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Purification and characterization of lactate dehydrogenase from *Geotrichum candidum*

Y. D. Hang* & E. E. Woodams

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

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An appreciable quantity of lactate dehydrogenase (EC 1.1.2.3) was present in the crude extract of *Geotrichum candidum* grown previously in sauerkraut processing waste effluents. The enzyme catalyses the oxidation of lactate to pyruvate with potassium ferricyanide and 2,6-dichlorophenol indophenol as electron acceptors. The enzyme was purified 64-fold by ammonium sulfate fractionation, gel filtration, and ion exchange chromatography with a yield of more than 5%. The specific activity of the purified enzyme was more than 6 units per mg protein. pH and temperature optima were 7·5 and 30°C, respectively. The enzyme was stable at pH values between 7·0 and 8·0, but lost most of its activity at 40°C in 10 min. The enzyme was specific for L(+)-lactate and its K_m value with potassium ferricyanide as an electron acceptor was 0·71 mM. The enzyme had a molecular weight of >200 000 as determined by gel filtration, and its pI was estimated to be 7·5. The enzyme activity was inhibited by heavy metals, and EDTA could be used to prevent heavy metal inhibition.

INTRODUCTION

Sauerkraut processing waste effluents are high in BOD, lactic acid, and NaCl and thus pose a serious environmental problem (Hang *et al.*, 1972). *Geotrichum candidum* has been reported to grow well in the wastewater without nutrient supplementation and completely utilized the lactic acid (Hang *et al.*, 1974). In our preliminary studies, we have found that the crude extract of *Geotrichum candidum*, previously grown in sauerkraut processing waste water, contained an appreciable amount of NAD-independent lactate dehydrogenase (EC 1.1.2.3) activity. This enzyme catalyzes the oxidation of lactate to pyruvate with cytochrome c and potassium ferricyanide as electron acceptors. The objective of the present investigation was to purify and characterize an NAD-independent lactate dehydrogenase from *Geotrichum candidum*.

* To whom correspondence should be addressed.

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MATERIALS AND METHODS

Growth and enzyme production

G. candidum ATCC34614 was grown in 500-ml Erlenmeyer flasks containing 100 ml of sauerkraut waste (acidity expressed as lactic acid, 0·76%) for 66 h at 30°C on a rotary shaker at 240 rpm.

Preparation of the crude extract

The cells harvested by centrifugation at 13 400 × *g* for 15 min were washed twice with 0·05 M phosphate–citrate buffer (pH 8·0). The cells were suspended in the same buffer and passed four times through a French press operated at 4°C at 1000 psi and high setting. The material was centrifuged at 17 200 × *g* for 20 min and the pellet was discarded. The supernatant was designated as the crude extract.

Enzyme purification

Ammonium sulfate fractionation

Solid ammonium sulfate was added to bring the crude extract to 40% saturation. The precipitate, devoid of enzyme activity, was discarded and the supernatant was

then brought to 60% saturation by an additional amount of ammonium sulfate. The precipitate, collected by centrifugation at 17 000 × *g* for 20 min, contained the enzyme activity.

Gel filtration
The enzyme was dissolved in a small amount of 0·05 M phosphate–citrate buffer (pH 8·0) and passed through a Sephadex G-200 column (2·5 × 47 cm). The enzyme activity was eluted with the same buffer. Fractions of 3 ml were collected.

DEAE Bio-Gel A agarose chromatography
The active fractions obtained from gel filtration were pooled and loaded on a DEAE Bio-Gel A agarose column (1·5 × 7 cm). The enzyme activity was eluted with 0·15 M phosphate–citrate buffer (pH 8·0)

CM-Sephadex chromatography
The active fractions from the DEAE Bio-Gel A agarose column were pooled and diluted with distilled water to a final concentration of 0·05M. The diluted enzyme fraction was then loaded on a CM-Sephadex C-25 column (1·5 × 6 cm). The enzyme activity was eluted with 0·1 M phosphate–citrate buffer (pH 8·0).

Enzyme assays

The enzyme activity was measured by the method of Decker (1977) and the rate of reduction of potassium ferricyanide was estimated continuously at 420 nm. The reaction mixture contained 1·9 ml of 0·1 M sodium pyrophosphate buffer (pH 7·4), 0·3 ml of 0·01 M EDTA, 0·5 ml of 0·5 M DL sodium lactate, 0·2 ml of 0·0083 M potassium ferricyanide and 0·1 ml of diluted enzyme. One unit reduces 1 μM of potassium ferricyanide per minute under assayed conditions. Protein was estimated by the method of Bradford (1976) with bovine serum albumin as the protein standard.

Enzyme characterization

Temperature and pH optima were determined by incubating the enzyme at different pH values and temperatures. Thermal stability was determined by measuring the residual activity after incubation at 30, 35 and 40°C for up to 60 min. pH stability was determined by measuring the residual activity after incubation in 0·1 M phosphate–citrate buffer (pH 4·0–8·0) for 3 h at 25°C. The K_m value of the enzyme for lactate was measured using different substrate concentrations. The molecular weight of purified enzyme was determined by gel filtration (Andrews, 1964), and the isoelectric point was estimated according to the method of Lampson & Tytell (1965) except that a DEAE Bio-Gel A agarose column and 0·12 M phosphate–citrate buffer were used.

RESULTS AND DISCUSSION

The results for purification of the NAD-independent lactate dehydrogenase of *G. candidum* 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 2-fold by ammonium sulfate fractionation (40–60% saturation) with a yield of more than 40%. Purification of the enzyme on a Sephadex G-200 column resulted in a marked increase in specific activity. The enzyme was further purified from the crude extract with a final yield of more than 5% after treatments with the DEAE Bio-Gel A agarose column and the CM Sephadex C-25 column. The purified enzyme preparation had a specific activity of more than 6 units per mg protein, representing a 64-fold purification from the crude extract.

The purified NAD-independent lactate dehydrogenase from *G. candidum* was specific for L(+)-lactate and could use 2,6-dichlorophenol indophenol and potassium ferricyanide as electron acceptors. Temperature and pH optima for the purified enzyme were observed at 30°C and at pH 7·5 respectively. The enzyme was found to be stable at pH values between 7·0 and 8·0 for up to 3 h at 25°C, but lost most of its activity at 40°C in 10 min (Table 2). The NAD-independent lactate dehydrogenase of *Rhizopus oryzae* showed highest activity between pH 7·2 and 8·4 and was almost inactive

Table 1. Purification of lactate dehydrogenase from *Geotrichum candidum*

	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	36·8	392·5	0.09	—	—
Ammonium sulfate fractionation	14·9	79·5	0·19	2.0	40.1
Sephadex G-200	13·0	16·8	0·77	8·2	35·2
DEAE-Bio-Gel A chromatography	6·0	1·5	3·91	41·7	16·2
CM-Sephadex C-25 chromatography	2·0	0·33	6·03	64·3	5·4

Table 2. Thermal stability of *Geotrichum candidum* lactate dehydrogenase

	Enzyme activity (%)				
Temp (°C)	0 min	10 min	20 min	30 min	60 min
30	100	100	100	82	82
35	100	82	61	70	36
40	100	9	0	0	0

Experimental conditions were the same as the standard method except that the enzyme was incubated at different temperatures for varying periods of time.

at pH 5·2 (Pritchard, 1971). The activity of NAD-independent lactate dehydrogenase from baker's yeast was highest between pH 7·0 and 8·5 (Decker, 1977).

The K_m value for *G. candidum* lactate dehydrogenase using potassium ferricyanide as an electron acceptor was found to be 0·71 mM (Fig. 1). The K_m value of lactate dehydrogenase from *G. candidum* for lactate is thus much lower than the published K_m values of other microorganisms. The K_m value for *Rhizopus oryza* NAD-independent lactate dehydrogenase using dichlorophenol indophenol as an electron acceptor has been reported by Pritchard (1971) to be 3·85 mM. The K_m value for the NAD-independent lactate dehydrogenase of baker's yeast, *Saccharomyces cerevisiae*, with potassium ferricyanide as an electron acceptor was 1·6 mM (Decker, 1977). The data obtained in this work thus indicate

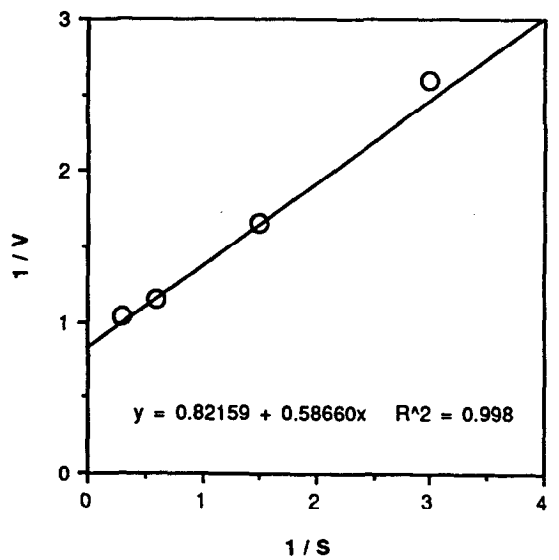

Fig 1. Effect of lactate concentration on the activity of *G. candidum* lactate dehydrogenase. Lineweaver-Burk plot of $1/V$ against $1/S$ (S, lactate concentration (mM); V, rate of ferricyanide reduced (mM/min).

that lactate is the most reactive substrate for the NAD-independent lactate dehydrogenase of *G. candidum*. The purified enzyme had a molecular weight of greater than 200 000 as determined by Sephadex gel-filtration. The molecular weight of the NAD-independent lactate dehydrogenase of baker's yeast has been reported to be 228 000 (Decker, 1977). The pI of the enzyme as determined by the modified method of Lampson & Tydell (1965) was estimated to be 7·5.

The activity of *G. candidum* NAD-independent lactate dehydrogenase was inhibited by heavy metals such as mercury, copper, zinc, and cadmium. Chelating agents such as EDTA could be used to prevent heavy metal inhibition. Similarly, the NAD-independent lactate dehydrogenase activity of baker's yeast has been reported to be inhibited by heavy metals (Decker, 1977).

CONCLUSION

The crude extract of *Geotrichum candidum* previously grown in sauerkraut processing waste effluents was found to contain an appreciable amount of NAD-independent lactate dehydrogenase activity. The enzyme has been purified more than 60-fold and its properties have been studied. The purified enzyme catalyzes the oxidation of L-lactate to pyruvate with potassium ferricyanide and 2,6-dichlorophenol indophenol as electron acceptors and could thus be particularly useful as a diagnostic tool for the specific determination of lactic acid in foodstuffs, beverages, and other biological materials.

REFERENCES

Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91**, 222–33.

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

Decker, L. A. (1977). *Worthington Enzyme Manual.* Worthington Biochemical Corporation, Freehold, NJ.

Hang, Y. D., Downing, D. L., Stamer, J. R. & Splittsttoesser, D. F. (1972). Wastes generated in the manufacture of sauerkraut waste. *J. Milk Food Technol.*, **34**, 432–5.

Hang, Y. D., Splittsttoesser, D. F. & Landschoot, R. L. (1974). Propagation of *Geotrichum candidum* in acid brine. *Appl. Microbiol.*, **27**, 807–8.

Lampson, G. P. & Tytell, A. A. (1965). A simple method for estimating isoelectric points. *Anal Biochem.*, **11**, 374–7.

Pritchard, G. G. (1971). An NAD-independent L-lactate dehydrogenase from *Rhizopus oryzae. Biochim. Biophys. Acta*, **250**, 25–31.