Food Chemistry **41** (1991) 219–225

Purification and Characterization of NAD-dependent Lactate Dehydrogenase from *Rhizopus oryzae*

Roch-chui Yu & Y. D. Hang*

Department of Food Science & Technology, Cornell University, Geneva, New York 14456, USA

(Received 21 May 1990; accepted 21 June 1990)

ABSTRACT

NAD-dependent lactate dehydrogenase (EC 1.1.1.27) of Rhizopus oryzae *NRRL 395 was purified 175-fold with a yield of 63% by ammonium sulfate fractionation and Polybuffer exchanger 94 chromatofocusing. The purified enzyme had a specific activity of > 15 units per mg protein, and its sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) pattern showed only one protein band. As estimated by gel filtration, the molecular weight of the native enzyme was 131 000 daltons, and as determined by SDS–PAGE, that of a subunit was 36 000 daltons. The purified enzyme was stable at room temperature. The optimal pH was about 7·5 and the isoelectric point (pI), as determined by chromatofocusing, was about 5·2. The* K_m *values for NADH, pyruvate, and 2-oxobutyrate were 1·48, 6·40, and* $54{\cdot}4 \times 10^{-4}$ M, *respectively. Activity was inhibited by* Cd^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+} *and* Zn^{2+}, *but not by EDTA.*

INTRODUCTION

Lactate dehydrogenases have been studied extensively in animals (Everse & Kaplan, 1973), plants (Davies & Davies, 1972; Hoffman & Hanson, 1986), and a variety of microorganisms (Haugaard, 1959; Yoshida & Freese, 1965; Soler *et al.*, 1982). However, the NAD-dependent lactate dehydrogenase (EC 1.1.1.27) of *Rhizopus oryzae* has not heretofore been amenable to high purification because of its instability. Obayashi *et al.* (1966) reported that the

* To whom correspondence should be addressed.

Food Chemistry 0308-8146/91/$03·50

purification of the enzyme was not successful beyond a 3-fold level, and Pritchard (1973) achieved only a 10-fold purification with a yield of 16% by ammonium sulfate fractionation and DEAE cellulose chromatography. This report describes the isolation, 175-fold purification, and characterization of an NAD-dependent lactate dehydrogenase from *R. oryzae*.

MATERIALS AND METHODS

Chemicals

Ammonium sulfate was obtained from Fischer Scientific Co. (Fair Lawn, NJ, USA). Polybuffer exchanger 94 and Polybuffer 74 were obtained from Pharmacia LKB (Uppsala, Sweden). Chemicals for polyacrylamide gels, Coomassie Blue R-250, and Bio-Gel P-300 were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Sodium pyruvate, NADH, and Sephadex G25-80 were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Culture

A lactic acid-producing strain of *Rhizopus oryzae* NRRL 395 (C. W. Hesseltine, Northern Regional Research Center, USDA, Peoria, IL, USA) was maintained on potato dextrose agar slants. Fungal spores were produced on steamed rice by the method of Wang *et al.* (1975).

Enzyme production

Standard enzyme production experiments were conducted in 500-ml Erlenmeyer flasks containing 1 g of ground rice plus 100 ml of distilled water (sterilized at 121°C for 15 min). Each flask was inoculated with 10^7 viable spores and incubated at 30°C for 24 h on a shaker operated at 240 rpm. After fermentation, the mycelia were harvested by filtration and washed twice with cold 20 mM phosphate buffer (pH 7·5) containing 1 mM EDTA.

Preparation of the crude extract

Washed mycelia (6·8 g) were suspended in 300 ml of 20 mM phosphate buffer (pH 7·5) and blended in a Sorvall homogenizer for 1 min (16 000 rpm) at 4°C. The homogenized material was centrifuged at 13 000 × *g* for 15 min at 4°C and the pellet discarded. The supernatant was designated as the crude extract.

Enzyme purification

Solid ammonium sulfate was added to bring the crude extract to 30% saturation. The precipitate, devoid of enzyme activity, was discarded and the supernatant was then brought to 60% saturation by addition of an appropriate amount of solid ammonium sulfate. The second precipitate, collected by centrifuging at 13 000 × *g* for 15 min, was dissolved in 20 ml of 20 mM phosphate buffer (pH 7·5) and desalted by passing it through a Sephadex G25-80 column (2 cm × 10 cm). The active fractions were pooled and applied to a Polybuffer exchanger 94 column (1 cm × 10 cm) previously equilibrated with 25 mM imidazole–HCl buffer (pH 7·4). The pH gradient was formed by the addition of 12·5% Polybuffer 74 (pH was adjusted to 4·0 with HCl). Fractions of 2 ml were collected at a flow rate of 20 ml/h.

Molecular weight determination

The molecular weight of NAD-dependent lactate dehydrogenase from *R. oryzae* was determined by the gel filtration method of Andrews (1964). The molecular weight standards (Bio-Rad, Richmond, CA, USA) were: γ globulin (158 000), ovalbumin (44 000), myoglobin (17 000), and vitamin B_{12} (1350).

Determination of enzyme purity

The purity and molecular weight of the purified enzyme were estimated by sodium dodecylsulfate–7·5% polyacrylamide gel electrophoresis (SDS–PAGE) using the Laemmli system (1970). The reference proteins used (Diversified Biotech., Newton Centre, MA, USA) were lactoglobulin (18 400), carbonic anhydrase (29 000), lactate dehydrogenase (36 000), ovalbumin (43 000), glutamate dehydrogenase (55 000), and phosphorylase b (95 500).

Standard enzyme assays

The activity of NAD-dependent lactate dehydrogenase was assayed by measuring the decrease in the absorbance at 340 nm associated with NADH oxidation in a Hitachi model 200 spectrophotometer equipped with a recorder. The standard assay mixture as described by Hoffman & Hanson (1986) contained 0·1M Tris–HCl buffer (pH 8·0), 15 μmoles of sodium pyruvate, 150 μg of NADH, and appropriately diluted enzyme solution. The reaction was started by the addition of pyruvate after measuring any NADH oxidase in the enzyme solution. One unit of NAD-dependent lactate

dehydrogenase is defined as the amount of the enzyme that causes 1 μmole of NADH oxidation per min under assayed conditions. The specific activity is expressed as units per mg protein.

Protein determination

The protein content of the enzyme solution was determined by measuring the absorbance at 590 nm using the protein-dye binding method of Bradford (1976) with bovine serum albumin as the protein standard.

Enzyme properties

The effect of pH on enzyme activity was studied using the standard method except that 0·1 M of phosphate–citrate buffer was used in the pH range of 3·0–10·0.

The thermal stability of the enzyme was studied using the standard method except that the enzyme was treated for 0 to 60 min at 30, 45 and 55°C. Heat-treated enzyme was cooled immediately in an ice bath and the remaining activity then measured.

The Michaelis–Menten constants (K_m) of the enzyme for NADH, sodium pyruvate, and 2-oxobutyrate were studied at pH 8·0 in 0·1 M Tris–HCl buffer containing different concentrations of a substrate. The K_m values were derived by Lineweaver–Burk plots of initial velocity for different concentrations of a substrate.

The effect of various compounds on the enzyme activity was tested by addition of 1 mM concentrations to the reaction mixture. Residual activity was assayed as described above.

RESULTS AND DISCUSSION

Results for purification of NAD-dependent lactate dehydrogenase from *R. oryzae* are summarized in Table 1. The specific activity of the crude extract was less than 0·1 units per mg protein. The enzyme was purified only about 3-fold with a yield of 78% by ammonium sulfate fractionation (30–60% saturation). Further purification of the enzyme was achieved by chromatofocusing on a Polybuffer exchanger 94 column. The enzyme eluted as a sharp single peak (Fig. 1), and had a specific activity of more than 15 units per mg protein. The enzyme was further purified about 175-fold with a yield of 63% after treatment with the Polybuffer exchanger 94 column. Maximal enzyme activity occurred at pH 5·2 (Fig. 1) and the isoelectric point (pI) of the NAD-dependent lactate dehydrogenase of *R. oryzae* is thus about 5·2. The pI of

TABLE 1
Lactate Dehydrogenase Purification by Ammonium Sulfate Fractionation and Chromatofocusing on Polybuffer Exchanger 94

Step	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification (fold)*	*Yield (%)*
1. Crude extract	84	7·35	0·088	1	100
2. Ammonium sulfate (30–60%)	24·1	5·74	0·238	2·7	78
3. Chromatofocusing	0·30	4·63	15·4	175	63

lactate dehydrogenase from potato tubers has been reported to be 5·1 (Davies & Davies, 1972).

The SDS–PAGE pattern of the purified enzyme obtained by chromatofocusing on Polybuffer exchanger 94 showed a single protein band with a molecular weight of 36 000, identical to the lactate dehydrogenase standard. The molecular weight of the purified NAD-dependent lactate dehydrogenase of *R. oryzae* as determined by gel filtration on Bio-Gel P-300, was 131 000. These results suggest that the native NAD-dependent lactate dehydrogenase of *R. oryzae* is a tetramer and the single protein band obtained in SDS–PAGE corresponds to the monomeric form of the enzyme. Lactate dehydrogenases with a molecular weight range of 135 000–150 000 consisting of four subunits have been found in *Bacillus subtilis* (Yoshida & Freese, 1965), *Phycomyces blakesleeanus* (Soler *et al.*, 1982), and many animal tissues (Everse & Kaplan, 1973).

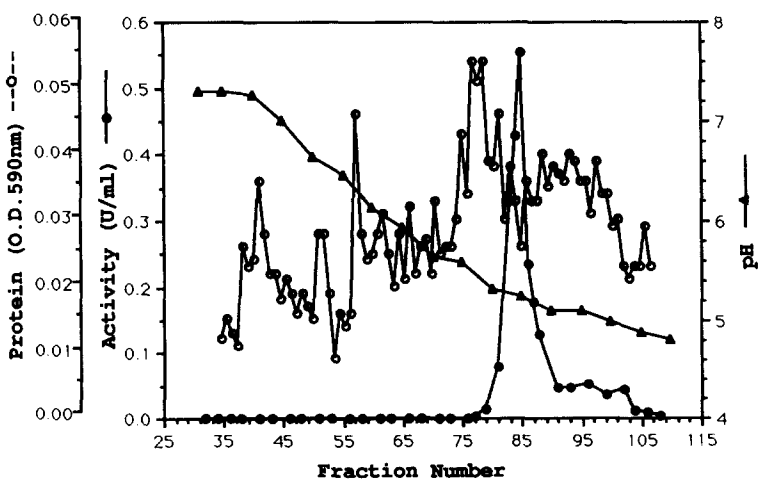

Fig. 1. Chromatofocusing of *R. oryzae* lactate dehydrogenase on Polybuffer exchanger 94. Protein —○—; pH —▲—; Lactate dehydrogenase activity —●—.

The optimal pH of the purified enzyme was found to be about 7·5. Pritchard (1973) reported that the activity of lactate dehydrogenase from *R. oryzae* was optimal at pH 7·2 in 0·1M phosphate buffer.

The purified enzyme was found to be very stable at 30°C. At 45°C, activity was reduced to 55% after 1 h, and at 55°C, activity was almost completely lost after 30 min. Obayashi *et al.* (1966) reported that the same enzyme in 0·1M buffer at 4°C lost its activity completely for only 7h.

The K_m values of the NAD-dependent lactate dehydrogenase of *R. oryzae* for pyruvate and 2-oxobutyrate were 6·4 and $54{\cdot}4 \times 10^{-4}$ M, respectively, indicating that pyruvate was a more suitable substrate than 2-oxobutyrate for the enzyme. The K_m value for NADH was found to be $1{\cdot}48 \times 10^{-4}$ M. The K_m values of the purified enzyme for pyruvate and NADH obtained in this work are in accord with those of Pritchard (1973).

The activity of the purified enzyme was completely inhibited by Hg^{2+}, Pb^{2+}, Fe^{2+}, Zn^{2+} and Cd^{2+}. EDTA had little effect on the activity. Lack of inhibition by EDTA indicates that metal ions may not be required for activity.

ACKNOWLEDGEMENT

Roch-chui Yu was supported by a scholarship from the Ministry of Education, Republic of China, Taipei, Taiwan.

REFERENCES

Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.*, **91**, 222.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–54.

Davies, D. D. & Davies, S. (1972). Purification and properties of L(+)-lactate dehydrogenase from potato tubers. *Biochem. J.*, **129**, 831–9.

Everse, J. & Kaplan, N. O. (1973). Lactate dehydrogenase, structure and function. *Adv. Enzymol.*, **37**, 61–133.

Haugaard, N. (1959). D- and L-lactic acid oxidases of *Escherichia coli*. *Biochim. Biophys. Acta*, **31**, 66–72.

Hoffman, N. E. & Hanson, A. D. (1986). Purification and properties of hypoxically induced lactate dehydrogenase from barley roots. *Plant Physiol.*, **82**, 664–70.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature (London)*, **227**, 680–5.

Obayashi, A., Yorifugi, H., Yamagata, T., Ijichi, T. & Kanie, M. (1966). Respiration in organic acid forming molds. Part I. Purification of cytochrome c, coenzyme Q9 and L-lactic dehydrogenase from lactate-forming *Rhizopus oryzae*. *Agric. Biol. Chem.*, **30**, 717–24.

Pritchard, G. G. (1973). Factors affecting the activity and synthesis of NAD-dependent lactate dehydrogenase in *Rhizopus oryzae*. *J. Gen. Microbiol.*, **78**, 125–37.

Soler, J., De Arriaga, D., Busto, F. & Cadenas, E. (1982). Lactate dehydrogenase in *Phycomyces blakesleeanus*. *Biochem. J.*, **203**, 381–91.

Wang, H. L., Swain, E. W. & Hesseltine, C. W. (1975). Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. *J. Food Sci.*, **40**, 168–70.

Yoshida, A. & Freese, E. (1965). Purification and chemical characterization of lactate dehydrogenase of *Bacillus subtilis*. *Biochim. Biophys. Acta*, **99**, 56–65.