正常酸素分圧条件におけるイガイヘモリンパ液の酸塩基平衡

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Acid-base Balance of the Hemolymph in Hard-shelled Mussel *Mytilus coruscus* in Normoxic Conditions

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Abstract : We examined hemolymph 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 hard-shelled mussel *Mytilus coruscus* in normoxic conditions. The hemolymph was collected anaerobically through a cannula by pretreatment of the adductor muscle by catheterization. The mean values of the hemolymph pH and Tco_2 were 7.617 and 1.44 mM/*l*, respectively. The CO_2 solubility coefficient (αco_2) was 40.6 μM/*l*/mmHg. The apparent dissociation constant of carbonic acid (pKapp) was able to be expressed using the estimated equation as follows: $pKapp = -6371.321 + 3923.163 \cdot pH - 856.100 \cdot pH^2 + 82.978 \cdot pH^3 - 3.014 \cdot pH^4$. Using αco_2 and pKapp determined in this study, hemolymph Pco_2 and $[HCO_3^-]$ were calculated as 0.57 mmHg and 1.42 mM/*l*, respectively. The non-bicarbonate buffer value (β_{NB}) was 0.44 Slykes.

Key words : *Mytilus coruscus*, acid-base balance, cannulation, dissociation constant of carbonic acid, CO_2 partial pressure, hemolymph

Introduction

The hard-shelled mussel *Mytilus coruscus* is a Mytilidae bivalve classified in the Mytiloida, PTERIOMORPHIA.[1)] *Mytilus coruscus* is distributed in East Asia and is cultivated commercially as food in China and Korea. In Japan, *M. coruscus* inhabits the rocky bottom of intertidal zones up to 20 m deep from Hokkaido to Kyushu,[1)] and it is caught as a local specialty of the littoral region. *Mytilus coruscus* has been a subject of previous research in terms of the morphology of larvae,[2)] polymorphic microsatellite loci,[3)] microsatellite markers,[4)] biochemical response to heavy metal exposure,[5)] the effect of natural biofilm on the settlement mechanism[6)] and immune activities of hemocytes.[7)] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid-base balance in *M. coruscus*. Research into the acid-base status 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 *M. coruscus* is useful for evaluation of fishery environments, and of the effects of ocean acidification and increase in CO_2 level. In some bivalves in normoxic and normocapnic conditions, the CO_2 partial pressure (Pco_2) of the hemolymph was 0.9 mmHg in blue mussel *Mytilus edulis*,[8)] 1.7-2.3 mmHg in akoya pearl oyster *Pinctada fucata*,[9,10)] and 1.55 mmHg in noble scallop *Mimachlamys nobilis*.[11)] Because the Pco_2 values of bivalves are very low, it was supposed that the Pco_2 in *M. coruscus* would also be similarly low; however, the direct measurement of Pco_2 is difficult. 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 estimates.[12)] 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. Therefore, we examined *M. coruscus* hemolymph pH, total CO_2 content, CO_2 partial pressure, and bicarbonate concentration using the hemolymph αco_2 and pKapp, which were determined in this study. By pretreatment

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with adductor muscle catheterization, the hemolymph was anaerobically from *M. coruscus* underwater.

Materials and Methods

Experimental animals and conditions

The experiments used 40 hard-shelled mussels *Mytilus coruscus* (shell length: 123.1 ± 2.2 mm (mean ± SE), shell height: 58.5 ± 0.9 mm, total wet weight: 186.1 ± 6.3 g). The animals were collected from the coastal sea area of Tana marine biological laboratory of the National Fisheries University in the Seto Inland Sea, Yamaguchi Prefecture, Japan. After cleaning the shell valves, they were reared for 3 months at 24℃ in aerated seawater with added cultivated phytoplankton.[13-15] Twenty-four hours before collecting hemolymph, the mussels were transferred to particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 32 psu, water temperature 24℃, O_2 saturation 99%, pH 8.15, and Tco_2 1.2 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). The small hole (2 mm diameter) was made adjacent to the shell valves near the adductor muscle at the posterior margin. A cannula with a stylet was inserted through the hole into the adductor muscle and was advanced 0.3-0.5 cm 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 with denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent any effect of the movement of the shell valves. This surgical operation was completed within 8 minutes. The cannulated mussel was transferred to a darkened respiratory chamber and was allowed to recover for 3 h at 23.7 ± 0.3℃ 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 0.3-0.4 m*l*.

Hemolymph properties analysis

The hemolymph pH and Tco_2 (mM/*l*) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments) using glass and reference electrodes (E301, E351; Cameron Instruments) at 23.7±0.3℃. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5; Cameron Instruments). The hemolymph CO_2 partial pressure (Pco_2, mmHg) and bicarbonate concentration ($[HCO_3^-]$, mM/*l*) were calculated by rearranging the Henderson-Hasselbalch equation.[16] In the equation, the αco_2, μM/*l*/mmHg) and pKapp of the *M. coruscus* hemolymph were required. The determinations of the αco_2 and pKapp were performed by *in vitro* experiments.

The αco_2 was determined using *M. coruscus* hemolymph adjusted to pH 2.5 by the addition of the 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, 15.0%; O_2, 20.9%; N_2 Balance) using the equilibrator (DEQ-1; Cameron Instruments) at 23.7±0.3℃, and subsequently the total CO_2 content of each equilibrated sample was measured using the total CO_2 analyzer. The CO_2 partial pressure of the equilibrated sample was calculated from a known CO_2 concentration standard gas (15.0%), prevailing barometric pressure, and water vapor pressure at the experimental temperature. The αco_2 was calculated using the equation:

$$\alpha co_2 = \text{Total } CO_2 \text{ content} \bullet CO_2 \text{ Partial pressure}^{-1}$$

For determination of the pKapp, hemolymph was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.2, 0.5, 1.0, 2.0 and 5.0%; O_2, 20.9%; N_2 balance) using an equilibrator at 23.7 ± 0.3℃. After equilibration, the pH and total CO_2 content of the sample were measured with the blood gas meter and the total CO_2 analyzer. Using the sample pH, total CO_2 content and αco_2 calculated using the above equation, the pKapp was determined by rearrangement of the Henderson-Hasselbalch equation[16] as follows:

$$pKapp = pH - \log [(\text{total } CO_2 \text{ content} - \alpha co_2 \bullet CO_2 \text{ partial pressure}) \bullet (\alpha co_2 \bullet CO_2 \text{ partial pressure})^{-1}]$$

where CO_2 partial pressure is calculated from the known CO_2 concentration of standard gases.

The αco_2 and pKapp obtained in this study were used for the calculation of hemolymph Pco_2 from measured pH and Tco_2:

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

The hemolymph $[HCO_3^-]$ was calculated from Tco_2, αco_2, and Pco_2 using the following equation[23] :

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

The non-bicarbonate buffer value (β_{NB}, Slykes), which is usually described at the absolute value, was calculated as the regression coefficient relating $[HCO_3^-]$ and pH in *in vitro* experiments with the standard gases.

Statistical analysis

All data are expressed as means ± standard error. Normality of distribution in hemolymph properties was assessed through use of the Shapiro-Wilk test. The homoscedasticity of variance was assessed using Bartlett's test for comparison the properties of hemolymph, which was equilibrated with standard CO_2 gases. One-way analysis of variance (ANOVA) was performed for changes in hemolymph properties using the standard CO_2 gases. Statistically significant differences were set at $P<0.01$.

Results

Hemolymph samples were collected from the adductor muscles of *M. coruscus* through cannulae. The collection volume was 0.3-0.4 *ml* from each individual. The hemolymph pH and Tco_2 in normoxic conditions were 7.617 ± 0.0225 and 1.44 ± 0.047 mM/*l*, respectively (Table 1). In *in vitro* experiments, the hemolymph αco_2 was 40.6 ± 0.37 μM/*l*/mmHg. 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 mean value of all pKapp was 6.2609. However, the pH was statistically significantly lowered with the rise in Pco_2, and the values of pKapp with each CO_2 standard gas were statistically significantly different (Table 2). Therefore, the interaction between pKapp and pH was analyzed, and the estimated equation of pKapp was obtained as follows:

Table 1. Hemolymph pH, total CO_2 content (Tco_2), CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of *Mytilus coruscus* at 24℃ in normoxic conditions

		Mean	SE	N
pH		7.617	0.0225	16
Tco_2	mM/*l*	1.44	0.047	16
Pco_2	mmHg	0.57	0.158	16
$[HCO_3^-]$	mM/*l*	1.42	0.043	16

Mean temparature 23.7 ℃; αco_2 40.6 μM/*l*/mmHg ;
see the details of the pKapp equation in the Result section

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

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (mmHg)	pH	Tco_2 (mM/l)	pKapp	N
0.203	1.51	7.483	1.549	6.10449	24
0.509	3.79	7.290	1.660	6.31157	24
0.993	7.39	7.074	1.984	6.33207	24
1.99	14.8	6.732	2.156	6.33513	24
4.97	37.0	6.336	3.569	6.22109	24

Water temperature 23.7 ℃; barometric pressure 765.7 mmHg ; water vapor pressure 21.98 mmHg

$$pKapp = -6371.321 + 3923.163 \cdot pH - 856.100 \cdot pH^2 + 82.978 \cdot pH^3 - 3.014 \cdot pH^4$$

Pco_2 and $[HCO_3^-]$ were calculated by substitution of the hemolymph αco_2 and pKapp in the rearranged Henderson-Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \cdot [0.0406 \cdot (1+10^{(pH-pKapp)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - 0.0406 \cdot Pco_2$$

where the units of the parameters in the equations were mmHg for Pco_2 and mM/l for Tco_2 and $[HCO_3^-]$.

In *in vivo* and *in vitro* experiments, Hemolymph Pco_2 and $[HCO_3^-]$ at 23.7℃ in normoxic conditions were 0.57 mmHg and 1.42 mM/l, respectively (Table 1). The mean values of Tco_2 and $[HCO_3^-]$ of hemolymph with known Pco_2 standard gases are shown in Table 3, and the non-bicarbonate buffer value (β_{NB}) which was obtained as the regression coefficient relating $[HCO_3^-]$ and pH was 0.44 Slykes.

Discussion

We collected *M. coruscus* hemolymph from the adductor muscle, and examined hemolymph pH, Tco_2, Pco_2, and $[HCO_3^-]$ in order to evaluate the acid-base balance of *M. coruscus* in normoxic conditions. The hemolymph was collected anaerobically through a cannula from animals kept underwater after pretreatment by adductor muscle catheterization. The mean values of pH and Tco_2 measured immediately after hemolymph collection were 7.617 and 1.44 mM/l, respectively. Previously reported mean values of hemolymph pH include 7.65 in blue mussel *M. edulis* at 12℃,[8] 7.36 in Pacific oyster *Crassostrea gigas* at 15℃,[17] 7.55 in *M. galloprovincialis* at 18℃,[18] 7.284–7.375 in *P. fucata* at 28℃,[9-10] 7.563 in *P. margaritifera* at 26℃,[19] and 7.442 in noble scallop *Mimachlamys nobilis* at 24℃.[11] Although there are few descriptions of hemolymph Tco_2 in marine bivalves, Handa and Yamamoto (2012, 2015, 2016) reported the mean values of Tco_2 in *P. fucata*, *P. margaritifera*, and *M. nobilis* as 1.90–2.10 mM/l,[10] 2.04 mM/l[19], and 1.50 mM/l,[11] respectively. The hemolymph pH in *M. coruscus* was almost the same as that in *M. edulis* and higher than that in other marine bivalves, and the contents of carbonic acid and CO_2 in *M. coruscus* hemolymph appeared to be less than in pearl oysters.

Cameron (1986) reported the CO_2 solubility as a function of temperature and salinity, and the solubility coefficients were 39.2–42.3 μM/l/mmHg at 22–24℃ and 30–35 salinity (psu).[20] The hemolymph αco_2 in *M. coruscus* (40.6 μM/l/mmHg) was in the range of the coefficient reported in Cameron (1986). The mean value

Table 3. Mean values of measured total CO_2 content (Tco_2) and calculated bicarbonate concentration ($[HCO_3^-]$) of hemolymph in the adductor muscle of *Mytilus coruscus* with known Pco_2 standard gases

Standard gas		Hemolymph		
CO_2 (%)	Pco_2 (mmHg)	Tco_2 (mM/*l*)	$[HCO_3^-]$ (mM/*l*)	N
0.203	1.51	1.55	1.49	24
0.509	3.79	1.66	1.51	24
0.993	7.39	1.98	1.69	24
1.99	14.8	2.16	1.56	24
4.97	37.0	3.57	2.07	24

Water temperature 23.7 ℃; water vapor pressure 21.98 mmHg; αco_2 40.6 μM/*l*/mmHg

of hemolymph pKapp in this study was 6.2609, whereas the hemolymph pKapp values of other marine bivalves were 5.8191 in the *P. fucata* at 28℃,[10] 5.9987 in *P. margaritifera* at 26℃,[19] 6.0641 in *M. nobilis* at 23℃,[11] and 6.114 in *M. edulis* at 12℃.[8,21] The pKapp is equal to the pH at which it is most effective as a buffer.[22] The most effective buffer pH in *M. coruscus* hemolymph seemed to be similar to the value in *M. edulis.*

Using the hemolymph αco_2 and pKapp in this study, Pco_2 and $[HCO_3^-]$ of the hemolymph of *M. coruscus* were calculated. The pKapp was estimated by the relational expression corresponding to the change in pH because the pH significantly with an increase in Pco_2 in standard gases. The mean values of hemolymph Pco_2 and $[HCO_3^-]$ in *M. coruscus* were 0.57 mmHg and 1.42 mM/*l*, respectively (Table 1). In other marine bivalves, the mean values of hemolymph Pco_2 and $[HCO_3^-]$ were 0.9 torr (0.9 mmHg) and 1.8 mM/*l* in *M. edulis* at 12℃,[8] 1.15 mmHg and 1.62 mM/*l* in *M. galloprovincialis* at 18℃,[18] and 1.50 mmHg and 1.98 mM/*l* in *P. margaritifera* at 26℃,[19] and 2.08–2.33 mmHg and 1.83–2.04 mM/*l* in the *P. fucata* at 28℃.[10] The acid-base status of *M. coruscus* approached that of *M. edulis.*

The β_{NB} of *M. coruscus* hemolymph (0.44 Slykes) was lower than that of *P. fucata* (1.45 Slykes)[17] and *C. gigas* (0.88 Slykes),[17] and was in the same range as *M. edulis* (0.4–0.622 Slykes).[8, 21] The non-bicarbonate buffer value is decided by the buffer capacity of the non-bicarbonate buffer system (for example, protein buffer system), and used to quantify the amount of buffering of the solution component. The interaction of the CO_2 and bicarbonate buffer systems with non-bicarbonate buffers is particularly advantageous when nonvolatile H^+ ions are to be buffered in a buffer system.[23] Therefore, the *M. coruscus* would experience a large change in hemolymph pH with a slight fluctuation of Pco_2. *Mytilus coruscus* seems to be sensitive to environmental changes in comparison with *P. fucata* and *C. gigas* from the viewpoint of acid-base balance of the hemolymph.

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正常酸素分圧条件におけるイガイヘモリンパ液の酸塩基平衡

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要　旨

イガイ（*Mytilus coruscus*）の酸塩基平衡を解明するため、供試貝の閉殻筋にヘモリンパ液を採取する為のカニュレーション手術を行った。手術から回復した供試貝から、カニューラを通じてヘモリンパ液を嫌気的に採取し、正常酸素分圧条件におけるイガイヘモリンパ液の酸塩基平衡を分析した。その結果、ヘモリンパ液のpH 7.617、全炭酸含量1.44 mM/*l*、二酸化炭素分圧0.57 mmHg、炭酸水素イオン濃度1.42 mM/*l*を示した（環境水の酸素飽和度99 %、pH 8.15、全炭酸含量 1.2 mM/*l*、水温24.0 ℃）。