Semester – II

19BTP212 IMMUNO - AND ENZYME TECHNOLOGY – PRACTICAL IV 4H-2C

Instruction Hours / week: L: 0 T:02 P: 4 **Marks:** Internal: **40** External: **60** Total:**100**
End Semester Exam: 3 Hours

Course Objectives:

- To make students familiar with principles of enzyme activity, analysis of enzyme.
- To impart knowledge on the immune system and characterization of immune aspects.

Course Outcomes:

1. Students will get an knowledge on enzyme characteristic analysis.

Immuno-technology

1. ABO blood grouping, Preparation of serum from blood
2. Methods of immunization, Methods of bleeding, Hemolysis
3. Single and Double radial immunodiffusion
4. Immunoelectrophoresis
5. Rocket Immunoelectrophoresis
6. Counter Current Immunoelectrophoresis
7. WIDAL test
8. DOT-ELISA

Enzyme technology

1. Purification of an enzyme from any natural resource
2. Quantitative estimation of proteins by Bradford/Lowry's method.
3. Perform assay for the purified enzyme.
4. Calculation of kinetic parameters such as Km, V_{max}, K_{cat}

SUGGESTED READINGS

1. Nigam, A. & Ayyagari, A. (2008). *Lab Manual in Biochemistry, Immunology and Biotechnology.* McGraw-Hill Education Publishers, London, United Kingdom.
2. Bisen, P.S. (2014). *Laboratory Protocols in Applied Life Sciences* (1st ed.). CRC Press, Florida, United States.
3. Vashist, S.K. & Luong, J.H.T. (2018). *Handbook of Immunoassay Technologies: Approaches, Performances, and Applications* (1st ed.). Academic Press, London, United Kingdom.
4. Crowley, T.E. & Kyte, J. (2014). *Experiments in the Purification and Characterization of Enzymes: A Laboratory Manual* (1st ed.). Academic Press, London, United Kingdom.
5. Bisswanger, H. (2017). *Enzyme Kinetics: Principles and Methods* (3rd ed.). Wiley-VCH Publishers, New Jersey, United States.

SYLLABUS

EX NO. 1A. ABO Blood Grouping

Aim

To understand the basic concept of Blood Grouping.

Principle:

It was in 1901, that Austrian-American immunologist and pathologist **Karl Landsteiner** discovered human blood groups. Karl Landsteiner's work helps to determine blood groups and thus opened a way for blood transfusions which can be carried out safely. He was awarded the **Nobel Prize in Physiology or Medicine** in 1930 for this discovery.

Death of the patient was the result in most cases before 1900, when blood transfusion was attempted. Blood transfusion was made much safer by the discovery of blood groups, as blood of the same ABO group could be chosen for each patient. However, there were still many cases of unexplained blood transfusion reactions. Biologists still went in search of these unexplained questions.

In 1902, the fourth main type, AB was found by Decastrello and Sturli. It was the observations of Levine and Stetson in 1939, and Landsteiner and Weiner in 1940, that laid the foundations of our knowledge about the remaining major blood group- the Rhesus system. Once reliable tests for Rhesus grouping had been established, transfusion reactions became rare! For this discovery Landsteiner was awarded the Nobel Prize in Physiology or Medicine in 1930.

The Components of Blood

The circulatory system distributes about 4-6 liters of blood to the adult human body. The blood mainly has 2 portions: **the Plasma** and **the Blood Cells**. Plasma is mainly composed of water, but contains different types of proteins and other chemicals such as: enzymes, glucose, fat particles, salts ,hormones , antibodies etc. It constitutes about 60% of the blood. Blood cells can be observed under a microscope on staining. The formation of blood cells occur in the bone marrow by the 'Hematopoietic stem cells'. They can be divided into 3 basic cell types:

Erythrocytes- Red Blood Cells (RBC):

As the name suggests, these red coloured cells give blood its red colour. (The word erythrocyte is from erythro-Gk.meaning red and Latin-cytos meaning cell.) 1 ml of blood contains approximately 5 million RBCs! The proportion of blood occupied by red blood cells is referred to as the hematocrit, and is normally about 45%. Mature RBCs are biconcave in shape, lack a Nucleus and many other organelles. They circulate in the system for about 120 days, carrying out their job, i.e., to supply oxygen.
This function is carried out by them most efficiently. since they are rich in an Iron-containing biomolecule called haemoglobin. Haemoglobin has high affinity for oxygen, thus binds to it and is transported from the alveoli (in lungs) to every part of the body. There is constant replenishment of RBCs in order to remove old cells that break down. (This process is carried out in organs like the liver, also producing by-products like bile pigments.) Millions of cels are released into the bloodstream from the bone marrow each day.

Leukocytes- White Blood Cells:

These cells are key players in our immune system. They are of different types such as neutrophils, lymphocytes, eosinophils, monocytes, basophils. Each of them have a variety of functions in our immunity. 1 ml of blood of an adult human contains about 4,000-11,000 leukocytes. Basically, they destroy and remove old or aberrant cells and clear cellular debris, as well as attack foreign substances and infectious agents (pathogenic entities).

Thrombocytes- Platelets:

The coagulation or blood clotting process is taken care of by them. They act on clotting proteins like Fibrinogen, converting it into Fibrin. They create a mesh onto which RBCs collect and form a clot. This prevents excessive blood loss and also checks the entry of pathogens into the body. 1 ml of blood of an adult human contains about 200,000-500,000 platelets.

Materials Required:

- Monoclonal Antibodies (Anti-A, B and D)
- Blood Lancet
- Alcohol swabs
- Tooth picks
- Sterile cotton balls
- Clean glass slide
- Ice tray

- Biohazard disposal container

Procedure:

1. Set the table with all the materials required. Remember to place the Monoclonal Antibody (Mab) kit in an Ice tray.
2. Open an Alcohol swab, and rub it at the area from where the blood will be sampled (finger tip). (Discard the swab)
3. Open the Lancet cover, put pressure at the tip of the finger from where blood will be sampled (maintain it). Prick the finger tip with the opened Lancet.(Discard the Lancet)
4. As blood starts oozing out, make 1 drop fall on the three depressions of the glass slide. (in clinical setup, there will be a fourth well used as a control).
5. Place a cotton ball at the site where it was pricked. Using the thumb, put pressure on the area to stop blood flow.
6. Take the Anti-A (blue) bottle, resuspend the content and use the dropper to place a drop of the Mab in the 1st spot. Place the bottle back in ice.
7. Take the Anti-B (yellow) bottle, resuspend the content and use the dropper to place a drop of the Mab in the 2nd spot. Place the bottle back in ice.
8. Take the Anti-D (colorless) bottle, resuspend the content and use the dropper to place a drop of the Mab in the 3rd spot. Place the bottle back in ice.
9. Take a tooth pick and mix the content in each well. Discard the tooth pick after using in one well (take a new one for the next well).
10. After mixing, wait for a while to observe the result.

Result:

1B. Preparation of serum from blood

Aim

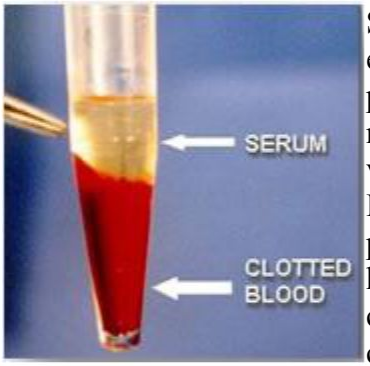
To understand the various techniques applied to process a blood sample and obtain serum.

Principle

Blood serum can be defined in a number of ways. Clear, watery fluid of the blood that separates when blood clots or blood plasma from which the protein fibrinogen or clotting factors, has been removed. Clotting factors are the proteins which causes the clotting of blood. when the blood is allowed to clot after its withdrawn from a vein, the clot slowly shrinks and a clear watery fluid squeezed out from the clot is known as serum.

$$Serum = Plasma - Clotting\ Factor$$

Why use Serum for Studies?



Serum includes antibodies, antigens, electrolytes, hormones and any exogenous substances such as drugs and microorganisms and all proteins expect that used in blood clotting. Thus it has applications not only in the field of clinical diagnostics, but also in research. A very simple example could be their use in the different types of ELISA tests. They are therefore used to detect the presence of a particular antibody, antigen, hormone, exogenous substances, etc. It has been proposed in several papers that observing changes in the curve of the ultra-violet absorption spectra of blood serum and certain amino acids may give us valuable information about pathological conditions.

Blood serum and plasma are biofluids that are increasingly important in NMR-based metabolomics analysis. Metabolite analysis of fluids from the circulatory system provides a view of the metabolic state of an organism. Unlike urine analysis, which measures an organism's waste products, serum or plasma analysis measures homeostatic levels of metabolites throughout the organism.

Following the rise of "Omics" in advanced research, Biomarkers for various pathologic conditions needs a lot of interest in researches. Introduction of Array techniques has enhanced the output in work. Other than elevated or lower protein levels, MicroRNA profiling from blood serum and plasma is emerging as a new class of blood-based biomarkers. Abnormal levels of hormones and various other indicators can also be detected from serum, and are being used in clinical diagnostic labs for routine tests. The presence of elevated levels of antibodies indicates that the body is not at homeostasis.

It is important to study the parameters like pharmacokinetics (what the body does to the drug) and pharmacodynamics (what the drug does to the body) while conducting clinical trials. Also it is necessary to know; how the drug is metabolized, what the half-life of the drug is, and after metabolism in what chemical from it persists in the body. All these studies are important because when new agent is being studied, literally data from its 'death to birth' needs to be collected (with scientific evidences).

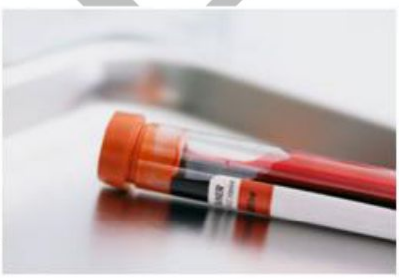
Pharmacological studies are among the most difficult realms to conquer. Blood serum is used in these studies to obtain information regarding all the critical parameters mentioned above. For example: in order to learn about the rate at which a molecule is being metabolized or to learn about the half-life, typically; serum from the patient is collected at various time intervals. Then, an HPLC (high-pressure liquid chromatography) is conducted to isolate the drug from rest of the content. Next, a TANDEM-Mass spectrometric analysis is conducted to learn about the concentration or amount present in a fixed amount of sample.

The serum chemistry profile is one of the most important initial tests that are commonly performed. The functions of various organs and body systems can be assessed by using these measurements. Sometimes a specific diagnosis may be made on the basis of a blood chemistry profile alone. More often than not, however, the profile provides information on a variety of body organs and systems, giving the doctor an indication of where a problem might be located. The profile can be extremely helpful in determining which of the many other diagnostic tests might be beneficial. Tests are performed to check Glucose, Blood Urea Nitrogen (BUN), Calcium, Total Protein, Cholesterol, Creatine Phosphokinase (CPK), Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Chloride, Potassium, Sodium levels, etc.

Serum Collection

Blood sampling-

Blood obtained from the patient should be handled extremely carefully. Normally, blood is collected from the Intravenous route. It is directly transferred to a sterile container that is stoppered and also has a label on it. It depends on the purpose of collection whether the blood has been Heparinised (anti-coagulant) or not. When serum is desired to be collected, it is not heparenised, since we want the clot to form and remove the clotting factors from the sample!



It is critical to take care when an individual is dealing with blood since blood is a biohazardous material. The container must be stoppered to prevent spillage, contamination, etc. The container must be properly labeled, since samples from multiple patients might be dealt with and there is high chance of confusion while conducting the tests. A wrong diagnosis can have devastating effects!! The clinician looking at the result makes a wrong conclusion, thus making his diagnosis also wrong; at the end of the day putting the life of the patient 'on the line'.

Agents Used for Serum Preservation

The simplest method is refrigeration. Even though this is acceptable when the application is in a clinical and diagnostic lab, this is not a fool proof method. Due to the activity of the various substances present, the sample can either become inactive, or improper functioning may not occur during reuse. Thus the result cannot be completely trusted or dependable, neither might it be reproducible! Thus direct refrigeration method is not acceptable in terms of research applications. The chances of generating false, un reproducible data are very high.

Thus purification processes, prior to storage are recommended. Filter sterilization is the most commonly used method. Filters of various pore sizes are available. There are chances for the

filter getting clogged. The filter can be fitted onto a sterile collection container, onto this filter; the container into which the sample will be poured is attached. At the side of the filter there is an outlet to which a vacuum pump is attached. After transferring the sample into the top container, the lid is sealed and the vacuum pump is turned on. Gradually, filtered serum gets collected at the bottom container. This can either be directly stored in the refrigerator or other preservative methods can be carried out using it.

Merthiolate is an organomercury compound. It is used as a well established antiseptic and antifungal agent. Also used as a used as a preservative in vaccines, immunoglobulin preparations, skin test antigens, antivenins, ophthalmic and nasal products, etc. It is very toxic by inhalation, ingestion, and in contact with skin, with a danger of cumulative effects. For aquatic organisms also, it is a very toxic agent and may cause long-term adverse effects in aquatic environments. When merthiolate reaches the body, it is metabolized to thiosalicylate and ethylmercury (C2H5Hg+). Thus, the chemical should be handled only in the fume hood and the user should always wear disposable gloves.

Glycerol stocks are prepared for transportation purposes. This is because, glycerol acts as an inherit layer between the fluid and the atmosphere. It thus preserves the cells, and also acts as a shock absorber, preventing damage of cells due to physical stress.

Procedure

1. Collect the blood into a glass container and allow it to clot at room temperature for 1h.
2. Once the clot has formed, loosen it from the walls of the container to aid retraction.
3. Keep at 4^{o}C and leave it there overnight.
4. Collect the expressed serum and centrifuge at 150 g for 5 min (to sediment erythrocytes) and then at 350g for 15 min.
5. Transfer the serum (straw-colored supernatant) to containers suitable for long-term storage and heat at 56^{o}C for 30 min to destroy the heat-labile components of complement.

Technical notes

1. Serum may be frozen at -20^{o}C for long-term storage but continuous freezing and thawing should be avoided.
2. Protein denaturation at room temperature is minimal if serum is purified by filtration (0.22-µm pore size). For filter sterilization of volumes of serum greater than 20 ml use a combination of filters; 0.45-µm prefilter pad, 0.22-µm filter. A single 0.22-µm filter will block very rapidly.
3. Alternatively, storage at 4^{o}C is possible after the addition of merthiolate (0.01% w/v, final concentration) as a preservative agent.

4. Preservation of non-sterile serum for transport without refrigeration can be achieved by the addition of 50% v/v glycerol with no interference with its immune reactivity.

For centrifugation conditions, it must be specified in terms of relative centrifugal force (RCF) which is expressed in units of gravity (times gravity or x g). Many microcentrifuges only have settings for speed (revolutions per minute, RPM), not relative centrifugal force. Thus a formula for conversion is required to ensure that the appropriate setting is used in an experiment. The relationship between the revolutions per minute(RPM) and relative centrifugal force(RCF) is as follows:

$$g = (1.118x10^{-5})RS^2$$

Where g is the relative centrifugal force, R is the radius of the rotor in centimetres, and S is the speed of the centrifuge in revolutions per minute. Values of RCF in units of times gravity (x g) for common microcentrifuge rotor radii appear in the following conversion table. As an example, centrifugation of a sample at 5,000 RPM in a microcentrifuge that has a rotor with a radius of 7 cm will deliver a centrifugal force of 1,957 x g.

Differences Encountered in Real Laboratory

1.Always wear gloves and coat while performing the experiment in order to minimize exposure hazards..
2. Sterilize all the glass equipments and keep neatly in the lab table before doing the experiment.
3. You should ensure that the water bath is set to 56 degree celsius before performing the experiment.
4. All parts of the syringe coming into contact with body should be kept free of contamination.
5. Before collecting the blood, take care the individual position, ie., the person should be either sit in a chair, lie down or sit up in a bed.
6. Approach the individual in a friendly calm manner, provide for their comfort as much as possible and gain the individual cooperation.
7. Palpate and trace the path of veins with index fingers.
8. Prepare the venipuncture site using appropriate antiseptics. For example use alcohol wipes ie 70% isopropyl alcohol to clean the area. Cleanse the area in circular fashion and allowed to air dry .
9. Do not palpate venipuncture site after cleansing.

10. The needle should form a 15-30 degree angle with the surface of the arm. Swiftly insert the needle through the skin and into the lumen of the vein.
11. Remove the needle from the person's arm using a swift backward motion.
12. During blood collection to the syringe, make sure that you do 'Not replunge the syringe into the vein'. If you do so it will results in air embolism.
13. Press down on the gauze once the needle is out of the arm, applying adequate pressure to avoid formation of a hematoma.
14. Discard the used needles and syringe immediately after use which virtually eliminates transmission of blood borne pathogens.
15. Wash your hand frequently and properly after blood collection.
16. Make sure to separate serum from red blood cells within 60 minutes (1 hr) of venipuncture.
17. During the collection of serum using pasteur pipette, you should take care the tip of the pipette do not touch the lysed cells settled at the bottom of the tube.
18. Before using the syringe /Disc filters to purify the serum, ensure that you are using the correct membrane filter to optimize the flow rate and throughput.
19. Avoid repeated freezing and thawing of serum sample during its long term storage.

EX. No.2. Methods of Immunization, bleeding and hemolysis

EX NO 2A Methods of Immunization

Aim

To demonstrate the effect of the immune response o experimental animals by various means of infection. For immunization of animals there are various routes available specific for specific type of the antigen used. The main consideration while selecting the route is the form and nature of the antigen . Given below are the different routes of administration of antigen.

Intradermal

The route is generally used for injecting viscous and emulsing type of anigen (Freund's adjuvant). This route provides rapid access to lymphatics. Intradermal injections are made into the flank of rodents, guinea pigs , rabbits, into the site of the neck of the sheep and goat, into the ear of pigs and fore arm of higher primates. The above said region should be carefully wiped with alcohol prior to injection. Fill solutions to be injected into the syringe making sure that no air bubble is trapped in the barrel of the needle. Hold a fold of skin between the fore fingers and thumb, inserted the point of needle forward into the dermis parallel into the skin surface. Take care that the tip of the needle is just visible, below the surface. Release the skin holding the needle at the point of entry and injected 5-10µl of antigen and withdraw the needle, compressing

simultaneously along the back of the needle with the fore finger and thumb. A heart pea like swelling at the site of injection indicated a true intradermal injection.

While with drawing the needle after the injection care was taken not to release the plunger of the syringe.

Subcutaneous

This route is suitable for emulsions, precipitate, viscous material and antigen while spread a little more. However, antigen is slowly absorbed into the circulation compared to intraperitonial injection.

Hold the tail of the mouse with right hand between the palm and fourth and fifth finger of the left hand. Grasp a fold of skin over the back near the neck region. Release the tail and lift the mouse in left hand. Clean the skin surface over the belly with 70% alcohol. Hold the fold between the thumb and the fore finger. Inserted the needle into the pocket of skin lying behind skin fold taking care not to piearce the peritoneum. Injected the desired volume (50µl) with drawn the needle and pinch the needle tract to avoid loss of antigen due to leakage.

Intramuscular

It is one of the most frequently adopted route of immunisation suitable for alum precipitates and absorb the antigen. The needle was inserted right angle to the skin surface of a point half way along the femur, so that its point lies within the muscle. The injection was then made into the muscle. The needle with drawn and the site was massaged for about 0.1ml of antigen can be injected by this way.

Intravenous

Extra care is needed in this step, as the antigen enters directly into the blood circulation which may cause anaphylactic shock and ultimately death of the animal. But it is a good route for particular antigen in the case of rodents tail was used for intravenous injection. The antigen was introduced through mouth of the mouse. Holder and cork was replaced so that the tail lies v-groove. The base of the tail was wiped with cotton bag and soaked in hot water. In the hot water it was left for 1-2 min was for vasodilation to occur.

Alternatively, tail veins were rubbed with cotton wool and soaked in xylene for vaso dilation of veins. The syringe was filled with antigen to be injected. Care was taken not to have any air bubble within syringe or needle. The level of the needle should be on same as graduation on the barrel. The cotton 4was removed and the tail was held in left hand and needle was inserted so that it flow directly along the line of the vein and come within the lumen. The plunger of the needle was gently pressed without any resistance as it was in the vein. As the antigen enters the vein it should be visible. If an intravenous injection is not achieved at the first attempt then a higher point towards the base can be tried after rewarming. 0.1-2ml antigen can be injected by this way. Dorsal view could be used in case of male rats and guinea pigs.

Intra peritoneal

Injection of antigen into intra peritoneal cavity causes immediate attraction of macrophages and monocytes . It is therefore more suitable for complex and particulate antigen. Eg. Cell suspension of antigen. The mouse was held as required for subcutaneous injection and inserted the needle into the abdomen about 6mm deep. Deeper the injection may damage other organs and blood vessels. Injected the required volume (0.1ml) and withdrawn the needles.

Footpad

This method can be used to inoculate particular antigen upto 15µl in mouse and guinea pigs. The only disadvantage is the development of granuloma with certain form of antigen which leave the animal in a painful and immobile condition. Injection is made only in hind limb as the forelimb is used by the animal for good manipulation of food. The footpad was cleaned with 70% ethanol and needle was inserted in distal or proximal direction through a depth of 5mm into pad. In guinea pigs the needle was inserted through inter digital space to the depth of about 10mm. A volume of up to 25 µl can be injected by this method.

EX NO. 2B Methods of bleeding

Aim

To bled the laboratory experimental animals for obtaining serum.

Principle

To obtain clear serum or plasma, animals should be bled before feeding. Blood is collected in a tube with or without anti coagulant. Blood can be collected by one of the following procedure.

1. Bleeding from ear
2. Retro orbital bleeding
3. Cardiac puncture
4. Bronchial vein
5. From the external jugular vein

Materials required

- Laboratory animals
- Razor blade or electric shaver
- Scalpel, cotton wool, table lamp
- 78% alcohol, hypodermic needle
- Collecting tubes or bottles.

Procedure

Blood can be collected by any one of the following procedure

1. **Bleeding from ear**

About 10-50ml of blood can be collected in a single dose from a healthy rabbit at an interval of one month. Rabbit was restrained in a rabbit box. The downward pressure was applied at the back. Lateral margin of the ear was dried, Shaved and cleaned with 70% alcohol with a help of a scalpel blade.A diagonal incision was made across the vein. The blood was collected immediately using the hypodermic needle. Bleeding was stopped by pressing the vein at the site of cut with dry cotton.

2. Retro orbital bleeding

Blood can be collected from the orbital socket of mice, rat, guinea pigs and even from rabbits. It is an good method of collecting small amount of blood samples. Small amount of blood samples can be repeatedly collected at 1-2 week interval from the same immunized animal by this method. The mouse was held against a wire grid under the left hand and restrained by holding its tail with right hand. It was immobilized by pressing down into the back with second, third and forth finger of left hand. With the help of the forefinger and the thumb, scalp of neck was held. A fold of skin was trapped down from the back of the mouse between the thumb and the second finger and curled remaining finger into the palm and released the tail simultaneously. The friction exerted by the thumb and the forefinger on skin close to superficial venus returns back which leads to the bulging of eye.

It was slided under the eyeball at 45deg and over the bony socket to rupture the fragile venus plexus. The passage was about 5mm in the mouse, 10mm in rat, 12mm in guinea pig and 15-20mm in rabbit. The tip of the capillary was slightly retracted and the blood collected in the orbital cavity followed by the flowing out from the capillary and was collected in the collecting tube.

3. Cardiac puncture.

From Rabbit

The rabbit was laid down in supine position on a dissecting table. The head of the animal was held backward, so as to avoid the sideway movement of the skull. The hind limb are also stretched to get a " barrel chest". Hairs are shaved off from the chest area avoiding and aberrations of skin or thoracic nipples. Inserted 21g needle through the gap between the last sternal on the left side of the midline xiphoid process, penetrated downward for a few mm, slanted forward at an angle of 30 deg C to the chest well till the heart beat can be felt and pushed forward approximately 3-6mm and simultaneously collect the blood using syringe.

4. Bronchial vein

5- 10ml blood can be collected from domestic animals from the bronchial vein which run over the proximal end of the radius and ulne. Soft and fine hairs are plucked in order to expose the vein and cleaned it with 70% ethanol. Inserted 25G needle in the vein and collected the desired volume by withdrawing the

plunger's slowly. Remove the needle, pressed the side of the entry of the needle with a dry cotton to stop bleeding.

5. From the external jugular vein

Large quadrapiles like horses, sheeps and goats can be bled from the external jugular vein. 500-600ml of blood can be collected from a healthy sheep or goat in a single bled. The legs of the animal are tied with the hel[p of a rope and the head was raised to expose external jugular vein. Hairs at the site of the vein are dried, shaved and cleaned with 70% alcohol and venous returns to the vein was blocked by pressing the jugular groove with left thumb to distant the vein. 18G needles attached to 20 or 50 ml syringe was inserted along the lumen of the vein pointing towards the head.

EX. No. 2C **Haemolysis**

Aim:

To lyse the RBC using haemolysis buffer

Materials Required:

15ml centrifuge tube, 2 clean glass test tubes, 3ml disposable syringe with needle, colorimeter incubator.

Reagents

150mM/L AmmoniumChloride
12mM/L Sodium bicarbonate
Normal saline (0.85% NaCl)
Heparin

Preparation of haemolysis buffer

100ml of 150mM/L ammonium chloride and 12mM/L sodium bicarbonate was prepared. Mix these two solutions in such a way to get pH of 7.4.

Procedure

1. 1ml of blood was drawn (with sterile disposable syringe) by clean veins punch tube, using heparin as anticoagulant.
2. The heparinized blood was transferred into a clean centrifuge
3. double the volume of saline was added and centrifuged at 1000rpm for 10 min
4. This was repeated 2-3 times to get 100% red blood cell pellet.
5. Two clean centrifuge tube were taken
6. To one tube, 2ml of haemolysis buffer and to the other tube 2ml of normal saline were added respectively.
7. 10mlof erythrocytes was added to both the tubes

8. Both the tube were incubated at 37C for 15mins to achieve complete haemolysis
9. After incubation both the tubes were centrifuged at 10,000 rpm to 10 min and supernatant was taken
10. To both the supernatant equal volume of dis.H_2O was added and read at against dis.H_2O as the blank.

EX NO. 3

3A. SINGLE RADIAL IMMUNODIFFUSION

Aim

To estimate the amount of antigen present in the given sample against an antibody.

Introduction

Single radial immunodiffusion technique is used to estimate the amount of antigen or antibody present in the serum samples.

Principle

The antigen-antibody precipitation is made more sensitive than in double immunodiffusion by the incorporation of the antiserum in the agarose solution before the gel is made. Thus the antibody remains uniformly distributed throughout the gel. Antigen is then allowed to diffuse from the wells cut in the gel. This is an example of single immunodiffusion. Initially as the antigen diffuse out of the well, the antigen concentration is relatively high and an insoluble antigen antibody precipitation is formed. However as antigen diffuses further from the well and the antigen antibody complex reacts with more amount of antibody to form lattice, that precipitate to form precipitin ring. The concentration of antigen is directly proportional to the diameter of the precipitin ring.

Materials Required

- Agarose
- PBS
- Commercially available antigens
- Test antigen
- Antiserum
- Gel punch with syringe
- Microscopic slides
- Template
- Semi log graph sheet
- Micropipette with tips and moist chamber

Procedure

The microscopic slide should be cleaned properly with a cotton for even spreading of agarose, 1% of agarose is dissolved in PBS (100 ml) by heating the solution. From this 3ml of

1% agarose solution was transferred to a clean dried test tube, which was kept at 55°C in a waterbath. To this agarose solution 75µl of antiserum (antibody solution) was added and mixed well. The agarose solution containing antibody was poured onto a microscopic slide and allowed to solidify at room temperature. Using the template and gel punch the wells were made (4mm in diameter) on the gel.

The standard antigen (Known concentration) is added to wells as shown in the table. The test antigen was added to the well and marked as 'A'. Then, the microscopic slides were incubated in a moist chamber for 24 hrs. After 24 hrs of incubation, the slides were observed for the precipitin ring formation and the diameter of the ring was measured.

Result

3B. DOUBLE RADIAL IMMUNODIFFUSION

Aim:

To study the reaction pattern of an antigen with a set of antibodies by Ouchterlony Double Diffusion method.

Introduction:

Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. Gel immunodiffusion can be classified into two groups: 1.Single Immunodiffusion2.Double ImmunodiffusionIn the Ouchterlony double diffusion, both the antigen and the antibody diffuse toward each other in a semisolid medium to a point till their optimum concentration is reached. A band of precipitation occurs at this point. The qualitative Ouchterlony Test can simultaneously monitor multiple Antibody-Antigen system and can be used to identify particular antigens in a preparation. This procedure was developed by Örjan Ouchterlony in 1948.

Principle:

When soluble antigen and antibody samples are placed in adjacent wells in agarose gel, they diffuse radially into the agarose gel and set up two opposing concentration gradients between the wells. Once the gradients reach to an optimal proportion, interactions of the corresponding molecules occur and a line of precipitation will form. Using such a technique, the antigenic relationship between two antigens can be analyzed. Distinct precipitation line patterns are formed against the same anti-sera depending on whether two antigens share all antigenic epitopes or partially share their antigenic epitopes or do not share their antigenic epitopes at all. The Ouchterlony test also can be used to estimate the relative concentration of antigens. When an antigen has a relatively higher concentration, the equivalent zone will be formed a little bit away from the antigen well. When an antigen has a relatively lower concentration, the equivalent zone will be formed a little bit closer the antigen well.

The pattern of lines that form can be interpreted to determine the relationship between the antigens and antibodies.

Pattern of Identity: X

Pattern of identity occurs when the antigens in the two wells are identical and specific for the antibody in the antiserum present in the third well. The concentration of the two antigens being the same, they will diffuse at the same rate resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens i.e. the two antigens are immunologically identical.

Pattern of Partial Identity: Y

Pattern of partial identity occurs when the antigens in the two wells share some epitopes which are same for both, yet each of the two antigens also have unique epitopes. In this case antiserum contains polyclonal antibodies specific for each epitope. When one of the antigen has some of the same epitopes compared to other, the polyclonal antibody population will respond differently to the two antigens and the precipitin line formed for each antigen will be different. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen.

A similar pattern of partial identity is observed if the antibodies are cross reactive with an epitope on one of the antigen that is similar, but not identical to that present on the other antigen.

Pattern of Non-Identity: Z

Pattern of non-identity occurs when the antigens in the two wells are totally different. They are neither cross reactive, nor do they have any epitopes which are same. In this case the antiserum containing the antibodies is heterogeneous as some of the antibodies react with antigen in one well while some react with antigen present in the other well. So the two antigens are immunologically unrelated as far as that antiserum is concerned.

Materials/Reagents Required

Antiserum X, Y and Z, Measuring cylinder, Alcohol, Distilled Water, Incubator (37°C), Microwave or Bunsen burner, Vortex mixer, Spatula, Micropipettes, Tips, Moist chamber (box with wet cotton).

Procedure

1. Prepare 10 ml of 1% agarose (as given in important instructions).
2. Cool the solution to 55-60oC and pour 5 ml/plate on to grease free glass plates placed on a horizontalsurface. Allow the gel to set for 30 minutes.
3. Place the glass plate on the template provided.
4. Punch wells with the help of the gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming of rugged wells.
5. Add 10 µl each of the antiserum and the corresponding antigens to the wells.

6. Keep the glass plate in a moist chamber overnight at 37°C.

7. After incubation, observe for opaque precipitin lines between the antigen and antiserum wells.

Observation and Result:

Ex No. 4. IMMUNOELECTROPHORESIS

Introduction

Immunoelectrophoresis is a powerful technique to characterize antibodies. It combines antigen seperation technique of electrophoresis and immunodiffusion of the separated antigen against antiserum. This technique is extensively to check antibodies for their presence, specificity and homogeneity.

Principle

The technique is based on the technique of technique of electrophoresis of antigens and Immunodiffusion of the electrophoresed antigens with a polyspecific antiserum to form precipitin bands.

Procedure

Preparation of gel plate

- Prepare 10ml of agarose (1.5%) in 1X reservoir buffer and heating slowly till it is about to boil.
- Mark the end of the slide that will be positive during the electrophoresis
- Place the slide on a leveled tabletop. Pipette and spread 10ml of agarose solution onto 50x75 mm slide. Solidify for 15 minutes.
- Place the gel plate on the template holder provided in ETS-2 and fix the template. Punch a 3mm well with a gel punch.
- Cut two troughs with the gel cutter provided but do not remove the gel from the trough.

Electrophoresis

- Add 12-15l of antigen to the well.
- Place the slide in the electrophoresis tank and pour the buffer such that it covers the gel.
- Set the voltage to 50-100 V and electrophorese until the blue dye travels 3-4cms from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

Immunodiffusion

- Remove gel from both troughs and keep the slide at room temperature for 15 minutes. Add 250 µl of antiserum –A in one of the troughs and antiserum-B in the other.
- Place the slide in the moist chamber and allow diffusion at room temperature overnight (24 to 48 hours).
- Observe for precipitant lines between antiserum troughs and the central antigen well.

Result and Interpretation

- Presence of precipitant line indicates the presence of antibody in the antiserum to the antigen, which moves to the position during electrophoresis.
- Presence of more than one line indicates the heterogeneity of the antibodies in the antiserum to the antigen.
- Absence of precipitant indicates the absence of antibodies to any of the antigen present in the antigen mixture.

Ex No. 5. Rocket immunoelectrophoresis

1. Aim

To demonstrate antigen-antibody interaction by double immunodiffusion technique.

2. Introduction

There are wide varieties of techniques involving antigen-antibody immunodiffusion in gels. The key reaction of these techniques is the binding of antibodies to antigens and forming large macromolecular complexes. The principle behind these techniques is to react an increasing amount of antigen with a fixed amount of antibody. This results in precipitation due to formation of cross links as antibody binds with more than one antigen. The bonds formed between antigen and antibody, include hydrogen bonds, ionic bonds, hydrophobic interactions, and Vander Waals interactions.

An immunodiffusion bioassay where both antigen as well as antibody diffuses into the gel is called 'double-diffusion'. Thus, Ouchterlony's double diffusion technique is a free diffusion method and differs in speed and sensitivity: some are strictly qualitative, others are quantitative. It provides a very useful tool for illustrating and clarifying the principles of antibody heterogeneity and specificity and is widely used in diagnosing diseases, monitoring the level of the humoral immune response, and identifying molecules of biological or medical interest.

3. Materials Required

3.1. Biological Materials: Test antigens (Ag1 and Ag2), antiserum

3.2. Chemicals/Reagents: Agarose, 1X assay buffer, alcohol
3.3. Equipment: Micropipette, pipette tips, petri plate, incubator
3.4. Glassware/Plastic ware: Glass slide, conical flask, measuring cylinder
3.5. Miscellaneous: Distilled water, cotton

4. Procedure

4.1. Preparation of agarose plate

i. Take 5 mL normal saline and add 50 mg of agarose to prepare 1% agarose solution. Heat the solution in boiling water bath or in oven till agarose dissolves completely and no particles of agarose remain in suspension. After complete dissolution, cool the hot agarose solution to 40-50 °C.

ii. Keep a clean glass microscopic slide on a horizontally level surface.

iii. Gently pour 4.5 mL agarose solution on the slide using a glass pipette.

iv. Pour the agarose solution such that it does not flow out of the edges of the slide but remains over the slide surface to form a 3-4 mm thick Layer.

Allow the agarose to solidify at room temperature and keep it aside covered in a Petri dish.

vi. Store the agarose slide at 4 ºC, if not to be used immediately.

4.2. Cutting walls in the agarose

i. Prepare a paper template for wells using a square white paper or using a graph paper, cut the edges equal to the width of the slide.

ii. Mark positions of the central well in the centre of the paper and six outer wells at 60° angle and 5 mm away from the edges of the central well.

iii. Place the slide over the template such that the marking on the paper visible through the agarose layer.

iv. Before cutting the wells, keep the slide at 2-4 ºC for a short period so that edges of the wells do not break when the agarose plugs are removed.

v. Use a 3 mm cork borer (Fig. 1) to cut wells after the agarose gel is hardened sufficiently,

vi. Cut1well in the center and 6 outer wells on the Gel, as marked on the template.

vii. Prepare one more set of gel (Set 2) with six wells as explained above.

viii. Use a marker and label the wells on the lower side of the slide.

ix. In Set 1 gel, label the central well as Ag (for undiluted antigen), and the six outer wells serially as Ab (for undiluted antibody), and diluted antibody as 1:2, 1:4, 1:8, 1:16, and NS (normal saline) for the sixth well.

4.3. Filling wells and incubation of agarose slides

i. Dilute the antibody two-fold (1:2), four-fold (1:4), eight-fold (1:8) and sixteen-fold (1:16) with normal saline.

ii. In the Set 1 Gel, add 10 µL undiluted antigen in the central well (marked 'Ag') by using a micropipette.

iii. Add 10 µL of undiluted antibody in the first well (marked 'Ab') and add 10 µL of diluted antibody 1:2, 1:4, 1:8, 1:16 sequentially, in each outer well of agarose slide.

iv. Add 10 µL of normal saline to the sixth well.

v. Dilute the serum two-fold (1:2), four-fold (1:4), eight-fold (1:8) and sixteen-fold (1:16) with normal saline.

In the Set 2 Gel, add 10 µL undiluted antibody in the central well (marked 'Ab') by using a micropipette.

vii. Add 10 µL of undiluted serum in the first well (marked 'Ser') and add 10 µL of diluted serum 1:2, 1:4, 1:8, 1:16 sequentially, in each outer well of agarose slide.

Keep the slide in a petri dish lined with filter paper moistened with distilled water.

ix. Cover the petri dish with its lid and keep at room temperature overnight, undisturbed (lower temperature may slower the formation of precipitation line and prolong the test).

x. Observe the slide after overnight incubation by keeping it on a black paper or black tile and note the white precipitin line, along the radius, between the edges of central and outer wells

Ex No. 8 DOT ELISA

Aim

To perform sandwich Dot ELISA test for antigen.

Principle

Dot ELISA (Enzyme Linked Immunosorbent Assay) is an extensively used immunological tool in research as well as analytical/diagnostic laboratories. In sandwich DOT-ELISA the antigen is sandwiched directly between two antibodies which react with two different epitopes on the same antigen. Here one of the antibodies is immobilized onto a solid support and the second antibody is linked to an enzyme. Antigen in the test sample first reacts with the immobilized antibody and then with second enzyme linked
antibody. The amount of enzyme linked antibody bound is assayed by incubating the strip with an appropriate chromogenic substrate which is converted to a coloured insoluble product. The latter precipitates onto the strip in the area of enzyme activity, hence the name Dot ELISA. Enzyme activity is indicated by intensity of the spot which is directly proportional to the antigen concentration.

Materials Required

- Glasswares: test tubes or 1.5ml vials
- Reagent: Distilled water
- Other Requirements: Micropipette, Tips

Procedure

- In a test tube/vial, take 1 ml of 1X assay buffer and 50µl of serum sample. Mix thoroughly. Insert a Dot- ELISA strip.
- Allow the reaction to occur at room temperature for 20 minutes.
- Wash the strip 3 times by dipping it in 1ml of 1X assay buffer for about 5 minutes each. Replace the buffer each time.
- Add 1ul of antibody-HRP conjugate to it. Mix thoroughly and dip the strip, allow the reaction to take place for 20 minutes.
- Wash the strip as in step 3, 3 times.
- In a fresh tube/vial take 0.1ml of 10X TMB/H_2O_2 and 0.9 ml of distilled water, mix thoroughly. Dip the strip in substrate solution
- Observe the strip after 10-20 minutes for appearance of blue/grey spot.
- Rinse the strip with distilled water.

ENZYME TECHNOLOGY

1. **Purification of enzyme from any natural resource**
2. **Quantitative estimation of Proteins by Bradford / Lowry's method**
3. **Perform assay for the purified enzyme**
4. **Calculation of Kinetic Parameters such as Kmax Vmax, Kcat**

EX. NO-1 Purification of an enzyme from any natural resource

Aim: To identify the enzymes present in different solutions

Principle: Different enzymes will identify using following reactions

Starch $\xrightarrow{Amylase}$ Simple sugars

Sucrose $\xrightarrow{Invertase}$ Glucose + fructose

H_2O_2 $\xrightarrow{Catalase}$ water + oxygen

Sources: Dry leaves, fresh leaves, raw potatoes, Boiled potatoes, Sprouted seeds, apple, banana and yeast.

Apparatus required: Glass wares, Pestle and Mortar, Water bath, Centrifuge, Testubes, Beaker.

Reagents Required: 2% solution of Glucose, Maltose , Starch and Sucrose, Benedicts reagent and Iodine solution

Control preparation

- Take 3 test tubes
- Add 2.5ml of 2% solution of starch to 1 test tube maltose to the second and glucose to third test tube
- Add 1 ml of 2% iodine solution to each test tube.
- Add 1 ml of Benedicts reagent to all test tubes and vertex
- Use this as reference for the color change in the sample.

Procedure

Sample preparation for Amylase and Catalase Assay

- Take 5 g of each sample and homogenize using distilled water in Pestle and Mortar
- Transfer homogenate to centrifuge tubes and centrifuge for 3 min.
- Collect the supernatant in test tubes

Amylase assay

- Take 2.5 ml of different samples in different test tubes.
- Add 2.5ml of 2% solution of starch,1 ml of 2% iodine solution and 1 ml of Benedicts

reagent to all test tubes and vertex
- Place the test tubes in boiling water bath for few minutes.
- Take out the test tubes from water bath and compare the color with control.

Invertase assay
- Take 2.5 ml of different samples in different test tubes.
- Add 2.5ml of 2% solution of sucrose and add 1 ml of Benedicts reagent to all test tubes and vertex.
- Place the test tubes in boiling water bath for few minutes.
- Take out the test tubes from water bath and compare the color with control.

Catalase assay
- Take 5 ml of H_2O_2 in different test tubes
- Crush different samples and add it to the different test tubes
- Observe the test tubes for effervescence

Observation table

Source	Amylase	Invertase	Catalase

EX. NO-2

Quantitative estimation of proteins by Bradford/Lowry's method

Aim: To determine Specific activity of œ Amylase from different source

Principle: Specific activity is calculated by determining amount of proteins present in I mg in 1 ml of enzyme source and dividing it by the enzyme activity.

Materials and Reagents: Lowry's reagent, Folin's reagent, BSA standard solution **Procedure**

1. Take 7 test tubes.
2. Pipette 0,0.2,0.4,0.6,0.8 and 1ml of working BSA solution to 6 test tubes and number it from 1-6.
3. Make the volume as 1 ml in each test tube by adding water
4. Add 1ml of diluted enzyme to 7 th test tube.
5. Add 5 ml of Lowry's reagent to all test tubes.
6. Incubate the test tubes at room temperature for 15 min.

7. After incubation add 0.5 ml of FC reagent to all test tubes
8. Keep the test tubes in dark at room temperature for 30 minutes.
9. Measure the OD and calculate the concentration of protein in 1ml of enzyme.

.

Tabular column

Sl.no	Vol of BSA (ml)	Vol of water (ml)	Concentration of protein in µg	Lowry's reagent 5 ml		FC reagent		OD at 660nm
1							Incubation at roomtemperaturefor15min	
2								
3								
4								
5								
6								
7								

Calculations:

Enzyme activity of œ Amylase =…….mmole/min

1ml of 1:25ml diluted enzyme consists of…… µg of protein 1ml of undiluted enzyme has ……..mg of protein

Specific activity=enzyme activity/mg of proteins= ……..µmol/min/mg

Result: Specific activity of enzyme is …….

EX. NO-4 Calculation of kinetic parameters such as Km, Vmax, Kcat

Aim: to determine Km and Vmax of α Amylase

Reagents required :Citrate buffer (pH 5.3),Enzyme extract, Starch solution, DNS reagent.

Procedure:

1. Clean and dry 10 testubes
2. Mark test tubes as C1 T1 to C10 T10 depending on substrate concentration.(c-control without enzyme).
3. Add o.5 ml of diluted (1:5) enzyme to test tubes marked as T
4. Add substrate (in the range of 0.1-1) to different test tubes
5. Add buffer to make the volume as 2ml
6. Vertex the test tubes and incubate at room temperature for 15 min
7. Add 1ml of DNS and keep it in boiling water bath for 5 min.
8. Cool all test tubes and add 4 ml of distilled water to all test tubes.
9. Vertex the contents in test tube and read the absorbance at 540nm.
10. Calculate the activity for each test tube.
11. Plot a graph and determine the constants by using Michalis-Menton plot and Line-weaver Burk plot.

Sl.no	Test tube	Vol of enzyme (ml)	Vol of substrate (ml)	Vol of buffer(ml)	Incubate at room temperature for 15 min	Vol of DNS	Keep it in boiling water bath for 5 min .Add 4 ml of distilled water	OD at 540nm	Activity	I/V	[S]	I/[S]
1	C1 T1											
2	C1 T1											
3	C1 T1											
4	C1 T1											
5	C1 T1											
6	C1 T1											
7	C1 T1											
8	C1 T1											
9	C1 T1											
10	C1 T1											