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Purification and Properties of Thermostable L-lactate Dehydrogenase Produced by *Escherichia coli*

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Abatract

The 4.3-kb gene coding for L-lactate dehydrogenase of *Bacillus stearothermophilus* has been subcloned and expressed in *E. coli* cells. The enzyme was purified 200-fold with 25% yield by heat treatment, DEAE-Sephadex, and NAD^+-Sepharose CL-4B affinity chromatography followed by gel filtration through Sephadex G-200. The molecular weight of the purified enzyme was estimated to be about 35,000 and 140,000 on SDS-polyacrylamide gel electrophoresis and gel filtration, respectively, indicating that the enzyme is composed of four identical subunits. The enzyme for pyruvate reduction and lactate oxidation was stable at 60 and 75° C for 30min, and the optimal temperatures for both reactions were 60 and 70° C, respectively. The enzyme had an optimal pH at 5.5 and 8.5 in pyruvate reduction and lactate oxidation, respectively. The pH stability of enzyme for pyruvate reduction was stable between pH 5 and 7. More than 90% of enzyme activity was lost at 1mM $FeSO_4$ and *p*-chloromercuribonzoate. The maximal activation of the enzyme was obtained with 0.8mM fructose 1,6-bisphosphate.

Key words : thermostable lactate dehydrogenase, enzyme purification

INTRODUCTION

L-lactate dehydrogenase (LDH ; L-lactate : NAD$^+$ oxidoreductases, EC 1.1.1.27) is enzyme which catalyzes the reversible reaction of pyruvate to lactate using NADH or NAD$^+$ as cofactor under anaerobic condition. LDHs have been found in a wide variety of organisms, from bacteria to vertebrates[1,2], and various kinds of bacterial LDHs showing genus-specific or species-specific properties were reported[2]. The structure and function of LDHs from skeletal muscle and heart of vertebrates have been studied in great detail[1]. Vertebrate LDHs are non-allosteric enzyme, while some bacterial LDHs are known to be allosteric enzymes activated by fructose 1,6-bisphosphate (FBP)[1,2]. All these enzymes are tetramers in the active state, being composed of identical subunits with molecular masses of 30~35kDa. In the microorganisms, LDHs have been found in *Streptococci*[3-5], *Lactobacilli*[6,7], *Thermus* sp.[8,9], *Bifidobacterium longum*[10] and others[2]. However, because amount of enzyme produced was comparatively of small quantity, the study of bacterial LDHs has not been performed in detail. In the recent years, nucleotide sequences of some bacterial LDH genes and two tertiary structures of them have been reported[11-15]. Thermostable enzymes are of use in studies of the relationship between the stability and structure of proteins, and are stable catalysts for various practical applications. LDH gene from thermophilic *Bacillus stearothermophilus* UK 788 has been cloned[16], but enzymatic characterization of LDH produced in *E. coli* cells was not investigated.

In this paper, we describe the expression of LDH gene from *Bacillus stearothermophilus* UK788, purification and enzymatic properties of LDH produced in *E. coli* cells.

MATERIALS AND METHODS

Reagents

DEAE-Sephadex A-50, NAD^+-Sepharose CL-4B and Sephadex G-200 were purchased from Pharmacia LKB Biotechnology. NAD^+, NADH, sodium py-

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ruvate and L-lactate were obtained from Sigma Chemical Co. T4 DNA ligase and restriction enzymes were obtained from Takara Shuzo, Toyobo Biochemicals. Molecular weight marker proteins were from Pharmacia LKB Biotechnology. All other chemicals used were of analytical grade and obtained commercially.

Strain, media and cultivation

E. coli JM109 (*recA* 1, *endA* 1, *gyrA* 96, *thi*, *hsdR* 17, *supE* 44, *relA* 1, *Δ*(*lac-proAB*)/F' [*traD* 36, *proAB*$^+$, *lac* [^{q}, *lacZΔM* 15]) was used as a host for expression of LDH and the recipient for transformation. *E. coli* JM 109 harboring recombinant plasmids was aerobically grown at 37°C overnight in the LB medium (pH 7.2) containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 100μg/ml of ampicillin. For efficient expression of LDH, an overnight culture was inoculated (1%) into the same medium and cultured at 37°C for 15h. The cells were collected by centrifugation at 10,000×g for 5min, and stored in a freezer at −20°C until use.

Preparation of cell-free extract

The frozen cell was thawed and suspended in 50 mM potassium phosphate buffer (pH 6.5) and then sonicated for 2min three times in succession. After centrifugation at 10,000×g for 10min, the supernatant was heated at 55°C for 30min to remove other impure proteins. The supernatant obtained by centrifugation at 10,000×g for 20min was used as crude enzyme for purification.

Purification of thermostable L-lactate dehydrogenase

All purification procedures were carried out at 4°C and 50mM sodium phosphate buffer (pH 6.5) was used throughout the purification.

Step 1. Ammonium sulfate precipitation : To the cell-free extract, ammonium sulfate was slowly added to 80% saturation with stirring. The precipitate formed was collected by centrifugation at 10,000×g for 20min, dissolved in the buffer and dialyzed against the same buffer.

Step 2. DEAE-Sephadex A-50 column chromatography : The dialyzed enzyme solution was loaded onto a DEAE-Sephadex A-50 column (2.5×60cm) equilibrated with the same buffer. Proteins were eluted with a linear gradient of NaCl from 0 to 0.5M in the same buffer. The enzyme fraction eluted from 0.2 to 0.35M NaCl were combined and dialyzed against the same buffer.

Step 3. NAD^+-Sepharose CL-4B affinity chromatography : The enzyme solution obtained was applied to Sepharose CL-4B column (1.0×20cm) coupled to NAD^+ after equilibration of column with the same buffer. Nonadsorbed proteins which had not affinity against NAD^+ were eluted in the same buffer. When NADH solution was added to the same buffer to final concentration of 0.2mM, the enzyme was recovered by stronger affinity of NADH than NAD^+.

Step 4. Gel filtration column chromatography : The enzyme fraction was put on a column (1.0×20cm) of Sephadex G-200 equilibrated with the same buffer to remove the remaining NADH and impure proteins. Proteins were eluted by the same buffer at the flow rate of 0.1ml per minute and the active enzyme fraction gave a single peak of protein. The purified enzyme was stored at −20°C until use.

Enzyme assay

The assay of LDH activity was performed by measuring the change in absorbance of NADH or NAD^+ at 340nm with LKB recording spectrophotometer. The standard assay solution contained 50mM sodium phosphate buffer (pH 6.5), 10mM sodium pyruvate, 0.4 mM NADH and 0.5mM fructose 1,6-bisphosphate. The reaction was usually initiated by the addition of the enzyme (0.5μg) and carried out at 30°C unless otherwise noted. One unit is the amount of enzyme required to catalyze either the oxidation of 1μmol NAD^+ per min at 30°C. Specific activity was expressed as units per mg of protein.

Protein measurement

Protein concentration was determined by the method of Lowry *et al.*[17] with bovine serum albumin as the standard. The protein concentration in the column chromatography was determined by optical density at 280nm.

Measurement of molecular mass

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli[18] with 12% polyacrylamide gel. The molecular mass of subunits was determined by comparing its mobility on SDS-PAGE with those of the following marker proteins (obtained from Pharmacia) : α-lactalbumin (M.W. 14,400), soybean trypsin inhibitor (M.W. 20,100), carbonic anhydrase (M.W. 30,000), ovalbumin (M.W. 43,000), bovine serum albumin (M.W. 67,000), and phosphorylase b (M.W. 94,000). The molecular mass of the native enzyme was measured by gel filtration on a column (1.0×20cm) of Sephadex G-200 at a flow rate of 1.0ml/min. The purified LDH and standard proteins, bovine thyroglobulin (M.W. 670,000), bovine γ-globulin (M.W. 158,000), chicken ovalbumin (M.W. 44,000) and horse myoglobin (M.W. 17,000) were applied to the column equilibrated with the same buffer.

RESULTS AND DISCUSSION

Subcloning and expression of the cloned LDH gene in *E. coli*

As described previously[16], *E. coli* harboring the hybrid plasmid pLD3 containing 4.3kb *Hind* III fragment of LDH gene produced an active L-lactate dehydrogenase. Subcloning experiments were performed to identify the minimum region of the insert DNA necessary for the production of LDH. As shown in Fig. 1, DNA was prepared from pLD3 and cleaved with various restriction enzymes. These fragments were then subcloned into the corresponding sites of pUC18, respectively, and transformed into *E. coli* JM109. *E. coli* cells harboring hybrid plasmids, pLD 30, 31 and 32 did not produce an active LDH, but in the case of a hybrid plasmid pLD33 with the 2.4kb DNA fragment between *Sac*I site and *Sph*I site, active enzyme was produced in *E. coli* cells harboring it. These results indicate that an open reading frame coding for LDH gene is contained in 2.4kb fragment. Furthermore, *E. coli* cells harboring pLD33 produced active LDH without addition of inducer (IPTG) of *lac* promotor in pUC18, suggesting that the promotor and the ribosome-binding sequence for the LDH gene, both of which can function in *E. coli*, are present in 2.4kb fragment, and the LDH gene is expressed constitutively without control of *lac* promotor. When *E. coli* cells harboring pLD33 were grown at 37° C, the highest LDH activity was observed after late logarithmic phase of growth (data not shown).

Purification of LDH produced in *E. coli*

L-lactate dehydrogenase was purified from *E. coli* cells by the procedure described in "Materials and Methods" (data not shown). The purification steps and specific activities of LDH from the crude extract

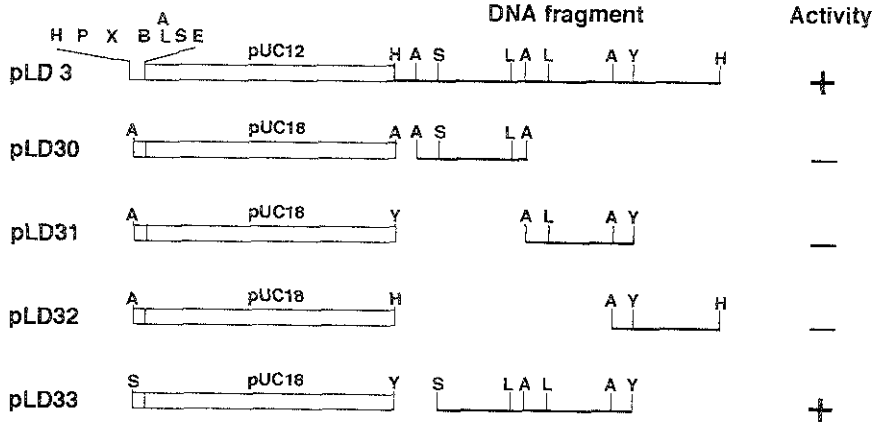

Fig. 1. Subcloning of hybrid plasmid pLD33 harboring L-lactate dehydrogenase gene of *Bacillus stearothermophilus* UK788. Open box indicates the plasmid pUC12 or pUC18.
Solid line represents the inserted DNA fragment of *Bacillusstearothermophilus*. The following abbreviations were used for restriction sites : A, *Acc* I ; B, *Bam*H I ; E, *Eco*R I ; H, *Hind* III ; P, *Pst* I ; L, *Sal* I ; S, *Sac* I ; X, *Xba* I ; Y, *Sph* I.

are summarized in Table 1. The specific activity of the most purified sample was 200 times that of the crude extract, and the yield of the enzyme was 25%. After gel filtration chromatography, the purified enzyme preparation gave a single protein band having a molecular mass of about 35,000 on SDS-polyacrylamide gel electrophoresis (Fig. 2). The molecular mass of the native enzyme was estimated as approximately 140,000 by gel filtration on Sephadex G-200 (data not shown). This suggests that the native enzyme has a tetrameric structure, like the other known L-lactate dehydrogenases[2].

Effect of pH on enzyme activity and stability

The effect of pH on the enzyme activity was examined for both lactate oxidation and pyruvate reduction at 30° C under standard assay condition. The optimum pH for reduction of pyruvate was 5.5 and that for oxidation of lactate was 8.5 (Fig. 3). This difference of pH optima shows the strong dependence of both reaction on the protonation or nonprotonation, respectively, of a base in the active site, as in the case of

Table 1. Purification of L-lactate dehydrogenase from *E. coli* harboring pLD33

Purification step	Total protein(mg)	Total activity (U)	Specific activity (U/mg)	Activity yield (%)
Crude extract	3,600	23,400	6.5	100
Heat treatment	1,760	16,850	9.6	72
$(NH_4)_2SO_4$ precipitation	1,620	16,300	10.1	69
DEAE-Sephadex A-50	150	12,870	86	55
Affinity chromatography	8	8,190	1,024	35
Gel filtration	4.5	5,850	1,300	25

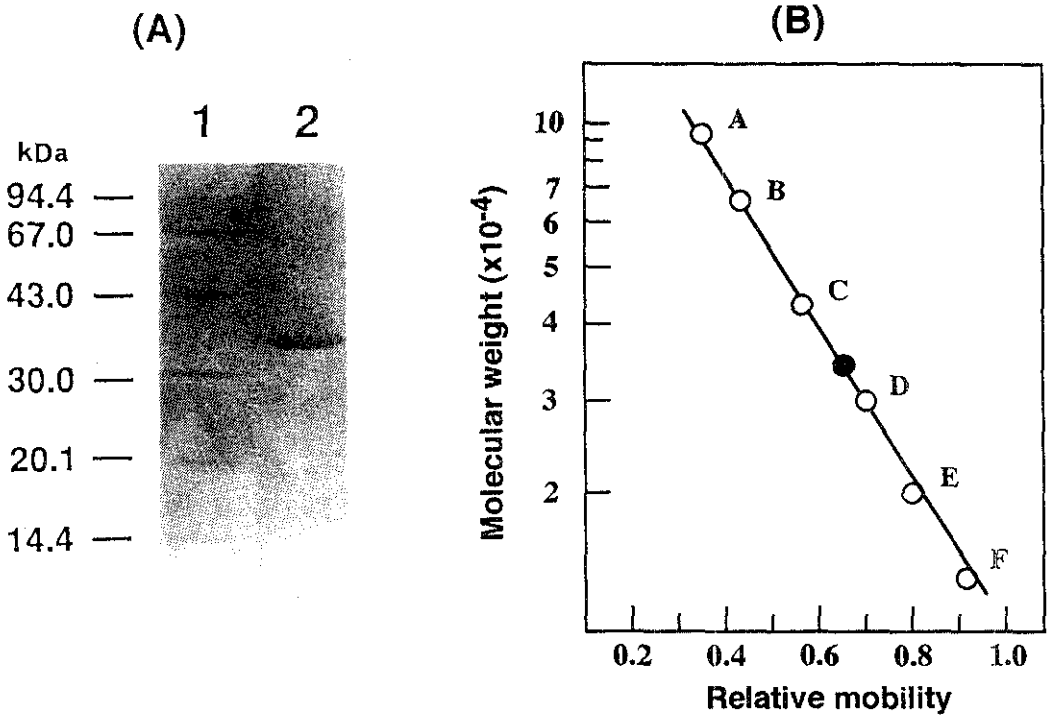

Fig. 2. Determination of the molecular weight of the purified enzyme.
(A) SDS-polyacrylamide gel electrophoresis(12% gel) ; lane 1. molecular weight standard markers, lane 2. the purified enzyme. (B) Calibration curve ; A. phosphorylase b (M. W. 94,00), B. bovine serum albumin (M. W. 67,000), C. ovalbumin (M. W. 43,000), D. carbonic anhydrase. (M. W. 30,000), E. soybean trypsin inhibitor (M. W. 20,100) F. α-lactalbumin (M. W. 14,400). ● ; purified enzyme.

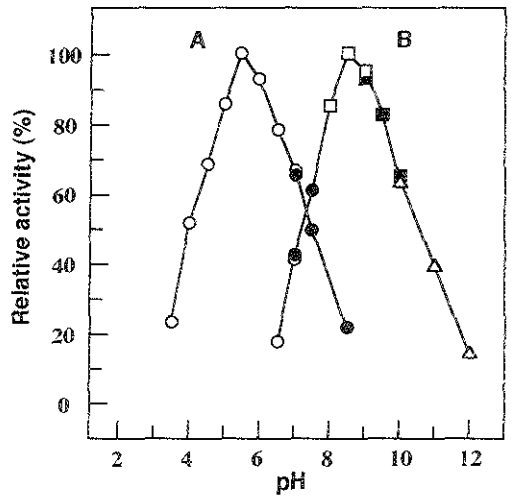

Fig. 3. Effect of pH on pyruvate reduction (A) and L-lactate oxiation (B) by L-lactate dehydrogenase.
Each assay mixture contained 10mM sodium pyruvate, 0.4mM NADH in the reduction and 0.5M L-lactate, 2 mM NAD^+ in the oxidation, except for 0.5μg of enzyme. The enzyme reaction was carried out at 30°C. The following buffers (at 50mM) were used ; sodium citrate buffer (○), sodium HEPES buffer (●), sodium borate-HCl (□), sodium borate-NaOH (■) and Na_2HPO_4-NaOH (△)

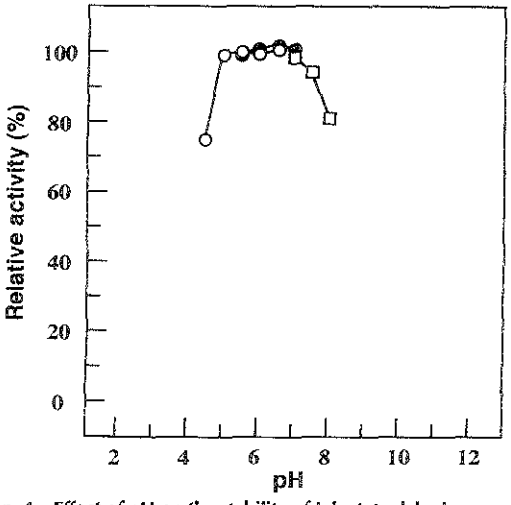

Fig. 4. Effect of pH on the stability of L-lactate dehydrogenase for pyruvate reduction.
The enzyme (0.1mg of protein/ml) was incubated at 4°C for 24h in the following buffers containing 0.5 mM fructose 1,6-bisphosphate ; 50mM sodium citrate buffer (○), 50mM sodium phosphate buffer (●) and 50mM sodium HEPES buffer (□). Enzyme assay mixture contained 0mM sodium pyruvate, 0.4mM NADH and 0.5 μg of enzyme, and enzyme reaction was carried out at 30°C.

the vetebrate LDH[1]. The enzyme was completely stable in the pH range of 5.0 to 7.0 at 4°C for 24h (Fig. 4).

Effect of temperature on enzyme activity and stability

The optimum temperatures of enzyme reaction was 60°C for pyruvate reduction and 70°C for lactate oxidation (Fig. 5). This difference for optimal temperature in forward and reverse reactions has also been observed in the enzymes from mesophilic and thermophilic bacilli[2] as well as *Thermus*[8]. Fig. 6 shows the activity of the enzyme remaining after treatment at the indicated temperatures for 30min. The enzyme was stable upto 60 and 75°C for pyruvate reduction and lactate oxidation, respectively.

Effect of metal ions and chemical reagents on enzyme activity

The effect of metal ions on the LDH activity was

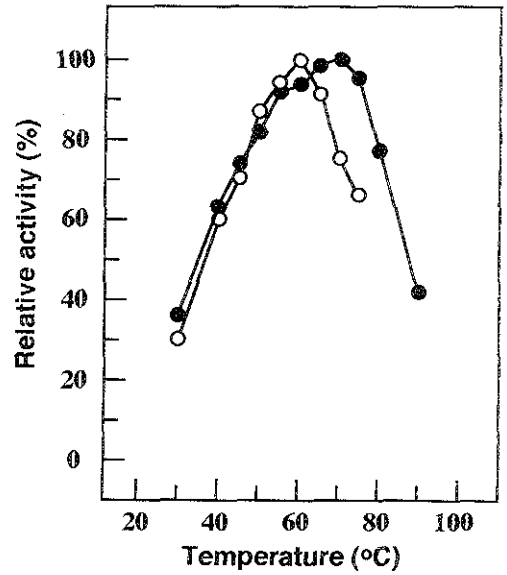

Fig. 5. Effect of temperature on pyruvate reduction (○) and L-lactate oxidation (●) by L-lactate dehydrogenase.
Reaction mixture for pyruvate reduction contained 10mM sodium pyruvate, 0.4mM NADH, 0.5mM fructose 1,6-bisphosphate and 50mM sodium phosphate buffer (pH 6.5). L-lactate oxidation was performed in the reaction mixture containing 0.5M L-lactate, 2mM NAD^+ and 50mM sodium phosphate buffer (pH 6.5). Enzyme solution (5 μg of protein) was added to reaction mixture previously warmed at the indicated temperatures, and then enzyme activity at 340nm was measured.

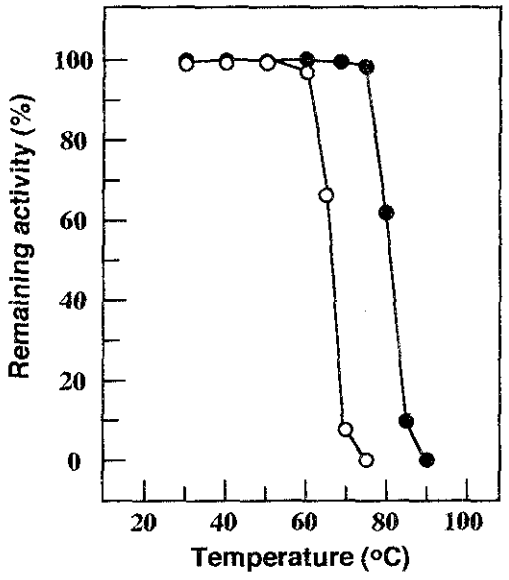

Fig. 6. Effect of thermostability of L-lactate dehydrogenase on pyruvate reduction (○) and L-lactate oxidation (●).
The enzyme (5μg of protein) was treated for 30min at the indicated tempeatures in 50μM sodium phosphate buffer (pH 6.5). After solutions were cooled on ice bath, enzyme activity with reaction mixtures were measured at 340nm. Pyruvate reduction was carried out in the reaction mixture containing 10mM sodium pyruvate, 0.4mM NADH, 0.5mM fructose 1,6-bisphosphate and 50mM sodium phosphate buffer (pH 6.5). L-lactate oxidation was performed in the reaction mixture containing 0.5M L-lactate, 2mM NAD^+ and 50mM sodium phosphate buffer (pH 6.5). The remaining activity was compared with the activity of the untreated enzyme under standard assay conditions.

Table 2. Effect of metal ions and chemical reagents on the activity of L-lactate dehydrogenase

Metal ions and chemical reagents	Final concentration	Relative activity (%)
None		100
$CaCl_2$	1mM	85
	2mM	83
$MgSO_4$	1mM	74
	2mM	69
$CuSO_4$	1mM	35
	2mM	22
$FeSO_4$	1mM	8
	2mM	2
Iodoacetate	1mM	52
	2mM	38
p-Chloromercuribonzoate	1mM	10
	2mM	5

The enzyme was incubated in 50mM sodium phosphate buffer (pH 6.5) containing various metal ions or chemical reagents for 30min at 30°C, and then the enzyme activity was assayed under the standard conditions in the presence of the same metal ions or chemical reagents.

examined. As shown in Table 2, Ca^{2+} and Mg^{2+} had slightly effect on the enzyme activity, whereas the activity was inhibited by iodoacetate and Cu^{2+} (less than 50% inhibition). However, more than 90% of enzyme activity was lost at 1mM $FeSO_4$ and *p*-chloromercuribonzoate (pCMB), indicating that Fe^{2+} and pCMB have a great effect on the enzyme activity. Inhibition of the enzyme activity by iodoacetate and pCMB, which specifically bind to thiol group, suggests that amino acid residues with -SH group such as cysteine or methionine play an important role in enzyme activity, and possibility that these residues may be contained in the active site of LDH. But, the effect of Cu^{2+} and Fe^{2+} on the LDH activity is not clear. The inhibition of enzyme activity by sodium oxalate was decreased against the increase of pyruvate concentration (Fig. 7). This result suggests that sodium oxalate, an analogue of pyruvate, reacts as competitive inhibitor in the interaction between substrate and enzyme, as oxaloacetate in LDH from *Bacillus subtilis*[2].

Dependence of the enzyme activity on fructose 1,6-bisphosphate (FBP)

Bacterial LDHs, which are usually allosteric enz-

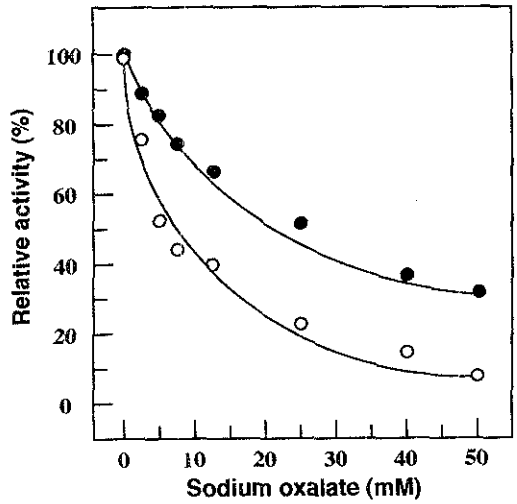

Fig. 7. Effect of sodium oxalate on the activity of L-lactate dehydrogenase.
Reaction mixtures contained 0.5mM fructose 1,6-bisphosphate, 0.4mM NADH, 1mM (○) or 10mM (●) sodium pyruvate, as well as various concentration of sodium oxalate, in 50mM sodium phosphate buffer (pH 6.5). Enzyme assay was carried out under standard assay conditions.

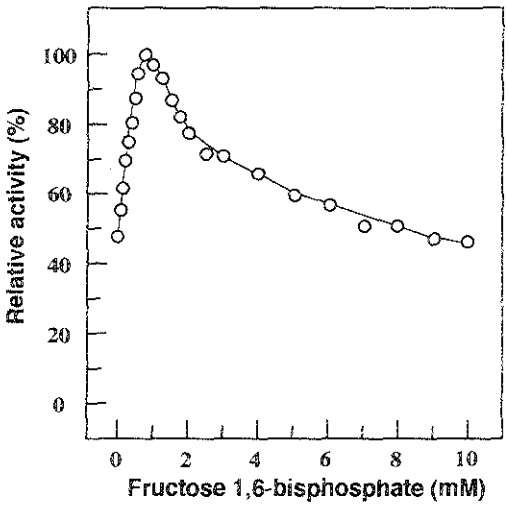

Fig. 8. Effect of fructose 1,6-bisphosphate on pyruvate reduction by L-lactate dehydrogenase.
Reaction mixtures contained 0.4mM NADH, 10mM sodium pyruvate and various concentration of fructose 1, 6-bisphosphate in 50mM sodium phosphate buffer (pH 6.5). Enzyme assay was carried out under standard assay conditions.

yme, are activated by FBP[2]. Fig. 8 showed the effect of FBP on the enzyme activity under the standard assay condition. The maximal activation was obtained with 0.8mM FBP, but the enzyme activity seemed to be inhibited by higher concentration of FBP. About 50% of the maximal activation was seen with 10mM FBP. This result indicates that FBP has an effect on the enzyme activity as a kind of allosteric activator, like in other bacterial LDH[2,8,15]. FBP not only activates the enzyme reaction, but also increases the thermostability of the native enzyme[8]. The allosteric activation mechanism by analysis of the three-dimensional structure of LDH clarified that FBP binding induced the conformational change at the effector site and, therefore, resulted in the substrate affinity change at the activity site[15]. Morever, chemical modification and site-directed mutagenesis showed that arginine at 173 and histidine at 188 in bacterial LDHs were essential to FBP-induced activation[19-22], and recent structure analysis demonstrated that these two residues were FBP-binding site[15].

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대장균으로 부터 생산된 L-lactate Dehydrogenase의 정제 및 특성

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요 약

*Bacillus stearothermophilus*의 유산탈수소 효소에 대한 4.3kb인 유전자를 subcloning하여 대장균에서 생산된 효소의 특성을 조사하였다. 본 효소는 25%의 수율로 200배 정제되었으며, 열처리, DEAE-Sephadex A-50, NAD^+-Sepharose CL-4B affinity chromatography, gel filteration을 거쳐서 정제되었다. Gel filteration에 의해 정제된 효소의 분자량은 약 140,000이었으며, SDS-polyacrylamide 전기영동 결과, 분자량 35,000의 단일 band를 나타내어 4개의 subunit로 구성된 효소임을 알 수 있었다. 정제효소에 대한 pyruvate 환원 반응과 lactate 산화 반응에 있어서의 최적 pH는 각각 5.5와 8.5이었고, pH 안정성은 pyruvate 환원 반응에 있어서 5에서 7 사이였다. 효소활성에 대한 최적온도 및 열안정성은 pyruvate 환원 반응에서 60°C, lactate 산화 반응에서 70°C와 75°C이었다. 본 효소는 FeSO4와 pCMB에 의해 강한 저해 반응을 나타내었으며, sodium oxalate는 경쟁적 저해제로 작용하였다. 또한 fructose 1,6-bisphosphate에 의한 효소활성 촉진은 0.8mM에서 최대의 활성을 나타내었다.