Pergamon

PII: S0031-9422(96)00629-2

Phytochemistry, Vol. 44, No. 4, pp. 571–574, 1997

0031-9422/97 $17.00+0.00

EVALUATION OF THE ROLE OF LACTATE DEHYDROGENASE IN OXALATE SYNTHESIS

NOBUO SUGIYAMA* and NAOKI TANIGUCHI

Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

(*Received* 8 *July* 1996)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; lactate dehydrogenase; oxalate.

Abstract—The kinetic properties of lactate dehydrogenase (LDH) (EC 1.1.1.27) from spinach leaves were studied in order to evaluate the possible roles of LDH in the production of oxalate. LDH was purified by affinity chromatography on affigel blue and oxamate agarose columns. The pH optimum for the reduction of pyruvate was 7.25, while that for the reduction of glyoxylate was 7. The rate of reduction of pyruvate and glyoxylate at the optimum pH indicated substrate inhibition at concentrations above 8 and 40 mM, respectively. The maximum activity of LDH with pyruvate was about three times greater than with glyoxylate. The pH optimum for the oxidation of lactate was 9, with very low activity below pH 8. Substrate inhibition was apparent at lactate concentrations above 10 mM. LDH was inactive with glyoxylate in the oxidative reaction, which would lead to the biosynthesis of oxalate. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Lactate dehydrogenase (LDH) (EC 1.1.1.27), which catalyses the oxidation of lactate and the reduction of pyruvate, is a terminal enzyme in the anaerobic glycolysis in vertebrates [1]. In most higher plants, however, the terminal enzyme of glycolysis is alcohol dehydrogenase (EC 1.1.1.1) [2] with the exception that lactate glycolysis by LDH contributes to anaerobic metabolism in barley aleurone layers [3].

Some green leaves contain LDH at low levels under aerobic conditions [4–8]. Davies [2] assumed that lactate produced by LDH lowers cytoplasmic pH, which in turn triggers alcohol dehydrogenase under anaerobic conditions. *In vivo* NMR studies on corn roots support this proposal [9]. However, the function of LDH in providing the signals that trigger alcohol dehydrogenase has been questioned. This is because (i) anaerobic conditions do not stimulate the flow of carbon to lactate glycolysis in barley roots [10], and (ii) lactate production does not precede ethanol production in rice seedlings after transfer to anoxia [11].

LDH from plant sources has been reported to catalyse the oxidation of glyoxylate to oxalate as well as mammalian LDH [1, 7]. Davies and Asker [6] proposed a physiological function of constitutive LDH in the biosynthesis of oxalate in plant leaves. They postulated that glyoxylate, if produced in excess in the peroxisomes, leaks into the cytosol where LDH converts it to glycolate or oxalate. The aim of the present study was to examine the possible role of constitutive LDH in the biosynthesis of oxalate in spinach leaves containing large amounts of oxalate.

RESULTS

The purification protocol and typical purification data are shown in Table 1. A modification of the method of Hoffman and Hanson [12] was necessary to purify LDH from spinach leaves because LDH elutes from the affigel blue column at 0.3 M KCl in equilibration buffer. The overall yield of LDH at the step of affigel blue chromatography was 42% with a 35-fold purification, while that at the step of oxamate–agarose chromatography was about 55% with a 74-fold purification (Table 1). The final preparation was assessed by SDS–PAGE to have only one band of M_r 38 000 (data not shown).

The optimum pH for LDH reduction of pyruvate was 7.25 and that for the reduction of glyoxylate was 7 (Fig. 1(A)). At pH 7.25, the rate of pyruvate reduction increased as the concentration of pyruvate increased up to 4 mM, but above 8 mM the rate of reduction decreased (Fig. 2). Substrate inhibition in the reduction of glyoxylate was also apparent above 40 mM (Fig. 3). As the concentration of NADH increased, the rate of pyruvate and glyoxylate reduction increased and reached a plateau between 0.3 and 0.5 mM (Fig. 4). The maximum rate of pyruvate and glyoxylate reduction was about 550 and 170 nkat mg^{-1} protein, respectively.

* Author to whom correspondence should be addressed.

Table 1. Purification of lactate dehydrogenase from spinach leaves

Purification step	Protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Yield (%)	Purification factor
Crude extract	300	59.5	0.2	100	1
Affigel blue	3.54	24.7	7.0	42	35
Oxamate–agarose	0.026	13.5	519	23	2600

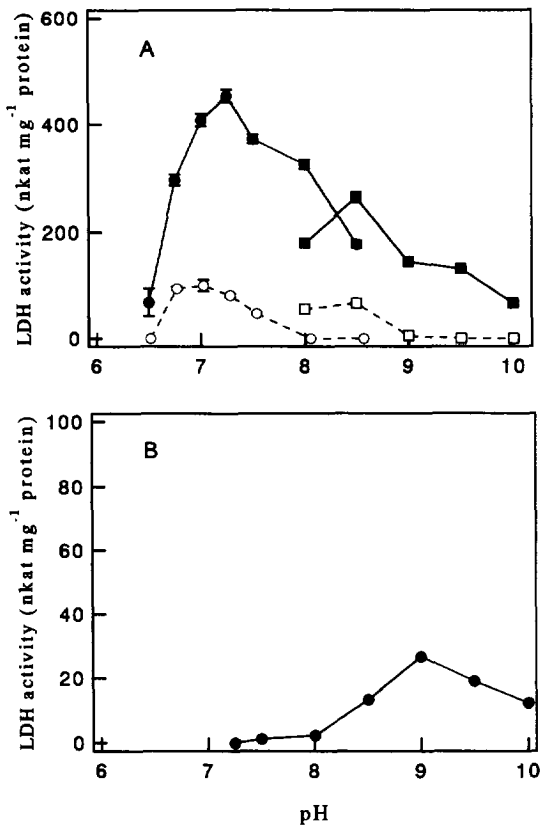

Fig. 1. Effect of pH on the activity of LDH. (A) The reaction was measured as the reduction of pyruvate (closed symbols) and glyoxylate (open symbols). The assay mixture contained 0.1 M phosphate buffer (circles) or 0.1 M Tris–acetate (squares), 0.5 mM NADH and substrate. Concentrations of sodium pyruvate and sodium glyoxylate were 4 and 40 mM, respectively. Data are the means of five determinations ± S.D. (B) The reaction was measured as the oxidation of lactate. The assay mixture contained glycine buffer (0.1 M), 0.5 mM NADH and 0.01 M lithium lactate. Data are the means of five determinations ± S.D.

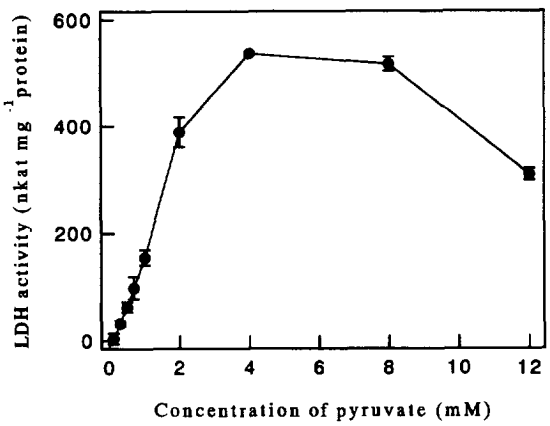

Fig. 2. Effect of pyruvate concentration on the activity of LDH catalysing the reduction of pyruvate at pH 7.25. The assay mixture contained phosphate buffer (0.1 M), 0.5 mM NADH and sodium pyruvate at various concentrations. Data are the means of five determinations ± S.D.

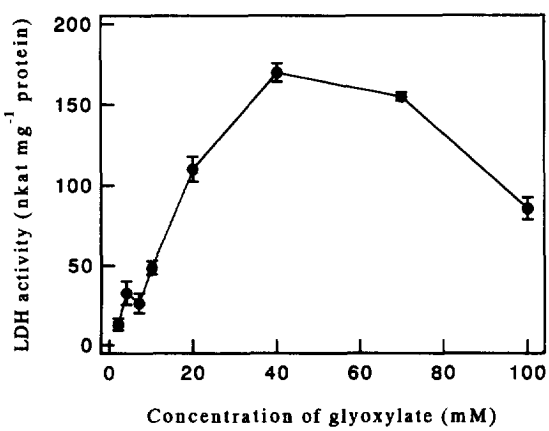

Fig. 3. Effect of glyoxylate concentration on the activity of LDH catalysing the reduction of glyoxylate at pH 7. The assay mixture contained phosphate buffer (0.1 M), 0.5 mM NADH and sodium glyoxylate at various concentrations. Data are the means of five determinations ± S.D.

The optimum pH for LDH lactate oxidation was 9 (Fig. 1(B)). The rate of lactate oxidation was almost negligible at pH below 8, indicating clearly that this is a side reaction of no physiological importance. The rate of lactate oxidation at pH 9 increased as lactate concentrations increased to 10 mM, but substrate inhibition became apparent at higher concentrations (Fig. 5). The rate of lactate oxidation was maximum at 0.5 mM NAD, although it showed only a slight response to NAD over 0.1 or 6 mM (data not shown). The maximum rate of lactate oxidation was about 33 nkat mg^{-1} protein, which corresponds to about 1/17 of the rate for pyruvate reduction.

LDH failed to catalyse the conversion of glyoxylate to oxalate in the pH range 7–10 or at glyoxylate concentrations up to 100 mM (data not shown).

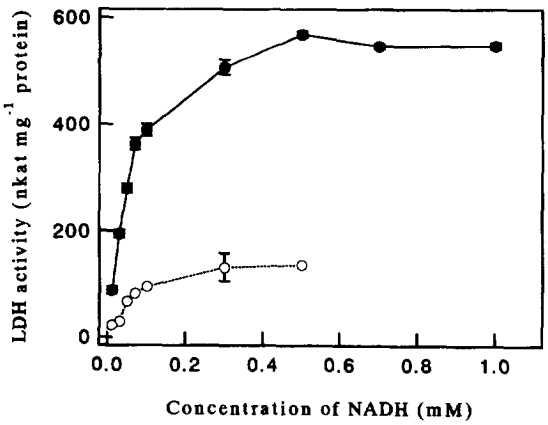

Fig. 4. Effect of NADH concentration on the reduction of pyruvate (closed symbols) by LDH at pH 7.25 and glyoxylate (open symbols) at pH 7. The assay mixture contained phosphate buffer (0.1 M), 4 mM sodium pyruvate or 40 mM sodium glyoxylate, and NADH at various concentrations. Data are the means of five determinations ± S.D.

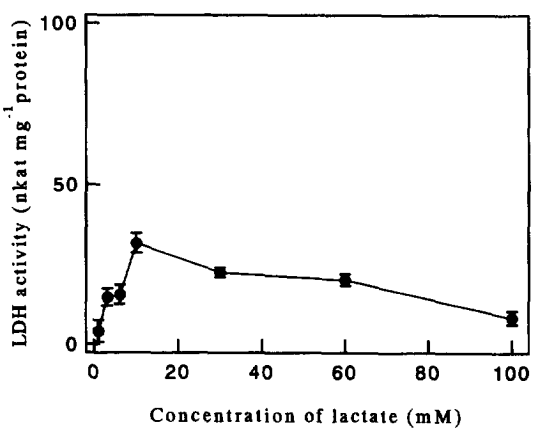

Fig. 5. Effect of lactate concentration on the activity of LDH catalysing the oxidation of lactate. The assay mixture contained glycine buffer (0.1 M), 0.5 mM NADH and lithium lactate at various concentrations. Data are the means of five determinations ± S.D.

DISCUSSION

Oxalate comprises more than 10% of the dry matter in several plant species, including spinach, sugarbeet and amaranth [13]. Oxalate may be produced by the action of glycolate oxidase (EC 1.1.3.1) [14, 15] or LDH [6] on glyoxylate, although Raven *et al.* [16] disputed the possibility that oxalate is formed via glyoxylate because $^{18}O_2$ supplied to spinach leaves is incorporated into glycolate and glycerate but not into oxalate. Based on studies of the kinetic properties of LDH from lettuce leaves, Davies and Asker [6] proposed a model in which glyoxylate produced in excess in peroxisomes leaks into the cytosol where it is converted by LDH into oxalate or glycolate. However, their model is inconsistent with our results, which show that LDH from spinach leaves does not catalyse the oxidation of glyoxylate to oxalate. Contradictory results have been reported for LDH from potato tubers. Rothe [17] reported that LDH from potato tubers is unable to oxidize glyoxylate. On the other hand, Poerio and Davies [1] and Asker and Davies [18] demonstrated that LDH from potato tubers oxidizes glyoxylate into oxalate as in the case of LDH from lettuce leaves.

Glyoxylate inhibits photosynthesis and the activation of ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39) in intact chloroplasts [19]. The ability of LDH to catalyse the conversion of glyoxylate to glycolate indicates that cytosolic LDH may represent an auxiliary system for eliminating glyoxylate that sometimes escapes from the peroxisomes into the cytosol [6]. However, Betsche [4] claimed that glyoxylate is unlikely to be a natural substrate for LDH since the affinity of LDH for glyoxylate during glycolate production is unfavourably low. In the present study, LDH from spinach leaves did not show normal Michaelis–Menten kinetics, which made it impossible to determine the Michaelis constants for pyruvate and glyoxylate in the reductive reaction. Therefore, it is open to question whether LDH might play a role in the reduction of glyoxylate to glycolate. Heupel *et al.* [20] considered that cytosolic glyoxylate reductase (EC 1.1.1.79) acts to eliminate glyoxylate. Recently, glyoxylate reductase has been purified and characterized [21].

EXPERIMENTAL

Spinach plants (*Spinacia oleracea* L. var. Okame) were grown hydroponically in a naturally lit growth room (25° from 6 a.m. to 7 p.m. and 20° during the other period) for 50–60 days in winter. The composition of the nutrient soln was the same as that used in ref. [22]. Purification of LDH was performed by a modification of the method in ref. [12]. Briefly, spinach leaves (15 g) were ground in an ice-cold mortar and pestle in 30 ml of 0.1 M Tris–HCl (pH 8.8) with 10 mM Na borate, 5 mM DTT and 2 mM EDTA. The cellular debris was removed by centrifugation for 15 min at 12 000 *g*. The supernatant was filtered through Whatman No. 4 paper. An affigel blue column (40 ml bed vol.) was equilibrated by washing with 200 ml of buffer (pH 8.8) containing 40 mM Tris–acetate, 2 mM EDTA and 0.5 mM DTT. A filtered extract containing less than 400 mg protein was run on the column at a flow rate of 2 ml min^{-1}. After washing the column with 200 ml of 40 mM Tris–acetate (pH 7.9) containing 2 mM EDTA, 0.5 mM DDT and 0.2 M KCl, the LDH was eluted with 100 ml of 40 mM Tris–acetate (pH 7.9) containing 2 mM EDTA, 0.5 mM DDT, 0.35 M KCl and 0.7 mM NADH at a flow rate of 1.5 ml min^{-1}. Fractions of 5 ml were collected and assayed for LDH activity. Active fractions were combined and concd on an ultrafiltration membrane (Molcut L membrane, M_r cut-off 30 000, Millipore Japan). The concd active fractions were applied to an oxamate agarose column equilibrated with 50 mM K-P_i buffer (pH 7.4) containing 0.3 M KCl, 0.5 mM DTT and

0.07 mM NADH. Proteins were eluted using the same buffer without NADH. The eluate was used immediately for the study of kinetic properties or concd by ultrafiltration for the determination of M_r. The M_r of LDH subunit was determined by SDS–PAGE according to the method in ref. [23].

The standard assay was carried out at 25°. The activity of LDH in the pyruvate/lactate direction was measured by the decrease in A at 340 nm with NADH oxidation. The assay mixture consisted of 0.1 M K-P_i (pH 7.25), 0.5 mM NADH, 4 mM Na pyruvate, 2.3 mM 4-methypyrazole, and 2.3 mM NaCN. When the activity was measured in the direction of lactate/pyruvate, the assay mixture contained 0.1 M glycine (pH 9), 10 mM lithium lactate and 1 mM NAD. Protein was determined by the method in ref. [24] using bovine serum albumin as a standard.

REFERENCES

1. Poerio E. and Davies, D. D., *Biochemical Journal*, 1980, **191**, 341.
2. Davies, D. D., in *The Biochemistry of Plants*, Vol. 2, eds P. K. Stumpf and E. E. Conn. Academic Press, New York, 1980, pp. 581–611.
3. Hanson, A. D. and Jacobsen, J. V., *Plant Physiology*, 1984, **75**, 566.
4. Betsche, T., *Biochemistry Journal*, 1981, **195**, 615.
5. Betsche, T., Bosbach, K. and Gerhardt, P., *Planta*, 1979, **146**, 567.
6. Davies, D. D. and Asker, H., *Plant Physiology*, 1983, **72**, 134.
7. Davies, D. D. and Davies, S., *Biochemical Journal*, 1972, **129**, 831.
8. O'Carra, P. and Mulcahy, P., *Phytochemistry*, 1996, **42**, 581.
9. Roberts, J. K. M., Callis, J., Wemmer, D., Walbot, V. and Jardetzky, O., *Proceedings of the National Academy of Science, U.S.A.*, 1984, **81**, 3379.
10. Hoffman, N. E., Bent, A. F. and Hanson, A. D., *Plant Physiology*, 1986, **82**, 658.
11. Rivoal, J., Ricard, B. and Pradet, A., *Plant Physiology and Biochemistry*, 1989, **27**, 43.
12. Hoffman, N. E. and Hanson, A. D., *Plant Physiology*, 1986, **82**, 664.
13. Libert, B. and Franceschi, V. R., *Journal of Agricultural and Food Chemistry*, 1987, **35**, 926.
14. Richardson, K. E. and Tolbert, N. E., *Journal of Biological Chemistry*, 1961, **236**, 1280.
15. Fujii, N., Watanabe, M., Watanabe, Y. and Shimada. N., *Soil Science and Plant Nutrition*, 1993, **39**, 627.
16. Raven, J. A., Griffiths, H., Glidewell, S. M. and Preston, T., *Proceedings of the Royal Society, London, Series B*, 1982, **216**, 87.
17. Rothe, G. M., *Archives of Biochemistry and Biophysics*, 1974, **162**, 17.
18. Asker, H. and Davies, D. D., *Planta*, 1984, **161**, 272.
19. Campbell, W. J. and Ogren, W. L., *Photosynthesis Research*, 1990, **23**, 257.
20. Heupel, R., Markgraf, T., Robinson, D. G. and Heldt, H. W., *Plant Physiology*, 1991, **96**, 971.
21. Givan, C. V. and Kleczkowski, L. A., *Plant Physiology*, 1992, **100**, 552.
22. Okutani, I. and Sugiyama, N., *HortScience*, 1994, **29**, 1019.
23. Laemmli, U. K., *Nature*, 1970, **227**, 680.
24. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.