การทำให้บริสุทธิ์และคุณสมบัติของเอนไซม์ Lactate Dehydrogenase จากเชื้อกลายพันธุ์ *Rhizopus oryzae*

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บทคัดย่อ

เอนไซม์ Lactate Dehydrogenase จากเชื้อกลายพันธุ์ *Rhizopus oryzae* สายพันธุ์ 3 เอ็น 6 ได้รับการทำให้บริสุทธิ์โดยวิธีการตกตะกอนด้วยแอมโมเนียมซัลเฟต โครมาโตกราฟฟีแบบแลกเปลี่ยนอิออนและแบบดูดซับ และโครมาโตโฟกัสซิ่ง พบว่ามีความเข้มข้นเพิ่มขึ้น 39 เท่า และมีผลได้ 11 เปอร์เซ็นต์ เอนไซม์ที่บริสุทธิ์แสดงการทำงานสูงสุดในช่วงพีเอชระหว่าง 7.0 ถึง 7.5 และในช่วงอุณหภูมิระหว่าง 25 ถึง 30 องศาเซลเซียส นอกจากนี้เอนไซม์มีความคงตัวได้ดีในช่วงพีเอชระหว่าง 7.0 ถึง 8.0 และสูญเสียความสามารถในการทำปฏิกิริยาที่อุณหภูมิเกินกว่า 30 องศาเซลเซียส ค่าคงที่ของไมเคิลลิส-เมนเทนของเอนไซม์ต่อสารตั้งต้นโซเดียมไพรูเวทและเอ็นเอดีเอชมีค่า 0.61 และ 0.10 มิลลิโมล ค่าความเร็วสูงสุดในการทำปฏิกิริยาของเอนไซม์ต่อโซเดียมไพรูเวทและเอ็นเอดีเอชมีค่า 23.5 และ 34.5 มิลลิโมลต่อมิลลิกรัมโปรตีนต่อนาที ในการศึกษาด้วยวิธีอิเล็คทรอโฟริซิสภายใต้สภาวะที่ทำให้เสียสภาพด้วยโซเดียมโดเดซิลซัลเฟต และภายใต้สภาวะไม่ทำให้เสียสภาพ พบว่าเอนไซม์มีน้ำหนักโมเลกุลประมาณ 38 และ 150 กิโลดาลตันตามลำดับ จากการวิเคราะห์ด้วยวิธีโครมาโตโฟกัสซิ่ง พบว่าเอนไซม์มีค่าไอโซอิเล็คตริค ประมาณ 5.2

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Purification and Properties of NAD-Dependent L-Lactate Dehydrogenase from a *Rhizopus Oryzae* Mutant

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Abstract

NAD-dependent L-lactate dehydrogenase from *Rhizopus oryzae* mutant 3N6 was purified 39-fold with an 11% yield by ammonium sulfate fractionation, DEAE-Sephadex chromatography, hydroxyapatite chromatography, and chromatofocusing. The enzyme showed optimal pH and temperature of 7.0 to 7.5 and 25 to 30°C. respectively. It was stable over a pH range of 7.0 to 8.0, but lost its activity above 30°C. The K_m for sodium pyruvate and NADH were 0.61 and 0.10 mM, respectively. The Vmax for sodium pyruvate and NADH were 23.5 and 34.5 mM per mg protein per min, respectively. The molecular weight was 38 kDa as determined by SDS-PAGE and 150 kDa by non-denaturing PAGE. The enzyme had an isoelectric point (pI) of 5.2 as determined by chromatofocusing.

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Introduction

NAD-dependent L-lactate dehydrogenase (EC 1.1.1.27) (LDH) is an enzyme that catalyzes the reduction of pyruvate by NADH to L(+)-lactic acid. It has been found in animals [1], plants [2-4] and microorganisms [5-7]. The enzyme was also detected in ***Rhizopus oryzae*** mycelia at a high level during the rapid growth phase when L(+)-lactic acid was produced [6]. The purification and characterization of lactate dehydrogenase from this organism was reported [8-9]. The purified enzyme can be commercially applied to determine L(+)-lactic acid in foods and clinical analysis [10-12].

Recently, we have isolated a number of lactic acid-and glucoamylase-overproducing ***R. oryzae*** mutants by treatment of a parent strain (NRRL 395) [13]. Mutant 3N6 was found to produce highest yield of lactate dehydrogenase. The objectives of this study were to purify and characterize its NAD-dependent L-lactate dehydrogenase.

Materials and Methods

A buffer used was 50 mM Tris-HCl buffer, pH 7.2, containing 2 mM dithiothreitol (DTT), 5 mM disodium ethylenediaminetetraacetic acid (EDTA) and 20% glycerol except otherwise noted.

Enzyme Preparation

LDH from ***Rhizopus oryzae*** mutant 3N6 was produced in 500-mL Erlenmeyer flasks containing 1 g of rice in 100 mL of distilled water. All flasks were sterilized at 121°C for 15 min. After cooling, each flask was inoculated with 2x10' spores and incubated at 30°C on a rotary shaker at 250 rpm. After 48 h of fermentation, mycelia were filtered on Whatman paper no.4, and then washed twice with ice-cold distilled water. The mycelia (68 g wet wt.) were suspended in 150 mL of buffer plus 1 mM phenylmethanesulfonyl fluoride (PMSF) and passed twice through a pre-chilled French pressure cell (SLM Instruments, Inc., Urbana, IL) at an internal cell pressure of 16,000 psi. After centrifugation at 23.000 x g for 2 min, the supernatant was used as the crude extract.

Enzyme Purification

All subsequent purification steps were carried out at 0-4°C.

Ammonium sulfate fractionation

Proteins in the crude extract were fractionated by adding solid ammonium sulfate into the following saturation ranges: 0-20%, 2040%. 40-60% and 80-100%. The precipitates collected by centrifugation at 23,000 x g for 20 min were dissolved in a minimal volume of the buffer.

Ion-exchange column chromatography

A most active fraction was desalted on a Sephadex G25-80 column (1.5 x *17* cm) equilibrated with the buffer. Salt-free active fractions were pooled. The pooled fraction was applied to a diethyaminoethyl (DEAE)-Sephadex A-50 column (2 x 10 cm) pre-equilibrated with the buffer. The column was initially eluted with a two-bed volume of the buffer to wash out unadsorbed proteins. The adsorbed proteins were then eluted with a linear gradient of O-O.5 M NaCl in the same buffer at the flow rate of 30 mL/h. The salt concentration in the fractions was also determined. Active fractions were combined, concentrated by ultrafiltration with a 10 kDa MW-cutoff membrane, and desalted on a Sephadex G25-80 column.

Hydroxyapatite column chromatography

The sample obtained from the previous step was introduced into a hydroxyapatite (Bio-Gel HT Hydroxyapatite. Bio-Rad) column (1.5 x 11 cm) equilibrated with buffer. The column was eluted with two-bed volumes of buffer followed by a linear gradient of O-O.5 M phosphate buffer (pH 8.8) in the same buffer at a flow rate of 30 mL/h. The salt concentration in the fractions was measured.

Active fractions were pooled, concentrated by ultrafiltration with a 10 kDa MW-cutoff membrane and desalted on a Sephadex G 25-80 column equilibrated with 25 mM imidazole-HCl buffer, pH 7.4. The enzyme was eluted from the desalting column with the same buffer at a flow rate of 30 mL/h.

Chromatofocusing

Polybuffer Exchanger 94 (PBE 94) and Polybuffer 74 were used to purify the enzyme at the final step. Following the directions given by Pharmacia. a PBE 94 column (1.0 x 20.5 cm) was pre-equilibrated with 25 mM imidazole-HCl buffer, pH 7.4. The pooled salt-free sample was applied to the column and eluted with 1:8 diluted Polybuffer 74 (adjusted to pH 4.0 with 1 N HCl and degassed) at a flow rate of 30 mL/h. The pH of the fractions was also monitored.

Analytical Methods

Protein was determined either by measuring the absorbance at 280 nm or by the protein-dye binding method of Bradford [14]. The activity of LDH was determined by the method of Pritchard[6]. One unit of enzyme was defined as the amount of enzyme catalyzing the oxidation of 1 μmole of NADH/min under the assay conditions.

Enzyme Properties

Molecular weight determination

Two methods were used for molecular weight determination. Non-denaturing PAGE was used to determine the native molecular weight of LDH according to Hedrick & Smith [15]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli[16] on a 0.75 mm thick polyacrylamide slab gel (7 cm x 8 cm) consisting of 12% separating and 4% stacking gels by using the minigel system (Mini-Protein II) of Bio-Rad. The molecular weight standards used (Bio-Rad) were phosphorylase B (112 kDa), bovine serum albumin (84 kDa), ovalbumin (53.2 kDa), carbonic anhydrase (34.9 kDa), and lysozyme (20.5 kDa).

Isoelectric point determination

The isoelectric point of LDH was determined by measuring the pH of the most active fraction eluted from the chromatofocusing column.

Effect of pH and temperature

Studies on the effect of pH and temperature on enzyme activity were performed like the enzyme assay except 0.1 M phosphate citrate buffer (pH 3.0-9.0) was used and a range of temperatures from 25-90°C. The experiments on the effect of pH and temperature on enzyme stability were carried out by incubating the enzyme solution over the pH range of 3.0-9.0 and the temperature range of 25-90°C for 60 min and then the activities were determined.

Kinetic studies

The Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of purified enzyme for pyruvate and NADH were determined like the enzyme determination except different substrate concentrations, measuring the reaction velocity, and establishing the Lineweaver-Burk double reciprocal plot.

Results and Discussion

As shown in Table 1, the enzyme was purified approximately 39-fold with a yield of 11% by ammonium sulfate fractionation, DEAE-Sephadex column chromatography, hydroxyapatite adsorption chromatography, and chromatofocusing. The enzyme was purified to homogeneity by SDS-PAGE (data not shown).

The enzyme had a molecular weight of 38 kDa as determined by SDS-PAGE. The molecular weight of native LDH obtained by non-denaturing PAGE was 150 kDa (Figure 1). The results suggest that the enzyme was a tetramer consisting of 4 identical subunits. Similar results were reported previously [8]. The LDH of most vertebrate tissue extracts and bacteria had a molecular weight of 140 kDa and contained 4 subunits [1, 5. 11].

The isoelectric point (pI) of the enzyme was found to be 5.2 by chromatofocusing. The pI of LDH from the parental *R. oryzae* strain was also reported to be 5.2 [8], while those from soybean and potato tubers were reported to be 6.0-6.5 and 5.1, respectively [2,4].

As shown in Figure 2, the enzyme was most active at pH 7.0-7.5 and stable over a pH range of 7.0-8.0. It lost nearly 40% and 60% of its activity after 60 min of incubation at pH 6.0 and 9.0. respectively. The property of this enzyme was similar to that of the parent strain [8]. Obayashi *et al* [9] reported the optimum pH of the *R. oryzae* enzyme was 6.5-6.7. Hoffman & Hanson [3] found the optimum pH of LDH from barley roots and potato tubers was 7.0.

The enzyme had an optimum temperature of 25-30°C and was stable over a temperature range of 25-30°C (Figure 3). But it partially lost activity after 60 min of incubation in the range of 40-60°C and totally lost its activity over 80°C. The activity of LDH from the parent strain was also stable at 30°C. reduced to 55% after 1 hour of incubation at 45°C and nearly completely lost after 30 min of incubation at 55°C [8].

The K_m values for pyruvate and NADH were found to be 0.61 and 0.10 mM, respectively, while those from the parent strain for puruvate and NADH were reported to be 0.64 and 0.15 mM, respectively [8]. The K_m values of LDH from plant sources for pyruvate varied in a range of 0.18-0.57 mM and those for NADH were from 29 to 77 μM [2-3]. The K_m values of mammalian and bacterial LDH for pyruvate were in a range of 0.14-8.0 mM and those for NADH were 10-74 μM [5].

The V_{max} values of the enzyme for pyruvate and NADH were found to be 23.5 and 34.5 mM per mg protein per min, respectively. The V_{max} of frog liver and beef LDHs for pyruvate were reported to be 0.75 and 0.26 mM per mg protein per min. respectively [17-18], while the V_{max} of Streptococcus LDH for NADH was 1.67 mM per mg protein per min [19].

Conclusion

The overall characteristics of LDH from the mutant were similar to those of the enzyme from the parent (Table 2). This suggests that the structural gene of the mutant LDH might not be changed from the gene in the parent strain by the action of the mutagen.

Table 1 Purification of lactate dehydrogenase from Rhizopus oryzae mutant 3N6

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2265.5	390.6	5.8	1.0	100
30% $(NH_4)_2SO_4$ fractionation	1904.4	121.3	15.7	2.7	84
DEAE-Sephadex	1256.0	41.5	30.3	5.2	55
Hydroxyapatite	667.8	8.4	79.5	13.7	30
Chromatofocusing	247.2	1.1	224.7	38.7	11

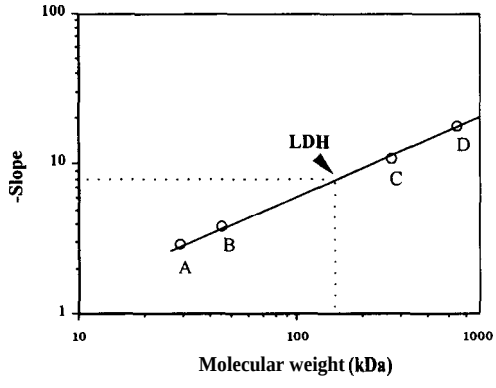

Figure 1 Determination of lactate dehydrogenase molecular weight of Rhizopus oryzae mutant 3N6 by non-denaturing PAGE. Standard proteins: A, Carbonic anhydrase (29 kDa); B, Chicken egg albumin (45kDa); C, Urease, trimer (272 kDa); D, Urease, hexamer (545 kDa)

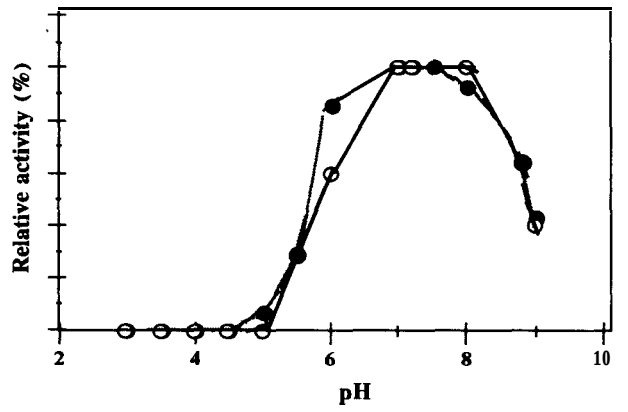

Figure 2 Effect of pH on the activity (●) and stability (○) of lactate dehydrogenase from Rhizopus oryzae mutant 3N6. Relative activity is expressed in comparison with the activity at pH 7.2 which is taken as 100%.

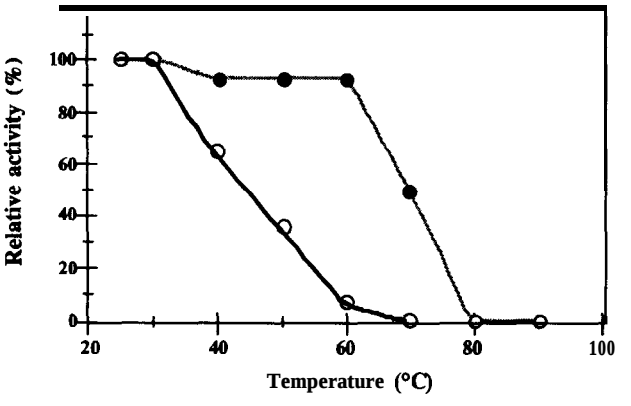

Figure 3 Effect of temperature on the activity (●) and stability (○) of lactate dehydrogenase from *Rhizopus* oryzae mutant 3N6. Relative activity is expressed in comparison with the activity at **30 C** which is taken as 100%.

Table 2 Comparison on the properties of LDH from Rhizopus oryzae mutant 3N6 and the parent (Rhizopus oryzae NRRL 395)

Enzyme properties	*Rhizopus oryzae* mutant 3N6	*Rhizopus oryzae NRRL 395* [8]
Molecular weight (kDa)	150	131
Subunits	4	4
Isoelectric point	5.2	5.2
Optimum temperature (°C)	25-30	30
Optimum pH	7.0-7.5	7.5
K_m for pyruvate (mM)	0.61	0.54
K_m for NADH (mM)	0.10	0.15

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