Acid-Base Balance of Hemolymph in Pacific oyster Crassostrea gigas in Normoxic Conditions

Acid–Base Balance of Hemolymph in Pacific oyster *Crassostrea gigas* 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 ability of the acid–base balance of the Pacific oyster *Crassostrea gigas* in normoxic conditions. Hemolymph was collected anaerobically through a cannula inserted into the adductor muscle of Pacific oyster submerged in experimental seawater. The mean values of hemolymph Po_2, pH, and Tco_2 were 62.0 torr, 7.414, and 1.87 mM/*l*, respectively. The apparent dissociation constant of carbonic acid (pKapp) was estimated using the following equation: pKapp = 33.462 – 13.032 • pH + 2.065 • pH^2 – 0.1088 • pH^3. Using αco_2 (40.51 μM/*l*/torr) and pKapp determined in this study, the hemolymph Pco_2 and $[HCO_3^-]$ were calculated as 2.18 torr and 1.78 mM/*l*, respectively. The non-bicarbonate buffer value (β_{NB}) was 0.732 Slykes. These hemolymph properties were compared with those of other marine bivalves. The Pacific oyster should have an acid–base balance that is similar to Pectinidae and Pteriidae bivalves, but which is different from Mytilidae bivalves.

Key words : *Crassostrea gigas*, hemolymph, acid–base balance, normoxia, cannulation, adductor muscle

Introduction

Pacific oyster *Crassostrea gigas* inhabits the intertidal and subtidal gravel to mud bottom of brackish-water embayments and often forming oyster reefs.[1)] Pacific oyster is an important cultured species, and worldwide production amounted to 662,513 tons in 2010.[2)] In Japan, the production volume in 2016 was 106,111 tons in Hiroshima, 19,366 tons in Okayama and 11,581 tons in Miyagi prefectures.[3)] Pacific oyster has been a subject of a previous study in terms of anatomy and respiratory physiology. The anatomical structures of the digestive diverticula, ctenidium and circulatory system were clarified recently.[4,5)] The regulation of the ventilation volume, oxygen uptake, and ciliary movement of the ctenidium in normoxic, hypoxic, hypotonic, anathermal, and feeding conditions have been studied.[6-10)] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid–base balance in Pacific oyster *C. gigas*. Handa et al. (2017A) developed surgical procedures, cannulation of the adductor muscle of Pacific oyster, and examined the hemolymph oxygen and acid–base status postoperation.[11)] The Pacific oyster temporarily showed slight hypoxemia without acidosis just after surgery, but the fluctuation disappeared 1 h after surgery. In this study, we elucidated the hemolymph acid–base balance of Pacific oyster in normoxic conditions. 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 Pacific oyster is useful for evaluation of 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 was 0.57–2.3 torr.[12-18)] The hemolymph Pco_2 of Pacific oyster was supposed to be low as in other bivalves; therefore,

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direct measurement of Pco_2 would be 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 such estimates.[19] 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 determined hemolymph αco_2 and pKapp of Pacific oyster, and evaluated acid–base balance of hemolymph in normoxic conditions.

Materials and Methods

Experimental animals and conditions

The experiments used 54 Pacific oysters *Crassostrea gigas* (shell length: 60.3 ± 2.2 mm (mean ± SE), shell height: 120.2 ± 6.1 mm, total wet weight: 112.8 ± 7.6 g). The animals were obtained from a marine farm in the western sea area of Hiroshima Prefecture, Japan. After cleaning the shell valves, they were reared for 1 month at 23°C in aerated seawater with added cultivated phytoplankton.[6,9,20] Twenty-four hours before collecting hemolymph, the Pacific oysters were transferred to particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 32 psu, water temperature 23 °C, O_2 saturation 98%, pH 8.19, and total CO_2 content 1.4 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).[11] 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 5 mm 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., Ltd.) in order to prevent effects from movement of the shell valves. This surgical operation was completed within 7 minutes. The cannulated oyster was transferred to a darkened acrylic respiratory chamber and was allowed to recover for 2 hr at 23.0 ± 0.2° 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 0.5 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 23.0°C. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5, Cameron Instruments). The hemolymph CO_2 partial pressure (Pco_2, torr) and bicarbonate concentration ($[HCO_3^-]$, mM/*l*) were calculated by rearranging the Henderson–Hasselbalch equation.[19,21] In the equation, the CO_2 solubility coefficient (αco_2, μM/*l*/torr) and apparent dissociation constant of carbonic acid (pKapp) of Pacific 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, 15.0%; O_2, 20.9%; N_2 Balance) using an equilibrator (DEQ-1, Cameron Instruments) at 23.0°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 CO_2 concentration standard gas (15.0%), prevailing barometric pressure, and water vapor pressure at 23.0°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, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 23.0°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, and pKapp was determined by rearrangement of Henderson–Hasselbalch equation[19,21] as follows:

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

where Pco_2 was calculated from known CO_2 concentration standard gases. The αco_2 and pKapp obtained in this study were used for calculation of hemolymph Pco_2 from measured pH and Tco_2:

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

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

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

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 was assessed using Bartlett's test or F-test. Kruskal–Wallis test was performed for changes in hemolymph properties using the standard gases. The comparison of two parameters used unpaired *t*-test in normal distribution and homoscedasticity. Statistically significant differences were set at $P<0.05$.

Results

Hemolymphs were collected anaerobically from the adductor muscles of Pacific oysters through cannulae. The mean values of hemolymph Po_2, pH, and Tco_2 in normoxic conditions were 62.0 torr, 7.414, and 1.87 mM/*l*, respectively (Table 1). The hemolymph αco_2 was 40.51 ± 0.24 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 6.07343 ± 0.01756. Hemolymph Pco_2 and $[HCO_3^-]$ were calculated by substitution of the mean value of αco_2 and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \bullet [0.04051 \bullet (1+10^{(pH-6.07343)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - 0.04051 \bullet Pco_2$$

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 23°C in normoxic conditions were 2.18 torr and 1.78 mM/*l*, respectively (Table 3). In *in vitro* experiments (Table 2), the changes in pH, Tco_2, 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:

$$pKapp = 33.462 - 13.032 \bullet pH + 2.065 \bullet pH^2 - 0.1088 \bullet pH^3$$

For comparison, Pco_2 and $[HCO_3^-]$ were estimated using the mean value of pKapp and the correction equation. There was no significant difference in hemolymph Pco_2 and $[HCO_3^-]$ calculated by the two methods (Table 4). The mean values of pH and $[HCO_3^-]$ in *in vitro* experiments and the non-bicarbonate buffer value (β_{NB}), which was obtained as the regression coefficient relating pH and $[HCO_3^-]$, was 0.732 Slykes (Table 5).

Discussion

We collected Pacific oyster hemolymph from the adductor muscle, and examined hemolymph Po_2, pH, Tco_2, Pco_2 and $[HCO_3^-]$ in order to evaluate the acid–base

balance of Pacific oyster in normoxic conditions. The hemolymph was collected anaerobically through a cannula from submerged experimental animals after pretreatment by adductor muscle catheterization. The hemolymph Po_2 in this study was 62.0 ± 6.86 torr at 23℃ (Table 1). Allen and Burnett (2008) and Tran et al. (2008) reported Pacific oyster hemolymph Po_2 in the adductor muscle by direct puncture as 7.09 ± 0.53 kPa (53.17 ± 3.98

Table 1. Hemolymph oxygen partial pressure (Po_2), pH and total CO_2 content (Tco_2) of the Pacific oyster *Crassostrea gigas* in normoxic condition

		Mean	SE	N
Po_2	torr	62.0	6.86	10
pH		7.414	0.0592	10
Tco_2	mM/*l*	1.87	0.104	10

Water temperature. 23.0 ± 0.2℃ (Mean ± SE)

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 Pacific oyster *Crassostrea gigas* with known Pco_2 standard gases

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/*l*)	pKapp	N
0.102	0.758	7.476	1.05	5.98178197	8
0.203	1.509	7.278	1.13	6.04599521	8
0.515	3.831	7.031	1.48	6.10528476	8
1.01	7.519	6.804	1.84	6.10272056	8
2.00	14.860	6.579	2.31	6.12683108	6
5.00	37.190	6.202	3.38	6.10923144	6

Barometric pressure, 764.1 torr; water temperature, 23.0 ℃; αco_2, 40.51 μM/*l*/torr

Table 3. Hemolymph CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of the Pacific oyster *Crassostrea gigas* in normoxic condition

		Mean	SE	N
Pco_2	torr	2.18	0.314	10
$[HCO_3^-]$	mM/*l*	1.78	0.097	10

Water temperature. 23.0 ± 0.2 ℃ (Mean ± SE)

Table 4. The comparison 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	2.18 ± 0.314	1.78 ± 0.097	10
pKapp calculated by the correction equation	1.95 ± 0.346	1.79 ± 0.097	10

Data are shown mean ± SE. No statistically significant difference (unpaired *t*-test, $P>0.05$)

Table 5. Mean values of measured pH and calculated bicarbonate concentration ($[HCO_3^-]$) of the hemolymph in adductor muscle of with known Pco_2 standard gases

Standard gas		Hemolymph		
CO_2 (%)	Pco_2 (torr)	pH	$[HCO_3^-]$ (mM/*l*)	N
0.102	0.758	7.476	1.023	8
0.203	1.509	7.278	1.071	8
0.515	3.831	7.031	1.323	8
1.01	7.519	6.804	1.538	8
2.00	14.860	6.579	1.711	6
5.00	37.190	6.202	1.880	6

Barometric pressure, 764.1 torr ; water temperature, 23.0 °C.
Non-bicarbonate buffer value (β_{NB} : the regression coefficient relating pH and bicarbonate), 0.732

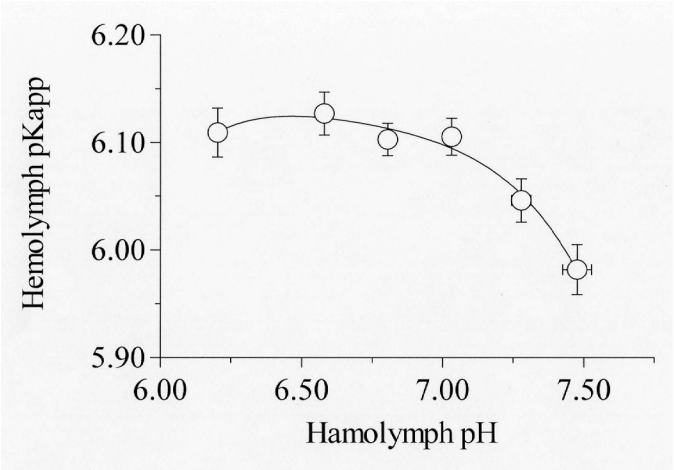

Fig 1. Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) of hemolymph collected from the adductor muscle of Pacific oyster *Crassostrea gigas* at 23°C. Values are means ± standard error (N=44). Solid line fitted to the data and the equation: $pKapp = 33.462 - 13.032 \bullet pH + 2.065 \bullet pH^2 - 0.1088 \bullet pH^3$ ($r^2 = 0.9790$)

torr) at 18°C and 3.8–5.4 kPa (28.5–40.5 torr) at 26°C, respectively.[2,23] In marine blue mussel *Mytilus edulis*, the adductor muscle comprises a large fraction of the total hemolymph volume,[24] and the hemolymph samples collected from the adductor muscle probably contain a mixture of pre- and post-branchial hemolymph from various regions of the circulatory system.[12] Pacific oyster hemolymph, which was collected from the adductor muscle, would circulate around various regions and perfuse to the adductor muscle.

Pacific oyster hemolymph pH and Tco_2 measured immediately after hemolymph collection were 7.414 and 1.87 mM/*l* at 23.0°C, respectively. Previously reported hemolymph pH values include pH 7.65 in *M. edulis* at 12°C,[12] pH 7.55 in Mediterranean mussel *Mytilus galloprovincialis* at 18°C,[13] pH 7.617 in hard-shelled mussel *Mytilus coruscus* at 24°C,[18] pH 7.284–7.375 in akoya pearl oyster *Pinctada fucata martensii* at 28°C,[14,15] pH 7.563 in black-lip pearl oyster *Pinctada margaritifera* at 26°C[16] and pH 7.442 in noble scallop *Mimachlamys nobilis* at 24°C.[17] Handa and Yamamoto (2012, 2015, 2016) and Handa et al. (2017B) reported hemolymph Tco_2 in *P. fucata martensii, P. margaritifera, M. nobilis*, and *M. coruscus* as 1.90–2.10 mM/*l*,[15] 2.04 mM/*l*,[16] 1.50 mM/*l*[17] and 1.44 mM/*l*,[18] respectively. The hemolymph pH in Pacific oyster was almost the same as that in *M. nobilis* and lower than that in *M. edulis*, *M. galloprovincialis* and *M. coruscus*. The contents of carbonic acid and CO_2 was approximately the same as *P. fucata martensii* and *P. margaritifera*, and higher than in *M. nobilis*, *M. galloprovincialis* and *M. coruscus*. The Pacific oyster should have the acid–base status that is similar to Pectinidae and Pteriidae bivalves, and which is different from Mytilidae bivalves.

Cameron (1986) reported CO_2 solubility as a function of temperature and salinity, and the solubility coefficients were 39.2–42.3 μM/*l*/torr at 22–24°C and 30–35 salinity (psu).[25] The hemolymph αco_2 in Pacific oyster (40.51 μM/*l*/torr) was in the range of the coefficient reported in Cameron (1986). The mean value of hemolymph pKapp in this study was 6.07343, whereas the hemolymph pKapp values of other marine bivalves were 5.8191 in the *P. fucata martensii* at 28°C,[15] 5.9987 in *P. margaritifera* at 26°C,[16] 6.0641 in *M. nobilis* at 23°C,[17] 6.2609 in *M. coruscus* at 24°C[18] and 6.114 in *M. edulis* at 12°C.[12,26] The pKapp value is equal to the pH value at which it is most effective as a buffer.[27] The most effective buffer pH in Pacific oyster hemolymph seemed to be similar to the value in *P. margaritifera* and *M. nobilis*.

Using the hemolymph αco_2 and pKapp in this study, Pco_2 and $[HCO_3^-]$ of the hemolymph of Pacific oyster were calculated. The pKapp was estimated by the relational expression corresponding to the change in pH because the pH changed significantly with an increase in Pco_2 in standard gases. The mean values of hemolymph Pco_2 and $[HCO_3^-]$ in Pacific oyster were 2.18 torr and 1.78 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,[12] 1.15 torr and 1.62 mM/*l* in *M. galloprovincialis* at 18°C,[13] and 0.57 torr and 1.42 mM/*l* in *M. coruscus* at 24°C,[18] 1.50 torr and 1.98 mM/*l* in *P. margaritifera* at 26°C,[16] and 2.08–2.33 torr and 1.83–2.04 mM/*l* in *P. fucata martensii* at 28°C.[15] The hemolymph acid–base status of Pacific oyster approximated that of *P. fucata martensii*.

The β_{NB} of Pacific oyster hemolymph (0.732 Slykes) was lower than that of *P. fucata martensii* (1.35–1.45 Slykes)[15] but higher than those of *M. edulis* (0.4–0.622 Slykes),[12,26] *M. galloprovincialis* (0.65 Slykes),[28] and *M. coruscus* (0.44 Slykes).[18] The non-bicarbonate buffer value was 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.[29] Therefore, Pacific oyster *C. gigas* could experience a change in hemolymph pH with an anaerobic metabolism or a slight fluctuation of seawater pH. Pacific oyster *C. gigas* seems to be sensitive to environmental changes in comparison with *P. fucata martensii*, but not more sensitive than Mytilidae bivalves from the viewpoint of acid–base balance of the hemolymph.

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