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CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

1. Acrylamide, N, N methylene bis acrylamide ,ion exchanger - PEAE cellulose of Serva, West Germany, were used.
2. Bio-gel P - 100 was obtained from Bio-rad Laboratories, California, U.S.A.
3. Tris (hydroxy methyl) amino ethane from Fluka, Germany, was used.
4. Drugs like streptomycin and penicillin forted with procaine of Alembic Chemical Works, Bombay, India.
5. Folin phenol reagent was obtained from Sisco Research Laboratories, Bombay, India.
6. All other chemicals of highest purity, acids, salts, solvents and EDTA were used. Starch was prepared from potatoes, sucrose, maltose, glucose were of Analar grade.

2.2 METHODS

2.2.1 Preparation of Buffers

1] 0.2 M Sucrose - 2 mM EDTA solution of pH 7.2

The 0.25 M sucrose -2mM EDTA buffer was prepared by dissolving 99.7 gm sucrose and 0.744 gm of EDTA in 1000 ml distilled water, the pH of solution was adjusted to pH 7.2.

2] 5 mM Tris-2mM EDTA - 5 mM β-mercapto-ethanol

The 5 mM Tris-2mM EDTA -5mM-β-mercapto - ethanol buffer was made by dissolving 0.605 gm of Tris, and 0.744 gm EDTA and 0.35 ml β-mercapto ethanol in 1000 ml distilled water. The pH was adjusted to 7.0.

3] 10 mM Tris-HCl buffer

The 10 mM Tris-HCl buffer was prepared by dissolving 1.211 gm of Tris (hydroxy methyl amino ethane) in 1000 ml of distilled water. The pH of the buffer was adjusted to pH 7.0 with 0.2 M HCl solution.

2.2.2 Quantitative Determination of Protein

Protein estimation was carried out using Lowry et al (60) procedure. The intensity of colour is dependent on the biurate reaction of protein with copper ions in alkali and the reaction of phosphomolybdate - phosphotungstic reagent by tyrosine and tryptophan present in the proteins.

1) Lowry A. :- 10 % sodium carbonate in 0.1 N NaOH
2) Lowry B_1 :- 1 % Copper sulphate solution
3) Lowry B_2 :- 2 % Sodium citrate solution
4) Lowry C :- It is prepared by mixing freshly prepared 100 ml of Lowry A to 1 ml Lowry B_1 and 1 ml of Lowry B_2
5) Folin-phenol Reagent :- Commercial preparation was diluted 3 times
6) 20 µgm/ml of bovine serum albumin was prepared in water

The standard protein (5-25 µgm in 1.5 ml) was mixed with 3 ml of Lowry C. After 15 minutes 0.5 ml Folin-phenol reagent was added to each tube with immediate mixing. After 30 minutes colour developed was read at 660 nm. The graph of optical density was plotted as shown in Fig. 1.

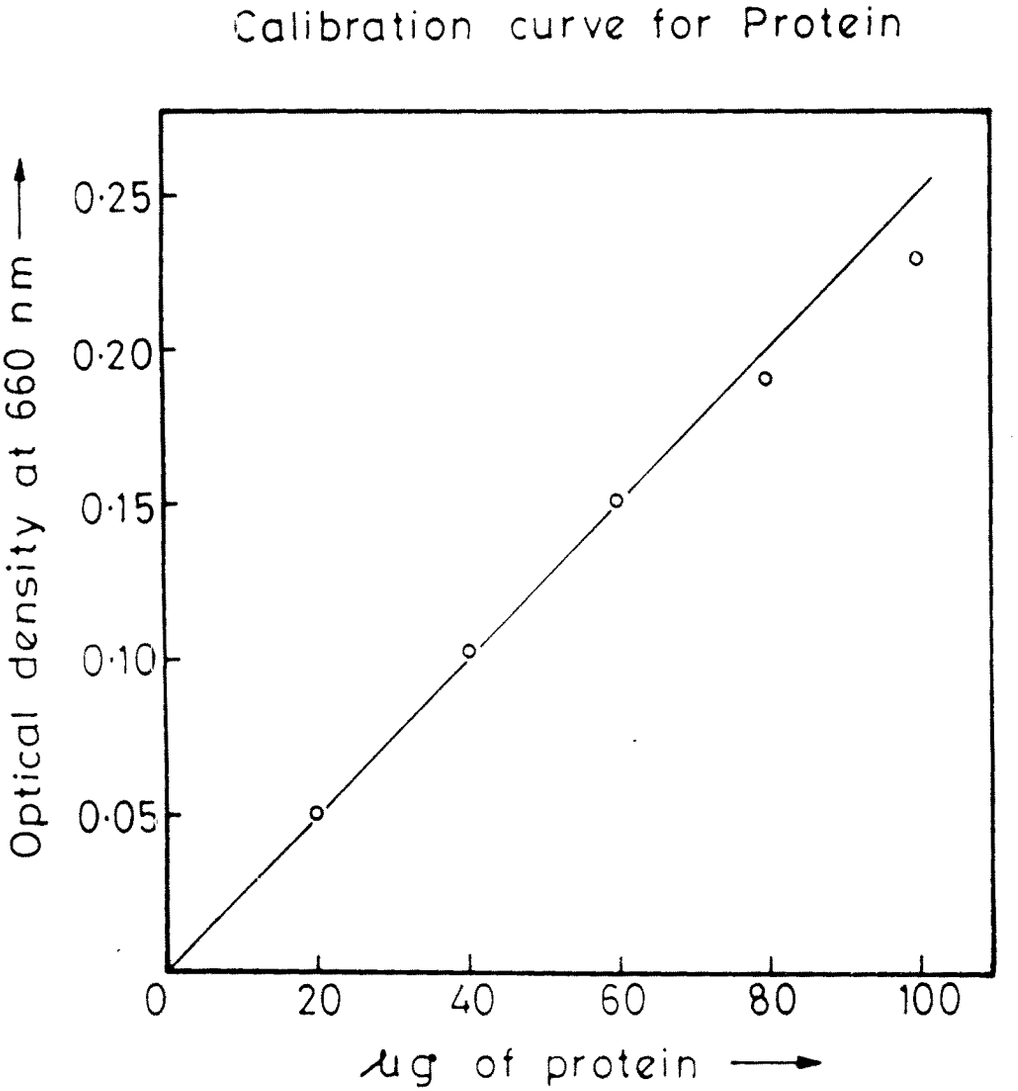
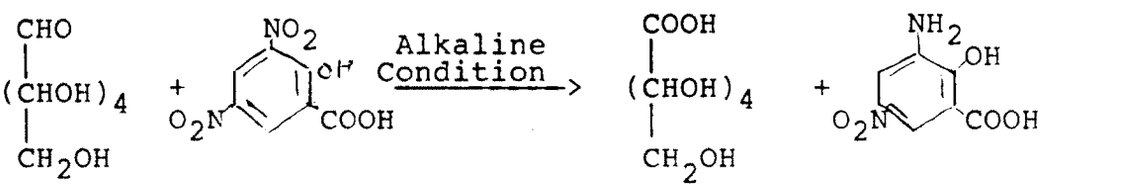


Fig.1 The technique of lowry et al was used The reaction mixture contains protein (BSA 20 to 100 µg/ml) + 3 ml lowry C and 0·5 ml Folin-phenol reagent The blue colour was read at 660 nm.

2.2.3 Quantitative Estimation of Glucose by 3,5 dinitro Salicylic Acid Method

The glucose estimation was carried out using 3, 5 dinitrosalicylic acid method (61). Glucose is a reducing sugar which reduces 3,5 dinitro salicylic acid to 3 amino 5 nitro salicylic acid giving redish brown colour. The intensity of colour is directly proportional to the number of reducing groups i.e. number of glucose molecules.



Glucose | 3, 5 dinitro Salicylic acid | Gluconic acid | 3, amino-5nitro Salicylic acid

Reagents : •

1) 3,5 dinitro salicylic acid reagent :- 10 gms of 3,5 dinitro salicylic acid is dissolved in 20 ml 2 N NaOH with constant stirring and 30 gms of sodium potassium tartarate was then added. The volume of reagent was made 100 ml with distilled water.

2) Working standard glucose solution :- 500 µg/ml of glucose was prepared in water.

Calibration curve for Glucose

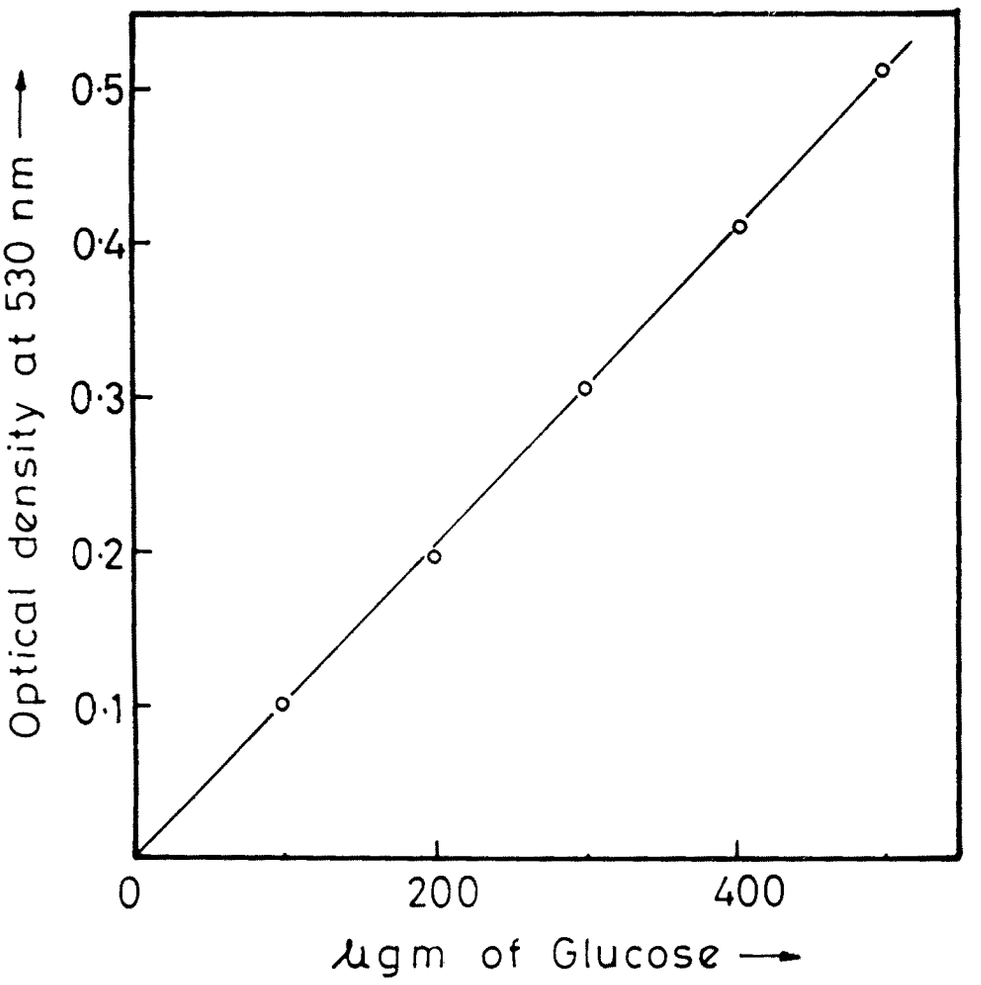


Fig.2 3,5 Dinitro-salicylic acid reagent method. The reaction mixture consists of glucose (100 to 500 µgm / ml) + 2·5 ml distilled water + 3 ml DNSA reagent colour developed was read at 530 nm.

The standard glucose (50-500 Ug/3 ml) was mixed with 3 ml 3,5 dinitro salicylic acid reagent. The contents were mixed thoroughly and kept in boiling water bath for 5-10 minutes. The tubes were cooled under running tap water. The colour developed was read at 530 nm. The graph of optical density against concentration of glucose was plotted as shown in Fig. 2.

2.2.3.1 Estimation of Glucoamylase Activity

The enzyme activity was estimated using starch as substrate. Glycoamylase hydrolyzes the starch to glucose by breaking α -1,4 and α -1,6 glycosidic linkages. The liberated glucose molecules were quantitatively determined using 3,5 dinitro salicylic acid reagent method. The quantity of glucose released is directly obtained from glucose calibration curve.

Reagents :

1) 2 % Starch Solution :- It was prepared by dissolving 2 gms of soluble starch in 100 ml distilled water.

2) Buffers

a) 0.1 M Potassium Acetate Buffer Containing 0.25 M Potassium Chloride :- This was prepared by dissolving 9.81 gm potassium acetate and 14.9 gm potassium chloride in 1000 ml distilled water, the pH was adjusted to pH 4.2 using dilute acetic acid.

b) 0.2 M Tris Buffer :- 0.2 M Tris buffer was prepared by dissolving 24.22 gm Tris (hydroxy methyl) amino ethane in 1000 ml distilled water, the pH was adjusted to 8.0 using 0.2 M hydrochloric acid.

3) 3,5 dinitro salicylic acid reagent

Experimental

The reaction mixture contains 1 ml 2 % starch solution and 1 ml of enzyme in 0.1 M potassium acetate buffer. The mixture was incubated at 37°C for 20 minutes. The reaction was stopped by adding 2 ml 0.2 M Tris buffer pH 8.0. The reaction mixture was centrifuged to remove coagulated protein. The glucose content was determined by using 3,5 dinitro salicylic acid reagent method. 3 ml of 3,5 dinitro salicylic acid reagent was added to 1 ml of

above enzyme reaction mixture and volume is made to 6 ml with distilled water. The contents were held in a boiling water bath for 5 - 10 minutes and rapidly cooled under running tap water. The optical density of the solution was determined at 530 nm (22). A blank was made containing all other components except enzyme solution. The amount of glucose produced in enzymic hydrolysis was determined from calibration curve. The enzyme activity is expressed as mg of glucose liberated per ml of enzyme solution at 37°C per 20 minutes.

2.3 ISOLATION AND PURIFICATION OF GLUCOAMYLASE

A simple procedure was used to isolate and to purify glucoamylase from human liver.

2.3.1 Isolation and Fractionation of Cellular Components

All operations were carried out at 4°C except where stated. The human liver was washed by transferring to fresh portions of 0.25 M sucrose, 0.2 mM EDTA buffer pH 7.2 until the exterior surface was free of blood. The liver tissue (about 180 gm) was cut with scissors into small pieces and homogenised with 1000 ml ice cold sucrose - EDTA buffer

using Remi mixer at 1000 speed for one minute (the operations were carried out in parts). The homogenate was centrifuged at 7000 rpm for 25 minutes. The supernatent containing glucoamylase activity was processed further to achieve purification of glucoamylase (22).

2.3.2 Ammonium Sulphate Fractionation

The protein in solution is salted out by adding a definite quantity of ammonium sulphate (22). The fractional precipitation of protein at varying concentrations of ammonium sulphate was used in seperating the glucoamylase fraction from protein solution i.e. the fraction between 42% and 84% ammonium sulphate saturation showed glucoamylase activity.

Experimental

The soluble extract was saturated to 42% with ammonium sulphate at 4 - 5°C by adding 243 mg of solid ammonium sulphate per ml of solution, taking care to stirr the solution continuously during the addition of ammonium sulphate. The pH was maintained at approximately at 7 by addition of thr required quantity of 1 M potassium hydroxide solution. After standing for one hour a small amount of

precipitate formed was removed by centrifugation at 6000 rpm for 30 minutes.

The clear supernatent was saturated to 84 % by adding 283 mg of solid ammonium sulphate per ml of solution. After adjusting the pH to 7, and allowing the mixture stand for one hour, the precipitate was collected by centrifugation at 6000 rpm for 30 minutes.

2.3.3 Dialysis

The precipitate was dissolved in minimum quantity of 5 mM Tris - 2 mM EDTA - 5 mM β-mercapto ethanol buffer pH 7.2 and dialysed with five litres of same buffer. Two operations removed most of the ammonium sulphate. The resulting solution could be stored for at least one month in frozen state without significant loss of activity.

2.3.4 Ion-exchange Chromatography with DEAE-Cellulose (22)

The separation of a protein from a mixture of proteins is possible in making the use of its ionic characteristics. Ion-exchange chromatography is one of the choiced method for the separation of proteins. The elution of proteins was effected by applying the salt gradient

buffer system. The nature of sample determines whether the separation should be performed on a cation exchanger or anion exchanger. In present investigation, the DEAE cellulose anion exchanger was used.

Experimental :- Preconditioning of DEAE Cellulose

The DEAE cellulose was reconditioned by suspending it in 0.2 M HCl. The suspension was kept for half an hour with intermediate stirring. The DEAE cellulose was repeatedly washed with distilled water until pH was neutral. The DEAE cellulose was suspended in 0.5 M NaOH for half an hour with intermediate stirring. Again the DEAE cellulose was repeatedly washed with distilled water until pH is reduced to neutral. The DEAE cellulose was equilibrated with the Tris-HCl buffer pH 7.

Column Preparation

The chromatographic column 24 x 2 cm was filled by transfering the activated DEAE-cellulose slurry. The column was washed with chilled fresh Tris-HCl buffer. The pH of effluent and inffluent was checked to see that they were the same.

Column Operation

The 5 ml sample solution (dialysed glucoamylase protein fraction) containing 40 mg protein was applied onto the surface of DEAE cellulose with the help of a pipette. It was washed twice with 20 ml chilled Tris-HCl buffer pH 7.0. The fraction of 5 ml each were collected at a flow rate of 40 ml/hr so as to get good resolution.

The Tris-HCl 0.25 M KCl buffer was used to elute enzyme. The fractions were collected of same volume with the same flow rate. After completion of the run the DEAE - cellulose was removed from the column and regenerated for the future use.

Analysis of Effluent Fractions

The protein estimation of each fraction was carried out with U. V. spectrophotometer at 280 nm.

The glucoamylase activity of the fractions was determined using 3,5 dinitro salicylic acid reagent method.

The profile of DEAE cellulose column was obtained by plotting optical density at 280 nm against fraction number and mgms of glucose released as shown in Fig. 3.

2.3.5 Lyophilization of Glucoamylase Fractions

Lyophilization is the process of removing water from thermally labile biological materials. It removes water by sublimation of ice from the frozen sample at a temperature between 0 - 60°C at a pressure 0.1 - 0.001 mm Hg.

Experimental

The fractions from DEAE cellulose column chromatography, containing highest glucoamylase activity were pooled and dialyzed against distilled water. The resulting enzyme solution was lyophilized and stored in freeze.

2.3.6 Molecular Sieving of Enzyme Preparation

Gel chromatography separation components based on their size and molecular weight. The larger molecules will emerge first from the column while the smaller molecules are retarded.

The chromatographic gel, in gel filtration does not need regeneration and can be used repeatedly. The polyacryl amide gel (Bio-gel P-100) employed for gel chromatography is

stable in the pH range 2 - 10 due to its inert matrix chain. The Bio-gel P-100 was used in the present study.

Experimental

The Bio-gel P-100 was swollen in hot distilled water and kept at overnight. The fine gel beads particles were removed and resuspended in the 0.1 M potassium acetate, 0.2 M potassium chloride pH 4.2 buffer; and equilibrated. It was deaerated and filled in a LKB column of size 70 x 1.6 cm. After the height of gel column becomes constant, the flow rate was adjusted to 12 ml/hr. The protein solution of lyophilized powder in acetate buffer pH 4.2, 1 ml was loaded onto the top of column, only after then sample entered the gel-bed. The connections were made to the reservoir containing buffer via peristaltic pump. One ml fractions were collected at the flow rate 12 ml/hr. The protein content in each fraction was estimated by Lowry technique. The glucoamylase activity of fraction containing protein was determined by 3,5 dinitro salicylic acid reagent method. Using protein concentration and mgms of glucose liberated the elution profile was obtained as shown in Figure 4.

2.3.7 Polyacrylamide Gel Electrophoresis (63)

The tertiary and quaternary structure of any protein depend upon its primary structure i.e. number of amino acids and their sequence. As amino acids are amphoteric in nature, consequently contribute towards inducing charge on the proteins. These charged protein molecules move towards cathode or anode under the applied potential difference, depending upon their net charge. By using this electrophoretic mobility the proteins can be separated from each other. The presence of single band in the electrophoretic gel indicates the protein is pure and hence this technique is used as a criteria of purity and homogenity.

Experimental

The following reagents were used :

0.05 M Glycine - NaOH Buffer :- The Glycine-NaOH buffer was prepared by dissolving 3.88 gms of glycine and adjusting the pH to 8.5 by NaOH solution. The buffer is then diluted to 500 ml.

Stock Solution A :- This was prepared by mixing 0.05 M Glycine-NaOH buffer pH 8.5 with 0.23 ml of TEMED.

Stock Solution B :- 28 gms of acrylamide is dissolved in 100 ml of distilled water.

Stock Solution C :- 0.735 gms N-N' methylene bis acrylamide dissolved in 100 ml of distilled water.

Ammonium Persulphate Solution (0.14%) :- 140 mg of ammonium persulphate was dissolved in 100 ml of distilled water.

Bromophenol blue Solution (0.002%) :-
Approximately 0.2 to 0.5 mg bromophenol blue dye was dissolved in 10 ml of 50% glycerol solution.

Amidoblack Solution :- 1.5 gms of amidoblack was dissolved in a mixture of 250 ml of methanol, 50 ml glacial acetic acid and 250 ml of distilled water.

Acetic acid Solution (7%) :- 140 ml of glacial acetic acid, 140 ml methanol were mixed and resulting volume was made up to 2 litres.

Glycerol (50%) :- 5 ml of glycerol was mixed with 5 ml of distilled water.

The solutions A, B, and C are kept in brown coloured bottle and remain stable for one month at 4°C.

Preparation of Gel

The stock A, B and C were mixed just before the experiment as 1 part A + 2 parts B + 2 parts C. The resulting volume was made to 40 ml. The polymerisation was initiated by addition of a pinch ammonium persulphate which acts as a catalyst. Generally the time required for polymerisation is about 20 - 30 minutes. The resulting solution was poured into previously cleaned and dried gel tubes of dimensions 9.5 X 0.5 cms by the help of a pipette and set aside for half an hour. All operations are carried out at room temp.

Application of Sample

The 100 U l of protein sample containing 35 Ug of protein was loaded on top of the gel column. The sample was prepared in 50% glycerol. Bromophenol blue dye (1 drop) was added to one of the tubes as the tracking dye.

Electrophoresis Operation

The gel tubes were fitted in the electrophoresis apparatus. Glycine - NaOH buffer is poured in the lower and upper tank till both the ends are immersed in the buffer. Electrophoresis is carried out at constant current i.e. 3 mA per tube (36 mA) for two and half hours or till the tracking dye reaches the bottom of the tubes at room temperature. After the electrophoresis is complete the gels are removed from the tubes with the help of a syringe filled with ice cold water and subjected to staining.

Staining :- 0.25% amido black is used as the staining dye. The gels are left standing in the dye for a period of 30 minutes.

Destaining :- Destaining is done by 7% glacial acetic acid, prepared in 7% methanol. The first two to three washings were given rapidly and the consequent washings were given at longer periods till the protein bands are clearly visible.

The position of the bands with respective peak fractions is shown in figure 5.

2.4 IMMOBILIZATION OF THE ENZYME

Immobilization is a process of limiting the movement of the enzyme by linking it to a neutral support. During immobilization conformational changes occur which may alter the kinetics and characteristics of the free enzyme.

There are several methods utilized to immobilize the enzyme. The simplest method of immobilization, involving covalent linking is utilized. CNBr activated agar-pectin is used to immobilize the enzyme.

Experimental : Fractionation of agar into agarose and agaropectin

2 - 4% agar was dissolved in 10 M urea by heating the solution, till the agar solubilizes completely. After cooling the resulting agar-urea solution to 55°C absolute alcohol is added with mechanical stirring till the alcohol concentration reaches 60%. It is kept overnight. Then the solution is centrifuged, the precipitate is agarose while the supernatent contains agaropectin. The supernatent is made up to 100% with alcohol with constant mechanical stirring. The precipitate of agaropectin is washed till it is free of urea and attains neutral pH.

Activation of agaropectin : CNBr treatment

Immobilization of the enzyme is carried out at 4°C. The agaropectin is suspended in water and the pH is adjusted to 11.2 by the addition of Sodium hydroxide solution. This pH of 11.2 is maintained during the addition of CNBr and also in later operations. The reaction mixture is constantly stirred for two hours. The solution is then refrigerated for 5 - 6 hours. The agaropectin sample is washed to remove the excess of cyanogen bromide. The washing is continued till the pH comes to neutral.

The activated agaropectin was mixed with the enzyme solution, the mixture is intermitently for two hours and the supernatent is checked for the abscence of protein and enzyme activity.

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Similar immobilization technique was carried out to immobilize Rhizopus glucoamylase. The immobilized enzyme is used to study the various kinetic parameters.

Assay Conditions to Immobilize Glucoamylase Activity

Reaction mixture in the tube contains 1 ml immobilized enzyme, 1 ml 2 % starch solution and 1 ml buffer.

The mixture was incubated at 37°C for 20 minutes. The activity of enzyme was stopped by adding 2 ml 2 M Tris buffer of pH 8.

One ml from this mixture was used to react with 3 ml 3,5 dinitro salicylic acid reagent. The colour was developed by putting it in boiling water bath. After cooling, the colour was read at 530 nm and activity was determined.

2.5 CHARACTERIZATION OF GLUCOAMYLASE ENZYME

The various parameters were studied and dependence of enzyme with respect to pH, temperature and substrate concentration were determined in free enzyme and immobilized enzyme form.

2.5.1 Dependence of Glucoamylase Activity on pH

Experimental :- The following buffers were used.

0.1 M Potassium Acetate 0.25 M Potassium Chloride

This was prepared by dissolving 9.81 gm potassium acetate and 14.9 gm of potassium chloride in 1000 ml

distilled water. The buffer of different pH values were made by adjusting the pH values with acetic acid in a range (pH 3 to 6).

Phosphate Buffer

The stock solution of Disodium hydrogen phosphate was prepared by dissolving 5.36 gms in 100 ml distilled water. The stock solution of sodium dihydrogen phosphate was prepared by dissolving 2.78 gms in 100 ml distilled water. The required quantities of both stock solutions were mixed to obtain buffer of pH in the range of 5 to 6.

Assay Condition

Buffer solutions with different pH values were made.

The reaction mixture contains 0.2 ml free enzyme, 1 ml 2 % starch solution, and 1.8 ml buffer. Similarly, other reaction mixture contains 1 ml immobilized enzyme, 1 ml 2 % starch solution, and 1 ml buffer.

The reaction mixtures were incubated at 37°C for 20 minutes. After incubation enzyme activity was stopped by

adding 2 ml of 0.2 M pH 8 Tris buffer. One ml of the content was used to determine the amount of glucose released.

The plots of pH against enzyme activity were then plotted and depicted in Figures 6 and 7.

2.5.2 Dependence of Glucoamylase Activity on Temperature

The reaction mixture contains the buffer free and immobilized enzyme and substrate similar to pH dependence assay. The difference was only in the temperature of each tube in incubation period. The temperature was in the range of 10°C to 70°C.

The plots of temperature against activity were plotted and are shown in the Figures 8 and 9.

2.5.3 Dependence of Glucoamylase Activity on Substrate Concentration

Two percent stock solution was used as stock substrate solution. The concentration of starch in increasing order was present in the reaction mixtures. The

volume of the reaction mixture was adjusted with buffer, and contains 0.2 ml free enzyme or 1 ml immobilized enzyme. The reaction mixtures were incubated at 37°C for 20 minutes. The reaction was stopped by adding 2 ml of 0.2 M Tris buffer of pH 8. The amount of glucose liberated was determined using 3,5 dinitro salicylic acid reagent.

The graphs of $\frac{1}{S}$ against $\frac{1}{V}$ were plotted and are shown in Figures 12, 13, and 15.

2.5.4 Dependence of Glucoamylase Activity on Drugs

The glucoamylase activity was altered by the presence of toxic compounds. The release of glucose due to effect of toxic compounds or drugs may create problem to patients suffering from the disease. Therefore, it is important to study the effect of drugs on glycoamylase activity. In the present investigation the drugs used are penicillin and streptomycin.

Experimental

The enzyme was pre-incubated with 60 µg drug, then it was mixed with increasing concentration of substrate and the reaction was carried out under standardised conditions of assay. The product formed was estimated using 3,5

dinitro salicylic acid reagent. The same experiment was repeated with drug concentration of 120 µg and the results were plotted in the Figures 15, 16, and 17.