Oxygen and Acid-Base Status of Hemolymph in the Pacific oyster *Crassostrea gigas* after Cannulation of the Adductor Muscle

Takeshi Handa†, Akira Araki and Ken-ichi Yamamoto

Abstract : We examined hemolymph O_2 partial pressure, pH, total CO_2 content, CO_2 partial pressure and bicarbonate concentration in order to evaluate the acid-base balance of the Pacific oyster *Crassostrea gigas* after pretreatment of the adductor muscle by cannulation. The hemolymph O_2 and acid-base properties changed just after surgery. The temporary and significantly fluctuation of hemolymph properties disappeared at 1 h after surgery in this study, and the O_2 and acid-base status was stable afterwards in normoxic conditions. The results in this study showed the possibility that sampling with a cannula can collect hemolymph as required. This sampling may be useful, when respiratory and endocrine function must be monitored in minimally disturbed animals without the effects of handling.

Key words : *Crassostrea gigas*, Pacific oyster, cannulation, hemolymph, acid-base balance, adductor muscle

Introduction

Pacific oyster *Crassostrea gigas* is a Ostreidae bivalve classified in the Pterioida, Pteriomorphia,[1] and is widely distributed in Japan and East Asia.[1] Pacific oyster inhabits the intertidal and subtidal gravel to mud bottom of brackish-water embayments, and it often forms oyster reefs.[1] The Pacific oyster is an important cultured species for food, and it is cultivated in many countries. In Japan, the production volume of Pacific oyster is greatest in Hiroshima, Okayama, Hyogo, and Miyagi prefectures. Pacific oyster has been the subject of previous research in terms of anatomy and respiratory physiology. The anatomical structures of the digestive diverticula, ctenidium, and circulatory system were clarified recently.[2,3] The regulation of ventilation volume, O_2 uptake, and ciliary movement of the ctenidium in normoxic, hypoxic, hypotonic, anathermal, and feeding conditions has been studied.[4-8] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid-base balance in Pacific oyster. 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 Pacific oyster was useful for the evaluation of cultivation environments, and of the effects of ocean acidification and increasing CO_2 levels. 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 (mmHg).[9-15] The hemolymph Pco_2 of Pacific oyster was supposed be low and similar to other bivalves, and, therefore, direct measurements of Pco_2 would be difficult. The estimation of 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 the 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 were required for experimental animals. Therefore, we determined hemolymph αco_2 and pKapp, and estimated hemolymph Pco_2 and bicarbonate concentration ($[HCO_3^-]$). In order to accurately measure the hemolymph properties (O_2 and acid-base status), hemolymph was collected from submerged Pacific oysters. We developed a hemolymph withdrawal method using a cannula. The

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surgical procedure for collecting hemolymph involved cannulation of the adductor muscle, and hemolymph was collected anaerobically through the cannula from submerged Pacific oysters. This study evaluated the effect of the surgical procedures on the basis of the change of the hemolymph properties, O_2 and acid–base status, in the Pacific oyster *C. gigas*. The technical knowledge proposed in this study may contribute to the advances in research on respiratory physiology and homeostasis in Pacific oyster.

Materials and Methods

Experimental animals and conditions

The experiments used 29 Pacific oyster *Crassostrea gigas* (shell length: 58.9 ± 1.4 mm (mean ± SE), shell height: 123.6 ± 3.1 mm, total wet weight: 113.2 ± 5.2 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 10℃ in aerated seawater with added cultivated phytoplankton.[3–5] 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 10℃, O_2 saturation 99%, pH 8.18, and total co_2 content 1.3 mM/L.

Surgical procedures

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). A 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 5 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 right shell valve using denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent any effect of the movement of the shell valves. This surgical procedure was completed within 7 minutes. The cannulated oyster was transferred to a respiratory chamber at 10.1 ± 0.2℃ in normoxic conditions.

Hemolymph collection

Multiple collections of hemolymph were conducted at 0 h (initial collection), 0.5 h, 1 h, 2 h, 3 h, 24 h, and 48 h after surgery (N=6). Single collection of hemolymph was conducted at 24 h after surgery (N=6). A hemolymph sample was drawn through the cannula using a gas-tight micro syringe (Model 1750, Hamilton Co.). The volume of hemolymph collected was 0.3–0.4 mL in each time.

Hemolymph properties analysis

The hemolymph O_2 partial pressure (Po_2, torr), pH, and total CO_2 content (Tco_2, mM/L) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments) with glass and reference electrodes (E301, E351; Cameron Instruments) at 10.0±0.2℃. 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.[17] In the equation, the αco_2 and pKapp of the Pacific oyster hemolymph were required. The determinations of αco_2 and pKapp were performed by *in vitro* experiments.

The αco_2 was determined using Pacific oyster hemolymph 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 the equilibrator (DEQ-1; Cameron Instruments) at 10.0 ± 0.3℃, and subsequently the Tco_2 of each equilibrated sample was measured using the total CO_2 analyzer. The Pco_2 of the equilibrated sample was calculated from a known CO_2 concentration standard gas (15%), prevailing barometric pressure, and water vapor pressure at the experimental

temperature. αco_2 was calculated using the equation:

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

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%, 5.0%, and 15%; O_2, 20.9%; N_2 balance) using an equilibrator at 10.1 ± 0.3℃. After equilibration, the pH and Tco_2 of the sample were measured using the blood gas meter and the total CO_2 analyzer. Using the sample pH, Tco_2 and αco_2 calculated using the above equation, pKapp was determined by rearrangement of the Henderson-Hasselbalch equation[17] 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 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[18] :

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

Statistical analysis

All data are expressed as mean ± standard error. Normality of distribution in hemolymph properties was assessed through use of the Shapiro-Wilk test. For the test of the fluctuation of the hemolymph properties in multiple collections, two-way repeated measures analysis of variance and one-way analysis of variance were used with normal distributions. Multiple comparison test used the Tukey-Kramer's test. Unpaired *t*-test was used for the comparison of mean values of hemolymph properties between the multiple collections and a single collection. For non-normal distributions, multiple comparison test used the Friedman test. Wilcoxon rank-sum test was used for the comparison of hemolymph properties between the multiple collections and a single collection. In *in vitro* experiments, one-way analysis of variance was performed for changes in hemolymph properties using the standard CO_2 gases. Statistically significant differences were set at $P<0.01$.

Results

Hemolymph was collected from the adductor muscles of Pacific oyster through cannulae. The mean values of hemolymph Po_2 were statistically significantly increased from 0 h to 0.5 h, and Po_2 was 57.6-67.1 torr at 1 h or later (Fig. 1). The hemolymph pH and Tco_2 were 7.576-7.760 and 1.36-1.50 mM/L, respectively (Figs. 2-3). In *in vitro* experiments, the hemolymph αco_2 was 59.11 ± 0.98 μM/L/torr (N=9), and the hemolymph pKapp was 6.3158 ± 0.0374 (N=8). Pco_2 and $[HCO_3^-]$ were calculated by substitution of the mean value of hemolymph αco_2 and

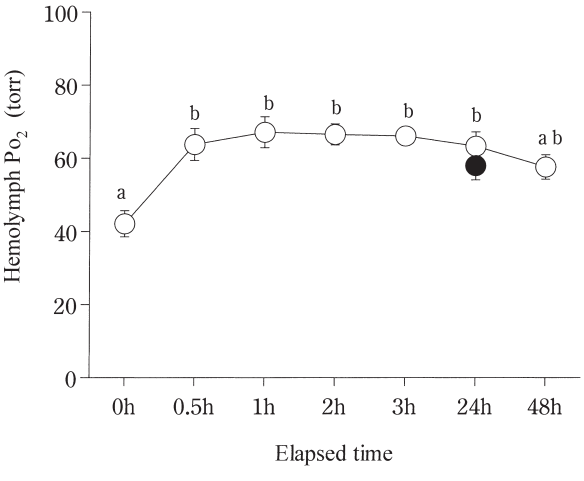

Fig. 1. Hemolymph oxygen partial pressure (Po_2, torr) in Pacific oyster *Crassostrea gigas* after surgical procedures (cannulation of the adductor muscle). The time indicates the time elapsed from when the surgical procedure and the transfer of the experimental animal to a respiratory chamber were completed. The values shown are means ± SE. Each value from multiple and single collections is shown as open circles and closed circles, respectively. Different letters indicate statistically significant differences from the other values ($P<0.01$).

pKapp in the rearranged Henderson-Hasselbalch equation. The hemolymph Pco_2 decreased slightly from 0 h to 0.5 h and was 0.82-1.05 torr at 1 h or later (Fig. 4). $[HCO_3^-]$ was 1.33-1.44 mM/L (Fig. 5). The fluctuation of hemolymph properties in the multiple collection were not statistically significant, except for Po_2 at 0 h. There was no significant difference in the multiple collections and the single collection (Figs. 1-5).

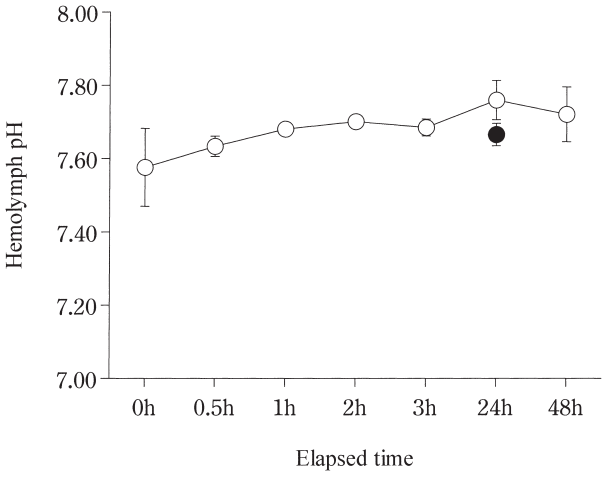

Fig. 2. Hemolymph pH in Pacific oyster *Crassostrea gigas* after surgical procedures (cannulation of the adductor muscle). The time indicates the time elapsed from when the surgical procedure and the transfer of the experimental animal to a respiratory chamber were completed. The values are shown means ± SE. The symbols are the same as in Fig. 1. There were no statistically significant differences in each value.

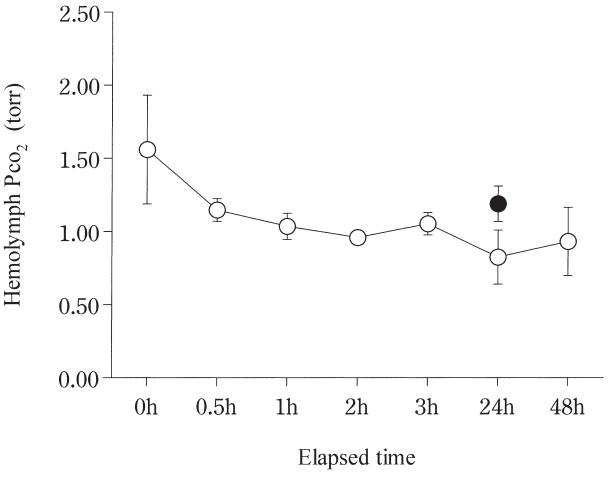

Fig. 4. Hemolymph CO_2 partial pressure (Pco_2, torr) in Pacific oyster *Crassostrea gigas* after surgical procedures (cannulation to the adductor muscle) . The time indicates the time elapsed from when the surgical procedure and the transfer of the experimental animal to a respiratory chamber were completed. The values are shown means ± SE. The symbols are the same as in Fig. 1. There were no statistically significant differences in each value.

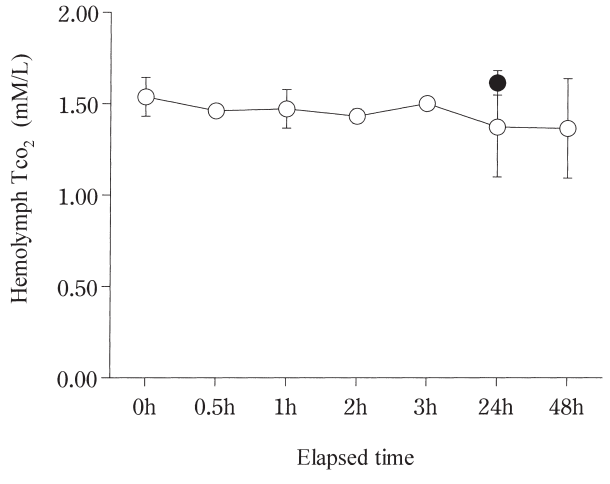

Fig. 3. Hemolymph total CO_2 content (Tco_2, mM/L) in Pacific oyster *Crassostrea gigas* after surgical procedures (cannulation of the adductor muscle). The time indicates the time elapsed from when the surgical procedure and the transfer of the experimental animal to a respiratory chamber were completed. The values are shown means ± SE. The symbols are the same as in Fig. 1. There were no statistically significant differences in each value.

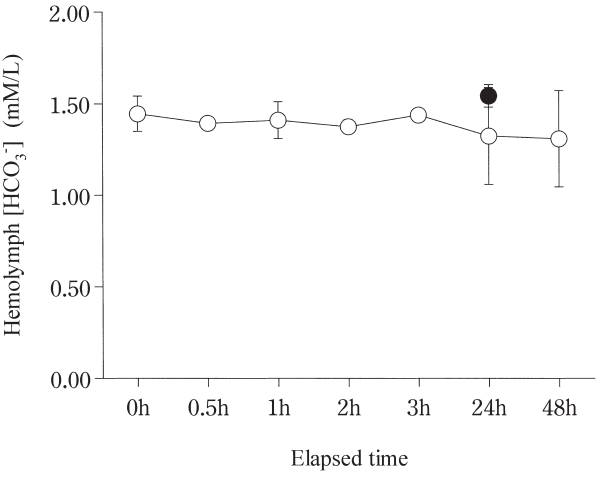

Fig. 5. Hemolymph bicarbonate concentration ($[HCO_3^-]$, mM/L) in Pacific oyster *Crassostrea gigas* after surgical procedures (cannulation of the adductor muscle). The time indicates the time elapsed from when the surgical procedure and the transfer of the experimental animal to a respiratory chamber were completed. The values are shown means ± SE. The symbols are the same as in Fig. 1. There were no statistically significant differences in each value.

Discussion

We carried out repeated collections of hemolymph from the adductor muscle of the Pacific oyster through cannulae and measured hemolymph Po_2, pH, and Tco_2. The hemolymph αco_2 and pKapp were determined by *in vitro* experiments using a tonometer and CO_2 standard gases. The αco_2 and pKapp obtained in this study were used for the calculation of hemolymph Pco_2 and $[HCO_3^-]$. The Po_2, pH, Tco_2, Pco_2, and $[HCO_3^-]$ of hemolymph in the multiple collections were stable by 1 h after the surgical procedure. The hemolymph properties in the multiple collections were not significantly different from those in the single collection. These findings indicated the O_2 and acid–base status were not greatly influenced at 1 h after the surgical procedure in these experimental conditions.

The hemolymph Po_2 in Pacific oyster just after surgery (0 h) was significantly lower than that at 0.5 h or later because the experimental animals were exposed to air and closed shell valves during surgery. When marine bivalves[9,11,19–21] and freshwater bivalve[22] close their shell valves or are exposed to the air, the O_2 partial pressure of body fluid decreased rapidly but pH decreased slowly. Therefore, the hemolymph in this study appeared to undergo temporary mild hypoxemia just after surgery. Pco_2 just after surgery (0 h) was slightly higher than that after 0.5 h and later. Handa and Yamamoto (2011) indicated that akoya pearl oyster *Pinctada fucata martensii* increased hemolymph Pco_2 during cannulation surgery of the anterior aorta, and the possibility of inhibition of CO_2 discharge during the surgery.[11] In some bivalves, the discharge of CO_2 was inhibited and accumulated during air exposure.[9,11,19–21] The accumulated CO_2 titrated toward acidity and reduced the hemolymph pH in akoya pearl oyster *P. fucata martensii*, and the akoya pearl oysters showed temporary respiratory acidosis during air exposure for the surgery.[11] However, the Pacific oyster did not show acidosis, although temporary mild hypoxemia was shown in this study. The experimental temperature in this study was 10℃, which was lower than the previous study (28℃) in akoya pearl oyster.[11] The time required for the surgical procedure was 7 minutes in Pacific oyster adductor muscle and 15 min in akoya pearl oyster anterior aorta. In this study, CO_2 production and accumulation in Pacific oyster should be lower because of the differences of the experimental conditions. Therefore, the Pacific oyster showed only temporary mild hypoxemia without respiratory acidosis in this study.

In the Pacific oyster, a temporary and significantly fluctuation in hemolymph properties disappeared at 1 h after surgery in this study, and the O_2 and acid–base status were stable afterwards in normoxic conditions. Pacific oyster required 1 h for a process to return to preoperative levels or a stable state. Hemolymph collection through a cannula from the adductor muscle was useful for research on the respiratory physiology of Pacific oyster *C. gigas*. The results in this study showed the possibility that sampling with a cannula can collect body fluids under various conditions. This sampling may be useful, for example, when the hemolymph containing gases and hormones must be monitored in minimally disturbed animals without the effects of handling. The technical knowledge proposed in this study may contribute to the advances in research on homeostasis and environmental tolerance in Pacific oyster.

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