Manual of Biochemical Methods for Determining Stress and Disease Status in Crustaceans

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Preface

Unlike land-based animals, aquatic farmed animals demand more attention in order to monitor their health and disease status. The terrestrial farm animals are stationed at one place, which is not the case with regard to animals reared in aquatic environment. Similarly, feed consumption and mortalities can be clearly monitored in terrestrial farm animals whereas this is not possible in the case of farmed aquatic organisms. Disease is now recognised as one of the formidable challenges facing aquaculture sector. White spot syndrome, one of the most serious viral diseases, affecting shrimps is perhaps the most serious among them.

The complexity of aquatic ecosystem makes it difficult to distinguish between the healthy, organisms under sub-optimal activities and those having diseases. In most of the cases, stress precedes the state of disease. Hence regular monitoring of the organism for stress and health status assumes importance in preventing mortality and subsequent losses. Diagnostics play significant role in aquatic animal health management and disease control.

Currently haematology is widely used as a diagnostic tool for specific evaluation of health in human beings as well as animals. But in aquaculture sector, especially for shrimps, haematology has not been widely used. This manual is a comprehensive guide, which helps in detecting stress and monitoring health status in crustaceans using hematological and biochemical methods. I hope this manual will be of use to the researchers in the field and thereto farmers. This also opens up scope for utilizing the immunological and metabolic parameters in disease diagnosis of crustaceans.

Dr. K. Devadasan

Director

Contents

Abbreviations

A/G	Albumin / Globulin Ratio
ALT	Alanine aminotransferase
ANSA	Amino Naphthol Sulphonic Acid
AST	Aspartate aminotransferase
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CDNB	1, Chloro 2,4 dinitro Benzene
DCPI	2,6, Dichloro Phenol Indo Phenol.
DNA	Deoxy Ribose Nucleic Acid
DNPH	Dinitro Phenyl Hydrazine
DTNB	5' 5 –Dithio Bis –2- Nitrobenzoic acid
EC	Enzyme Commission
EDTA	Ethylene Diamine Tetra Acetic Acid
FDP	Fructose 1,6 –Diphosphate
GOT	Glutamic Oxaloacetic Transferase
GPT	Glutamic Pyruvic Transferase
GPX	Glutathione Peroxidase
GSH	Total Reduced Glutathione
GST	Glutathione – S – Transferase
HPLC	High Performance Liquid Chromatography
LDH	Lactate Dehydrogenase
L-Dopa	L-3,4 Dihydroxy Phenyl Alanine
NAD^+	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Di nucleotide Phosphate, reduced form
OPA	O-phthalaldehyde
PBS	Phosphate Buffer Saline
PMS	Phenazine Metho Sulphate
PO	Phenol Oxidase
Pro PO	Pro Phenol Oxidase
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis
SGOT	Serum Glutamic Oxaloacetic Transferase
SGPT	Serum Glutamic Pyruvic Transferase
SOD	Superoxide Dismutase
TBA	Thio Barbituric Acid
TEMED	N N N' N' Tetra Methyl Ethylene Diamine
TEP	Tetra Ethoxy Propane
THC	Total Haemocyte count
WSSV	White Spot Syndrome Virus

1. Introduction

Diseases are causing much havoc today in shrimp aquaculture, which can be tackled only through systematic scientific studies on immunology and metabolism of Crustacea. In general, the metabolism of Crustacea does not differ from that of higher animal phyla. It is now established that the principal pathways are more or less similar in Crustacea as in vertebrates and the similarities overshadow the differences. There are evidences for the existence of common metabolic pathways like glycolytic, pentose phosphate etc in various crustacean species. Hence it is very clear that many of the immunological and metabolic parameters can be used as health or disease markers as we do in higher animals. Though some studies on immunological parameters have been initiated very recently, practically no attention has been paid on shrimp metabolism during the onset of diseases. This manual is a compilation of the methods standardized in our laboratory to determine main metabolic indicators directly correlated with the stress and disease status of crustaceans. Monitoring the changes in metabolic and immunological processes (brought about by stress and disease) can be a useful tool in the control of epidemics in aquaculture.

2. Sample Preparation

Haemolymph

Collect the haemolymph by inserting a glass capillary tube in the anterior side of cephalothorax between the eyes. The needle should be pre-rinsed with an anticoagulant solution (10mM Tris-HCl, 250mM sucrose, 100mM sodium citrate, pH 7.6). Invert the prawn slowly so that the haemolymph drains into the tube. Transfer the collected haemolymph into a graduated eppendorf tube containing 100 µl of anticoagulant solution. Store the collected haemolymph at 4°C.

Hepatopancreas and Muscle

Dissect the animals after the collection of haemolymph and remove hepatopancreas and abdominal muscle and store separately at –20°C for biochemical analysis. For the analysis, prepare extracts of muscle and hepatopancreas in Tris-HCl Buffer (pH 7.4).

All the chemicals to be used should be of analytical grade and the standards used should be from reliable sources.

3. Haematological parameters

Crustacean haemocytes are normally produced in the haematopoietic tissues. Haematopoiesis is regulated in crustaceans probably by several physiological processes, for example moulting, reproduction, and diseases, and also by environmental conditions. Stress as well as chronic infections and diseases induce haematopoiesis. There are three basic types of haemocytes in most crustacean species, hyalinocytes, granulocytes and semigranulocytes. .

Hyalinocytes can initiate or contribute to haemolymph clotting in crustaceans, whereas granulocytes are responsible for host defense mechanisms. Granulocytes also take part in cuticle hardening by producing proteins which crosslink with diphenols. Granulocytes may also have an important role in membrane formation and wound healing. Semigranular cells have some phagocytic activities. These cells, together with granular cells, granulate spontaneously and participate in cellular defense reactions. Haemocytes play crucial role in defense reactions against disease causing agents and thus enhance disease resistance of the organism.

The main haematological parameters which are monitored for stress include haemolymph volume, clotting time of haemolymph, total haemocyte count, glucose level, creatinine level and albumin/globulin ratio of haemolymph.

3.1 Haemolymph volume

Collect haemolymph as per the procedure mentioned above. Repeat the procedure till entire haemolymph is collected from the prawn.

Haemolymph volume can be obtained by measuring the volume of haemolymph collected in the eppendorf tubes and by subtracting the volume of anticoagulant. Care should be taken to draw maximum amount of haemolymph from the animal each time, while collecting the haemolymph for measuring the total volume.

3.2. Measurement of Clotting time

The haemolymph clotting time can be measured as per Jussila *et al.* (2001). Collect the haemolymph directly into a pre-cooled (4°C) glass capillary tube of 1.2 mm diameter and 50mm length. Keep the haemolymph in motion, by keeping the tube vertically. Once the haemolymph reaches the end, invert the tube. Repeat the action till the haemolymph ceases to flow and note the clotting time.

3.3. Measurement of Total Haemocyte Count

Total haemocyte count includes the count of haemocytes including granular, semi granular and hyaline cells. Total haemocyte count (THC, cells ml^{-1}) is determined using a Burker haemocytometer (Le Moullac *et al.,* 1997). Dilute the fixed haemolymph 2, 4, 8, 16, and 32 times with ice-cold phosphate buffer saline (PBS, 20 mM, pH 7·2). Place a drop of haemolymph in the grid of haemocytometer. Observe the haemocytes on the haemocytometer under microscope and count manually in all 25 squares (= 0.1 mm^2).

3.4. Estimation of Glucose in Haemolymph

Glucose, a simple monosaccharide, is one of the most important carbohydrates and is used as a source of energy in animals and plants. Glucose is involved in the production of ATP, the cell's energy carrier. In addition, it is critical in the production of protein and in lipid metabolism. Glucose represents a potential source of stimulants for immune response.

Glucose is the major sugar in the circulating haemolymph in crustacea and it originates from different sources, either directly from the absorption of dietary glucose through hepatopancreatic and intestinal epithelial cells or from the hepatopancreas where it is stored as glycogen or synthesized by gluconeogenic pathway. Stress affects the qualitative and quantitative nature of circulating carbohydrates. Glucose levels in the haemolymph are strongly affected by stress and infection (Racotta and Palacois, 1998; Hall and van Ham, 1998).

Glucose in haemolymph is estimated by the method of Sasaki *et al.* (1972) using o-toluidine reagent .

Reagents

a. Trichloro acetic acid (TCA) : 10%

b. o-toluidine reagent: Dissolve 12.5 g of thiourea and 12.0 g of boric acid in 50 ml of distilled water by heating over a mild flame. Mix exactly 75 ml of redistilled o-toluidine and 375 ml of acetic acid with thiourea-boric acid mixture and make up the total volume to 500 ml with distilled water. Keep the reagent in a refrigerator overnight and filter.

c. Standard glucose solution: Dissolve 10 mg of pure glucose in 100 ml of 0.2% boric acid in water.

Procedure

Mix 0.1 ml of very fresh haemolymph with 1.9 ml of TCA solution to precipitate protein and then centrifuge. Mix 1.0 ml of the supernatant with 4.0 ml of o-toluldine reagent and keep in a boiling water bath for 15 minutes. Read the green colour developed at 600 nm in a spectrophotometer. Treat a set of standard glucose solutions of appropriate concentrations similarly.

Haemolymph sugar levels are arrived at by comparison with the standard curve and expressed as mg / dl.

3.5 Creatinine in Haemolymph

Creatinine is the major form of nitrogenous waste in the circulation in crustaceans. It may originate either from the urea cycle or from the break down of creatine phosphate. Low levels of creatinine may result from decreased hepatic production due to severe hepatic damage and inadequate dietary protein. During viral infection, there could be severe hepatopancreatic damage, resulting in low levels of creatinine. Also during infection, feeding rate comes down and hence metabolic processes are reduced resulting in less nitrogenous waste in the form of creatinine in the haemolymph. Creatinine in haemolymph is estimated by the method of Slot (1965).

Reagents

a. Picric acid: Dissolve 1.2g of picric acid in 1l of distilled water.

b. Sodium hydroxide: Dissolve 30g in 1l water.

c. Alkaline picrate reagent: Mix equal volumes of solutions (1) and (2) just before use.

d. Sodium tungstate solution : 50g / l of water

e. Sulphuric acid : 0.33 M

f. Creatinine standard : Dissolve 100 mg of creatinine in l00ml of 0. 1M HCl. Before use, dilute this stock standard to 10 fold with water.

g. Glacial acetic acid.

h. Trichloro acetic acid (TCA) : 10%

Procedure

Mix 0.1 ml of haemolymph with 3.9 ml of 10% TCA to precipitate protein. To 3.0 ml of deproteinised supernatant add 2.0ml of alkaline picrate solution. Treat blank containing 3.0ml of water and aliquots of standard in 3.0 ml of water in a similar manner. After 30 minutes, measure the colour at 520nm against the reagent blank.

Express the values as mg/dl of haemolymph

3.6. Estimation of Albumin/Globulin Ratio

Albumin is the major constituent of serum protein (usually over 50%). It is produced by liver from the amino acids taken through the diet. It helps in osmotic pressure regulation, nutrient transport and waste removal. High levels are seen in liver disease, shock, dehydration, or multiple myeloma. Lower levels are seen in poor diets, diarrhoea, fever, infection, inadequate iron intake, and edemas or hypocalcemia.

Globulin, a larger protein than albumin, is important for its immunologic responses, Globulins have diverse functions such as, the carrier of some hormones, lipids, and metals. Elevated globulin levels are observed during chronic infections, liver disease, rheumatoid arthritis, and myelomas. Low levels of globulin are seen in immune compromised patients, poor dietary habits, malabsorption and liver or kidney diseases.

The albumin/globulin (A/G) ratio in plasma is an indicator of health status of the organism. Low A/G ratio may be due to overproduction of gamma-globulin or due to low production of albumin as in cirrhosis or excessive loss of albumin as in nephrotic syndrome or protein losing enteropathy. Therefore significantly low albumin/ globulin ratio points to severe damage of hepatopancreas.

The albumin and globulin content of the haemolymph can be estimated by the method of Varley (1980).

a. Sodium sulphite solution : Dissolve 25 g of anhydrous sodium sulphite in 100 ml of distilled water

b. Diethyl ether

c. Biuret reagent : Dissolve 1.5 g copper sulphate and 6g sodium potassium tartarate in 50 ml distilled water, add 300 ml 10% sodium hydroxide. Make up the solution to 1l. Add 1.0 g potassium iodide and store in plastic containers.

Procedure

To 0.05 ml of haemolymph, add 4 ml of sodium sulphite solution and 4 ml of ether and centrifuge. After centrifugation take 2.0 ml of lower layer and add 2.0 ml of freshly prepared biuret reagent. The blank contains 2.0 ml of sodium sulphite solution. Leave all the tubes at room temperature for 15 minutes. Read the colour developed at 540 nm in a spectrophotometer. Determine the albumin content from the absorbance and is calculated by substracting the albumin content from total protein. From the values of albumin and globulin, calculate albumin / globulin ratio.

4. Assays of parameters pertaining to immunity and antioxidant status

4.1. Assay of Phenol Oxidase (PO) (E.C 1.14.18.1)

The PO system is one of the best-studied immune systems in crustaceans. The PO system is involved in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation and phagocytosis. In crustaceans phagocytosis is the most common reaction of cellular defense and a decrease in the activity of phenol oxidase enzyme during virus attack can lead to the failure of the defense system of prawn.

PO activity can be assayed according to procedure described by Sung *et al.* (1994) using L-3, 4- dihydroxyphenylalanine (L-dopa) as substrate.

Reagents

a. L-Dihydroxy phenyl alanine (L-Dopa) – 10 mM

b. Tris Buffer – 100 mM pH 7.8 (Dissolve 2.48g Tris-HCl, 0.2033 g $MgCl_2$ and 0.147 g $CaCl_2$ in distilled water, adjust pH to 7.8 and make up the volume to 200ml)

c. Sodium dodecyl sulphate (SDS) - 0.4g in 10ml water.

Procedure

Measure phenol oxidase activity spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA). To 100µl of sample, add 1.0ml of L-DOPA (10mM), followed by 1.9ml of 100 mM Tris HCl buffer (pH 7.8) containing 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.2% SDS. After a 10-min incubation at 25°C, read the optical density at 492 nm using a spectrophotometer.

Express the specific activity as units calculated from the change of absorbance per min per mg protein.

4.2. Assay of Xanthine Oxidase (E.C.1.2.3.2)

Xanthine oxidase is the enzyme which catalyzes oxidation of xanthine and hypoxanthine by molecular oxygen. Unlike most oxidases, which generate water from oxygen, xanthine oxidase converts oxygen to oxygen radical (O_2^-) and H_2O_2. So it is similar to NADPH oxidase of the plasma membrane, which reduces oxygen to superoxide. This enzyme is a unimolecular, multicomponent electron transport system. Xanthine is metabolized in the peroxisome and the degree to which it is metabolized depends on the animal. Primates, birds, reptiles, and insects degrade xanthine to uric acid, while other vertebrates metabolize it to allantoin. It can be metabolised even further in some animals, with marine invertebrates metabolizing it all the way to ammonia. During the state of stress or disease conditions, the levels of xanthine oxidase get drastically reduced. Xanthine oxidase can be estimated by the method of Bray (1962).

Reagents

1. Phosphate buffer 0.05 M pH 7.8 : 89.6 ml of 0.05 M Na_2HPO_4 is added to 10.4 ml of 0.05 M NaH_2PO_4 and adjust the pH to 7.8.
2. Hypoxanthine 1.5g/l - 100 ml.

Procedure

To 0.1ml of sample, add 1.9ml of buffer, 1.0ml of distilled water and 1.0ml of hypoxanthine solution as substrate. Measure the change in absorbance at 250nm and calculate the activity by the formula (change in absorbancex1000)/ ($1.22x10^4$xmg Protein/ml reaction mixture)˙ and express as units / mg protein.

3.3 Determination of Total Reduced Glutathione (GSH)

Glutathione (GSH) is a tripeptide formed from the amino acids

cysteine, glycine, and glutamic acid. GSH has multiple functions in disease prevention and in detoxification of chemicals and drugs and its depletion is associated with increased risks of toxicity and disease. GSH is estimated by the method of Ellman (1959).

Reagents

1. DTNB : 0.6mM in 0.2M phosphate buffer, pH 8.0
2. Phosphate buffer : 0.2M, pH 8.0 (47.35ml of 0.2M Na_2HPO_4 is mixed with 2.65ml of 0.2M NaH_2PO_4 and made upto 100ml with distilled water.)
3. TCA : 5%
4. Standard glutathione : Stock : 61.4mg in 100ml 0.02M EDTA. Dilute stock 50 times to prepare the working standard.

Procedure

Precipitate protein in the homogenates of hepatopancreas and tissue (0.5ml) with 0.1ml 5% TCA and 0.4ml water. Mix the contents well for complete precipitation of proteins and centrifuge. To 0.5ml of clear supernatant, add 2.5ml of 0.2M phosphate buffer and 50µl of DTNB. Read the absorbance at 412 nm against a blank containing TCA instead of sample. Run a series of standards along with blank treated in a similar way to determine the glutathione content.

Express the amount of glutathione as µg glutathione/mg protein.

4.4. Assay of Glutathione Peroxidase (GPX) (EC 1.11.1.9)

Glutathione peroxidase (GPX) is important in the prevention of cell damage by oxidants. GPX, being an antioxidant enzyme, removes precursors of free oxygen radicals and is necessary for the conversion of hydrogen peroxide to molecular oxygen and water.

GPX reduces reactive oxygen species (ROS) and intervene in hyperoxide detoxification. GPX reduces peroxides to their corresponding alcohols or water. The method of Paglia and Valentine (1967) is used for the assay of glutathione peroxidase.

Reagents

1. 0.4 M Phosphate buffer, pH 7.0 : Mix 5.48g KH_2PO_4 with 0.93g of NaOH in 100ml distilled water, adjust pH to 7.0.
2. EDTA: 0.4mM
3. Tris buffer 0.4M pH 8.9. : Dissolve 4.846g of tris in distilled water, adjust pH to 8.9 and make up final volume to 100ml.

4. GSH: 61.4 mg GSH in 100ml 0.02M EDTA
5. Sodium azide: 10mM solution in water
6. H_2O_2 solution: Mix 0.01ml of 30% solution with 100 ml phosphate buffer.
7. TCA : 10%
8. DTNB : 99mg in 25ml methanol.
9. Glutathione reductase (2.4 units/ml)
10. NADPH

Procedure

To 0.2 ml of sample add 0.2 ml of phosphate buffer, 0.2 ml of EDTA, 0.1 ml sodium azide and 0.1ml of glutathione reductase (0.24 units). Mix the reaction mixture well and add 0.2ml GSH, 0.1ml of hydrogen peroxide and 0.1ml NADPH. Incubate the mixture for 10 minutes at 37 ^{o} C and add 0.5ml 10% TCA. Centrifuge at 10,000 rpm for 5 minutes. To 1ml of supernatent, add 2ml tris buffer and 50µl DTNB and read the absorbance at 412nm. Do the blank along with each sample, by adding 10% TCA in the beginning. To different concentrations of standard, add 2ml tris buffer and 50µl of DTNB and measure the absorbance.

Express the enzyme activity as unit / min / mg protein (n mole glutathione oxidised per minute per mg protein)

4.5. Assay of Glutathione-S-Transferase (GST) (EC 2. 5.1.18)

Glutathione S-transferase (GST) catalyses the conjugation of glutathione to numerous potentially toxic compounds, including aliphatic, aromatic, heterocyclic radicals, epoxides, and arene oxides.

Glutathione-S-transferase activity can be determined by the method of Habig *et al.,* (1974)

Reagents

1. Phosphate buffer: 0.3 M, pH 6.5 (Add 47.35 ml of 0.3 M Na_2HPO_4 to 2.65 ml of 0.3 N NaH_2PO_4 and adjust the pH to 6.5).
2. 1-chloro-2, 4-dinitrobenzene (CDNB) : 30mM. Dissolve in 1ml methanol and make up to the required volume with water
3. Reduced glutathione (GSH): 30mM in water.

Procedure

Make up the reaction mixture containing 1.0 ml of buffer, 0.1ml of CDNB and 0.1 ml of tissue homogenate to 2.5 ml with water. Pre-incubate the reaction mixture at 37°C for 5 minutes. Add 0.1 ml of GSH and measure the change in absorbance at 340 nm for 3 minutes at 30 seconds intervals.

Express the enzyme activity as m moles of CDNB conjugate formed / min / mg protein.

4.6 Assay of Superoxide Dismutase (SOD) (EC 1.15.1.1)

Superoxide Dismutase (SOD) is an enzyme which plays a major role in the cellular fight against infection and protects cells from damage. Superoxide, peroxide, hydroxyl radical and other free radicals derived from oxygen are highly reactive and therefore threatening to the integrity of essential biomolecules such as DNA and RNA, enzymes and other proteins and phospholipids responsible for membrane integrity. The activity of the enzyme usually increases as an adaptive response to free radical overload.

The enzyme is assayed according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine - adrenochrome transition by the enzyme

Reagents

1. Carbonate -bicarbonate buffer: pH 10.2 (0.1M) containing 57mg/ 100ml EDTA (Prepare by mixing 75 ml of 0.1 M Na_2CO_3.10 H_2O (28.62 g in 1 l distilled water) and25 ml 0.1 M $NaHCO_3$ (8.4 g in 1 l distilled water) and adjust the pH to 10.2. Add 57 mg of EDTA to this solution).

2. Epinephrine : 3 mM in water

Procedure

To 0.1 ml of the sample, add 1.5ml of the carbonate-bicarbonate buffer. Initiate the reaction by the addition of 0.4ml of epinephrine and measure the change in optical density per minute at 480 nm in a UV - visible spectrophotometer.

One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation.

4.7. Assay of Catalase (EC 1.11.1.6)

The formation of highly reactive oxygen species is a normal

consequence of essential biochemical reactions including mitochondrial and microsomal electron transport systems, phagocytosis, xenobiotic enhanced redox cycling etc. Catalase is an enzyme, which promotes the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent to water and diatomic oxygen.

Catalase is assayed according to the method of Takahara *et al.,* (1960)

Reagents

1. Phosphate buffer: 50mM, pH 7.0 (Prepare 80ml of KH_2PO_4 50mM solution, adjust pH to 7.0 and make up to 100ml with distilled water).
2. Hydrogen peroxide: 30mM solution in the above buffer

Procedure

To 1.2ml of phosphate buffer, add 0.2ml of the enzyme source and start the enzyme reaction by the addition of 1.0 ml of H_2O_2 solution. Measure the decrease in absorbance at 240nm at 30 seconds intervals for 3 minutes. Run the enzyme blank simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

Express the enzyme activity as nanomoles of H_2O_2 decomposed / min / mg protein.

4.8. Estimation of Lipid peroxidation

A direct consequence of failure of antioxidant system is the accumulation of ROS in the system, which leads to higher rate of formation of lipid peroxides and consequent tissue damage. Unsaturated fatty acids are more susceptible to attack by hydroxyl free radicals, generating lipid peroxides. Peroxides of unsanturated fatty acids are more hydrophilic and consequently alter the structure of the membrane, thereby disturbing normal membrane function.

The lipid peroxide content can be estimated by thiobarbituric acid reaction as described by Ohkawa *et al.* (1979).

Reagents

1. Acetic acid glacial: 20%
2. 0.8% thiobarbituric acid (TBA) in 20% acetic acid. Prepare freshly by boiling, just before use.
3. SDS: 8%
4. Standard: Tetraethoxypropane at different concentrations Prepare in distilled water.

Procedure

To the sample (0.1ml), add 1.5 ml acetic acid, 1.5 ml TBA reagent, 0.2 ml SDS and make up to 5ml using distilled water. Treat standards (Tetraethoxypropane,TEP) and blank similarly. Incubate the tubes for 1h in boiling water. Centrifuge the tubes at 10,000rpm for 10 min. Measure the pink colour developed at 532nm.

Record the results as ng of TBA reactive substances / mg protein.

4.9. Assay of Alkaline Phosphatase (EC 3.1.3.1)

The acid and alkaline phosphatases are used as indicators of exposure to toxic metal ions. These enzymes also are indicators of ROS production. Alkaline and acid phophatases are intrinsic plasma membrane enzymes, found in almost all animal cells. Acid and alkaline phosphatases (E.C.3.1.3.1) catalyse the hydrolysis of various phosphate containing compounds and act as transphosphorylases at acid and alkaline pH's.

Alkaline phosphatase is a broad term associated with non-specific phosphomonoesterases with activity optima at alkaline pH. Alkaline phosphatases are enzymes involved in the process of minerailzation of calcium carbonate in invertebrates.

Alkaline phosphatase is assayed by the method of King (1965a) using disodium phenyl phosphate as the substrate.

Reagents

1. 0.1 M carbonate - bicarbonate buffer, pH 10.0 (Dissolve 28.62 g Na_2CO_3 in 1 llitre distilled water. Dissolve 8.4g $NaHCO_3$ in 1 l distilled water. Mix 75 ml of first solution with 25 ml of second solution and add 5g EDTA and adjust pH to 10)
2. Substrate: 0.01 M disodium phenyl phosphate solution in water.
3. Folin's Ciocaltau reagent: Diute with distilled water in the ratio 1:10.
4. 15% sodium carbonate in water.
5. 0.1 M magnesium chloride in water.
6. Standard phenol solution: A solution of distilled crystalline phenol in water, containing 5.0 μg in 0.1 ml is prepared.

Procedure

Mix 1.5 ml of carbonate-bicarbonate buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride and requisite amount of the enzyme source (0.05 ml haemolymph) together. Incubate the reaction mixture at 37°C for 15 minutes. Terminate the reaction by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appears, centrifuge the tubes. Incubate the controls without enzyme sources and add the enzyme source after the addition of Folin's-phenol reagent. Then add 1.0 ml of 15 % sodium carbonate solution and incubate for a further 10 minutes at 37°C. Read the blue colour developed at 640 nm against a blank. Treat the standards also similarly.

Express the activity of the enzyme as ng of phenol liberated / mg protein / h.

4.10. Assay of Acid Phosphatase (EC 3.1.3.2)

Acid phosphatase is ubiquitous in nature. This enzyme is involved in a variety of metabolic processes such as membrane permeability, growth and cell differentiation and steroidogenesis. In ecotoxicology this enzyme may serve as an indicator of intoxication because of its sensitivity to metallic salts. The acid phosphatase can be estimated by the method of King (1965b)

Reagents

1. Citrate buffer 0.1M pH 4.9. (mix 37.5ml 0.1M citric acid with 62.5 ml of 0.1M trisodium citrate and adjust pH to 4.9.)
2. Substrate: 0.01 M disodium phenyl phosphate solution.
3. Folin's Ciocaltau reagent: Diute with distilled water in the ratio 1:10.
4. 15% sodium carbonate in water
5. Standard phenol solution: Prepare a solution of distilled crystalline phenol in water, containing 5.0 μg in 0.1 ml.

Procedure

The procedure adopted for the assay of acid phosphatase is the same as described above for alkaline phosphatase, excepting that a citrate buffer (0.1M, pH 4.9) should be used and omit magnesium chloride from the incubation mixture.

The activity of the enzyme is expressed as ng of phenol liberated / h / mg protein.

5. Assays for determining metabolic and energy status

5.1. Assay of Aldolase (EC 4.1.2.13)

Aldolase is an enzyme that is involved in the breakdown of glucose, fructose, and galactose, a process used by cells to generate energy in the form of ATP (adenosine triphosphate). Aldolase catalyses a key reaction in glycolysis and energy production. The enzyme is assayed by the method of King (1965 b).

Reagents

1. Tris HCl buffer: 0.1 M, pH 8.6.(Dissolve 1.21 g of Tris HCl in 100 ml of distilled water and adjust pH to 8.6)
2. Substrate: Fructose 1, 6-diphosphate, 0.05 M.
3. Hydrazine sulphate: 0.56N in water.
4. Trichloro Acetic acid (TCA): 10% in water
5. Colour reagent: 10% solution of 2,4-dinitrophenyl hydrazine (DNPH) in 6N hydrochloric acid.
6. Sodium hydroxide : 0.75N in water.
7. Standard: Dissolve 12.3 mg of DL-glyceraldehyde in 100 ml of distilled water and keep at room temperature for 4 days for de - polymerization.

Procedure

Incubate a mixture containing 0.25 ml of substrate, 0.25 ml hydrazine sulphate, 1 ml tris-HCl buffer and 0.1 ml of tissue homogenate at 37°C for 15 minutes. Terminate the reaction by the addition of 1 ml of 10% TCA and centrifuge. Transfer one ml of the supernatant to a tube containing 1 ml of 0.75N sodium hydroxide. Leave the tube at room temperature for 10 minutes. Then add 1 ml of 2,4-dinitrophenyl hydrazine reagent and incubate at 37°C for 60 minutes. Read the colour developed after the addition of 7 ml of 0.85 N sodium hydroxide at 540 nm using a spectrophotometer. Treat Standard DL-glyceraldehyde solution in a similar manner.

Express the enzyme activity as mg of glyceraldehyde 3-phosphate formed / h / mg protein.

5.2. Assay of Glucose 6-Phosphate Dehydrogenase (EC 1.1.1.49)

Glucose 6-phosphate dehydrogenase catalyses the entry step of Glucose 6 Phosphate into the pentose phosphate shunt. This anaerobic

pathway for glucose metabolism is the source for NADPH and the maintenance of the level of reduced glutathione. Glucose 6-phosphate dehydrogenase activity can be determined by the method of Ellis and Kirkman (1961)

Reagents

1. Tris - HCl buffer: 0.05M, pH 7.5 (Dissolve 0.6 g of Tris HCl in distilled water and adjust pH to 7.5 and make up to 100 ml)
2. $MgCl_2$: 1.0 M in water
3. NADP: 1.0 M solution in water
4. Phenazine methosulphate (PMS) : 0.005% in water
5. 2,6-dichlorophenol indophenol (DCPI) : 0.01% in water
6. Glucose 6-phosphate: 0.02M in water

Procedure

The incubation mixture in a total volume of 2.2 ml containing 1.0 ml of Tris-HC1 buffer, 0.5 ml of PMS, 0.4 ml of DCPI solution, 0.1 ml of magnesium chloride, 0.1 ml of NADP and a requisite amount of enzyme preparation (0.1ml) is allowed to stand at 30 – 35°C for 10 minutes to permit the oxidation of endogenous materials. Initiate the reaction by the addition of 0.5 ml of glucose 6-phosphate. Measure the change in optical density continuously at 340 nm for 60 minutes against a water blank in a spectrophotometer.

Express the enzyme activity as units/mg protein. Calculate the unit of activity from the formula (Change in absorbance/min)/ (6.22 x mg protein/ ml reaction mixture).

5.3. Assay of Aspartate Aminotransferase (EC 2.6.1.1) (AST)

Aspartate aminotransferase is also known as glutamic-oxaloacetic transferase (GOT), serum glutamic-oxaloacetic transferase (SGOT), aspartate transaminase, and glutamic-aspartic transaminase. When an organ or body tissue is injured, increased levels of AST are released into the blood. The greater the degree of tissue damage, the greater the quantity of AST that is released. The activity of aspartate aminotransferase is assayed by the method of Mohur and Cook (1957).

Reagents

1. Phosphate buffer: 0.10 M, pH 7.5 (Dissolve 1.5g K_2HPO4 and 0.2g KH_2PO_4 in distilled water. Adjust the pH to 7.5.)

2. Substrate: Dissolve 300 mg of L-aspartic acid and 50 mg of α- ketoglutaric acid in 20-30 ml of the phosphate buffer and add 10% sodium hydroxide to bring the pH to 7.5. Make up to 100 ml with phosphate buffer.

3. 2,4-dinitrophenyl hydrazine (DNPH) reagent: Dissolve 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and make up to 1 l with distilled water.

4. 0.4 N sodium hydroxide.

5. Standard pyruvate: Disolve 11.01 mg of sodium pyruvate in 10 ml of distilled water. Dilute this further to 100 ml with distilled water and prepare fresh each time.

Procedure

To 1.0 ml of the substrate, add 0.1 ml of sample and incubate for one hour at 37°C. Add 1.0 ml of DNPH reagent and leave for 20 minutes. At the end of 20 minutes, add 10 ml of 0.4 N NaOH and keep for 10 min and read the colour developed at 540 nm in a spectrophotometer. Treat the blank and standards also similarly. Express the enzyme activity as μ moles of pyruvate liberated / h / mg protein.

5.4. Assay of Alanine Aminotransferase (EC 2.6.1.2) (ALT)

Alanine aminotransferase is also known as glutamic-pyruvic transferase (GPT), serum glutamic-pyruvic transferase (SGPT), and alanine transaminase. When an organ or body tissue is injured, increased levels of ALT are released into the blood. When ALT is high, the most common cause is hepatic tissue damage.

The activity of alanine aminotransferase can be assayed by the method of Mohur and Cook (1957)

Reagents

1. Buffered substrate solution: Dissolve 1.5 g K_2HPO_4, 0.2 g KH_2PO_4, 0.03g α ketoglutaric acid and 1.78 g DL- alanine in distilled water. The pH is adjusted to 7.4 with 1N NaOH, if necessary, and make up to 100 ml.

2. 2,4-dinitrophenyl hydrazine (DNPH) reagent: As described previously.
3. 0.4 N sodium hydroxide
4. Standard pyruvate: As described previously.

Procedure

To 1.0 ml of the buffered substrate, add 50µl of sample and incubate at 37°C for 30 minutes. Arrest the reaction by adding 1.0 ml of DNPH and leave aside for 20 minutes at room temperature. Read the colour developed by the addition of 10 ml of 0.4 N NaOH at 540 nm in a spectrophotometer against the reagent blank.

Express the enzyme activity as µ moles of pyruvate liberated / h / mg protein.

5.5. Assay of Lactate Dehydrogenase (EC 1.1.1.27) (LDH)

Lactate dehydrogenase mediates the inter-conversion of lactate and pyruvate, depending on the availability of NAD. LDH is an enzyme found in many body tissues and organs. Injury to organs and tissues often causes a release of LDH into the haemolymph, which raises the level of this enzyme.

The enzyme activity can be assayed as per the method of King with slight modification (1965a). The amount of pyruvate formed is measured colorimetrically.

Reagents

1. 0.1 M glycine buffer: Dissolve 7.5 g of glycine and 5.85 g of sodium chloride in 1 l of distilled water.
2. Buffered substrate: Dissolve 2.76 g of lithium lactate in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide to adjust the pH to 10.0. Prepare this just prior to use.
3. 0.4 N sodium hydroxide.
4. Dissolve 5.0 mg of NAD^+ in 1.0 ml of distilled water. Prepare this just before use.
5. 2,4-Dinitrophenyl hydrazine (DNPH) reagent: Dissolve 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and make up to 1 litre with distilled water.
6. Standard pyruvate solution: As described in 5.3

Procedure

To 1.0 ml of the buffered substrate, add 0.2 ml of sample and incubate at 37°C for 15 minutes. After adding 0.2 ml of NAD^+ solution, continue the incubation for another 30 minutes and then add 1.0 ml of DNPH reagent. Incubate the mixture for a period of 15 minutes at 37°C. Then add 7.0 ml of 0.4 N sodium hydroxide solution and measure the colour developed at 520 nm in a spectrophotometer. Treat standards also in the same manner along with blank.

Express the enzyme activity as μ moles of pyruvate liberated / h / mg protein.

5.6. Assay of Glucose 6-Phosphatase (EC 3.1.3.9)

Glucose 6-phosphatase is the liver enzyme that converts glucose 6-phosphate into glucose. It is vital for release of glucose into the bloodstream from glycogen breakdown i.e. glycogenolysis. Low levels Glucose 6-phosphatase activity will cause disorder of glycogen metabolism. This will lead to the organisms' inability to break down glycogen into usable glucose molecules during periods of fasting, precipitating metabolic disarray and chronic stress conditions. Glucose 6-phosphatase is assayed according to the method of King (1965 b).

Reagents

1. Citrate Buffer : 0.1M, pH 6.5 (Mix 30.75 ml 0.1 M Citric acid and 69.25 ml of 0.2M Na_2HPO_4 and adjust the pH to 6.5)
2. Substrate :Glucose 6-phosphate, 0.10M in water.
3. Ammonium molybdate reagent: Mix 25g of ammonium molybdate in 200 ml distilled water. Add this to 300ml 10N H_2SO_4, and dilute to 1l with distilled water.
4. Amino naphthol sulphonic acid (ANSA): Grind 0.2g of ANSA with 1.2g of Na_2SO_3 and 1.2g of sodium bisulphite ($NaHSO_3$). Keep the mixture in the freezer. At the time of use, dissolve 0.25g in 10ml distilled water.
5. TCA: 10%

Procedure

The incubation mixture in a total volume of 1 ml contains 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of the enzyme preparation. Carry out Incubation at 37°C for 60 minutes. Arrest the reaction by the addition of 1 ml of TCA and centrifuge. The phosphorus content of the supernatant is estimated by the method of Fiske and Subbarow (1925) (Refer 7.4.b).

Express the enzyme activity as μg of inorganic phosphorous liberated / min/mg protein.

5.7. Assay of Fructose 1, 6-Diphosphatase (EC 3.1.3.11)

Fructose 1,6-diphosphatase (also termed fructose 1,6-biphosphatase) is an important enzyme in gluconeogenesis because it facilitates conversion of fructose 1,6-diphosphate (FDP) to fructose 6-phosphate, which permits endogenous glucose production from gluconeogenic amino acids (eg, alanine and glycine), glycerol, or lactate. Low activity of fructose 1,6-diphosphatase will lead to accumulation of intrahepatocellular fructose 1,6-diphosphatase, which inhibits gluconeogenesis. The inability to convert lactic acid or glycerol into glucose leads to hypoglycemia, lactic acidosis, and glyceroluria.

The content of fructose 1, 6-diphosphatase is determined by the method of Gancedo and Gancedo (1971)

Reagents

1. Tris-HCl buffer: 0.1M, pH 7.0 (Dissolve 1.21 g of Tris HCl in distilled water and adjust the pH to 7.0 with NaOH and make up to 100 ml)
2. Substrate, Fructose 1,6-diphosphate : 0.05M
3. $MgCl_2$: 0.1M
4. KCl: 0.1M
5. EDTA:0.001M
6. TCA: 10%
7. Ammonium molybdate reagent (Prepare as mentioned earlier. Refer 5.6)
8. ANSA. (Prepare as mentioned earlier. Refer5.6)

Procedure

The assay medium in a final volume of 2 ml contains 1.2 ml of buffer 0.1 ml of substrate, 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.l ml of tissue homogenate. Carry out the incubation at 37°C for 15 minutes. The reaction is terminated by the addition of 1 ml of 10% TCA. The suspension is centrifuged and the phosphorous content of the supernatant is estimated according to the method of Fiske and Subbarow (1925)(Refer 7.4.b)

The enzyme activity is expressed as μg of phosphorous liberated / min / mg protein.

6. Assay of Proteins and aminoacids

6.1 Estimation of Proteins

Protein content of haemolymph is of special significance because of its important role in the immune system. Total protein is determined spectrophotometrically based on the method of Lowry *et al.* (1951).

Reagents

1. Reagent A. Dissolve 0.5g copper sulfate ($CuSO_4.5H_2O$) and 1g sodium citrate (tri sodium citrate) in 100ml water. This solution can be kept indefinitely.
2. Reagent B: Dissolve 20g sodium carbonate (Na_2CO_3) and 4g sodium hydroxide in 1litre of water.
3. Reagent C : To 50 ml Reagent B, added 1ml Reagent A.
4. Reagent D : To 10 ml Folin – Ciocalteau reagent, add 10 ml water
5. Standard bovine serum albumin (BSA): Dissolve 100 mg of BSA in 100 ml water in a standard flask. 10 ml of the stock is diluted to 100 ml to get a working standard containing 100 μg / ml.

Procedure

Pipette out 0.01 ml of haemolymph or 0.1 ml of tissue homogenate and standard BSA in the range of 20-100 μg into test tubes, and make up the total volume to 1.0 ml with water. The blank contains 1.0 ml of water. Add 2.5 ml Reagent C, mix and keep for 5 –10 min. Add 0.25 ml of Reagent D and keep for 20 –30 min. Read the blue colour developed at 660 nm against the reagent blank, in a spectrophotometer.

Protein values are expressed as g / dl (haemolymph) or mg / g (tissue).

6.2. Estimation of Amino Acids

Total aminoacids and free amino acids in the haemolymph, hepatopancreas and muscle are determined as per the procedure of Ishida *et al.* (1981)

Reagents

1. 6N HCl
2. 0.05M HCl
3. Buffer A (Dissolve sodium citrate(58.8g) in 2l of double distilled

water,add 210ml ethanol of 99.5%, adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 l using double distilled water).

4. Buffer B (Dissolve tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4N NaOH, and make up the volume to 1 l using double distilled water.

5. o phthalaldehyde (OPA) Buffer (Dissolve 122.1 g of Na_2CO_3, 40.7 g of H_3BO_3 and 56.4 g of K_2SO_4 in double distilled water and make up the volume to 3 l).

6. O-Phthalaldehyde solution (OPA) – Dissolve 400 mg OPA, 7 ml ethanol, 1 ml of 2-Mercapto ethanol and 2 ml of 10 % Brij- 35 solution in 500 ml OPA buffer)

7. Sodium hypochlorite solution: 0.16% Sodium hypochlorite in OPA buffer.

6.2.1. Total Aminoacids

Sample Preparation

Weigh about 100 mg sample accurately into a heat-sealable test tube. Add 10ml 6N HCl and heat seal the tube after filling with pure nitrogen gas. Carry out the hydrolysis at 110° C for 24h. After the hydrolysis is over, open the test tube. Remove the contents quantitatively and filter into a round bottom flask through Whatman filter paper No 42, wash the filter paper 2-3 times with distilled water. Flash evaporate the contents of the flask to remove all traces of HCl, the process should be repeated for 2-3 times with distilled water. Dissolve the residue and make upto 10ml with 0.05M HCl.

HPLC Analysis

Filter the sample thus prepared again through a membrane filter of 0.45μm and inject 20μl of this to an aminoacid analyzer equipped with cation exchange column and fluorescence detector. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60° C. The amino acid analysis can be done with non – switching flow method and fluorescence detection after post – column derivatization with o – phthalaldehyde. In the case of proline and hydroxyproline, imino group is converted to amino group with hypochlorite. Run an amino acid standard also to calculate the concentration of aminoacids in the sample. Calibration of equipment

using standards needs to be done before the start of analysis.

The amount of each aminoacid is expressed as mg aminoacid /16g nitrogen.

Free Aminoacids

Preparation of Trichloroacetic acid extract

Weigh the sample accurately (1g) and extract with 10% trichloroacetic acid by grinding in mortar . Filter the content quantitatively through Whatman filter paper No: 1 and make up the filtrate to 10ml. Use the TCA extract to measure free amino acids.

Determination of Free Aminoacids

Pipette out about 1.0 ml TCA extract accurately and bring the pH to below 3.0 with NaOH and make upto definite volume with buffer. Inject 20µl of the above into the amino acid analyzer and determine the amino acid composition. The amount of each free amino acid is expressed as percentage of total amino acids.

6.3. Electrophoretic Separation of Haemolymph Proteins

Haemolymph proteins can be separated by SDS-PAGE technique as described by Laemmli (1970). In this discontinuous buffer system, the separating (resolving) gel is prepared at 7.5 % gel concentration.

Reagents

1. Acrylamide – bis acrylamide solution: 30 g of acrylamide and 0.8g of bisacrylamide in l00 ml of double distilled water.
2. Separating gel buffer: 1.5 M Tris-HCl, pH 8.8
3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8
4. SDS solution: 10%
5. Ammonium persulphate: 10%
6. N,N,N',N'-tetramethylethylenediamine (TEMED)
7. Electrode buffer: 3.0g of Tris, 14.4 g of glycine and l0 l of 10% SDS in one litre of double distilled water.
8. Sample buffer: Make up 2.5ml of stacking gel buffer, 4 ml of 10 percent SDS, 1 ml of 2-mercaptoethanol, 2g of sucrose, and 1 ml of 0.4% bromophenol to 10 ml with water.
9. Staining solution: Dissolve 1.2 g Coomassie blue R-250 in 500 ml of methanol and mix with 200 ml of glacial acetic acid and

500 ml water. Filter it through Whatmann No: 1 filter paper.

10. Destaining solution: Mix 70 ml of glacial acetic acid with 1000 ml of water.

Procedure

Prepare separating gel by mixing 10.5 ml of stock acrylamide – bis acrylamicle, 7.5ml of separating gel buffer, 0.3ml of 10% SDS solution, 6 ml of water, 0.15 ml of ammonium persulphate and 20µl of TEMED. Pour this solution quickly between molds formed by two glass plates, separated by spacers. Gently overlay with about l00 µl of distilled water using a micropipette. After polymerization is complete, remove the overlay and rinse with water. Then, insert a comb and pour stacking gel consisting of 1.0 ml of gel solution, 3.75 ml of stacking gel buffer, 0.15 ml of SDS, 9.15 ml of water 0.1 ml of ammonium per sulphate and 10 µl of TEMED.

Once polymerization is complete, remove the comb and the gel is ready for electrophoresis. Dilute the haemolymph to a protein concentration of 5mg/ml and mix with sample buffer in 1:4 ratio and heat for 3 minutes at 95-100°C. Chill the treated sample and load into the wells using a micropipette. Then carry out electrophoresis at 200 V and 300milli ampere current. After completion of the run, remove the gels from the plates, stain with coomassie brilliant blue solution and destain using destaining solution. Staining can be done for a period of 30 minutes and destaining can be done repeatedly till the gel becomes clear.

7. Assay of Lipids

7.1 Estimation of total Lipids

Any stress or disease can alter the lipid content of haemolymph and tissues.The total lipid content of the muscle, hepatopancreas and haemolymph can be estimated by the method of Folch *et al.,* (1957).

Reagents

1. Chloroform
2. Methanol
3. Dried anhydrous sodium sulphate

Procedure

Take an exactly weighed/measured sample and add about 15 volumes of chloroform –methanol mixture (2:1, v/v). Rinse the residue with chloroform – methanol mixture to extract the lipids quantitatively.

To the filtrate, add 20% water and allow to stand overnight in a separating funnel. Separate the lower chloroform layer, dry over sodium sulphate, filter and concentrate by flash evaporation and store under nitrogen in a deep freezer (-20ºC). Take a small volume in a pre-weighed vial and evaporate to constant weight to determine the percentage of lipid in the samples.

7.2 Estimation of Cholesterol

Total Cholesterol is estimated as per the method of Rudel and Morris (1973)

Reagents

1. Cholesterol Standard Stock – 1 mg / ml in chloroform
2. Working standard – Make up 1ml stock to 10ml with chloroform
3. Ferric chloride solution- Dissolve 840 mg of ferric chloride hexahydrate in 10 ml acetic acid.
4. Concentrated sulphuric acid
5. Methanol
6. KOH - 150% in water
7. Diethyl ether

Procedure

a. Saponification

Transfer suitable aliquot of lipid into a saponification flask, add 30ml methanol and 1.5ml of 150% KOH (Volume of reagents is sufficient for 1.2g of fat). Carry out saponification by refluxing with occasional swirling on steam bath for 30 minutes. Care should be taken to prevent the loss of alcohol during saponification. Transfer quantitatively the alcoholic soap solution, still warm, to 250 ml separating funnel. Extract with 120ml diethyl ether (30ml each) in 4 extractions and wash the combined ether extracts with distilled water repeatedly till the washings are neutral to phenolphthalein. Measure the total volume of ether extract and take an aliquot for the estimation of cholesterol.

b. Estimation of cholesterol

Transfer an aliquot of ether extract in duplicates to test tubes and evaporate the ether under nitrogen. Standards of cholesterol and blank should be taken simultaneously. Add 1.5 ml of ferric chloride solution, mix thoroughly and allow to stand for 10 minutes. Add concentrated

sulphuric acid, mix well and keep the tubes in the dark for 45 minutes. Determine absorbance at 560 nm.

The amount of cholesterol in the samples can be expressed as mg%.

7.3. Estimation of Triacylglycerides

The level of triacylglycerides in muscle, hepatopancreas and haemolymph is determined by the method of Rice (1970).

Reagents

1. Alumina – activated
2. KOH – Dissolve 50g KOH in 600ml water and add 40ml isopropanol
3. Sodium metaperiodate – Dissolve 77g of ammonium acetate (anhydrous) in 700ml distilled water ,add 60ml glacial acetic acid and 600mg sodium meta periodate . Make upto 1l using distilled water. This solution is stable for 6 months.
4. Acetyl acetone: Add 7.5ml acetyl acetone to 200ml isopropanol, mix and and make upto 1l. This is stable for 6 months.
5. Standard - Tripalmitin

Measure 0.1ml lipid extract, standard and distilled water (blank) into screw-capped tubes. Add 3.9ml isopropanol to each tube, mix well and then add 400mg washed alumina. Place in a mechanical rotator for 15 minutes, centrifuge and transfer 2ml supernatants to another test tube. Add 0.6ml KOH, stopper and incubate at 60 – 70°C for 15 min. Cool, add 1ml metaperiodate solution, mix and add 0.5ml acetyl acetone reagent. Incubate at 50°C for 30 min. Cool and read the absorbance at 405 nm against the reagent blank.

The amount of triacylglycerides in the samples can be expressed as nmol tripalmitin / g of lipid.

7.4.a. Estimation of Phospholipids

Phospholipid content of muscle, hepatopancreas and haemolymph is estimated by the method of Fiske and Subbarow (1925) as inorganic phosphorus liberated after digesting with perchloric acid as outlined by Bartlette (1959)(Refer 7.4.b).

Procedure

Digestion of lipids

Mix 0.1g of lipid after removing solvent with 0.5ml perchloric acid and add a drop of concentrated sulphuric acid. Heat in a sand bath till it becomes a clear solution and make upto 3ml with distilled water. Determine the inorganic phosphorous formed as described in 7.4.b.

The amount of phospholipids in the samples can be expressed as mg of inorganic phosphorous per 100g of tissue.

7.4.b. Estimation of Inorganic Phosphorus

Phosphorus compounds perform vital functions in all known forms of life. Inorganic phosphorus plays a key role in biological molecules such as DNA and RNA where it forms part of those molecules' molecular backbones. Living cells also utilize inorganic phosphorus to store and transport cellular energy via adenosine triphosphate (ATP). Phosphorus is also an important element in cell protoplasm and nervous tissue.

Inorganic phosphorus is estimated by the method of Fiske and Subbarow (1925).

Reagents

1. Ammonium molybdate reagent: Add 25g of ammonium molybdate to 200 ml distilled water. To 300ml 10NH_2SO_4, add molybdate solution and dilute to 1l with water.
2. Amino naphthol sulphonic acid (ANSA): Grind 0.2g of ANSA with 1.2g of Na_2SO_3 and 1.2g of sodium bisulphite ($NaHSO_3$). Keep the mixture in the freezer. At the time of use, dissolve 0.25g in 10ml distilled water.
3. Standard phosphorus: Weigh 35.1mg of potassium dihydrogen phosphate accurately, dissolve and make up to 100 ml with distilled water. One ml contains 80 μg phosphorus.

Procedure

To suitable aliquots of the sample, add 1.0 ml of ammonium molybdate reagent. Add 0.1ml of ANSA after 10 minutes of incubation at room temperature. Treat standards and blank also in the above manner. Read the blue colour developed after 20 minutes at 660nm in a UV-Vis spectrophotometer. The values are expressed as μg phosphorous per g tissue.

8. Assay of Membrane Bound Enzymes

8.1 Assay of Na^+/K^+ -Dependent ATP ase

The sodium, potassium ATPase , also known as sodium pump is a membrane bound protein that hydrolyses ATP in order to drive the coupled extrusion and uptake of sodium and potassium ions across the plasma membrane, which is vital for proper cellular function.

Na^+/K^+ dependent ATPase activity is measured according to the method of Bonting (1970)

Reagents

1.	Tris buffer, pH 7.5	:	184 mM (Dissolve 2.2g Tris HCl in 85ml distilled water, adjust pH to 7.5 and make up volume to 100ml.
2.	Magnesium sulphate	:	50 mM in water
3.	Potassium chloride	:	50 mM in water
4.	Sodium chloride	:	600 mM in water
5.	EDTA	:	1.0 mM in water
6.	ATP	:	40 mM in water
7.	Trichloro acetic acid (TCA)	:	10% in water

Procedure

Mix One ml of Tris-buffer with 0.2ml each of magnesium sulphate, potassium chloride, and sodium chloride and 0.1ml each of EDTA and ATP. After 10 minutes equilibration at 37°C in an incubator, start reaction by the addition of 0.2ml of the enzyme preparation. Incubate the assay medium for 30 minutes and at the end of the incubation period stop the reaction by the addition of 1ml of ice cold 10% TCA. Estimate the inorganic phosphorous liberated by the method of Fiske and Subbarow (1925) (Refer 7.4.b).

The enzyme activity is expressed as μ g of inorganic phosphorous liberated/ mg protein/ h.

8.2 Assay of Ca^{2+}dependent ATP ase

Ca^{2+} ATPase is assayed by the method of Hjerten and Pan (1983)

Reagents

1.	Tris-HCl buffer	:	0.125 M, pH 8.0 (Dissolve 1.5g tris HCl in 85ml of distilled water, adjust pH to 8.0 with NaOH and make up volume to 100ml.
2.	Calcium chloride	:	0.05M in water
2.	ATP	:	0.01M in water
3.	TCA	:	10% in water.

Procedure

Take Tris-HCl buffer 0.1ml, calcium chloride 0.1ml, ATP solution, 0.1ml and distilled water 0. 1ml. Add 0. 1ml of enzyme preparation and incubate the mixture at 37°C for 15 minutes. Arrest the reaction by the addition of 10% TCA to the incubation mixture. The reaction mixture treated similarly, but where TCA is added in the beginning is taken as blank for each sample. Centrifuge the contents. Use the supernatant for the estimation of inorganic phosphorous by the method of Fiske and Subbarow (1925) (Refer7.4.b)

The enzyme activity can be expressed as µg of inorganic phosphorous liberated/ minute / mg protein.

9.Bibliography

Bartlette, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234 : 466-468.

Bonting, S. L. 1970.Sodium-potassium activated adenosine triphosphatase and cation transport In: Bittar EE, editor. Membrane and ion transport. London: Wiley-Interscience; , New York, pp. 257–363.

Bray, R.: 1962 Spectrophotometric Studies on the Reduction of Xanthine Oxidase, *Biochem J 83*, 11P,

Ellis, H.A. and Kirkman, H.N., 1961. A colorimetric method for assay of erythrocyte glucose-6-phosphate dehydrogenase. *Proc. Natl. Exp. Biol. Med.* 106, 607–609

Ellman, G.L. 1959 .Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70-77

Fiske, C.H., and Subbarow, Y. 1925. the colorimetric determination of phophorus. *J. Biol. Chem.* 66 : 375-400.

Folch, J, M. Lees, and G.H. Sloane-Stanley 1957. A simple method for the isolation and purification of total lipids from animal tissues *J. Biol. Chem* 226 : 497-506

Gancedo, J. M., and C. Gancedo. 1971. Fructose-1,6-diphosphatase, phosphofructokinase and glucose-6-phosphate dehydrogenase from fermenting and non fermenting yeast. *Arch. Mikrobiol.* 76:132-138.

Habig, W.H., Pabst, M.N. and Jacoby, W.B 1974. Glutathione S Transferase, the first enzymatic step in mercatpopunc acid formation. *J. Biol. Chem.* 249 : 7130-7138.

Hall, M.R. and van Ham, E.H. 1998. The effects of different types of stress on blood glucose in the giant tiger prawn *Penaeus monodon*. *J.World. Aquacult.Soc.* 29 : 290-299.

Hjerten S, and Pan H. 1983 Purification and characterization of two forms of low affinity Ca2+ATPase from erythrocyte membranes. Biochem Biophys Acta ,728: 281-288.

Ishida, Y., Fugita, T. and Asai, K. 1981. New detection and separation method for amino acid by high performance liquid chromatography. *J. Chromato.* 204 : 143-148.

Jussila , J. , McBride, S.,. Jago, J and Evans,L.H., 2001. Hemolymph clotting time as an indicator of stress in western rock lobster (*Panulirus cygnus* George). *Aquaculture.* 199, 185–193.

King J 1965 b The hydrolases-acid and alkaline phosphatases. In: D. Van (ed). Practical Clinical Enzymology. Nostrand Co., London, 1965, pp 191– 208

King J. 1965 a The dehydrogenases or oxidoreductases. Lactate dehydrogenase In: Practical Clinical Enzymology. London: Van Nostrand, D.Company Ltd; 1965. pp. 83–93.

Klein, J. 1990. *In Immunology* Publ: Blackwell Scientific, Boston.pp:1-240

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 : 680-685.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, and P. Aquacop., 1997. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with molt cycle : protection against vibriosis. *Fish Shellfish Immunol.* 7: 227-234.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Misra, H.P.; Fridovich, I. 1972. The role of superoxide anion in the antoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 217: 3170-3175.

Mohur, A and Cook, I.J.Y. 1957. Simple methods for measuring serum levels of glutamic-oxalo acetic and glutamic-pyruvic transaminase in routine laboratories. *J. Clin. Pathol.* 10: 394-399.

Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissue by TBA. Anal. Biochem. 95: 351–358.

Pagila, D.E, Valentine, W.N 1967. Studies on the quantitative and qualitative characterisation of erythrocyte glutathione peroxidase. *J Lab Clin Med;*70:158.

Racotta, I.S and Palacios, E. 1998. Haemolymph metabolic variables in response to experimental manipulation stress and serotonin injection in *Penaeus vannamei. J. World Aquacult.Soc.* 29: 351-356.

Rice, E.W. 1970. Triglycerides in serum, *In* Standard methods in Clinical Chiemistry (Eds : Roedrick, P. and Mc DonaldO, R.P.) Academic Press, New York . p 215.

Rudel, L.L. and Morris, M.D. 1973. Notes on methodology, determination of cholesterol using O-phthalaldehyde. *J. Lipid Res.* 14: 364-366.

Sasaki T, Matsuv S, Sanne A., 1972. Effect of acetic acid concentration of the colour reaction in the o-toluidine boric acid for blood glucose determination. *Rinsho Kagaku.* 1,346-53.

Slot C., 1965. Plasma Creatinine determination. *Scand J Clin Lab Invest.* 17, 381-385

Slot C., 1965. Plasma Creatinine determination. *Scand J Clin Lab Invest.* 17, 381-385

Sung , H.H,, G.H. Kou and Y.L.Song 1994. Vibriosis Resistance induced by Glucan Treatment in Tiger Shrimp (*Penaeus monodon*) Fish Pathol. 29 (1) : 11-17.

Takahara, S., Hamilton, B.H., Neil, J.V., Kobra, T.Y., Ogawa, Y. and Nishimura, E.T. 1960. Hypocatalasemia : A new genetic carried state. *J. Clin. Invest.* 29 : 610-619.

Telford, M. 1968 Changes in blood sugar composition during the molt cycle of the lobster *Homarus americanus. Comp. Biochem. Physiol.* 26: 917-926.

Varley, H., Gowenlock, A.H, and Bell, M., 1980. *In* Varley, H., Gowenlock, A.H. and Bell, M.(eds) *Practical Clinical Biochemistry* 5th edition. Arnold-Heinemann, 535-595.