UNIVERSITY OF NAIROBI

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL FOR BACHELOR OF SCIENCE IN BIOCHEMISTRY (BSc II)

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September 2015

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BSc. Biochemistry Year 2.

1. Aims and objectives of the course

When you have finished this course you should:-

a) Be familiar with a range of biochemical techniques and understand the theoretical basis of each technique
b) Be confident in using the apparatus and applying these techniques with accuracy and efficiency
c) Be able to predict which technique might be useful for solving a particular problem in biochemistry
d) Understand how practical results can complement and extend our theoretical knowledge
e) Be able to write a concise, logical report of an experiment
f) Be able to manipulate experimental results and perform simple biochemical calculations
g) Have an appreciation of the limitations inherent in any biochemical method
h) Hopefully find the work interesting and possibly even enjoy yourself.

2. General instructions on laboratory practice

Become familiar with the following instructions. They are intended to help you to improve your practical ability and to ensure that the experiment runs smoothly for the whole class.

(a) Preparation

Before the practical period read the relevant schedule. Have in your mind a clear idea of what you are trying to achieve and the details of the methods involved.

b) Cleanliness.

Neatness and cleanliness are particularly important in the Biochemistry laboratory, where you are dealing with small amount of sensitive biological materials. Srnoking and eating in the laboratory are forbidden.

Also, follow these guidelines.

i. Do not pipette from a stock solution – you may contaminate it.
ii. Do not pour any excess solution back into the stock solution estimate the amount you require before hand.
iii. Do not remove a special reagent from the front bench to your own bench – other students will want to use it.
iv. Treat concentrated acids and alkalis with respect – if necessary, wash them down the sink with a strong stream of water
v Clean up immediately any material spilt on working surface or on instruments.
vi Solid waste material belongs in the waste boxes, not the sinks. Let the technician clear up any broken glass
vii After the experiment clean your glassware and leave your bench as you would wish to find it
viii The technicians are always available for giving advice on minor technical problems

c) Working notebook

An introduction to each experiment and the practical details are given in the schedule. Why should you write them out again? You should record all observations, results and preliminary calculations in a workbook at the time of the experiment. Omit unnecessary detail from your report (e.g., mass of weighing paper = xg) and also omit extensive arithmetic in calculations.

Complete your report as soon as possible after the experiment, otherwise you will forget the details. Try to be as concise and accurate as possible; comments on errors and limitations associated with a particular method are often useful. Include answers to questions in your reports.

After your report has been marked and returned to you, keep it for revision later. The grades for practical reports will be used for assessing your performance.

1. Carbohydrates – Folin-Wu assay for reducing sugar

Reducing sugar can be quantitatively determined colorimeteically by the Folin-Wu method. In this method copper (II) ions in alkaline solution are reduced by the sugar to give copper (I) oxide, which forms a blue complex with the phosphomolybdic acid reagent:

$$2Cu^{2+} + RCHO + H_2O \longrightarrow 2Cu^{+} + RCOOH + 2H^{+}$$

The colour intensity is proportional to the quantity of reducing sugar and so a standard curve can be constructed using a glucose solution of known concentration. The reducing sugar concentration of any unknown solution can then be found by applying the same method and comparing with the standard curve, just as you did for the Biuret assay of protein. Not all monosaccharide sugars have the same "reducing power" as glucose – the reaction with the alkaline copper reagent is not instantaneous and some sugars react more quickly than glucose.

Coffee bean contains a wide variety of carbohydrates, including small amounts of the free monosaccharides (such as glucose), and disaccharides (such as sucrose), together with relatively large amounts of polysaccharides and sugars bound to other organic, non-carbohydrate groups (glycosides). These carbohydrates may be extracted from coffee bean using distilled water. Extracts of natural products usually contatin many other substances (particularly protein), but the low levels of these contaminants in coffee bean do not interfere with the sugar analysis.

Disaccharides, polysaccharides and glycosides are hydrolysed by dilute acid to the constituent monosaccharides. Use the Folin-Wu method to determine the reducing sugar concentrations of the unhydrolysed and hydrolysed extracts of coffee bean.

Reagents and materials

Standard glucose solution (0.25 mM)
Standard solutions of galactose and fructose (0.25mM)
Folin-Wu alkaline copper reagent
Phosphmolybdic acid reagent
Unhydrolysed and hydrolysed extracts of coffee bean.

Procedure

1. Add standard glucose solution at suitable intervals over the range 0-1.0 ml to separate test-tubes, making the volume up to 1.0 ml in each case with water. Do this in duplicate.
2. Add the alkaline copper reagent (2.0 ml) to each tube. Mix well and heat in a boiling water bath for 8 min.
3. Add phosphomolybdic acid reagent (1.0 ml) to each tube and mix well. After 30 min measure the absorbance at 540 nm.
4. Construct a rough standard curve relating absorbance to quantity of reducing sugar (0-0.25 micromoles), in order to determine the linear range.

5. Compare the "reducing power" of galactose and fructose with that of glucose by measuring the absorbances of samples (0.2 ml) of the two sugar solutions treated as in steps 1 to 3.
6. At the same time, determine the reducing sugar concentration of appropriately diluted samples (1.0 ml) of the hydrolysed and unhydrolysed extracts of coffee bean. Approximate concentrations for the hydrolysed and unhydrolysed extracts are 1 00 micromoles per ml and 10 micromoles per ml respectively.

7. Include an accurate standard curve with your report. Express your results for the two extracts as micromoles of reducing sugar per gram of coffee bean (1.0 ml of each extract was obtained from 2 g coffee bean).

Discussion

Comment on your results, relating them to the information given in the introduction to this experiment.

1. Why is the copper reagent prepared in alkaline solution?
2. Why does the glucose solution not have the same "reducing power" as the galactose and fructose solutions of the same concentration, although all three monosaccharides have one reducing group per molecule?
3. Over what range of quantity of reducing sugar may your standard curve be used with confidence?
4. Draw the structures (ring-form) of α-L-galactose α-L-fructose and D-sucrose.
5. In the laboratory the glycosides were hydrolysed with dilute acid. How is this hydrolysis achieved in the coffee bean?

2. Carbohydrates – Isolation of glycogen

Living organisms often contain storage carbohydrates in the form of polysaccharides e.g. starch (plants) and glycogen (animals). In this and the next practical we shall examine some of the properties of the polysaccharide glycogen, which we shall obtain from rat liver. When rat liver is homogenized in trichloroacetic acid (TCA), many charged high molecular weight compounds such as proteins and nucleic acids are precipitated leaving glycogen (a neutral polymer) in solution, together with the simple sugars and other low mol. wt.compounds. Glycogen can be separated from sugars by precipitation with ethanol. Purified glycogen is obtained from aqueous solution by subsequent reprecipitation with ethanol.

Keep your sample of glycogen for analysis during the next practical

reagents and materials

Rat liver
10% TCA (20 ml) and 5% TCA (20 ml).
95% ethanol (50 ml)
Washed sand(0.5 g)
Diethyl ether (3 ml)
NOTE: See instructions below:

Procedure

1. The liver sample has already been weighed and cut into small pieces. Put the pieces in an already cooled mortar containing one volume (1 ml per gram of tissue) of 10% TCA and 0.5 g of washed sand. Grind the liver with the pestle. **CARE:** Avoid TCA splashing on hands 1.0 ml-TCA
2. Centrifuge the homogenate for 5 min at top speed. Decant the supernatant into a 100 ml measuring cylinder.
3. Rinse the mortar and pestle with one more volume of 5% TCA. Pour these washings into the centrifuge tubes containing the residue from the first extraction.
4. Stir the mixtures and allow to stand for about 2 min. Centrifuge as before. Discard the precipitates. Add the supernatants to the first TCA extract in the measuring cylinder and record the total volume.
5. While stirring the combined TCA extracts, slowly add 2 volumes of 95% ethanol per volume of TCA extract. Allow to stand until the precipitate coagulates. (You may have to add a little NaCl and warm the suspension).

NOTE:

Chill the following:-

- Pestle and motar
- T.C.A'S
- Avail ice cubes

6. Centrifuge as before.Discard the supernatant. Dissolve the white precipitate in about 5 ml of water and precipitate it by adding 2 volumes of 95% ethanol.

7. Collect the precipitate by centrifugation and wash it in the centrifuge tube with about 3 ml of 95% ethanol by dispersing it with a glass rod.
8. After centrifugation wash the precipitate with about 3ml of diethyl ether. Air-dry the preparation by spreading the suspension in a thin layer over a watch glass.
9. Weigh the dry glycogen and record the yield.

Discussion

Calculate the content of glycogen in the liver as grams of glycogen per 100 grams of fresh liver.Compare your results with those of other students to see if liver size affects glycogen content. Comment on your results.

QUESTIONS

1. What factors might affect the percentage of glycogen in a rat liver?
2. Why is it necessary to wash the glycogen preparation with ethanol and ether?
3. Why are time and temperature important in the initial stages of the isolation but not in the later steps?
4. Draw the structure of a small portion of the polysaccharide chain of glycogen
5. Would you expect glycogen to have reducing properties?
6. Why does ethanol precipitate the polysaccharide glycogen but not the simple sugars?

MC/6/76

3. Carbohydrates – Characterization of glycogen

Glycogen has a branched structure with linear chains of consecutive glucose residues joined by alpha-1, 4 linkages and with alpha-1,6 linkages at the branch points. The result is a fun-like structure with only one terminal reducing end and many non-reducing ends. Hydrolysis of these glycosidic bonds may be catalysed in a random fashion by acid or in a more specific fashion by certain enzymes e.g. salivary amylase. This enzyme hydrolyses internal alpha-1,4 bonds but not alpha-1,6 bonds or maltose. The mixture of products includes dextrins, maltose, isomaltose and some glucose.

The progress of hydrolysis is determined by measuring the increase in reducing sugar assayed by the Folin-Wu method. The percentage at any time with the amount of sugar present after total acid hydrolysis.

Reagents and material

Rat liver glycogen (from previous practical)
2M HCl
1.2M NaOH
0.02 M sodium phosphate buffer, pH 6.9, containing 5mM NaCl.
Folin-Wu alkaline copper reagent
Diluted saliva.
Phosphomolibdic acid reagent

Procedure

1. Dissolve your glycogen (10 mg) in 10 ml of buffered saline. Use this solution for both the acid hydrolysis and the enzymic hydrolysis.

2. For the acid hydrolysis add aliquots (0.2 ml) of the glycogen solution to each of 7 test-tubes (numbered 2-8). Fill tube 1 with water (0.2 ml) as a blank.

3. Add 2 M HCl (0.3 ml) to each tube and record the time. Immediately add 1.2 M NaOH (0.5 ml) to tubes 1 and 2 place tubes 3-8 in a boiling water bath.

4. Remove tubes 3-6 at 2 minute intervals and neutralize with 1.2 M NaOH (0.5 ml). Remove tube 7 after 20 min and tube 8 after 30 min, neutralizing the contents as before.

5. Determine the reducing sugar content of each tube by using the Folin-Wu assay method.

6. For the enzymic hydrolysis prepare 7 tubes each containing an aliquot (0.2 ml) of glycogen solution and one tube with water (0.2 ml) as before. Add distilled water (0.5 ml) to each tube and allow the tubes to incubate at room temperature by adding diluted saliva (0.3 ml) to each tube.

7. Immediately after adding the saliva, stop the reaction in the tube 2 by adding the standard amount of Folin-Wu reagent (Zero time sample)

8. Stop the reaction in tubes 3-6 at 2 min intervals, in tube 7 after 20 min and tube 8 after 30 min. Add Folin-Wu reagent to the water blank (tube 1).

9. Complete the assay for reducing sugar.

10. The free glucose released in the complete hydrolysis of glycogen (tube 8 of the acid hydrolysis) represents 100% conversion of glycogen to glucose. With this as a standard, plot the % hydrolysis against time for the acid and enzymic hydrolysis on the same graph.

Discussion

Comment on your results, explaining why you did not get 100% hydrolysis with the enzyme.

Questions

1. Another enzyme, beta-amylase, also catalyses the hydrolysis of glycogen by cleaving alpha-1,4 bonds from the non-reducing ends of glucose chains and thereby releasing maltose units. It does not hydrolyse alpha-1,6 bonds or alpha-1,4 bonds beyond an alpha-1,6 branch point. What would you expect to be the final products formed by the action of beta-amylase on glycogen?
2. What other methods could you use to follow the progress of hydrolysis of glycogen by salivary amylase? Would they give exactly the same results as the measurement of reducing sugar give?
3. Would you expect salivary amylase to catalyse the hydrolysis of cellulose? Explain.

MC/6/76

4. Amino acids – Separation by paper chromatography

A mixture of low mol. Wt. Compounds may often be separated by means of partition chromatography. This method is based on the relative distribution of the compound between two solvents in which it has different solubilities. This partitioning effect may be carried out in solution (as in counter current distribution) or, more commonly, on an inert supporting medium.

The medium in paper chromatography consists of a network of hydrophilic cellulose fibres. The solvent system usually contains an organic alcohol, water and either a strong acid or a strong base. The cellulose binds a layer of water, which represents the stationary phase. The mobile phase is the remaining solvent, which migrates up the paper by capillary action, the organic component having the greatest mobility. A compound applied as a sample will distribute itself between the stationary water phase and the mobile organic phase depending on its relative affinity (solubility) for the two solvents. This portioning effect is repeated many times and since the relative affinity is characteristic for a given compound, a separation is achieved. The more hydrophobic (non-polar) components migrate the furthest, the more hydrophilic (polar) remain near the origin.

In a given solvent system each amino acid migrates with a characteristic Rf value. Usually the migration of an unknown amino acid compared with that of known amino acids run as standards. Following the chromatography the amino acids are revealed as purple spots on the paper by spraying with a solution of ninhydrin and heating at 100^0.

Paper chromatography is often combined with paper electrophoresis in a two-dimensional form to the resolution of complex mixtures of amino acids and peptides, such as results from the hydrolysis of a protein, Electrophoresis in one dimension is followed by chromatography in the second dimension. You will apply each of these techniques in the following two experiments to the separation of amino acids.

Reagents and materials

Solution (1mg/ml in water) of glycine, alanine, leucine, tryptophan, glutamic acid, lysine.

A mixture of three of the above (labeled X)

Casein hydrolysate (20 mg/ml) (casein protein hydrolysed in 5M H_2SO_4 at 100^0 for 4h. then neutralized and deionised with $Ba(OH)_2$)
Whatman No. 1 paper (20 cm x 18 cm)
Chromatography solvent (n-propanol-0.88 ammonia, 7:3, v/v)
Ninhydrin reagent (1% in acetone)
Procedure

1. Pour the solvent (about 50ml) into the chromatography bottle and replace the lid. CARE: Avoid the fumes and spillage.
2. Using a pencil draw a line across each of two papers about 2cm from the lower edge. Mark on the line 8 points at roughly 2cm. Intervals, taking care to start well in from the left hand edge. Avoid touching the paper with your fingers except by the extreme edges.
3. Apply a sample of glycine solution to the first spot on one paper. Touch the end of the capillary tube containing glycine solution to the paper lightly in order to transfer as small a volume as possible. Label the spot in pencil.
4. Repeat the above procedure with each of the other amino acids, the unknown mixture and the casein hydrolysate. Use the individual capillary tubes supplied with each solution so as to avoid contamination. Allow the spots to dry completely.
5. Form the paper into a cylinder and staple the edges together so that they do not overlap. Place the cylinder, sample side down, in the chromatography bottle, taking care not to let the paper touch the glass walls. Replace the lid of the tank firmly.
6. Allow to run for about 1½ hr. Remove the paper, undo the staples and mark the solvent front in pencil. Allow the paper to dry.
7. Spray the paper lightly with ninhydrin solution inside the fume cupboard. Do not allow the paper to become completely soaked. Develop the chromatogram by incubation at 100^0 for a few minutes.
8. Outline each spot in pencil and mark the center of the spot. Calculate the Rf value of each amino acid. Include the chromatogram (or a copy) in your report.

Discussion

Comment on the separation of the amino acids by single dimension ascending paper chromatography as evidenced by your own results. Can you identify any of the constituent amino acids of casein? Which amino acids were present in the unknown mixture?

Questions

1. In preparing the casein hydrolysate what is the precipitate formed by adding barium hydroxide to the hydrolysed mixture? Why is this step necessary?

2. Why is it necessary to add the solvent to the chromatography bottle well before starting the experiment?

2. Why should you not mark the sample spots on the paper with ink and why should you avoid touching the paper with your fingers?
3. Account for the Rf value of each amino acid in terms of its structure. For separating the amino acids in a protein hydrolysate? How could you improve the separation?

5. Proteins – Separation by ion-exchange chromatography I

Theory

Proteins may be separated by differential adsorption to ion-exchange materials. Several such materials are conveniently prepared by introducing ionized groups into cellulose. Diethylaminoethyl cellulose (DEAE-C) is such a material and contains $-O-CH_2$ $CH_2.N(C_2H_5)_2$ groups in place of some of the –OH groups of the original cellulose. These groups acquire a positive charge when the nitrogen atom combines with a proton to become – NH^+R_2.

A column may be packed with the DEAE-C, the solution of proteins to be separated is passed through solutions of different proteins are eluted by passing through it, and different compositions. Lowering the pH elutes more proteins, because this destroys the negative charges on the protein molecules, and it is by these negative charges that the protein is held to the positively charged DEAE-C. Similarly raising the concentration of salt elute more proteins, since there will then be more anions (negative ions) in the medium to compete with the protein for the positive charges of the DEAE-C/

This process is often known as chromatography, but it should be noted that it differs from the usual process. Chromatography usually means separation of substances which travel at different fractions of the speed of a moving phase. They do this because they have different partition coefficients between moving and stationary phases. The molecules of a substance that partitions between a moving and a stationary phase spend on average a fixed fraction of the time in the moving phase, and so the substance moves at that fraction of the speed of the moving phase. In true chromatography it is therefore necessary to have long columns to obtain good separations. In the process used in this experiment, however, the proteins change from being completely held by the stationary phase to being completely released. Good separations can often be obtained in this way, Since different conditions release different proteins. Thus short columns can be used.

N.B. In some instances proteins of low molecular weight can be chromatographed in the sence of moving at a fixed fraction of the speed of the eluent on ionic derivatives of cellulose. However, we do not use this technique in this experiment. Here we study the separation of the green protein catalase, an enzyme which catalyses the reaction.

$$H_2O_2 \longrightarrow H_2O + 1/2O_2$$

From the yellow (flavo) protein, glucose oxidase, an enzyme which catalyses the overall reaction

$$C_6H_{12}O_6 + O_2 + H_2O \longrightarrow C_6H_{12}O_7 + H_2O_2$$

REAGENTS AND MATERIALS

0.1m Na-acetate
20mM Na-acetate
40mM Na-acetate
5mM Acetic acid
40mM Acetic acid
0.1M acetic acid
10% H2O2
O-dianizidine
Glucose peroxidase
Column parked with DEAE-C of height 3.0cm
0.1M sodium phosphate buffer pH 7.0
Catalase
Glucose oxidase

Experiment

A suspension of DEAE-cellulose (DEII) is provided. It is necessary first to remove any 'fines' (fine particles) that may have been produced by prior handling, else the column may run too slowly. This is done by discarding particles that sediment slowly. The suspension should give half its volume of packed bed. Hence, to obtain 4 ml of packed bed take about 8 ml of suspension and make it up to about 50ml with the buffer of 20 mM-sodium acetate and 5 mM-acetic acid and stir. Carefully time its settling, and pour off the supernatant when all the particles that settle at 2 cm/min or more have had time to pack down. Use a squat vessel for this, so that it does not take long, and repeat until there are few fines being rejected. It is important that the packed bed should not be more than about a tenth of the total volume, because if the suspension is too thick, the particles that should settle fast are delayed by colliding with fine particles, and no fractionation results.

Suspend the packed DEAE-C in an equal volume of starting buffer, and pour the slurry into a column of 1 cm diameter. Pour a column of height 3.0 + 0.5 cm. Ensure that the top of the column is flat. Use great care in adding any liquid to the column, pipetting it down the walls, so that the top is not disturbed.

It is important that the column should not go so dry that bubbles of air enter it. It is usually all right for such a short column to run dry for a minute or two, since the height of liquid in it gives insufficient head to pull air into the top.

Dissolve the 0.3 g. catalse sample provided of mould extract in about 2 ml. Water, using a glass rod to break lumps. Dilute with water to 30 ml. Filter through a fast, strengthened (No. 114) paper using it fluted. Pass the filtered extract through the column, observe the concentration of coloured material at the top of the column. After the mould extract has been applied, wash in carefully with 5 ml. Of the starting buffer (20 mM sodium acetate and 5 mM acetic acid).

Before changing to a new buffer make sure that the top of the column is flat, if necessary stirring the top few mm in the old buffer. Add the new eluent buffer of 40 mM soldium acetate and 40mM acetic acid very carefully so that it does not mix with the previous buffer but forms a sharp front. Look for a faint olive-green band that passes down the column at this front. It will only be visible if the front is sharp. When the band comes to the bottom change tubes so as to collect it in as small a volume as possible

If it is not visible, collect 2 samples each of 5 ml. After adding the second buffer. After 10 min in all of this buffer with column running change to a buffer of 0.1M sodium acetate and 0.1M acetic acid. Look for a yellow band which passes down the column. Again collect this band in the minimum volume.

Study of the coloured proteins

Divide each coloured fraction of effluent into two parts. To one add a drop of 10 vol. H_2O_2.. Then test each of the remaining portions for glucose oxidase activity, as follows:To 2 ml of glucose-peroxidase reagent add 0.5 sample, and mix immediately. Then add about 1 drop 0-dinisidue and shake immediately What do you observe?

6. Proteins – Separation by ion-exchange chromatography II

When purifying a protein by ion-exchange chromatography it is necessary to choose certain important operating variables, including: Operating temperature, type of separation (column or batchwise), dimensions of column, pH and composition of elution buffers, and elution flow rate. For all experiments it is useful to know the capacity of the chosen exchanger and the likely time course of adsorption and desorption.

Where high resolution is the major factor, ion-exchange chromatography should be with a linear as lt or pH gradient. One problem associated with the packing of columns is that "classification" of the ion-exchange particles to pack the column. The flow rate used for elution should be less than that used in column packing. The flow rate is directly proportional to the pressure drop across the column and its cross-sectional area, and inversely proportional to its length.

Samples should be equilibrated with the starting buffer and excess salts should be removed by prior dialysis. Application of the sample should be such as not to disturb the top layer of ion-exchanger. Elution may be carried out in one of the three ways:

(a) Using the starting buffer if the component on interest is not adsorbed at that pH; (b) using gradient elution in which the pH or salt concentration is varied linearly over a suitable range;

(c) using stepwise elution, so as to produce a sharp change in eluting power which moves down the column as a front. Gradient elution has a higher resolving power than stepwise elusion and is less likely to cause denaturation of labile proteins.

In this experiment you will perform ion-exchange column chromatography of the proteins of egg while using CM-cellulose and eluting with a stepwise increase in salt concentation.

REAGENTS AND MATERIALS

CM – cellulose (Whatman CM II) in 0.01M
Sodium phosphate buffer, pH 6.0
2% Egg white solution (2% in 0.01M phosphate buffer, pH 6.0)
NaCl solutions (0.05M, 0.01M, 0.2M, 1.0M in 0.01M phosphate buffer pH6.0)
Standard ovalbumin solution (10 mg/ml)
Folin ciocateau Reagent
Column fitted with glass wool plug, rubber tubing and clamp
Filter paper circle (same diameter as column)
0.01M sodium phosphate buffer, pH 6.0

NOTE: CM – cellulose (caboxymethyl – cellulose)

Procedure

1. Stir the suspension of CM – cellulose well and pour the slurry into the column. Allow the excess liquid to drain off. Add more slurry until the height of the packed bed is about 8 cm. Do not allow the ion-exchanger to become dry at any time.
2. Add egg white solution (10 ml) to the column. Adjust the flow rate to about 1 ml/min (about one drop every 3 sec.) and collect the effluent.

3. Wash off any unadsorbed protein with an aliquot (=10 ml) of starting buffer. Collect the effluent and combine it with the previous effluent.
4. Elute the bound protein by adding successive 10 ml portions of buffered 0.05M, 0.2M and 1.0M, NaCl, collecting the effluent at each separate salt concentration. Measure the volume of each fraction.
5. Dilute a sample of the egg white solution ten times. Determine the protein concentration of the diluted egg white solution, the ovalbumin standard and each fraction by the Folin method.

Discussion

Draw a histogram showing the amount of protein recovered at each salt concentration. Account for your chromatographic separation and the yield of protein.

Questions

1. How else might you have eluted the bound protein?
2. Draw the likely pattern of protein bands if you subjected the fractions to electrophoresis at pH6.0. What precaution would you take before electrophoresing the fractions containing bound proteins?
3. How might you improve the resolution of the protein components of egg white by cation-exchange chromatography?
4. Which component of the NaCl solution actually elutes the bound protein?

7. Adsorption chromatography

Some insoluble inorganic gels have the property of adsorbing proteins to the surface of the gel particles. The nature of the interactions concerned have not been well characterized, but it is likely that ionic binding and possibly hydrogen bonding are involved. The adsorbents often used are calcium phosphate gel and, to a lesser extent, aluminium hydroxide gel.

Adsorption chromatography is normally a batchwise process applied to the purification of enzymes. An aliquot of adsorbent gel is added to the crude solution (e.g a homogenate) containing the enzyme in question and after equilibration, the gel is removed. Enzyme activity is measured in the remaining solution to see if it has been adsorbed. If not, increasing amounts of adsorbent are added in a stepwise manner until a gel concentration is reached where most of the enzyme is adsorbed. One generally finds that adsorption of a particular protein occurs over a rather narrow range of gel concentrations. This can be exploited to remove contaminating proteins from our enzyme and thus achieve a purification.

The adsorbed enzyme is recovered by elution from the adsorbent. This may often be accomplished by slightly alkaline buffer solution, either with or without ammonium sulphate. The volume of the eluent used should not be too large and is often not greater than the volume of the centrifuged gel. It is better to elute several times in succession with small volumes than once with a large volume.

In this experiment you will purify the enzyme lactate dehydrogenase (LDH) from an aqueous extract of heart muscle using calcium phosphate gel as the adsorbent. As in most enzyme purifications a low temperature is used so as to avoid denaturation.

Reagents and materials

10% aqueous extract of heart muscle (5.5 ml)
Calcium phosphate gel suspension
0.2M sodium phosphate buffer, pH 7.2
0.1M sodium phosphate buffer, pH 7.4
0.01M pyruvate
NADH solution (2.5 mg/ml)
Biuret reagent
Ovalbumin protein standard (5 mg/ml)

PROCEDURE

1. Keep a small sample (0.5 ml) of the muscle extract for LDH and protein assays. Treat the remainder as follows, keeping the temperature at 0° whenever possible.
2. To the extract (5.0 ml) add calcium phosphate gel suspension (1.0 ml) After 20 min. With occasional stirring, centrifuge at maximum speed. In the bench centrifuge for about 5 min. and discard the supernatant.
3. Elute the enzyme by suspending the gel in ice – cold 0.2 M phosphate buffer, pH 7.4 (1.0 ml) with the aid of a glass rod. Centrifuge as before and keep the supernatant.
4. Wash the gel again as in step 3 and combine the supernatants. Measure the volume of the combined supernatants. Dilute a small sample (0.1 ml) to 3.0 ml with 0.1 M phosphate, pH 7.4.
5. Dilute a sample (0.1 ml) of the muscle extract as in step 4.

6. Assay the protein concentration of the diluted muscle extract and diluted combined supernatants by the Biuret method.
7. Measure the activity of LDH as follows. To a spectronic tube add 0.1 M pho sphate buffer, pH 7.4 (1.5 ml), 0.01M pyruvate 0.2 ml), NADH solution (0.1ml) and water to make up the total volume to 3.0 ml.
8. Start the reaction by addition of enzyme solution (0.05 ml). Measure the absorbance at 340 nm at 30 sec intervals up to 3 min.
9. Calculate the activity of LDH (micromol pyruvate reduced/min/ml) and its specific activity (micromol/min/mg of protein) for the muscle extract and for the combined supernatants. Calculate the yield of enzyme activity in the combined supernatants and the purification factor (specific activity after adsorption/specific activity before adsorption).

Discussion (additions)

From your results discuss whether adsorption chromatography is a suitable method for the purification of LDH from heart muscle.

8. Gel filtration I

In previous experiments you separated proteins on the basis of charge (ion-exchange chromatography), solubility (ethanol fractionation) and charge plus molecular size (electrophoresis). In the following two experiments you will study gel filtration, a technique based only on molecular size.

Sephadex consists of linear polysaccharide chains of alpha-glucose residues joined by chemical cross-links. It takes the form of very small spherical beads which absorb a characteristic amount of water when placed in aqueous solution. The degree of swelling depends on the extent of the size depending also on the degree of cross-linking. Molecules which are smaller than the pores can penetrate the swollen beads but larger molecules are excluded. The Sephadex G-25 used here excludes molecules of mol. wt. greater than about 2,500.

The swollen sephadex is normally packed into vertical columns for chromatogrpy. After application of the sample the column is eluted with buffer at a suitable flow rate and fractions of the eluate of convenient volume are collected. Normally the fractions are analysed for protein, enzyme, ions etc. Molecules which are totally excluded from the Sephadex beads appear in the eluate at a characteristic point depending on the type of Sephadex and the size of the column; this is referred to as the Void volume, Vo. Smaller molecules will beretarted to a greater or lesser extent depending on their size, they have a characteristic elution volume, Ve. The elution volume of a very small molecule, such as an inorganic ion, marks the internal volume, Vi, of the column.

Gel filtration is a versatile technique with several uses. The major applications are in protein purification, determination of mol. wt. of proteins, removal of a salt from protein solution (desalting) and buffer exchange. In this experiment you will consider the last two uses. Haemoglobin will be separated from inorganic ions. The separation may be followed by studying the appearance and disappearance of the characteristic colours produced by the interaction of the haemoglobin and the particular ions used.

Reagents and materials:

Sephadex G-25 (equilibrated in 0.01M Na phosphate buffer, pH 7.0).
Haemogloin solution (10% red blood cell haemolysate) containing ferricyanide (5m g/ml)
Ferricyanide (5mg/ml)
Sodium dithionite solution (10mg/ml) (freshly prepared) whitish/milkish is assign of expiring
KCN solution (2 mg/ml)

Haemoglobin solution (in 0.1M glycine/HClbuffer, pH 2.8) adjust pH with HCl -
Containing 0.1M $(NH_4)_2SO_4$.
Barium hydroxide solution (10%)
Column fitted with glass wool plug and rubber tubing with a clamp.

Procedure

During any periods of waiting refer to the Pharmacia booklet for further information on the theory and practice of gel filtration with Sephadex.

1. Stir the Sephadex suspension until it is uniform and pour the slurry into the column. Allow excess buffer to drain through until the bed of Sephadex gel is about 10 cm high. The column must not be allowed to run dry at any time.
2. Add dithionite solution (0.2 ml) carefully to the top of the column and let it run into the bed. Carefully add buffer (0.3 ml) and let that run in.
3. Add haemoglobin solution containing ferricyanide (0.5 ml) and let it run into the bed. Repeat with buffer (0.5 ml). Fill the column with buffer and continue the elution. Adjust the flow rate to about 1 ml/min or less.
4. During the elution collect fractions of 40 drops each in separate test tubes. Also make a note of the colour changes that occur as the haemoglobin band passes down the column.
5. Estimate the tube with the highest concentration of haemoglobin by its colour and similarly for ferricyanide. Measure the volume of any one fraction.
6. Repeat the experiment but add carefully and in order KCN(0.1ml) buffer (1.0 ml), ferricyanide(0.2 ml) and buffer (1.0 ml). Then proceed as before from step 2.
7. Note any colour changes that occur as the haemoglobin band passes down the column

Discussion

Account for the colour changes that you observed in part 1 (steps 2-5) and part 2 (steps 6-7) of the experiment. From your results determine the values of Vo and Ve for your column of Sephadex G-25. Account for your results in part 3 (steps 8-9) of the experiment.

9. Gel filtration II

In this experiment you will study the ability of a highly porous dextran gel, Sephadex G-100, to fractionate a mixture of proteins on the basis of molecular size. Such a procedure is widely applied in the purification of enzymes and in the determination of their mol. wt. The latter application is simpler than most other procedures for estimation of mol. wt. of enzymes, since the technique is applicable to unpurified preparation of the enzyme. Purification by gel filtration is usually a mild technique resulting in little loss of enzyme activity.

For determination of mol. wts. a Sephadex gel is chosen which is known to have an exclusion limit greater than the mol. wt. of the enzyme concerned. A column of the swollen gel is prepared in a suitable buffer and a sample containing proteins of unknown mol. wt. is applied to the gel. The sample also normally contains markers for the void volume, Vo (Blue Dextran 2000) and for the internal volume, Vi (sucrose or inorganic ion) of the column. Elution is performed with buffer at a suitable flow rate and fractions of appropriate volumes are collected and analyzed for absorbance, protein or enzyme activity. The peak of enzyme activity corresponds to the elution volume, Ve, of that enzyme. A graph of log mol. wt. against elution volume should be linear within the effective fractionation range of the gel used. Alternatively, kav may be plotted against log mol, wt., where kav = (Ve – Vo)/(Vt – Vo) and Vt is the total volume of gel bed. In this way the column of Sephadex gel is calibrated and the mol. wt. of an uncharacterized enzyme can be determined by referring its elution volume on the same column to the calibration curve. Alternatively, and less accurately, the mol. wt. may be calculated from an equation relating Ve. To Vo = for the sephdex G-100 it is. 10g mol. wt. = 5,911 – 0.847 (Ve/Vo)/

Reagents and materials

0.01M Na phosphate buffer, pH 7.0, containing 0.1M NaCl
Sephadex G-100, equilibrated in the above buffer
Alkaline phosphatase solution (1 mg/ml in buffer)
Blue Dextran 2000 solution (5 mg/ml in buffer)
Cytochrome c solution (5 mg/ml in buffer)
5mM 4-nitrophenyl phosphate (4NPP) in water
Chromatography tube with rubber tubing and clamp.

Procedure:

1. Prepare a column of sephadex G-100 of about 10 cm bed height. Allow the column to settle by passing one bed volume of buffer through it. Measure the diameter of the column and its height and calculate the total volume (Vt) of the gel bed.
2. Prepare a sample mixture containing Blue Dextran (0.1 ml) and cytochrome c (0.1 ml). Apply the sample to the top of the column and elute it at a suitable flow rate with buffer. Collect numbered fractions of 40 drops each.
3. When all the cytochrome c has emerged, stop the elution, measure the volume of any one fraction.
4. Determine the elution profile of the Blue Dextran by measuring the absorbance of each fraction at 600 nm. Determine Ve for Blue Dextran – this represents the void volume, Vo, of the column.

5. Determine Ve for the cytochrome c similarly but estimate it by its absorbance at 410mn. Calculate Kav for cytochrome c and compare its known mol, wt. (12,400) with that derived from the pharmacia chart (Kav against log mol. wt.) and from the equation given in the introduction.
6. Apply a sample (0.2 ml) of alkaline phosphatase to the column, eluting and collecting fractions as before. Assay enzyme activity in the fractions by incubating a sample (0.1ml) with glycine buffer (0.2 ml) and 4NPP (0.3ml) at 37^0c. for 15 min. Stop the reaction with 0.1M NaOH (2.5 ml) and measure the absorbance at 420 nm.
7. Determine Ve for alkaline phosphatase and its Kav value. Estimate the mol. Wt. of the enzyme by reference to the Pharmacia chart and by calculation using the equation given in the introduction. Do not discard the Sephadex gel.

Questions:

1. Which type of Sephadex might you use for determining the mol. wt. of each of the following proteins:- insulin ($6x0^3$), haemoglobin ($6.8x0^4$), Urease ($4x10^5$) and glutamate dehydrogenase ($1.2x10^6$)?
2. Why is 0.1M NaCl included in the eluting buffer?
3. Would you expect the gel filtration behaviour of a fibrous protein like fibrogen to resemble that of a typical globular protein?
4. some enzymes, particularly α-glucosidase and dextranase, bind to Sephadex gel and are not eluted. Why might this be so? How could you determine the mol. wt. of α-glucosidase by gel filtration?

11. Agar gel electrophoresis

Agar is a natural product extracted from seaweed. It is an anionic polysaccharide containing galactose and galactose substituted with sulphate residues, the sugars being linked by glycosidic bonds. A neutral fraction containing no sulphate can also be isolated from agar which is called agarose. This is widely used as a gel medium for electrophoresis. When dissolved in hot aqueous solution, both agar and agarose form a three dimensional lattice of polysaccharide chains which, on cooling, sets to form a semi-solid gel. Low concentrations (1-3%) are sufficient to form a suitable gel. The sample is applied in a small well cut in the middle of the gel applied to a microscope slide.

After electrophoresis, the proteins may be detected on the gel with Amido Black or commacie-blue

Reagents and materials

Difco Noble agar – Agar Rose or (Agar agar)
5 mM sodium phosphate buffer pH 7.0
Shandon electrophoresis tank filled with 4.0 mM
Sodium phosphate buffer pH 7.0
Microscope slides
Amido Black solution (0.5% in 10% acetic acid) commussie blue or hena – blue
7% acetic acid solution
Normal human serum
Pathological human serum

Procedures

1. To a 50 ml conical flask add agar (0.2 g) and 5 mM phosphate buffer pH 7.0 (10 ml). Heat the mixture on a hot plate with continuous swirling until boiling, then simmer for about 2 min.
1. Pipette the hot gel (2.5 ml onto a microscope slide, making sure the gel is spread evenly over the surface. Prepare 2 gels and discard any gel remaining.
2. When the gels are cool, use the tip of a Pasteur pipette to remove two small plugs of gel from the center of each slide. Remove one conner of gel on the right-hand side to mark the anode end.
3. Fill one well with the normal human serum and the other with the abnormal serum on each slide.
4. Place the two slides in the electrophoresis tank and cover the ends with the paper wicks saturated with buffer. (Make sure that the marked end of the gel is at the anode).
5. Carry out electrophoresis at room temperature for 45 min at a constant voltage of 90V (2.5 M per gel).
6. After electrophoresis, remove the slide and place it in amido bland solution for 5 minutes. Then remove it and destain in 7% acetic acid
7. Draw the pattern of the bands formed on the gel in your lab report

10. Proteins – Isoelectric precipitation

Protein molecules have on their surface a large number of charged groups contributed by the acidic and basic amino acids. A soluble protein is maintained in solution mainly by two kinds of force:

a) mutual repulsion of similarly charged protein molecules,

b) many hydrogen bonds formed between the charged groups and water molecules.

Addition of an inorganic salt to a protein solution reduces both these forces. If the salt concentration reaches a high enough value, the protein may become insoluble and precipitate. This effect is called "salting out". Different proteins are usually salted out at different salt concentrations and we can exploit this property to fractionate a mixture of soluble proteins. In the case of plasma the globulins (alpha, beta- and gamma-globulin classes) are mostly insoluble in 40% saturated ammonium sulphate solution, whereas albumin is soluble. The globulin precipitate can be centrifuged off and redissolved in water; the albumin remains in solution.

A pure protein in solution has an overall charge contributed by the total of the acidic and basic groups of the appropriate amino acids on the surface of the protein. Each protein has a characteristic isoelectric point, pI, at which pH it is electrically neutral. At the pI the forces of mutual repulsion between similarly charged protein molecules are at a minimum and the protein is least soluble. This property is exploited in the technique of isoelectric precipitation, which may be used to separate one protein from a mixture of proteins in solution. In this experiment you will demonstrate the isoelectric precipitation of casein, the major protein of milk.

Reagents and materials

Normal human plasma (2 ml)
Saturated ammonium sulphate solution (5 ml).
Biuret reagent.
Casein solution (1 mg/ml in water) containing 0.1 M sodium acetate.
0.1 M acetic acid solution
BSA (10mg/ml) in water

Procedure

1. Add plasma (1.0 ml) to a bench centrifuge tube. Add saturated ammonium sulphate solution to a final concentration of 40% - 0.67 mls of saturation. Mix the solution well using a dropper with a "pumping" action.
2. After about 15 min centrifuge the mixture in a bench centrifuge for about 5 min at maximum speed. Make sure that your tube is counterbalanced and if necessary labeled before operating the centrifuge.
   100g – 100ml water = 100% saturated $(NH_4)_2SO_4$
   60ml plasma – 40ml saturated $(NH_4)_2SO_4$
3. Draw off the supernatant with the dropper, taking care not to disturb the precipitate. Transfer the supernatant to a clean tube and add about 2 ml of water.
4. Add about 3 ml of water to the precipitate and dissolve it completely. Use a 5 ml pipette to measure the volume of the two fractions.
5. Assay the protein concentration of each fraction by the Biuret method. You will have to dilute the fractions appropriately before assay.

When doing this, assume an albumin/globulin ratio of one and remember that the plasma protein concentration is about 80mg/ml.

6. While waiting for the colour to develop, continue with the isoelectric precipitation experiment. Prepare 6 tubes containing water and 0.1 M acetic acid as follows:

Water (ml)	9.0	8.75	8.5	8.0	7.0	5.0
Acetic acid (ml)	0	0.25	0.5	1.0	2.0	4.0

7. To each tube add casein solution (1.0 ml). Mix well and determine the turbidity (cloudiness) of each solution by measuring the absorbance at 450 nm.
8. Calculate the pH of each solution from the Henderson-Hasselbalch equation (pKa for acetic acid = 4.7). Determine the approximate pI of casein.
9. Determine the concentration of albumin and globulin in plasma and express your results in the form of the albumin/globulin ratio. Calculate the yield of protein from the ammonium sulphate fractionation procedure (use the plasma protein concentration you obtained in experiment 2).

Discussion

Comment on your results, and errors involved in the two procedures used. Why might the albumin/globulin ratio be useful value to measure in clinical medicine?

Questions

1. Why might the pH values of the acetate buffer solutions used in the isoelectric precipitation experiment differ slightly from the calculated values?
2. Why can you use the absorbance of a cloudy solution as a measure of turbidity?
3. If you wanted to remove the ammonium sulphate from the albumin fraction before the protein assay, how could you do this?
4. You want to prepare a purified sample of casein from milk. How might you do this using the techniques that you have encountered in today's experiment

12. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and hence always migrate towards the anode. Proteins and nucleic acids are electrophoresed within a matrix or "gel". Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. The gel itself is composed of either agarose or polyacrylamide, each of which has attributes suitable to particular tasks:

Agarose: Is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "stiffer" the gel. Agarose gels are extremely easy to prepare: Simply mix agarose powder with buffer solution, melt it by heating, mix and pour onto a gel-casting mould. At room temperature the gel should form within an hour. It is also non-toxic. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques.

Polyacrylamide: Is a cross-linked polymer of acrylamide monomers which, when formed, turns into a gel. A polyacrylamide gel is not solid but is made of labyrinths of tunnels through a meshwork of fibers. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders).

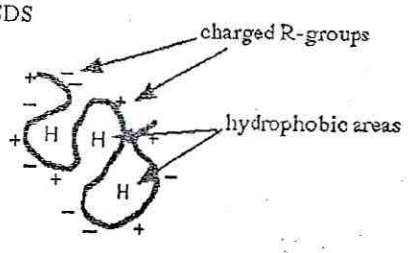
***Acrylamide is a potent neurotoxin and should be handled with care!* Wear disposable gloves when handling solutions of acrylamide, and a mask when weighing out powder. Polyacrylamide is considered to be non-toxic, but polyacrylamide gels should also be handled with gloves due to the possible presence of free acrylamide.

Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 base-pairs (bp). However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins. Polyacrylamide Gel Electrophoresis (PAGE) can be performed under Native conditions or under denaturing conditions.

SDS-PAGE

The purpose of this method is to separate proteins according to their size, and no other physical feature. Since we are trying to separate many different protein molecules of a variety of shapes and sizes, all of which influences migration in gel, we first want to get them to be linear so that the proteins no longer have any secondary, tertiary or quaternary structure (i.e. we want them to have the same linear shape). Consider two proteins that are each 500 amino acids long but one is shaped like a closed umbrella while the other one looks like an open umbrella. If you tried to run down the street with both of these molecules under your arms, which one would be more likely to slow you down, even though they weigh exactly the same? This analogy helps point out that not only the mass but also the shape of an object will determine how well it can move through an environment. So we need a way to convert all proteins to the same shape - we use an ionic detergent, sodium dodecyl sulphate (SDS).

BEFORE SDS



AFTER SDS

Now we are ready to apply the mixture of denatured proteins to the gel and turn on the current. If all the proteins enter the gel at the same time and have the same force pulling them towards the other end, which ones will be able to move through the gel faster? Think of the gel as a tiny forest with many branches and twigs throughout the forest but they form tunnels of different sizes. If we let children and adults run through this forest at the same time, who will be able to get through faster? The children of course. Why? Because of their small size, they are more easily able to move through the forest. Likewise, small molecules can maneuver through the polyacrylamide forest faster than big molecules.

PRACTICAL PROCEDURES

(a) Stock Acryl amide solution

Acrylamide	30% (wt/v)	75.0g
Bis acrylamide	0.8% (wt/v)	2.0g

(make up to 250ml in dH_2O)

(b) Buffers

(i) 1.875 M Tris-HCl, pH 8.8 - 113.5g/500ml : adjust pH with HCl

(ii) 0.6 M M Tris-HCl, pH 6.8 - 36.6g/500ml: adjust pH with HCl

(c) Gel polymerizers

(i) 10% (wt/v) Ammonium persulphate (1.0 g/10 ml fresh before use) Always prepare (ii) 10% SDS (in water)

(d) Electrode Buffer

Tris 0.05M	(12g)
Glycine 0.384M	(57.6g)
SDS (0.1%)	(2g)

(Make up to 2 Litres with water, No pH adjustment)

(e) Sample Buffer (50 ml)

0.6 M	Tris-HCl	pH	6.8
Sucrose	5.0g		
SDS	0.5g		
Mercaptoethanol	0.25 ml		
Bromophenol blue	5.0 ml (from a 0.5% stock)		

GEL PROCEDURE

Gel Cassette Assembly (To be demonstrated)
Clean and completely dry the glass plates, combs, and any other pertinent materials.

1. Place a short plate on top of a spacer plate.
2. Insert both plates into the green casting frame on a flat surface. Be sure that the "legs" of the casting frame are down. Clamp the casting frame and check that the plates are level on the bottom.
3. Place the cassette assembly on a flat surface so the plates are level. Insert the well forming comb into the opening between the glass plates.
4. Determine the appropriate gel type and composition for your experiment. Combine all reagents (except TEMED) in small beaker in the order listed.

Acrylamide gel under SDS-PAGE conditions

Mix the following in a 100ml flask.

NB: The amount under each % is sufficient for pouring a mini gel

For adding µl quantities use Gilson's pipettes

Gel Composition	10%	15%
Water (ml)	2.85	4.45
1.875M Tris (ml)	2.0	2.0
30% acrylamide (ml)	3.4	5.0
10% SDS (µl)	100	100
10% APS (µl)	50	50
TEMED** (µl)	10	10
Total Volume (ml)	5	5

*** Degas to remove air just before adding TEMED. Oxygen inhibits the polymerization step.

When ready to pour the gel, quickly add the TEMED, mix by swirling gently, draw the solution into a 10 ml syringe and gently dispense the solution between the glass plates. Continue adding until it reaches a position 0.3 cm from the bottom of the placed comb.

5. Eject the remaining acrylamide solution back into the small beaker. Polymerization of this solution indicates the completeness of polymerization of the gel between the plates.
6. Allow gel to polymerize (Not less than 30 min)
7. While the separating gel is setting, prepare the following **stacking gel** (4%) solution in a 50ml flask.

Stacking Gel Composition	4%
Water (ml)	3.75
1.875M Tris (ml)	0.5
30% acrylamide (ml)	0.68
10% SDS (µl)	50
10% APS (µl)	25
Total Volume (ml)	5

**Degas as before.

8. When separating gel has set, remove the overlaid water (use a Whatman No. 1 Blotting paper). Add 5 µl TEMED to the stacking gel solution and then pour it into the cassette above separating gel until the solution reaches the cutaway edge of the plate. Place the well-forming comb into the solution and leave to set. (Takes about 20 min).

Running the Gel

1) Remove the "gel cassette"from the casting stand, remove comb and place it in the electrode assembly with the short plate on the inside. Place buffer dam plate opposite the gel cassette assembly.
2) Provide a slight upward pressure on the gel cassette and buffer dam while clamping the frame to secure the electrode assembly. This step is important to minimize potential leakage during the electrophoresis experiment.
3) Place the assembly into the electrophoresis tank
4) Prepare 500 ml 1X electrophoresis buffer
5) Completely fill the inner chamber with 1X electrophoresis buffer so that it floods over and fills the wells. Check for leaks.
6) Use a gel-loading tip or syringe to pipette buffer into each well to remove debris.
7) When all wells are sufficiently cleaned, using a gel loading pipette tip, slowly pipette a maximum of 10 µl of sample or molecular weight marker into individual wells. A yellow guide can be placed on top of the electrode assembly to aid in loading the gel.
8) Gently add enough 1X electrophoresis buffer to the bottom tank to cover the bottom of the gel cassette assembly.
9) Cover the tank with the lid aligning the electrodes (black or red) appropriately.
10) Connect the electrophoresis tank to the power supply.
11) Run the gel at the appropriate voltage, power or current for your particular application.
12) When electrophoresis is complete (when the bromophenol blue reaches the bottom of the gel) turns off the power supply, disassemble the apparatus, and remove the gel and stain (below) using Coomassie stain. Be gentle so as not to cut gel.

Staining of proteins in gels

Procedure:

Stain: 0.1% Coomassie Brilliant Blue R250 dissolved in 50% methanol, 10% glacial acetic acid.

Destain: 10% methanol, 7% glacial acetic acid in water

(i) Pour enough stain solution (50 ml) into a plastic/glass tray (lunch box can do). Immerse the gel into Coomassie solution, just enough to cover the entire gel.
(ii) Shake gently for 30min-1 hour. Pour back the stain into the container- is reusable.
(iii)Pour enough destain solution and continue gentle shaking until completely destained. Several changes of the destain may be necessary for complete destaining to obtain blue protein bands against a clear background.

procedure we use Coomassie Brilliant Blue stain.

13. Native /non-denaturingpolyacrylamide gel electrophoresis

SDS PAGE is probably the most commonly used gel electrophoretic system for analyzing proteins. However, this method separates denatured proteins. Sometimes one needs to analyze native proteins in the gel by its biological activity (eg enzymatic activity) hence the need to use a non-denaturing system.

Procedure

The procedure for setting up a Native gel is as for SDS PAGE except that SDS is (excluded) replaced with water.

NB: The amount is sufficient for pouring two mini gels (7.5% Acrylamide)

Gel Composition	Volume
Water (ml)	14.85
1.875M Tris (ml)	7.5
30% acrylamide (ml)	7.5
10% APS (µl)	150

*** **Degas** as before to remove air then add 15 µl of TEMED to initiate polymerization. (Oxygen inhibits the polymerization step).

Pour into the gel cassette as explained above.

While the separating gel is setting, prepare the following **stacking gel** (4%) solution in a 50ml flask.

Stacking Gel Composition	4%
Water (ml)	6.9
Stacking gel buffer (ml)	3.0
30% acrylamide (ml)	2.0
10% APS (µl)	100

**Degas as before add 15 µl of TEMED and pour it above the separating gel as explained for the SDS PAGE.

Staining and distaining is done as above.

14. Isolation of genomic DNA

INTRODUCTION

To clone genes from DNA, total cellular DNA is first prepared from a culture of either bacteria or animal cells or a sample of plant material. The type of DNA isolated from these samples is genomic DNA. The procedure for total genomic DNA isolation from bacterial cells requires several steps. Firstly, cells are grown and harvested by centrifugation and a lysate made by gentle disruption of the cells. After this, the extract is treated to remove all the impurities from the DNA. Lastly, the DNA is concentrated by precipitation, dried and dissolved in an appropriate buffer.

Bacterial cells are grown overnight in broth culture, usually Luria Bertani medium (LB medium = 10gm Tryptone, 5gm Yeast extract and 10gm NaC per litre). The cells are harvested by centrifugation. DNA is then isolated by digestion with proteinase K in prescence of EDTA and a detergergent such as SDS, followed by extraction with phenol/chloroform and then precipitation with absolute ethanol.

A cell lysate is first made by disrupting the cell wall with lysozyme. This cell extract contains a mixture of DNA, RNA and proteins. To purify the DNA from this mixture, two methods can be used, an ion exchange chromatography using a resin that binds DNA or removal of contaminants by enzyme digestion and organic extraction. The latter method is the most widely used. The proteins are treated with a protease such as proteinase K followed by phenol/chloroform extraction. The RNA is removed by the use of the enzyme Ribonuclease A (RNAse A). The DNA is then precipitated with ethanol and can be removed by spooling with a glass rod.

This method yields DNA whose size (100-150 Kb) is adequate for Southern analysis and construction of genomic libraries. The DNA concentration can be accurately accurately measured by Ultraviolet (UV) absorbance spectrophotometry. The amount of of UV absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. The absorbance is measured at 260 nm and an absorbance (A260) of 1.0 corresponds to 50 ng of double stranded DNA/ml. A ratio of absorbance at 260 and 280 (A260/A280) is used to check for purity of the DNA sample. A pure sample should have a ratio of more than 1.8. A ratio of less than 1.8 indicates that the DNA preparation is contaminated with proteins and phenol. In this case a repeat of the extraction with phenol/chloroform should be performed.

MATERIALS:

E. coli bacteria

LB medium

Lysozyme (10 mg/ml)

0.5 M EDTA

SDS (10% stock solution)

Proteinase K (10 mg/ml)

Phenol:Chloroform:Isoamyl alcohol 25:24:1)

RNAse A (5 mg/ml stock solution in TE)

3 M Sodium acetate pH 5.2

Cuvettes

Absolute ethanol

70% ethanol

TE buffer (10mM Tris.Cl pH 8.0, 1 mM EDTA pH 8.0)

Deionised distilled water

Pipette tips and pipetters

Gloves

Water bath at 37°C and 50°C

EXTRACTION PROCEDURE

1. Grow cells overnight in 50 ml LB medium.

2. Pellet cells by centrifugation, and resuspend in 0.5 ml of 50 mM Tris.Cl (pH 8.0), 50 mM EDTA.

3. Freeze cell suspension briefly at -20°C.

4. Add 100 µg/ml Lysozyme (10 mg/ml stock) to frozen suspension and thaw at room temperature. When thawed place on ice for 15 min.

5. Add SDS up to 0.5% (from 10% stock). Incubate at 37°C for 15 min.

6. Add 100 µg Proteinase K (from10 mg per ml stock). Incubate at 50°C for at least 1 hour.

7. Extract with 0.6 ml Tris-equilibrated Phenol:Chloroform:Isoamyl alcohol. Mix by shaking and aliquot in 1.5 ml eppendorf tubes. Centrifuge at 14,000 rpm for 15 min. Transfer the top layer (avoid interface) to a new tube. This step can be repeated once to remove all proteins in the sample.

8. Extract with an equal volume of Chloroform. Mix by shaking and centrifuge at 14,000 rpm for 5 min. Transfer the top layer into a new eppendorf tube.

9. Add 0.1 volumes of 3 M Sodium acetate and 2.5 vol. 95% ethanol. Mix and leave at -20 for at least 30 min.

10. Spool out the DNA using a bent twisted pasture pipette (or pellete by centrifugation at 12,000 rpm for 15 min if concentration is low and you cannot see the DNA strands after precipitation with ethanol). Dry the DNA in air for about 10 min and resuspend in 50 µl TE. Add 2.5 µl RNAse A (from 5mg/ml stock in TE). Store the DNA at 4°C.

11. Make an appropriate dilution (eg 1:100) and take absorbance reading at 260nm. Determine the yield of the DNA by calculation of the DNA concentration (OD of 1.0 at A260 = 50 µg/ml.

12. Check purity of DNA by electrophoresis and by calculating the ratio of A260/A280. If ratio is < 1.8, then sample is contaminated with proteins and phenol. Repeat from the phenol/chloroform extraction step.

CAUTION: Phenol is corrosive. Wear plastic gloves when handling it!

15. Agarose gel electrophoresis

INTRODUCTION

Gel electrophoresis is a technique that separates different sizes of nucleic acid molecules (RNA, DNA) according to shape and weight. DNA being negatively charged due to the phosphate groups migrates towards the positive pole (anode). Gel electrophoresis of nucleic acids is done on a support matrix such as polyacrylamide or agarose gel. The gel comprises a complex network of pores through which DNA molecules moves through to reach the positive electrode. The smaller the DNA molecule the faster it migrates through the gel while large molecules move slower.

To make a gel, the agarose is melted in an appropriate buffer and allowed to cool to about 50°C and poored into a gel chamber. Combs are inserted into the gel before it polymerizes. The gel is then submerged in an electrophoresis tank containing 1X TAE buffer. After removing the combs, samples are mixed with a loading dye (final concentration 1X) and loaded into the wells. An electric current of about 50–100 volts is applied until the bromophenol blue dye front reaches near the end of the gel.

Materials needed
Agarose
Buffer (usually 1X TAE)
Gel casting chamber, with comb
Electrophoresis chamber
Power pack
DNA samples
Loading buffer
DNA size markers

Procedure

1. Clean and set up the the gel casting apparatus.
2. Prepare a 0.8% agarose gel by dissolving 0.8g agarose into 100 mL 1X TAE buffer. Heat to the boiling point, let all the agarose melt, and then allow to cool to approximately 60°C (hand hot), add ethidium bromide to a final concentration of 0.5 μg/mL (form a 10 mg/mL stock), swirl to mix, and por into the cleaned electrophoresis apparatus. Allow the gel to solidify (about 30 min).
3. Take a 5 μL aliquot of the DNA to be electrophoresed, and put it in a sterile microfuge tube. Add 5 μL of loading buffer, mix and spin for a few seconds. This sample is now ready to be loaded.
4. After the gel had solidified, place the gel in the electrophoresis tank, put buffer (1X TAE) to slightly cover the gel, gently remove the comb. Add buffer to cover the gel, then carefully load the samples into the wells using a miro pipette. Make sure not to touch the bottom of the well with the pipette tip.
5. Load a DNA size marker on the last well.
6. Electrophorese the gel, when the dye front is near the bottom of the gel, stop the electrophoresis. Retrieve the gel and place in a container for transportation to the photography room. **Note: Be careful with handling of ethidium bromide (EtBr) stained gels and avoid contaminating surfaces with it because ethidium bromide is highly mutagenic (wear gloves) and mildly toxic.** Wipe out surfaces that come into contact with EtBr, and discard any gels or solutions that have EtBr as directed.

Photography

Stained gels should be observed under UV light at 254 -302 nm and photographed with a Polaroid camera with Polaroid film. Never look at UV light directly with naked eyes. Always wear protective goggles/glasses.

16. Production of polyclonal antibodies

Antibodies are glycoproteins, synthesized by B-cells and are part of the humoral immune response. The traditional method for making polyclonal antibodies of desired specificity is to immunize animals with appropriate antigen and then prepare antisera from their blood. The specificity and quality of such antisera is highly dependent upon the purity of the immunizing antigen preparation because antibodies will be made against the entire foreign component it contains.

Polyclonal antibodies are antibodies that are obtained from different B cell resources. They are a combination of immunoglobulin molecules secreted against a specific antigen, each identifying a different epitope.Although the advances offered by the development of monoclonal antibody techniques have revolutionized the specificity, uniformity, and quantity of antibodies, there remain many circumstances in which polyclonal antibodies are more desirable than monoclonal antibodies. Production of polyclonal antisera takes less time and effort than production of monoclonal antibodies, requires relatively simple and readily available equipment, and produces reagents that can be used for immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assays (ELISAs).

Reagents and materials

Young rabbits (2.5-3.0 kg; 10-16 weeks of age)

Bovine Serum Albumin

Freund's complete adjuvant

Freund's incomplete adjuvant

25 gauge sterile needles

Sterile syringes

Serum collection tubes

70% ethanol

Scalpel blade

Procedures

A. Preimmune test bleed on rabbits – marginal ear vein

1. Restrain the rabbit in an appropriate manner.
2. The marginal vein is found on the inner edge of the dorsal surface of the ear and should be visible.
3. Using a scalpel blade gently shave a patch over the vein about two-thirds of the distance from the head to the tip of the ear.
4. Clean the area with 70% alcohol.
5. Hold the ear out horizontally and stabilize.
6. Penetrate the vein with the needle bevel up.
7. Draw 1ml of blood using a sterile needle and syringe.
8. Following completion ofblood withdrawal, remove the needle and cover the vein penetration site with a cotton ball. Apply gentle pressure to the site for up to two minutes to ensure adequate homeostasis.
9. Verify that no furtherbleeding is occurring and return rabbit to its cage.

B. Collection of serum

1. Transfer the blood into aneppendorftube and allow it to clot at 4°C overnight.
2. Next day, centrifuge at 350xg for 15 minutes to sediment the erythrocytes.
3. Transfer the serum and store frozen at -20°C. Alternatively store at 4°C after adding methiolate 0.01% w/v final concentration or 0.1% sodium azide.

C. Immunization of a rabbit

1. Prepare the antigen (bovine serum albumin (BSA)) as follows; Dissolve 0.2mg/ml of BSA in 0.85% NaCl solution (physiological saline). Combine 0.5ml (0.1mg) of this BSA solution with 0.5ml of Freund's complete adjuvant and mix until an emulsion is formed.
2. Inject approximately 250µl of the antigen-adjuvant emulsion into four subcutaneous sites two on the right thighand two on the left thigh of the rabbit (Day 0).
3. After two weeks boost with antigen mixed with incomplete Freund's adjuvant.The booster antigen is prepared by dissolving 0.2mg of BSA in 0.85%NaCl and mixingwith incomplete Freund's adjuvant to form emulsion.
4. Inject approximately 250µl of the antigen-adjuvant emulsion into four subcutaneous sites two on the right thigh and two on theleft thigh of the rabbit (Day 14).
5. After another two weeks boost with antigen mixed with incomplete Freund's adjuvant.The booster antigen is prepared as in step 3 and injected into the rabbit as in step 4 (Day 28).
6. After another two weeks (Day 42) bleed the rabbit to obtain the antiserum
7. Confirm the immunization success using Ouchterlony.

17. Double immunodiffusion (Ouchterlony)

The key reaction of immunology and immune defense is the interaction of antibodies and antigens. The body's defense mechanism recognizes foreign substances, or antigens, and raises specific antibodies against them. The specificity of antigen-antibody interactions has led to the development of a variety of immunologic assays, which can be used to detect the presence of either antibody or antigen.

Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. An antigen reacts with a specific antibody to form an antigen-antibody complex, the composition of which depends on the nature, concentration and proportion of the initial reactants.

Immunodiffusion in gels are classified as single diffusion and double diffusion. In Ouchterlony double diffusion, both antigen and antibody are allowed to diffuse towards each other in a horizontal agar or agarose gel. They are applied via sample wells or as a template on the surface of the gel. Each diffuses radially outward from its application point, with their concentration decreasing geometrically with increasing distance from the application well. Where the antibody and antigen meet in equivalence an insoluble precipitate appears. Each combination of antigen and antibody will give its own precipitin line.

The precipitates formed may be visualized directly by transmitted or diffused light or stained. The Ouchterlony method can be used to detect the presence of either antigen or specific antibody and to determine antigenic relationship and specificities among a series of antigens or antibodies. An idea of relative concentration can also be obtained by position distance.

Reagents and Materials

1% agar in PBS (Phosphate Buffered Saline)

Antigen

Anti-serum

Pre-immune serum

Micro-pipettes and tips

Pasteur pipettes

Humid-incubation box

Microscope slides

Procedure

1. Prepare agarose or purified agar solution (1% (w/v) in Phosphate Buffered Saline).
2. Using a 10 ml pipette pour perpendicularly 7 mls of the molten agarose onto a level microscope slide and allow to cool at room temperature.
3. Punch wells using a micropipette in the desired pattern, using a drawn pattern under the gel or a template laid on top.
4. Place the prepared gel on a level surface. Fill the reactant with antiserum and antigen solutions as desired. Do not overfill. (Usually the antiserum is placed in the central well and different antigen solutions- or dilutions of a single one- are placed in the outer four wells in a six-well pattern.
5. Prepare another slide (control) with pre-immune serum in the central well.
6. Place slides in a humid chamber.
7. Allow diffusion to proceed overnight or as long as necessary for precipitin bands to form. Large proteins have longer diffusion distances and lower temperatures increase the required diffusion time.
8. View and photograph precipitation lines using indirect light.

Post lab questions

1. In what situation would you expect to see more than one precipitation line between wells on an Ouchterlony plate?
2. State 5 applications of double diffusion.
3. Why is it necessary to maintain humidity in the chamber during incubation?
4. Why is agarose used as a base to study this interaction?