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Purification and Characterisation of Phosphoenolpyruvate Carboxykinase in *C. jejuni*

Adnan Ayna
Department of Chemistry, Faculty of Sciences and Arts, Bingol University, Bingöl, 12000, Turkey

Abstract*: Analysis of the Campylobacter jejuni genome showed that it lacks two glycolytic enzymes. It did appear to have a full set of gluconeogenic enzymes [1]. This is consistent with experiments that showed C. jejuni cannot use carbohydrates as a carbon source, and instead uses amino acids. These make gluconeogenesis more important than glycolysis for C. jejuni. Despite this unique feature of the microorganism, to date no study was focused on phosphoenolpyruvate carboxykinase in C. jejuni. The objective of this study was to purify and characterize the gluconeogenic enzyme PEP carboxykinase from enteric pathogen C. jejuni. The data revealed that the protein was purified with single chromatographic step at high purity. It was also concluded that the protein is alpha helical as its homologues in other organisms and the enzyme was stable with a temperature of around 42°C.*

1. Introduction

Gluconeogenesis is a metabolic pathway that synthesizes glucose from certain non-carbohydrate carbon substrates such as glucogenic amino acids, triglycerides, pyruvate and lactate. It is a central metabolic process in organisms ranging from bacteria to humans. One of the key enzyme of the pathway is phosphoenolpyruvate carboxykinase (PCK; E.C. 4.1.1.49) which catalyses the decarboxylation reaction and concomitant phosphorylation of oxaloacetate to phosphoenolpyruvate [2]. PCKs studied until date can be divided into two main groups, those which utilises ATP as a substrate, and those which use GTP. Enzymes of the ATP and GTP-dependent classes show varying degrees of primary sequence homology with enzymes within the same group, but no significant similarities exists between PCKs of the two classes. The GTP-dependent PCK enzymes are found to be in monomeric form, whereas the ATP-dependent enzymes may be in different oligomerisation state which could be monomeric (*E. coli;* [3]), dimeric (*Trypanosoma brucei*; [4]), tetrameric, (*Saccharomyces cerevisiae*; [5]) or hexameric (plant; [6]). In addition, PCK of *S. cerevisiae* together with numerous other enzymes within the GTP-dependent class generally contain a highly reactive cysteine residue in their active site [7-9].

C. jejuni cannot metabolise glucose as carbon source as it lacks the gene encoding key metabolic enzyme 6-phosphofructokinase [1]. Initial physiological studies regarding the substrate utilization demonstrated inability of *C. jejuni* to use glucose and other 6-carbon carbohydrates as a carbon source for growth and since then *Campylobacter* species have been considered as asaccharalytic microorganism as reviewed by Hofreuter [10]. A study carried out by Velayudhan and Kelly, showed that phosphoenolpyruvate carboxykinase (PEPCK, Cj0932c) is found to be located in a connection between catabolic and anabolic reactions [11]. The aim of the work described in this paper was to overexpress and purify the PEPCK in *C. jejuni* (cjPEPCK) using recombinant DNA technology and investigate secondary structure and thermal stability of the enzyme via Circular Dichroism.

2. Material and Method

2.1. Preparation and Purification of CjPEPCK

For the expression of cjPEPCK, the constructed vectors were transformed into *E. coli* (Rosetta DE3). A streak of colonies were added into LB media and grown overnight at 37 °C. 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were added into the LB media as *Rosetta* is chloramphenicol resistant and the vector is ampicillin resistant. In the following morning, the culture was diluted with LB medium adding ampicillin and chloramphenicol with the same concentration as overnight culture and grown at 37 ^{o}C. Once the OD600 of the cell suspension reached to 0.1, the temperature was dropped to 20 ^{o}C and Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 200 µM for induction and the cells were grown overnight at 20 ^{o}C in shaking incubator. Bacterial cells were harvested by centrifugation at 4°C for 15 minutes at 4000 rpm next day and suspended in lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole pH 8, 1 mM DTT and 1 Roche complete EDTA free protease tablet). The cell suspension was sonicated and the cell debris was removed by centrifugation at 20000 rpm for 20 min. Subsequent to that, the obtained supernatant was loaded into 2 ml slurry Ni-

NTA (Nickel-Nitrilotriacetic acid) and left on roller at 4 oC for 5 minutes to facilitate binding protein to the resin. Following the binding step, the solutions were centrifuged at 4000 rpm for 2 min and both supernatant and resin were collected to run on the gel. The resin was washed in wash buffer (50 mM Tris-HCl, 200 mM NaCl, 40 mM imidazole pH 8 and 1 mM DTT) to remove the protein that is non-specifically bound to the resin. The proteins were eluted with a buffer containing high imidazole concentration (50 mM Tris-HCl, 200 mM NaCl, 400 mM imidazole pH 8, and 1 mM DTT). The supernatant was filtered with 0.45 µm filter and the concentration of the protein was measured using Bio-Rad assay (800 µl H_2O, 200 µl Bio-Rad assay reagent, and 2 µl protein) after elution.

2.2. Determination of secondary structure and stability of cjPEPCK

All CD experiments were carried out at room temperature using Chirascan-Plus CD spectrometer (Applied PhotoPhysics, UK). The spectra were recorded over a wavelength range of 200-260 nm in the far UV region with a bandwidth of 1nm using a quartz cuvette with 10 mm path length. 5 scans were averaged and baseline corrected. For thermal stability experiments, the changes in the CD spectra over a range of temperature from 5 ^{o}C to 90 ^{o}C, increasing in 5 ^{o}C increments were recorded. The experiments were carried out in buffer containing 20 mM Tris-HCl, 50 mM NaCl for cjPEPCK.

3. Results and Discussions

3.1. Purification

The intact pepck gene was overexpressed in E. coli (*Rosetta* DE3) and purified as described in material and method section. The purity of the proteins judged at >95% based on SDS-PAGE analysis (Figure 1). The expression and purification of enteric pathogen cjPEPCK is shown in Figure 1. The short 6xHis residues remain to be fused to the wild-type cjPEPCK and this has the added advantage of being small enough to prevent interference during enzymatic characterization.

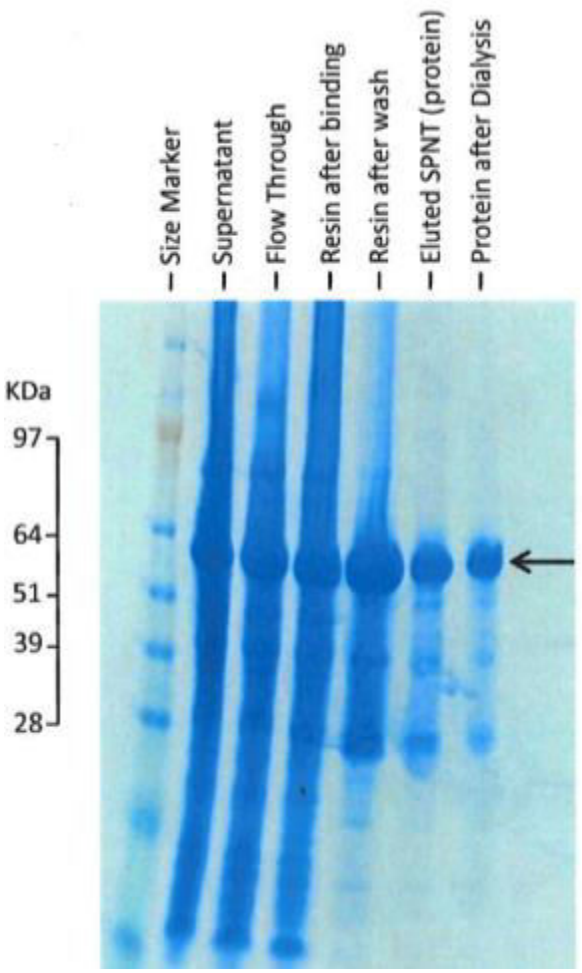

Figure 1. Expression and purification of 6xHis tagged cjPEPCK induced at 20 °C.

3.2. Secondary structure and thermal stability experiments

The enzyme was characterized by far UV Circular Dichroism (CD) to address whether the protein is folded and to estimate the secondary structure of the cjPEPCK as described in material and method. Circular dichroism (CD) is a technique that is commonly used for the determination of the secondary content and to determine whether the protein of interest is folded properly in solution. Additionally, it can also be used to investigate protein-protein and protein ligand interactions. In circular dichroism spectroscopy, differences in the absorption of left-handed polarized light is measured versus right-handed polarized light, which is arisen due to structural asymmetry. All secondary structural elements show a characteristic CD spectrum, if a protein is α-helical dominant then negative bands at 222 nm, 208 nm and a positive band at 193 nm is acquired. While if it is a β sheet, negative band around 218 nm and positive bands at 195 nm is resulted. Lastly, disordered protein has positive band at 210 nm, negative bands near 195 nm [13]. The

Circular Dichroism (CD) spectrum was used to study the secondary structure and thermal stability of the cjPEPCK. The spectrum shown in Figure 2 obtained for the cjPEPCK revealed that the protein is alpha helical as indicated by two intense negative (CD) peaks at approximately 211 and 219 nm suggesting the protein is folded. This finding is consistent with the theoretical finding and also with the data deposited in PDB for all homologues which are all alpha helical [12].

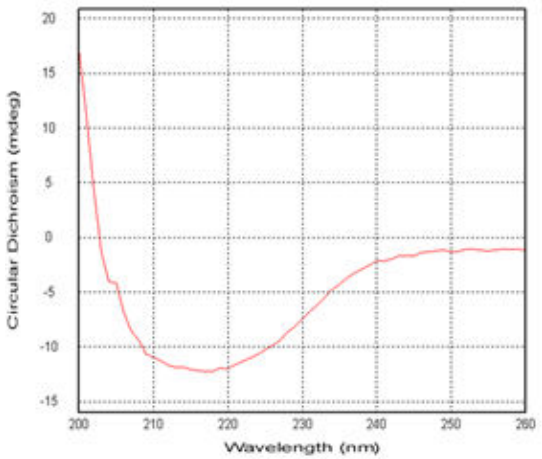

Figure 2. CD spectrum of Apo cjPEPCK. A final concentration of 0.2 mg/ml of the enzyme was used to predict secondary structure, using wavelengths with a range between 200-260 nm. The experiments were carried out in buffer containing 20 mM Tris-HCl pH 8, 50 mM NaCl. A CD spectrum is obtained as a function of wavelength and is reported in mdeg.

Thermal stability of the enzyme was also assessed through CD experiments by following the changes in the CD spectrum over a range of temperature from 5 °C to 90°C in increments of 5°C as explained in material and method. Thermal stability showing ratio of stable folded protein to unstable/denatured protein. The point at which these two traces intersect can be considered as the melting temperature of the protein. An initial change in stability of the protein is seen at 40-45 °C with less than 10% of it begin to unfold. At 60 °C, 100% of the protein has become unfolded or denatured. The thermal denaturation curve revealed a sigmoidal type curve suggesting a stable well folded protein. The native protein is stable up to at least 42°C, with a midpoint of around 50°C (Figure 3). This result is consistent with the literature. Campylobacter species are generally mesophilic, with a growth temperature range from 30 °C to 45 °C and optimal growth at 37 °C or 42 °C. The mesophilic nature of the pathogen is associated with the lack of the gene encoding a cold shock protein which is vital for low temperature adaptation [14]. Bearing this finding in mind, the stability up to 42°C is logical.

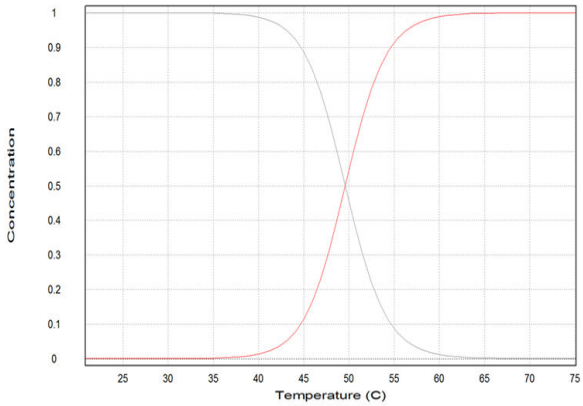

Figure 3. Thermal unfolding of recombinant cjPEPCK. The changes in the CD spectra over a range of temperature from 5 ^{o}C to 90 ^{o}C, increasing in 5 ^{o}C increments were recorded. The change in protein structure is determined at wavelength, 222 nm.

4. Conclusions

The presence of the PEP carboxykinase and absence of 6-phosphofructokinase in the genome of *C. jejuni* makes gluconeogenesis more important than glycolysis for the pathogen as it cannot utilize but can produce glucose. One important enzyme of gluconeogenesis as mentioned previous sections is Phosphoenolpyruvate carboxykinase (PEPCK), an enzyme in the gluconeogenic pathway converting oxaloacetate into phosphoenolpyruvate and carbon dioxide. Herein we aimed to overexpress and purify the enzyme of *C. jejuni* using recombinant technology and study secondary structure and thermal stability of the enzyme. The enzyme was purified at high purity assessed by SDS-PAGE. The CD analysis suggested the protein is folded and stable up to 40°C.

5. References

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