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Comparision of the Activity and Characteristics of Lactate Dehydrogenase Isolated from Different Parts of Soybean Seedling

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발아초기의 콩 부위별 Lactate Dehydrogenase 활성변화 및 효소성질 비교

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抄 錄

發芽初期에 콩(大豆)의 部位別로 Lactate dehydrogenase(LDH)의 Isozyme 存在可能性을 調査하기 위한 基礎的인 硏究가 遂行되었다. 발아가 進行됨에 따라 자엽부위의 酵素活性變化에는 큰 유의성이 나타나지 않았으나, 배축이나 뿌리 部位에서는 감소하는 경향을 보였으며 4~7°C에서 배축이나 뿌리로부터 얻은 LDH는 자엽에서 얻은 LDH에 比해 不安定했다.

전기영동상의 Rm value가 자엽으로부터 얻은 酵素에서는 0.25인데 비하여, 배축으로 부터 분리된 LDH는 0.29였다. 배축이나 뿌리로 부터 분리된 LDH는 Biphasic으로 0.45mM과 0.014mM의 두 km 값을 보이고 자엽부위에서는 0.45mM 값만 관찰할 수 있었다.

이상의 결과는 자엽부위의 LDH와는 다른 性質을 가진 LDH가 배축이나 뿌리部位에 存在할 可能性을 보여주고 있다.

Introduction

The lactate dehydrogenase(EC 1.1.1.27, Oxidoreductase; LDH) from various animal origins has been studied in detail by many investigators. Now there seems generally to agree with that LDH is tetrameric molecule made up with four subunits of two parent subunits such as H type and M type. A different composition of H type and M type for tetrameric molecule gives rise to five isozyme forms; H_4, MH_3, M_2H_2, M_3H, and M_4.[1~3] The different LDH isozymes show a characteristic distribution, a given kinetic and immunological properties with respect to different tissues.[4~6]

In contrast to detailed studies with animal LDH, a few reports have described the characterization of plant LDH which may play an important role in the metabolism of the anaerobic condition, in particular, when oxygen into seed is limited by seed coats or imbibition[7]. The evidence accumulated so far with plant LDH[8~12] is similar to animal LDH in terms of the stru-

ctural and kinetic charateristics. But two roles of plant LDH, the formation of lactate during the phase of germination and the degradation of lactate after testa has ruptured, are little understood with its structural characteristics as compared with animal LDH. Also, LDH isozyme have not been identified with plants as enzyme sources even if the possibility of the existence of isozymes has been speculated.

Therefore, this investigation was undertaken to examine the possibility of the existence of LDH isozymes as well as the roles of those isozymes in relation to germination metabolism of soybean.

Materials and methods

Plant materials

Seeds of soybean(Glycine max. L; Gwang-kyo) were germinated for a different time periods in a growth chamber in dark at 25°C by soybean sprout method[13] with the application of tapwater 4 times a day. Depending upon germination time, soybean sprout was divided into the as classified in Table 1.

Chemicals

All buffers were made of glass-redistilled water and adjusted to the required pH. Dialysis-tubing was obtained from Thomas laboratories. DEAE-52 cellulose was purchased from Whatman Ltd. N,N,N′, N′-Tetramethylenediamine(TEMED) and ammonium persulfate were supplied from Canal co. Electrophoretic reagents were procured from Bio-Rad laboratories. All other reagents were obtained from Sigma chemical co.

Enzyme extration and Isolation

Each enzyme sources were ground in a motar with two volumes(W/V) of 10mM potassium phosphate-1mM ethylenediaminetetraacetic acid (EDTA)-0.1% 2-mercaptoethanol, pH 7.4, and then were centrifiuged at 10,000×g for 10 minutes. The supernatant was collected and was refered to crude homogenate.

Solid ammonium sulfate was slowly added to crude homogenate to 30% saturation stirring with magnetic-stirrer. The precipitate formed by ammonium sulfate was removed by centrifugation at 10,000×g for 15 minutes. The supernatant was made 40% saturated with ammonium sulfate. The second precipitate collected by centrifugation at 10,000×g for 15 minutes was dissolved in 10mM potassium phosphate buffer, pH 7.4. The dissolved protein solution was dialyzed for 3 hours against 10mM potassium phosphate buffer, pH 5.0. The dialyzed solution was centrifuged at 12,000×g for 30 minutes, and then solid ammonium sulfate was added to the supernatant as mentioned above(between 25 and 40% saturation). The precipitate was dissolved in a small volume of 10mM potassium phosphate buffer, pH 6.5, and then was again dialyzed against same buffer. The dialyzed solution was centrifuged at 12,000×g for 30 minutes, and the supernatant was applied to DEAE-cellulose column had been previously equilibrated with 10mM potassium phosphate buffer, pH 6.5. The protein retained on the column were eluted at a rate of 0.8ml per minutes by increasing concentration of buffer linearly from 10mM to 750mM. The active fraction obtained from the column was concentrated with ammonium sulfate, and was dialyzed against 50mM Tris-HCl-1mM EDTA-1% 2-mercaptoethanol, pH 7.4. The dialyzed solution was stored at 2~5°C and it was used for experiment. These all steps were carried out arround 4°C, and potassium phosphate buffer containing 1mM

Table 1. Classification of parts from soybean sprout in relation to germination time.

Part of soybean sprout	Cotyledon	Cotyledon, Hypocotyl	Cotyledon, Hypocotyl, Root
Germination time(hours)	4, 20	40	60, 80, 100, 120

EDTA and 0.1% 2-mercaptoethanol was used throughout all steps of purification, unless specified otherwise.

Enzyme assay

The standard assay was carried out at 30°C by measuring the decreasing rate in E_{340} associated with NADH oxidation. The assay mixture contained 2.7ml of 50mM sodium phosphate buffer, pH 7.4, 0.1ml of 10mM sodium pyruvate, 0.1ml of enzyme solution, and 0.1ml of 2mM NADH solution in a total volume of 3.0ml. A unit of enzyme activity is defined as the oxidation of 1.0μM NADH per minute and the specific activity is expressed as the units of enzyme per mg of protein. The molar extinction coefficient of NADH was taken as $6.22 \times 10^3 M^{-1}$ [14].

The protein concentration was determined by the method of Lowry *et al*[15].

Polyacrylamide disc gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by the slight modification of the procedure of Davis[16]. The sample containing 150~200μg of protein(to show Protein band) or 28~33 units of LDH activity(to show enzyme activity band) was applied on the top of stacking gel, and then electrophorized at constant current of 3 miliamphere per tube. The localization of LDH activity band appeared when the electrophorized gels were incubated with the incubation medium (Table 2) at 25°C for 2~3 hours as described by Dietz[17].

Results and Discussion

Changes of LDH activity

At the various time intervals samples of germinated soybean were taken and LDH activity of their crude homogenate was measured. The results are given in Fig. 1. The enzyme activity was little changed in cotyledon throughout the early stage of germination, except the slight increase at the time of about 40 hours after imbibition when radicle had emerged.

Table 2. Incubation medium for stainning of lactate dehydrogenase activity band.

Reagents	Final conc.
Tris-HCl buffer, pH 7.4	50mM
(±)-Lactate	50mM
NAD^+	2.0mM
Nitroblue tetrazolium	0.1mM
Phenazine methosulfate	0.1mM

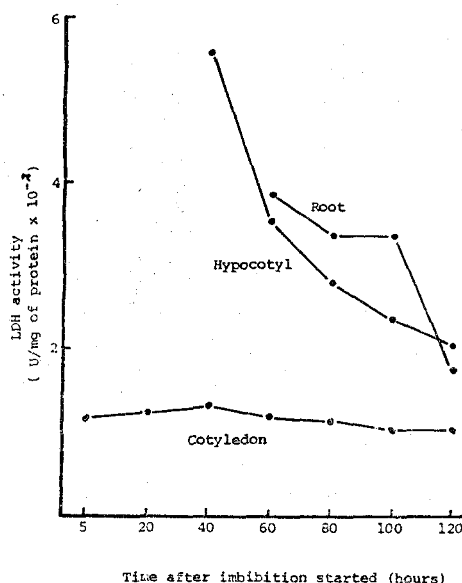

Fig. 1. Time course of lactate dehydrogenase activity during germination of soybean seeds. Each points are the average values of the enzyme activity obtained from three or more experiments.

This observation is somewhat similar to the results of early reports[18~21] that the enzyme activity reached at the maximum in 32~36 hours after imbibition of soybean. The slight discrepancy of the time to show the highest enzyme activity may be attributed to the enzyme source employed for the experiment. In the present investigation, studies of time course of the enzyme activity were performed with separation of cotyledon and hypocotyl from soybean sprout, while germinated soybean as a whole was employed in the early reports.

On the other hand, the crude homogenate of

Table 3. Summary of purification of LDH from cotyledons of soybean seeds germinated for 40 hours.

Fraction	*T.A [U]	Protein [mg]	Specific activity [U/mg of protein]	Fold purification
Crude homogenate	66. 1	4, 890	1.35×10^{-2}	1
30~40% $(NH_4)_2SO_4$	34. 4	1, 180	2.92×10^{-2}	2. 16
pH 5-precipitate	14. 8	371	4.00×10^{-2}	3. 00
DEAE-52	12. 8	14. 5	87.9×10^{-2}	65. 1

*T.A indicates the total enzyme activity. A unit of enzyme activity[U] is defined as 1μM of NADH oxidation per minute. The details of the experiments are given in the text. All data are average values obtained from at least three experimental results.

hypocotyl and root showed the continuous decline of the enzyme activity as germination time went on. In particular, rapid changes of the enzyme activity occurred between 40 and 60 hours in hypocotyl and between 100 and 120 hours in root.

Enzyme isolation

Soybean cotyledons germinated for 40 to 60 hours were subjected to enzyme purification in order to establish the purification procedure for LDH from different parts of soybean sprout and characterize the enzyme in terms of kinetic behavior and stability. The enzyme purification procedure included ammonium sulfate fractionation, pH 5. 0 precipitation, and DEAE-52 cellulose column chromatography. The enzyme was purified 65 fold through the above procedure as presented in Table 3. However, the purified LDH was not homogenous when judged by gel electrophoresis. Surprisingly, satisfactory result was not obtained when crude homogenates of hypocotyls and roots were subjected to the enzyme purification, using the procedure employed for that of cotylendons. This result suggests a possibility that LDH from hypocotyls and roots is quite unstable as compared with that of cotyledons.

Stability of soybean LDH

To determine stability of LDH from various sources, the enzyme activity of fraction was measured in 5, 15, 25, and 60 hours after storage in refrigerator at 4~7°C. Fig. 2 shows that partially purified enzyme from cotyledons was the most stable, and that crude homogenate of cotyledons was more stable than that of hypocotyls or roots. The results also indicate that the stability of the enzyme was not directly correlated to the content of protein since partially purified enzyme fraction containing 5. 8mg/ml of protein showed higher relative enzyme activity than crude homogenate of cotyledon, hypocotyl, and root, containing 46. 9, 16. 2, and 99. 8mg/ml of protein, respectively. This observation also supports a possibility that LDH in hypocotyls or roots is quite different from that in cotyledon.

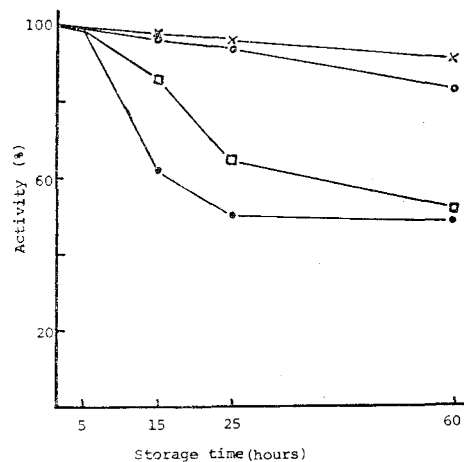

Fig. 2. Effect of temperature(at 4~7°C) on the stability of LDH activity. Partially purified enzyme of the cotyledon from soybean sprout germinated for 40 hours (×). Crude homogenate of the cotyledon (○), hypocotyl(□), and root(●) from soybean sprout germinated for 60 hours.

Electrophoretic study

Each enzyme fractions from different parts were employed to investigate the existence of isozymes in soybean sprout by means of polyacrylamide disc gel electrophoresis. The results are given in Fig. 3. Each enzyme fraction showed a single enzyme activity band. However, Rm valae of the enzyme from cotyledons was quite different from that of enzyme from hypocotyls or roots. The Rm value of LDH from cotyledons was estimated to be 0.25 which is similar to the value reported by King[8], while that from hypocotyls or roots to be 0.29.

These results give another important evidence that soybean may have LDH isozymes which play physiologically an important role at an early stage of germination.

Michaelis-Menten constant determination

A specific requirement for pyruvate and NADH in lactate formation reaction was reported with LDH from soybean by King[8] and Davis[9]. Thus the apparent Michaelis-Menten constant(Km) for LDH was determined by sodium pyruvate

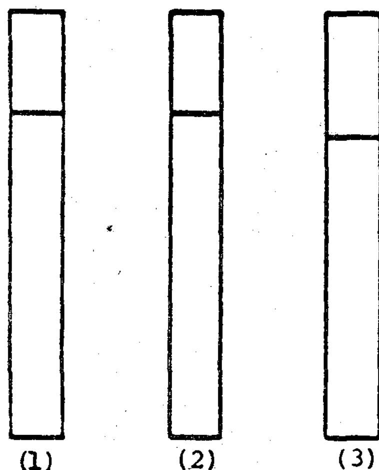

Fig. 3. LDH activity band on polyacrylamide gel. Partially purified enzyme of the cotyledon from soybean sprout germinated for 40 hours(1). Crude homogenate of the cotyledon(2), hypocotyl and root (3), from soybean sprout germinated for 60 hours. Protein was migrated from top(cathode) to bottom(anode).

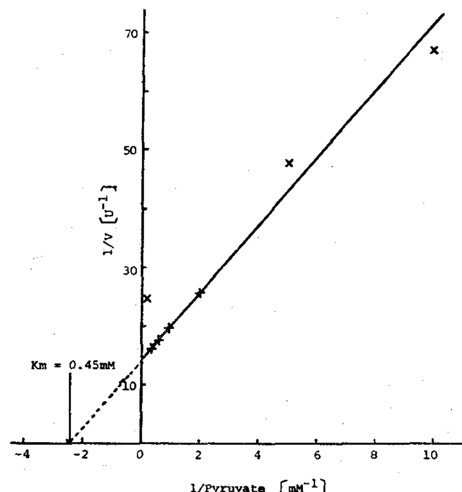

Fig. 4. Double reciprocal plots of LDH from cotyledon of soybean sprout germinated for 60 hours. A crude homogenate of 32×10^{-3} units of enzyme activity was used.

with 10 different concentration. The Km value was estimated by a double reciprocal plot suggested by Lineweaver-Burk[18]. As shown in Fig. 4, the apparent Km value for LDH from cotyledons was 0.45mM. A salient observation was the fact that enzyme reaction did not take place when sodium pyruvate concentration was kept low as much as 0.05mM. This Km value is essentially identical to the earlier results[8,9,19]. However, when the enzyme reaction rate was determined with crude homogenate from hypocotyls or roots, the biphasic phenomenon of enzyme reaction was observed. That is, the two different linear relationships were achieved in a wide range of sodium pyruvate concentration; low concentration region(0.01mM to 0.2mM) and high concentration region(0.5mM to 10mM), respectively. The Km value was calculated to be 0.014mM, while the Km value from high concentration region was 0.45mM, which is identical to that of cotyledon enzyme.

These results indicate that hypocotyls and roots have a different LDH isozyme from the enzyme obtained early from cotyledons which is predominant in soybean and has been exten-

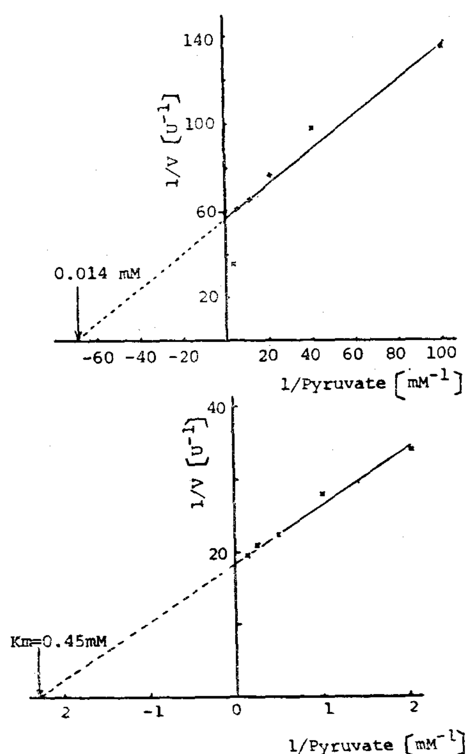

Fig. 5. Double reciprocal plots of LDH from hypocotyls and roots of soybean sprout germinated for 60 hours.
(A); low concentration region of pyruvate(0.01mM to 0.2mM). (B); high concentration region of pyruvate(0.5mM to 10mM). A crude homogenate of 31.5 $\times 10^{-3}$ units of enzyme activity was used.

sively studied by other investigators[8~11,19]. It is very interest to know that LDH isozyme from hypocotyls and roots has higher affinity for pyruvate than LDH from cotyledons, and that LDH with higher affinity for pyruvate exists in hypocotyls and roots at particular time when radicle emerges from seed coat. At the present, the physiological role of the LDH isozyme existence is not well understood yet.

Abstract

The change of lactate dehydrogenase(LDH) activity and the possibility of the existence of LDH isozyme were examined with different parts of soybean sprout. The enzyme activity was little changed in cotyledons throughout the early stage of germination. However, hypocotyls and roots showed the continuous decline of the enzyme activity since the radicle emerged from seeds. It was found that LDH from hypocotyls and roots was unstable as compared with LDH from cotyledons, even at low temperature. The enzyme from hypocotyls and roots was not purified with a good yield when the purification procedure developed for LDH from cotyledons was employed. LDH from hypocotyls and roots has the Rm value of 0.29, and 0.25 from cotyledons. The apparent Km value for LDH from cotyledons was 0.45mM with sodium pyruvate, while crude homogenate of hypocotyls or roots showed biphasic phenomenon with two Km values 0.014 and 0.45mM.

These results indicate the possibility that crude homogenate of hypocotyls or roots may contain a different LDH isozyme from the LDH of soybean reported previously.

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