PARTIAL PURIFICATION AND KINETIC CHARACTERIZATION OF L-LACTATE DEHYDROGENASE FROM *RHIZOPUS ORYZAE*

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ABSTRACT

PARTIAL PURIFICATION AND KINETIC CHARACTERIZATION OF L-LACTATE DEHYDROGENASE FROM *Rhizopus oryzae*

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L-Lactate dehydrogenase (EC 1.1.1.27), L-lactate:NAD^+ oxidoreductase (LDH), catalyzes the reduction of pyruvate in the presence of NADH, in the final step of lactic acid fermentation. It is present in all vertabrates, many invertabrates, plants and diverse microorganisms

LDH has an application area in microbiology, food, cosmetics and paper industries. It is also an important auxillary enzyme for the determination of many enzymes. Microbial LDH, has caught great attention in order to determine the parameters of lactic acid production: Global lactic acid demand is estimated as more than 100.000 tons per year.

In this study, LDH was partially purified and characterized from the filamentous fungi, *Rhizopus oryzae* (ATCC 9363), a lactic acid producing fungus.

LDH has been partially purified by ammonium sulfate fractionation and chromatofocusing techniques. The enzyme was precipitated between 30-60 % saturation, by which a 2.4 purification fold

with 76 % recovery was achieved. After desalting, the sample was applied to a PBE 94 column and the purification fold was 1.5 with 10 % recovery. The enzyme activity was determined by monitoring NADH oxidation at 340 nm, as pyruvate is reduced to lactate. The optimum pH and temperature for LDH activity in crude extract was determined as 7.5 and 35 °C, respectively. The K_m and V_{max} values of the enzyme for pyruvate has found as 6.44×10^{-3} M, and 0.223 μ mol min^{-1} mL^{-1}. The molecular weight of monomer unit of LDH was estimated as 36 kDa with SDS-PAGE.

Keywords: Lactate dehydrogenase, *Rhizopus oryzae*, Lactic acid fermentation

ÖZ

Rhizopus oryzae'den L-LAKTAT DEHİDROGENAZ ENZİMİNİN KISMİ SAFLAŞTIRILMASI VE KİNETİK KARAKTERİZASYONU

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L-Laktat dehidrogenaz (EC 1.1.1.27), L-laktat: NAD^+ oksidoredüktaz (LDH), laktik asit fermentasyonunun son aşamasında, NADH varlığında pürivatın laktata indirgenmesini katalize eder. LDH, tüm omurgalılarda, bir çok omurgasızlar ile bitkilerde ve çeşitli mikroorganizmalarda bulunur.

LDH nin mikrobiyoloji, gıda, kozmetik ve kağıt endüstrilerinde yaygın kullanım alanları mevcuttur. Bir takım enzimlerin saptanması sırasında da varlığına gereksinim duyulan aksüler bir enzimdir. Mikrobiyal LDH, laktik asit üretimindeki parametrelerin belirlenmesi amacıyla da cok dikkat çekmiştir; küresel laktik asit talebi yıllık 100.000 ton' dan fazladır.

Bu çalışmada, LDH enzimi laktik asit üreten filamentli bir küf suşu olan *Rhizopus oryzae* (ATCC 9363), den kısmen saflaştırılmış ve karakterize edilmiştir.

LDH amonyum sülfat fraksiyonu ve kromatofokusing teknikleri ile saflaştırılmıştır. Enzim % 30-60 amonyum sülfat fraksiyonunda çökmüş ve % 76 lık bir verimle 2.4 kat saflaştırılmıştır. Fraksiyon tuzlardan uzaklaştırıldıktan sonra, PBE 94 kolonuna tabi tutulmuş ve % 10 luk bir verimle 1.5 kat

saflık derecesi elde edilebilmiştir. Enzim aktivitesi pürivatın laktata indirgenmesi sırasındaki NADH oksidasyonunun 340 nm dalga boyunda takip edilmesi ile saptanmıştır. Ham özütte optimum pH ve sıcaklık değerleri sırasıyla 7.5 ve 35 °C olarak bulunmuştur. Enzimin K_m ve V_{max} değerleri Pürivat için 6.44×10^{-3} M, and 0.223 μ mol min^{-1} mL^{-1} olarak bulunmustur. SDS-PAGE kullanılarak enzimin monomer formunun moleküler ağırlığı 36 kDa olarak tahmin edilmiştir.

Anahtar kelimeler: Laktat dehidrogenaz, *Rhizopus oryzae*, Laktik asit fermentasyonu

Dedicated to Gülistan & Hıdır Arslan...

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE:

LIST OF TABLES

Table

CHAPTER I

INTRODUCTION

Lactate Dehydrogenase (EC 1.1.1.27), L-lactate:NAD^+ oxidoreductase (LDH), a common enzyme among vertebrates, invertabrates, plants and microbes, was discoverd in the early period of enzymology. It catalyzes the reduction of pyruvate by NADH, through the final reaction of glycolysis, forming L(+)-lactic acid. Among some animal species and several microorganisms, D(-)-lactate is oxidized by another stereoselective LDH (EC 1.1.1.28; D(-)-lactate:NAD^+ oxidoreductase), as well. Three types of LDH were found in yeast which are quite different from those mentioned above and which are accepted as metal-containing flavoproteins. All of these three enzymes catalyze the conversion of lactate to pyruvate irreversibly and they are, L(+)-lactate:cytochrome c reductase or cytochrome b_2 (EC 1.1.2.3), D(-)-lactate:cytchrome c reductase (EC 1.1.2.4) and D(-)-lactate dehydrogenase (EC 1.1.2.8). (Gleason, 1966)

The discovery of LDH catalysis was first in cell-free muscle extracts, which were determined to oxidize L-lactate to pyruvate (cited in Kopperschläger, 1996). The enzyme was then purified by Straub in 1940 and the first crystallized form of the enzyme was obtained by Kubovitz and Ott in 1943.

A great attention has been drawn to LDH due to its significant metabolic role. Its widespread occurance is due to its crucial function in the glycolytic pathway. After glucose has been broken down to pyruvic acid, through glycolysis, the two molecules of pyruvic acid formed, are reduced by two molecules of NADH to form two molecules of lactic acid catalyzed by lactate dehydrogenase. It is a reversible reaction for mammals, but in many bacteria and fungi it is irreversible (Pritchard, 1973). In muscle cells such a reaction means that, it permits organisms to overcome a temporary

oxygen debt in the form of accumulated L-lactate to be later discharged by the reoxidation reaction to pyruvate when oxygen becomes available. So, lactic acid fermentation in higher animals is a way of overcoming oxygen insufficiency temporarily as an alternative to aerobic respiration. However, one should not always expect this fermentative pathway to occur in the absence of oxygen. Unlike muscle cells of animals, retina, brain and erytrhocytes and many microorganisms produce lactic acid under aerobic conditions. The reaction does not require oxygen; it may happen under aerobic or anaerobic conditions. Although the fungi are generally an aerobic group of organisms, many of them grow under microaerobic conditions, or as in the case of yeasts, anaerobic conditions. The energy required for anaerobic growth is obtained by fermentation of carbohydrates, with the production of lactic acid or ethyl alcohol. Although alcoholic fermentation has been studied extensively in yeasts, only a few investigators have dealt with lactic acid fermentation in fungi. Regulation of the glycolytic flux in yeast is under the control of several metabolic circumstances, and are well documented. On the other hand, in the case of many other fungi and thus *R. oryzae*, by which factors the fate of the glycolytic flux (between aerobic respiration and lactic acid fermentation), is regulated are not known. In fact fungal lactate dehydrogenase has a coenzyme of nicotinamide-adenine dinucleotide (NAD^+) rather than a flavin of yeast.

Animal LDH was found to be a tetrameric molecule and this molecule were determined as different isoenzymes (Kopperschläger, 1996). The dissociation of oligomeric enzyme can be done by freezing and thawing, by high salt concentrations or by treatment with denaturating agents. LDH occurs in vertabrate tissues as at least five different isozymes separable by electrophoresis. All LDH isozymes contain four polypeptide chains but the five isozymes contain different ratios of two kinds of polypeptides that differ in composition and sequence.

Only about a dozen fungal genera produce significant quantities of lactic acid during fermentation, but these are distributed randomly among the major groups of fungi. A NAD^+-dependent lactate dehydrogenase has been found in two of these genera, *Rhizopus* and *Blastocladiella* (Gleason, 1966). D(-)- lactate stereospecific LDH, was also detected in high concentrations in some lactic acid producing fungi belonging to Class *Oomycetes* (Gleason, 1966).

Obayashi *et. al.* (1966) reported that the purification of LDH from *Rhizopus oryzae* belonging to class Zygomycetes, was only 3-fold level and, Pritchard (1973), achieved a 10-fold purification. In 1991, Yu & Hang isolated LDH from this fungus with a 175- fold purification. Recently, the genes involved in synthesis of lactic acid by *R. oryzae*, has been isolated and characterized (Skory, 2000). It was reported that at least three different LDH are produced by *R. oryzae*. Two of these enzymes, *LdhA* and *LdhB*, require cofactor NAD^+, while the third one is probably a mitochondrial NAD^+-independent LDH that is used for oxidative utilization of lactate. However, there are still many areas in glycolytic pathway to be discussed, such that, carbohdrate metabolism of fungi may confine one, who attempt to review their metabolism, with a lack of information.

On one hand, global lactic acid production is estimated to be more than 100.000 tons per year, on the other hand, *Rhizopus orzyzae*, its physiology, genetics and metabolism has recently caught a great attention in that this fungus is a valuable lactic acid producer. . In this study, L-(+)-lactate dehydrogenase from *R. oryzae*, was attempted to purify by Ammonium Sulfate precipitation, and by Chromatofocusing on PBE 94 column.

1.1. *Rhizopus oryzae*: An Overview

Rhizopus oryzae are filamentous fungi and are known as moulds, those dusty like spots often found spreading over bread, cheese, books and many other things at home, cause the loss of millions of dollars to our economy every year, and even worse may be a damage to our health. The beneficial activities of yeasts and other fungi however, are also of great economic significance. They have long been exploited as food, in processing food, and in brewing. With the progressive outcomes of fermentation technology, by this century, they have been used for ultimate source for antibiotics, vitamins and enzymes. With the advances in genetic engineering, they are also being used to produce hormones and proteins available only from mammals. So, fungi are important source for biotechnology issues.

Moulds are microscopic plant like organisms, belonging to fungal species, and composed of long filaments called **'hyphae'**. Mould hyphae grow over the surface and inside nearly all substances of plant or animal origins. Because of their filamentous construction and consistent lack of chlorophyll they are considered by most biologistS to be separated from the plant kingdom and accepted as the members of kingdom fungi.

When mould hyphae are numeorous enough to be seen by the naked eye, they form a cottony mass called **'mycelium'**. It is the hyphae and resulting mycelia that invade things in our homes and cause them to decay.

Moulds reproduce by spores, by sexually or asexually. Sexually initiated spores usually result from a mating between two different organisms or hyphae, where as asexual spores result from a simple internal division or external modification of hyphae. The recognition of a mating and subsequent spore formation is often difficult for an observer, and is usually reserved for patient specialists. However for practical purposes one can learn sexual process namely, the four kind of sexually determined spores that appear in mould fungi.

(1) Oospores

(2) Zygospores

(3) Ascospores

(4) Basidiospores

Asexual spores usually occur either in **sporangia** or as **conidia.** Sporangia are modified hyphae or cells containing numerious spores. They never have more than a single connecting hyphae and the spores do not constantly occur in groups of four or eight as do ascospores. Conidia are the most difficult group to characterize because of their great diversity of form. The only feature that most conidia have in common is that they occur externally on the cells that produce them. These conidium bearing cells may occur within rather specialized or characteristic structures that resemble those that frequently bear asci, however, and it is often necessary to break them open to

confirm that the spores are truly conidia and not ascospores. Conidia that are borne on the hyphae without any kind of compound fruiting the structure, are the most commonly encountered type. Structure that completely enclose conidium-bearing cells are called pychidia, and those broad or sporodochia if broader than long.

1.1.1. Morphology, Classification and Physiology *R. oryzae*

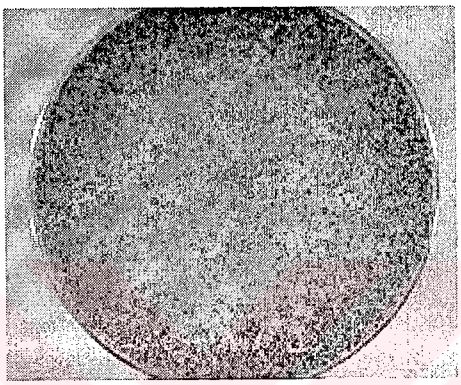

Figure 1.1: culture of *R. oryzae* (downloaded from mycology online; www.mycology.adelaide.edu.au)

On Sabouraud's dextrose agar, colonies of *R. oryzae* (Figure 1.1), are very fast growing at 25°C, reaching 5-8 mm in height, with some tendency to collapse, white cottony at first, becoming brownish grey to blackish-grey depending on the amount of sporulation. Sporangiophores up to 1500 μm in length and 18 μm in width, smooth walled, non-septate, simple or branched, arising from stolons opposite rhizoids usually in groups of 3 or more. Sporangia are globose, often with a flattened base, greyish black, powdery in appearance, up to 175 μm in diameter and many spored. Columella and apophysis together are globose, subglobose or oval, up to 130 μm in height and soon collapse to an umbrella-like form after spore release. Sporangiospores are angular, subglobose to

ellipsoidal, with ridges on the surface, and up to 8 um in length. No growth at 45°C; good growth at 40°C.

Rhizopus oryzae belong to the kingdom Fungi, Division Eumycota, Subdivision Zygomycotina, Class Zygomycetes, Order Mucorales. Mucorales are usually saprotrophs, and are common in soil and on droppings of rodents and large herbivores. Others cause rots of fruits and some occur on the decaying fruiting bodies of mushrooms and toadstools. The saprotrophic members of the Mucorales usually have little ability to attack refractory substrates such as polysaccharides, but have large hyphae that spread rapidly. They, then, 'get there first' and can exploit readily assimilable nutrients such as sugars before other fungi arrive. A few members of the Mucorales are mycoparasites, attacking other fungi, particularly saprotrophic Mucorales growing on dung. Their hyphae are small and many are obligate parasites and can not be cultured. The largest genus in the Mucorales is Mucor itself, many species of which are common in soil and on decaying plant materials.

The principal characteristics that distinguishes class zygomycetes of R. oryzae, is the production of a thick-walled resting spore (large nearly spherical, dark brown or black). Zygospores resemble the Oomycetes in having hyphae that usually lack crosswalls or septa, but differ in having motile spores. Asexual reproduction is by sporangia or conidia as described before. The members of this group are usually terrestrial and will be encountered only occasionally in aquatic conditions. Zygomycetes are easy to observe. They will invariably grow if soil is sprinkled over strawberries or rasperries. Any attempts to isolate fungi from soil will reveal various zygomycetes.

The zygospore develops within a zygosporangium that is formed after fusion of two gametogania. Another typical characteristic is the presence of a coenocytic mycelium (mycelium that lacks regular septation) which is a characteristic of lower fungi.

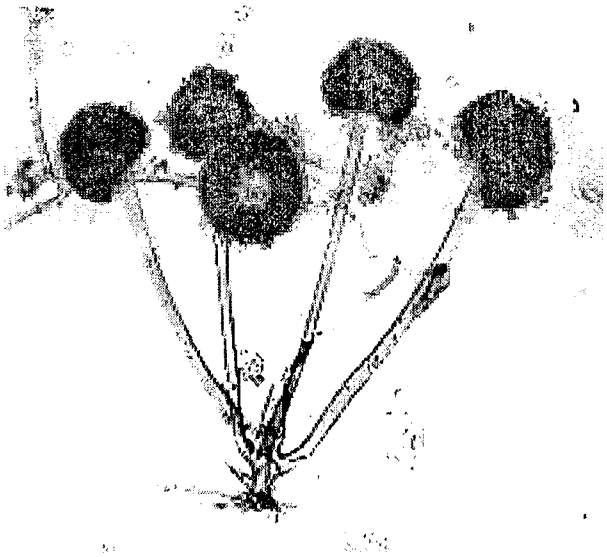

Figure 1.2: Sporangiophores, rhizoids and sporangia of *R.oryzae*. (Downloaded from mycology online www.mycology.adelaide.edu.au)

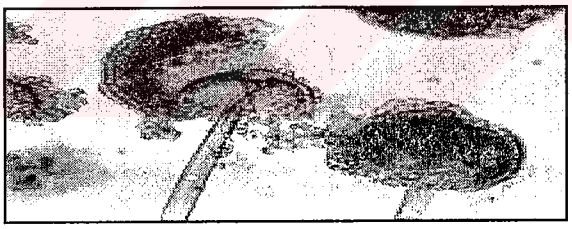

Figure 1.3: Sporangium with collapsed columella and sporangiospores of *R. oryzae*. (Downloaded from mycology online www.mycology.adelaide.edu.au)

Asexual reproduction is usually by sporangia spores, growth starts by the germination of the spore and followed by spread and penetration of the vegatative hyphae to the substratum. Released of the produced lactic acid is mainly from hyphal tips that are active

1.1.2 Clinical Significance

Rhizopus oryzae (=R. arrhizus) is the most common causative agent of zygomycosis, accounting for some 60% of the reported culture positive cases, and nearly 90% of the rhinocerebral forms of infection. *R. oryzae* has a world-wide distribution with a high prevalence in tropical and subtropical regions. It has been isolated from many substrates, including a wide variety of soils, decaying vegetation, foodstuffs, and animal and bird dung. *R. oryzae* is often used in the production of fermented foods and alcoholic beverages in Indonesia, China and Japan. However, it also produces the ergot alkaloid agroclavine which is toxic to humans and animals. (mycology online, www.mycology.adelaide.edu.au)

1.1.3. *R. oryzae* in Biotechnology

Although *R. oryzae* has been recently investigated for industrial applications, fungal microorganisms have had a great application area in the field of biotechnology. They are used for the production of many 1° and 2° metabolites, e.g., organic acids, antibiotics and enzymes.

1.1.3.1 Traditional Applications of *R. oryzae*

Traditional application of *R. oryzae* can be seen in Indonasian kitchen. Tempe is a fermented soybean food traditionally made in Indonasia. Usually it is consumed during the day of its production. It is cut into strips and deep-fat fried or boiled in a soup. Its production involves, first soybeans to be soaked in water (12-48 hrs, 28 C), which causes bacteria multiply. (*L. casei, E.*

faecium), then soybeans are boiled for 40 – 60 mins, before cooling they are drained. After the cooling they are dried and the dried mat is inocculated with fungal spores (the principal species of Indonasian tempe is *R. oligosporus* but *R. oryzae* can also be detected.) Traditionally tempe molds are grown on leaves of *Hibicus similis.* Besides to this, in China, starch breakdown in rice wine manufacture is also due to *R. oryzae.*

1.1.3.2. Fermentation Technology and Lactic Acid Production

Fermentation technology is a crucial industry for the large-scale culture of microorganisms to produce alcohol by yeast, acetone, butanol, or lactic acid from bacteria. Global demand for lactic acid, is estimated to be more than 100.000 tons per year and approximately 75 % of the lactic acid is used in the food industry or as an antimicrobial agent (Raber, 1998). Lactic acid is an important industrial consumable which is used in the production of cheese, or yogurt from milk, rye bread from sugar and grain, sauerkraut from cabbage and summer sausage from meat in the food industry. More recent uses for lactic acid have also ecological importance and involve production of the nonchlorinated solvent ethyl lactate and the biodegradable plastic polylactic acid. Polylactic acid is a polymer whose properties are similar to those of polyolefins, and it could replace a significant portion of the polyethylene terephthalate-based polymers, which has a demand of 15 millions tons per year worldwide (Skory, 2000). Lactic acid can be synthesized chemically, but such a process will result in a racemic mixture (Skory, 2000). The bacterial fermentation is another alternative to obtain this consumable and is employed in the industry, by the use of, *Lactobacillus, Streptococcus, Lactobacillus bulgaricus, Peidococcus.* The results of microbial fermentation depend on the organism used, and may be a mixture of two isomers of lactic acid or one of the stereoisomer, only. Although the desired stereotype is related to intended use, L(+)-form of the lactic acid is desired for most of the applications.

Rhizopus oryzae, was first found as a lactic acid producer, in 1936, by Lockwood et. al. in that it produces the L(+)- isomer of the lactic acid, in a chemically defined medium under aerobic conditions. An increasing attention has been drawn to Rhizopus spp, because of the ability of this

fungus to utilize both complex carbohydrates and pentose sugars, to form only L-form lactic acid, in that, the product purification becomes easier, and it can grow on less expensive media. *Lactobacilli*, in contrast, require medium components supplemented with complex additives, which makes the cost higher and the process of product purification complicated.

Rhizopus oryzae cells can produce more than 1.5 moles of lactic acid from 1 mol of glucose under aerobic conditions (Skory, 2000). The remaining glucose is converted to mycelial mass, ethanol, glycerol, or fumarate. Although the production yield of *R. oryzae* based on the total carbohydrate consumed is relatively low when compared to that produced by lactic acid bacteria (Hang, 1989), those competitive advantages of *R. oryzae*, described above, motivated the researches to focus on this fungus.

The fungus was immobilized in continuos or batch reactors, to study lactic acid production (Hang *et. al.*, Kristofikoya *at al*, respectively). Submerged culture was also reported (Rosenberg *et. al.*). Hamamci *et. al*, in 1993, has reported an efficient production of lactic acid by immobilization of this microorganism in Ca^{2+}-alginate gels, using glucose as the carbon source.As a result, *Rhizopus oryzae* seem as an important potential producer of many industrially important enzymes, lactic acid, chitosan, and thus this microorganism has become a target for various research projects.

1.2. Lactate Dehydrogenase

1.2.1 Kinetics

The enzyme L-Lactate Dehydrogenase (E.C. 1.1.1..27) (LDH) catalyzes the final reaction of glycolysis, the formation of L-Lactic acid. The enzyme catalyzes a straightforward equilibration shown in Figure 1.4.

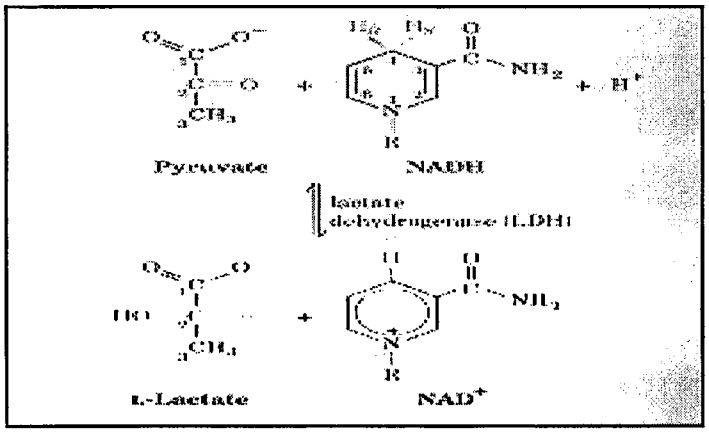

Figure 1.4: Reaction catalyzed by LDH

1.2.1.1. Substrate Specificity

L-Lactate Dehydrogenase is specific for L(+)-lactate and does not react with D(-)-lactate. It has known that, many LDH slowly oxidizes glyoxalate, glycerate and 3-halogen derivatives of L-lactate and 2,4-diketoacids. The potential of the enzyme to reduce 2-oxobutyrate, depends on the enzyme source. Hang *et.al.*, 1991, reported that, LDH from *Rhizopus oryzae*, have Km values of 6.4 and and 54.4 x 10^{-4} M for pyruvate and 2-oxybutyrate, respectively, indicating that the pyruvate was a more suitable substrate than 2-oxybutyrate for the enzyme.

LDH is specific for NADH not for the phosphorylated derivative NADPH (Koppenschaler, 1996).

The Km values for L-lactate and NAD^+ of the vertabrate enzyme are significantly higher than those for pyruvate and NADH, respectively. Yet, for *R. oryzae* the reaction is already irreversible (Pritchard, 1973).

1.2.1.2. pH and Temperature optimum

The pH-optimum of LDH is between pH 6 and pH 8 and depends in this range on the direction of the reaction and on the origin of the sample. For LDH from microorganisms, the pH optimum is shifted more to acidic range (pH 4.5 to pH 6). The optimum temperature was determined for most of the enzymes between 39 and 45 °C. The optimal pH for LDH from *R. oryzae* was reported as 7.5 by Hang *et al.*, 1991, and Pritchard in 1973 reported that the LDH of *R. oryzae* was optimal at pH 7.2 in 0.1 M phosphate buffer. In 1966, Obayashi, *et. al.* has found that, LDH of *R. oryzae*, lost its activity completely in 7 hours at 4 °C in phosphate buffer. The enzyme has found to be very stable at 30 °C, and activity was reduced to 55 % after 1 hour at 45° C, and at 55 °C activity was almost completely lost after 30 min (Hang, *et. al.*, 1991).

1.2.1.3. Activation

Mammalian LDH is activated by 2-amino-2-methyl-1-propanol, diethanolamine, fluoride, and heparin (Kopperschläger, 1996). Sodium sulphite protects the enzyme form the inhibitory effects of thiol attacking reagents, while mercaptans reverse the inhibitory effects of them. LDH from various bacteria, is activated by fructose 1,6-biphosphate, fructose 2,6-biphosphate, and glucose 1,6-biphosphate (Kopperschläger, 1996). Bivalent cations like Mn^{2+}, Co^{2+}, Ca^{2+}, situmulates some enzymes from microbial sources.

1.2.1.4. Inhibition

Mammalian LDH is generally inhibited by, pyruvate, high concentrations of lactate, NAD^+ and various carboxylic acids, particularly oxamate and oxalate.

The susceptibility of lactate dehdrogenase to inhibition by pyruvate was noted by Kubowitz and Ott. All enzymes isolated are inhibited by pyruvate, although, in general the effect is more pronounced with the heart than the muscle isozymes. The pyruvate concentration required for

inhibition is less at low pH than at high pH. Molecular investigation of the inhibition was initiated by Fromm's discovery that the enzyme forms a complex with NAD^+ and pyruvate which has an absorption band at 325 nm and a protein fluorescence of 0.12 relative to apo-protein. A similar compound can be formed if NAD^+ is replaced by 3-acetylpyridine-AD^+ which has an absorption band at 355nm. Subsequent structural investigations have been summarized by Kaplan and co-workers. Two observations show that formation of LDH:NAD-pyruvate complex is the cause of inhibition by high concentrations of pyruvate. First, the dissociation constant of pyruvate from the complex is similar to the inhibition by pyruvate appears slowly and correlates well with the formation of the 325-nm absorbing compound Coulson and Rabin showed that enol-pyruvate was the effective inhibitor and suggested that it forms a compound with enzyme-bound NAD^+ (The enzyme-bound complex is extremely stable). Even up to 0.1 M pyruvate there does not appear to be a site on the enzyme-NAD^+ complex which reversibly binds enolpyruvate (Kopperschläger, 1996).

LDH is inhibited by very high concentrations of lactate. As with most inhibitors, the H_4 isozyme (K_i = 26 mM) is more sensitive than the M_4 isozyme (K_i=130 mM). The variation of this inhibition with pH has not been well studied, and at low pH the situation is unclear since pyruvate is a common impurity in lactate. Taking typical assay conditions (10 mM lactate and 10 mM NAD^+ at pH 6), then only 15 mM pyruvate and NADH are produced at equilibrium. Hence, even slight impurities of pyruvate at this pH will give reduced initial rates. Inhibition may result from the formation of abortive ternary complex NADH-E-lactate. NADH in this complex is twice as fluorescent as in E-NADH.

Oxamate was found to be a competetive inhibitor of pyruvate, and oxalate competes with lactate. Both inhibitors can form ternary complexes (1:1:1) with LDH-NAD^+ and LDH-NADH, respectively. No inhibition was observed in the presence of cyanide and EDTA (Kopperschläger, 1996). LDH of *R. oryzae* was inhibited completely by Pb^{2+}, Fe^{2+}, Zn^{2+}, and Cd^{2+} but not by EDTA indicating that metal ions may not be required for the activity (Hang, 1991).

The oxidation of lactate to pyruvate is inhibited by NAD^+-derivatives which form complexes with enzyme bound NAD^+. The reduction of pyruvate is inhibited by NADH-derivatives which can

be formed, in particular during storage of phosphate buffered NADH solutions (Kopperschläger, 1996).

1.2.1.5 Reaction Mechanism

The mechanism of dehydrogenases is related to chemistry of the pyridine coenzymes. α-Hydroxy acid dehydrogenases, such as lactate dehydrogenase, catalyze the oxidation of alcohol functionalities. This group of dehydrogenases is most extensively studied, and their mechanism should be applicable to the machinery of catalysis for other enzymes.

The phrase 'induced fit' could stand for the mechanism of LDH, since the substrate binding triggers an essential conformational change in the formation of the active ternary complex. In fact, the studies about LDH catalytic mechanism show that how a protein catalyzes a normally unfovarable reaction.

As a result of kinetic and equilibrium binding experiments, it was reported that, first the coenzyme binds then the substrate binds to the active site of the enzyme. The rate limiting step in the oxidation of lactate at saturating concentrations of substrates is the rate of dissociation of NADH from the binary complex. In the reverse direction, the rate limiting step is either associated with the redox reaction or with with the dissociation of the enzyme-NAD^+ complex (Kopperschläger, 1996).

A schematic summary for the reaction mechanism of lactate dehydrogenase is shown in Figure 1.5, below. According to this figure, The imidazole ring of His-195 interacts with substrates and serves for two main functions: (1) proton donor / acceptor in the redox step, as stated in the paragraph above and (2) proper substrate orientation for its interaction with the C_4 of NADH. Whenever the substrate are bound, Asp-168 interacts with His-195 to stabilize the protonated form. Arg-171 provides a strong two-point interaction with the sustrate carboxylate. Arg-109 serves to polarize the carbonyl bond of ketoacid promoting the hydride transfer to the carbon and proton transfer to the oxygen. Gln-102 and Thr-246 form the environment of the substrate chain and play a

role in the discrimination of the substrate. One of the features of the coenzyme pocket in LDH is the hydrophobicenvironment of the nicotinamide ring represented bye Ile250.

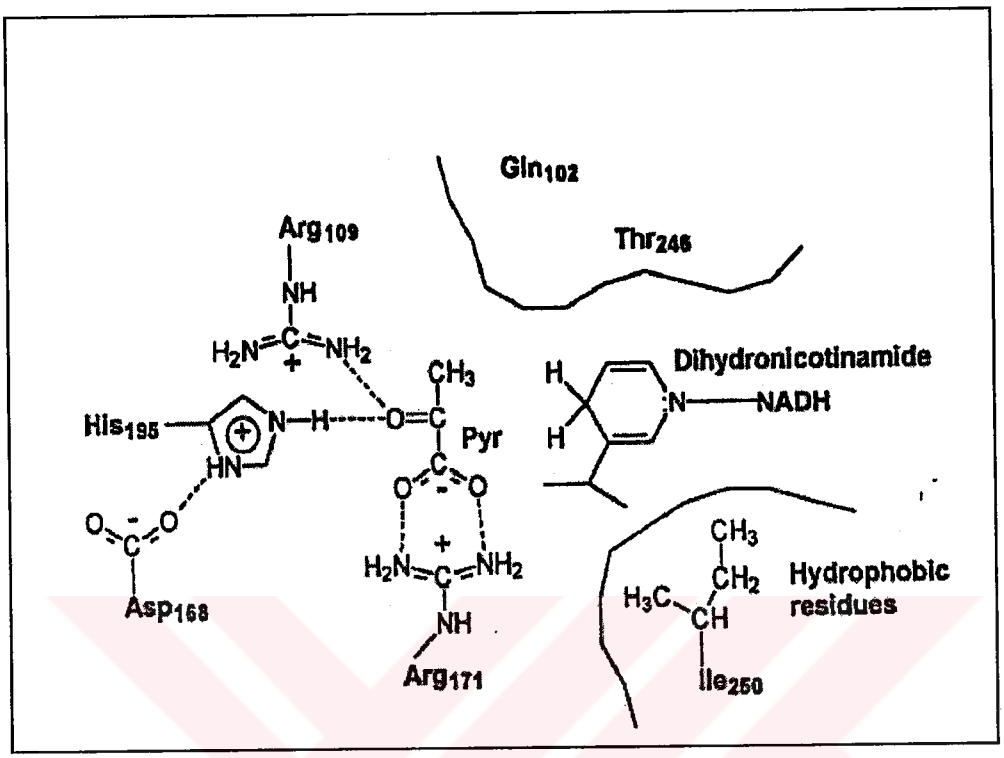

Figure 1.5: Catalytic mechanism of LDH, (Journal of Chromatography B, 684 (1996) Kopperschläger, *et. al.*)

This has been considered as an explanation for the tighter binding of the neutral NADH over the charged NAD^+.

As shown by fluorescence spectroscopy, the enzyme passes through the eight different kinetically identified intermediates, during the catalysis. The crystal structure of these intermediates, i.e., the enzyme, the enzyme-NADH complex, and the enzyme-NADH-substrate complex were analyzed. A number of essential elements concerning the reaction mechanism are well documented. Most of the rate acceleration performed by dehydrogenases is due to two key steps. In the direction of alcohol oxidation, dehydrogenases first promote formation of the equivalent of an alkoxide.

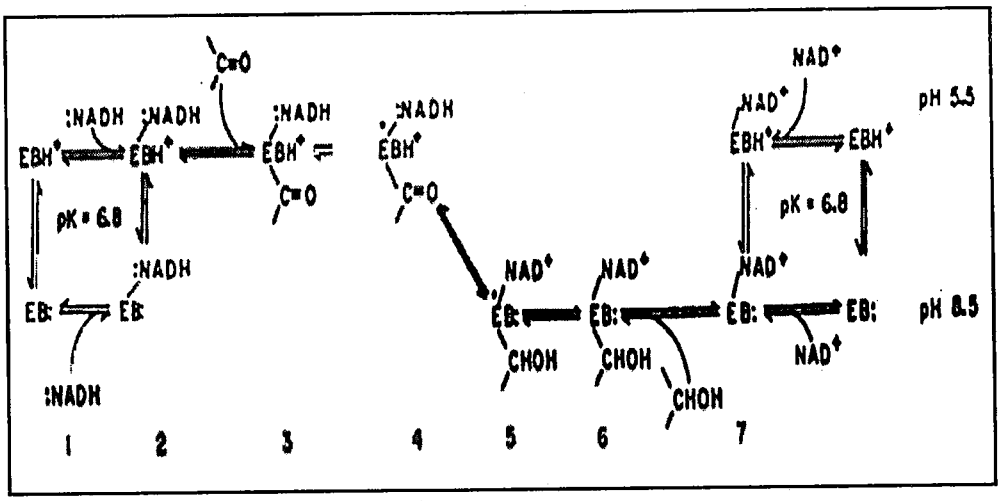

Figure 1.6: Reaction Mechanism for LDH activity

Then, that alkoxied, is juxtaposed to a nicotinamide ring. The mechanistic motif described for LDH, utilizes imidazole as an acid/base to promote transfer to or from the oxygen functionality, like the motif of glyceraldehyde-3-phosphate dehydrogenase. In carbonyl reduction, the conjugate acid of the imidazole increases the electron deficiency of the carbonyl through hydrogen bonding to the oxygen, that promotes hydride transfer from the dihydronicotinamide ring (Oppenheimer & Handlon , The Enzymes, Vol. XX, 1992). At the first stage, the affinity of enzyme for NADH is actually not much sensitive to hydrogen ion concentration over the pH range between 5.5 and 8.5. Binding of the coenzyme becomes weaker above pH 8.5 though there has not been any structural explanation for this, yet. Although crystallographic experiments show a conformational change on binding NADH, the kinetics data show that this change must be very fast. For a simplification, those fast conformational changes were not included in the **Figure 1.6 above.**

The binding of pyruvate to the binary E-NADH complex in the stage two, leads to the protonation of histidine-195 residue. The pk for this group is 6.8 in the apoenzyme and also in the e-NADH complex. Stabilization of this structure is shown in figure34b and according to this figure

N1 of histidine 195 residue act as a hydrogen bond donor to the main chain carbonyl of glycine-164 in the LDH:NAD^+-pyruvate complex. During the third stage in the Figure 1.6., the carbonyl group of pyruvate seems to form an ion pair with arginine-171 and the keto group of pyruvate forms a hydrogen bond with N2 of histidine-195. The formation of inactive ternary complex is fast. Because of similarities in fluorescence behavior, Holbrook and Stinson suggested that the structure of the active ternary complex in stage 4 is the same as that of the inhibitor complex with oxamate and thus identical with one of those complexes studied crystallographically. According to indirect evidences, once the inactive ternary complex is formed there is a slow process yielding the active complex. It could be argued that the fast formation of complex 3 is the binding of of the pyruvate to the binary complex and that the slow isomerization reflects the extensive conformational changes in the protein and coenzyme released by the movement of the loop.The chemical interconvension between the active ternary complex with NADH and pyruvate and the one with NAD^+ and lactate is a rapid equilibrium and needs only a transfer of electrons. Although the steps after the initial reduction of NAD^+ bound to the enzyme are less well understood, it seems plausible that the enzyme must undergo a reverse isomerization and conformational changesequivalent to steps from 4 to 5.

Now, lets consider some characteristic points that are useful in identifying the catalytic properties of LDH enzyme. One of these points is the 'activation of coenzyme by hydride transfer'. This activation is carried out by some interactions taking place during the process. These interactions involve, nonpolarity of dihydronicotinamide ring, solvation, direct electrostatic interactions, conformation of the side chain at C-3, conformation around the glycosyl bond,and the inductive effects directed at sugar moiety. If the propositions are true, then the nonpolarity of dihydronicotinamide ring 'puckering', seems to have an important impact. Since it was suggested by Vennesland and Levy, the potential involvement of nonplanar, puckered conformations of the dihydronicotinamide ring has been under considiration. Dehydrogenases can potentially activate the desired C-4 proton by forcing the ring to pucker in a direction that specifically weakens the C-H bond of the hydride to be transferred. Such a model has both experimental and computational support (Holbrook). However the nature of the distortion is unknown. Is the dihydronicotinamide ring intrinsically planar, but easily distorted, or is it intrinsically puckered, but with only a small

energy barrier between two rapidly interconverting forms. Calculations indicate that the dihydronicotinamide requires only 1.4 kcal/mol to distort from planarity to 160°.

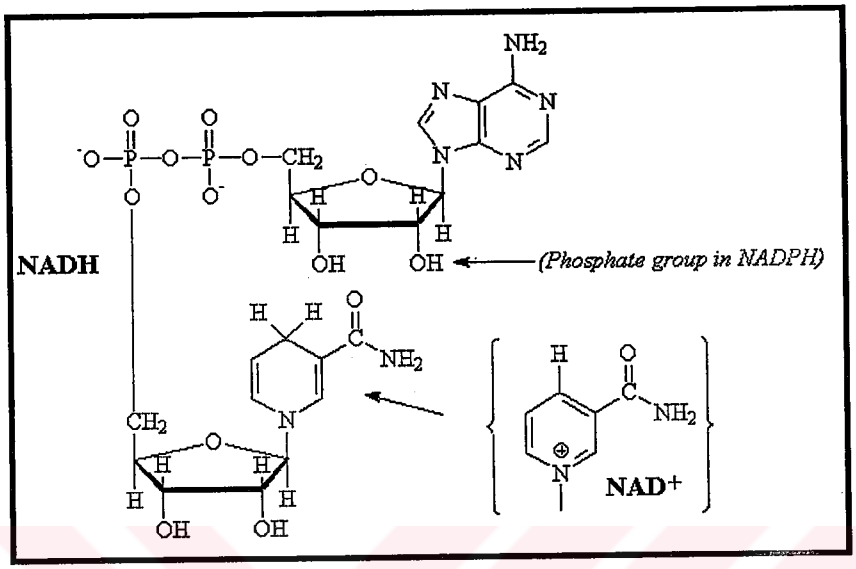

Figure 1.7: Structure of Nicotinamide adenine dinucleotide (NADH).

Puckering of the dihydronicotinamide ring can lead to activation of the axial proton at the C-4 position.

With such a small difference in energy there is little hope to distinguish the two models experimentally; even the unambiguous observation of a puckered from in an active site could be accounted for by either model.No significant departure of the dihydronicotinamide ring from planarity has been observed from X-ray crystallography studies. These negative results are inconclusive. The molecules studied all are symmetrical to internal reflection through the plane of dihydronicotinamide ring; thus in the absence of highly stable puckered forms, both faces will have identical interactions in the achiral crystal lattice, which decreases the likelihood of observable

puckering of the ring and stereoselectivity of hydrogen abstraction are observed for model compounds where dihydropyridine ring is rigidly held against a planar group. The current resolution of X-ray crystallographic structures of reduced coenzyme bound to dehydrogenases is sufficient to provide direct evidence either for or against puckering. Furthermore, the methods of refinement used in previous analyses mathematically suppress puckering even if it were to occur.

1.2.2. Molecular Properties:

If we compare and contrast the primary and tertiary structures of LDH form different sources, we may conclude that all of these enzymes belong to an evolutionary family with a common ancestor (Rossmann, *et. al.*, 1975). Complete aminoacid sequences of LDH from various species are available in respecitve protein data banks. Not only the sequence but also the secondary structure, geometry concerning dihedral angles and bond lengths are available and can even be downloaded from 'structure explorers' or protein databanks through the Internet.

1.2.2.1 Composition:

The aminoacid composition of a number of lactate dehydrogenase are well documented (Rossmann, *et. al.*, 1975). Chemical modification of one cysteine per subunit results in a loss of activity. Hence cysteine in LDH has received a particular attention. No disulphide bridges have been reported for LDH. The enzyme does not contain any metals.

1.2.2.2. Aminoacid sequence; Primary Structure:

The complete sequence of dogfish M_4 LDH consisting of 329 aminoacid residues has been determined. Various N- and C- terminal sequences have been determined and show good homology. However residues from 298 to 315 in pig H_4 LDH show little homology with the dogfish M_4 LDH sequence and are difficult to accomodate in the dogfish LDH structure. Heart enzymes may all have one extra residue at the C-terminus compared to muscle enzyme Arginine peptides isolated from pig M_4 and H_4 as well as from chicken M_4 are complete agreement with the sequences from Arginine 101 to arginine 115 in the dogfish M_4 enzyme. Possible homologies, where only composition is known, are also inidicated. Many types of reagents have been used to identify residues in the active site of the enzyme have modified an essential Histidine residue in the pig H_4 enzyme and isolated the labelled peptide. The sequence of this peptide is in very close agreement with residues 191-203 in the dogfish M_4 enzyme.

1.2.2.3. Secondary Structure

The enzyme has an extensive amount of secondary structure. Ramachandran diagrams are also documented (Oppenheimer, 1992).

Estimates of the proportion of aminoacid residues located in the helices depend on the precise definition of secondary structure and vary around 40 %. Three different β structures are found in LDH and account for around 23 % of all residues. The amino terminal half of the molecule contains a six stranded parallel sheet whereas the carboxy terminal half of the subunit has two three-stranded antiparallel β structures. The parallel sheets which is mainly in the interior of the molecule, exposes two edges to the solvent and one to a subunit interface. Thus most of the residues in this structure are hydrophobic. LDH has a number of reverse turns (β Bends, $3_{10}-$ bends).

1.2.2.4. Tertiary Structure

Three dimensional structures of LDH have been solved from, dogfish, porcine mose, *Bacillus stearothermophillus*, and *Lactobacillus casei* (Kopperschläger, 1996). An unusual feature of the subunits is the N-terminal 20 residues that extend from the main 'body' of the subunit. This 'arm' is important in the interaction between subunits.

Subunit structure will be described in terms of (a) the amino terminal arm, (b) that part which binds the coenzyme and (c) tha part containing catalytic residues. The dinucleotide binding part is subdivided into two mononucleotide binding domains (B1 and B2) and these have been observed in other dehydrogenases. The catalytic part is also subdivided in to two domains.

The dinucleotide binding part consists of two mononucleotide binding domains each of similar structures. Domain B1 is associated with AMP binding and B2 with NMN binding. It is suggested that the two domains are the result of gene duplication. (Rossmann, *et. al.*, 1975). Domains B1 and B2 are releted by an approximate twofold rotation axis with a small translational element. This axis is located between β A and β D. Domain B1 is associated with AMP binding and B2 with NMN binding. It suggested that 2 domains are the result of gene duplication. Domains B_1 and B_2 are related by an approximate twofold rotation axis with a small translational element. This axis is located between Ba and BD. The loop and helix α D, which are between B D and alpha E are only found in the second domain and are intimately involved with the binding of phosphate moiety of the coenzyme.

The 3rd and 4th domains also show some degree of similarity as indicated by the distance. Each of these two domains could be described as a three-stranded antiparallel β structure with immediate returns from one strand into the next. This piece of polypeptide chain is preceeded and

followed by a helix. An important active site residue, Histidine-195, occurs between the two first strands of structure in domain C_1. There is no corresponding residue in domain C_2.

1.2.2.5. Quarternary Structure

The querternary structure varies depending on the source of the enzyme and sometimes, on the presence of structure- stabilizing effectors like the coenzyme or fructose-1,6-biphosphate. Both tetrameric and dimeric forms of the enzyme has found *in vivo*.

With respect to LDH of *R. oyzae,* it was reported that, this LDH is a tetramer, with a molecular weight of 131.000 and each of the subunits have a molecular weight of 36.000 (Hang, 1991). LDH, consisting of four subunits, with a molecular weight ranging form 135.000 to 150.000 have been found in *B. subtilis, P. blakesleeanus* and in many animal tissues (Yoshida, *et. al.;* 1965, Soler *et. al.*; 1982, Everse and Kaplan, 1973, respectively).

The first description of *ldh* genes isolated from a fungus, has been obtained from *R. oryzae,* recently (Skory, 2009). In this study, a unique phylogeny of these genes with those of other LDH's has been reported: At least three different LDH are produced by *R. oryzae.* Two of them *ldhA* and *ldhB*, are NAD^+ dependent, where as the third one is estimated as a mitochondrial NAD^+-independent LDH, which is used for oxidative utilization of lactate. It was also reported that *ldhA* is most probably responsible for most of the lactic acid produced by *R. oryzae.* It was also hypothesized that *ldhB* gene, may function in conversion of storage sugars to lactic acid, since a transcript of *ldhA* was present even with nonfermentable carbon sources, such as glycerol and ethanol (Skory, 2001).

1.2.2.6. Isoenzymes

The concept of multiple molecular forms of LDH (isozymes) by Market & Moller has stimulated many investigations into the nature, function and control of isozymes.

Although there are only two major structural genes corresponding to the M and H chains, there is a complex variety of other *ldh* genes which can be expressed in some tissues at certain stages of development.

Those isozymes are distinguishable by their chemical composition and their kinetic and immunological properties.

The difference in isozyme composition in various tissues has been coprrelated to different metabolic requirements.

1.2.3. Methods for the Purification of LDH

As being an intra-cellular enzyme, LDH is located in the cytoplasm, and can be released in to solutions by mechanical disruption of the cells or osmatic shock. Since the first isolation of the enzyme by Straub, 1940, applying Ca^{2+}-phosphate adsorption, acetone and ammonium sulfate precipitation, many purification protocols has been improved continuously.

1.2.3.1. Ion Exchange Chromatography

The introduction of ion exchange chromatography has been considered as a milestone in the purification of the enzyme. For instance, by using QAE-cellulose as adsorbent and an elution buffer of pH 7.4, a stepwise fractionation of all five LDH isoenzymes can be performed by increasing the

ionic stregth. Alternatively, the five isoenzymes have been eluted from DEAE cellulose columns with increasing salt concentration and decreasing pH.

The HPLC version of protein ion-exchange chromatography has also been applied for the purification of LDH, such that a higher resolution and a faster implication was obtained. For example, LDH isoenzymes were successfully fractioned by anion-exchange chromatography on Synchropak AX-300 (Kopperschläger, 1996).

Chromatofocusing, a recent ion-exchange choromatography technique in protein purification, has been expoited for the LDH purification in that a high resolution and an efficient purification yield of the enzyme from LDH, was obtained (Hang, 1991). Pritchard (1973), reported a three fold level of purification from DEAE-cellulose column following Ammonium sulfate fractionation.

1.2.3.2. Affinity Chromatography:

Studies, concerning the spacer length between the ligand and the matrix, were well documented (Kopperschläger, 1996), and for the binding of LDH in to an affinity column, an extension length of 0.7-1.0 nm was found optimal. This was achieved by coupling the nucleotide via a 6-amino-hexanoyl-group to the sepharose matrix. As an alternative, Oxamate, the pyruvate analogue, has also been coupled to sepharose resulting in a similar binding of LDH, as in the case of NAD^+.

The bound LDH can be released from the column, by the addition of NADH (0.1-1 mM) or NAD^+, but the former is better. On the other hand the effect of the latter, can be drastically enhanced when the NAD^+-containing buffer is completed with sulphite, lactate pyruvate or oxamate that give rise to form strong ternary complexes with the enzyme (Kopperschläger, 1996).

Differentiation of various dehydrogenases can be achieved by choosing the correct nucleotide and and concentration to desorb the enzymes specifically from columns which were loaded with a rough tissue extract. For instance, LDH and glyceraldehyde-3-phosphate dehydrogenase have been separated in a column of N6-(6-aminohexyl)-NAD^+-sepharose by eluting the former enzyme with 0.15 mM NADH, and 0.15 mM NAD^+, respectively.

The differences in the affinities of LDH isoenzymes for the nucleotide substrate can also be exploited for the separation of multiple molecular forms of LDH. The relative affinity of NADH in the solution correlates with the order of the elution of the five LDH isoenzymes.

Although affinity chromatography with immobilized ligands, exhibits a fascinating, effective, and contemporary approach, its large scale application, however is confined by the high costs of labelled matrices and instability of most of the ligand molecules.

A special branch of affinity separation technique, on the other hand, has been developed with the discovery of reactive textile dyes exhibiting a pseudo-biospecific affinity to many enzymes and other proteins. These dyes are, fortunately, available as low cost chemicals in large amounts. Since they are reactive, they are easy to couple to varios matrices under mild conditions. Many of these dyes show a certain bioaffinity especially to dehydrogenases, kinases and other nucleotide-dependent enzymes.

The affinity of LDH to Blue Dextran, a conjugate of dextran and Cibacron Blue F3G-A, was first reported by Stellwagen et. al. (Kopperschläger, 1996), who could demonstrate the reversibility of binding by eluting the enzyme from a Blue Dextran-Sepharose column with NADH.

Another group of ligands are immobilized metal-chelates, and a procedure describing the purification of LDH from chicken muscle in one step by appliying a Ni^{2+}-IDA-Sepharose column has been reported (Kopperschläger, 1996).

1.2.3.3. Affinity Precipitation:

This technique combines a classic step of protein enrichment with the principle of biorecognition. The use of this approach has been demonstrated with a bifunctional NAD^+-derivative, bis-NAD^+. This compund was mixed with certain NAD^+-dependent dehydrogenases and the result is the quantitative precipitation of the enzymes. LDH's tetrameric structure seems to fit well to this technique in that, each of the four subunit has potentially the ability to form complex with the half of bis-dinucleotide. Then, the aggregate formed, becomes insoluble and precipitated. LDH of bovine heart, can be precipitated directly form the crude extract by this way (Kopperschläger, 1996). In comparison with other affinity separation techniques, the equipment demand for affinity precipitation is low, the operationspeed high and the specificity is satisfactory.

However, the large scale applicability of this technique is restricted by the expense and lability of the NAD^+-derivative.

Another affinity precipitation of LDH was developed by Guoqiang et.al. and called as affinity thermoprecipitation. In this technique, a copolymer, Eudragit, was used as a carrier for the covalent binding of Cibacron blue F3G-A. The solubility properties of this polymer depends on the pH of the medium, being soluble above pH 5.8 and insoluble at pH 4.8 and below. Moreover, the polymer can be precipitated with Ca^{2+}, of high concentarion and this effect was enhanced, at higher temperatures. After coupling the dye to the matrix, the efficiency of precipitation at low pH could be enhanced by temperature increase with low Ca^{2+} concentrations. Affinity precipitation of LDH has been also successfully elaborated with commercial soluble Blue Dextran. After the complexes are formed, they were precipitated, by addition of the lectin, concavalin A ConA, which binds the dextran residues and induces precipitation. The efficiency of precipitating enzyme was 85 %. Dissociation of the bound LDH was carried out using 1.5 M KCl.

1.2.3.4. Affinity Partitioning in Aqueous two-phase Systems:

Affinity two-phase systems have been used for more than three decades in biochemical research as tools for fractionation of proteins, nucleic acids, cell particles and cells. The partitioning of the proteins depends on the chemical nature of two phase forming polymers, their molecular weights, and their concentrations as well as ionic strength and pH of the medium. In addition, conformation, size and hydrophobicity of proteins affect the partitioning behavior of proteins. A number of reactive dyes was screened to optimise affinity partitioning of LDH for a purification. Therefore, Procion Yellow HE-3G coupled to PEG 8000 has been selected as ligand, and LDH from crude muscle extract was purified in a single step, with a 20.6 fold purification, and 79 % yield (Kopperschläger, 1996).

In conclusion, besides to many mammalian tissues, LDH , was purified and characterized from many bacterial species, particularly in the genera Streptococcus, Lactobacillus, Leucocnostoc, Pediococcus, which are known as lactic acid bacteria. A number of purification protocols for microbial LDH comprise such as ion-exchange chromatography, and affinity chromatography appliying AMP, oxamate and dyes as ligands.LDH from many plants like potato tubers, roots and lettuce have also been purified and characterized (Kopperschläger, 1996) .

1.2.4.The Importance of LDH

Commercial LDH from muscle or heart is prefarably used for the quantitative determination of various metabolites like L-alanine, citrate, creatine, creatinine, succinate, triglycerides, lecithin, D-sorbitol, sialic acid, glycerol, D-glycerate, L(+)-lactate and pyruvate. The lactate/pyruvate quotient is an important parameter in the diagnosis of peripheral hypoxia (shock, respiratory insufficiencies). Moreover, the determination of lactate is important in clinical biochemistry indicating lactic acidosis or liver and kidney insufficiencies, and for the medical control of athletes in competetive sports. In addition to these LDH is necessary for microbiology, food, cosmetics and paper industries.

Moreover, LDH is an important auxillary enzyme for the determination of many enzymes such as alanine aminotransferase, ATPases, adenylate kinase and pyruvate kinase.

1.3. Aim of the Study

Our aim is to test and optimise the previously used methods for the purification of LDH from *R. oryzae*. Our study is necessary as a crucial part of understanding the glycolytic flux of filamentous fungi, by which lactic acid is produced. Moreover optimization of enzyme assays will lead other investigations to become clarified.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

NADH and Pyruvate were obtained from Roche. Polybuffer exchanger 94, Polybuffer 74 and Sephadex G-25 were supplied from Sigma Chem. Ltd, Germany. All other chemicals used for analytical grade were ither from Merck or Sigma Chem. Corp.

2.1.2. Microrganisms

A strain of *Rhizopus oryzae*, ATCC 9363 was used in the present study. Organism was sporulated on agar at 30 C. After the completition of sporulation, the spores are then, transferred to 500 mL erlenmayer flasks containing 200 mL nutrient broth, with a 2 % glucose. The flasks are then incubated by shaking at 175 rpm and 30 °C for 18 hrs. The medium compositions are given in Table 2.1 and Table 2.2

Table 2.1: Nutrient Agar medium composition for *R. oryzae*

Chemical	% (w/v)
Ammonium sulfate	0.2
Potassium phosphate	0.065
Magnesium sulfate	0.025
Zinc sulfate	0.005
Glucose	0.5
Agar	2

Table 2.2: Nutrient broth medium composition for *R. oryzae*

Chemical	% (w/v)
Sodium chloride	0.5
Tryptone	1
Yeast extract	0.5
Glucose	0.5

2.2. Methods

2.2.1 Purification of LDH from *R. oryzae*

Purification of LDH from *R. oryzae* have been performed by Ammonium Sulfate precipitation, Gel Filtration and Chromatofocusing techniques.

2.2.1.1. Preparation of Crude Extract

All steps during preparation of crude extract was carried out in ice bath, assuring of a temperatureof 0-4°C.

Mycelia were harvested by filtration and washed twice by ice-cold distilled water. Following the absorbance of excess humidty by a filter paper, 5.8 g mycelia were suspended in 86 mL of 0.1 M pH 7.5 potassium phospahate buffer containing 10 % glycerol, 2mM EDTA and, 5mM DTT (Extraction Buffer). PMSF was added freshly to obtain 1 mM of final concentration before homogenization of fungal cells by sonication. Then, the mixture was sonicated with a 10 μ amplitude of sonicator Soniprep 150, for 3 x 60 seconds with thirty seconds intervals. Cell homogenate was then clarified by centrifugation at 23.000 x g, for 20 mins at 4°C by a Sorvall GSA 10 type centrifuge rotor. To obtain a completely clarified solution, the supernatant was finally filtered by cheese-clot and referred to as 'crude extract'.

2.2.1.2. Ammonium Sulfate Fractionation

The crude extract was brought to 30 % saturation by addition of ammonium sulfate, and stirred for at least 6 hours at 4° C. Then the solution was centrifuged at 23.000 x g for 30 mins at 4 °C. The pellet discarded and solution was then, brought to a 60 % saturation and stirred overnight at

4° C. The precipitant was collected by centrifugation at 23.000 for 30 minutes at 4° C. The pellet can be stored at 4° C for at least 2 weeks for furter treatments.

2.2.1.3 Sephadex G-25 Gel Filtration

The 30-60 % Ammonium Sulfate precipitant was suspended in extraction buffer and desalted through Sephadex G-25 column with a size of (2 x 14) cm, with a flow rate of 0.6 mL/min with 25 mM Imidazole-HCl buffer pH 7.4 (Starting Buffer) at 4 °C. The active fractions(each with a volume of 1.8 mL) were collected and applied to a PBE 94 column.

2.2.1.5. Chromatofocusing

All of the procedure was carried out at 4 °C. For chromatofocusing, a 1x 16 cm chromatography column was packed by Polybuffer Exchanger 94 (PBE 94), and equilibrated by starting buffer described above, until the pH in the outlet of the column is the same as the buffer. For elution, Poly buffer 74 (Elution buffer) diluted to 1:8, and equilibrated to pH 4 by 1M HCl, was used. Before the application of the desalted sample, 5 mL of Polybuffer 74 was applied to the column, to prevent sample from the extreme pH levels during elution. After this 10 mL of the sample was aplied to column and eluted with polybuffer 74 with a flow rate of 25 mL/hour. Fractions of 2 mL were collected and assayed for LDH activity, besides, protein profile and pH profile of fractions were also determined.

2.2.3. Analytical Methods

2.2.3.1 Enzyme Assays

Lactate Dehydrogenease activity was detected by measuring the decrease in the absorbance at 340 nm associated with NADH oxidation by a Shimadzu 2100 model spectrophotometer. The

standard assay system described by Hoffman and Hanson (1986) was composed of, 0.1 M tris HCl buffer pH 8, 5 mM of Na-pyruvate, 0.07 mM of NADH and appropriately diluted enzyme solution in 3 mL reaction mixture. The reaction was started by the addition of pyruvate to reaction mixture after the elimination of any NADH oxidase activity in the reaction mixture at 30 °C for 15 minutes.One unit of L-Lactate dehydrogenase activity was defined as the amount of the enzyme that causes 1 µmole of NADH oxidation per minute under assayed conditions. The specific activity of the enzyme is expressed as units per mg of protein.

2.2.3.2. Protein Determination

Determination of protein concentrations of the samples during purification procedure, was performed in accordance with the method of Bradford's Dye Binding Method (1980). BSA was used as a standard protein. The composition of reagents and the details for standard curve is given in Appendices A and B.

2.2.4.Characterization of LDH

Preliminary characterization of LDH is achieved by the use of kinetic and physical data.

2.2.4.1. Kinetic Parameters

K_m and V_{max} values of LDH was determined by measuring initial velocities at different substrate concentrations . Na-pyruvate, ranging from 0.02 mM to 1 mM concentrations, was studied in the presence of 0.167 mM NADH concentration in a 3 mL assay reaction mixture. NADH, ranging from 0.01 mM to 0.2 mM concentrations, was studied in the presence of a constant concentration of 0.2 mM pyruvate. Assays were performed at 35 °C and pH 7.5 in the crude extract and enzyme concentration was 0.073 mg/mL.

2.2.4.2 Electrophoretic Analysis

SDS-PAGE, were exploited to determine the degree of purification at the end of the each fractionation step. The details of the procedures as well as reagents, buffers and solutions used during SDS-PAGE and are given in the Appendices C and D. In accordance with the procedure of Laemmli (1970), the native The SDS-PAGE gels were prepared as 4 % stacking and 12 % separating. Protein samples were solubilized in the sample buffer (Appendix D). Markers used for the molecular weight determination are given Appendix D. Markers were loaded in tracking buffer containing 0.0025 g bromophenol blue as tracking dye. Separation was achieved at constant current such that 10mA in stacking gel and 20 mA in separating gel at 4 °C.

2.2.4.3. Gel Processing

Before the slab gels were silver stained, they were immersed in fixative solution containing 50 % methanol, 12 % acetic acid, and 0.5 ml of 37 % formaldehyde. Shaking of the slab gels were performed on a platform shaker for at least 1 hour.

2.2.4.3.1. Silver Staining

The silver staining of gels was performed according to the procedure of Blum et. al. (1978). Reagents used and the experimental details were outlined in Appendix D. After the completition of the protocol the photos of the gels were taken and the measurements were done. The relative mobility (R_f) of each of the protein was determined by distance migrated by protein divided by distance migrated bye tracking dye from the top of the stacking gel. The R_f values were plotted against molecular weight of markers to obtain a standard curve.

2.2.4.3.2. Gel Drying

The gels were dried and stored. This was done by placing the gels between two cellophane sheets which were soked with tap water. Then they were hung on a frame o/n for a complete drying. After that, the cellophane sheet was cut off in the desired dimesions and stored in a dry place.

CHAPTER 3

RESULTS

3.1. Purification of LDH

The L-Lactate dehydrogenase enzyme was isolated from *R. oryzae* by 30-60 % ammonium sulfate precipitation and chromatofocusing on PBE 94 column. Partially purified enzyme was characterized in terms of kinetic parameters, and molecular weight .

3.1.1. Enzyme Assay

The enzyme activity, determined spectrophotometrically by following NADH oxidation per minute, was specified at 30 °C and pH 7.5. One unit of L-Lactate dehydrogenase activity was defined as the amount of the enzyme that causes 1 μmole of NADH oxidation per minute under assayed conditions. The specific activity of the enzyme is expressed as units per mg of protein. In the Figure 3.1 the enzyme standard enzyme assay applied to the samples from crude extract, Ammonium sulfate Precipitant, G-25 Sephadex Gel Filtration, and Chromatofocusing are shown.

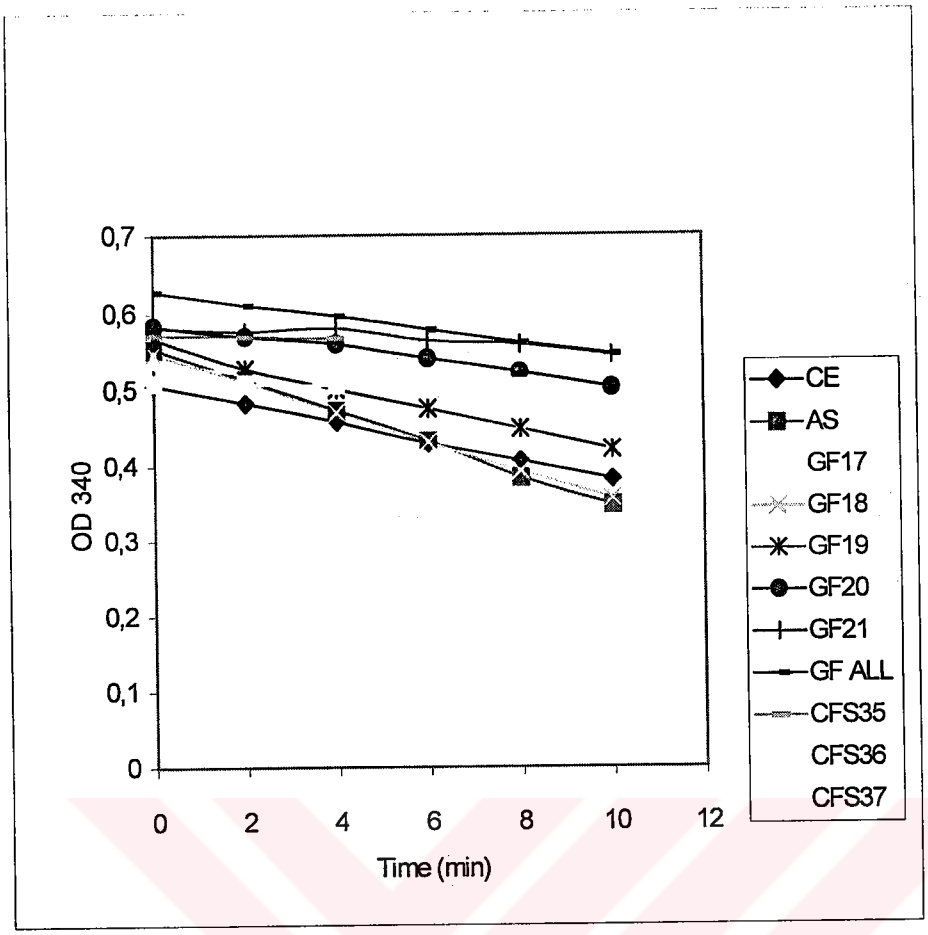

Figure 3.1: LDH activity determined at pH 7.5 and 30 °C in U/mL in crude extract (CE), 30-60 % ammonium sulfate fraction (AS), after Gel Filtration (GF), and Chromatofocusing (CFS). The numbers given in the left of the GF and CFS correspond to the fraction number pooled from each column. The assays were carried out at 30 °C and pH 7.5, with 0.07 mM NADH, 5 mM pyruvate, and 0.028 mg/mL enzyme sample.

3.1.2 Crude Extract

The Crude Extract, prepared as described in section 2, was assayed for enzyme activity and the specific activity of LDH was found as 0.0706U/mg of protein. Optimum temperature and pH determinations for the enzyme was carried out for the sample of crude extract, which are 35 °C and pH 7.5, respectively and shown in the Figures 3.2 and 3.3.

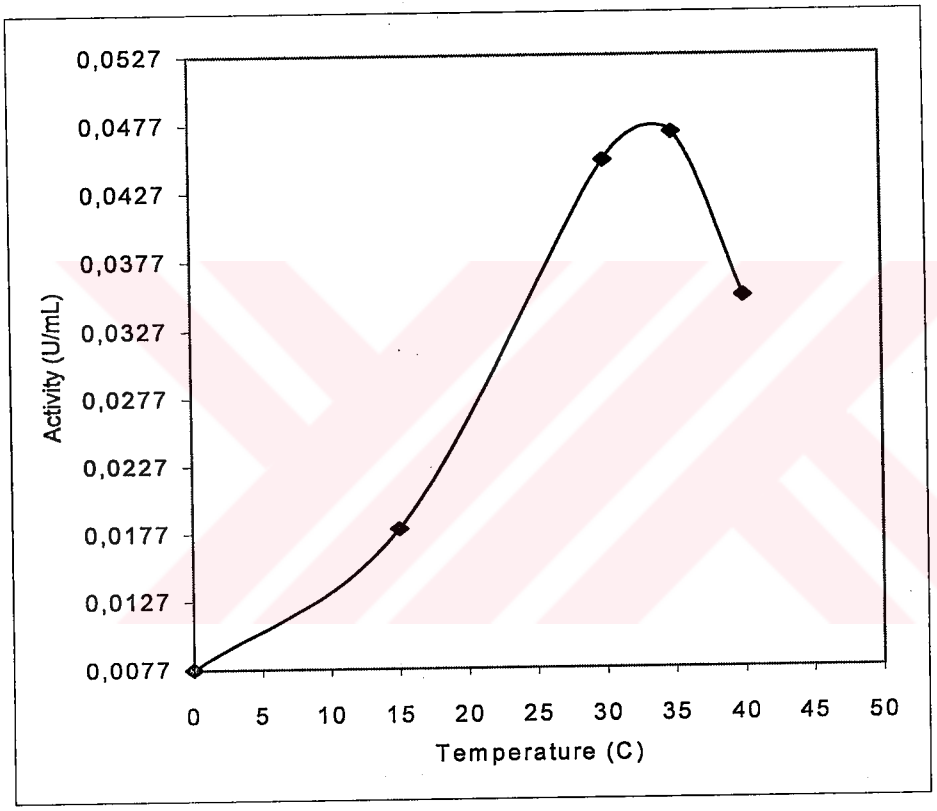

Figure 3.2: Optimum temperature for LDH Activity. Assay reactions were carried out at pH 7.5.

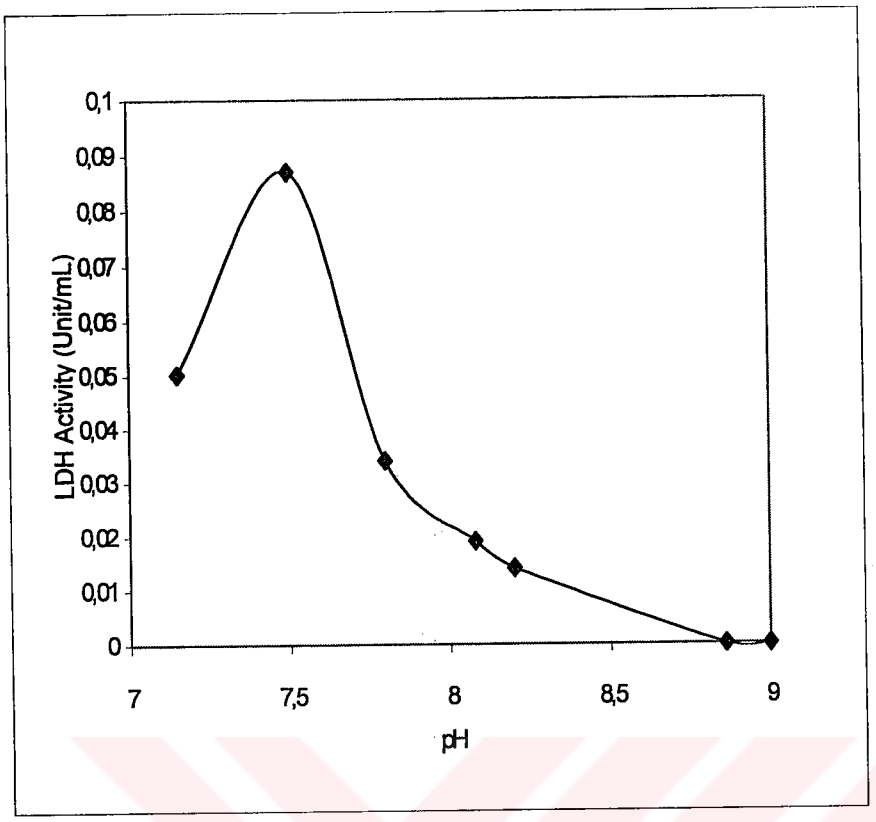

Figure 3 .3: Optimum pH for LDH Activity. Assay reactions were carried out at 30 °C.

3.1.3 Ammonium Sulfate Precipitation

30-60 % Ammonium sulfate precipitation was carried out to a total volume of 44 mL crude extract. The specific activity was found as 0.169 units/mg of protein, where a 2.39 fold of purification with a 75 % yield was achieved.

3.1.4. Gel Filtration

Sephadex G-25 gel filtration experiment was performed for preparation of the sample to chromatofocusing by removing ammonium sulfate and changing the buffer of the sample. The

starting buffer of chromatofocusing, 25 mM Imidazole-HCl, pH 7.4, was used both for column equilibration and elution during gel filtration. Elution conditions was described in Chapter 2 and the protein was eluted at the void volume. The specific activity was found as 0.15 units/mg of protein with a 2.13 purification fold. The Elution profile of the Sephadex G-25 Column is shown in the Figure 3.4.

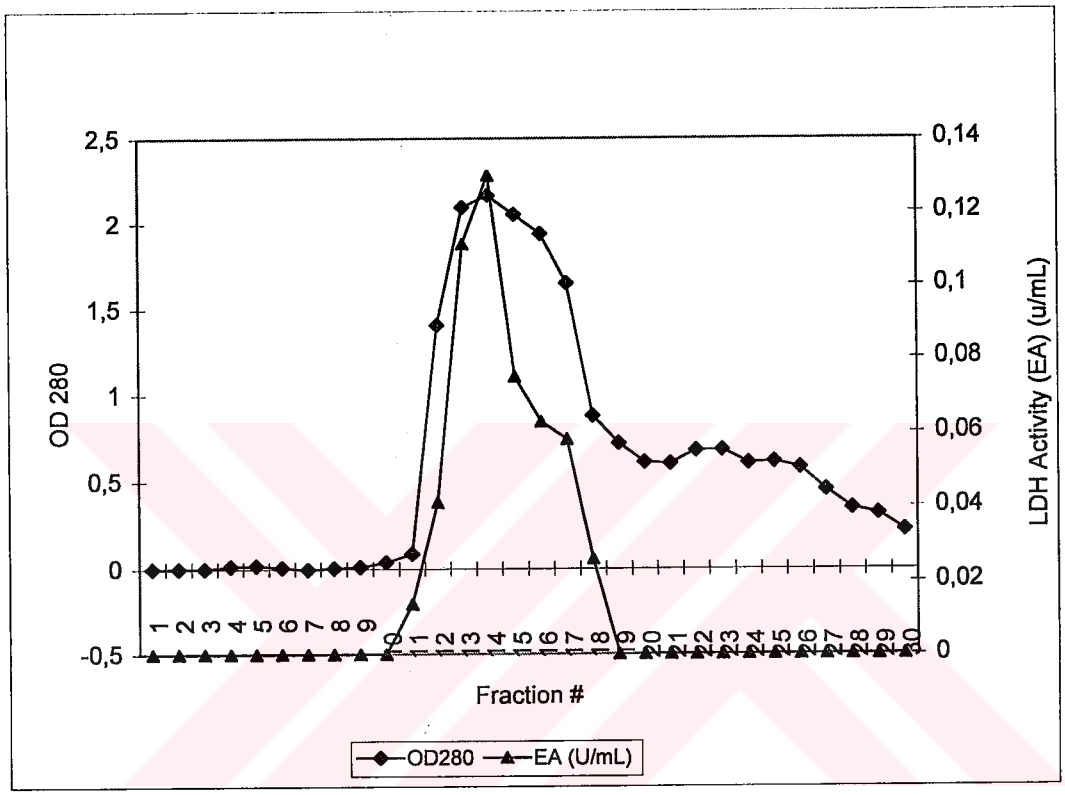

Figure 3 4 Elution Profile of Sephadex G-25 Gel Filtration, Flow rate: 0.7 mL/min, Column size: (2x 14) cm, Fraction volume1.6 mL

3.1.5 Chromatofocusing on PBE 94

The elution profile of Chromatofocusing on PBE 94 column was shown in the figure above, where the ezyme activity was detected at its isoelectric point, pH 5.2. The column was equilibrated with 25 mM Imidazole-HCl buffer, pH 7.4, Sample application was achieved by preliminary introduction of 5 mL of elution buffer to prevent sample from extreme pH levels during elution. Loaded sample was eluted by elution buffer: 12.5 % Polybuffer 74 pH 4. The purification table concerning all fractions are given in the Table 3.1.

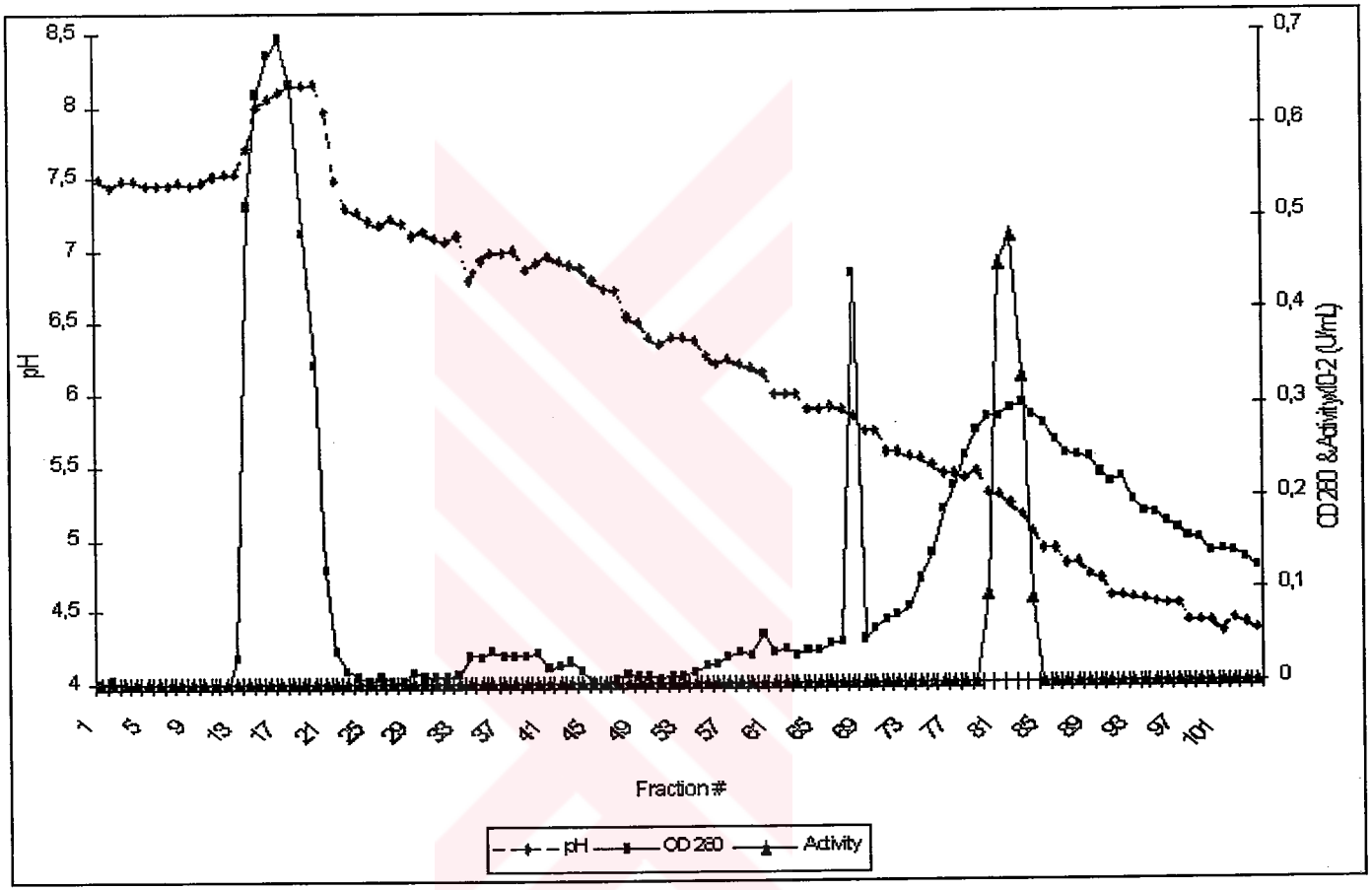

Figure 3.5: Chromatofocusing of LDH on PBE 94; Starting buffer 25 mM Imidazole-HCl pH 7.4, Elution Buffer 12.5 % PB 74, pH 4, Fraction volume: 2 mL, Flow rate:0.33 mL /min, Column size: (1x 16) cm. Column was equilibrated by 15 bed volumes of starting buffer. Regeneration is achieved by 2 -3 bed volumes of 1 M NaCl.

Table 3.1: Table of purification of LDH from *R. oryzae*

Fraction	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold	% Yield
1. Crude Extract	37.4	2.64	0.0706	1	100
2. 30-60 % ammonium sulfate	11.8	2	0.169	2.4	76
3. Sephadex G-25 Gel Filtration	3.24	0.49	0.15	2.12	18
4. Chromatofocusing	0.27	0.029	0.107	1.5	10

3.2. Characterization of LDH

3.2.1. Determination of Kinetic Parameters

3.2.1.1. Effect of Pyruvate Concentration on LDH Activity

Reaction velocity shows the following response to increasing pyruvate concentrations, in the Figure 3.6.

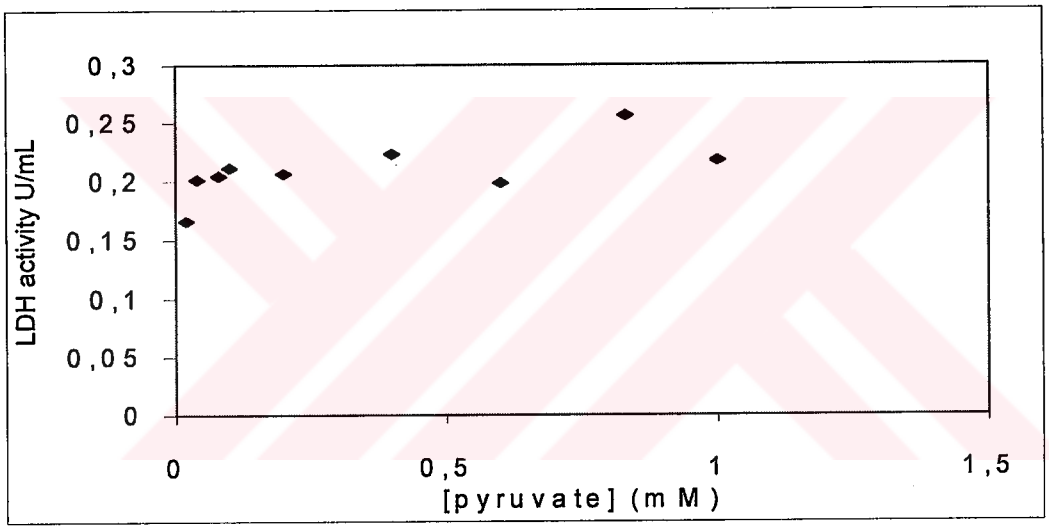

Figure 3.6: Effect of Pyruvate concentration on LDH Activity, in the presence of 0.167 mM NADH, at 35 °C , pH 7.5, in 3 mL reaction mixture.

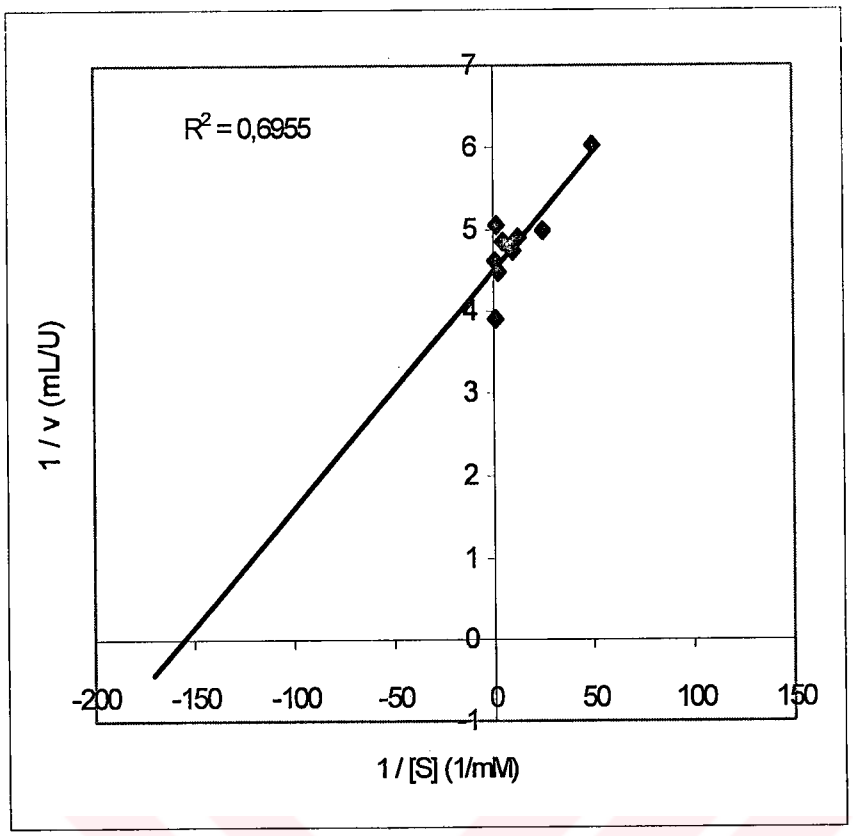

Figure 3.7. Lineweaver-Burk plot of LDH for pyruvate, in the presence of 0.167 mM NADH, at 35 °C, and pH 7.5.

From the Lineweaver-Burk plot shown in the Figure 3.7., V_{max} and K_m values were calculated as 0.223 U/mL, and 0.064 x 10^{-4} M, respectively.

3.2.1.2. Effect of NADH Concentration on LDH Activity

Reaction velocity reveals a sigmoidal response to NADH concentration as shown in the Figure 3.8. The reaction velocity deviates from Michaelis Menten kinetics so Lineweaver-Burk plot is not applicable. The Hill plot of the data is shown in Figure 3.9.

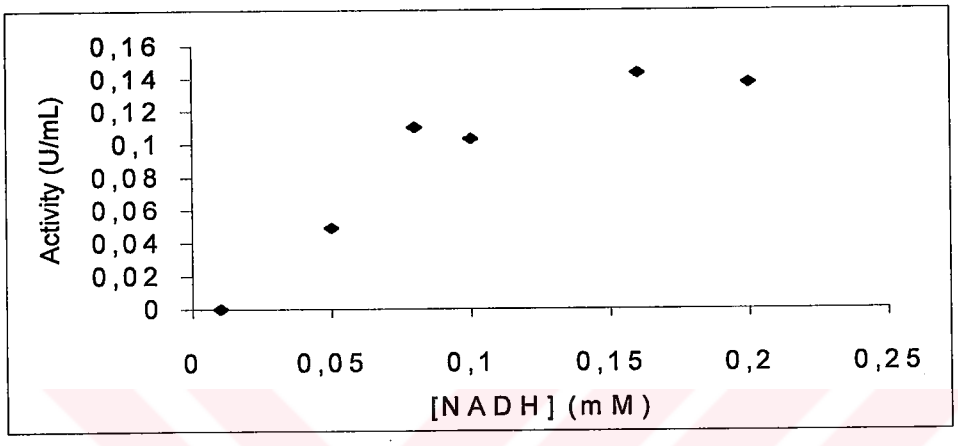

Figure 3.8. Effect of NADH concentraion on LDH activity in the presence of 0.2 mM Pyruvate at 35°C, pH 7.5 in 3 mL reaction mixture.

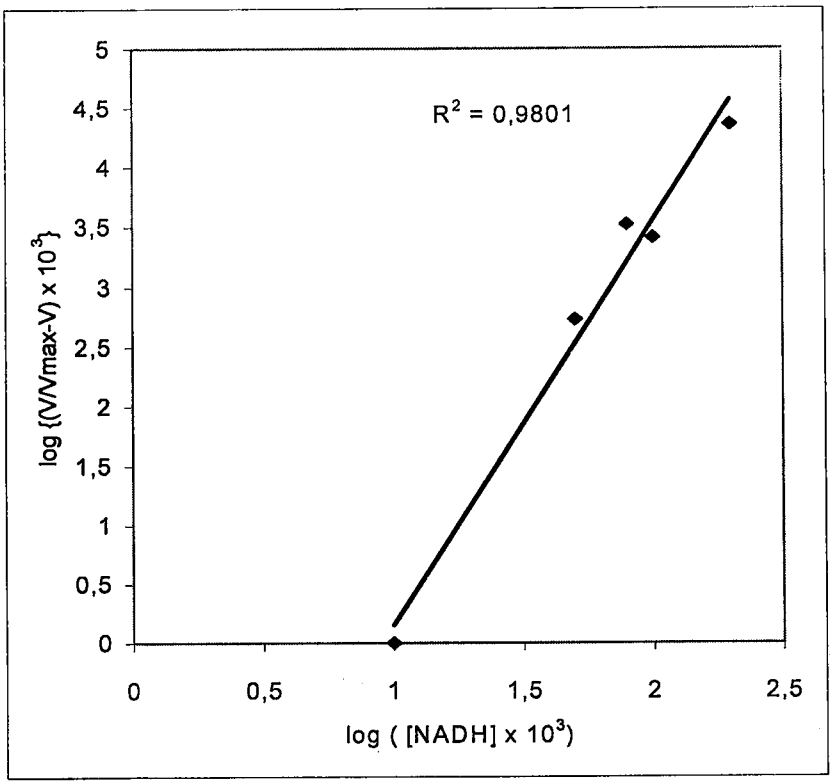

Figure 3.9: Hill plot of LDH activity in response to NADH concentration in the presence of 0.2 mM pyruvate, at 35 °C and pH 7.5.

Table 3.2. shows a summary of kinetic parametrs of LDH.

Table 3.2: Kinetic parameters of LDH

Substrate:	K_m (mM)	V_{max}(U/mL)
Pyruvate	6.44×10^{-3}	0.223

3.2.2. Determination of Physical Parameters

3.2.2.1. Electrophoretic Pattern of LDH Fractions

Samples obtained from crude extract, G-25 gel filtration and chromatofocusing fractions were loaded to SDS-PAGE gels and following pattern in the Figure 3.10 was obtained. Since the fractions of gel filtration and chromatofocusing are very dilute, only a little amount of protein could be loaded to wells for these samples. The details for SDS-PAGE and marker proteins are given in Appendix C. The standard curve for the marker proteins (lane 1) is shown in the Figure 3.11.

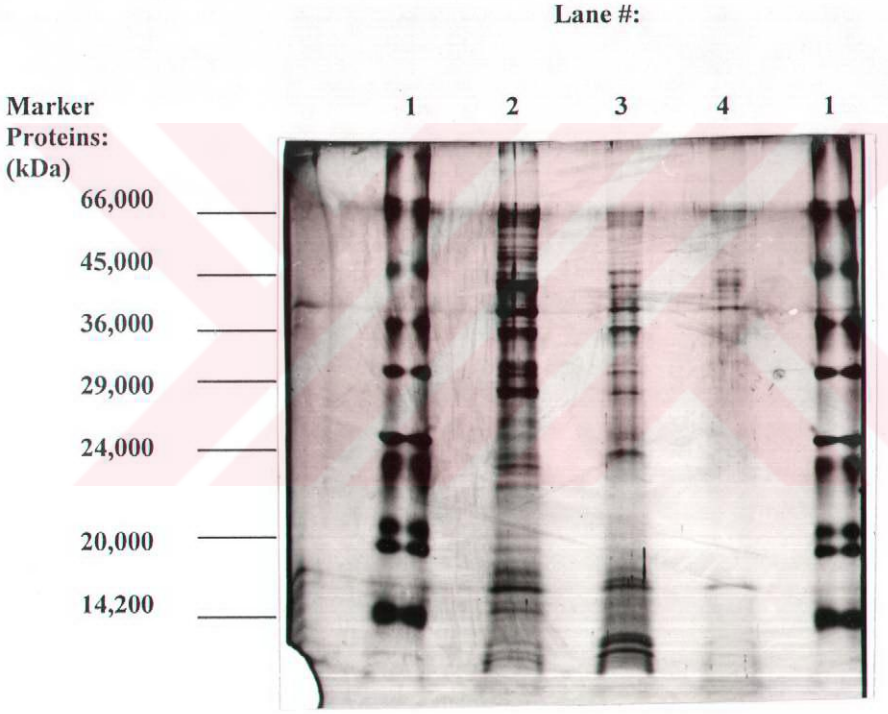

Figure 3.10: SDS-PAGE pattern of the samples after silver staining. Lane 1: Standard proteins: Albumin, Ovalbumin, Glyceraldehyde 3-phosphate dehdrogenase, Carbonic anhdrase, Trypsinogen, Trypsin inhibitor, α-Lactalbumin, Lane 2: Crude extract, Lane 3: Fraction of gel filtration, Lane 4: Chromatofocusing fraction. Amount of proteins applied to lane 2, 3 and 4 are 10.35 μg, 20.2 μg and 2.5 μg, respectively.

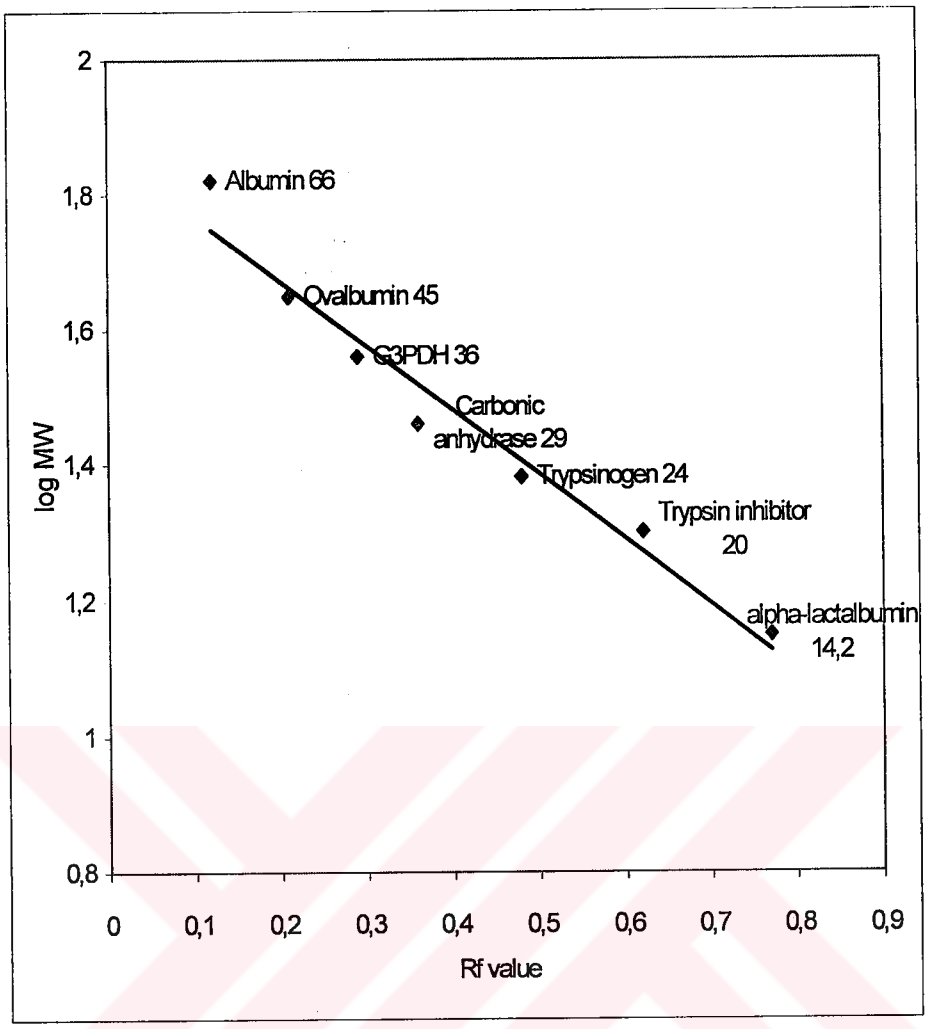

Figure 3.11: Standard curve for SDS-PAGE. Numbers given next to the names of molecular markers refer to the molecular weight of each marker protein in kDa. The circular point in the Figure represents the 36 kDa molecular weight, which seems to correspond to molecular weight of LDH monomer as well.

CHAPTER 4

DISCUSSION

In this study, LDH was partially purified from *R. oryzae* by a three step purification protocol, including ammonium sulfate fractionation, gel filtration and chromatofocusing. The characterization of partilly purified enzyme was achieved by SDS-PAGE. The kinetic parameters were determined by using crude extract. The critisism of each step was mentioned below.

4.1. Enzyme Assays

The general trend for measuring LDH activity is monitoring NADH oxidation at 340 nm when pyruvate is reduced to lactate. There are some assay sytems in the literature with slight differences in the compositions of reaxtion mixture. The reaction mixture used in this study was that of Hoffman and Hanson (1986), but the pH of the tris buffer used in the assay sytem was adjusted to 7.5 instead pH 8. In fact, the pH range in the assay system depends on the reaction direction and origin of the sample, and LDH from microbial sources, has a optimum pH shifted slightly acidic range (Kopperschläger, 1996). Some other researches have used phosphate buffer (Gleason 1966, Pritchard 1973, Skory 200) instead of tris buffer, in their assay systems and obtained higher values of specific activities in the crude extract. But such a difference, of course may not be necessarily a function of the assay buffer but also of the method of cell disintegration and ingredients of the extraction buffer used in the procedures, etc.

In addition to the UV method for the determination of LDH activity of this study, various colorimetric measurements utilizing different electron carriers and redox indicators were developed and (cited in Kopperschläger, 1996)

4.2. Purification of Lactate Dehydrogenase

The three step purification protocol of LDH was discussed below.

Mycelial cells were disrupted by sonication, and a specific activity of 0.0706 U/mg was obtained from the crude extract. This specific activity almost fits well with the results of Hang (1991), which was reported as 0.088 U/mg. However the specific activity obtained in the crude extract of this study, is still very low comparing others. For instance, Pritchard (1973) had obtained a specific activity of 0.49 U/mg, which is 6.94 times greater. Since the assay sytems used in this study is the same as that of Hang's system, the similar specific activities of both of the studies is interpretable. Methods of cell disintegration may on the other hand create a difference since sonication is a critical technique in terms of the shelflife of its probe and the maximum volume of the sample to be homogenized at a set. In most of the other cited studies glass bead homogenizers or Sorvall Homogenizers have been chosen for cell disintegration (Skory, 2000, Hang, 1991)

The instable nature of the enzyme makes critical the process of cell disintegration and homogenization and thus, a special attention should be drawn to cell disintegration and composition of the extraction buffer. Although Hang (1991) had not reported any use of additives, except EDTA, in their preparatons, the instability of LDH is a chalanging obstacle against its further purification. Many investigators had achieved only 3 (Obayashi, 1966) or 10 (Pritchard, 1973) fold of purification due to this problem. Therefore we have used serine protease inhibitor PMSF, DTT, EDTA and 10 % glycerol in the extraction buffer to overcome the instability. However, concerning Hang's system of cell disintegration and extraction, in which such ingredients we have used were not included, and mycelia were homogenized by a Sorvall homogenizer, still, a slightly greater specific activty was achieved. So, instead of sonication, another method of cell disintegration may be considered as an alternative approach.

As described in section 2.2.1.2, a range of 30-60 % ammonium sulfate saturation level has been chosen for the concentration of crude extract. 0.169 U/mg of specific activity with a 2.39 fold of purification and 76 % yield was achieved as a result of this step. These findings seem to fit well with the 2.7 purification fold and a 78 % of the recovery of the enzyme by Hang (1991). Pritchard (1973), obtained a 2.96 fold of purification with 101 % yield, by 35-55 % saturation.

On the other hand to obtain a desired precipitation of proteins, in the target level of saturation, much longer times than that mentioned in the literature required for our study. After the completition of Ammonium sulfate addition by 30-45 mins intervals in three sets, to bring the sample to an equilibrium , at least 6-8 hours stirring was necessary for 30% saturation, and for 60% saturation, the sample should be stirred for overnight at 4 C. This may be due to the composition of extraction buffer in the crude extract: 10 % glycerol composition of it, directly created a visible increase in the viscosity of the sample. The hydrophilic nature of the glycerol, forming hydrogen bonds with water molecules, seem to affect not the precipitation but the rate of the precipitation of the proteins by ammonium sulfate molecules. In fact, glycerol content of the buffer as a stabilizing agent, requires extra-time for not only salt fractionation of proteins but also for chromatographic techniques due to its viscous nature. Thus, the stabilization factor obtained by the glycerol must be higher than this time factor to make it worthwhile. Glycerol addition has had a positive effect on our system in that, the higher yield of purification was achieved.

The protein sample obtained from the fraction of 30-60 % saturation, could be stored in the form of a precipitant, at least 10 days at 4° C without any loss in the LDH activity. Therefore the stabilizing effect of ammonium sulfate salt seems to applicable for the case of LDH activity, as well.

It should be noted that ammonium sulfate precipitation in the protein purification procedures is actually a preparatory step: it has the property of concentrating the samples and almost 2 – 3 fold of purification could be achieved by it. Moreover, for further treatment of the sample, after concentration by ammonium sulfate, the salt should be removed effectively, by an extra step of dialysis or gel

filtration. Therefore, ultrafiltration seems to be an attractive alternative for concentration of the crude extract in that it requires less time and and it is not associated with an extra-step.

In order to remove salt from the 30-60 % ammonium sulfate fraction, a gel filtration experiment was designed. The efective removal of the salts from the sample is of primary importance in that, the ionic strength of the sample is a crucial parameter at the next step: Chromatofocusing. In addition to change the phosphate buffer (extraction buffer)of the sample and to make sample suspended in the target buffer (starting buffer of the next step Chromatofocusing) Imidazole-HCl, gel filtration was necessary.

Although, gel filtration dilutes the sample at the end of it, it requires much less time for application comparing to dialysis. Besides higher enzyme acivity was detected in the fractions eluted from Sephadex G-25 than an overnight dialysis of the sample.

However, not only to remove salts but also to generate a small fractionation range between the proteins of the loaded sample, Sephadex G-200 or G-150 may be an alternative for G-25, yet it took 18 hours for the elution of 6 mL of the sample,

When desalted sample was applied to PBE 94 column, a minute LDH activity was detected in the fractions eluted at pH 5-5.6, around the pI value of LDH which is 5.2 (Hang, 1991). The specific activity achieved in those fractions is maximum 0.107 U/mg with a 1.5 purification fold and 10 % of recovery. The experiment was repeated 4 times and results were too low comparing with that of Hang (1991) who had obtained a 175 fold of purification of LDH at the end of chromotafocusing on PBE 94 column.

There are many critical factors to be discussed for the application of chromatofocusing. The low purification fold achieved by this step may arise from the previous step and sample it self or the system of chromatofocusing already. The Ionic strength, pH and composition of the sample is of

primary importance. We tried to control the ionic strength under 0.05M as adviced in the literature. Effective removal of salts is essential to control the ionic stregth so a special attention has been drawn to optimise G-25 gel filtration procedure, of which outcomes could be correlated with the cited results quite well. Besides EDTA, may have been a competititor of proteins to bind the ion-exchanger PBE 94 and therefore may affect the proper resolution, however when the EDTA was excluded from the buffer systems, not a typical change in the outcome was achieved, and none of these manipulations in the sytem did not go beyond the present results.

Another important point is that, the fractions eluted from PBE 94 column actually had a quite dilute protein content: comparing to crude extract, the active fractions had a 0.046 mg/mL protein concentration which corresponds to a 94.6 % reduction in the protein content of the crude extract. From this context the activity of the enzyme, not the resolution capacity of the chromatofocusing, seems to need a focus.

Denaturation of most proteins can be seen when they are exposed to extreme pH, and elution buffer in chromatofocusing has a pH 4, but extereme pH exposure had already tried to be overcame as described above.

Catalytic site inactivation also took considiration so DTT or 2-Mercaptoethanol with EDTA was used in starting and elution buffers.

To block protease effect, PMSF inhibitors was used in the crude extract and in subsequent preparations, although it is known that PMSF irreversibly block those proteases. However, apart from PMSF, a serine protease inhibitor, Skory (2001) reported the use of inhibitors like Pepstain A, and leupeptin, too to avoid other types of proteases. None of them were used in the present study.

Considering the highly lost enzyme activity in this study at PBE 94 column, it is appropriate here to mention a few words about dilute enzyme solutions. It is well known that very dilute enzyme

solutions lose activity quickly, and through the end of the purification steps, more dilute protein contents are obtained, they even become more dilute when assayed in the reaction mixture. In fact, the enzyme assay of the fractions eluted from PBE 94 needs to be justified one more time in the presence of BSA as a stabilizer in the assay sytem. After that if still there is no a positive response than a special attention should be paid to whole procudure to manuplate or to change it completely.

4.3. Characterization of LDH

Optimum pH and temperature for LDH activity was studied in the crude extract and determined as 7.5 with tris buffer and 35 °C, respectively. These finding could be correlated with those of Hang (1991). Pritchard obtained a pH 7.2 with phospahate buffer.

Reaction velocity seems to show a sigmoidal response to increasing NADH concentrations, although the enzyme sample is not pure. Pritchard (1973), has also reported that reaction velocity showed a sigmoidal response to increasing concentrations of NADH with a Km value of 2.9×10^{-4} M and Hang (1991) reported a K_m value of 1.48×10^{-4} M. From Hill Plot of variying NADH concentrations, an 'n' value of 3.4 was calculated, indicated the positive cooperativity in NADH binding. From the same plot, K′ value was calculated as 5.75×10^{-4}.

A sigmoidal relationship was also found by Tarmy and Kaplan (1968) for the response of *E. coli* lactate dehydrogenase to increasing concentartions of pyruvate, and they also interpreted their data as the existence of two types of binding sites for pyruvate with different affinitics for this substrate, in that one of them with a higher afffinity for pyruvate is associated with the enzyme activation. Once this is saturated the co-operativity of the binding is no longer shown. A similar interpretation can be made for the response of LDH to NADH considering the literature (Pritchard, 1971) and present data.

Plot of reaction velocity versus pyruvate concentration is different than in the case of NADH response. The enzyme may have a hyperbolic saturation kinetics with respect to pyruvate. Impure

enzyme sample seems to hide the observation of this property, clearly. K_m of the enzyme was calculated as 0.64×10^{-4} M, with a V_{max} of 0.223 μ mole $min^{-1}.ml^{-1}$ for pyruvate. Hang (1991), and Pritchard (1973), reported the K_m for pyruvate as 6.40×10^{-4} and 5.5×10^{-4} M.

With respect to SDS-PAGE pattern of each fraction, the presence of the bands around the band corresponding to 36 kD of the lane 1 (marker lane) at lanes 2, 3, and 4 justifies the monomeric form of the LDH with a molecular weight of 36 kDa, as cited in the literature (Hang, 1991). Since the fractions of gel filtration (lane 3) and chromatofocusing (lane 4) are very dilute comparing to crude extract (lane 2), the intensity of the bands of the lanes are not the same in the electrophoretic pattern, although maximum volumes of the dilute samples were loaded to eliminate the differences between the total amount of protein in the crude extract sample and gel filtration & chromatofocusing fractions applied to the gel.

CHAPTER 5

CONCLUSION

The aim of this study was to optimise enzyme assay system and to develop a purification strategy for the enzyme.

The spectrophotometric enzyme assay for the determination of LDH activity is optimised. The optimum pH and temperature conditions for the enzyme were determined as 7.5 and 35 C. The purification protocol for LDH includes 30-60 % ammonium sulfate precipitation, G-25 Gel Filtration, and Chromatofocusing on PBE 94 column. The first two steps seem to work well within the present strategy, with a 2.4 and 2.12 purification folds respectively, and can be corelated with the literature. The third step of the protocol, chromatofocusing on PBE 94 column did not result in a desired purification fold. In fact the fold was only 1.5 with a low yield, 10 %.

A preliminary characterization of kinetic behavior of the enzyme was also achieved by the sample obtained from crude extract. The enzyme showed a hyperbolic response to increasing pyruvate concentrations which obeys Michealis-Menten Kinetics. However, a sigmoidal behavior was seen with respect to NADH at a fixed concentration of pyruvate. The Km for pyruvate was found to be 0.064×10^{-4} M and Vmax is 0.223 μ mol.min^{-1}.ml^{-1}. From Hill Plot of variying NADH an 'n' value of 3.4 was calculated indicated the positive cooperativity in NADH binding.

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APPENDIX A

COMPOSITION OF THE BRADFORD REAGENT AND STANDARD CURVE PREPARATION

5 X Stock solution:

500 mg (SERVA) Brillant Blue G

250 mL 95 % Ethanol

500 mL 85 % phosphoric acid

Complete the volume to 1000 Ml with distilled water and store at 4 C (In dark).

The reagent can be prepared with a 1:4 dilution of the stock solution by distilled water.

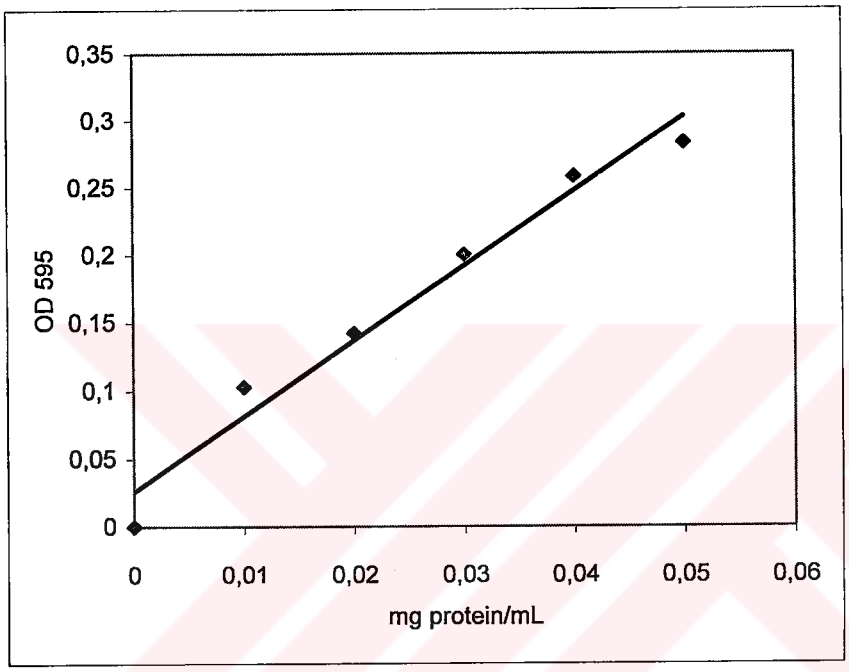
Standard Curve Table:

A stock solution of 1 mg/mL BSA was used.

Tube #	BSA(µl)	dH_2O (µl)	Bradford's Reagent (mL)
1	0	500	5
2	5	495	5
3	10	490	5
4	15	485	5
5	20	480	5
6	25	475	5

APPENDIX B

BRADFORD STANDARD CURVE WITH RESPECT TO BSA FOR DETERMINATION OF PROTEIN CONCENTRATION



APPENDIX C

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents:

a) 10 % SDS solution:

Prepare 50 mL of 10 % SDS solution by dissolving calculated amount of SDS in distilled water and mix gently to prevent foaming of SDS.

b) 30 % Acrylamide/bis solution:

Since acrylamide is a nerve toxin precautions should be taken by wearing gloves and mask during preparation of this solution.

1. Weigh 58.4 g. Acrylamide in a 500 mL beaker
2. Add 1.6 g NN-bis-methylene-acrylamide
3. Pour 150 mL dH_2O in to the beaker, cover the beaker with aluminium foil and mix on a magnatic stirrer
4. When it is completely dissolved, complete the volume to 200 mL by adding dH_2O
5. Store the solution at 4°C in a dark bottle

c) 1.5 M Tris-HCl, pH 8.8 buffer

Weigh out 54.45 g. Tris base and dissolve in 150 mL of dH20

Stir to dissolve and adjust pH to 8.8 with concentrated HCl

Complete the volume to 300 mL with dH2O and store at 4C

d) 5X stock running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3):

Weigh out 15 g Tris base and 72 g glycine and dissolve in 1 liter d H2O. Store at 4 ° C

e)Ready to use running buffer:

Pour 600 mL of 5x stock running buffer solution into the electrophoresis tank and dilute it to 3 liter total volume with d H2O.

Stir with magnetic stirrer and add 3 g SDS. Place the tank into the refrigerator and complete stirring gently.

f) 10 % Ammonium Persulfate (APS) (should be prepared freshly on the day of usage)

Weigh out 0.06 g APS İN AN Eppendorf tube and dissolve in 600 microliter d H2O by vortexing.

g) Tracking dye (0.5 % agarose containing 0.5 mg % bromophenol blue)

Prepare 100 mL of 0.5 % agarose containing 0.5 mg % bromophenol blue. This solution solidifies at room temperature and can be liquified in a boiling water bath before use

h) Sample buffer:

l) Fixer solution for silver staining

Pour 150 mL ethanol in a 500 mL graduated cylindir

Add 36 mL acetic acid and 150 microliter 37 % formaldehyde

Complete the volume to 300 mL with dH2O, mix throughly and pour into a pyrex or plastic container.

Procedure:

a) Preliminary praparation

Clean the surfaces of glasseswith pure ethanol, align the spacers at the two edges of the bigger glass and place the smaller glass on it. Adjust the bottom edges very carefully to prevent leakage. Install the clamps and fasten the screws.

Place the sandwich on the base and check that the bottoms of glasses are properly sealed.

b) Preparation of SDS-PAGE gel solution (7.5 % slab gel):

Stacking Gel:

Add the followings into an erlenmeyer flask and shake gently:

40 mL of 30 % acrylamide

33.5 mL distilled water

25 mL of 1.5 M Tris-HCl, pH 8.8

1 mL of 10 % SDS solution

500 microliter of 10 % APS

50 microliter TEMED

Immediately collect the solution into a glass pipette using a pump and discharge it in the space between the glasses (very gently not the form the bubbles). Fill the space upto 5 cm below the upper edge of the small glass.

With a pesteur pipette, pour dH2O on to the gel in order to cut off the contact of the gels with air which will prevent the polymerization.

Allow to stand it to complete polymerization.

Separating Gel:

Dry the uppper part of the gel by the help of a filter paper and blace the comb between the glass sandwich.

Add the followings into the small beaker (4 % gel)

1.6 mL of 30 % acrylamide/bis

6.1 mL of dH2O

2.5 mL of Tris-HCl, 0.5 M pH 6.8

100 µl 10 % (w/v) SDS

50 µl 10 % APS (fresh)

10 µl TEMED

Immediately pour the gel solution between the galsses and allow to stand for polymerization. After polymerization is completed take the comb off and fill the wells with 1 X loading buffer. With an Hamilton syringe inject 50 µl tracking dye into one corner of the gel.

Load the standard and samples into the wells in an order and keep note for them.

Install the slab gel sandwiches to the cooling core.

Fill the upper chamber of the core with 1 X loading buffer.

Gently place the cooling core into the electrophoretic tank. Be sure that there are no bubles trapped on the upper side of the glass sandwich; if there are, using a glass rod remove them. This is necessary as traqpped air bubles can act as an insulator.

Perform the separation at 4 C using 10 mA Per gel constant current for the first 30 minutes and then 25 mA Per gel for the rest of the run; until the tracking dye have run to 1 cm above the edge of the slab gel.

When the run is over exclude gels very carefully.

Immerse the gels in fixing solution containing 50 % methanol, 12 % acettic acid and 0.5 mL of 37 % formaldehyde Per liter and perform shaking on a platform shaker for at least 1 hour (gels can be soaked in this solution overnight)

The gels were then silver stained using the procedure of Blum *et. al.* (1987)

Molecular weight markers used in the SDS-PAGE procedure is given below:

Proteins	**Approx. Molecular Weight (kDa)**
α -Lactalbumin	14,200
Trypsin inhibitor	20,000
Trypsinogen	24,000
Carbonic anhydrase	29,000
Glyceraldehyde-3-phosphate dehdrogenase	36,000
Ovalbumin	45,000
Albumin	66,000

APPENDIX D

SILVER STAINING OF THE SLAB GELS

Reagents:

a)Fixer:

150 mL methanol

36 mL acetic acid

150 µl of 37 % formaldehyde

complete to 300 mL with distilled water

b) 50 % Ethanol:

Prepare 1 lt of 50 % ethanol

c) Pre-treatment solution (sodium thio sulfate solution):

Dissolve 0.08 g. Sodium thiosulfate (Na2S203.5H2O) in 400 mL distilled water,

mix by a glass rod and take 8 mL for further use in developing solution preparation.

d) Silver nitrate solution:

Dissolve 0.8 g. Silver nitrate in 400 mL distilled water and add 300 µl 37 %

formaldehyde.

e) Developing solution

In an erlenmeyer flask measure 9 g. Potassium carbonate and add 8 mL previously kept pretreatment solution and 300 µl 37 % formaldehyde. Complete the volume to 400 mL with distilled water

f) Stop solution:

In an erlenmeyer flask mix 200 mL methanol and 48 mL acetic acid and complete to 400 mL with distilled water.

Procedure:

The procedure given in the table were followed. All the steps were performed by constant shaking on a platform shaker.

Step	Solution	Time	Comments
Fixing	fixer	>1 hour	
Washing	50 % ethanol	3 x 20 min	
Pretreatment	Ppretreatment soln.	1 min	Time exact
Rinse	dH_2O	3 x 20 sec	Time exact
Impregnate	Silver nitrate soln.	20 min	
Rinse	dH_2O	2 x 20 sec	Time exact
Developing	Developing soln.	App 5 min*	
Wash	$d H_2 O$	2 x 2 min	
Stop	Stop soln.	> 10 min	

* Time is adjusted by oneself according to the color development