THIRD SEMESTER
BIOCHEMISTRY LABORATORY

Experiment: Determination of proteins by Bradford method

Principle

This is a rapid, simple and sensitive method for estimation of proteins in a sample extract. The color development is virtually complete in 2 min and the color is stable for about 1h. Unlike Lowry method, metal ions such as NH4, Na, K+, phenols and carbohydrates such as source do not interfere in this assay. The procedure is based on interaction of a dye, Coomassie Brilliant Blue, with proteins. The unbound dye has an absorbance maximum at 465 nm. However, on interaction with proteins the dye turns blue and their absorbance maximum is displaced to 595 nm. Thus from the absorbance at 595 nm the amount of protein in a sample solution can be quantitatively

estimated. However, as in Lowry procedure, detergents such as SDS, Triton X-100 etc. interfere in estimation of proteins by this method.

Materials and Reagents

1. Colorimeter
2. Sample extract: prepare as given in step 1 of experiment 4.4.2
3. Bradford reagent: dissolve 100 mg of Coomassie Brilliant Blue G 250 in 50 ml of ethanol, add 100 ml of 85% of phosphoric acid and make the volume to 1 L with water.
4. 0.1 M phosphate buffer (pH 7.5): see section 1.3.8 for preparation of this buffer.
5. Stanadard protin solution: Dissolve 5 mg of bovine serum albumin in 50 ml of 0.1 M phosphate buffer. This solution contains 100 μg protein/ml.

Proceduere:

1. Take 0.1 ml of sample solution (see step1, EXPERIMENT 4.4.2) and make the volume of 1 ml with 0.1 M phosphate buffer (pH 7.5).
2. Pipette appropriate aliquots of bovine serum albumin solutions containing 0-100 μg protein. Make the volume to 1 ml with 0.1 M phosphate buffer (pH 7.5)in all the tubes.
3. Add 5 ml of Bradford reagent to all the tubes and mix thoroughly.
4. Record the absorbance at 595 nm against the reagent blank.
5. Plot a standard curve of A_{595} versus μg of proteins in the standards (step 2).
6. Determine the protein content in the sample extract from the standard curve.
7. Calculate the amount of protein per ml of the sample preparation.

Experiment: Estimation of DNA by diphenylamine reaction

Principle

This is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of acid, is converted to ω-hydroxylevulinic aldehyde which reacts with diphenylamine to form a blue colored complex with absorbance maxima at 600 nm. Compounds such as furfuryl alcohol and arabinal, which can be converted in to ω-hydroxylevulinic aldehyde will also give this reaction. In DNA, since only deoxyribose of purine nucleotides is released, the value obtained represents half of the total deoxyribose in the sample. The reacting leading to the formation of the colored complex are as follows:

Materials and Reagents

1. Colorimeter or spectrophotometer.
2. Standard DNA solution: Dissolve calf thymus DNA (100 µg/ml) in 1N $HClO_4$ by heating at 70^0C for 15 min. make different dilutions of this stock solution ranging from 20-100 µg DNA/ml using 0.5 N $HClO_4$.
3. 1.6% (w/v) acetaldehyde: prepare by dissolving 1 ml of ice cold acetaldehyde in 50 ml of distilled water.
4. Diphenylamine solution: Dissolve 1.5 g of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of conc. H_2SO_4.
5. Diphenylamine reagent: prepare by mixing 0.5 ml of 1.6% acetaldehyde and 100 ml of diphenylamine solutions. This solution must be prepared fresh.

Procedure:

1. Take 2.0 ml aliquot of the sample in which DNA has to be estimated in a test tube.
2. In another set of test tubes, pipette 2.0 ml of standarad DNA solution of different dilutions. In one of the test tube 2.0 ml of 0.5N $HClO_4$ as a reagent blank.
3. Add 4.0 ml diphenylamine reagent (Reagent no. 5) to all the tubes, mix the contents properly and keep at room temperature in dark for 16-18h or overnight. Alternatively, keep the tubes in boiling water for 10 min and cool them under running tuning tap water.
4. Record the absorbance at 600 nm.
5. Draw a standard curve of A_{600} vs DNA concentration. From absorbance of the sample, determine the amount of DNA in it. Express the results as mg of DNA/g fresh weight of tissue.

Experiment: Determine of RNA by Orcinol method.

Principle

Orcinol, in the presence of ferric chloride as a catalyst, react with furfural producing a green colored compound with absorbance maxima at 665 nm. DNA gives a limited positive reaction with orcinol test. The reactions leading to the formation of a green colored complex are as follows:

Materials and Reagents

Colorimeter or spectrophotometer.

1. Boiling water bath.

2. 5% $HClO_4$

3. Standard RNA solution: Dissolve yeast RNA (500 μg/ml) in 5% $HClO_4$. Make different dilutions to obtain solutions containing 100-500 μg RNA/ml with 5% $HClO_4$.

4. Orcinol reagent: Dissolve 100 mg of ferric chloride (FeCl3.6H20) in 100 ml of conc. HCl and then add 3.5 ml of 6% solution of orcinol prepared in alcohol.

Procedure

1. Take 2.0 ml solution of each of the dilutions of RNA standard solutions, test sample and 2.0 ml of 5% $HClO_4$., as a blank, in different test tubes.

2. Add 3.0 ml orcinol reagent to all the tubes and mix properly.

3. Keep the test tubes in a boiling water bath for 20 min.

4. After cooling them, add 7.0 ml of n-butanol to each tube and measure the A_{665} against blank.

5. Plot a graph between A_{665} vs amount of RNA and from this standard curve determine the amount of RNA in the provided sample.

Experiment: Quantitative determination of DNA and RNA by spectrophotometric method.

Principle

Spectrophotometric method can also be employed for judging purity of DNA and RNA extract and these have absorption maximum at 280 nm. The ratio of absorbance at 260 and 280 nm, hence, provides a rough idea about the extent of contamination in the preparations. A ratio between 1.8-2.0 is indicative of fairly pure DNA and RNA preparations but values less than 1.8 signify presence of proteins as impurities.

Materials and Reagents

1. UV Spectrophotometer.

2. Saline sodium citrate (SSC) solution: Prepare 0.015M solution of sodium citrate (pH 7.0) and dissolve NaCl so that its final concentration in solution is 0.15M.

Procedure

1. Switch ON Spectrophotometer and allow it to warm up for about 10 min.adjust the wavelength at 260 nm and put ON the UV-lamp.

2. Set the instrument at zero absorbance with SSC solution.

3. Read absorbance of solution of the provided sample. If O.D. is too high appropriately dilute the sample solution with SSC and again take the reading.

4. Calculate the concentration of DNA and RNA in the sample from following formulae:

For double standard DNA:

Concentration of DNA in sample solution (μg/ml) = 50 x A_{260} x Dilution factor

For RNA:

Concentration of RNA in sample solution (μg/ml) = 40 x A_{260} x Dilution factor

Experiment: determination of melting temperature and base composition of DNA from thermal denaturation characteristics

Principle

When a dilute aqueous solution of double stranded DNA is heated, the two strands get separated due to disruption of H-bonds between the complementary bases. Such DNA is referred to as denatured DNA and the process is called denatuartion.

Denatuartion is a reversible process. If the above solution is allowed to cool slowly to room temperature, the complementary strands of DNA resemble to give a duplex DNA. DNA is now said to be reannealed and process is called reanealing of renatuaration of DNA.

Upon denatuartion, the absorbance of DNA at 260 nm increase by 30-40% due to exposure of bases. This is called hyperchromic effect. Heat treatment at temperatures of upto 80^0C causes only a very slight increase in absorbance at 260nm.

Materials and Reagents

1. UV-spectrophotometer with thermoprogrammer.

2. Saline sodium citrate (SSC) solution: Prepare 0.015M solution of sodium citrate (pH 7.0) and dissolve NaCl so that its final concentration in solution is 0.15M.

3. DNA: Dissolve 50 µg DNA/ml of SSC.

Procedure A

1. Switch ON Spectrophotometer and after allowing sufficient period for warming up, set it zero absorbance at 260 nm with SSC.

2. Measure A260 of the DNA samples at p-nitophenol.

3. Heat the DNA solution at a rate of 1^0C rise /min upto 100^0C with the help of a thermoprogrammer. Record the absorbance values manually or with the primer.

4. Calculate A260 (T^0C)/A260 at 25^0C for each of the following temperatures: 25, 35, 50, 70, 75, 80, 90, 95 and 100^0C and plot the absorbance ratio against the temperature.

5. Determine the midpoint of increase in absorbance and by extrapolation find the corresponding temperature which represents Tm for the DNA sample.

6. Calculate % (G+C) content of the DNA using the following equation:

$$\% (G+C) = (Tm-69.3) \times 2.44$$

Procedure B

If UV- Spectrophotometer with thermoprogrammer is not available, then the following procedure can be adopted.

1. Arrange a series of constant temperature water baths maintained at 25, 50, 75, 80, 85, 90, 95 and 100^0C respectively.

2. Record absorbance at 260 nm of DNA sample solution 50 μg/ml kept at 25^0C.

3. Distribute the above solution into eight test tubes and put one test tube each in the water baths maintained at different temperatures. Allow the tubes to stand for 15 min. after incubation, quickly cool all the tubes (except one at 25^0C) by placing them in ice bath for 10 min.

4. Record the A_{260} of these samples.

5. Then proceed as in steps 4 to 6 of the procedure A described earlier.

Experiment: To study time course of the reaction catalysed by alkaline phosphatase (EC 3.1.3.1)

Principle

Phosphatase is a broad term used for non-specific phosphomonoeserases which hydrolyze organic phosphatase esters librating in alcohol derivative of the substrate and inorganic phosphate (Pi). These enzyme catalyze the following reaction:

Orthophosphoric monoester + H2O – alcohol + Pi

Depending on their pH optima, phosphatases have been classified into two groups' viz. acid phosphatases and alkaline phosphatases. Acid phosphatases function optimally at acidic pH (4.0 – 5.5) whereas alkaline phosphates give maximum activity at alkaline pH (8-10). For assaying phosphatases, p-nitophenyl phosphatase can be used as substrate which is hydrolysed to p-nitophenyl and Pi p-nitophenyl is colorless at acidic or neutral pH but at alkaline pH of 11 it gives yellowish colour with absorbance maxima at 410 nm. Hence the activity of alkaline or acid phosphatases can be conveniently determined calorimetrically by determining the amount of p-nitophenyl produced. The reaction catalysed by alkaline phosphates:

Materials and Reagents

1. Water bath at 35^0C
2. Calorimeter
3. Refrigerated centrifuge
4. Glycine-NaOH buffer (0.005 M, pH 10.5): Dissolve the 375 mg glycine in small volume of water, add 42 ml of 0.1N NaOH solution and adjust the pH 10.5
5. NaOH solution (0.085N): prepare by dissolving 340 mg of NaOH in 100 ml of distilled water.
6. MgCl2 solution (10.5mM): weigh 10 mg of MgCl2 and dissolve it in 10 ml of distilled water. 0.1 ml of this solution is used in 3.5 ml of reaction mixture so that the final conc. Of Mg^{++} during the assay is 0.3mM.
7. p-nitophenyl phosphatase (35mM): take 38.8 mg of p-nitophenyl phosphatase and dissolve it in 5 ml of 0.05 M glycine –NaOH buffer pH 10.5.
8. Standard solution of p-nitophenyl (100mM): weigh 69.75 mg of p-nitophenol and dissolve it in 5 ml of distilled water. This solution contains 100 µ moles p-nitophenol/ml.
9. Plant material: use 5 days old germinating pea seeds which have been germinated at 25^0C in petri plates lined with two layers of whatman no. 1 filter paper. Ensure that the petri plates contain sufficient amount of water throughout this period.

Procedure

1. All the operations for preparation of the tissue extract have to be carried out in cold at 0-4^{0}C. Weigh 1 g of the germinating seeds and grind them in chilled pestle and mortar in presence of 10 ml of glycine-NaOH buffer (0.05 m, pH 10.5). a small amount of acid washed river sand may be used as an abrasive to facilitate complete breakage of the cells.

2. Centrifuge the homogenate in a refrigerated centrifuge at 10,000 x g for 20 min. Decant the supernatant and use it as the enzyme preparation.

3. Take nine numbered test tubes and add 3.0 ml of glycine-NaOH buffer, 0.1 ml of MgCl2 and 0.3 ml of the enzyme preparation into each of them.

4. Transfer these tubes to awater bath maintained at 37^{0}C. After 3 min start the reaction in seven of the above tubes by adding 0.1 ml p-nitophenyl phosphate. Note down the time of starting the reaction for each tube.

5. Exactly after 5, 10, 15, 20, 25, 30 and 45 min stop the reaction by adding 9.5 ml of 0.085 N NaOH to each tube. In the eight tube add NaOH followed by 0.1 ml p-nitophenyl phosphate. This represents 0 min control. In the ninth tube, instead of p-nitophenyl phosphatase add 0.1 ml of 0.05 M glycine –NaOH buffer (pH 10.5) and this serves as the reagent blank.

6. To prepare a standard curve, take 0-1 ml (0-100) μ moles of p-nitophenyl. Add 3 ml of 0.05 M glycine –NaOH buffer (pH 10.5) to all the tubes and make the final volume to 3.5 ml with distilled water. pipette 9.5 ml of 0.085N NaOH into each tube, mix the contents and record the absorbance at 410 nm using the tube without p-nitophenol for setting the instrument to 100% transmission or zero absorbance.

7. Plot a graph of A410 vs μ moles of p-nitophenol to obtain a standard curve.

8. Determine the amount of μ moles of p-nitophenol in the tubes 1-8 of step 5.

9. Draw a graph of μ moles of p-nitophenol produced vs reaction time.

10. From this graph note down the maximum assay period upto which the production of p-nitophenol is linear. In all the subsequent experiments the duration of reaction should be well within this time limit.

11. Using the experimental data plot another graph of velocity of reaction vs reaction time.

Experiment: To determine the temperature optima for alkaline phosphatase

Principle

All the enzymes have a narrow temperature range for their efficient functioning. The reasons for decline in velocity at temperature beyond optimum temperature have been discussed in section 7.3.5.4. For determining enzyme activity in the sample, assay should be carried out a optimum temperature. So before undertaking further investigations on an enzyme, temperature optima for its activity ought to be determined in a preliminary experiment. While carrying out any enzyme assay it is essential that appropriate controls must be run simultaneously.

Materials and Reagents

1. All the requirements listed in experiment 7.8.1

2. In addition several water baths maintained at the chosen temperatures (say 20, 25, 30, 35, 40, 45 and 50^0C) will be needed.

Procedure

1. Prepare the enzymes extract as outline in experiment 7.8.1 (steps 1 and 2)

2. Take two sets of tubes (one set for zero minute control and the other set for actual enzyme assays) and pipette 3.0 ml of 0.05M glycine NaOH (PH 10.5) and 0.1ml of Mgcl2 into each tube.

3. Add 0.3 ml of the enzyme extract to the assay tubes. Keep one assay tube and one control tube at the temperature at which the enzyme activity has to be determined (20, 25, 30, 35, 40, 45 and 50^0C) and after 3 min add 0.1 ml of p-nitophenol phosphate to all the tubes.

4. Allow the reaction to proceed for 30 min or any other suitable time period which falls within linear region of curve of the graph constructed in experiment 7.8.1 and stop the reaction with 9.5 ml of 0.085N NaOH. After this add 0.3 ml of the enzyme preparation to control tubes.

5. Record the absorbance of all the tubes at 410 nm.

6. Deduct the obtained A_{410} for the controls from the A_{410} of the corresponding assay tubes and from the standard curve determine the amount of p-nitophenol formed in the assay tubes.

7. Draw a graph of amount of p-nitophenol produced at different temperatures.

8. From the graph determine the optimum temperature for the activity of alkaline phosphatase.

Experiment: To examine the effect of pH on activity of alkaline phosphatase.

Principle

The influence of pH on activity of enzymes has been discussed in details in section 7.3.5.3 for measurement of enzyme activity in biological sample; the assay ought to be conducted at its optimum pH.
For determining the pH optima the enzyme activity is measured at varying pH of the reaction mixture. Each buffer has a limited range over which it can effectively fulfill the buffering role. In many instances two different buffers may have to be tried to ascertain effect over a broader range of pH. In such cases it is advisable to select at least one common pH for both these buffers so that from this overlapping pH the variation in enzyme activity due to nature of the buffer can be distinguish from the actual effect of pH.

Materials and Reagents

1. All the requirements as in experiment 7.8.1 except that 0.05 M glycine NaOH of different pH (8.6, 9.0, 9.6, 10, 10.6, 11, 11.5) by adjusting the pH to the desired level with increasingly higher volumes of 0.1M NaOH to 50 ml 0.1 M solution of glycine and then making the final volume to 100 ml with water.

2. 0.05M Tris-HCl buffer: prepare 0-.05M Tris-HCl of pH 7.2, 7.6, 8.0, 8.6, and 9.0 as described in section 1.3.8.

Procedure

1. Prepare the enzyme extract accordingly to the method given in steps 1 and 2 of experiment 7.8.1.

2. Arrange two sets of tubes (one for assay and the other for zero min control). Pipette 3.0 ml of different buffers in succession to control and assay tubes followed by 0.1 ml MgCl2 to all tubes.

3. Add 0.3 ml of enzyme preparation in the assay tubes and incubate them at 37^0C (or more appropriately at the optimum temperature as determined in experiment 7.8.3). Start the reaction by pipetting 0.1 ml of p-nitophenol to all the tubes.

4. After 30 min, stop the reaction with 9.5 ml of 0.085N NaOH in all the tubes. Now add 0.3 ml of the enzyme extract in the control tubes.

5. Note the absorbance at 410 nm against distill water.

6. Deduct absorbance of the controls from the corresponding assay tubes and using the standard curve (see experiment 7.8.1, step 7). Determine the amount of p-nitophenol formed in the assay tubes.

7. Since the enzyme extract was prepared in a buffer of pH 10.5, the actual pH of the reaction mixture might deviate from that of the assay buffer. Hence prepare a mixture containing

extraction buffer and assay buffer in the same ratio as used for measuring enzyme activity and using pH meter determine its pH which will represents the actual pH of the reaction mixture.

Experiment: to study the effect of substrate concentration on activity of alkaline phosphatase and determine the Km and Vmax of the reaction.

Principle

The enzyme activity is measured at varying concentrations of the substrate under optimal conditions on the basis of the information obtained in experiment 7.8.1-7.8.4. The Km and Vmax of the reaction are then determined by processing the data and drawing Lineweaver-Burk(1/v vs 1/[S], Eadie-hostee (v/vs v/[S]). Hanes ([S]/v vs v) or eisenthal and Corrnish-Bowden (v vs [s] plots.

Materials and Reagents

All the requirements as mentioned in experiment 7.8.1 except for reagents 7. For this experiment prepare 5mM solution by dissolving 38.8 mg p-nitophenyl phosphate in 35 ml of 0.05 M glycine-NaOH buffer (pH 10.5).

Procedure

1. Prepare the enzyme extract from the germinating pea seeds as outlined in experiment 7.8.1 (step 1 and 2).
2. Take eight numbered test tubes and add 0.0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 and 0.50 ml of p-nitophenol phosphate in successive tubes.
3. Add calculate amount of 0.05M glycine-NaOH buffer (pH 10.5). So that the total volume of p-nitophenol phosphate and the buffer in each tube is 3.0 ml. pipette 0.1 ml of 10 mM MgCl2 to the tubes and keep them in water bath at 37^0C.
4. After letting them to warm up for 3 min, start the reaction with 0.3 ml of the enzyme extract in each tube.
5. Stop the reaction after 30 min with 9.5 ml of 0.085N NaOH.
6. Record the absorbance of the color formed at 410 nm against distilled water.

Experiment: to prepare various sub-cellular fractions of rat liver cells.

Principle

Liver is one of the vital organ in an animal body and its function is to process the incoming nutrients from alimentary canal and maintain them at steady level in the blood. In addition it performs excretory functions by catabolizing the amino acids and the other nitrogen containing compounds via urea cycle and degradation of protoporphyrin IX to metal devoid open chain tetrapyrroles. The bile pigmebnts such as bilirubin. It also serves as the principal storage organ for glycogen which may account for about 10% fresh weight of the liver in normal adult.

Materials and Reagents

1. Experimental animal: a young adult rat
2. Scissors
3. Potter-Elvejhem Homogenizer
4. Cheese cloth
5. Wide mouthed canula attached to a syringe.
6. Refrigerated high speed centrifuge.

Procedure

1. Anaesthetize the rat by injecting pentabarbitone sodium (6mg/ml) intraperitoneally using 1 ml for 100 g of the rat weight. Decapitate to kill the rat and let it bleed from the neck to drain out the blood.
2. Dissect the rat and open the abdomen with amid line incision, displace the intestine to right and excise the liver and transfer it to pre weight chilled beaker containing 20 ml of the homogenate media. After weighing the liver, drain out the media and slices in to small pieces with scissors. Add calculated volume of ice cold homogenization media (4ml/g) liver). Preparation of the liver slices and all the subsequent step should be carried out at 0-4^0C.
3. Transfer the liver slice along with homogenization media into chilled Potter-Elvejhem Homogenizer. Operate the Homogenizer at about 500 rpm and push the glass tube and down 6-8 times to ensure proper breakage of the cells. Filter the homogenate through 3-4 layer of cheese cloth which has been wetted with homogenization media. Stir the homogenate with glass rod to facilitate quicker filtration.
4. Pour the filtrate into 50 ml centrifuge tubes and centrifuge at 1.000 x g for 10 min to sediment the heaviest material (Pi). Since the pellet formed is not well packed and is fluffy. Remove the supernatant carefully with the help of a wide bore cannula attached to a syringe. Centrifuge this supernatant at 3, 000 x g for 10 min to obtain P2 which is again a

loose packed one. Recover the pellet by withdrawing the supernatant with a syringe as described above.

5. Subject the supernatant from the preceding step to centrifugation at 10,000 x g for 30 min. gently recover the pellet and label it as P3.

6. Finally centrifuge the remaining supernatant from step 5 at 10,000 x g for 40 min to obtain pellet (P4). Decant the supernatant into chilled beaker and store it on ice.

7. Suspend the pellet (P1+P4) in original volume of the homogenization buffer and store them in cold on ice.

Experiment: Elecroblotting (western blooting) of proteins from SDS-polyacrylamide gel.

Procedure

When an electric field is applied, proteins migrate from cathode to anode and as they come in contact with nitrocellulose sheet.

Materials and Reagents

1. Western blot apparatus consisting of gel holder, sponge and transfer tank.
2. Power pack and electrical leads.
3. Slab gel containing separated proteins
4. Nitrocellulose sheet cut to the size of the gel.
5. Whatman 3mm paper cut to the size of the gel.
6. Transfer buffer: it consist of
    a. Tris 3.0 g
    b. Glycine 14.7 g
    c. Metanol 200 ml

    After mixing the above components, adjust the pH of the solution to 8.3 and make the final volume to 1L with distilled water.

Procedure

1. Take the gel obtained after electrophoresis in experiment 10.4.3. Do not stain it and mark it by cutting of the one corner. Place the gel form 30 min in transfer buffer for equilibration.
2. Take a nitrocellulose sheet, cut to the size of the gel and dip it in the transfer buffer by carefully wetting one edge and then slowly lowering the sheet into the buffer. Leave it under the buffer for 30 min.
3. Soak the sponge in transfer buffer and place the wet sponge on the gel holder. Now keep a sheet of Whatman 3mm paper on the sponge.
4. Place the equilibrated gel carefully on the filter paper avoiding trapping of any air bubbles.
5. Now lay down carefully the Nitrocellulose membrane with shining side towards the gel on top of the gel. Gently roll a sterile 10 ml pipette over the membrane to remove air bubbles for ensuring a good contact between the membrane and the gel.
6. Complete the sandwich by placing wet Whatman 3mm filters paper and a second sponge on the filter paper. Close the gel holder and place it in the transfer tank containing sufficient transfer buffer to completely cover the blot.

7. Connect to the power supply and run for 5 h at 60v or at 30v over night.

8. When the transfer is complete, lift the membrane from the gel. Stain and destain it as in experiment 10.4.1 using Coomassie Brilliant Blue R-250 stain. Examine the nitrocellulose sheet for the presence of blue bands of the transferred proteins.

Experiment: To precipitate proteins from protein solution, using ammonium sulphate.

Principle
Solubility of proteins is lowered at high salt concentration and the protein is precipitated out. This phenomenon is called salting out. Hydrophobic region generally are protected from hydrophilic region which interact with water when salting take place. Water is generally attacked by thr higher salt concentration this generally increase protein –protein interaction and result in coagulation with each other.

Requirements
Protein solution, suspension buffer (20 mM tris + 100 mM NaCl pH 7.5) ammonium sulphate, centrifuge, stirrer, etc.

Procedure
Protein solution, is taken out from 4^0C and suspension buffer is added to dissolve the pellet and thaw it. Make the solution upto 20 ml and then add 2.2 g of ammonium sulphate so as to make the solution 20% satuartion. The solution is stirred continuously in the presence of ice to dissolve the salt in solution. The solution is centrifuged at 15 k rpm for 15 min at 4*C. the pellet id collected and stored at 4*C. the supernatant is agin mixed with suspension buffer to make the volume upto 20 ml than again add 2.2 g of ammonium sulphate to make solution 40% saturation. The salt is added very slowly keeping the solution continuously in ice. The solution is again centriguged at 15 k rpm for 15 min at 4 *c. pellet is collected and dissolved in suspension buffer while supernatant is processed same way but with 60% ammonium sulphate saturation. This process is repeated with 80% ammonium sulphate saturation. Pellet and supernatant are stored at -80^0C.

GTPase Assay

Expression and purification of GTPase (dynamin):
10 grow tetrahymena (harboring TAP tagged GTPase under MTT1 promotor) culture in CHELAX treated SPP media upto cell density $3x10^5$

1. Prepare SPP

    1. Make SPP

        a. Proteose petone-1%

        b. Glucose-0.2%

        c. Yeast extract- 0.1%

        d. EDTA (ferric sodium salt) 0.003%

    2. Autocalve

2. Prepare SPPC (chelax-100 treated SPP)

    1. Chelax-100 (sodium form) biorad # 143-2832

    2. Add 50 g of Chelax-100 to 1000 ml of SPP

    3. Shake the stir for 2 hours

    4. Decant or spin down (230xg, 4 min)

3. Prepare SPPCT (SPPC + 3 essential trace metals)
make a 100 x solution containing 3 trace metals

    1. FeCl3.6H20 ---1 mg/ml

    2. Co(No3)2.6H20 --- 0.05mg/ml

    3. MnSo4.4H2o---0.16 mg/ml

Filter sterilize
Add the trace metals solution at a 1:100 dilution to SPPC

2. Add 2µg/ml CdCl2 to the culture media and incubate at 30^0C for 3 h

3. Pellet down the cells in appropriate bottle at 1100 x g for 10 min

4. Add 20 ml of 20mM Tris-cl pH7.5, 200 mM NaCl, 1.05NP40, protease inhibitor cocktail and incubate for 30 min on ice.

5. Centrifuge at 15000 rpm for 30 min

6. Collect the supernatant and incubate with 1 ml of rabbit IgG Agarose resin for 1 h

7. Centrifuge at 3000 rpm for 5 min

8. Wash the resin with 10 ml with lysis buffer for 5 times

9. Incubate with TEV protease in TEV cleavge buffer for I h at room temp.
10. Centrifuge at 3000 rpm for 5 min and collect the supernatant that contains purifies dynamain protein
11. Use this protein for GTPase assay or further pyurify by calmodulin-sepharose column.