Methods for the Detection of D-Amino-Acid Oxidase

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ABSTRACT

Four methods (an enzyme activity assay, western blotting, RT-PCR, and northern hybridization) to detect the enzyme D-amino-acid oxidase are described.

INTRODUCTION

D-Amino-acid oxidase catalyzes oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding 2-oxo acids, producing ammonia and hydrogen peroxide in the course of the reaction (1). This enzyme is present in a wide variety of organisms (2). However, the physiological role of this enzyme has been unclear because its substrate D-amino acids have been considered to be rare in eukaryotic organisms (2, 3). In previous work, my colleagues and I concluded that D-amino-acid oxidase is not present in the mouse liver (13). This paper examines in greater detail the methods used in reaching that conclusion.

MATERIALS AND METHODS

Assay of D-amino-acid oxidase activity

D-Amino-acid oxidase activity can be measured by various methods. One of them is the colorimetric method of Watanabe et al. (4). Organs, tissues, or tissue culture cells were homogenized in 7 mM pyrophosphate buffer (pH 8.3). The homogenates were centrifuged at 550 x g for 5 minutes. The supernatant solutions were used for the assay. The reaction mixture consisted of 0.3 ml of 0.133 M pyrophosphate buffer (pH 8.3) containing catalase at 700 IU/ml, 0.3 ml of 0.1 M D-alanine, 0.2 ml of 0.1 mM FAD, and 0.1 ml of 70% (V/V) methanol. The reaction was started by the addition of 0.1 ml of the supernatant solution. The reaction was carried out at 37°C for 15-60 minutes depending on the activity of the samples. It was terminated by the addition of 1 ml of 10% trichloroacetic acid. In a blank, trichloroacetic acid was added to the reaction mixture before the enzyme reaction was started. The precipitate was removed by centrifugation (700 x g, 20 min). To 0.5 ml of the supernatant solution were added 0.5 ml of 5 N KOH and 0.5 ml of 0.5% 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole in 0.5 N HCl. The mixture was kept standing at room temperature for 15 min. After 0.5 ml of 0.75% KIO_4 in 0.2 N KOH were added to the mixture with vigorous shaking, absorbance at 550 nm was measured.

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D-Amino-acid oxidase activity is calculated with this formula: $Activity\ (\mu mol\ min^{-1}) = \frac{2.584A}{t}$, where *A* is a differential absorbance at 550 nm between the sample and the blank, and *t* is the reaction time in minutes. This activity value was further divided by the quantity of the protein present in the first reaction. The protein concentration in the supernatant solution was determined according to the method of Lowry et al. (5) using bovine serum albumin as a standard. It was also determined using a Protein Assay Kit (Bio-Rad, Hercules, CA). D-Amino-acid oxidase activity is finally expressed as the amount of D-alanine oxidized per min per milligram of protein. Hog kidney D-amino-acid oxidase (Sigma, St. Louis, MO or Boehringer Mannheim, Germany) was used as a control.

Western blotting

Organs, tissues, or tissue culture cells were homogenized in distilled water and centrifuged at 550 x g for 5 min. The supernatant solutions were mixed with an equal volume of the sample buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) and were heated in a boiling water for 3 min. The samples (5~50 µg protein per lane) were electrophoresed according to the method of Laemmli (6) on a polyacrylamide-gradient (8-16%) slab gel (Tefco, Tokyo) with the electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) at 20 mA for about 1.5 hr. Hog kidney D-amino-acid oxidase (10 ng, Sigma) and prestained protein standards (SeeBlue, Novex, San Diego, CA) were run together. The proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (BA85, Schleicher and Schuell, Dasel) with the blotting buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol) at 180 mA for 1 hr.

The membrane was incubated in 5% non-fat dried milk (Bio-Rad) in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) for 1 hr and then quickly rinsed twice with TBS-T and washed in TBS-T once for 15 min then twice for 5 min. It was incubated for 1 hr in TBS-T containing rabbit anti-hog D-amino-acid oxidase IgG (1/3,000 dilution) and quickly rinsed twice with TBS-T and further washed in TBS-T once for 15 min then twice for 5 min. The membrane was incubated for 1 hr in TBS-T containing horseradish peroxidase-labeled antibody against rabbit IgG raised in a donkey (ECL western blotting detection set, Amersham, Buckinghamshire) (1/1,000 dilution). Following this, the membrane was quickly rinsed three times with TBS-T and further washed in TBS-T once for 15 min then twice for 5 min. After draining excess TBS-T solution, the membrane was covered with a mixture of 2.5 ml each of Detection solution 1 and 2 (ECL western blotting detection set) for 1 min. After the excess solution was drained off, the membrane was wrapped with Saran Wrap and exposed to an autoradiography film (Hyperfilm-ECL, Amersham) for about 5~30 sec. D-Amino-acid oxidase protein was detected as a 39 kDa band under these conditions.

PCR amplification of D-amino-acid oxidase cDNA fragment

Total RNA was extracted from organs, tissues, or tissue culture cells basically by the method of Chomczynski and Sacchi (7). Isogen (Nippon Gene, Tokyo) was used for this extraction. The first strand of cDNA was synthesized using Superscript Preamplification System (BRL, Gaithersburg, MD).

A sense primer (F: 5'-GGTTAACTGAGAGGGGAGTGAA-3') and an antisense primer (R: 5'-CCATAGTTGTGGATGACCTCTG-3') were designed from the sequence conserved in cDNAs encoding human, mouse, rabbit, and pig D-amino-acid oxidases. These primers were synthesized by Greiner Japan (Tokyo). The PCR reaction mixture (20 µl) contained 0.8 µl of the first strand of cDNA solution, 0.2 µM each of the sense and antisense primers, dNTP mix (0.2 mM each), 0.5 units of Expand High Fidelity (Boehringer Mannheim) and the reaction buffer. In place of Expand High Fidelity, LA Taq (Takara, Ohtsu), AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) were also used satisfactorily. After an initial denaturation at 94°C for 1 min, 30 cycles of PCR (denaturation: 94°C, 30 sec; annealing: 55°C, 30 sec; extension: 72°C, 45 sec) were performed using a thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer Cetus, Norwalk, CT). A final extension was done at 72°C for 5 min and the reaction was kept at 4°C. 10 µl of the reaction solution were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining. A DNA fragment of about 480 base-pairs was amplified under these conditions.

In this procedure, cDNA synthesis and PCR were carried out separately. However, these two steps could be done in a single tube using Titan One Tube RT-PCR System (Boehringer Mannheim) or One Step RNA PCR Kit (Takara). Both gave satisfactory results.

Northern hybridization

Total RNA was extracted from organs, tissues, or tissue culture cells as described above. Poly(A)$^+$ RNA was purified using oligo(dT) latex beads (Oligotex dT30 Super, Takara) according to the method specified by the supplier. Poly(A)$^+$ RNA was finally recovered in the TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). In place of Oligotex dT30 Super, the FastTrack mRNA Isolation System (Invitrogen, San Diego, CA) also yielded satisfactory results.

Poly(A)$^+$ RNA (2~10 µg) were electrophoresed on a 1.2% denaturing agarose gel according to the method of Sambrook et al. (8). Markers (0.24-9.5 kb RNA Ladder, BRL) were run together. The RNAs were capillary-transferred overnight to a nylon membrane (Hybond N, Amersham). This transfer could be done within 1 hr using a vacuum/pressure blotter (Model RB-30S, Nippon Genetics, Tokyo). The RNA was UV-crosslinked to the membrane (UV crosslinker, Stratagene, La Jolla, CA). The membrane was incubated at 65°C for 1 hr in a prehybridization solution [5 x SSPE (1 x SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.7), 5 x Denhardt's solution (8), and 0.5% SDS] containing 20 µg/ml denatured, fragmented salmon sperm DNA. The D-amino-acid oxidase cDNA fragment amplified above by PCR was labeled with [-32P]dCTP (~110 TBq/mmol, Amersham) basically by the method of Feinberg and Vogelstein (9). DNA Labeling Kit (Takara) or Multiprime DNA Labeling System (Amersham) was used. After being heat-denatured, the probe was added to the prehybridization solution. Hybridization was done at 65°C for about 16 hr in a roller bottle in a hybridization oven (Maxi 14, Hybaid, Middlesex). The membrane was washed twice for 10 min each in a solution of 2 x SSPE and 0.1% SDS at room temperature, once for 15 min at 65° in a solution of 1 x SSPE and 0.1% SDS, and twice for 10 min each at 65°C in a solution of 0.1 x SSPE and 0.1% SDS. It was wrapped with Saran Wrap and exposed to an imaging plate (Fuji Film, Tokyo). The plate was read in an imaging analyzer (BAS 2000II, Fuji Film). In place of the imaging plate and imaging analyzer, conventional autoradiography could be used to detect the hybridizing signal. However, this process

required more time. Under these conditions, a hybridizing 2-kb band was detected in the kidney and cerebellum of the mouse, and in the kidney, liver, and hindbrain of the rat.

RESULTS AND DISCUSSION

D-Amino-acid oxidase activity was detected in the kidneys of human, monkey, rat, mouse, chicken, frog, carp, dace, crucian carp, cat fish, rainbow trout, and electric ray (10, 11). D-Amino-acid oxidase protein was detected by western blotting in the kidney and brain of the mouse, and in the kidney of the rat (12-14). RT-PCR amplified a D-amino-acid oxidase cDNA fragment from RNA extracted from the kidney and brain of the mouse, and from the kidney, liver, and cerebellum of the rat (13, 14). Northern hybridization showed the presence of mRNA for D-amino-acid oxidase in the kidney and cerebellum of the mouse, and in the kidney, liver, and hindbrain of the rat (13, 14).

All the vertebrates examined so far have D-amino-acid oxidase in their livers (1, 2, 15, 16). However, the mouse liver did not show positive results in the D-amino-acid oxidase enzyme activity assay, western blotting, RT-PCR, and northern hybridization. Therefore, we concluded that the mouse does not have this enzyme in its liver (13). The mouse is a very unique animal in this respect.

Northern hybridization, RT-PCR, and western blotting gave positive results for the presence of D-amino-acid oxidase in the ddY/DAO$^-$ mice (12, 17). However, the kidney and brain homogenates of these mice did not show D-amino-acid oxidase activity (18, 19). Therefore, we concluded that they produced the D-amino-acid oxidase protein without enzyme activity. This lack of activity was due to a single-base substitution in the coding region of the cDNA for this enzyme (17).

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