Haemoglobin structure and biochemical characteristics of the sulphide-binding component from the deep-sea clam *Calyptogena magnifica*

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Abstract: *Calyptogena magnifica* is a large heterodont clam belonging to the family vesicomyidae that lives at a depth of ca. 2600 m, near deep-sea hydrothermal vent areas on the East-Pacific Rise and Galapagos Rift. This species harbors abundant autotrophic sulphide-oxidizing bacteria contained in bacteriocytes and located in the gills. Unlike the tube-worm *Riftia pachyptila*, which possesses extracellular haemoglobins that bind sulphide and oxygen simultaneously and reversibly at two different sites, *Calyptogena magnifica* possesses in its haemolymph two different types of molecules for these functions: an intracellular circulating haemoglobin (Hb) for oxygen binding and a sulphide-binding component (SBC) dissolved in the serum. To elucidate its quaternary structure, the haemoglobin was purified by gel chromatography (FPLC) and then analysed by electrospray ionization mass spectrometry (ESI-MS). FPLC analysis revealed a molecular mass of approximately 68 kDa. By mass spectrometry, under native condition, we found that this molecule contains two subunits of molecular masses 16134.0 Da (\(\alpha\)) and 32513.1 Da (\(\beta\gamma\)). After reduction, the \(\beta\gamma\) subunit corresponds to a covalent heterodimer consisting of chains \(\beta\) and \(\gamma\) with \(M_r\) of 16148.0 and 16371.0, respectively. Our data suggest that *C. magnifica* intracellular Hb is a tetrameric molecule with three possible associations: \((\beta\gamma)_2\), \((\beta\gamma)_1\) \((\alpha)_2\), and/or \((\alpha)_4\). The electron micrographs show that the sulphide-binding serum component is a dumbbell-shaped molecule consisting of two globular subunits of 74 ± 5 nm. It is a glycosylated molecule (non-haeme) that also possesses a protein moiety. Its absorbance peak shifts from 280 nm to 208 nm when it binds sulphide. This molecule moreover possesses unusual solubility properties suggesting that it may be a lipoprotein. It has a high zinc content and there is a 1:1 ratio between zinc and sulphide bound suggesting that zinc is the sulphide-binding site of this compound.

Résumé : Structure de l’hémoglobine et caractéristiques biochimiques de la molécule liant le sulfure d’hydrogène chez le bivalve hydrothermal *Calyptogena magnifica*. *Calyptogena magnifica* est un bivalve hétérodonte de grande taille, appartenant à la famille des Vesicomyidae et vivant à des profondeurs de 2600 m autour des sources hydrothermales profondes localisées sur les dorsales océaniques du Pacifique Est et des Galapagos. Cette espèce abrite une grande quantité de bactériés autotrophes sulfo-oxidantes contenues dans des bactériocytes situés au niveau des branches. Contrairement au Vestimentifère *Riftia pachyptila* qui possède des hémoglobines extracellulaires capables de lier simultanément et réversiblement sur deux sites différents l’oxygène et le sulfure d’hydrogène, *Calyptogena magnifica* possède dans son hémostyme deux molécules différentes assurant ces fonctions: une hémoglobine intracellulaire circulante (Hb) pour lier l’oxygène, et un composé dissous dans le sérum (SBC) et destiné à transporter le sulfure d’hydrogène. Afin de caractériser sa structure quaternaire, l’hémoglobine purifiée par chromatographie basse pression (FPLC) a été analysée par spectrométrie
Introduction

The hydrothermal vent clam, *Calyptogena magnifica* Boss & Turner, 1980, is one of the some 50 species belonging to the family Vescomyidae associated with sulphidic environments (Peek et al., 1997). This clam possesses a thick white shell (up to 30 cm in length) and is one of the most abundant members of the fauna that inhabits deep-sea hydrothermal vents on the Galapagos Rift and East-Pacific Rise (2600 m depth) (Hessler et al., 1985). It colonizes active venting areas and its foot is wedged in cracks in the basalt where warm vent water continually exits carrying sulphide, iron, and other metals (Arp et al., 1984). The chemical characteristics of its environment are extremely variable, ranging between that of mixed vent water (up to 22°C, 350 µM H₂S, pH 6.5 and anoxic) and ambient sea water (2°C, 0 µM H₂S, pH 7.5 and 110 µM O₂) (Corliss et al., 1979).

This animal possesses a reduced digestive system indicative of a limited ability to feed on particulate organic matter (Boss & Turner, 1980; Fiala-Medioni & Metivier, 1986; Fiala-Medioni & Le Pennec, 1987) and most of its energy requirements, for growth and metabolism, seems satisfied with chemosynthetic sulphur-oxidizing bacteria harboured within the gills (Felbeck et al., 1981; Cavanaugh, 1986; Fiala-Medioni & Metivier, 1986; Fisher et al., 1988).

*C. magnifica* has an erythrocytic, circulating, haemoglobin, as well as a cytoplasmic gill haemoglobin (Terwilliger et al., 1983; Wittenberg, 1985). Earlier studies of the erythrocytic Hb revealed a tetrameric structure with different associations of dimeric subunits composed of globin chains of *Mr* 13400 and 14200 (Terwilliger et al., 1983; Terwilliger & Terwilliger, 1985a). *C. magnifica* Hb has a relatively high oxygen affinity (P₅₀ of 7.6 torr at 10°C and pH 6.78), low cooperativity (n₅₀ₓ=1.24) and a slight temperature effect (∆H=-5.3 kJ/mol) (Terwilliger et al., 1983).

In contrast to the extracellular Hbs of the tube-worm *Riftia pachyptila* Jones, 1981 (Arp et al., 1987; Zal et al., 1997b, 1998), the *C. magnifica* Hb is not involved in the binding and transport of sulphide. Moreover the oxygen-binding site is sensitive to sulphide: as in many other intracellular Hbs (National Research Council, 1979), hydrogen sulphide completely blocks oxygenation of the Hb in vitro (Arp et al., 1984). In addition to this intracellular Hb, Wittenberg (1985) has shown that the *C. magnifica* gills contained a cytoplasmic low molecular weight Hb at a concentration of 250 µmol kg⁻¹ tissue. This Hb may be involved in facilitating the transport of oxygen to the symbionts or supplying sulphide upon transformation to metHb, as shown in other bivalves housing chemosynthetic symbionts in their gills, such as *Solemya velum* (Say, 1822) and *Lucina pectinata* Gmelin, 1791 (Wittenberg, 1985, Doeller et al., 1988; Kraus et al., 1990).

To supply sulphide to its symbionts, *C. magnifica* possesses an unidentified sulphide-binding component (SBC) dissolved in the haemolymph which in vitro binds sulphide with high affinity but does not readily release this bound sulphide (Arp et al., 1984, Childress et al., 1991, 1993). A freely-dissolved SBC with similar properties was detected in the haemolymph of other vescomyid clams including *Calyptogena elongata* Dall, 1916, *C. ponderosa* (Boss, 1968) and *Vesicomya cordata* Boss, 1968 (Childress et al., 1993 and Childress, unpublished observations).

Although very few data are available in the literature on these compounds, some data suggest that *C. elongata* SBC possesses a high molecular weight of several million of daltons and contains a large amount of zinc (review in Childress & Fisher, 1992). The SBC may protect *Calyptogena* spp. from sulphide toxicity, in particular at the level of the cytochrome-c oxidase and the enzymes involved in carbon fixation of the symbiont, as proposed for *Riftia pachyptila* Hbs (Powell & Somero, 1983, 1986; Childress et al., 1991).

Key words: haemoglobin, structure, sulphide, sulphide-binding component, hydrothermal vent, *Calyptogena*
The experiments presented in this article were designed to identify and purify *C. magnifica* SBC and intracellular Hb and determine their molecular and biochemical characteristics.

**Materials and methods**

**Animal Collection**

Specimens of *Calyptogena magnifica* were collected on the East-Pacific Rise at 13°N (12°46′N-103°56′W and 12°50′N-103°57′W) and 9°N (9°51′N-104°18′W and 9°48′N-104°17′W) at an average depth of 2600 m by the French and American submarines *Nautil* and *Alvin* during the research expeditions HERO ’91 and HOT TIME’97, respectively. Upon collection, clams were stored in a thermally insulated container and brought to the surface 2-8 hours after capture. After recovery, the animals were transferred quickly to cold sea water on board ship and kept in a cold room before dissection.

**Haemolymph and Serum collection**

Haemolymph samples were taken with a 50 ml syringe inserting the needle through the gap of the shells from the anterior end of the clams into the heart region, as previously described by Childress et al. (1991). This method permits the collection of approximately 30 ml of haemolymph from a clam of about 15 cm in length. These samples were frozen in liquid nitrogen and stored at -80°C in the laboratory until further use.

**Purification methods**

Haemolymph samples were thawed and centrifuged at low speed to remove insoluble materials in a cold room at 4°C. The freezing-thawing process was sufficient to lyse the erythrocytes and release the Hb. Analytical gel filtration was performed on a 1 x 30 cm Superose 6-C column (Pharmacia LKB Biotechnology Inc., fractionation range from 5 to 5000 kDa) using a low-pressure FPLC system (Pharmacia). The column was equilibrated with the following saline buffer: Hepes 50 mM, 420 mM NaCl, 53.5 mM KCl, 50.1 mM MgCl₂.₆H₂O and 27.8 mM Na₂SO₄ (pH 7.5) (Childress et al., 1991). Flow rates were typically 0.5 ml min⁻¹ and the absorbance of the eluate was monitored at 280 nm and 414 nm. The peaks were collected separately and concentrated with a microconcentrator Centricon-10 or -100 (Amicon), depending on the molecular weight of the fraction eluted. Further purification of the sulphide-binding component (SBC) was accomplished using a column containing Sephacryl S-500 HR gel of a 2.5 x 100 cm (Amersham Pharmacia Biotech, fractionation range from 40 to 20,000 kDa). One ml of the SBC obtained from the first purification step on Superose 6-C was dialysed in 3 ml saline buffer (described above), loaded onto the Sephacryl S-500 HR gel column, and eluted at a flow rate of 0.5 ml min⁻¹. The absorbance was measured at 280 nm with a Gilson UV/VIS detector spectrophotometre. During the first purification, the column was calibrated with the following protein-markers: bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), α-lactalbumin (14.4 kDa), blue dextran (=2000 kDa) and purified *Riftia pachyptila* haemoglobin (3503 ± 13 kDa) (Zal et al., 1996). The Sephacryl S-500 HR column was calibrated with the following protein-markers (HMW, Amersham Pharmacia Biotech): aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), blue dextran 2000 (=2000 kDa) and purified *Arenicola marina* (Linnaeus) haemoglobin (3648 ± 24 kDa) (Zal et al., 1997a).

**Sulphide-Binding Analysis**

Sulphide-binding by the purified SBC was measured by equilibrium dialysis against deoxygenated saline buffer, described above, after addition of Na₂S to 1mM. After 48 h dialysis, the samples and buffer contained in the dialysis container were analysed by gas chromatography for total sulphide content (ΣH₂S, the sum of the different species of sulphide) and the difference between the saline buffer and sample measurements was taken to be SBC bound sulphide (Childress et al., 1984; Arp & Childress, 1983). We used as a positive and negative controls of sulphide-binding ability, *Riftia pachyptila* and *Lumbricus terrestris* Hbs, respectively (Zal et al., 1998). Furthermore, we followed the difference in absorbance of this component before and after contact with sulphide (48 hours after dialysis against 1mM sulphide solution) using a Shimadzu UV 160 U spectrophotometre.

**Transmission Electron Microscopy (TEM)**

TEM was performed on purified SBC fractions diluted (1:600) into a Tris-Cl 0.05 M buffer (pH 7.4). Negatively stained molecules were prepared on carbon-support films following a previously described technique (Valentine et al., 1968). The preparations were observed with a Jeol JEM-1200 EX electron microscope.

**Electrospray Ionization Mass spectrometry**

Purified Hb samples were extensively dialysed against distilled water and lyophilized. One mg of each of these samples was dissolved (at a concentration of 10 µg µl⁻¹) in distilled water. Ten µl aliquots of these solutions were diluted with 45 µl of a 2% HCOOH solution in distilled water and 45 µl of acetonitrile to make the working solutions for the analyses (final concentration, 1 µg µl⁻¹ in 50/50 H₂O/CH₃CN plus 1% HCOOH). Reduction was carried out at room temperature, by addition of DTT (2 mM) and letting samples stand for 15, 30 and 60 min. ESI-MS
analysis was performed on a Quattro II triple quadrupole mass spectrometre (Micromass, Altrincham, UK) scanning over the m/z range 800-3800 at 15 sec scan−1 and 24 scans summed over approximately 6 min from 30 µl aliquots of the working solutions. The flow rate into the electrospray source was 5 µl min−1. The extraction cone potential was ramped from 30 V at m/z 800 to 150 V at m/z 3800 during each scan. Mass scale calibration was carried out using multiply-charged ion series consisting of horse heart myoglobin (Mr = 16951.5; Sigma Cat. n° M-1882). Molecular masses are based on the following atomic weights of the elements: C=12.011, H=1.00794, N=14.00674, O=15.9994 and S=32.066 (IUPAC, 1993).

Metal content and protein determination of the SBC

Zinc and cadmium concentrations of purified SBC were determined by flame atomic absorption at the UCSB MSI analytical facility, on a Control Corporation model 240XA elemental analyser.

Carbohydrate content of the SBC

The method of Dubois et al. (1956) was used for the determination of carbohydrate content. A 2.5 mg sample of purified SBC was dissolved in 0.1 N NaOH. The pH was measured with a model PSC-56 auto sampler. Protein concentrations of purified SBC were determined by the methods of Lowry and Bradford using bovine serum albumin as standard (Lowry et al., 1951; Bradford, 1976).

Amino Acids, Carbon, Hydrogen and Nitrogen contents of the SBC

The amino acid content was determined on a Gilson HPLC Model 303 coupled with a Gilson Model 121 Fluorometre detector using a precolumn fluorescence with o-phthalaldehyde (OPA) and a Beckman Ultrasphere ODS reverse phase column. We used a binary mobile phase composed of A:90/10 sodium-citrate buffer pH 7.5/methanol and B: methanol. Complete protein hydrolysis with tryptophan recovery was obtained using 5 mg of purified dried SBC, washed thrice with deionized water, and dried at 60°C during 48 h. The SBC was then dissolved in 200 µl 12 N HCl (6 N HCl failed to hydrolyse the molecule) during 24 h and neutralized at the end with NaOH. The sample was then placed under vacuum in an amino acid analysis tube and stored in a sample drying oven at 100°C for 24 h until used. For amino acids recovery determination, standard stock solutions were supplied from Pierce.

Results and Discussion

The gel filtration of the pinkish C. magnifica haemolymph provided two major fractions (Fig. 1). The first fraction associated with the excluded volume had a cloudy white appearance and did not absorb at 414 nm. However, it showed an absorbance at 280 nm characteristic of proteins, and its molecular weight was greater than 5000 kDa corresponding to the upper exclusion limit of the Superose-6C column. The second peak, which absorbed at both wave-
lengths, corresponded to the intracellular haemoglobin with an apparent molecular mass of ca. 68 kDa. Hence, during this purification process we did not observe the presence of the minor haemoglobin peak with a relative molecular mass of 28 kDa observed by Terwilliger et al. (1983). This Hb fraction has been suggested to be an artefact due to the tetramer dissociation into dimers as a result of either metHaemoglobin formation or sample thawing (Terwilliger et al., 1983). Consequently, *C. magnifica* Hb seemed to be solely a tetrameric molecule.

The cloudy fraction was subjected to further gel filtration on the Sephacryl S-500 HR column. Unfortunately, no protein-markers with a sufficiently high molecular weight are commercially available to estimate the molecular weight of this fraction. However, the fraction eluted as a unique component in the exclusion volume of the column, distant from purified *Arenicola marina* haemoglobin (3648 ± 24 kDa) (Zal et al., 1997a). Consequently, this fraction possesses a very high molecular weight roughly around 15000 kDa. Electron micrographs of this molecule (Fig. 2) revealed dumbbell-shaped molecules made of the association of two globular subunits, which seem to have different electron densities (Fig 2, inset). The dimension of each globular subunit is 74 ± 5 nm (n=10).

Mass spectrometry analyses were performed on the two fractions purified by gel chromatography in order to determine the complete constituents of these molecules. The nomenclature used to label the different constituents of *C. magnifica* Hb accords with that originally used by Terwilliger et al. (1983). However, the assignment of these names to the chains is probably different in the two studies (i.e. SDS-PAGE vs ESI-MS).

The raw, multi-charged ESI mass spectra, observed for the *C. magnifica* haemoglobin is shown in Fig. 3. Fig. 4 shows the deconvoluted mass spectrum obtained after MaxEnt analysis. Deconvoluted mass spectrum of the haemoglobin (Fig 4A) revealed two groups of components. The first group around $M_r$ 16-17000, comprised one peak, named $\alpha$ with a $M_r$ of 16134.0 ± 1.2. The second group, around $M_r$ 32-33000, similarly comprised one major component, named $\beta\gamma$ with a $M_r$ of 32513.1 ± 2.8. The component $\alpha$ represents about 30 % of the intensity of the component $\beta\gamma$.

Upon DTT treatment (i.e. reduction of disulphide bridges), $\beta\gamma$ decreased in relative intensity and two new components appeared $\beta$ ($M_r = 16148.0 \pm 5.9$) and $\gamma$ ($M_r = 16371.0 \pm 5.2$), clearly indicating that $\beta\gamma$ was a covalent heterodimer made by the association of two polypeptide chains $\beta$ and $\gamma$ (i.e. $16148.3 + 16372.2 - 8.0 = 32511.7$, 8.0 Da corresponding to the eight protons added to the Cys after reduction) (Fig. 4B). No modification was observed for the mass of $\alpha$ showing that this component was a single monomeric globin chain.

The very heavy fraction highlighted by gel filtration was also submitted to ESI-MS and MALDI-MS analysis. In both cases, no ions were produced by the mass spectrometers. Several non-exclusive hypotheses can explain this result: i) the size of the ions generated by ESI-MS could possess a mass vs charge ratio (i.e. m/z) larger than 8000, which exceeds the detection range of the MS used; ii) the ions produced by MALDI-MS are generally monocharged whereby the m/z generated is too high to be detected by this technique; iii) the complexity of this molecule, characterized by a large size and high molecular weight involving heterogeneities and/or several non-covalent
adducts (e.g. phosphates, calcium, sodium, etc.) which could be difficult to remove by a dialysis. Therefore, this component generates a high number of unresolved peaks with weak intensities undetectable either by ESI-MS or MALDI-MS.

By comparing mass spectra obtained after carbamidomethylation, with or without reduction, we have determined the numbers of free Cys and disulphide bonds in the haemoglobin (Fig. 4C). No free Cys residue was detected in the Hb (data not shown). The monomeric chain $\alpha$ contained 2 Cys involved in an intra-chain disulphide bond ($Mr = 16337.5 \pm 6.9$). The heterodimer $\beta\gamma$ contained 5 Cys on $\beta$ ($Mr = 16424.9 \pm 5.0$) and 3 Cys on $\gamma$ ($Mr = 16539.5 \pm 6.1$). Two assemblages can be proposed for this heterodimer (Fig. 5): i) one inter-chain disulphide bond between $\beta$ and $\gamma$ in addition to three intra-chain disulphide bridges, two on $\beta$ and one on $\gamma$, ii) three inter-chain disulphide bonds between $\beta$ and $\gamma$ and one intra-chain disulphide bridge on $\beta$. The MS data combined with the native mass of the Hb as determined by gel chromatography (i.e. around 68 kDa), revealed a tetrameric molecule with

![Image](33x545 to 273x704)

**Figure 2.** Electron micrographs of *Calyptogena magnifica* sulphide-binding component, negatively stained with 2% uranyl acetate. Arrows point on the dumbbell-shaped molecule. Inset showing the molecule at higher scale.

**Figure 2.** Micrographies électroniques du composé liant le sulfure d’hydrogène chez *Calyptogena magnifica*, coloration négative avec 2 % d’acétate d’uranyle. Les flèches montrent la molécule en forme d’haltère. A gauche : la molécule à plus grande échelle.

![Image](54x136 to 504x443)

**Figure 3.** Raw ESI mass spectrum of *Calyptogena magnifica* intracellular haemoglobin. This spectrum is composed of two series of multiply-charged ions (relative intensity of each ion as a function of m/z ratio; m/z = (M + nH)/n, where M is the mass of the component, H is the mass of the proton and n is a series of consecutive integers. $\alpha$ corresponds to a monomeric globin chain and $\beta\gamma$ to a subunit constituted by the covalent association of the $\beta$ and $\gamma$ globin chains.

**Figure 3.** Spectre brut ESI-MS de l’hémoglobine intracellulaire de *Calyptogena magnifica*. Ce spectre est composé de deux séries d’ions multi-chargés (intensité relative de chaque ion en fonction du rapport m/z ; m/z = (M + nH)/n, où M représente la masse du composé analysé, H la masse du proton et n un nombre entier de charges. $\alpha$ correspond à une chaîne monomérique de type globine et $\beta\gamma$ à une sous-unité dimérique constituée des chaînes de type globine $\beta$ et $\gamma$ liées entre elles par des liaisons covalentes.

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various possible associations, i.e. \((\beta\gamma)^2\) \(Mr = 67493\), \((\beta\gamma)^4\) \(Mr = 67247\), \((\alpha)^4\) \(Mr = 67002\); these masses comprise the four haem groups.

Intracellular haemoglobins have been found in the erythrocytes of molluscs, annelids, echiurans and echinoderms (Terwilliger et al., 1985). These Hbs can be mono-, di-, tetra- or even polymeric. Among the molluscs, the coelomic fluid of some clams contain nucleated erythrocytes showing a remarkable diversity in subunit structure and constituent polypeptide chains. This is in contrast to the homogeneous tetrameric structure of vertebrate Hbs (Bonaventura & Bonaventura, 1983; Nagel, 1985; Terwilliger & Terwilliger, 1985b). For instance, the Hbs of the clams *Anadara broughtoni* (Schrenck, 1867) and *Scapharca inaequivalvis* (Bruguière 1789) contain both an homodimeric and an heterotetrameric Hb (Furuta et al., 1977; Chiancone et al., 1981; Mozzarelli et al., 1996), whereas *Noetia ponderosa* (Say, 1822) and *Barbatia virescens* (Reeve, 1844) have only a heterodimeric Hb (San George & Nagel, 1985; Suzuki et al., 1989a).

The quaternary haemoglobin structure of *Calyptogena magnifica*, as determined by mass spectrometry, is in good agreement with the results published previously except for the masses of the polypeptide chains (Terwilliger et al., 1983). Hence, the *C. magnifica* Hb appears to contain three subunits with a molecular weight higher than previously determined by SDS-PAGE (Terwilliger et al., 1983). In contrast to previous works, we have shown that two of the globin chains are involved in a covalent heterodimer complex (i.e. \(\beta\gamma\)) with only three possible tetrameric

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**Figure 4.** MaxEnt processed ESI spectra of *Calyptogena magnifica* Hb. A native haemoglobin (Hb), B reduced Hb with 2 mM DTT for 30 min, the \(\alpha\) component is not visible on this spectrum since the resolution cannot resolved the \(\alpha\) and \(\beta\) peaks at the same time and since the intensity of the \(\beta\) peak is higher than the \(\alpha\). C reduced Hb with 2 mM DTT for 30 min and carbamidomethylated. Note: the peaks higher than those of the components \(\alpha\), \(\beta\) and \(\gamma\) correspond to phosphate adducts.

**Figure 4.** Analyse par le système de traitement MaxEnt des spectres de masse ESI de l’hémoglobine de *Calyptogena magnifica*. A Hémoglobine (Hb) native, B Hb réduite avec 2 mM de DTT pendant 30 min, le composé \(\alpha\) n’est pas visible sur ce spectre car la résolution de l’appareil n’est pas suffisante pour résoudre simultanément les pics correspondant à \(\alpha\) et \(\beta\) et que l’intensité du pic \(\beta\) est plus importante que celle du pic \(\alpha\). C Hb réduite avec 2 mM de DTT pendant 30 min et carbamidométhylée. On peut noter que les pics supérieurs à \(\alpha\), \(\beta\) et \(\gamma\) correspondent à des adduits de phosphates.
associations inside erythrocytes, i.e. (βγ)2, (βγ)1(α)2, or (α)4. Consequently, as previously shown in Barbatia spp. (Grinich & Terwilliger, 1980; Suzuki et al., 1989a), the high diversity of subunit associations seems also to exist in Calyptogena spp.. Indeed, Suzuki and co-workers revealed in C. soyoae Okutani, 1957 the presence of two homodimeric haemoglobins (Hb I and Hb II) constituted by the non-covalent association of two globin chains (Suzuki et al., 1989b; 1989c), whereas in C. magnifica only one tetrameric Hb is present.

In order to determine if the high molecular weight component purified on Sephacryl S-500 HR column was the sulphide-binding component, we performed sulphide-binding experiments on this molecule. Our results show a sulphide-binding capacity of 10.80 ± 2.4 mM \( \sum H_2S \ g^{-1} \) protein \((n=5)\), confirming its sulphide-binding potential. This value is in good agreement with previous data published by Childress and co-workers (i.e. 8.4 mM \( \sum H_2S \ g^{-1} \) protein) (Childress et al., 1991). In addition, after dialysis against 1mM sulphide, the absorption maximum of this molecule showed a shift from 280 to 208 nm (Fig. 6). Flame atomic absorption analysis revealed the presence of cadmium at 16.30 ± 1.45 \( \mu g\) mg\(^{-1}\) DWPSBC \((n=4)\) and zinc at 60.0 ± 8.23 \( \mu g\) mg\(^{-1}\) DWPSBC \((n=5)\), respectively. The protein and carbohydrate concentrations were 16.24 ± 1.38 \( \mu g\) mg\(^{-1}\) DWPSBC \((n=5)\) and 5.1 ± 1.00 \( \mu g\) mg\(^{-1}\) DWPSBC \((n=5)\), respectively and the carbon, hydrogen and nitrogen contents were 141.8 ± 15.20, 45.6 ± 4.42 and 60.0 ± 8.23 \( \mu g\) mg\(^{-1}\) DWPSBC \((n=5)\), respectively. Furthermore, starting at neutral pH in the saline buffer (cf. Material and methods), the addition of HCl in a purified solution of sulphide-binding component slightly dissolved the fraction at pH 2.2 and completely dissolved it at pH 1.8. Starting at neutral pH, addition of NaOH resulted in SBC slightly dissolved at pH 10.5 and completely dissolved at pH 12.3. These characteristics seem to be related to lipoproteins as observed for apolipoproteins. Indeed, the apolipoproteins derived from high density lipoprotein, apoA-I, apoA-II are readily dissolved in aqueous buffers of low molarity at neutral to basic or to acid pH (Edelstein & Scanu, 1986). In addition, the sulphide-binding component containing sulphide was dissolved at basic pH and the addition of EDTA formed a white precipitate and provoked the release of sulphide gas. In contrast, under acid pH the addition of EDTA to dissolved SBC containing sulphide does not form a precipitate or cause a sulphide release. This set of

![Figure 5](image1.png)

**Figure 5.** Schematic representation of Cys residues located on the β and γ globin chains which form the heterodimer βγ of *Calyptogena magnifica* haemoglobin (based on data from Fig. 4). A one inter-chain disulphide bridge between β and γ in addition of three intra-chain disulphide bridges, two on β and one on γ. B three inter-chain disulphide bridges between β and γ and one intra-chain disulphide bridge on β. Note: the locations of the Cys residues on the chains are arbitrary.

![Figure 6](image2.png)

**Figure 6.** Absorbance spectrum of the sulphide-binding component of *Calyptogena magnifica.*
experiments demonstrated that the sulphide is bound to a metallic ion, most likely zinc, due to its predominance in comparison to the cadmium content.

Table 1 summarizes the amino acid composition determined on the purified SBC. This analysis revealed a high percentage of amino acids with charged polar side chains, such as arginine, histidine and lysine. The non-polar amino acid side chains that include glycine, alanine, valine, leucine, isoleucine, methionine and phenylalanine are present in smaller amounts. No proline and tryptophan residues were detected probably due to the strong hydrolysis performed. Amino acids with uncharged polar side chain are present with roughly the same percentage, except for tyrosine, which appeared at a higher percentage, as did amino acids with charged polar chains. In this last amino acid category, no cysteine residues were detected. We also identified the presence of taurine. No proteins with amino acid compositions closely related to the SBC were found in the protein data banks.

In conclusion, the sulphide-binding component of *Calyptogena magnifica* appears to be an unusual molecule (Arp et al., 1984; Childress et al., 1991, 1993). Although it is not destroyed by boiling or by proteases (Arp et al., 1984), we have shown in this study that this component contains a protein moiety and that purified SBC exhibits a peak of absorption at 280 nm, a usual characteristic of proteins. Although, we do not have an explanation for the absorbance shift from 280 nm to 208 nm after sulphide treatment, this property can be used for its identification. We also found that SBC in *Calyptogena magnifica*, as in *C. elongata*, has unusual solubility properties which may be indicative of a lipoprotein molecule (Arp et al., 1984; Childress et al., 1993). Similarly to *C. elongata*, we found an extremely high concentration of zinc associated with this component, 16.30 ± 1.45 µg mg⁻¹ DWPSBC or 11.16 mmol l⁻¹, versus 8.95 mmol l⁻¹ for *C. elongata* (Childress et al., 1993). The high concentration of zinc in comparison to cadmium content, the 1:1 ratio between bound sulphide and zinc (10.8 mmol l⁻¹: 11.16 mmol l⁻¹), and the sulphide release after EDTA treatment strongly suggest that zinc is the sulphide-binding site in this molecule.

In conclusion, this study documents a simple way to isolate and purify the intracellular haemoglobin and the sulphide-binding component contained in the haemolymph of *Calyptogena magnifica* for further biochemical and/or physiological studies. Furthermore, we reported for the first time the polypeptide chain composition of a mollusk intracellular Hb, obtained by ESI-MS. A tetrameric haemoglobin is an exception among the heterodont clams. On the other hand, the preliminary biochemical data obtained on *Calyptogena magnifica* sulphide-binding component revealed the unusual nature of this giant dumbbell-shaped molecule, a glycosylated molecule possessing a protein moiety, a high zinc content, probably corresponding to its sulphide-binding site. Further investigations are needed to identify this molecule more precisely.

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<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molecular Weight (dalton)</th>
<th>Molar percentage</th>
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<tbody>
<tr>
<td>alanine</td>
<td>89.09</td>
<td>3.49</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>133.10</td>
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<tr>
<td>glutamic acid</td>
<td>147.10</td>
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<td>histidine</td>
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<td>threonine</td>
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<td>arginine</td>
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<td>asparagine</td>
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References


