Current Topics

D-Amino Acid Biosystem

Physiological Function and Metabolism of Free D-Alanine in Aquatic Animals

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Aquatic crustaceans and some bivalve mollusks contain a large amount of free D-alanine (up to 100 μmol/g wet wt.) in their tissues. Under high salinity stress, crustaceans and bivalve mollusks largely accumulate D- and L-alanine irrespective of species examined, together with L-glutamine, L-proline, and glycine of which increases are species dependent. These data indicate that D-alanine is one of the major compatible osmolytes responsible for the intracellular isosmotic regulation in the tissues of crustaceans and bivalves. Alanine racemase has been proven to catalyze the interconversion of D- and L-alanine in these invertebrates. The enzyme has been isolated to homogeneity from the muscle of black tiger prawn *Penaeus monodon* and its cDNA has been cloned from the muscle and hepatopancreas of kuruma prawn *Penaeus japonicus* for the first time in eukaryotes other than yeast. Several fish species fed on crustaceans and mollusks contain D-amino acid and D-aspartate oxidases that catalyze the decomposition of D-amino acids. A cDNA of D-amino acid oxidase has been cloned from the hepatopancreas of omnivorous common carp *Cyprinus carpio*. During oral administration of free D-alanine to carp, the activity and mRNA of D-amino acid oxidase increased rapidly in hepatopancreas and the increases were highest in intestine followed by hepatopancreas and kidney. These data suggest that D-amino acid oxidase is inducible in carp and an important enzyme responsible for the efficient utilization of carbon skeleton of D-alanine in their feeds.

Key words D-alanine; alanine racemase; invertebrate; osmoregulation; D-amino acid oxidase; fish

1. INTRODUCTION

Organisms on this planet maintain homochirality by employing only L-enantiomers as proteinaceous amino acids. As the results, D-amino acids had long been considered not to exist in organisms other than eubacteria. Due to the recent advance of analytical techniques, however, free D-amino acids are clarified to exist even in mammals and the research data on the metabolism and physiological roles of D-amino acids in eukaryotes have been accumulated rapidly in this decade.

In invertebrates, however, D-amino acid research has unexpectedly long history. In insect species, D-alanine and D-serine has long been known to exist in milkweed bug and silkworm, respectively.[1] Serine racemase was recently purified partially from silkworm.[2] In aquatic invertebrates, D'Aniello and Giuditta[3] discovered over 10 μmol/g wet wt. of D-aspartate in the brain of octopus *Octopus vulgaris* in 1977 and thereafter found 1—9 μmol/g of D-alanine in several crustacean muscles in 1980.[4] After that the report on invertebrate D-amino acid occurred sporadically in 1980s. In 1984, Matsushima *et al.*[5] found 25 μmol/g of D-alanine in the mantle muscle of a small brackish water clam *Corbicula japonica* and reported that a high concentration of D-alanine existed in the mantle and foot muscles of short-necked clam *Tapes philippinarum* and hard clam *Meretrix lamarckii* but not in mussel *Mytilus edulis* and oyster *Crassostrea gigas*. They also detected alanine racemase activity in the foot muscle of *C. japonica*. On bivalve mollusks, Felbeck and Wiley[6] determined D- and L-enantiomers of alanine and aspartate using HPLC for the first time and reported that the foot muscle of bivalves, *Solemya reidi*, *M. edulis*, and *Crassostrea virginica* contained no D-alanine but the former two species contained a considerable amount of D-aspartate. Aspartate racemase was also detected in *S. reidi*.[7] Moreover, they found an extremely large amount of D-alanine, 22—187 μmol/g, in a lucinid clam *Codakia obicularis*.

In these research works until the end of 1980s, D-amino acids had been determined by the methods using D-amino acid or D-aspartate oxidase. Thus, the methods were not specific to each D-amino acid. In 1990s, several highly sensitive HPLC have made possible to separate the enantiomers of amino acids. The authors developed a high-resolution HPLC method enabled the separation of all free D- and L-amino acids and other physiological amino acids using (+)-1-(9-fluorenyl)ethyl chloroformate as a pre-labeling reagent and reversed-phase ion-pair HPLC.[8]

2. DISTRIBUTION OF D-AMINO ACIDS IN AQUATIC INVERTEBRATES

The distribution of D-alanine in crustaceans is shown in Table 1.[8,9] D-Alanine exists in all crustacean species and tissues. The percentage of D-alanine to total alanine does not exceed 50% in crustaceans with some exceptions. D-Alanine is also found in the gills and hemolymph of crustaceans but the content is low compared with the other tissues. Other than D-alanine, several D-amino acids were also detected in crustacean tissues, although the contents are below 1 μmol/g.

The distribution of D-alanine in several bivalve mollusks is also shown in Table 1.[10] Bivalves belonging to subclass Pteriomorphia, such as ark (blood) shell *Scapharca broughtonii*, oyster *C. gigas*, and scallop *Patinopecten yessoensis*, contain almost no D-alanine in their tissues, corresponding to the above mentioned data.[5,6] Recently, *S. broughtonii* was reported to have a large amount of D-aspartate and aspartate racemase activity in the mantle and foot muscles,[11] and the aspartate racemase was isolated quite recently from foot

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Table 1. Distribution of Free D-Alanine in Aquatic Crustaceans and Mollusks

Species	Tissues	D-Alanine (μmol/g wet wt.)	D/(D+L) (%)
Crustaceans			
Kuruma prawn (n=35)	Tail muscle	3.43	38.2
Penaeus japonicus	Heart muscle	4.15	37.9
	Hepatopancreas	6.96	40.9
Crayfish (n=20)	Tail muscle	3.23	32.1
Procambarus clarkii	Heart muscle	3.97	29.8
	Nervous tissue	3.43	35.3
Rock lobster (n=5)	Tail muscle	6.07	45.6
Jasus lalandi	Heart muscle	14.6	60.1
	Hepatopancreas	11.4	50.4
Snow crab (n=3)	Leg muscle	11.9	44.4
Chionoecetes opilio	Hepatopancreas	6.92	34.7
	Nervous tissue	9.74	33.9
Japanese mitten crab (n=5)	Leg muscle	16.8	41.2
Eriocheir japonicus	Nervous tissue	9.72	24.1
	Eye	4.39	37.5
Mollusks (Pterimorphia)			
Ark shell (n=5)	Adductor muscle	0.06	0.1
Scapharca broughtonii	Foot muscle	ND	—
Oyster (n=12)	Mantle muscle	ND	—
Crassostrea gigas	Mid-gut gland	ND	—
Scallop (n=5)	Adductor muscle	ND	—
Patinopecten yessoensis	Mid-gut gland	0.02	0.3
Mollusks (Heterodonta)			
Hard clam (n=3)	Adductor muscle	29.4	54.0
Meretrix lusoria	Mantle muscle	18.1	48.3
	Foot muscle	31.2	48.1
Short-necked clam (n=38)	Adductor muscle	13.3	47.8
Ruditapes philippinarum	Siphon	8.68	38.8
	Mid-gut gland	7.01	32.9
Sakhalin surf-clam (n=2)	Mantle muscle	25.0	67.8
Pseudocardium sachalinensis	Siphon	26.7	74.8
	Gonad	4.97	37.7
Otter shell (n=1)	Mantle muscle	32.4	80.7
Tresus keenae	Siphon	50.6	83.6
	Gonad	15.8	62.0

muscle.[12] In contrast, bivalves belonging to subclass Heterodonta contain an extremely large amount of D-alanine in all tissues. In particular, otter shell *Tresus keenae* has over 50 μmol/g of D-alanine in siphon of which content sometimes reaches 100 μmol/g and the percentage of D-alanine to total alanine reaches over 80%. The other Heterodonta species also show high percentage of D-alanine. There also exist some other D-amino acids such as D-arginine, D-aspartate, D-proline in these tissues but their contents and percentages are not high. One exception is the above *S. broughtonii* where D-aspartate content reaches up to 5 μmol/g and the percentage falls within 40—77%.[10,11]

In invertebrate species other than crustaceans and mollusks, several species of annelids contain a large amount of unspecified D-amino acid.[13] We found 40 μmol/g of D-alanine in an annelid (unpubl. data). D-Alanine is also detected in sipunculid *Phascolosoma arcuatum*[14] and the percentage of D-alanine exceeds 50%. Sea urchin gonad has also been reported to contain D-alanine that decreased during development after fertilization, suggesting its involvement in the embryonic development.[15] D-Alanine content in sea urchin gonad sometimes exceeds 30 μmol/g (unpubl. data).

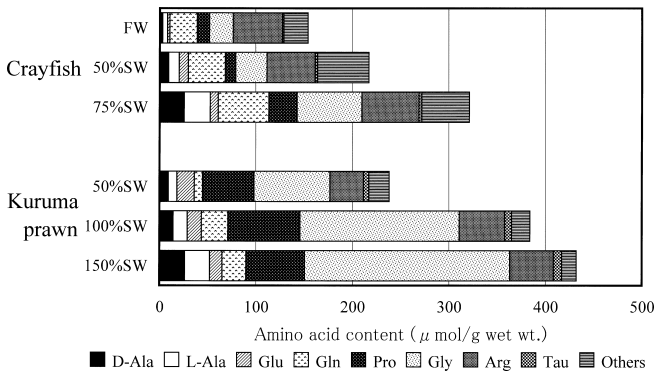


Fig. 1. Changes of Free Amino Acid Contents in Crustacean Muscle during Acclimation to Different Salinity

3. PHYSIOLOGICAL FUNCTIONS OF D-ALANINE UNDER OSMOTIC STRESS

From the distribution patterns of D-amino acids described above, D-alanine is possible to have important physiological roles in these animals. When red-swamp crayfish *Procambarus clarkii* was acclimated from freshwater to full seawater, the increase of alanine reached twice of glycine increase and a half of the alanine increase was attributed to D-form.[16] Thus, D-alanine is one of the most effective osmolytes responsible for the intracellular isosmotic regulation or cell volume regulation in this species. In crayfish acclimated to 0, 50, and 75% seawater, total free amino acid increased over twice from freshwater to 75% seawater (Fig. 1). The most responsible amino acids were D- and L-alanine which increased to 6.7-fold, and the percentage of D-alanine to total alanine also elevated from 38% in freshwater to 48% in 75% seawater. Next to D- and L-alanine, the glycine and L-glutamine contributions were large and L-proline largely increased only in 75% seawater. From these data, the above five amino acids are concluded to be responsible to increase the muscle osmotic pressure in crayfish. Such increase of amino acid osmolytes was also found in the other tissues such as cardiac muscle, hepatopancreas, and nervous tissues except for gills and hemolymph.

Kuruma prawn *Penaeus japonicus* was transferred from 100% seawater to 75 and 125% and to 50 and 150% seawater, respectively, after 2 d acclimation. The prawn acclimated to 50 and 150% seawater for 2 d showed the twice increase of total free amino acid (Fig. 1). In this species, glycine was the largest in amount and occupied almost half of the total amino acid in 150% seawater. Next to glycine, D- and L-alanine increased largely and the percentage of D-alanine was almost 50% regardless of the salinity. L-Glutamine and L-proline decreased in hyposalinity seawater. Thus, the most effective osmolytes in hypersalinity acclimation of this species are attributed to glycine and D- and L-alanine.

The most intriguing behavior of D- and L-alanine was found in Japanese mitten crab *Eriocheir japonicus*. This crab has a unique life cycle since it is a euryhaline catadromous species. Post-larval juvenile crabs migrate upstream from brackish water area, and grow up in freshwater river for three to five years. After maturation, adult crabs migrate downstream toward the sea from autumn to early winter for mating and spawning. This species is a strong osmoregulator and the

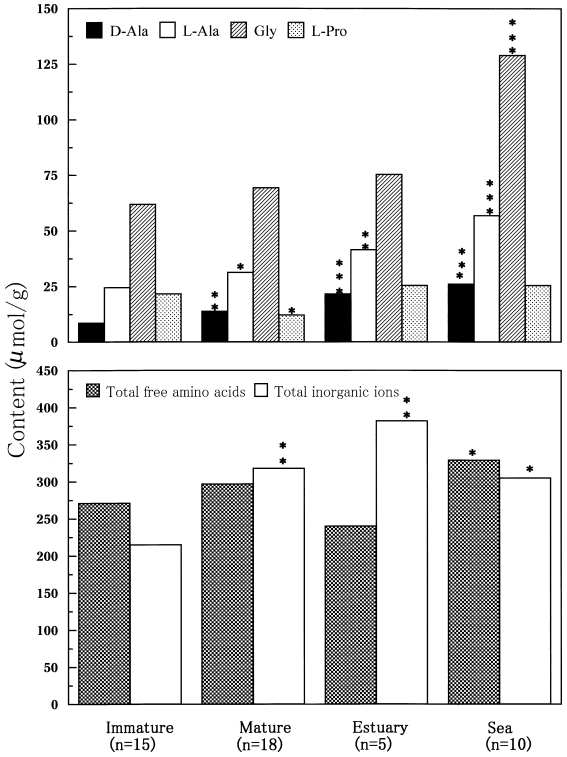


Fig. 2. Changes in Muscle Free Amino Acids and Inorganic Ions during Maturation and Downstream Spawning Migration of Japanese Mitten Crab *Eriocheir japonicus*[17]

Upper panel: Major free amino acids in muscle. Lower panel: Total free amino acids and total inorganic ions (Na^+, K^+, and Cl^-). Only mean values were shown for individuals written below. $*p<0.05$, $**p<0.01$, $***p<0.001$ compared with immature specimens.

hemolymph osmolality only increased from 660 mOsm/kg water in freshwater to 860 in full strength seawater. It is of interest whether even such a powerful osmoregulator utilizes D-alanine during their downstream migration. The crabs were captured at about 30 km upstream from the mouth of the river once a month from April to October. Brackish water and sea samples were collected in October in the estuaries and coastal shallow water regions, respectively, during downstream migration. The freshwater crabs were divided into two groups, immature (from April to June) and mature (from July to October), according to the gonad maturation.

As seen in Fig. 2, only D- and L-alanine levels were significantly higher in the mature group than in the immature group.[17,18] Total alanine level and the percentage of D-form to total alanine were also higher in the mature group. These values were much higher in the estuarine and sea specimens. Glycine level increased dramatically only in the sea specimens. Hepatopancreas also showed the same tendency. Along with the maturation, sodium, potassium, and chloride ions were incorporated into muscle even in freshwater environment (Fig. 2, lower panel). The inorganic ion content in the mature group was 1.5-fold higher than in the immature group. The ions in muscle further increased in estuaries but decreased in the sea in response to the increase of glycine. From these data, it is clear that the crabs adjust their intracellular salinity tolerance simply by increasing D- and L-alanine and incorporating inorganic ions from hemolymph prior to the downstream migration toward the sea. In estuaries, they accumulate more D- and L-alanine and uptake more ions. After reaching the sea, the crabs increase more D- and L-alanine and glycine in place of harmful inorganic ions for the muscle cell. Thus, in even such a powerful osmoregulator, D-alanine together with L-form is a major compatible osmolyte and responsible to acquire salinity tolerance during maturation.

The accumulation of D- and L-alanine under osmotic stress was also confirmed in clam species *C. japonica*[5] and *M. lusoria* in which only D- and L-alanine largely increased during acclimation from 100 to 150% seawater.[10] In an intertidal sipunculid *P. arcuatum*, D- and L-alanine levels decreased significantly during the acclimation from 50 to 30% seawater.[14] Therefore, D-alanine together with L-alanine is a major osmolyte responsible for the intracellular isosmotic regulation for not only in crustaceans but also in invertebrates having a large amount of D-alanine.

4. ROLE OF D-ALANINE UNDER ANOXIC STRESS

Aquatic invertebrates encounter periodical or long-lasted environmental hypoxia or anoxia and well survive the severe conditions by metabolic arrest and/or anaerobic metabolism. During anaerobiosis, bivalve mollusks are well known to produce several anaerobic end products such as alanine, alanopine, succinate, and propionate.[19] The above crayfish acclimated to freshwater, 50, and 75% seawater were exposed to anoxia ($P_{O_2}<0.1$ mg/l) for 12 h under constant bubbling of nitrogen gas. After anoxia, the animals were recovered in normoxic water for 8 or 12 h.[20] Under anoxia, ATP dramatically decreased and changed to AMP in the muscle of crayfish regardless of salinity. Along with the decrease of muscle glycogen level, the significant increase of lactate was found in muscle (Fig. 3A). The lactate level returned almost to the control level during recovery in all salinity levels.

Anaerobic end product of crustaceans has been considered to be only L-lactate as is the case in mammals. However in crayfish muscle, both D- and L-alanine also increased significantly and the increase was remarkable in seawater (Fig. 3B). After recovery, D- and L-alanine levels decreased almost to the control level in 75% seawater but were still high in freshwater and 50% seawater. These changes of lactate and D- and L-alanine were also found in hepatopancreas. The increase of D- and L-alanine during anaerobiosis has also been reported in hard clam *Meretrix lusoria*,[10] annelid *Arenicola marina*,[21] and sipunculid *Sipunculus nudus*.[22] Thus, D- and L-alanine is quite possible to be anaerobic end products even in crustaceans and other invertebrates.

5. D-ALANINE SYNTHESIS IN AQUATIC INVERTEBRATES

The origin of free D-amino acids in mollusks had been controversial. The exogenous D-amino acids have been postulated to be incorporated from external media[13,23] or from symbiotic bacteria.[7] However, alanine racemase [EC 5.1.1.1], catalyzing the interconversion between D- and L-alanine, has been detected both in bivalves[5,24] and crustaceans.[25,26] Eukaryotic alanine racemase has recently been purified to homogeneity from a fungus *Tolypocladium niveum*,[27] crayfish muscle,[28] mantle muscle of *C. japonica*,[29] hepatopancreas[30] and muscle[31] of black tiger prawn

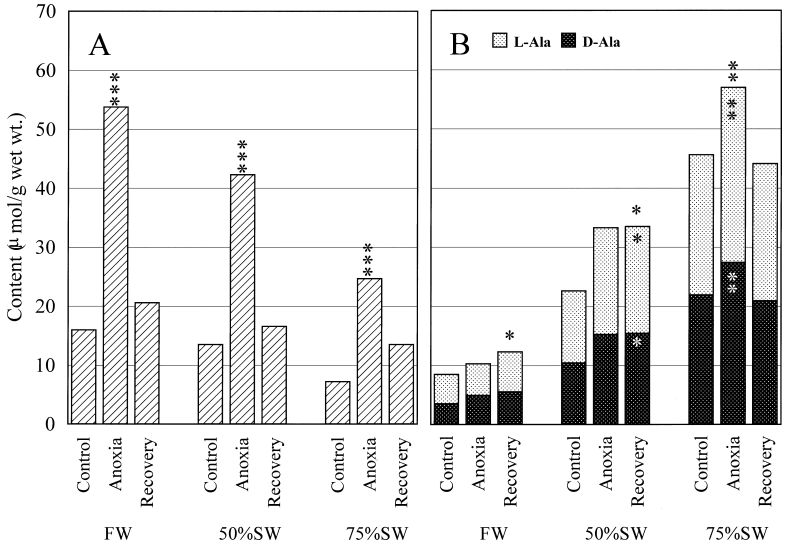


Fig. 3. Changes of L-Lactate (A) and D- and L-Alanine (B) in Crayfish Muscle during Anoxia and Recovery in Freshwater (FW), 50, and 75% Seawater (SW)

Only mean values were shown for five animals. $*p<0.05$, $**p<0.01$, $***p<0.001$.

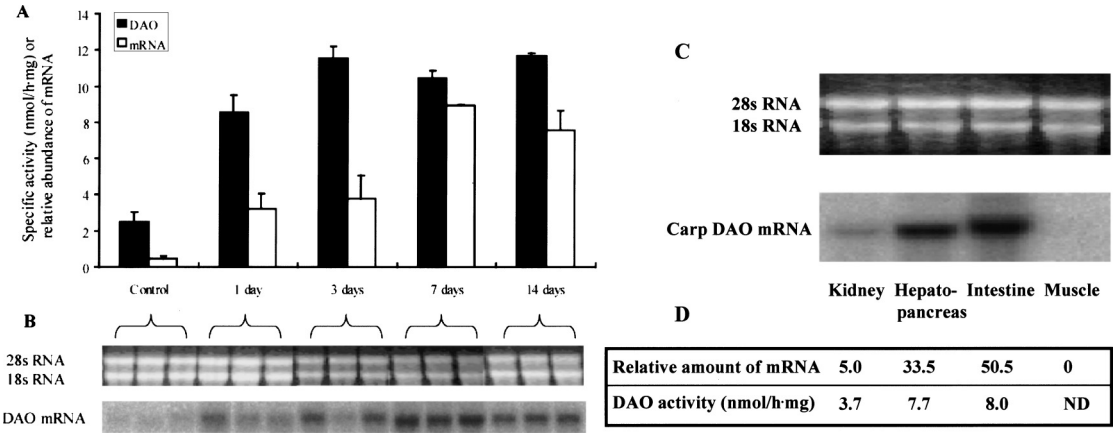
Table 2. Properties of Alanine Racemase Isolated from Aquatic Invertebrates

Species and tissue	Molecular mass (kDa)	Reaction direction	V_{max} (μmol/min/mg)	K_m (mM)	k_{cat} (s^{-1})	Optimal pH	Reference
Crayfish	58	L→D	7763	171	7504	9.0	28)
Tail muscle	(Monomer)	D→L	3386	73.5	3273	8.5	
Black tiger prawn	41	L→D	460	150		9.0	30)
Hepatopancreas	(Dimer)	D→L	94	24		10.0	
Black tiger prawn	46	L→D	3502	167	2568	9.5	31)
Tail muscle	(Dimer)	D→L	3155	179	2314	10.0	
Corbicula japonica	41	L→D	626	22.6		9.5	29)
Mantle muscle	(Tri or tetramer)	D→L	286	9.2		9.5	

Penaeus monodon (Table 2). We purified the enzyme to homogeneity from the muscle of *Penaeus monodon*.[31] The molecular size of the monomer (around 40 kDa) is almost identical for all enzymes thus far isolated from both prokaryotes and eukaryotes with some exceptions (Table 2). The purified enzyme is highly specific to D- and L-alanine and did not catalyze the racemization of other amino acids. Apparent K_m values for both L to D and D to L directions were over 160 mM which is typical for crustacean enzymes at least toward L-alanine.[28,30,31] However, V_{max} and k_{cat} of the prawn enzyme were high and almost the same for both directions. Thus, the catalytic efficiency (k_{cat}/K_m) was also not different for both directions. The equilibrium constant (K_{eq}) was calculated to be 0.84 from the Haldane equation, (k_{cat}/K_m for D-alanine)/(k_{cat}/K_m for L-alanine), and the value was only slightly below the theoretical one for racemization, *i.e.* 1.0. From these data, the enzyme may well regulate the intracellular concentrations of D- and L-alanine in crustaceans, in spite of the low content in tissues and high K_m values.

All known prokaryotic alanine racemase require pyridoxal 5′-phosphate (PLP) as a cofactor to form a Schiff base with the substrate. In contrast, the invertebrate enzyme showed activity even in the absence of PLP.[28—31] On the other hand, the enzyme was strongly inhibited by several inhibitors specific to PLP-dependent enzymes, suggesting the tightly binding of PLP to the enzyme, probably with a covalent bond as is shown in alanine racemase of *Bacillus stearothermophilus*, where PLP is covalently linked *via* an aldimine linkage to lysine 39.[32]

Based on the partial amino acid sequences obtained from the purified alanine racemase from black tiger prawn, we have cloned its cDNA from the muscle and hepatopancreas of kuruma prawn for the first time in animals.[33,34] The nucleotide sequence of 1798 bp including polyadenylation signal and poly(A)$^+$ tail contained an open reading frame of 1263 bp flanking by 5′- and 3′-non coding regions of 459 and 73 bp, respectively. The open reading frame encodes an identical protein of 421 amino acid residues both for muscle and hepatopancreas with a calculated molecular weight of 45770. The sequence shared about 25 and 27% of amino acids with that of *Bacillus stearothemophilus* and *Schizosaccharomyces pombe* enzymes, respectively. The prawn enzyme contained a signal peptide of 19 residues at the N-terminus and common amino acid residues proposed to be important in the catalytic mechanism of bacterial enzymes. They are an active site lysyl residue that binds PLP and a tyrosine residue constituting the catalytic moiety. These data indicate that alanine racemase gene is conserved from bacteria to invertebrates during a long evolutional time scale.



Relative amount of mRNA	5.0	33.5	50.5	0
DAO activity (nmol/h·mg)	3.7	7.7	8.0	ND

Fig. 4. Changes of Enzyme Activities and the Expression of mRNA of D-Amino Acid Oxidase in Carp Tissues during D-Alanine Ingestion (5 μmol/g Body Weight/d) for 14 d[40]

(A) D-Amino acid oxidase activity and relative mRNA expression in hepatopancreas of carp, (B) levels of mRNA of D-amino acid oxidase, (C) levels of mRNA in several tissues of carp fed D-alanine for 14 d, (D) relative mRNA expression and activities of D-amino acid oxidase.

6. METABOLIC UTILIZATION OF EXOGENOUS D-ALANINE IN FISH

Several fish species have been known to feed on juvenile and adult invertebrates such as crustaceans and mollusks containing a large amount of D-alanine. However, the contents of free D-alanine and other D-amino acids were only below 0.5 μmol/g in some fish tissues.[35] Thus, we were interested in the fate of orally ingested D-alanine in fish. It has been well documented that as D-amino acid catabolizing enzymes, D-aspartate oxidase [EC 1.4.3.1] specific to acidic amino acids and D-amino acid oxidase [EC 1.4.3.3] specific to neutral and basic ones distributed widely in nature from bacteria to mammals. These enzymes catalyze the oxidative deamination of D-amino acids to hydrogen peroxide and the corresponding imino acid, which is non-enzymatically hydrolyzed to 2-oxo acid and ammonia. The enzymes have been suggested to work as detoxifying agents that eliminate D-amino acids derived from both exogenous and endogenous sources.[36] In aquatic animals, D-aspartate oxidase has been known to exist in several fish liver[35] and several tissues of octopus,[36—38] and the latter also have D-amino acid oxidase.[36] Both enzyme activities are detected in kidney, liver (hepatopancreas), and intestine of fishes.[39] However, both enzyme activities are highly species specific and invertebrate-eaters have higher activities of both enzymes, especially D-amino acid oxidase, than piscivores. The oral administration of D-alanine to carp which often feed on crustaceans and mollusks increased D-amino acid oxidase activity about 8-, 3-, and 1.5-fold in intestine, hepatopancreas, and kidney, respectively, but no increase was found in brain. In contrast, there was no increase in the acitivty of D-aspartate oxidase as well as D-amino acid oxidase in any organs following the ingestion of D-aspartate or D-glutamate. These data suggest that D-amino acid oxidase is an inducible enzyme in fish and D-alanine is a physiological substrate for the enzyme.

To confirm the inducibility of D-amino acid oxidase in fish, we cloned a cDNA of the enzyme from the hepatopancreas of carp *Cyprinus carpio*.[40] A 1294 bp clone contained an open reading frame of 1041 bp which corresponded to a predicted polypeptide of 347 amino acid residues. The predicted molecular mass of carp enzyme is 39 kDa. Carp enzyme displayed around 60% sequence identity to mammalian enzymes and about 21—29% to yeast and bacterial ones. The deduced amino acid sequence contained an FAD binding consensus sequence, GXGXXG, which was GAGVIG commonly in the enzyme from animals including carp. The active site three residues, Tyr224, Tyr228, and Arg283,[41] were also conserved in carp enzyme. A conserved C-terminal sequence, Ser-(Lys/His/Arg)-Leu, is thought to be a peroxisomal signal[42] and the carp enzyme is also localized in peroxisomes.[39]

D-Alanine was orally administered to carp (5 μmol/g body weight/d) through feed for 14 d and the increase of both enzyme activity and mRNA level was examined on hepatopancreas (Fig. 4A, B).[40] The enzyme activity increased rapidly to a plateau after 3 d and the level of mRNA also increased up to 7 d. After 14 d ingestion of D-alanine, tissue specific expression of mRNA and enzyme activity were also examined. As seen in Fig. 4C, D, a strong hybridization signal was detected in intestine followed by hepatopancreas and kidney. These data clearly show that D-amino acid oxidase is inducible in carp tissues with exogenous D-alanine. In mammals, D-amino acid oxidase is not an inducible enzyme[43] while D-aspartate oxidase is inducible in mouse liver after oral administration of D-aspartate.[44] Thus, D-amino acid oxidase in carp is not merely a detoxifying agent but may be an important enzyme responsible for the effective utilization of the carbon skeleton of exogenous D-amino acids in intestine and hepatopancreas and finally in kidney. This may reflect the fact that the distribution of D-alanine in carp feed is wider and larger in amount than D-aspartate or D-glutamate.

As described up to this point, D-amino acid metabolizing enzymes distribute widely in aquatic animals. Crustaceans and bivalve mollusks apparently synthesize D-alanine actively by alanine racemase. Other than alanine racemase, only aspartate racemase in ark (blood) shell[11,12] and serine racemase in mammals[45] and silkworm[2] are known in an animal kingdom until now. However, it is highly possible to exist other D-amino acid metabolizing enzymes in animals.

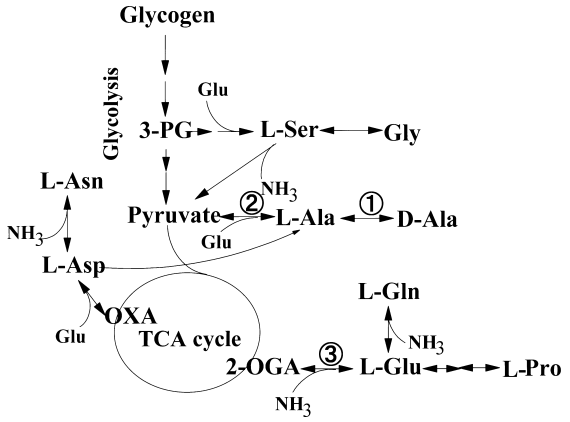


Fig. 5. Metabolic Relationship of Free Amino Acid Osmolytes Increased in the Tissues of Invertebrates during Hypersalinity Acclimation

① Alanine racemase; ② alanine aminotransferase; ③ glutamate dehydrogenase. 3-PG, 3-phosphoglycerate; OXA, oxaloacetate; 2-OGA, 2-oxoglutarate, TCA, tricarboxylic acid.

7. CONCLUSION

At this point, it is relevant to ask why free D-alanine is employed to play an important role in aquatic invertebrates. Figure 5 shows the anabolic pathway of some amino acids utilized as osmolytes for intracellular isosmotic regulation in invertebrates.[46] These amino acids are non-essential and easily biosynthesized from glycogen *via* anaerobic glycolysis. Apart from aspartate and glutamate, these amino acids are neutral amino acids having no additional group interacting with proteins. These amino acids may not give harmful effects on protein functions or structures even if they are accumulated in considerable amounts in the cell. Such intracellular osmolytes are referred to 'compatible osmolytes'.[47] Even these amino acids, however, will perturb the micro-environment in the cell if accumulated in large amounts. Thus, it is necessary for the cell to avoid the large accumulation of only one osmolyte. As seen in Fig. 1, most invertebrates avoid this situation by accumulating several osmolytes in the cell under hyperosmotic stress. L-Alanine, for instance, is considered to be the best 'compatible osmolyte' with glycine but it is an inhibitor of pyruvate kinase and a substrate for several enzymes such as alanine dehydrogenase and alanine aminotransferase. If it is accumulated in large amount in the cell, metabolic homeostasis of the cell may be disrupted. D-Alanine, however, do not interact with enzymes in the cell.[46] Thus, if L-alanine is transformed into D-form, the excess accumulation of L-alanine would be avoided and the cell microenvironment would be maintained. From these reasons, it is considered that invertebrates have retained alanine racemase gene and utilized D-alanine for specific cell functions.

From these considerations, it is clear that D-amino acids exist in various animals including mammals and play important roles in the cell. Thus, organisms of the earth are dependent not only on L-amino acid biosystems but also on D-amino acid biosystems for maintaining life-support system. The research on D-amino acid biosystems has just started and many subsystems will be clarified in the future even on aquatic animals.

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