20BTC501J – BIOMOLECULES LABORATORY

Offered to

I YEAR M.Tech – BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIOENGINEERING

SRMIST

KATTANKULATHUR

2020

SYLLABUS

20BTC501J - BIOMOLECULES LABORATORY

PURPOSE

To establish the basics of practical biochemistry and to provide a platform for understanding and analyzing the biomolecules

INSTRUCTIONAL OBJECTIVES

1. To learn laboratory safety and standard operating procedures of common laboratory equipments
2. To impart skills in preparation of solutions and biological buffers
3. To extend sound knowledge in analysis, estimation and comparison of biomolecules in normal and diseased conditions
4. To offer exposure on modern separation techniques for Biomolecules

LIST OF EXPERIMENTS

1. Estimation of proteins by Bradford's method
2. Estimation of proteins by Lowry's method
3. Estimation of proteins by Biuret method
4. Salting in, salting out and dialysis of proteins
5. Estimation of Cholesterol by Zak's Method
6. Preparation of DEAE-cellulose column
7. Separation of proteins by Ion Exchange Chromatography
8. Determination of molecular weight of protein using SDS-PAGE
9. Assay of Alkaline Phosphatase Activity
10. Estimation of Protease activity in commercially available detergent

REFERENCE BOOKS: Laboratory Manual

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Ex. No. – 1
Date:

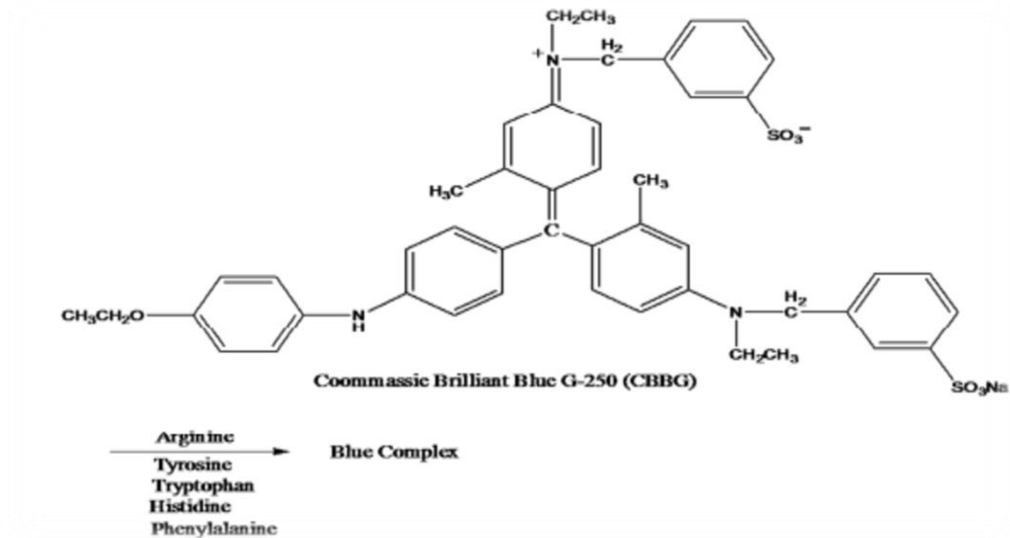
Estimation of proteins by Bradford's method

Aim:

To quantify the amount of protein present in the sample by Bradford's method.

Principle:

The assay is based on the observation that the maximum absorbance of an acidic solution containing Coomassie Brilliant Blue G-250 dye shifts from 465nm to 595nm when the dye binds to the protein. Both hydrophobic and ionic interactions stabilize the ionic form of dye, causing a visible colour change. When the given sample is treated with Coomassie Brilliant Blue dye, the dye binds to the lysine, arginine and aromatic amino acid residues of the proteins which lead to formation of blue coloured complex. The intensity of the colour is directly proportional to the concentration of protein present in the given sample.



Reagents Required

1) Coomassie Brilliant Blue G-250 dye

- Dissolve 100mg of Coomassie Brilliant Blue G250 in 50ml of 95% ethanol.
- Mix with 100ml of 85% orthophosphoric acid and make it up to 1 litre using

distilled water. The reagent should be filtered through Whatman filter paper No.1, and then stored in brown bottle at room temperature.

2) Standard solution

Bovine serum albumin 1mg dissolve in 1mL of distilled water and this is served as a standard (1mg/mL concentration)

Procedure

1) Pipette out 0.02 – 0.1ml of protein in per ml of total volume in a test tube with water.

2) Add 3 ml of reagent to each test tube and mix it.

3) Mix the solutions

4) Measure the absorbance at 595 nm.

5) Plot the graph of OD versus protein concentration and determine the concentration of unknown protein sample.

Result

The graph of OD values versus protein concentration is plotted, and the concentration of ( ) protein is determined to be .

Observations

	Vol. of working standard (µl)	Vol. of sample (µl)	Vol. of Distilled Water (µl)	Conc. Of BSA (µg/ml)	Volume of CBB dye	OD at 595 nm
Blank	0	-	1000	0	3	0
S_1	20	-	980	20		
S_2	40	-	960	40		
S_3	60	-	940	60		
S_4	80	-	920	80		
S_5	100	-	900	100		
T1	-	500	500			
T2						

Observations

	Vol. of std. solution (ml)	Vol. of sample	Vol. of water (ml)	Conc. Of BSA (µg/ml)	Vol. of Complex-forming Reagent (ml)	Incubate at Room Temperature for 10 minutes	Vol. of Folin's reagent	Incubate at Room Temperature for 30 minutes	OD at 660nm
B	0	-	1	0	4.5		0.5		
S_1	0.05	-	0.95	50					
S_2	0.1	-	0.9	100					
S_3	0.150	-	0.85	150					
S_4	0.2	-	0.8	200					
S_5	0.250	-	0.75	250					
T_1	-	0.5	0.5						
T_2	-	1	0						

Ex. No. – 2
Date:

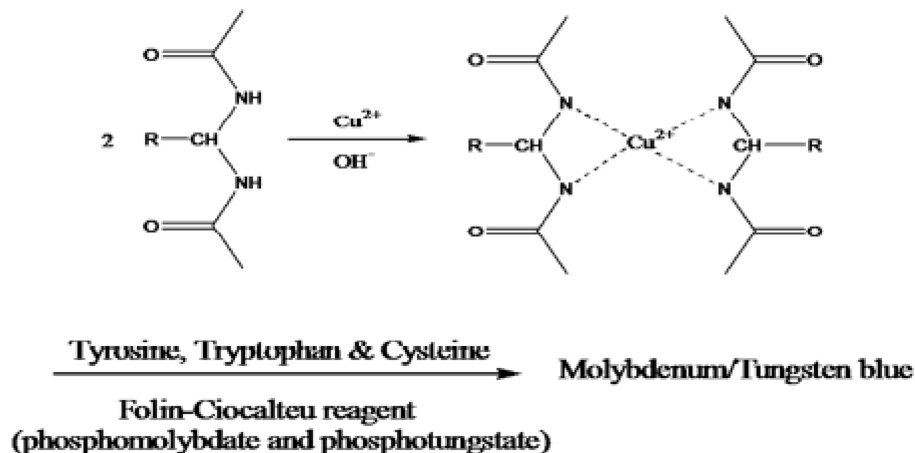
Estimation of protein content by Lowry's method

Aim

To estimate the amount of protein present in the given sample by Lowry's method

Principle

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The method combines the reactions of Copper ions with peptide bond under alkaline conditions, with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01-1mg/ml, and is based on the reaction of Cu^{2+} produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent. This reaction involves reduction of Folin's reagent and oxidation of aromatic (mainly Tryptophan and Tyrosine). Experiments have shown that Cysteine is also reactive towards the reagent. The concentration of the reduced Folin's reagent is measured by absorbance maximally at 750nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of Trp and Tyr residues that reduce the reagent.



Requirements

1. Complex forming Reagents

   A. 2% Na_2CO_3 in 0.1 N NaOH
   B. 1% NaK Tartrate in H2O
   C. 0.5% $CuSO_4$ 5 H2O in H2O
   D. **Reagent I:** 48 ml of A, 1 ml of B, 1 ml C

2. Folin's Reagent

   It is prepared in the ratio 1:1 with distilled water just before use.

3. Standard solution

   Prepare the standard 1mg/mL concentration of BSA.

Procedure

1. Take a series of test tubes and add increasing volume of working standard solution to each tube.
2. Take 1ml of water as blank, and 0.5ml and 1ml of unknown protein solution in tubes T1 and T2, respectively.
3. Make up the volume in all the tubes to 1ml using distilled water.
4. Add 4.5ml of complex-forming reagent to all the tubes, and incubate the tubes at room temperature for 10 minutes.
5. Add 0.5ml of Folin's reagent to each tube, and incubate at room temperature for 30 minutes.
6. After incubation, read the absorbance at 660nm.
7. Plot a graph of OD versus protein concentration, and determine the concentration of the unknown protein sample from the graph.

Result

The graph of OD vs. protein concentration is plotted, and the concentration of the unknown protein sample (   ) is found to be   .

Observations

<table>
<tr><th></th><th>Vol. of std. solution (ml)</th><th>Vol. of sample</th><th>Vol. of water (ml)</th><th>Conc. Of BSA (μg/ml)</th><th>Vol. of Complex-forming Reagent (ml)</th><th rowspan="9">Incubate at Room Temperature for 10 minutes</th><th>Vol. of Folin's reagent</th><th rowspan="9">Incubate at Room Temperature for 30 minutes</th><th>OD at 660nm</th></tr>
<tr><td>B</td><td>0</td><td>-</td><td>1</td><td>0</td><td rowspan="8">4.5</td><td rowspan="8">0.5</td><td></td></tr>
<tr><td>S_1</td><td>0.05</td><td>-</td><td>0.95</td><td>50</td><td></td></tr>
<tr><td>S_2</td><td>0.1</td><td>-</td><td>0.9</td><td>100</td><td></td></tr>
<tr><td>S_3</td><td>0.150</td><td>-</td><td>0.85</td><td>150</td><td></td></tr>
<tr><td>S_4</td><td>0.2</td><td>-</td><td>0.8</td><td>200</td><td></td></tr>
<tr><td>S_5</td><td>0.250</td><td>-</td><td>0.75</td><td>250</td><td></td></tr>
<tr><td>T_1</td><td>-</td><td>0.5</td><td>0.5</td><td></td><td></td></tr>
<tr><td>T_2</td><td>-</td><td>1</td><td>0</td><td></td><td></td></tr>
</table>

Ex. No. – 3
Date:

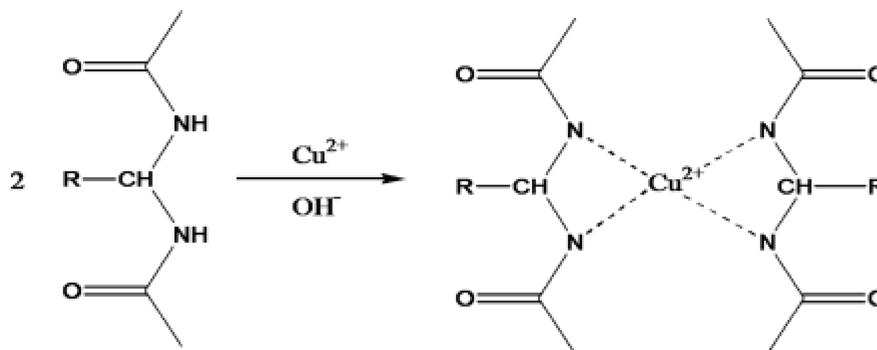
Estimation of proteins by Biuret method

Aim

To estimate the amount of protein present in the given sample.

Principle

The Biuret is a general protein assay for batches of material for which yield is not a problem. In Biuret reaction peptides containing three or more amino acid residues form a colored chelate complex with cupric ions (Cu^{2+}) in an alkaline environment containing sodium potassium tartrate. This became known as the biuret reaction because it is chemically similar a complex that forms with the organic compound biuret (NH_2-CO-NH-CO-NH_2) and the cupric ion. Biuret, a product of excess urea and heat, reacts with copper to form a light blue tetradentate complex.



Copper (II) Ions + Protein ----------> Biuret Complex (PURPLE).

Single amino acids and dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce the light blue to violet complex that absorbs light at 540 nm. One cupric ion forms a colored coordination complex with four to six nearby peptide bonds. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. Thus, the biuret reaction is the basis for a simple and rapid colorimetric reagent of the same name for quantitatively determining total protein concentration.

Materials Required:

1) Standard solution

Transfer 5mg of Bovine serum albumin to a 1ml of distilled water. Shake gently to prevent froth formation.

2) Biuret Reagent

- **Solution A** – 9g of sodium potassium tartarate is dissolved in 40ml of 0.2N NaOH in 100ml volumetric flask. 1.5gm of $CuSO_4$ is added to this is and dissolved completely. 0.5gm of KI is added to this, dissolved, and the solution is made up to the mark by 0.2N NaOH.

- **Solution B** – Dissolve 5g of KI in 100ml flask using distilled water.

To prepare Biuret Reagent, mix 20ml of solution A and 8ml of solution B and make up to 100ml using 0.2N NaOH.

Procedure:

1. 8 test tubes are taken and marked as B, S1, S2, S3, S4, S5, T1 and T2.

2. 2.5ml of distilled water to be pipette into T1, T2. 0.2, 0.4, 0.6, 0.8, 1 ml of standard solution is taken in different tubes and volume made up to 2.5ml by distilled water.

3. 4ml of Biuret Reagent is added to all the tubes and mixed well.

4. Test tubes are kept at RT for 20min.

5. The OD of test and standard solution to be measured calorimetrically using beam filter at 540nm.

6. From standard graph, concentration of unknown sample is determined.

Result

The graph of OD values versus protein concentration is plotted, and the concentration of given protein is determined to be .

Observations

<table>
<tr><th></th><th>Volume of std. sol. (ml)</th><th>Vol. of sample (ml)</th><th>Vol. of Distilled water (ml)</th><th>Conc. of BSA (mg/ml)</th><th>Vol. of Biuret Reagent (ml)</th><th rowspan="9">Incubate at Room Temperature at 20 minutes</th><th>OD at 540nm</th></tr>
<tr><td>B</td><td>0</td><td>-</td><td>1</td><td>0</td><td rowspan="8">4</td><td>0</td></tr>
<tr><td>S_1</td><td>0.2</td><td>-</td><td>0.8</td><td>1</td><td></td></tr>
<tr><td>S_2</td><td>0.4</td><td>-</td><td>0.6</td><td>2</td><td></td></tr>
<tr><td>S_3</td><td>0.6</td><td>-</td><td>0.4</td><td>3</td><td></td></tr>
<tr><td>S_4</td><td>0.8</td><td>-</td><td>0.2</td><td>4</td><td></td></tr>
<tr><td>S_5</td><td>1</td><td>-</td><td>0</td><td>5</td><td></td></tr>
<tr><td>T_1</td><td>-</td><td>0.5</td><td>0.5</td><td></td><td></td></tr>
<tr><td>T_2</td><td>-</td><td>1</td><td>0</td><td></td><td></td></tr>
</table>

Summary of first three experiments

Ex. No. – 4
Date:

Salting in, salting out and dialysis of proteins

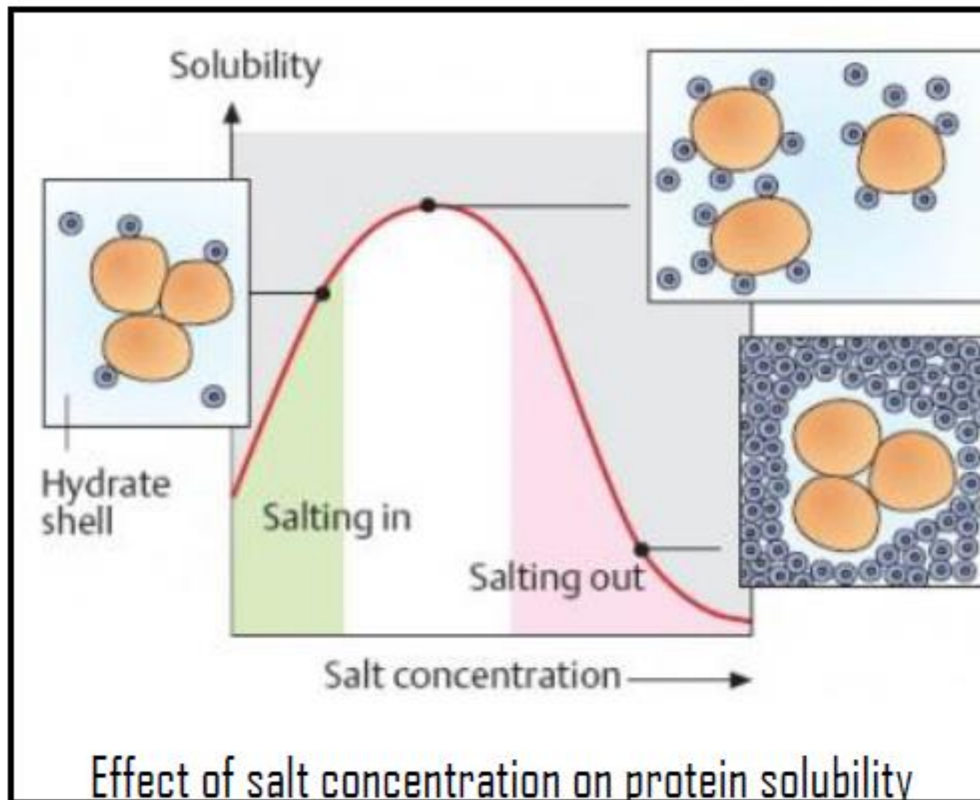
Aim:

To learn the techniques of Extraction of protein on basis of their solubility in water

Principle:

A protein contains multiple charged groups, its solubility depends on the concentrations of dissolved salts, the polarity of the solvent, the pH, and the temperature. Some or all of these variables can be manipulated to selectively precipitate certain proteins while others remain soluble. The solubility of a protein at low ion concentrations increases as salt is added, a phenomenon called "salting in". The additional ions shield the protein's multiple ionic charges, thereby weakening the attractive forces between individual protein molecules (such forces can lead to aggregation and precipitation). However, as more salt is added, particularly with sulfate salts, the solubility of protein again decreases. This "salting out" effect is primarily a result of the competition between the added salt ions and the other dissolved solutes (protein molecules) for molecules of solvent (water). At very high salt concentrations, so many of the added ions are solvated that there is significantly less bulk solvent available to dissolve other substances, including proteins. Since proteins precipitate at different salt concentrations, salting out is the basis of one of most commonly used protein purification procedures

A typical protein solubility curve where the log of the protein solubility is plotted as a function of AS concentration. The main features of this curve are a region at low salt where the solubility increases (called ‘‘salting in’’), and then a region where the log solubility decreases linearly with increasing AS concentration (called ‘‘salting out’’).The latter part of the curve can be described by the equation $\log10S = \beta - Ks(T /2)$ where S in the solubility of the protein in mg/ml of solvent, T /2 is the ionic strength, and β and Ks are constants characteristic of the protein in question. Ks is a measure of the slope of the line and β is the log of the solubility if the salting-out curve is extrapolated to zero ionic strength. In general, most proteins have similar Ks values but vary considerably in their β value.



Effect of salt concentration on protein solubility

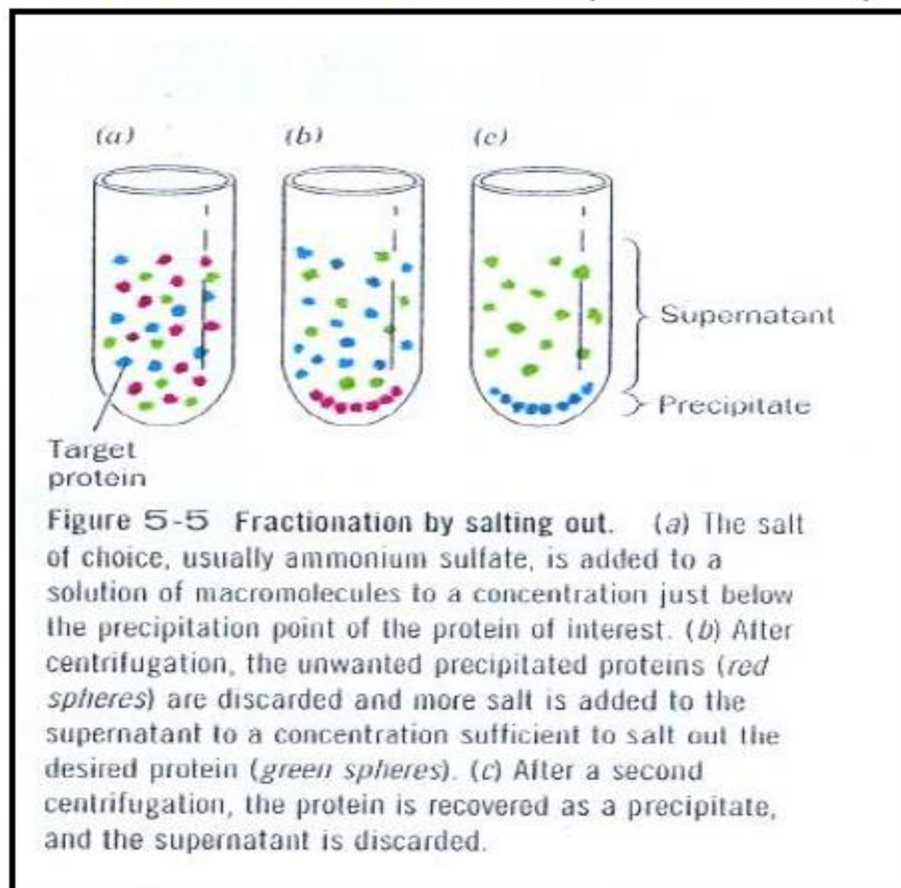


Figure 5-5 Fractionation by salting out. (*a*) The salt of choice, usually ammonium sulfate, is added to a solution of macromolecules to a concentration just below the precipitation point of the protein of interest. (*b*) After centrifugation, the unwanted precipitated proteins (*red spheres*) are discarded and more salt is added to the supernatant to a concentration sufficient to salt out the desired protein (*green spheres*). (*c*) After a second centrifugation, the protein is recovered as a precipitate, and the supernatant is discarded.

Solvent Precipitation: (Ethanol and acetone precipitation)

The alcoholic extraction process after dehulling and conventional deoiling has a high efficiency of protein recovery. Aqueous alcohols (ethanol, isopropyl alcohol, butanol) are widely used on a commercial scale to remove desired compounds. Care must be taken to carry out precipitations at very cold temperatures to avoid protein denaturation.

Isoelectric precipitation:

Proteins are less soluble at their isoelectric point where they have zero net charge and can most easily approach each other with minimal charge repulsion. Since proteins are also less soluble at very low ionic strength, isoelectric precipitation is usually done at very low or no salt.

0.1M Phosphate buffer Preparation (pH 7.4):

1) Prepare sodium phosphate dibasic by adding 2.1g for100 mL of distilled water in a suitable container.
2) Prepare sodium phosphate monobasic by adding 1.8g for100 mL of distilled water in another suitable container.
3) Finally prepare 0.1M phosphate buffer by mixing 92.6 mL of sodium phosphate dibasicsolution and 7.4 mL of sodium phosphate monobasic solution.
4) Adjust solution to final desired pH using HCl or NaOH.

Method:

1) Prepare 150 ml of 2 g/L bovine serum albumin solution.
2) Transfer 50 ml of the BSA solution into a beaker. Place the beaker in anice bath.
3) If the sample is milk follow the step 4 directly.
4) Slowly add the appropriate amount of ammonium sulfate until 35% saturation.
5) Stir the mixture for 30 minutes.
6) Centrifuge the sample for 10 minutes at 8000 rpm at 4°C.
7) Decant the supernatant into a beaker and slowly re-suspend the pellet in 5 ml of 0.1M phosphate buffer pH7.2.
8) If there is no pellet obtained, use the supernatant for further analysis.
9) Repeat the same procedure for 60% and 80% ammonium sulfate saturation.
10) Place it in dialysis bag overnight.

Calculation:

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulphate to add (grams) per liter of solution at +20 °C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Result:

Ex. No. – 5
Date:

Estimation of Cholesterol by ZAK's Method

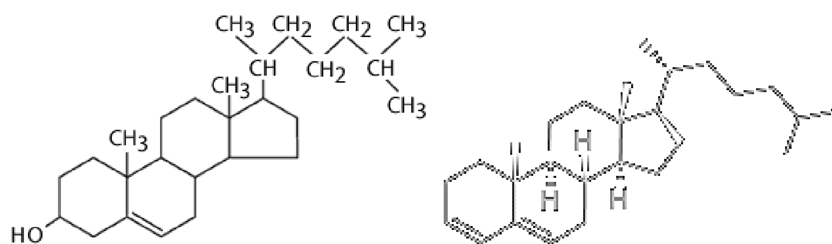
Aim

To estimate the amount of cholesterol present in the given sample using Zak's method

Principle

Cholesterol is a steroid lipid, amphipathic in nature. It consists of basic cyclopentanoperhydrophenothrene nucleus. It is synthesized in liver from Acetyl CoA. It acts as a precursor for steroid hormones and vitamin D.

In Zak's method, the proteins present in the sample are first precipitated by adding Fecl3-CH3 COOH reagent. The protein-free filtrate is treated with conc. H_2SO_4. In the presence of excess conc. H_2SO_4, cholesterol present in the sample gets dehydrated to form cholesta-3,5-diene, and by the catalytic action of Fe3+ ions a red coloured complex is formed. The intensity of red colour is measured at 560 nm.



Requirements

1) Glacial Acetic Acid

2) Concentrated Sulphuric Acid

3) 0.05% (w/v) $FeCl_3$ solution

4) Stock Solution of Cholesterol

Dissolve 100mg of cholesterol in 100ml of glacial acetic acid to prepare stock solution of concentration 1mg/ml

5) Working Standard

Mix 4ml of stock solution with 96ml of glacial acetic acid to prepare working standard having concentration 0.04mg/ml

6) UV-Spectrophotometer

7) Glassware

Procedure

1) 0.5 ml to 2.5 ml of working standard was pipetted out into clean test tubes.

2) 0.5 ml and 1 ml of unknown sample supernatant was taken in test tubes.

3) The volume was made up to 5.0 ml with ferric chloride and 3.0 ml of concentrated sulphuric acid were added.

4) The test tubes were kept at room temperature for 15 minutes.

5) The absorbance was read at 540 nm.

6) Standard graph of OD vs. concentration of cholesterol was drawn for the values obtained. From the standard graph the amount of cholesterol present in the food sample was calculated.

Result

The amount of cholesterol present in the given food sample is

Observations

<table>
<tr><th></th><th>Vol. of std. (ml)</th><th>Vol. of test (ml)</th><th>Vol. of $FeCl_3$ (ml)</th><th>Vol. of H_2SO_4 (ml)</th><th rowspan="9">Incubate at Room Temperature for 15 minutes</th><th>OD at 560 nm</th></tr>
<tr><td>B</td><td>0</td><td></td><td>5</td><td rowspan="8">3</td><td></td></tr>
<tr><td>S_1</td><td>0.5</td><td></td><td>4.5</td><td></td></tr>
<tr><td>S_2</td><td>1.0</td><td></td><td>4</td><td></td></tr>
<tr><td>S_3</td><td>1.5</td><td></td><td>3.5</td><td></td></tr>
<tr><td>S_4</td><td>2.0</td><td></td><td>3</td><td></td></tr>
<tr><td>S_5</td><td>2.5</td><td></td><td>2.5</td><td></td></tr>
<tr><td>T_1</td><td></td><td>0.5</td><td>4.5</td><td></td></tr>
<tr><td>T_2</td><td></td><td>1.0</td><td>4</td><td></td></tr>
</table>

Ex. No. – 6
Date:

Preparation of DEAE-cellulose column

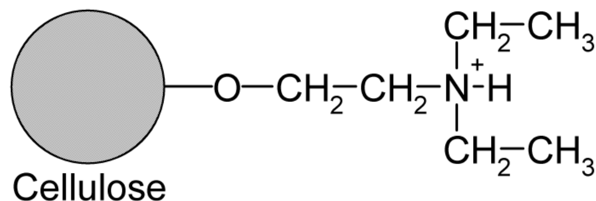
Aim:

To prepare DEAE-cellulose in the column for the separation of proteins.

Principle:

Diethylaminoethyl cellulose (DEAE-C) is a positively charged resin used in ion-exchange chromatography, a type of column chromatography, for the separation and purification of proteins and is notable for their high yields of proteins. Gel matrix beads are derivatized with diethylaminoethanol (DEAE) and lock negatively charged proteins or nucleic acids into the matrix. The proteins are released from the resin by increasing the salt concentration of the solvent or changing the pH of the solution as to change the charge on the protein.

DEAE ion exchangers are based on 4% crosslinked agarose beads. The gels show negligible shrinking or swelling with changes in pH or ionic strength, which permit regeneration of the gel in the column. The macroporous nature of the gel allows both small and large molecules to access the exchange sites located throughout it.



Requirements:

Materials: GlassColumn, glass wool

Chemicals: Diethylaminoethyl (DEAE), NaCl, NaOH, HCl.

Buffer: 1X Sodium acetate buffer (pH 4)

243mg/L of sodium acetate (Use acetic acid for reducing pH)

10X Sodium acetate buffer (pH 4)

2.430g/L of sodium acetate

Procedure:

1. Suspend dry resin in 5 volumes of distilled water and allow to settle 30-45 minutes.
2. Filter the resin and wash with 0.1 M NaOH containing 0.5 M NaCl for 10 minutes.
3. Allow the resin to get settle and later remove the supernatant.

4. Wash the resin with 0.5 M NaCl and again repeat the step 3.
5. Wash the resin with 0.1 M HCl containing 0.5 M NaCl and later repeat the step 3.
6. Wash with the resin with distilled water until the effluent pH is 5 or greater and after each wash repeat the step 3.
7. Wash with 1 M NaCl and adjust the pH of slurry to 7-8 with NaOH after each wash repeat the step 3.
8. Wash with 10X sodium acetate buffer and again repeat the step 3.
9. Wash 2 times with 1X sodium acetate buffer and finally pack the column.

Observations:

Result:

Ex. No. – 7
Date:

Ion Exchange Chromatography

Aim:

To separate the protein from the given sample using DEAE ion exchange chromatography.

Principle:

Ion exchange chromatography is a separation technique used for purification or analysis of molecules based their charge. The method can be used to separate charged molecules from uncharged ones or it can separate molecules of different charge from one another.

Ionizable chemical groups are immobilized on a solid support such as cellulose. The support, or resin, is usually maintained in a column. Molecules of opposite charge can bind the column by electrostatic interaction while uncharged residues will pass through. Once bound to the column, molecules can be released with salt (NaCl is commonly used). The salt ions compete for interaction for the column, and the molecule of interest is released. Hence, the term called "ion exchange".

Molecules having different charges can be separated from one another by gradually increasing the salt concentration. This is achieved with a gradient of increasing salt concentration in the solution being passed through the column. Lower charged groups are released at low salt concentrations because they are weakly bound. Highly charged molecules are more tightly bound and require higher salt concentration to release them. Thus molecules are released from the column according to the magnitude of their charge. It should be noted that pH of the column buffers can have a profound effect on ion exchange chromatography. Both the ion exchange resin and the molecule binding to it are charged molecules with a defined pKa.

The charged resin can be of two types: cation exchangers and anion exchangers. Cation exchangers bind positively charged molecules and anion exchangers bind negative ones.

Requirements:

Materials: GlassColumn, syringe filter, glass wool

Reagents: Protein sample, Diethylaminoethyl (DEAE).

Buffer: 1X Sodium acetate buffer (pH 4)

243mg/L of sodium acetate (Use acetic acid for reducing pH)

Gradient buffer (1M)

58.44g/L of NaCl

Procedure:

1. Prepare a column of DEAE-cellulose by placing glass wool at the bottom of a column to serve as a plug.
2. Add a slurry of DEAE-cellulose equilibrated in 1X sodium acetate buffer.
3. The final height of DEAE-cellulose in the column should be between 7 to 8 cm. Drain the column to just above the top of the resin (do not let resin go dry).
4. Add 1 ml of the compound mixture and allow it to run into the column.
5. Cap the column, and start collecting effluent.
6. Have a test-tube rack ready with 20 numbered test-tubes. Collect ~2 ml of effluent in each tube.
7. Measure the absorbance of each tube at 260 nm.
8. Once the absorbance reaches to zero. Add gradient buffer for the release of bounded protein.
9. Again collect ~2 ml of effluent in each tube and measure the absorbance at 260 nm.

Observations:

Result:

Ex. No. – 8
Date:

Determination of molecular weight of protein using SDS-PAGE

Aim

To determine the molecular weight of unknown protein sample using SDS-PAGE

Principle

Proteins are held together by noncovalent, interactions such as hydrogen bonds and salt bridges. These can be disrupted to denature proteins and then to separate them electrophoretically by electrophoresis. Such experiments are useful with proteins because they have an especially varied range of tertiary structures. Electrophoretic mobility in denaturing conditions is altered in comparison to that in nondenaturing conditions. This results from altered charge and/or shape because the polypeptide now migrates as an unstructured monomer through the gel.

The detergent sodium dodecyl sulphate (SDS) contains a 12-carbon hydrophobic chain and a polar sulphated head and is also a powerful denaturant of protein structure. The hydrophobic chain intercalates into hydrophobic parts of the protein by detergent action, disrupting tertiary structure. The sulphated head remains in contact with water, maintaining the solubility of the detergent protein complex. This disrupts the folded structure of single polypeptides as well as subunit–subunit (i.e., quaternary structure) and protein-membrane interactions. SDS coats proteins more or less uniformly with a "layer" of negative charge. They therefore always migrate towards the anode when placed in an electrical field, regardless of their original intrinsic charge. The negative charge gives a charge-density largely independent of the primary structure or protein mass (*M*r) of the polypeptide. For this reason, there is a close relationship between mobility of SDS-protein complexes in polyacrylamide gels and the protein *M*r. This is called SDS PAGE, the most widely-used form of protein electrophoresis.

Requirements:

1. 30% acrylamide
2. Resolving and separating gel buffer (10%)
3. Stacking gel buffer
4. Electrophoresis tank buffer

5. Ammonium Persulphate (APS)
6. N-Tertramethyl ethylene amide(TEMED)
7. Sample buffer
8. Staining solution
9. Destaining solution
10. Base plate
11. Notch plate
12. Vaseline or Petroleum Wax
13. Clamps

Procedure:

1) The glass plate was thoroughly cleaned with SDS and washed. The plates were then dried. The gel plates were assembled and the volume of gel mould was determined. The acrylamide separating gel was prepared.

2) The gels are prepared by mixing the gel components followed by addition of APS and TEMED. The mixture was then poured into the glass mould and allowed to polymerize (for approximately 20 minutes).

3) The components of the stacking gel was mixed and poured on top of polymerized separating gel. The comb was inserted immediately and the gel was allowed to polymerize.

4) The gel mould was placed in the electrophoretic chamber. The chamber was filled with tank buffer and the comb was removed carefully.

5) Preparation of the Sample:

    a. The samples are prepared with equal volumes of sample buffer. It was mixed thoroughly.

    b. The samples were heated in boiling water bath to denature the protein.

6) Electrophoresis:

i. The samples were loaded and current was applied.

ii. The gel was run until the Bromophenol Blue dye reached the bottom of the resolving gel.

iii. The power supply was turned off and the gel mould was removed from the apparatus.

iv. The plates were separated using a spatula.

v. Fixing, staining and Destaining:

   a. The gel was immersed in a tray containing 5 volumes of staining solution. The gel was stained at room temperature for 4 hours.

   b. The stain was removed and the gel was destained.

   c. The gel was stored in 7% acetic acid.

   d. The bands were viewed under illuminator.

Result:

The molecular weight of the unknown protein, as determined using SDS-PAGE is

Observations

Composition of Solutions

1) 2X Sample Buffer

Volume = 1ml

2) Tank Buffer

Volume =

Tris HCl	1.5g
Glycine	7.2g
SDS	0.5g

3) Stacking Gel (5%)

Volume = 3ml

Tris HCl	0.75ml
30% Acrylamide-Bis-Acrylamide	0.5ml
10% ApS	0.015ml
10% SDS	0.03ml
TEMED	0.005ml
Water	1.7ml

4) Separating Gel (10%)

Volume = 6ml

Tris HCl	1.5ml
30% Acrylamide-Bis-Acrylamide	2ml
10% ApS	0.020ml
10% SDS	0.060ml
TEMED	0.01ml
Water	2.41ml

Ex. No. – 9
Date:

Assay of Alkaline Phosphatase Activity

Aim:

To estimate the amount of phosphate present in the given simple by alkaline phosphatase mediated enzyme assay.

Principle:

Alkaline phosphatase (ALP) catalysis the hydrolysis of para-nitro phenol phosphate (pNPP) at pH 10.9, liberating para nitro phenol (PNP) and phosphate. The rate of PNP formation is proportional to the catalytic concentration of alkaline phosphatase present in the sample.

Materials required:

Alkaline phosphatase(enzyme), para-nirophenol phosphate(substrate), test tubes, micropipette,NaOH solution tris-cl, distilled water, weighing balance, spectrophotometer.

Reagents preparation

Enzyme preparation-Stock

10 mg of the lyophilised enzyme was dissolved in 20 ml of distilled water to obtain a stock of 1 U of Enzyme solution.

$$U= A * m/V$$

Where U= units of enzyme required

A= specific activity of enzyme

m= mass of lyophilised powder weighed

V= volume of distilled water

Preparation of Tris- cl Buffer(0.05M) pH=10.5:

606 mg of tris-cl was weighed and dissolved in 50 ml of distilled water

The pH was adjusted to 10.5 using 1N NaOH

Procedure:

1. 0.6 ml of substrate(p-NPP) was taken in varying concentrations in different test tubes.
2. 0.5 ml of 0.05M tris-cl buffer, pH-10.5 was added to the all the test tubes.
3. 100ul of alkaline phosphatase was taken from the enzyme stock solution and added to various test tubes.
4. The total volume was made upto 1.45 ml using distilled water.
5. Another separate test tube containing 0.6 ml of substrate, 0.5 ml of tris-cl buffer and 350ul of distilled water was prepared kept aside.
6. All the test tubes were incubated for 60 minutes in dark.
7. After 60 minutes 100ul of 3N of NaOH (stop solution) was added to all the test tubes
8. The absorbance was measured at 405nm.

Observation:

Results:

Ex. No. – 10
Date:

Estimation of Protease activity in commercially available detergent

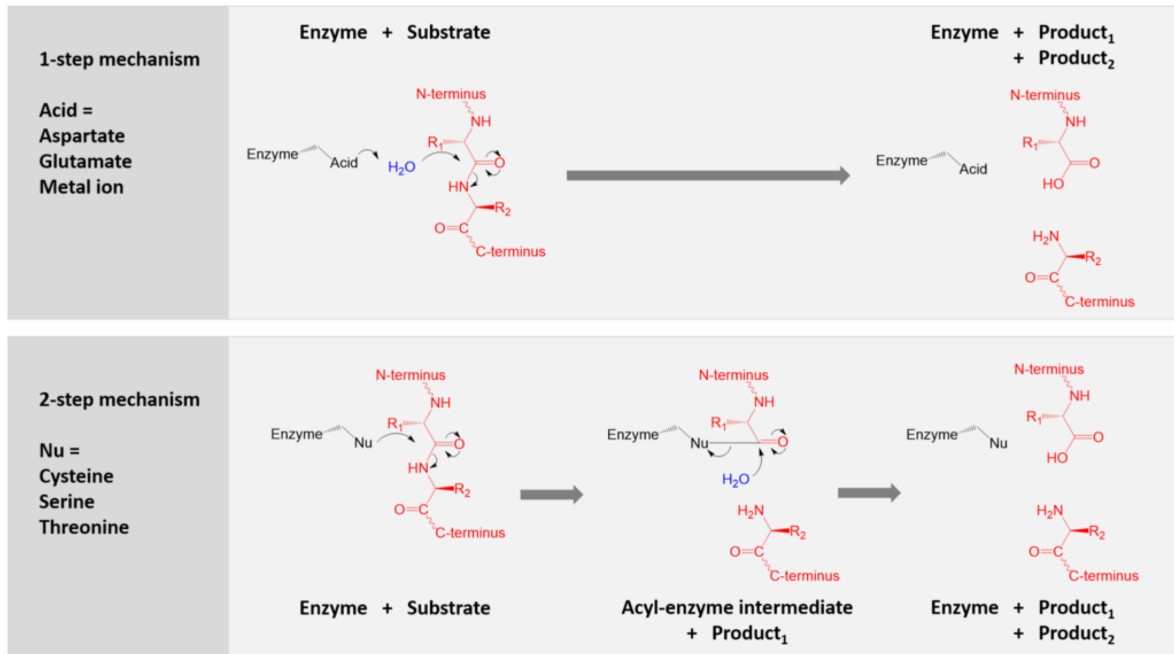
Aim:

To estimate the protease activity in the given sample of the detergent using casein as the substrate

Principle:

Protease is a type of enzyme that cuts the peptide bonds between proteins using a process called proteolysis or proteolytic activity. Some detach the terminal amino acids from the protein chain (exopeptidases, such as aminopeptidases, carboxypeptidase) others attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, elastase). Protease activity is essential to many biological functions, including immune system defense, cell death and growth, and blood clotting. Proteolytic enzymes are very important in digestion as they breakdown the the peptide bonds in the protein foods to liberate the amino acids needed by the body. In this assay casein acts as a substrate. In today's laundry detergents, enzymes such as proteases and amylases are some of the active ingredients. Almost all powdered bleach additives now contain enzymes to help break down stains that are otherwise hard to remove with conventional surfactants alone. A common enzyme used in detergent making proteases, helps in hydrolysis of large protein molecule into smaller polypeptides and individual amino acid units.

When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease.



Materials Required:

1. Casein- 0.1% (0.1gm powder dissolved in 100ml (50 mM) Citrate Phosphate Buffer)
2. 10% TCA
3. Standard Solution:

   0.0181g (1mM) tyrosine of stock is dissolved100ml distilled water.
4. Preparation of Reagent A:

   6g of NaOH and 14.5g Na_2CO_3 are mixed together in 500ml of water.
5. Preparation of Reagent B:

   Folin phenol and water were mixed in the ratio 1:1 respectively, for 10ml.

Procedure:

Sample Preparation:

1. To 0.5 ml of substrate (casein) add 0.5ml of sample detergent is added and subjected to 1 hour incubation at room temperature.
2. 1ml of cold TCA is added to the above solution after incubation

3. The mixture is subjected to centrifugation at 5000RPM for 5 min.
4. The supernatant is used as sample solution.

Assay procedure:

1. 7 test tubes are taken and marked as B, S1, S2, S3, S4, S5 and T1.
2. Add increasing volume of working standard solution to each tube
3. Take 0.5ml of water as blank, and 0.5ml of sample solution in tubes T1.
4. Make up the volume in all the tubes to 0.5ml using distilled water.
5. 2.5ml of reagent A and 0.5ml of reagent B is added to all the tubes.
6. Test tubes are kept at room temperature for 20min.
7. The OD of test and standard solution was measured calorimetrically using beam filter at 650nm.
8. From standard graph, concentration of unknown sample is determined.

Result:

The activity of enzyme present in a given sample using casein as a substrate was estimated and is found to be.

Observation:

SL NO	Volume Of Stock (µl)	Concentration of stock (µ mol)	Volume Of Distilled Water(µl)	Volume Of Reagent A (ml)	Volume Of Reagent B (ml)	Incubate at room temperature for 20 min	OD AT 650 nm
B				**2.5**	**0.5**		
S1				**2.5**	**0.5**		
S2				**2.5**	**0.5**		
S3				**2.5**	**0.5**		
S4				**2.5**	**0.5**		
S5				**2.5**	**0.5**		
Test 1							
Test 2							