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Improved *o*-chlorobenzoylformate bioreduction by stabilizing aldo-keto reductase YtbE with additives

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ABSTRACT

Asymmetric reduction of methyl *o*-chlorobenzoylformate (CBFM) using aldo-keto reductase YtbE is a potentially cost-effective and green technology in manufacturing methyl (*R*)-*o*-chloromandelate which is a key intermediate for synthesizing (*S*)-clopidogrel (a popular medicine for treating atherosclerosis). At the moment, large scale application of YtbE has been complicated by uncertain thermal and operational stabilities. Consequently, we endeavored possible enzyme inactivation mechanism, and showed that (a) unfolding of YtbE explains enzyme activity loss, and (b) YtbE dimerization has a less significant effect owing to a small quantity detected. The effects of substrate and temperature on YtbE are mostly upheld by a one-step inactivation model, whereas the effect of product by a 2-step activity reduction modality. Partially based on these new understandings, a multi-factor experimental strategy was rationalized for improving the YtbE stability. For instance, glycerol was introduced to reduce enzyme unfolding whilst dithiothreitol to suppress its dimerization. This improved substrate conversion from 62.9% to 98.7%, and from 70.5% to 96.6% at 0.1 M and 1.0 M CBFM, respectively, with YtbE half-life being increased from 46.6 min to 159 min.

1. Introduction

Methyl (*R*)-*o*-chloromandelate [(*R*)-CMM] is a key chiral synthon for manufacturing (*S*)-clopidogrel bisulfate [e.g. trade name Plavix®], the world second best-selling drug widely administered to atherosclerotic patients [1]. Regardless of practicability, there are usually 3 known routes for preparing (*R*)-CMM. Both the first and the second routes are common by, first of all, obtaining the precursor chiral (*R*)-carboxylic acid: (a) enzymatic and enantioselective hydrolysis of a racemic nitrile compound mixture to produce solely a required chiral (*R*)-carboxylic acid [2,3]; or alternatively (b) the (*R*)-carboxylic acid is obtained through tedious fractional crystallization from its racemic mixture [4]. The (*R*)-carboxylic acid from the either route then undergoes methyl esterification to produce (*R*)-CMM. The third route (c), termed as asymmetric reduction, is to convert the non-chiral compound, CBFM, directly to chiral (*R*)-CMM using a single or a mixed enzyme system involving invariably a carbonyl reductase [1,5–8].

Compared to the first and the second routes, the third route obviously is more straightforward and so commercially more desirable. With large-scale application in mind, our group has recently identified and developed a new aldo-keto reductase, YtbE (EC1.1.1.X), for realizing this reaction. Compared to the efforts made previously, our own enzyme YtbE exhibited a high activity toward the substrate CBFM, showed a remarkable substrate tolerance, and resulted in excellent chiral selectivity with >99% *ee* for (*R*)-CMM [8]. In view of the documented outcome for enduring high substrate concentration by YtbE, the prospect appears to be positive for synthesizing enantiopure clopidogrel intermediate using this enzyme on industrial scales. This set of enzyme reactions is composed of two coupled reactions (see Scheme 1).

However, the inherent, rather generic enzyme instability may well complicate large scale application using this enzyme. This is so despite of encouraging advantages of this enzyme in product stereo-purity, in compelling activity under mild conditions, in high reaction turnover number, and environmentally friendly [9].

In order to compensate for the loss of YtbE activity during manufacturing processes, an excess amount of this enzyme will be required and this will inevitably result in a heightened burden on downstream processing and so on the overall production cost.

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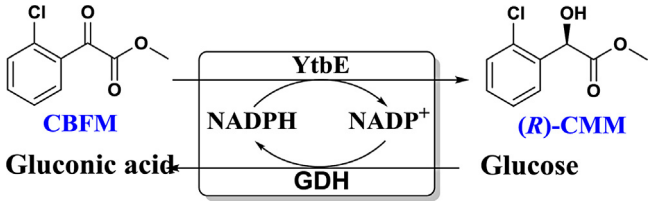
Nomenclature

ANS	8-anilino-1-naphthalene-sulphonate A hydrophobic fluorescent molecule for probing YtbE conformational change during loss of activity
DTT	dithiothreitol reducing agent to prevent YtbE intermolecular disulfide bonds
Glc-Na and Glc-K	sodium gluconate and potassium gluconate, respectively, Alkalines Na_2CO_3 and K_2CO_3 can be used to maintain the pH of reaction (Scheme 1), but Glc-Na and Glc-K affected YtbE stability differently.
YtbE	aldo-keto reductase asymmetrically reducing CBFM into (*R*)-CMM



Scheme 1. CBFM was directly reduced to chiral (*R*)-CMM using a mixed enzyme system involving the reductase YtbE and a glucose dehydrogenase (GDH).

To manage this problem, a proposed solution was to lower the reaction temperature from 30 °C to 20 °C [8]. Conceivably, this approach is at the costs of decreased productivity (owing to an increased reaction time) and increased refrigeration energy.

This present work however sought an alternative approach in which the loss of enzyme activity could be minimized through molecular interactions. Based on an understanding of enzyme inactivation mechanism, Chen et al. improved the thermal stability of maltose-binding protein Heparinase I [10]. Aided by enzyme kinetic analysis, Dib and coworkers considerably reduced thermal inactivation of D-amino acid oxidase [11]. For the present work, we thus desire to understand the mechanism of YtbE inactivation and to quantify its activity loss kinetics.

Two complementary approaches are usually seen for studying such enzyme interactions. Biochemical analysis could elucidate the effect of external factors on secondary or tertiary structures of an enzyme whilst mathematical simulation focuses on quantitative effects of these factors on enzyme activity [12,13]. Thus, the first objective of this work was to gain an insight into plausible YtbE inactivation mechanism using both the biochemical and the kinetic simulation approaches. Partially guided by these results, the second objective was to work out an improved YtbE reaction system for recommending to industrial uses.

2. Experimental and modeling methods

2.1. Materials

CBFM (≥98%) was prepared as reported previously [8] and (*R*)-CMM (≥99%) was then synthesized through CBFM bioreduction in our laboratory. ANS was purchased from Tokyo Chemical Industry Shanghai (China). $NADP^+$ and NADPH were obtained from Roche Shanghai (China). All other chemicals were of analytical grade. The enzyme YtbE was produced by *Escherichia coli* BL21 cells genetically engineered to synthesize YtbE through plasmid pET28a. This recombinant YtbE has an N-terminal His-tag and so, as described before [14], an immobilized metal affinity chromatograph was used to purify it to a specific enzyme activity of 6.47 U/mg.

2.2. Enzymatic activity assay

To assay YtbE activity, an appropriate amount of the enzyme was added to 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 2 mM CBFM and 0.1 mM NADPH at 30 °C and then decrease of the absorbance at 340 nm was monitored by a Beckman DU 730 Nucleic Acid/Protein Analyzer. One unit of enzyme activity was defined as oxidizing 1 μmol NADPH per minute.

2.3. Native SDS–PAGE

Gel electrophoresis was performed on 12% SDS–PAGE with Tris–glycine buffer (pH 8.3), and a stacking gel containing 5% acrylamide was also used. All the enzyme samples (unless specified in figure legend) were processed without 2-mercaptoethanol and boiling treatments.

2.4. Detecting YtbE conformational change by UV scanning and by a fluorescence probe

In order to reveal temperature-caused conformational change of YtbE during an inactivation process, 0.08 mg/ml YtbE in 10 mM sodium phosphate buffer (pH 7.0) was incubated at 50 °C for a time period (to be specified in figure legend) and following 4 °C for 1 h, and then 240–400 nm UV spectra were taken using a Beckman DU 730 Nucleic Acid/Protein Analyzer.

This conceivable configuration shift was investigated further using a fluorescence probing molecule ANS. This molecule binds to hydrophobic regions of proteins and therefore exhibits increased fluorescence intensity upon binding to proteins. Following addition of 0.1 mM ANS to 100 mM sodium phosphate buffer (pH 7.0) containing 0.12 mg/ml YtbE, incubation at 50 °C was for a time period (to be specified in figure legend) and then at 30 °C for 30 min. Mixtures of YtbE and (*R*)-CMM were made by swiftly combining their concentrated stocks. Upon further fast mixing with an ANS concentrated stock, incubation at 30 °C was made for 30 min. At the end of a designated incubation, the ANS fluorescence emission was scanned in the range 400–600 nm at 380 nm excitation wavelength using an F-4600 fluorescence spectrophotometer (Hitachi, Japan).

2.5. Kinetic and thermodynamic modeling

The enzyme concerned, YtbE, is assumed to take three forms [15]: the native (*N*), the unfolded (*U*), and the fully inactivated (*D*), which are connected by 2 first-order inactivation reactions,

$$N \xrightarrow{k_1} U \xrightarrow{k_2} D \tag{1}$$

where

$$\frac{dN}{dt} = -k_1 N \tag{2}$$

and

$$\frac{dU}{dt} = k_1 N - k_2 U \tag{3}$$

and

$$\frac{dD}{dt} = k_2 U \tag{4}$$

at $t = 0$, $N = N_0$, $U = D = 0$; otherwise $N + U + D = N_0$ (5)

The residual enzyme activity is defined as,

$$R_a = \frac{(N + \beta U)}{N_0} \tag{6}$$