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Note

Quantitative thin-layer chromatographic assay of amino acid decarboxylase activity

ZORKA PROŠTENIK, BOŽENA ŠVIGIR-ŠTENGL and ZLATA ŠTEFANAC

Institute for Medical Research, Yugoslav Academy of Sciences and Arts, Moše Pijade 158, 41000 Zagreb (Yugoslavia)

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Activity assays for amino acid decarboxylase are based on the quantitative determination of one of the products formed, carbon dioxide or amine[1,2]. In order to avoid the low sensitivity of the manometric measurement and the need for labelled substrates for $^{14}CO_2$ determination[1–3], the assay of DOPA decarboxylase activity with continuous spectrophotometric monitoring of the increase of CO_2 concentration was developed.

Quantitative determinations of the amine released during the enzyme-catalyzed reaction incorporate repeated extractions with organic solvents followed by washing of the extract and final fluorimetric or spectrophotometric measurement. Moreover, isotopic procedures have been described, as well as determinations based on the biological activity of the relevant amine[1,2,4,5].

If high sensitivity is not critical, the separation and quantitative determination of amines can be performed by thin-layer chromatography in a less complex manner than when ion-exchange[6,7] or paper chromatography[8,9] is applied to the assay of amino acid decarboxylase. The high separation capacity of thin-layer chromatography combined with photodensitometric evaluation of chromatograms offers an efficient and convenient procedure suitable for following the course of enzymic reactions by taking, at regular intervals, very small aliquots for analysis. By this method, which avoids the use of an extraction procedure, commercially available enzymic preparations of histidine, lysine, tyrosine and glutamic acid decarboxylase have been characterized.

EXPERIMENTAL

Materials and equipment

DOPA decarboxylase (E.C. 4.1.1.26): crude preparations obtained from homogenates of rat liver or kidney, ox kidney or adrenal gland as well as rabbit adrenal gland were used[1]. L-3-(3,4-Dihydroxyphenyl)alanine (L-DOPA), puriss grade, and pyridoxal phosphate were products of Fluka (Buchs, Switzerland). Sodium acetate, 0.1 *M* solution: the addition of 0.05 % aqueous neutral red solution (1.1 ml to 25.0 ml of 0.1 *M* sodium acetate) raised the absorbance at 460 nm to a value of about 0.7. L-Histidine decarboxylase (E.C.4.1.1.22, *Clostridium welchii* V 3656) was obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A.; L-tyrosine decarboxylase (E.C. 4.1.1.25)

from Mann Labs., New York, N.Y., U.S.A.; L-lysine decarboxylase (E.C. 4.1.1.18, *Bacillus cadaveris* ATCC 9760), L-glutamic acid decarboxylase (E.C. 4.1.1.15, *Escherichia coli* ATCC II 246), L-lysine and L-glutamic acid from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.; L-tyrosine, histamine hydrochloride, tyramine hydrochloride and tryptamine (all puriss grade) from Fluka, Buchs, Switzerland; cadaverine and L-aminobutyric acid from Bender & Hobein, Zürich, Switzerland, and L-histidine hydrochloride monohydrate from Sigma, St. Louis, Mo., U.S.A.

Amino acid and amine solutions, as well as enzyme suspensions, were prepared in Sörensen's 0.01 *M* phosphate buffer of pH 7.46.

TLC plates with a 0.1-mm thick layer of Cellulose F were used (Kemika, Zagreb, Yugoslavia). The solvent system was absolute ethanol–25% ammonia solution (4:1)[10].

Amine spots were detected by immerging the air-dried plates in 0.3% ethanolic ninhydrin solution, drying in air and heating for 30 min at 120°. Scanning of the spots was performed with a Camag-T scanner (Camag, Müttenz, Switzerland) and the photodensity was measured with a Vis lamp (850) and secondary filter (No. 812) only.

Procedures

Continuous spectrophotometric assay of DOPA decarboxylase activity. Pre-incubation of enzyme preparations with pyridoxal phosphate before addition of substrate afforded slightly higher activities. For this reason, 0.3 ml of the crude preparation suspended in sodium acetate solution (20–50 mg/ml) was incubated with 0.2 ml of pyridoxal phosphate solution (0.1 mg/ml) for 1 h at 37°. The mixture was then transferred into a spectrophotometric cell and 2.0 ml of sodium acetate solution coloured with neutral red was added. One millilitre of the substrate solution in sodium acetate (5 mg/ml of L-DOPA) was injected with a syringe into the bottom of the cell followed immediately by time and absorbance readings. Instead of the substrate, sodium acetate solution was injected into the reference cell.

Thin-layer chromatographic separation and quantitation of amines. Calibration graphs were constructed with solutions containing 0.1–0.5 mg/ml of the amine in question (hydrochlorides were neutralized with sodium hydroxide) and enzyme at the same concentration that was used later for the enzymic reactions. The addition of enzyme ensured the compensation of the error caused by the amine possibly present in the enzyme preparation.

Decarboxylase activity assay. The enzymic decarboxylation was performed at pH 7.46 and 37°. At fixed time intervals, aliquots of 10 μl were taken and the increase in amine concentration was determined by thin-layer chromatography .

RESULTS AND DISCUSSION

For the assay of the DOPA decarboxylase of animal tissues with low activity, a system sensitive to small increases in carbon dioxide concentration was necessary. Neutral red solution in sodium acetate, showing changes in absorbance at 525 nm proportional to the increase of carbon dioxide concentration, seemed suitable. Continuous recording of the changes in absorbance with time reflected directly the course of the enzyme-catalyzed reaction. Sensitivity to pH changes regardless of origin represented a severe limitation to the applicability.

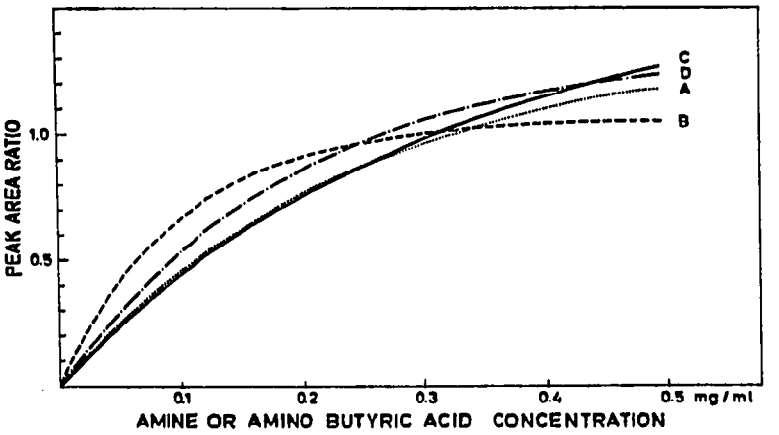

Fig. 1. Plots of the fitted function: A, calibration curve for tyramine (variance 0.011); B, calibration curve for histamine (variance 0.0016); C, calibration curve for cadaverine (variance 0.009); D, calibration curve for 4-aminobutyric acid (variance 0.0016). Amine was separated on Cellulose F layer, 0.1 mm thick, with solvent system absolute ethanol–25% ammonia solution (4:1)[10]. The air-dried chromatogram was dipped into a 0.3% ethanolic ninhydrin solution and the colour fully developed by heating the plate for 30 min at 120°. Scanning was performed at 405 nm.

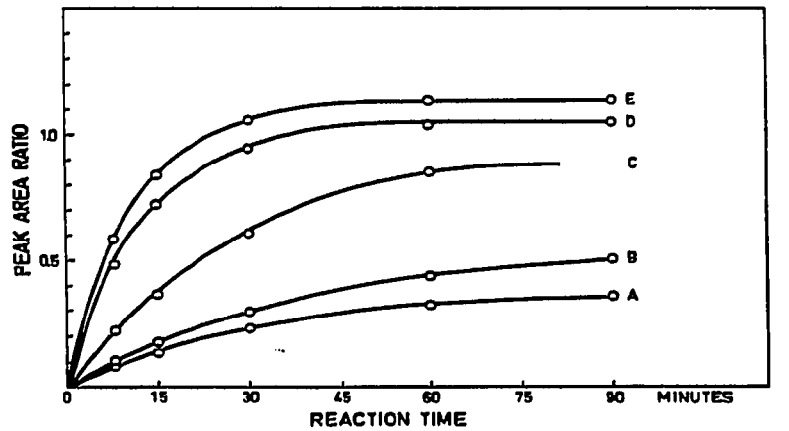

Fig. 2. Plots of the fitted function. Course of enzyme-catalyzed decarboxylation of tyrosine with different concentrations of enzyme preparation: A, 0.100 mg/ml (variance 0.0004); B, 0.125 mg/ml (variance 0.0006); C, 0.250 mg/ml (variance 0.00022); D, 0.400 mg/ml (variance 0.004); E, 0.500 mg/ml (variance 0.0015).

Thin-layer chromatographic assay showed no limitations in this respect. All enzyme–substrate systems tested could be assayed satisfactorily with results presented graphically. Uniform spreading of the colour on the spot area was achieved by dipping the chromatogram into the ninhydrin solution instead of spraying. Quantitative evaluation of peak areas was performed by the Monte Carlo method[11] with all peak area calculations reported representing the average of 3–5 determinations. The experimental variations were eliminated by running each time a standard of mean concentration and taking as the determining value the ratio of the peak area of the unknown to the peak area of the standard.

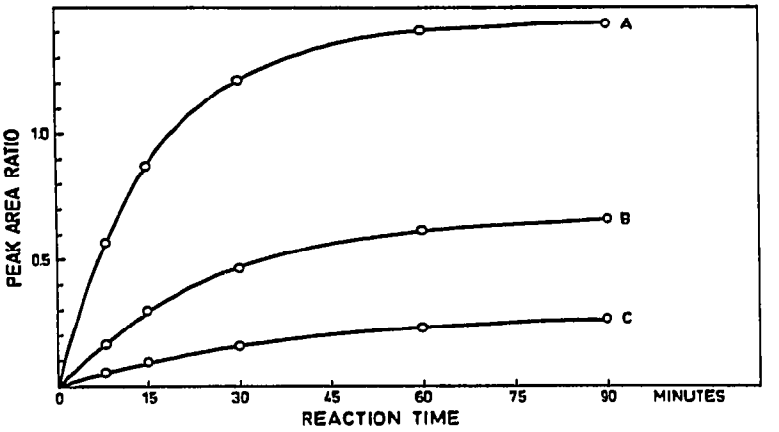

Fig. 3. Plots of the fitted function. Tyrosine decarboxylase activity assays performed with enzyme suspensions allowed to stand for different time intervals: A, 40 min (variance 0.0092); B, 130 min (variance 0.001); C, 220 min (variance 0.0011).

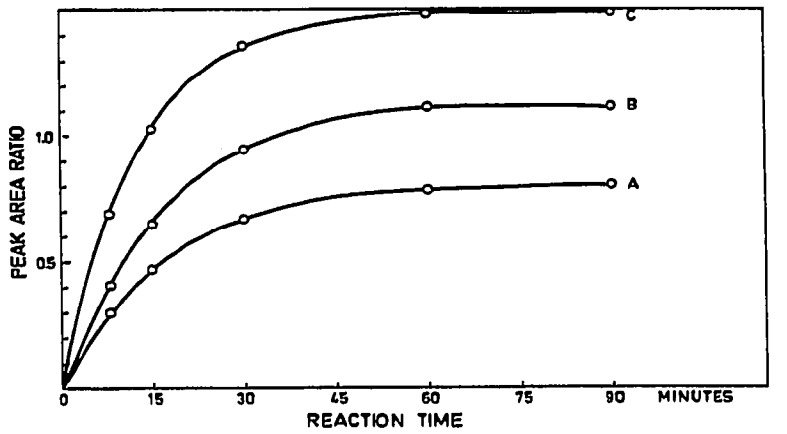

Fig. 4. Plots of the fitted function. Course of enzyme-catalyzed decarboxylation of lysine with different concentrations of enzyme preparation: A, 0.5 mg/ml (variance 0.011); B, 1.5 mg/ml (variance 0.0043); C, 2.5 mg/ml (variance 0.0095).

The increase in amine concentration in the course of the enzymic reaction was calculated by using the calibration curves in Fig. 1, and the results are given in Figs. 2–7.

The activity of the enzyme preparation suspension in phosphate buffer was found to be time dependent. For tyrosine decarboxylase, a permanent decrease in activity was evident and for lysine and glutamic acid decarboxylase the initial increase in activity was followed later by a decrease (Figs. 3, 5 and 7). The histidine decarboxylase preparation obtained showed no measurable activity; even after 4 h, no increase in histamine concentration was detected.

The Michaelis–Menten constants were found to be $1.2 \cdot 10^{-3}$ for tyrosine decarboxylase, $4 \cdot 10^{-3}$ for lysine decarboxylase and $2.6 \cdot 10^{-3}$ for glutamic acid decarboxylase, which are approximately the same as the values given in the literature[1].

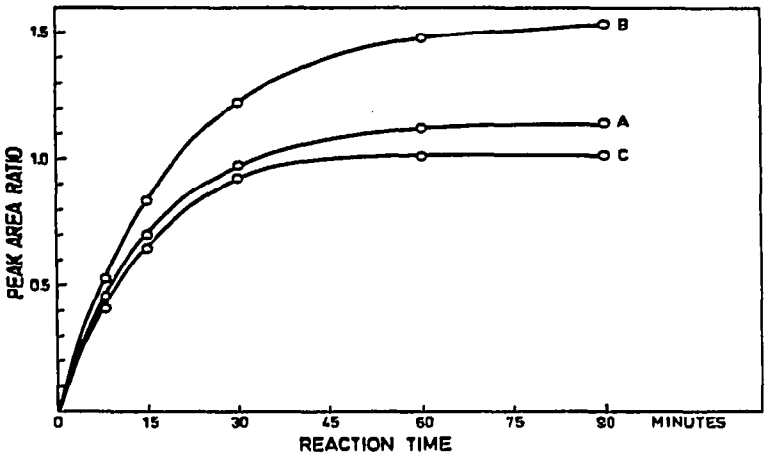

Fig. 5. Plots of the fitted function. Lysine decarboxylase activity assays performed with enzyme suspensions allowed to stand for different time intervals: A, 40 min (variance 0.028); B, 130 min (variance 0.0017); C, 220 min (variance 0.0041).

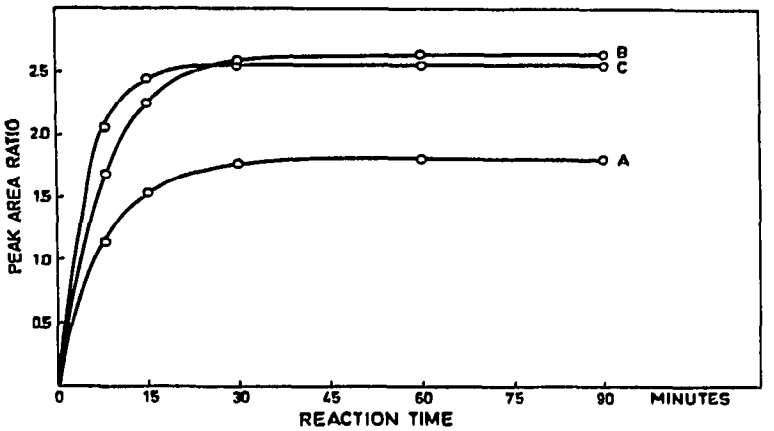

Fig. 6. Plots of the fitted function. Course of enzyme-catalyzed decarboxylation of glutamic acid with different concentrations of enzyme preparation: A, 0.5 mg/ml (variance 0.0017); B, 1.0 mg/ml (variance 0.0033); C, 1.5 mg/ml (variance 0.0041).

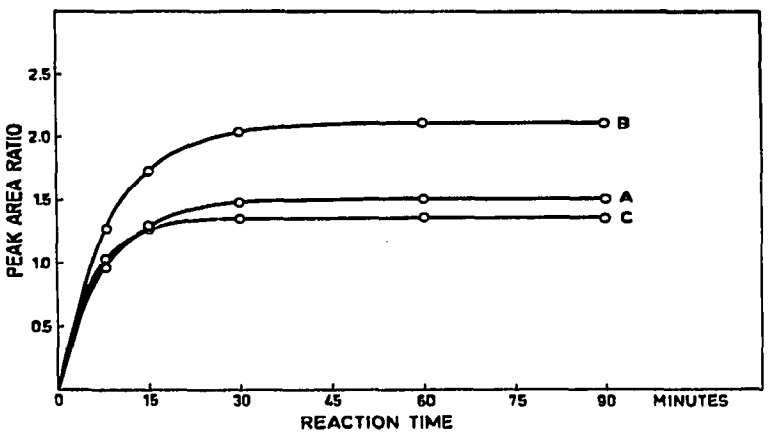

Fig. 7. Plots of the fitted function. Glutamic acid decarboxylase activity assays performed with enzyme suspensions allowed to stand for different time intervals: A, 40 min (variance 0.00055); B, 130 min (variance 0.00021); C, 220 min (variance 0.00021).

In all assays we used the same solvent system, absolute ethanol–25% ammonia solution (4:1)[10], which in all instances gave satisfactory separations of amine spots for densitometric measurements. However, in the case of one preparation of L-lysine decarboxylase, a satisfactory separation of the amine spot was achieved with 70% isopropanol in an ammonia atmosphere, but not until a series of different solvent systems had been tried unsuccessfully. Preliminary experiments of this type might be unavoidable in some instances.

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