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<th>Targeting glutamate synthase for tuberculosis drug development</th>
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Introduction

Tuberculosis is a contagious disease mainly caused by the infection of high guanine plus cytosine content Gram-positive mycobacteria, mainly Mycobacterium tuberculosis. According to the World Health Organization (WHO) 2007 Tuberculosis Facts, the annual incidences of tuberculosis are stable or falling in all six WHO regions and have peaked globally. Nonetheless, the total number of cases in developing countries is still increasing. Tuberculosis remains a leading cause of death worldwide and is a potentially lethal infectious disease in Hong Kong.

The complete genome sequence of M tuberculosis enables better understanding of this bacterium and the potential targets that may be useful in therapeutic interventions. By using postgenomic methods, identification of essential genes opened a window of opportunity to pursue novel therapeutic strategies to develop antituberculosis agents. Recent research identified a group of essential genes based on a postgenomic in silico approach, and suggested that the glutamate synthase is one of the essential genes in the M tuberculosis life cycle. As both large and small subunits of glutamate synthase, gltB and gltD respectively, are absent from the human genome, targeting the glutamate synthase may be an excellent means of treating tuberculosis. This study may provide the foundation for new therapeutic strategies against tuberculosis, by cloning and purifying both subunits of the M tuberculosis glutamate synthase.

Methods

This study was conducted from January to June 2007. The E coli strain TOP10 was used for gene cloning and deoxyribonucleic acid (DNA) sequencing, whereas BL21 (DE3) was used for protein expression. pGEM-T Easy Vector (Promega, USA) was used for cloning polymerase chain reaction (PCR) products, whereas pET-28a(+) vector (Novagen, USA) was used for protein expressions.

Oligonucleotide primers were purchased from Tech Dragon Limited in Hong Kong, and DNA sequencing was also performed by this company. Amplification of PCR was performed with Pwo SuperYield DNA Polymerase (Roche) using genomic DNA of M tuberculosis H37Rv provided by colleagues. The gltB and gltD open reading frames were amplified by PCR. The PCR products were cloned into pGEM-T Easy Vector (Promega) and then transformed into E coli strain TOP10 for propagating plasmids and then cloned into the NheI/HindIII site of pET-28a(+) Vector (Novagen) to yield plasmid pET28a-gltB, pET28a-gltD and pET28a-gltBD for protein expressions in E coli strain BL21 (DE3).

Plasmids pET28a-gltB and pET28a-gltD that encoded the α and β subunits of glutamate synthase were transformed into E coli BL21 (DE3). The cells were maintained in LB broth supplemented with 50 μg/mL of kanamycin. For protein expression, 1 M of IPTG was added to the culture to a final concentration of 0.5 mM IPTG to induce protein expression when the cells grow to the log phase (OD600 ≈0.6). The cells were further incubated at room temperature or 37°C for protein expression. For protein expression with addition of chemical chaperones, different concentrations of dimethyl sulfoxide and 4-phenylbutyric acid were added to the culture before IPTG induction.
After expression of proteins, cells were pelleted by centrifugation and extraction buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol, 1% Triton-X 100, 1X protease inhibitor cocktail [Roche]) was added to the pellet in a 1:100 ratio. The cell suspension was incubated on ice for 30 minutes and then sonicated by a tip probe sonicator. The soluble protein fraction and insoluble protein fraction were separated by centrifugation. For refolding of the α subunit, the insoluble fraction was dissolved in solubilising buffer (6 M urea, 50 mM Tris, pH 7.5, 20 mM imidazole, 0.3 M NaCl, 10 mM β-mercaptoethanol, 1X protease inhibitor cocktail [Roche]) in a 1:50 ratio. The solubilised proteins was then rapidly diluted in 1 L of extraction buffer for protein refolding. This protein solution was centrifuged at 24000x g to remove the insoluble fraction, and the supernatant was collected for protein purification.

Both of the subunits were purified by using HisTrap FF column (Amersham Biosciences). The column was equilibrated in a 3 bed volume of 20 mM imidazole in elution buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 10 mM β-mercaptoethanol, 1% Triton-X 100, 1X protease inhibitor cocktail [Roche]), and then the protein solution was loaded on the column. For the α subunit, a column was washed with 50 mM imidazole in elution buffer, and the α subunit was eluted using 300 mM imidazole in elution buffer. For the β subunit, a column was washed by 75 mM imidazole in elution buffer, and the β subunit was eluted using 100 mM imidazole in elution buffer. Regarding screening for the protein expressions with added chemical chaperones, Ni-NTA Magnetic Agarose Beads (Qiagen) were used for purification of the α subunit. The purification step was carried out according to the manufacturer’s instructions. Briefly, the soluble fraction of the cell extracts were loaded on the pre-washed beads and the beads were washed by extraction buffer. Subsequently, proteins were eluted using 500 mM imidazole in elution buffer. The purification of α and β subunits of glutamate synthase were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Glutamate synthase activity was detected by the assay described by Miller and Stadtman in 1972 with some modifications. Glutamate synthase activity was determined by measuring the rate of oxidation of NADPH at 340 nm. Standard assay mixtures (250 μL) contained 0.16 mM NADPH, 1 mM α-ketoglutaric acid, 2 mM L-glutamine, 1 mM EDTA, 50 mM potassium phosphate buffer, pH 7.5, and sufficient enzyme to produce an absorbance change at 340 nm at 30°C.

The absorbance at 280 nm and 440 nm were detected by spectrophotometer at room temperature. Also, for the detection of the flavin bound to the subunits, the emission spectrum of the purified subunits were recorded between 450 and 600 nm with excitation at 440 nm in a spectrofluorometer.

Results

The glutamate synthase consisted of two subunits, GltB and GltD, which were cloned and purified independently. The genes were cloned and ligated into a heterologous protein expression vector in E. coli using a hexahistidine tag for facile purification. The α subunit misfolded upon expression in E. coli, but refolding and purification of the alpha subunit of the protein was successfully performed (Fig 1a). The β subunit expressed in the soluble form under low temperature expression conditions and was expressed and purified successfully, using immobilised metal affinity chromatography (Fig 1b).

Discussion

Previous research had identified a group of essential genes using a postgenomic in silico approach, and suggested that the glutamate synthase was an essential gene for the M. tuberculosis life cycle. By comparing the deduced amino acid sequences of M. tuberculosis gltB and gltD with other
mycobacteria, it was inferred that these proteins were highly conserved. This suggested that the cloning and expression of glutamate synthase genes might provide a foundation for the development of inhibitors against *Mycobacterium* growth.

We aimed to express the relevant glutamate synthase in a soluble and functional form. However, only the β subunit could be expressed in a soluble form. Even with assistance of chemical chaperones, the α subunit was expressed as an insoluble fraction. We therefore refolded the protein before purification.

Previous studies suggested that glutamate synthase was a multicomponent iron-sulphur flavoprotein, composed of two dissimilar subunits (α and β). The α subunit was believed to contain one flavin mononucleotide and β subunit one flavin adenine dinucleotide (FAD). As the glutamate synthase activity depends on the cooperation of both flavin co-factors, which the β subunit serves to transfer reducing equivalents from NADPH to the FMN co-factor through the FAD co-factor and the iron-sulfur cluster. Therefore, loss of co-factor during refolding may lead to loss of glutamate synthase activity. We also noted that in future we should scan the atomic absorption spectrum of both subunits to assess whether the iron-sulfur clusters persist in the purified glutamate synthase.

In this project, we successfully cloned the subunits, *gltB* and *gltD*, of glutamate synthase and the proteins encoded by these two open reading frames were expressed in *E coli* BL21 (DE3). The α subunit was expressed in an insoluble form and the β subunit as a soluble fraction. We were able to purify the α subunit after refolding. Further studies are required to fully characterise the purified and reconstituted enzyme before targeting this protein as part of a therapeutic strategy against tuberculosis.

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**References**