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Purification and kinetic characterisation of lactate dehydrogenase from *Dioscorea cayenensis* tuber

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Abstract

Lactate dehydrogenase from yellow yam tuber (*Dioscorea cayenensis* Lam.) was isolated and purified using various chromatographic methods and electrophoresis. Only one form of the enzyme obtained, which obeyed Michaelis-Menten kinetics, was activated by Mg^{2+} and Ca^{2+} and inhibited by nucleotides and PEP. AMP, which activated the enzyme in the direction of pyruvate reduction, inhibited it in the direction of lactate oxidation. The enzyme is specific for pyruvate and L-lactate and uses only NADH and NAD^+ as the electron carriers. Polyacrylamide gel electrophoresis showed single band of lactate dehydrogenase activity. The average molecular mass obtained for the enzyme was 160 ± 1.2 kDa, while SDS gel electrophoresis indicated a dimer for the enzyme protein. The enzyme is very stable when frozen but its activity was hardly detectable when the tubers were stored in a well aerated place.

Additional key words: L-lactate, pyruvate, SDS gel electrophoresis, yam.

Introduction

Lactate dehydrogenase (LDH) (EC 1.1.1.27) is the enzyme that reversibly catalyses the conversion of pyruvate to lactate with NADH + H^+ as electron donor. The enzyme is present in appreciable amount in animals and bacteria and also in plants (Davies and Davies 1972, Oba *et al.* 1977, Ugochukwu and Anosike 1979). LDH from animals, fungi and bacteria has been often studied, but little attention has been paid to LDH from plants except that extracted from potato tuber (Davies and Davies 1972) and sweet potato roots (Oba *et al.* 1977). Since yam tubers produce lactate as well as alcohol under anaerobic condition (Ugochukwu and Anosike 1979), it is necessary to study the properties of LDH from this source in order to elucidate the nature and the role of this enzyme in sugar metabolism in yam tubers. This paper, describes the isolation, purification, and kinetic characterisation of LDH from yellow yam tubers.

Materials and methods

Materials: The yam (*Dioscorea cayenensis* Lam.) tubers used in this study were obtained from the author's garden and stored in the refrigerator at 4 °C for 48 h before use. All reagents and chemicals were supplied by *Sigma*, London, while *Sephadex* series were the products of *Pharmacia Fine Chemicals*, Uppsala, Sweden. Spectrophotometric measurements were made using *Pye Unicam SP 1800* (Cambridge, UK) spectrophotometer fitted with chart recorder. Temperature was stabilized by constant temperature circulator.

Calcium phosphate gel: Calcium phosphate gel was prepared by mixing 250 cm^3 of 1.07 M calcium chloride with 250 cm^3 of 1.56 M K_2HPO_4. The precipitate was filtered and washed with 50 cm^3 distilled water. The gel was sucked dry and stored at 4 °C.

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Abbreviations: CTP - cytidine triphosphate; DTT - dithiothreitol; GTP - guanosine triphosphate; LDH - lactate dehydrogenase; PEP - phosphoenolpyruvate.

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Enzyme extraction: Yellow yam tubers were peeled and 40 g each obtained from the head, middle and tail regions were combined and homogenized in 100 cm^3 of 30.0 mM phosphate buffer, pH 7.5, containing 1.0 mM dithiothreitol (DTT). The homogenate was squeezed through four layers of cheese cloth, and the extract centrifuged at 20 000 *g* for 25 min at 4 °C. The supernatant was brought to 20 % saturation with solid $(NH_4)_2SO_4$ (10.6 g per 100 cm^3), centrifuged and the precipitate discarded. The resulting supernatant was raised to 65 % saturation with solid $(NH_4)_2SO_4$ and centrifuged as before. The precipitated protein was dissolved in 50 cm^3 of the extraction medium and dialysed against 3 × 500 cm^3 distilled water. The dialysed sample was centrifuged and any precipitate discarded.

Purification: The dialysed sample (100 cm^3) was stirred while calcium phosphate gel (5 g) was added. After 10 min the gel was collected by centrifugation at 6 000 *g* for 5 min and discarded. The supernatant was then treated with 10 g calcium phosphate gel, left for 10 min and centrifuged. The gel was retained. The gel was resuspended in 40 cm^3 of 20 mM potassium phosphate buffer, pH 7.8, and left to stand for 10 min. The gel was collected by centrifugation as before and the supernatant was discarded. The gel was treated with 40 cm^3 of 0.1 M potassium phosphate buffer, pH 7.8, and after standing for 10 min, the calcium phosphate gel was removed by centrifugation and the supernatant which contained LDH was retained. The LDH from the above treatment was separated using $(NH_4)_2SO_4$ gradient solubilization as previously described (Oluoha and Ugochukwu 1991). The fractions constituting the peak showing LDH activity were pooled and desalted by passing through *Sephadex G -25* column (2 × 30 cm). The effluent containing LDH (18.5 cm^3) was applied to *DEAE - Sephadex A-25* ion exchange column (4.5 × 20 cm) previously equilibrated with 5 mM phosphate buffer, pH 7.5, containing 1.0 mM DTT, and 10 % sucrose. Then 200 cm^3 of the same buffer were passed through the column, followed by linear gradient formed from 400 cm^3 of the buffer in the mixing flask and 400 cm^3 of the buffer containing 0.4 M KCl in the reservoir flask at a flow rate of 1.0 cm^3 min^{-1}. 4.0-cm^3 fractions were collected and the active fractions were combined and concentrated. 1.4 cm^3 were layered on the top of *Sephadex* gel column (1.4 × 80 cm) pre-equilibrated with 5 mM phosphate buffer, pH 7.5, containing 10 % sucrose and 1.0 mM DTT. 50 cm^3 of the same buffer were passed through the column at a flow rate of 15 cm^3 h^{-1}. The active protein fractions were collected and concentrated using ethylene glycol powder (Oluoha 1990) and the concentrated sample was stored at -20 °C. No loss of activity occurred on storage for two months. DTT and sucrose were essential to stabilize the enzyme during purification.

Analytical methods: Protein was estimated using protein dye-binding method (Bradford 1976). Bovine serum albumin was used as the standard protein. Sodium was estimated using flame photometer, while NH_4^+ was determined employing phenol-hypochloride/nitroprusside reaction (Varley 1969).

Enzyme assay: Lactate dehydrogenase activity was assayed at pH 7.3 by measuring the decrease in absorbance at 340 nm associated with NADH oxidation. Assay mixture contained 0.3 mM NADH, 2.0 mM pyruvate and 0.1 cm^3 enzyme extract in a volume of 2.5 cm^3. The reaction was started by addition of pyruvate. The activity of the enzyme was also assayed in the direction of lactate oxidation by measuring the increase in absorbance of NADH produced at 340 nm. The buffer used was Tris-HCl, pH 8.5. Optimum pH of the LDH was determined in 60 mM phosphate buffer in the pH range of 5.0 to 7.0 and in the pH range of 7.1 to 9.5, using 60 mM Tris-HCl buffer.

Polyacrylamide gel electrophoresis in non-denaturing buffer system was carried out as described by Hedrick and Smith (1968). LDH activity in the gel was detected by staining each gel in 5.0 cm^3 of Tris-HCl buffer, pH 8.4, containing 10 mM lactate, 0.32 NAD^+, 0.4 mg of phenazine methosulphate, and 1.6 mg of *p*-nitro-blue tetrazolium for 1 h at 3 °C in the dark. The gels were removed and rinsed in distilled water and stained bands identified.

Molecular mass of LDH was determined by polyacrylamide disc gel electrophoresis in non-denaturing buffer system (Hedrick and Smith 1968) and gel filtration. SDS gel electrophoresis (Weber and Osborne 1969) was also performed to determine the subunit molecular mass. The following substances were used as protein markers: chymotrypsinogen (25.0 kDa), bovine serum albumin (67.0 kDa), hexokinase (96.0 kDa), alcohol dehydrogenase (150.0 kDa) and catalase (240.0 kDa).

Effect of modulators. LDH activity was assayed in the presence and absence of the modulators. In the direction of pyruvate reduction, pyruvate was varied from 0.5 to 5 mM, while in the direction of NAD^+ reduction, lactate was varied from 1.0 to 5 mM.

Results and discussion

Lactate dehydrogenase extracted from yellow yam tubers was separated by calcium phosphate gel and further purified using $(NH_4)_2SO_4$ gradient solubilization, gel filtration and ion exchange chromatography. The enzyme was eluted from *DEAE-Sephadex* ion exchange chromatography between 0.08 and 0.12 M KCl. One form of the enzyme was obtained which contrasts with 2 forms from roots of sweet potato (Oba *et al.* 1977) but is similar to one form from potato tubers (Davies and Davies 1972). The enzyme was purified 33.3 fold with specific activity of 100 nkat mg^{-1}(protein) and a recovery of 33.3 % (Table 1). The problem encountered in this study was the low activity of the enzyme from yellow yam tuber which necessitated concentration of the extract before purification.

Table 1. Summary of purification procedures.

Purification steps	Protein [mg]	Activity [nkat]	Specific activity [nkat mg^{-1}]	Purification [fold]	Recovery [%]
Crude extract	200	600	3.0	1	100.0
20 - 65 % $(NH_4)_2SO_4$ (precipitate)	100	400	4.0	1.2	66.6
$Ca_3(PO_4)_2$ gel desorption (supernatant)	20	350	17.5	5.8	58.3
Sephadex G-25	10	300	30.0	10.0	50.0
DEAE-Sephadex	4	250	82.5	20.6	41.6
Sephadex G-150	2	200	100.0	33.3	33.3

Substrate specificity: The enzyme is active with pyruvate but showed very little activity with glyoxylate, α-oxo-butyrate and insignificant activity with D-lactate. It showed no reduction activity with hydroxypyruvate and α-ketoglutarate and does not react with NADPH. It uses NADH and NAD^+. This indicates that the enzyme is specific for pyruvate and NADH and this is reflected in its low K_m and very high V_{max} in the reduction of pyruvate (Table 2). However, LDH from potato tuber (Davies and Davies 1972) and sweet potato roots (Oba *et al.* 1977) have been reported to be active with all the oxo acids (Table 2) and use NADH and NADPH as the electron donors. However, plants and animals contain NAD-linked glycerate dehydrogenase that can catalyse the reduction of hydroxypyruvate, glycerate and pyruvate (Davies and Davies 1972). Plant also contains glyoxylate reductase that can reduce pyruvate and hydroxypyruvate at low rate (Tolbert *et al.* 1970).

Table 2. Kinetic constants of LDH from yellow yam tuber using various substrates.

Substrate	K_m [mM]	V_{max} [nkat mg^{-1}(protein)]
Pyruvate	2.2	80.0
Hydroxypyruvate	-	-
Glycerated	16.0	8.0
α-oxo-butyrate	30.0	4.0
α-ketoglutarate	-	-
D-lactate	60.0	2.0
L-lactate	8.0	50.0

The K_m found for the yam enzyme in respect of NAD^+ and NADH are 0.13 and 0.12 mM, respectively. These values are about 10 times higher than those reported for sweet potato root LDH (Oba *et al.* 1977) and potato tuber LDH (Davies and Davies 1972). The K_m obtained for the enzyme in respect of L-lactate is 8.0 mM while that of D-lactate is 60 mM with V_{max} of 2.0 nkat mg^{-1}(protein). This indicates that the yam LDH is specific for L-lactate in the direction of NAD+ reduction.

High level of pyruvate has been demonstrated to inhibit sweet potato LDH (Oba *et al.* 1977). In this study, the yam LDH was not inhibited by high concentration of pyruvate. The yam LDH is stable when frozen and this is similar to the stability shown by LDH from sweet potato roots (Oba *et al.* 1977). pH optimum found for the enzyme is 7.5 in the direction of pyruvate reduction and 8.0 in the direction of lactate oxidation. These pH values were higher than those reported for potato tuber LDH (Davies and Davies 1972) but similar to those reported for sweet potato LDH (Oba *et al.* 1977).

Polyacrylamide gel electrophoresis in non-denaturing buffer system showed single band of LDH activity when performed at different gel concentrations, pH values and using various enzyme concentrations. Moreover, the enzyme was eluted from all chromatographic columns as single symmetrical peaks: the preparation seems to be homogeneous. The plots of log mobility against gel concentration showed a single line which supports single

band of LDH activity. This contrasts with two forms of LDH from sweet potato roots (Oba *et al.* 1977) which exist as charge isomers when separated by disc gel electrophoresis. The average molecular mass obtained for the enzyme using various methods is 160.0 ± 1.2 kDa. This molecular mass is higher than that reported for LDH isoenzymes from sweet potato roots (Oba *et al.* 1977). SDS gel electrophoresis showed subunit molecular mass of 78.5 ± 2.0 kDa, indicating a dimeric protein. This contrasts with LDH from animal tissues which consists of 4 polypeptide chains.

Effect of various compounds: The K_m and V_{max} obtained for the LDH in the direction of pyruvate reduction are 2.2 mM and 80 nkat mg^{-1}(protein), respectively (Table 3). In the presence of ATP and cytidine triphosphate (CTP), the K_m increased while the V_{max} decreased, indicating mixed-type inhibition. Guanosine triphosphate (GTP) competitively inhibited the enzyme as shown by increased K_m value but the V_{max} remained unchanged with K_i of 1.57 mM. In the presence of ADP both K_m and V_{max} decreased, showing uncompetitive inhibition.

Table 3. Effect of various modulators on the activity of lactate dehydrogenase from yam tuber in direction of pyruvate reduction.

Addition to assay	[mM]	K_m [mM]	V_{max} [nkat mg^{-1}(protein)]	K_i [mM]
None		2.2	80.0	-
ATP	0.5	4.5	31.0	0.48
AMP	1.2	1.5	100.0	-
ADP	1.2	1.6	60.0	3.40
GTP	2.0	5.0	80.0	1.57
CTP	2.0	8.0	60.0	9.45
PEP	2.0	2.2	40.0	2.00
Mg^{2+}	1.0	1.0	130.0	-
Ca^{2+}	1.0	1.1	110.0	-

Phosphoenolpyruvate (PEP) non-competitively inhibited the enzyme as is confirmed by decrease in V_{max} but without any change in the K_m. The K_i is 2.0 mM. AMP, Mg^{2+}, and Ca^{2+} activated the LDH as their presence lowered the K_m with highly increased V_{max}. The most potent inhibitor in this direction is ATP with the lowest K_i of 0.48 mM, while the most efficient activator is Mg^{2+}. The least inhibitory is CTP with the highest K_i of 9.43 mM. It has been reported that AMP inhibited LDH from sweet potato roots (Oba *et al.* 1977).

In the direction of lactate oxidation (Table 4), the K_m and V_{max} found for the enzyme were 8.0 mM and 50.0 nkat mg^{-1}(protein), respectively. The K_m reported here is higher than that reported for sweet potato root enzyme (Oba *et al.* 1977).

In the presence of ATP, ADP, and PEP, the K_m increased with decrease in V_{max}. This indicates mixed type inhibition by the effectors. However, CTP, AMP and GTP uncompetitively inhibited the enzyme as shown by decrease in both K_m and V_{max} with K_i of 8.0, 1.20, and 9.76 mM, respectively. Mg^{2+} and Ca^{2+} activated the enzyme as shown by reduced K_m and very high V_{max}. The most potent inhibitor is PEP with K_i of 2.0 mM, while GTP is the least inhibitory with K_i of 9.76. It has to be noted that the type of inhibitions shown by the modulators in the direction of pyruvate reduction (Table 3) are different from those exhibited by the modulators in the direction of lactate oxidation (Table 4).

Table 4. Effect of various modulators on the activity of lactate dehydrogenase from yam tuber in direction of lactate oxidation.

Addition to assay	[mM]	K_m [mM]	V_{max} [nkat mg^{-1}(protein)]	K_i [mM]
None		8.0	50.0	-
ATP	0.5	9.0	20.0	0.33
AMP	1.2	10.0	26.0	2.20
ADP	1.2	5.0	25.0	1.20
GTP	2.0	5.0	41.5	9.76
CTP	2.0	4.0	40.0	8.00
PEP	2.0	10.0	18.0	2.00
Mg^{2+}	1.0	0.6	100.0	-
Ca^{2+}	1.0	1.2	70.0	-

High activity of LDH from yellow yam tuber was observed when tubers were injured or subjected to anaerobiosis but its activity was extremely low and in some cases undetectable when the tubers were stored in a well aerated place. It seems available oxygen is unfavourable for LDH to operate, probably by increasing the operation of TCA cycle. However, similar increases in LDH and malate dehydrogenase have been reported when white yam tubers were stored under nitrogen (Ugochukwu and Anosike 1979), while Oba *et al.* (1977) reported high increased activity of sweet potato root LDH when the potatoes were injured or infected by pathogens. These results indicate that LDH can play an important role in regulation of glycolysis by oxidation of $NADH+H^+$ thus making NAD^+ available for glycolysis to continue during high sugar degradation occasioned by the injury or anaerobiosis.

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