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Distribution and Substrate Specificity of D-Amino Acid and D-Aspartate Oxidases in Marine Invertebrates

Mohammed Golam Sarower,[a,b*] Takashi Matsui[a] and Hiroki Abe[a]

[a] Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan.
[b] Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna-9208, Bangladesh.

* Corresponding author, E-mail: sarower@gawab.com

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ABSTRACT: The distribution of D-amino acid oxidase (DAO, EC 1.4.3.3) and D-aspartate oxidase (DDO, EC 1.4.3.1) activities was examined on several tissues of various marine invertebrate species. Only DAO activity was detected in kidney and midgut gland from most of the mollusks with some exceptions. Hepatopancreas or liver from kuruma prawn, crayfish, squid, and octopus and antenal gland from crayfish showed significant amount of activity of both enzymes. Squid demonstrated a higher activity of DDO than that of DAO. D-Proline was the best substrate for DAO in common flying squid liver followed by D-alanine, D-histidine, D-tyrosine, and D-phenylalanine. On the contrary, *meso*-2,3-diaminosuccinate was the best substrate for DDO followed by *N*-acetyl-DL-aspartate, D-glutamate, D-amino adipate, and D-homocysteic acid. These enzymes, at least in kidney and midgut gland, are believed to metabolize exogenous and endogenous free D-alanine, that is abundant in aquatic invertebrates such as crustaceans and bivalve mollusks.

KEYWORDS: D-Amino acid oxidase; D-Aspartate oxidase; marine invertebrates; D-Amino acid; and substrate specificity.

INTRODUCTION

D-Amino acid oxidase (DAO, EC 1.4.3.3) and D-aspartate oxidase (DDO, EC 1.4.3.1) are highly stereoselective flavoenzymes containing FAD as the prosthetic group. They catalyze oxidative deamination of D-amino acids, according to the following scheme:

$$H_2NCHRCOOH + E\text{-}FAD \rightarrow HN{=}CRCOOH + E\text{-}FADH_2 \quad (1)$$

$$E\text{-}FADH_2 + O_2 \rightarrow E\text{-}FAD + H_2O_2 \quad (2)$$

$$HN{=}CRCOOH + H_2O \rightarrow RCOCOOH + NH_3 \quad (3)$$

The reductive half-reaction, the only enzymatic step of the whole scheme, dehydrogenates the amino acid to the corresponding imino acid, coupled with the reduction of FAD (1). FAD reoxidizes spontaneously in the presence of molecular oxygen, which in turn is reduced to hydrogen peroxide (2). The imino acid then is hydrolyzed, nonenzymically, to the α-keto acid and ammonia (3).

DAO was discovered by Krebs [1] and has been known to distribute widely in nature from bacteria to mammals. DDO, specific to acidic D-amino acids, was first reported by Still *et al* [2] and has been examined extensively in mammals and microorganisms. On the basis of the common properties shared by these two enzymes and the homology of amino acid sequences, it has been suggested that they were derived from the divergent evolution of a single gene. The nucleotide sequences of DAO-encoding cDNAs have been determined in pig[3], human[4], mouse[5], rabbit[6], rat[7], carp[8] and microbes[9]. On contrary DDO has been cloned only from beef kidney[10] and human brain[11]. In spite of the extensive research on both enzymes in mammals and microorganisms, only a little information has been available for the other organisms including aquatic animals. Several results, however, have reported the presence of these enzymes in aquatic animals, such as the presence of both DAO and DDO in octopus[2, 13], and that of DDO in octopus [14] and in several fish species[15, 16].

Although these enzymes were discovered 70 years ago[1], their physiological function still remains unclear. Recently, significant quantities of D-amino acids have been detected in various animals including humans. For instance, a large amount of D-alanine has been found in various aquatic invertebrates such as crustaceans and bivalve mollusks[17]. Thus, aquatic animals are considered to have a high chance of intake of D-amino acids through predation compared with terrestrial animals where the D-amino acid source is rather limited to bacteria in foods and intestine or food consisting of aquatic invertebrates. These enzymes have several current and potential biotechnological

applications for the production of α-keto acids, chiral intermediates for antihypertensive drugs, antihuman immunodeficiency virus protease inhibitor, biosensors, and many semi-synthetic cephalosporins[18].

It is suggested that DAO and DDO are detoxifying agents that eliminate D-amino acids derived from both exogenous and endogenous sources[13]. Although several experimental data clarified that these enzymes metabolize D-amino acids, more positive evidence is required for verifying the physiological role of these enzymes. Information on the distribution and properties of these enzymes in aquatic animals would be useful in delineating the physiological function of the enzymes, because of the daily oral intake of D-amino acids by aquatic animals. In our previous study, we investigated distribution pattern of these enzymes in fish[16]. In this study, we examined on the distribution of DAO and DDO in the tissues of various marine invertebrates. We also determined the substrate specificity of these enzymes in liver from squid.

Materials and Methods

Chemicals

All chemicals were of analytical grade. Trichloroacetic acid, 2,4-dinitrophenylhydrazine, pyruvic acid, FAD, hydrogen peroxide, 2-oxoglutaric acid, H_2O_2, and glycylglycine were purchased from Wako Pure Chemical Industries Ltd., Osaka. *N*-Methyl-D-aspartic acid, D-aminoadipic acid, *meso*-2,3-diaminosuccinic acid, D-homocysteic acid, *N*-acetyl-DL-aspartic acid, D-hydroxyglutaric acid, *cis*-2,3-piperidinedicarboxylic acid, peroxidase, sodium 3,5-dichloro-2-hydro-xybenzenesulphonic acid, 4-aminoantipyrine, benzoic acid, bovine serum albumin, and NaN_3 were purchased from Sigma Chemical Co., Missouri. *meso*-Tartaric acid was from Tokyo Kasei Kogyo Co. Ltd., Tokyo.

Animal

All live marine invertebrate species were purchased from the Central Wholesale Fish Market of Tokyo. Species used were as follows: crayfish *Procambarus clarkii* (n = 6), kuruma prawn *Penaeus japonicus* (n = 10), geoduck *Tresus keenae* (n = 2), hard clam *Meretrix lusoria* (n = 6), scallop *Patinopecten yessoensis* (n = 5), mussel *Mytilus galloprovicialis* (n = 5), button shell *Tectus maximus* (n = 3), ark shell *Scapharca broughtonii* (n = 6), turban shell *Batillus cornutus* (n = 6), octopus *Octopus vulgaris* (n = 1), and common flying squid *Todarodes pacificus* (n = 7). These species were chosen because they belong to a variety of subclasses and orders of mollusks and crustaceans, and were readily available. They were transported to the laboratory and were dissected immediately under hypothermic anesthesia by dipping them in crushed ice bath. Tissues were removed quickly and combined together on the organ basis with those from numbers of animals and stored at – 20 °C until use.

Preparation of Crude Enzyme Solution

For enzyme assay, 1 g tissue sample was homogenized with 3 ml of 0.1 M Tris-HCl, pH 8.2, at 2-4 °C with a Polytron homogenizer (Kinematica, Switzerland) and centrifuged at 10,000 rpm for 15 min. The supernatant was dialyzed overnight against the same buffer and used as a crude enzyme solution after centrifugation.

Enzyme Assay

Activities of DAO and DDO were determined according to the 'keto acid method' described by D'Aniello *et al*[13]. In brief, the enzyme solution (200 µl) was incubated with equal volume of 0.1 M D-alanine dissolved in 0.1 M Tris-HCl, pH 8.2, for 30 min at 37 °C. The reaction was terminated by adding 200 µl of 25% trichloroacetic acid, followed by centrifugation. 400 µl aliquots of supernatant were mixed with 400 µl of 1 mM 2,4-dinitrophenylhydrazine in 1 M HCl and incubated at 37 °C for 10 min. Subsequently, 800 µl of 1.5 M NaOH was added. After a further incubation for 10 min, the absorbance was read at 445 nm. The activity of DDO was measured in the same way, except that D-alanine was replaced by D-aspartate as substrate.

The 'H_2O_2 method' was only employed to determine the substrate specificity of both enzymes as described previously[19] with the following modifications. A reaction mixture (400µl) contained 100µl each of the enzyme solution, solution A, 0.1 M D-amino acids and H_2O. Solution A consisted of 1.0 mL of 0.2 M pyrophosphate, pH 8.3, 40 µL of 0.5 mg/mL FAD, 40 µL of 5 mg/mL bovine serum albumin, 60 µL of 12 mg/mL peroxidase, 50 µL of 60 mg/mL sodium 3,5-dichloro-2-hydroxybenzenesulphonic acid, 5 µL of 90 mg/mL 4-aminoantipyrine, and 3 µL of 10 mM NaN_3. The incubation for H_2O_2 production coupled with chromogen was carried out at 37 °C for 10 min and the reaction was terminated by adding 1 mL of 0.1 M sodium borate, pH 10. After 2 min at room temperature, the resulting quinone-imine dye was quantified at 500 nm. The amount of substrate oxidized in the reaction in both methods was calculated based on the difference in absorbance of the reactions with and without substrate, and the calibration curve for keto acids or H_2O_2. One enzymatic unit of DAO or DDO was defined as the amount of enzyme that oxidized in 1 min 1 µmol of D-amino acids, to produce 1 µmol of keto acid or H_2O_2, under the above assay conditions. Protein concentration of the crude enzyme solution was determined by the method of Lowry *et al*[20] using bovine serum albumin standard.

Results and Discussion

Distribution of DAO and DDO in Marine Invertebrates

Eleven different marine invertebrate species were used to determine the distribution pattern of DAO and DDO in invertebrate. The results shown in Table 1 indicate that most of the mollusks had the activity of DAO in kidney and midgut gland. In the kidney of octopus, the activities of both enzymes were much higher than those in the other species, and DDO activity surpassed DAO one. Only little DAO activity was detected in the kidney and midgut gland of ark shell and scallop, while no DDO activity was detected. Turban shell and mussel had the activity of both enzymes in the kidney, but they had no DDO activity in midgut gland. Hard clam exceptionally had DDO in midgut gland but not in kidney.

No activity of both enzymes was found in muscle except for the species cited in Table 2. The activities of both enzymes in muscle of geoduck, hard clam, and octopus were negligible, whereas DDO activity in scallop muscle was noticeable. Table 2 also indicates the activities of both enzymes in the gills of scallop, octopus, and crayfish. Scallop and octopus had the activities of both enzymes, whereas crayfish contained only DAO activity.

Table 1. DAO and DDO activities in the kidney and midgut gland of invertebrates.

Species	Kidney		Midgut gland	
	DAO	DDO	DAO	DDO
Turban shell	0.34±0.01	0.13±0.01	3.33±0.04	ND
Ark shell	0.13±0.01	ND	0.2±0.01	ND
Scallop	0.53±0.03	ND	0.13±0.01	ND
Mussel	0.73±0.07	0.43±0.03	1.35±0.02	ND
Geoduck	0.55±0.02	ND	ND	ND
Hard clam	0.26±0.05	ND	ND	0.67±0.02
Octopus	3.72±0.03	4.93±0.05	—	—

DAO = D-amino acid oxidase (nmol/h/mg)
DDO = D-aspartate oxidase (nmol/h/mg)
ND = not detected
— = not tested
Mean±SD was calculated from three separate analyses

Table 2. DAO and DDO activities in the muscle and gill of invertebrates.

Species	Muscle		Gill	
	DAO	DDO	DAO	DDO
Scallop	ND	0.4±0.02	0.13±0.01	0.67±0.04
Geoduck	0.1±0.01	ND	—	—
Hard clam	ND	0.07±0.01	—	—
Octopus	0.1±0.01	0.2±0.03	0.17±0.01	0.13±0.02
Crayfish	ND	ND	0.20±0.02	ND

See the legend of table1 for abbreviations

Table 3. DAO and DDO activities in hepatopancreas or liver and antenal gland of invertebrates.

Species	Hepatopancreas/Liver		Antenal gland	
	DAO	DDO	DAO	DDO
Kuruma prawn	2.63±0.06	ND	ND	0.13±0.01
Crayfish	0.13±0.01	0.13±0.01	0.13±0.01	1.07±0.05
Squid	2.70±0.03	11.86±0.70	—	—
Octopus	1.83±0.04	11.90±0.90	—	—

See the legend of table1 for abbreviations

As shown in Table 3, hepatopancreas or liver of kuruma prawn, crayfish, squid, and octopus contained noticeable amounts of activities of both enzymes. Squid and octopus had a higher activity of DDO than that of DAO, whereas kuruma prawn produced only DAO activity. DDO activity was much higher than DAO one in the liver of squid and octopus. Hepatopancreas of the other species was not examined for enzyme activities. Antenal gland of kuruma prawn and crayfish (Table 3) showed the activities of these enzymes. The antenal gland of crayfish contained higher DDO activity than DAO one, but kuruma prawn showed only DDO activity. Mantle muscle, adductor muscle, foot muscle, and gonad were taken from most of these species to determine the activity, but no activity was detected in these organs except for octopus and scallop. Octopus contained the activities of both enzymes in gonad, mantle muscle, and stomach, while scallop contained them in gonad (data not shown). The activities of these enzymes at least in kidney and midgut gland are considered to be involved in the metabolism of D-amino acids.

Of these species used in this study, all crustaceans and some mollusks were reported to contain some D-amino acid, especially D-alanine, in their tissues[17]. Muscle, gill, and hepatopancreas of kuruma prawn and crayfish contained considerable amounts of D-alanine and small amounts of D-arginine, D-aspartate, and D-glutamate[21], but no activity of these enzymes was detected in muscle and gill except for DAO activity in the hepatopancreas of kuruma prawn and both enzyme activities in crayfish hepatopancreas and antenal gland. Several crustaceans contained a sizable amount of D-alanine in muscle, gill, nervous tissues, and hepatopancreas [17]. These crustaceans have also been reported to have alanine racemase in these tissues[22]. Thus, at least in hepatopancreas of crustaceans, DAO and/or DDO might be expected to have some roles in the metabolism of D-amino acids. Adductor, foot, and mantle muscles and mid gut gland of hard clam and geoduck contained copious amounts of D-alanine and small amounts of D-arginine, D-aspartate, and D-proline[17]. In contrast, almost no activity of DAO and/or DDO was detected in these organs. In

the kidney of both species, however, weak activity of DAO was observed. Thus, DAO in kidney may work to recover keto acids from D-amino acids before excretion. In muscle and midgut gland in these bivalves, the level of D-alanine may be regulated mainly by alanine racemase.

Ark shell, scallop, and oyster as well as squid and octopus contained only trace amounts of D-amino acids in the muscle tissues[14, 17]. All organs of octopus showed substantial activities of both enzymes. Thus, at least in octopus, D-amino acids might be decomposed effectively by these oxidases. In the other species, however, no clear inverse relationship was found between the activities of DAO and DDO and D-amino acid contents. Thereby, these enzymes should be considered to be involved in the regulation of small amounts of D-amino acids in invertebrates.

At present, the catabolic pathway known for D-amino acids is either through racemization catalyzed by a racemase enzyme or through oxidative deamination catalyzed by DAO or DDO. The properties of DDO of *Octopus vulgaris* have been studied extensively[23]. Both of these enzyme activities are commonly high in kidney and liver and low in intestine of fish, and are believed to eliminate D-amino acids from fish tissues[16]. In crustaceans and mollusks tested in this study, only squid liver contained the activities of both oxidases equally matched with those in octopus liver. Thus, squid liver enzymes merit further study.

Substrate Specificity

Substrate specificity of DAO and DDO in common flying squid liver were determined, because the animal showed substantial activities of both enzymes among the species examined. Substrate specificity of DAO is illustrated in Fig 1. D-Proline was the best substrate for DAO in this species, followed by D-alanine, D-histidine,

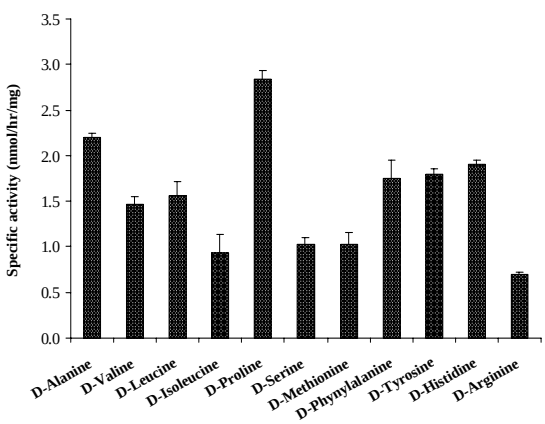

Fig 1. Substrate specificities of DAO in liver of common flying squid. Mean±SD was calculated from triplicate determinations.

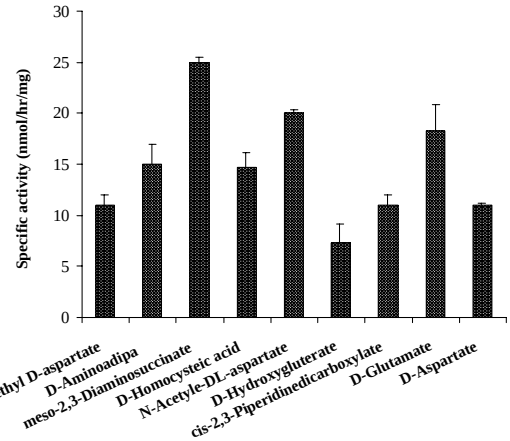

Fig 2. Substrate specificities of DDO in liver of common flying squid. Mean±SD was calculated from triplicate determinations.

D-tyrosine, D-phenylalanine, D-leucine, and D-valine. On the other hand, *meso*-diaminosuccinate was found to be the best substrate for DDO, followed by *N*-acetyl-DL-aspartate, D-glutamate, D-amino adipate, and D-homocysteic acid (Fig 2). Although both enzymes are restricted in their action to the D-isomers of their substrate, substrate specificity is strikingly different. DAO oxidizes many monocarboxylic D-amino acids and their derivatives, but not the dicarboxylic ones and their derivatives. DDO, on the other hand, oxidizes only dicarboxylic D-amino acids and their derivatives. Flying squid liver DAO demonstrated almost the same order of preference for substrate as DAO[16] found in rainbow trout kidney, common carp kidney and hepatopancreas. D-Proline was found the best substrate for mammalian DAO[24]. D-Proline, D-methionine, and D-phenylalanine were reported to be better substrates than D-alanine for DAO from hog kidney[12].

Meso-2,3-diaminosuccinate has been reported to be a good substrate at high pH (9.5) for a partially purified DDO from beef kidney[25]. However, the oxidation of the compound by the avian DDO was considerably slower, irrespective of species. This trend is in good agreement with mammalian data on tissue homogenates from rat and human[26]. It was also a good substrate for DDO from fish[16]. A good performance for D-glutamate of DDO from flying squid liver is in agreement with those of octopus[23], microorganism[27], and carp kidney enzyme[16], and in marked contrast with those of the mammalian[26], and amphibian enzyme[28], in which D-glutamate was a poor substrate. D-Glutamate was also reported to be a poor substrate in comparison with D-aspartate for DDO from the liver of carp, crucian carp, rainbow trout, yellowtail, and red sea bream[15], but flying squid liver showed the opposite result. D-

Aspartate was the best substrate for DDO from beef kidney and octopus liver, followed by D-glutamate, *N*-methyl D-aspartate (NMDA), and D-asparagine[12]. In comparison with D-aspartate, NMDA was a better substrate for DDO from fish[16]. This is common to DDO of many vertebrates such as amphibians[28] and birds[29], although the opposite relationship is reported for rat renal cortex[2] and human liver[25]. It is noteworthy that substrate specificity of DAO or DDO from microbial to mammalian sources is greatly varied. No common trend of preference order of these enzymes toward D-amino acids has been found yet among mammals or microbes. Although primary structures of DAO or DDO from various sources are significantly different from each other, the catalytic residues in the active site are conserved[3-11]. The variation of DAO or DDO structures also provides different conformations of active site cavities[30]. Similarly plasticity of active site cavity is required for binding a broad range of substrate. So, the affinity order of these enzymes toward substrate is highly subjected to the conformation of active site cavity and the mode of substrate orientation. However, the reason is not clear why both of these enzymes in squid liver prefer derivative substrates than their native ones. Thereby, study on three-dimensional structures of these enzymes with particular substrate will be able to address the aforementioned reason.

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