Hemolymph Acid-Base Balance of Akoya Pearl Oyster Pinctada fucata martensii with Cannulated Adductor Muscle in Normoxic Conditions

Hemolymph Acid–Base Balance of Akoya Pearl Oyster *Pinctada fucata martensii* with Cannulated Adductor Muscle in Normoxic Conditions

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Abstract : We collected the hemolymph anaerobically through a cannula inserted into the adductor muscle of Akoya pearl oyster *Pinctada fucata martensii* submerged in experimental seawater, and examined hemolymph pH, total CO_2 concentration (Tco_2), CO_2 partial pressure (Pco_2), and bicarbonate concentration ($[HCO_3^-]$) in order to evaluate the ability of the acid–base balance of *P. fucata martensii* in normoxic conditions. The mean values of hemolymph pH and Tco_2 were 7.544 and 2.21 mM/L, respectively. The apparent dissociation constant of carbonic acid (pKapp) was estimated using the following equation: pKapp = 225.712 – 97.096 • pH + 14.313 • pH^2 – 0.7035 • pH^3. Using mean values of pKapp (5.99585) and CO_2 solubility coefficient (αco_2, 37.02 μM/L/torr) determined in this study, the hemolymph Pco_2 and $[HCO_3^-]$ were calculated as 1.69 torr and 2.14 mM/L, respectively. The non-bicarbonate buffer value (β_{NB}) was 0.967 slykes.

Key words : *Pinctada fucata martensii*, Akoya pearl oyster, hemolymph, acid–base balance, adductor muscle, buffer value

Introduction

Akoya pearl oyster *Pinctada fucata martensii* is a filibranchial bivalve classified in the Pteriidae and is endemic to Japan.[1)] *P. fucata martensii* is distributed from the Boso Peninsula on the Pacific coast and the Oga Peninsula on the Japan Sea coast south to Okinawa.[1)] *P. fucata martensii* has nacreous aragonite in the inner layer of its shell valves, and it is used for the production for Akoya pearls. The process of pearl production is similar to the growth of the shell valves and is directly related to metabolism. The metabolism of *P. fucata martensii* has been studied in terms of regulation of gill ventilation volume and oxygen uptake in normoxic, hypoxic, anathermal, and feeding conditions.[2-6)] The anatomical structures of the digestive diverticula, ctenidium, and circulatory system were clarified recently.[7,8)] Handa and Yamamoto (2011, 2012) developed surgical procedures, cannulation of the anterior aorta in *P. fucata martensii*, and examined the hemolymph oxygen and acid–base status in the anterior aorta.[9,10)] In this study, we developed cannulation of the adductor muscle in Akoya pearl oyster and examined its hemolymph properties, including acid–base balance. Research into the acid–base balance may contribute to efficient CO_2 utilization, which is related to respiration and calcification for the formation of the shell valves. The CO_2 dynamic phase of *P. fucata martensii* 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, the CO_2 partial pressure (Pco_2) of the hemolymph is 0.57–2.3 torr in normoxic and normocapnic conditions.[11-17)] The estimation CO_2 partial pressure 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.[18)] 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. Therefore, we determined hemolymph αco_2 and pKapp of *P. fucata martensii*, and evaluated the hemolymph acid–base balance in comparison with the other bivalves.

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Materials and Methods

Experimental animals and conditions

The experiments used 16 Akoya pearl oysters *P. fucata martensii* (total wet weight: 42.6 ± 2.6 g (mean ± SE)). The animals were obtained from a marine farm in Tsushima, Nagasaki Prefecture, Japan. After cleaning the shell valves, they were reared for 1 month at 26°C in aerated seawater with added cultivated phytoplankton.[3,19,20] Twenty-four hours before collecting hemolymph, *P. fucata martensii* was transferred to particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 33 psu, water temperature 26°C, O_2 saturation 100%, pH 8.19, and total CO_2 concentration 1.7 mM/L.

Surgical procedures and hemolymph collection

Hemolymph was collected from the adductor muscle using a cannula (PE-50; Clay-Adams polyethylene tubing, 0.96 mm outer diameter, 0.58 mm inner diameter, Becton Dickinson Co., US). A small incision was made in the shell valve from the center of the posterior margin. A cannula with a stylet was inserted through the incision into the adductor muscle and advanced toward the center of the adductor muscle. The stylet was removed, and the outside of the cannula was closed. The cannula was gently fixed to the left shell valve using denture adhesive (Kobayashi Pharmaceutical Co., JP) in order to prevent effects from movement of the shell valves. The cannulated oyster was transferred to the acrylic respiratory chamber and was allowed to recover for 2–3 h at 26.0 ± 0.2°C in normoxic conditions. A hemolymph sample was then drawn through the cannula using a gas-tight micro syringe (1750LTN; Hamilton Co., US). The volume of hemolymph collected was 0.3–0.4 mL.

Hemolymph analysis

Hemolymph pH and total CO_2 concentration (Tco_2) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments Co., US) with glass and reference electrodes (E301, E351; Cameron Instruments Co., US) at 26°C. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5; Cameron Instruments Co., US). Hemolymph total protein concentration (Tpro, g/L) was determined with a test kit (Micro TP-test; Wako Pure Chemical Co., JP) and a spectrophotometer (Spectronic 20A; Shimadzu Co., JP).

The hemolymph Pco_2 and bicarbonate concentration ($[HCO_3^-]$) were calculated by rearranging the Henderson–Hasselbalch equation.[21,22] In the equation, αco_2 and pKapp of *P. fucata martensii* were required. The determinations of αco_2 and pKapp were performed using *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 Co., JP.). 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 Co., US) at 26°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 CO_2 concentration standard gas (10.0%), prevailing barometric pressure, and water vapor pressure at 26°C. For the determination of pKapp, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.1%, 0.2%, 0.5%, 1.0%, and 2.0%; O_2, 20.9%; N_2 balance) using an equilibrator at 26°C. After equilibration, the pH and Tco_2 of the sample were measured using a blood gas meter and total CO_2 analyzer. Using the sample pH, Tco_2, and αco_2, pKapp was determined by rearrangement of Henderson–Hasselbalch equation.[18,21]

Calculation

αco_2 of the experimental animals was calculated using the equation:

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

where the units of the parameters are mM/L/torr for αco_2, mM/L for Tco_2 and torr for Pco_2.

Using the sample pH, Tco_2, and αco_2 calculated using the above equation, pKapp was determined by

rearrangement of Henderson–Hasselbalch equation[18,21] as follows:

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

where Pco_2 is calculated from known CO_2 concentration standard gases.

The hemolymph Pco_2 and $[HCO_3^-]$ were calculated using the equations:

$$Pco_2 = Tco_2 \bullet [\alpha co_2 \bullet (1+10^{(pH-pKapp)})]^{-1}$$
$$[HCO_3^-] = Tco_2 - \alpha co_2 \bullet Pco_2$$

where Tco_2 and pH were measured values, and αco_2 and pKapp were obtained in *in vitro* experiments.

The non-bicarbonate buffer value (β_{NB}, slykes) was obtained as the regression coefficient relating pH and $[HCO_3^-]$ from the *in vitro* experiment.

Statistical analysis

All data are expressed as means ± standard error. Kruskal–Wallis test was performed for changes in pH and pKapp using the CO_2 standard gases. The comparison of two parameters used Mann–Whitney *U* test. Statistically significant differences were set at $P<0.05$ (KyPlot 5.0, KyensLab Inc., JP).

Results

Hemolymph samples were collected anaerobically through a cannula from the adductor muscle of *P. fucata martensii*. The mean values of hemolymph pH and Tco_2 in normoxic conditions were 7.544, and 2.21 mM/L, respectively (Table 1). The hemolymph αco_2 was 37.02 μM/L/torr. The hemolymph pKapp at known CO_2 partial pressures (standard gases) and the corresponding measured pH and Tco_2 values are shown in Table 2. The calculated pKapp from all hemolymph samples was 5.995853 ± 0.051311. Hemolymph Pco_2 and $[HCO_3^-]$ were

Table 1. Hemolymph pH, total CO_2 concentration (Tco_2), CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of the Akoya pearl oyster *Pinctada fucata martensii* at 26°C under normoxic condition

		Mean	SE	N
pH		7.544	0.039	8
Tco_2	mM/L	2.21	0.066	8
Pco_2	torr	1.69	0.164	8
$[HCO_3^-]$	mM/L	2.14	0.062	8

αco_2: 37.02 μM/L/torr; pKapp: 5.995853 (Mean values)

Table 2. Mean values of measured pH, total CO_2 concentration (Tco_2), and calculated apparent dissociation constant of carbonic acid (pKapp) of the hemolymph in adductor muscle of the Akoya pearl oyster *Pinctada fucata martensii* with known Pco_2 standard gases

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	N
0.102	0.75	7.521	1.478	5.80686	8
0.203	1.49	7.389	1.654	5.91215	8
0.515	3.78	7.185	1.977	6.06691	8
1.01	7.41	6.964	2.356	6.08460	8
2.00	14.7	6.714	2.750	6.10875	8

Water temperature 26.0°C

calculated by substitution of the mean value of αco_2 and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \bullet [0.03702 \bullet (1+10^{(pH-5.995853)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - 0.03702 \bullet Pco_2$$

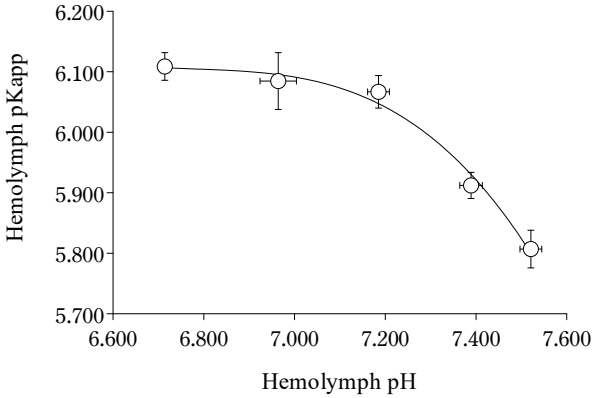

Fig. 1. Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) of hemolymph collected from the adductor muscle of Akoya pearl oyster *Pinctada fucata martensii* at 26°C. Values are means ± standard error. Solid curve fitted to the data and the equation: pKapp = 225.712 – 97.096 • pH + 14.313 • pH^2 – 0.7035 • pH^3 (R^2 = 0.9871)

where the units of the parameters in the equations are torr for Pco_2 and mM/L for Tco_2 and $[HCO_3^-]$.

Hemolymph Pco_2 and $[HCO_3^-]$ at 26°C in normoxic conditions were 1.69 torr and 2.14 mM/L, respectively (Table 1). In *in vitro* experiments (Table 2), the changes in pH and pKapp were statistically significant with the increase in Pco_2 (P<0.05). At the same time, the interaction between pKapp and pH was analyzed (Fig. 1), and the correction equation for pKapp was obtained as follows:

$$pKapp = 225.712 - 97.096 \bullet pH + 14.313 \bullet pH^2 - 0.7035 \bullet pH^3$$

For comparison, Pco_2 and $[HCO_3^-]$ were estimated using the mean value of pKapp and the correction equation (Table 3). The hemolymph Pco_2 estimated by the correction equation was lower than that from the mean value of pKapp (P<0.05). The non-bicarbonate buffer value (β_{NB}), which was obtained as the regression coefficient relating pH and $[HCO_3^-]$ in the *in vitro* experiment, was 0.967 slykes (Table 4). The hemolymph Tpro was 1.6 ± 0.04 g/L.

Table 3. The compariosn of the values calculated by the correction equation and by the mean pKapp in hemolymph Pco_2 and $[HCO_3^-]$

	Pco_2 (torr)	$[HCO_3^-]$ (mM/L)	N
the mean value of pKapp	1.69	2.14	8
pKapp calculated using the correction equation	1.06 *	2.17	8

* : statistically significant difference (Mann–Whitney U test, P<0.05)

Table 4. Mean values of measured pH and calculated bicarbonate concentration ($[HCO_3^-]$) of the hemolymph of Akoya pearl oyster *Pinctada fucata martensii* with known Pco_2 standard gases

Standard gas		Hemolymph		
CO_2 (%)	Pco_2 (torr)	pH	$[HCO_3^-]$ (mM/L)	N
0.102	0.750	7.521	1.45	8
0.203	1.49	7.389	1.60	8
0.515	3.78	7.185	1.84	8
1.01	7.41	6.964	2.08	8
2.00	14.7	6.714	2.21	8

Water temperature: 26.0°C; Non-bicarbonate buffer value (β_{NB}): 0.967

Discussion

We collected the hemolymph of *P. fucata martensii* anaerobically through a cannula from submerged experimental animals after pretreatment by adductor muscle catheterization, and examined hemolymph properties in order to evaluate the acid–base balance of *P. fucata martensii* in normoxic conditions.

P. fucata martensii hemolymph in the adductor muscle exhibited pH of 7.544 and Tco_2 of 2.21 mM/L at 26.0°C. Previously reported hemolymph pH values include pH 7.65 in *Mytilus edulis* at 12°C,[11] pH 7.55 in *M. galloprovincialis* at 18°C,[12] pH 7.414 in *Crassostrea gigas* at 23°C,[16] pH 7.617 in *M. coruscus* at 24°C,[15] pH 7.442 in noble scallop *Mimachlamys nobilis* at 24°C,[14] pH 7.576 in *Ostrea denselamellosa* at 24°C,[17] and pH 7.563 in *P. margaritifera* at 26°C.[13] The hemolymph pH in *P. fucata martensii* was almost the same as that in *M. galloprovincialis*, *O. denselamellosa*, and *P. margaritifera*. Handa and Yamamoto (2015, 2016) and Handa et al. (2017, 2018) reported hemolymph Tco_2 in *P. margaritifera*, *C. gigas*, *M. nobilis*, *M. coruscus*, and *O. denselamellosa* as 2.04 mM/L, 1.87 mM/L, 1.50 mM/L, 1.44 mM/L, and 1.22 mM/L, respectively.[13-17] The content of carbonic acid and CO_2 was approximately the same as *P. margaritifera*, and higher than that in *C. gigas*, *M. nobilis*, *M. coruscus*, and *O. denselamellosa*. *P. fucata* martensii had an acid–base status that was similar to *P. margaritifera* (Pteriidae bivalve), but seemed to be different from Mytilidae, Pectinidae, and Ostreidae bivalves.

Cameron (1986) reported CO_2 solubility as a function of temperature and salinity, and the solubility coefficients were 37.28–38.12 μM/L/torr at 26°C and 30–35 salinity (psu).[24] The hemolymph αco_2 of *P. fucata martensii* at 26.0°C (37.02 μM/L/torr) was similar to the coefficient reported by Cameron (1986). The mean value of hemolymph pKapp in this study was 5.995853, whereas the hemolymph pKapp values of other marine bivalves were 6.114 in *M. edulis* at 12°C,[11,23] 6.07343 in *C. gigas* at 23°C,[16] 6.2609 in *M. coruscus* at 24°C,[15] 6.0641 in *M. nobilis* at 24°C,[14] and 5.9987 in *P. margaritifera* at 26°C.[13] The pKapp value is equal to the pH value at which it is most effective as a buffer.[24] The most effective buffer pH in *P. fucata martensii* hemolymph seemed to be similar to the value in *P. margaritifera*, *C. gigas*, and *M. nobilis*.

In *in vitro* experiments, the pH decreased significantly with an increase in Pco_2 (Table 2). The hemolymph Pco_2 estimated using the correction equation was lower than that from the mean value of pKapp, nevertheless there was no statistical difference in $[HCO_3^-]$ (Table 3). αco_2 and pKapp vary with ionic strength and temperature,[18] and the estimation of Pco_2 may be affected by temperature and salinity. Therefore, it is necessary to examine these parameters at various temperatures and salinities in order to increase the accuracy of the calculation of Pco_2 and to formulate the correction equation.

Using the mean values of hemolymph αco_2 and pKapp in this study, Pco_2 and $[HCO_3^-]$ of the hemolymph in adductor muscle of *P. fucata martensii* were calculated. The mean values of hemolymph Pco_2 and $[HCO_3^-]$ in this species were 1.69 torr and 2.14 mM/L, respectively. In other marine bivalves, the mean values of hemolymph Pco_2 and $[HCO_3^-]$ were 0.9 torr and 1.8 mM/L in *M. edulis* at 12°C,[11] 1.15 torr and 1.62 mM/L in *M. galloprovincialis* at 18°C,[12] 2.18 torr and 1.78 mM/L in *C. gigas* at 23°C,[16] 1.55 torr and 1.44 mM/L in *M. nobilis* at 23°C,[14] and 1.50 torr and 1.98 mM/L in *P. margaritifera* at 26°C.[13] The hemolymph Pco_2 and $[HCO_3^-]$ of *P. fucata martensii* were approximately the same as those of *C. gigas* and *P. margaritifera*.

The non-bicarbonate buffer value (β_{NB}) is provided by the buffer capacity of the non-bicarbonate buffer system by mainly protein and protein residues,[25] and used to quantify the amount of buffering of the solution component. In *P. fucata martensii*, the hemolymph β_{NB} and Tpro were 0.967 slykes and 1.6 g/L, respectively. The hemolymph β_{NB} and Tpro of *M. edulis* were 0.4–0.622 slykes and 0.79 g/L. The hemolymph β_{NB} and Tpro in *P. fucata martensii* were higher than that in *M. edulis*. The high β_{NB} in *P. fucata martensii* was probably as a result of the high concentration of the total protein in the hemolymph. Although there are few reports on the relationship between non-bicarbonate buffer value and total protein concentration in the marine bivalves, β_{NB}

was 0.65 slykes in *M. galloprovincialis*,[12] 0.44 slykes in *M. coruscus*,[15] and 0.732 slykes in *C. gigas*.[16] The hemolymph β_{NB} of *P. fucata martensii* was higher than that of other marine bivalves. Therefore, *P. fucata martensii* should have a relatively high buffering capacity of the non-bicarbonate buffer system of the hemolymph in comparison with *M. edulis*, *M. galloprovincialis*, *M. coruscus*, and *C. gigas*. The changes in hemolymph pH in *P. fucata martensii* could be relatively smaller than that of Mytilidae and Osteridae bivalves, even if environmental CO_2 concentrations increase.

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