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Recombinant Hemoglobin II From *Lucina pectinata*: A Large-Scale Method For Hemeprotein Expression in *E. coli*

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Abstract

Hemoglobin II from the clam *L. pectinata* is an O₂ reactive protein that remains oxygenated in the presence of other molecules. To determine the mechanism of ligand selection in this hemoglobin, rHbII was expressed in large quantities using an improved fermentation process. The highest protein yield was obtained by: transforming HbII into the BLi5 cells, inducing and supplementing the culture during the mid-log phase with 1 mM IPTG, 30 µg/mL hemin chloride and 1% glucose, and decreasing the temperature to 30 °C after induction. In addition, cell culture density was greatly enhanced by using glycerol, adding MgSO₄, supplementing the media with glucose after the glycerol was consumed and maintaining the dissolved oxygen at 35%. Under these conditions the maximum protein yield obtained was ~2,300 mg/L. The results indicate that rHbII is similar to the native protein. The protocol was validated with other hemoglobins, indicating that it can be extended to other hemeproteins.

Keywords

Lucina pectinata; Recombinant HbII; O₂ binding; Stopped-flow kinetics; Fermentor

1 Introduction

The clam *Lucina pectinata* is an intriguing invertebrate organism characterized by the presence of three highly concentrated hemoglobins with distinct structural and functional features [12]. Hemoglobin I (HbI) is a sulfide reactive protein that transports H₂S to chemoautotrophic bacteria, which live in symbiosis with the mollusk. The other two hemoglobins (HbII and HbIII) are believed to transport O₂ to the clam as well as to the endosymbiont. HbI is unique in the sense that it binds and transports this well known toxic gas in the ferric heme iron center to maintain the symbiotic relationship with the bacteria as well as to protect the clam from H₂S toxicity [13]. The affinity of H₂S for ferric HbI is exceptionally high and is believed to be achieved through fast association ($k_{\text{on}} = 2.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) and very slow dissociation processes ($k_{\text{off}} = 2.2 \times 10^{-4}\text{s}^{-1}$). Structural studies of the HbI active site have shown that the hemoglobin contains a distinctive distal structural organization involving residues GlnE7, PheB10, and PheE11 [22]. Based on the peculiar amino acid composition, it has been suggested that this environment is responsible for the high affinity of HbI for H₂S. In fact, by employing site-directed mutagenesis technology along with spectroscopic approaches, we have identified the role of key residues in the affinity of H₂S. Regarding this, it has been shown that Gln at position E7 possess a flexibility that allows H₂S to bind rapidly to the ferric heme [18]. Sulfide is then stabilized by hydrogen bonding and electrostatic interactions with GlnE7 and PheB10, respectively. Stabilization of the bound H₂S through H-bonding interaction with GlnE7 can in turn induce some heme reduction and H₂S delivery as a function of H₂S concentration [19].

Hemoglobins II and III, on the other hand, remain oxygenated in the presence of H₂S and have been regarded as oxygen carrier proteins [12]. It has been shown that both proteins are structural analogous and that similar to HbI, they have GlnE7 and PheE11 in their distal heme environment [1,21]. However, the B10 position in these hemoglobins is occupied by a Tyr instead of the Phe residue found in HbI. Indeed, as Fig. 1 shows, the presence of these residues was recently confirmed by the X-ray crystal structure of HbII [9]. The structure of HbII also reveals that oxygen is tightly bound to the heme through hydrogen bonds with TyrB10 and GlnE7, and that the heme group is buried farther than in HbI. These findings led to the suggestion that the combined effects of the hydrogen bonding interactions involving TyrB10 and GlnE7, and the localization of the heme pocket, confer stability to the oxy-HbII complex and prevent oxidation of the ferrous iron center [9]. This explains in part the capability of HbII to selectively bind oxygen and at the same time, its inability to coordinated H₂S. This ligand discrimination process facilitates the relationship between the mollusk and its endosymbiont. Nonetheless, to prove this hypothesis and to define the role of the heme pocket environment in the ligand discrimination events, mutations of the HbII active site residues are required. Moreover, mutation of TyrB10 and GlnE7 pair in the HbII heme pocket can provide a better understanding about the role of these residues in other hemoproteins such as the truncated hemoglobins (trHbs). These hemoglobins are found in unicellular organisms as well as higher plants and, as in HbII, they contain TyrB10 and GlnE7 in their heme active site [7]. Similar to HbII, their extremely high O₂ affinity has been attributed to a strong hydrogen bond network between GlnE7, TyrB10 and the bound ligand. On this basis, it has been suggested that the unique structural features of these trHbs preclude O₂ transport as a major function, and that they may be involved in NO reactions [7]. Interestingly, we have reported recently that HbII shows little reactivity toward NO [5], suggesting that besides the GlnE7 and TyrB10 motif, other factors may contribute to ligand discrimination in both HbII and trHbs. Therefore,

engineering the HbII distal pocket can not only provide relevant information about ligand selection in *Lucina pectinata* hemoglobins but in other trHbs as well.

Herein, we report for the first time the expression of the recombinant HbII (rHbII) as a first step toward the production of HbII mutants. The protein was successfully expressed in large quantities by developing a controlled fermentation process. The highest cell densities were obtained when Terrific Broth (TB) medium was enriched with 50 mM of glycerol and 2 mM of magnesium sulfate (MgSO₄), glucose was used as an alternate carbon source and the dissolved oxygen (dO₂) within the culture was maintained at 35%. The structural and functional characteristics of the recombinant HbII are also presented here and the results indicate that the protein thus obtained exhibit similar properties to those of its native counterpart. In addition, the fermentation protocol was tested and validated with rHbI and several HbI mutants, indicating that the method can also be extended to other hemoglobins. The improved methodology shows that there is an 80% enhancement in cell culture density, which directly results in a significant increase in the concentration of the hemeproteins obtained during the process.

2 Materials and Methods

2.1 Construction and Cloning

The cDNA of HbII was cloned into the pET-28a(+) vector (Novagen) using the procedure described for rHbI [14]. Briefly, the gene coding for HbII was obtained and amplified from the total *L. pectinata* gill RNA by RT-PCR [26]. The amplified HbII cDNA product was cloned into the pET-28a(+) vector using the *Hind*III and the *Xho*I restriction sites of the vectors polylinker region. The resulting pET-28a(+) vector, containing HbII cDNA, was further transformed into competent BLi5 or BL21 *E. coli* cells. Recombinant colonies of transformed cells (BLi5 or BL21) were selected in media containing kanamycin (70 µg/mL) and chloramphenicol (30 µg/mL) (Sigma, St. Louis, MO) and stored as frozen stocks in sterilized glycerol (Sigma, St. Louis, MO) at -80 °C. These frozen stocks were then used for cell growth and rHbII expression. As previously reported, a similar procedure was used to construct and clone HbI cDNA into the pET-28a(+) vector [14]. However, the restriction site *Nde*I was used instead of *Hind*III. Similarly, the HbI PheE11Val, GlnE7His and PheB10Val mutants were obtained by introducing single amino acid substitutions into the HbI coding region, using the Quick Change Site-directed mutagenesis kit (Stratagene, La Jolla, CA), and cloned into the pET28(a+) vector. As in HbII, the expression constructs were transformed in *E. coli* cells and stored until used.

2.2 Expression in Fernbach Flasks

The expression of rHbII was evaluated first in Fernbach flasks following the protocol used for rHbI and the HbI mutants [14]. First, 100 mL of TB (Sigma, St. Louis, MO) was inoculated with a frozen stock of the *E. coli* cells transformed with the rHbII plasmid, and incubated overnight on a shaker at 125 rpm with 70 µg/mL kanamycin and 30 µg/mL chloramphenicol at 37 °C. This overnight culture was then used to inoculate 900 mL of fresh TB medium with the corresponding antibiotics. The new culture was grown and induced with 1 mM IPTG, followed by supplementation with 1% w/v glucose and 30 µg/mL hemin chloride (Sigma, St. Louis, MO). To determine the optimal conditions for the expression, different induction times, expression temperatures (25, 30 or 37 °C) and *E. coli* strains (BL21 or BLi5), were examined. The experiments at different induction times were carried out first by adding IPTG to either BL21 or BLi5 cells during the beginning of the log phase (optical density, OD₆₀₀ of ~1.0) or later in this stage (OD₆₀₀ between 1.5 and 2). The best induction time was then used in both strains to evaluate the effect of temperature after the addition of IPTG on the expression of the

recombinant protein. In all cases, the cells were harvested in the stationary phase by centrifugation at 4,000 rpm for 20 min at 4 °C and stored until further protein purification.

2.3 Large-Scale Expression

The growth of the transformed *E. coli* cells to high culture densities was performed using a 5-Liter BioFlo 110 Modular Benchtop Fermentator (New Brunswick, Edison, NJ). The protocol was tested and optimized for the *E. coli* cells transformed with HbII cDNA, applying the best expression conditions obtained in the Fernbach flask experiments. Prior to the fermentation process, a flask containing 50 mL of Luria–Bertani (LB) medium (Sigma, St. Louis, MO) was inoculated with a frozen stock of rHbII and incubated overnight with 70 µg/mL kanamycin and 30 µg/mL chloramphenicol at 37 °C on a shaker at 125 rpm. This preculture was then used to inoculate a 2.5 L flask containing 500 mL of fresh LB medium and grown overnight under similar conditions. The fermentation process was initiated by adding this second overnight culture to 4.5 L of TB media supplemented with 30 µg/mL of chloramphenicol, 70 µg/mL of kanamycin, and 100 µL/L of antifoam agent (Sigma, St. Louis, MO). Recombinant protein expression was induced by adding 1 mM of IPTG in the mid-log phase (OD₆₀₀, of 1.5–2.0) along with glucose (1%) and hemin chloride (30 µg/mL). The temperature was maintained at 37 °C before induction and at 30 °C after the addition of IPTG. The culture pH was controlled at 7.0 throughout the process using 1.5 M KH₂PO₄ and 30% NH₄OH (Sigma, St. Louis, MO).

To increase the population of transformed *E. coli* cells, oxygen supply was analyzed and regulated first by evaluating the optimum dO₂ content within the cell culture. In this regard, a dO₂ content of 100% was initially investigated by supplying the gas constantly and setting the agitation at 350 rpm. Then, a concentration of dO₂ of 35% was tested using a cascade from 300 to 700 rpm and increasing the agitation as needed.

Growth of the cells was also evaluated by increasing the concentration of glycerol and MgSO₄ (Sigma, MO) in the TB medium (TB + Glycerol + MgSO₄) and by controlling glucose feeding during the bacterial growth. In this respect, expression of rHbII in this enriched media was induced as before, with the exception that glucose feeding was set to respond to dO₂ level.

The optimized fermentation protocol was then applied to the recombinant HbI protein and the HbI mutants. Similar to the Fernbach expression, the cells were harvested in the stationary phase by centrifugation for 20 min at 4 °C.

2.4 Purification of the Hemoglobins

In both processes (Fernbach and fermentation), the cells containing either rHbII, rHbI or the HbI mutants were lysed and purified by metal affinity chromatography using an AKTA FPLC system (GE Healthcare, Piscataway, NJ). Initially, the bacterial pellets, obtained by centrifugation, were weighed and resuspended in 2.6 mL of buffer/per g of bacteria (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 68 mM NaCl, pH 7.5). One milligram of lysozyme (Sigma, St. Louis, MO) per gram of bacteria and one tablet of protease inhibitor cocktail (Roche Applied Science) per 50 mL of buffer were then added to the resuspended cells. The mixture was incubated on ice for 1 h, sonicated and homogenized for 1 min several times until a liquefied solution was obtained. To separate the soluble proteins from the bacterial pellets the mixture was centrifuged at 4 °C, 15,000 rpm for 1 h. To purify the proteins, an automated FPLC method based on His-tagged affinity chromatography, with a step elution profile, was used. As a first step, an FPLC high performance column (Tricorn, GE Healthcare, Piscataway, NJ) was packed with 10 mL of cobalt resin (Talon, Clontech, Palo Alto, CA) and equilibrated with 10 bed volume (BV) of the equilibrating buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.5) at a flow rate of 4.0 mL/min. Approximately 20 mL of the soluble proteins were loaded into the column. Unbound proteins were eluted first with two column volume (CV) of the equilibrating buffer.

Subsequently, the resin was washed with 10 CV of buffer containing 50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole at pH 7.0, to remove weakly bound and unwanted proteins. The desired protein was then eluted using the step elution profile with 10 CV of 50 mM NaH_2PO_4 , 300 mM NaCl, and 150 mM imidazole and automatically collected in a fraction collector. The fractions containing rHbII were identified in the chromatogram, which showed two peaks representing unwanted samples and the purified protein. To eliminate the excess of imidazole, an AMICON ultra-filtration cell with an YM-10 membrane was employed. The proteins were stored in phosphate buffer, pH7, at -80°C until further characterization. During the purification process, aliquots of each step were stored for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE on 10% acrylamide gel) analysis, to verify the purity of the proteins. Protein concentrations were estimated using the Bradford method [2] or by measuring their optical absorption spectra.

2.5 Spectral Properties and Kinetic Measurements of rHbII

The spectral properties of the purified rHbII, deoxy rHbII, rHbII- O_2 and metaquo-rHbII derivatives were evaluated by analyzing their UV–Vis spectra using Agilent 8453 spectrophotometer. The deoxy-rHbII complex was obtained by adding a slight excess of sodium dithionite (50-fold) to a solution of deoxygenated rHbII under anaerobic conditions. Likewise, the active oxy form of rHbII was obtained by exposing the deoxy rHbII sample to an O_2 atmosphere. On the other hand, the metaquo-rHbII derivative was formed by titrating the rHbII- O_2 complex (3.40 μM , pH 7.5) with a 10% excess of potassium ferricyanide and by decreasing the pH from 7.5 to 5.0. The oxygen association rate constants, k_{on} , for the recombinant HbII and the native HbII protein (nHbII) were measured using a rapid scanning monochromator with a stopped-flow apparatus (Olis-Online Instrument Systems, Bogart, GA). The reactions were followed under pseudo-first order conditions ($[\text{O}_2] \gg [\text{HbII}]$) in which deoxygenated solutions of HbII (15 μM of either nHbII or rHbII) were mixed rapidly with solutions of O_2 at different concentrations ranging from 0.01 to 0.33 mM. The reactions of the deoxygenated HbII with the O_2 solutions were monitored at 414 nm, the maximum absorption in the static, UV–Vis spectra of the HbII- O_2 complex.

The O_2 dissociation constants, k_{off} , for rHbII and nHbII were determined by monitoring the decay of the HbII- O_2 absorption signal at 414 nm and the spectra associated with these decays, using a UV–Vis spectrophotometer (Agilent 8453). Small volumes (100 μL) of the HbII- O_2 proteins (5 μM) were mixed rapidly with large volumes of deoxygenated sodium dithionite solutions (2 mL) and the kinetic traces were acquired for a period of 1 h.

Similarly, the auto-oxidation constants, k_{ox} , were determined by following the decay of the HbII- O_2 absorption signal at 414 nm. The spectra associated with these kinetics were also recorded. Briefly, 5 μL of HbII- O_2 samples (0.2 mM based on heme) was mixed with 2 mL of degassed and deoxygenated phosphate buffer and the changes associated with the auto-oxidation process were monitored during 24 h.

3 Results

3.1 Expression of rHbII

The first approach to produce the recombinant HbII protein at high levels was to determine the optimal expression conditions by evaluating induction time and temperature as well as *E. coli* strains. Table 1 and Fig. 2 show the effect of induction time on rHbII expression. In Fig. 2, lane 1 of the 10% polyacrylamide gel contains the broad range SDS–PAGE standards, and lanes 2–5 show the purified rHbII samples induced at different times. Accordingly, lanes 2 and 3 show the purified rHbII induced at an OD_{600} of ~ 1 , while lanes 4 and 5 contain the purified samples induced at an OD_{600} 1.5–2.0. Inspection of Fig. 2 and Table 1 clearly demonstrate

that the highest yield in both strains was obtained when the culture was induced in the mid-log phase at an OD_{600} of 1.5–2.0 (lanes 4, 5). The amount of protein achieved under this condition was 117 mg/L for the BL21 strain and 135 mg/L for the BLi5 cells, respectively. Table 1 also summarizes the effect of induction temperature on rHbII production. In this scenario, the protein was induced in the mid-log phase and different expression temperatures were tested. Under this circumstance the highest level was attained when the temperature was reduced to 30 °C during the induction period, showing protein concentrations of 130 and 155 mg/L for the BL21 and BLi5 cells, respectively. The data also demonstrate that in both sets of experiments (induction time and temperature) the BLi5 strain produced more protein than the BL21 cells. In general, the results show the feasibility of expressing high levels of rHbII by inducing the BLi5 cells in the mid-log phase and by decreasing the temperature from 37 to 30 °C during this process. To increase the production of rHbII, these conditions were therefore maintained during the development of the fermentation protocol while different oxygen supply and media formulation were evaluated.

3.2 Optimization of the Fermentation Protocol for rHbII, rHbI and HbI Mutants

In all aerobic fermentations, maintaining an adequate oxygen supply is not only important to ensure sufficient O_2 transfer, but it is also fundamental when the ultimate goal is to obtain high cell densities and large quantities of the recombinant product [8,15]. Hence, to determine the optimal condition of O_2 supply, the bacterial growth in pure TB medium was evaluated by adding O_2 to maintain a concentration of dO_2 at 100% at 350 rpm, and by using an agitation cascade to control the content of dO_2 at 35%. As Fig. 3 shows, the density of the BLi5 cells transformed with rHbII increased from an OD_{600} of ~12–18 when the dissolved oxygen was maintained at 35% with an agitation cascade of 300–700 rpm. This implies that increasing the agitation, instead of the amount of dO_2 , improves O_2 transfer and consumption throughout the medium resulting in turn in higher cell culture density.

The medium formulation and carbon feeding conditions within the cell culture were then analyzed using this optimal O_2 supply. Figure 4 shows the fermentation profiles when pure TB was used and glucose was supplemented during the induction period, and when the TB medium was fortified (TB + Glycerol + $MgSO_4$) and glucose was added in response to the dO_2 level. As Fig. 4 clearly demonstrates, there was a substantial increase in the OD_{600} , from ~18 to 60, when the medium was modified with 50 mM of glycerol and 2 mM of $MgSO_4$, and glucose was added in response to O_2 consumption. Indeed, a 4.8-fold increase in the cell wet weight (from 114 to 553 g) was also obtained under these conditions (Table 2). Here glycerol was used as the carbon source in the initial phase of the bacterial growth. When a rise in the dO_2 was observed, which indicates reduction of O_2 consumption due to depletion of glycerol, glucose was then added. Increasing the amount of glycerol or $MgSO_4$ did not enhance any further the density of the cell culture. Nevertheless, adding these two nutrients to the TB media greatly improved the growth of the BLi5 cells transformed with the rHbII plasmid. High optical densities and cell wet weights were also obtained for the recombinant HbI protein and several HbI mutants using these optimized conditions, as shown in Fig. 4 (inset) and Table 2. In fact, Fig. 5 (lanes 6, 7) demonstrates that purification of the proteins using the one step, automated FPLC method yielded ~90% purified samples. In all cases the amount of protein expressed per gram of wet weight was ~1,500 to 2,300 mg/L, indicating that the increase in protein production was mainly due to the increase in cell density.

3.3 Functional Properties of rHbII

The identity of the expressed rHbII was evaluated first by measuring the UV–Vis absorption spectrum of the purified protein. Figure 6a shows that the spectrum of the recombinant protein is characterized by a Soret band at 414 nm and Q bands at 540 nm and 576 nm, which correspond to the low spin HbII- O_2 form of the native protein [12]. To corroborate that the

protein is expressed in the oxy form, rHbII was reduced with dithionite and exposed to an O₂ atmosphere. As Fig. 6a demonstrates, reduction of the protein with dithionite produces a deoxy-like species with characteristics Soret and Q bands at 432 and 556 nm, respectively. Exposure of this deoxy rHbII complex to O₂ induces displacement of the Soret band to 414 nm and the appearance of new Q bands at 540 and 576 nm, indicating that indeed, the recombinant protein is expressed in the oxy form and that the native HbII-O₂ and rHbII-O₂ complexes have identical absorption spectra. The optical properties of the oxidized rHbII were also evaluated by analyzing the absorption spectrum of the metaquo derivative. Figure 6b shows that titration of the purified rHbII with potassium ferricyanide did not induced full oxidation of the protein. In fact, similar to nHbII [9], to oxidize the rHbII heme center a decrease in pH from 7.5 to 5 was required. Taken together, these results suggest that both, nHbII and rHbII have similar heme electronic and distal structural properties.

To define the reactivity of rHbII with its natural ligand, O₂ bimolecular binding and dissociation kinetics were further evaluated and compared to the native HbII protein. Figure 7 shows the microsecond bimolecular time courses for O₂ binding to rHbII at different ligand concentrations. The traces followed simple pseudo-first order kinetics and were therefore fitted with single exponential functions. The observed constants thus obtained, k_{obs} , were then plotted as a function of O₂ concentration to determine the association rate constant. The calculated k_{on} is very similar to that of the native HbII ($0.19 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in rHbII, and of $0.22 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for nHbII), indicating that the aspects influencing O₂ binding are the same in both proteins.

Figure 8a show the O₂ dissociation traces obtained by monitoring the decay of the rHbII-O₂ and nHbII-O₂ complexes at 414 nm. Fitting these traces with single-exponential functions yielded calculated dissociation constants of 0.053 s^{-1} for rHbII and 0.056 s^{-1} for nHbII. Likewise, the measured auto-oxidation kinetic shown in Fig. 8b produced a k_{ox} of 0.03 h^{-1} , which is analogous to that of the native protein (0.01 h^{-1}) [5]. Overall, the results indicate that the recombinant HbII protein retains the functional and structural properties of its native counterpart and that it can be produced in large quantities.

4 Discussion

4.1 Over-Expression of rHbII, rHbI and HbI Mutants

With the recent advances in DNA technology, the structural factors regulating the function of many hemeproteins have been elucidated. For instance, the development of a large number of site-directed mutants has helped to build a detailed knowledge about the structural factors that regulate O₂ affinity and ligand discrimination in myoglobin and hemoglobin [4,6,16,17,23, 24]. Motivated by these advances, we have initiated a thorough investigation aimed at elucidating the mechanisms by which the hemoglobins from the clam *Lucina pectinata* control ligand selection. Indeed, using mutagenesis and spectroscopic approaches, we have defined in part the structural aspects controlling H₂S binding in the hemoglobin I from *L. pectinata* [18]. Here, we have extended these studies by successfully expressing the other hemoglobin (HbII) in large quantities as a first step toward the production of HbII mutants. As shown in this study, expression of rHbII in Fernbach flasks yielded up to 154 mg/mL of purified protein by transforming HbII cDNA into the BLi5 cells, inducing and supplementing the culture during the mid-log phase with 1 mM IPTG, 30 µg/mL hemin chloride and 1% glucose, and by decreasing the temperature to 30 °C after induction. In addition, the amount of rHbII was significantly increased by developing a controlled fermentation process using the above conditions as the foundation for the optimization of the large scale protocol. Moreover, the results presented here show that cell culture density and rHbII production was greatly enhanced during the fermentation process by simply modifying the culture medium and controlling the growth conditions of the *E. coli* cells, without using laborious and expensive molecular biology

techniques. Overall, cell growth was improved by 80% when glycerol was used as the initial carbon source, MgSO_4 was added to the culture to reduce metabolic stress during induction with IPTG, the media was supplemented with glucose after exhaustion of glycerol and the dO_2 was maintained at 35%.

Supplementation of the culture with additional glycerol appears to fortify the nutrient-rich TB media by providing sufficient carbon source to the bacteria during the early phase of cell proliferation. Also, supplying more carbon source to the culture by adding glucose after the primary source was completely consumed (see Fig. 4) seems to increase further cell growth, thereby improving cell density and the amount of protein expressed. Furthermore, the combined effect of using glycerol at the beginning of the bacterial growth and glucose at a later stage of the process, may avoid large accumulation of acetate that can otherwise be extremely harmful to the cells [1,3,8,20]. Acetate, which inhibits cell growth, is formed as a major by-product when glucose is used as the limiting substrate during the fermentation process. Consequently, the results suggest that regulating the amount of carbon source added to the medium not only provides sufficient nutrients to the cells but it also minimizes acetate production, increasing in turn cell population and protein production.

Similarly, fortification of the media with additional MgSO_4 appears to improve cell growth and protein expression. Magnesium is an essential element involved in many metabolic reactions, including mRNA stabilization when *E. coli* cells undergo metabolic stress and it is thus introduced in many commercially available media in the form of yeast extract [11,25]. However, it has been found that even in these media, the amount of Mg^{+2} is not enough to assure maximum cell growth. Therefore, these results reiterate the need of increasing the amount of MgSO_4 in commercially available, nutrient-rich media to ensure high cell culture density.

In general, the optimized protocol involves the use of a modified media along with a controlled carbon feeding procedure based on the amount of dissolved oxygen within the culture. This fermentation process was successfully applied to rHbI and other HbI mutants, indicating that the method can also be extended to other hemoglobins. This study also shows that the recombinant HbII protein obtained with this fermentation protocol is functional and structurally analogous to the native protein.

The presence of an oxy derivative in the expressed rHbII protein was the first evidence suggesting that similar to nHbII, the recombinant protein has the ability to bind O_2 tightly in the ferrous iron center. This was further confirmed by the kinetic analyses, which showed that both, recombinant and native proteins have high and similar affinity constants ($3.58 \times 10^6 \text{ M}^{-1}$ for rHbII and $3.93 \times 10^6 \text{ M}^{-1}$ for nHbII), indicating that rHbII maintains the oxygen binding properties of its native counterpart. In addition, the fact that to fully oxidize rHbII required a decrease in pH, indicates that, as in nHbII, the recombinant protein is resistant to oxidation of the iron heme moiety due to the architecture of the heme distal environment. Regarding this, previous results have demonstrated that the orientation of TyrB10, reduces the heme cavity of HbII and the HbI PheB10Tyr mutant and that this reduction may help to prevent oxidation of the ferrous iron center by impeding the access of external water molecules into the distal environment [9]. Hence, this finding along with the slow auto-oxidation kinetics observed here strongly suggests that architecture of the rHbII heme distal environment is similar to that of the nHbII and that mutations of the rHbII distal residues can provide relevant information about ligand discrimination in *Lucina pectinata* as well as other organisms. This work also set the basis for the development of rHbII site-directed mutants to further evaluate its use as a potential artificial O_2 carrier.

In this respect, the development of an ideal hemoglobin based-oxygen carrier has been hampered in part by the inability of many proteins to carry oxygen due to spontaneous auto-oxidation, generation of radical oxygen species, which leads to heme degradation and high NO reactivity [5,10]. Although some of these issues have been addressed by site-directed mutagenesis, heme auto-oxidation and degradation appear to be the major challenges. Nonetheless, we have recently shown that nHbII has low reactivity toward NO and that it oxidizes very slowly in air [5]. Additionally, interaction of nHbII with radical oxygen species (i.e., H₂O₂) shows that this hemoglobin is extremely resistant to heme degradation. Like nHbII, the results presented here show that the recombinant protein has a low auto-oxidation rate, thus making rHbII an excellent prototype for an artificial O₂ carrier.

Abbreviations

HbI, HbII, and HbIII	Hemoglobins I, II and III
rHbII	Recombinant HbII
nHbII	Native HbII
<i>L. pectinata</i>	<i>Lucina pectinata</i>
IPTG	Isopropyl β-D-1-thiogalactopyranoside
k_{on}	Association rate constant
k_{off}	Dissociation rate constant
Gln	Glutamine
Phe	Phenylalanine
Tyr	Tyrosine
RT-PCR	Reverse transcription polymerase chain reaction
TB	Terrific broth
<i>E. coli</i>	<i>Escherichia coli</i>
cDNA	Complementary DNA
OD	Optical density
dO ₂	Dissolved oxygen

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Fig. 1. Orientation of GlnE7 and TryB10 with respect to bound O₂ in the heme pocket of HbII from *L. pectinata*. PDB: 20LP, PyMOL (DeLano Scientific LLC)

Fig. 2. SDS-PAGE analysis of rHbII expression at different induction time. *Lane 1* molecular weight markers; *lanes 2–3* purified rHbII induced at an OD_{600} of ~ 1 ; and *lanes 4–5* purified rHbII induced at an OD_{600} 1.4–2.0

Fig. 3. Density of the *E. coli* BLi5 cells using different dissolved O₂ concentrations within the culture media. TB was used as a media and protein expression was induced with IPTG at an OD₆₀₀ of 1.5–2.0 and supplemented glucose and hemin chloride

Fig. 4. Growth of the *E. coli* BLi5 cells using TB versus TB + Glycerol + MgSO₄ (fortified TB). Expression of proteins in the enriched media was induced as before, with the exception that glucose feeding was set to respond to dissolved O₂ level

Fig. 5.

Analysis of the purified rHbII by SDS-PAGE on 10% polyacrilamide gel after Coomassie blue staining. rHbII purification was performed using a one step, automated FLPC method based on metal affinity chromatography. *Lanes 1 and 8* molecular weight markers; *lane 2* crude lysate; *lane 3* unbound proteins eluted with equilibrating buffer; *lane 4* weakly bound proteins eluted during the wash step; *lane 5* fractions collected before rHbII elution and; *lanes 6 and 7* rHbII after purification

Fig. 6.

a UV-Vis spectra of the purified rHbII and the deoxy rHbII complex. **b** Oxidation of rHbII with potassium ferricyanide. *Solid line*, purified rHbII; *dashed line*, rHbII after addition of 50 μ M potassium ferricyanide, pH 7; *dotted line*, rHbII after addition of 50 μ M potassium ferricyanide, pH 5

Fig. 7.

Association kinetics of rHbII with oxygen using different ligand concentrations. The traces were collected at 414 nm, the maximum absorption in the static UV–Vis spectra of the HbII-O₂ complex

Fig. 8.
a Oxygen dissociation kinetic traces of rHbII and nHbII. **b** Time course associated with rHbII auto-oxidation. Both kinetics were monitored at 414 nm

Table 1

Yield of rHbII in the Fernbach flasks experiments using different induction times and temperature as well as *E. coli* strains

Conditions	Protein yield (mg/L)	
	BL21	BLi5
Induction time		
OD ₆₀₀ of ~1.0	34	42
OD ₆₀₀ of 1.5–2.0	117	135
Induction temperature		
25 °C	18	19
30 °C	130	155
37 °C	102	104

Table 2

Cells densities and weights, grown in the fermentor with different media

Protein	OD ₆₀₀		Cell wet weight (g)	
	Grown in TB ^a	Grown in fortified TB ^b	Grown in TB ^a	Grown in fortified TB ^b
rHbII	18	60	114	445
rHbI	20	59	116	553
HbI-PheE11Val	20	60	152	585
HbI-GlnE7His	12	55	95	474

^aTB media used as purchased^bTB media fortified 50 mM of glycerol and 2 mM of MgSO₄