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Purification and partial characterization of D-(−)-lactate dehydrogenase from *Lactobacillus helveticus* CNRZ 32

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SUMMARY

D-(−)-Lactate dehydrogenase (LDH) was purified to homogeneity from a cell-free extract of *Lactobacillus helveticus* CNRZ 32. The native enzyme was determined to have a molecular weight of 152 000 and consisted of four identical subunits of 38 000. This enzyme was NAD dependent, fructose 1,6-diphosphate (FDP) and ATP independent. It was most active on pyruvate followed by β-hydroxypyruvate as substrates. The K_m values for pyruvate and D-(−)-lactate were 0.64 and 68.42 mM respectively, indicating that the enzyme has a higher affinity for pyruvate. The enzyme activity was completely inhibited by *p*-chloromercuribenzoate (1 mM) and partially by iodoacetate, suggesting the involvement of the sulfhydryl group (-SH) in catalysis. Optima for activity by the purified enzyme were pH 4.0 and 50–60°C. Limited inhibition of D-(−)-LDH was observed with several divalent cations. Additionally, $HgCl_2$ was observed to strongly inhibit enzyme activity. The purified enzyme was not affected by dithiothreitol or any of the metal chelating agents examined.

INTRODUCTION

Lactic acid is the major end product of carbohydrate fermentation by homofermentative lactic acid bacteria, an industrially important group of microorganisms. Two isomeric forms of lactic acid, dextrorotatory [D(−)] and levorotatory [L(+)], are produced by distinct stereospecific NAD-dependent lactate dehydrogenases (LDH). Electrophoretic separation of LDHs followed by histochemical staining has been used for the identification and differentiation of dairy lactic acid bacteria [9]. Lactobacilli produce either only D-(−)-, only L-(+)-, or a mixture of both lactate isomers. The presence of both D-(−)- and L-(+)-LDH in several lactobacilli [7,9–11] suggests that these enzymes are responsible for the isomeric forms of lactic acid produced; however, a lactate racemiase, an enzyme which interconverts D-(−)- and L-(+)-lactate, has been detected in *Lactobacillus sake*, *Lactobacillus casei* ssp. *pseudoplantarum* and *Lactobacillus curvatus* [9]. Presence of only L-(+)-LDH in *Lactobacillus bavaricus* [22] and *Lactobacillus helveticus* [5] has also been reported. L-(+)-LDH from prokaryotes including lactic acid bacteria, and eukaryotes have been studied extensively. However, only limited reports are available regarding D-(−)-LDH. Recently, the D-(−)-LDH genes of *Lactobacillus plantarum* [23] and *Lactobacillus delbrueckii* ssp. *bulgaricus* [3] have been cloned and sequenced. Both groups [3,23] observed high sequence homology between D-(−)-LDH and D-hydroxyisocaproate dehydrogenase of *L. casei* but not with L-(+)-LDHs, suggesting that D-(−)- and L-(+)-LDHs have evolved from different ancestors. Although D-(−)- and L-(+)-LDH have long been recognized in lactic acid bacteria, no detailed studies have been done on their physiological role, evolutionary relationships, and regulation. *Lactobacillus helveticus* CNRZ 32 was chosen for this study because of its importance in cheese ripening [1,2] and the proposed involvement of D-(−)-lactic acid in the formation of white crystals on cheese surfaces during ripening [18]. This paper describes the purification and partial characterization of D-(−)-LDH (EC 1.1.1.28) from *L. helveticus* CNRZ 32.

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MATERIALS AND METHODS

Organism and growth condition

L. helveticus CNRZ 32 was obtained from Center for Dairy Research, University of Wisconsin-Madison, and routinely propagated in MRS broth [6] for 14 h at 37°C.

Preparation of cell-free extract

Cells grown in MRS broth (5 L) were harvested by centrifugation at 5520 × *g* for 10 min at 4°C and washed three times with cold (4°C) saline (0.85% NaCl). Cells were disrupted by grinding with alumina (Sigma Chemical Co., St Louis, MO, USA) in a mortar and pestle at 4°C for 30 min and suspended in standard buffer (400 ml; 20 mM Tris-HCl, 0.5 mM 2-mercaptoethanol, pH 8.6). Cell debris and alumina were removed by centrifugation at 5520 × *g* for 10 min at 4°C and the supernatant used as the cell-free extract.

D-(−)-LDH assay

D-(−)-LDH activity in the cell-free extract was assayed according to the method of Vassault [24] using D-(−)-lactate

(52 mM) and pyruvate (1.6 mM) as substrates and NAD (6.5 mM) and NADH (0.2 mM) as coenzymes. D-(−)-LDH activity was monitored using D-(−)-lactate as susbtrate and NAD as coenzyme during purification. Pyruvate and NADH were used in all other experiments unless otherwise indicated. The specific activity was calculated according to the method of Vassault [24] and expressed as mmol of NAD reduced or NADH oxidized min^{-1} mg^{-1} of protein at 30°C.

Substrate specificity

Substrate specificity of D-(−)-LDH was determined using L-(+)-lactate, β-hydroxypyruvate, β-phenylpyruvate, α-ketobutyrate, α-ketocaproate, and D-(−)-3-phosphoglycerate as substrates by the standard D-(−)-LDH assay procedure as described above. NAD was used as a coenzyme with L-(+)-lactate and D-(−)-3-phosphoglycerate and NADH with the other substrates.

Protein determination

Protein concentrations were determined by the method of Lowry et al. [16] using bovine serum albumin as the standard.

Purification of the D-(−)-LDH

All of the following purification steps were conducted at 4°C. Protein concentration of each fraction was monitored by measuring the absorbance at 280 nm and D-(−)-LDH activity was followed by measuring the reduction of NAD at 339 nm using D-(−)-lactate as substrate. The cell-free extract was prepared as described above.

(a) Protamine sulfate precipitation. To remove nucleic acids from the cell-free extract, one milliliter of a 10% (w/v) solution of protamine sulfate per absorbance at 260 nm (A_{260}) of 1500 was added [19]. The precipitate was removed by centrifugation at 12 100 × *g* for 15 min.

(b) Ammonium sulfate fractionation. The supernatant obtained from the previous step was fractionated by ammonium sulfate at 60–80% saturation and the precipitate was collected by centrifugation at 22 100 × *g* for 15 min. The pellet containing D-(−)-LDH activity was dissolved in standard buffer and dialyzed (cellulose type, pore size 4.8 nm, Arthur H. Thomas Co., Philadelphia, PA, USA) against the same buffer for 24 h with several changes.

(c) Rotofor fractionation. The dialyzed sample (34 ml) from the previous step was mixed with glycerol (0.1% v/v), ampholytes of pH 3–10 range (2% v/v, Bio-Rad Laboratories, Richmond, CA, USA) and standard buffer to adjust the volume to 60 ml and applied to the rotofor chamber (Bio-Rad). Isoelectric focussing was carried out at 12 W constant power for 4 h at 4°C. The initial conditions were 454 V and 26.7 mA. At equilibrium, the values were 970 V and 12.4 mA. Twenty fractions were harvested. pH value, protein content and D-(−)-LDH activity of each of the fractionation were determined. Fractions containing D-(−)-LDH activity were pooled and dialyzed.

(d) Cibacron Blue F3GA affinity chromatography. The dialyzed sample obtained from the previous step was applied to a Cibacron Blue F3GA (Sigma Chemical Co., St Louis, MO, USA) column (1 × 5 cm) previously equilibrated with the standard buffer. The column was washed as follows and 5-ml fractions collected (1 ml min^{-1}):

(1) The column was washed with 25 ml of standard buffer.
(2) The column was then washed with 5 ml of standard buffer containing D-(−)-lactate (1 mM) and NAD (1 mM), pH 8.6.
(3) The column was further washed with 10 ml of buffer containing Tris-HCl (10 mM) and 2-mercaptoethanol (0.5 mM), pH 8.6.
(4) The column was washed with 5 ml of buffer containing Tris-HCl (10 mM), 2-mercaptoethanol (0.5 mM) and NADH (1 mM), pH 8.6.
(5) The column was finally washed with 10 ml of buffer containing Tris-HCl (10 mM) and 2-mercaptoethanol (0.5 mM), pH 8.6.

Fractions containing active D-(−)-LDH were pooled, dialyzed and kept for further analysis.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was done according to the method of Laemmli [15] using a 12% running gel and 4% stacking gel in the presence of sodium dodecyl sulfate (SDS). Proteins were stained with Coomassie Blue. Non-denaturing PAGE was done using 6% gel in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer without any stacking gel as reported previously [4]. Protein bands were visualized using silver staining according to the manufacturer's instruction (Bio-Rad).

Detection of LDHs

Following non-denaturing PAGE, D-(−)- and L-(+)-LDH activities were detected using D-(−)- and L-(+)-lactate respectively, according to the method of Gasser [10].

RESULTS

Identification of LDHs

Two distinct NAD-dependent D-(−)- and L-(+)-LDH active bands were detected in the cell-free extract of *L. helveticus* CNRZ 32 as shown in Fig. 1. Both enzymes could be detected only when NAD was added in the reaction mixture.

Purification of D-(−)-LDH

Results of the purification of D-(−)-LDH from *L. helveticus* CNRZ 32 are summarized in Table 1. The enzyme was purified approximately four-fold with a yield of 0.39% from the cell-free extract. The fractionation profile of Rotofor (preparative isoelectric focusing) and elution profile of D-(−)-LDH from a column of Cibacron Blue F3GA are shown in Figs 2 and 3, respectively. The SDS–PAGE results

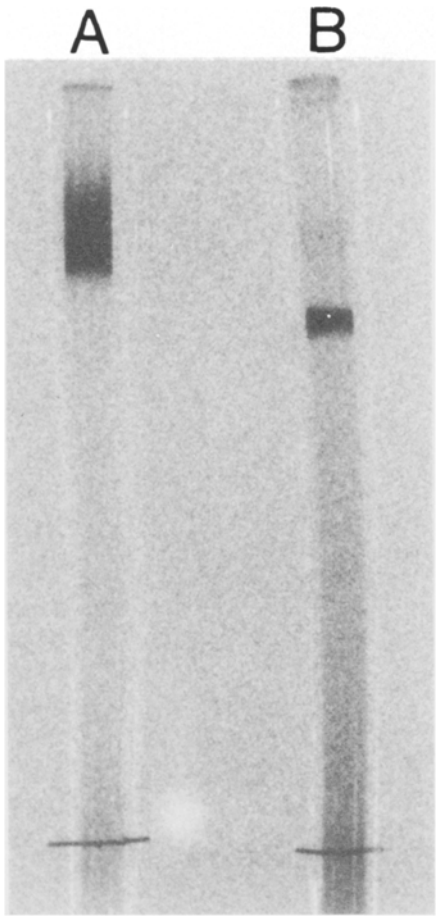

Fig. 1. Identification of D-(−)-LDH (A) and L-(+)-LDH (B) activity by histochemical staining of a cell-free extract of *L. helveticus* CNRZ 32.

TABLE 1

Purification of D-(−)-LDH from *L. helveticus* CNRZ 32

Procedure	Total protein (mg)	Specific activity ($\times 10^3$)[a]	Yield (%)	Purification (fold)
Cell-free extract	1483	1.38	100	1.00
Protamine sulfate	1100	1.47	79	1.06
Ammonium sulfate (60–80%)	368	1.73	31	1.24
Rotofor (pH 3–10)	20	3.85	3.74	2.78
Affinity Chromatography	1.41	5.70	0.39	4.11

[a]Specific activity expressed as mmols of NAD reduced min^{-1} mg $protein^{-1}$ at 30°C.

of the preparation after affinity chromatography (Fig. 4) indicate that D-(−)-LDH had been purified to homogeneity.

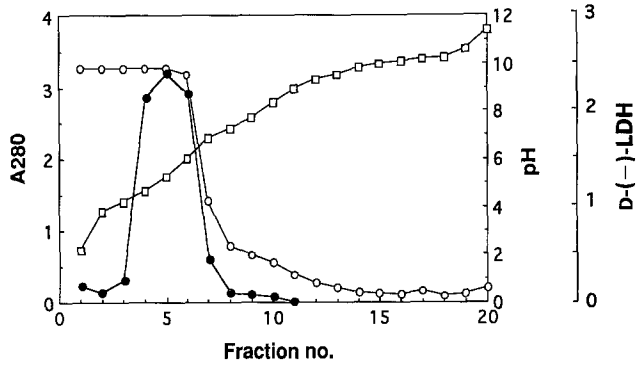

Fig. 2. Analysis of rotofor fractions. -○-, protein (280 nm); -□-, pH; -●-, D-(−)-LDH activity (339 nm).

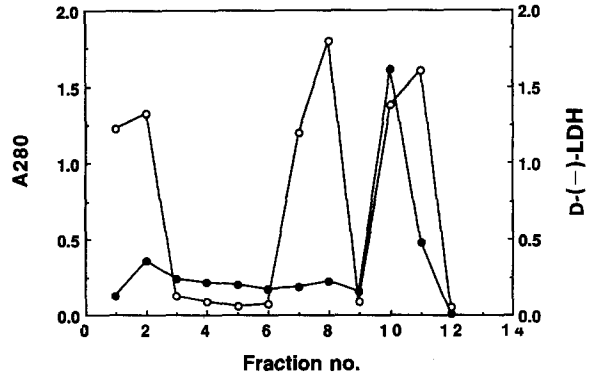

Fig. 3. Chromatography of the enzyme fraction obtained by rotofor fractionation on Cibacron Blue F3GA column. -○-, Protein (280 nm); -●-, D-(−)-LDH activity (339 nm).

Detection of D-(−)-LDH

The purified enzyme was also analyzed for purity and activity by native PAGE. Electrophoretic pattern of the purified enzyme after silver staining showed a single protein band which had LDH activity when D-(−)-lactate was used as the substrate and both bands had a similar relative mobility (R_f) value (data not shown).

Molecular weight

The molecular weight of the D-(−)-LDH was determined by SDS–PAGE and native PAGE using appropriate molecular weight standards. The molecular weight of the enzyme was estimated to be approximately 38 000 by SDS–PAGE (Fig. 4) and 152 000 by native PAGE (data not shown) using molecular weight markers for non-denaturing PAGE (Sigma Chemical Co., St Louis, MO, USA).

Effect of pH on enzyme activity

The effect of pH from 3.0 to 9.0 on D-(−)-LDH activity was determined at 30°C using 20 mM 2-*N*-morpholinoethanesulfonic acid (MES; pH 3–6) and 20 mM Tris-HCl, 0.5 mM 2-mercaptoethanol (pH 7–9). The optimum pH for activity was 4.0 (Fig. 5).

Effect of temperature on enzyme activity

The effect of temperature (4–80°C) on D-(−)-LDH activity was determined using the standard assay procedure. The

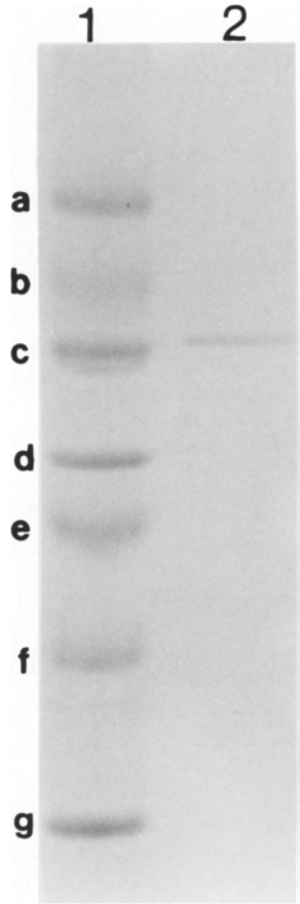

Fig. 4. SDS–PAGE of the purified D-(−)-LDH from *L. helveticus* CNRZ 32 (lane 2). Electrophoresis was carried out in the presence of 12% polyacrylamide with 10 μg of protein. Lane 1 molecular weight standards: a, 66 000; b, 45 000; c, 36 000; d, 29 000; e, 24 000; f, 20 000; g, 14 200.

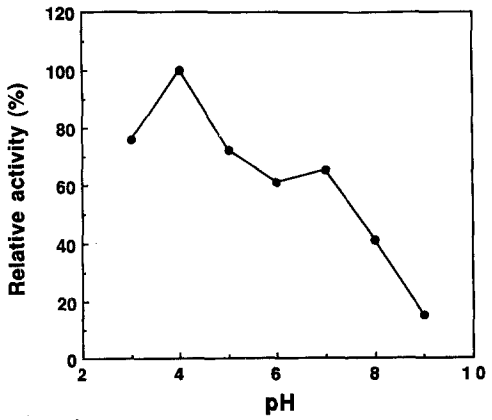

Fig. 5. Effect of pH on D-(−)-LDH activity.

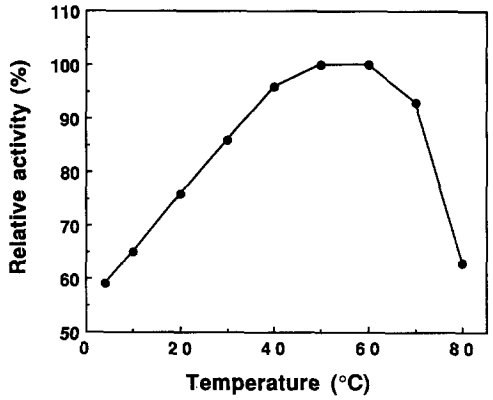

Fig. 6. Effect of temperature on D-(−)-LDH activity.

enzyme was active over a broad range of temperature with optimum activity between 50°C and 60°C (Fig. 6).

Effect of metal ions on enzyme activity

The effect of metal ions on enzyme activity was examined using standard assay conditions, except KCl was omitted. The results are presented in Table 2. The D-(−)-LDH was significantly inhibited (25–85%) by $HgCl_2$, $CoCl_2$, $CuCl_2$ and $ZnCl_2$; $CaCl_2$, $MgCl_2$ and $MnCl_2$ apparently had no effect on enzyme activity. All metal ions were added as chlorides to prevent any influence of anions.

Effect of alkylating, reducing and metal chelating agents

The effect of several chemical reagents on D-(−)-LDH activity was investigated using standard assay conditions, except the purified enzyme was dialyzed extensively with standard buffer without 2-mercaptoethanol. The D-(−)-LDH was sensitive to alkylating agents, with the degree of inhibition being reagent dependent (Table 3). It was completely inhibited by *p*-chloromercuribenzoate (PCMB) at 1 mM while only 50% inhibition was observed with iodoacetamide and iodoacetic acid at 2 mM. The inhibitory effect of PCMB was reversed by the addition of dithiothreiotol (Table 3). No significant effect by reducing (Table 3) or metal chelating agents such as EDTA and 1,10-phenanthroline (5 and 10 mM) was observed (data not shown).

TABLE 2

Effect of metal ions (2 mM) on D-(−)-LDH activity

Metal	Relative activity (%)[a]
None	100
$CaCl_2$	87
$MgCl_2$	96
$MnCl_2$	97
$HgCl_2$	15
$CoCl_2$	75
$CuCl_2$	50
$ZnCl_2$	44

[a]The enzyme activity assayed according to standard conditions containing no metal ions served as a control (i.e. 100% activity).

TABLE 3

Effect of alkylating and reducing agent(s) on D-(−)-LDH activity

Agents	Concentration (mM)	Relative activity (%)[a]
None	0	100
PCMB[b]	0.5	25
PCMB (1 mM) + DTT[c] (10 mM)		100
Iodoacetamide	0.5	81
Iodoacetamide	1.0	62
Iodoacetamide	2.0	49
Iodoacetic acid	0.5	58
Iodoacetic acid	1.0	53
Iodoacetic acid	2.0	53
DTT	1.0	100
DTT	10.0	100

[a]The enzyme activity assayed according to standard conditions containing no oxidizing or reducing agents served as a control (i.e. 100% activity).
[b]PCMB = *p*-chloromercuribenzoate.
[c]DTT = dithiothreitol.

Effect of ATP and FDP

No significant effect by ATP or FDP on D-(−)-LDH activity was observed (Table 4).

Substrate specificity and kinetics

Using standard assay conditions the oxidoreductase activity of purified D-(−)-LDH was examined using several substrates and appropriate coenzymes. Purified D-(−)-LDH was most active on pyruvate (100% rel. act.) followed by β-hydroxypyruvate (59% rel. act.). No activity was detected when L-(+)-lactate, β-phenyl pyruvate, α-ketobutyrate, α-ketocaproate or D-(−)-3-phosphoglycerate were used as substrates. The K_m values were determined from measurement of reaction velocities at different substrate concentrations ranging from 0.3 to 7 mM for pyruvate and 25 to 200 mM for D-(−)-lactate. The K_m values for pyruvate and D-(−)-lactate were calculated, from Lineweaver–Burke plots, to be 0.64 and 68.42 mM respectively.

TABLE 4

Effect of ATP and FDP on D-(−)-LDH activity

Agent	Concentration (mM)	Relative activity (%)[a]
None	0	100
ATP	1	86
ATP	2	77
ATP	4	78
FDP	1	100
FDP	3	100

[a]The enzyme activity assayed according to standard conditions served as a control (i.e. 100% activity).

DISCUSSION

D-(−)-LDH activity has been detected in mollusks, polychaete worms, some arthropods and prokaryotes [21]. In prokaryotes, knowledge concerning the occurrence, structure, function, regulation, and physiological role of D-(−)-LDH is limited. The presence of D-(−)-LDH in several lactic acid bacteria has been reported [9]; however, detailed studies on D-(−)-LDH have not been conducted. *L. helveticus* is an industrially important organism which produces both D-(−)- and L-(+)-lactic acid via stereospecific LDHs. The D-(−)-LDH from *L. helveticus* CNRZ 32 was purified to initiate studies on its structure, regulation, substrate specificity, and physiological role.

The purification of D-(−)-LDH was accomplished by a four-step procedure, with rotofor fractionation and affinity chromatography being particularly effective. Cibacron Blue has been reported to have structural similarities to NADH, therefore it likely served as a D-(−)-LDH ligand [20]. Both the SDS–PAGE and native PAGE results indicate that the enzyme has been purified to homogeneity. The reasons for relatively low yield (0.39%) and fold purification (4×) cannot be explained. However, the partial inactivation of the enzyme during purification cannot be excluded.

The monomeric molecular weight of the purified D-(−)-LDH was estimated to be 38 000 on the basis of its electrophoretic mobility on SDS–PAGE. The molecular weight of the native enzyme was determined to be 152 000 by native PAGE. These results suggest that the CNRZ 32 D-(−)-LDH is a tetrameric enzyme. The tetrameric structure and molecular weight of the CNRZ 32 D-(−)-LDH is not in agreement with results reported for other lactic acid bacteria. Results suggesting a dimeric structure of D-(−)-LDHs from *L. plantarum* [7], *Leuconostoc lactis* [14], and *Pediococcus cerevisiae* [13] have been reported. Additionally, native molecular weight ranging from 64 000 to 80 000 for D-(−)-LDHs from *L. plantarum* [7], *Lactobacillus leichmanni* [11], *Lactobacillus fermenti* [11], *Lactobacillus jensenii* [11], *Lactobacillus acidophilus* [11], *L. lactis* [14], *Leuconostoc mesenteroides* [8] and *P. cerevisiae* [13] have been reported. In general, the monomeric molecular weight of the D-(−)-LDH from CNRZ 32 agrees with previous reports; however, the tetrameric structure and hence native molecular weight differ.

The D-(−)-LDH purified from *L. helveticus* CNRZ 32 was most active at pH 4.0. The pH optimum of D-(−)-LDHs from other lactic acid bacteria such as *L. plantarum* [7], *Leuconostoc* [8,14], and lactococci [17] have been reported to range from 8 to 8.5. However, Gasser et al. [11] reported the optimum pH of D-(−)-LDH from several lactobacilli ranged from 7.7 to 8.6 and suggested that the buffer and substrate used had a significant impact on observed pH optima. This conclusion is supported by the observation that the optimum pH of D-(−)-LDH from *P. cerevisiae* shifted from 8.0 to 3.6 when the pyruvate concentration was lowered from 5 to 0.5 mM [13].

The D-(−)-LDH purified from CNRZ 32 was active over a broad range of temperature with an optimum at 50°C, which again differs markedly from results obtained with other

D-(−)-LDHs from lactic acid bacteria. Rapid inactivation of D-(−)-LDH from *Leuconostoc* [8,14], lactobacilli [11], lactococci [17], and pediococci [13] at or above 40–45°C has been reported.

Significant inhibition of D-(−)-LDH from *L. helveticus* CNRZ 32 by a low concentration of PCMB suggests the involvement of a sulfydryl (-SH) group(s) in catalysis. Similar results have also been reported in *Leuconostoc* [8,14], and a low level of inhibition in a case of pediococci [13]. The addition of DTT did not affect CNRZ 32 D-(−)-LDH enzyme activity, even when added at 10 mM, suggesting that disulfide bonds are not essential for activity. However, it is noteworthy that no cysteine residues were detected in the DNA sequencing data of D-(−)-LDH genes from *L. plantarum* [23] and *L. delbrueckii* ssp. *bulgaricus* [3].

Among the divalent metal cations tested, $CoCl_2$, $ZnCl_2$, $CuCl_2$, and $HgCl_2$ inhibited D-(−)-LDH activity, with $HgCl_2$ being the strongest inhibitor. Severe inhibition of D-(−)-LDH by $HgCl_2$ has also been observed in *L. lactis* [17] and *P. cerevisiae* [13]; additionally, limited inhibition by $CuSO_4$ in both organisms has been reported. Inhibition of D-(−)-LDH by $HgCl_2$ supports the conclusion that a sulfydryl group(s) is involved in catalysis. No significant effect of metal chelating agents on D-(−)-LDH activity was observed, suggesting that metal ions are not involved in enzyme activity. Similar information is not available for D-(−)-LDHs from other lactic acid bacteria.

The substrate specificity of the CNRZ 32 D-(−)-LDH was determined with a variety of 2-ketocarboxylic acids. Highest activity was obtained with pyruvate, with lower activity observed with β-hydroxypyruvate. No activity was detected with L-(+)-lactate, β-phenylpyruvate, α-ketobutyrate, α-ketocaproate or D-(−)-3-phosphoglycerate. In contrast, the D-(−)-LDH from *L. plantarum* had a broad substrate specificity which included β-phenyl pyruvate [23].

LDH activity is frequently regulated by glycolytic intermediate(s) such as FDP and ATP. L-(+)-LDH from several lactic acid bacteria including lactobacilli have been determined to be activated by FDP [9]; however, no such information is available regarding D-(−)-LDH. D-(−)-LDH from *L. helveticus* CNRZ 32 was found not to be affected by FDP. It has been reported that D-(−)-LDH from *P. cerevisiae* [12], *L. lactis* [17], and *L. plantarum* [7] were inhibited by ATP and it has been suggested that ATP might control D-(−)-LDH activity in vivo. However, no inhibition of the CNRZ 32-D-(−)-LDH by ATP was observed. The lack of inhibition of the CNRZ 32-D-(−)-LDH by either ATP or FDP suggests that this enzyme is either unregulated or regulated in an unknown manner.

The D-(−)-LDH from *L. helveticus* CNRZ 32 differs from other reported lactobacilli D-(−)-LDHs in several aspects, such as native molecular weight, optimum pH and temperature for activity, involvement or sulfydryl group(s) in catalysis, substrate specificity and regulation. These differences are likely to be due to structural and/or evolutionary differences. Further research is needed to elucidate the physiological role, regulation, and evolutionary relationships between D-(−)-LDHs of lactic acid bacteria.

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