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Reverse Reaction of Malic Enzyme for HCO_3^- Fixation into Pyruvic Acid to Synthesize L-Malic Acid with Enzymatic Coenzyme Regeneration

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Malic enzyme [L-malate: $NAD(P)^+$ oxidoreductase (EC 1.1.1.39)] catalyzes the oxidative decarboxylation of L-malic acid to produce pyruvic acid using the oxidized form of NAD(P) ($NAD(P)^+$). We used a reverse reaction of the malic enzyme of *Pseudomonas diminuta* IFO 13182 for HCO_3^- fixation into pyruvic acid to produce L-malic acid with coenzyme (NADH) generation. Glucose-6-phosphate dehydrogenase (EC1.1.1.49) of *Leuconostoc mesenteroides* was suitable for coenzyme regeneration. Optimum conditions for the carboxylation of pyruvic acid were examined, including pyruvic acid, NAD^+, and both malic enzyme and glucose-6-phosphate dehydrogenase concentrations. Under optimal conditions, the ratio of HCO_3^- and pyruvic acid to malic acid was about 38% after 24 h of incubation at 30 °C, and the concentration of the accumulated L-malic acid in the reaction mixture was 38 mM. The malic enzyme reverse reaction was also carried out by the conjugated redox enzyme reaction with water-soluble polymer-bound NAD^+.

Key words: HCO_3^- fixation; malic enzyme; NADH regeneration; L-malic acid

Abundant atmospheric CO_2 is one factor causing the greenhouse effect. The concentration of CO_2 is far higher than that of other gasses, such as CH_4, NOx, and CFC (fluorocarbons). About 2,000 to 3,500 million tons of CO_2 naturally exist in the air, but 5,500 million tons of artificial CO_2 gas is annually discharged. One important proposal to reduce atmospheric CO_2 is to develop new sources of energy, but it will take time and money to replace the current energy system. New technologies are quickly needed at least to reduce the huge volume of CO_2 from thermal power plants and chemical plants. There have been various attempts to fix CO_2, including physical and chemical processes. Especially, there is currently great interest in the fixation of CO_2 with an enzymatic reaction system, because the enzymatic method is an environmentally friendly process and that can be applied in industry.[1)]

Reverse reactions of malic enzymes have been designed and used in CO_2 fixation with coenzyme regeneration such as photochemical and electrochemical reactions.[2–5)] Malic enzyme (L-malate:$NAD(P)^+$ oxidoreductase (decarboxylating) EC 1.1.1.38-40) is known as a catalyst of the decarboxylation reaction of L-malic acid, producing pyruvic acid with $NAD(P)^+$ reduction. Malic enzyme also catalyzes the fixation of carbon dioxide, forming L-malic acid,[6)] but it has proved to be difficult to control CO_2 fixation given the unfavorable *in vitro* situation. In addition, the malic enzyme reaction consumes the coenzyme at the same rate as the substrate. Regeneration of coenzyme is important in carrying out the malic enzyme reverse reaction.

We have found that *Pseudomonas diminuta* IFO13182 produced malic enzyme (EC 1.1.1.39), and we have investigated its applications, focusing on the production of a reduced form coenzyme, such as NAD(P)H.[7,8)] We succeeded in L-alanine production combined with malic enzyme and alanine dehydrogenase in a NADH regeneration system.[9)] In this case, the malic enzyme reaction favored a forward reaction for decarboxylation of L-malic acid with NAD^+ reduction. In this study, we investigated the reverse reaction of the malic enzyme of *P. diminuta* IFO 131832 with enzymatic NADH regeneration in a preliminary study of CO_2 fixation, as shown Fig. 1.

Materials and Methods

Chemicals. Glucose-6-phosphate dehydrogenase (G-6-PDH, EC1.1.1.49, 400 units·mg^{-1}) of *Leuconostoc mesenteroides* was obtained from Oriental Yeast

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid, monohydrate

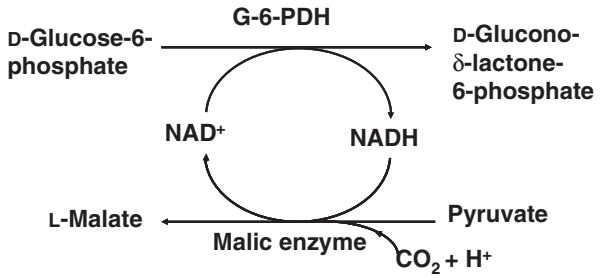

Fig. 1. Design of Malic Enzyme Reverse Reaction with Coenzyme Regeneration Conjugated with G-6-PDH.

(Tokyo). Pig heart lactate dehydrogenase of (EC1.1.1.27, 350 units·mg^{-1}) was obtained from Toyobo (Tokyo). NAD^+ was from Oriental Yeast. N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and 2-morpholinoethanesulfonic acid, monohydrate (MES) were from Nacalai Tesque (Kyoto). The other reagents and compounds were of analytical grade and were used without further purification.

Preparation of malic enzyme. Pseudomonas diminuta IFO 13182 was cultured in a 30-liter jar-fermenter and prepared as a cell-free extract with partial purification, as described previously.[10] Malic enzyme was purified 44-fold over the cell-free extract with a final yield of 49% and a specific activity of 0.989 units·mg^{-1} protein.

Assay of malic enzyme activity for reverse and forward reactions. The enzyme activity for the forward reaction was measured as previously described.[8] The reverse reaction was measured in a $KHCO_3$ solution as a carbon source *via* the NADH-conversion of L-malic acid from pyruvic acid. Activity was measured spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 nm. The substrate mixture contained 60 µmol of pyruvic acid, 30 µmol of $MgCl_2$, 0.6 µmol of NADH, 60 µmol $KHCO_3$, and 100 µmol of HEPES-KOH buffer, pH 7.4, in a total volume of 2.0 ml. The reaction was started by adding 1.0 ml of the enzyme solution, and the mixture was incubated at 30 °C. After exactly 2 min, 1.0 ml of 10% (w/v) sodium dodecyl sulfate (SDS) was added to stop the reaction, and the absorbance at 340 nm was measured. In a blank test, 10% SDS was added before the enzyme solution. One unit of activity was defined as the amount of the enzyme that produced 1 µmol of NADH per min at 30 °C. In calculating the amount of NADH produced, a molar absorption coefficient for NADH of 6.22×10^3 M^{-1}·cm^{-1} was used.

Synthesis of L-malic acid by coenzyme regeneration using G-6-PDH. The reverse reaction of malic enzyme with NADH regeneration using G-6-PDH was also carried out using glucose-6-phosphate (G-6-P) and G-6-PDH.

The standard reaction mixture for the malic enzyme reverse reaction with NADH regeneration using G-6-PDH contained 1.0 µmol of NAD^+, 100 µmol of G-6-P, 100 µmol of pyruvic acid, 15 µmol of $MgCl_2$, 100 µmol $KHCO_3$, 0.5 unit of malic enzyme, and 0.5 unit of G-6-PDH in 1.0 ml of 55 mM potassium phosphate buffer (pH 7.4). The reaction mixture was incubated at 30 °C.

Preparation of water-soluble macromolecular polymer immobilized NAD^+. Water soluble polymer (sodium alginate) immobilized NAD^+ (Alg-NAD^+) was prepared as previously described.[11] Samples of 13.8 mg of sodium alginate and 70 mmol 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were dissolved in 15 ml of water. The final pH was adjusted to 4.7, and the solution was stirred for 40 min at room temperature. NAD^+ was added to the solution to 70 mmol. After readjustment of the pH to 4.7, the resulting solution was stirred for 12 h at room temperature. The reaction mixture was dialyzed with 10 mM Tris–HCl buffer (pH 7.0) for 12 h, and then dialyzed with water for 12 h at 5 °C. Alg-NAD^+ was recovered by lyophilization. Lyophilized products were stored at −20 °C in the dark. The coenzyme activity of Alg-NAD^+ was determined enzymatically by the alcohol dehydrogenase system.[12]

HPLC analysis. HPLC analysis of L-malic acid, pyruvic acid, and L-lactic acid in the reaction mixture was performed using a Hitachi L-7100 and Shodex® Asahipak ES-502N (100 × 7.6 mm i.d.) (Showa Denko, Tokyo) at 50 °C. The elution buffer consisted of 0.1 M potassium dihydorogenphosphate (KH_2PO_4) buffer (pH 4.2) at a flow rate of 1.0 ml·min^{-1} at 50 °C. The detector was a Hitachi U2010 ultraviolet/visible spectrophotometer (Hitachi, Tokyo) at 210 nm. The retention times of L-malic acid and pyruvic acid were 21.8 min and 11.1 min respectively.

Results and Discussion

Reverse reaction activity of malic enzyme

The reverse reaction (carboxylation) and forward reaction (decarboxylation) activities of malic enzyme were measured at different pH levels. As shown in Fig. 2, the optimum pH for the reverse reaction was at 6.0, and carboxylation was strongly dependent on the pH value, but the reverse reaction activity was 9.7 µM·min^{-1}, representing only 5% of the forward reaction activity. Considering the results for the optimum pH for the malic enzyme reverse reaction and the stability of both the reduced form (NADH) and the oxidized form (NAD^+) coenzyme,[13,14] pH 7.4 was used thereafter in malic enzyme reverse reaction mixtures.

The reverse reaction of the malic enzyme with potassium ions was slightly better in terms of CO_2 fixation (5% of the forward reaction) than with sodium ions (2% of the forward reaction). $KHCO_3$ and potassium pyruvate were used in further investigation.

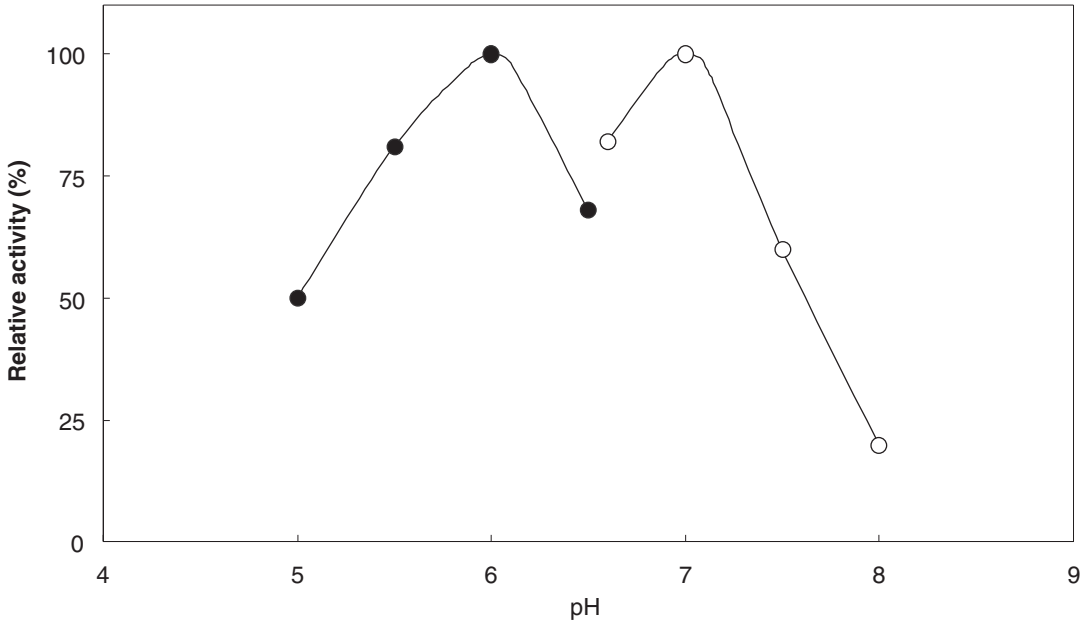

Fig. 2. Effect of pH on Reverse Reaction (carboxylation) Activity of Malic Enzyme.
Enzyme activity was measured at pH 5–8. Symbols: ●, MES buffer; ○, HEPES buffer.

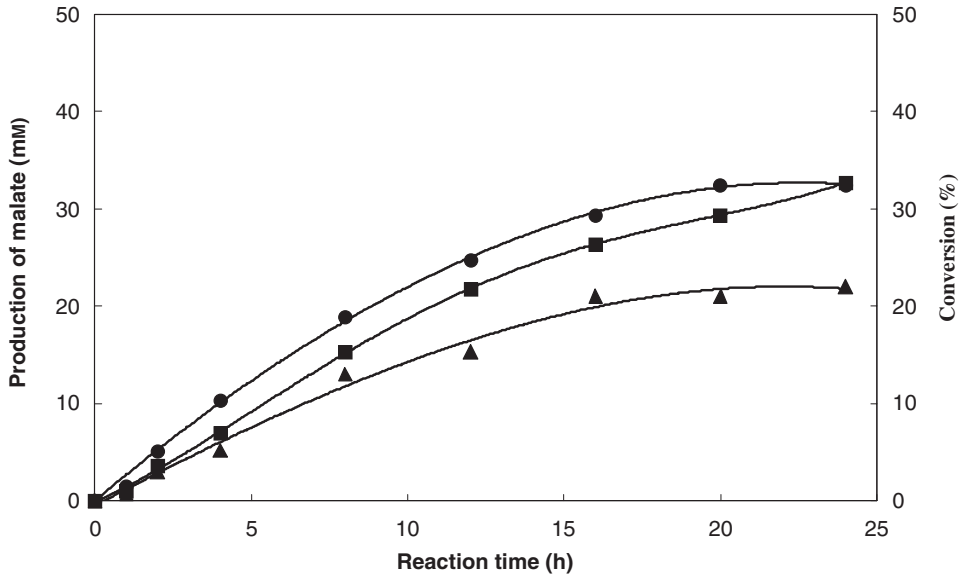

Fig. 3. Effect of NAD^+ Concentration on Malic Enzyme Reverse Reaction System.
The pyurvic acid concentration was 500 mM and the concentration of other components was the same as for the standard reaction mixture. Symbols: ●, 1.0 mM NAD^+; ■, 0.5 mM NAD^+; ▲, 0.1 mM NAD^+.

Reverse reaction of malic enzyme with coenzyme regeneration using G-6-PDH

Initially, LDH was used in the malic enzyme reaction for NADH regeneration,[15] but L-malic acid, an indicator of HCO_3^- fixation into pyruvic acid in the reaction system, was not produced in the reaction mixture (data not shown). The equilibrium of the LDH reaction for NADH regeneration is not favorable in the neutral pH region, and L-lactic acid causes a decrease in the catalytic activities of the malic enzyme.[3] LDH was thus not suitable for the regeneration reaction combined with the reverse reaction of malic enzyme.

Two substrate systems were designed to improve coenzyme regeneration. The G-6-PDH reaction was used as the NADH regenerator in the system, since G-6-PDH is useful in the regeneration of NADH from NAD^+ in the neutral pH region[16,17] (Fig. 1).

The effects of several conditions on the malic enzyme reverse reaction were investigated. The effects of the NAD^+ concentration on the malic enzyme reverse reaction are shown in Fig. 3. Under concentrations of 0.5 and 1.0 mM NAD^+ in the reaction mixture, 39 to

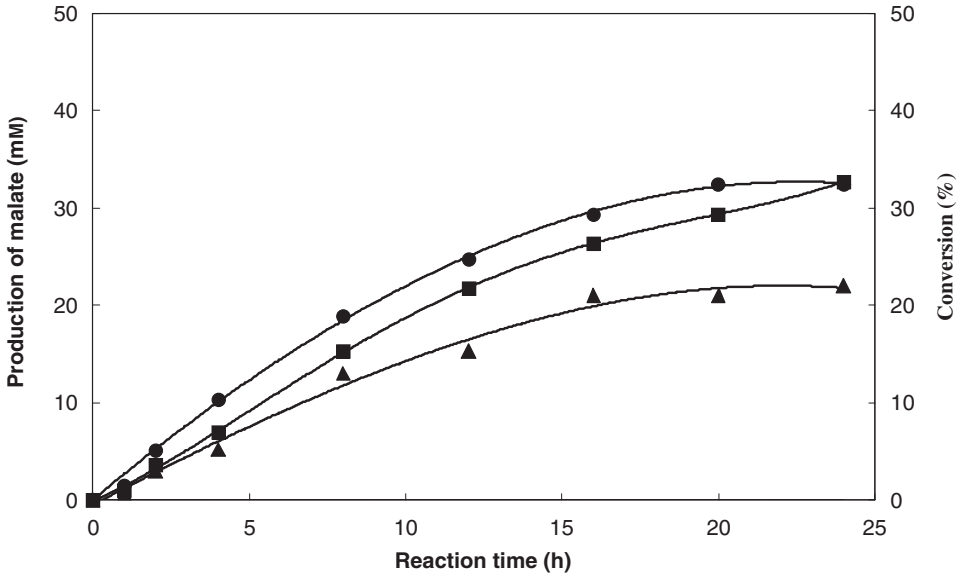

Fig. 4. Malic Enzyme Reverse Reaction System with Coenzyme Regeneration Using Alg-NAD^+.

The reaction was performed under standard conditions, except for the indicated Alg-NAD^+. The concentrations of other components were the same as for the standard reaction mixture. Symbols: ●, 1.0 mM Alg-NAD^+; ■, 0.5 mM Alg-NAD^+; ▲, 0.1 mM NAD^+. The concentration of Alg-NAD^+ indicates an equimolar concentration of free NAD^+.

40 mM L-malic acid was produced.

When a reaction mixture containing 100 mM pyruvic acid and 0.5 mM NAD^+ was used in the malic enzyme reverse reaction, L-malic acid production was 38 mM after 24 h of incubation (data not shown). The ratio of HCO_3^- and pyruvic acid to L-malic acid was about 38%. On the other hand, the synthesis of L-malic acid combined with malic enzyme reverse reaction and photochemical coenzyme regeneration has been reported.[5,18] The ratio of HCO_3^- and pyruvic acid to L-malic acid was from 1.1 to 6.6%. Using 100 mM pyruvic acid and 0.5 mM NAD^+ in the reaction mixture was suitable for the production of L-malic acid.

Reverse reaction of malic enzyme with Alg-NAD^+ as coenzyme

In a previous study, we successfully prepared Alg-NAD^+ coupling amino groups of NAD^+ and the carboxy group of alginic acid with water soluble carbodiimide.[11] Immobilization of the coenzyme as well as the enzyme is necessary for continuous or repeated use. We also investigated the reverse reaction of malic enzyme with coenzyme regeneration using Alg-NAD^+ as a coenzyme. As shown in Fig. 4, both cases of Alg-NAD^+ concentration (equimolar of 0.5 and 1.0 mM free NAD^+) produced L-malic acid, accumulating to about 33 mM. Alg-NAD^+ is also useful in L-malic acid production in the malic enzyme reverse reaction system. We have found that the NAD^+ moieties on Alg-NAD^+ work as an electron transfer carrier in the conjugated two oxidoreductase reaction.[11] In the reverse reaction of malic enzyme using the coenzyme regeneration with G-6-PDH, Alg-NAD^+ also showed improvement in the reaction at 100 mM pyruvic acid and 0.1 mM coenzyme concentration in the reaction mixture; the NAD cycling number (moles of L-malic acid produce per NAD^+ in the reaction mixture) was calculated to be 200. When 0.1 mM free NAD^+ was used in the reaction, the NAD cycling number was 76 (data not shown).

In conclusion, we carried out HCO_3^- fixation into pyruvic acid to produce L-malic acid in the reverse reaction of the malic enzyme of *P. diminura* IFO 13182 with coenzyme regeneration. Recently, several types of enzymatic HCO_3^- and CO_2 fixation using the dehydrogenase reverse reaction have been developed. For example, several series reactions of three enzymes (formate dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase) have been applied in CO_2 fixation into methanol.[19] Isocitrate dehydrogenase[20,21] and malic enzyme[3] can also be applied in carboxylation. However, in these cases, CO_2 fixed from 0.007–1.1 mM of the products. In contrast, the present study indicates 40 mM $KHCO_3$ fixation into L-malic acid, which is about 300 times more efficient than in other studies. This improvement of the conjugated coenzyme regeneration system warrants further investigation and refinement. Since G-6-P is expensive and unstable, it is not suitable for practical use. We also developed a modified electrode using a viologen derivative as mediator and Alg-NAD(P)$^+$ immobilized on the electrode surface for efficient NADH regeneration. Enzyme reaction conjugated with electrochemical coenzyme regeneration has been accomplished.[22] HCO_3^- fixation into pyruvic acid combined with electrochemical regeneration of coenzyme is now under study.

Acknowledgments

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