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Effect of IPTG amount on apo- and holo- forms of glycerophosphate oxidase expressed in *Escherichia coli*

Chunyang Zheng [a], Zhanyong Zhao [b], Yan Li [c], Lin Wang [a,b], Zhiguo Su [c,*]

[a] Center for structural and molecular biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China
[b] Biotrand Incorporation, Beijing 100101, PR China
[c] National key laboratory of biochemical engineering, Institute of Process Chinese Academy of Sciences, Beijing 100190, PR China

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ABSTRACT

Escherichia coli has proved to be a successful host for the expression of many heterologous proteins, and much efforts have been made toward improving recombinant protein expression including the usage of strong promoters and co-expression with chaperones. But little attention was paid on the relation between expression level and function of the target protein. Glycerophosphate oxidase (GPO) is a protein with FAD cofactor (without free cysteine and disulfide bonds).It was observed that the specific activity of GPO dramatically decreased with the increase of inducer IPTG. In addition, the stability of it decreased correspondingly. The structural difference of samples expressed under varying IPTG was investigated using size-exclusion and reverse-phase high performance liquid chromatography, together with CD spectrum. It was found that the conformation of peptide and organization of subunits were not affected. The loss of specific activity and stability were correlated to incomplete attachment of FAD onto GPO. These results revealed that synthesis speed should be controlled either by reduction of IPTG amount or using weak promoters in the production of GPO.

Introduction

Glycerophosphate oxidase (GPO)[1] catalyses the reaction in which dihydroxyacetone phosphate and one molecule of hydrogen peroxide are formed from L-α-glycerophosphate and one molecule of oxygen. The enzyme has clinical and industrial interests, e.g., for the measurement of lipase activity and determination of triglyceride, glycerol, ATP and the like. Also, it is used in the enzymatic preparation of dihydroxyacetone phosphate [1]. Conventionally, GPO is produced by purification from the natural sources with low contents, resulting in considerable cost [2]. With the development of DNA technology, the industrial production of many proteins with high additional value has been achieved. GPO of *Enterococcus casseliflavus* was once expressed in *Escherichia coli* for the determination of sequence homology with the membrane-associated dehydrogenase and kinetic analysis [3].

However, for large-scale production, three problems could not been overlooked. GPO is a flavoprotein with flavin adenine dinucleotide (FAD) cofactor, therefore: (1) whether the over-expression of GPO has effect on the attachment of FAD on GPO; (2) whether apo- and holo-enzyme have differential bioactivity or stability; (3) whether the difference, if exists, affects the downstream processing (fermentation, purification, storage, etc.).

D-Amino acid oxidase (DAAO) from human, one of the flavoproteins, was found to be expressed as holo-protein form in *E. coli* and had weak binding with FAD cofactor [4]. Arroyo et al. reported that His-tagged DAAO from *Trigonopsis variabilis* was largely expressed as apo-protein (about 90%), and the stability of this protein was dependent upon the binding of FAD cofactor. The addition of exogenous FAD converted apo-protein to holo-protein with full activity [5]. Porphobilinogen deaminase, another flavoprotein, was expressed as apo-protein in *E. coli*. The apo-protein appeared to exist in a conformationally mobile state similar to the denatured state of holo-protein. Partial activity of the enzyme was reconstituted by the addition and binding of its cofactor [6]. However, 6-hydroxy-D-nicotine oxidase (6-HDNO), when expressed in *E. coli*, part of this protein was present as 6-HDNO apo-protein [7]. A complex process was needed to convert the apo-form to its enzymatically active holo-form, which requiring an energy-generating system consisting of ATP, phospho*enol*pyruvate and pyruvate kinase [8]. The investigation on FAD binding in glycine oxidase from *Bacillus subtilis* showed that the apo-enzyme had high exposure of hydrophobic surfaces and low temperature stability as compared to the holo-enzyme. The incubation with FAD recovered partially

* Corresponding author. Address: 1 Beiertiao Road, Zhongguancui District, Beijing 100190, PR China. Fax: +86 10 62561813.
E-mail address: zgsu@home.ipe.ac.cn (Z. Su).

[1] *Abbreviations used:* GPO, glycerophosphate oxidase; FAD, flavin adenine dinucleotide; DAAO, D-Amino acid oxidase; MTHF, methenyltetrahydrofolate; iNOS, inducible nitric oxide synthase; IPTG, isopropyl-β-d-thiogalactopyranoside; CD, circular dichroism.

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(50%) activity [9]. Dimethylglycine dehydrogenase (Me2GlyDH), a mitochondrial enzyme with FAD cofactor, and its precursor were expressed in *E. coli*. Both apo- and holo- forms presented, while its precursor with mitochondrial presequence was mainly in apo-form. Further study on the apo-Me_2GlyDH indicated that the binding of FAD had significant effect on stability against trypsin [10,11]. For GPO, Koditschek and Umbreit separated GPO from *Streptococcus faecium*, and found that FAD cofactor was quite firmly bound and could not be easily removed as for DAAO. Removal of FAD cofactor from GPO was accomplished with 10% cold trichloroacetic acid. The apo-form of GPO was completely reactivated by incubation with FAD [12,13]. The addition of FAD to holo-form of GPO gave no stimulation of activity [14].

Other related reports include: Xu et al. found in their work about *E. coli* DNA photolyase that its methenyltetrahydrofolate (MTHF) cofactor could be removed at neutral pH by washing with deionized water and its FAD cofactor could be removed by treating the holo-enzyme with 0.5 M imidazole pH 10.0[15,16]. Fossetta et al. expressed active full-length human inducible nitric oxide synthase (iNOS) in *E. coli*. They found that the expression required co-expression with calmodulin, a particularly tight-binding cofactor. They postulated that the extremely tight association of calmodulin with iNOS served a structural purpose in allowing iNOS to fold correctly [17]. Cheesman et al. reported that the mutation of certain histidine caused compromised FAD cofactor retention [18]. Therefore, it is reasonable that the gene engineering, expression components, expression/fermentation condition, purification protocol, and the storage should be considered as a whole in the production of such proteins with cofactors either covalently or non-covalently bound.

In this article, GPO gene of *Enterococcus faecium* was cloned and expressed in *E. coli*. It was found that with increase of the amount of inducer isopropyl-β-D-thiogalactopyranoside (IPTG) the specific activity of GPO decreased correspondingly. Spectral and chromatographic methods were used to characterize GPO obtained under certain expression condition. Moreover, the strategies to set up producible production process were discussed.

Materials and methods

Materials

All chemicals were, if not otherwise specified, from Sigma–Aldrich. Chelating Sepharose Fast Flow was from GE Healthcare. Restriction endonucleases were from New England Biolabs. Water used for the experiments was Ultrapure water from Milli-Q supplied by Millipore, USA.

Expression

The recombinant GPO is expressed in *E. coli* BL21 (DE3) cells using pET21b expression vector. Bacteria were grown on LB medium containing 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride per liter of water. Ampicillin, when required, was added at 100 μg/ml. The cell was cultured at 16 °C following induction with varying IPTG during the exponential phase of growth.

Purification

The cells were disrupted by sonication at 4 °C (pulse for 4 s with a 6 s pause for 99 cycles at 300 W). The soluble and the insoluble cell fractions were separated by centrifugation of the cell lysate at 15,000*g* for 20 min at 4 °C. The supernatant, containing the soluble GPO, was used for further protein purification.

The impurity other than GPO was removed by Chelating Sepharose Fast Flow column, previously charged with 150 mM $NiCl_2$, according to the producer's protocol, and equilibrated with the start buffer. The column was first washed after protein loading with start buffer, and then developed with 500 mM imidazole in the same buffer. Proteins eluted at 500 mM imidazole were collected and desalted to Tris–HCl 50 mM pH 8.5. At each step the purity of the GPO protein was checked by SDS–PAGE.

Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco170 spectropolarimeter (Japan Spectroscopic Co. Ltd., Japan) and analyzed by means of Jasco software. The cell path was 0.1 cm for the measurements in the far field region (200–250 nm).

Activity assay

The activity of GPO was determined by the quantity of hydrogen peroxide. Peroxidase was used to catalyze the reaction of hydrogen peroxide and 4-aminoantipyrine phenol, forming quinoneimine which could be measured at 500 nm by spectrophotometry. One unit was defined as the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute in the presence of 100 mM of DL-α-glycerophosphate at 37 °C. All results were expressed as units per milligram of enzyme protein, to compare the specific activity.

SEC-HPLC analysis

Samples were loaded onto a Sephadex 200 HR10/30 column (GE Biosciences) and run at 0.25 mL/min on an AKTA FPLC workstation (GE Biosciences). The chromatography buffer contained 50 mM sodium phosphate (pH 7.5) and 150 mM NaCl. UV absorbance was measured at 280 and 456 nm.

Reverse-phase-HPLC analysis

GPO purified was analyzed by reverse-phase HPLC on Agilent 1100 with Proteonavi S5 column (SHISEIDO, Tokyo, Japan). A linear gradient elution was carried out from 5% to 95% acetonitrile (V/V) in 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. the detection absorbance was set at 280 nm.

Other procedures

Protein concentration during the purification procedures was measured by the method of Bradford [19], by using bovine serum albumin as reference protein. Electrophoresis was performed by 12% or 15% SDS–PAGE, according to Laemmli [20]. Protein bands were stained with Coomassie bright blue R-250. Marker proteins were SDS–PAGE molecular-mass standard. The content of FAD bound on GPO was identified by the characteristical absorbance of flavoprotein at 456 nm [21,22].

Results and discussion

Effect of IPTG inducer on specific bioactivity of GPO

Data presented in Fig. 1A showed that GPO was expressed as soluble protein and the expression level of GPO was promoted with the increase of IPTG inducer. After purification by Chelating Sepharose Fast Flow column, the purified GPO was subjected to determination of activity. By comparing the specific activity of all four samples induced under varying IPTG concentration (Fig. 1B),

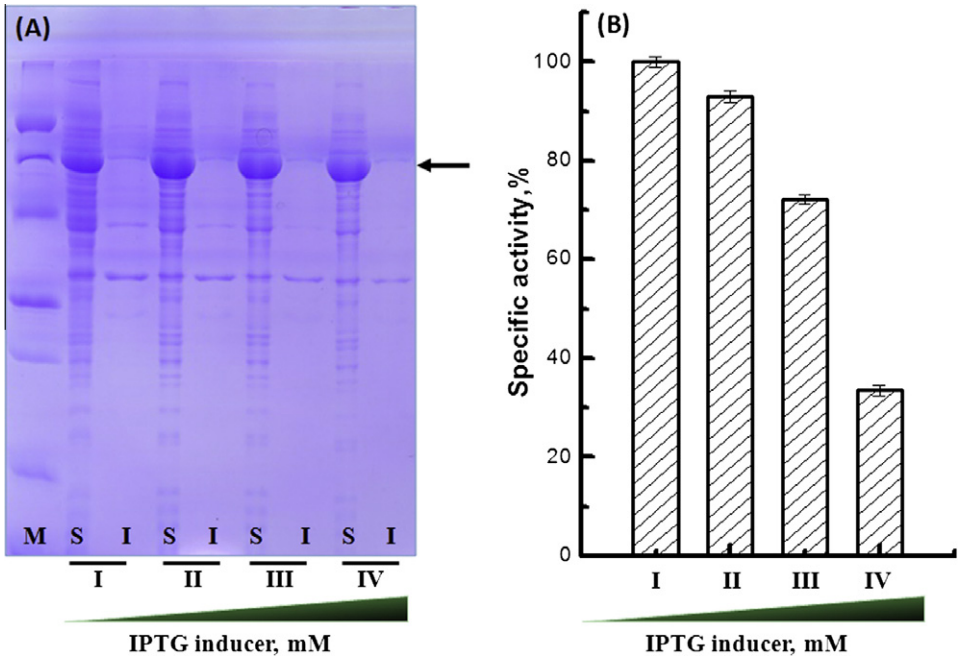

Fig. 1. Relationship between amount of IPTG inducer and specific bioactivity of GPO. (A) SDS–PAGE analysis of soluble (S) and insoluble (I) cell fractions expressed in *E. coli*. The arrow indicated the position of GPO. Lane M was the molecular weight standards included rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg whit lysozyme (14.4 kDa). Sample I–IV was induced by 0.005, 0.015, 0.15, 0.5 mM IPTG, respectively. (B) The soluble cell fractions were purified by Chelating Sepharose Fast Flow column. Pure GPO was collected and subjected to specific activity determination. The specific activity of GPO expressed under induction by 0.005 mM IPTG was set as 100%.

it was clear that there was a direct influence on the function of GPO by IPTG inducer and corresponding increase of synthesis rate. The specific activity of GPO induced by 0.5 mM IPTG was only 34% of that induced by 0.005 mM IPTG.

The cellular environment, folding machinery and conformational quality control checkpoints of prokayotes are quite different from those of eukaryotes [23]. As the target gene was synthesized at high rate, it was possible that GPO over-expressed in *E. coli* misfolded, which resulted in partial loss of function. Further study of GPO induced under 0.005 mM and 0.5 mM IPTG (designated as sample I and IV, respectively) by chromatographic and spectral analysis were performed. Reverse-phase HPLC of sample I was similar to that of sample IV, indicative of structural similarity of the peptide portion (as shown in Fig. 2). The secondary structures of both samples were studied by measuring their circular dichroism spectra in the far-UV region. Both spectra showed quite similar features as illustrated in Fig. 3, which also confirmed the structural similarity of the peptide portion.

However, sample I and IV significantly differed in elution profile of size-exclusion HPLC. The chromatographic process was monitored at 280 nm for peptide portion and 456 nm for FAD portion. As shown in Fig. 4, both samples exhibited similar elution profile at 280 nm, again suggesting structural similarity of the peptide portion. But pronounced difference in elution profiles at 456 nm was found. First, the peak at 456 nm appeared earlier than that at 280 nm, indicating that only partial GPO was attached by FAD and the GPO with FAD attachment featured as close structure. Secondly, in contrast to sample I, the GPO with FAD in sample IV accounted for about third of total GPO. Moreover, inset of Fig. 4 showed that with the increase of IPTG inducer, the content of FAD on GPO dropped correspondingly. It was evident that the content of FAD was correlated to bioactivity of GPO. When induced under IPTG of high concentration, the high synthesis rate might result in incomplete attachment of FAD onto GPO and further impact the bioactivity of GPO.

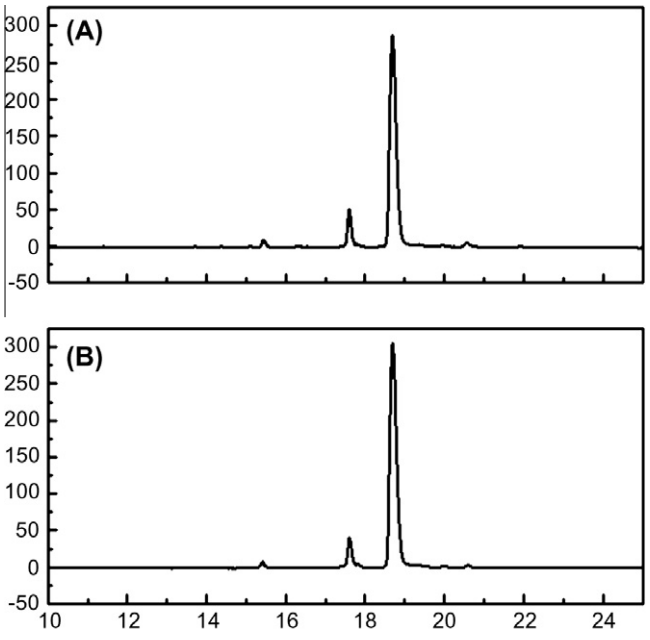

Fig. 2. Reverse-phase HPLC of the GPO using Proteonavi S5 column (SHISEIDO, Tokyo, Japan). (A) and (B) represented sample I and IV, respectively.

Effect of IPTG inducer on stability of GPO

As can be seen in Fig. 4, the GPO with FAD possessed closer structure than that without FAD cofactor. There was also potential difference in stability. Therefore, stability of sample I and IV was assayed. Both samples were incubated at 37 °C for 24 h, after which their activities were determined. The results were summarized in Fig. 5. 53% of activity was left for sample I. Comparatively,

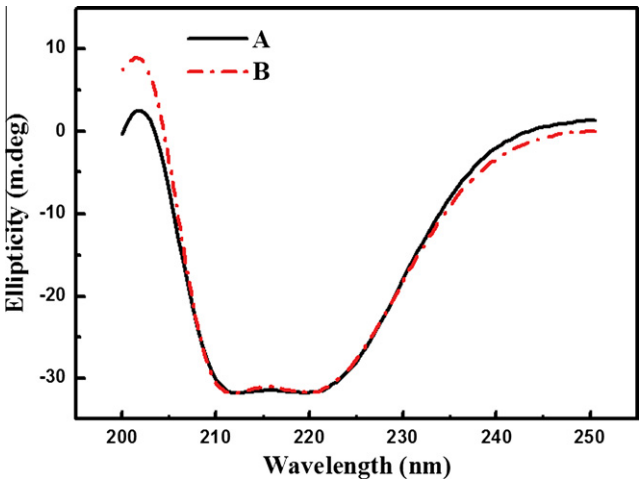

Fig. 3. Far-UV CD spectra of purified GPO were recorded in 20 mM PB, pH7.4, in the range of 200–250 nm. Cell of path length 0.1 cm was used for recording the Far-UV spectrum. Ellipticity was represented in millidegrees. (A) and (B) represented sample I and IV, respectively.

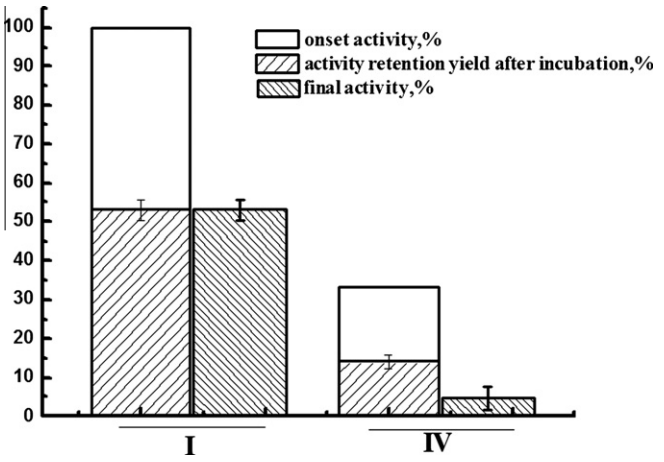

Fig. 5. Stability comparison of sample I and IV to stimulate the storage process. The samples were incubated at 37 °C. The activity after incubation was determined as described in Materials and Method. The onset activity of sample I and IV was determined, and the onset activity of sample I was set as 100%. The final activity of sample I and IV was determined after incubation at 37 °C. Based on the onset activity and final activity of each sample, the activity retention yield after incubation was calculated.

14% of activity was left for sample IV. Taken the onset difference of activity into consideration, only 4.7% of activity was left at the end of the experiment. We also found that GPOs purified from the different IPTG conditions could reach the same level of bioactivity when FAD was excessively added, incubated and then removed by SEC column (data not shown).

It was postulated that the apo-GPO had both lower bioactivity and lower stability than holo-GPO, which definitely had profound influence on the subsequent downstream production.

Conclusion

With the development of recombinant technology, increasing proteins were cloned, expressed and purified from *E.coli*. However, little attention was paid for potential questions in large-scale production. For the first time, GPO was cloned, and the effect of IPTG inducer concentration on expression was investigated. It was found that with the increase of IPTG apo-GPO formed as a result of high synthesis rate. Moreover, the activity and stability of apo-GPO were much lower than holo-GPO.

These results revealed that the cloning, expression and purification should be considered systematically, for the production of GPO and other proteins with similar structure. Weak promoter was preferred in the choice of elements when cloning; Together with reducing inducer in expression, synthesis rate might be controlled to alleviate the formation of apo-GPO; the process should

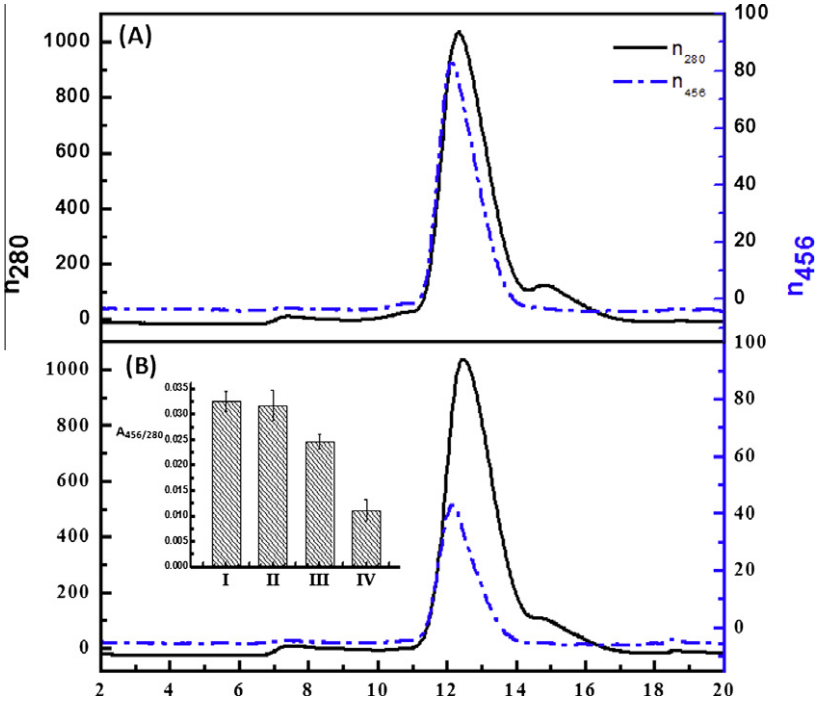

Fig. 4. Size-exclusion HPLC of the GPO using Superdex 200 column (HR 10/30). (A) and (B) represented sample I and IV, respectively. The elution profiles were monitored at 280 nm for peptide portion and at 456 nm for FAD portion. The inset was the ratio of absorbance at 456 and 280 nm of samples I-IV, to represent the content of FAD on GPO expressed under varying IPTG concentration.

be gentle and rapid during purification. Alternatively, addition of FAD during the process might contribute to batch-to-batch reproducibility.

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