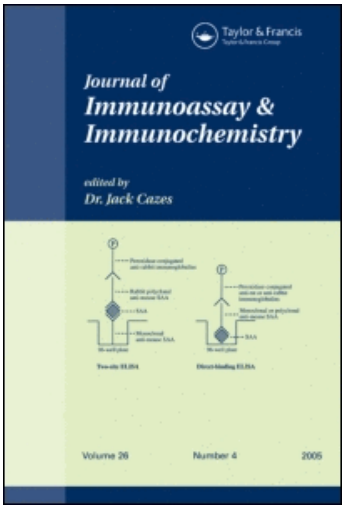

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SANDWICH ENZYME IMMUNOASSAY FOR ORNITHINE DECARBOXYLASE

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ABSTRACT

A sensitive enzyme immunoassay (EIA) was developed for the determination of ornithine decarboxylase (ODC, EC 4.1.1.17), in the range of 0.02-10 ng, using an affinity-purified anti-ODC-Fab'-peroxidase conjugate. The amount of ODC protein was determined in crude extracts from the kidney of testosterone-treated mice, regenerating rat liver and human thyroid carcinoma, with purified mouse kidney or rat liver enzyme as standard. In all these tissues, similar activity/protein ratios were found for ODC: 1.2×10^6-1.9×10^6 nmol CO_2/h/mg of ODC protein, which were roughly equivalent to the final specific activity of purified enzymes. ODC inactivated by α- difluoromethylornithine (DFMO) could also be assayed with this method similarly to active ODC protein. However, ODC-antizyme complex gave a somewhat lower value than free ODC protein.

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INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17), the first and key enzyme of polyamine biosynthesis, exhibits dramatic fluctuations in response to many different stimuli, owing to its extremely short half-life (1). Much attention has therefore been paid to the regulatory mechanism of ODC. However, the extremely low enzyme content in most animal tissues has caused serious difficulty in its accurate determination in these tissues (1). Thus far, the measurement of ODC has been performed by radioimmunoassay of two different kinds. The method of Seely et al. with ^{3}H-DFMO-labeled ODC has a rather low sensitivity, although it has the advantage that purified enzyme is not needed because of the specific binding of DFMO (2). Another competitive immunoassay with ^{125}I-labeled ODC reported by Isomaa et al. (3) is about ten-fold more sensitive than that of Seely et al. This method needs, however, frequent preparation of purified enzyme labeled with ^{125}I.

In the present paper, we describe the development of a highly sensitive and convenient sandwich enzyme immunoassay for ODC, using the hinge method introduced by Ishikawa et al. (4). We also present results obtained with this method on the fluctuation of the amount of ODC protein, as compared with that of its activity level, in regenerating rat liver and in the

kidney of testosterone-treated mice. We further compared the reactivity of various forms of ODC protein, namely free ODC, DFMO-inactivated ODC and ODC-antizyme complex in the present EIA system.

MATERIALS AND METHODS

Purification of mouse kidney ODC and rat liver ODC.

ODC was purified from mouse kidney or rat liver as described previously (5). The final specific activity of mouse kidney ODC was 1.2×10^6 units/mg, roughly identical with that of the rat liver ODC reported previously (6). When purified rat liver ODC was used as a standard, we calculated its amount from its activity assuming that one unit was equivalent to 0.83 ng ODC protein, because the amount of purified enzyme was too small for exact determination of protein.

Preparation of anti-mouse kidney ODC polyclonal antibody.

Rabbit antiserum was raised against mouse kidney ODC as described previously (5). IgG was prepared by ammonium sulfate fractionation, followed by a passage through a DEAE cellulose column.

Preparation of affinity adsorbent.

Affi-Gel 10 (Bio-Rad, Richmond, California) affinity column with bound ODC was prepared as follows: 400 μg/ml of purified mouse kidney ODC was treated with

0.4 ml of Affi-Gel 10 in the buffer of 0.1 M 3-[N-Morpholino] propanesulfonic acid (MOPS) (pH 7.5) containing 80 mM $CaCl_2$, 1 mM dithiothreitol, and 30 μM pyridoxal-5'-phosphate (PLP) at 4°C for 8 h with shaking. ODC bound to Affi-Gel 10 was 100% and 97% judging from activity and protein measurements, respectively.

Preparation of affinity-purified anti-ODC Fab' fragment and Fab' conjugated with horseradish peroxidase.

$F(ab')_2$ was prepared by the digestion of the IgG with pepsin (1:10000, Nakarai Chemicals Ltd., Kyoto, Japan), and adsorbed to an ODC-Affi-Gel 10 column. The $F(ab')_2$ was then eluted from the column with 0.2 M glycine-HCl (pH 2.3). Approximately 0.3 mg of affinity-purified $F(ab)'_2$ was obtained from 6.6 mg of total $F(ab)'_2$. In a preliminary experiment the titer of affinity - purified IgG was 200-400 units ODC/μg of IgG, while that of non-affinity IgG was 25 units ODC /μg of IgG. The specificity of the affinity purified IgG was checked by immunoblotting analysis of crude mouse kidney extracts or pure ODC as shown in Fig 1. This affinity- purified $F(ab)'_2$ was dissociated into Fab' by reduction with 2-mercaptoethylamine hydrochloride (Nakarai Chemicals Ltd., Kyoto, Japan). Fab'-peroxidase conjugate was prepared by using a thiol group, succinimidyl 4-(N-maleimidoethyl)-cyclohexane-1-

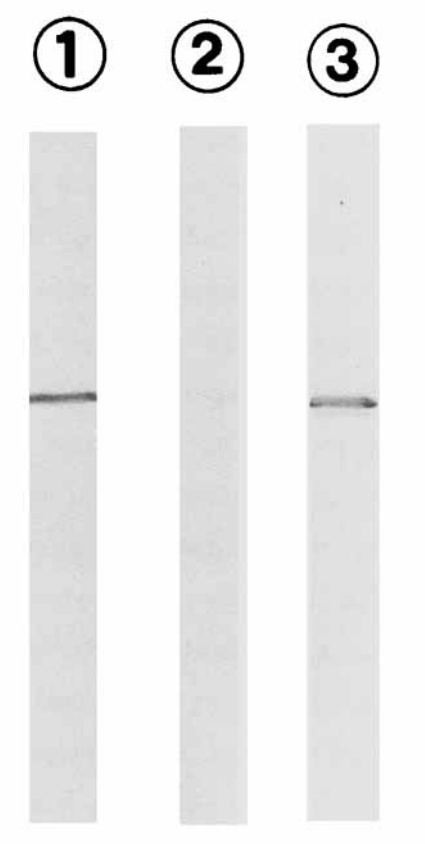

FIGURE 1
Specificity of the affinity-purified anti-ODC IgG as indicated by immunoblotting analysis after SDS-polyacrylamide gel electrophoresis. Lane 1, kidney extract of testosterone-treated mouse (20 units of ODC activity); lane 2, kidney extract of starved female mouse (0 unit of ODC activity); lane 3, same sample as in lane 2 plus 20 units of purified mouse kidney ODC.

carboxylate (Zieben Chemicals Tokyo, Japan), introduced in the hinge of the Fab', according to the maleimide method described by Ishikawa et al. (4).

Preparation of tissue extracts.

ICR male mice (Sankyo Labo Service Co., Tokyo, Japan) were injected subcutaneously with testosterone enanthate (Teikoku-Zoki Co., Tokyo, Japan) and killed 0, 1, 4, 7 and 11 days later to obtain their kidneys. Male Sprague-Dawley rats (Sankyo Labo Co., Tokyo, Japan) were partially hepatectomized after starvation for one day and killed 0, 4, 6 and 8 hours later to obtain regenerating liver. Human thyroid carcinoma tissue that had been resected surgically, quickly frozen in liquid nitrogen and stored at -80°C was provided by Dr. H. Takami (Department of Surgery, Teikyo University School of Medicine). Each tissue was immediately cooled on ice and homogenized with 25 mM Tris-HCl (pH 7.5), 0.2 M sucrose, 1 mM dithiothreitol, 0.1 mM EDTA. The homogenate was centrifuged at 100,000 x g for 60 min to obtain the crude extract. These crude extracts were used for assay of ODC activity or protein. Inactive ODC was obtained by a treatment with DFMO, a kind gift from Merrell Dow Research Institute, Cincinnati, OH, USA., at 37°C for 60 min in 25 mM Tris HCl (pH 7.5), 5 μM DFMO, 1 mM dithiothreitol, 40 μM PLP, and 0.5 mg/ml bovine serum albumin (BSA).

Purification of antizyme

Antizyme was prepared from rat liver and its activity assayed as described previously (7). One unit of antizyme activity was defined as the amount inhibiting one unit of ODC activity.

Assay of ODC activity and of protein.

The activity of ODC was assayed as described previously (5). One unit of enzyme activity was defined as the amount forming 1 nmol of CO_2 per hour. Protein was determined by the method of Lowry et al. (8) using BSA as a standard. Amounts of IgG, $F(ab')_2$, and Fab' of the rabbit were determined by using their extinction coefficients at 280 nm : E_{280}=1.5, 1.48, and 1.48 $g^{-1}.l.cm^{-1}$, respectively (4).

Sandwich enzyme immunoassay for ODC.

Polystyrene tubes (70 x 11 mm, Maxisorp Tube, A/S Nunc, Roskilde, Denmark) were coated with anti-mouse kidney ODC IgG by incubating with 0.75 ml each of 50 µg/ml IgG solution in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C overnight. After washing with 10 mM sodium phosphate buffer (pH 7.0), the tubes were kept with 1% BSA/0.02% NaN_3 in 0.1 M sodium phosphate buffer at 4°C to minimize nonspecific adsorption and washed with 10 mM sodium phosphate buffer (pH 7.0) immediately before use.

Procedure of sandwich enzyme immunoassay was essentially the same as that of Hashida et al. (9). Each crude extract or purified standard enzyme sample in a total volume of 0.2 ml was incubated overnight at 4°C in the anti-ODC IgG-coated polystyrene tube. Usually samples were diluted with 25 mM Tris-HCl (pH 7.5) containing 0.5 mg/ml BSA, 1 mM dithiothreitol and 40 μM PLP. After incubation, the polystyrene tube was washed twice with 10 mM sodium phosphate buffer (pH 7.0) containing 3 M NaCl and incubated further with affinity-purified anti-ODC Fab'-peroxidase conjugate (6.2 ng/tube) in 0.2 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.1% BSA and 0.1 M NaCl at room temperature for 5 hours. After removing the solution, the polystyrene tube was washed twice with 10 mM sodium phosphate buffer (pH 7.0). Bound peroxidase activity was assayed at room temperature for 60 min using 3-(p-hydroxyphenyl) propionic acid (Tokyo Kasei, Japan) as a substrate. After addition of 3.0 ml of 0.1 M glycine buffer (pH 10.3), fluorescence intensity was measured using 320 nm and 405 nm for excitation and emission wave lengths, respectively, with a Shimadzu fluorophotometer (RF-510, Shimadzu Seisakusho, Ltd.) and standardized by adjusting the scale to 100 with 1 μg/ml quinine sulfate in 1 N H_2SO_4. Blank values were expressed as the fluorescence intensity in the absence of ODC.

Sodium laurylsulfate (SDS)-polyacrylamide gel electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis was carried out essentially by the method of Lugtenberg et al. (10). ODC was detected by immunoblotting, according to the method of Towbin et al. (11) using the affinity-purified anti-ODC IgG.

RESULTS AND DISCUSSION

Standard curve for the ODC EIA system.

As shown in Fig. 2, the present EIA system could detect 0.02 ng to 10 ng of ODC protein. The minimum enzyme amount detectable with the system (0.02 ng) is lower than that of previously reported radioimmunoassay methods: 1 ng with the method of Seely et al. (2) and 0.1 ng with the method of Isomaa et al. (3). Linearity was obtained in the range of 0.5 ng to 4 ng. As the relative fluorescent intensity varied somewhat in different experiments, a standard curve was performed in each experiment.

Correlation between ODC activity and the amount of ODC protein in mouse, rat, and human tissues.

Fig. 3 shows excellent correlation between ODC activity and protein in crude extracts from mouse, rat, and human tissues. These results appear similar to

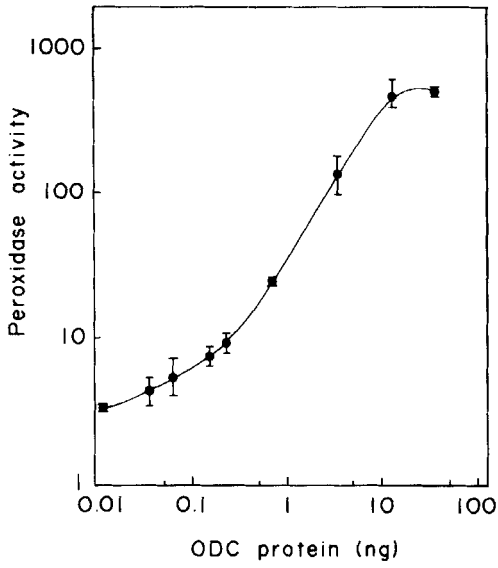

FIGURE 2
Standard curve for mouse kidney ODC by sandwich enzyme immunoassay. Each point is the mean of three determinations, and vertical bars indicate S.D.

results reported previously (12). The present findings indicate that ODC in these tissues has about the same activity/protein ratio: ca. 1.2×10^6 units/mg of ODC protein. We have recently shown that mouse kidney and rat liver ODCs have similar final specific activities (5).

Time courses of ODC induction determined by both activity and protein in the kidney of the testosterone-treated mouse and in regenerating rat liver.

As shown in Fig. 4-a, there were marked and apparently parallel increases in the amount of ODC

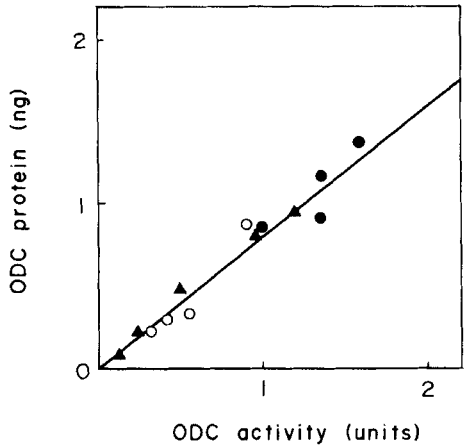

FIGURE 3
Correlation between ODC activity and protein in regenerating rat liver (●), untreated male mouse kidney (○), and human thyroid carcinoma (▲). The correlation coefficient ($r = 0.983$) between the two values was highly significant ($p < 0.001$).

protein and ODC activity during the course of ODC induction in the kidney of male mice upon treatment with testosterone enanthate. In partially hepatectomized rats, the amount of hepatic ODC protein increased to 1.22±0.09 ng/mg of protein, peaking at 6 hours after the operation (Fig. 4-b), and also exhibited close parallelism with activity. In both cases of ODC induction, the activity/protein ratio of ODC was calculated to be in the range of 1.2×10^6-1.9×10^6 units/mg of ODC protein.

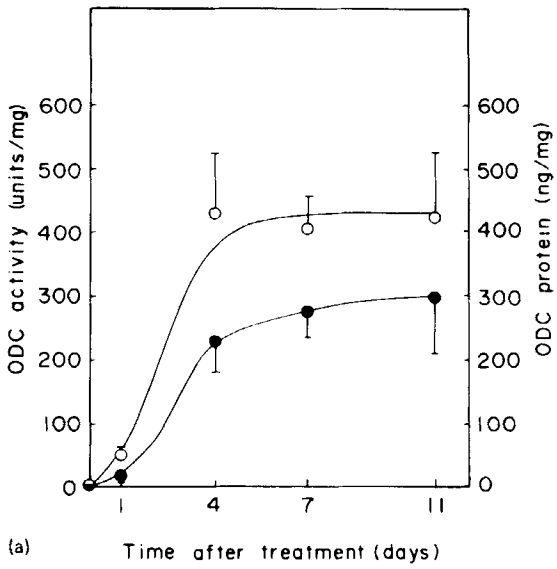

FIGURE 4-a
Time-dependent changes in renal ODC activity (O) and protein (●) of male mice upon testosterone enanthate treatment. Results are means ± S.D. for three animals of each group.

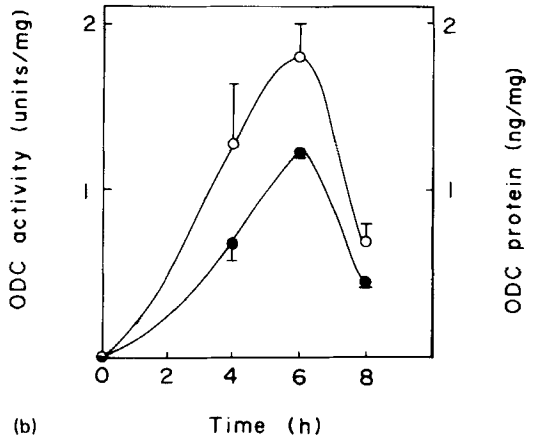

FIGURE 4-b
Time-dependent changes of ODC activity (O) and protein (●) in regenerating rat liver after partial hepatectomy. The rats were starved overnight before hepatectomy. Results are means ± S.D. for three or four animals of each group.

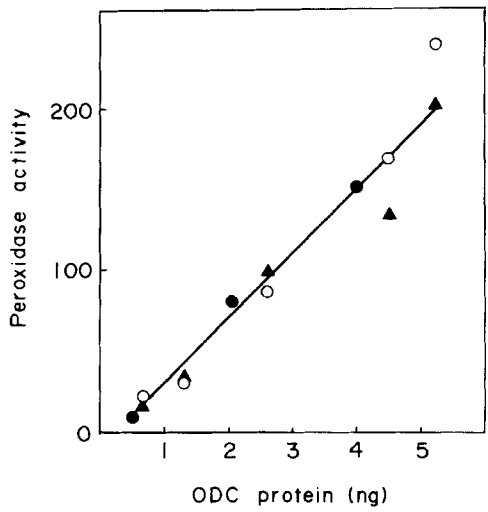

FIGURE 5
Comparison of active ODC (○) and DFMO-inactivated ODC (▲), both in crude rat liver extract, and purified rat liver ODC (●) in the present enzyme immunoassay system. Amounts of ODC in crude extracts were calculated assuming the activity/protein ratio of 1.2 x 10^6 units/mg of ODC protein.

Comparison of active ODC and DFMO-inactivated ODC in the present EIA system.

As shown in Fig. 5, crude ODC that had been inactivated with DFMO had the same reactivity both with active ODC in crude extracts and with purified ODC.

Comparison of free and complexed ODCs in the present EIA system.

It is known that part of cellular ODC is present as a complex with antizyme, a specific protein

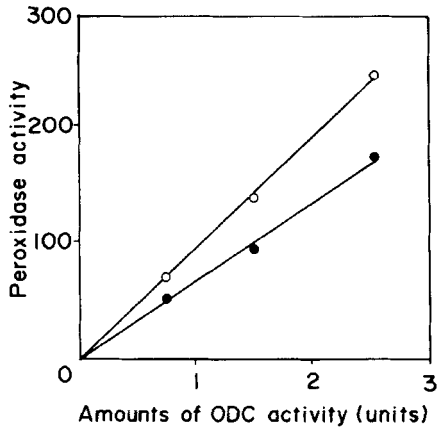

FIGURE 6
Comparison of free ODC and ODC-antizyme complex. Six units of highly purified antizyme (O) or saline (●) was added to partially purified ODC before the reaction with solid-phase anti-ODC antibody.

inhibitor (13). We examined whether the complexed form of ODC could be determined with the same sensitivity as free ODC in the present EIA system. As shown in Fig. 6, the complexed ODC gave somewhat lower values compared with free ODC. This should be kept in mind, therefore, when the EIA is applied to a sample with a high proportion of complexed ODC.

Concluding remarks.

Besides the high specificity (Fig. 1) and sensitivity (Fig. 2), the present EIA system has the advantage of simplicity in that it dose not require any radioactive material or purified ODC. A crude kidney

extract from testosterone-treated mice can be used as a standard ODC preparation assuming the activity/protein ratio of 1.2×10^6 units/mg of ODC protein, since it dose not contain any significant amount of either cross-reacting material (Fig. 1) or interfering substance (Figs. 3 and 5), and the proportion of ODC that is complexed with antizyme is less than one percent in such an extract (14).

ACKNOWLEDGMENTS

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