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Cloning and expression of phosphoenolpyruvate carboxykinase from a cestode parasite and its solubilization from inclusion bodies using L-arginine

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ABSTRACT

Phosphoenolpyruvate carboxykinase is an essential regulatory enzyme of glycolysis in the cestode parasite, *Raillietina echinobothrida*, and is considered a potential target for anthelmintic action because of its differential activity from that of its avian host. However, due to the unavailability of its structure, the mechanism of regulation of PEPCK from *R. echinobothrida* (rePEPCK) and its interaction with possible modulators remain unclear. Hence, in this study, the rePEPCK gene was cloned into pGEX-4T-3 and overexpressed for its characterization. On being induced by IPTG, the recombinant rePEPCK was expressed as inclusion bodies (IBs); hence, various agents, like different inducer concentrations, temperature, time, host cell types, culture media, pH, and additives, were used to bring the protein to soluble form. Finally, a significant amount (~46%) of rePEPCK was solubilized from IBs by adding 2 M L-arginine. Near-UV circular dichroism spectra analysis indicated that L-arginine (2 M) had no effect on the conformation of the protein. In this study, we have reported a yield of ~73 mg of purified rePEPCK per 1 L of culture. The purified rePEPCK retained its biological activity, and *K*m of the enzyme for its substrate was determined and discussed. The availability of recombinant rePEPCK may help in biochemical- and biophysical-studies to explore its molecular mechanisms and regulations.

1. Introduction

Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) is a key enzyme in glycolytic pathway in parasitic helminths including cestode parasites of avian and mammalian hosts [1–4]. In contrast, in higher vertebrates, PEPCK is considered to be the rate-limiting enzyme in gluconeogenesis [5]. Because of its differential activity in the glucose metabolism, this enzyme is considered a plausible anthelmintic target [6]. The enzyme has been purified biochemically and characterized from few helminths including *Raillietina echinobothrida* [7–13]. However, molecular and structural details of PEPCK in *R. echinobothrida* (rePEPCK) are not known so far; hence, the mechanism of regulation of the enzyme in the parasite remains unclear. Therefore, in the present study, an attempt was made to clone the rePEPCK gene and overexpress the enzyme for its molecular and structural details.

Fusion tags are generally tagged to recombinant proteins for improving their solubility and purification [14]. Glutathione-S-transferase (GST) from the blood fluke, *Schistosoma japonicum*, is used as a fusion tag for solubility and purification of the recombinant proteins [15]; however, not all GST-tagged fusion proteins are expressed in soluble form, thus warranting further approaches to bring recombinant proteins to soluble form [16]. Although the expression and production of many recombinant proteins are well established; in some cases, formation of inclusion bodies (IBs) can affect their expression and solubility. The host cells should possess essential genetic components for maintenance of the expression plasmid, and should not contain any proteases that are harmful to recombinant proteins [17]. *Escherichia coli* (*E. coli*) is the preferred host cell for expression of heterologous proteins. However, it has limitations at expressing some proteins due to lack of advanced molecular apparatuses for post-translational modifications, thereby resulting in their poor solubility and expressed as IBs [18]. Thus, more than 30% of recombinant proteins are expressed as IBs in *E. coli* [19]. Therefore, avoiding the formation or solubilization of IBs becomes the key for the production of heterologous proteins. The production of IBs in *E. coli* can be partially overcome by optimizing various agents, like inducer concentration, temperature, time, host cells, media, pH, additives (ethanol, glycerol, glycylglycine etc.), and coexpression of molecular chaperones [20–26]. Heterologous proteins, expressed as IBs,

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can also be solubilized by adding solubilizing agents (guanidine hydrochloride (GdmCl), urea, N-lauroyl sarcosine, L-arginine, etc) [27,28].

Considering all the fore-mentioned factors, in this study, we cloned rePEPCK gene into pGEX-4T-3, which was expressed in *E. coli* BL21. Since most of the recombinant rePEPCK was found distributed among insoluble pellet fractions, various agents were used to bring the protein to soluble form; finally adding L-arginine solubilized rePEPCK to its biologically active form.

2. Materials and methods

2.1. Vectors and Chemicals

The vector, pGEX-4T-3 (Code No. 28-9545-52), was used for expression of rePEPCK and was procured from GE Healthcare, U.K. Thrombin protease (Code No. 27-0846-01) and glutathione Sepharose 4B (Code No. 52-2303-00AK) were purchased from GE Healthcare, U.K. The primary antibody, rabbit anti-PEPCK-C pAb (Code No. sc-74823) and the corresponding secondary antibody, goat anti-rabbit IgG-HRP conjugated (Code No. sc-2004) were purchased from SantaCruz Biotechnology, Inc, U.S.A. Amicon Ultra-15 centrifugal filters (Code No. UFC903008) were from Millipore, U.S.A. The restriction enzymes, Sal 1 (Code No. R3138S) and Not 1 (Code No. R3189S); alkaline phosphatase (Code No. M0290S); DNA ligase (Code No. M0202S); PCR reagents were purchased from New England Biolabs, U.K. PCR purification kit (Code No. NA1020), GenElute™ gel extraction kit (Code No. NA1111), isopropyl-beta-D-thiogalactopyranoside (IPTG), ampicillin (Code No. A9393), and L-arginine monohydrochloride (Code No. A5131) were obtained from Sigma, U.S.A. Bacterial culture media were from Himedia Laboratories, India. Other reagents and chemicals, supplied either from Sigma, U.S.A. or Sisco Research Laboratories (SRL), India, were of the highest analytical grade.

2.2. Cloning and overexpression of rePEPCK

In order to sub-clone the ORF of rePEPCK (GenBank acc. No. KC252609.1) into pGEX-4T-3 vector, the pGEM-PEPCK construct, originally cloned in our laboratory, was amplified using the forward primer having Sal I cut site (5′-GAGGAGAAGTCGACTAAGCCCA AGCC-3′) and a reverse primer having Not I cut site (5′-TTG GGAAGAGCGGCCGCAACTTGGT-3′). PCR was performed using 50 ng of pGEM-PEPCK construct with the following cycle parameters: initial denaturation temperature of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min followed by a final extension of 72 °C for 10 min. 1× PCR reaction buffer contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs and 200 nM of each primer. The 1.8 kb rePEPCK amplicon was gel eluted and purified using Sigma GenElute gel extraction kit following manufacturer's instructions.

The pGEX-4T-3 vector was digested with Sal I and Not I for 2 h at 37 °C, dephosphorylated by alkaline phosphatase, and purified using PCR purification kit. Similarly, the insert was digested and purified. The PCR amplicon and the digested pGEX-4T-3 vector were ligated at 3:1 molar ratio using DNA ligase. Presence of rePEPCK sequence and its orientation were confirmed by colony PCR, double digestion using restriction enzymes, and direct sequencing.

The construct was then induced by IPTG, and the overexpressed protein was isolated and analyzed by 10% polyacrylamide SDS–PAGE. In brief, the construct was transformed into *E. coli* BL21 cells and the next day a single colony was picked from the Luria–Bertani (LB) agar plate and inoculated in 5 mL LB broth supplemented with 100 μg/mL ampicillin. The culture was incubated at 37 °C with continuous shaking at 220 rpm on Excella E25 shaking incubator (New Brunswick Scientific) overnight. 5 mL of this primary culture was inoculated in l L culture, and incubated at 37 °C with shaking until the OD_{600} reached about 0.8. The cells were subsequently induced with 1 mM IPTG, and grown for 4 h at 37 °C. An uninduced culture containing only the recombinant plasmid served as the control. Proteins were isolated and the fractions (supernatant and pellet) were analyzed by 10% polyacrylamide SDS–PAGE.

2.3. Optimization of rePEPCK solubility using various agents

Since most of the recombinant rePEPCK was distributed among insoluble pellet fractions, various agents, such as, inducer concentration (0.2 mM, 0.5 mM, 1 mM), temperature (16 °C, 20 °C, 24 °C, 37 °C, 42 °C), time course experiments (30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h, 24 h), host cells (Rosetta, BPL, Codon+, Tuner, C41, Origami), media (terrific broth (TB), 2xYT and auto induction media), pH (pH 5, pH 8, pH 10), and additives (ethanol, glycylglycine, glycerol), were used for soluble expression of rePEPCK. Since these procedures failed to bring the protein to soluble form, various solubilizing agents (6 M GdmCl, N-lauroyl sarcosine, and L-arginine) were used to solubilize the protein from IBs.

2.4. Solubilization of rePEPCK from IBs

L-Arginine was used to solubilize rePEPCK from IBs. In brief, the construct was induced and cells were harvested by centrifugation at 6000 rpm for 15 min, and the cell pellet (~5.22 g) was resuspended in 50 mL of ice cold 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM DTT, 10% glycerol, 150 mM NaCl, and 0.1 mM EDTA (buffer A) supplemented with 100 μg/mL lysozyme and 0.01 mM PMSF. After incubation at 4 °C for 30 min, the cells were lysed by sonication (3 cycles @10 s at 10 amps; Soniprep 150) at 4 °C. The lysed cells were then centrifuged at 10,000 rpm for 30 min. To remove the contaminants, the pellet containing the IBs was washed twice in 10 mL of buffer A containing 2% Triton X-100 followed by centrifugation at 10,000 rpm for 30 min, and finally the IBs were washed in 10 mL of buffer A without Triton X-100. The washed IBs (~ 2.9 g wet weight containing 387 mg of protein) was then resuspended in 30 mL of buffer A containing 2 M L-arginine overnight at 4 °C. Solubilized rePEPCK was centrifuged at 10,000 rpm for 30 min and the protein fractions were analyzed by 10% polyacrylamide SDS–PAGE.

2.5. Purification and refolding of rePEPCK

The recombinant protein was incubated with 16 mL of pre-equilibrated glutathione Sepharose 4B resin for 4 h at 4 °C. The resultant glutathione Sepharose 4B resin containing the recombinant protein was loaded into the Econo-Column chromatography columns (BioRad, 1.0 × 50 cm) having 40 mL bed volume. The unbound protein was washed with two column volumes of buffer A until the A_{280} of the flow through was less than 0.1. On-column cleavage of the GST-tag protein was performed by adding 20 U of thrombin per mg of the recombinant protein, and incubated overnight at 4 °C. The protein was subsequently eluted from the resin using 10 mL of elution buffer (50 mM Tris–HCl, pH 7.4, and 1 mM DTT). The purification procedure was performed at 4 °C.

The eluted protein was diluted in buffer A to a final concentration of ~1 mg/mL, and then added dropwise into a 20 fold volume of refolding buffer (50 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 250 mM L-arginine, 5 mM β-mercaptoethanol and 5% glycerol) overnight at 4 °C. The refolded protein was concentrated to a final volume of ~ 5 mL. The protein was buffer exchanged at 4 °C by dialysis against a 50 fold volume of the buffer containing