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Note

Rapid assay for oxalate oxidase using reversed-phase high-performance liquid chromatography

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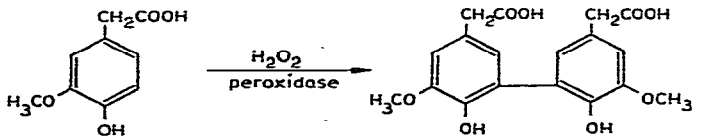
Oxalate oxidase from barley seedlings (oxalate: oxygen oxidoreductase, E.C. 1.2.3.4) has been recently purified and used[1] for the assay of oxalic acid in biological fluids. The activity of this enzyme is usually measured by the colorimetric determination of hydrogen peroxide enzymatically produced according to

$$(COOH)_2 + O_2 \xrightarrow{\text{oxalate oxidase}} 2\,CO_2 + H_2O_2$$

$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2\,H_2O + D$$

where DH_2 are different chromogenic systems, such as 3,5-dichloro-2-hydroxybenzenesulphonic acid–4-aminophenazone[2], 3-methyl-2-benzothiazolinone hydrazone hydrochloride–N,N-dimethylaniline[1] and *o*-dianisidine[3].

High-performance liquid chromatography (HPLC) represents a useful alternative to the colorimetric methods for enzyme assays, because of its selectivity, sensitivity and reliable quantitation[4–6]. Therefore we have developed an HPLC method for the assay of oxalate oxidase, which measures the formation of 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (II) from 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, I)[7]:



I II

This analytical approach, based upon reversed-phase HPLC, is of wide utility, as demonstrated by its application to other oxidases in the presence of different chromogenic systems[8].

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EXPERIMENTAL

A Model 6000A solvent delivery system, Model U6K universal injector, Model 440 ultraviolet detector and Model 730 data module, all from Waters Assoc. (Milford, MA, U.S.A.), were used in all determinations.

A prepacked, stainless-steel column of μBondapak C_{18} (30 cm × 4.0 mm I.D., particle size 10 μm) was obtained from Waters Assoc. A precolumn of Bondapak C_{18} Corasil (2 cm × 4 mm I.D.) was used.

Reagents

Homovanillic acid (I), nicotinic acid, tris(hydroxymethyl)aminomethane (Tris) and peroxidase (Type II) were purchased from Sigma (St. Louis, MO, U.S.A.); reagent-grade diammonium hydrogen phosphate and succinic acid were from E. Merck (Darmstadt, G.F.R.), and hydrogen peroxide was from Montedison–Carlo Erba (Milan, Italy).

Water used for the preparation of eluents and solutions was distilled, deionized and filtered through Millipore membrane filters, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.). Methanol was of HPLC (Chromasolv; Riedel-De Haën, Hannover, G.F.R.).

A homovanillic acid solution (2.4 mg/ml) was prepared by dissolving the acid in water. The buffer 0.1 *M* Tris, was obtained by adding the amine (1.211 g) to 100 ml of water and adjusting the pH to 7.8 with 0.1 *M* HCl. A 10 m*M* hydrogen peroxide solution and an aqueous peroxidase solution (1.15 mg/ml) were employed. Nicotinic acid, as internal standard (IS), was dissolved in water to give a concentration of 50 μg/ml. This solution was used within 24 h.

Oxalate oxidase from barley seedlings

Barley seed (*Hordeum vulgare* L. var. distichon Alefeld) was supplied by Malteria Adriatica (Mestre, Italy) and seedlings were used as enzyme source after 8 days at 24°C.

Barley seedlings (1 kg) were homogenized and extracted with 1 l of water. The extract was heated at 80°C for 3 min at 4°C. To the resulting supernatant was added solid ammonium sulphate to give a 60% saturated solution which was allowed to stand for 1 h at 0°C. After centrifugation at 100 *g* for 15 min at 4°C, the precipitate was collected and dissolved in distilled water. The enzyme solution was dialyzed overnight against distilled water at 4°C and then lyophilized. Yield: 100 mg. Specific activity: 0.03–0.1 units per mg of protein. K_M (Michaelis constant, from the Lineweaver–Burk plot) = $4 \cdot 10^{-4}$ *M*.

Chromatographic conditions

Samples were eluted isocratically using 0.01 *M* diammonium hydrogen phosphate buffer–methanol (68:32). After mixing, the buffer was brought to pH 4.9 with 20% phosphoric acid and filtered through a 0.45-μm membrane filter (Type HA, Millipore).

The flow-rate was 1.5 ml/min, and the temperature was ambient in all cases. Chromatographic peaks were monitored at 254 nm. The range setting was fixed at 0.005 a.u.f.s.

Calibration curve

A 50-μl volume of a solution of I and 25 μl of peroxidase solution were added to 1.35 ml of 0.1 *M* Tris buffer, spiked with hydrogen peroxide in the range of 0.0–

0.18 μmol. Then 0.25 ml of nicotinic acid solution were added. Replicate injections of 5 μl were made for each sample.

Oxalate oxidase activity

A 100-μl volume of enzyme solution (10 mg/ml) was added to 100 μl of oxalic acid solution (11 m*M* in 50 m*M* succinate buffer, pH 3.6). The resulting mixture was incubated at 37°C for different times. The reaction was stopped by the addition of 1.35 ml of 0.1 *M* Tris, 50 μl of the solution of I and 25 μl of peroxidase solution. After adding 0.25 ml of nicotinic acid solution, the solution was filtered through a 0.45-μm filter (Millipore), and 5 μl of the filtrate were injected into the chromatograph.

RESULTS AND DISCUSSION

For the determination of II (2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid) an isocratic elution mode of reversed-phase HPLC was used. The separation of a synthetic mixture of the reference compounds I, II and IS detected at 254 nm is shown in Fig. 1. The minimum detection limits were ≈30 ng for I, ≈30 ng for II and ≈5 ng for IS. A series of standard samples each containing 0–0.18 μmol hydrogen peroxide were prepared according to the procedure described under *Calibration curve*. Chromatographic results showed that the peak area ratio, II/IS, *versus* amount of hydrogen peroxide amount is linear up to 0.20 μmol.

The amount of the enzymatically liberated hydrogen peroxide can be obtained directly from

$$y = 0.11\,x + 0.189;\ r = 0.998$$

where y and x represent the μmol of hydrogen peroxide and the peak area ratio, respectively.

Prior to the chromatographic assay, the enzymatic reaction was stopped by raising the pH from 3.6 (optimum value for oxalate oxidase activity) to 7.8 and the samples were filtered to avoid column contamination.

The activity of oxalate oxidase was calculated from the amount of the en-

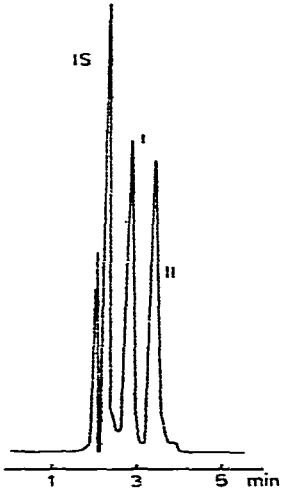

Fig. 1. High-performance liquid chromatogram of homovanillic acid (I), 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (II) and nicotinic acid (IS).

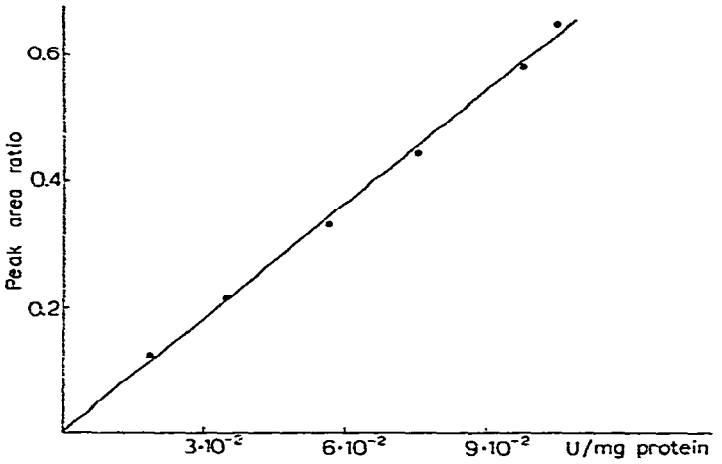

Fig. 2. Assay of oxalate oxidase activity.

zymatically produced hydrogen peroxide, using the following formula:

$$\text{Activity (U/mg protein)} = \frac{\mu\text{mol } H_2O_2}{\text{incubation time} \cdot \text{mg protein}}$$

As shown in Fig. 2, there is a good correlation of the amount of II produced in 5 min to oxalate oxidase activity, in the range of $2 \cdot 10^{-2}$–$1 \cdot 10^{-1}$ U/mg of oxalate oxidase.

CONCLUSIONS

The described reversed-phase HPLC determination of oxalate oxidase activity offers several advantages over colorimetric assays. The analysis time, after incubation, is short (4 min) and the separation is free from interference.

Moreover, the II produced might be measured flurometrically (λ_{ex} 315 nm; λ_{em} 425 nm), which makes the assay even more sensitive ($\approx$5 ng) than the UV detection.

Finally, this reversed-phase HPLC procedure can be successfully extended to the determination of small amounts of other oxidases by the use of synthetic substrates with UV or fluorometric absorption[8].

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