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Colorimetric Enzymatic Assay of L-Malic Acid Using Dehydrogenase from Baker's Yeast

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Summary

A colorimetric method has been developed and optimized to measure L-malic acid in samples of fruit juices and wine. This method is based on oxidation of the analyte, catalyzed by malate dehydrogenase (MDH) from dry baker's yeast, and in combination with the reduction of a tetrazolium salt (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). In the present study, the method exhibited sensitivity in the range of 500––4000 μM of L-malic acid in the reaction cuvette, with the lower detection limit of $6.7 \cdot 10^{-2}$ g/L, the upper limit of $53.6 \cdot 10^{-2}$ g/L and a maximum standard deviation of only 2.5 % for the analyzed samples. The MDH activity from baker's yeast was also optimized, the enzyme showed a high stability at pH=8.0–9.0 and the activity was maintained completely at temperatures up to 40 °C for 1 hour. The results show that the colorimetric method using enzymatic preparations from dry baker's yeast is a simple and low-cost method with possibility of wide application.

Key words: colorimetric assay of L-malic acid, fruit juices, wine, malate dehydrogenase (MDH), baker's yeast

Introduction

Organic acids are commonly present in food, beverages, pharmaceuticals and a variety of compounds of analytical interest. Carboxylic acids in grape juice and beverages are often separated to eliminate interference from other components, and determined by chromatographic methods (*1*). However, the instruments required are expensive and not available in every laboratory (*2*). A great number of other methods of L-malic acid analysis are reported in the literature (*3–5*) and the enzymatic methods are advantageous in terms of selectivity and sensitivity (*3*). L-Malic acid is one of the major organic acids found in fruits (*6*) and the most important organic acid present in wine (*7*), affecting the sensory characteristics (taste and flavour) of wines and fruit juices. Its content is associated with microbial changes during aging and handling of wine (*8*), or adulteration of fruit juice (*4*). It is also present in other products such as vinegar (*9*), coffee (*10*) and vegetables like potato (*11*) and tomato (*12*).

The determination of L-malic acid in food samples by commercial 'kits' based on enzymatic assay has become rather expensive due to the need for a large number of purification stages, as well as the low yield usually associated with the purification process. Dehydrogenases have been widely used in the enzymatic assays of diverse compounds of industrial interest, including L-malic acid. These enzymes require nicotinamide adenine

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dinucleotide (NAD^+), and the simplest way to follow the reactions catalyzed by them is to measure the formation or consumption of the reduced form of the nucleotide (NADH). Malate dehydrogenase (MDH; L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) isoenzymes are located in different cellular compartments and fulfill important functions in intermediate metabolism. In *Saccharomyces cerevisiae*, three MDH genes, MDH1, MDH2 and MDH3, encoding mitochondrial, cytosolic and peroxisomal variants respectively, have been identified (*13*), which catalyze the interconversion of malate plus NAD^+ to oxaloacetate plus NADH.

A simple plate assay has been developed to screen microorganisms for L-malic acid production. Acid-producing organisms were identified, after microbial colony growth on solid media, by the appearance around the colony of a dark halo of formazan produced by reduction of a tetrazolium salt (*14*). This reaction has proved very useful in a great number of enzymatic assays (*15–18*). L-Malic acid is converted to oxaloacetate by malate dehydrogenase, and the formed NADH transfers its hydrogen to the phenazine methosulphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (PMS-MTT) system to produce the purple-coloured MTT formazan by the reduction of MTT. Since the MTT colorimetric technique was developed (*19*), the MTT assay has been widely used to measure the toxicity of various substances in cell cultures (*20*). MTT enters the cells by endocytosis and is reduced to formazan, which can be measured spectrophotometrically, by NADH reductase and other enzymes (*21*). Dehydrogenases, using NADH or NADPH as coenzymes, can convert the yellow form of the MTT salt to insoluble purple formazan crystals (*22*).

In the present paper a colorimetric method has been developed for determining L-malic acid in fruit juices (apricot, apple, banana, pear, papaya, orange) and wine, based on the oxidation reaction catalyzed by malate dehydrogenase (MDH) using dry baker's yeast as the source of enzyme and the reduction of a tetrazolium salt. The optimization of the activity of the MDH from baker's yeast is described, and the pH and temperature stability have also been determined.

Materials and Methods

Microorganism

Dry baker's yeast used in this study was obtained from AB Brasil Indústria e Comércio de Alimentos Ltda (Pederneiras, SP, Brazil).

Samples of juices and wine

Apricot (*Prunus armeniaca*), apple (*Malus* sp.), banana (*Musa* sp.), pear (*Pyrus* sp.), papaya (*Carica papaya* L.) and orange (*Citrus* sp.) were purchased from local markets. The juice of each fruit was prepared by homogenizing 13 g of the fruit in 100 mL of water in an electric liquidizer and it was clarified by centrifugation (12000×*g*, 20 min). Samples of Estate sweet white wine (Brazil) and fruit juices were used in the determination of L-malic acid.

Cell disruption and crude cell extract preparation for enzymatic assay

The cells (100 mg) were disrupted, as described by Gattás and Peres (*23*), in a 5-mL screw-capped tube (flat bottom) containing 1 mL of 2 mM sodium citrate buffer (pH=6.2) with 2 mM β-mercaptoethanol, and 1.5 g of glass beads (425–600 μm). The cells were disrupted by six periods of 30-second vortexing at intervals, and the tubes were kept in an ice bath during the resting periods. The supernatant separated by centrifugation (10 min at 12000 rpm in Eppendorf 5415 R centrifuge) was used as the source of MDH in L-malate assays.

Malate dehydrogenase assay

An enzymatic procedure for the assay of lactate dehydrogenase described in the literature (*24*) was adapted and optimized for the malate dehydrogenase assay. Background reductions were eliminated from the assay by using blank cuvettes containing all the constituents of the reaction, except the malic acid (substrate of the enzymatic reaction), which was replaced by water. The colour developed was measured at the test wavelength of 570 nm using a reference wavelength of 655 nm. The assay measures the production of the reduced derivative (formazan) of an artificial electron acceptor, MTT. A molar absorption coefficient of $8.15 \cdot 10^3$ M/cm for the MTT formazan was used to calculate the enzyme activity (*25*). One unit of enzyme activity (U) caused the reduction of 1 μmol of MTT per min. MDH activity was expressed as mU/mg protein.

Effects of pH on malate dehydrogenase activity and stability

The optimum pH for MDH activity was determined by carrying out assays at 37 °C, at various pH values, between 6.0 and 10.0 (phosphate buffer, pH=6.0–7.0; Tris-HCl buffer, pH=8.0; glycine/NaOH buffer, pH= 9.0–10.0). The pH stability of the enzyme was determined by measuring the enzymatic activity under standard enzyme assay conditions after incubating the enzyme at various pH for 8 days at 4 °C in the following 0.1 M buffer systems: for pH=4.0–5.0, acetate buffer; for pH=6.0–8.0, phosphate buffer; and for pH=9.0–10.0, Tris buffer.

Effects of temperature on malate dehydrogenase activity and thermal stability

The optimum temperature for MDH activity was determined by assaying the activity at pH=9.7 in 0.1 M glycine/NaOH buffer, at temperatures of 30, 37, 40, 45, 50, 55 and 60 °C. The thermal stability of the enzyme was determined by measuring the activity under standard enzyme assay conditions after incubating the enzyme solution for 1 hour at various temperatures: 0, 30, 40, 50, 53, 56, 60 and 70 °C.

L-malic acid assay

The L-malic acid was measured in fruit juices and wine by measuring the reduced derivatives (formazan)

of an artificial electron acceptor (MTT). The following reaction mixture was used: 100 μL of 25 mg/mL of NAD^+, 100 μL of 600 μM MTT dissolved in phosphate buffered saline (PBS, pH=7.4), 1 μL of 100 μM PMS dissolved in PBS, 40 μL of sample, 759 μL of 0.1 M glycine/NaOH buffer (pH=9.7). A volume of 0.30 mL of aliquot was taken from the mixture and added to 0.26 mL of 0.1 M glycine/NaOH buffer (pH=9.7) and 40 μL of the enzyme preparation (a total of around 32 mU/mg protein) to start the reaction. This mixture was incubated for 30 min at 40 °C, and the reaction was interrupted by the addition of 0.6 mL of a solution containing 50 % dimethylformamide and 20 % sodium dodecyl sulphate (DMF/SDS). The colour developed was measured at a test wavelength of 570 nm using a reference wavelength of 655 nm.

Protein assay

Total protein was assayed according to the method of Lowry, modified by Layne (*26*), using bovine serum albumin as the standard protein. The levels of total protein in crude cell extracts were 32–35 mg/mL.

Results and Discussion

Optimization of the malate dehydrogenase assay

The catalytic activities vary in response to the concentrations of various substances. Therefore, we sought optimum concentrations of MTT, PMS and NAD^+ in the reaction mixture. The finally chosen concentrations were as follows: 600 mM of MTT, 2.5 mg/mL of NAD^+ and 100 μM of PMS. The effects of adding metals on malate dehydrogenase activity were also tested, but no activator was found (data not shown). The linear range of the assay was found adjusting the reaction time and the dilution of the enzyme, and maximal reaction velocity was determined (Figs. 1 and 2). We found that a 30-minute reaction, up to 15-fold dilution of the enzyme and a substrate concentration of 0.4 g/L were the best conditions for determination of the MDH activity.

Effects of pH and temperature on malate dehydrogenase activity

The effects of pH (6.0 to 10.0) and temperature (30 to 60 °C) on the activity of the MDH can be observed in Fig. 3. The highest activity found was at pH=9.7 (optimum pH) and the optimum temperature was 40 °C. The optimum pH (*27–29*) and temperature (*28*) for the assay system were established, and the values obtained in this study are similar to the literature values.

Thus, the following reaction mixture was chosen: 100 μL of 25 mg/mL NAD^+, 100 μL of 600 μM MTT dissolved in phosphate buffered saline (PBS, pH=7.4), 1 μL of 100 μM PMS dissolved in PBS, 40 μL of 0.4 g/L of L-malic acid, 759 μL of 0.1 M glycine/NaOH buffer (pH=9.7). A volume of 0.30 mL of this mixture was added to 0.26 mL of 0.1 M glycine/NaOH buffer (pH=9.7), and 40 μL of the enzyme preparation (diluted 15-fold) to start the reaction, which proceeded for 30 min at 40 °C. The reaction was interrupted by adding 0.6 mL of a solution containing 50 % of dimethylformamide and 20 % sodium dodecyl sulphate (DMF/SDS).

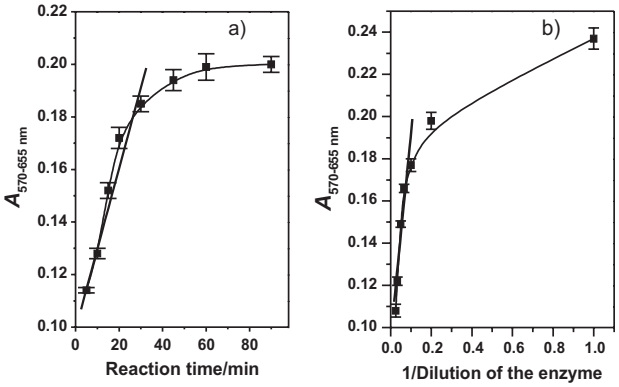

Fig. 1. Absorbance values obtained for a standard solution of L-malic acid at various (a) reaction time and (b) dilutions of the cell extract. Each point represents the average of three determinations

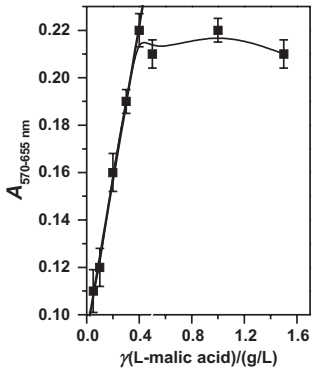

Fig. 2. Absorbance values obtained for various concentrations of L-malic acid. Each point represents the average of three determinations

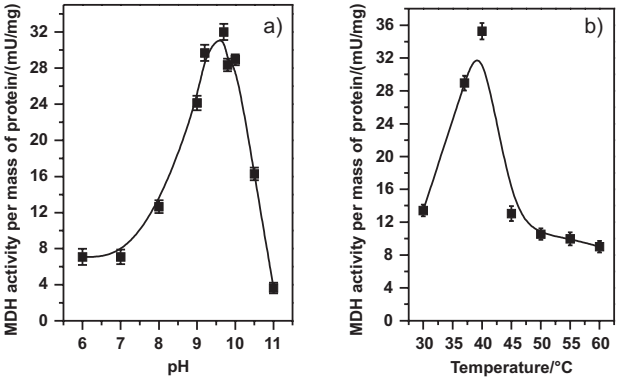

Fig. 3. Influence of (a) pH and (b) temperature on malate dehydrogenase activity. The MDH was assayed in the pH range of 6.0–10.0, using various buffers (phosphate buffer, pH=6.0–7.0; Tris-HCl buffer, pH=8.0; glycine/NaOH buffer, pH=9.0–10.0). The optimum temperature of malate dehydrogenase activity was determined by assaying enzyme activity at pH=9.7 in 0.1 M of glycine/NaOH buffer at 30, 37, 40, 45, 50, 55 and 60 °C. Each point represents the average of two determinations

Stability of malate dehydrogenase activity at various pH and temperatures

Thermal stability of MDH activity at optimum pH and a range of temperatures was assayed. For the stability at pH from 4 to 10, the activity was determined after 8 days at 4 °C, as shown in Fig. 4a. Fig. 4b shows the activity after the incubation of the enzyme for 1 h at temperatures from 0 to 70 °C. The results obtained showed

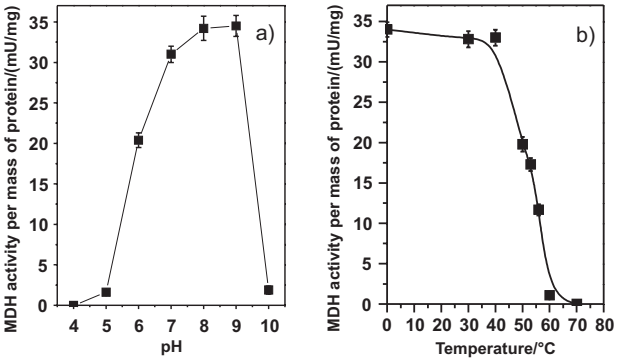

Fig. 4. Stability of malate dehydrogenase activity at various (a) pH and (b) temperatures measured at the optimum pH and temperature. MDH was stored for 8 days at 4 °C in buffers at various pH. The thermal stability of the enzyme was determined by incubating the enzyme solution for 1 h from 0 to 70 °C. Each point represents the average of two determinations

that the enzyme was most stable at pH=8.0–9.0 and temperatures up to 40 °C, above which its activity gradually decreased. These results coincided with the data in the literature for cytoplasmic and mitochondrial isoenzymes, which were stable for more than 48 h at 20 °C and 2 weeks at 4 °C (*28*), and for mitochondrial MDH from isolated rat liver mitochondria, which showed a 20–45 % loss of MDH activity from 60–120 min at 37 °C, but more than 90 % activity within 30 min (*30*).

Analytical curve for the assay of L-malic acid

The method was sensitive in the range of 500–4000 μM of L-malic acid in the reaction mixture, as observed in Fig. 5, with the lower detection limit of $6.7 \cdot 10^{-2}$ g/L and the upper limit of $53.6 \cdot 10^{-2}$ g/L, and a linear correlation coefficient of 0.998. This sensitivity was compared with the UV method (*31*) using the same enzymatic preparation, which gave a lower detection limit of $8.04 \cdot 10^{-3}$ and an upper limit of $16.08 \cdot 10^{-3}$ g/L, showing that the UV method was more sensitive. Nevertheless, the results obtained here are perfectly compatible with the UV method, and there is the added advantage that they can be quantified in a single colorimeter, simply and rapidly.

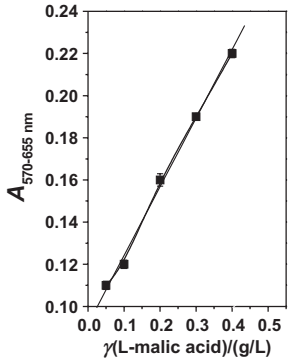

Fig. 5. Analytical curve for L-malic acid. Each point represents the average of two determinations

L-Malic acid determination

In this study, the concentration of L-malic acid was measured in samples of wine and juices, and a maximum standard deviation of only 2.5 % was obtained, as can be observed in Table 1. The L-malic acid content found was similar to that described in the literature for some samples of fruit juices (*4*) and wine (*3,7*) and similar standard deviations were obtained by using proton nuclear magnetic resonance spectroscopy for the determination of L-malic acid (*15*).

Table 1. Results obtained for the determination of L-malic acid in fruit juice and wine

Samples	γ(L-malic acid) / g/L
Apricot juice	0.79±0.02
Apple juice	3.99±0.03
Banana juice	0.64±0.01
Pear juice	1.28±0.02
Papaya juice	5.10±0.05
Orange juice	2.15±0.01
Sweet white wine (Estate)	2.39±0.04

Conclusions

We estimated that a 30-minute reaction, up to 15--fold dilution of the extracted enzyme and a concentration of the substrate of the 0.4 g/L are the best conditions for the determination of the MDH activity. The highest activity was found at pH=9.7 (pH optimum) and the optimum temperature was 40 °C. The enzyme was highly stable at pH=8.0–9.0 and the thermal stability was constant up to 40 °C, after which the activity gradually decreased. The method was sensitive in the range of 500–4000 μM of L-malic acid in the reaction cuvette, and the colour was proportional to the amount of L-malic acid present in the sample, with relatively small errors. The method developed here is very precise and linear and could readily be applied to analyses of real food samples, being a simple methodology of low cost.

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