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THE HIGH-THROUGHPUT SCREENING SYSTEM FOR INHIBITOR *MYCOBACTERIUM TUBERCULOSIS* COMPOUNDS BASED ON ATP HYDROLYSIS ACTIVITY OF RECOMBINANT PROTEIN CLPC1

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SUMMARY

The global Tuberculosis (TB) rate continues to increase by 1% per year with the widespread of drug-resistant TB. Therefore, the development and research to find new anti-TB drugs are becoming an extremely urgent mission. To be able to screen lead anti-tuberculosis drugs, currently, researchers have to carry out directly on the cells of *Mycobacterium tuberculosis* and to be performed in bio-security facilities level 3 or 4, to prevent infection from pathogens. However, our results demonstrated that the screening of anti-TB drug candidates can be implemented in bio-security facilities level 1 laboratory with the *Escherichia coli* cell extraction and recombinant ClpC1 protein - an integral part of the *Mycobacterium tuberculosis* genome. We focused on the ATP hydrolysis activity of ClpC1 to create a specific research direction for the high-throughput anti-tuberculosis drug screening system. ClpC1 protein was overexpressed and purified with functionally characterized (93.5 kDa). The steady-state growth of recombinant ClpC1 protein in Luria-Bertani (LB) broth High Salt medium was maintained and stabilized after extraction. The determined ATPase activity of ClpC1 was performed by measuring the released phosphate from the reaction. Ecumicin was chosen to be a control compound with expected ATP hydrolysis activities (Hill coefficient = 1,19 ± 0,217; K_d value = 0,52 ± 0,275). We tested this high throughput screening system with ten anti-TB drugs to evaluate the effectiveness of our screening system. Based on these results, we built a complete high-throughput screening system anti-tuberculosis drug safety and quickly.

Keywords: ATPase; ClpC1; High throughput screening system; *Mycobacterium tuberculosis*; Recombinant protein

INTRODUCTION

Tuberculosis is supposed to be one of the deadliest human diseases but has ever been recognized as such. Globally, TB incidence is falling at about 1% per year, with an estimated 54 million lives were saved through TB diagnosis and treatment between 2000 and 2017 (WHO, 2018). However, the distraction in TB control programs, the outbreak of the HIV/AIDS epidemic, and migration have prompted the rise of tuberculosis. Although many drugs have been developed recently and there are special courses for preventing and reducing TB, it has still a huge threat to the world's population (Wermuth, 2006; Zumla *et al*., 2013). Thus, ending the TB

epidemic by 2030 is among the health targets of the Sustainable Development Goals (WHO, 2018).

The Clp ATPase is a member of the AAA + superfamily ATPase with the characteristics of one or two nucleotide-binding domains (Hoskins *et al*., 1998; Martin *et al*., 2008). In *Escherichia coli*, Clp ATPase is the most widely studied objects, with increase intrinsic ATPase activity with the presence of ClpP and substrates (Kim *et al*., 2000; Botos *et al*., 2004; Pavan *et al*., 2016). Many Clp ATPases also shows the chaperone activities independently of their roles in protein degradation, such as ClpC. The purified ClpC forms as a monomer-dimer mixture, but under the effect of magnesium, ATP or ATP analogs will promote the association of ClpC to form a hexamer (Hoskins *et al*., 1998). ClpC has low intrinsic ATPase activity and depends on co-factors to get the action of chaperones. ClpC has two ATP-binding sites, one of the binding sites plays a role in hexamer formation, while the other position needs for ATP hydrolysis (Meliana, 2009; Mary *et al*., 2019).

The purpose of this study, based on fluorescence indications in cellular biological responses -ATPase, was to screen for active anti-TB drugs. Initially we tested with some anti-TB compounds of First-line and Second-line, and positive control drug Ecumicin - a powerful antibiotic discovered at Myongji University, Korea (Gao *et al*., 2015; Lee, Suh, 2016).

MATERIALS AND METHODS

Bacterial Strains and Medium Cultures

TOP10 and Rosetta 2 (DE3) of *Escherichia coli* (Novagen) were used in cloning, protein expression, and purification experiments. LB (Luria-Bertani) Broth High Salt medium (MB cell, Korea), LB (Luria-Bertani) Agar High Salt medium (MB cell, Korea) were used for expressing recombinant protein. Fifty µg/mL of Kanamycin (Sigma, USA) was supplemented.

Cloning, Expression and Purification Recombinant Protein ClpC1

The *M*. tuberculosis ClpC1 was amplified with the primer pair of 5'-TTCACCATGGGCTTCGAACGATTTACCG ACC-3' and 5'-GTAAAAGCTTCGCTCCAGCCTTGGCCAG ATC-3'. The amplified fragments were sequentially cloned into pET28a(+) at the cognate restriction sites and generated pET-ClpC1. The recombinant ClpC1 protein was overexpressed in *Escherichia coli* Rosetta 2 (DE3) by heat shock method. Expression protein by IPTG (isopropyl 1-thio-β-D galactopyranoside -Sigma) after O.D of the culture was reached to 0,7 at A_{600} nm according to Martinou *et al*. (2003) and Pavan *et al*. (2016).

Ni-TED spin column (Macherey-Nagel) was used to purify the recombinant ClpC1 protein. Using SDS PAGE Mini-PROTEAN Electrophoresis System (Bio-rad, USA) gel electrophoresis, insoluble fraction, soluble fraction and the eluted protein were analyzed with 12 % of gel. The gel was then incubated with a staining solution (EZ - Gel Staining solution, DOGEN) (Nagel, 2011). The protein concentration was determined before use.

Measurement Concentration Protein and Exchanged Buffer

Protein concentration was measured by the Bradford method in a Microliter plate. We prepared three dilutions of a protein standard (Bovine Serum Albumin - BSA), which is representative of the protein solution to be tested. The linear range of the assay was 8 µg/mL to approximately 80 µg/mL. Protein solutions were normally assayed in triplicate. We measured the absorbance at 595 nm on an Infinite® 200 PRO (Tecan, Switzerland) (Bradford, 1976). The buffer containing 50 mM Tris-HCl, 100 mM KCl, 8 mM $MgCl_2$, pH 7,5 was used for elution for using PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB, Sweden).

ATPase Activity Assay

BIOMOL® GREEN Reagent: The determination ATPase activity of ClpC1 was measured by monitoring the released phosphate level using BIOMOL® Green (Enzo life Science, Farmingdale, NY). The development of the green color intensity was monitored by the absorbance at 620 nm. We converted to the amount of phosphate released using the phosphate standard curve. A graph of phosphate released versus concentration of ATP was plotted with the OriginPro 8 program. The curve was fitted by Hill equations as follows; y = START + (END - START) * x^n / (k^n + x^n) (Ito, Akiyama, 2005).

The Anti-tuberculosis Drugs and Ecumicin

Ten anti-tubercular compounds have been developed and used in different combinations and circumstances. All of the drugs were provided by the Institute for Tuberculosis Research (Table 1). In addition, Ecumicn was isolated from a methanolic mycelial extract of *Nonomuraea* sp. MJM 5123, and supported by the Center for Nutraceutical and Pharmaceutical Materials.

Table 1. First- and second-line anti-tuberculosis drugs based on the World Health Organization classification (Sacchettini *et al*., 2008).

	Antibiotic	**Mechanism and target**	**The mutation associated with resistance**
First-line	Isoniazid (INH)	Inhibits mycolic acid synthesis; The primary target is InhA and secondary objectives are KasA and DfrA	*katG* (required for drug activation); *inhA* (promoter mutations)
	Rifampicin (RPM)	Inhibits transcription; RNA polymerase β-subunit	*rpoB*
	Ethambutol (EMB)	Inhibits arabinogalactan synthesis; possibly EmbB	*embB*
	Pyrazinamide (PZA)	Unknown (possible inhibits FAS-I or alters membrane energetics)	*pncA* (required for drug activation)
	Streptomycin (SM)	Inhibits protein synthesis 30S ribosomal subunit	*rpsL* and *rrs*
Second-line	Moxifloxacin (MOX)	Inhibits DNA gyrase	*gyrB*
	Clofazimine (CLF)	Inhibits uncharacterized oxidoreductase CzcO-like; Intercalation DNA	unknown
	Linezolid (LIZ)	Inhibit 23S ribosomal RNA	*rrn4* and *rrn5*
	Pretomanid (PA-824)	Unknown (possible inhibits FAS or Nucleoid associated protein Lsr2)	*fas* and *Isr2*
	Capreomycin (CAP)	Inhibits protein synthesis	*tlyA* and *rrs*

RESULTS AND DISCUSSION

Cloning, Expression and Purification of the Recombinant ClpC1 Protein

LB (Luria-Bertani) Broth High Salt is the most commonly used medium for culturing *E. coli* and easy to make. Besides, the LB Broth medium was also suitable to be used to deter the growth of plasmid-free cells (Sezonov *et al.*, 2007). In this study, we used 6x–His tag vectors to express recombinant ClpC1 protein, thus, this medium is even more relevant. His-tagged proteins can be recovered by immobilized metal ion affinity chromatography using Ni^{2+} or Co^{2+} - loaded nitrilotriacetic acid-agarose resins. Due to the above reasons, Luria-Bertani (LB) broth medium was selected for use as the culture medium for this high-throughput screening system.

Our PCR product was initially cloned into pET28a(+) expression vector. After expression with *E. Coli* Rosetta 2 (DE3) and identification on 12 % gel of SDS – PAGE, Protino® Ni-TED was used to purification. Ni-TED product enabled fast and convenient purification of recombinant polyhistidine-tagged proteins by immobilized metal ion affinity chromatography. Binding of protein was based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni^{2+} ions. This process was reproducible and did not affect the target protein structure (Loughran *et al.*, 2017). As a result, the target protein of excellent purity was eluted from the column by elution buffer containing Imidazole 250 mM. The purified recombinant protein ClpC1 has a predicted molecular weight of approximately 93.5 kDa (Figure 1).

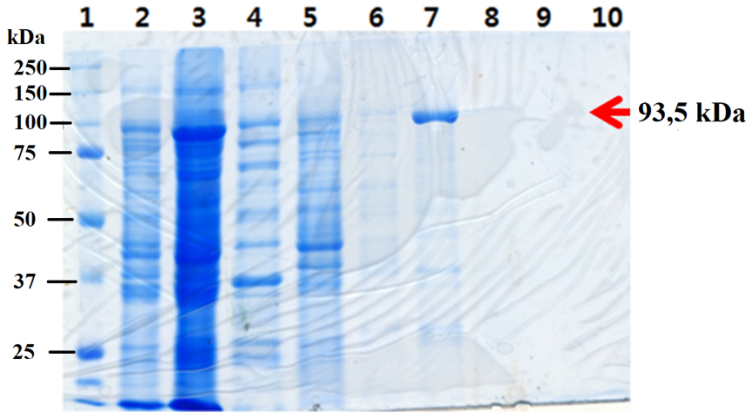

Figure 1. Electrophoretic analysis of affinity chromatography using Ni-TED resin for purification of recombinant *Mycobacterium tuberculosis* ClpC1. Lane 1, BIO-RAD Precision Plus Protein™ Standards; lane 2, Non-induced pET28a(+)/ClpC1; lane 3, Induced pET28a(+)/ClpC1; lane 4, Loading flow; lane 5, Washing flow fraction 1; lane 6, Washing flow fraction 2; lane 7, elution fraction 1; lane 8, elution fraction 2; lane 9, elution fraction 3; lane 10, elution fraction 4.

ClpC1 Displays Basal ATPase Activity

By the association with ClpP1/ P2 complex, ClpC1 supported ATP-dependent protein degradation. The recombinant ClpC1 was determined for analysis if it had an inherent ATPase activity (Kar *et al.*, 2008; Fraga *et al.*, 2019). We used the radioactive of ATP as a substrate and quantified the radioactive inorganic phosphate generated upon its enzymatic hydrolysis by ClpC1. Our recombinant protein was found to contain significant ATPase activity with the basal ATPase activity of ClpC1. The ATP hydrolysis activity of ClpC1 was recorded when the ATP concentration in the reaction gradually

increased from 10 µM, and reached the hight value at 1 mM ATP (Figure 2). Therefore, our recombinant ClpC1 protein has been shown to be fully active in ATP hydrolysis.

In addition, the determination of the stability of ATPase activity in ClpC1 protein showed that 10 days after ClpC1 protein was purified by Ni-TED column, the ATPase activity was not changed significantly and remained the original activity (Figure 3).

Testing the ATPase Activity of Fluorescence Free - phosphate Released Assay with Ecumicin and Anti-TB Drugs

Ecumicin (Formula: $C_{83}H_{134}N_{14}O_{17}$) is a macrocyclic peptide produced, including 13 natural and highly methoxylated unnatural amino acids. Previous experiments identified the *M. tuberculosis* ClpC1 as the putative target with the function is ATP hydrolysis, and this was confirmed by a drug affinity response test under the effect of Ecumicin. In the presence of low concentrations of Ecumicin, ATP hydrolysis by ClpC1 increased up to 2- to 3- fold (Gao *et al.*, 2015). This effect of Ecumicin on ATP hydrolysis was highly cooperative and showed a Hill coefficient = 1,19 ± 0,217 and indicated positive cooperative binding of ATP with the dissociation constant value K_d = 0,52 ± 0,275 (Table 2). The data demonstrated that the killing seemed to result from binding to ClpC1, with probably little or no barrier to Ecumicin's entry into the bacteria. Thus, Ecumicin was used as a positive compound for anti-TB drug screening in our screening system.

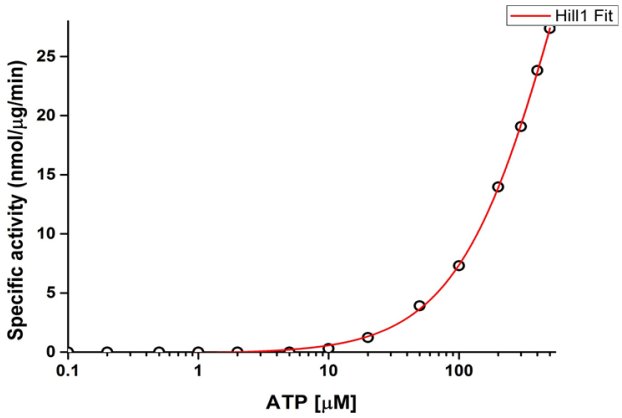

Figure 2. Basal ATPase activity of recombinant ClpC1 protein.

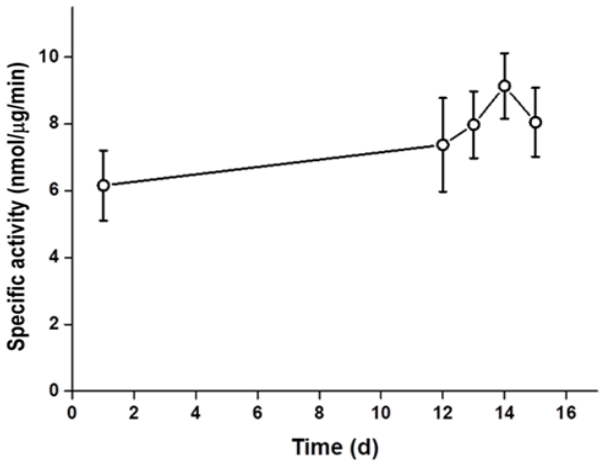

Figure 3. The stability of recombinant ClpC1 protein in ATPase activity.

Table 2. ATPase activity of recombinant ClpC1 protein.

	Value	Standard error
K_d	50,7 µM	2,7
Hill Coefficient (n_H)	1,7	0,14

Table 3. ATPase activity of ClpC1 with Ecumicin.

	Value	Standard error
K_d	0,52 µM	0,275
Hill Coefficient (n_H)	1,19	0,217

To measure ATPase activity of ClpC1, there are many ATPase assays available, such as based on the amount of free-phosphate released or ADP generated from the reaction. The previous study indicated that ADP-Glo™ reagent, which measures ADP, was relatively insensitive but has the advantage, allowing us to measure low amounts of ADP production by using the slope of the progress curve and compatible with high ATP concentrations (Worzella, Larson, 2006; Cali *et al*., 2008). While BIOMOL® GREEN reagent, which is an endpoint assay to measure free-phosphate, can be sensitive in the single-digit micromolar range. It can suffer from interference by compounds that interact with the dye and can have trouble with high ATP concentrations because of background levels of phosphate and the acidity of the reagent causes spontaneous ATP hydrolysis or interference caused by compound absorbance at 620 nm. However, with the advantage not only the price cheaper, faster screening time, but also more comfortable to carry out than other commercial ADP detection reagents, BIOMOL® GREEN reagent was selected to develop the screening potential anti-TB drugs system.

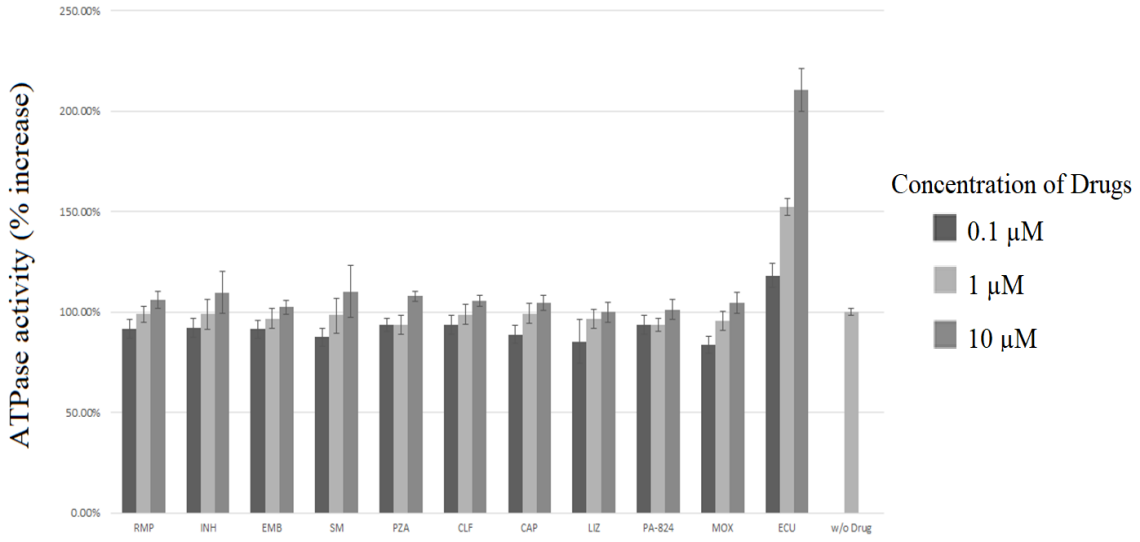

Figure 4. The ClpC1 ATPase activity in response to drugs treatment.

We implemented both of these residues with final concentrations of Ecumicin (0,1; 1 and 10 µM), and also investigated the effects of 10 anti-TB drugs: Rifampicin (RMP); Isoniazid (INH); Ethambutol (EMB) dihydrochloride; Streptomycin (SM) sulfate; Pyrazinamide (PZA); Clofazimine (CLF); Capreomycin (CAP) sulfate; Linezolid (LIZ); Pretomanid (PA-824); Moxifloxacin (MOX). The results show that most drugs also affect the ATP

hydrolysis of ClpC1 in the gradual increase in drug concentration. However, these activities are much lower when compared to Ecumicin and not significantly change with the non-treatment case. This data has been demonstrated which is entirely consistent with all of the previous view, Rifampicin inhibits transcription RNA polymerase B-subunit, Isoniazid inhibits mycolic acid synthesis, Ethambutol inhibits arabinogalactan synthesis, or both of Capreomycin and Streptomycin inhibits protein synthesis (Sacchettini *et al*., 2008). It also clearly indicates that the ClpC1 protein is not the target to inhibit by current first-line and second-line antituberculosis drugs. This result confirmed that our screening system worked well (Figure 4).

CONCLUSION

First, recombinant ClpC1 protein was successfully overexpressed, purified and functionally characterized (93.5 kDa). The steady-state growth in Luria-Bertani (LB) broth High Salt medium are maintained and stabilized after extraction with concentrations approximately ClpC1 = 0,523 ± 0,021 mg/ml. In addition, *E. coli* Rosetta 2 (DE3) proved suitable to express recombinant protein ClpC1. Second, the ATPase activity of ClpC1 was carried out by measuring the released phosphate (BIOMOL® GREEN reagent). This activity was not changed significantly and remained the original activity in 10 days. Ecumicin was chosen becoming control compounds with expected ATP hydrolysis activities (Hill coefficient = 1,19 ± 0,217, K_d value = 0,52 ± 0,275). Notably, with the advantage not only of the effective cost, faster screening time but also easier to carry out than other commercial ADP detection reagents, thus, BIOMOL® GREEN reagent was selected to develop the screening potential anti-TB drugs system. Third, Ecumicin analogues and 10 anti-TB drugs were implemented with this screening system. Ecumicin analogues also present the result in interaction with recombinant protein ClpC1. However, under the effect of 10 anti-TB drugs, the ATPase activity does not significantly change with the non-treatment. This indicated that the ClpC1 protein was not targeted to inhibit by current first-line and second-line anti-tuberculosis drugs. These results confirmed that our screening system worked well.

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NGHIÊN CỨU PHÁT TRIỂN HỆ THỐNG SÀNG LỌC CÁC HỢP CHẤT CÓ TIỀM NĂNG KHÁNG LAO DỰA TRÊN HOẠT ĐỘNG THỦY PHÂN ATP CỦA PROTEIN CLPC1

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SUMMARY

Tỷ lệ lao toàn cầu tiếp tục tăng 1% mỗi năm với tình trạng lao kháng thuốc lan rộng. Do đó,

việc phát triển và nghiên cứu để tìm ra các hợp chất có tiềm năng kháng lao mới đang trở thành một nhiệm vụ cực kỳ cấp bách. Kết quả nghiên cứu của chúng tôi đã chứng minh rằng việc thực hiện sàng lọc các hợp chất kháng lao có thể thực hiện trong phòng thí nghiệm an toàn sinh học cấp 1 với tế bào *Escherichia coli* và protein ClpC1 tái tổ hợp. Chúng tôi đã tập trung vào hoạt động thủy phân ATP của ClpC1 để tạo ra một hướng nghiên cứu phát triển hệ thống sàng lọc các hợp chất có tiềm năng. Protein ClpC1 tái tổ hợp đã được biểu hiện thành công và tinh sạch với đầy đủ chức năng (93,5 kDa). Sự tăng trưởng ổn định của protein ClpC1 tái tổ hợp trong môi trường nuôi cấy Luria-Bertani (LB) được duy trì ổn định sau khi tách chiết. Hoạt tính ATPase xác định của ClpC1 được thực hiện bằng cách đo lượng phosphate giải phóng từ phản ứng. Ecumicin được chọn trở thành hợp chất đối chứng với các hoạt động thủy phân ATP tích cực (n_H = 1,19 ± 0,217; K_d = 0,52 ± 0,275). Đề tài đã thử nghiệm với mười loại thuốc kháng lao hiện nay để đánh giá hiệu quả của hệ thống sàng lọc của chúng tôi. Dựa trên những kết quả này, chúng tôi đã nghiên cứu một hướng đi mới nhằm phát triển hệ thống sàng lọc các hợp chất kháng lao hoàn chỉnh, an toàn và nhanh chóng.

Từ khoá: *ATPase; ClpC1; Hệ thống sàng lọc thông lượng cao; Mycobacterium tuberculosis; Protein tái tổ hợp.*