Recombinant Fructosyl Peptide Oxidase from Eupenicellium terrenumand: Periplasmic Secretion Could Improve the Solubility and activity of Enzyme?

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Abstract

HbA1c enzymatic method has been considered as a popular method for determination of blood glucose. In this study, the methods for decreasing the formation of Fructosyl Peptide Oxidase (FPOX) inclusion bodies in E. coli and secretion of the enzyme into periplasmic space using PelB signal peptide were investigated. Recombinant FPOX was mainly expressed as inclusion bodies. The inclusion bodies of FPOX were only soluble in urea 8 M, while was not soluble in any of used concentrations of DMSO solvent. By freeze-thawing method, the inclusion bodies was soluble in urea 8 M, but was not soluble in urea 0.5 M. By using the stabilizers on solubility of rFPOX we found that the effect of sorbitol was higher than arginine. The rFPOX was successfully secreted in the periplasmic space using PelB signal peptide that shown the amount of periplasmic protein in shuffle was higher than of BL21. For rFPOX activity, TMB was used as coloring agent for the first time in HbA1c enzymatic method as peroxidase substrate. In conclusion, FPOX inclusion bodies are heterogenous in size and can be decreased using signal peptide. The Freeze-thawing method can be used to solubilize FPOX inclusion bodies at lower concentrations of urea.

1 Introduction

Amadori compounds are formed from reducing sugars, such as glucose, and an amine by a non-enzymatic glycation reaction. In addition to food samples, this reaction takes place also in vivo, i.e. proteins of blood such as albumin and hemoglobin that are undergone the glycation based on glucose concentration in blood. HemoglobinA1c (HbA1c) is produced by stable connection of glucose to the N-terminal section of the hemoglobin β-subunit and took into consideration as a key indication for the lasting control of the glycemic state of diabetic persons. The average level of glucose in blood is reflected by HbA1c concentrations in the stages corresponding to the protein half-life [1, 2]. In comparison to other assay procedures, the newly developed HbA1c enzymatic assay has been considered as a popular method because of easy management on customary auto-analyzers and low cost. Two HbA1c enzymatic assay: i) HbA1c is denatured by detergents and digested by protease to produce the fructosyl dipeptide part, fructosyl valyl histidine (F-ValHis), and ii) the generation of glucosone, valyl histidine (ValHis) and hydrogen peroxide (H_2O_2) by the reaction of the released F-ValHis with Fructosyl peptide oxidase (FPOX) [3]. FPOX belongs to the flavo-enzymes family and catalyzes the oxidative deglycation of F-ValHis as a substrate derived from HbA1c by proteolytic digestion [4]. FPOX which acts specifically on glycated proteins has a great potential for using as a diagnostic enzyme for diabetes mellitus [5]. Most penicillium enzymes exhibit high activities toward F-ValHis [6]. Also, FPOX from *Eupenicillium terrenum* shows high activity toward FruValHis [5, 7] and is expected to be applicable in HbA1c measurement [8].*E.coli* as an expression system for production of recombinant protein has several advantages including, high expression level, fast growth in an inexpensive culture medium, simplicity of genetic manipulation, well known genetics, and, availability of various strains [9, 10]. However, there are several limitations in producing recombinant proteins by *E. coli* such as low biological activity, complicated

downstream processing, low solubility and stability of the product. Moreover, recombinant proteins are largely formed as inclusion bodies, in form of inactive and insoluble molecules, and so, need refolding in vitro [10]. Although, the considerable capacity of *Bacillus* species for secretion of protein is well known, but, the use of *Bacillus* for the production of heterologous protein often have contains limitations [11]. For improvement these drawbacks different strategies have been applied to resolve protein secretion in *E. coli.* These approaches include using of different signal peptides [12], periplasmic and extracellular expression of protein [13] and, conditions optimization of expression [14].Theperiplasmic space creates an oxidative location that is suitable for the formation of disulphide bond, protein stability and folding [15, 16]. The use of signal tags can mediate the recombinant proteins to the periplasmic space [17]. In this study, we investigated the methods for decreasing the formation of FPOX inclusion bodies in *E. coli* and secretion of the enzyme into periplasmic space using PelB signal peptide.

2 Materials and Methods

The cDNA sequence of wild-type *Eupenicillium terrenum* FPOX (Accession Number: AB116146) in the form of sub-clone in pET22b(+) was synthesized by Generay Company (Hongkong). Horseradish peroxidase (HRP), TMB (3,3',5,5'-Tetramethylbenzidine) and protein marker were provided by Applied Biotechnology Center (Iran, Kermanshah). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Sigma (St. Louis, MO, USA). *Escherichia coli* strains BL21 (DE3) and sHuffle were obtained from IBRC (Tehran, Iran). Arginine, sorbitol, glycerol and DMSO were purchased from Merck (Germany). All other chemicals and reagents were of the highest commercial grade available.

2.1 Codon optimization of fpox gene

For the heterologous expression of recombinant FPOX in *E. coli*BL21 (DE3) and shuffle strains, the *fpox* gene sequence from*E. terrenum* was retrieved from NCBI and the codons of *fpox*sequence were optimized using Jcat server (http://www.jcat.de/). Different physicochemical properties of FPOX protein were investigated by protparam server (https://web.expasy.org/protparam/).The optimized *fpox* gene was evaluated using the GeneScript rare codon analysis server (https://www.genscript.com/tools/rarecodon-analysis). The codon-optimized sequence of FPOX,with *NcoI* and *XhoI*restriction sites, was chemically synthesized sub-cloned in pET22b(+) and, transformed into *E. coli* shuffle and BL21 strains.The pET22b(+)-FPOX- *E. coli* construct was cultured on LB-agar plate containing ampicilin (100 µg/mL) at 37 C overnight. The positive clone selection, plasmid stability and glycerol stocks preservation were done.

2.2 Cloning and Expression of recombinant protein

The recombinant plasmid pET22b-*fpox* was transformed into*E.coli* BL21 (DE3) and shuffle strains using calcium chloride-heat shock method [18]. Transformants were inoculated in the LB medium supplemented with 100 µg/mL ampicilin and incubated at 37 °C overnight. Protein expression was conducted by the inoculation of 1 mL into 100 mL LB medium containing antibiotic and incubated at 37 °C to achieve an optical density at 600 nm (OD_{600}) of 0.6. The FPOX protein expression was induced by adding a final concentration of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 16 °C for 24 h. Cells were harvested by centrifugation at 4000g for 12 min and were washed twice with PBS buffer (1 X). The pellets were stored at -20 °C. The pellets were suspended in lysis buffer containing 20 mM Tris–HCl pH 8.0, 40 mM NaCl [7]and lysozyme 1 % w/v and then incubated in 37 °C for 1 h followed by sonication, and then centrifuged at 13000g for 20 min. The insoluble inclusion body was washed twice with PBS buffer and in order to remove cell debris from insoluble protein; it re-suspended in washing buffer (Tris 20 mM, NaCl 300 mM, EDTA 1 mM, Triton X-100 1%, urea1 M, pH 8.0) and was washed three times with this buffer. Lastly, the inclusion bodies was washed with PBS buffer to eliminate contaminating detergent and then centrifuged at 12,000 g for 20 minutes and it was applied for following solubilization [19].

2.3 Purification of soluble and inclusion bodies of recombinant FPOX

For purification of soluble FPOX, the supernatant from the cell lysiswas applied onto a 15 mL column containing 1 mL of Ni–NTA agarose (Qiagen) equilibrated with lysis buffer (20 mMTris–HCl pH 8.0, 40 mM

NaCl) and washed with wash buffer (20 mM Tris–HCl pH 8.0, 40 mM NaCl, and 20 mM imidazole).His-tagged FPOX was eluted using the elution buffer containing 300 mM imidazole [7]. In order to achieve a purified state of inclusion bodies, after cell lysis by lysozyme and sonication, it centrifuged at low speed (4000g for 10 min) for sedimenting the bacterial cells that are not broken by sonication and also separating the large-size particels of inclusion bodies. The presence of large-size particels of inclusion bodies in the pellet was analyzedby SDS-PAGE. The supernatant of this stage was again centrifuged at high speed (13000g for 20 min) and the supernatant was removed and the pellet containing the small-size particels of inclusion body was stored at -20°C for analysis with SDS-PAGE. The pellet from centrifugation at low speed contained intact cells was lyzed and centrifuged at high speed (13000g for 20 min) and the supernatant was analyzed.

2.4 Solubility of FPOX inclusion bodies

Two different methods including traditional urea-denatured method and freeze-thawing method were used to solubilize inclusion bodies of FPOX in urea. In the urea-denatured method, purified inclusion body was suspended in buffer ($NaH_2PO_4.2H_2O/Na_2HPO_4$100 mM, tris-HCl 100 mM, DTT 5 mM) with different concentrations of urea (1, 2, 4, 8 M) and stirred for 30 min at room temperature followed by a centrifugation at 12000g for 20 min, and supernatant was analyzed by SDS-PAGE. The solubility of purified inclusion body also studied in different concentrations of DMSO (5, 10, and 20 %). In the second method, the purified inclusion body of FPOX was suspended in PBS buffer at different concentrations of urea (0.5, 1, 2, 8 M) and the suspension was frozen at -20°C and thawed at room temperature, finally, the supernatant was obtained by a centrifugation at 12000 g for 20 min and analyzed by SDS–PAGE[19]. At the end of this experiments the supernatant was dialysis against refolding buffer (20 mMTris, 100 mM NaCl, 10 mM CaCl2, 0.2mM ZnCl2,pH 7.5) at 4°C for 3 hours.

2.5 Arginine and sorbitol stabilizers and their effects on solubility of rFPOX

Arginine and sorbitol with concentrations of 0.2 and 0.3 M, respectively, were added to the LB medium and the production of recombinant FPOX was performed in the presence of these compounds. The induction of protein expression was done by adding 0.1 mM IPTG and incubation at temperature of 16 °C for overnight.

2.6 Periplasmic extraction

After protein expression, culture media were centrifuged at 4000g for 12 min to harvest cells and the extraction of periplasmic fraction was done by osmotic shock method. Briefly, cells were resuspended in a hypertonic solution (Tris 30 mM, sucrose 20% w/v, EDTA 1 mM, pH 8) and incubated for 30 min at 4 °C. Cells were harvested by centrifugation (4000g for 12 min) and the supernatant collected. Cells were re-suspended in a hypotonic solution containing 5 mM MgSO4 that incubated 30 min at 4 °Cthen,the periplasmic fraction was obtained by centrifugation at 13000g for 20 min [20]. For improvement of periplasmic extraction, the effect of sonication and lysozyme were investigated. In case of sonication, after resuspending the cells in hypotonc solution, it sonicated and incubated at at 4 °C for 30 min.

2.7 The investigation of the effect of different proteases on hemoglobin as substrate

For this purpose, different proteases including Neutral protese, Alkaline protease, Pepsin, Papain and Chymotrypsin (1:100 W/W of enzyme to substrate) were applied to digest hemoglobin in order to produce substrate (f-ValHis) for FPOX enzyme. The digestion of hemoglobin by the proteases was evaluated by SDS-page analysis.

2.8 HbA1c enzymatic assay

For HbA1c enzymatic assay three reagents were used. The reagent 1 was consisted of 0.1% Triton X-100, reagent 2 was composed of Alkaline protease, 3,3-5,5-Tetramethylbenzidine (TMB 0.5 mM), and suitable buffer and, reagent 3 was included purified FPOX and Peroxidase (5 U/ml).The blood cells were hemolyzed by mixing 500 µL of reagent 1, and 25 µL of blood sample. Torelease Fru-ValHis, 12 µL of hemolyzed sample was added to the 180 µL of reagent 2 and incubated at 37 °C, and after 5 min its absorbance was read at

652 and 800 nm (sub wavelength), next, 60 µL of reagent 3 was added to the sample then incubated at 37 °C for 20 minand the absorbance was read at the same wavelengths.

3 Results

3.1 Expression of FPOX

Recombinant FPOX, approximately 47 KDa, was mainly expressed as inclusion bodies in BL21 and shuffle strains under different conditions of IPTG and induction temperature (Fig.1) and no considerable soluble protein was observed in supernatant. The level of protein expression was same in both of strains. The different physicochemical characteristics were evaluated by protparam tool, and shown that, FPOX composed of 437 amino acids with a molecular weight of about 48 KDa and contained a theoretical pI of 6.68. This molecule consisted of 229 hydrophobic amino acids (52.4 %) and 208 hydrophilic residues (47.6 %) and, the acidic and basic amino acids were 51 (11.7 %) and 62 (14.2 %), respectively.

3.2 Purification of inclusion bodies

Recombinant FPOX was expressed as both soluble form and inclusion bodies. FPOX contained a His-tag in the C-terminal of the protein to enable protein purification by Ni-NTA column. Purified soluble FPOX was evaluated by SDS-page and has been shown in Fig.1A with a band of 47 KDa. For inclusion bodies, after cell lysis with sonication, it was centrifuged in low speed (4000g for 10 min) and the supernatant was separated and then the supernatant was centrifuged in high speed (13000g for 20 min) and in each case, the pellets were investigated for the presence of inclusion bodies. The pellet from low speed stage was sonicated to lyze the intact cells in the pellet. As shown in Fig.2 B FPOX protein has been expressed around 47 KDa. The soluble protein in the supernatant from the first and second stages of cell lyses was low and was mainly formed as inclusion bodies. The results showed that inclusion bodies of FPOX have been consisted of small and large particles. The large particles were sedimented with centrifugation at 4000g for 10 min that is corresponding with the sedimentation of the bacterial cells in the same speed, while the small particels were sedimented in 13000g for 20 min. The main part of FPOX inclusion bodies were particles with large size.A band of insoluble FPOX with purity about 95 % was observed in analysis with SDS-PAGE that was related to the small particles of inclusion bodies that sedimented in high speed of centrifugation of supernatant obtained in low speed stage.

3.3 Solubilizing the inclusion body in Urea

According to theSDS-PAGE analysis, inclusion bodies of FPOX were only soluble in urea 8 M with a slight solubilization in the presence of 4 M urea by traditional method while was not soluble in any of used concentrations of DMSO solvent (Fig.3). In freeze-thaw method, the inclusion bodies were not soluble in urea 0.5 M while were soluble in urea 1 and 2 M with a concentration of 1mg/mL and 1.4 mg/mL, respectively, while it was not soluble with these concentrations of urea in traditional method. However, the major part of inclusion bodies of FPOX was soluble in urea 8 M by freeze-thaw method. The dialysis method was used to refold soluble inclusion bodies; which gradually permitted the alteration from denaturing to native buffer conditions. However, considerable precipitation was observed after dilution to the refolding buffer without urea. Supernatant containing refolded FPOX was collected after centrifugation, and used for the activity assay.

3.4 The effect of arginine and sorbitol in culture medium on the formation of inclusion body

The effect of arginine and sorbitol on protein solubility was investigated in shuffle strain. Results show that nearly a similar amount of protein expression was observed in the presence of sorbitol compared to control (Fig.4). In the other side, in the presence of arginine in the culture media the expression of rFPOX was very low and concluded that arginine had the least effect on solubility compared to sorbitol. The concentration of total protein in the presence of sorbitol (2.43 mg/ml) was higher than arginine and control culture media (2.13 mg/ml).

3.5 Periplasmic secretion of FPOX by signal PelB

FPOX successfully expressed in *E. coli* by connecting PelB sequence peptide in the protein upstream. PelB signal peptide acts as a targeting and recognition signal that mediated the transfer of recombinant FPOX across the inner membrane to periplasm space. Within the periplasm, the PelB signal peptide was cut by specific signal peptidase and the recombinant protein has about 2.3 KDa lower weight after cutting signal peptide. The recombinant FPOX was secreted in the periplasmic space, however, the main part of FPOX remained in the inclusion bodies. As showed in Fig.5, the amount of periplasmic protein in shuffle was higher than of that in BL21. The most level of protein in the periplasmic space was observed in sample treated with lysozyme in shuffle that contributes to break the cell wall.

3.6 Selection of protease for Fru-ValHis production from HbA1c

The effect of several proteases on hemoglobin as substrate was evaluated by SDS-page analysis (Fig 6). Among them, alkaline protease and pepsin were properly able to digest hemoglobin.

3.7 Enzyme assay

The activity of FPOX was evaluated. Results from this study shown that rFPOX was an active enzyme. In FPOX activity, was used as coloring agent for peroxidase substrate. The activity of our FPOX enzyme was compared with FPOX enzyme from HbA1c Kit (SEKISUI, Japan). For blood samples from diabetic patients, similar results were obtained but our results were gained after 20 mins instead of 5 mins obtained by the kit.

4 Discussion

Inclusion bodies are mainly produced by over expression of recombinant proteins, which intricate and costly denaturation and refolding procedures are needed to recover biologically active proteins. Besides, the final soluble refolded protein yields are typically very low because of protein aggregation forming by interactions between the proteins hydrophobic areas. In this study, the amino acid sequence of FPOX was evaluated by protparam server as a bioinformatics tool. The evaluation of FPOX amino acid sequence showed that it contains 437 amino acids with about 52% of hydrophobic amino acids that is prone to form inclusion body. It has been reported that a protein with a more hydrophobic part, it will probably produces more aggregates [21]. The aggregation behavior of a protein could be slowly changed by alteration of the non-polar composition by only a few percent [22]. Under different conditions of expression including the use of various temperatures (16-37 °C) and IPTG concentrations (0.1-1 mM), FPOX was predominantly expressed as inclusion body aggregates. The major section of FPOX inclusion bodies are considered as large particles. It has been shown that inclusion bodies can be formed intracellularly with a distinctive size range of 0.2-1.5 μm as refractile particles [23]. Inclusion bodies can intensify to more than 1 μm in diameter [24], a big part of a single bacterial cell, and therefore can obviously be visible under a microscope. Inclusion bodies are mainly composed of recombinant protein (up to 99%), but contain also both chaperones and membrane parts that join to the inclusion bodies during formation [24, 25] and is in agreement with our study that the major part of inclusion body is composed of recombinant FPOX based on SDS-PAGE analysis. Typically, high concentrations of chaotropes and denaturants such asGdnHCl and urea are used to solubilize inclusion bodies. While, the use of high concentrations of these compounds leads to disrupt completely the structure of protein that sometimes, causes protein aggregation during refolding process [26]. In the present work, FPOX inclusion bodies were solubilized by high concentration of urea (8 M), however, the soluble FPOX showed no activity. Therefore, for depletion of urea and refolding process FPOX was dialyzed against lower concentrations of urea. However, the whole of protein aggregated during the elimination of urea that can be attributed to the structure disruption of protein in the presence of high level of urea. In this case, by traditional urea-denatured procedure, FPOX was only soluble in urea 8 M, while, recently, Qi et al. proposed a freeze-thawing method that can be used to directly solubilize a considerable of inclusion body in low concentration of urea [19]. In this context, by traditional urea-denatured procedure, inclusion bodies of enhanced green fluorescent preotein (EGFP) and catalytic domain of human macrophage metalloelastase (MMP-12-CAT) could be solubilized in urea 5-8 M and are not soluble in lower concentrations of urea (0-3 M). While, by freeze-thawing method, both proteins inclusion bodies could highly solubilize in different

concentrations of urea (1-8 M), and maximum solubilization of them was observed in urea 2 M that the solubility of these proteins in urea 2 M by freeze-thawing method is comparable with their solubility in urea 8 M by traditional urea-denatured procedure [19]. In this study, by freeze-thawing method, FPOX was soluble by about 30 % in low concentrations of urea (1-2 M) while was not soluble in urea 0.5 M. The alkaline pH away from protein isoelectric point plays an important role in aggregation destabilizing of inclusion body, therefore contributes to the solubility of protein in the presence of low concentration of urea [19]. It has been reported that the important agent of inclusion body solubilization by freeze-thawing procedure is cold temperature stress and the formation of ice crystals during freezing process compared to other factors [19]. However, after dialyzing the soluble FPOX in urea 1 and 2 M, it showed a weak activity. Totally, refolding efficiency is low, leading to decreased yield of the final product[27]. In both strains of *E. coli,fpox* gene was expressed as inclusion body; however, the presence of FPOX in the soluble phase was more in the shuffle strain. *E. coli* BL21 (DE3) which no contains pivotal proteases such as OmpT is the greatest broadly used prokaryotic expression host thatapplied as an important standard among other expression hosts [10]. The reteplase was produced in*E. coli* BL21strain and it was found that all of the recombinant protein is expressed as insoluble form [28] that is in agreement with ourresult. In addition, by resolving codon bias problem in Rosetta-gami B and SHuffle strains of *E. coli* , these strains are also used to increase the solubility of heterologous proteins which have been manipulated to create correct folding of proteins containing disulfide bonds in *E. coli* [10, 29]. However, by expressing reteplase with 9 disulfide bonds in these strains, no expression was observed in the soluble form [28]. In case of reteplase, it was formed as inclusion body in BL21, Rosetta-gami B and SHuffle strains of*E. coli* which the greatest level of inclusion bodies was produced in *E. coli* BL21 [28]. In another study, the greatest level of FGF-1was produced in Shuffle strain with a maximum of soluble/insoluble ratio than the two other strains [30] that is in accordance with the solubility of FPOX in Shuffle strain compared to BL21 in our study. However, the amount of FPOX in cytoplasmic fraction as soluble phase was yet low; therefore, we applied other methods for increasing the solubility of FPOX. It has been showed that the solubility of recombinant proteins can be increased by adding compatible solutes during protein expression [27]. In this study, we added sorbitol and arginine to the culture medium during the protein expression. In the presence of sorbitol, the solubility of FPOX had a negligible increase compared to arginine and control. In agreement with our study, the solubility of green fluorescent protein (GFP) was increased in the presence of sorbitol in culture medium [27]. Sorbitol is applied frequently as proteins stabilizer *in vitro* [31, 32]. The inhibition of the native conformations unfolding to the misfolded/unfolded states can be took place by sorbitol using a mechanism similar to that of other polyhydric alcohols [33, 34]. Finally, sorbitol by conversion to fructose-6-phosphate is entered into the glycolysis and helps to the ATP production [35, 36]. It has been indicated that solutes which could potentially help to ATP production by the cell and interact favorably with the side chains of protein and stabilize them against inactivation, are effective solubilizers [27]. In the presence of arginine in the culture medium, the growth of bacteria and thus the level of FPOX expression were decreased. In this context, Prasad et al. showed that the growth of bacteria in the presence of arginine was decreased.TheGFP activity in the soluble phase enhanced till 0.2 M arginine and then decreased [27]. In another study, The solubilization of active GFP from insoluble phase was obtained in higher concentration of arginine at 1 and 2 M. So, in our study the used level of arginine may be low and should be optimized according to the expression conditions [37]. In the present work, we used pET22b as expression vector for the expression of *fpox* gene which contains pelB signal peptide for the secretion of expressed protein into periplasmic space in *E.coli* . Transfer of protein from the inner membrane to periplasm is mediated by PelB signal peptide [38]. In the periplasm, the signal peptide is cut by signal peptidase and the mature protein is folded and transported across the outer membrane [39]. In different studies, PelB has successfully been used to secrete various enzymes into periplasmic space in *E. coli* . In a study, active nattokinase was successfully secreted into periplasmic space in *E. coli* using PelB and the native signal peptide of nattokinase [40]. The level of inclusion bodies formation of lipase from *Pseudomonas fuorescens* BJ-10 was 20.8 % of that formed by non-tag expression during the use of PelB signal peptide [12]. It seems that periplasmic space has much less protease activity compared to the cytoplasm. Additionally, because of less contaminating proteins in the periplasmic space, the purification of recombinant proteins is easier than cytoplasmic fraction. Besides, since periplasmic space

has a more oxidative environment compared to the cytoplasm, thus, the correct formation of disulfide bonds can be facilitated [41]. According to our results, among proteases, alkaline protease efficiently produced Fru-ValHis from HbA1c. In this context, Hirokawa et al. selected an *Aspergillus* protease as the most effective enzyme for releasing Fru-ValHis as substrate for FPOX based on screening among different proteases [5]. In another study by Hirokawa et al. different proteases with various sources of plant, bacterial, fungal and yeast were investigated that they concluded among different proteases, neutral protease with bacterial origin (*Bacillus polymyxa*) could efficiently liberate Fru-ValHis from HbA1c [4]. It seems that proteases from bacterial source (*Bacillus* genus) are a suitable enzyme for producing the substrate of FPOX. In our study, FPOX showed activity for using in HbA1c enzymatic method. In traditional HbA1c kits, 10-(Carboxymethyl amino carbonyl)-3,7-bis (dimethylamino) phenothiadine sodium is mainly used as coloring agent. However, we used TMB as coloring agent in HbA1c enzymatic method for the first time. TMB has been used as a more accurate, sensitive, cheap and non mutagenic coloring agent as peroxisae substrate [42]. Thus, the use of TMB in HbA1c enzymatic method can be economically useful and can be used to detect low concentrations of H_2O_2. The activity of our FPOX enzyme was compared with FPOX enzyme from HbA1c commercial Kit. For blood samples from diabetic patients, similar results were obtained but our results were gained after 20min instead of 5min obtained by the commercial kit. The higher time can be attributed to the lower concentration of our FPOX compared to the kit FPOX. Because of,more enzymes are available to bind to the substrate is caused to increase the reaction speed [43].

Concluding remarks

In conclusion, recombinant FPOX is prone to form a high level of inclusion body. FPOX inclusion bodies are heterogenous in size and can be decreased using signal peptide. Freeze-thawing method can be used to solubilize FPOX inclusion bodies at low concentrations of urea. Compounds such as arginine and sorbitol at concentrations of 0.2 and 0.3 M, respectively, showed no considerable effect on protein solubility. FPOX can be secreted into periplasmic space as active form.

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5 References

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Legends to the figures

Fig.1. Different expression condition in Shuffle and BL21 strains of *E.coli* . **M** : Marker, **A**) Expression with IPTG 1 mM at 37 degC in BL21, 1: negative control, 2: expressed protein, 3:supernatant, 4: inclusion body solution in urea 8 M.**B**) expression with IPTG 1 mM at 30 degC in BL21, 1:before induction of IPTG, 2: expressed protein, 3: negative control4: supernatant.**C**) Expression with IPTG 0.5 mM at 30 degC in BL21, 1: negative control, 2: expressed protein, 3: supernatant.**D**) Expression with IPTG 0.5 mM at 26 degC in BL21, 1: before induction by IPTG, 2: expressed protein, 3:negative control, 4: supernatant (cell lysis using lysozyme and sonication), 5: supernatant (cell lysis using sonication and without lysozyme), 6: inclusion body in urea 8 M.**E**) Expression with IPTG 0.1 mM at 16 degC in BL21, 1:before induction by IPTG, 2 ,3: expressed protein, 4:negative control, 5: supernatant. **F**) Expression with IPTG 0.5 mM at 26 degC in Shuffle, 1: expressed protein, 2: negative control, 3: supernatant.**G**) Expression with IPTG 0.1 mM at 26 degC in Shuffle, 1: before induction by IPTG, 2: expressed protein, 3: negative control, 4: supernatant, 5: inclusion body in urea 8 M. **H**) Expression with IPTG 0.1 mM at 16 degC in Shuffle, 1: negative control, 2: inclusion body in urea 8 M, 3:expressed protein, 4: supernatant.

Fig.2. The purification of soluble and inclusion bodies of FPOX. A) Purification of FPOX B) The purification of inclusion body of FPOX by the centrifugation at low and high speeds.**M** : marker,**1** : negative control, **2** : expression of FPOX before cell lysis, **3** : supernatant from centrifugation at high speed,**4** : supernatant after pellet lysis from the stage with low speed centrifugation, **5** : inclusion bodyobtained at low speed of centrifugation, **6** : purified inlcusion body from the centrifugation of supernatant at high speed,**7** : inlcusion body after pellet lysis from the stage with low speed centrifugation.

Fig.3. SDS–PAGE analysis of solubilized FPOX from inclusion bodies in different concentrations of urea by two different methods and also the solubility of FPOX in DMSO solvent. A) **M** :marker,**1** : urea 1M, **2** : urea 2M, **3** : urea 4 M,**4** : urea 8 M, **5** : DMSO 5% ,**6** : DMSO 10 %,**7** : DMSO 20%, B)**M** : marker, **1** : urea 0.5 M,**2** : urea 1M, **3** : urea 2 M, **4** : urea 8 M,

Fig.4. TheSDS-PAGE analysis of the effect of sorbitol and arginine on the solubility and expression of FPOX. M: marker, 1: negative control, 2: expressed FPOX in control(without sorbitol and arginine), 3: expressed FPOX in the presnce of sorbitol, 4: expressed FPOX in the presence of arginine, 5: supernatant from control, 6: supernatant from cells growed in the presence of sorbitol, 7: supernatant from cells growed in the presence of arginine.

Fig.5. Periplasmic extraction of recombinant FPOX produced in*E. coli* BL21 and Shuffle strains.M: marker, 1: negative control, 2: expression of FPOX in shuffle, 3: expression of FPOX in BL21,4: periplasmic fraction in shuffle, 5: periplasmic fraction in BL21, 6: supernatant from lysis of cells after periplasmic extraction in shuffle, 7: supernatant from lysis of cells after periplasmic extraction in BL21, 8: periplasmic fraction in shuffle treated by sonication, 9: periplasmic fraction in BL21 treated by sonication.10: periplasmic fraction in shuffle after adding lysozyme, 11: periplasmic fraction in BL21 after adding lysozyme.

Fig. 6. The effect of different proteases on hemoglobin as substrate. 1: Control, 2: Alkaline protease, 3: Neutral protease, 4: Pepsin, 5: Papain, 6: Chymotrypsin.

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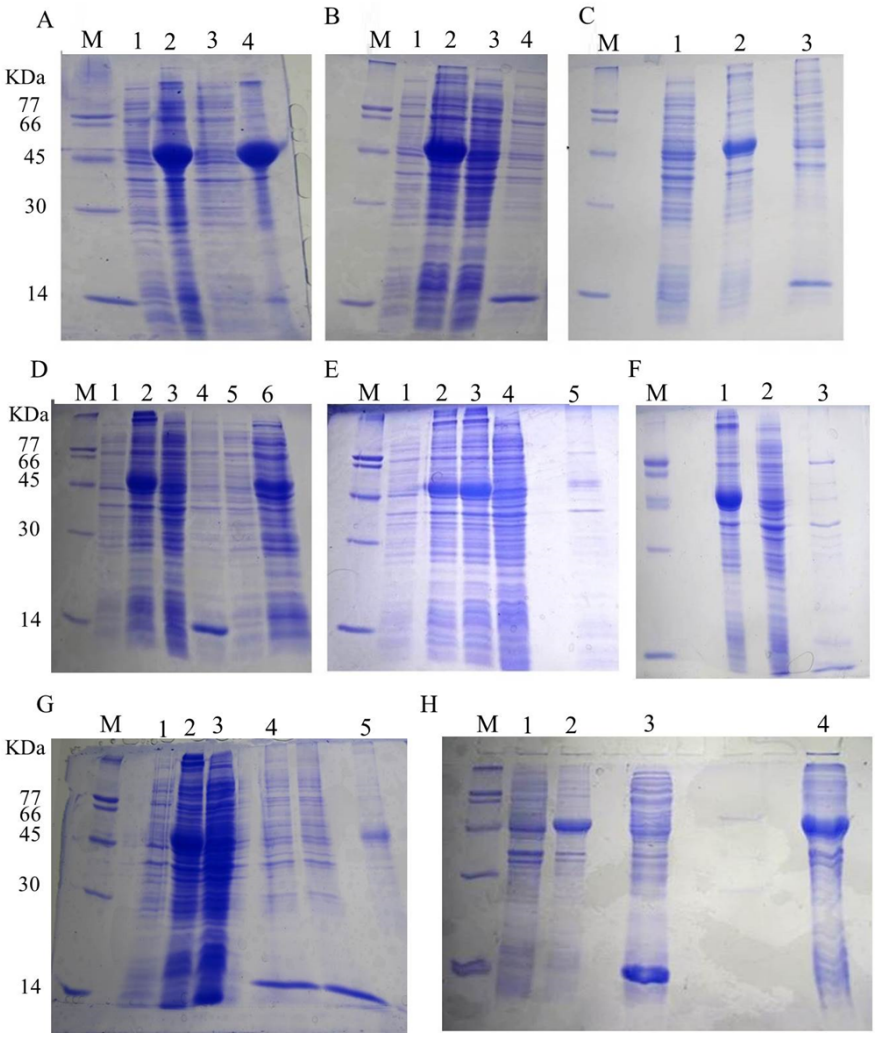

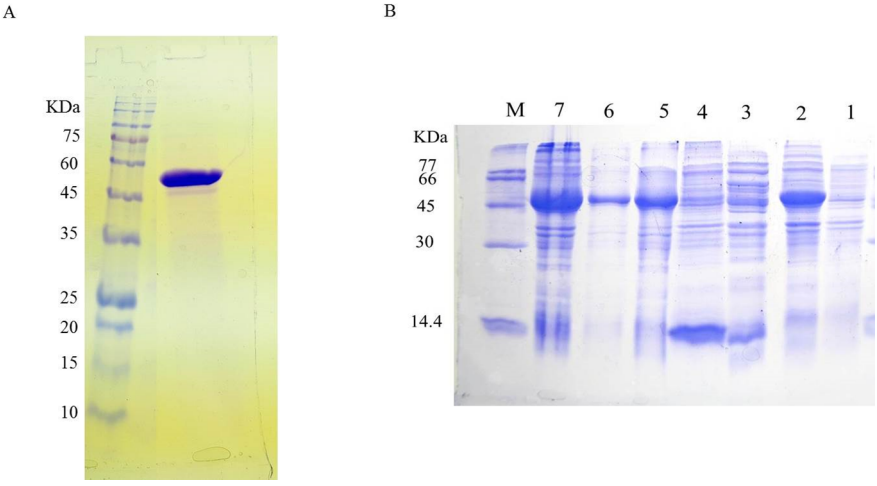

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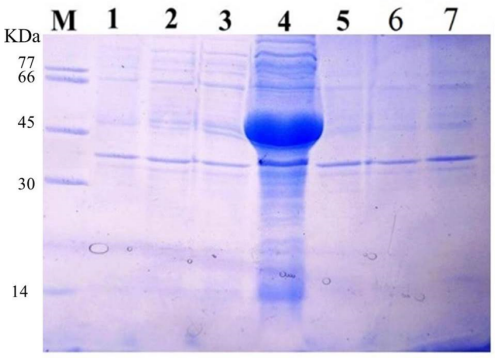

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