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Optimization of Hnox Protein Production in *Escherichia Coli*

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Abstract: Hemeproteins carry a variety of different functions in organisms ranging from steroid biosynthesis to respiration, signaling to drug metabolism. In industry, hemeproteins are used for production of drugs such as: pravastatin for lowering cholesterol, progesterone for hormonal treatment of cancers of uterus and cervix, and cortisone used against allergy and inflammation. Hemeproteins can also be used in drug development and biological remediation. The industrial applications of hemeproteins will expand with development of molecular biology and protein design techniques. One of the obstacles to the widespread use of hemeproteins is difficulties in production of high levels of heme bound protein. This study aims to maximize the amount of heme cofactor bound hemeprotein produced. Here, three important factors affecting hemeprotein production in bacteria are examined: induction by isopropyl β-D-1-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (ALA), precursor for heme biosynthesis, and expression temperature. Effects of these factors on production of thermophilic hemeprotein *Tt*HNOX are investigated. Since ALA is an expensive molecule, optimization of the amount of ALA used is important. The most suitable conditions to produce *Tt*HNOX is at low temperature, 0.5 mM IPTG and 1 mM ALA. This study concludes that ALA concentration and expression temperature are important in production of heme bound hemeproteins.

***Keywords*:** Hemeproteins, protein expression, heme biosynthesis, δ-aminolevulinic acid.

HNOK Proteinlerinin *Escherichia Coli*'de Üretiminin Optimizasyonu

Özet: Hemoproteinler canlılarda steroid biyosentezinden solunuma, sinyalizasyondan ilaç metabolizmasına kadar pek çok farklı biyolojik süreçlerde önemli görevler üstlenirler. Endüstride, hemoproteinler kolesterol düşürücü pravastatin, rahim ve rahim ağzı kanserlerinin hormonal tedavisinde kullanılan progesteron, alerji ve yangı'ya karşı kullanılan kortizon gibi ilaçların üretiminde kullanılmaktadır. Bunların yanı sıra hemoproteinlerin ilaç geliştirilmesi, biyolojik iyileştirme gibi alanlarda kullanılması da planlanmaktadır. Moleküler biyoloji ve protein tasarımı tekniklerinin gelişmesi ile bu proteinlerin endüstriyel uygulama alanları da genişleyecektir. Hemoproteinlerin bu alanlarda yaygın kullanımı karşısındaki en önemli engellerden biri Hem kofaktörüne bağlı bir şekilde yüksek miktarda hemoprotein üretilememesidir. Bu çalışmada Hem kofaktörüne bağlı bir şekilde üretilen hemoprotein miktarını en yüksek seviyeye getirebilecek koşullar araştırılmıştır. Bu çalışma kapsamında bakteride hemoprotein üretimini etkileyen en önemli üç etken olan indükleyici izopropil β-D-l-tiyogalaktopiranosid (IPTG), ve Hem öncül molekülü δ-aminolevülinik asit (ALA), ve ekspresyon sıcaklığı incelenmiştir. Bu etkenlerin termofilik hemoprotein *Tt*HNOK üretimine etkisi araştırılmıştır. Özellikle, ALA pahalı bir molekül olduğu için hemoproteinlerin üretiminde kullanılan ALA miktarının optimizasyonu önemlidir. Bu çalışma sonucunda *Escherichia coli* bakterisinde Hem kofaktörüne bağlı TtHNOK proteinin üretimi için en uygun koşulların düşük sıcaklık, 0,5 mM IPTG ve 1 mM ALA olduğu gösterilmiştir. Bu çalışmada çıkan sonuçlar Hem kofaktörüne bağlı hemoprotein üretiminde ALA konsantrasyonun ve ekspresyon sıcaklığının önemli olduğunu göstermiştir.

Anahtar Kelimeler: Hemoproteinler, protein ekspresyonu, hem biyosentezi, δ-aminolevülinik asit.

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1. INTRODUCTION

The heme cofactor is a porphyrin molecule that contains an iron ion which coordinates four nitrogen atoms (Figure 1). The proteins bound to the heme cofactor are called hemeproteins. The most commonly known hemeproteins are oxygen-carrying hemoglobin and myoglobin. Hemeproteins play a variety of roles in living organisms ranging from steroid biosynthesis to respiration, signaling to drug metabolism. The fact that the same heme cofactor can carry such different functions in nature arises from the rich chemistry of the cofactor. The chemical versatility of the heme cofactor makes hemeproteins an attractive target for protein design [1].

The development of molecular biology and protein design techniques, have led to an increase in the industrial applications of hemeproteins. One such example is utilization of cytochrome P450 (P450) oxygenases which use the heme cofactor to catalyze oxidation of hydrocarbons with high efficiency and selectivity. Indeed, P450s are currently used in pharmaceutical industry in the synthesis of societally important drugs such as progesterone used in treatment of uterine and cervical cancer, cortisone used in treatment of allergy and inflammation, and pravastatin used for lowering blood cholesterol [2-4]. In addition, current studies are underway for utilization of hemeproteins in the synthesis and development of other novel drugs and biological remediation [5]. These include synthesis of artemisinin in the treatment of malaria, paclitaxel in the treatment of cancer, and many other medicines such as testosterone, the important steroid hormone [6], [7]. The potential applications of hemeproteins are not limited to drug production. These proteins can also be used in biosensor applications, biofuel cells and electrochemical immunoassays [8-10].

For heme proteins binding to the heme cofactor is an absolute requirement for activity. However, under physiological conditions the free heme concentration in *Escherichia coli* is very low. Therefore, hemeproteins expressed in *E. coli* are usually expressed as heme free apo-proteins. Only proteins that have an extremely high affinity for the heme can be expressed with the heme cofactor bound, as holo-proteins, under physiological conditions [11, 12]. One of the most important problems that limits the widespread use of hemeproteins is difficulties in production of high levels of holo-hemeproteins. The goal of this study is to determine the conditions under which maximum amount of holo-protein can be produced for hemeproteins.

1.1. Heme Biosynthesis in *E. coli*

Cells must produce sufficient quantities of the heme cofactor and related compounds for the respiratory chain during normal growth. Heme synthesis in *E. coli* and similar bacteria occurs with a complex and branched pathway. As seen in Figure 1, the first step in the main pathway of heme biosynthesis is the formation of δ-aminolevulinic acid (ALA) from glutamyl-tRNA and glutamate [13]. Following this step, a porphobilinogen is formed from condensation of two ALA molecules. The head-to-tail condensation and deamination of four porphobilinogen molecules yields hydroxymethylbilane. Cyclization of hydroxymethylbilane yields uroporphyrinogen III. Uroporphyrinogen III can be used for the synthesis of various tetrapyrrole-based cofactors. For heme biosynthesis, uroporphyrinogen III is converted into protoporphyrin IX in three steps. Addition of ferrous (Fe^{2+}) iron to protoporphyrin IX results in formation of the heme cofactor (Figure 1).

In humans ALA is obtained from the combination of glycine and succinyl-CoA. ALA is synthesized from different reactions in mammals and bacteria. However, in all these organisms ALA is the key precursor molecule for the synthesis of heme. Therefore, ALA biosynthesis is tightly regulated by feedback inhibition, and ALA formation is the rate-determining step for heme biosynthesis [14]. Accumulation of heme and porphyrins in *E. coli* cells was observed by addition of ALA [15]. For this reason,

ALA is added to the media during expression of hemeproteins to increase the production of holo-proteins [14]. However, ALA is an expensive molecule (1 g about 290 €), so optimization of the amount of ALA used in the production of hemeproteins is important.

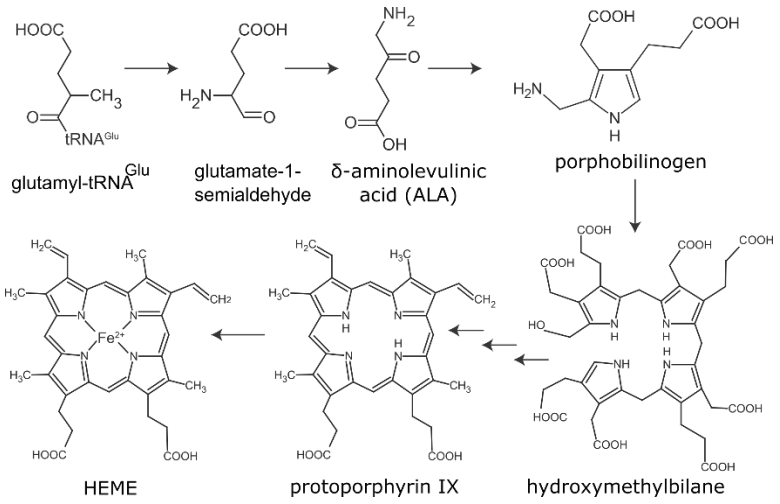

Figure 1. Main heme biosynthesis pathway in *E. coli.*

1.2. HNOX Proteins

Nitric oxide (NO) is a signaling molecule that plays important roles in the cardiovascular system, the nervous system and the immune system. NO performs these physiological tasks by binding to the heme cofactor found in the enzyme soluble receptor guanylate cyclase (sGC). Recently a family of hemeproteins homologous to the region containing the heme cofactor of sGC enzyme has been discovered [16]. Studies have shown that these proteins can bind nitric oxide (NO) and oxygen [17]. This family is called Heme-Nitric Oxide / Oxygen (HNOX) proteins because they can sense gas molecules such as NO and O_2 using the heme cofactor. They are prevalent in prokaryotes and higher eukaryotes. They play a large variety of roles in prokaryotes such as regulation of motility, biofilm formation, quorum sensing, and symbiosis [18]. HNOX proteins have a unique fold, and are all about 190 amino acids in length. In these proteins, the proximal ligand for the iron ion in the heme cofactor is histidine (His102) (Figure 2). The YSR (tyrosine, serine, arginine) motif, which plays a role in the binding of the cofactor, is found in all HNOX proteins [19]. Prokaryotic HNOX proteins obtained from obligatory anaerobes generally produce highly stable oxygen (O_2) complexes. These HNOX proteins are usually found as domains of methyl-accepting chemotaxis proteins. One of the most striking members of this family is the HNOX protein (*Tt*HNOX) (Figure 2) obtained from *Thermoanaerobacter tengcongensis* (*Tt*). *T. tengcongensis* is a bacterium that lives in an oxygen-free environment in the springs (ideal temperature 75°C), and is adapted to high temperatures [20]. Therefore, *Tt* proteins show resistance toward heat inactivation. Furthermore, studies have shown that *Tt*HNOX protein is resistant to mutations [21, 22]. Taken together, *Tt*HNOX proteins are an ideal building block for protein design for many industrial applications such as pharmaceutical applications and biocatalysis.

Although the use of the *Tt*HNOX protein is currently limited to laboratory studies, there are many potential applications. For example, *Tt*HNOX can be used for therapeutic purposes in carbon monoxide poisoning because it exhibits the ability to coordinate with gases similar to globulins

(such as CO, NO and O_2) [23]. It has also been shown that both *Tt*HNOX can also be used as an MRI contrast agent by substitution of the native heme with unnatural porphyrin groups [24]. Similarly, *Tt*HNOX has been developed for use in imaging applications by modification of the heme cofactor [25]. To be used in these applications, it is necessary to produce high amounts of *Tt*HNOX protein. The aim of this study is to examine the factors affecting the production of *Tt*HNOX in *E. coli* and determine the optimal conditions to obtain maximum yield for the holo-protein. This study will also shed light on the factors that should be considered in the production of hemeproteins as *Tt*HNOX can be used as a model hemeprotein.

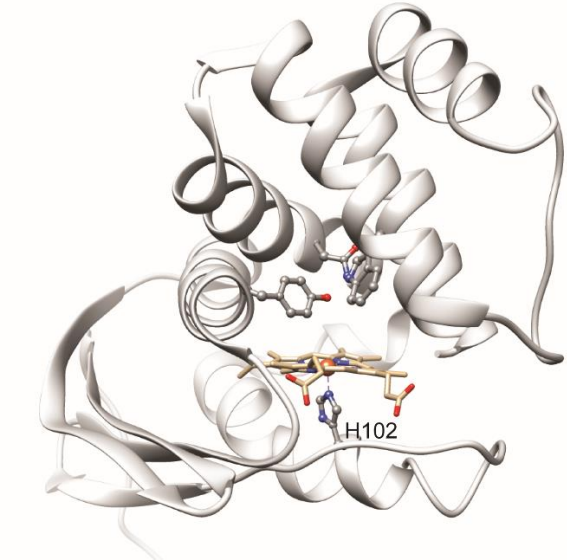

Figure 2. *Tt*HNOX crystal structure with histidine (H102) ligating the heme cofactor (PDB number: 1U55).

2. MATERIALS AND METHODS

2.1. Materials

Isopropyl β-D-1-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (ALA) and sodium chloride were obtained from Sigma Aldrich; α-toluenesulfonyl fluoride (PMSF) and Coomassie Brilliant Blue from VWR; glycerol, ampicillin and tryptone from Fisher Chemical; Triethanolamine (TEA), benzamidine hydrochloride and yeast extract were obtained from Merck. The purity level of all other chemicals used was analytical. The pET20b vector encoding the *Tt*HNOX protein was obtained from Prof. Dr. Michael M. Marletta's laboratory (University of California, Berkeley, U.S.A.). The sequence coding *Tt*HNOX protein was confirmed by sequence analysis using the T7 primer prior to the beginning of the study. This plasmid in pET20b vector was transformed into BL21 (DE3) *E. coli* by standard chemical methods and experiments were carried out using these recombinant bacteria.

2.2 *E. coli* Expression of *Tt*HNOX

Cell culture procedures were performed, as previously described, with some modifications [17, 26, 27]. Recombinant *E. coli* was cultured in 10 mL of culture in Terrific Broth media (12 g / L tryptone, 24 g / L yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) in a 50 mL sterile centrifuge tube and they were grown with 50 µg/mL ampicillin. Cell cultures were incubated at 37°C with shaking at 220 rpm until optical densities were OD600 = 0.8. After this point, the temperature of the cell cultures was lowered to the expression temperature, 25°C or 16°C. After incubation of the cultures for 30 minutes at this temperature, IPTG (0 to 1 mM final concentration) and ALA (0 to 1 mM, final concentration) were added to the cells at the same time. Cells were harvested by centrifugation 16-18 hours after addition of IPTG and ALA. The harvested cell pellet was frozen at -80°C.

2.3. Isolation of TtHNOX Protein

Isolation of *Tt*HNOX was performed as previously described with some modification [26]. The frozen cell pellet was dissolved in the buffer solution containing 50 mM TEA, 300 mM NaCl, 0.2 mM PMSF, and 1 mM benzamidine hydrochloride at pH 7.5. Cells were lysed in this solution by sonication. Following the disruption of the cells, the expression of the *Tt*HNOX protein was examined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE). For precipitation of unwanted *E. coli* proteins in the cell lysate, the cell lysates were incubated at 70°C for 40 minutes then protein aggregates were removed by centrifugation at 14,000 rpm for 30 minutes. After centrifugation, the supernatant was used. The proteins in the final solutions obtained

(*Tt*HNOX samples) were examined by SDS PAGE. The bands observed on the SDS PAGE were analyzed with the GelAnalyzer (www.gelanalyzer.com) program. The ratio of the *Tt*HNOX protein band to the other bands and the protein purity level were determined using this program.

2.4. UV-Vis Spectroscopy

All spectra were recorded using a UV-1600PC UV/Vis scanning spectrophotometer. *Tt*HNOX samples were diluted 10-fold in the buffer solution containing 50 mM TEA and 300 mM NaCl at pH 7.5 for spectroscopic measurements.

2.5. Determination of Total Protein Concentration

The total amount of protein in the *Tt*HNOX samples were determined using the standard Bradford assay [28]. Briefly, after addition of the Bradford reagent, the absorbance at 595 nm was determined by plate reader and the amount of protein was determined using bovine serum albumin standards.

2.6 Calculation of the Ratio for the Heme Bound *Tt*HNOX Obtained to Total Protein

The concentration (mg/mL) of the *Tt*HNOX protein bound to the heme cofactor was calculated using the Soret maximum absorbance at 400 nm with the extinction coefficient of 89 $mM \bullet cm^{-1}$ and molecular weight of 23077 g/mol protein [17]. For the determination of the heme binding ratio, this number is divided by the total amount of protein obtained from the Bradford assay described above (Equation 1).

$$\%heme\ bound\ TtHNOX = \frac{heme\ bound\ TtHNOX\ (mg/mL)}{total\ TtHNOX\ (mg/mL)} \times 100 \quad (1)$$

3. RESULTS

3.1. Expression of *Tt*HNOX Protein in *E. coli*

In this study, the effects of IPTG, ALA, and temperature on the expression of *Tt*HNOX protein in *E. coli* was investigated. For this purpose, after the cell cultures reached 0.8 optical density (at 600 nm), protein expression was induced with 0-1 mM IPTG. At the same time as IPTG, 0-1 mM ALA was added to induce heme biosynthesis. Cells were harvested after 16-18 h growth under these conditions. The harvested cells were frozen at -80°C and then lysed in the buffer solution using sonication. Following the disruption of the cells, the expression of the *Tt*HNOX protein was examined by SDS PAGE analysis (Figure 3). The molecular weight (MW) of *Tt*HNOX is 23 kDa so it can be observed on SDS PAGE at this MW (Figure 3). As shown in Figure 3, the expression of *Tt*HNOX protein is induced by addition of IPTG, and even at the lowest IPTG concentrations used induction of *Tt*HNOX expression was observed.

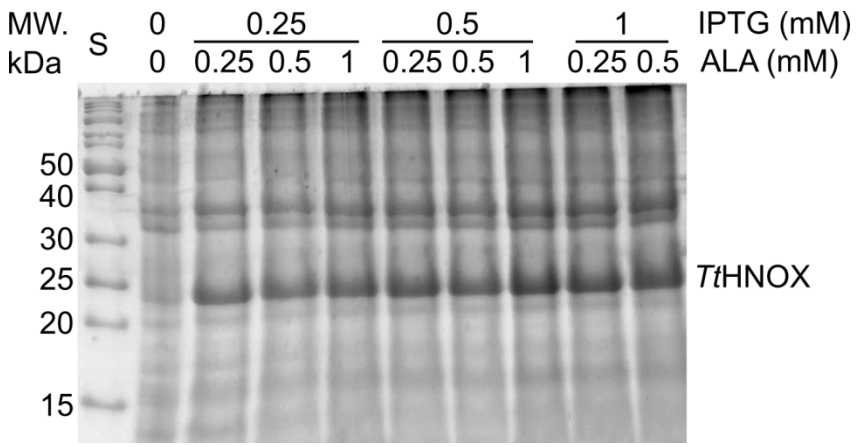

Figure 3. Expression of *Tt*HNOX protein in *E. coli* at 16°C at various IPTG and ALA concentrations.

3.2. Isolation of *Tt*HNOX Protein

The thermophilic nature of the *Tt*HNOX protein was used to easily separate it from other *E. coli* proteins. For this process, the lysate from the cells grown under different conditions were incubated at 70°C for 40 minutes. At this temperature, the *E. coli* proteins form aggregates while the *Tt*HNOX remains soluble. The *E. coli* proteins in the cell lysates were precipitated by centrifugation and experiments continued with the supernatant. Isolation of *Tt*HNOX was followed by SDS PAGE analysis which showed *Tt*HNOX protein was obtained in 86 % or higher purity (Figure 4).

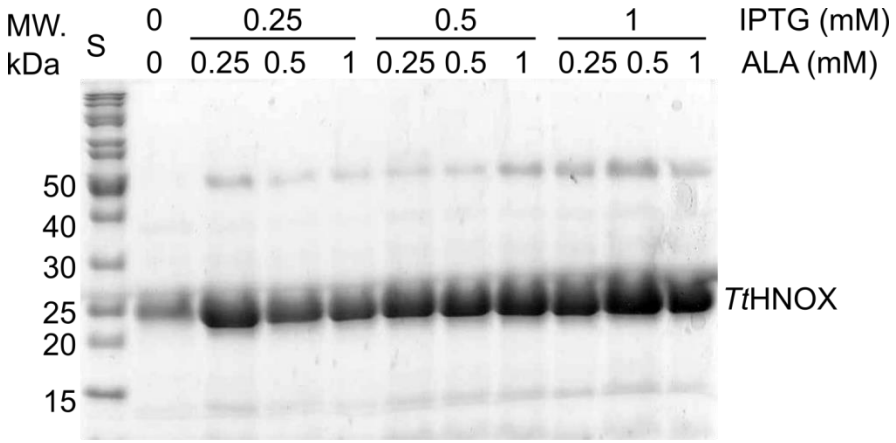

Figure 4. Purity of *Tt*HNOX protein produced at various IPTG and ALA concentrations in the supernatant after removal of aggregates by centrifugation.

3.3. Effects of IPTG and ALA on the Production of *Tt*HNOX

*Tt*HNOX protein expression was performed under various conditions as described above to determine the effect of IPTG and ALA on the total amount of *Tt*HNOX produced. The amount of total protein (both apo- and holo- *Tt*HNOX) in the supernatant after the precipitation of *E. coli* proteins were determined by the Bradford assay, on the lysates from cell cultures grown under various conditions as described above. As seen in figure 5, higher amounts of protein were obtained from cellular expression at 25°C compared to expression at 16°C. The amount of total protein obtained increased by increasing the concentration of IPTG at 25°C expression (Figure 5). Increased ALA concentration also affected the total protein content, although the effect was less substantial compared to the effect of IPTG at same temperature.

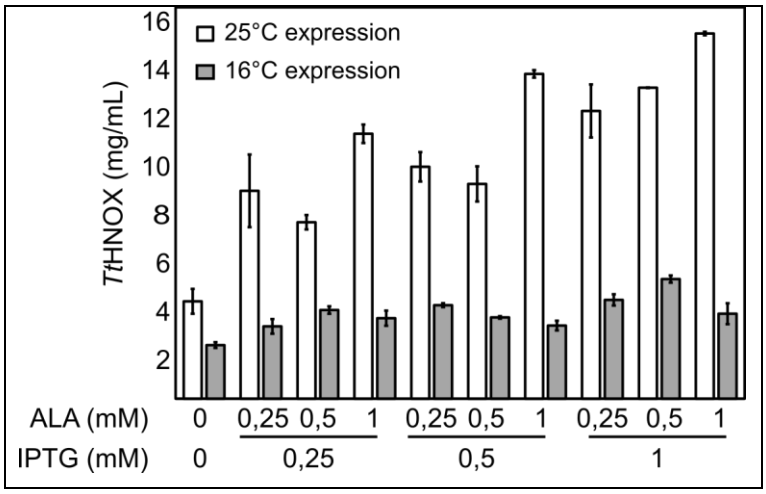

Figure 5. Effect of IPTG and ALA on the total amount of *Tt*HNOX protein produced in *E. coli* at different temperatures.

3.4. Effect of IPTG and ALA on the Production of Holo-*Tt*HNOX

Concentration of (heme bound) holo-*Tt*HNOX was determined using UV Visible spectrophotometer. The heme specific Soret absorbance of the *Tt*HNOX was observed at 400 nm (Figure 6). The amount of holo-*Tt*HNOX can be determined using the known extinction coefficient at this wavelength as described in the Materials and Methods section.

The effect of the inducer IPTG and heme precursor molecule ALA on the amount of holo-*Tt*HNOX produced in bacteria was determined by this method. As seen in figure 6A, increasing the ALA concentrations (0.25 - 1 mM) at 0.25 mM IPTG increases the amount of holo-*Tt*HNOX obtained. Similarly, increasing the concentration of IPTG (0.25 - 1 mM) at 0.25 mM ALA also increased the amount of holo-*Tt*HNOX produced (Figure 6B).

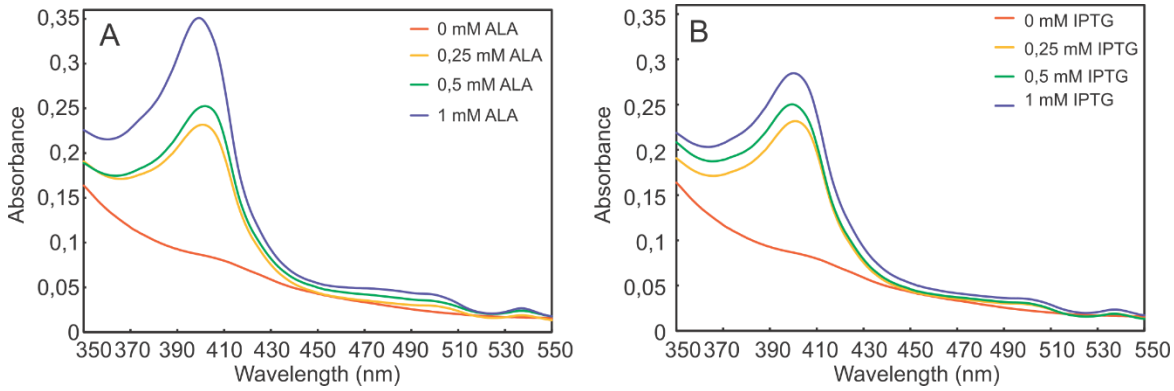

Figure 6. UV Visible spectra of *Tt*HNOX protein obtained at various IPTG and ALA concentrations (A: 0,25 mM IPTG, 0-1 mM ALA, B: 0,25 mM ALA, 0-1 mM IPTG).

3.5. Comparison of holo-*Tt*HNOX production at different temperatures

The amount of holo-*Tt*HNOX obtained under different conditions was determined using the Soret absorbance as described above. Using this method final holo-*Tt*HNOX concentration was determined under various expression conditions. As seen in Figure 7, the highest yields at both temperatures (25°C and 16°C) were obtained at 1 mM IPTG and 1 mM ALA concentrations. In addition, when expression is performed at 16°C ALA concentration has a more substantial effect on holo-*Tt*HNOX production compared to IPTG (Figure 7).

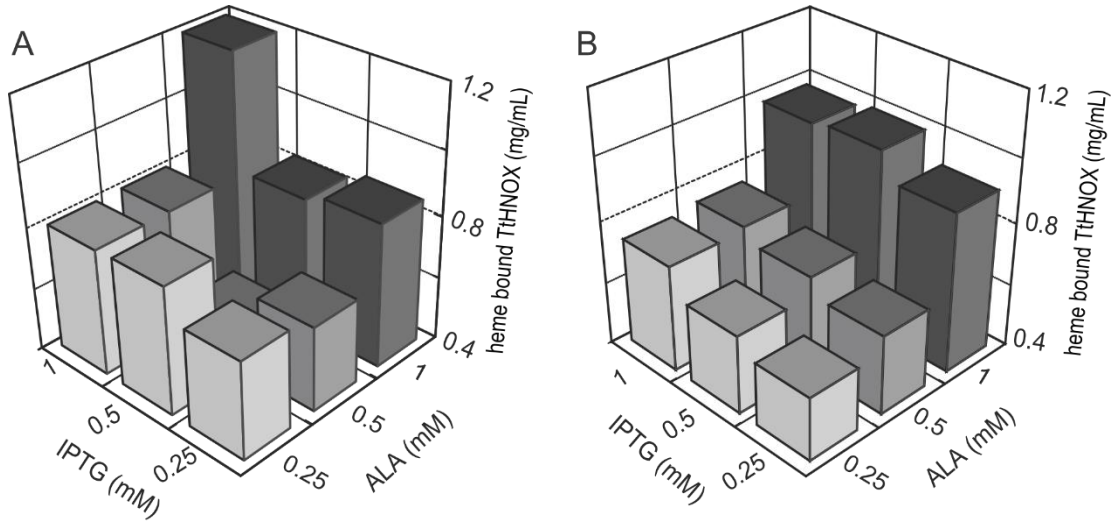

Figure 7. Effect of IPTG and ALA on holo-*Tt*HNOX obtained at different temperatures (A:25°C, B: 16°C expression).

3.6. The Ratio of Heme Bound *Tt*HNOX vs Total Protein Obtained

The percentage of heme binding in the resulting *Tt*HNOX protein obtained was calculated using equation 1, which is described in the Materials and Methods section. As seen in Figure 8, rate of the heme binding under expression at 25°C is very low compared to expression at 16°C. The percent ratio of heme binding after expression at 16°C, increased with increasing ALA concentrations (Figure 8B). As seen in Figure 8, the highest heme binding ratio was observed at 16 °C with 0.5 mM IPTG, and 1 mM ALA.

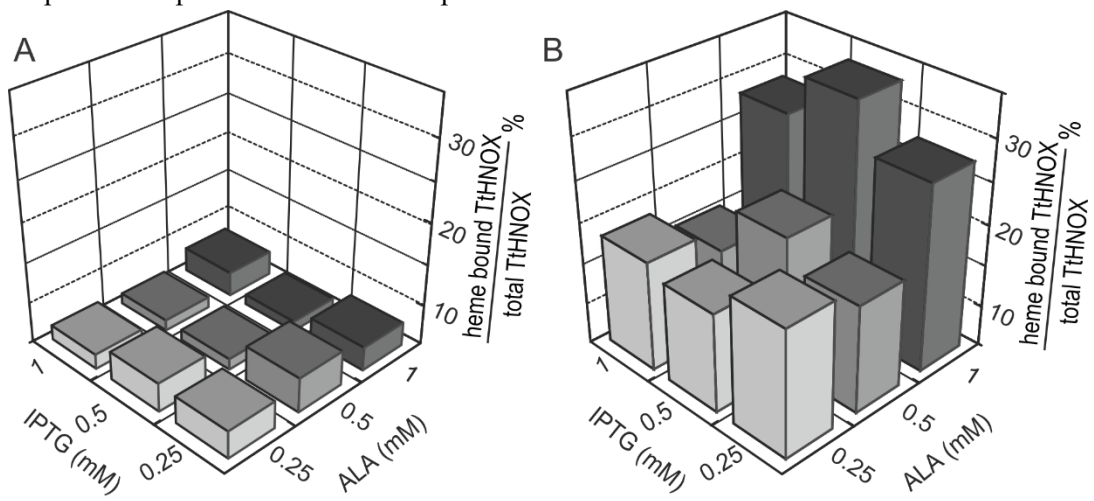

Figure 8. Effects of IPTG and ALA on the percent the heme bound *Tt*HNOX protein obtained at different temperatures. (A: 25°C, B: 16°C expression).

4. DISCUSSION

In this study, factors affecting bacterial production of hemeproteins were investigated on *Tt*HNOX protein, a thermophilic hemeprotein. Hemeproteins are widely found in nature and exhibit a variety of activities in biological systems. Hemeproteins carry many important functions in living organisms such as electron transfer (cytochrome c), oxygen binding and transport (hemoglobin), signaling (soluble guanylate cyclase), and catalysis [1]. With the development of molecular biology and protein design techniques, these proteins have found industrial applications. Hemeproteins can be used: as biocatalysts in drug production, in biological remediation, biosensor applications, and biofuel cells [29]–[33]. In order to increase the widespread use of hemeproteins in these areas, it is necessary to produce a high amounts of heme bound (holo-) hemeproteins [34], [35]. The aim of this study was to determine the optimum conditions to produce holo-hemeproteins. For this purpose, the three most important factors affecting the production of bacterial hemeprotein, inducer IPTG, heme precursor molecule ALA, and expression temperature were investigated.

The heme cofactor is crucial for the activity of hemeproteins. The total amount of protein expressed is easily monitored by standard SDS PAGE analysis; however, the heme cofactor binding ratio of a hemeprotein can only be measured after laborious isolation steps. The thermophilic property of the *Tt*HNOX facilitates the measurement of heme binding ratio. *Tt*HNOX is easily separated from *E. coli* proteins by incubation at + 70°C and the amount of protein bound to heme cofactor can be determined rapidly using UV visible spectroscopy.

This study shows that, in parallel with previous observations, it is important to use ALA at high concentrations for holo-protein production [14]. It was also observed that the temperature at which protein expression is performed is also important. As seen in Figures 5 and 7, higher amounts of protein are obtained at higher temperatures, but the rate of heme binding under this condition protein is very low. Instead, inactive apo-protein is obtained. As seen in Figure 8, at high ALA concentrations it is possible to obtain high amounts of holo-protein even at low IPTG levels.

Another method that can be used in the production of hemeproteins involves expression of these proteins in apo-form then reconstitution by addition of free heme [36]. In this case, proteins can be produced at high temperature and without addition of ALA. There are mainly two problems with this method. First, the proteins obtained by this method may not subsequently fold correctly around the heme cofactor which will lead to loss of activity [37], [38]. Secondly, many hemeproteins require the heme cofactor to properly fold and retain water solubility during translation. In the absence of heme, these apo-proteins form clustering inclusion bodies in the cell. Protein isolation from these inclusion bodies is much more laborious and costly than isolation of soluble proteins. Thus, the production of holo-hemeproteins is the most advantageous method.

Although the use of the *Tt*HNOX protein is currently limited to laboratory studies, there are in fact many potential applications of these proteins. For example, they can be used for treatment of carbon monoxide poisonings such as neuroglobin [23]. They can also be used as MRI contrast agents by replacement of the heme cofactors with unnatural porphyrins [24]. The production of *Tt*HNOX protein in high amounts is important for its use in these applications. At the end of these studies, the most promising condition for obtaining the highest amount of holo-*Tt*HNOX protein in the bacteria were determined to be at low temperature (16 °C) with 0.5 mM IPTG and 1 mM ALA. To reduce cost, the ALA amount can be reduced by half, in which case a yield reduction of 30% can be expected. If production apo-*Tt*HNOX is necessary, then high temperature (25 °C), 1 mM IPTG and 0.5 mM ALA conditions will lead the highest amount of apo-protein. Under these conditions, only 6% of the *Tt*HNOX protein is bound to the heme cofactor. At elevated temperatures (25 °C), ALA did not affect the rate of binding to the heme cofactor (Figure 8) but it increased the total protein produced (Figure 5). This effect can be explained

that stimulation of the production of respiratory chain proteins by the increase in heme production, thereby triggering cell metabolism and proliferation.

5. CONCLUSION

This study shows that the optimal conditions for the production of holo-*Tt*HNOX in *E. coli* is at low temperature (16 °C) with 0.5 mM IPTG and 1 mM ALA. In order to reduce cost, the ALA amount can be reduced by half, in which case a yield reduction of 30% can be expected. If apo-*Tt*HNOX is desired, higher temperature (25 °C), 1 mM IPTG, 0.5 mM ALA conditions can be used to obtain the highest amount of protein. This study shows that the precursor molecule ALA and expression at low temperature are two important factors in achieving high levels of holo-hemeproteins.

6. Acknowledgement

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