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High-level Production of Recombinant *Arenicola Marina* Globin Chains in *Escherichia Coli*: A New Generation of Blood Substitute

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Abstract: This work reports for the first time the expression of a soluble B2 globin chain that is part of the extracellular hexagonal-bilayer haemoglobin from *Arenicola marina*. Two recombinant B2 globins were produced, one fused with glutathione S-transferase (B2-GST) and the other without a fusion tag (RecB2) and requiring a different purification procedure. We also describe a new method for the expression of globin that uses Studier's auto-induction medium together with the heme precursor δ -aminolevulinic acid. Media supplementation with the heme precursor δ -aminolevulinic acid in the culture increased heme synthesis by *E. coli* leading to the expression of the recombinant B2 globins in their active form. RecB2 and B2-GST were expressed with a yield of up to 105 mg/l of *E. coli* culture. Our approach is rapid and requires only one chromatographic purification step for B2-GST and three purification steps for RecB2. The overall results on RecB2 and B2-GST show that the recombinant globins exhibit similar properties to those of *Arenicola marina* native HBL-Hb with a great stability and a strong oxygen binding. The results and methodologies described in this paper are the beginning of a work aiming at reconstituting a recombinant HBL-Hb by genetic engineering in order to produce an innovative oxygen carrier for therapeutic applications.

Keywords: Annelid, extracellular haemoglobin, over-expression, blood substitute

INTRODUCTION

The polychaete *Arenicola marina* inhabits the intertidal zone and forms large populations on sandy shores in

Brittany. This lugworm possesses a giant, extracellular, hexagonal bilayer hemoglobin (HBL-Hb), typical of annelids, and circulating in the vascular system. A model of the quaternary structure of *Arenicola marina* HBL-Hb

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has been proposed by Zal et al. based on electrospray ionization mass spectrometry (ESI-MS) analysis and Multi-Angle-Laser-Light-Scattering (MALLS) measurements [1]. These authors reported that this macromolecule was composed of eight different globin chains and two structural linker chains. Recently, five globin and one linker cDNAs have been sequenced by Zal's group, encoding for A2a, A2b, A2c, B1, B2, and L2 chains, and in agreement with the masses determined by ESI-MS [2]. Royer et al. highlighted a remarkable hierarchical organization of *A. marina* HBL-Hb by crystallography [3], following that of *Lumbricus terrestris*, another annelid [4]. According to this model, the HBL-Hb has a molecular mass of ~3.6 MDa, and is made up of two hexagonal layers comprising a total of 144 globin subunits (15–17 kDa) and 36 structural linker chains (24–27 kDa). The various globin subunits are arranged into one or several types of trimers, and with monomeric chains form 12 dodecamers. These latter assemble onto a central core formed by linker subunits. Consequently, this giant HBL-Hb could bind up to 144 oxygen molecules on a single molecule. Interestingly, this HBL-Hb has recently been proposed as a new class of oxygen carriers for use as a red blood cell substitute [5]. Indeed, this molecule possesses interesting properties, placing this biopolymer in the second generation of blood substitutes. This macromolecule is reported to be resistant to auto-oxidation and subunit dissociation under physiological conditions. During pre-clinical trials, no apparent signs of toxicity were observed after the infusion of HBL-Hb to mice and rats [5].

Another interesting property is the property of reassociation of the subunits after dissociation by chemical reagents of *A. marina* HBL-Hb, similarly to *L. terrestris* HBL-Hb [5,6]. Consequently, one of the possible ways of HBL-Hb production in order to use it as a blood substitute would be to assemble a recombinant molecule by genetic engineering using this special self-assembling property on individually expressed subunits.

With recent advances in recombinant DNA technologies, native or specifically modified hemoglobins have been produced by microorganisms, transgenic plants, or animals [7,8]. Many studies described the expression or coexpression of human intracellular α - and β -globin in *Escherichia coli*. Different experiments showed the difficulty of expressing the monomers α - and β -globin in bacteria. Expression of fusion α -globin alone involves the formation of unstable proteins and inclusion bodies in the bacterial cytoplasm [9]. It has recently been shown that the coexpression of fusion α -globin with α -hemoglobin-stabilizing protein (AHSP) allows the production of recombinant soluble α -globin that is stable and functional [10]. Others methods, such as the co-expression with methionine aminopeptidase (MAP), allows also the production of soluble α -globin or α/β -globin [11,12]. For most of the culture media or the expression systems,

the yields of recombinant proteins are moderate or low and require many purification steps.

In marine annelid polychaetes, only the intracellular haemoglobin of the *Glycera dibranchiata* has been expressed in bacteria [13]. Recently, Léon et al. expressed an invertebrate hemoglobin from *Lucina pectinata* (HbI) in fusion with a His tag [14]. This fusion HbI was soluble, stable, and produced at a high yield of proteins without the need for the coexpression of MAP or molecular chaperone.

In this paper, we describe, for the first time, the expression of a soluble *Arenicola marina* B2 globin chain using an *E. coli* expression system, the first step toward the production of an artificial HBL-Hb by genetic engineering. Two recombinant B2 globins were produced, one fused with glutathione S-transferase (GST) and the other without a fusion tag and requiring a different purification procedure. This study also reports a new method for the expression of globin, using the Studier's auto-induction medium [15], together with the heme precursor δ -aminolevulinic acid, allowing the production of soluble B2 globin with a high yield.

MATERIALS AND METHODS

Construction of the pGEX-B2 and pET-Duet-B2 Plasmids, and *E. coli* Transformation

The coding sequence of *Arenicola marina* B2 globin cDNA was cloned into the prokaryotic expression vectors pET-Duet 1 and pGEX-5X3 (Novagen) to produce a recombinant B2 globin. For the construction in pGEX-5X3, the desired sequence was amplified by PCR with two primers, 5'-CGGGATCCCGATGGACGACTGTTG TACCACCGAG-3' and 5'-TCCCCCGGGCTAATCCTC GAGGAGGGAAGCAAT-3', which are complementary with the 5'- and 3'- end sequence of the *A. marina* B2 globin cDNA and contain a *Bam*HI and a *Sma*I site, respectively.

For construction in pET-Duet1 site 1, the desired sequence was amplified by PCR with two primers, 5'-CATGCCATGGACGACTGTTGTACCACC-3' and 5'-CCCAAGCTTAATCCTCGAGGAGGGAAGCA-3', which are complementary with the 5'- and 3'- end sequence of the *A. marina* B2 globin cDNA and contain a *Nco*I and a *Hind*III site, respectively. The PCR products were cloned using the TOPO®-TA cloning kit (Invitrogen), following the manufacturer's instructions. The positive recombinant clones were isolated and plasmid DNA was prepared with the Flexiprep kit (Amersham Pharmacia Biotech). Purified plasmids containing the B2 globin insert were used in a dye-primer cycle sequencing reaction to check that no mutations were introduced. The sequencing was performed with the universal primers T3

or T7, and the Big Dye[®] Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). PCR products were subsequently run on 31000 Genetic Analyser (Applied Biosystems).

The positive B2 globin recombinant clones and the pET-Duet 1 and pGEX-5X3 vectors were doubly digested with endonucleases *NcoI/HindIII* or *BamHI/SmaI* in separate reactions. Digestion products were gel-purified using the Qiaquick gel purification kit. The *A. marina* B2 globin insert and the pET-Duet and 1 pGEX-5X3 vectors were ligated using T4 DNA ligase in duplicate, and these mixtures were used to transform competent TOP10 *E. coli* cells. Positive clones were used for minipreps in order to obtain enough plasmid to transform the expression bacteria (*E. coli* BL21/pGEX-B2 or *Origami B*/pET-Duet-B2).

Expression and Solubilization of Different B2 Globin Proteins

E. coli cells, containing the various plasmids, were grown overnight at 37°C in 4 ml Luria-Bertani medium supplemented with 100 µg/ml ampicillin. ZYP-50-52 auto-induction medium [15] used for the expression of the B2 globins contained 1X ZY (10 g tryptone, 5 g yeast extract, 925 ml water), 20X NPS (1M Na₂HPO₄, 1M KH₂PO₄, 500 mM (NH₄)₂SO₄), 50X 5052 (25% glycerol, 2.5% glucose, 10% α-lactose monohydrate), and 2 mM MgSO₄.

These cultures were supplemented with ampicillin (100 µg/ml), kanamycin (30 µg/ml), tetracycline (25 µg/ml), and with 1 mM 5-aminolevulinic acid (ALA, haem precursor) [16]. The cells were grown 3–4 days in 2-L flasks containing 500 ml ZYP-50-52 at 32°C until the optical density at 600 nm reached 10. Expression of B2 globins was induced automatically by α-lactose monohydrate present in the media after 2–3 days of growth. There was then a clear change in the medium's color to red. The bacterial cells were harvested by centrifugation at room temperature.

The cell pellets were resuspended in lysis buffer (sucrose 20%; glycerol 10%; Tris-HCL (pH 8.0) 50 mM, Na₂S₂O₅ 0.2 mM, MgCl₂ 2 mM, anti-protease cocktail and DNase I), lysed in a French press (Constant Cell Disruption Systems) at 1.6 Kbar. The cell debris was removed again by centrifugation at 4°C for 40 min at 10 000 rpm (Eppendorf) and the soluble fractions were saved.

Purification of Fusion B2-GST and In Vitro Binding Assays

The blood-red supernatant containing the GST-B2 globin was mixed with glutathion-Sepharose 4B beads (Amersham Biosciences). The beads were first washed by 10 (bead) volumes of PBS. The beads were then washed

by 3 (bead) volumes of NaCl 1M and the beads were finally washed by 10 (bead) volumes of PBS. The blood-red beads were stored frozen at –20°C or eluted by the addition of five bead volumes of elution buffer (50 mM Tris-HCl pH 8.0; reduced glutathione 10 mM). The GST fusion protein elute was concentrated by centrifugation on an Amicon Ultra-30 concentrator, cut-off molecular weight 30 kDa (Millipore, Billerica, Massachusetts). In a second experiment, the B2 globin was cleaved from the GST proteins by addition of factor Xa (50 U/mg protein), and the incubation was carried out for 7 hours at room temperature. The cleavage mix was concentrated by centrifugation on an Amicon Ultra-10 concentrator and was purified by HPLC on a Superdex 75 (Amersham Biosciences) equilibrated with 25 mM Tris-HCl pH 7.5 at room temperature.

Purification of “Native” B2 Globin

The blood-red supernatant containing the B2 “native” globin was then fractionated with ammonium sulphate at 60% saturation. The supernatant was collected by centrifugation at 10000 g for 20 min and dialysed against 25 mM Tris-HCl pH 7.5 overnight at 4°C.

The dialysate was then loaded onto a 2 cm X 10 cm column of DEAE-Sepharose (Pharmacia), equilibrated with 25 mM Tris-HCl pH 7.5 at room temperature. The column was washed with the same buffer until the A_{280 nm} was less than 0.001. The “native” B2 globin was then eluted with a linear NaCl gradient of 0 mM at 500 mM NaCl. The fraction containing the “native” B2 globin, identified and collected by measuring the absorbance at 414 nm and 280 nm, were concentrated by centrifugation on an Amicon Ultra-10 concentrator. The proteins were then separated through a 10/300 Superdex 75 gel-filtration column (Pharmacia) equilibrated with 25 mM Tris-HCl pH 7.5 at room temperature. The colored fractions containing the “native” B2 globin, identified and collected by measuring the absorbance at 414 nm and 280 nm, were concentrated by centrifugation on an Amicon concentrator as described above.

The different recombinants globins (B2-GST or “native” B2) were washed by centrifugation on an Amicon Ultra-10 concentrator and stored in a specific saline buffer developed in the laboratory (400 mMol l⁻¹ NaCl, 2.95 mMol l⁻¹ KCl, 32 mMol l⁻¹ MgSO₄, 11 mMol l⁻¹ CaCl₂ and 50 mMol l⁻¹ Tris-HCl at pH 7.0, [17]).

Protein Analysis

Different aliquots were withdrawn during the expression, solubilization, and purification steps of the expression of the recombinant proteins. The samples were analyzed by

SDS/PAGE (Ready Gels Tris-HCl Glycine 4–20% BioRad). The gels were stained with Coomassie brilliant blue R-250 and dried onto Whatman 3MM paper.

Protein concentrations of “native” B2 (hereafter referred to as Rec-B2) or B2-GST were determined using haem concentration as a proxy with Drabkin’s method [18] using Drabkin’s reagent (Sigma). In this method all the hemoglobin in the sample is converted into methe-moglobin and then converted into cyanomethemoglobin. The absorbance of cyanomethemoglobin is then measured in a spectrophotometer at 540 nm.

Spectrophotometric Studies

UV and visible spectral measurements were carried out in a UVmc2 spectrophotometer (SAFAS, Monaco).

The spectra of the oxidized and deoxy- forms of the rec B2 and B2-GST were measured at 25°C in 100 mM potassium phosphate buffer at pH 7.0. Deoxygenation of the B2 recombinant globins was achieved by adding an excess of sodium dithionite (=hydrosulfite). The B2 recombinant globins were oxidized in the presence of 1 mM potassium ferricyanide at pH 7.0 for 20 min resulting in metB2 recombinant globin spectra.

Measurement of Auto-oxidation

Auto-oxidation rate constants were measured at 37°C in 100 mM potassium phosphate buffer pH 7.0, with 1 mM EDTA. An excess of sodium dithionite was added to obtain the ferrous protein. The solution of reduced protein was immediately loaded onto a small G-25-medium gel filtration column and eluted quickly with 100 mM potassium phosphate pH 7.0, with 1 mM EDTA to eliminate the sodium dithionite. The protein spontaneously bound oxygen from the atmosphere. The different solutions were then added to a sample cuvette and the auto-oxidation kinetics was followed by measuring the complete absorption spectra between 500 and 620 nm.

Oxygen Binding Properties

Oxygen equilibrium curves were determined on 3- μ L samples of HbA (human adult Hb), AmHb (*Arenicola marina* HBL-Hb), B2-GST, and Rec-B2 using a thermostated diffusion chamber linked to a mass-flow meter gas mixing system [19]. The diffusion chamber was placed in the light path of a diode array spectrophotometer (Optic Ocean) and the absorbance was measured at 430 nm. Oxygenation data based on at least six equilibrium steps between 0.3 and 0.7 fractional saturation (Y) were

converted to Hill plots $\{\log [Y/(1 - Y)]$ against $\log PO_2$, (where PO_2 is the oxygen partial pressure) for the estimation of the half-saturation oxygen partial pressure (P_{50}) and Hill’s cooperativity coefficient at half-saturation (n_{50}) [20].

Intact Protein Mass Determination

Mass determination was performed on the purified proteins. Electrospray (ESI) mass-spectrometry data were acquired on a LCQ-Advantage ESI-ion trap system (Thermo-Fisher), scanning over the m/z range 500–2000. Samples were desalted by ultrafiltration on an Amicon-5kDa against 32 mL of MilliQ water, at 4°C prior to analysis. A solution of protein (0.1 mg.mL⁻¹) in acetonitrile/water (1:1, v/v) containing 0.1 % (v/v) formic acid was introduced into the electrospray source at 5 μ L.min⁻¹.

Protein Identification by LC-MS/MS Analysis

In-gel digestion. After separation on SDS/PAGE, the band corresponding to the M_w of globin was excised and in-gel digested with Trypsin, essentially as described elsewhere [21]. Briefly, the gel band was washed with 100 μ L of 25 mM NH₄HCO₃, followed by 100 μ L of 50% acetonitrile in 25mM NH₄HCO₃. Proteins were reduced by incubation with 10 mM DTT (1 h at 57 °C), and alkylated with 100 μ L of 55 mM iodoacetamide (45 min at room temperature). Gel spots were further washed and dried in a vacuum centrifuge. The proteins were digested overnight at 37°C by the addition of 10 μ L of Trypsin (12.5 ng/ μ L in 25mM NH₄HCO₃; modified Trypsin purchased from Promega, Madison, WI).

LC-MS/MS analysis. Nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of the digested proteins were performed using a Switchos-Ultimate II capillary LC system (LC Packings/ Dionex, Amsterdam, the Netherlands) coupled to a hybrid quadrupole, orthogonal, acceleration time-of-flight mass spectrometer (Q-TOF Global, Waters, Manchester, UK). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75 mm i.d., 15 cm length, LC Packings) at a flow rate of 200 nL.min⁻¹. The gradient consisted of a linear increase from 2% to 40% of acetonitrile in 50 min, followed by a rapid increase to 50% of acetonitrile within 10 min.

Mass data acquisitions were piloted by the Mass Lynx software (Waters) using a data dependent acquisition mode: MS data were recorded for 1 sec on the mass-to-charge (m/z) range 400–1500, after which the three most intense ions (doubly or triply charged ions) were

selected and fragmented in the collision cell (MS/MS measurements).

Protein identification. Collected data were searched against the TrEMBL database (release 35) using both MASCOT 2.2 (Matrix Science, London, <http://www.matrixscience.com>) and Protein Lynx Global Server (version 2.1, Waters). Mass accuracy was set to 150 ppm for parent ions and 0.3 Da for MS/MS fragments. One missed trypsin cleavage was allowed per peptide. This led to the unambiguous identification of Q53164 entry. In order to increase the sequence coverage, a *de novo* sequencing of the remaining peptides was performed and the sequence stretches were aligned against Q53164. Results were validated and exported in an analytical report using the OVNIp software developed by INRA Nantes (<http://www.appli.nantes.inra.fr:8180/OVNIp>).

RESULTS AND DISCUSSION

Expression and Purification of Recombinant B2-GST and RecB2 Globins

The sequence coding for *A. marina* B2 globin was cloned from purified cDNA as described in the Materials and Methods. The globins B2-GST and RecB2 were expressed in auto-induction medium in *E. coli* BL21 (DE3). Association of two methods, auto-induction medium [15] and addition of ALA, allowed us to obtain soluble B2-GST and RecB2 globins.

In a first experiment, the *A. marina* B2 globin was expressed as a fusion protein with GST under the control of a tac promoter (Figure 1). The pGEX-B2 construct was derived from the pGEX-5X3 expression system. The B2-GST recombinant protein was expressed at a high-level in *E. coli* BL21 (DE3). The B2-GST recombinant protein allows a purification of the protein in a single step. The SDS-PAGE analysis (Figure 2a) shows the different steps of purification of recombinant B2-GST. The recombinant protein was expressed at a high level and obtained in the soluble fraction (Figure 2a, lane 2). After purification by affinity chromatography on glutathione Sepharose 4B and elution by addition of reduced glutathione, the SDS-PAGE revealed the presence of a single band corresponding to B2-GST (Figure 2a, lane 3). Gel permeation chromatography on Superdex 75 10/300 revealed the presence of single peak pure at 98.7% (data not shown) and a concentration of 105.6 mg/l of culture according to the Drabkin's method.

After Factor Xa cleavage of the fusion protein B2-GST, SDS-PAGE revealed the presence of three bands (Figure 2a, lane 4). The first band of low intensity represents the uncleaved fusion B2-GST protein (44 kDa), the second band corresponds at the GST subunit only (27 kDa), and the last band to the B2 globin (17 kDa). Purification of the cleavage mix by gel filtration on Superdex 75 10/300 (Figure 2b) showed the presence of two peaks. The analysis of the second fraction by SDS-PAGE (Figure 2a, lane 5) revealed the presence of a single band at 17 kDa corresponding at the overexpressed B2 globin. This protein is pure at 95% and at a yield of 43

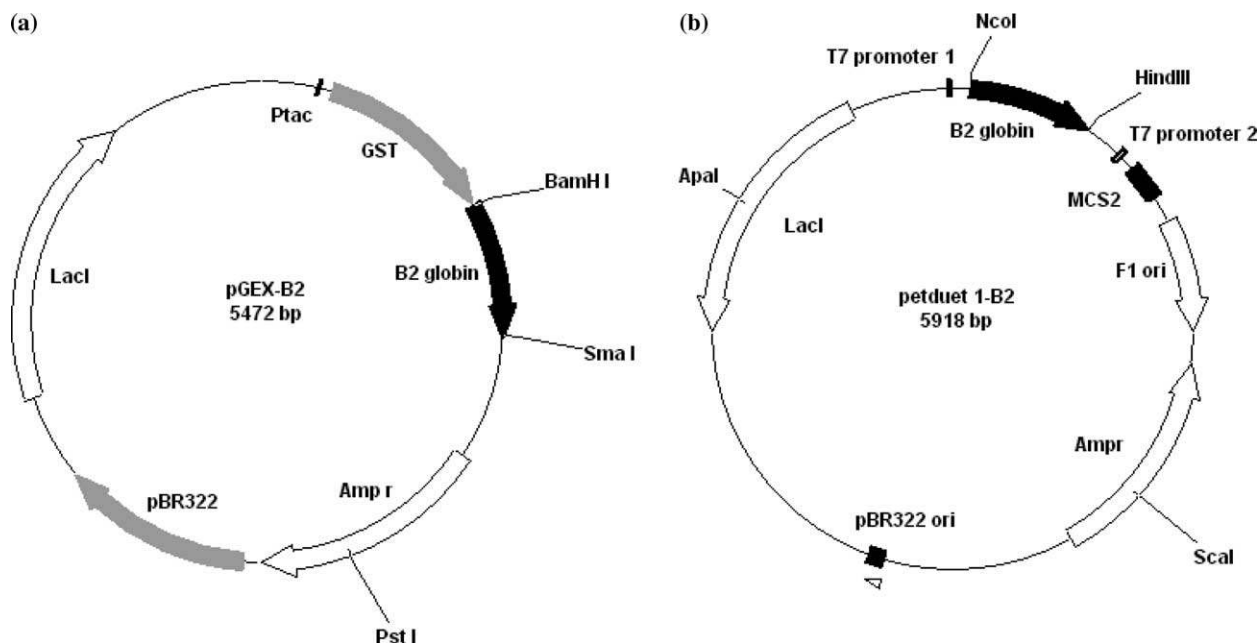


Figure 1. Construction of plasmids pGEX and pET-Duet 1 containing *Arenicola marina* B2 globin cDNA for the production in BL21 *E. coli*. Map of pGEX-B2 (a) and pET DUET-1 B2 (b) plasmids.

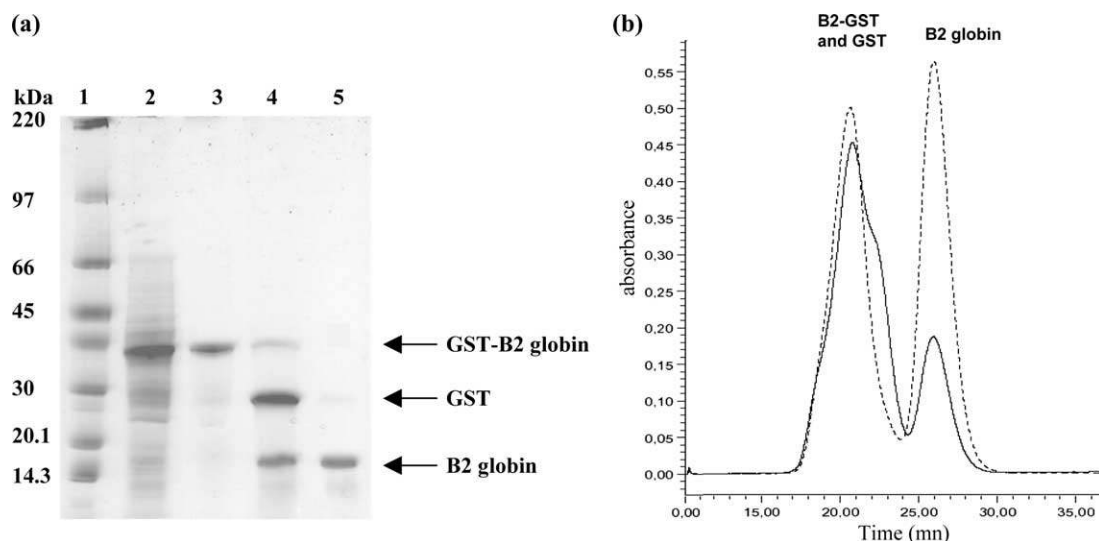


Figure 2. Expression and purification of recombinant B2-GST globin. (a) Analysis of the purified B2-GST globin by SDS/PAGE on a gradient 4–20% polyacrilamide gel after Coomassie blue staining. Lane 1, molecular weight markers (kDa shown on the left); lane 2, total fraction of soluble proteins from cells containing the pGEX-B2 plasmid; lane 3, soluble B2-GST after purification and elution from the bound glutathione; lane 4, cleavage B2-GST by factor Xa; lane 5, purification by gel filtration Superdex 75. (b) Elution profile of the B2-GST globin on a superdex 75 10/300GL column after cleavage by factor Xa. The elution was followed by the absorbance at 280 nm (solid line) and 414 nm (dashed line).

mg/l of culture (Table 1). The first fraction of the gel filtration (Figure 2b) contains the uncleaved B2-GST protein and the subunit GST (verified by SDS-PAGE).

These first results show the solubility and stability of *A. marina* B2 globin as a GST fusion protein. In addition, this protein was produced at a higher yield (Table 1) than those obtained when GST- α Hb was co-expressed with GST-ASHP [10] and for His-rHbI from *Lucina pectinata* [14].

In a second experiment, the *A. marina* B2 globin was without a fusion protein. The recombinant B2 globin (RecB2) was produced when the cloned gene was placed under the control of the T7 RNA polymerase promoter in

the pET-Duet-1 expression vector (Figure 1b). The recombinant RecB2 was purified to near homogeneity by a three-step procedure, whose progress was followed by SDS-PAGE (Figure 3a). The association of auto-induction media with ALA for the culture allowed us to obtain a high-level expression in the soluble fraction (Figure 3a, lane 2). After the first steps of purification (ammonium sulphate fractionation 60% and DEAE-sepharose anion-exchange chromatography), the SDS-PAGE showed a decrease of contaminant protein (Figure 3a, lanes 3 and 4). The purification of the DEAE fraction by gel filtration on Superdex 75 10/300 (Figure 3b) showed the presence of two peaks. The analysis of the

Table 1. Comparison of the recombinant globins yield

Expressed proteins	Hemin	ALA	Protein Yield (mg/1 culture)	Ref
β -Hb	+	–	3–4	[Yamaguchi et al. (1998)]
Neuroglobin	+	–	5	[Nancy et al. (2000)]
α -Hb	+	–	3	[Shen et al. (1997)]
chloroplastic Hb	–	+	1–2	[Couture et al. (1996)]
His-HbI	+	–	32	[Léon et al. (2004)]
<i>Lucina pectinata</i>				
GST- α -HB ^a	+	–	12–20	[Vasseur-Godbillon et al. (2006)]
GST-B2 ^a	–	+	43	
<i>Arenicola marina</i>				
B2 globin	–	+	11	
<i>Arenicola marina</i>				

a: this quantity corresponds to the α -Hb and B2 globin after cleavage

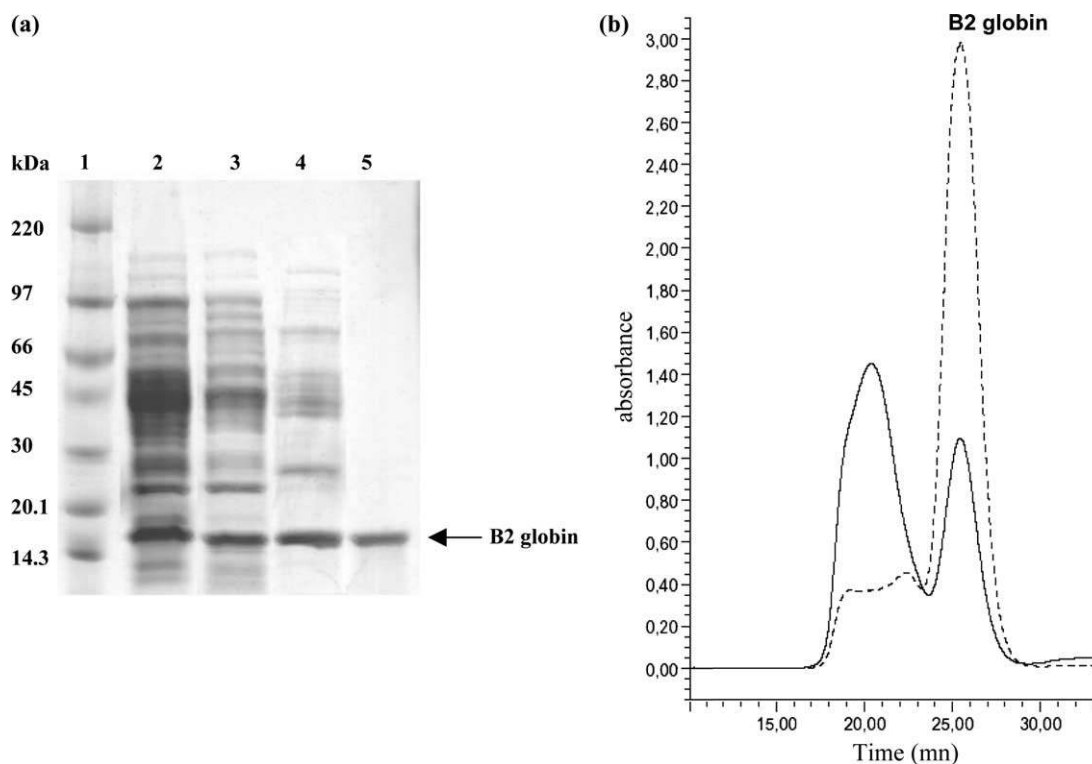


Figure 3. Expression and purification of recombinant RecB2 globin. (a) Analysis of the purified RecB2 globin by SDS/PAGE on a gradient 4–20% polyacrilamide gel after Coomassie blue staining (1–2 μ g proteins/lane). Lane 1, molecular weight markers (kDa shown on the left); lane 2, total fraction of soluble proteins from cells containing the pET-Duet-B2 plasmid; lane 3, supernatant fraction obtained after ammonium sulfate fractionation 60%; lane 4, first purification by DEAE-Sepharose; lane 5, second purification by gel filtration Superdex 75. (b) Elution profile of the RecB2 globin on a superdex 75 10/300GL column after purification on DEAE Sepharose. The elution was followed by the absorbance at 280 nm (solid line) and 414 nm (dashed line).

second fraction by SDS-PAGE (Figure 3a, lane 5) revealed the presence of a single band at 17 kDa corresponding to the overexpressed B2 globin. After a second analysis by gel filtration (Figure 3a, b), this protein was found to be pure at 96.8% and a yield of 11mg/l of culture using Drabkin's method (Table 1).

These results also indicate the solubility and stability of recombinant *Arenicola marina* B2 globin. In addition, this protein was produced with a higher yield than those obtained when α -Hb was co-expressed with methionine aminopeptidase [11] or Chloroplastic Hb with ALA [22] (Table 1).

Analysis of Proteins by Mass Spectrometry

The goal of the ESI-MS analysis of the intact purified protein was to determine its mass and compare it to the 17033 Da previously reported by Zal et al. [1], and to the calculated mass based on its primary sequence [2]. The raw multicharged spectrum (Figure 4a) and the deconvoluted mass spectrum (Figure 4b) show the presence of an abundant protein with an Mw of 17257.55 ± 0.78 (relative abundance of 75%) and ad-

ducts of +18 Da, which could be attributed to water molecules. This is 93.5 Da higher than the expected 17164 Da for the intact protein (17033 Da without the initiator methionine (M) of 131 Da).

An LC-MS/MS analysis was performed on the tryptic digest of the excised SDS-PAGE band corresponding to the protein of interest in order to confirm that it corresponds to the B2 globin. The mass data (Figure 4c) were confronted to the TrEMBL database (release 35) using the MASCOT and PLGS2 search engines. An unequivocal assignment could be made to haemoglobin B2 of *Arenicola marina* (Q53I64), the identified peptides leading to a sequence coverage of 83% (Figure 4c).

One peptide at 1410.6 Da (doubly charged at 706.3 m/z) remained non-identified after the MASCOT search and was submitted to *de novo* sequencing. It could be assigned to the N-terminal fragment "MDDCCTT EDRK" (carrying the initiator methionine) without any ambiguity (Figure 4d). However, the measured mass for this peptide (1410.6 Da) together with the MS/MS spectrum suggest that the N-terminus methionine is modified (it displays an extra mass of 38 Da). We failed to determine more precisely the nature of this modification. However, it may contribute to

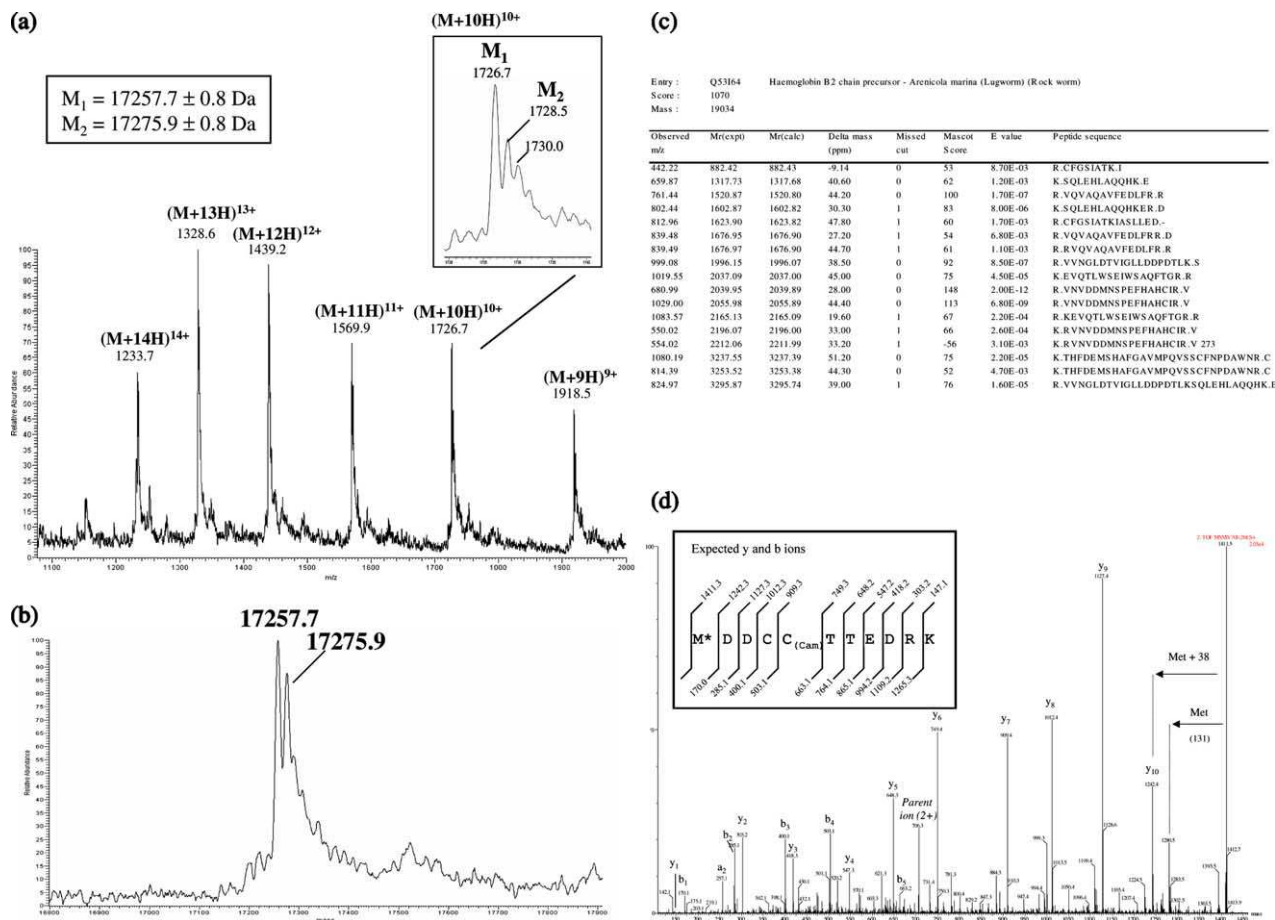


Figure 4. ESI-MS of overexpressed B2 globin. (a) Smoothed, multiply charged ESI mass spectrum. (b) Corresponding deconvoluted spectrum. (c) List of the peptide sequences identified using the Mascot search engine in *Arenicola marina* globin B2 (Q53I64) (from the LC-MS/MS analysis of the digested 1D-SDS PAGE band). (d) Product ion spectra of the doubly charged precursor ion at 706.25 m/z (the spectrum is deconvoluted into $(M+H)^+$ species). The proposed amino acid sequence for the N-terminus peptide (including a modified Met having a side chain mass of 169 Da) and the expected y_n (C-terminus ions) and b_n (N-terminus ions) fragment-ions are represented on the spectrum.

part of the delta mass observed for the intact protein (mass difference of +93.5 Da compared to the expected mass, taking into account the initiator methionine).

Spectral Characterization of Recombinant Proteins B2-GST and RecB2 Globins

RecB2 and B2-GST were characterized by their light-absorption spectra. Qualitative comparisons show that the spectra of Rec-B2 and B2-GST measured between 300 and 700nm (Figure 5a, b) are very similar to each other, and this for different ligand and iron states. The oxy-B2 globin has a characteristic Soret band at 418 nm, with α and β bands at 572 nm and 540 nm, respectively. When the solution is treated with sodium dithionite, the α and β bands disappear and are replaced by a single broad band with a peak at 558 nm, while the Soret band shifts towards the visible region at 426 nm. This shift in the Soret band

indicates that after the treatment with sodium hydrosulfite there is no ligand on the heme and the iron is now penta-coordinated. The ferric form of the recombinant globins was obtained after treatment with potassium ferricyanide. The α and β peaks disappear and the Soret band shifts to 414 nm. These results show that the Rec-B2 and B2-GST globins have spectral features characteristic of a typical globin. These results were similar to those observed for the recombinant globin of *Lucina pectinata* [14].

Auto-oxidation and Functionality of B2-GST and RecB2 Globins

The auto-oxidation kinetic of the RecB2 and B2-GST globins in the oxygenated form was compared to those obtained for HbA and HbAm. Figure 6 illustrates the time courses of auto-oxidation. The transition from the oxy- to the met- spectrum of RecB2, B2-GST, HbA and HbAm

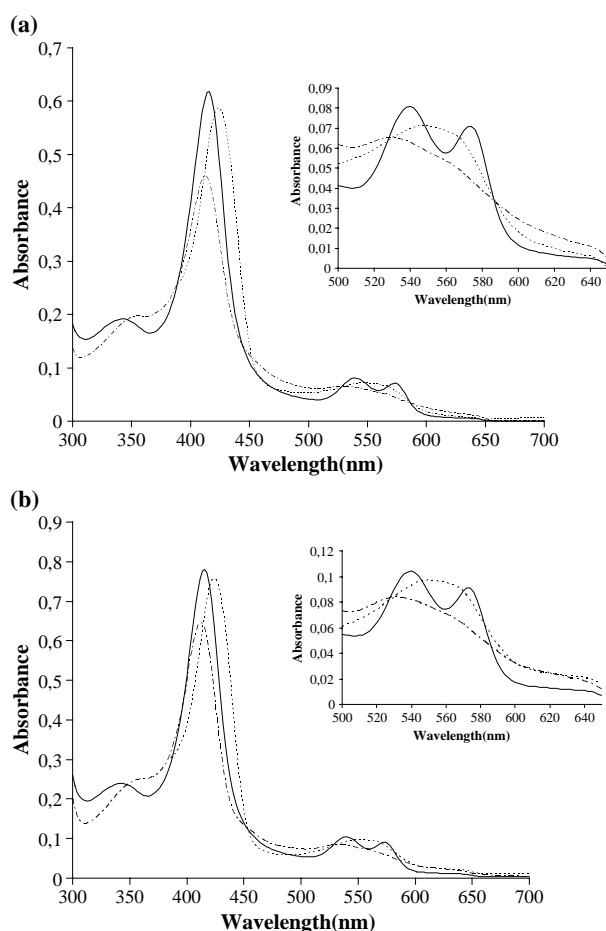


Figure 5. UV/Vis Spectra of RecB2 (a) and B2-GST (b). Absorption spectra were measured in saline buffer buffer (see Materials and Methods) at 25°C for ferric hexacoordinated form (long-dashed line), deoxy ferrous pentacoordinated form (short-dashed line) and oxy form (solid line).

exhibits well-defined isosbetic points at 522 and 593 nm (Figure 6b). The auto-oxidation mechanism for haemo-proteins developed by Weiss involves the dissociation of the superoxide anion O_2^- and the formation of the ferric ion [23]. All auto-oxidation curves could be simulated as a monoexponential process (Figure 6a). The data are summarized in a table (Figure 6c). RecB2 and B2-GST globins have a k_{ox} of 0.008 h^{-1} and 0.004 h^{-1} , respectively, with a half-life ($t_{1/2}$) of 78 h and 148 h at 37°C, and pH 7.0 (Figure 6c). The auto-oxidation of RecB2 seems faster than for B2-GST, which could be explained by the fusion GST protein thus avoiding the auto-oxidation of this recombinant globin. These values of half-life were two or three times greater than the value for HbA obtained under the same conditions as reported in the literature.

The functional properties of HbA, AmHb, B2-GST, and RecB2 were determined using a thermostated diffusion chamber linked to a mass-flow meter gas mixing

system. Oxygen binding molecules are usually compared by means of their oxygen dissociation curve, which gives access to characteristic parameters such as the O_2 affinity (P_{50} , the partial pressure of oxygen necessary to saturate half of the oxygen binding sites and consequently the higher the P_{50} , the weaker the oxygen is bound) and cooperativity (n_{50}) (Table 2). Under the same conditions, AmHb and HbA (purified) have P_{50} similar (11.5 mmHg and 11.09 mmHg) and the recombinant monomeric form RecB2 and B2-GST globins have P_{50} of 0.98 mmHg and 1.02 mmHg. These results show, as reported in the literature, that recombinant monomeric forms have a greater oxygen affinity than native Hb.

CONCLUSION

We describe the first expression of a soluble *Arenicola marina* B2 globin chain using an *E. coli* expression system. Two recombinant B2 globins were produced: one fused with glutathione S-transferase (GST), and the other one without a fusion tag (and requiring a different purification procedure). In addition, we describe a new method for the expression of globin that involves Studier's auto-induction medium [15], and the heme precursor δ -aminolevulinic acid. Media supplementation with the heme precursor δ -aminolevulinic acid in the culture increased the ability of *E. coli* to produce heme, leading to the expression of the recombinant B2 globins in the active form. High culture densities and high yield of recombinant B2 globins were obtained in the bioreactor or scale with Studier's auto-induction medium. Furthermore, RecB2 or B2-GST fusion proteins were expressed alone in bacteria without the need for the coexpression of MAP or of a molecular chaperone. With these methods RecB2 and B2-GST were expressed yielding up to 105 mg/l of *E. coli* culture. Our procedure is rapid and requires only one chromatographic purification step for B2-GST and three purifications steps for RecB2. The expression system described here may be especially interesting for the study of recombinant globin, stable or not. UV-Vis spectral analysis of RecB2 and B2-GST showed absorption maxima identical to those of the recombinant globin His-HbI of *Lucina pectinata* [14]. The overall results on RecB2 and B2-GST show that the recombinant globins exhibit similar properties to those of *Arenicola marina* native HBL-Hb with a great stability and a strong oxygen binding. The results and methodologies described in this paper are the beginning of a work to reconstitute a recombinant HBL-Hb by genetic engineering using the auto-assembling property of HBL-Hb already demonstrated [24]. The second step will be to produce a recombinant dimer globin B2-B1 or B2-A2b in the expression vector pET-Duet 1. The polycistronic expression system pST39 kindly provided by Dr. Song Tan

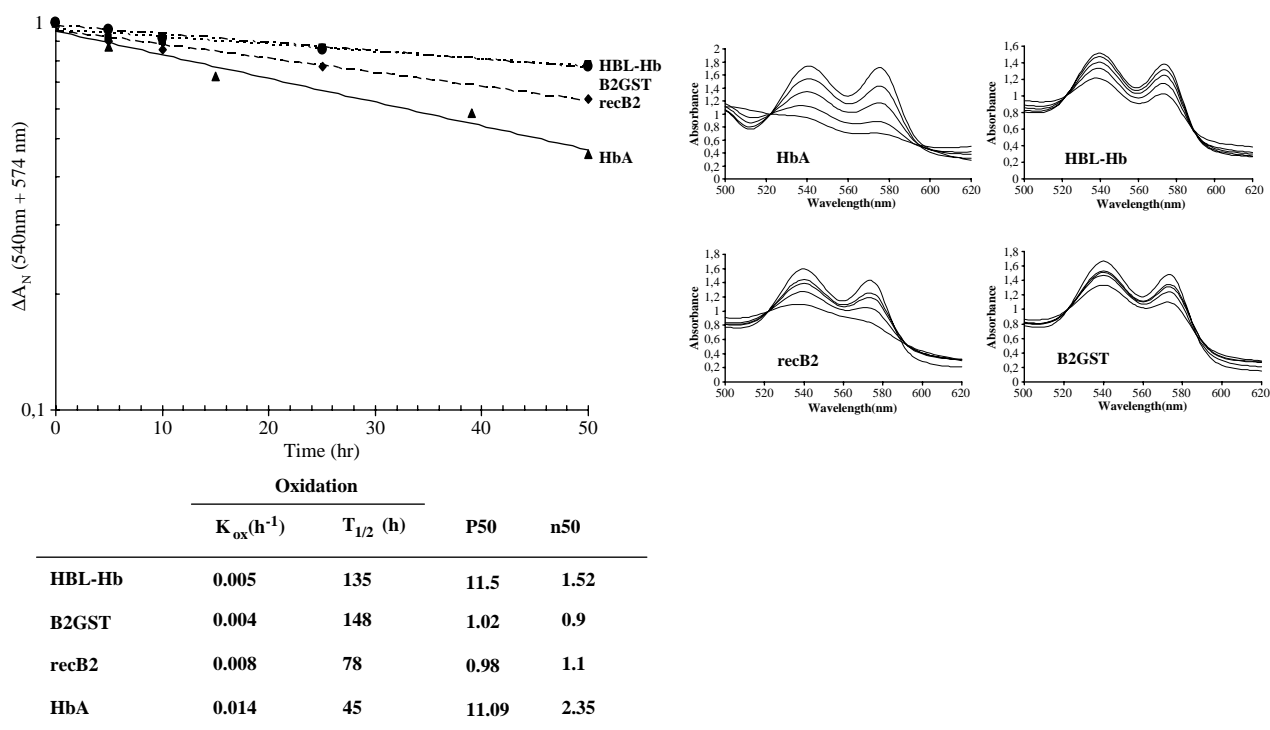


Figure 6. Auto-oxidation kinetics of RecB2 (◆) and B2-GST (●), compared with those of Hba (▲) and HBL-Hb (■). The transition of the ferrous oxy to ferric hexacoordinated form is shown on the graph (b). Experimental conditions were 100 mM potassium phosphate buffer pH 7.0, with 1 mM EDTA.

Table 2. Functional properties

	Oxidation			
	$K_{ox}(h^{-1})$	$T_{1/2}(h)$	P50	n50
HBL-Hb	0.005	135	11.5	1.52
B2GST	0.004	148	1.02	0.9
recB2	0.008	78	0.98	1.1
HbA	0.014	45	11.09	2.35

could be used for overexpressing recombinant trimer or dodecamer with the production of three or four globins in the same bacteria [25]. The production of GST fusion globin such as B2-GST may be useful *in vitro* to study the property of reassociation of HBL-Hb by GST pull-down approach. This work will be pursued in the laboratory using the polycistronic vectors in order to express several globins of *Arenicola marina*. This approach associated with the techniques of production presented in this paper would allow us to produce a synthetic HBL-Hb.

REFERENCES

- Zal, F., Green, B.N., Lallier, F.H., Vinogradov, S.N., Toulmond, A. (1997). Quaternary structure of the extra-
- cellular haemoglobin of the lugworm *Arenicola marina*: a multi-angle-laser-light-scattering and electrospray-ionisation-mass-spectrometry analysis. *Eur J Biochem* **243**: 85–92.
- Chabasse, C., Bailly, X., Rousselot, M., Zal, F. (2006). The multigenic family of the extracellular hemoglobin from the annelid polychaete *Arenicola marina*. *Comp Biochem Physiol B Biochem Mol Biol.* **144**:319–25.
- Royer, W.E., Omartian, M.N., Knapp, J.E. (2007). Low resolution crystal structure of *Arenicola* erythrocrucorin: Influence of coiled coils on the architecture of a megadalton respiratory protein. *J Mol Biol.* **365**:226–36.
- Royer, W.E., Strand, K., van Heel, M., Hendrickson, W.A. (2000). Structural hierarchy in erythrocrucorin, the giant respiratory assemblage of annelids. *Proc Natl Acad Sci U S A* **97**:7107–7111.
- Rousselot, M., Delpy, E., Drieu La Rochelle, C., Lagente, V., Pirow, R., Rees, J-F., Hagege, A., Le Guen, D., Hourdez, S., Zal, F. (2006a). *Arenicola marina* extracellular hemoglobin: a new promising blood substitute. *Biotechnol. J.* **1**:333–345.
- Sharma, P.K., Kuchumov, A.R., Chottard, G., Martin, P.D., Wall, J.S., Vinogradov, S.N. (1996). The role of the dodecamer subunit in the dissociation and reassembly of the hexagonal bilayer structure of *Lumbricus terrestris* hemoglobin. *J Biol Chem.* **271**: 8754–8762.
- Chang, T.M.S. (1997). Blood Substitutes: Principles, Methods, Products and Clinical Trials. Vol.1, Karger, Basel 1–141.

8. Tsai, A., Vandegriff, K., Intaglietta, M., Winslow, R. (2003). Targeted oxygen delivery by cell-free Hb: a new basis for oxygen therapeutics. *Am. J. Physiol.* **285**:1411–1419.
9. Jessen, T.H., Komiyama, N.H., Tame, J., Pagnier, J., Shih, D., Luisi, B., Fermi, G. and Nagai, K. (1994). Production of human hemoglobin in *Escherichia coli* using cleavable fusion protein expression vector. *Methods Enzymol.* **231**: 347–364.
10. Vasseur-Godbillon, C., Hamdane, D., Marden, M. C., Baudin-Creuzat, V. (2006). High-yield expression in *Escherichia coli* of soluble human alpha-hemoglobin complexed with its molecular chaperone. *Protein. Eng. Des. Sel.* **19**:91–97.
11. Adachi, K., Yamaguchi, T., Yang, Y., Konitzer, P.T., Pang, J., Reddy, K.S., Ivanova, M., Ferrone, F., Surrey, S. (2000). Expression of functional soluble human α -globin chains of hemoglobin in bacteria. *Prot. Expr. Purif.* **20**:37–44.
12. Shen, T.J., Ho, N.T., Zou, M., Sun, D.P., Cottam, P.F., Simplaceanu, V., Tam, M.F., Bell, D.A., Ho, C. (1997). Production of human normal adult and fetal hemoglobins in *Escherichia coli*. *Protein Eng.* **10**:1085–1097.
13. Zafar, R.S., Weber, R.E., Sharma, P.K., Vinogradov, S.N., Walz, D.A. (1993). Purification and characterization of recombinant polymeric hemoglobin P1 of *Glycera dibranchiate*. *Protein Expr Purif.* **4**:547–51.
14. Léon, R.G., Munier-Lehmann, H., Baudin-Creuzat, V., Pietri, R., López-Garriga, J., Cadilla, C.L. (2004). High-level production of recombinant sulfide-reactive hemoglobin I from *Lucina pectinata* in *Escherichia coli*. High yields of fully functional holoprotein synthesis in the BLi5 *E. coli* strain. *Protein Expr Purif.* **38**:184–95.
15. Studier, F.W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif.* **41**:207–34.
16. Pesce, A., Nardini, M., Dewilde, S., Ascenzi, P., Burmester, T., Hankeln, T., Moens, L., Bolognesi, M. (2002). Human neuroglobin: crystals and preliminary X-ray diffraction analysis. *Acta Crystallogr D Biol Crystallogr.* **58**:1848–50.
17. Zal, F., Lallier, F.H., Wall, J.S., Vinogradov, S.N., Toulmond, A. (1996). The multi-hemoglobin system of the hydrothermal vent tube worm *Riftia pachyptila*: I- Reexamination of the number and masses of its constituents. *J. Biol. Chem.* **271**:8869–8874.
18. Drabkin, D.L., Austin, J.H. (1935). Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide haemoglobin and sulphemoglobin. *J. Biol. Chem.* **112**:51.
19. Sick, H., Gersonde, K. (1969). Method of continuous registration of O₂ binding curves of hemoproteins by means of a diffusion chamber. *Anal Biochem* **32**:362–376.
20. Weber, R.E. (1981). Cationic control of O₂ affinity in lugworm erythrocytes. *Nature* **292**:386–387.
21. Devouge, V., Rogniaux, H., Nesi, N., Tessier, D., Gueguen, J., Larre, C. (2007). Differential proteomic analysis of four near-isogenic *Brassica napus* varieties bred for their erucic acid and glucosinolate contents. *J. Proteome Res.* **6**:1342–1353.
22. Couture, M., Guertin, M. (1996). Purification and spectroscopic characterization of a recombinant chloroplastic hemoglobin from the green unicellular alga *Chlamydomonas eugametos*. *Eur J Biochem.* **242**:779–87.
23. Weiss, J.J. (1964). Nature of the iron-oxygen bond in oxyhaemoglobin. *Nature* **202**:83–84.
24. Rousselot, M., Le Guen, D., Zal, F. (2006b). Novel dissociation mechanism of a polychaetous annelid extracellular haemoglobin. *FEBS J.* **273**:1582–96.
25. Tan, S. (2001). A modular polycistronic expression system for overexpressing protein complexes in *Escherichia coli*. *Prot. Expr. Purif.* **21**:224–234.