THE ENZYME ACTIVITIES OF OPINE AND LACTATE DEHYDROGENASES IN THE GILLS, MANTLE, FOOT, AND ADDUCTOR OF THE HARD CLAM MERETRIX LUSORIA

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Key words: hard clam, opine dehydrogenase, tissues, lactate dehydrogenase.

ABSTRACT

Alanopine dehydrogenase (ADH) and both ADH and strombine dehydrogenase (SDH) are respectively the major opine dehydrogenases in the gills and mantle of hard clam under normoxia. However, ADH, octopine dehydrogenase (ODH), and SDH are the predominant opine dehydrogenases in the foot and adductor of the hard clam under normoxia. After anoxic exposure, significant increases in the activities of opine dehydrogenases in the gills were observed for ADH as well as SDH and lactate dehydrogenase (LDH). However, the activities of ADH and SDH in the mantle were not responsive to anoxic exposure. Activities of ADH, ODH, SDH, and LDH in the foot were not affected by 3 h of anoxic exposure, but were significantly increased after 6 h of anoxic exposure. The major species of opine dehydrogenases in the adductor were the same as those in the foot. The activities of LDH and all opine dehydrogenases except taurine dehydrogenase (TDH) in the adductor were significantly stimulated by anoxic exposure, and their maximum activities were found by 3 h of anoxic exposure. After anoxic exposure, the rates of increases in the activities of ADH, ODH, and SDH in the adductor ranged from 150 to 230 mU/mg/h were much higher than those in the gills, mantle, and foot. Therefore, these opine dehydrogenases play an important role in the anaerobic metabolism of the adductor in the hard clam.

I. INTRODUCTION

Hard clam *Meretrix lusoria* are benthic marine organisms and naturally inhabit estuaries. However, most hard clam sold in the market is cultured in ponds in the southwestern part of Taiwan. The salinity of pond water varies with the site and is maintained at approximately 20‰ in the Taishi area of Yunlin County, Taiwan [18]. Normally, the food of juvenile clam is supplied by natural production which may provide insufficient food to meet the needs of clam in the middle and late stages of culture. Therefore, powdered fish meal and fermented organic matter are added to clam ponds to directly feed the clam and enrich natural production. The addition of organic matter to ponds can result in the formation of a reduced layer in the sediments [14]. The reduced layer in the sediment consumes a lot of dissolved oxygen (DO) and results in low levels of DO in the bottom layer of pond water under stagnant conditions [22]. Therefore, hard clam are likely to experience environmental hypoxia in aged ponds.

Physiological functions of organisms are performed by consuming chemical energy, the production of which is achieved by the oxidation of organic matter with consumption of oxygen molecule. There is no problem for land-based animals to extract oxygen molecules from the air. However, marine aquatic organisms, especially those with habitats in sediments, must face the problem of the sources of DO due to the low solubility of oxygen in seawater: 210 ml O_2/L in air vs. 5.5 ml O_2/L in seawater at 20°C [12]. A decrease in the supply of and a lack of oxygen will result in hypoxia and anoxia, respectively.

Anaerobiosis is divided into two categories, environmental anaerobiosis and functional anaerobiosis; the former is defined as exposure of the entire organism to anoxic conditions, while the latter characterizes the conditions of oxygen consumption in tissues which exceeds the oxygen supply [2].

Anaerobic metabolism in invertebrates is divided into four classes: the aspartate-succinate pathway, the glucose-succinate pathway, the opine pathway, and the lactate pathway (Fig. 1, top panel) [8, 19, 20]. The former two pathways produce energy output at relatively high efficiencies (5~7 ATP molecules per glucose unit) compared to that in the latter two pathways (3 ATP molecules per glucose unit). However, the latter two pathways produce energy at a higher rate than do the former two pathways. The former two pathways were found to exist in hard clam [17]. However, how the latter two path-

Glucose (glycogen)

↓

↓ Glycolysis

↓

pyruvate

Anaerobic pathway	Opine pathway	Aspartate-succinate pathway	Glucose-succinate pathway	Lactate pathway

End product	Opines	Alaine, succinate propionate	Succinate propionate	Lactate

Opine dehydrogenases

Strombine dehydrogenase
Glycine + pyruvate + NADH ⟷ Strombine + NAD^+ + H_2O

Alanopine dehydrogenase
Alanine + pyruvate + NADH ⟷ Alanopine + NAD^+ + H_2O

Octopine dehydrogenase
Arginine + pyruvate + NADH ⟷ Octopine + NAD^+ + H_2O

Tauropine dehydrogenase
Taurine + pyruvate + NADH ⟷ Tauropine + NAD^+ + H_2O

β-alanopine dehydrogenase
β-alanine + pyruvate + NADH ⟷ β-alanopine + NAD^+ + H_2O

Lysopine dehydrogenase
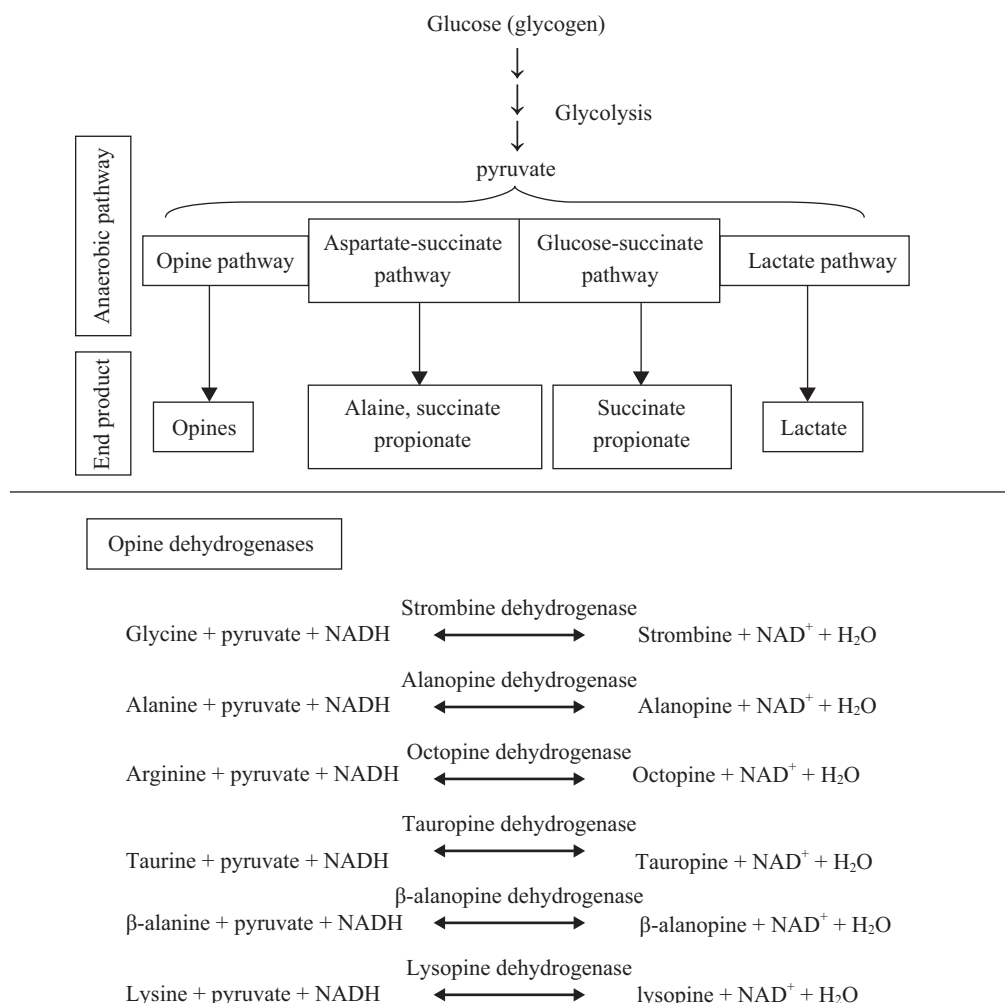Lysine + pyruvate + NADH ⟷ lysopine + NAD^+ + H_2O

Fig. 1. Four kinds of anaerobic pathways (top panel) and dehydrogenases in opine pathways in mollusks [4, 5, 19, 20].

ways operate in tissues of hard clam under anoxia is still unknown, although the activities of opine dehydrogenases and lactate dehydrogenase (LDH) were found in the foot and adductor of the hard clam [25].

Opine dehydrogenases are a family of enzymes which can catalyze one mole of amino acids and pyruvate to form one mole of opine as well as oxidize one mole of NADH (Fig. 1, bottom panel). The function of opine dehydrogenase is similar to that of LDH. These dehydrogenases can oxidize NADH produced by glycolysis in the cytosol and promote glycolysis to yield more ATP for physiological functioning under anoxia. The first opine dehydrogenase discovered was alanopine dehydrogenase (ADH) which was found in the adductor of the Pacific oyster [9]. Thereafter, several kinds of opine dehydrogenases were found in many kinds of organisms, such as strombine dehydrogenase (SDH) in *Mytilus edulis* [7], octopine dehydrogenase (ODH) in *Cerebratulus lacteus* [13], β-alanopine dehydrogenase (BADH) in *Scapharca broughtonii* [24], and tauropine dehydrogenase (TDH) in *Arabella iricolor* [16].

Because hard clam are often exposed to hypoxia in aged culture ponds, understanding their anaerobic metabolism is very important to be able to evaluate their resistance to anoxia/hypoxia. The aim of this study was to determine the activities of opine dehydrogenases (ADH, BADH, lysopine dehydrogenase (LYDH), ODH, SDH, and TADH) and LDH in the gills, mantle, foot, and adductor of hard clam and to discuss the importance of these enzymes in their physiological function.

II. MATERIAL AND METHODS

1. Animals

Hard clam (16.2 ± 0.5 g) was purchased from clam farms in the Taishi area (Yunlin County, southwestern Taiwan) and starved for 2 days in 2000-L tanks containing 1300 L of 20‰ artificial seawater (ASW) prepared by dissolving 1:50 (w/v) of synthetic sea salts (Meersaltz®, Heinsberg, Germany) in tap water. No food was provided during the starvation period. The dissolved oxygen (DO) in the seawater was maintained at ≥ 7 mg/l.

2. Normoxic and Anoxic Exposure of Hard Clam

After starvation, six clams were sampled as a replicate, and there were six replicates at 0 h (under normoxia). A plastic reservoir with 12 L of 20‰ ASW and 36 glass jars were bubbled with N_2 for 2 h. After bubbling, 108 clams were placed in the plastic reservoir with N_2 gas blowing over them. Six clams were sealed in a glass jar inside the plastic reservoir. Eighteen glass jars were placed in a water bath at 25°C. Six glass jars were respectively sampled at 3, 6, and 24 h (anoxic exposure).

3. Collection of Specimens and Preparation of Crude Extracts from Clam Tissues

The gills, mantle, foot, and adductor of six clams in each glass jar were respectively dissected and stored in liquid nitrogen until use. The frozen tissues of six clams from a jar were homogenized in a ratio of 1:10 (w/v) with homogenization buffer containing 50 mM Tris, 2 mM EDTA, and 1 mM DTT at pH 7.5 [7]. The homogenate was centrifuged at 48,000 g for 30 min at 4°C. The supernatant was collected and passed through a Sephadex G-25 column to remove the salt and small molecules.

4. Determination of the Activities of Opine Dehydrogenases and Protein

1) Activities of Opine Dehydrogenases

The assay for the activity of opine dehydrogenases was based on the method described by de Zwaan and Zurburg [7]. The assay buffer for the activities of opine dehydrogenases was 100 mM triethanolamine-HCl, 1 mM KCN, 5 mM pyruvate, 0.24 mM NADH, and specific amino acids at pH 7.5. The specific amino acids for ADH, ODH, SDH, TDH, BADH, and LYDH were 100 mM alanine, 5 mM arginine, 100 mM glycine, 80 mM taurine, 100 mM β-alanine, and 100 mM lysine, respectively [1]. The activity of LDH was assayed as described above except in the absence of a specific amino acid. The activities of LDH and opine dehydrogenases were monitored by changes in the absorbance at 340 nm at 25°C. One unit of activity was defined as the amount of enzymes which consumed one micromole of NADH per minute.

2) Protein

Samples were precipitated using 10% trichloroacetic acid to remove DTT which interferes in the protein assay. The resulting precipitate was assayed by a BCA Protein Assay Kit.

5. Analysis of Data

All data were statistically analyzed by analysis of variance (ANOVA) [26] using SigmaStat statistical software from SPSS. Duncan's test for pairwise comparisons was used at the 5% significance level.

III. RESULTS

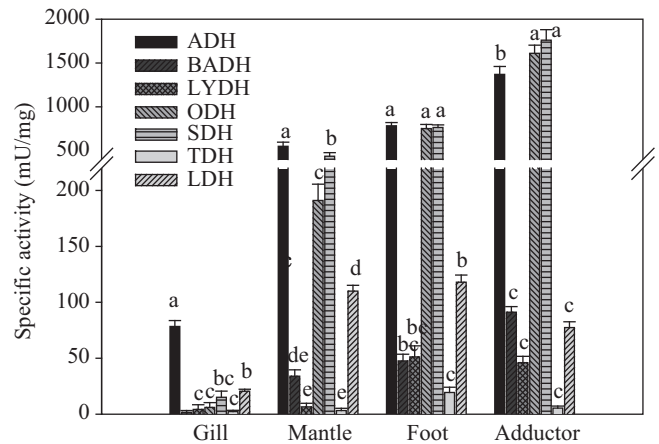ADH is the major opine dehydrogenase in the gills of hard

Fig. 2. Activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the gill, mantle, foot and adductor of hard clam under normoxia. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. Significant (< 0.05) differences in the tissues are denotes by different letters.

clam under normoxia. Its specific activity was 78.4 mU/mg protein. The activity of the other opine dehydrogenases in the gill was ≤ 15.1 mU/mg protein and was much lower than that of ADH (Fig. 2). ADH and SDH are the major opine dehydrogenases in the mantle of hard clam kept in aerated seawater, followed by the activities of ODH and LDH. However, the activities of BADH, LYDH, and TDH among mantle opine dehydrogenases were minor. It is notable that ADH, ODH, and SDH predominate the opine dehydrogenases in the foot and adductor of hard clam under normoxia. The activities of ODH and SDH were significantly higher than that of ADH in the adductor, while the activity of the latter was significantly higher than those of the former in the gills and mantle (Fig. 2).

In gills of the hard clam subjected to anoxic exposure, significant increases in the activities of opine dehydrogenases were observed in ADH as well as in SDH and LDH. Their activities were the highest at 3 h of anoxic exposure: 284 mU/mg protein for ADH, 78.8 mU/mg protein for SDH, and 94.4 mU/mg protein for LDH. Their activities at 6 h and 24 h of anoxic exposure were significantly lower than those at 3 h of anoxic exposure, but they were still higher than those under normoxia (Fig. 3). No significant changes in the activities of BADH, LYDH, ODH, and TDH in the gill were observed after anoxic exposure.

Although the activities of ADH and SDH, at 549 and 436 mU/mg protein, in the mantle were much higher than those, at 78.4 and 15.1 mU/mg protein, respectively, in the gills (Fig. 2), their activities were not responsive to anoxic exposure (Fig. 4). In addition, there were no significant changes in the activities of the other opine dehydrogenases in the mantle of hard clam exposed to anoxia (Fig. 4). The activity of LDH in the mantle was not affected by 6 h of anoxia exposure, while 24 h of anoxia exposure stimulated a significant change in its activity.

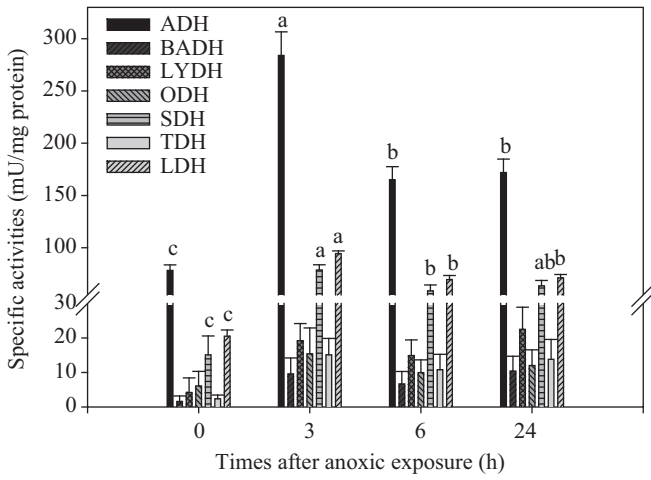ADH, ODH, and SDH predominated in the foot not only

Fig. 3. Activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the gill of hard clam after anoxic exposure. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. Significant (< 0.05) differences in a specific dehydrogenase are denotes by different letters.
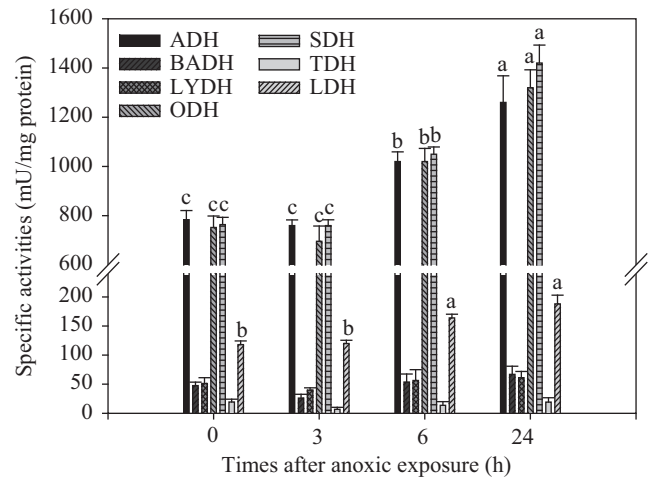

Fig. 5. Activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the foot of hard clam after anoxic exposure. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. Significant differences (< 0.05) in a specific dehydrogenase are denotes by different letters.
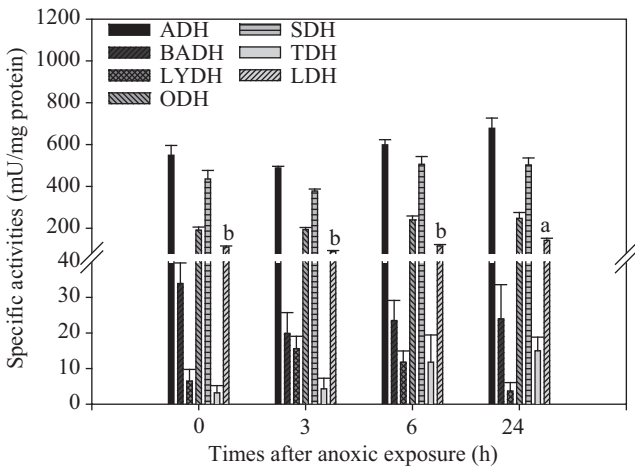

Fig. 4. Activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the mantle of hard clam after anoxic exposure. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. Significant differences (< 0.05) in a specific dehydrogenase are denotes by different letters.
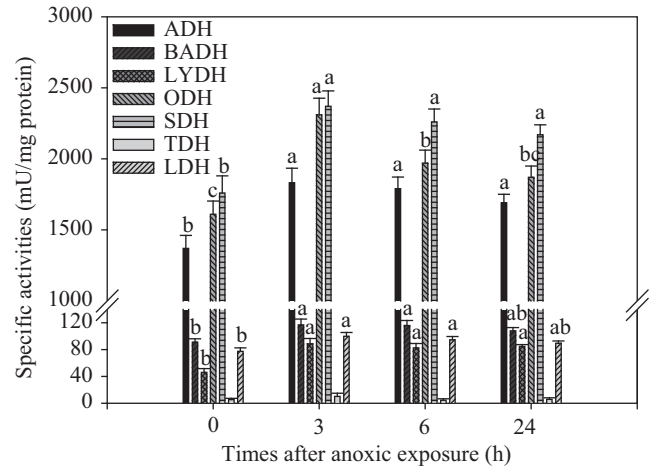

Fig. 6. Activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the adductor of hard clam after anoxic exposure. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. Significant differences (< 0.05) in a specific dehydrogenase are denotes by different letters.

under normoxia but also after anoxic exposure (Fig. 5). Their activities were not affected by 3 h of anoxic exposure, significantly increased after 6 h of anoxic exposure, and reached a maximum after 24 h of anoxic exposure. The activity of LDH in the foot did not significantly increase until by 6 h of anoxic exposure. After anoxic exposure, no significant changes in the activities of BADH, LYDH, and TDH in the foot were observed.

The major species of opine dehydrogenases, ADH, ODH, and SDH, in the adductor were the same as those in the foot (Fig. 6). The activities of LDH and all opine dehydrogenases except TDH in the adductor were significantly stimulated by

anoxic exposure, and their maximum activities were found at 3 h of anoxic exposure. Although the activities of BADH, LYDH, and LDH responded to anoxic exposure, their total activities were approximately 5% of one of the major opine dehydrogenases. Therefore, they have little importance in anaerobic metabolism of the adductor.

Maximum activities of lactate and opine dehydrogenases in the gills, mantle, foot, and adductor were seen at 3, 24, 24, and 3 h of anoxic exposure, respectively. After anoxic exposure, the rates of increases in the activities of ADH, ODH, and SDH, 150~230 mU/mg protein/h, in the adductor were much higher than those in the gills, mantle, and foot (Fig. 7). The species of
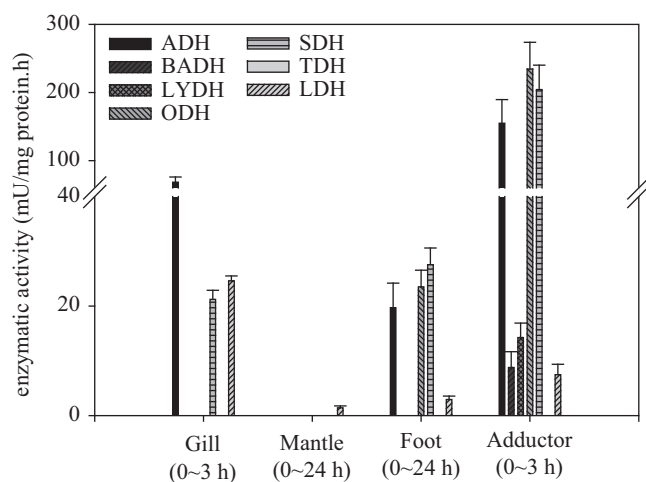
Fig. 7. Enzymatic activities of ADH, BADH, LYDH, ODH, SDH, TDH, and LDH in the tissues of hard clam after anoxic exposure. Names of dehydrogenases were abbreviated as those in Fig. 1. Data are given as the mean with the standard error. The number in parenthesis indicates anoxic exposure period for calculating enzymatic activities.

opine dehydrogenases with a higher increase rate in the gill was ADH, while those in the foot and adductor were ADH, ODH, and SDH. It was interesting that the rate of increase in the activity of SDH in the gill was comparable to that in the activity of LDH.

IV. DISCUSSION

Under environmental hypoxia/anoxia, organisms cannot take up sufficient oxygen molecules for physiological function. This results in a decrease in the pO_2 of the hemolymph of bivalves. After *Mytilus edulis* was exposed to air, the pO_2 of hemolymph sharply declined within the first few hours from 80 to 40 mmHg and was maintained at this level for 24 h of anoxic exposure [6]. A similar observation was also found in the hard clam. The hemolymph pO_2 of hard clam decreased from 23.8 mmHg initially to 18.3 mmHg after 1 min of exposure at 15°C. Subsequently, it significantly dropped to 5.9 mmHg after 2 min of exposure, and thereafter, low-level values were maintained until 3 min of exposure [15]. Huang *et al.* [15] also determined the effect of Mg^{+2}-free artificial seawater, K^+, and KCN on the hemolymph pO_2 of hard clam. A constant value of their hemolymph pO_2 of > 10 mmHg was observed for the entire exposure period of 48 h. *M. edulis* and *M. lusoria* have no hemoglobin, while a minor amount of hemocyanins is present in the hemolymph of hard clam [12, 15]. Possible explanations for the reason why hemolymph pO_2 does not drop to zero may be due to air gaping, impacting oxygen uptake by tissues, or the onset of anaerobiosis.

In mollusks, both opine dehydrogenases and LDH are well known as terminal enzymes for regulating the cytosolic redox balance in glycolysis under anoxia [4]. The catalytic mechanisms of both dehydrogenases are simple, and their favorite

reaction direction totally depends on the equilibrium constant. Their reaction rates are rapid, but the efficiency of energy production is relatively low. The function of vigorous muscular activity, characterized by sudden energy demands, is performed by the organs of foot and adductor of mollusks. High activities of ADH, ODH, and SDH were found in the foot and adductor of hard clam and presumably account for the instant energy supply in functional anaerobiosis. This observation is consistent with results of high activities of ADH, ODH, and SDH in the foot and adductor of the bivalves *Meretrix lusoria*, *Scapharca inequivalvis,* and *M. edulis* [7, 20, 25]. However, the gills are responsible for respiratory function and, unlike the foot and adductor, do not have "bursts" of contractions. The gills having low activities of ADH, ODH, and SDH could also explain the role of these dehydrogenases in functional anaerobiosis. Similarly, the gills having low activities of these dehydrogenases were also observed in the sea mussel *M. edulis* and oyster *Gryphea angulata*, while no activity of ODH was found in gills of *G. angulata* [7].

Opine dehydrogenases require pyruvate and amino acids as their substrates. Therefore, free amino acids in tissues are required for the reactions catalyzed by these dehydrogenases. In kinetics studies, high concentrations of alanine and glycine are respectively required for appreciable enzyme activities of ADH and SDH, and the apparent Km values for alanine and glycine are on the order of 170~240 mM [11]. The role of free amino acids in osmoregulation of marine invertebrates is well established [27]. Due to the osmotic balance, the concentrations of free amino acids in the tissues of marine invertebrates are much higher than those in their freshwater counterparts. This is why no opine dehydrogenases were found in freshwater bivalves [21]. Chiou *et al.* [3] determined the concentrations of 22 species of amino acids in the edible meat of hard clam. Among them, amino acids with concentrations of > 0.5 mg/L·g wet weight were taurine (6.5 mg/L·g wet weight), glutamic acid (1.5 mg/L·g wet weight), glycine (0.6 mg/L·g wet weight), alanine (2 mg/L·g wet weight), and arginine (0.9 mg/L·g wet weight). The low concentration of substrates explains the low activities of BADH and LYDH in tissues of the hard clam. It is interesting that the concentrations of taurine and glutamate in tissues of the hard clam are high, but their respective opine dehydrogenase activities are low and undetectable (data not shown). This phenomenon indicates that the sole role of glutamate and taurine is in osmoregulation. Concentrations of arginine in the gills, mantle, foot, and adductor were 0.5, 0.7, 0.5, and 1 mg/g wet weight, while the activities of ODH in these organs were 6.08, 191, 752, and 1610 mU/mg protein, respectively [23]. This indicates that the activity of ODH in the gills is tissue-specific rather than being related to the concentration of arginine. However, the activity of ADH depends on the concentration of alanine in tissues since concentrations of alanine in the gills, mantle, foot, and adductor were 1.5, 4, 6.1, and 7.03 mg/g wet weight, respectively [23]. Concentrations of glycine in the adductor and gills were respectively the highest and lowest and were comparable to the trend of the

activity of SDH in both organs.

Although the physiological role of opine dehydrogenases is analogous to that of LDH, anaerobic opine pathways are widely distributed in marine invertebrates instead of the well-known lactate pathway which is predominant in vertebrates [25]. In vertebrates, LDH is used to maintain the cytosolic redox balance in anaerobiosis, while opine dehydrogenases are used to regulate this redox balance in some marine invertebrates. On the other hand, the opine pathway and lactate pathway predominate in less-mobile animals with poorly developed organs and in much more-mobile animals with well-developed organs, respectively [25]. Therefore, the lactate pathway selected by vertebrates might have been due to the development of circulatory systems and other means of removing acidic lactate end-products. The reason that the lactate pathway occurs in phylogenically higher animals may partly be that the energy production rate of the lactate pathway is greater because its operation is only limited by the size of the carbohydrate pool. The reason that the opine pathway is widely distributed in phylogenically lower animal can be explained by a study using a computer simulation in which the cytosolic redox ratio ($NADH/NAD^+$) with octopine as the end product was small compared to that with lactate as the end product [10]. The activities of LDH in the foot and adductor of hard clam were respectively 5% and 1.6% of the activity of all opine dehydrogenases in our study. This is consistent with the finding of Sato *et al.* [25]. Therefore, the role of opine dehydrogenases is more important than that of LDH in anaerobic metabolism of bivalves.

ACKNOWLEDGMENTS

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