Malolactic Enzyme of *Lactobacillus plantarum*

**PURIFICATION, PROPERTIES, AND DISTRIBUTION AMONG BACTERIA***

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The malolactic enzyme of *Lactobacillus plantarum* was purified from 5.5 units/mg to a specific activity of 265 units/mg of protein. The enzyme has an isoelectric point of pH 4.4. The molecular weight is $M_r = 140,000$ as determined by gradient gel electrophoresis. The enzyme consists of two probably identical subunits ($M_r = 70,000$) that were observed after treatment with sodium dodecyl sulfate. Malolactic enzyme catalyzes the NAD- and manganese-dependent reaction $\text{L-malate} \rightarrow \text{CO}_2 + \text{L-lactate}$. Therefore, this enzyme can be distinguished from the well known malic enzymes (L-malate: NAD$^+$ oxidoreductase, oxalacetate-decarboxylating EC 1.1.1.38 or 1.1.1.39). Malolactic enzyme is found in most lactic acid bacteria (*Lactobacillaceae*); it has not been detected in other bacteria.

The list of the International Enzyme Commission (1) contains the following "malic enzymes" that are specific for L-malate: 1) L-malate:NAD$^+$ oxidoreductase (oxalacetate-decarboxylating), EC 1.1.1.38, first observed in *Lactobacillus arabinosus* (synonym with *Lactobacillus plantarum*, the now accepted name (2); 2) L-malate:NAD$^+$ oxidoreductase (decarboxylating), EC 1.1.1.39, isolated from *Ascaris lumbricoides* (3) and from *Streptococcus faecalis* (4), this enzyme does not decarboxylate oxalacetate; 3) L-malate:NADP$^+$ oxidoreductase (oxalacetate-decarboxylating), EC 1.1.1.40, originally isolated from pigeon liver (5). The activity of the malic enzymes EC 1.1.1.38 and EC 1.1.1.40 can be determined either by measuring spectrophotometrically the formation of NAD(P)H or by measuring the formation of carbon dioxide. However, the activity of the enzyme from the lactic acid bacterium *L. plantarum* (synonym for *L. arabinosus*) can only be determined by the measurement of carbon dioxide. Neither NADH nor pyruvate is found as an end product of the reaction of malate that yields L-lactate and CO$_2$ only (2). The occurrence of lactate instead of pyruvate has been originally regarded as the result of the joint action of a malic enzyme and a constitutive L-lactate dehydrogenase in *L. arabinosus* (= *L. plantarum*), because this latter enzyme could not be separated from the malate decarboxylating activity during its partial purification (6, 7).

Later on it was shown that L-lactate dehydrogenases are not involved in the formation of L-lactate from L-malate by *L. plantarum* (8). The enzyme involved in the reaction was shown to have a molecular weight of about 150,000. Recently, a malolactic enzyme from *Leuconostoc mesenteroides* was partially purified (9), with a molecular weight of 235,000.

This paper describes the purification of the malolactic enzyme of *L. plantarum*. Some characteristics of this enzyme are determined. Of particular interest was obtaining information about the occurrence of malolactic enzyme among lactic acid bacteria and related bacteria.

**EXPERIMENTAL PROCEDURES**

**Materials**

Phenyl-Sepharose CL 4B, Sephadex IEF, gradient gel PAA 4/30, and HMW and LMW protein calibration kits were purchased from Pharmacia, Freiburg; TEAE$^-$ 23-cellulose, sodium dodecyl sulfate, L-cysteine-HCl, Ampholine pH 4–6, and P, marker protein No. 9 were obtained from Serva, Heidelberg. Yeast extract was supplied by Ohly, Hamburg. All the other chemicals were purchased from Merck, Darmstadt.

**Microorganisms**

*L. plantarum* B 38 (10) is very similar to *L. arabinosus* 17.5 (synonym for *L. plantarum*). All bacterial strains were from the collection of this institute. The strains were originally obtained from various sources. We have to thank all colleagues who supplied these strains.

**Preparation of Cell-free Extracts**

The cells of *L. plantarum* were grown in *Leuconostoc oenos* medium (DSM, Catalogue of strains 1977: casein peptone, tryptic digest, 10 g; yeast extract, 5 g; glucose, 10 g; fructose, 5 g; MgSO$_4$·7H$_2$O = 0.2 g; MnSO$_4$·H$_2$O = 0.05 g; diammonium citrate, 3.5 g; Tween 80, 1 ml; tomato juice, filtered 100 ml; cysteine-HCl, 0.5 g; distilled water, 900 ml), modified by the addition of 10 g of L-malic acid, final pH 5.5–6.0. Three liters of medium in a 5-liter flask were sterilized by steaming for 30 min, inoculated with 1% of a preculture, and incubated with slight agitation at 30 °C for 14 h. The cells (about 15 g) were washed, suspended in 0.01 M phosphate buffer, pH 6.0, containing 0.03% sodium azide, and aerated for 2.5 min in the CO$_2$-cooled cell homogenizer (MSK, Braun, Melsungen) at 4,000 rpm (2.5 ml of buffer and 0.5 g of glass beads, diameter 0.11–12 mm, per g of cells, fresh weight). Supernatant and rinse liquid were centrifuged at 10,000 × g for 30 min to remove cells and debris. The cell-free extract (91.6 ml) contained 19.1 mg of protein and 105 units of malolactic enzyme per ml.

**Purification of Malolactic Enzyme from *L. plantarum***

All procedures were carried out at 4 °C.

**Step 1: Precipitation with Protamine Sulfate**—The cell-free extract was treated with protamine sulfate (1 ml of 1% (w/v) solution per 100 mg of protein).

**Step 2: Ion Exchange Chromatography**—One-third of the enzyme preparation after Step 1 (310 mg of total protein) was loaded onto a TEAE 23-cellulose column (diameter, 36 mm; volume, 40 ml; prepared from 6 g of TEAE-cellulose in 0.01 M phosphate buffer, pH 6.5), washed with phosphate buffer, and eluted (4.7 ml·cm$^{-2}$·h$^{-1}$) with a linear
gradient of KCl (0-0.5 M) over a 380-ml volume phosphate buffer. Fractions of 6 ml were collected. The highest specific activity of malic enzyme was observed at the conductivity of the eluant of 8 mS (see Fig. 1).

Step 3: Hydrophobic Chromatography.—The most active fractions obtained from Step 2 were dialyzed with a PM 10 ultrafilter (Amicon), adjusted to 1 M (NH₄)₂SO₄, and applied to a column of phenyl-Sepharose 4B (diameter, 15 mm; gel volume, 8 ml; equilibrated with 1 M ammonium sulfate in 0.01 M phosphate buffer, pH 6, and 0.03% sodium azide). Then the column was washed with equilibrating solution. For elution (22 cm² h⁻¹), a decreasing gradient of 1 to 0 M ammonium sulfate was used. The highest specific activity of malolactic enzyme observed in one fraction at pH 5.5-7.0.

Polyacrylamide Gradient Gel Electrophoresis

The molecular weight of malolactic enzyme was estimated by polyacrylamide gradient gel electrophoresis with PHARMACIA PAA 4/10 gradient gel and the flat bed apparatus (PHARMACIA FBE 3,000). Before applying the samples, the gels were equilibrated for 20 min at 70 V. 0.68 mg of lyophilized protein was dissolved in 100 µl of electrode buffer (0.09 M Tris, 0.08 M boric acid, 0.933 g/liter of Na₂EDTA, pH 8.4). Ten µl of this solution was loaded onto the gel. Electrophoresis was lasted 16 h at 150 V. A mixture of proteins (HMW Calibration kit) was run simultaneously (fixing and staining, 15 h in 0.02% Coomassie blue in 7% acetic acid; desaining, 24 h in 7% acetic acid).

SDS-Polyacrylamide Gradient Gel Electrophoresis

The procedure used was similar to the method of Weber and Osborn (11). The same equipment and staining procedures as for gradient gel electrophoresis were employed. 0.68 µg of lyophilized protein was dissolved in 100 µl of electrode buffer (0.04 M Tris, 0.02 M sodium acetate, pH 7.4, 2 mM EDTA, 0.2% [w/v] sodium dodecyl sulfate). To 50 µl of this solution, 5% (v/v) mercaptoethanol was added and the mixture was immersed in boiling water for 5 min. Ten µl of the protein samples were applied to the gradient plates.

Enzyme Assay

The activity of malolactic enzyme was determined by a modified method of Kaufmann et al. (7) by manometric measurement of CO₂ formation at 37 °C. The two-armed Warburg-vessels contained in the (a) main vessel: 0.2 ml of MnSO₄ (2 µmol), 0.1 ml of NAD (5 µmol), 0.01-0.2 ml of enzyme solution, and 0.05 ml phosphate buffer, pH 6, added to a final volume of 2.7 ml; (b) first side arm: 0.1 ml of l-malic acid (100 µmol), pH 6; (c) second side arm: 0.2 ml of 7 N sulfuric acid. After starting by adding the malic acid, the reaction was stopped after 5-7 min by the addition of the sulfuric acid to liberate 4% CO₂. The activity of malolactic enzyme was determined manometrically at pH 4.6 according to the method of London and Meyer (4). The specific activities of malolactic enzyme and of malic enzyme (units/mg) are expressed in micromoles of CO₂ formed mg of protein⁻¹ min⁻¹. The total activity (units) is defined as micromoles of CO₂ formed min⁻¹.

Determination of Cell Weight and Protein

The weight of the cells (fresh weight) was determined by weighing the sediment after centrifugation at 6,000 × g for 15 min. The protein content of the extracts was determined by the biuret reaction or spectrophotometrically at 280 and 260 nm according to Warburg and Christian (17).

Measurement of Conductivity

The conductivity (recorded as milliSiemens) of the fractions after chromatography was measured with the conductivity meter CDM 2B of Radiometer, Copenhagen.

RESULTS

Purification of Malolactic Enzyme.—For the purification of malolactic enzyme, the lactic acid bacterium L. plantarum B38 was used. This strain is very similar to the strain used by Korkes and Ochoa (2) in their paper describing "malic enzyme." L. plantarum B38 shows a particular high activity of malolactic enzyme when grown at inducing conditions (8).

Malolactic enzyme was purified 48-fold from a cell-free extract of L. plantarum with a specific activity of 5.5 units/mg. The purification of malolactic enzyme from L. plantarum is summarized in Table I (for details see "Experimental Procedures"). A preparation with a specific activity of 265 units/mg was obtained after isoelectric focusing. The isoelectric point was pH 4.4. Polycrylamide gradient gel electrophoresis and staining with Coomassie blue showed that this sample of malolactic enzyme yielded one protein band (Fig. 3A). By comparison with the known molecular weights of the "kit"-proteins, the malolactic enzyme showed a molecular weight of about M₀ = 140,000 (Fig. 4).

After treatment with SDS to separate malolactic enzyme into polypeptide subunits, gradient gel electrophoresis revealed only one protein band by staining with Coomassie blue (see Fig. 3B). According to its Rf value and by comparison with the "calibration kit" proteins, the molecular weight of the polypeptide subunit from malolactic enzyme was about M₀ = 70,000 (see Fig. 5). This is about one-half of the molecular weight of the entire malolactic enzyme. Therefore, it can be assumed that malolactic enzyme consists of two subunits that are apparently (but not necessarily) identical.
Malolactic Enzyme of *L. plantarum*

**TABLE I**

| Fraction          | Total protein | Total activity | Specific activity | Purification fold | Yield % |
|------------------|---------------|----------------|-------------------|-------------------|---------|
| Cell-free extract| 1750          | 9600           | 5.5               | 1                 | 100     |
| Protamine sulfate | 930           | 9000           | 9.7               | 1.8               | 94      |
| TEAE-cellulose, PM 10 | 100       | 5300           | 53.0              | 9.6               | 55      |
| Phenyl-Sepharose  | 11.8          | 2450           | 208               | 37.8              | 26      |
| Isoelectric focusing | 3.4       | 900            | 265               | 48.2              | 9.4     |

*All three preparations.*

**Reactions of Malolactic Enzyme**—Using a purified preparation of malolactic enzyme, the previously described (8) stoichiometry of the reaction of L-malate → CO₂ + L-lactate was confirmed, by enzymatic determination of malate and lactate and manometric determination of CO₂ (5 μmol of L-malate consumed yielded 4.8 μmol of L-lactate and 5.5 μmol of CO₂). When purified malolactic enzyme (1.4 units) was used in the usual spectrophotometric tests (596 nm), no activity (i.e., less than 0.1%) of malate dehydrogenase (substrate oxalacetate and NADH₂), malic enzyme (substrate L-malate and NAD), or lactate dehydrogenase (substrate pyruvate and NADH₂) was detected. Oxalacetate is decarboxylated to pyruvate at pH 6 at a similar rate as malate.

**Substrate Affinity of Malolactic Enzyme**—The substrate affinities for L-malate, NAD, and Mn⁺⁺ ions of malolactic enzyme of *L. plantarum* were determined with an enzyme preparation with a specific activity of 297 units/mg. Except for the substrate tested, the composition of the test system was the same as in the enzyme assay. From the Lineweaver-Burk plots, the following *Km* values were obtained: L-malate, *Km* = 9.5 mM; NAD, *Km* = 59·10⁻³ mM; Mn⁺⁺, *Km* = 12·10⁻³ mM.

**Distribution of Malolactic Enzyme in Bacteria**—Among 59 strains of lactic acid bacteria belonging to 25 species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, the occurrence of malolactic enzyme present was determined by polyacrylamide gradient gel electrophoresis using the subunits of malolactic enzyme of *L. plantarum* as markers.

**TABLE II**

| Genus          | Species |
|----------------|---------|
| Malolactic enzyme present | Lactobacillus alimentarius, brevis (9), buchneri (3), casei (7), cellobiosus, coryneformis, delbrueckii, farcininis, fermentum (4), fructosus, hilgardii, lactis, plantarum (9), salivarius, viridescens, xylopus |
|                 | Leuconostoc citrovorum, dextranicum, mesenteroides, oenos |
|                 | Pediococcus cereisai (5) |
|                 | Streptococcus cremoris, diacetilactis, faecalis (2), lactis (3) |
| Malolactic enzyme absent | Lactobacillus brevis (1), fermentum (1) |
|                  | Pediococcus cereisai (2) |
|                  | Streptococcus faecalis (2) |

*The numbers in parentheses are the number of strains tested.*
**Malolactic Enzyme of *L. plantarum***

tococcus, only 6 strains did not contain malolactic enzyme (see Table II). These "negative" strains belonged to four species, other strains of these species (except *S. faecalis*) contained the enzyme. A further investigation of several Gram-positive bacteria that may be regarded to show some relatedness to lactic acid bacteria did not detect malolactic enzyme. This enzyme was not found in strains of the following species: *Propionibacterium acidipropionicium, Propionibacterium thoenii, Aerococcus viridans, Bifidobacterium bifidum, Sporolactobacillus inulinus, Peptococcus variabilis, Peptostreptococcus anaerobius, Eubacterium limosum.*

**DISCUSSION**

The malolactic enzyme from *L. plantarum* described in this paper is identical with the enzyme from *L. arabinosus* (synonym of *L. plantarum*) that was reported by Korkes et al. (2) and called malic enzyme by these authors. In contrast to the first reported malic enzyme from pigeon liver (EC 1.1.1.40) that yields CO$_2$, pyruvate, and reduced coenzyme from L-malate and NAD (5), the end products of the reaction of malolactic enzyme from *L. plantarum* are CO$_2$ and L-lactate. Therefore, malolactic enzyme is different from the malic enzyme of pigeon liver (EC 1.1.1.40) and also from the other true malic enzyme (EC 1.1.1.39) that has been found in *S. faecalis* and *Lactobacillus casei* (4).

In this paper, the molecular weight of malolactic enzyme from *L. plantarum* as determined by gradient gel electrophoresis was found to be about $M_w = 140,000$. The malolactic enzyme of *Lc. mesenteroides* that was partially purified by Lonvuo-Funel and Strasser de Saad (9) has a molecular weight of about $M_w = 235,000$. This shows that the malolactic enzyme of *L. plantarum* ($M_w = 140,000$) and *Lc. mesenteroides* are somewhat different. This is in agreement with our own immunological investigations. An antiserum prepared against the malolactic enzyme of *L. plantarum* did react against the enzymes of other *Lactobacillus* species and *Pediococcus* by showing a homogenous, continuous precipitin band with the agar double diffusion technique. It reacted also against the malolactic enzyme of *Lc. mesenteroides* but showed the presence of different determinants that were indicated by a different band and additional spur formation.

The substrate affinities for the "malic enzyme" of *L. arabinosus* 17-S had been determined with partially purified preparations: malate, $K_m = 9.8 \times 10^{-3}$ mM, NAD, $K_m = 10.6 \times 10^{-3}$ mM, Mn$^{2+}$, $K_m = 0.29$ mM (6). With a purified preparation of malolactic enzyme of *L. plantarum*, we found the following values: malate, $K_m = 9.5$ mM; NAD, $K_m = 59 \times 10^{-3}$ mM; Mn$^{2+}$, $K_m = 12 \times 10^{-3}$ mM. With our purified preparation of malolactic enzyme, a very low $K_m$ for manganese was observed.

The treatment of malolactic enzyme with sodium dodecyl sulfate led to the separation of the enzyme into its subunits: gradient gel electrophoresis revealed only one band that showed a molecular weight of 70,000. Therefore, it is assumed that malolactic enzyme consists of two probably identical subunits.

Alizade and Simon (12) and Kraus et al. (13) have investigated the fermentation of labeled L-malate by intact cells of *Lc. mesenteroides*. Their results led to the assumption that a multienzyme complex catalyzes the steps L-malate $\rightarrow$ oxaloacetate $\rightarrow$ pyruvate $\rightarrow$ L-lactate. It was further assumed that the intermediary compounds oxaloacetate and pyruvate are not liberated from the enzyme complex. The direct decarboxylation of L-malate to L-lactate was regarded as rather unlikely.

The results of our paper do not support the hypothesis that malolactic enzyme is an aggregation of several different enzymes. Its two subunits are apparently identical. Therefore, if malolactic enzyme is regarded as a multienzyme complex, then the components must be covalently linked for they cannot be separated by sodium dodecyl sulfate.

The necessity of NAD ($K_m = 59 \times 10^{-3}$ mM) for the reaction of malolactic enzyme is a typical characteristic of an oxireductase. However, no reduction of NAD can be observed by the usual spectrophotometric methods. If not the activating compound but the end products of the reaction of malolactic enzyme are being considered, then this enzyme could be regarded as a carboxylase. In this case, the original designation L-malate:NAD$^+$ oxireductase (oxalacetate-decarboxylating) (EC 1.1.1.38) could be transferred to the malic enzyme (sensu stricto) of Schizosaccharomyces pombe, that reacts with L-malate and NAD, yields pyruvate and NADH, and decarboxylates oxalacetate (14).

The reaction catalyzed by malolactic enzyme that is very frequent in lactic acid bacteria (but is limited to this group of organisms) does not yield metabolizable end products, reduction equivalents, or energy equivalents. The significance of malolactic enzyme that many lactic acid bacteria contain in high activities probably rests in the compensation of the increase in hydrogen ions that are formed during the fermentation of carbohydrates (15, 16). This decrease of acidity caused by malolactic enzyme (the dicarboxylic acid is converted to the monocarboxylic lactic acid) is a selective advantage for lactic acid bacteria that occur in environments with a high sugar content in the presence of malic acid. The malolactic fermentation of wine is of considerable importance for the wine industry and the malolactic enzyme is the key enzyme in this process.

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