Purification of Lactate Dehydrogenase And Electrophore tic separation of Isozymes of LDH

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CERTIFICATE

This is to certify that the work entitled, "Purification of Lactate Dehydrogenase and electrophoretic separation of isozymes of LDH" submitted by Gurinder Bir Singh and Shivangi Sharma in partial fulfillment for the award of degree of Bachelor of Technology in -Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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TABLE OF CONTENT

ABBREVIATIONS

1. ATP: - Adenosine Triphosphate.
2. BSA:-Bovine serum albumin.
3. DNPH: - Dinitrophenylhydrazine.
4. DDW: Double distilled water.
5. LDH: - Lactate Dehydrogenase.
6. NAD^+:- Nicotinamide adenine dinucleotide.
7. NADH: - Reduced form of NAD^+.
8. SDS-PAGE: - Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
9. LP: - Low Pressure.
10. Kd: - Kilo Daltons.
11. O.D:- Optical Density.

ABSTRACT

The techniques employed in protein purification utilize important biochemical ideas about the structure, catalytic reactivity and other characteristics of the protein of interest. Any cell, whether prokaryotic or eukaryotic, contains tens of thousands of different proteins with a wide range of biological activities. Purification of individual proteins is therefore central to any detailed biological analysis of their structure and function. There is no single procedure whereby any and every protein can be isolated in pure form, but rather a set of procedures each of which attempts to distinguish between proteins on the basis of specific structural or functional characteristics. By selecting appropriate procedures and applying these in a carefully considered sequence of steps, it is usually possible to obtain a high degree of purification of the desired protein and in an acceptable yield. There is no standard order in which these procedures should be used to obtain optimal purifications. Because of structural and functional differences between proteins, an ideal sequence of steps for one protein will, quite possibly, be unsuccessful for another. Proteins can be separated from each other on the basis of differences in size, solubility properties, electric charge, adsorption behavior and biological affinity of a protein for a specific ligand.Various purification procedures were adopted for LDH.Chicken liver, brain, muscle, heart and stomach tissues were homogenized and centrifuged. Enzyme assay of all the five tissues was done using colorimetric method. SDS page was performed as an analysis for checking the presence of enzyme and to check purity after every purification step. The purification step that was performed was Ion Exchange Chromatography using LP system. Lowry's method of protein estimation was also performed. Various parameters which was measured after each purification step:

- Quantization of total protein.
- Assay of enzymatic activity.

CHAPTER 1

INTRODUCTION

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical property and binding affinity An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isolectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight. Choice of a starting material is key to the design of a purification process. In a plant or animal, a particular protein usually isn't distributed homogeneously throughout the body; different organs or tissues have higher or lower concentrations of the protein.

The isolation in pure form of a given protein from a given cell or tissue may appear to be difficult task, particularly since any given protein may exist in only small conc. In the cell, along with thousand of others. Despite these difficulties a great many different proteins have been isolated in pure form. Early methods for isolation of proteins were emperical, slow and very laborious.Current methods for separating proteins have exceptionally high resolving power i.e higher purification and fast.

Protein purification involves **(1)** availability of a specific assay method **(2)** a method to release the protein from the cell **(3)** extraction of the protein from the organelle, if needed **(4)** use of sequence of different fractionation procedures until maximum and constant specific activity is not attained.

Enzymes Enzyme is mainly defined as protein or a proteinaceous substance which catalyzes a specific reaction necessary for the maintenance of life. They are proteins specialized to catalyze biological reactions. Their catalytic activity is a consequence of their primary, secondary and tertiary structure. They are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic power.Urease was the first enzyme isolated in crystalline form by J.B Sumner in 1926 from extracts of the jack bean. Enzymes are mainly classified according to the types of reaction catalyzed.The six groups are:

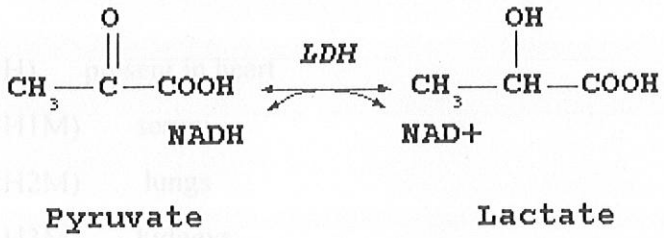
- Oxido-reductases. (EC 1): catalyze oxidation/reduction reactions.
- Transferases. (EC 2): transfer a functional group (*e.g.* a methyl or phosphate group).
- Hydrolases. (EC 3): catalyze the hydrolysis of various bonds.
- Lyases. (EC 4): cleave various bonds by means other than hydrolysis and oxidation.
- Isomerases. (EC 5): catalyze isomerization changes within a single molecule
- Ligases. (EC 6): catalyze isomerization changes within a single molecule

Enzymes are true catalysts that enhance the rate of reaction and are regenerated during the course of reaction. They are highly specific, catalyzing only one reaction.

Isozymes Enzymes that catalyze the same reaction but which differ in structure. They are multiple forms of given enzymes that occur within a single cell or in single species of organism. They are coded by diff. genes, so they differ in amino acid composition and thus in their isoelectric pH values. These enzymes usually display different kinetic properties and different regulatory function.Isozymes represent enzymes from different genes whose products catalyse the same reaction.Isozymes are variants of same enzyme. Isozymes were first described by Hunter and Markert (1957) who defined them as *different variants of the same enzyme having identical functions and present in the same individual.* This definition describes (1).Enzyme variants that are the product of different genes and thus represent different loci (*isozymes*) (2).Enzymes that are the product of different alleles of the same gene

(*allozymes*).Isozymes are usually the result of gene duplication, but can also arise from polyploidisation or hybridization.

Lactate Dehydrogenase LDH is an enzyme present in wide variety of organisms. It catalyses the interconversion of pyruvate and lactate with combined conversion of NADH AND NAD^+.Lactate Dehydrogenase (LDH) is an important enzyme in the anaerobic metabolism of glucose for the generation of ATP.The activity of this enzyme is primarily responsible for explosive anaerobic athletic activity.



The regeneration of the NAD+ permits continued metabolic flux down the glycolytic pathway. LDH is a tetramer of 35 Kd subunits. There are 2 types of subunits: a H form which predominates in the heart and an M form that predominates in the muscle and liver. These subunits can associate to form five types of tetramers, all with LDH activity but with different substrate affinities and different responses to allosteric effectors. All five isozymes have the same molecular weight of 134 Kd and all contain four polypeptide chains of H and M subunits.

Lactate dehydrogenase (LDH) has been purified and crystallized from yeast, bacteria, and various animal and plant tissues (Sweetlove and others, 2000; Vassault, 1983). Kidney has the highest LDH activity followed by heart and skeletal muscle in human tissue extracts. Skeletal muscle LDH is a tetrameric enzyme that exists in five isoforms due to the existence of two subunits of either M (muscle) or H (heart) form, both of which favor the production of lactate (Spriet and others, 2000). As the names indicate, the M_subunit is produced most likely in tissues capable of anaerobic

metabolism, such as skeletal muscle, while the H subunit is mainly found in tissues with aerobic metabolism, such as cardiac muscle.

The enzyme is located in both cytosol and mitochondria of skeletal muscle cells (Brooks, 2002; Gladden, 2001; Vassault, 1983).

Lactate produced in the cytosol, by glycolysis, can be taken up directly into mitochondria, oxidized to pyruvate through LDH, and then utilized by the TCA cycle under aerobic exercise conditions. Through this intracellular lactate shuttle, the reducing equivalents (NADH) are produced and transported to the electron transport chain (ETC). There are five isozymes formed due to combination of H and M polypeptide chains:

- LDH-1 (4H) present in heart
- LDH-2 (3H1M) serum
- LDH-3 (2H2M) lungs
- LDH-4 (1H3M) kidneys
- LDH-5 (4M) liver and striated muscle

(Thomas **Wuntch, Raymond F. Chen,Vol 67, no 3914)**

Single M and H chains have been isolated and found to differ significantly in amino acid content and sequence.

LDH4 and LDH5 are the most abundant among all five isoenzymes from skeletal muscle. Isoenzyme LDH5 is the predominant enzyme accounting for over 87% of the total LDH activity (Stalder and others, 1987). LDH rapidly binds NAD before binding lactate, which induces movement within LDH, trapping both substrate and coenzyme in the active site (Branden and Eklund, 1980). Two major interactions are involved in the association between lactate and LDH (Branden and Eklund, 1980). First, lactate forms a salt bridge with an arginine located on LDH. Second, oxygen binds to lactate's center carbon coordinated with the imadazole ring of a histidine located on LDH. These enzyme-substrate interactions are responsible for distinguishing between D-lactate, less likely to be metabolized by LDH, and L-lactate, the preferred substrate. The hydrogen on the center carbon of L-lactate is

positioned close to nicotinamide, which allows easy transfer of hydrogen to NAD. In contrast, D-lactate orients with its methyl group toward NAD, therefore hydrogen points away from NAD.

The LDH isozymes differ depending on muscle types. White muscles contain primarily the M4 isozyme of LDH, while the H4 isozyme predominates in red muscles (Collins and others, 1991a). Different LDH activity, which implies a different utilization rate of NADH, depends on which muscle fiber type is dominant within a muscle (Stalder and others, 1991). Semimembranosus muscle (1476 U) had a high level of LDH activity, the longissimus (1439 U) was intermediate, and the psoas major had the lowest level of LDH activity (644 U). Postmortem aging increased LDH activity (Table 5), while freezing and freezing-thawing markedly decreases activity (Collins and others, 1991b).

During postmortem aging of muscles, enzymatic degradation of the muscle structure occurs, and thus soluble proteins including LDH may be released from sarcoplasmic protein. High temperature (> 63) and low pH (4.8) conditions significantly deactivate LDH mainly due to the denaturation of muscle proteins (Stalder and others, 1991).

The M and H subunits are encoded by two different genes.M subunit is encoded by LDH-A, located on chromosome 11 p15.4.H subunit is encoded by LDH-B located on chromosome 12 p12.2-12.1.Thus,the biosynthesis of the two types o chains and thus the relative amounts of the LDH isozymes present in given cell is under genetic regulation. The major catalytic difference is explained by replacement of alanine in M chain with a glutamine in H chain in the vicinity of binding site. Sequence LDH-A consist of 332 amino acids. Sequence LDH-B consists of 334 amino acids. The differences in proporties of LDH isozymes are dependent on their subunit composition and are most prominent between the homotetramers M4 and H4.LDH isozymes differ significantly in their Km values for their substrates as well as their Vmax values, when pyruvate is present. Differences in catalytic properties of H and M isozymes arise due to differences in the amino acid chains decorating the highly conserved structure of polypeptide chains.

There is lot of difference between M and H subunit. Turnover no. of M4 isozyme is generally about twice that of H4 isozyme.The activity of H4 isozymes is more readily diminished by modification of carboxyamide group on 3-position of nicotinamide.

The forward reaction lactate to pyruvate is inhibited to far greater extent by high conc. of pyruvate in H4 than in the M4 isozyme.H4 isozyme binds oxidized or reduced coenzyme better than M4 isozyme.Both enzymatic activity and the affinity of substrate for enzyme are reduced to greater extent in M4 isozyme as length aliphatic chain of @-keto acid increases.

CHAPTER 2

PURIFICATION OF ENZYME

Source of enzyme and preparation of crude extract For the practical purpose,chicken was taken as source of enzyme.LDH was extracted from five different parts like brain, heart, liver, stomach and muscle.The different chicken parts were brought fresh and stored at -20^{0}C.For the preparation of homogenate, different parts were cut into very small pieces and kept at low temperature.Na-Phosphate buffer having ph.8 was prepared for the preparation of crude extract.Homogenation of different tissues was done keeping in ice bucket (to maintain low temperature) with the help pf homogenizer. The rotor of homogenizer was washed before and after with extraction buffer every time after working with each tissue. The crude extract from different tissues was then centrifuged at approximately 8500 rpm to extract the enzyme of interest i.e LDH out the tissue cells. The centrifugation was done for 30 min under low temperature conditions. The supernatants from different tubes were collected and stored in different tubes as LDH would be present in supernatant and pellet consist of just cell mass. The tubes were marked properly and stored at -20^{0}C to inhibit any enzymatic activity and protein denaturation.

Enzymatic activity assay The activity was determined spectrophotometrically by determining the amount of NAD+ converted to NAD.This method takes advantage of the fact that NADH has an absorbance peak at 340 nm while NAD+ exhibits little absorbance at this wavelength.

Lactate + NAD^+ ----------> Pyruvate + NADH

The reagents used were:

- 1.4 ml of 99mM lithium lactate in 10mM Tris-HCl at pH 8.6
- 0.7 ml of 0.7mM NAD in 10mM Tris-HCl at pH 8.6
- 0.4 ml of NaCl in 19mM NaHCO3.

The required volume was adjusted by adding DDW to take OD correctly in the cuvette.

The tubes taken were:

- Blank: Homogenate (enzyme) + 1.4 ml lactate + 0.4 ml $NaCl/NaHCO_3$ + 2 ml of DDw.
- Test: Homogenate (enzyme) + 1.4 ml lactate + 0.4 ml $NaCl/NaHCO_3$ + 0.7 ml of NAD^+ (just before taking the reading).

The absorbance was measured at interval of 15sec at 340nm by taking volume 10ul, 20ul, 40ul, 60ul and 80ul of crude extract (Homogenate) for every tissue..

The readings are:

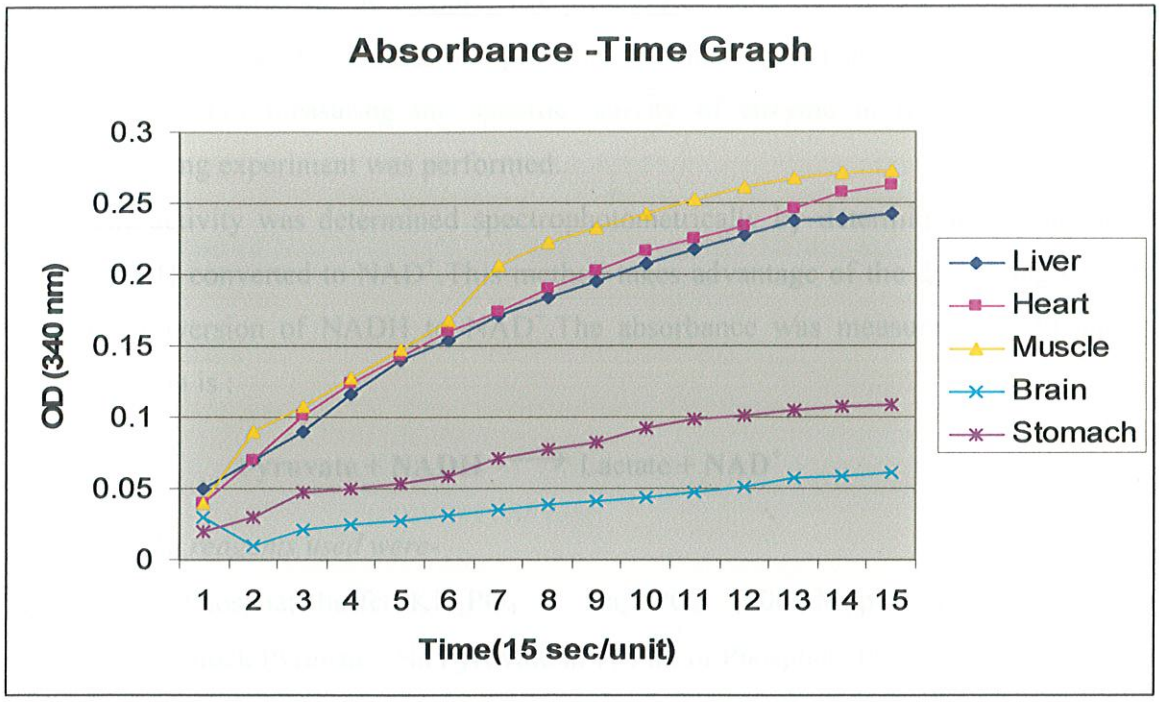
I. <u>At 80 ul of Homogenate</u> :

Liver	Heart	Muscle	Brain	Stomach
0.05	0.06	0.01	0.05	0.02
0.104	0.09	0.129	0.018	0.041
0.13	0.118	0.167	0.024	0.047
0.142	0.137	0.191	0.029	0.052
0.155	0.153	0.209	0.037	0.06
0.168	0.166	0.223	0.041	0.069
0.182	0.178	0.234	0.043	0.07
0.196	0.187	0.246	0.048	0.074
0.205	0.197	0.253	0.052	0.08
0.212	0.205	0.261	0.056	0.086
0.225	0.212	0.266	0.061	0.091
0.234	0.216	0.271	0.065	0.096
0.246	0.223	0.271	0.068	0.104

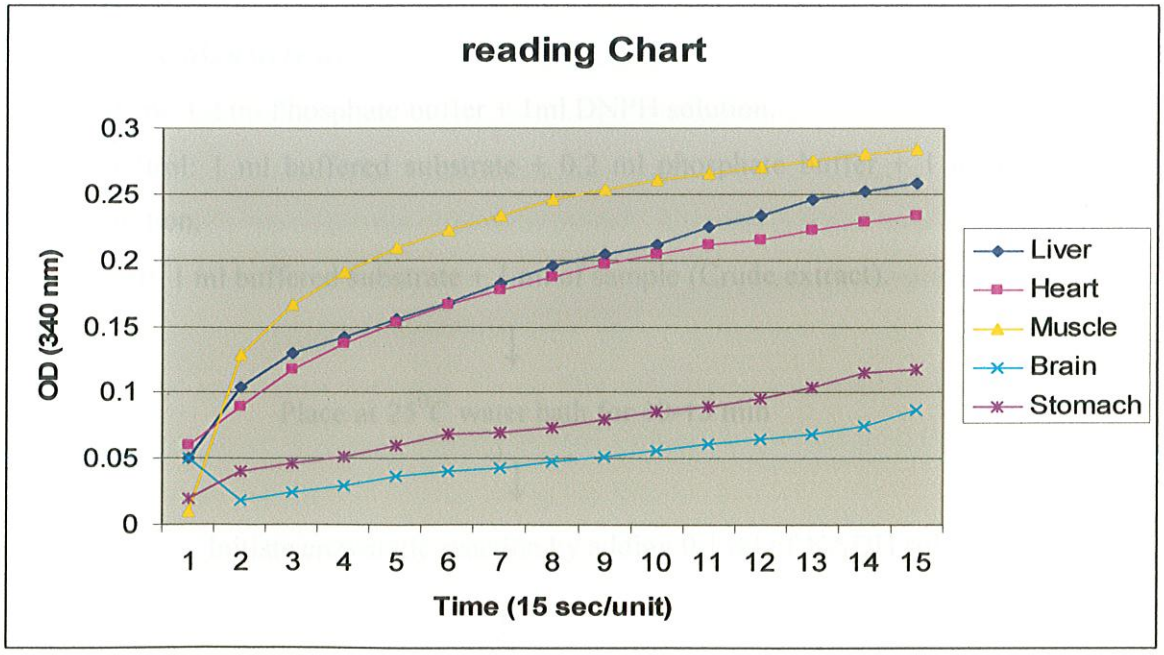
II. <u>At 40 ul of Homogenate</u> :

Liver	Heart	Muscle	Brain	Stomach
0.05	0.041	0.04	0.03	0.02
0.077	0.071	0.096	0.018	0.036
0.097	0.101	0.108	0.021	0.047
0.166	0.124	0.127	0.025	0.05
0.14	0.142	0.147	0.028	0.054
0.154	0.16	0.168	0.031	0.059
0.171	0.174	0.188	0.035	0.071
0.184	0.19	0.206	0.039	0.078
0.195	0.203	0.223	0.041	0.082
0.207	0.216	0.232	0.044	0.092
0.218	0.225	0.242	0.047	0.099
0.228	0.234	0.252	0.051	0.101
0.236	0.242	0.261	0.057	0.105
0.244	0.258	0.281	0.059	0.107

Graph (Using 40 ul of Homogenate)



Graph (Using 80 ul of Homogenate)



This experiment was mainly done to check the presence of enzyme of interest i.e LDH.The change in O.D by adding different concentration of homogenate determines the presence of LDH and conversion of lactate to pyruvate with the combined conversion of NAD^+ to NADH. The presence of LDH was determined By analyzing the conversion of substrates into products, which are responsible for change in O.D.

For measuring the specific activity of enzyme in the homogenate, the following experiment was performed.

The activity was determined spectrophotometrically by determining the amount of NADH converted to NAD^+.This method takes advantage of the decreasing O.D due to conversion of NADH to NAD^+.The absorbance was measured at 520 nm.The reaction is :

Pyruvate + NADH $\longrightarrow$ Lactate + $\mathbf{NAD^+}$

The reagents used were-

- Phosphate buffer: KH_2PO_4 + Na_2HPO_4 (100mM, pH 7.4).
- Stock Pyruvate: Na Pyruvate in 100 ml of Phosphate buffer. (37.5 mM).
- Buffered Substrate: 0.5 ml of stock in 25 ml buffer (prepare fresh).
- $NADH_2$ solution: 10 mg/ml in Phosphate buffer.
- 2, 4- Dinitrophenylhydrazine: 20 mg of 2, 4 DNPH in 4.2 ml conc.HCl.
- NaOH: 0.4 N.

The tubes taken were as:

- Blank: 1.2 ml Phosphate buffer + 1ml DNPH solution.
- Control: 1 ml buffered substrate + 0.2 ml phosphate buffer + 1 ml DNPH solution.
- Test: 1 ml buffered substrate + 1 ml of sample (Crude extract).

↓

Place at 25^0C water bath for 10-15 min

↓

Initiate enzymatic reaction by adding 0.1 ml of NADH solution.

↓

Incubate for exactly 15 min at 25^0C.

↓

Remove tube and add 1 ml of DNPH solution.

↓

Mix thoroughly and put for 15-20 min at room temperature.

↓

Put 10 ml of NaOH in each tube and mix, wait for 10 min and then take O.D at 520 nm.

Calculation

If O.D of control is 'X' then concentration is 'Y' umoles.

If O.D of control is 1, than conc is Y/X umoles.

Then conc of Test is (Y/X)*O.D of Test.

So, let conc of Test be Z umoles.

Then, the number of substrate molecules converted to product by enzyme is (Y-Z) umoles.

Let (Y-Z) = A

In 15 min, numbers of substrate mol. converted are A umoles.

So in 1 min, it is A/15.

In this way, Specific Activity is *(A/15) umol* $mg^{-1}min^{-1}$.

[The *Specific Activity* is the moles converted per unit time per unit mass of enzyme (enzyme activity / total mass of protein). The SI units are katal kg^{-1}, but more practical units are μmol mg^{-1} min^{-1}. Specific activity is a measure of enzyme efficiency, usually constant for a pure enzyme]. Specific activity is a convenient method of communicating the amount of enzyme activity you have in each milligram of protein. Specific activity is calculated after each purification step and can be used to monitor the success of that step. To do this calculation you need to know much enzyme activity there is in your sample as well as how much total protein is present. You can calculate the specific activity using the formula below:

Specific Activity = (units/ml)/ (mg total protein/ml)

Quantization of protein The concentration of total protein in the sample was estimated through *Lowry Method*. The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids [Dunn, 13].The cupric amino acid complexes thus obtained have absorption maxima at 670 nm.

The reagents used were:

Na-K Tartarate (2%).

Na_2CO_3 (2%).

NaOH (1N)

BSA (standard protein). [conc = 0.2mg/ml]

$CuSO_4$ (1%).

Lowry's Reagent.

Folin Ciocalteau solution.

The tubes taken were:

	Test	Standard	Blank
Sample	0.1 ml	0.1 ml	0 .1 ml H_2O
Lowry's reagent	5 ml	5 ml	5 ml
	Mixed and allowed to stand at 37^0C for 10 min.		
Folin reagent	0.5 ml	0.5 ml	0.5 ml
	Mixed and allowed to stand for 30 min at 37^0C.		

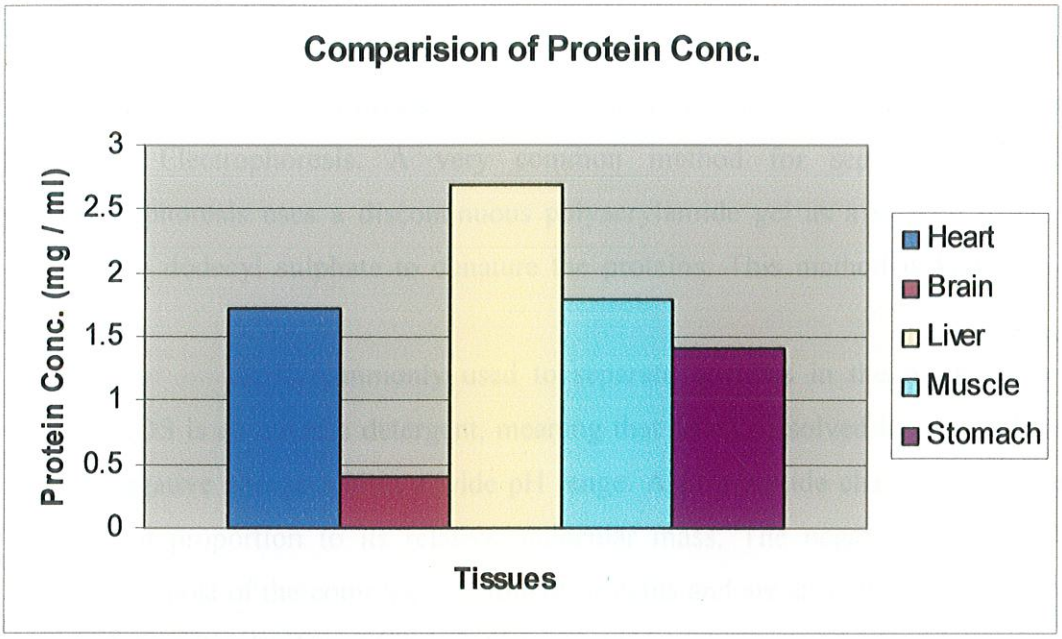
The absorbance of blank was set at 0, and then O.D of standard and test samples was taken.

The protein conc. was calculated through following formula:

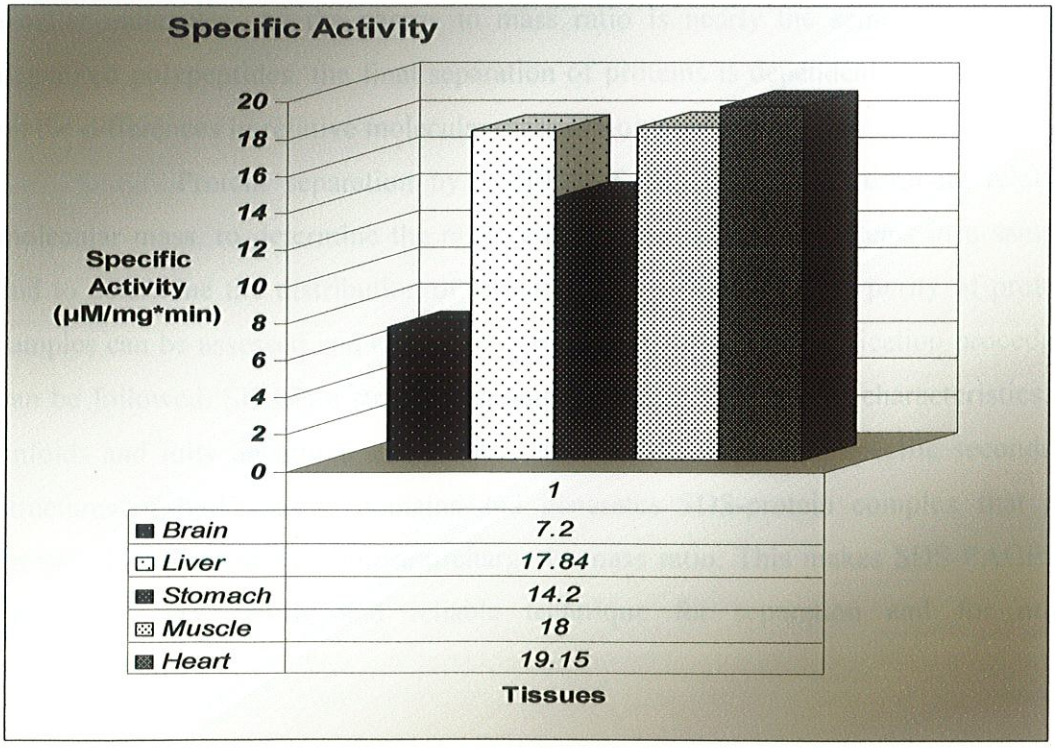
Protein Conc. = [(O.D of Test – O.D of blank) / O.D of standard] * conc. of std.

Graphs

Protein concentration in different tissue samples (homogenate).



Specific activity of enzyme present in samples.



For monitoring the purity and presence of enzyme in the sample, SDS-PAGE is done which is both rapid and sensitive and separates proteins solely on the basis of size.Suitabe marker proteins with known molecular weights are run along with the samples for comparison and determination of molecular weight of unknown protein.

SDS PAGE Electrophoresis The separation of macromolecules in an electric field is called Electrophoresis. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulphate to denature the proteins. This method is known as SDS-PAGE.

It is commonly used to separate proteins in the mass range 1-100 kDa.SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins and are strongly attracted toward an anode (positively charged) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. As the charge to mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. SDS is a strongly anionic detergent with unique characteristics. It unfolds and fully denatures all proteins, essentially disregarding specific secondary structures or hydrophobic domains and generates SDS-protein complex that are mostly characterized by a uniform charge to mass ratio. This makes SDS-PAGE in general a very simple and reliable technique for separation and for mass determination.

Ion Exchange Chromatography By choosing different buffers, the same protein can adsorb to both anion exchangers (that bind negatively charged molecules) and cation exchangers (that bind positively charged molecules). Remember that proteins are composed of amino acids and amino acids have different overall charges at different pH values. The matrix material for the column is formed from beads of some inactive material, often a carbohydrate such as cellulose or dextrans. This matrix is in the form of tiny beads that can be slurried in buffer and poured into a vertical column formed by a glass tube. A screen at the bottom keeps the beads from flowing out with the buffer and the column is "packed" in this manner. When the protein solution is pumped onto the top of the column, the beads adsorb the proteins as they flow past. The proteins are then eluted fro the column according to the tenacity with which they bind. Those that are the most highly charged at that pH will bind the tightest. To release the proteins in the order of binding tenacity we can either increase the salt concentration or change the pH.The elution of ion exchange column is done through salt gradient. The proteins then "elute" or come off the column matrix when the ionic strength of the buffer neutralizes their charge. The least charged molecules come off first and the most highly charged come off last.

Purification of LDH The purification of LDH was done through Cation Exchange Chromatography. The optimum pI of the enzyme is 7.2, so phosphate buffer with pH 6 was used to run in the column. As pI > pH, so our protein will have positive charge suitable for running in cation exchanger. The phosphate buffer (0.25 M) was prepared for equilibration i.e to bring the pH of cation exchanger equal to 6.For this continuous washing was done of the exchanger with the buffer for 2-3 days.

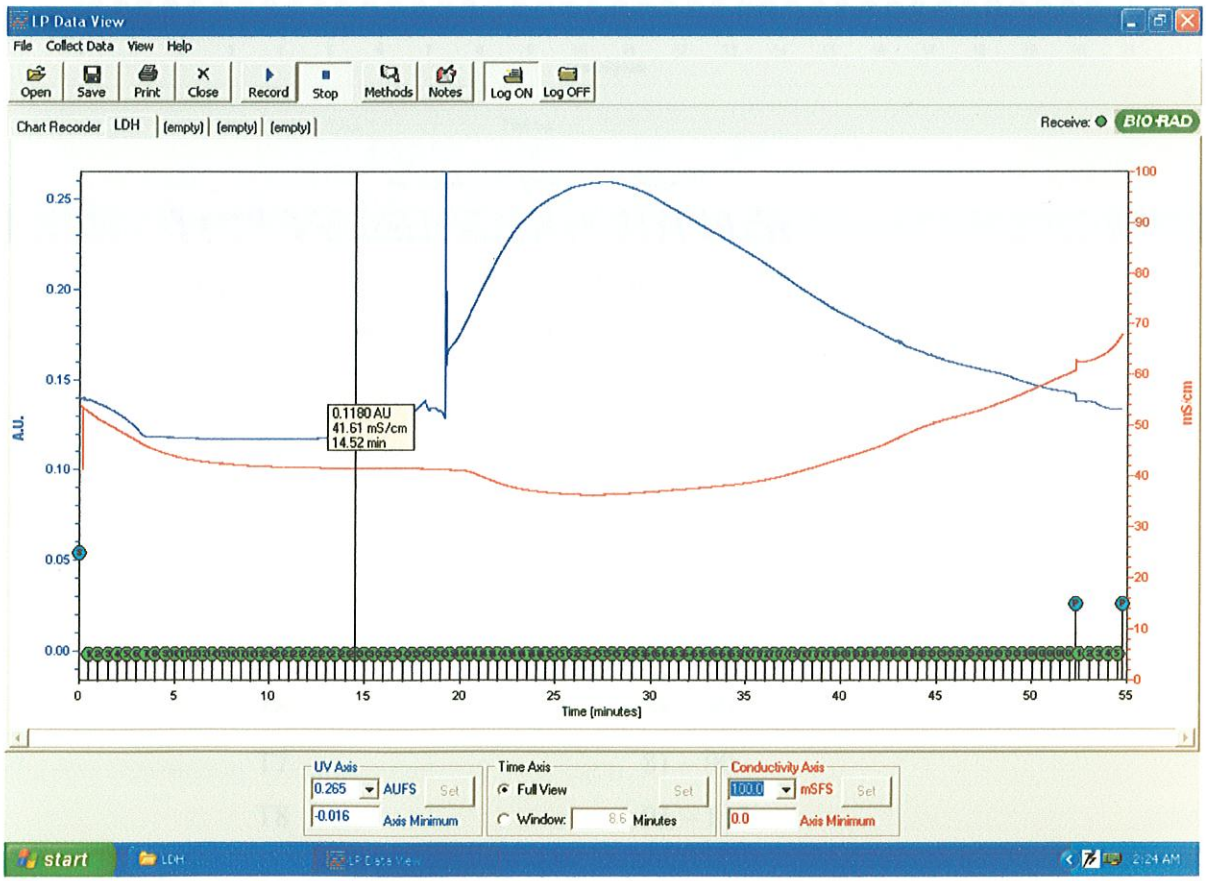
Two other phosphate buffer samples were prepared with salt concentration (NaCl) of 0.1 M and 1 M, which were to be used as salt gradient for elution of proteins in cation exchanger.

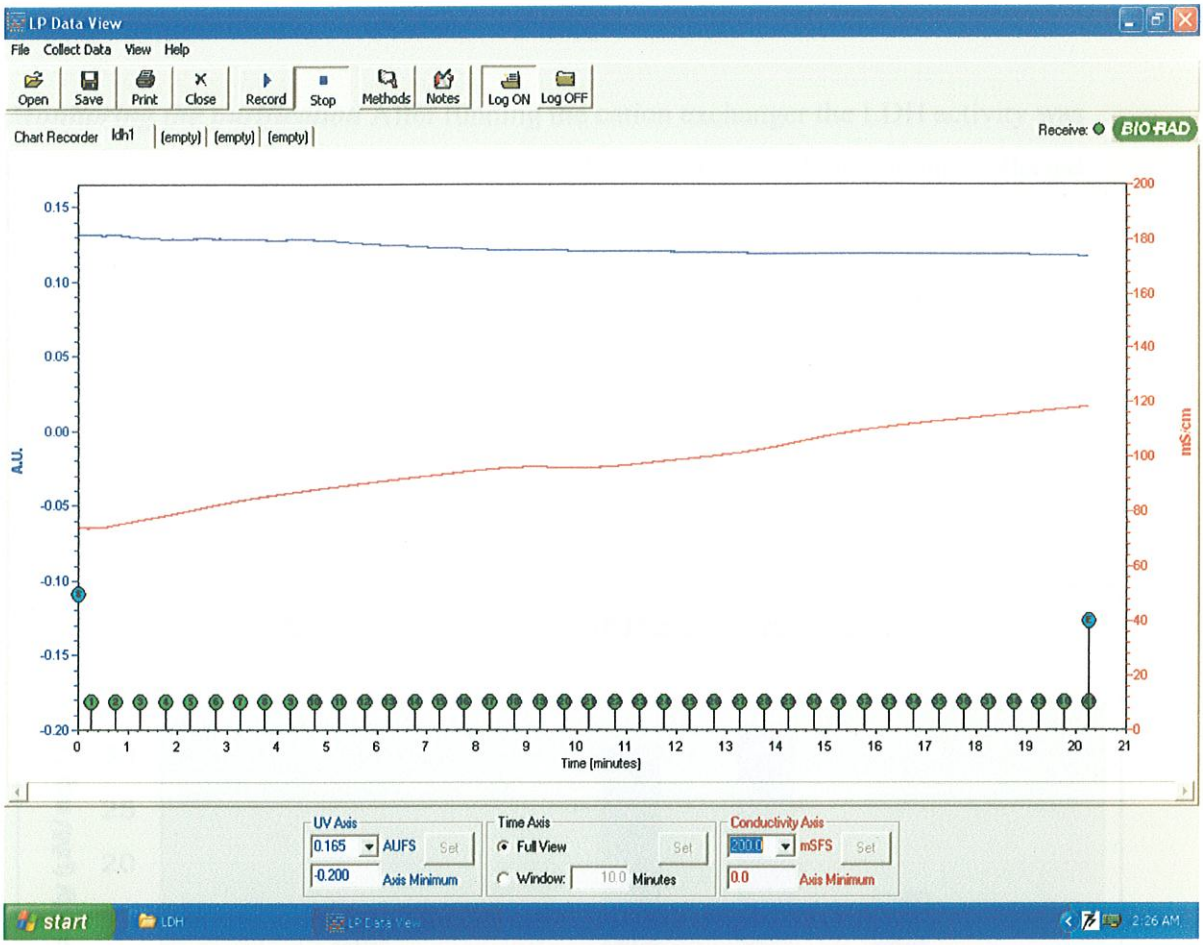
Preparation For LP system Before using LP system, the sample to be chosen for running in cation exchanger was chose. The activity was highest in heart and muscle, so these two samples were chosen for further purification.

The molecular weight of LDH is approximately 140 kDa and molecular weight of each subunit is 35 Kda.So before using cation exchanger, the samples from heart and muscle were further filtered by using tube having filter of pore size 10 kDa.The samples in this tube was centrifuged for 30 min at 5000 RPM.All the proteins with molecular weight less than 10 kDa was filtered out of the sample. The left over sample having LDH was collected and stored in tubes at low temperature.

The heart sample was run in Cation Exchange Chromatography. The buffers were used as salt gradient. The elution sample was collected in different tubes. The tubes showing highest activity were collected and pooled together for checking LDH activity.

The data plot





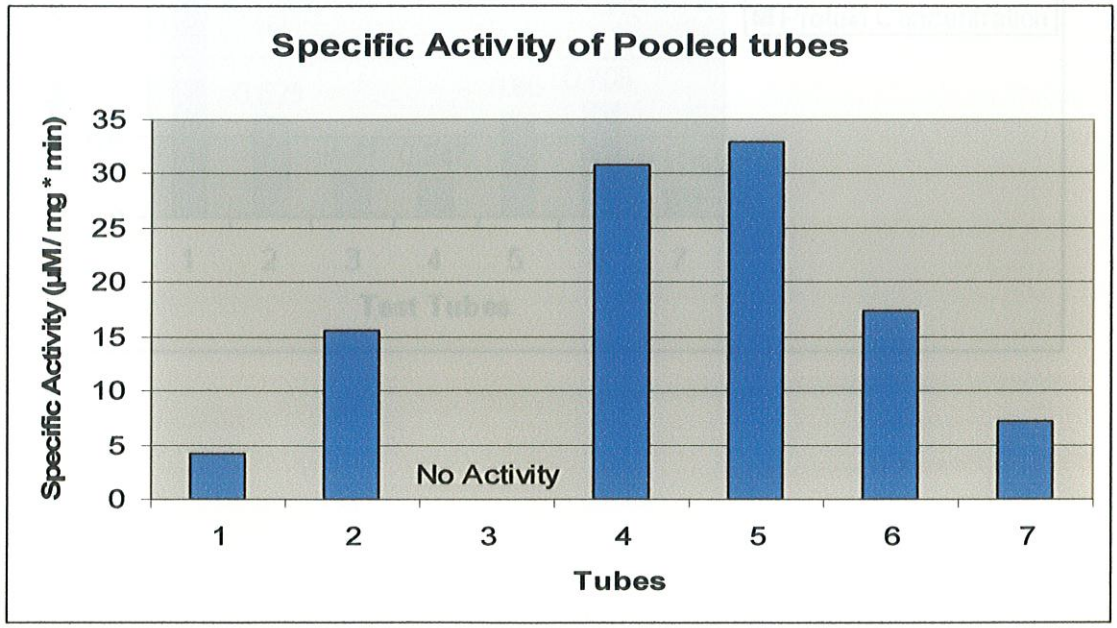
The elution sample was collected in different tubes. The tubes showing high activity were pooled together and marked as T1, T2, T3, T4, T5 ,T6, T7 AND T8.

Tube	Pooled Tubes.
T1	1 – 10
T2	11 – 20
T3	21 – 36
T4	37 – 47
T5	48 – 58
T6	68 – 80
T7	81 – 90
T8	91 – 115

Monitoring the purification After running the cation exchanger the LDH activity was again measured, protein quantization and SDS-PAGE was done on the collected tubes.

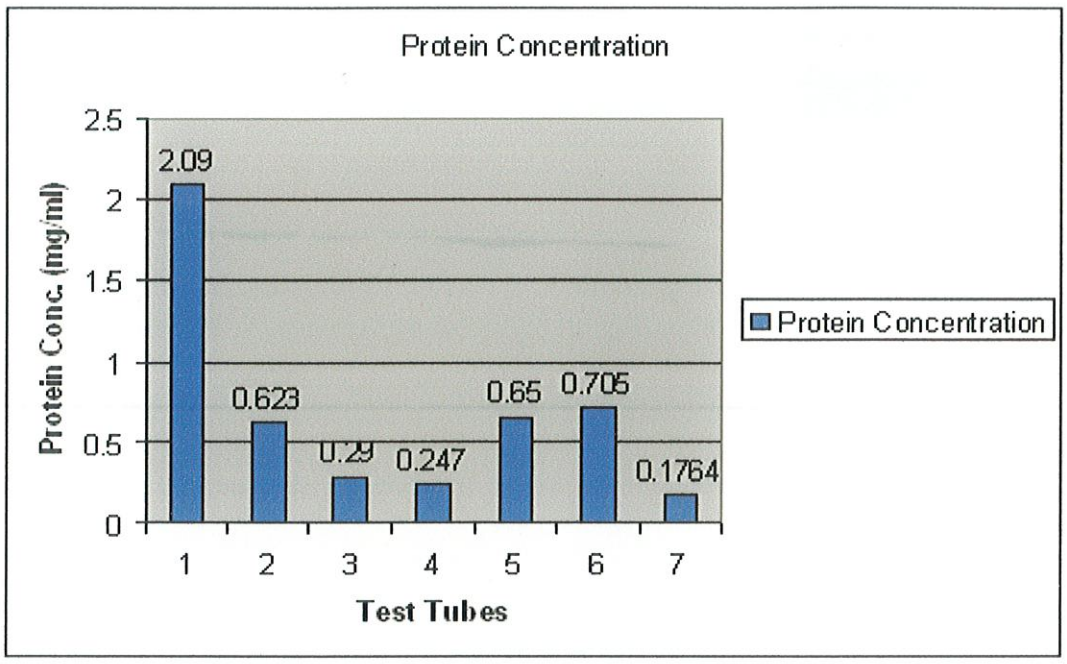
GRAPHS

Specific activity of different tubes.



The protein concentration was measured through Lowry Method and subsequently, the specific activity was measured and calculated.

Protein Concentration Of tubes



SDS-PAGE was done on the tube sample:

CONCLUSION

The LDH was successfully extracted from the tissue samples.

The increase in specific activity has been observed from 19.15 $umolmg^{-1}min^{-1}$ to 32.88 $umolmg^{-1}min^{-1}$ i.e. **an increase of 68.2 % and purification fold of ~2 was obtained.**

The total protein concentration in the sample has decreased from 1.719 mg/ml to 0.65 mg /ml i.e **a decrease of** 61.7 **%.**

There was decrease in number of bands in SDS-PAGE before and after the purification.

Some of the bands were still present even after purification in range of molecular weight 30 kDa to 40 kDa, thus confirming the presence of LDH subunits.

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