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D-Amino acid oxidase and presence of D-proline in *Xenopus laevis*

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ABSTRACT

We purified D-amino acid oxidase (EC 1.4.3.3, DAO) from *Xenopus laevis* tadpoles. The optimal temperature and pH for enzyme activity were 35–40 °C and 8.3–9.0, respectively, depending on the substrate amino acids available to the enzyme; the highest activity was observed with D-proline followed by D-phenylalanine. Activity was significantly inhibited by *p*-hydroxymercuribenzoate, but only moderately by *p*-chloromercuribenzoate or benzoate. Enzyme activity was increased until the final tadpole stage, but was reduced to one-third in the adult and was localized primarily in the kidney. The tadpoles contained high concentrations of D-proline close to the final developmental stage and nearly no D-amino acids were detected in the adult frog, indicating that D-amino acid oxidase functions in metamorphosis.

1. Introduction

D-Amino acid oxidase (EC 1.4.3.3, DAO) is one of the most extensively investigated flavoenzymes with FAD as a prosthetic group. DAO catalyzes the oxidative deamination of a neutral free D-amino acid, producing an imino acid, which is subsequently hydrolyzed nonenzymatically to a 2-oxo acid and ammonia (Hafner and Wellner, 1971). The reduced enzyme is reoxidized by molecular oxygen, generating hydrogen peroxide. DAO is a peroxisomal enzyme present in the liver, kidney, and brain, among other organs (Krebs, 1951; Weimar and Neims, 1977). The enzyme has been found in a variety of organisms, ranging from microbes to mammals (Krebs, 1935; Corrigan et al., 1963; Pilone Simonetta et al., 1989; Abe et al., 2005).

Xenopus laevis is an anuran amphibian species that remains aquatic after metamorphosis and can be maintained in a homogenous laboratory environment. *X. laevis* is a nonmammalian model animal of the NIH *Xenopus* Initiative that has established many genetic and genomic resources to provide insights into all areas of biomedical research (Klein et al., 2002). In this project, the amino acid sequence of the *X. laevis* DAO is reported as one of these genomic resources; however, its enzymatic characteristics and biological function are unknown. In this study, we purified DAO from the tadpole and investigated basic enzymatic characteristics. Furthermore, we investigated the contents of several D-amino acids in *X. laevis* and DAO activity on developmental stages of *X. laevis* to gain insight into the biological role of DAO. Since we found a substantial amount of D-proline in the tadpole, we examined the role of DAO in the metamorphosis from tadpole to frog.

2. Materials and methods

2.1. Animals

Male and female adult frogs of *X. laevis* were purchased from Xenopus Aquaculture for Teaching Materials (Koga, Ibaraki, Japan). To obtain embryos, 0.10 mL and 0.15 mL of human chorionic gonadotrophin, Gonatropin 3000 (Aska, Tokyo, Japan) were injected into the body cavity of male and female adults, respectively. The following day, the jelly layer around the oviposited eggs was dissolved with 142 mM L-cysteine hydrochloride monohydrate to remove the layer. After hatching, tadpoles were reared in the laboratory, fed an XL No. 3 artificial diet (Oriental Yeast, Tokyo) every 2 days and maintained under controlled light-dark (16-h:8-h) and temperature (20–25 °C) conditions in a bat containing aerated chlorine-free tap water. At each stage, some tadpoles were stored at −80 °C.

Tadpole stages were defined by the emergence and length of the hindlimb, forelimb, and tentacle, according to the external stage criteria described by Nieuwkoop and Faber (1967).

2.2. Reagents

Chemical reagents used as DAO inhibitors included benzoic acid (Wako, Osaka, Japan), *p*-hydroxymercuribenzoic acid (PHMB; Sigma-Aldrich, St. Louis, MO, USA), *p*-chloromercuribenzoic acid (PCMB;

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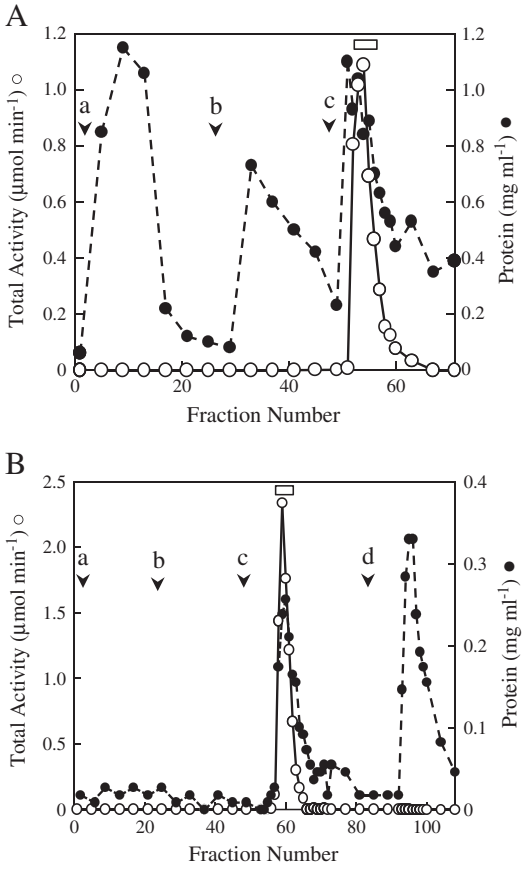

Fig. 1. Purification of *X. laevis* DAO. Cell-free extract from *X. laevis* tadpoles at stage 54–57 was purified by chromatography using Toyopearl butyl and Q Sepharose. A, Elution pattern of the Toyopearl butyl 650 M column chromatography. Proteins were eluted with Buffer A (pH 8.2) containing a, 30%-; b, 10%-; and c, 0%-saturated ammonium sulfate. B. Elution pattern of the Q Sepharose column chromatography. Proteins were eluted with Buffer B (pH 9.0) containing a, 0 mM; b, 20 mM; c, 150 mM; and d, 300 mM NaCl. ○, DAO activity (μmol min^{-1}); ●, protein concentration (mg mL^{-1}). White bar, active fractions used for the next purification step. Representative results are shown.

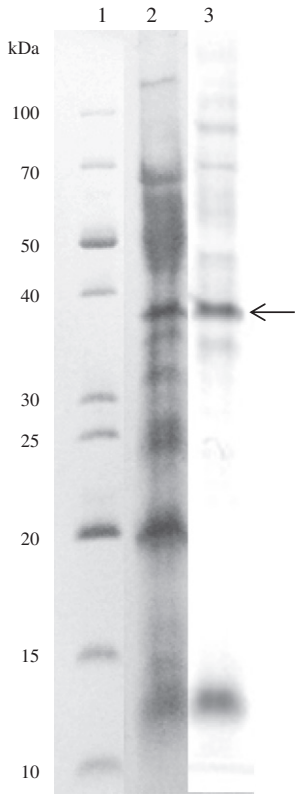

Fig. 2. SDS–PAGE of DAO-containing protein fractions. Lane 1, molecular marker proteins; lane 2, eluate from Toyopearl butyl column; lane 3, enzyme fraction purified by the second Q Sepharose column chromatography. Arrow, protein band analyzed for the N-terminal amino acid sequence.

Sigma), iodoacetamide (Wako), and iodoacetic acid (Kanto Chemical, Tokyo, Japan). Other reagents were of analytical or HPLC grade.

2.3. Purification of DAO

All experimental procedures were carried out at 4 °C. Tadpoles (stage 54–57, approximately 60 g) or adult organs (kidney, liver, gut, and stomach, each 70 to 80 g) were minced with scissors, and homogenized with an equal volume or four volumes (w/v) of 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, and 0.4% protease inhibitor cocktail (Sigma) using a polytron-type homogenizer (Ultra Turrax T25; IKA, Staufen, Germany) at 11,000 rpm for 10 min, and then centrifuged at 14,500 ×*g* for 20 min. The supernatant, which included the cell-free extract, was further centrifuged at 140,000 ×*g* for 40 min. Finely ground solid ammonium sulfate was added to the supernatant, and the pellets with 30–45%-saturated ammonium sulfate were collected by centrifugation. Pellets were dissolved in a small volume of 50 mM Tris-HCl buffer (pH 8.2) and applied to a Toyopearl butyl 650 M (Tosoh, Tokyo) column (12 mm i.d. × 83 mm), which had been equilibrated with Buffer A [50 mM Tris-HCl buffer, pH 8.2, 10% glycerol, and 1 μM flavin adenine dinucleotide (FAD)] containing 30%-saturated ammonium sulfate. After washing the column with Buffer A containing 30%, followed by 10% saturated ammonium sulfate, the active enzyme fractions were eluted with Buffer A. Active fractions were combined and dialyzed against 500 mL of Buffer B composed of 50 mM Tris-HCl buffer (pH 9.0), 10% glycerol, 1 μM FAD, and 2 mM EDTA, four times each for 2 h. The dialysate was applied to a Q Sepharose column (25 mm i.d. × 30 mm) equilibrated with Buffer B. After washing the column with Buffer B, proteins were eluted with Buffer B containing 20 mM, 150 mM, and 300 mM NaCl, in this order. The effluent with Buffer B containing 150 mM NaCl was dialyzed against Buffer B and Q Sepharose column purification (column size, 12 mm i.d. × 58 mm) was repeated as described above.

Protein concentration was determined using Bradford's method (Bradford, 1976) with bovine serum albumin as a standard.

Table 1
Purification of DAO from *Xenopus laevis* tadpole.

	Specific activity (nmol min^{-1} mg^{-1})	Total activity (nmol min^{-1})	Yield (%)	Purification (-fold)
Cell-free extract	1.8[13.5]	198,018	100	1.0[1.0]
140,000 × *g*-Supernatant	6.6[14.3]	92,335	46.6	3.6[1.1]
Ammonium sulfate pellet	54.8[61.0]	24,065	26.1	30.4[4.5]
Toyopearl butyl	1162[413]	13,577	6.9	646[31]
Q Sepharose, 1st	2997[1199]	5,135	2.6	1665[89]
Q Sepharose, 2nd	6414[2225]	3,685	1.9	3563[165]

The reaction was carried out with 20 mM D-proline in 20 mM Tris-HCl (pH 8.5) at 37 °C for 10 min. Values in [] are for purification of DAO from kidneys of adult frogs.