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Novel mutations in *katG* gene of a clinical isolate of isoniazid-resistant *Mycobacterium tuberculosis*

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Abstract: Most of isoniazid-resistant *Mycobacterium tuberculosis* evolved due to mutation in the *katG* gene encoding catalase-peroxidase. A set of new mutations, namely T1310C, G1388T, G1481A, T1553C, and A1660G, which correspond to amino acid substitutions of L437P, R463L, G494D, I518T, and K554E, in the *katG* gene of the L10 clinical isolate *M. tuberculosis* was identified. The wild-type and mutant KatG proteins were expressed in *Escherichia coli* BL21(DE3) as a protein of 80 kDa based on sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis. The mutant KatG protein exhibited catalase and peroxidase activities of 4.6% and 24.8% toward its wild type, respectively, and retained 19.4% isoniazid oxidation activity. The structure modelling study revealed that these C-terminal mutations might have induced formation of a new turn, perturbing the active site environment and also generated new intramolecular interactions, which could be unfavourable for the enzyme activities.

Key words: *katG*; catalase-peroxidase; isoniazid resistance; clinical isolate; *Mycobacterium tuberculosis*.

Abbreviations: INH, isoniazid; LB, Luria-Bertani; MDR, multidrug resistant; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TB, tuberculosis; t-BHP, tert-butylhydroperoxide.

Introduction

Tuberculosis (TB) contributes to the causes of deaths of two million people annually by infectious diseases (Ernst et al. 2007). The infection is caused by *Mycobacterium tuberculosis*, which consistently develops resistance to anti-TB drugs (Atalay et al. 2004). This developing resistance contributes to the rapid progression of TB. According to World Health Organization report (Global tuberculosis control: tuberculosis facts; http://www.who.int/tb/data/; 2010) about 2% of new cases are already involving multidrug resistant (MDR). So far, isoniazid (INH) has been one of the front-line and the most effective drugs, which is widely used in the treatment of TB (Wengenack et al. 2004). However, development of new anti-TB drugs is constantly required to cope with *M. tuberculosis* developing resistance.

An interesting fact is that 98% of TB cases occur in tropical countries (Gordon & Alimuddin 2008). Indeed, on the list of countries with the largest number of TB cases since 2009, Indonesia ranks fifth, where the TB prevalence was estimated as ∼285 cases per 100.000 people population. Upon the TB treatment with INH, the recent nationwide investigation on the primary INH resistance shows that the national frequency for MDR-TB is at 28.2–34.6% (Massi et al. 2011). Despite the percentages may vary greatly due to geographic regions, this report urges the evaluation on the use of INH as the primary treatment for TB.

INH is taken up by *M. tuberculosis* in a prodrug form through passive diffusion. The prodrug is then activated by endogenous KatG protein encoded by the *katG* gene (Atalay et al. 2004). KatG protein demonstrates both catalase and peroxidase activity. Activated INH targets the NADH-dependent enoyl-acyl carrier protein reductase (InhA) and β-ketoacyl acyl carrier protein synthase (KasA) (Mdluli et al. 1998; Atalay et al. 2004), which are involved in the biosynthesis of mycolic acid, a cell wall component of mycobacteria (Pretorius et al. 1995). In turn it will inhibit *M. tuberculosis* growth.

M. tuberculosis develops resistance against INH by mutating its *katG* gene (Pretorius et al. 1995). The mu-

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tation results in distortion of the KatG protein activity (Rouse et al. 1996; Yu et al. 2009; Cade et al. 2010), as demonstrated in the study using *katG* gene-deficient *M. tuberculosis* strain (Zhang et al. 1992; Rouse et al. 1996). The most common mutation of *katG* occurs at the codon 315, resulting in a conversion of the amino acid serine to threonine (Pretorius et al. 1995; Rahimi et al. 2009). This S315T KatG mutant has been proposed to be responsible for *M. tuberculosis* resistance against INH (Wengenack et al. 2004; Ghiladi et al. 2005; Kapetanaki et al. 2007). Nevertheless, INH-resistant *M. tuberculosis* strain that undergoes mutation at *katG* codons other than 315 have also been reported (Pretorius et al. 1995; Atalay et al. 2004). A study on the position of *katG* mutation is prerequisite to resolve this issue.

KatG occurs as a homodimeric and heme-dependent protein, where each of its monomer is composed of two peroxidase-like domains (Bertrand et al. 2004; Mo et al. 2004; Baker et al. 2006). Both the heme binding and bifunctional catalase-peroxidase active sites are located at its N-terminal domain, whilst the C-terminal domain is important for subunit interactions (Saint et al. 1999). Intramolecular interactions between the N- and C-terminal domains of KatG are also important for its enzymatic function (Wilming & Johnsson 2001).

Clinical isolates of *M. tuberculosis* that demonstrate INH-resistant characteristic were obtained from various patients of a clinic in Bandung (Indonesia) who were diagnosed with pulmonary TB. Among these isolates, the so-called isolate L10 exhibited resistance to INH at 1 μg/ mL and has no mutation at codon 315 (Noviana et al. 2007), but interestingly mutations in *katG* gene, which lead to amino acid changes at the C-terminal of KatG. In order to understand the effect of the *katG* mutation on the INH-resistance characteristic of this isolate, we performed biochemical characterization and *ab initio* structural study of the KatG. We conclude that the amino acid changes found at the C-terminal domain have a significant effect on the catalase-peroxidase activity, which then explained the INH-resistant phenotype of the L10 clinical isolate.

Material and methods

Bacterial strains and plasmids

The isolate L10 was obtained from the culture collection of the Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Indonesia. The INH-sensitive *M. tuberculosis*, named as H37Rv strain and containing the wild-type *katG* gene, was a gift from the Health Research Center, Bandung, Indonesia. The TOP10 (Invitrogen, Maryland, USA) and BL21(DE3) (Promega, Madison, USA) *Escherichia coli* strains were employed for cloning of the gene and expression of the protein, respectively. The pGEM® T vector (Invitrogen, Maryland, USA) was used in the cloning, whilst pCold II DNA in the expression of the protein.

Polymerase chain reaction

Chromosomal DNA of *M. tuberculosis* was used as a template for polymerase chain reaction (PCR). The DNA was prepared by incubating the *M. tuberculosis* cells in 5 mM Tris-Cl buffer, pH 8.5, containing 05% (w/v) Tween-20 and 0.2 mg/mL proteinase K at 50 ℃ for 60 min. The disrupted *M. tuberculosis* cells were then heated at 95 ℃ for 5 min. Cellular debris was removed by cold centrifugation at 12,000×*g* for 10 min and the chromosomal DNA of *M. tuberculosis* in the supernatant was subjected to PCR for the amplification of the full-length *katG* gene, using FG and RG primers (Table 1). The FG primer comprises six nucleotides adapter for an *Eco*RI restriction site and subsequent nucleotide sequences that encode the first six N-terminal amino acids of KatG. The RG primer contains a nucleotide sequence that encodes the last six C-terminal amino acids of the KatG protein and a subsequent *Not*I restriction site with six nucleotides adapter. PCR was performed in a total reaction volume of 50 μL, which consists of 50 ng chromosomal DNA, PCR buffer (20 mM Tris-Cl, pH 8.4, 50 mM KCl), 0.1 mM of each FG and RG primers, 200 μM dNTPs, 1.5 mM $MgCl_2$, and 0.25 unit of *Taq* DNA polymerase (Amersham, New Jersey, USA). The PCR reaction was done with the following steps: pre-denaturation at 94 ℃ for 4 min, 25 cycles of denaturation at 94 ℃ for 1 min, annealing at 57 ℃ for 1 min, and an extension at 72 ℃ for 3 min, and finally post-elongation at 72 ℃ for 7 min. The PCR products were analyzed in agarose gel electrophoresis and purified by GFX purification kit (Amersham, New Jersey, USA).

Table 1. The oligonucleotide primers for amplification and sequencing of the *katG* gene of *M. tuberculosis*.

Name of primer	Nucleotide sequences of primers (5'→3')
SP6 promoter	catacgatttaggtgacactatag
T7 promoter	taatacgactcactataggg
FG	gttattgaattcgatgcccgagcaacacccac
RG	ttcatagcggccgcgcgcacgtcgaacctgtc
KF	gcagatggggctgatctacg
FDPRK	cgacgagttcgccaaggc
*katG*F	ggtcatatgaaatacccgtcgagggcg
*katG*R	cgtctagactcagcgcacgtcgaacctgtc

Cloning and clone characterizations

The *katG* gene from PCR was inserted to the pGEM®T vector, and the resulted plasmid was then transformed into *E. coli* TOP10 using the $CaCl_2$ method (Sambrook & Maniatis 1989). The transformed cells were transferred to an agar plate containing Luria-Bertani (LB) medium, 100 μg/mL ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside. The colonies were selected and subjected to a resctriction analysis of the recombinant plasmid.

Restriction analysis

The selected *E. coli* colonies were grown in a LB medium containing ampicilin at 37 ℃ with shaking for 16 hours. The *E. coli* cells were collected by cold centrifugation at 12,000×*g* for 10 min. The recombinant plasmid was extracted from the cells using QIAgen Spin Plasmid Miniprep Test Kit (Qiagen, Santa Clarita, USA), and digested with the restriction enzymes, *Eco*RI and *Not*I. The obtained DNA fragment was then purified on an agarose gel. The DNA fragment band with the expected size for *katG* gene was recovered from the gel and used for sequencing.

DNA sequencing
The *katG* gene fragment in the recombinant plasmid was sequenced by an automatic nucleotide sequencer (ABI PRISM, Macrogen, Seoul, Korea). The confirmed recombinant pGEM®-T was designated as pGEM-T-katG. All oligonucleotide primers used in the sequencing of *katG* gene are presented in Table 1.

Alignment analysis
The*katG* genes from the wild-type and L10 were analyzed *in silico* by aligning their nucleotide and deduced amino acid sequences. The alignment was performed by the DNA star program SeqManTMII for DNA sequence and MegAlignTM for the amino acid sequence (Lasergene, Biocomputing software for windows; DNASTAR, Inc., Madison, USA, 1997).

Subcloning of the katG gene
The *katG* gene fragment from pGEM-T-*katG* was modified to facilitate its insertion to the expression vector using PCR method. The modification was done to introduce *Nde*I site at 5' end and *Xba*I sites at 3' end after stop codon, using *katG*F and *katG*R primers (Table 1). To check whether the modification is successful, the PCR product was digested with *NdeI*I and *Xba*I. The digestion product was purified and inserted into plasmid pCold II DNA, which previously has been digested with the same restriction enzymes. The ligation product was transformed to *E. coli* BL21(DE3) and the transformed bacteria were then grown on a selective LB agar plate.

Expression and purification of recombinant KatG protein
A single colony of *E. coli* BL21(DE3), designated as pCold II DNA-katG, was cultured at 37℃ with shaking at 200 rpm to an optical density (λ 600 nm) of 0.4. The culture was immediately cooled at 15℃ for 30 min. The recombinant protein expression was stimulated by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.1 mM. The cells were harvested after 24 hours of induction at 15℃. The cells were washed and re-suspended in 50 mM potassium phosphate buffer (pH 7.0), and then disrupted by sonication. The cellular debris was removed by centrifugation at 12,000×*g* for 15 min. The protein crude extract was purified in a 1 mL HisTrap^{TM} HP column (GE Healthcare, Freiburg, Germany) according to the manufacturer's protocol. The KatG protein was recovered from the column with an elution at a flow rate of 1 mL/min, applying a linear imidazole gradient of 50–200 mM in 50 mM potassium phosphate buffer, pH 7.0. The purity of the protein was monitored by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Catalase and peroxidase activity assays
The catalase activity was measured according to the method by Patti & Bonet (1953), which was based on the generation of titanium colour for hydrogen peroxide. Briefly, 1.25 µg of protein was incubated in the 50 mM potassium phosphate buffer, pH 7.0, containing 12.5 mM H_2O_2 at 37℃ for eight min. Titanium reagent was added to stop the enzymatic reaction and to develop a stable yellow colour of pertitanic acid. The absorbance of this reaction mixture was recorded at 410 nm. One unit of catalase activity was defined as the amount of enzyme that decomposes 1 mmol of H_2O_2 per minute.

Peroxidase activity was measured based on the conversion of 100 µM *o*-dianisidine into *o*-dianisidine quinonediimine in the presence of 25 mM tert-butylhydroperoxide (t-BHP) in 50 mM potassium buffer (pH 4.5) with 12.5 mM H_2O_2 as the substrate (Wengenack et al. 2004). The absorbance of *o*-dianisidine quinonediimine was measured at 460 nm. The amount of tBHP was calculated using an extension coefficient of ε_{460} = 11.3 mM^{-1} cm^{-1}. One unit of peroxidase activity was defined as the amount of enzyme that catalyzes the formation of one µmol product per min at 30℃.

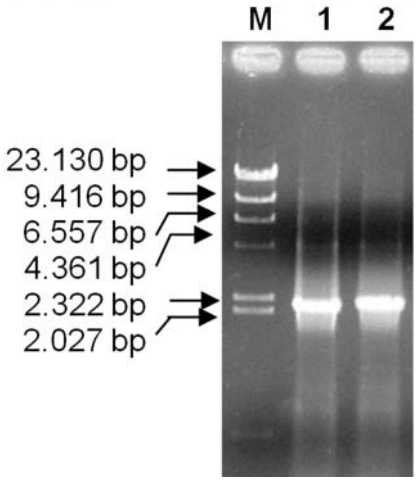

Fig. 1. Amplification products of the *katG* gene using PCR. Lane 1, fragment DNA (2.2 kb) of INH-sensitive *M. tuberculosis* H37RV; lane 2, fragment DNA (2.2 kb) of INH-resistant *M. tuberculosis* L10; lane M, marker DNA λ/*Hind*III.

Isoniazid oxidation assay
Catalase-peroxidase of *M. tuberculosis* could catalyze the oxidation of INH to produce OH radical. The INH-dependent OH radical detection could be performed by sequential phenol hydroxylation and its oxidation into benzo-quinone chromophore that absorbs at about 444 nm. Briefly, a mixture of 13.0 mM phenol, 0.65 mg of KatG, and 6.5 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0, was incubated at 37℃. After 7 min, INH at final concentration of 2.5 mM was added to the mixture. The absorption of the benzo-quinone was recorded at 444 nm (Shoeb et al. 1985). One unit of the activity was defined as the amount of enzyme that catalyzes the formation of benzo-quinone product per minute at 37℃.

Structure alignment
Three-dimensional structure of KatG of L10 strain was generated by the automated protein homology modelling SWISS-MODEL server (Schwede et al. 2003), using the crystal structure of KatG from the wild-type (Protein Data Bank Code: 1SJ2; Bertrand et al. 2004) as the template. The minimization of the effect in the rigid structure of wild-type and mutant KatG was done with the program AMBER 9 (Case et al. 2006). The superposition of the generated and 1SJB structures was done using SuperPose version 10 (Maiti et al. 2004). The software was also used to calculate the root mean square deviations. The model structures of mutant KatG was visualized in PyMOL 1.3 (DeLano 2002).

Results and discussion

The complete *katG* open reading frame of 2.2 kb from L10 of INH-resistant (L10) and INH-sensitive *M. tuberculosis* (H37Rv) were amplified by PCR (Fig. 1) and

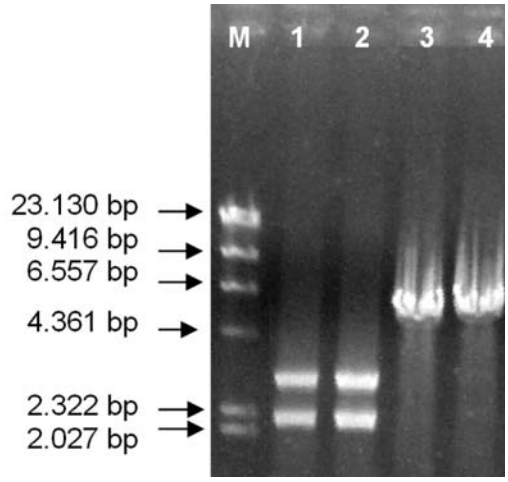

Fig. 2. The profile of restriction analysis of recombinant pGEM-T-*katG*. Lane M, marker DNA λ/*Hind*III; lane 1, pGEM-T-*katG* (wild-type) was simultaneously digested by two enzymes, *Eco*RI and *Not*I; lane 2, pGEM-T-*katG* (clinical isolate L10) was simultaneously digested by two enzymes, *Eco*RI and *Not*I. Both digestions resulted in two DNA fragments, the first fragment exhibited 3.0 kb in size and the second 2.2 kb. Lanes 3–4, the recombinant pGEM-T-*katG* (wild-type and L10, respectively) was digested by a restriction enzyme, *Eco*RI, and yielded a DNA fragment of 5.2 kb.

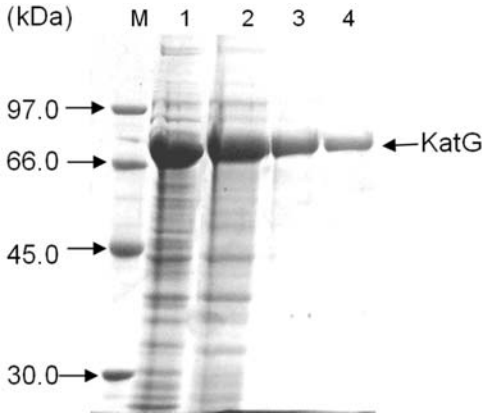

Fig. 4. Electrophoresis analysis with 10% SDS-PAGE of the wild-type KatG and L10 KatG. Lane 1, marker protein; lane 2, crude extract protein from wild-type *katG* expression (8 μg protein); lane 3, crude extract protein from clinical *katG* L10 expression (8 μg protein); lane 4, wild-type KatG (1.3 μg) following HisTrap^{TM} column purification; lane 5, L10 KatG (1.0 μg) following HisTrap^{TM} column purification. Band corresponding to KatG is indicated.

the PCR products were cloned into pGEM-T vector. Both pGEM-T containing either wild-type or mutant *katG* were characterized by single digestion with *Eco*RI to give a fragment of 5.2 kb (Fig. 2, lanes 3–4), which corresponds to the size of the plasmid harbouring the *katG* gene. Double digestion with *Not*I and *Eco*RI produced two DNA fragments of 2.2 kb and 3.0 kb, which correspond to *katG* gene and pGEM-T empty plasmid, respectively (Fig. 2, lanes 1–2). These results showed that *katG* genes from both the INH-resistant and INH-sensitive *M. tuberculosis* strains have successfully been inserted to the pGEM-T vector.

The nucleotide sequences of the wild-type *katG* from the INH-sensitive *M. tuberculosis* H37Rv strain exhibited 100% identity to that of *katG* deposited in the GenBank (Acc. No.: X68081; CAA48213.1), however, the *katG* nucleotide sequences from L10 showed differences of five-nucleotide to both *katG* sequences from GenBank and from the H37Rv strain. The deduced amino acid sequences of the L10 *katG* suggested a complete *katG* open reading frame. The mutations in *katG* gene from L10 were observed at T1310C, G1388T, G1481A, T1553C, and A1660G (Fig. 3), corresponding with amino acid substitutions L437P, R463L, G494D, I518T, and K554E, respectively. All five mutations are found at the C-terminal domain of its protein.

KatG proteins from each H37RV and L10 strains were expressed as protein with a molecular mass of 80 kDa and the purification on a Ni-sepharose affinity column has resulted in the KatG proteins with over 95% purity (Fig. 4). The catalase and peroxidase activities of KatG protein from L10 were about 4.6% and 24.8%,

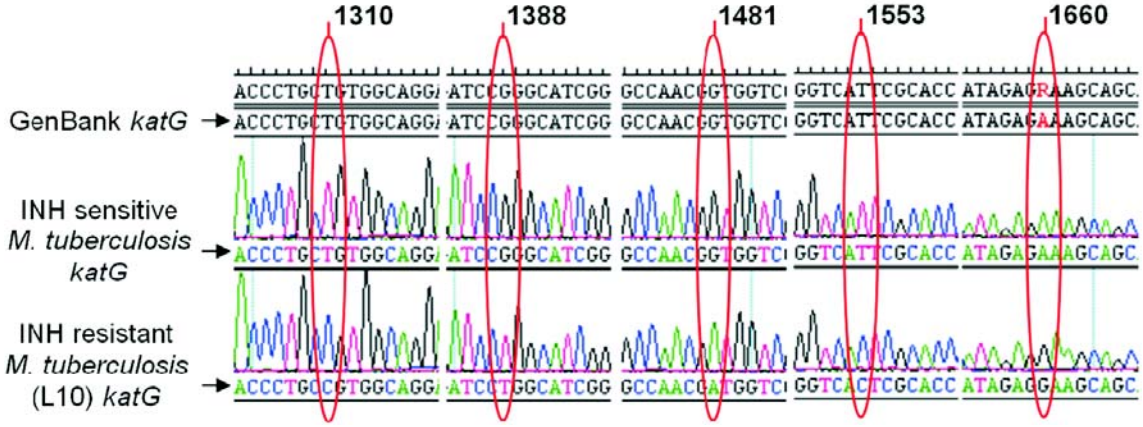

Fig. 3. The alignment of *katG* from INH resistant and sensitive *M. tuberculosis* toward *katG* from GenBank. Compared with the GenBank and wild-type *katG*, the *katG* of clinical L10 showed five nucleotide differences (red underline), consisting of T1310C, G1388T, G1481A, T1553C, and A1660G, corresponding to amino acid substitutions Arg437Pro, Arg463Leu, Gly494Asp, Ile518Thr, and Lys554Glu, respectively.

Table 2. Catalytic activities of KatG.

M. tuberculosis strain[a]	Activity of KatG		
	Catalase (U/mg)	Peroxidase (U/mg)	INH oxidation (U/mg)
H37RV (INH sensitive)	518	1.7×10^3	850
L10 (INH resistant at 1 μg/mL)	24	4.2×10^2	165

[a] This column means the INH sensitivity.

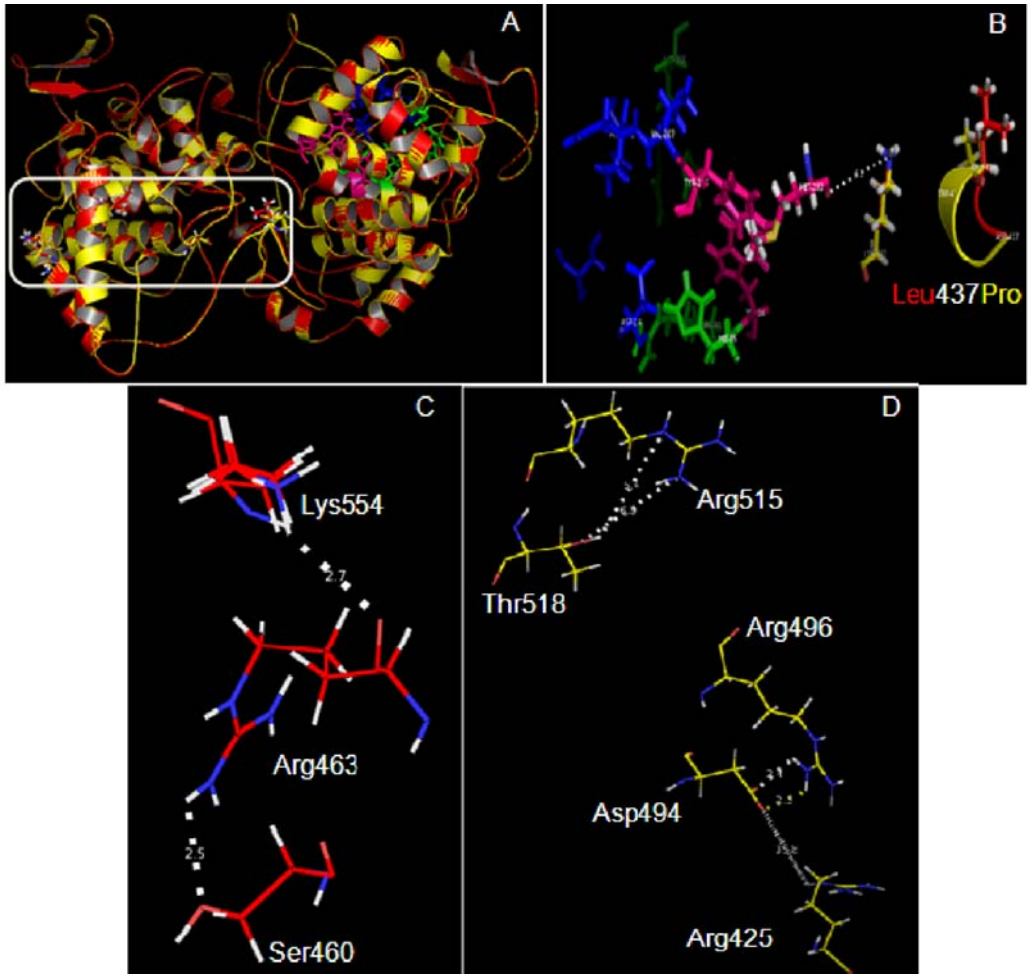

Fig. 5. Structural alignment of the wild-type and clinical L10 KatG. (A) The superposed structure of the wild-type KatG (red) and L10 KatG (yellow). The green, blue, and magenta residues in the structures represent residues of active sites, substrate binding sites, and adduct triad sites, respectively. Five mutations are shown in the white box. (B) The turn conformation is formed by Leu437Pro replacement. (C) The wild-type KatG shows hydrogen bonds which link Arg463 to Ser460 and Lys554. The bonds are absent in the L10 KatG. (D) The L10 KatG shows close interaction of Asp494 with Arg425 and Arg496, and Thr518 to Arg515. These interactions are absent in the wild type KatG.

respectively, of the wild-type activities (Table 2). Furthermore, the KatG from L10 demonstrated only 19.4% activity of the wild-type KatG in INH oxidation (Table 2). The k_{cat}/K_M value for catalase activity of the wild-type KatG was 8.62×10^4 $M^{-1}s^{-1}$, whereas that of KatG from L10 was 1.79×10^4 $M^{-1}s^{-1}$, which is 80% lower. The k_{cat}/K_M for the peroxidase activity of the L10 KatG was 5.0×10^4 $M^{-1}s^{-1}$, which was 75% lower than that of the wild type KatG.

The mutations found in the L10 *katG* differ from most multiple-mutations of *katG* gene from INH-resistant *M. tuberculosis*, which predominantly contains amino acid change at position 315 (Rahimi et al. 2009). The *katG* gene from L10 suffers no mutation in that particular position, despite having resistant phenotype to INH. Three mutations in the *katG* gene from L10 that results in three amino acid changes L437P, I518T, K554E have not been previously reported (Table 2) whilse the other two amino acid changes G494D and R463L have already been found in other KatG of INH-resistant *M. tuberculosis* from France (Brossier et al. 2006).

It has also been reported that KatGs lacking the C-terminal 41, 119, 221, and 334 amino acids are in-

active and unable to restore INH sensitivity (Devito & Morris 2003). Furthermore, other C-terminal mutations, namely R619P, L639F, and D735A (Wei et al. 2003) as well as L587M, G629S, and D735N (Cade et al. 2010) exhibited lower catalase-peroxidase and INH activation compared to that of the wild-type KatG.

As an attempt to understand the effect of amino acid substitutions on the L10 KatG structure, a preliminary structure modelling study has been performed. The L10 KatG shares 99.3% identity of its amino acid sequence to the wild-type KatG. The C_α-backbone superposition of the structures of the wild-type KatG and that of generated model for KatG from L10 gave root mean square deviations (RMSD) of 0.30 Å, suggesting highly similar structures. However, the structure of L10 KatG has a new turn conformation in the area of residues 437–440 due to a leucine replacement by proline in the position 437 (Fig. 5A,B), instead of loop in the wild-type KatG. In addition to this, new interactions in the L10 KatG have also been observed. Salt bridge network might have been formed by the mutant Asp494 to Arg425 and to Arg496, and a hydrogen bond might have occurred between the mutant Thr518 and cationic residue Arg515. Moreover, the L10 KatG might have lost hydrogen bonds that connect Arg463 to polar residues Ser460 and Lys554, due to R463L substitution (Fig. 5C,D). These new turn and interactions might contribute to the formation of KatG with less active structure.

It has been hypothesised that the C-terminal domain is essential for catalase-peroxidase activities (Devito & Morris 2003; Cook et al. 2009). It has an important role in KatG dimerization (Saint et al. 1999; Bertrand et al. 2004; Baker et al. 2006; Cook et al. 2009). The changes in the interdomain interactions due to the D419H and M420T substitutions resulted in loss of enzymatic activities of KatG (Ando et al. 2010). The residues of KatG at positions 494 and 518 appear to be located in the interconnecting of subunits (Bertrand et al 2004; Yu et al 2007). Hence, amino acid substitutions of G494D and L518T may affect dimerization of KatG.

The residue Leu437 in the wild-type KatG lies adjacent to the Trp107-Tyr229-Met255 adduct, a conserved conformation in many catalase-peroxidase that plays a crucial role for the catalytic activity (Bertrand et al. 2004; Kapetanaki et al. 2007). The cross-linked adduct contributes to the optimization of heme-pocket geometry to enhance the binding and reactivity of substrates. Disruption of the adduct, which was induced by R104L and H108Q substitutions reduced the catalase activity (Ghiladi et al. 2005). In the L10 KatG protein, a turn helix that could be induced by L437P substitution (Fig. 5B) may perturb the adduct and then impair the catalase/peroxidase activities of this mutant.

Taken together, our study has revealed several amino acid residues at the C-terminal domain, which are important for catalase-peroxidase activity. In the future, site-directed mutagenesis study should be performed to identify the role of each amino acid mutation in the catalase-peroxidase and KatG crystal structure determination to elucidate molecular interactions precisely.

Table 3. Kinetics parameter of wild type and L10 KatG.

Kinetics parameter	KatG	
	Wild type	L10
Catalase activity		
k_{cat} (s^{-1})	272.3	142.9
K_M (mM)	3.16	8.01
k_{cat}/K_M (M^{-1} s^{-1})	8.62×10^4	1.79×10^4
Peroxidase activity		
k_{cat} (s^{-1})	49.0	7.0
K_M (mM)	0.25	0.13
k_{cat}/K_M (M^{-1} s^{-1})	1.99×10^5	0.50×10^5

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