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Short communication

Catalytic promiscuity of *Escherichia coli* BioH esterase: Application in the synthesis of 3,4-dihydropyran derivatives

Ling Jiang[a], Bo Wang[b], Rong-rong Li[c], Sa Shen[c], Hong-wei Yu[a], Li-dan Ye[a,*]

[a] *Department of Chemical and Biological Engineering, Zhejiang University, 310027 Hangzhou, PR China*
[b] *College of Materials and Chemical Engineering, Hainan University, 570228 Haikou, PR China*
[c] *School of Chemical and Material Engineering, Jiangnan University, 214122 Wuxi, PR China*

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ABSTRACT

Enzymatic catalytic promiscuity has received increasing attention in the past decade. In this research, ten enzymes were investigated for the promiscuous activity in catalysis of the Michael addition-cyclization cascade reaction of *p*-nitrobenzalacetone with 1,3-cyclohexanedione to prepare 2-hydroxy-2-methyl-4-(4-nitrophenyl)-3,4,7,8-tetrahydro-2*H*-chromen-5(6*H*)-one in anhydrous media, and control experiments were conducted to exclude false positive results. The highest yield (46.1%) was observed with *Escherichia coli* BioH esterase and the optimal reaction condition was: 1 mmol α,β-unsaturated ketone, 1 mmol 1,3-dicarbonyl compound, 20 mg *E. coli* BioH esterase, 20 ml N,N-dimethylformamide at 37 °C for 120 h. To preliminarily investigate the mechanism, site-directed mutagenesis was performed on the hydrolysis catalytic triad of BioH, and the results indicated "alternate-site enzyme promiscuity". When a series of substituted benzalacetones and 1,3-cyclic diketones were used as the reactants, yields of up to 76.3% were achieved. These results imply the potential industrial application of *E. coli* BioH in the preparation of dihydropyran derivatives.

1. Introduction

Dihydropyran derivatives have attracted significant attention due to their diverse biological activities [1]. Generally, 3,4-dihydropyran derivatives are prepared by inverse-electron-demand hetero-Diels–Alder reactions of α,β-unsaturated carbonyl compounds with electron-rich alkenes [2]. In 2008, Franke et al. used pyrrolidine derivatives as catalysts to prepare 3,4-dihydropyrans [3]. After that, Yu et al. designed and utilized a fluorinated diarylprolinol silyl ether to catalyze the reaction between 1,3-dicarbonyl compounds and α,β-unsaturated aldehydes [4]. Then, Liu et al. applied chiral diamine catalysts to synthesize 3,4-dihydropyran derivatives using cyclic dimedone and α,β-unsaturated ketones as reactants [5]. Recently, Ray and coworkers reported a facile method to synthesize 3,4-dihydronpyrans through chiral pybox-diph-Zn(II) complex catalyzed Michael addition of cyclic 1,3-dicarbonyls to 2-enoylpyridine *N*-oxides [6]. Despite the great progresses made in the employment of organocatalysts, many reported methods for the synthesis of 3,4-dihydropyran derivatives suffer from drawbacks such as high cost, harsh reaction conditions, toxicity and laborious workup procedure. Therefore, the development of a new method with a simpler and more convenient procedure using a cost-effective, eco-friendly catalyst is still in demand.

Owing to the potential application of enzyme catalytic promiscuity in organic synthesis, considerable effort has been made over the past decade [7]. For instance, enzymes have been used to catalyze Aldol reaction [8], Baylis–Hillman reaction [9], Mannich reaction [10], Michael addition [11] and Knoevenagel reaction [12]. This kind of catalytic promiscuity largely expands the application scope of enzymes in organic synthesis.

In continuation of our work on enzyme catalytic promiscuity [13], herein we describe another example of the catalytic promiscuity of *Escherichia coli* BioH esterase and its application in the synthesis of 3,4-dihydropyran derivatives via the Michael addition-cyclization cascade reaction of α,β-unsaturated ketones and 1,3-dicarbonyl compounds (Fig. 1). The reaction reported here employed the economical and eco-friendly *E. coli* BioH esterase as the catalyst and successfully provided access to a diverse range of 3,4-dihydropyran derivatives. This protocol provides an interesting insight into enzyme catalytic promiscuity and may have the potential for industrial application.

* Corresponding author. Tel.: +86 571 87951873; fax: +86 571 87951873.
E-mail address: yelidan@zju.edu.cn (L.-d. Ye).

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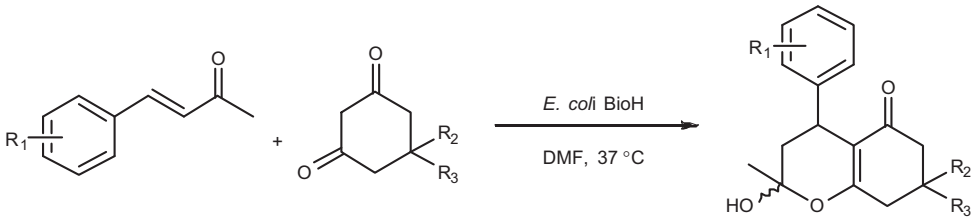

Fig. 1. *E. coli* BioH esterase-catalyzed synthesis of 3,4-dihydropyran derivatives via the Michael addition-cyclization cascade reaction between substituted benzalacetones and 1,3-cyclic diketones.

2. Materials and methods

2.1. Materials

"Amano" lipase AK, "Amano" lipase AS, "Amano" lipase AYS and "Amano" lipase DF were purchased from Amano Enzyme Inc. Lipase from *Candida rugosa* was purchased from Sigma–Aldrich. Lipozyme RMIM and lipozyme TLIM were purchased from Beijing Gaoruisen Technology Co. Ltd. Lipase from *Rhizomucor miehei*, esterase from *Rhodobacter sphaeroides* (RspE) were expressed in *E. coli* [14,15]. Bovine serum albumin (BSA) was purchased from Aladdin Chemicals. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification.

2.2. Construction of mutants by site-directed mutagenesis

pET-30a plasmid harboring the recombinant BioH gene (*E. coli* K12) was used as a template for polymerase chain reaction (PCR)-based site directed mutagenesis using the QuikChange™ method (Stratagene, La Jolla, CA). The synthetic primers used to construct the mutants are listed in Table S1 in Supplementary materials. The mutations were confirmed by DNA sequencing.

2.3. Protein expression and purification

The resulting recombinant cells were grown in 500 ml LB media containing 50 μg/ml kanamycine at 37 °C. The culture was allowed to reach an OD_{600} of 0.4–0.6 before induced with 0.5 ml isopropyl β-D-1-thiogalactopyranoside (0.1 mM) for 4 h at 37 °C. The cells were then harvested by centrifugation (2150 × *g*, 10 min, 4 °C), and washed twice with 100 ml phosphate-buffered saline (0.1 M, pH 7.4). Then the cells were resuspended in 50 ml Tris–HCl buffer (50 mM, pH 8.0) and disrupted by sonication. The supernatants were purified on a Nickel column as reported [16]. The purity of enzyme was checked by SDS-PAGE (Supplementary materials, Fig. S1).

2.4. Typical enzymatic procedure for the formation of 3,4-dihydropyran derivatives

A mixture of the 1,3-dicarbonyl compound (1 mmol), α,β-unsaturated ketone (1 mmol), pure enzyme powder of *E.* coli BioH esterase (20 mg) in DMF (20 ml) was shaken at 200 rpm, 37 °C for specified time. The reaction progress was monitored by thin layer chromatography (TLC). After the reaction was completed, the enzyme was filtered off (Whatman® qualitative medium flow filter paper, Ø15 cm, ash ≤ 0.06%, pore size 11 μm) and the filtrate was washed with water (20 ml) and extracted with dichloromethane (2× 20 ml). The organic phase was combined, dried with $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (200–300 mesh silica gel) with an eluent consisting of petroleum ether/ethyl acetate (2:1, v/v). Product-containing fractions were pooled, concentrated and dried. All the compounds were spectroscopically characterized (^{1}H NMR and ^{13}C NMR).

2.5. Analytical methods

Analytical thin layer chromatography (TLC) was performed on Haiyang precoated TLC plates (silica gel GF254), eluted with petroleum ether/ethyl acetate (1/1, v/v). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Advance 2B 400 instrument. Chemical shifts (*δ*) were quoted in ppm using $CDCl_3$ (^{1}H NMR *δ* 7.26, ^{13}C NMR *δ* 77.16) or DMSO-d_6 (^{1}H NMR *δ* 2.5, ^{13}C NMR *δ* 39.52) as solvent and tetramethylsilane (TMS) as an internal reference. The coupling constants (*J*) were quoted in Hz. The structures of the products were confirmed by comparing the ^{1}H NMR and ^{13}C NMR spectral data with those reported in the literature [5].

3. Results and discussion

The reaction of *p*-nitrobenzalacetone **1a** and 1,3-cyclohexanedione **2a** was chosen as a model reaction (Fig. 2). A wide range of enzymes were rapidly screened for catalytic activity in this Michael addition-cyclization cascade reaction (Table 1). When the reactants were incubated without any protein or with a non-catalytic protein such as bovine serum albumin (BSA), no product was formed even after 120 h (entries 1–2, Table 1). "Amano" Lipase Ak, "Amano" Lipase AS, "Amano" Lipase AYS and "Amano" Lipase DF all showed very low activity toward this cascade reaction (entries 3–6, Table 1). Lipase from *R. miehei*, Lipozyme TLIM from *Thermomyces lanuginosus*, Lipozyme RMIM from *R. miehei* and lipase from *C. rugosa* also showed low catalytic activities in this reaction, giving product yields of only 2.6%, 3.7%, 4.5% and 3.4%, respectively (entries 7–10, Table 1). When the *R. sphaeroides* esterase (RspE) was applied, the reaction proceeded slowly and the target compound was obtained in a yield of 8.7% (entry 11, Table 1). *E. coli* BioH esterase, which contains a classical Ser-His-Asp catalytic triad and belongs to the group of carboxylesterases [16], was identified as the most effective biocatalyst for this cascade reaction and the target compound was obtained in a yield of 46.1% (entry 12, Table 1). In the past few years, some studies found that the previously reported enzyme promiscuous activity was actually not catalyzed by the enzymes [17,18]. As a control experiment, the reactants were incubated

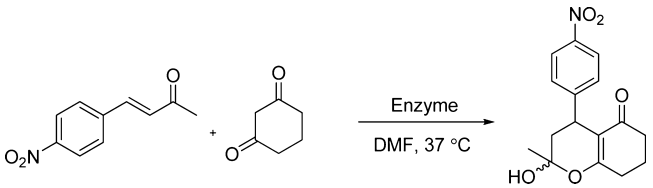

Fig. 2. Method of biocatalyst screening for the Michael addition-cyclization cascade reaction between *p*-nitrobenzalacetone and cyclohexane-1,3-dione.

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Table 1
Biocatalysts screened for the Michael addition-cyclization cascade reaction between *p*-nitrobenzalacetone and cyclohexane-1,3-dione and their product yields.[a]

Entry	Catalyst	Yield (%)[b]
1	No enzyme	0
2	Bovine Serum Albumin	0
3	"Amano" Lipase Ak from *Pseudomonas fluorescen*	<1
4	"Amano" Lipase AS from *Aspergillus niger*	<1
5	"Amano" Lipase AYS from *Candida rugosa*	<1
6	"Amano" Lipase DF from *Rhizopus oryzae*	<1
7	Lipase from *Rhizomucor miehei*	2.6
8	Lipozyme TLIM from *Thermomyces lanuginosus*	3.7
9	Lipozyme RMIM from *Rhizomucor miehei*	4.5
10	Lipase from *Candida rugosa*	3.4
11	Esterase from *Rhodobacter sphaeroides*	8.7
12	BioH esterase from *Escherichia coli*	46.1
13	Denatured BioH[c]	0
14	BioH esterase Ser82Ala	43.0
15	BioH esterase His235Ala	44.5

[a] Experimental conditions: *p*-nitrobenzalacetone (200 mg, 1 mmol), cyclohexane-1,3-dione (110 mg, 1 mmol), DMF (20 ml) and enzyme (20 mg) was shaken at 200 rpm at 37 °C for 120 h.
[b] Determined by HPLC.
[c] Pre-treated with urea at 100 °C for 8 h.

with the urea-denatured *E. coli* BioH esterase, where no conversion was observed (entry 13, Table 1), confirming that the catalysis was indeed mediated by the bioactive enzyme. However, it remains unknown whether the hydrolysis site in *E. coli* BioH esterase is also responsible for its promiscuous activity, as believed for other hydrolases [19]. In order to clarify this question, site-directed mutagenesis studies were performed on the Ser-His-Asp catalytic triad. The two mutants "BioH esterase Ser82Ala" and "BioH esterase His235Ala" showed catalytic activities similar to that of the wide-type (entries 14–15, Table 1), indicating an active site other than this catalytic triad. In 2007, Reetz and co-workers proposed a viewpoint called "alternate-site enzyme promiscuity", suggesting that the reaction catalyzed by promiscuous enzyme neither involves any of the catalytic amino acids of the natural enzymatic process, nor appears to occur in the natural binding pocket [20]. To some extent, our study confirms this view. These results implied that perhaps the tertiary structure and the special spatial conformation rather than the hydrolysis site of the BioH esterase are responsible for this cascade reaction. Other reaction conditions such as enzyme loading and temperature were also investigated. The results showed that yields of up to 46.1% were achieved when 20 mg of pure enzyme powder of BioH esterase were used in 20 ml of DMF at 37 °C for 120 h.

Encouraged by our initial study results, some more α,β-unsaturated ketones and 1,3-dicarbonyl compounds were tested in order to investigate the generality and the substrate spectrum of this *E. coli* BioH-catalyzed Michael addition-cyclization cascade reaction (Table 2). It was noted that the reaction activity of 1,3-cyclohexanedione was higher than that of 5,5-dimethyl-1,3-cyclohexanedione (entries 1–3 versus entries 5–7, Table 2). This was probably due to the steric hinderance of methyl. In the case of α,β-unsaturated ketones, the electronic properties and substituent position at the aromatic ring played an important role in the product yields. For example, α,β-unsaturated ketones with electron-withdrawing groups could enhance the reactivity of the substrate (entries 5–8, entries 11–12, Table 2), and the substituent in the 4-position of benzalacetone led to a higher yield than the same substituent in the 2- or 3-position (entry 1 versus entries 2–3, entry 5 versus entries 6–7, entry 8 versus entry 12, Table 2). This might be due to the stablilization of electron-withdrawing groups on the phenyl ring of the benzalacetones by the amino acid residues of the *E. coli* BioH via hydrogen bond, which was in favor of nucleophilic attack by carbanion.

Table 2
E. coli BioH-catalyzed Michael addition-cyclization cascade reaction between α,β-unsaturated ketones and 1,3-dicarbonyl compounds[a] (please refer to Fig. 1 for the reaction schematic).

Entry	R_1	R_2	R_3	Time (h)	Yields (%)[b]
1	4-NO_2	H	H	120	46.1
2	3-NO_2	H	H	120	27.5
3	2-NO_2	H	H	120	17.0
4	4-H	H	H	120	14.1
5	4-NO_2	CH_3	CH_3	144	76.3
6	3-NO_2	CH_3	CH_3	144	66.4
7	2-NO_2	CH_3	CH_3	168	47.0
8	4-F	CH_3	CH_3	144	68.7
9	4-Me	CH_3	CH_3	168	31.9
10	4-OMe	CH_3	CH_3	168	19.2
11	2-Cl	CH_3	CH_3	168	35.8
12	2-F	CH_3	CH_3	168	41.4

[a] Experimental conditions: α,β-unsaturated ketone (1 mmol), 1,3-dicarbonyl compound (1 mmol), DMF (20 ml), pure enzyme powder of BioH esterase (20 mg), shaken at 200 rpm, 37 °C.
[b] Isolated yields.

4. Conclusions

In conclusion, we have developed a novel *E. coli* BioH esterase-catalyzed method to synthesize 3,4-dihydropyran derivatives with moderate to high yields via the Michael addition-cyclization cascade reaction. The two mutants BioH esterase Ser82Ala and BioH esterase His235Ala showed a catalytic activity similar to that of the wide-type, confirming the view proposed by Reetz and co-workers called "alternate-site enzyme promiscuity". In addition, because this enzyme could be prepared by large scale fermentation of *E. coli* with a low cost, this protocol could provide an economic route to preparation of dihydropyran derivatives and might be potentially useful for industrialization. Further studies on improving the activity and enantioselectivity of this enzyme by both directed evolution and rational design are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.03.020.

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