. Since incubation period of 60 minutes showed the maximum activity, it is therefore favorable to incubate the enzyme within this duration (Table 3; Figure 4).

4.2.4 Phenoloxidase Activity of Crude Protein with Different Incubation Temperature of Activator

Incubation temperature of 10^0C-30^0C was sufficient to induce an increase in activity between phenoloxidase, its activator, and its substrate. Precipitate formed at much higher temperature that caused inconsistency in absorbance readings. It is therefore sufficient to conclude that 30^0C is the optimum incubation temperature due to its high absorbance (Table 4, Figure 5).

4.2.5 Phenoloxidase Activity of Crude Protein with Different Buffer pH

Ideal pH for sodium phosphate buffer ranges from pH 6 to pH 8. Buffers with pH lower than pH 4 and higher than pH 8 showed very low relative absorbance due to possible denaturization of phenoloxidase enzyme (Table 5, Figure 6).

4.3 SDS-PAGE of Crude Phenoloxidase

Molecular weight of prophenoloxidase was determined with techniques of SDS-electrophoresis (Plate 1). Lane 1 shows the protein molecular weight standard used from while lane 2 and 3 shows 15μg and 30μg crude protein respectively. The dark stains of lane 2 and 3 pointed by the red arrows possibly shows phenoloxidase based on their molecular weight. The inactive form, prophenoloxidase has a molecular mass of 70-80 kDA while the active form has a molecular mass of 60-70 kDa (Table 6). The pointed protein

bands both have a molecular weight of 88.55 kDa which is relatively near the molecular weight of prophenoloxidase of other arthropods studied.

4.4 Partial Purification Using Ion-Exchange Chromatography

4.4.1 Anion Chromatography

Fractions 1 to 24 contain proteins that did not bind to the column. Fractions 25 to 40 contain proteins that were bounded in the column while fractions 41 to 60 contain strongly bounded proteins. From the absorbance reading at 280nm, most proteins did not bind to DAEA cation column. Low enzyme activities at 520nm of fractions with salt gradient and pure 0.5 NaCl salt are observed as compared to the very prominent activity of the unbound fractions (Table 7; Figure 7).

The molecular weights of proteins from fractions with high absorbance reading were determined using SDS-PAGE (Plates 2 to 5). Best-fit lines of the markers of the different gels were computed (Figures 8 to 11). Fractions 1-22 were loaded into two minigels and have shown 4-5 protein bands in each tunnel. The 5 bands and their ranges are: a) 143-158 kDa, b) 77-85 kDa, c) 62-67 kDa, d) 46-50 kDa, and e) 43-46 kDa (Tables 8 to 11). There was no protein band formed from the fractions eluted with a salt gradient and pure salt. A crude sample is loaded in one tunnel of each of the SDS PAGEs. There were two bands formed the highest one weighing 131-140 kDa, while the other one weighs 85-92 kDa.

4.4.2 Cation Chromatography

Fractions 1-20 are unbound proteins. Fractions 21-40 are fractions using a salt gradient. The fractions eluted using pure 0.5M NaCl are fractions 41-61. Absorbance at 280nm and enzyme activity was highest in the unbound fractions. However, there are relatively high readings at fraction 48, 50, 51, and 52, which were eluted by pure salt (Figure 12; Table 12).

Best-fit lines of SDS-PAGE gels (Plates 6 to 10) were computed for each gel (Figures 13 to 17). In the SDS-PAGE of the first few unbound fractions (fractions 4-12) there are 2 protein bands visible. However, in the following fractions with high absorbance there is only one band visible. The two bands have the following ranges of molecular weight: a) 133-140 kDa, b) 85-92 kDa (Tables 13 to 17). The protein band that is present in all the SDS-PAGE throughout the column is the second type, which taking into account all of the SDS-PAGEs ranges from 85-92 kDa.

5.0 DISCUSSION

The hemolymph obtained from the larvae is a whitish sticky fluid containing the phenoloxidase enzyme. During the hemolymph collection from the larvae, serine protease had become activated thus converting prophenoloxidase to its active form which is phenoloxidase (Brivio *et a.l*, 1996). This was shown by the melanization reaction of the larvae after incision and hemolymph collection. The anticoagulant that was injected into the larvae contains an amount of salt that increases the low activity of phenoloxidase.

Phenoloxidase is a biological catalyst or enzyme needed for melanization and wound healing of insect. As an enzyme, it has an active site that contains contact residues for immobilizing the substrate, which are the substances that enzyme act upon to proceed with the reaction (Nicolas, 2000). The scope of visible spectrophotometric enzyme assays can be extended by the use of artificial substrates and by the production of colored derivatives of the substrate or product (Wilson and Goulding, 1986). 4-methylcatechol proved to be superior substrate for phenoloxidase because of its high absorption. Since the increase in the substrate concentration will increase over all rate of the enzyme reaction, it maybe inferred that 4-methylcatechol is originally present in the hemolymph. The addition of 4-methylcatechol *in vitro* increases the substrate concentration thus an increase in the activity as shown by the high absorption spectra. Phenoloxidase is also an oxidoreductase. 4-methylcatechol demonstrates an oxygen consuming

process, which is faster than other substrates during phenoloxidase activity. 4-methylcatechol can also come closer to the active site of phenoloxidase compared to other substrates with lower activity. Catechol and L-dopa followed the speed of oxidation reaction. Many studies of insect phenoloxidase used L-dopa routinely for assay but 4-methylcatechol proved to be a better substrate (Hall *et al.*, 1995). The inefficiency of the enzyme to oxidize L-tyrosine and hydroquinone maybe due to the presence of acidic group in the substrates (Nellaiappan and Ramalingam, 1980).

The phenoloxidase enzyme in the hemolymph is in its inactive form which is the pro-phenoloxidase. The activation of prophenoloxidase maybe of two mechanisms: proteolytic cleavage of the pro-enzyme and detergent activation (Hall *et al.*, 1995). Activation is the consequence of the conformational change of the enzyme molecule brought about by the aggregation caused by the activator (Ashida and Ohnishi, 1967). However, the study on Tarantula hemocyanin shows that activation of phenoloxidase by limited proteolysis seemed to be based not on a rearrangement of the active site but by providing free access for various substrates to the active site following removal of a non-active analogue which is phe-49 (Decker and Rimke, 1998). The activation of prophenoloxidase is accompanied by the reduction of cupric copper. Prophenoloxidase can be activated by detergents, such as sodium oleate and SDS, or by a protease such as ∝-chymotrypsin (Ashida, 1971). In our study, trypsin showed better promises as an activator because of its consistent high absorbance readings (Figure 3). Trypsin

activates pro-phenoloxidase to phenoloxidase. Incubation period which ranges from 20 to 60 minutes allowed enough equilibriation time for the activator and the enzyme (Figure 4).

The rate of enzyme reaction also varies with temperature since the relationship between the reaction rate and temperature is exponential. This means that for every 10^0C rise in temperature (Q_{10} value), the rate of enzyme reaction doubles. The temperature of 30^0C showed maximum activity for the enzyme (Figure 5). However, at temperature 40^0C to 60^0C, the enzyme was denatured and the activity was lost (Wilson and Goulding, 1986).

One factor that affects enzyme activity is the pH of the buffer used. Most enzymes have optimum pH needed before the reaction can proceed. The catalytic activity relies to the specific state of ionization of amino acid residues, which in turn is pH dependent. The optimum pH for the phenoloxidase ranges from pH 6 to 8 (Figure 6). The pH values outside this may result to denaturation or alteration of the native condition of the enzyme.

The target protein (prophenoloxidase) did not bind to either column. The result proves that the anticoagulant used, which contains an amount of salt, has led to the weak binding ability of many of our proteins. The random pattern of enzyme activity and the absorbance at 280nm of the cation (SP) column fractions is due to the oxidation of the hemolymph solutions as a result of the long experiment. However, phenoloxidase is still partially purified since the SDS-PAGE of the last fractions of the second column shows only a single protein band of 85-92 kDa. This weight of

prophenoloxidase is near the molecular weights of other insects studied, which are around 70-80 kDa (refer to 2.0 Review of Related Literatures). In the anion column fractions' PAGEs, there are several bands with slightly lower weights than prophenoloxidase. The 62-67 kDa bands are phenoloxidase, which are cleaved by a serine protease. The serine protease, which may be a prophenoloxidase-activating enzyme (PPAE) are the 43-46 kDa bands. It was shown in previous experiments that prophenoloxidase is sometimes activated when it is under SDS-PAGE. This is concluded since it has been identified in another coleopteran. Bands with molecular weights above 100 kDa have not yet been identified but the bands can be involved in melanization like the 165 kDa protein extracted from the coleoptera *Tenebrio* (Lee, K.M. et al., 2000). However, further study is still needed to be certain that the band is analogous since the computations with reference to the marker is different for each SDS-PAGE gel.

6.0 SUMMARY

Phenoloxidase is present in the hemolymph of coconut beetle larvae which is the most destructive and widely distributed pest of the coconut. The enzyme is responsible for the sclerotization, melanization, development and immunity of the insect. The purification and characterization of the enzyme will provide more information regarding the properties of phenoloxidase. This study may serve as the basis of further pest control researches which will focus on inhibition of immune response of the insect.

The study was guided by three objectives: (1) to isolate and partially purify phenoloxidase in Philippine *Oryctes rhinoceros* L. larvae hemolymph plasma, (2) to measure phenoloxidase activity as a function of varying substrate, activators (with different incubation period and temperature), and buffer pH; and (3) to determine the molecular weight of partially purified phenoloxidase.

A hundred coconut beetle larvae were collected from Tayabas, Quezon. Experiments were done in National Institute of Molecular Biology and Biotechnology. Prior to partial purification of the enzyme using ion-exchange chromatography, the optimum assay conditions were determined. Based from results of spectrophotometric assay, 4-methylcatechol appeared as the best substrate for the enzyme. Trypsin was the most efficient activator under 20-60 minutes incubation period and 30^0C incubation temperature. Sodium phosphate buffer is effective at pH ranging from 6-8.

Partial purification of the hemolymph was done using DEAE and SP ion exchange chromatography. Different gradient used to flow through the columns in the following order were 1) sodium phosphate buffer pH 7, 2) salt gradient (0.5M NaCl), and 3) pure salt (0.5M NaCl). Hemocyte-lysate was first loaded on DEAE anion chromatography. Fractions were read at 280nm for protein content and at 520nm for enzyme activity using UV-Vis spectrophotometer with the predetermined optimum conditions. When absorbance reading at 280nm zero or was relatively very low, the gradient used was changed. The fractions that have high absorbance reading at 280nm or high enzyme activity were loaded on SDS-PAGE. Fractions from the anion column having high absorbance readings were then mixed and loaded in the SP cation column. The same procedure was done in reading absorbances at 280nm and 520nm using the same three gradients. Fractions with high absorbance readings were also loaded in SDS-PAGE. Spectrophotometric readings showed that the proteins did not bind with either column since high absorbance reading were shown in the first few fractions using pure buffer as gradient. This was due to the salt content of the anticoagulant used. However, other materials are eluted and the clear bands shown in the SDS-PAGEs of the cation chromatography showed partial purification. Results showed the presence of prophenoloxidase with a molecular weight of 85-92 kDa and its active form (phenoloxidase) has a molecular weight of 62-67 kDa. Bands with 43-36 kDa shown in some of the PAGE gels are probably serine protease that cleaves prophenoloxidase.

7.0 CONCLUSION

Study of enzyme characteristics is essential before its reaction mechanism will be thoroughly known. Characterizing phenoloxidase by inducing it under different conditions and determining where its highest activity is pertinent before an effective inhibitor can be identified.

- Phenoloxidase in *Oryctes rhinoceros* L. has an optimum reaction activity with 4-methylcatechol as the substrate and with trypsin as the activator. Increase in reaction will be further observed if trypsin is incubated for 20 to 60 minutes at a temperature of 30°C using buffers having pH ranging from pH 6 to pH 8.
- SDS-PAGE of the partially purified prophenoloxidase showed that it has a molecular weight slightly different weight than other insect studied. It has a molecular weight ranging from 85-91 kDa while its active form phenoloxidase has a molecular weight of 62-67 kDa.
- A serine protease, which is probably essential for the activation of prophenoloxidase, has a molecular weight of 43-46 kDa which is similar to those found in other coleopterans already studied. From the present of this serine protease, it could be concluded that prophenoloxidase is activated in the hemolymph. Since phenoloxidase is responsible for the activation of melanization, the enzyme may be activated first in the hemolymph and carried in an activated form to the cuticle.

8.0 RECOMMENDATIONS

Coconut beetle in their pupal stage and other stages aside from the larval stage should be used as test subjects for another study to determine the difference using those in their larval stage. Other enzymes may be present in the pupal stage, which are not yet developed when in the larval stage. Instars of the larvae should be taken into account in follow-up studies. Phenoloxidase in other species could also be studied.

In purifying phenoloxidase using any ion-exchange column, we recommend that the sample should undergo dialysis to remove any salt present. NaCl present in anticoagulants increase enzyme activity but the salt should be removed before loading into an ion-exchange column. SDS-PAGE set-up should be run in a cold cabinet. Electric current should be monitored to be as constant as possible throughout all SDS-PAGE runs to attain a more consistent Rf value for the different markers of the gels. We suggest purifying phenoloxidase using other columns or other different purifications techniques. Structure-function relationship should also be studied.

A DNA analysis of coconut beetle phenoloxidase would also be a good study in comparing it in other species and finding its inhibitor for pest control. Determination of N-terminal and internal amino acid sequences maybe useful to further understand phenoloxidase. CDNA cloning of pheboloxidase will lead to easier production of protein to be studied. Inhibitors for phenoloxidase both present in the insect and artificial should be studied.

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Tables

TABLES

TABLE 1. Phenoloxidase Activity of Crude Protein with Different Substrates

TIME	CONTROL	4-METHYL-CATECHOL	L-DOPA	CATECHOL	HYDRO-QUINONE	L-TYROSINE
0.5 min	0.0666	0.2049	0.2172	0.1953	0.1882	0.1317
1.0 min	0.0672	0.3206	0.2987	0.2247	0.1936	0.1194
1.5 min	0.0685	0.4113	0.3941	0.2707	0.1946	0.1171
2.0 min	0.0663	0.4813	0.4269	0.3153	0.2002	0.1192
2.5 min	0.0673	0.5252	0.4707	0.3527	0.1975	0.1141
3.0 min	0.0693	0.5589	0.5224	0.3919	0.2001	0.1177
3.5 min	0.0693	0.5789	0.5566	0.4293	0.2023	0.1178
4.0 min	0.0698	0.5953	0.6039	0.4681	0.2056	0.1173
4.5 min	0.0688	0.6111	0.6333	0.5007	0.2052	0.1164
5.0 min	0.0677	0.6049	0.6043	0.5343	0.2082	0.1168

TABLE 2. Phenoloxidase Activity of Crude Protein with Different Activators

TIME	ENZYME(E) ONLY	E + SUBSTRATE (S)	E + S + TRYPSIN	E + S + CHYMO-TRYPSIN	E + S + PROPANOL
0.5 min	0.0539	0.0616	0.1152	0.0867	0.2577
1.0 min	0.0589	0.0698	0.1656	0.0983	0.2982
1.5 min	0.0526	0.0909	0.1914	0.1212	0.3097
2.0 min	0.0515	0.1213	0.2563	0.1445	0.3327
2.5 min	0.0507	0.1459	0.3406	0.1692	0.3902
3.0 min	0.048	0.1671	0.3885	0.194	0.3804
3.5 min	0.0493	0.1904	0.4435	0.2181	0.383
4.0 min	0.0539	0.2248	0.4645	0.2469	0.4364
4.5 min	0.0461	0.2449	0.4891	0.2691	0.4518
5.0 min	0.054	0.2665	0.4917	0.2914	0.4933

TABLE 3. Phenoloxidase Activity of Crude Protein with Different Incubation Period of Trypsin

TIME	W/O TRYPSIN	DIFFERENT INCUBATION PERIODS OF TRYPSIN				
		0 min	10 min	20 min	30 min	1 hr
0.5 min	0.1354	0.1435	0.1275	0.1626	0.1646	0.2501
1 min	0.1354	0.1496	0.1458	0.1793	0.1814	0.2703
1.5 min	0.1417	0.1492	0.1528	0.1929	0.1904	0.2891
2 min	0.1415	0.152	0.1618	0.2098	0.2059	0.3014
2.5 min	0.1453	0.1538	0.1707	0.225	0.2206	0.3245
3 min	0.1574	0.1562	0.1795	0.2478	0.2378	0.3408
3.5 min	0.1462	0.1674	0.1917	0.2617	0.2532	0.3587
4 min	0.1491	0.1616	0.2019	0.2782	0.2692	0.3695
4.5 min	0.1538	0.1583	0.211	0.2953	0.2882	0.3831
5 min	0.1544	0.1618	0.219	0.3137	0.3044	0.3948
Total	1.4602	1.5534	1.7617	2.3663	2.3157	3.2829
Mean	0.14602	0.15534	0.17617	0.23663	0.23157	0.32829
Activated-nonactivated		0.00932	0.03015	0.09061	0.08555	0.18227

TABLE 4. Phenoloxidase Activity of Crude Protein with Different Incubation Temperature of Trypsin

TIME	TEMPERATURE									
	4°C		10°C		25°C		30°C		40°C	
	w/o T	w/ T	w/o T	w/ T	w/o T	w/ T	w/o T	w/ T	w/o T	w/ T
0.5	0.0686	0.0778	0.0627	0.1134	0.0644	0.1284	0.0802	0.1889	0.1122	0.2256
1	0.0721	0.0825	0.0664	0.1612	0.0632	0.1778	0.0876	0.289	0.1455	0.2942
1.5	0.0782	0.0942	0.0642	0.2051	0.0677	0.2327	0.1103	0.3955	0.2026	0.3759
2	0.0747	0.1276	0.0658	0.2677	0.0708	0.2995	0.1082	0.4699	0.2613	0.4632
2.5	0.0745	0.1428	0.0739	0.3257	0.0779	0.3524	0.1314	0.5216	0.3142	0.5335
3	0.083	0.1597	0.0838	0.3827	0..0833	0.4648	0.1427	0.5618	0.362	0.5667
3.5	0.0767	0.1767	0.0885	0.429	0.0888	0.4188	0.1809	0.5851	0.4382	0.5985
4	0.0776	0.1888	0.0863	0.4906	0.0982	0.4988	0.1846	0.6075	0.4866	0.6228
4.5	0.0788	0.2024	0.0862	0.5129	0.1227	0.5224	0.2008	0.6165	0.5108	0.6318
5	0.0816	0.2243	0.0876	0.5221	0.101	0.5473	0.219	0.6252	0.535	0.6408
Total	0.7658	1.4768	0.7654	3.6429	0.838	3.6429	1.4457	4.861	3.3684	4.953
Mean	0.0766	0.1477	0.0765	0.3643	0.0838	0.3643	0.14457	0.4861	0.3368	0.4953
*A-nA	0.0711		0.2645		0.28049		0.34153		0.15846	

TABLE 5. Phenoloxidase Activity of Crude Protein with Different Buffer pH

	DIFFERENT BUFFER pH							
	pH 2.6		pH 3		pH 4		pH 5	
TIME	w/o T	w/ T	w/o T	W/ T	W/o T	w/ T	w/o T	W/ T
0.5	0.1329	0.1491	0.0464	0.1065	0.0556	0.1290	0.0362	0.1515
1	0.1332	0.1470	0.0372	0.1042	0.0588	0.1349	0.0351	0.1603
1.5	0.1321	0.1442	0.0405	0.1050	0.0615	0.1394	0.0362	0.1705
2	0.1293	0.1425	0.0459	0.1132	0.0606	0.1421	0.0370	0.1764
2.5	0.1263	0.1438	0.0422	0.1070	0.0649	0.1414	0.0378	0.1874
3	0.1247	0.1443	0.0324	0.1087	0.0632	0.1430	0.0404	0.1954
3.5	0.1247	0.1450	0.0464	0.1082	0.0703	0.1451	0.0377	0.2058
4	0.1293	0.1456	0.0405	0.1086	0.0688	0.1456	0.0398	0.2132
4.5	0.1286	0.1458	0.088	0.1086	0.0668	0.1456	0.0382	0.2191
5	0.1352	0.1454	0.088	0.1075	0.0699	0.1443	0.0389	0.2259
Total	1.2963	1.4527	0.5075	1.0775	0.6404	1.4104	0.3773	1.9055
Mean	0.12963	0.1453	0.0508	0.1078	0.0640	0.1410	0.03773	0.19055
*A-nA	0.01567		0.0580		0.077		0.15282	

TIME	pH 6		pH 7		pH 8		pH 9	
	W/o T	W/ T	w/o T	w/ T	w/o T	w/ T	w/o T	W/ T
0.5	0.0541	0.1631	0.0422	0.1463	0.0603	0.2054	0.0632	0.3093
1	0.0539	0.1743	0.047	0.2011	0.0686	0.3543	0.0925	0.451
1.5	0.0559	0.1911	0.059	0.2931	0.093	0.5111	0.1063	0.5216
2	0.0607	0.2089	0.077	0.3921	0.1166	0.6398	0.1157	0.5455
2.5	0.0626	0.2283	0.0905	0.4869	0.1336	0.7324	0.1121	0.486
3	0.0659	0.248	0.1074	0.5576	0.1551	0.7589	0.1107	0.4112
3.5	0.0707	0.2785	0.1299	0.5953	0.1764	0.7598	0.1065	0.3276
4	0.0774	0.3042	0.1368	0.6194	0.1935	0.7593	0.1029	0.2446
4.5	0.0805	0.3162	0.1528	0.6382	0.2108	0.7577	0.0974	0.2173
5	0.0846	0.3389	0.1736	0.6468	0.2314	0.7575	0.0904	0.199
Total	0.6663	2.4515	1.0162	4.5768	1.4393	6.2362	0.9977	3.7131
Mean	0.06663	0.2452	0.1016	0.4577	0.1439	0.6236	0.09977	0.37131
*A-nA	0.17857		0.3561		0.4797		0.27154	

TIME	pH 10		pH 11		pH 12	
	w/o T	W/ T	w/o T	w/ T	w/o T	w/ T
0.5	0.1483	0.1375	0.0643	0.2591	0.0533	0.1745
1	0.141	0.2017	0.1001	0.2611	0.0588	0.1557
1.5	0.1268	0.1985	0.1341	0.2804	0.0454	0.1838
2	0.0695	0.1381	0.1527	0.3549	0.041	0.1852
2.5	0.0497	0.078	0.1603	0.3243	0.0411	0.1796
3	0.0225	0.0398	0.1623	0.3269	0.0423	0.1775
3.5	0.0101	0.0141	0.1628	0.3244	0.0426	0.1743
4	0.0038	-0.001	0.1602	0.3308	0.0496	0.1759
4.5	0.0003	-0.0164	0.1597	0.3212	0.0568	0.1783
5	-0.0021	-0.0246	0.1634	0.3151	0.0553	0.1784
Total	0.5699	0.7657	1.4199	3.099	0.4862	1.7632
Mean	0.0570	0.0766	0.14199	0.3099	0.04862	0.17632
*A-nA	0.0196		0.16791		0.1277	

*A-nA = Activated (with Trypsin) – non activated (without trypsin)

TABLE 6. Computation of Crude Protein SDS-PAGE

MW kda)	Rf	log MW
200	0.0431	2.3
116.25	0.1379	2.07
97.4	0.1724	1.99
66.2	0.2759	1.82
45	0.4569	1.65
31	0.6293	1.49
21.5	0.8362	1.33
14.4	0.9569	1.16

Bands	Rf	MW
(tunnel 2)		
w/o arrow	0.1034	126.71
w/ arrow	0.2414	88.55
(tunnel 3)		
w/o arrow	0.1207	121.12
w/ arrow	0.2414	88.55

TABLE 7. Anion Chromatography Fractions' Absorbance Reading and Enzyme Activity

FRACTION	280 nm	520 nm	FRACTION	280 nm	520 nm	FRACTION	280 nm	520 nm
1	0.049	0.0007	21	0.111	0.0129	41	0.059	0.0005
2	0.105	0.0008	22	0.08	0.014	42	0.079	0.0005
3	0.038	-0.0026	23	0.179	0.0074	43	0.02	0.0007
4	0.269	-0.003	24	0	0.0071	44	0.015	0.0007
5	9.076	0.0025	25	0.148	0.0013	45	0.015	0.0006
6	13.943	0.1615	26	0.301	0.0023	46	0.02	0.0005
7	16.511	0.2026	27	0.746	0.0027	47	0.012	0.0005
8	2.682	0.0898	28	0.535	0.0021	48	0.01	0.0006
9	2.613	0.016	29	0.209	0.0049	49	0.009	0.0004
10	2.1538	0.0064	30	0.176	0.0017	50	0.008	0.0004
11	1.082	0.0038	31	0.355	0.0006	51	0.006	0.0004
12	0.7305	0.0021	32	0.255	0.0021	52	0.004	0.0003
13	1.056	0.0009	33	0.214	0.0009	53	0.003	0.0004
14	0.405	0.001	34	0.294	0.0008	54	0.004	0.0003
15	0.157	0.001	35	0.384	0.0001	55	0.002	0.0002
16	0.332	0.0016	36	0.576	0.0003	56	0.001	0.0002
17	0.33	0.0012	37	0.477	0.0003	57	0	0.0002
18	0.361	0.0009	38	0.539	0.0004	58	0	0.0002
19	0.138	0.0074	39	0.593	0.0012	59	0	0.0001
20	0.294	0.0119	40	0.555	0.0026	60	0	0.0002

Fractions
1 - 24 – unbound fractions
25 - 40 – bound fractions
41 – 60 – Pure salt gradient fractions

TABLE 8. Computations of SDS-PAGE of Unbound Fractions (1st Gel) from Anion Chromatography

Marker	Rf	log MW
200	0.125	2.30103
116.25	0.2578	2.065393
97.4	0.3203	1.988559
66.2	0.4453	1.820858
45	0.6684	1.653213
31	0.8672	1.491362

Bands	Rf	log MW	MW	bands	Rf	log MW	MW
1	0.189	2.156422	143.358	26	0.5079	1.823554	66.61223
2	0.3937	1.942756	87.65081	27	0.6349	1.690991	49.08981
3	0.1811	2.164668	146.1059	28	0.6825	1.641307	43.7831
4	0.3937	1.942756	87.65081	29	0.1587	2.188049	154.1874
5	0.188	2.157466	143.7029	30	0.254	2.088575	122.6238
6	0.4173	1.918122	82.81753	31	0.381	1.956012	90.36749
7	0.5188	1.812177	64.88982	32	0.4127	1.922924	83.73822
8	0.6299	1.69621	49.68329	33	0.5079	1.823554	66.61223
9	0.6693	1.655085	45.1944	34	0.6349	1.690991	49.08981
10	0.1496	2.197548	157.5968	35	0.6667	1.657799	45.4777
11	0.4252	1.909876	81.25989	36	0.176	2.169991	147.9078
12	0.5276	1.802991	63.53179	37	0.256	2.086487	122.0358
13	0.6614	1.663331	46.06072	38	0.368	1.969582	93.23556
14	0.1575	2.189302	154.6328	39	0.416	1.919479	83.07669
15	0.4252	1.909876	81.25989	40	0.512	1.819274	65.95905
16	0.5354	1.794849	62.35187	41	0.632	1.694018	49.43316
17	0.6614	1.663331	46.06072	42	0.68	1.643916	44.04697
18	0.1575	2.189302	154.6328	43	0.1694	2.17688	150.2728
19	0.4409	1.893489	78.25076	44	0.2661	2.075945	119.1091
20	0.5354	1.794849	62.35187	45	0.3629	1.974905	94.38543
21	0.6614	1.663331	46.06072	46	0.4194	1.91593	82.40058
22	0.1587	2.188049	154.1874	47	0.5161	1.814995	65.31228
23	0.254	2.088575	122.6238	48	0.6613	1.663435	46.07179
24	0.381	1.956012	90.36749	crude A	0.2119	2.132519	135.6809
25	0.4286	1.906327	80.59857	crude B	0.3984	1.93785	86.66626

TABLE 9. Computations of SDS-PAGE of Unbound Fractions (2nd Gel) from Anion Chromatography

Marker	Rf	log MW
200	0.1852	2.30103
116.25	0.3852	2.065393
97.4	0.4667	1.988559
66.2	0.6	1.820858
45	0.8222	1.653213

Bands	Rf	log MW	MW	Bands	Rf	log MW	MW
1	0.2941	2.168848	147.5189	26	0.6567	1.796566	62.59881
2	0.5294	1.927265	84.57948	27	0.7761	1.673978	47.20393
3	0.6618	1.79133	61.84861	28	0.806	1.64328	43.98249
4	0.7794	1.67059	46.8371	29	0.2761	2.187328	153.9317
5	0.8088	1.640405	43.69231	30	0.5075	1.94975	89.07375
6	0.2794	2.18394	152.7355	31	0.5373	1.919154	83.01453
7	0.5	1.95745	90.66716	32	0.6567	1.796566	62.59881
8	0.5662	1.889482	77.53226	33	0.7761	1.673978	47.20393
9	0.6618	1.79133	61.84861	34	0.806	1.64328	43.98249
10	0.7794	1.67059	46.8371	35	0.2761	2.187328	153.9317
11	0.8088	1.640405	43.69231	36	0.5	1.95745	90.66716
12	0.2963	2.166589	146.7536	37	0.5299	1.926752	84.47957
13	0.5185	1.938456	86.78727	38	0.6493	1.804164	63.70356
14	0.5481	1.908066	80.92184	39	0.7612	1.689276	48.8963
15	0.6593	1.793897	62.21523	40	0.791	1.65868	45.57013
16	0.8074	1.641842	43.83716	41	0.6567	1.796566	62.59881
17	0.2836	2.179628	151.2265	42	0.7985	1.65098	44.76927
18	0.5	1.95745	90.66716	crude A	0.3164	2.145952	139.9433
19	0.5373	1.919154	83.01453	crude B	0.5294	1.927265	84.57948
20	0.6568	1.796463	62.58402				
21	0.7836	1.666278	46.37435				
22	0.8134	1.635682	43.21975				
23	0.2836	2.179628	151.2265				
24	0.5075	1.94975	89.07375				
25	0.5363	1.920181	83.21101				

TABLE 10. Computations of SDS-PAGE of Bound Fractions (1st Gel) from Anion Chromatography

Marker	Rf	log MW
200	0.0362	2.30103
116.25	0.087	2.065393
97.4	0.1159	1.988559
66.2	0.1812	1.820858
45	0.3188	1.653213
31	0.4783	1.491362
21.5	0.6522	1.332438
14.4	0.7754	1.158362
6.5	0.8333	0.812913

Bands	Rf	log MW	MW
Crude A	0.05	2.12969	134.8
Crude B	0.1714	1.947566	88.62693

TABLE 11. Computations of SDS-PAGE of Bound Fractions (2^{nd} Gel) from Anion Chromatography

Marker	Rf	log MW
200	0.0331	2.30103
116.25	0.0661	2.065393
97.4	0.0992	1.988559
66.2	0.1818	1.820858
45	0.3471	1.653213
31	0.5455	1.491362
21.5	0.7769	1.332438
14.4	0.9339	1.158362

bands	Rf	log MW	MW
crude A	0.0163	2.115804	130.5581
crude B	0.1545	1.964917	92.23949

TABLE 12. Cation Chromatography Fractions' Absorbance Reading and Enzyme Activity

FRACTIONS	280 nm	520 nm	FRACTIONS	280 nm	520 nm	FRACTIONS	280 nm	520 nm
1	0.0035	0.0021	22	0.0102	0.0001	43	0.0098	0.0018
2	0.001	0	23	0.0065	-0.0009	44	0.0123	0.0103
3	0.0043	0.0008	24	-0.0043	-0.0008	45	0.0113	-0.0013
4	0.0716	0.0034	25	-0.0035	0	46	0.0114	-0.0016
5	0.1315	0.0244	26	0.0147	0	47	0.0057	0
6	0.4593	0.0508	27	0.0125	-0.0025	48	0.0089	-0.0021
7	0.4701	0.0733	28	0.0203	0.002	49	0.0182	-0.0006
8	0.5228	0.0571	29	0.0272	0.0011	50	0.0566	0.0184
9	0.3219	0.0292	30	0.0382	0.0038	51	0.0541	0.0284
10	0.2039	0.0047	31	0.0361	0.0032	52	0.0422	0.0173
11	0.0895	0.0028	32	0.0157	0	53	0.029	0.0124
12	0.0671	0.003	33	0.0036	-0.0002	54	0.0279	0.0073
13	0.0483	0.0027	34	-0.0027	-0.0009	55	0.0277	0.0001
14	0.0477	0.0023	35	-0.0061	0.0006	56	0.0208	-0.001
15	0.0332	0.0018	36	-0.0067	0.0006	57	0.0152	-0.0003
16	0.0255	0.0015	37	-0.0103	-0.0007	58	0.0112	-0.0025
17	0.0219	0.001	38	-0.012	-0.0014	59	0.0142	-0.003
18	0.0226	0.0012	39	-0.0119	-0.001	60	0.0089	-0.0007
19	0.0208	0.0001	40	-0.0116	-0.001	61	0.0069	0.0007
20	0.0202	0.0025	41	0.0082	0.0035			
21	0.0028	0.0023	42	0.0094	0.0005			

Fractions
1 - 20 – unbound fractions
21 - 40 – bound fractions
41 – 61 – Pure salt gradient fractions

TABLE 13. Computations of SDS-PAGE of Unbound Fractions (1st Gel) from Cation Chromatography

Marker	Rf	log MW
200	0.1418	2.30103
116.25	0.2837	2.065393
97.4	0.3404	1.988559
66.2	0.4539	1.820858
45	0.6525	1.653213
31	0.9078	1.491362

Bands	Rf	log MW	MW	Bands	Rf	log MW	MW
1	0.2128	2.14575	139.8782	11	0.2199	2.138411	137.5344
2	0.4113	1.94058	87.21282	12	0.4113	1.94058	87.21282
3	0.234	2.123838	132.9957	13	0.2199	2.138411	137.5344
4	0.4113	1.94058	87.21282	14	0.4113	1.94058	87.21282
5	0.234	2.123838	132.9957	15	0.3972	1.955154	90.18911
6	0.4113	1.94058	87.21282	16	0.3972	1.955154	90.18911
7	0.227	2.131073	135.2299	Anion A	0.2128	2.14575	139.8782
8	0.4113	1.94058	87.21282	Anion B	0.3972	1.955154	90.18911
9	0.2199	2.138411	137.5344				
10	0.4113	1.94058	87.21282				

TABLE 14. Computations of SDS-PAGE of Unbound Fractions (2nd Gel) from Cation Chromatography

Marker	Rf	log MW
200	0.0966	2.30103
116.25	0.2207	2.065393
97.4	0.2759	1.988559
66.2	0.3862	1.820858
45	0.5793	1.653213
31	0.8483	1.491362

bands	Rf	log MW	MW
1	0.3333	1.957501	90.67789
2	0.3403	1.950207	89.16767
3	0.3403	1.950207	89.16767
4	0.3427	1.947707	88.65569
5	0.338	1.952604	89.66109
6	0.338	1.952604	89.66109
7	0.3404	1.950103	89.14627
8	0.3286	1.962399	91.70622
9	0.3286	1.962399	91.70622
10	0.3286	1.962399	91.70622
Anion A	0.1857	2.111301	129.2113
Anion B	0.3429	1.947498	88.61316

TABLE 15. Computations of SDS-PAGE of Bound Fractions (1st Gel) from Cation Chromatography

Marker	Rf	log MW
200	0.0811	2.30103
116.25	0.2162	2.065393
97.4	0.2568	1.988559
66.2	0.4324	1.820858
45	0.5946	1.653213
31	0.8649	1.491362
21.5	0.9459	1.332438

bands	Rf	log MW	MW
1	0.3611	1.932411	85.58768
2	0.3497	1.943943	87.89081
3	0.3521	1.941516	87.40085
4	0.3662	1.927252	84.57696
Anion A	0.1756	2.120063	131.8448
Anion B	0.3571	1.936458	86.38884

TABLE 16. Computations of SDS-PAGE of Bound Fractions (2nd Gel) from Cation Chromatography

Marker	Rf	log MW
200	0.1096	2.30103
116.25	0.2603	2.065393
97.4	0.3356	1.988559
66.2	0.4795	1.820858
45	0.6986	1.653213

Bands	Rf	log MW	MW
1	0.4028	1.937545	86.60548
2	0.4028	1.937545	86.60548
3	0.4028	1.937545	86.60548
4	0.4097	1.930067	85.12696
5	0.4028	1.937545	86.60548
Anion A	0.2394	2.114638	130.2082
Anion B	0.4155	1.923781	83.9037
Anion C	0.5211	1.809332	64.46616

Table 17. SDS-PAGE of Pure Salt Gradient Fractions from Cation Chromatography and Computations

Marker	Rf	log MW
200	0.1216	2.30103
116.25	0.25	2.065393
97.4	0.3243	1.988559
66.2	0.473	1.820858
45	0.6554	1.653213
31	0.9257	1.491362

bands	Rf	log MW	MW
1	0.4085	1.935459	86.1904
2	0.3972	1.946494	88.40859
3	0.3972	1.946494	88.40859
4	0.3857	1.957725	90.72467
Anion A	0.2214	2.118181	131.2746
Anion B	0.4071	1.936826	86.46217
Anion C	0.5	1.8461	70.16168

Figures

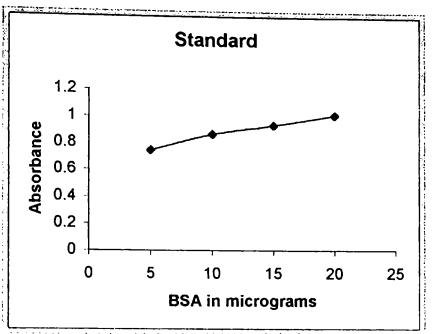


Figure 1. Standard Curve of Protein Concentration (Bradford Assay)

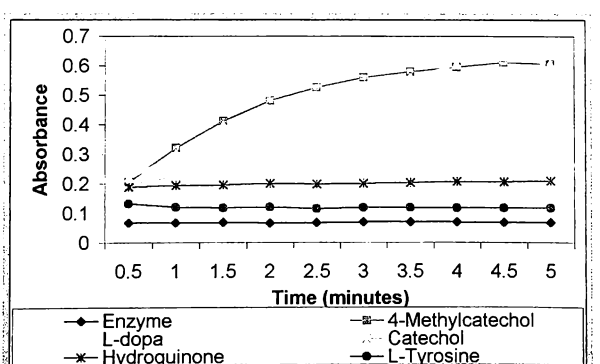


Figure 2. Phenoloxidase Activity of Crude Protein with Different Substrates

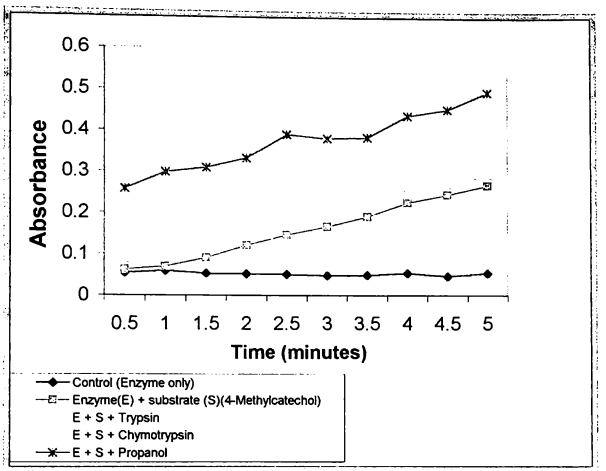


Figure 3. Phenoloxidase Activity of Crude Protein with Different Activators

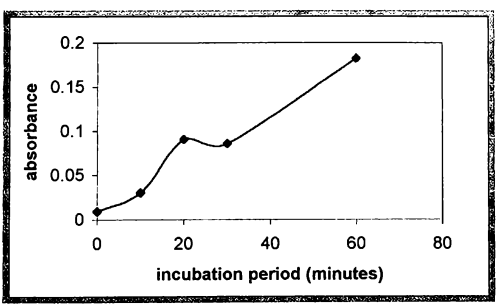


Figure 4. Phenoloxidase Activity of Crude Protein with Different Incubation Period of Trypsin

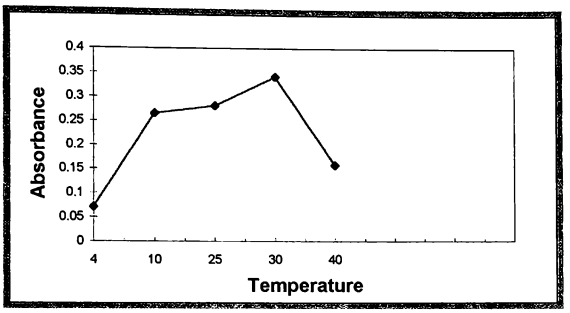


Figure 5. Phenoloxidase Activity of Crude Protein with Different Incubation Period of Trypsin

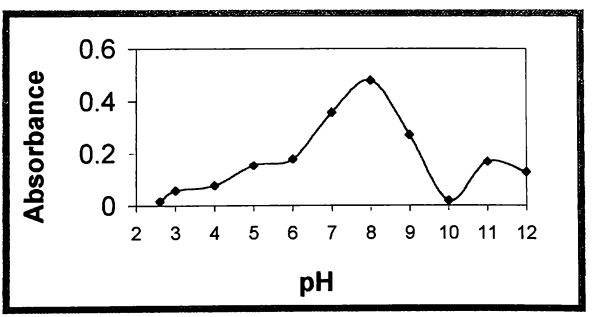


Figure 6. Phenoloxidase Activity of Crude Protein with Different Buffer pH

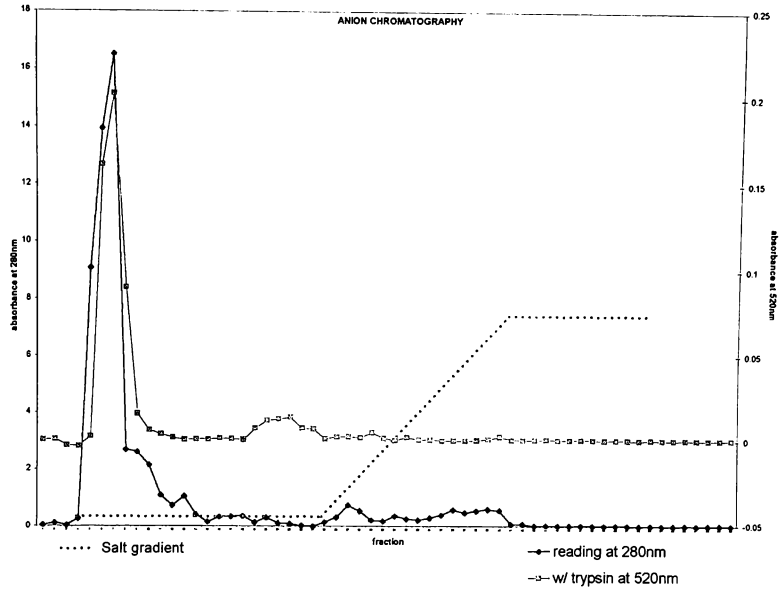


Figure 7. Anion Chromatography Fractions' Graph of Absorbance and Enzyme Activity

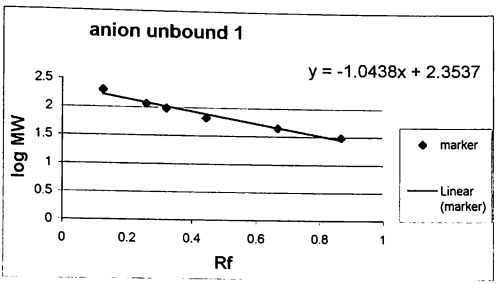


Figure 8. Best-Fit Line of Protein Standards from SDS-PAGE of Anion Chromatography Unbound Fractions (1st Gel)

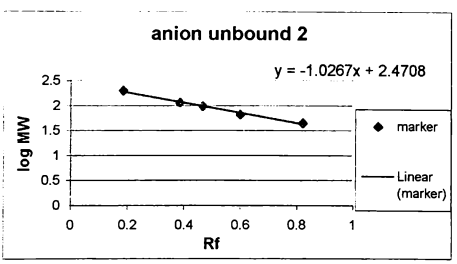


Figure 9. Best-Fit Line of Protein Standards from SDS-PAGE of Anion Chromatography Unbound Fractions (2nd Gel)

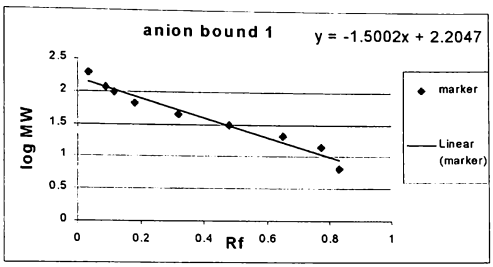


Figure 10. Best-Fit Line of Protein Standards from SDS-PAGE of Anion Chromatography Bound Fractions (1st Gel)

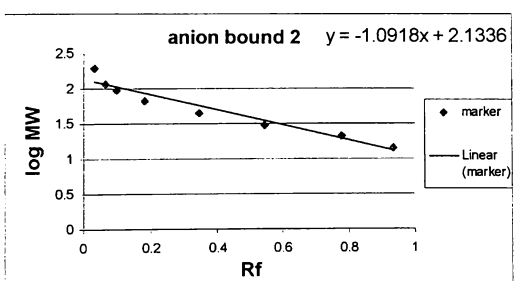


Figure 11. Best-Fit Line of Protein Standards from SDS-PAGE of Anion Chromatography Bound Fractions (2nd Gel)

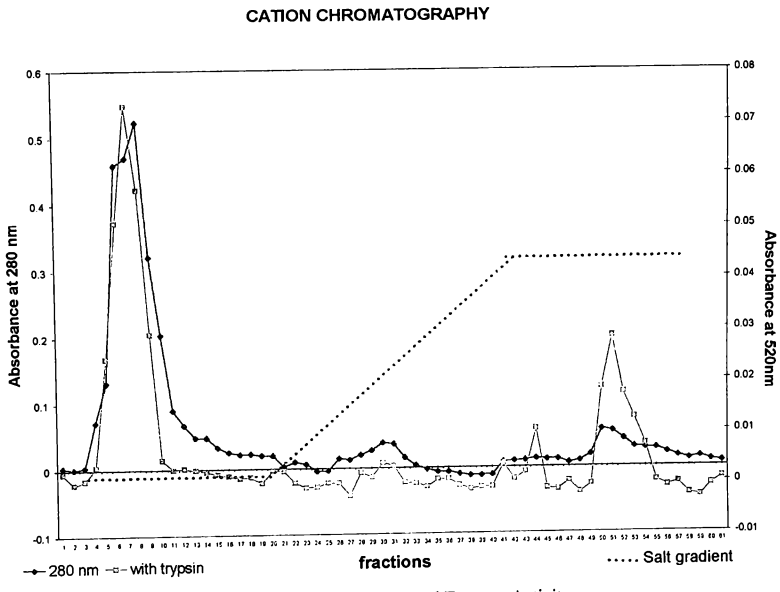


Figure 12. Cation Chromatography Fractions' Graph of Absorbance and Enzyme Activity

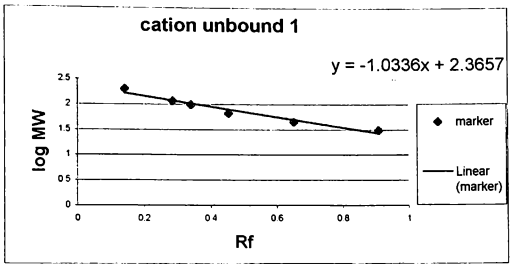


Figure 13. Best-Fit Line of Protein Standards from SDS-PAGE of Cation Chromatography Unbound Fractions (1st Gel)

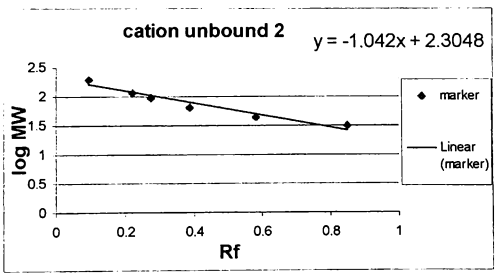


Figure 14. Best-Fit Line of Protein Standards from SDS-PAGE of Cation Chromatography Unbound Fractions (2nd Gel)

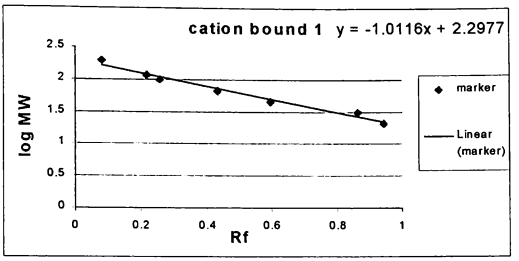


Figure 15. Best-Fit Line of Protein Standards from SDS-PAGE of Cation Chromatography Bound Fractions (1st Gel)

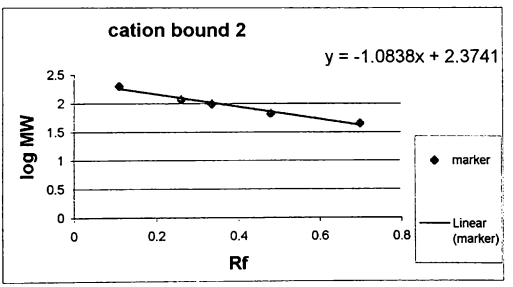


Figure 16. Best-Fit Line of Protein Standards from SDS-PAGE of Cation Chromatography Bound Fractions (2nd Gel)

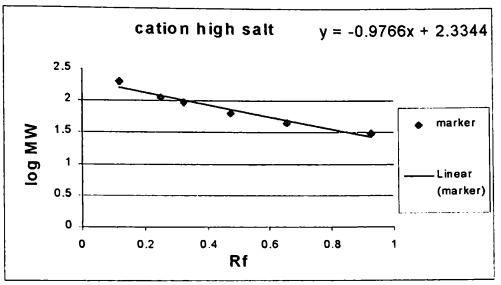


Figure 17. Best-Fit Line of Protein Standards from SDS-PAGE of Cation Chromatography Pure Salt Gradient Fractions

Plates

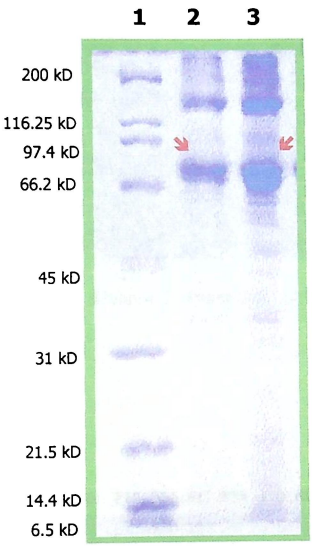


Plate 1. SDS-PAGE of Crude Protein

- lane 1 - Protein molecular weight standards, lane 2 - 15mg crude protein, lane 3 - 30mg crude protein

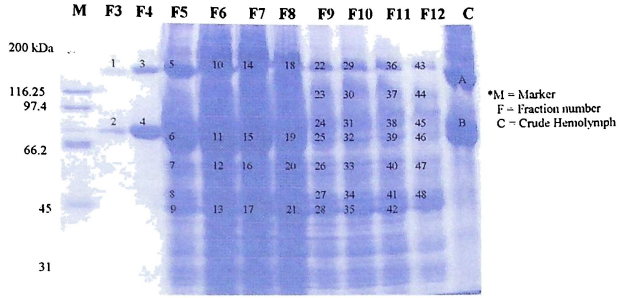


Plate 2. SDS-PAGE of Unbound Fractions (1st Gel) from Anion Chromatography

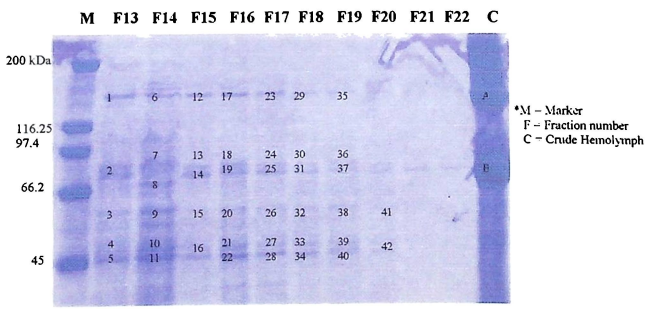


Plate 3. SDS-PAGE of Unbound Fractions (2nd Gel) from Anion Chromatography

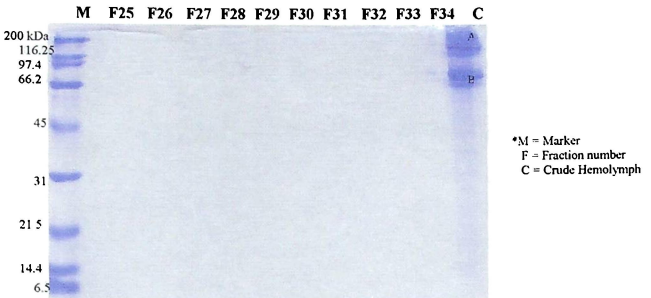


Plate 4. SDS-PAGE of Bound Fractions (1st Gel) from Anion Chromatography

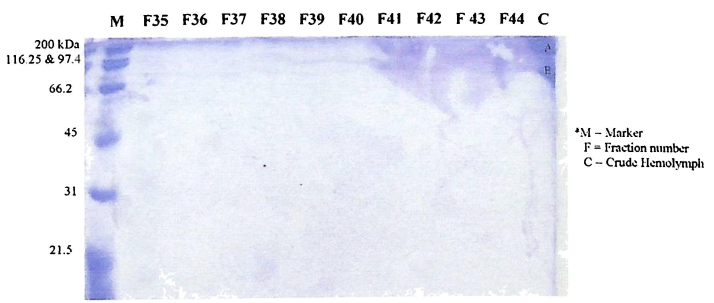


Plate 5. SDS-PAGE of Bound Fractions (2nd Gel) from Anion Chromatography

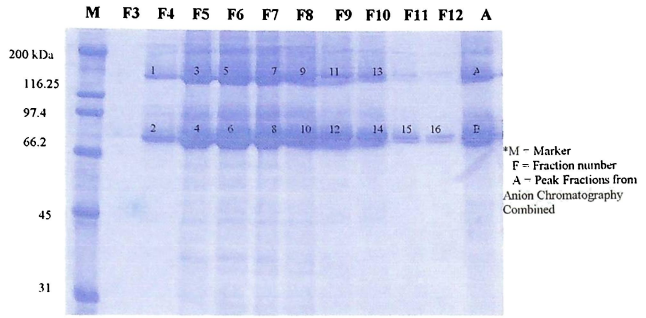


Plate 6. SDS-PAGE of Unbound Fractions (1st Gel) from Cation Chromatography

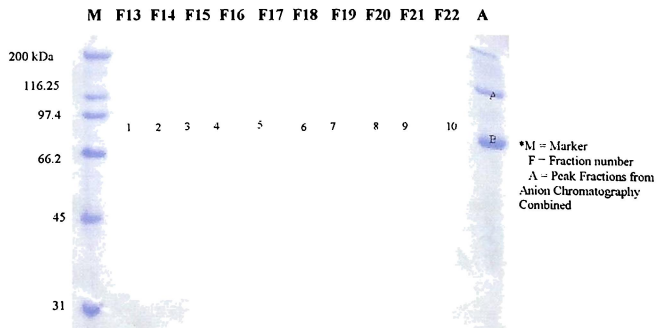


Plate 7. SDS-PAGE of Unbound Fractions (2nd Gel) from Cation Chromatography

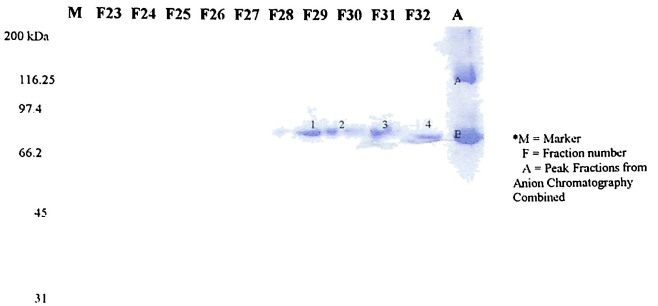


Plate 8. SDS-PAGE of Bound Fractions (1st Gel) from Cation Chromatography

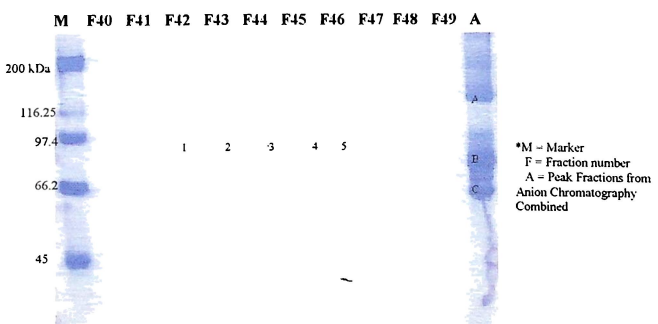


Plate 9. SDS-PAGE of Bound Fractions (2nd Gel) from Cation Chromatography

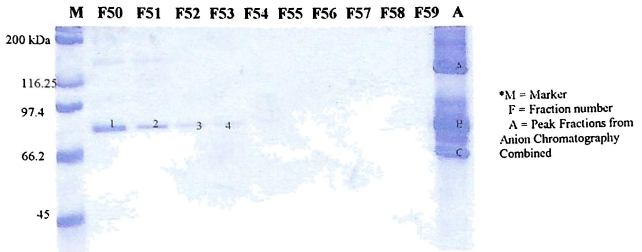


Plate 10. SDS-PAGE of Pure Salt Gradient from Cation Chromatography

Appendix A
(Reagents)

APPENDIX A (REAGENTS)

A. Stock Solutions

1. **monomer solution (30.8% T, 2.7%Cbis)**
   60 g acrylamide (FW 71.08)
   1.6grams bis-acrylamide
   dd H_2O to 200 mm
   store up to 3 months at 4^0C in the dark

2. **4X running gel buffer (1.5M Tris-Cl, pH 8.8)**
   36.3 g Tris (FW 121.1)
   add 150 ml ddH_2O
   adjust pH to 8.8 with HCl
   dd H_2O to 220 ml
   store up to 3 months at 4^0C in the dark

3. **4X stacking gel buffer (0.5M Tris-Cl, pH 6.8)**
   3.0 g Tris (FW 121.1)
   add 40 ml ddH_2O
   adjust pH to 6.8 with HCl
   dd H_2O to 50 ml
   store up to 3 months at 4^0C in the dark

4. **10% SDS**
   10 g of SDS
   ddH_2O to 100 ml
   store up to 6 months at room temperature

5. **10% ammonium persulfate (APS)**
   0.1g APS
   ddH_2O to 1.0 ml
   use fresh, do not store

6. **running gel overlay (0.375M Tris-Cl, 0.1% SDS, pH 8.8)**
   25 ml running gel buffer
   1.0ml 10% SDS
   ddH_2O to 100 ml
   store up to 3 months at 4^0C in the dark

A. Stock Solutions (continuation...)

7. **2X treatment buffer (0.125 M Tris-C;, 4% SDS, 20% v/v glycerol, 0.2 M DDt, 0.002% bromophenol blue, pH 6.8)**
   215 ml 4X stacking buffer gel
   4.0 ml 10% SDS
   1.0 ml glycerol
   1.0 mg bromophenol blue
   0.31g DTT (FW 154.2)
   ddH_2O to 100 ml

8. **tank buffer (0.025 M Tris, 0.192 M glycine, 0.1%SDS, pH 8.3)**
   30.38 g Tris (FW 121.1)
   144.13 g glycine
   10.0 g SDS
   ddH_2O to 10 L
   store at room temperature up to a month

9. **water saturated n-butanol**
   50 ml n-butanol
   5 ml ddH_2O
   combine in a bottle and shake
   store at room temperature indefinitely

10. **Additional reagents**
    a. **protein standard**
       BIORAD broad range standard, diluted to 1:20
    b. **tetramethylethylenediame (TEMED)**

B. Running Gel and Stacking Gel Recipes

RUNNING GEL

	MINIGEL				**SE 600/400 GELS**			
REAGENTS	6%	7.5%	8%	10%	6%	7.5%	8%	10%
ddH_2O	8 ml	7.25ml	7 ml	6 ml	16 ml	14.5ml	14 ml	12 ml
4X Running Gel Buffer	3.75ml	3.75ml	3.75ml	3.75ml	7.5 ml	7.5 ml	7.5 ml	7.5 ml
10% SDS	150µl	150µl	150µl	150µl	300µl	300 µl	300 µl	300 µl
Monomer solution	3 ml	3.75ml	4 ml	5 ml	6 ml	7.5 ml	8 ml	10 ml
TEMED	22.5 µl	22.5µl	22.5µl	22.5µl	45 µl	45 µl	45 µl	45 µl
10% ammonium persulfate (freshly prepared)	100µl	100µl	100µl	100µl	200 µl	200 µl	200 µl	200 µl

STACKING GEL

REAGENTS	**MINIGEL**	**SE 600/400 GEL**
ddH_2O	2.4 ml	6 ml
4X running gel buffer	1 ml	2.5 ml
10% SDS	40 µl	40 µl
Monomer solution	530 µl	1.33 µl
TEMED	9 µl	22.5 µl
10 % ammonium persulfate (fresly prepared)	40 µl	100 µl

Appendix B
(Pictures)

APPENDIX B (PICTURES)

A. Collection Site (Tayabas, Quezon)

B. Infested Coconut Trees

C. Coconut Beetle Larvae in Infested Coconut Tree

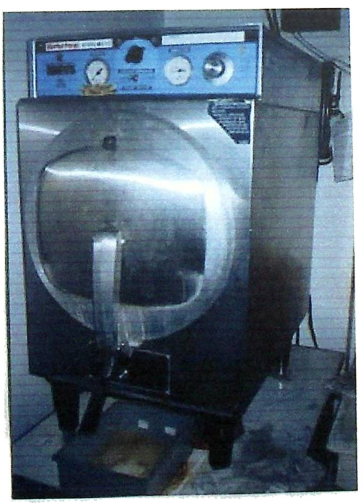
D. Collection of Coconut Beetle Larvae from Infested Coconut Tree Trunks

E. National Institute of Molecular Biology and Biotechnology

F. Coconut Beetle Larva

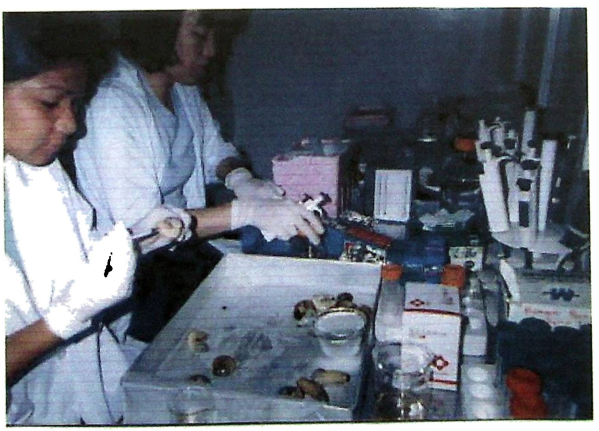
G. Breeding of Coconut Beetle Larva

H. Autoclave

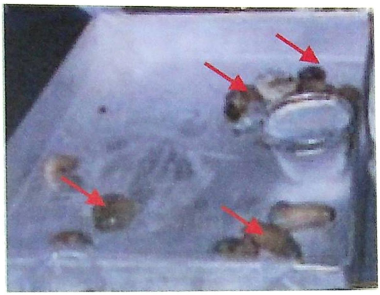


I. Preparation of Sample for Hemolymph Extraction

J. Hemolymph Extraction



K. Melanization of Coconut Beetle Larva After Hemolymph Extraction

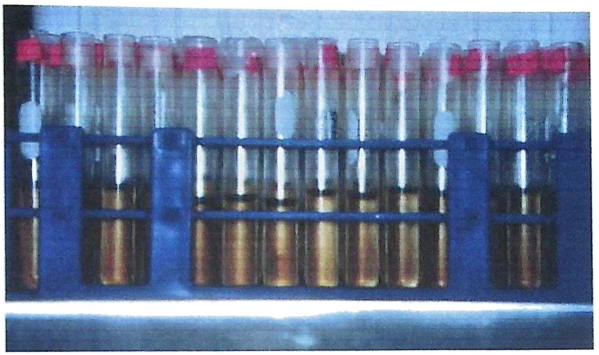


L. Sorvall RC 28S Centrifuge

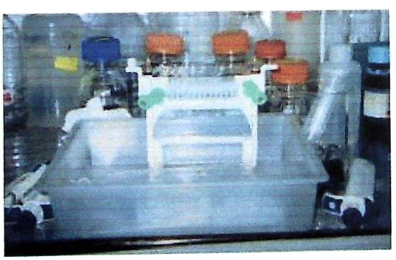
M. Incubator

N. Beckman DU 650 UV-Vis Spectrophotometer

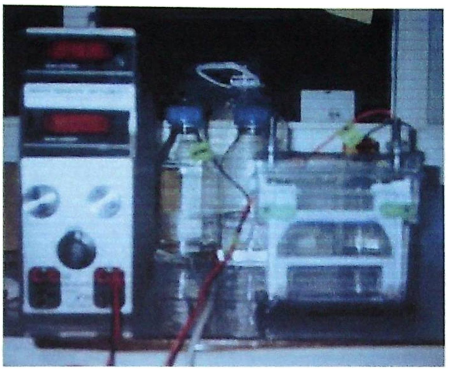
O. Coloring of Hemolymph Solution After Oxidation



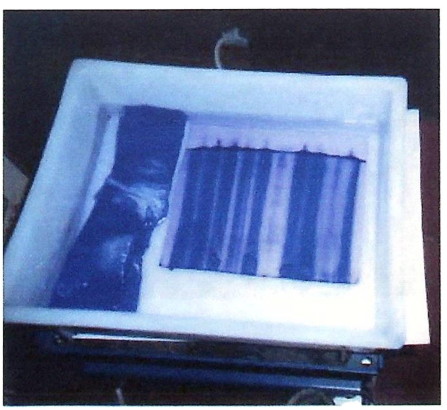
P. Vertical Slab Gel Unit (SDS-PAGE)



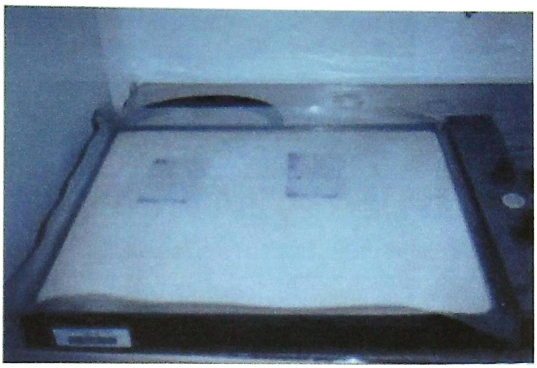
Q. Running SDS-PAGE

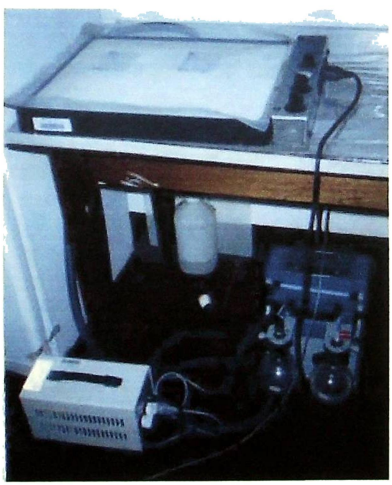


R. Staining SDS-PAGE

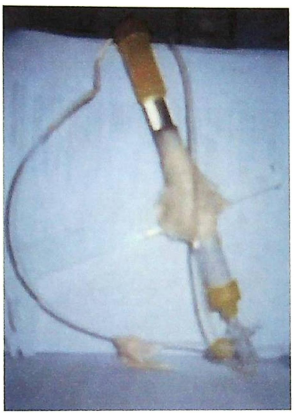


S. Drying of SDS-PAGE Using Hoefer Drygel Sr.

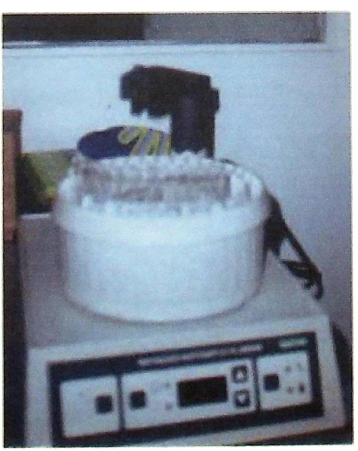




T. Column Used in Ion-Exchange Chromatography



U. Fraction Collector



V. Ion-Exchange Chromatography Elution Set-up

W. Fractions from Ion-Exchange Chromatography