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Purification and characterization of a functionally active *Mycobacterium tuberculosis* pyrroline-5-carboxylate reductase

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Abstract

Pyrroline-5-carboxylate reductase (P5CR) plays an important role in the survival of *Mycobacterium tuberculosis and* is related to virulence of this pathogen. RT-PCR analysis indicated that *proC*, encoding P5CR, was expressed at the transcriptional level cultured in vitro. The His-rMtP5CR with an N-terminal His-tag (His-rMtP5CR) was firstly purified in *Escherichia coli* and rMtP5CR was obtained by removal of the N-terminal fusion partner using enterokinase. His-rMtP5CR had considerable β-pleated sheet analyzed by circular dichroism spectroscopy. The effect of pH, temperature, cations, denaturants, and detergents on the purified enzyme activity and stability was characterized. The N-terminal fusion partner was found to have very little effect on the biochemical properties of P5CR.

Keywords: *Mycobacterium tuberculosis*; Pyrroline-5-carboxylate reductase; *Rv0500*; *proC*; Protein expression

Tuberculosis remains one of the world's greatest health problems whose morbidity and mortality is increasing. The appalling extent of infection by *Mycobacterium tuberculosis*, the exclusively human pathogen, covers one of every three people, a total of 2 billions persons worldwide [1,2]. About 8 million people develop active tuberculosis, and more than 2 million die of this disease each year, and over 95% of those are in developing countries.

The increasing global health threat of tuberculosis is due to both the synergistic pathology of coinfection with the human immunodeficiency virus (HIV), and the continued evolution of multi-drug resistant (MDR) strains [3,4].

According to the 2004 Global tuberculosis (TB) Control Report of the WHO, there are 300,000 new cases per year of MDR-TB worldwide, and 79% of MDR-TB cases are now "super strains" resistant to at least three of the four main drugs used to treat TB [5].

The spread of resistant disease is also an economic trouble. Drugs have to be given for 18–24 months, compared with 6 months for standard treatment. The cost of treating an infected patient of MDR-TB is, on average, 100 times greater than that for susceptible patient. So, if resistant TB continues to disseminate, the costs of treating these cases would be astronomical.

Hence, faster acting and potent new drugs to better combat TB, including MDR-TB, are needed badly.

Mycobacterium tuberculosis has the potential to make all the essential amino acids, vitamins, and enzyme cofactors, to survive in the host.

Pyrroline-5-carboxylate reductase (P5CR)[1] belongs to the P5CR family, catalyzes the NADPH-dependent conversion of pyrroline-5-carboxylate (P5C) to proline:

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[1] *Abbreviations used:* P5C, pyrroline-5-carboxylate; P5CR, pyrroline-5-carboxylate reductase; His-rMtP5CR, *M. tuberculosis* P5CR expressed as a His-tagged protein in *E. coli*; rMtP5CR, *M. tuberculosis* P5CR expressed as a His-tagged protein in *E. coli* after removal of the His tag.

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The third and terminal step in proline biosynthesis, and this reaction is reversible at certain condition.

P5CR is correlation with intermediary metabolism and respiration of *M. tuberculosis*. *ProC* (*Rv0500*), the gene encoding P5CR, was demonstrated to have essential role in *M. tuberculosis* virulence and be required for mycobacterium growth. Targeted disruption of the *proC* gene resulted in auxotrophic mutant that had reduced intracellular survival in macrophages in vitro. In immunocompromised SCID mice, the *proC* mutant showed significant attenuation avirulent. In contrast, *proC* mutant was avirulent in immunocompetent mice. In vaccination experiments for mice, *proC* mutant showed protection equivalent to or greater than that of BCG, the current vaccine [6,7]. Therefore, P5CR may represent a good drug-design target site for more effective agents against tuberculosis.

In this manuscript, we detected the expression of P5CR at transcriptional level in cultured *M. tuberculosis*, first purified His-rMtP5CR protein about 35.6-kDa from *M. tuberculosis* in *Escherichia coli* and obtained a rMtP5CR about 32-kDa by cleaving the N-terminal fusion partner from the purified fraction using enterokinase. The enzyme properties of rMtP5CR and His-rMtP5CR were compared in detail, which could provide needed data for the further design of anti-TB drugs with high potency and selectivity efficacy.

Materials and methods

All the chemicals were purchased from Sigma (Sigma–Aldrich) unless otherwise specified. DNA manipulation enzymes, including polymerase, restriction endonucleases and ligase were from New England Biolabs (Beijing, China). pET30a vector and enterokinase cleavage capture Kit were supplied by Novagen (USA). pUC18 vector was purchased from TaKaRa (Dalian, China). The Ni–NTA His-binding resin and columns were bought from Qiagen (Germany). *M. tuberculosis* H37Rv (ATCC 93009), streptomycin, geomycine and kanamycin were supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Custom oligonucleotide primers were commercially synthesized by Shanghai Sangon Biological Engineering and Technology services (Shanghai, China). Gel extract, bacterial genome extract, and plasmid extract kits were provided by Watson Biotechnologies (Shanghai, China). Nondenatured protein molecular weight maker kit and apoferritin (native dimer) (Sigma–Aldrich).

Bacterial strains and culture conditions

Escherichia coli strains DH5α and BL21 (DE3) were used for cloning and expression respectively, both were cultured in Luria–Bertani (LB) medium and on agar added in 25 μg/ml kanamycin. *M. tuberculosis* was grown in Middlebrook 7H9 broth and on Middlebrook 7H10 agar, added in 10% (v/v) oleic acid–albumin–dextrose complex.

Primers design

Coding sequence for *proC*, in accordance with the published genome (GenBank Accession No. NC_000962), was used to design primers for polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) amplification. The upper primer is: 5′-AAG GAT CCA TGC TTT TCG GCA TGG CAA G-3′, and the down primer is: 5′-TAT AAG CTT TCA TTC CGG TGT AAT TCT GAG CTG C-3′, including *Bam*HI and *Hind*III restriction endonucleases sites, respectively.

Duplex RT-PCR

One microgram RNA was added to 1 μl of random hexamer primer (0.2 μg), and water was supplemented to 12 μl. Being cultured at 70 °C for 5 min, the chilled mixture was added to 4 μl of 5× reaction buffer (Bio Basic, Canada), 1 μl of RNase inhibitor (20 U), and 2 μl dNTP mixture (10 mM). The samples were cultured at 25 °C for 5 min and 1 μl of AMV reverse transcriptase (20 U) (Bio Basic, Canada) was added. The mixture was cultured at 25 °C for 10 min and then at 42 °C for 60 min, heated to 70 °C for 10 min, and chilled on ice. Control reaction to monitor DNA contamination was conducted with prepared RNA in a mock RT reaction without the presence of AMV reverse transcriptase.

Fifty microliter PCR solutions consist of 5 μl of 10× reaction buffer, 5 μl of dNTP (2 mM, respectively), template nucleic acid from the appropriate RT reaction mixture, 1 μl of *Taq* polymerase, and 250 pmol primers. PCR amplifications were done with 20 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1.5 min.

Construction of proC gene containing plasmid

The 50 μl PCR mixture containing 5 μl of 10× reaction buffer, 5 μl of dNTP (2 mM respectively), 2.5 μl of *M. tuberculosis* H37Rv genomic DNA (50 ng/μl), 1 μl of *Taq* polymerase (2 U/μl), and 250 pmol primers, was performed using the same temperature condition as RT-PCR, but the reaction conducted with 34 cycles instead of 20.

The purified PCR product and pUC18 vector both were digested by *Bam*HI and *Hind*III, extracted with gel extract, and then ligated together using T4 DNA ligase overnight at 4 °C. The ligation solution was transformed into *E. coli* strain DH5α and screened on LB plates containing 100 μg/ml ampicillin. One positive combinant clone was selected and sequenced to confirm that *proC* was in the proper