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Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Reduction of alpha-keto acids†

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D (−)- and L (+)-lactate dehydrogenases of Lactobacillus plantarum *(WSO) are the probable enzymes which catalyze the reduction of the alpha-keto acids (pyruvate, alpha-ketobutyrate, alpha-ketovalerate, and alpha-ketocaprylate) during anaerobic mannitol metabolism. This is indicated by the fact that each of these compounds are substrates for purified D (−)-* and L (+)-lactate dehydrogenases. There was no increase in the alpha-ketobutyrate reduction activity when cells were grown on mannitol with alpha-ketobutyrate as an electron acceptor compared to cells grown on glucose. HPLC ion exchange chromatography of cell extracts of L. plantarum *grown on mannitol with alpha-ketobutyrate at pH 7·5 and 5·5 revealed no enzymes capable of alpha-ketobutyrate reduction other than lactate dehydrogenases.*

Introduction

McFeeters and Chen (1986) have demonstrated that a group of aliphatic alpha-keto acids including pyruvate, alpha-ketobutyrate, alpha-ketovalerate, and alpha-ketocaprylate serve as electron acceptors for mannitol fermentation by *Lactobacillus plantarum* (WSO). The reduced products of pyruvate and alpha-ketobutyrate were identified as lactate and alpha-hydroxybutyrate, respectively. However, enzymes responsible for the reduction of these alpha-keto acids were not determined. The enzymes most likely to be involed in the reduction of these compounds are the D (−)- and L (+)-lactate dehydrogenases, which are constitutive enzymes present in high levels in all lactic acid bacteria. It was also possible that alpha-ketobutyrate, alpha-ketovalerate, and alpha-ketocaprylate might be reduced by an inducible enzyme with a greater specificity for longer chain alpha-keto acids similar to the L-2-hydroxyisocaproate dehydrogenase recently characterized in *Lactobacillus confusus* (Schutte et al. 1984).

The objective of this study was to determine which enzymes catalyze the reduction of aliphatic alpha-keto acids during mannitol fermentation by *L. plantarum*.

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Materials and Methods

Micro-organism and medium

Lactobacillus plantarum (WSO and chemically defined basal medium used were the same as described by McFeeters and Chen (1986).

Growth conditions

Cells were grown on basal medium with 55·5 mM glucose or mannitol under anaerobic conditions at 30°C. Pyruvate or alpha-ketobutyrate was added as an electron acceptor at a 20 mM concentration to the basal medium with mannitol. Other procedures have been described (McFeeters and Chen 1986).

Enzyme extraction

The procedures for the extraction of enzymes from the bacterial cells were the same as described previously (Chen and McFeeters 1986).

Enzyme assays

Enzyme assays were performed on duplicate fermentation samples. Variation of enzyme activity in duplicate fermentations within an experiment was 12% or less.

Lactate dehydrogenase activity was determined by the method of Mizushima et al. (1964), except that KCN was not added because NADH oxidase activity was low compared to lactate dehydrogenase activity. Enzyme activity data were corrected for NADH oxidase activity by subtracting the rate of NADH oxidation in the absence of pyruvate or alpha-ketobutyrate from the reaction rate in the presence of these substrates. One unit of enzyme is defined as that amount of activity which catalyzes the oxidation of 1 μmol of NADH min^{-1} ml^{-1} under the assay conditions.

Identification of D (−)- and L (+)-lactate dehydrogenase

D (−)- and L (+)-lactate dehydrogenases were differentiated by assaying for the ability of purified enzyme preparations to oxidize either D (−)- or L (+)-lactate with NAD as a cofactor. The procedure described in Sigma Technical Bulletin No. 826-UV (Sigma Chemical Company, St Louis, MO, USA) was used.

Protein determinations

The protein concentrations were measured either by the Lowry method (Lowry et al. 1951) or by the absorbance at 235 and 280 nm as described by Whitaker and Granum (1980).

Separation and purification of enzymes

All enzyme separations were done at room temperature by elution with a linear salt gradient from a Mono Q HPLC anion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ, USA). The HPLC consisted of two Waters M6000A pumps, a model 660 solvent programmer (Waters Associates, Milford, MA, USA), a Pharmacia injector, and a Varichrom UV/visible detector (Varian, Palo Alto, CA, USA). Fractions (0·5 ml) were collected manually for measurement of enzyme activities after the sample had passed through the detector.

To determine which enzymes in *L. plantarum* cells could catalyze the reduction of pyruvate or alpha-ketobutyrate with NADH as a cofactor, cell-free extract (500 μl) from cells grown on mannitol with alpha-ketobutyrate as the electron acceptor was injected onto the column. The column was initially equilibrated with either pH 7·5, 20 mM Tris-HCl buffer or pH 5·5, 20 mM histidine buffer. After equilibration, a 20 min gradient at a flow rate of 1·0 ml min^{-1} was run from 0 to 0·5 M NaCl in the appropriate equilibration buffer. Fractions for enzyme assays using both pyruvate and alpha-ketobutyrate as substrates were collected from the time of sample injection until the end of the salt gradient.

Purification of the D (−)- and L (+)-lactate dehydrogenases for substrate specificity studies was carried out with a series of three gradient elutions from the Mono Q column. Cell-free extract from cells grown on glucose in pH 7·5, 20 mM Tris-HCl buffer was chromatographed at pH 7·5 in 20 mM Tris-HCl as described above. Fractions containing the two lactate dehydrogenases were collected separately. Elution times for the two enzymes were 13 and 14·5 min from the start of the 20 min gradient. The two forms of lactate dehydrogenase were dialyzed separately in 20 mM, pH 6·5 histidine buffer and then chromatographed at pH 6·5 again with a 1·0 ml min^{-1}, 20 min linear NaCl gradient from 0 to 0·5 M NaCl. Fractions with high levels of enzyme activity were collected, dialyzed in

pH 5·5, 20 mM histidine buffer and chromatographed a third time with the same salt gradient as above, but at pH 5·5. After the third chromatography step, the enzyme solutions were concentrated in a dialysis bag using Ficoll (Sigma Chemical Company, St Louis, MO, USA) to remove water from the sample.

Electrophoresis

The purity of D (−)- and L (+)-lactate dehydrogenase was evaluated by disc gel electrophoresis in 7·0 × 0·5 cm tubes containing 7·5% acrylamide gels with pH 8·3 Tris glycine electrode buffer (Gabriel 1971). Gels were stained with Coomassie Brilliant Blue G250 (Blakesley and Boezi 1977).

K_m *and* V_{max} *determinations*

K_m and V_{max} were determined for pyruvate and alpha-ketobutyrate for both D (−)- and L (+)-lactate dehydrogenases. Purified enzymes, free of NADH oxidase activity, were used. Initial rate data were analyzed according to the procedure of Wilkinson (1961).

Results

Anion exchange chromatography of cell-free enzyme extracts

Extracts from cells grown on basal medium with mannitol and alpha-ketobutyrate were fractionated on an anion exchange column at pH 7·5 and 5·5 by the procedures described above. Two clearly separated peaks of enzyme activity centred at 13·0 and 14·5 min after the start of the pH 7·5 gradient were observed with pyruvate as a substrate [Fig. 1(B)]. Use of alpha-ketobutyrate as the substrate indicated that it was reduced only by the enzymes located in the fractions which reduced pyruvate, but at considerably lower reaction rates [Fig. 1(a)]. Chromatography of the crude enzyme extract at pH 5·5 gave the same pattern of activities for the two substrates except that the elution times were 11·5 and 13·0 min.

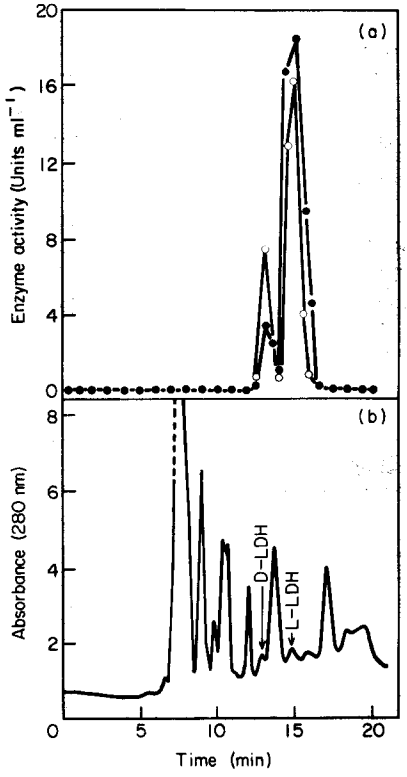

Fig. 1. Chromatography of the crude extract of *L. plantarum* on a Mono Q ion exchnage column. The flow rate was 1·0 ml min^{-1}, and the fraction size was 0·5 ml. The substrate concentration used for enzyme activities measurement was 3·3 mM for pyruvate (--○--) and 6·7 mM for alpha-ketobutyrate (--●--). The activity for alpha-ketobutyrate reduction was multiplied by ten to put it on the same scale. The absorbance at 280 nm was multiplied by 10^2. (a) = enzyme activity; (b) = absorbance at 280 nm.

Effect of growth medium on alpha-keto acid reduction activity

The effects of carbon source and electron acceptor on lactate dehydrogenase activity in *L. plantarum* cells were investigated. Results in Table 1 show there was little change in lactate dehydrogenase activity, as determined by the rate of pyruvate reduction, whether glucose or mannitol was the carbon source and whether pyruvate or alpha-ketobutyrate was the electron acceptor.

Table 1. Reduction of pyruvate and alpha-ketobutyrate by cell-free extracts from cells grown on different media.

	Specific activity (units mg^{-1} protein)					
	Growth media					
Substrate	Mannitol/ pyruvate	Glucose/ pyruvate	Glucose	Mannitol/alpha ketobutyrate	Glucose/alpha ketobutyrate	Glucose
Pyruvate	5·5	5·3	5·2	6·1	6·3	5·6
Alpha-ketobutyrate	–	–	–	0·3	0·3	0·3

Table 2. Reduction of alpha-keto acids by purified D (−)- and L (+)-lactate dehydrogenases.

	Specific activity (units mg^{-1} protein)	
Substrate	D(−)-Lactate dehydrogenase	L(+)-Lactate dehydrogenase
Pyruvate	5·90	32·92
Alpha-ketobutyrate	0·11	2·23
Alpha-ketovalerate	0·004	0·024
Alpha-ketocaprylate	0·004	0·022

Table 3. K_m and V_{max} for substrates of D (−)- and L (+)-lactate dehydrogenases.

	D(−)-Lactate dehydrogenase		L(+)-Lactate dehydrogenase		D(−)-Lactate dehydrogenase		L(+)-Lactate dehydrogenase	
Substrate	K_m (mM)	S.E. K_m	K_m (mM)	S.E. K_m	V_{max} (mM min^{-1})	S.E. V_{max}	V_{max} (mM min^{-1})	S.E. V_{max}
Pyruvate	2·3	0·27	0·48	0·06	4·9	0·17	7·1	0·26
Alpha-ketobutyrate	101·0	5·0	38·01	0·90	7·7	0·17	2·40	0·03

To determine whether there was any increase in alpha-ketobutyrate reduction activity in cell-free extracts when cells had to reduce alpha-ketobutyrate to grow on mannitol, the rate of reduction of alpha-ketobutyrate in the cell-free extracts from cells grown on glucose, glucose/alpha-ketobutyrate and mannitol/alpha-ketobutyrate was compared. No change in alpha-ketobutyrate reduction activity occurred when alpha-ketobutyrate reduction was required for growth on mannitol (Table 1).

Substrate specificity of purified D (−)- and L (+)-lactate dehydrogenases

After the third step of purification on the Mono Q column, the lactate dehydrogenase which eluted at 13 min at pH 7·5 [Fig. 1(b)] was found to oxidize only D

(−)-lactate. The enzyme which eluted at 14·5 min oxidized only the L (+)-lactate isomer. Disc gel electrophoresis of the D (−)-lactate dehydrogenase preparation showed it contained one major protein band with several minor bands. Only one protein band was visible on disc gels of the L (+)-lactate dehydrogenase preparation.

Substrate specificity of the two purified lactate dehydrogenases for reduction of substrates was studied by means of the standard assay system in which the same concentration (3·3 mM) of alpha-ketobutyrate, alpha-ketovalerate and alpha-ketocaprylate were substituted for pyruvate. The results indicated that all of these alpha-keto acids were reduced by both purified D (−)- and L (+)-lactate dehydrogenases (Table 2). The rate of reduction decreased rapidly as the chain length of alpha-keto acids increased from pyruvate to alpha-ketovalerate. Identical rates of reduction were measured for alpha-ketovalerate and alpha-ketocaprylate.

Kinetic properties of lactate dehydrogenase

K_m and V_{max} values were determined for both purified D (−)- and L(+)-lactate dehydrogenases with pyruvate and alpha-ketobutyrate as substrates (Table 3). The K_m values for alpha-ketobutyrate were over 45-fold higher for the D (−)-lactate dehydrogenase and 75-fold higher for the L (+)-lactate dehydrogenase than for pyruvate. On the other hand, the V_{max} values of D (−)- and L (+)-lactate dehydrogenases for pyruvate and alpha-ketobutyrate were similar.

Table 4. Specific fermentation rate of mannitol fermentation with pyruvate or alpha-ketobutyrate as an electron acceptor.

Specific fermentation rate Growth media	
Mannitol/pyruvate	Mannitol/ alpha-ketobutyrate
0·32	0·22

Specific fermentation rate

The specific fermentation rate for mannitol utilization by *L. plantarum* as measured by the increase in lactate concentration (Koch 1981) was compared using either pyruvate or alpha-ketobutyrate as the electron acceptor. The amount of lactate produced from mannitol in the mannitol/pyruvate fermentation was calculated by subtracting the amount of pyruvate disappearance from the total lactate measured. Results indicated that the specific fermentation rate of mannitol/alpha-ketobutyrate-grown cells was about 70% of that of mannitol/pyruvate-grown cells (Table 4).

Discussion

Pyruvate, alpha-ketobutyrate, alpha-ketovalerate, and alpha-ketocaprylate are substrates for lactate dehydrogenases from various muscle tissues (Meister 1950). However, only pyruvate and alpha-ketobutyrate have been demonstrated to serve as substrates for partially purified D (−)- and L (+)-lactate dehydrogenases from *L. plantarum* (Dennis and Kaplan 1960). The reduction of alpha-ketovalerate and alpha-ketocaprylate by enzymes from *L. plantarum* has not been reported. However, an enzyme, L-2-hydroxyisocaproate dehydrogenase, isolated from *L. confusus* can reduce the alpha-keto acids, pyruvate, alpha-ketobutyrate, and alpha-ketovalerate (Schutte et al. 1984). The difference between this enzyme and lactate dehydrogenase is that it has a lower K_m for longer chain alpha-keto acids than for pyruvate. *Lactobacillus*

plantarum could use a series of alpha-keto acids as electron acceptors during mannitol fermentation. We wanted to determine whether the constitutive D (−)- and L (+)-lactate dehydrogenases were the only enzymes involved in reduction of all four alpha-keto acids.

To determine which enzymes capable of alpha-ketobutyrate reduction were present in mannitol/alpha-ketobutyrate-grown cells, the cell-free extracts were chromatographed on an anion exchange column at two different pHs. Only two enzymes, which proved to be D (−)- and L (+)-lactate dehydrogenases, were detected that could reduce alpha-ketobutyrate. Alpha-ketobutyrate reduction activity did not increase in mannitol/alpha-ketobutyrate-grown cells compared to glucose-grown cells (Table 1) which suggests that there was no induction of additional enzymes to reduce alpha-ketobutyrate. We cannot completely exclude the possibility that other enzymes may be induced to reduce longer chain alpha-keto acids. If, for example, an enzyme which was very unstable or which had the same chromatographic properties as either D (−)- or L (+)-lactate dehydrogenase were formed, it may not have been detected in these experiments.

Substrate specificity studies showed that both purified lactate dehydrogenases were able to reduce all four alpha-keto acids tested. Comparison of K_m and V_{max} values for pyruvate and alpha-ketobutyrate indicate that binding of the longer chain compound was decreased, but that the rate of product formation was only slightly affected. K_m values for pyruvate were similar to those reported by Dennis and Kaplan (1960) which suggests that the rate of reduction of both compounds should be similar as long as the concentration of alpha-ketobutyrate available to the lactate dehydrogenases in the cell was sufficiently high. Since the specific fermentation rate when alpha-ketobutyrate was the electron acceptor was only slightly less than when pyruvate was added, it suggests that the cells can reduce the alpha-ketobutyrate at nearly the same rate as they reduce pyruvate during growth. The present results indicated that lactate dehydrogenases can use all the alpha-keto acids tested as substrates and that alpha-ketobutyrate can be reduced at reasonable rates by lactate dehydrogenases. Also, there was no evidence to indicate that other enzymes were induced to catalyze the reduction of alpha-keto acids. These results indicated that lactate dehydrogenases were the probable enzymes which carry out alpha-keto acid reduction during mannitol fermentation in *L. plantarum*.

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