FLOW INJECTION DETERMINATION OF MALIC ACID AND DOPA USING AN APPLE TISSUE REACTOR

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ABSTRACT

A reactor containing apple fruit tissue was used for flow injection measurements of both L-malic acid and L-Dopa. Malic acid was determined by the fluorescence of NADPH generated by the action of malate dehydrogenase in the tissue. Dopa was determined from the red color generated by polyphenol oxidase in the same tissue. The linear measurement ranges were 0.5-20 mM and 5-100 μM for malic acid and Dopa, respectively.

INTRODUCTION

Plant and animal tissues contain many kinds of enzymes, and the analytical use of tissues instead of purified enzymes has been previously investigated[1-3]. The biggest advantage of using tissues is lower cost compared to that of pure enzymes. However, it must be pointed out that the selectivity of such methods is limited due to the many enzymes present in tissue. While Rechnitz and Ho[2] suggested the possibility of multiple substrate analysis using one biocatalytic material as a source of several different enzymes, there has as yet been no published work which turns the enzyme multiplicity of tissues to advantage. This report presents the analysis of two substrates using one tissue reactor. Each analysis is based on different enzyme reactions. Photometric detections were applied to improve the selectivity.

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The substrates measured in this report are L-malic acid and L-Dopa (L-3,4-dihydroxyphenylalanine). Malic acid is one of the components of the TCA cycle, abundant in the tissue of apple and known to be an important indicator of the quality of wine and fruit. Dopa is the precursor of dopamine (an important neurotransmitter), and therapeutically effective for Parkinson's disease[4]. Moreover, it has recently been found that Dopa, rich in velbetbean, shows allelopathic activity to reduce weed population[5].

The enzymes in the tissue used are malate dehydrogenase (EC 1.1.1.37)[6] and polyphenol oxidase (EC 1.10.3.1)[7,8] for malic acid and Dopa analysis, respectively. The malate dehydrogenase reaction is:

$$\text{L-malate} + NAD^+ = \text{oxaloacetate} + NADH \qquad (1)$$

The NADH produced by this reaction was measured fluorometrically. Dopa is oxidized by polyphenol oxidase to produce a red compound called dopachrome. This red color can be measured colorimetrically. For flow injection analysis, the tissue was held in a reactor with fluorescent and photometric detection.

EXPERIMENTAL

Reagents

NAD^+ (free acid, 99%), L-Dopa, and L-malic acid were bought from Sigma (St. Louis, MO.). The glass beads used were 80-120 mesh (Wilkens Instrument & Research Inc., Walnut Creek, CA). 1 mM of Dopa stock solution was prepared daily.

Preparation of the tissue reactor

Fresh ripe apples (Red Delicious) were bought from a local market. The apples were cut into 2 mm cubes with a razor blade. About 150 mg of the cubes were packed into a glass column (3 mm inner diameter / 35 mm long) with 0.1 M phosphate buffer (pH 6.0). The spaces between the apple cubes were filled with the glass beads. The same buffer was passed through the reactor at a flow rate of 1 $ml\ min^{-1}$ more than 30 min before use to reduce the level of malate, polyphenols, ascorbate etc. present in the tissue.

Apparatus

The apparatus used was almost the same as described previously[9] except for the additional use of a spectrophotometric detector (JASCO, Model 875-UV, Tokyo, Japan). A carrier buffer was pumped by a HPLC pump (Shimadzu, LC-600, Kyoto, Japan) and passed through a Lo-pulse damper (Science System Inc., Model LP-21). The sample was injected using a injector (Rheodyne 7125) with a 20 μl sampling loop. The carrier buffer passed through the reactor and a detector, fluorescent (JASCO, Model 821-FP) or spectrophotometric, depending on what was being analyzed. Peak height was recorded with a chromato-integrator (Hitach, Model D-2500, Tokyo, Japan).

Measurement of malic acid and Dopa

Malic acid was measured under the following conditions. The carrier buffer used was 0.1 M pyrophosphate buffer (pH 8.0) and its flow rate was 0.2 $ml\ min^{-1}$. The temperature of the reactor was maintained at 25 °C by water flow from the thermostat. Sample solution was mixed with the same volume of NAD^+ solution (5 mM) and then the mixture was injected immediately into the reactor. The fluorescence of NADH was detected by the fluorescent detector at excitation and emission wavelengths of 360 and 460 nm, respectively.

Dopa was measured using 0.1 M phosphate buffer (pH 6.0) as the carrier. The samples were injected directly into the reactor and the absorbance of dopachrome was monitored by the spectrophotometric detector at a wavelength of 475 nm. The flow rate of the carrier buffer and temperature of the reactor were the same as those used for malic acid.

RESULTS AND DISCUSSION

Malic acid

The tissue of the apple was enough strong to be used as a rector at the pressure below 5 $kgf\ cm^{-1}$. In our conditions the pressure was usually about 2 $kgf\ cm^{-1}$, no problem was happened arising from the mechanical softness of the tissue.

The influence of pH was investigated with 0.1 M pyrophosphate buffers between pH 7.0-9.0. In this region, peak height became higher with increasing pH (Fig. 1). This effect is similar to the pH dependence of immobilized malate dehydrogenase[10]. While the signal is higher in alkaline cariers, pH

8.0 was chosen for further experiments, because tissue damage is likely to be more serious at higher pH.

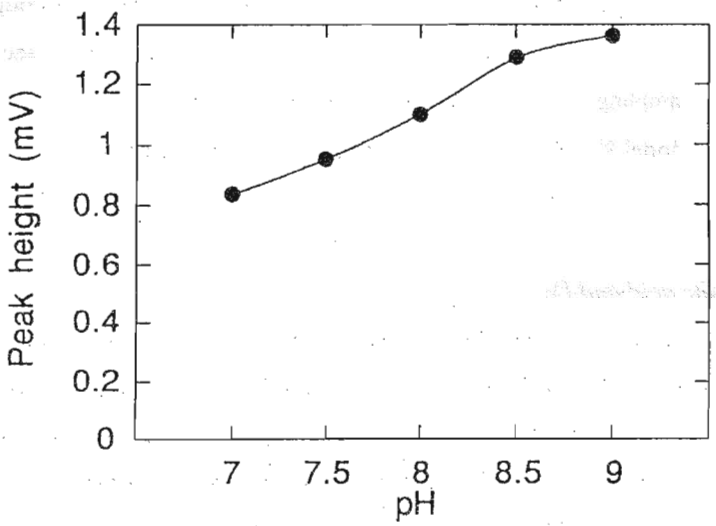

FIG. 1 Effect of carrier pH on the fluorescent intensity to 20 mM malic acid. This experiment was performed with 0.1 M pyrophosphate buffer as carrier at a flow rate of 0.2 m*l* min^{-1}. The temperature of the reactor was 25 °C.

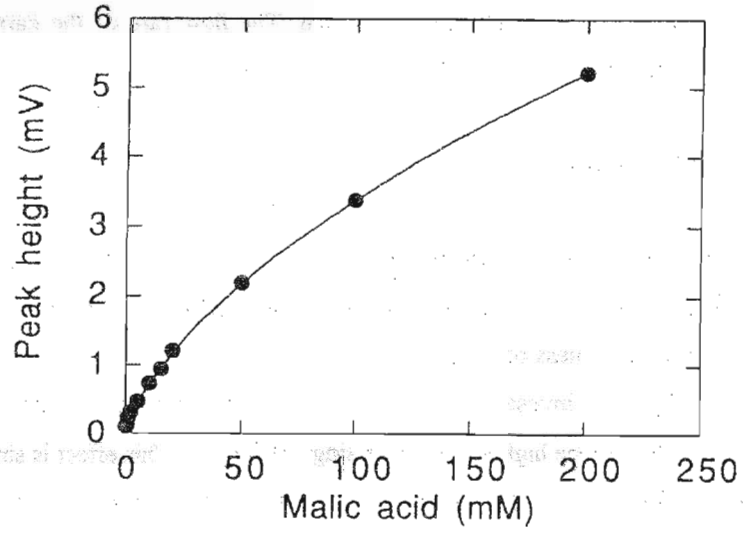

FIG. 2 Calibration curve for malic acid.

The temperature of the reactor was fixed at 25 °C, because above 30 °C the decrease in enzyme activity becomes significant. The signal is attenuated when flow rate is increased. A flow rate of 0.2 $ml\ min^{-1}$ was selected as a compromise between sensitivity and sample throughput. Under these conditions sample throughput was 15 samples hr^{-1}.

The calibration curve for malate is shown in Fig. 2. The detection limit was 0.5 mM and an almost linear relationship (r = 0.992, n = 9) was observed between 0 and 20 mM of malic acid. The relative standard deviation at 20 mM of malic acid was 2% (n=6). The sensitivity of this system is not as high as that using immobilized enzyme[10] because of the lower activity of the enzyme in the tissue.

Plant tissue contains many kinds of dehydrogenases. The selectivity of malate detection might be decreased by the presence of other enzymes. Several other compounds, e.g. citrate, succinate, lactate, pyruvate, 2-ketoglutarate and glutamate, were also tested. The peak heights for these compounds were less than 2% of the same concentration of malate, except for pyruvate (14.5 %) and glutamate (3.5 %).

The existence of a malic enzyme (EC 1.1.1.39) in apples has been reported[11], which requires $NADP^+$ instead of NAD^+ and the pH optimum is around 6.5-7.5. We could not observe signals strong enough to use for malic acid detection when $NADP^+$ was used instead of NAD^+. Therefore, the signal measured in all our experiments reported above is due to NAD^+ dependent malate dehydrogenase, and not due to $NADP^+$ dependent malic enzyme.

Dopa

Dopa is unstable under alkaline conditions, and below pH 6 the signal becomes smaller with decrease of the carrier buffer pH. Therefore pH 6.0 was chosen for further experiments. The other experimental conditions were the same as those for malic acid.

A calibration graph is shown in Fig. 3. The peak heights were linear between 0 and 100μM of Dopa (r=0.999, n=8). The peak was clearly detectable above 5 μM and the relative standard deviation at 50μM of Dopa was 2% (n=6).

Several reports[12-17] have already been published on the polyphenol analysis using a combination of plant tissues as a source of polyphenol oxidase and electrochemical detection.

However, our method is capable of measuring Dopa concentrations at 5 to 200 times lower levels by using photometric detection. Moreover, the peak heights of dopamine, chlorogenic acid and tyrosine were less than 30 % of that of the same concentration of Dopa as we selected suitable wavelength, whereas dopamine usually shows higher response than Dopa in electrochemical detection[14,15]. Thus, the proposed method shows both higher sensitivity and selectivity to Dopa than the electrochemical methods.

It was easy to change the detection mode from malic acid to Dopa and vice versa, since only changing the buffer and connecting the appropriate detector is required. The reactor was stored at 4 °C filled with pH 6 phosphate buffer when not in use. It retained 70 % of its initial activity to malic acid, but only 20 % of its activity to Dopa after one week of storage. The low cost and ease of preparation make the preparation of a new reactor feasible for each analysis day.

In this report the analysis of two substrates using one reactor was demonstrated. We showed separate analysis of malic acid and Dopa, but simultaneous analysis of these two substrates by using one reactor might be possible at the sacrifice of the sensitivity. Tissue reactors have the potential to simplify multi-component analysis system, since they can act as several different enzyme reactors. Many components must be analyzed to evaluate food quality. Several food components might be measured simultaneously by using one tissue reactor if suitable tissue could be selected.

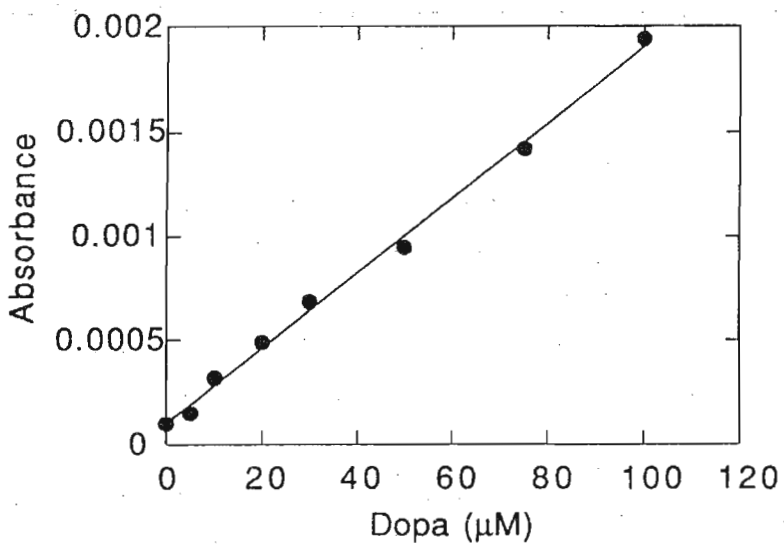

FIG. 3 Calibration graph for Dopa. This experiment was performed with phosphate buffer (pH 6.0) as the carrier at a flow rate of 0.2 m/ min^{-1} at 25 °C. The absorbance was monitored at a wavelength of 475 nm.

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