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# Purification, Characterization and Sequence Analyses of the Extracellular Giant Hemoglobin from *Oligobrachia mashikoi*

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**ABSTRACT**—We purified an extracellular hemoglobin with the molecular mass of ca. 440 kDa from the whole homogenates of *Oligobrachia mashikoi* (phylum Pogonophora) by a one-step gel-filtration. The preparation was pure to be crystallized. The $P_{50}$ values of the hemoglobin and the fresh blood prepared from *O. mashikoi* were about 0.82 Torr and 0.9 Torr, respectively, which were much lower than the $P_{50}$ value of human hemoglobin. However, the *n* values of the hemoglobin and the blood were about 1.2 and 1.1, respectively.Using the improved tricine SDS-PAGE, we could separate *O. mashikoi* hemoglobin into four kinds of the globin chains, A1, A2, B1 and B2, and succeeded for the first time in cloning and sequencing of the complete cDNA encoding B1 globin gene, in addition to A1, A2 and B2 globin genes in full length. We found that all globin genes have the extracellular signal sequences in each molecule and the distal His of the B1 globin chain is replaced to Gln. Finally, we constructed phylogenetic trees of the hemoglobins from Pogonophora, Vestimentifera and Annelida.

**Key words:** *Oligobrachia mashikoi*, extracellular giant hemoglobin, purification, crystallization, oxygen binding property

## INTRODUCTION

*Oligobrachia mashikoi* (phylum Pogonophora) lives in black mud smelling hydrogen sulfide on the shallow sea bottom at a 25 m in Tsukumo Bay of Noto Peninsula, Ishikawa Prefecture, Japan (Imajima, 1973). They lack a mouth, gut and anus (Sasayama *et al*., 2003) and Pogonophora as *O. mashikoi* possess easy closed blood-vascular system containing the extracellular giant hemoglobin (Ivanov, 1963). In general, the marine invertebrates as Pogonophora, Vestimentifera and Annelida have the extracellular giant hemoglobins (=erythrocruorins, widely including chlorocruorins), which are directly dissolved in blood or coelomic fluid (not in the erythrocyte) at high concentration (Weber and Vinogradov, 2001). There are two types of the giant respiratory proteins with the molecular masses of ca. 3000~3600 kDa (derived from Annelida and Vestimentifera) and ca. 350~440 kDa (derived from Pogonophora and Vestimentifera) (Svedberg and Hedenius, 1934; Vinogradov, 1985; Terwilliger *et al*., 1987; Suzuki *et al*., 1988; Yuasa *et al*., 1996; Zal *et al*., 1996a; Weber and Vinogradov, 2001; Suzuki and Vinogradov, 2003). Recently, it has been reported that the ca. 3000~3600 kDa hemoglobin found in blood from Annelida and Vestimentifera consists of many heme-containing globin chains and heme-deficient linker proteins with a hexagonal-bilayer quaternary structure proposed as “bracelet model” (Vinogradov, 1985; Vinogradov *et al*., 1986; de Hass *et al*., 1996; Weber and Vinogradov, 2001). Moreover, the X-ray crystal structural analyses at a 5.5 Å resolution of the Annelida *Lumbricus terrestris* erythorocruorin (ca. 3000~3600 kDa) clearly indicate that this hemoglobin is assembled from 144 globin chains (twelve dodecamers) and 36 linker chains (twelve trimer linker subunits) with a hexagonal-bilayer quaternary structure (Royer Jr *et al*., 2000). However, the three-dimensional structure of the ca. 350~440 kDa hemoglobin found in blood from Pogonophora and Vestimentifera have not been reported until now, although this hemoglobin has been proposed to be a dimer of dodecamers (Zal *et al*., 1996b; Weber and Vinogradov, 2001) and possess a small ring-shaped structure based on the analyses of a transmission electron microscopy (Zal *et al*., 1996a).

On the other hand, it is generally accepted that Pogonophora and Vestimentifera keep endosymbionts in the poste-

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rior specific organ called as “trophosome” or “bacteriocyte” (Cavanaugh *et al*., 1981; Southward, 1982; Matsuno and Sasayama, 2002; Kimura *et al*., 2003). The extracellular hemoglobins from Pogonophora and Vestimentifera show high oxygen binding affinities (Wells and Dales, 1976; Terwilliger *et al*., 1987; Arp *et al*., 1990) and can simultaneously transport not only oxygen with heme for mitochondrial aerobic respiratory chain but also hydrogen sulfide for the symbiotic bacteria which utilize sulfides as primary electron donor in an anaerobic respiratory chain (Felbeck, 1981; Arp *et al*., 1983, 1987; Nelson and Fisher, 1995; Zal *et al*., 1997, 1998; Weber and Vinogradov, 2001). Therefore, it is quite likely that a ca. 350~440 kDa giant hemoglobin from Pogonophora *O. mashikoi* functions as not only oxygen transporter to the hypoxia posterior end of the self-body in mud but also sulfide transporter to the endosymbiotic bacteria in the bacteriocyte.

In 1996, Yuasa *et al.* reported that the *O. mashikoi* giant hemoglobin is composed of eight kinds of globin chains (a1~a5, b, c, d) based on the analyses of electrospray ionization mass spectrometry and sequenced the three major globin genes encoding A2 (a5), A1 (b), and B2 (c), respectively (Yuasa *et al*., 1996). However, until now, the sequence of B1 (d) globin gene and the sequences of four globin genes from initiation codon to stop codon have not been reported. In the present study, we purified the *O. mashikoi* giant hemoglobin to be crystallized and characterized oxygen affinity. Furthermore, we succeeded in the cloning and sequencing of the four globin genes in the full length and constructed the phylogenetic trees from Pogonophora, Vestimentifera and Annelida hemoglobins including *O. mashikoi* B1 globin chain identified in the present study.

## MATERIALS AND METHODS

### Beard worm collections

Specimens of *O. mashikoi* were dredged from the shallow sea bottom at a depth of 25 m in Tsukumo Bay of the Noto peninsula in Japan (37°18'N, 137°14'E). We carefully collected the beard worms living in smelly mud including hydrogen sulfide with a special dredger made 7 mm-thick iron into a box type, 40×40×70 cm, about 80 kg weight on land (Sasayama *et al*., 2003) and transferred them into the fresh seawater on the ship. After the several collections, we took their bodies out of a self-made chitinous tube into the seawater by a syringe. All their bodies were immediately frozen in liquid nitrogen and stored at –80°C until use.

### Physical and chemical measurements

The spectra of the hemoglobin were recorded with UV-265FS recording spectrophotometer (Shimadzu, Co., Kyoto, Japan). The protein content was determined by the methods of Lowry (Lowry *et al*., 1951) with bovine serum albumin as a standard. The heme content was determined on the basis of the molar coefficient of pyridine ferrohemochrome; $\varepsilon_{550nm}$=34,400 $M^{-1}cm^{-1}$ for the ferrohemochrome of heme *b* (Falk, 1964). The molecular mass of *O. mashikoi* hemoglobin was estimated with a HiPrep 16/60 Sephacryl S-300 HR (Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer, pH 7.5 containing 0.2 M NaCl. The apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and carbonic anhydrase (29 kDa) were used as molecular mass calibrants.

### Crystallization of *O. mashikoi* hemoglobin

*O. mashikoi* hemoglobin was crystallized by hanging drop vapor diffusion method. The mixture of the hemoglobin solution and the reservoir solution were stilly incubated at 10°C for a month.

### Measurements of oxygen binding properties of the purified hemoglobin and the blood

Oxygen equilibrium curve of *O. mashikoi* hemoglobin was obtained by the spectrophotometric method with slight modifications (Matsukawa *et al*., 1979). The heme concentration of the hemoglobin solution was adjusted to be 50 μM heme with 0.5 M phosphate buffer, pH 7.0. We obtained the deoxygenated hemoglobin by repeating evacuations and flushing with Argon gas (99.99%) in a Thunberg-type cell with a 1 cm light path. It should be noted that *O. mashikoi* hemoglobin is denatured during evaculation in 0.1 M phosphate buffer, pH 7.0. So, we prepared the hemoglobin solution with 0.5 M phosphate buffer, pH 7.0. Then the small amount of air was added to the hemoglobin for oxygenation and the absorption spectra were measured from 450 nm to 650 nm at 25°C. The pH of the hemoglobin solution was always measured after the end of each experiment. The $P_{50}$ value and the Hill coefficient, *n* value, were calculated from the Hill plot. The measurement of the oxygen dissociation curve was also made with the whole blood, which was freshly prepared from the unfrozen body and diluted to be 50 μM heme with 0.5 M phosphate buffer, pH 7.0. Met-hemoglobin was not produced during oxygen equilibrium experiments because an absorption peak at 630 nm was not detected.

### Electrophoresis analyses

Improved tricine SDS-polyacrylamide gel electrophoresis (PAGE) system for good separation of the proteins with low molecular mass was adapted to the methods of the references (Schägger and von Jagow, 1987; Fountoulakis *et al*., 1998). The disk tricine SDS-PAGE (18.2%T, 3%C containing 5.8M urea) was performed with the separating gel of 18 cm high, 7 mm diameter and the stacking gel of 1 cm high. The hemoglobin sample was boiled with 2% (w/v) SDS plus 0.05% (v/v) β-mercaptoethanol for 5 min. The disk tricine SDS-PAGE was performed at 5 mA constant current per one disk at room temperature. The protein bands were fixed and stained with 18.3% (v/v) methanol solution containing 6.4% (v/v) acetic acid, 6% (w/v) trichloroacetic acid and 0.02% (w/v) Coomassie brilliant blue R-250 for overnight by shaking. The stained gels were completely destained with 28.6% (v/v) methanol solution containing 4.8% (v/v) acetic acid for overnight by shaking.

The slab tricine SDS- PAGE (20%T and 3%C containing 4.89 M urea) was performed with the separating gel of 110 mm high, 135 mm wide and 1.0 mm thick, and the stacking gel of 10 mm high. After the hemoglobin solution was boiled with 2% (w/v) SDS plus 0.05% (v/v) β-mercaptoethanol for 5 min, solid urea was added to the sample to be 1 M as final concentration. The slab tricine SDS-PAGE was performed at 20 mA constant current in the stacking gel and at 30 mA constant current in the separating gel at room temperature. The protein bands were fixed and stained with 45% (v/v) methanol solution containing 10% (v/v) acetic acid and 0.25% (w/v) Coomassie brilliant blue G-250 for 30 min by shaking. The stained gels were completely destained with 26% (v/v) methanol solution containing 8.2% (v/v) acetic acid for 3 h by shaking and the protein bands were transferred to polyvinylidene fluoride membranes (Sequi-Blot™ PVDF membrane, Bio-Rad, USA). The N-terminal amino acid sequences of all globin chains were determined with the protein sequencer model 476A (Applied Biosystems, USA).

### Cloning and sequencing techniques

All general DNA manipulations were performed according to Sambrook *et al*. (Sambrook *et al*., 1989). Total RNA was purified

from *O. mashikoi* with ISOGEN (NIPPON GENE, Japan) by the method as described in the instructions attached. The degenerate oligonucleotide of B1 globin chain was designed based on the N-terminal amino acid sequences (E-V-V-I-S-E-W and E-W-D-Q-V-F-N) as follows; Degenerate-B1-f1: 5'-GARGTNGTNATHAGYGA-RTGG-3', Degenerate-B1-f2: 5'-GARGTNGTNATHTCNGARTGG-3', and Degenerate-B1-f3: 5'-GARTGGGAYCARGTNTTYAAY-3', (where R represents A and G; Y represents C and T; H represents A, C and T; N represents A, C, G and T.) Reverse transcriptase-PCR (RT-PCR) was performed with these three primers and oligo-$dT_{20}$ primer by using Revertra Ace reverse transcriptase (TOYOBO, Japan) and KOD-plus DNA polymerase (TOYOBO, Japan). The PCR-products were inserted to pUC119 vector digested with *Sma*I. These recombinant plasmids were transformed into *Escherichia coli* XL-1 Blue MRF' (STRATAGENE, USA) and identified with ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) by using a multi capillary DNA sequencer, ABI PRISM 3100 DNA Analyzer (Applied Biosystems, USA).

The rapid amplification of 5'cDNA ends (5'-RACE) of A1, A2, B1, and B2 globin genes was performed with 5'-Full RACE Core Set (TAKARA, Japan). For the first-strand cDNA synthesis using AMV Reverse Transcriptase XL (Life Sciences, USA) and Revertra Ace reverse transcriptase (TOYOBO, Japan), the oligonucleotides of the globin chains were designed with 5'-phosphate as follows; 5'pRACE-A1-r: 5'p-TTAGCCGGAAATACCGCTAGC-3', 5'pRACE-A2-r: 5'p-TTAACCAGAAATGCCGCTGAC-3', 5'pRACE-B1-r: 5'p-CATCGATGTGAGCGCTAGGAA-3', and 5'pRACE-B2-r: 5'p-TTA-CAAGCCTGCTGAGATGC-3'. The first-strand cDNAs were ligated with T4 RNA ligase after dealing with RNase H, and formed the concatemer or circular DNAs. The oligonucleotides for the inverse-PCR were designed as follows; InversePCR-A1-f: 5'-TGATCT-TGCTCACCTTGCTGG-3', InversePCR-A2-f: 5'-TGAATTCTCAG-CATGACTCTCG-3', InversePCR-B1-f: 5'-CCTGCTGTCACATTT-GAACGC-3', InversePCR-B2-f: 5'-TGCTCATTTCGATGTGATG-GC-3', InversePCR-A1-r: 5'-GAGCTGACTGATCAGACGGTTAAG-3', InversePCR-A2-r: 5'-GCGTTGAGGACAGGTACGTC-3', InversePCR-B1-r: 5'-CCAGAAACCACACGGGATAC-3', and InversePCR-B2-r: 5'-TCGTTGAGCATGTTAATGGCG-3'. The oligonucleotides for the nested-PCR were designed as follows; NestedPCR-A1-f: 5'-TTTG-GATGCATCCAACTTCG-3', NestedPCR-A2-f: 5'-ATATCCAGCAT-TCGTCGCTTC-3', NestedPCR-B1-f: 5'- CTGTTACCCATTTAAGT-CAGGC-3', NestedPCR-B2-f: 5'-TGTGATGGCTGAGGCTTTCG-3', NestedPCR-A1-r: 5'-TAAGAGCTCCAGTGACACGAAGG-3', Nested-PCR-A2-r: 5'-CACACATGTCAAGACCACCC-3', NestedPCR-B1-r: 5'-GTTGTTACTGTCACCACCACCG-3', and NestedPCR-B2-r: 5'-TCGAGACCGTTAACAACGCG-3'.

Inverse-PCR and nested-PCR were performed with KOD-plus DNA polymerase (TOYOBO, Japan). The subcloning and DNA sequencing were carried out by the same method as described above.

**Informative search, multiple alignment and phylogenetic analyses**

The amino acid sequences and DNA sequences of the globin chains from *O. mashikoi* were analyzed with BLAST program (http://www.ncbi.nih.gov/BLAST/) for homology search. The amino acid sequences of the extracellular giant hemoglobins from Pogonophora, Vestimentifera, Annelida and Mollusca, human hemoglobin and myoglobin were obtained from the Entrez Protein program (http://www.ncbi.nlm.nih.gov/entrez/index.html) and from the reference (Suzuki *et al*., 1995). The structures of hemoglobins were observed with MMDB and Entrez Structure program (http://www.ncbi.nih.gov/Structure/). Signal sequences were predicted with PSORT II program in ExPASy (http://psort.nibb.ac.jp/from2.html). The multiple alignment of the hemoglobins from Pogonophora, Vestimentifera, Annelida and Mollusca, human hemoglobin and myoglobin was constructed with Clustal X 1.83 program (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html). PHYML 2.4.1 program (http://atgc.lirmm.fr/phyml/) was used for construction of the maximum likelihood (ML) tree with an input generated tree of BIONJ and with the calculated pairwise ML distance using WAG amino acid substitution model (Whelan and Goldman, 2001). PHYML 2.4.1 program also computed a discrete gamma distribution of eight variable rate categories for rate-across-site variation containing the estimated optimum shape parameter (alpha) and invariable sites, and with the bootstrap resampling at 100 times, respectively. The tree was drawn by NJ-plot program (http://pbil.univ-lyon1.fr/software/njplot.html).

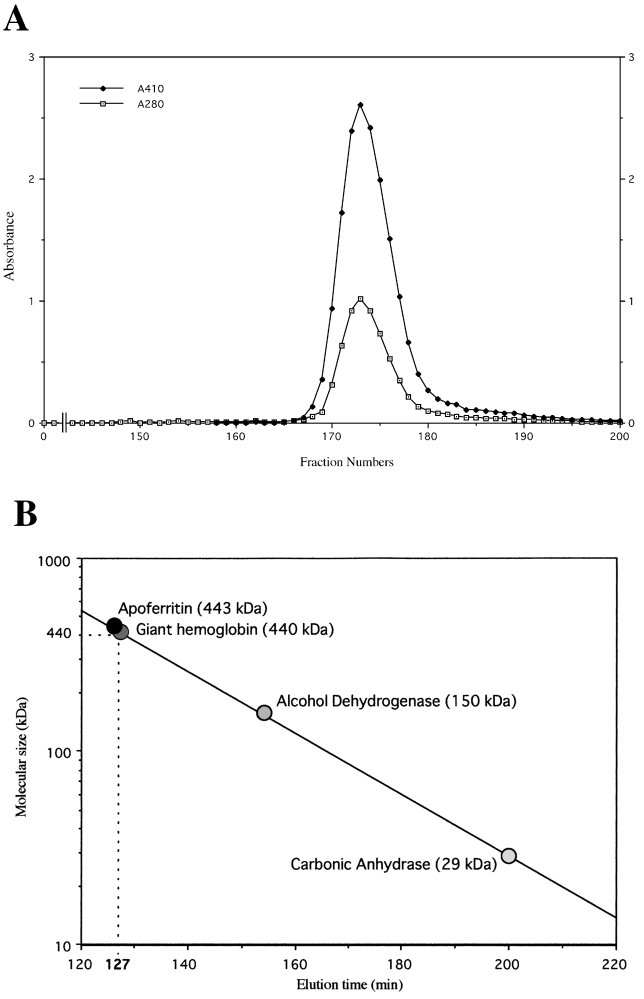


**Fig. 1.** Elution profile and molecular mass estimation of *O. mashikoi* hemoglobin. (A) Elution profile of the *O. mashikoi* hemoglobin by gel filtration with a Sephacryl S-300 column. The column (95×2.2 cm) was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2M NaCl. The flow rate was 1.5 ml/ 15 min in each tube. The hemoglobin fractions with a constant ratio of A415 / A280 =2.5~3.0 were collected (fraction numbers; 170~178) and used for the experiment of crystallization, oxygen binding assay, and SDS-PAGE. (B) Molecular mass estimation of the *O. mashikoi* giant hemoglobin, using gel filtration with a Sephacryl S-300 column. The elution buffer was 50mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. The proteins used as the markers were: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and carbonic anhydrase (29kDa).

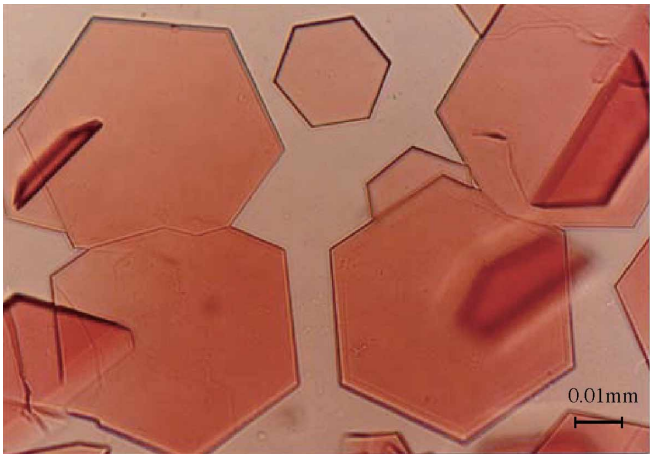


**Fig. 2.** The photomicrograph of *O. mashikoi* hemoglobin crystals. The crystallization of *O. mashikoi* hemoglobin was performed with hanging drop vapor diffusion method. The well-shaped hexagonal crystals with the size of about 50 μm width were prepared from the *O. mashikoi* hemoglobin solution.

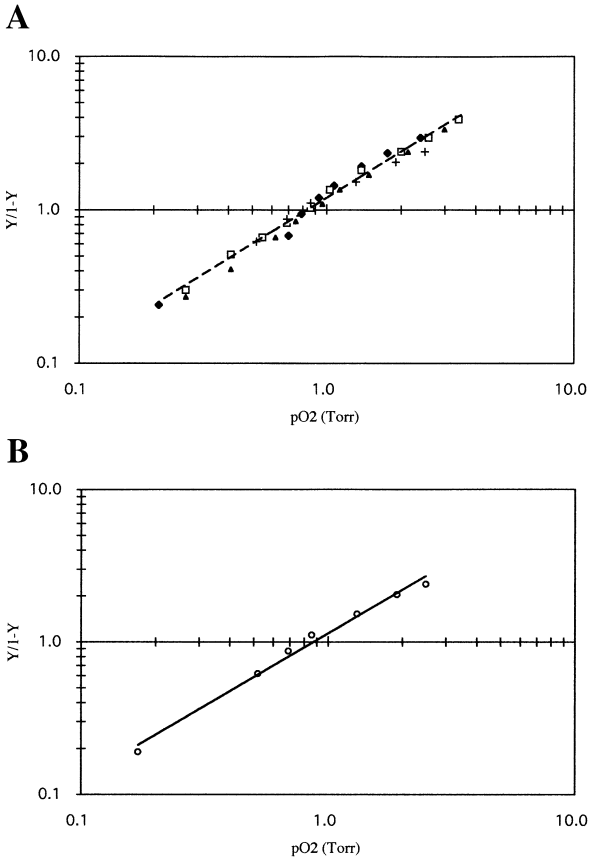


**Fig. 3.** The oxygen dissociation curves of the purified hemoglobins and the blood of *O. mashikoi*. (A) Hill plot of the *O. mashikoi* hemoglobins. The hemoglobins purified were diluted with 0.5 M phosphate buffer, pH 7.0 to 50 μM heme. Four oxygen dissociation experiments were independently performed by the method as described in Materials and Methods (□, ◆, ▲, +). (B) Hill plot of the blood from *O. mashikoi*. The blood was freshly prepared from the about fifty individuals of *O. mashikoi* and diluted with 0.5 M phosphate buffer, pH 7.0 to 50 μM heme. The titration was performed by the method as described in MATERIALS AND METHODS.

## RESULTS

### Purification and crystallization of the giant hemoglobin from *O. mashikoi*

All purification steps were conducted at 4°C. After the *O. mashikoi* bodies were homogenized with a tefron homogenizer on ice, the homogenate was centrifuged at 22,000×*g* for 1 h. The supernatant obtained was concentrated by ultrafiltration with 100,000 MW cut membrane (VIVASPIN 20 ml CONCENTRATOR, 100,000 MW cut, VIVASCIENCE, U.K.). The concentrated blood was subjected to gel filtration with a Sephacryl S-300 HR (Amersham Biosciences) column (95×2.2 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5 containing 0.2 M NaCl. Fig. 1 (A) shows the elution profile by a Sephacryl S-300 column chromatography. We observed one peak with high absorbances at 280 and 410 nm on the column chromatography. Following each of the absorbances at 280 nm and 410 nm, the fractions with a constant ratio of the absorbance at 410 nm / 280 nm of 2.5 to 3.0 were collected and concentrated to be ca. 50 mg protein/ml by ultrafiltration with 10,000 MW cut membrane (MICROCON YM-10, 10,000 MW cut, MILLIPORE, USA) and stored at –80°C until use. The molecular size of *O. mashikoi* hemoglobin was estimated to be about 440 kDa by a Sephacryl S-300 column chromatography (Fig. 1 (B)).

To confirm the purity of the *O. mashikoi* hemoglobin, we tried crystallization by the hanging drop vapor diffusion method. Fig. 2 shows a photomicrograph of the crystals of the *O. mashikoi* hemoglobin. The crystals were obtained

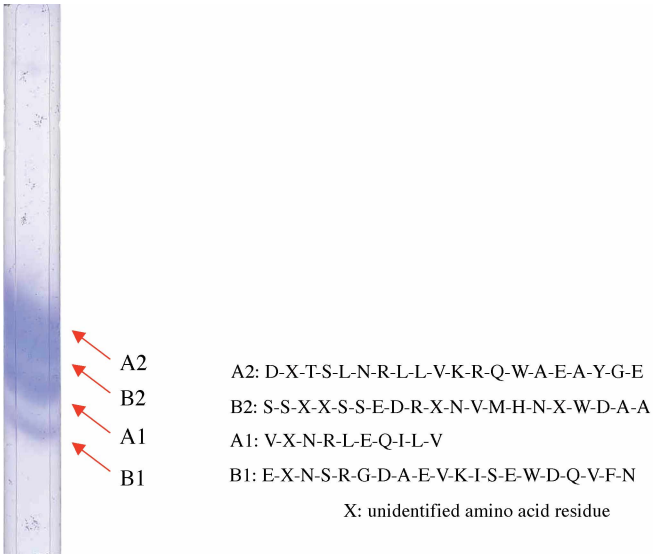


**Fig. 4.** Improved disk tricine SDS-PAGE of the *O. mashikoi* hemoglobin. The SDS-PAGE (18.2%T, 3%C containing 5.8 M urea) was performed by the method as described in MATERIALS AND METHODS. The hemoglobin sample was boiled with 2% (w/v) SDS plus 0.05% (v/v) β-mercaptoethanol for 5 min. The protein bands were calibrated with Precision Plus Protein Standards marker (Bio-Rad, USA). The N-terminal amino acid sequences of four globin chains determined by the protein sequencer are shown in the figure. X: unidentified amino acid residue.

under the experimental condition as follows; the reservoir solution containing 0.1 M imidazole, pH 6.5 and 1.0 M sodium acetate trihydrate and the hemoglobin solution (85 mg/ml) in 50 mM Tris-HCl buffer, pH 8.0 containing 0.2 M NaCl were suspended at the ratio 1:1 and stilly incubated at 10°C for a month.

### Oxygen binding properties of the purified hemoglobin and the blood freshly prepared from *O. mashikoi*

The respiratory properties of *O. mashikoi* hemoglobin and the blood were analyzed by the method as described in Materials and Methods. Fig. 3 (A) shows the Hill plot of the purified *O. mashikoi* hemoglobin. The $P_{50}$ value and *n* value were estimated to be about 0.82 Torr and 1.2. Fig. 3 (B) shows the Hill plot of the fresh blood prepared from *O. mashikoi*. The $P_{50}$ value and *n* value were estimated to be about 0.9 Torr and 1.1, respectively.

### Subunit structure and the complete amino acid sequences of four globin chains from *O. mashikoi* hemoglobin

The subunit structure of the *O. mashikoi* hemoglobin was analyzed by disk tricine SDS-PAGE. Fig. 4 shows the results of disk tricine SDS-PAGE. These apparent molecular sizes were estimated to be ca. 14–16 kDa. After the slab tricine SDS-PAGE, the four protein bands were transferred from the gel to the PVDF membrane and then, their partial N-terminal amino acid sequences were determined by the protein sequencer. As shown in Fig. 4, the N-terminal amino acid sequences of four globin chains were consistent with the registered sequences of the A1, A2, B1, and B2 globin chains in the database.

In the present study, we succeeded in sequencing four globin cDNAs in full length by RT-PCR and 5' RACE methods and deduced the full amino acid sequences (Accession numbers; A1; AB185392, A2; AB185391, B1; AB185394 and B2; AB185393. Fig. 5 shows the alignment of the deduced complete amino acid sequences of four globin chains. Gaps have been inserted where necessary to provide maximum alignment of these sequences. The N-terminal amino acid sequence of the A2 (a5) globin chain was determined to be D-X-T-S-L-N-R-L-L-V-K-R-Q-W-A-E-A-Y-G-E by protein sequencer, suggesting that the 16 amino acid residues from first Met to Ala (M-K-S-L-I-V-F-A-C-L-V-A-Y-A-A-A) may be a signal peptide. Therefore, the A2 (a5) globin chain consists of 142 amino acid residues with the molecular mass of 15,312 Da. Similarly, the A1 (b) globin chain has a signal peptide (M-K-V-L-I-I-F-A-C-L-V-V-M-A-S-A) and consists of 140 amino acid residues with the molecular mass of 15,174 Da. The B2 (c) globin chain has a signal peptide (M-I-A-L-F-V-L-M-G-L-M-A-A-A-S-A) and consists of 147 amino acid residues with the molecular mass of 15,606 Da. The B1 (d) globin chain has a signal peptide (M-T-I-L-V-L-F-L-S-C-A-A-L-A-S-A) and consists of 145 amino acid

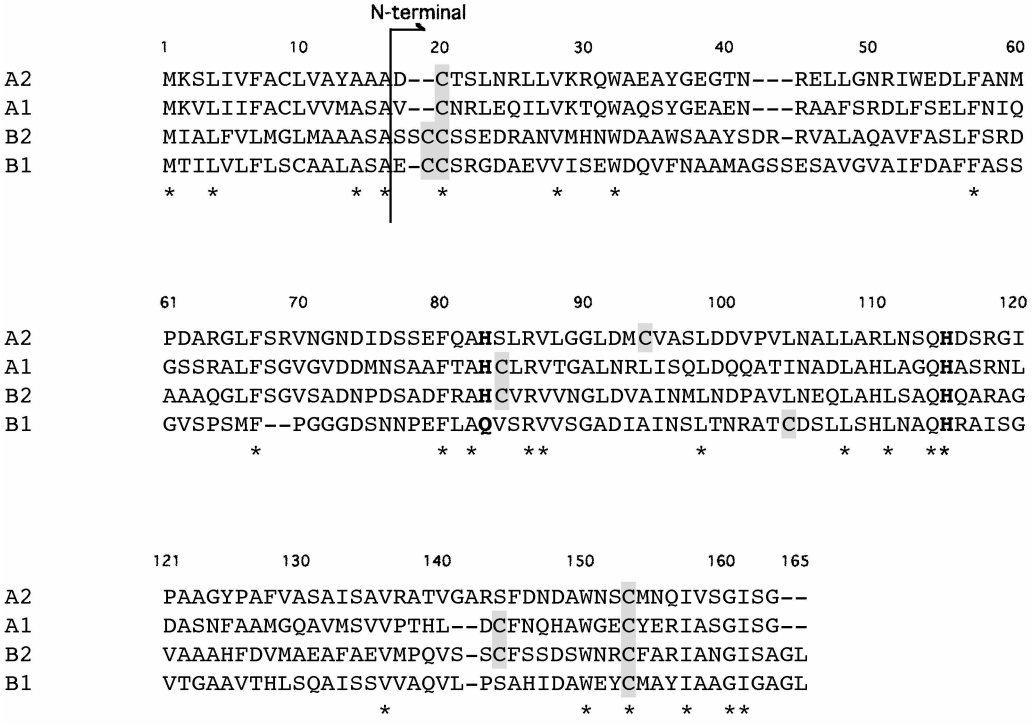


**Fig. 5.** Multiple alignment of amino acid sequences of four globin chains from *O. mashikoi* hemoglobin. The amino acid sequences were deduced from the cDNAs encoding the A1, A2, B1 and B2 globin genes. The amino acid residues of the four globin chains from 1st to 16th are predicted to be the extracellular secretory signal peptides. The conserved amino acid residues of the four globin chains are indicated by asterisks (*). The specific cysteine residues are shaded in gray, and the proximal and distal histidine residues are bold. These four globin chains were registered in DDBJ data bank with the accession numbers of A1:AB185392, A2:AB185391, B1:AB185394, and B2:AB185393.

residues with the molecular mass of 14,778 Da.

## DISCUSSION

### Purification and characterization of the *O. mashikoi* giant hemoglobin

In the present study, we could purify the *O. mashikoi* hemoglobin from the whole homogenates in a few days, using a mild column chromatography as gel filtration. As shown in Fig. 1 (A), a symmetric peak was observed in the elution profile. Furthermore, any small proteins and free hemes were not observed in the elution profile. It should be noted that Vestimentifera *Riftia pachyptila* hemoglobins were easily dissociated into the small proteins when the blood was stored at –40°C (Zal *et al*., 1996a). On the other hand, Zal *et al*. reported that Vestimentifera *Riftia pachyptila* has two kinds of the giant hemoglobin with the molecular mass of 440 kDa, which are assembled with different globin chains and localized in its blood (V2) and coelomic fluid (C1), respectively (Zal *et al*., 1996a, b). However, the *O. mashikoi* hemoglobin solution was highly pure enough to be crystallized as shown in Fig. 2. Therefore, it is concluded that pogonophoran *O. mashikoi* has one kind of 440 kDa hemoglobin different from Vestimentifera *R. pachyptila*.

The subunit structure of the *O. mashikoi* hemoglobin was analyzed by the improved SDS-PAGE system. We identified the four kinds of the globin chains, A1 (b), A2 (a5), B1 (d), and B2 (c). Yuasa *et al.* reported that the *O. mashikoi* giant hemoglobin is composed of eight kinds of globin chains (a1~a5, b, c, d) based on electrospray ionization mass spectrometer (Yuasa *et al*., 1996). However, the intensities of a1~a4 globin chains in the MaxEnt processed spectra are lower than that of a5 (A2), and all of the molecular masses of a1~a4 globin chains are lower than that of a5 (A2). Moreover, these spectra of a1~a4 globin chains were disappeared in the reduced and carbamidomethylated condition. Therefore, the minor a1~a4 globin chains might be the degradated products from a5 (A2).

As shown in Fig. 3 (A) and 3 (B), the $P_{50}$ values of the purified hemoglobin and the blood are about 0.82 Torr and 0.9 at pH 7.0, respectively. These values are much lower than that of human hemoglobin A (Nagai *et al*., 1972). In general, pogonophores transport the oxygen to the hypoxia posterior end of the self-body in the reductive and anaerobic black mud (Wells and Dales, 1976; Terwilliger *et al*., 1987). Therefore, the high affinity for oxygen of the hemoglobin seems to be physiologically important for growth. On the other hand, the Hill coefficient, *n* value, of the purified hemoglobin is estimated to be about 1.2, suggesting that the *O. mashikoi* hemoglobin has little co-operative oxygen binding property. However, it should be noted that the *O. mashikoi* hemoglobin is not denatured during the purification because the fresh blood has the same the $P_{50}$ and *n* values.

### Amino acid sequences of four globin chains from *O. mashikoi* hemoglobin

Fig. 5 shows that multiple alignment of amino acid sequences of four globin chains from *O. mashikoi.* We analyzed the amino acid sequences of four globin chains of *O. mashikoi* hemoglobin, using PSORT II program, and found that they have secretory signal sequences in the N-terminal region. The 16 amino acid residues of four globin chains were mainly occupied with the hydrophobic amino acid residues and especially, the signal sequences of A1 and A2 are well-defined consensus sequence; the hydrophobic core is preceded by basic residues as K and followed by a cleavage site for signal peptidase. These results are consistent with the localization of *O. mashikoi* hemoglobin.

The identity and homology of these four globin chains were calculated to be 13.5% and 46%, respectively. These are much lower than those of human hemoglobins $\alpha$ and $\beta$, 42% and 78.4%. However, the amino acid residues participating in the globin folding were highly conserved in the four globins. The proximal His at position 115, which occupies one of the axial positions of the heme and is in direct contact with Fe of the heme, was completely conserved in all the globin chains. The distal His at position 83, which is sandwiched between the ring nitrogen of the His and the iron atom of heme, was also conserved in A1, A2 and B2 globin chains, excepting B1 globin chain replaced by Gln. The human $\alpha$ globin chain variant, Hb Boghe, replaced to Gln at the distal His site, shows the normal potential of the $O_2$ and CO affinity and the normal oxygen equilibrium curves (Lacan *et al*., 1999). Furthermore, the distal His residues of the globin chains from *Calyptogena species* hemoglobin and *R. pachyptila* B1c globin chain are also replaced by Gln (Kawano *et al*., 2003; Bailly *et al*., 2002). Therefore, it is quite likely that the B1 globin chain bind oxygen as same as the A1, A2 and B2 globin chains.

Fig. 6 shows the multiple alignments of amino acid sequences of extracellular giant hemoglobins from Pogonophora, Vestimentifera including the partial amino acid sequences of *R. pachyptila* A1, B1a~B1c and B2 globin chains and Annelida, and those of intracellular hemoglobins from Annelida, Mollusca and vertebrata. The globin chains of the extracellular giant hemoglobins, interestingly, have a few Cys residues in the molecule. Recently, Strand *et al.* has been reported the crystal structure of the hemoglobin dodecamer from *Lumbricus* erythrocruorin (Strand *et al*., 2004). The structure reveals inter-disulfide bridges between A1 and B2 and between B1 and B2, respectively. Therefore, the dodecamer has A1, B1 and B2 trimeric structure, while A2 is not disulfide linked to other chains. Furthermore, they have found the intra-disulfide bridges in all globin chains. The Cys residues participated in such intra- and inter-disulfide bridges are also conserved in the globin chains of *O. mashikoi* hemoglobin. Therefore, the *O. mashikoi* hemoglobin may have the similar structure as that of the hemoglobin dodecamer from *Lumbricus* erythrocruorin.

On the other hand, *R. pachyptila* hemoglobins have

ability to bind sulphide and it is proposed that free Cys residues of the Hb are concerned with the sulphide binding (Zal *et al*., 1998). Figure 6 shows the $^{78}$Cys residues of B2 globin chains and $^{88}$Cys of A2 globin chains are conserved in the *R. pachyptila, Lameribrachia* sp. and *O. mashikoi* hemoglobins. Furthermore, these Cys residues do not participate in intra- and inner-disulfide bridges. Therefore, both of the $^{78}$Cys and $^{88}$Cys residues may be the putative sulphide binding sites.

An intracellular monomeric hemoglobin I (HbI) of Mollusca *Lucina pectinata* with the molecular mass of 14,812 Da has two physiological ligands of oxygen and hydrogen sulfide, and transfers the hydrogen sulfide ($H_2S$) to symbiotic bacteria. In 1994, Rizzi *et al*. determined the crystal structure of the sulfide-reactive *L. pectinata* hemoglobin and reported highly sulfide binding affinities through the hydrogen bonding to the distal Gln and the aromatic-electrostatic interactions with Phe surrounding the heme pocket (Rizzi *et al*., 1994). Interestingly, B1 (d) globin chain of *O. mashikoi* also has a similar motif of Gln as distal His site at position 77, Phe at position 40, and Phe at position 55 except that Phe was substituted to Val at position 81. Therefore, B1 (d) globin chain of *O. mashikoi* may bind the hydrogen sulfide as same as *L. pectinata* hemoglobin.

In the present study, we first constructed the phylogentic tree including B1 (d) globin chain of *O. mashikoi* hemoglobin and a new amino acid substitution model of WAG matrix, valid for soluble proteins (Whelan and Goldman, 2001). As shown in Fig. 7, the clade of *O. mashikoi* B1 globin chain was branched at first from the strains B with relatively high bootstrap values, suggesting that B1 globin of

**Fig. 6.** The multiple alignment of amino acid sequences of the globin chains from Pogonophora, Vestimentifera, Annelida, Mollusca and Vertebrata hemoglobin. The conserved amino acid residues of globin chains are indicated by asterisks (*). The specific cysteine residues used for intra-disulfide bridges (positions 7 and 152), inter-disulfide bridges (positions 6 or 143), and inferred sulfide-binding sites (positions 78, 88, and 98) are shaded in gray. The proximal and distal histidine residues are bold. The accession numbers of globin chains from Pogonophora, Vestimentifera, Annelida, Mollusca and Vertebrata as follows; Pogonophora *Oligobrachia mashikoi*: A1 (AB185392), A2 (AB185391), B1 (AB185394), B2 (AB185393). Vestimentifera *Riftia pachyptila*: A1 (CAD29154), A2 (P80592), B1a (CAD29156), B1b (CAD23157), B1c (AB29158), B2 (AB29159). Vestimentifera *Lamellibrachia* sp: the amino acid sequences of each globin chains (A1, A2, B1, B2) were obtained from the reference (Suzuki *et al*., 1995). Annelida *Lumbricus terrestris*: A1 (B28151), A2 (A29134), B1 (C28151), B2 (A28151). Annelida *Tylorrhynchus heterochaetus*: A1 (P02219), A2 (P09966), B1 (P02220), B2 (P13578). Annelida *Saballa spallanzanii*: B2-1 (CAC37410), B2-2 (CAC37411), A2 (CAC37412). Annelida *Macrobdella decora*: A1 (BAC82447), A2 (BAC82445), B1-1 (BAC82446), B1-2 (BAC82448). Annelida *Tubifex tubifex*: A1 (P18202). Annelida *Pheretima sieboldi*: A1 (P11740). Annelida *Glycera dibranchiata*: P1 (CAA37995), P2 (AAA29160), P3 (AAA29161), GMH4 (P15447). Mollusca *Lucina pectinata*: Hb1 (P41260), Hb2 (P41261), Hb3 (P41262). Human: α-globin (AAH08572), β-globin (AAH07075), myoglobin (P02144).

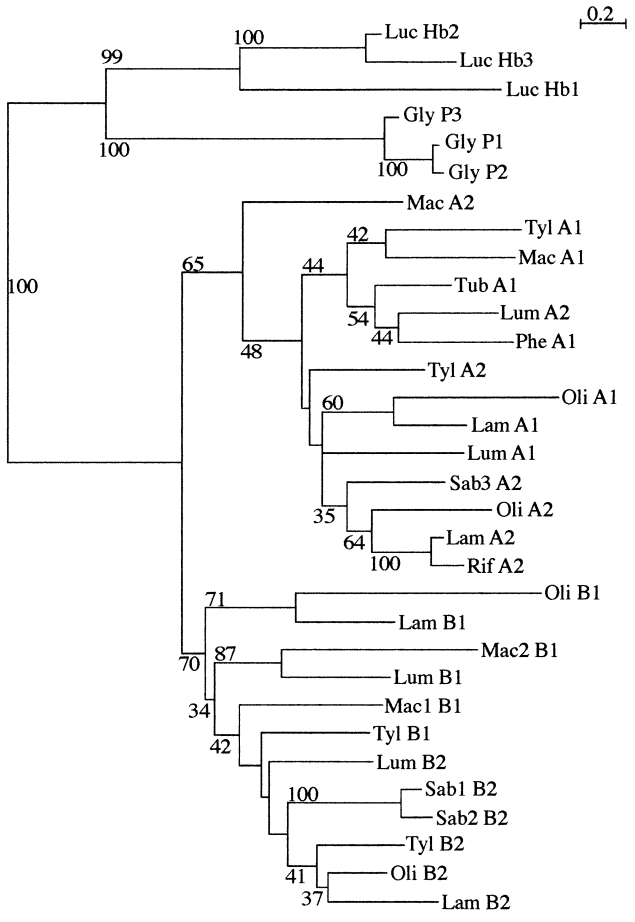


**Fig. 7.** The maximum likelihood consensus tree of globin chains composed of giant hemoglobin from Pogonophora *Oligobrachia*, Vestimentifera *Lamellibrachia*, Annelida *Tylorrhynchus*, *Lumbricus*, *Sabella*, *Tubifex*, *Pheretima*, and *Macrobdella*. The intracellular polymeric globin chains (P1~P3) of Annelida *Glycera dibranchiata*, intracellular monomeric globin chains (Hb1) and homotetramer globin chains (Hb2 and Hb3) of Mollusca *Lucina pectinata* were used as outgroup for rooting. The scores of branching point shows the bootstrap values derived from the 100 replicants and the scale bar shows the rate of substitution per site.

*O. mashikoi* might have the oldest properties in the strains B. Furthermore, B1 globin chains of ca. 350~450 kDa hemoglobins of Pogonophora *O. mashikoi* and Vestimentifera *Lamellibrachia* sp. were clearly separated into the cluster different from the another B1 globin cluster of ca. 3000~3600 kDa hemoglobins of Annelida *Lumbricus terrestris* and *Macrobdella decora*. The comparison between the crystal structures of *O. mashikoi* hemoglobin and *Lumbricus* erythrocruorin will provide quite important clues for elucidating the evolution of the extracellular giant hemoglobins.

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