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ASSAY OF ORNITHINE DECARBOXYLASE ACTIVITY BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Ornithine decarboxylase (L-ornithine carboxylase; EC 4.1.1.17; ODCase) is a key enzyme in the biosynthesis of polyamines. It catalyzes the decarboxylation of L-ornithine to putrescine. The high-performance liquid chromatographic (HPLC) method described here for determining ODCase activity combines the sensitivity of radiochemical detection with the separative capacity of HPLC without the necessity of generating a pre-column derivative. In this study, [1,2-^{3}H]putrescine was separated from L-[2,3-^{3}H]ornithine using reversed-phase HPLC eluted isocratically. This method was used to study ODCase from both prokaryotic and mammalian sources. With the ODCase from *Escherichia coli* we found the reaction rates to be linear for 5 min with an apparent Michaelis constant (K_M) of 20 m*M*. After 1 h this activity had produced approximately four-fold more product at pH 5.0 than at pH 7.3. In contrast, the initial rate of ODCase from submandibular glands was linear for 60 min. Also, the rate of putrescine synthesis was ten-fold higher in the embryonic gland than in the adult which was 8–80 times lower than that of *E coli*.

INTRODUCTION

Ornithine decarboxylase (L-ornithine carboxylase; EC 4.1.1.17 ODCase), a key enzyme in the polyamine biosynthetic pathway, catalyzes the decarboxylation of L-ornithine to putrescine. This enzyme is involved in the regulation of cell proliferation, differentiation, and growth and is a marker of cellular proliferation [1–3].

The assay of this activity is traditionally performed using L-[1-^{14}C]ornithine as the substrate. One reaction product, $^{14}CO_2$, is trapped in hyamine hydroxide

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[4] or on cation-exchange paper soaked with potassium hydroxide [5] and the amount of radioactivity determined by scintillation counting. While the use of radiochemicals confers a great deal of sensitivity to this assay, care must be taken to avoid loss of the gaseous product.

The advantages of employing a high-performance liquid chromatographic (HPLC) method in enzymatic analysis have been described [6] and ODCase activity has been assayed using this technique. Haraguchi et al. [7] used reversed-phase chromatography with a linear gradient to separate ornithine from the synthesized product, putrescine. Kvannes and Flatmark [8] developed an ODCase activity assay using reversed-phase chromatography with isocratic elution to separate ornithine from putrescine. The major disadvantage of both these methods is that the detection of putrescine required its conversion to a fluorescamine derivative prior to HPLC analysis.

The HPLC method described here for determining ODCase activity combines the sensitivity of radiochemical detection with the separative capacity of HPLC without the necessity of generating a pre-column derivative. In the present study, [1,2-^{3}H]putrescine was separated from L-[2,3-^{3}H]ornithine using isocratic reversed-phase HPLC with a mobile phase containing sodium dodecyl sulfate (SDS). This method, tested here with ODCase from both prokaryotic and mammalian sources, was found to be equal or superior in sensitivity to other methods for measuring ODCase with the added advantage that a separate derivatization step is not required. Also, to test its correlation with growth, the ODCase activity in submandibular glands from adult mice was compared to that from embryonic animals.

EXPERIMENTAL

Materials

CD-1 Swiss mice were supplied by Charles River Labs. (Wilmington, MA, U.S.A.). Hank's balanced salt solution (HBSS), *Escherichia coli* ODCase [Stock No. 03001, 0.05–0.2 U/mg of protein (1 U releases 1.0 μmol CO_2 per min at pH 5.2 and 37°C)], DL-dithiothreitol, pyridoxal 5′-phosphate, L-ornithine hydrochloride, and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum was obtained from Gibco (Grand Island, NY, U.S.A.). Sodium phosphate and disodium ethylenediaminetetraacetic acid (EDTA) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). SDS was from BioRad Labs. (Richmond, CA, U.S.A.). HPLC-grade acetonitrile and a Radnoti straight-sided, ground-glass tissue homogenizer were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). L-[2,3-^{3}H]Ornithine (118 mCi/mmol) and Aquasol universal LSC cocktail were obtained from New England Nuclear (Boston, MA, U.S.A.) and [1,4-^{14}C]putrescine dihydrochloride (55 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.).

MF-Millipore cellulose acetate and nitrate 0.45 μm pore size (HA) filters were obtained from Millipore (Bedford, MA, U.S.A.).

Preparation of ODCase from submandibular glands

ODCase was prepared from both adult and embryonic murine submandibular gland as follows. Fourteen day timed pregnant adult female CD-1 Swiss mice were sacrificed by cervical dislocation and embryos were removed and placed in HBSS. The embryos were decapitated and dissections were performed in the presence of HBSS containing 10% fetal bovine serum. With the aid of a dissecting microscope, the submandibular glands were removed and placed in 0.5 ml ice-cold solution containing 20 m*M* sodium phosphate buffer (pH 7.3), 1.25 m*M* dithiothreitol, and 10 μM disodium EDTA. The tissues were transferred to a 0.5-ml hand-held ground-glass tissue grinder and disrupted with 20–30 strokes of the pestle at 4°C. Disruption of the tissues and cells was confirmed by microscopic examination of the homogenates. The homogenates were centrifuged at 20 000 *g* for 5 min at 4°C. The supernatant solutions were recovered and assayed immediately for ODCase activity. Protein content was determined according to the method of Bradford [9] using bovine serum albumin as standard.

Reversed-phase ion-pair HPLC

Equipment. HPLC was performed using a Model 6000 solvent delivery system (Waters, Milford, MA, U.S.A.) and a Model 7125 syringe loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). A guard column packed with Co:Pell ODS reversed-phase pellicular chromatographic support (Whatman, Clifton, NJ, U.S.A.) preceded a 300 mm × 3.9 mm I.D. μBondapak C_{18} column (Waters), on which chromatographic separations were accomplished. An LKB Wallac 1214 Rackbeta liquid scintillation counter (Pharmacia LKB Biotechnologies, Gaithersburg, MD, U.S.A.) was used for detecting radiolabelled compounds in eluted fractions.

Chromatographic conditions. The chromatographic mobile phase contained 0.05 *M* sodium phosphate buffer (adjusted to pH 3.9 with orthophosphoric acid, 85%), 0.01 *M* SDS, and 36% acetonitrile and was prepared by adding 6.9 g sodium phosphate monohydrate salt and 2.88 g SDS to 640 ml of degassed, glass-distilled water. The pH was adjusted to 3.9 with concentrated orthophosphoric acid, and the solution was filtered through a 0.45-μm pore diameter mixed cellulose acetate and nitrate membrane. To this filtered solution were added 360 ml filtered acetonitrile; the solution was stirred thoroughly. The eluent was pumped at a flow-rate of 1.0 ml/min and 0.5-ml fractions were collected every 0.5 min. The sample injection volume was 50 μl.

Standardization. The retention times of ornithine and putrescine were determined by adding either radiolabelled ornithine ($2 \cdot 10^5$ cpm) or putrescine ($1 \cdot 10^5$ cpm) to 1 ml of 20 m*M* sodium phosphate buffer (pH adjusted to 5.0

with orthophosphoric acid, 85%) containing 2.5 m*M* dithiothreitol and 0.2 m*M* pyridoxal 5′-phosphate and injecting 50 μl of this mixture onto the column. Fractions (0.5 ml) were collected in scintillation vials and 4.5 ml of Aquasol universal LSC cocktail were added to each sample and the radioactivity determined by scintillation counting. With our counting conditions, approximately 50 cpm above background were detectable. Using this value we calculated a lower limit of detection for putrescine to be approximately 2.5 pmol.

Assay of ODCase by HPLC

Activity of ODCase was determined by measuring the rate of formation of [1,2-^{3}H]putrescine from L-[2,3-^{3}H]ornithine. The reaction mixture contained in a final volume of 1 ml, 20 m*M* sodium phosphate buffer (pH adjusted to 5.0 with orthophosphoric acid, 85%), 2.5 m*M* dithiothreitol, 0.2 m*M* pyridoxal 5′-phosphate, 0.4 μCi L-[2,3-^{3}H]ornithine, 100 m*M* L-ornithine hydrochloride and 0.3 ml of either *E. coli* ODCase or tissue homogenate. The *E. coli* ODCase solution was prepared by dissolving 3 mg of the partially purified, lyophilized enzyme in glass-distilled water. One unit (1 U) of *E. coli* ODCase was defined as the generation of 1 μmol of CO_2 per min at pH 5.0 and 37°C. As supplied, the enzyme was expected to produce between 0.05 and 0.2 U of activity. For the assay of mammalian ODCase, the pHs of the reaction mixtures were adjusted to 7.3 with sodium hydroxide prior to addition of the tissue homogenates.

Reaction mixtures were incubated at 37°C in a reciprocal shaking water bath and the reactions were terminated by injecting 50 μl of the incubation mixture onto the HPLC column.

Quantitation of putrescine synthesis

The amount of putrescine synthesized during the reaction was determined from the amount of radioactivity recovered following the separation. The moles of putrescine were calculated from the radioactivity in the following manner. The specific activity of the substrate was determined directly from the sum of the radioactivity recovered as substrate and, when present, product. This value divided by the total amount of non-radioactive ornithine in the reaction mixture gives the value in cpm/μmol. This value when multiplied by the radioactivity of putrescine gives the amount of putrescine synthesized per unit of volume injected (50 μl). Since this was 1/20 of the volume of the reaction mixture, this amount was multiplied by 20 to give the amount in the total reaction mixture. Finally, this product was normalized to 1 mg protein. The resulting number is expressed as moles of putrescine generated per unit of time per mg of protein. One unit of ODCase activity is defined as 1 μmol putrescine generated in 1 min at 37°C.

RESULTS

Determination of optimal conditions for the separation of ornithine from putrescine

Fig. 1 shows the retention times of L-[2,3-^{3}H]ornithine and [1,4-^{14}C]putrescine after elution with 0.05 *M* sodium phosphate buffer (pH 3.9) containing 0.01 *M* SDS and 36% acetonitrile. Ornithine was eluted between 4 and 5 min and putrescine was eluted between 7 and 9 min. Separation of ornithine from putrescine was completed in 10 min and the level of radioactivity declined to the baseline after 5.5 min. No other peaks of radioactivity were detected even after extending the elution time to 20 min. By increasing the acetonitrile from 36 to 40%, the retention time for putrescine was increased to 12.5 min. At 30% acetonitrile, the putrescine co-eluted with ornithine. When SDS was omitted from the mobile phase, no separation of putrescine with ornithine was achieved.

Activity of E. coli ODCase

The HPLC ODCase assay was tested using a commercially available preparation of *E. coli* ODCase. A reaction mixture containing 20 m*M* sodium phosphate buffer (pH 5.0), 2.5 m*M* dithiothreitol, 0.2 m*M* pyridoxal 5′-phosphate, 0.4 μCi L-[2,3-^{3}H]ornithine, 100 m*M* L-ornithine hydrochloride, and 0.15–0.6 U of activity was prepared and samples were removed at suitable intervals after the start of the reaction. A chromatogram of a representative sample (Fig. 2)

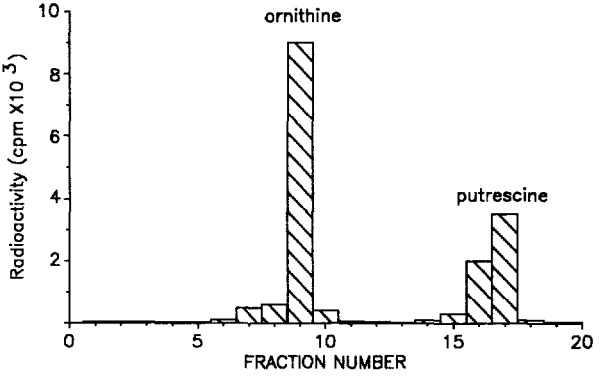

Fig. 1. Composite histogram of the elution positions of radiolabelled ornithine and putrescine. The retention times of radiolabelled ornithine and putrescine were determined by injecting 0.02 μCi of L-[2,3-^{3}H]ornithine or 0.005 μCi of [1,4-^{14}H]putrescine in incubation buffer [20 m*M* sodium phosphate buffer (pH 5.0) containing 2.5 m*M* dithiothreitol and 0.2 m*M* pyridoxal 5′-phosphate]. The elution positions were first determined by injecting ornithine and putrescine separately, then the two radiolabelled substances were added concurrently to incubation buffer and injected to confirm separation and retention times. Fractions (0.5 ml) were collected.

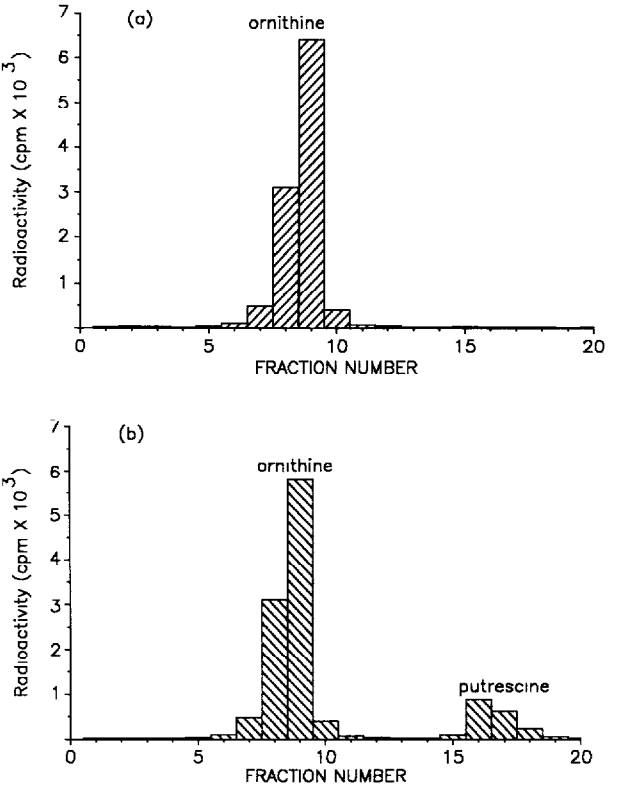

Fig. 2. Assay of *E coli* ODCase activity. The incubation mixture contained 20 m*M* sodium phosphate buffer (pH 5.0), 2.5 m*M* dithiothreitol, 0.2 m*M* pyridoxal 5′-phosphate, 4 m*M* ornithine, and 0.4 μCi L-[2,3-^{3}H]ornithine (10 500 cpm per 200 nmol). Representative chromatograms are shown for samples analyzed prior to addition of 3 mg (0.15–0.6 U) enzyme protein (a) and after a 1-h incubation period (b).

showed that prior to the addition of enzyme, radioactivity only eluted between 4 and 5 min, consistent with the recovery of ornithine; no putrescine was recovered (Fig. 2a). In contrast, the analysis of a sample taken from the same incubation mixture 1 h after the addition of enzyme revealed not only radioactivity eluting at the ornithine position, but also at a position similar to that of authentic putrescine (Fig. 2b).

Effect of substrate concentration on E. coli ODCase activity

Fig. 3 shows ODCase activity as a function of substrate concentration. For these experiments, reaction mixtures were prepared with concentrations of substrate ranging between 1 and 200 m*M*. Reactions were started by the addition of enzyme and samples removed for analysis at indicated time points to generate rate curves for each concentration of substrate. Reactions were found

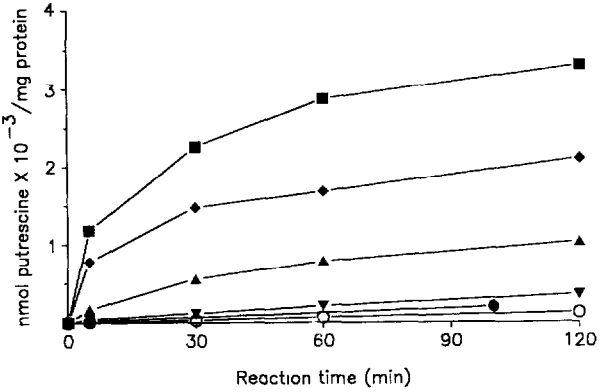

Fig. 3. ODCase activity as a function of substrate concentration. L-Ornithine hydrochloride was added to the reaction mixture in increasing concentrations to determine optimal conditions for the assay. The concentrations were 1 m*M* (○), 2 m*M* (●), 4 m*M* (▼), 20 m*M* (▲), 100 m*M* (◆), and 200 m*M* (■) in a 1-ml reaction volume.

to be linear of up to 5 min, then gradually declined. When initial rates were calculated from these data at 200 μM ornithine we found that 1.18 μmol (0.236 U) of putrescine were synthesized. This value is in agreement with 0.05–0.2 U/mg given by the manufacturer. When these data were replotted in double-reciprocal form, a Michaelis constant (K_M) of 20 m*M* was calculated. The activity from *E. coli* was measured at pH 5.0 and compared to that determined at pH 7.3. As expected from previous studies [7,10], the rate of putrescine synthesis was found to decline at the higher pH.

Developmental changes in ODCase activity in mammalian tissues

To test the relationship between ODCase activity and growth, the ODCase activity in a representative embryonic tissue was compared to the same tissue obtained from adult animals. For these experiments, submandibular glands were removed from mice, homogenized, and added to a reaction mixture containing radiolabelled ornithine. Initial rate measurements were performed and the results presented in Fig. 4 show the embryonic tissues have more activity than the adult. Note also that when compared to the activity of the *E. coli*, the specific activity of the ODCase from both mammalian tissues was 8–80 times lower. Also note that production of putrescine by ODCase activity in tissue homogenates was constant for at least 60 min of incubation in contrast to the results for the *E. coli* activity in which the ODCase activity remained constant for only 5 min.

Finally, Fig. 5 shows that enzyme activity from mammalian tissue homogenates was directly proportional to protein concentration in mixtures containing up to 3 mg of protein.

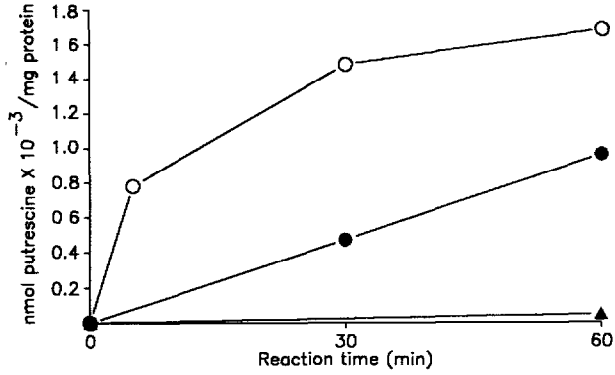

Fig. 4. ODcase activity as a function of tissue source. In the assay of *E. coli* ODCase, the incubation mixture contained 20 m*M* sodium phosphate buffer (pH 5), 2.5 m*M* dithiothreitol, 0.2 m*M* pyridoxal 5′-phosphate, 100 m*M* ornithine, 0.4 μCi L-[2,3-^{3}H]ornithine, and 0.15–0.6 U of enzyme. In the assay of mammalian ODCase, the incubation mixture contained 20 m*M* sodium phosphate buffer (pH 7.3), 2.5 m*M* dithiothreitol, 0.2 m*M* pyridoxal 5′-phosphate, 100 m*M* ornithine, 0.4 μCi L-[2,3-^{3}H]ornithine and, 1–3 mg/ml tissue homogenate (fourteen-day embryonic or adult submandibular gland). Activity was derived from *E coli* (○), embryonic submandibular gland (●) or adult submandibular gland (▲).

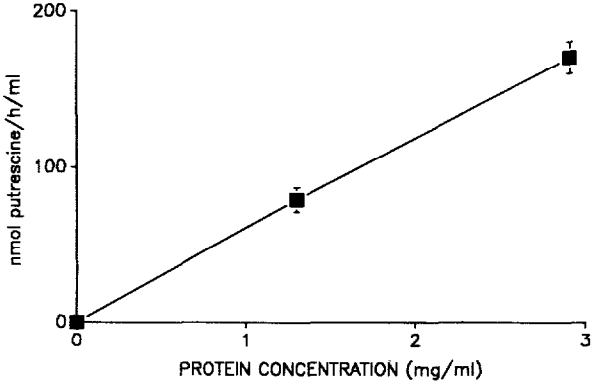

Fig. 5. Mammalian ODCase activity as a function of protein concentration. Data taken from Fig. 4.

DISCUSSION

This paper describes a new HPLC method for the measurement of ODCase activity. Other assays for this activity measured the production of radiolabelled CO_2 from labelled ornithine [4,5] or require the derivatization of the generated putrescine prior to detection [7,8]. Endo and co-workers [11,12] utilized open-column ion-exchange chromatography for the separation of ornithine from

putrescine, but the low degree of sensitivity of their method restricted them to non-continuous enzymatic determinations in which ODCase was assayed only once at a 3-h incubation time point. The method described here is an improvement over other methods because it combines the sensitivity of radiochemical detection, the rapid separative capacity of HPLC, and the benefits of continuous enzymatic analysis.

In the method described here, SDS was used for the separation of putrescine from ornithine [13]. We found that the addition of SDS to the mobile phase was crucial for baseline resolution of putrescine from ornithine. During the development of this assay is was noted that the concentration of acetonitrile was critical to the chromatographic profile. A large shift in the putrescine elution pattern was seen with changes from 36% in acetonitrile concentration to 30–40%. It was noted that the eluent must be very thoroughly mixed prior to use and that tightly sealed vessels were required to prevent evaporation of the acetonitrile. Putrescine was retained for longer periods of time when the room temperature increased or when precautions were not taken to prevent acetonitrile evaporation.

The ODCase reaction was dependent on the presence of pyridoxal 5′-phosphate and dithiothreitol in the incubation mixtures. It had been noted previously that spontaneous decarboxylation of ornithine occurs in the presence of pyridoxal 5′-phosphate [14]. In incubation mixtures lacking protein, this spontaneous decarboxylation was sometimes noted, but the addition of denatured, inactivated protein to reaction mixtures showed no activity or spontaneous decarboxylation. It may be that the presence of protein somehow discourages the non-enzymatic decarboxylation.

When equilibrating the column for the assay, washing the column with 60 ml of mobile phase was required. During periods of heavy use, the column was maintained in the experimental eluent overnight at flow-rates of 0.1 ml/min with no ill effects. The column performed consistently under the experimental conditions for over 300 analyses.

The assay described here has a lower limit of detection for putrescine of 2.5 pmol. This is comparable to that of 5 pmol previously reported [7]. The assay was standardized by utilizing *E. coli* ODCase. The manufacturer's estimates of 0.05–0.2 U of activity per mg of protein min at 37°C were used to determine the efficiency of the assay. It was determined that, under optimal conditions for this assay, that 0.236 U/mg of protein per min at 37°C could be measured.

Not only was the assay able to detect activity in partially purified *E. coli* ODCase, but activity from mammalian tissue homogenates could be measured. The embryonic submandibular glands have higher ODCase activity than their adult counterparts. This is consistent with other studies that suggested ODCase activity increased in cells that are proliferating and decreased when cells are in a quiescent state.

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