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Study the modern biochemical analysis techniques of proteins and alkaline phasphtase enzyme system from biological sample chicken liver

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ABSTRACT

The objective of the study was the biochemical analysis of proteins and Alkaline Phosphatase enzyme system from biological sample Chicken Liver using modern biochemical analysis techniques, including protein extraction, fractionation and electrophoresis separation technique and enzyme analysis. The amino acids in a polypeptide chain are linked by peptide bonds. Once linked in the protein chain, an individual amino acid is called a residue, and the linked series of carbon, nitrogen and oxygen atoms are known as the main chain are protein backbone .The end of the protein with a free carboxyl group is known as the C-terminus or carboxyl terminus, whereas the end with a free amino group is known as the N-terminus or amino terminus.

Keywords: Proteins, Amino acids, Protein chains, Biochemical analysis, Enzymes etc.

INTRODUCTION

Protein

Proteins are organic compound made of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acids residues. The sequence of amino acids in a protein is defined by the sequence of a gene, which is encoded in the genetic code. Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essentials parts of organisms and participate in virtually every process with in cell. Proteins were first described and named by the Swedish chemist Jons Jakob Berzelius in 1838. However, the central role of proteins in living organisms was note fully appreciated until 1926, when James B. Sumer showed that the enzyme urease was a protein. The first protein to be sequence was insulin, by Frederick Sanger, who one the Nobel Prize for this achievement in 1958.[1]

Protein Synthesis

Proteins are assembled from amino acids using information encoded genes. Each protein has its own unique amino acids sequence that is specified by nucleotide sequence of the gene encoding this protein. The genetic code is a set of three nucleotide sets called codons and each three nucleotide combination designates amino acids, for example AUG (Adenine- uracil- guanine) is the code for methionine. Because DNA contains four nucleotides, the total number of possible codons is 64; hence, there is some redundancy in the genetic code, with some amino acids specified by more than one codon. Genes encoded in DNA in first transcribed into pre-mRNA by proteins such as RNA polymerase. The process of synthesizing apple protein from an mRNA template is known as translation. The mRNA is loaded on to the ribosome and is read three nucleotides at a time by matching each codon to its base

pairing anticodon located on a transfer RNA molecule, which carries the amino acid corresponding to the codon it recognizes. Protein is always biosynthesize from N-terminus to C-terminus. [2]

Structure of proteins

Most proteins fold into unique 3-dimensional structures. The shape into which a protein naturally folds is known as its native conformation.

Secondary structure: regularly repeating local structures stabilized by hydrogen bonds. The most common examples are the alpha helix and beta sheet.

Tertiary structure: the overall shape of a single protein molecule; the spatial relationship of the secondary structure to one another. Tertiary structure is generally stabilized by non local interactions, most commonly the formation of a hydrophobic core, but also through salt bridges, hydrogen bonds, disulfide bonds, and even post-translation modifications.

Quarternary structures: the structure that results from the interaction of more then one protein molecule , usually called protein subunits in this context ,which function as part of the larger assembly or protein complex .

Cellular functions

Proteins are the chief actors within in the cell, said to be carrying out the duties specified by the information encoded in genes. The chief characteristic of proteins that also allows their diverse set of function is their ability to bind other molecules specifically and tightly. The region of the protein responsible for binding another molecule is knows as the binding site and is often a depression or “pocket” on the molecular surface.

Structural proteins

Most structural proteins are fibrous protein; for example, actin and tubulin are globular and soluble as monomers, but polymerise to form long , stiff fibers that comprise the cytoskeleton , which allows the cell to maintain its shape and size . Collagen and elastin are critical components of connective tissue such as cartilage, and keratin is found in hard or filamentous structures such as hair , nails, feathers ,hooves and some animal shells.

Protein purification

In order to perform in vitro analysis, apple protein must be purified away from other cellular components. This process usually begins with cell lysis. in which a cell’s membrane is disrupted and its internal contents released into a solution known as crude lysate. The resulting mixture can be purified using ultracentrifugation , which fractionates the various cellular components into fraction containing soluble protein ; membrane lipid and proteins ;cellular organelles, and nucleic acid .Precipitation by a method known as salting out can concentrate the protein from this lysate. Various types of chromatography are then used to isolate the protein or proteins of interest based on properties such as molecular weight, net charge and binding affinity. The level of purification can be monitored using various types gel electrophoresis if the desired protein’s molecular weight and isoelectric point are known, by spectroscopy if the protein has distinguishable spectroscopic features, or by enzyme assays if the protein has enzymatic activity .Additionally proteins can be isolated according their charge using electro focusing. [3]

Cellular localization

The study of protein in vitro is often concern with the synthesis and localization of the protein with in the cell. Although many intracellular proteins are synthesized in the cytoplasm and membrane- bound or secreted proteins in the endoplasmic reticulum, the specifies of how proteins are targeted to specific organelles or cellular structures is often unclear. Apple useful technique for assessing cellular localization uses genetic engineering to express in a cell apple fusion protein or chimera consisting of the natural protein of interest linked to a “reporter” such as GFP (green fluorescent protein). The fused protein’s position within the cell can be cleanly and efficiently visualized using microcopy, as shown in the figure opposite. In these cases, additional fluorescent chimeric proteins are generally required to prove the inferred localization.

Nutrition

Most microorganisms and plants can biosynthesize all 20 standard amino acids, while animals (including humans) must obtain some amino acids from the diet. In animals, amino acids are obtained through the consumption of foods containing protein. Ingested proteins are broken down through digestion, which typically involves denaturation of

the protein through exposure to acid and hydrolysis by enzymes called proteases. Some ingested amino acids are used for protein biosynthesis, while others are converted to glucose through gluconeogenesis.

Types of proteins

Based upon chemical composition, proteins are divided into two major classes: simple proteins, which are composed of only amino acids, and conjugated protein, which are composed of amino acids and additional organic and inorganic groupings, certain of which are called prosthetic groups. Conjugated proteins include glycoprotein, which contain carbohydrates; lipoproteins, which contain lipids; and nucleoproteins, which contain nucleic acids.

Protease

A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bond that linked amino acids together in the polypeptide chain, which form molecule of protein.

Occurrence

Proteases occur naturally in all organisms. These enzymes are involved in apple multitude of physiological reaction from simple digestion of food protein to highly- regulated cascades (e.g., the blood- clotting cascade). Bacteria also secrete proteases to hydrolyse the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. Proteases are also a type of exotin, which is virulence factor in bacteria pathogenesis. Bacteria exotoxic proteases destroy extra cellular structures. Protease enzymes are also used extensively in the bread industry in bread improver. [4]

Inhibitors

The function of peptidases is inhibited by protease inhibiter enzyme. Examples of protease inhibiters are the class of serpins (serine protease or peptidase inhibiters), incorporating alpha 1- antitrypsin. Other serpins are complement 1- inhibiter, antithrombin, alpha 1- antichymotrypsin, plasminogen activator inhibitor 1 and the recently discovered neuroserpin.

Peroxidase

Peroxidase (EC number 1.11.1x) are a large family of enzymes that typically catalyzed a reaction of the form:
ROOR' + electron donor (2e) + 2H ROH + R'OH

For many of these enzymes the optimal substrates the hydrogen peroxide, but others are more active with organic hydro peroxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or redox-active cysteine or selenocysteine residus.

Application

Peroxidase can be used for treatment of industrial waste water. For examples, phenols, which are important pollutants, can be removed by enzyme – catalized polymerization using horseradish peroxides. Thus phenols are oxidized to phenoxy radicals which participate in reactions where produced polymers are and oligomers that are less toxic than phenols.[5]

Acid phosphatase

Acid phosphatase is a phosphatase, a type of enzyme, used to free attached phosphste groups from other molecules during digestion. It is basically a phosphomonoesterase. Different forms of acid phosphatase are found in different organs, and their serum levels are used as a diagnostic for disease in the corresponding organs. Acid phosphatase catalyzes the following reaction at an optimal pH below 7. Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients.[6]

Alkaline phosphatase

Alkaline phosphatase is a hydrolase enzyme responsible roe removing phosphate groups from many types of molecules, including nucleotides, protein, and alkaloids. The process of removing the phosphate group is called dephosphorylation. In bacteria, alkaline phosphatase is located in the periplasmic space, external to the cell membrane. Since this space is much more subject to phosphatase is comparatively resistant to inactivation, denaturation, and degradation, and also has a higher rate of activity. Alkaline phosphatase has become useful tool in molecular biology laboratories, since DNA normally possesses hen groups on the 5' end. Removing these phosphates prevents the DNA from ligating, thereby keeping DNA molecules linear until the next step of the

process for which they are being prepared; also, removal of the phosphate groups allows radio labeling in order to measure the presence of the labeled DNA through further steps in the process. One common use in the dairy is as a maker of pasteurization in cow's milk. The molecule is denatured by elevated temperatures found during pasteurization, and can be tested for via colour change of a para-nitro-phenol phosphate substrate in a buffered solution. Raw milk would typically produce a yellow colouration within a couple of minutes, whereas properly pasteurized milk should no change.[7]

Inhibitors
All mammalian alkaline phosphatase isoenzymes except placental are inhibited by homoarginine and similarly all except the intestinal and placental ones are blocked by levamisole. Heating for~ 2 hours at 65^0 C inactivated most isoenzymes except placental isoforms.

Select a Sample: Chiken Liver
Select Enzyme system: Alkaline Phosphatase

PROCEDURES AND PROTOCOLS
The basis technique of this project requires preparation of tissue sample extract (i.e. protein extracts) followed by biochemical analysis of these extracts.

Preparation of Tissue Sample Extracts
1. Prepare duplicate tubes for each extraction. For animal tissue: weigh 50-60 mg fresh tissues; identical portions of 50 mg tissue each in separate grinding tube. Add 0.25 ml extraction buffer to each sample tube, as indicated for each Part.

2. Grind tissue in matching tube-pestle. Grind for 10-15 minutes each and until you have homogeneous tissue suspension (no clumps). Incubate 10 minutes in ice. Grind again for 5 minutes and then centrifuge at 12-15,000rpm for 15 minutes. Remove 200-**µ**l clear extract (without disturbing the sediments). Pool the extracts from two identical tubes. You will collect a total of 400-µl extract from each sample. Mark these extracts and store at 20.c until use (must be used within a week.){Note: Extracts are marked with serial number F1-----F10}.

3. Extract acidic (F1) neutral PBS (F2) & alkaline proteins (F3) & enzymes from each sample.[5]

Part 1
Using the following extraction buffer. One after another (as provided).
Acidic Extraction Buffer, PH = 5.5
Neutral Extraction Buffer, PH = 7.0
Alkaline Extraction Buffer, PH = 9.0

Part 2
1. Using a Protein Extraction Buffer (Ph 8.5): Extract soluble proteins (F-4) and insoluble membrane proteins (F5). Follow the extraction procedures described above.
2. The soluble protein extract (F-4) will be used for further fractionation with ammonium sulfate (A/S) (see below) followed by analysis of each fraction for enzyme activity and partial purification of enzyme.
3. Store the insoluble sediments (F-5) for later use. The insoluble membrane proteins (F-5) will be solubilized in Part-5.

Part- 3
1. Extract cytosolic (F-6), mitochondria (F-7), and nuclear fractions (F-8) of the samples. Use Sub-cellular Fractionation Kit and Protocol; for sub-cellular fractions each sample. Mark each fraction and store for later use.
2. Solubilize insoluble fractions (mitochondria (F-7) and nuclei (F-8) fractions) in Mild detergent Extraction Buffer as in Part-5.
3. These proteins extract (fractions) will also be used for analysis of enzyme activity. This will provide information for cellular compartmentalization of functional enzyme proteins.

Part-4

1. Extract Total cellular protein: with undiluted Strong Detergent Extraction Buffer (F-9)-this extract will be used for determining total protein in the tissue sample.
2. NOTE: For extraction of total cellular protein from tissue samples; grind the tissues for 10-15 minutes each in undiluted Strong Detergent Extraction Buffer and until you have homogeneous tissue suspension (no clumps). Incubate 10 minutes in hot water both (70-90 C). Centrifuge at 12-15,000 rpm for 15 minutes. Remove 200-µl clear extract (without disturbing the sediments).

PART-5

1. Solubilize insoluble cellular fractions (obtained in Part-2, F-5 and Part-3, F-7 & F-8) with Mild detergent extraction buffer insoluble proteins fractions are: Fraction F-5, mitochondria (F-7) & nuclear fractions (F-8).
2. Add 0.25 ml. Mild DETERGENT EXTRACTION BUFFER TO EACH TUBE CONTAINING Fraction F-5, F-7, & F-8.
3. Grind for 10-15 minutes each and until you have found homogeneous suspension. Centrifuge at 12-15,000 rpm for 15 minutes. Remove 200-µl clear extract (without disturbing the sediments). Pool the extract from two identical tubes. You will collect 400-µl extract from each sample. Mark these solubilized extracts F-5, F-7, & F-8, respectively and store at – 20^{o}C until use (must be used within a week).
4. Analyzer your extracts for proteins and enzyme activity, as follows

FRACTIONATE EXTRACTS FOR FURTHER ANALYSIS
ENZYME ASSAY

I. Find total enzyme units in soluble protein extract, express enzyme activity as units of O.D.
II. Find enzyme activity units in various fractions.

PROTEIN ESTIMATION

Determine protein concentration as percentage of total in various fractions. For total protein amount in samples use proteins extracted using strong detergent extraction buffer.

SDS-PAGE

Determine protein distribution in various fractions (F-1 TO F-9). Take photographs of your gels for your dissertation or thesis.

SULFHYDRIL GROUP ESTIMATION: (with Ellman's reagent)

Determine total SH-cellular proteins in various fractions (F-1 TO F-9).

Protein Electrophoresis

Protein Electrophoresis is a relatively simple, rapid and highly sensitive tool to study the properties of proteins. It is the principle tool in analytical chemistry, biochemistry and molecular biology. The separation of proteins by electrophoresis is based on the fact that charged molecules will migrate through a matrix upon application of an electrical field. The matrix for protein electrophoresis separation is polyacrylamide.[3]

MATERIALS FOR EACH GROUP

Each group (of 4 students) is supplied with the following components.
2.2ml 40% Acryl amide/ Bis-acrylamide solution, 150 µl 10% Ammonium Per sulfate, 1.5ml 10% Detergent solution (SDS), 0.7mL Stacking Gel Buffer, 2.2ml Separating Gel Buffer, 15 µl TEMED, 10 µl Protein Standard Marker, 25 µl 2x Sample Loading Buffer, 25 µl Chicken Liver Extract, 600 ml 1x Electrophoresis Running Buffer, 50 ml Lab Safe Gel Stain, 2 .15 ml Graduated tubes with cap for each group , 2 blotting paper sheets and 6 ml Water.

PROCEDURE

1. Preparation of a Polyacrylamide Gel (Stacking & Resolving Gels)
2. Assembly the gel plates and spacers on the gel stand for casting the gel, as shown by the instructor during the demonstration and described below.
3. Moisturize the sides and ends of the notched plate using tissue paper soaked with water.
4. Carefully stretch the rubber gasket around the sides and bottom of the notched plate. All three sides should be covered by the gasket.

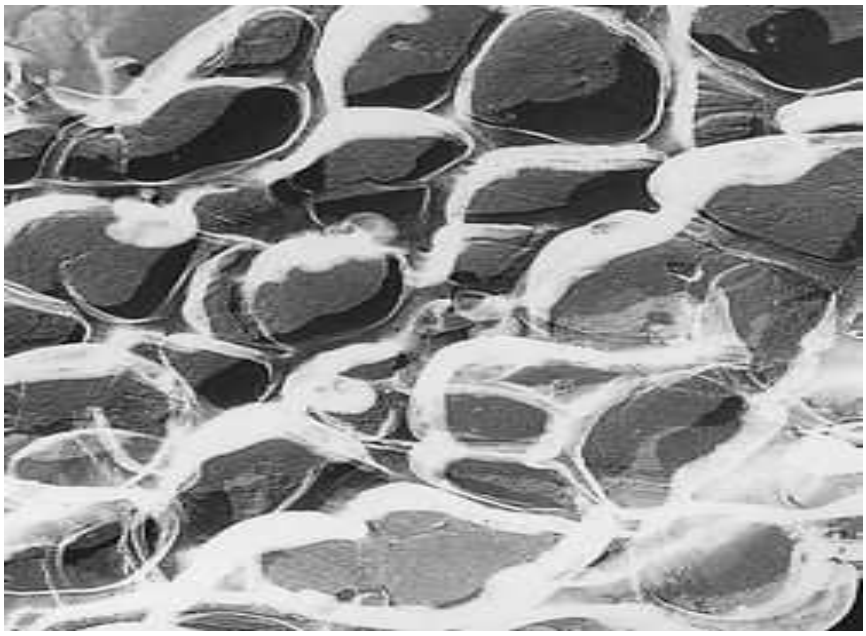
5. Align the spacers with the side and bottom edge of the notch plate. Ensure the curved spacer end is located at bottom of plate and that it follows the curve of the plate.
6. Place the notched plate with gasket on the bench with the thicker edge of the gasket facing upwards.
7. Align the blank plate over the notched plate and carefully lay on top of the gasket.
8. Place the spring clamps on the sides and bottom of the gel plates that are sealed with gasket. Place 2 clamps on the bottom and one on each sides.
9. Stand the gel casting unit upright using the bottom clamps.
10. Check for leaks by adding water to the top of the notched plate .Wait at least 10 minutes. If the level has dropped, disassemble and repeat assembly.
11. Pour off the water into a waste container and remove excess water with supplied blotting paper.[3]

Casting the Gel:

GEL COCENTRATION	12 % separating Gel	4 % Stacking Gel
Solution volume	5 ml	5 ml
40 % Acryl amide/Bis-acrylamide solution	1.5 ml	0.5ml
1M Separating Gel buffer	2ml	-
1M Stacking Gel buffer	-	0.6ml
10 % SDS	0.5 ml	0.5ml
10 % APS	100 µl	100 µl
Distilled water	To 5ml	To 5ml
TEMED	10 µl	10 µl

1. Label two 15 ml tubes for “Separating gel and Stacking Gel”. Add all the reagents, except TEMED, to the tube according to the table below. Mix the solution thoroughly.
2. The data provided in following is for making a 12% 8x10cm mini polyacrylamide gel.
3. Place the comb between the glass plates with the teeth facing down. With a marker pen, mark 1cm below the bottom of the comb’s teeth. Remove the comb.
4. Add TEMED to only the “Separating Gel” solution and mix well.
5. Using a 1 ml pipette swiftly add the running gel solution to the casting unit until the gel solution reach the mark, ensure you avoid the addition of air bubbles.
6. Slowly add 1ml 70% isopropanol to prevent evaporation and create an even surface on top of the gel. Let the gel polymerize for 30 minutes. Check the gel has polymerized by gently tilting to a 45^{o} angle.
7. Pour off the isopropanol the rinse the top layer of the resolving gel with 0.5 ml distilled water twice.
8. Add TEMED to the “Stacking Gel” solution and mix well. Using a 1ml pipette slowly add the “Stacking Gel” to the level of the lower plate.
9. Carefully put the comb between the 2 glass plates without introducing air bubbles, ensuring it is aligned in the middle of the plate. Let the stacking gel polymerize for 30 minutes. Now the gel is ready.
10. Slowly remove comb and rinse the wells twice with water to remove any unpolymerized acryl amide.
11. With a marker, mark the location of the wells on the larger blank plate.
12. Carefully remove the gasket and place the gel sandwich into the cassette with lower notched side facing inwards. Place a second gel sandwich or the blank White Plate into the opposite side of the cassette.
13. Place the cassette into the tank and ensure the red sign aligns with the red electrode side and black sign with black electrode.
14. Fill the inner buffer chamber with 1X SDS-PAGE running buffer, so that it is 1cm above the top edge of the notched plate. Add running buffer to the outer chamber until it is 1cm higher than the bottom of the gel plates.

Fig 1; TEM of a polyacrylamide gel



Running and Staining Electrophoresis Gel

1. Transfer 5µl tissue extract to a fresh tube. Add 5µl 2X Loading Buffer, vortex and briefly centrifuge.
2. Boil the protein samples and the supplied Protein Standard Marker for 5 minutes in a 100°C water bath or heat block.
3. Vortex the sample briefly and centrifuge for 30 seconds.
4. Load 10µl protein standard marker to the first lane, followed by 10µl protein samples to the remaining lanes.
5. Add coolant bags, apply the lid ensuring its correct orientation (red to red, black to black) and run the gel at maximum output for 100-120 minutes.
6. Once migration is completed, turn off power and remove gel sandwich from cassette. Place in the tank of distilled water. Press notched side against blank plate gentle shaking; remove the gel from blank plate.
7. Wash the gel twice in distilled water, 5 minutes each.
8. Remove all free water from the gel.
9. Add 50ml Lab Safe Gel Stain to cover the gel. Gently shake the gel for 60 minutes at room temperature.
10. Decant the Lab Safe Gel Stain and rinse the gel with distilled water. The gel can be stored in water. Longer destaining, such as overnight, in water will give a clearer view of the protein bands.

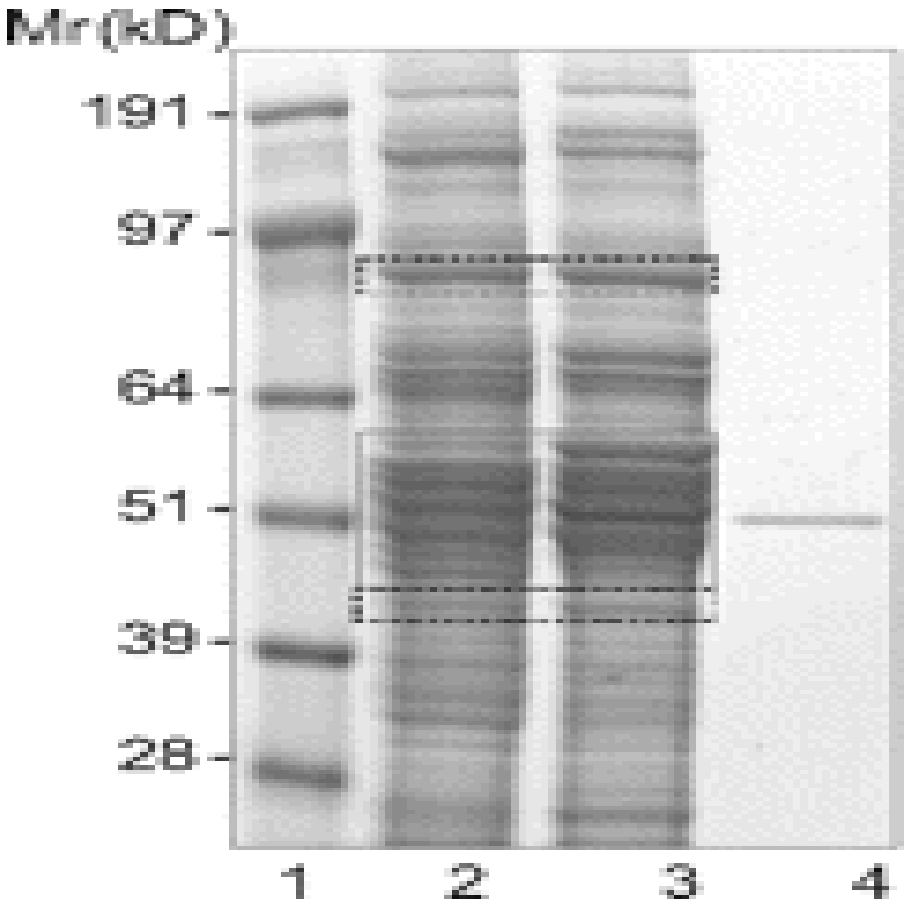


Fig2a; TLC of Sample;

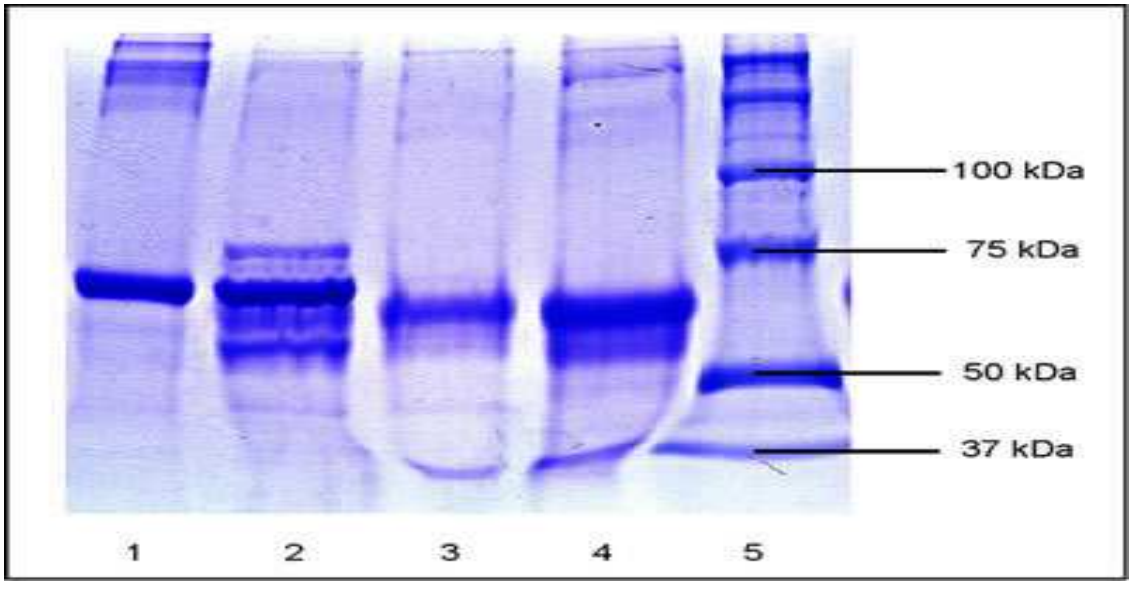


Fig2b; TLC of Sample

Alkaline Phosphatase Assays

STORAGE

Shipped at ambient temperature, Upon arrival, store at 4°C . The substrate is light sensitive and should be protected from direct sun light or UV sources.

Stop Solution. 3N Sodium hydroxide

PREPARATION BEFORE USE

This reagent must be handle carefully. Aliquot this reagent in to 0.5-1ml/vial and distribute to students as need and must be used under instructor's supervision.[4]

PROTOCOL

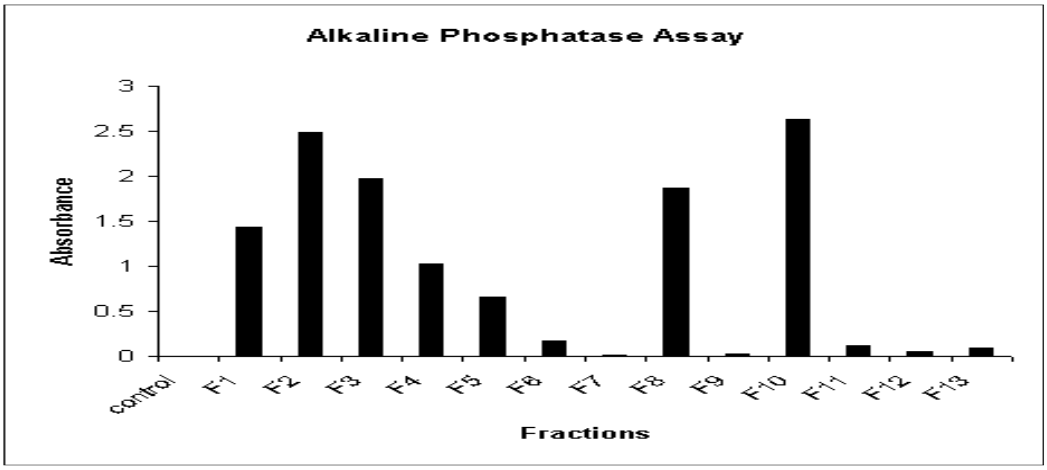
FOR: Alkaline Phosphates: Use alkaline extraction buffer.

Equilibrate the substrate and assay buffer to room temperature before use.

1. Set up of this assay in small 0.5 ml microfuge tubes. Set up one control tube, mark as control.
2. Transfer 45 µl of Ether Acidic or Alkaline Extraction Buffer into each assay tube.
3. Transfer 5µl test samples extracts into assay tubes . Do not add test samples in the control tube instead use 5 µl extraction buffer for the control.
4. Start the reaction with the addition of 50 µl PA substrate to each assay tube.
5. Incubate for 25 min. at room temperature.
6. At the end of the incubation, at 50 µl Stop Solution to each well to stop the reaction.
7. Measure the absorbance at 415nm .

RESULTS

(Fig3;)



SULFHYDRIL ASSAY

Description	Size
Ellman's Reagent	1 vial of 40 mg

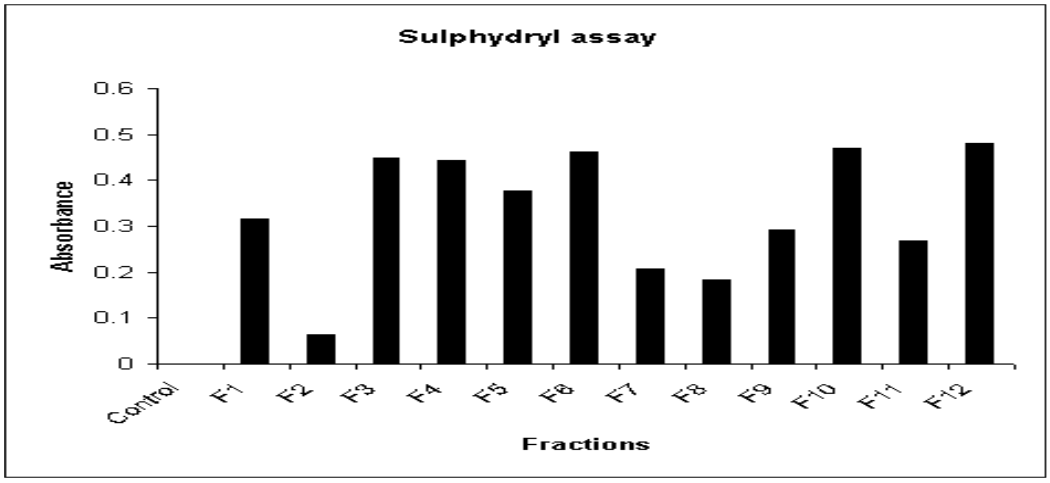
STORAGE CONDITION

Product is shipped at ambient temperature. Store in cold temperature, upon receiving. Stable for up to 2 years.

PROTOCOL

1. Make 10 mM DTNB Stock Solution by dissolving 40mg DTNB in 10ml 1X Tris-HCl pH 7.5 (Neutral Extraction Buffer). Incubate with periodic vortexing for 1h at room temperature. The Stock Solution can be stored at 4°C for 3 months.
2. Before use dilute the Stock Solution 100 fold with 1X Tris-HCl pH 7.5 (Neutral Extraction Buffer). To make 0.1 mM DTNB working solution- mix 100 µl 10mM DTNB in 10 ml 1X Tris- HCl pH 7.5.
3. Aliquot 950 µl of 0.1 mM DTNB work solution to each 1.5 ml centrifuge tube. Add 50 µl test sample extracts and mix by brief vortexing. Set a blank by adding 50 µl of 0.1M Tris-HCl pH 7.5 to 950 µl of 0.1 mM DTNB work solution.
4. Incubate 2 min. at room temperature.
5. Measure the absorbance of the test sample with a spectrophotometer against blank at 415nm.

RESULT



(Fig4;)

LOWRY PROTEIN ASSAY

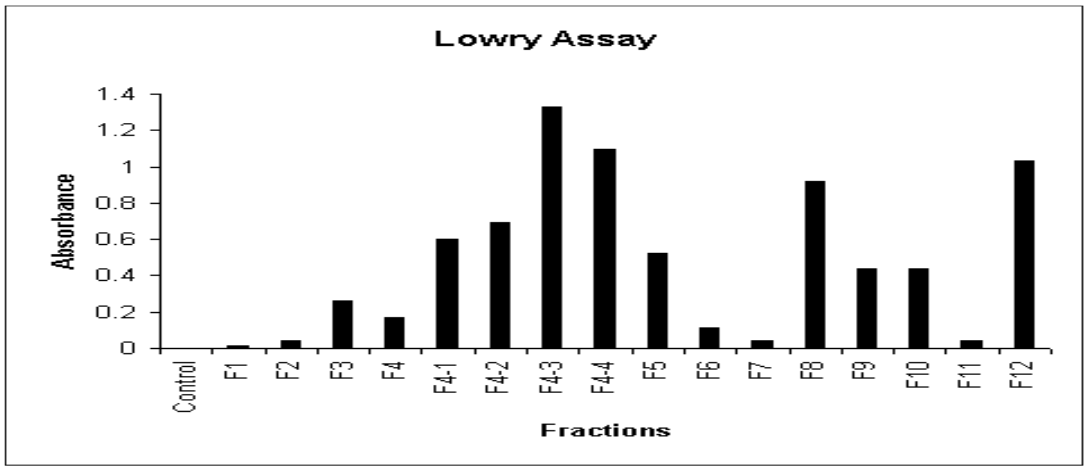
Prepare Prediluted Protein Standards (BSA)

1. As a group, add 0.5 ml BSA protein standard (2mg/ml) to a tube and dilute with 0.5ml protein extraction and Dilution Buffer to make 1mg/ml BSA solution.
2. As a group prepare a range of protein standards for a standard curve with the diluted BSA (1mg/ml) from step 1, as indicate in table:

BSA Concentration(mg/ml)	0.0	0.25	0.5	0.75	1.0
Diluted BSA(µl)	0	100	200	300	400
Protein Extraction and Dilution Buffer (µl)	400	300	200	100	0

3. As a group, label two sets of tubes with 0, 0.25,0.5,0.75 and 1.0 and transfer 50µl of the appropriate standard to the tubes. Save the remaining protein solutions For the Bradford Protein Assay.
4. Each student labels two tubes each for test sample-I, II and III and add 50 µl of each sample to the appropriate tube.
5. Add 0.1 ml Copper Solution to the standard and test sample tubes. Gently mix by vortexing.
6. Add 1ml Folin Ciocalteu Reagent and incubate at room temperature for 30 min..
7. Transfer 250 µl from each assay tube to a microtiter plate well. Read the absorbance at 650nm.
8. Measure the absorbance of the plate and record the value in the results section. [2]

RESULT



(Fig5;)

CONCLUSION

- Salt fractionation of proteins from chicken liver was carried out using salting out technique to precipitate the complete proteins. For salting out, ammonium sulphate solution was used. At 70% concentration of ammonium sulphate, complete proteins were precipitated out.
- Pellets were separated out at different fractions and marked as F1-F10 which was further used for the assessment of protein concentration.
- Lowry assay was done to estimate protein which shows the maximum concentration at the fraction no. F4-3. as observed from the absorbance at 650 nm.
- Alkaline phosphatase activity was assessed for the isolated protein, alkaline extraction buffer was utilized for the same. The absorbance at 415nm was found out to be F10 which showed the high concentration of alkaline phosphatase in chicken liver.
- Sulphydryl group detection was carried out using DTNB reagent assay from which it was observed that maximum Sulphydryl group was present at fraction no F12.

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