Metabolic Control Analysis of Reaction Catalysed by Chalcone Synthase to Inhibit Complex Lipid Formation in *Mycobacterium tuberculosis*

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**Authors’ contributions**

This work was carried out in collaboration between all authors. Author SM designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Author AS wrote the protocol and performed the computational work and analysis. Author PN managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

**Article Information**

DOI: 10.9734/SAJRM/2018/v2i29254

Editor(s):
(1) Dr. Muhammad, Ruqayyah Hamidu, Department of Microbiology, Federal University, Dutse Jigawa state, Nigeria.

Reviewers:
(1) Rachel Rocha Pinheiro Machado, Universidade Federal do Rio de Janeiro, Brasil.
(2) Germano Manuel Pires, National Institute of Health Mozambique, Mozambique.
Complete Peer review History: http://www.sciencedomain.org/review-history/27126

**Received 30 March 2018**
**Accepted 23 October 2018**
**Published 08 November 2018**

**ABSTRACT**

Tuberculosis is among the deadliest diseases occurring worldwide killing almost 2 million of people every year and the number increases every year. The drugs and medications used for treatment become ineffective due to the development of resistance in the causative pathogen, *M. tuberculosis* towards the drugs. This has led to the development of new approaches to study the diseases from different views. Chalcone Synthase is an enzyme involved in the flavonoid biosynthetic pathway occurring in plants and in certain bacterial species including *M. tuberculosis*. The enzyme is responsible for lipid biosynthesis in bacterial cell wall of *M. tuberculosis*. This study describes the kinetic behaviour of reaction; Chalcone Synthase catalyzes the first reaction in flavonoid biosynthesis and needs a certain threshold concentration of substrates for reaction to proceed. In this study, the steady state concentration of substrates is predicted by simulation experiment using Gepasi tool. Initial concentrations of substrates in *M. tuberculosis* are 4.78*10^-2 mMol for p-coumaroyl-CoA and 2.23*10^-7 mMol for malonyl-CoA, are taken as input which are essential for...
INTRODUCTION

In recent years, many researchers have become interested in combining experimental and computational techniques in cell biology. Several tools have been designed and implemented for modelling and simulation of the signal transduction and metabolic functions of the cell. Increasing availability of biochemical data and the complexity of cellular functions make the integrative approaches of systems biology even more important for future analysis. Metabolic Control Analysis provides a quantitative description of concentration variations with the change in system parameters. A Metabolic Control Analysis aids in the determination of threshold concentration of metabolites involved in a reaction and also helps in understanding role of various parameters in a reaction. MCA provides a focused approach to identify and characterise metabolic reactions in cell behaviour, which can be used as targets for effective therapy against various poorly understood disease processes [1,2].

Chalcone synthases are a family of polyketide synthase enzymes related with the making of chalcones, a class of organic compounds originates mainly in plants as natural defense mechanism and as synthetic intermediates that have been recently discovered in bacteria. Chalcone synthase is the enzyme which is involved in the fatty acid biochemical pathway in M. tuberculosis. As a ubiquitous enzyme in higher plants, CHS provides the first committed step in flavonoid biosynthesis by catalysing the sequential decarboxylative addition of three acetate units from malonyl-CoA to a p-coumaroyl-CoA starter molecule derived from phenylalanine via the general phenylpropanoid pathway. CHS catalyses flavonoid biosynthesis by leading carbon flux from overall phenylpropanoid metabolism towards flavonoid pathway [9]. The substrates in M. tuberculosis are provided by the bacteria itself by certain metabolic activities. In the same active site, CHS then forms chalcone via the intra- molecular cyclisation and aromatisation of the resulting linear phenylpropanoid tetraketide [10,11]. PKS synthesise polyketide by initialising a starter molecule which is followed by repetitive decarboxylation and condensation of Co-enzyme A analogue of simple carboxylic acid. PKS uses single active site for multiple condensation step in flavonoid biosynthesis.

Keywords: Naringenine chalcone; CHS; flavonoid biosynthesis; steady state kinetics; metabolic control analysis (MCA).

1. INTRODUCTION

Mycobacteria are classified in the phylogeny of the Actinomycetes, along with the Streptomycetes bacteria. Interestingly, these two actinomycete genera have received immense attention due to their contrasting effects on human society. Whereas Streptomycetes have provided a rich source of antibiotics and other therapeutic products for human diseases, Mycobacterium tuberculosis has been one of humankind’s greatest scourges. In 2007 a total of 13,293 cases of tuberculosis have been reported. The rate of TB decline to 4.4 cases per 100,000 populations, this was lowest recorded rate since national reporting commenced in 1953. The average annual percentage of TB rate has been decreased from 7.3% per year during 1993-2000 to 3.8% during 2000—2007 [3]. In parallel, the severe clinical problem of multiresistant M. tuberculosis is on the rise and Tuberculosis is in danger of becoming incurable [4,5].

The high concentration of lipids in the cell wall of M. tuberculosis has been attributed to its resistance to antibiotics. The lipid fraction of cell wall consists of three major components mycolic acids, cord factor and wax-D. The unique mycobacterial cell wall lipids are known to play an important role in pathogenesis, and therefore the genes responsible for their biosynthesis offer potential new targets [6,7,8].

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Chalcone synthases are a family of polyketide synthase enzymes related with the making of chalcones, a class of organic compounds originates mainly in plants as natural defense mechanism and as synthetic intermediates that have been recently discovered in bacteria. Chalcone synthase is the enzyme which is involved in the fatty acid biochemical pathway in M. tuberculosis. As a ubiquitous enzyme in higher plants, CHS provides the first committed step in flavonoid biosynthesis by catalysing the sequential decarboxylative addition of three acetate units from malonyl-CoA to a p-coumaroyl-CoA starter molecule derived from phenylalanine via the general phenylpropanoid pathway. CHS catalyses flavonoid biosynthesis by leading carbon flux from overall phenylpropanoid metabolism towards flavonoid pathway [9]. The substrates in M. tuberculosis are provided by the bacteria itself by certain metabolic activities. In the same active site, CHS then forms chalcone via the intra- molecular cyclisation and aromatisation of the resulting linear phenylpropanoid tetraketide [10,11]. PKS synthesise polyketide by initialising a starter molecule which is followed by repetitive decarboxylation and condensation of Co-enzyme A analogue of simple carboxylic acid. PKS uses single active site for multiple condensation
Literature shows that PKS is involved in the synthesis of PDIM and novel lipid formation. PDIM contain multimethyl branched mycocerosic acids which is esterified to phthiocerol and these occur in limited group of pathogenic mycobacteria [12].

2. METHODS

Gepasi is a software system for the simulation of chemical and biochemical kinetics. Kinetic modelling is about describing the evolution of a system in time. A particular case of kinetic modelling deals with finding a state in which the system stops evolving in time. Such states are called steady states. Gepasi has predefined kinetic types (i) the common Henry-Michaelis-Menten, (ii) inhibition or activation mechanism (iii) Hill Kinetics and its equivalent for reversible reaction (iv) several allosteric and multireactant mechanisms, extra kinetic types are also accepted.

This work is organised as follows. Firstly, a metabolic model of a CHS reaction is defined in Gepasi. Secondly, time series simulation is carried out through Gepasi. Finally, the work is concluded with the evaluation of simulation results.

The chalcone biosynthesis reaction in *M. tuberculosis* involving Chalcone synthase occurs via following reaction:

\[
P\text{-Coumaroyl-CoA} + 3\text{ Malonyl-CoA} \leftrightarrow \text{Naringenin chalcone} + 3\text{ CO}_2 + 4\text{ Coenzyme-A}
\]

The methodology of this work can be divided into following steps:

**Step 1:** Collection of data about chalcone synthase of *M. tuberculosis* from the existing literatures. The reaction is entered in standard notation to define model of reaction.

**Step 2:** Mass action (reversible) kinetics was selected from the list of all the predefined reactions in the model and list of possible kinetic type, where k1 and k2 are the rate constants for forward and backward reactions respectively and are taken as 1.

**Step 3:** The concentrations of the metabolites are entered to initialise the concentrations of the substrates and Products.

& steady state kinetics of the reaction is calculated by carrying out the simulation in which Concentration of metabolites is scanned from a minimum concentration (1 mMol) to maximum (2 mMol).

### Fig. 1. The biochemical model of CHS catalysed reaction is defined by entering reaction kinetic type (mass action (reversible) and metabolites [13]

### Fig. 2. The reaction to be simulated is entered in the add reaction page on Gepasi
the kinetic constants have been derived from the literature survey. The concentrations of p-coumaroyl-CoA and malonyl-CoA are taken as $5.00\times10^{-2}$ mMol and $2.20\times10^{-3}$ mMol respectively. The kinetic law of the reaction is taken as Mass Action reversible and $K_1$ and $K_2$ values are taken as 1, supposing that the rate of forward reaction equals to the rate of backward reaction. The model is defined in the Model Definition Page of gepasi by feeding the reaction and the data about the reaction. Initial concentration of p-Coumaroyl-CoA has been reduced from $5.000000\times10^{-2}$ mM to $4.914061\times10^{-2}$ mM, and for 3Malonyl-CoA, initial concentration has been reduced from $2.200000\times10^{-3}$ mM to $1.340605\times10^{-3}$ mM which is about half of the initial concentration.

Naringenin chalcone initial concentration is $0.000000\times10^{0}$ mM which shows product would have not been produced at initial stage, and final concentration of naringenin chalcone at $8.593946$ mM shows product formation in this reaction. Values of Rate constants for forward ($k_1$) and backward ($k_2$) reactions are $1.0000$ and $1.0000$ respectively. After 1 second, value of equilibrium constant $J(R1)$ was $6.587753\times10^{-5}$ mM*m/s (Table 1). After 5 seconds, at equilibrium constant $J(R1)$ $0.0000000+00$ mM*m/s, initial and final concentration of all the five components was $1.000000$ mM.

Eventually Malonyl Co-A has been increased upto $8.422792\times10^{-1}$ mM but product final concentration becomes low and stable which is $1.157721$ mM for both Naringenin chalcone as well as for Coenzyme A (Table 1).

Synthesis of Mycolic acid will not be sustained due to suppression of repetitive decarboxylation and condensation of Co-enzyme A. Inactivation of PKS results in loss of complex lipid in M.tuberculosis H37RY. Pthiocerol dimycorosate (PDIM) waxes and other lipid are essential for cell wall in M. tuberculosis.

### 3.2 Kinetic Analysis of the Steady State

Kinetic analysis of the Steady state shows that after 1 second, concentration of all products was $2.199777$ mM at chemical equilibrium $J(R1)$ - $7.898298\times10^{-1}$ mM*m/s. After 5 seconds, at equilibrium constant $J(R1)$ $0.000000$ mM*m/s, Initial and final concentration of all the five components was $1.000000$ mM, reaction rate was also $0.0000+00$ mM. After 5 seconds, at equilibrium constant $J(R1)$ $-4.201448\times10^{-10}$
mM*ml/s, concentration of Malonyl Co-A was 8.422792e mM and final product concentration was 1.157721 mM for both Naringenin chalcone as well as for Coenzyme A, which was corresponding to kinetic prediction. The concentrations obtained for p-coumaroyl-CoA and malonyl CoA after 5 seconds are 4.90*10^-2 mMol and 1.70*10-3 mMol respectively.

Table 1. Illustration of kinetic parameters and reaction components with initial and final concentrations of reactants and products of reversible reaction, R1 (Mass action (reversible)) at k1=1.0000e+000; k2 = 1.0000e+000, Compartment V(compartment) = 1.0000e+000

| Reaction Components | Initial concentration | Final concentration |
|---------------------|-----------------------|---------------------|
| After 1.00e+001 s | J (R1) = 6.587753e-005 mM*ml/s | |
| p-Coumaroyl-CoA | 5.000000e-002 mM | 4.914061e-002 mM |
| 3Malonyl-CoA | 2.200000e-003 mM | 1.340605e-003 mM |
| Naringenin chalcone | 0.000000e+000 mM, | 8.593946e-004 mM |
| 3CO2 | 0.000000e+000 mM | 8.593946e-004 mM |
| 4CoA | 0.000000e+000 mM | 8.593946e-004 mM |
| After 5.00e+000 s | J (R1) = 0.000000e+000 mM*ml/s | |
| p-Coumaroyl-CoA | 1.000000e+000 mM | 1.000000e+000 mM |
| 3Malonyl-CoA | 1.000000e+000 mM | 1.000000e+000 mM |
| Naringenin chalcone | 1.000000e+000 mM, | 1.000000e+000 mM |
| 3CO2 | 1.000000e+000 mM | 1.000000e+000 mM |
| 4CoA | 1.000000e+000 mM | 1.000000e+000 mM |
| After 5.00e+000 s | J (R1) = -1.065877e-008 mM*ml/s | |
| p-Coumaroyl-CoA | 2.000000e+000 mM | 1.842279e+000 mM |
| 3Malonyl-CoA | 1.000000e+000 mM | 8.422792e-001 mM |
| Naringenin chalcone | 1.000000e+000 mM | 1.157721e+000 mM |
| 3CO2 | 1.000000e+000 mM | 1.157721e+000 mM |
| 4CoA | 1.000000e+000 mM | 1.157721e+000 mM |

Fig. 5. Time course graph (after 2.6 seconds) of CHS catalysed reaction shows synthesis of 2 molecules of Naringenin and 1 molecule of CoA.
Table 2. Steady state solution for the reaction shows substrate concentration between 1 to 2 nmol, half-life and rate of reaction to attain chemical equilibrium

| Steady state solution (Results of integration) | Reaction components | Concentration | Tt (half-life) | rate |
|-----------------------------------------------|---------------------|---------------|---------------|------|
| After 1.00e+001 s (chemical equilibrium)      | p-Coumaroyl-CoA     | 4.780022e-002 mM | 6.051965e+01s | 7.898e-017 mM/s |
|                                               | 3Malonyl-CoA        | 2.226928e-007 mM | 2.819504e+00s | 7.898e-017 mM/s |
|                                               | Naringenin chalcone | 2.199777e-003 mM | 2.785128e+01s | -7.898e-017 mM/s |
|                                               | 3CO2                | 2.199777e-003 mM | 2.785128e+01s | -7.898e-017 |
|                                               | 4CoA                | 2.199777e-003mM | 2.785128e+01s | -7.898e-017 mM/s |
| After 5.00e+000 s)                            | p-Coumaroyl-CoA     | 1.000000e+000 mM | 1.797693e+30s | 0.000e+000 mM/s |
|                                               | 3Malonyl-CoA        | 1.000000e+000 mM | 1.797693e+30s | 0.000e+000 mM/s |
|                                               | Naringenin chalcone | 1.000000e+000 mM | 1.797693e+30s | 0.000e+000 mM/s |
|                                               | 3CO2                | 1.000000e+000 mM | 1.797693e+30s | 0.000e+000 mM/s |
|                                               | 4CoA                | 1.000000e+000 mM | 1.797693e+30s | 0.000e+000 mM/s |
| After 5.00e+000 s)                            | p-Coumaroyl-CoA     | 1.842279e+000 mM | 4.384867e+009s | 4.201e-010 |
|                                               | 3Malonyl-CoA        | 8.422792e-001 mM | 2.004735e+009s | 4.201e-010 mM/s |
|                                               | Naringenin chalcone | 1.157721e+000 mM | 2.755528e+009s | -4.201e-010 mM/s |
|                                               | 3CO2                | 1.157721e+000 mM | 2.755528e+009s | -4.201e-010 mM/s |
|                                               | 4CoA                | 1.157721e+000 mM | 2.755528e+009s | -4.201e-010 mM/s |
The steady state concentrations obtained are $4.78 \times 10^{-2}$ mMol for coumaroyl-CoA and $2.23 \times 10^{-7}$ mMol for malonyl-CoA at the transient time of 6.05 seconds and 2.82 seconds respectively (Table 2). The graphical view of simulation the simulation graph is observed by the time course data [Fig. 5] for various concentrations of the reactants. Gepasi has an additional feature for adjusting the speed of the plotting as per the user convenience.

These results show utilisation of these enzymes as a substrate for the synthesis of naringenin and related compounds needed for the synthesis of bacterial cell membrane of *M. tuberculosis* and its infection. Gene esA, drr B, PKS36 & PKS11 are involved in the synthesis of complex lipid in *M. tuberculosis*.

Biosynthesis of normal fatty acid precursors of Mycolic acids is done by FAS-I Malonyl-CoA, which synthesise C16 to C18 and C24 to C26 fatty acids, which was proposed by Bloch in 1970 [15]. According to them, type I fatty acid synthetase (FAS I), found in eukaryotes and eubacteria and type II(FAS II) found in plants and bacteria, interestingly both are present in *Mycobacterium segmantis*. According to G.S Besra, in *Mycobacterium tuberculosis*, similar system of FAS I and FAS II is found. In mycobacterial FAS I system substrate malonyl CoA and acetyl CoA are used for elongation of acetyl group butyryl-S-Enz by two carbon units, further elongation produces C16 and C18-Enz which is subsequently converted to CoA derivative specifically the C20 and V26-S-Enz products, required for the synthesis of membrane phospholipids [16,17].

C20 is the starting point where FAS II works to synthesise a very long chain mero segment of alpha methoxy and keto-mycolic acids. Products of FAS I system are converted into C50-C56 acyl products using elongation by FAS II. Combined products of FAS I and FAS II are used in synthesis of α-alkyl-β-hydroxy fatty acid [8]. Mycobacterium KAS III is involved in elongation of FAS I product C12 acyl–Co A [18].

4. CONCLUSION

Chalcone synthase is involved in the fatty acid synthesis in mycobacterial cell wall. The minimum concentrations of substrates of Chalcone synthase; p-coumaroyl-CoA and malonyl-CoA are predicted which are $4.78 \times 10^{-2}$ and $2.23 \times 10^{-7}$ mMol respectively. Naringenin chalcone initial concentration is $0.000000e+000$ mM and final concentration of naringenin chalcone at 8.593946e-004 mM shows product formation in this reaction. Values of Rate constants for forward ($k_1$) and backward ($k_2$) reactions are 1.0000e+000. After 1.00e+001 s, value of equilibrium constant $J(R1)$ is 6.587753e-005 mM*ml/s.

After 5 seconds at rate constant $J(R1)$ 0.000 mM*ml/s, initial and final concentrations of all the five components were 1.000000 mM, reaction rate was decreased upto 0.000e mM, but the final concentrations of the products become low and stable which is 1.157721e mM for both Naringenin chalcone as well as for Coenzyme A at equilibrium constant $J(R1)$ 1.157721e. 4.201448e mM*ml/s. This low concentration would not be able to induce repetitive decarboxylation and condensation of Co-enzyme A. This leads to inhibit Mycolic acid synthesis due to inactivation of PKS resulting in loss of complex lipid formation in *M. tuberculosis* H37RY.

Fatty acid synthesis (mycolic acid) will be inhibited below these predicted concentrations which may lead to suppression of lipid biosynthesis pathway, and pathogen will be rendered inactive because fatty acid synthesis is the main component of the mycobacterial cell wall, involved in pathogenicity. This study is useful in inhibiting the growth of *M. tuberculosis* if the drug is designed to minimise the concentrations of Malonyl CoA and p-Coumaryl CoA up to predicted value 1.157721 mM. The work brings a relevant approach about the possibility of the inactivation of mycobacterial growth by reducing mycolic acid production in the cell wall of mycobacteria. This new approach is cost effective and may be used to design vaccines which may function to control and regulate.

Metabolic control analysis can be used to predict genotype-phenotype correlation and genes can be ranked according to their importance in controlling and regulating cellular metabolic networks.

ACKNOWLEDGEMENTS

This Work has been carried out at Molecular Visualization and Simulation Laboratory CSJM University, Kanpur. We duly acknowledge the developers of Gepasi without which this work would have not been performed. Heartfelt gratitude is extended to reviewers who provided logical suggestions and added comments to improve the manuscript.
COMPETING INTERESTS

Authors have declared that no competing interests exist.

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