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Oxygen and Acid–Base Status of Hemolymph in the Densely Lamellated Oyster *Ostrea denselamellosa* in Normoxic Conditions

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Abstract : We examined hemolymph O_2 partial pressure (Po_2), pH, total CO_2 content (Tco_2), CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) in order to evaluate the acid–base balance of the densely lamellated oyster *Ostrea denselamellosa* in normoxic conditions. Hemolymph was collected anaerobically through a cannula inserted into the adductor muscle of *O. denselamellosa*. The mean values of hemolymph Po_2, pH, and Tco_2 were 64.7 torr, 7.576 and 1.22 mM/*l*, respectively. Using αco_2 and pKapp determined in this study, the hemolymph Pco_2 and $[HCO_3^-]$ were calculated as 1.10 torr and 1.17 mM/*l*, respectively. These hemolymph properties were compared with those of other marine bivalves. *Ostrea denselamellosa* seemed to efficiently discharge CO_2 and maintain high hemolymph pH in normoxic conditions.

Key words : *Ostrea denselamellosa*, hemolymph acid–base balance, normoxia, apparent dissociation constant of carbonic acid, CO_2 partial pressure, bicarbonate ion

Introduction

Densely lamellated oyster *Ostrea denselamellosa* is a Ostreidae bivalve classified in the Pterioida, Pteriomorphila.[1)] The densely lamellated oyster is distributed from the Boso Peninsula to Kyushu in Japan, and it inhabits sand and gravel at a water depth of 3–10 meters in inner bays.[1)] Densely lamellated oyster was caught as a local specialty food of the littoral region in the Seto Inland Sea, but it has considerably decreased and hardly been observed recently. Densely lamellated oyster has been the subject of a previous study in terms of the histology of the gonad,[2)] seedling production,[3)] reproduction,[4)] and DNA identification of the family Ostreidae.[5)] The regulation of the ventilation volume and oxygen uptake in normoxic and feeding conditions have been studied.[6)] The anatomical structures of the ctenidia were also clarified recently.[7)] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid–base balance in densely lamellated oyster. Handa et al. (2017) developed surgical procedures, cannulation of the adductor muscle of Pacific oyster *Crassostrea gigas*, and examined the hemolymph oxygen and acid–base status postoperation.[8)] In the present study, we applied the surgical procedures to the densely lamellated oyster *O. denselamellosa* and elucidated the hemolymph acid-base balance in normoxic conditions in this species. Research into the acid–base balance could contribute to efficient CO_2 utilization, which is related to respiration and calcification for the formation of the shell valves. The acid–base balance and CO_2 dynamic phase of densely lamellated oyster is useful for evaluation of the cultivation environments, and of the effects of ocean acidification and increases in CO_2 level. In some marine bivalves in normoxic and normocapnic conditions, the CO_2 partial pressure (Pco_2) of the hemolymph were 0.57–2.3 torr.[9-15)] The hemolymph Pco_2 of densely lamellated oyster was supposed to be low as in other bivalves. The estimation Pco_2 by application of the Henderson–Hasselbalch equation is practiced in studies of acid–base balance owing to the relative ease and accuracy of such estimates.[16)] In the equation, the characteristic values of the CO_2 solubility coefficient (αco_2) and apparent dissociation constant of carbonic acid (pKapp) in the hemolymph are required for the experimental animal.

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Therefore, we determined hemolymph αco_2 and pKapp of densely lamellated oyster, and evaluated the acid–base balance of hemolymph in normoxic conditions.

Materials and Methods

Experimental animals and conditions

The experiments used 46 densely lamellated oyster *Ostrea denselamellosa* (shell length: 103.9 ± 5.3 mm (mean ± SD), shell height: 82.2 ± 4.56 mm, total wet weight: 129.0 ± 30.3 g). The animals were obtained from marine farms in the Seto Inland Sea. After cleaning the shell valves, they were reared for 1 month at 24°C in aerated seawater with added cultivated phytoplankton.[17-19] Twenty-four hours before collecting hemolymph, the densely lamellated oysters were transferred to particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 32 psu, water temperature 24°C, O_2 saturation 98%, pH 8.20, and total CO_2 content 1.5 mM/*l*.

Surgical procedures and hemolymph collection

Hemolymph was collected from the adductor muscle using a cannula (polyethylene tubing, 0.96 mm outer diameter, 0.58 mm inner diameter, PE-50, Clay Adams).[8] A small hole (2 mm diameter) was made on adjacent shell valves, which was at the center of the posterior margin. The cannula with a stylet was inserted through the hole into the adductor muscle, and was advanced 4 mm toward the center of the adductor muscle. The stylet was removed, and the end of the cannula was closed. The cannula was gently fixed to the left shell valve using denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent effects from movement of the shell valves. This surgical operation was completed within 10 minutes. The cannulated oyster was transferred to an acrylic respiratory chamber and was allowed to recover for 3 hr at 24.0 ± 0.1°C in normoxic conditions. A hemolymph sample was then drawn through the cannula using a gas-tight micro syringe (Model 1750, Hamilton Co.). The volume of hemolymph collected was 0.4 m*l*.

Hemolymph analysis

The hemolymph oxygen partial pressure (Po_2, torr), pH, and total CO_2 content (Tco_2, mM/*l*) were measured immediately after each collection. Po_2 was measured using a blood gas meter (BGM200, Cameron Instruments) and Po_2 electrode (E101, Cameron Instruments). The pH was measured using the blood gas meter with pH glass and reference electrodes (E301, E351, Cameron Instruments). The Po_2 and pH electrodes were installed in a water jacket maintained at 24.0°C. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5, Cameron Instruments). The hemolymph Pco_2 (torr) and bicarbonate concentration ($[HCO_3^-]$, mM/*l*) were calculated by rearranging the Henderson–Hasselbalch equation.[16,20] In the equation, the CO_2 solubility coefficient (αco_2, μM/*l*/torr) and apparent dissociation constant of carbonic acid (pKapp) of densely lamellated oysters were required. The determinations of αco_2 and pKapp were performed by *in vitro* experiments.

The αco_2 was determined using hemolymph, which was adjusted to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Industries, Ltd.). The acidified sample was transferred to a tonometer flask, and equilibrated with humidified standard CO_2 gas (CO_2, 10.0%; O_2, 20.9%; N_2 balance) using an equilibrator (DEQ-1, Cameron Instruments) at 24.0°C, and subsequently the Tco_2 of each equilibrated sample was measured using a total CO_2 analyzer. The Pco_2 of the equilibrated sample was calculated from known a CO_2 concentration standard gas (10.0%), prevailing barometric pressure, and water vapor pressure at 24.0°C. The αco_2 was calculated using the equation:

$$\alpha co_2 = Tco_2 \bullet Pco_2^{\ -1}$$

For determination of the pKapp, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.5, 1.0, 2.0, and 5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 24.0°C. After equilibration, the pH and Tco_2 of the sample were measured using the blood gas meter and total CO_2 analyzer. Using the sample pH, Tco_2 and αco_2 calculated from the above equation, pKapp was determined by rearrangement of Henderson-Hasselbalch equation[16,20] as follows:

$$\mathrm{pKapp} = \mathrm{pH} - \log\left[(\mathrm{Tco_2} - \alpha\mathrm{co_2} \bullet \mathrm{Pco_2}) \bullet (\alpha\mathrm{co_2} \bullet \mathrm{Pco_2})^{-1}\right]$$

where $\mathrm{Pco_2}$ was calculated from known CO_2 concentration standard gases.

The $\alpha\mathrm{co_2}$ and pKapp obtained in this study were used for calculation of hemolymph $\mathrm{Pco_2}$ from measured pH and $\mathrm{Tco_2}$:

$$\mathrm{Pco_2} = \mathrm{Tco_2} \bullet [\alpha\mathrm{co_2} \bullet (1 + 10^{(\mathrm{pH-pKapp})})]^{-1}$$

$[\mathrm{HCO_3^-}]$ was calculated from $\mathrm{Tco_2}$, $\alpha\mathrm{co_2}$, and $\mathrm{Pco_2}$ using the equation:

$$[\mathrm{HCO_3^-}] = \mathrm{Tco_2} - \alpha\mathrm{co_2} \bullet \mathrm{Pco_2}$$

Statistical analysis

All data of hemolymph properties are expressed as means ± standard error. Normality of distribution in hemolymph properties was assessed through use of the Shapiro-Wilk test. Homoscedasticity was assessed using Bartlett's test. Friedman test was performed for changes in hemolymph properties using the standard gases. Statistically significant differences were set at $P<0.05$.

Results

Hemolymph samples were collected anaerobically from the adductor muscles of densely lamellated oysters through cannulae. The mean values of hemolymph $\mathrm{Po_2}$, pH, and $\mathrm{Tco_2}$ in normoxic conditions were 64.7 torr, 7.576, and 1.22 mM/*l*, respectively (Table 1). The hemolymph $\alpha\mathrm{co_2}$ was 38.69 ± 0.018 μM/*l*/torr. The hemolymph pKapp at known $\mathrm{Pco_2}$ (standard gases) and the corresponding measured pH and $\mathrm{Tco_2}$ values are shown in Table 2. The changes in pKapp were not statistically significant (P=0.528) with the increase in $\mathrm{Pco_2}$. The calculated pKapp from all hemolymph samples was 6.10825 ± 0.04340. Hemolymph $\mathrm{Pco_2}$ and $[\mathrm{HCO_3^-}]$ were calculated by substitution of the mean value of $\alpha\mathrm{co_2}$ and pKapp in the rearranged Henderson-Hasselbalch equation as follows:

$$\mathrm{Pco_2} = \mathrm{Tco_2} \bullet [0.03869 \bullet (1+10^{(\mathrm{pH}-6.10825)})]^{-1}$$

$$[\mathrm{HCO_3^-}] = \mathrm{Tco_2} - 0.03869 \bullet \mathrm{Pco_2}$$

where the units of the parameters in the equations are torr for $\mathrm{Pco_2}$ and mM/*l* for $\mathrm{Tco_2}$ and $[\mathrm{HCO_3^-}]$. Hemolymph $\mathrm{Pco_2}$ and $[\mathrm{HCO_3^-}]$ in normoxic conditions were 1.10 torr and 1.17 mM/*l*, respectively (Table 3).

Discussion

We collected hemolymph from the adductor muscle of densely lamellated oyster and examined hemolymph $\mathrm{Po_2}$, pH, $\mathrm{Tco_2}$, $\mathrm{Pco_2}$, and $[\mathrm{HCO_3^-}]$ in order to evaluate the acid-base balance of densely lamellated oyster in normoxic conditions. The hemolymph was collected anaerobically through a cannula from submerged experimental animals after pretreatment by adductor muscle catheterization. The mean value of hemolymph $\mathrm{Po_2}$ in this study was 64.7 torr. There are few descriptions of hemolymph $\mathrm{Po_2}$ in

Table 1. Hemolymph oxygen partial pressure ($\mathrm{Po_2}$), pH and total CO_2 content ($\mathrm{Tco_2}$) of the densely lammelated oyser *Ostrea denselamellosa* in normoxic conditions

		Mean	SE	N
$\mathrm{Po_2}$	torr	64.7	2.52	9
pH		7.576	0.0346	10
$\mathrm{Tco_2}$	mM/*l*	1.22	0.112	9

Water temperature. 24.0 ± 0.1 ℃ (Mean ± SD) densely lamellated oyster, but Handa et al. (2017) reported the hemolymph Po_2 in adductor muscle of *C. gigas* which is the related species of densely lamellated oyster was 62.0 torr at 23°C.[21] The hemolymph oxygen status of densely lamellated oyster was similar to that of *C. gigas*. Yamamoto et al. (2011) reported that oxygen uptake by densely lamellated oyster was 0.48 m*l*/min/kgWW (per wet weight of the soft body) before feeding, and 0.94 m*l*/min/kgWW during feeding.[6] In *C. gigas*, the amount of oxygen uptake before feeding was 0.25–0.26 m*l*/min/kgWW,[22,23] and during feeding it was 0.664 m*l*/min/kgWW.[23] It was considered that the metabolic rate of densely lamellated oyster is higher than that of *C. gigas*. The adductor muscle of marine blue mussel *Mytilus edulis* comprises a large fraction of the total hemolymph volume,[24] and hemolymph samples collected from the adductor muscle probably contain a mixture of pre- and post-branchial hemolymph from various regions of the circulatory system.[9] Densely lamellated oyster and *C. gigas* hemolymphs could circulate around various regions and perfuse the adductor muscle, and hemolymph Po_2 would be reduced because of oxygen consumption by various organs and tissues. Densely lamellated oyster and *C. gigas* have no respiratory pigments in their hemolymphs, and oxygen capacity of the hemolymph in both species should be the same. Therefore, densely lamellated oyster seemed to achieve high oxygen uptake by the regulation of hemolymph flow in the soft body parts, including the ctenidium, or by the specific structure of ctenidium. Yamamoto and Handa (2015) described the anatomical structure of the ctenidium of densely lamellated oyster,[7] and the ctenidial principal filament, ordinary filament, connective membrane and blood vessel of densely lamellated oyster had similar structures of the ctenidium in *C. gigas*. Therefore, densely lamellated oyster might achieve its high metabolic rate by high oxygen uptake with the regulation of hemolymph flow in the soft body, including the ctenidium.

Densely lamellated oyster hemolymph pH and Tco_2

Table 2. Mean values of measured pH, total CO_2 content (Tco_2) and calculated apparent dissociation constant of carbonic acid (pKapp) of the hemolymph in adductor muscle of the densely lamellated oyster *Ostrea denselamellosa* with known Pco_2 standard gases

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/*l*)	pKapp	N
0.515	3.831	7.067	1.31	6.15705443	9
1.01	7.519	6.894	1.88	6.17613741	9
2.00	14.860	6.652	2.34	6.20519384	9
5.00	37.190	6.302	3.65	6.15147474	9

Barometric pressure, 760.7 torr; water temperature, 24.1 °C; αco_2, 38.69 μM/*l*/torr

Non-bicarbonate buffer value (β_{NB} : the regression coefficient relating pH and bicarbonate), 1.29

Table 3. Hemolymph CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of the densely lamellated oyster *Ostrea denselamellosa* in normoxic conditions

		Mean	SE	N
Pco_2	torr	1.10	0.180	9
$[HCO_3^-]$	mM/*l*	1.17	0.110	9

Water temperature, 24 °C; αco_2, 38.69 μM/*l*/torr; pKapp, 6.10825±0.04340 (Mean±SE) measured immediately after hemolymph collection were 7.576 and 1.22 mM/*l*, respectively. Previously reported hemolymph pH values include pH 7.55 in Mediterranean mussel *Mytilus galloprovincialis* at 18°C,[10] pH 7.284–7.375 in akoya pearl oyster *Pinctada fucata martensii* at 28°C,[11,12] pH 7.563 in black-lip pearl oyster *Pinctada margaritifera* at 26°C,[13] and pH 7.442 in noble scallop *Mimachlamys nobilis* at 24°C[14] and pH 7.414 in *C. gigas* at 23°C.[21] The hemolymph pH of Densely lamellated oyster was higher than those of the other bivalves. Handa and Yamamoto (2012, 2015, 2016) and Handa et al. (2018) reported hemolymph Tco_2 values were 1.90–2.10 mM/*l* in *P. fucata martensii*,[12] 2.04 mM/*l* in *P. margaritifera*,[13] 1.50 mM/*l* in *M. nobilis*[14] and 1.87 mM/*l* in *C. gigas*.[21] The contents of carbonic acid and CO_2 were lower than those of other bivalves. Therefore, the densely lamellated oyster had the possibility of higher discharge of carbon dioxide, and the hemolymph Pco_2 and $[HCO_3^-]$ in densely lamellated oyster might be lower than those of the other bivalves.

The αco_2 and pKapp of the hemolymph in densely lamellated oyster were determined in *in vitro* experiments in this study. Cameron (1986) reported CO_2 solubility as a function of temperature and salinity, and the solubility coefficients were 37.28–42.33 μM/*l*/torr at 22–26°C and 30–35 salinity (psu).[25] The hemolymph αco_2 in densely lamellated oyster (38.69 μM/*l*/torr) was almost same as the coefficient reported by Cameron (1986). The mean value of hemolymph pKapp in this study was 6.10825, whereas the hemolymph pKapp values of other marine bivalves were 5.8191 in the *P. fucata martensii* at 28°C,[12] 5.9987 in *P. margaritifera* at 26°C,[13] 6.0641 in *M. nobilis* at 23°C[14] and 6.0734 in *C. gigas* at 23°C.[21] The pKapp is equal to the pH at which it is most effective as a buffer.[26] The most effective buffer pH in densely lamellated oyster hemolymph seemed to be slightly higher than those of the other bivalves. This high pKapp contributed to the higher pH of the hemolymph in densely lamellated oyster in this study.

Using the hemolymph αco_2 and pKapp in this study, Pco_2 and $[HCO_3^-]$ of the hemolymph of densely lamellated oyster were calculated. The mean values of hemolymph Pco_2 and $[HCO_3^-]$ in densely lamellated oyster were 1.10 torr and 1.17 mM/*l*, respectively. In other marine bivalves, the mean values of hemolymph Pco_2 and $[HCO_3^-]$ were 1.15 torr and 1.62 mM/*l* in *M. galloprovincialis* at 18°C,[10] 2.18 torr and 1.78 mM/*l* in *C. gigas* at 23°C,[21] 1.50 torr and 1.98 mM/*l* in *P. margaritifera* at 26°C[13] and 2.08–2.33 torr and 1.83–2.04 mM/*l* in *P. fucata martensii* at 28°C.[12] The hemolymph Pco_2 and $[HCO_3^-]$ in densely lamellated oyster were lower than those of the other bivalves; therefore, densely lamellated oyster which has a high metabolic rate seemed to be efficiently discharging the carbon dioxide in comparison with other bivalves.

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