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Purification and Properties of a Malolactic Enzyme from a Strain of *Leuconostoc mesenteroides* Isolated from Grapes

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An enzymatic complex able to transform L-malate to L-lactate was obtained from a *Leuconostoc mesenteroides* strain isolated from grapes. The molecular weight was about 235,000, the isoelectric point was at pH 4.35, and the optimal pH for activity was 5.75. The malolactic activity followed a sequential pattern concerning the involved substrates. At pH values substantially different from the optimum, a positive cooperativity between malate molecules was observed. Oxamate, fructose-1,6-diphosphate, and L-lactate acted as noncompetitive inhibitors, whereas succinate, citrate, and tartrate isomers produced a competitive inhibition.

Malolactic fermentation is indispensable for the production of certain types of red wines. It is accomplished by lactic acid bacteria, both lactobacilli and cocci. In the reaction, L-malate is converted exclusively to L-lactate and CO_2. The enzyme active in the fermentation is not the same as the malic enzyme described by Ochoa (8). Malic enzyme catalyzes the formation of pyruvate from L-malate with a concomitant reduction of NAD^+. Although the pyruvate can be converted to L- or D-lactate in the presence of the appropriate lactate dehydrogenase, some bacteria responsible for the conversion of L-malate to L-lactate in wine contain only D-lactate dehydrogenase (9). Thus, the malolactic transformation is catalyzed by a particular activity named "malolactic enzyme" which is active in the presence of NAD and Mn^{2+} and produces only L-lactate from L-malate (10; M. Lonvaud, thèse 3° cycle, Université de Bordeaux II, 1975).

In this study we describe the purification and properties of a malolactic enzyme from a strain of *Leuconostoc mesenteroides* isolated from grapes. Malolactic activity was inducible, and the strain did not contain an L-lactate dehydrogenase. Purification of the malolactic activity was simplified by the absence of L-lactate dehydrogenase, which is difficult to eliminate from malolactic enzyme by purification from bacterial extracts that contain both activities (5).

MATERIALS AND METHODS

Strain and culture medium. The strain of *L. mesenteroides* used for this study was preserved either in the culture medium at 4°C or lyophilized in a medium containing 5% inositol, 5% dextrane, and 2% sodium glutamate. The culture medium was modified Carr medium (J. G. Carr, Ph.D. thesis, University of Bristol, Bristol, England, 1956) containing tomato juice and 20 g of D-L-malic acid per liter. The pH was adjusted to 4.8 with sodium hydroxide. After autoclaving for 5 min at 120°C, 5 liters of medium was inoculated with 500 ml of a culture in the exponential phase of growth. After incubation for 40 h at 30°C, the cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet was suspended in 30 ml of a solution (pH 6.0) which contained monopotassium and disodium phosphate (0.05 M), KCl (0.1 M), and beta-mercaptoethanol (0.1%).

Preparation of cell-free extracts. The bacterial suspension was placed in a refrigerated container at 4°C, and the cells were disrupted with an ultrasonic disintegrator (MSE Scientific Instruments, Crawley, England) at 150 W for 15 min.

The cell-free extract was separated from the bacterial debris by centrifuging at 30,000 × *g* for 20 min at 4°C.

Protein determination. The protein concentration of the crude and purified extract was determined by the method of Lowry et al. (7), except for the first steps of purification for which the method of Warburg and Christian (11) was used.

Measurement of enzymatic activities. Malolactic activity was determined by measuring the CO_2 released from malate with specific CO_2 electrode (Eischweiler and Co., Kiel, Federal Republic of Germany) by the method of Lonvaud and Ribéreau-Gayon (6). The reaction mixture was at 25°C and contained 50 μmol of L-malate, 1 μmol of NAD, and 0.2 μmol of $MnCl_2$ in 2.3 ml of 0.2 M KH_2PO_4-Na_2HPO_4 buffer (pH 6).

The reaction was started by adding to the system the extract containing the enzyme, and the specific activity was expressed as micromoles of CO_2 released per minute per milligram of protein.

Malic enzyme activity (malate → pyruvate) was assayed in the same reaction mixture. The reduction of NAD^+ was measured at 340 nm in cuvettes of 1-cm light path, and the activity was expressed as micromoles of NADH produced per minute.

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TABLE 1. Purification of malolactic activity

Purification step	Sp act[a]
Crude extract	7.16
After heat plus $MnCl_2$ treatment	7.80
After protamine sulfate precipitation	8.00
After 75% $(NH_4)_2SO_4$ precipitation	34.40
Fraction 42 from the ACA 34 filtration	41.20

[a] Specific activity is expressed as micromoles of CO_2 released per minute per milligram of protein.

To determine the stereospecificity of the lactate dehydrogenase activity the oxidation of L- or D-lactate to pyruvate was determined by using a solution of 290 μmol of lactate and 5 μmol of NAD in 0.08 M Tris-hydrochloride buffer (pH 9). The final volume was 2.3 ml. Specific activity was expressed as micromoles of NADH formed per minute per milligram of protein. The rate of reduction of pyruvate to lactate was used to measure lactate dehydrogenase activity during purification. The assay solution contained 200 μmol of pyruvate and 0.1 μmol of NADH in 0.2 M KH_2PO_4-Na_2HPO_4 buffer (pH 6). Specific activity (micromoles of NADH formed per minute per milligram of protein) was determined by decrease in absorbance of NADH at 340 nm.

Purification. To each milliliter of crude extract, 0.13 ml of 1 M $MnCl_2$ and 0.06 ml of 2 M L-malate (pH 6) were added. After incubation for 10 min at 40°C with stirring, the mixture was cooled to 4°C, and the precipitate was removed by centrifugation at 28,000 × *g* for 10 min at 4°C.

The protein content of the supernatant was measured, and 0.15 mg of protamine sulfate was added per mg of protein. After 40 min at 4°C, the precipitate was removed by centrifugation at 30,000 × *g* for 20 min at 4°C.

The supernatant was brought to 40% saturation with ammonium sulfate. The precipitate was removed by centrifugation as in the previous step, and ammonium sulfate was added to the supernatant to 75% saturation.

The precipitate obtained by centrifuging as in the previous step contained all of the malolactic activity. The precipitate was dissolved in 2 ml of 0.02 M phosphate buffer (pH 6). The solution was applied to a column of Ultrogel ACA 34 (a gel of 3% acrylamide and 4% agarose for molecular weight separation in the range of 65,000 to 350,000; Pharmindustrie IBF) and eluted with 0.02 M phosphate buffer (pH 6) with 0.1 M KCl. The elution rate was 18 ml h^{-1}, and the volume of the fraction was 3.7 ml. After selecting the fractions containing protein by measuring the absorbance at 280 nm, their malolactic and malic enzyme activities were tested. Those showing maximal specific activities (fractions 40 to 47) were pooled and constituted the purified extract used for most of the experiment described in this report.

Determination of molecular weight. The ACA 34 gel column was calibrated by using a known protein mixture (Combitek calibration proteins; Boehringer Mannheim Corp.) The molecular weight of the malolactic enzyme was determined by using a graphic plot of the log of the molecular weights of the proteins against elution volume.

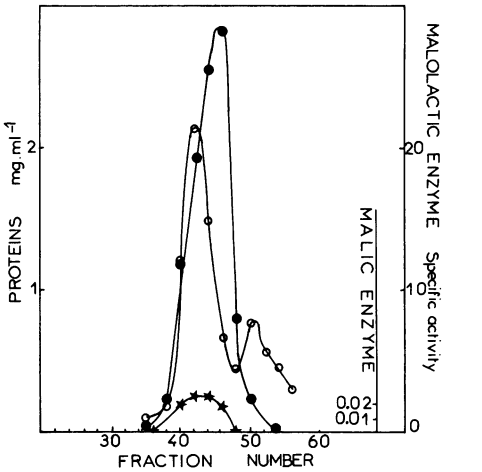

FIG. 1. Ultrogel ACA 34 column chromatography of the extract. Elution rate, 18 ml h^{-1}. Fraction volume, 3.7 ml. Eluant, 0.02 M KH_2PO_4-Na_2HPO_4 plus 0.1 M KCl (pH 6.0). Symbols: ○, proteins; ●, malolactic enzyme activity (micromoles of CO_2 released per minute per milligram of protein); ★, malic enzyme activity (micromoles of NADH formed per minute per milligram of protein).

Isoelectric focusing. The purified extract was dialyzed against 1% glycine and then electrofocused in a 110-ml LKB column. The ampholines, used at 2% final concentration, formed a pH gradient from 3.5 to 5. Stabilization was achieved by a glycerol density gradient. The temperature was maintained at 8°C. The focusing was done at about 5 W for 72 h. Fractions of 3.7 ml were collected, and the pH, absorbance at 280 nm, and the malolactic activity of each fraction were measured.

RESULTS

The crude extract showed a specific malolactic activity of 7.16 μmol of CO_2 released per min per mg of protein. However, neither a significant malic enzyme activity (malate → pyruvate) nor an L-lactate dehydrogenase could be demonstrated. Instead, it was possible to measure a D-lactate dehydrogenase in the range of 0.32 μmol of NADH formed per min per mg of protein. This D-lactate dehydrogenase was eliminated during the first steps of purification since it disappeared after removing the precipitate that formed at 40% ammonium sulfate concentration. In this precipitate a weak malic activity, 0.027 μmol of NADH formed per min per mg of protein, was detected.

Purification of the malolactic enzyme through the gel filtration step led to an approximately sixfold increase in specific activity (Table 1).

Figure 1 shows the elution profile of the gel filtration step. The purified extract pool was composed of fractions 40 to 47 and had a protein content of 1.4 mg ml^{-1}, a specific malolactic

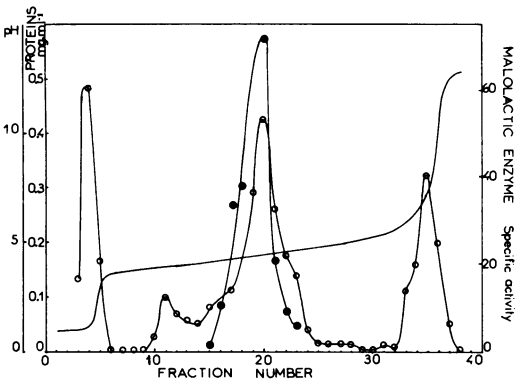

FIG. 2. Isoelectric focusing of malolactic enzyme. The pH gradient was made in a 110-ml LKB isoelectric focusing column with 2% carrier ampholines and 0 to 60% glycerol gradient. Fraction volume, 3.7 ml. Symbols: ●, malolactic enzyme activity (micromoles of CO_2 released per minute per milligram of protein); ○, proteins (milligrams per milliliter); ——, pH.

activity of 16.23 μmol of CO_2 released per min per mg of protein, and a very weak malic enzyme activity of 0.0085 μmol of NADH formed per min per mg of protein.

The molecular weight of the malolactic activity was found to be 235,000. The isoelectric point was determined by testing the malolactic activity of fractions obtained in the pH range of 3.5 to 5. The highest value, 72.5 μmol of CO_2 per min per mg of protein, was found in fraction 20 (Fig. 2) at pH 4.35, the isoelectric point of the malolactic activity.

Kinetic studies. The activity of the purified extract was studied as a function of the concentration of malic acid and NAD at 25°C and pH 6.0.

The Lineweaver-Burk curves (1/velocity as a function of 1/malate) resulting at different NAD concentrations showed that with all of them, the lines met the 1/velocity axis at the same point; this is characteristic of a sequential enzymatic mechanism. The results indicated that malate was the second substrate to bind to the protein.

The kinetics of the reaction when different NAD concentrations were used are depicted in Fig. 3, from which the following constant values were calculated: K_m^{malate}, 16.7×10^{-3} M; and K_m^{NAD}, 4.3×10^{-5} M.

From Fig. 3 it is also apparent that the point corresponding to 1 μl of NAD was always situated above the mean line. This fact is characteristic of a positive homotropic cooperativity activation of the reaction, which is due to the substrate itself. On the other hand, the position of the intersection point of the lines (negative ordinate) showed that malate impaired the binding of NAD.

Influence of pH. When 20, 50, 100, 150, and 200 μl of 0.25 M L-malate containing constant concentrations of NAD and $MnCl_2$ were brought to 2.3 ml with 0.2 M phosphate buffers at different pH and the velocity of the reactions was measured, it was found that the optimal pH was always 5.75 and independent of the L-malate concentration. However, when the velocity was plotted against malate concentration, the curves obtained demonstrated different behaviors depending on the pH: a sigmoidal pattern from pH 4.0 to 5.5 and a Michaelian pattern from pH 5.5 to 6.5 (Fig. 4).

Moreover, Lineweaver-Burk plots gave curves from pH 4.0 to 5.5 with a concavity, indicating a positive cooperativity of malate. To calculate the degree of cooperativity at different pH, Hill plots were used (Table 2).

This sigmoidal behavior of the substrate concentration strongly indicated the possibility that the enzyme was oligomeric.

Changes in pH and NAD concentration with constant malate (50 μmol in a 2.3-ml total volume) always produced reactions that followed

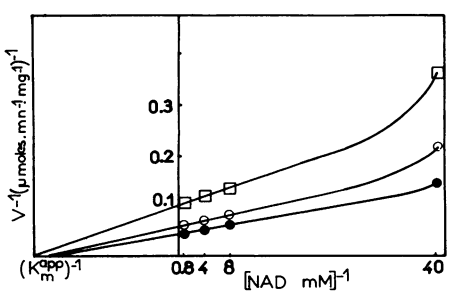

FIG. 3. Double-reciprocal plots of 1/velocity versus 1/NAD at several fixed concentrations of L-malate. Symbols for malate concentrations: □, 5.43 mM; ○, 10.86 mM; ●, 21.74 mM. K_m^{app}, Apparent Michaelis constant for the NAD.

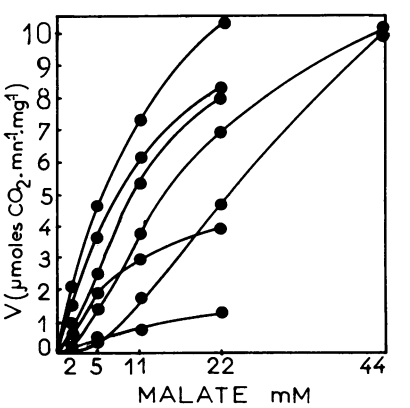

FIG. 4. Effect of the pH on malate kinetics (NAD, 0.5 mM; $MnCl_2$, 0.087 mM).

TABLE 2. Degree of cooperativity at different pH

pH	n^a
4.0	2.38
4.5	1.64
5.0	1.38
5.5	1.00
6.0	1.00
6.5	1.00

[a] n is the slope of Hill plots: log $v/(V_{max} - v)$ versus the logarithm of malate concentration.

Michaelian kinetics at all pH. From the results it was also evident that the enzyme affinity for NAD and the maximal velocity of the reaction were positively correlated with pH.

Influence of $MnCl_2$ concentration. The study of the kinetic behavior of the reaction as a function of the $MnCl_2$ concentration showed that Mn^{2+} acted as an allosteric activator. When the concentration of Mn^{2+} increased there was a shift from sigmoidal to Michaelian kinetics. The Hill coefficient decreased from 1.4 to 1.0. Thus, it seemed that there was positive cooperation between malate molecules only in the absence of Mn^{2+} since concentrations of this ion as low as 4.3×10^{-5} M were enough to suppress it. Under these conditions the binding of malate was increased by Mn^{2+}. It should also be noted that the kinetics of the reaction as a function of the $MnCl_2$ concentration were always hyperbolic whatever the concentration of malate employed and that the affinity constant for Mn^{2+} was 4.6×10^{-5} M.

From the above results the conclusion may be drawn that Mn^{2+} acted as an activator of the reaction and bound to the protein before and independently of malate. On the contrary, for the malic enzyme from pigeon liver the activating metal affected the K_m for the malate (2).

Influence of inhibitors. Fructose-1,6-diphosphate acted as a noncompetitive inhibitor with an inhibition constant (K_i) of 32×10^{-3} M.

Oxamic acid, a well known lactate dehydrogenase inhibitor, also produced noncompetitive inhibition with a K_i of 9×10^{-3} M.

Lactate, one of the products of the reaction, also acted as a noncompetitive inhibitor, but to a lesser extent than fructose-1,6-diphosphate and oxamic acid, with a K_i of 0.276 M (Fig. 5).

The influence of carboxylic acids normally present in wines and succinic, citric, and tartaric acids was also tested. In every case the inhibition was competitive, which is in accordance with the chemical structures of these substances. The K_i was 78×10^{-3} M for succinate and 11×10^{-8} M for citrate. The values of K_i of different isomers of tartaric acid were as follows: D-tartrate, 11×10^{-3} M; mesotartrate, 10×10^{-3} M; and L-tartrate 12×10^{-4} M. The affinity of L-tartrate for the enzyme was about 10-fold less than that of the other isomers. D-Tartaric acid, the only isomer present in wine, produced a strong inhibition of malolactic activity.

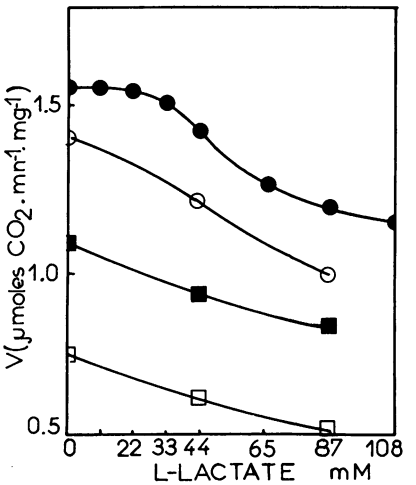

FIG. 5. Inhibition by L-lactate at various malate concentrations (NAD, 5 mM; $MnCl_2$, 0.087 mM). Symbols (malate concentrations): ●, 21.74 mM; ○, 16.37 mM; ■, 10.87 mM; □, 5.43 mM. K_i, Inhibition constant for L-lactate.

DISCUSSION

The reaction of the malolactic fermentation is not a direct decarboxylation as shown by Alizade and Simon (1).

Although the extracts used in this study produced L-lactate from L-malate, it was not possible to demonstrate any L-lactate dehydrogenase activity. A weak malic enzyme activity measured by the production of NADH was found which was ca. 2,000 times lower than malolactic activity; it was probably the same activity described by Kunkee (4) as the NADH-producing activity. The results obtained strongly indicate that malolactic activity was not a combination of malic enzyme and lactate dehydrogenase activities, but was carried out by a complex that produced the sum of these reactions without catalyzing the partial reactions. Stereochemical studies by Kraus et al. (3) pointed to the possibility that both oxalacetate and pyruvate might remain attached to the malolactic complex during the course of the reaction and not appear as free intermediates. This hypothesis is supported by the following experimental evidence obtained in the present investigation.

(i) NAD was essential for malolactic activity, but no net reduction of NAD occurred. The NADH produced during the first step(s) was probably immediately reoxidized by an L-lactate dehydrogenase contained within the complex.

To function, the complex must contain malic enzyme and L-lactate dehydrogenase components or, alternatively, malate dehydrogenase, oxalacetate decarboxylase, and L-lactate dehydrogenase components.

(ii) The kinetics studies of NAD, Mn^{2+}, and malate indicated that the NAD and Mn^{2+} attached first to the protein. Malate then was bound by the enzyme-NAD-Mn^{2+} complex. Moreover, the cooperativity observed between malate molecules at pH values substantially different from the optimum and also in the absence of Mn^{2+} pointed to the possibility that the protein of the complex which binds malate was oligomeric. It was also observed that the noncompetitive inhibitors fructose-1,6-diphosphate and L-lactate showed allosteric behavior, i.e., there was cooperative binding of the inhibitors to the protein. It should also be noted that the inhibition produced by oxamate was efficient even at the lower concentration tested. On the other hand, citric, succinic, and tartaric acids produced an effective competitive inhibition due to their structural similarity to malate.

The amount of inhibition produced by these acids at the usual concentrations encountered in wines were as follows: D-tartrate, 40%; succinate, 10%; and citrate, 5%. At the same concentrations, the inhibition was higher for whole cells than for the extracts prepared from them (unpublished data). These characteristics may partially account for the difficulties found in practice during the initiation and completion of the malolactic fermentation in wines when pH, temperature, and SO_2 are not limiting.

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