Enhanced Itaconic Acid Production by Self-Assembly of Two Biosynthetic Enzymes in Escherichia coli

Zhongwei Yang, Xin Gao, Hui Xie, Fengqing Wang, Yuhong Ren, Dongzhi Wei

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China; telephone: +86 21 6425 2163; fax: +86 21 6425 0068; e-mail: yhren@ecust.edu.cn; telephone: +86 21 6425 2078; fax: +86 21 6425 0068; e-mail: dzhwei@ecust.edu.cn

ABSTRACT: Here, we described a novel strategy for the production of itaconic acid in Escherichia coli by self-assembly of aconitase (ACO) and cis-aconitate decarboxylase (CAD) existing in the metabolic pathway of itaconic acid via the protein–peptide interactions of PDZ domain and PDZ ligand. Co-expression of ACO and CAD in E. coli (uCA) resulted in low levels of itaconate (117.25 mg/L) after 48 h fermentation while the itaconate titre was significantly improved up to 222.15 mg/L by self-assembly of ACO-PDZ (APd) and CAD-PDZlig (CPi) in E. coli (sPP) under the same conditions. To further confirm the effect of self-assembly, the itaconate catalyzed from sodium citrate was determined. The sPP was extra efficacious in the early catalytic period, showing approximately threefold itaconate yields increased after 2 h catalysis, when compared to uCA. Furthermore, the itaconate production of sPP was increased from 5 to 8.7 g/L after 30 h of reaction compared to uCA. This self-assembly strategy showed remarkable potential for the further improvement of itaconate production.


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KEYWORDS: itaconic acid; aconitase; cis-aconitate decarboxylase; self-assembly; protein–peptide interactions

Introduction

Itaconic acid (2-methylidenebutanedioic acid) is a C5-dicarboxylic acid with broad application as a building block for the production of plastics, resins, synthetic polymers, and so on (Miall, 1978; Okabe et al., 2009; Tate, 1981). Moreover, itaconic acid can be polymerized via its two carboxyl groups to generate a promising chemical polyacrylic acid (Nuss and Gardner, 2013).

Currently, itaconic acid is mainly fermented by Aspergillus terreus. There are only two enzymes in the itaconic acid pathway (Li et al., 2011). The first key enzyme is aconitase (ACO), which is found in the tricarboxylic acid (TCA) cycle for the reversible transformation of citric acid to cis-aconitic acid (Lauble and Stout, 1995). Then cis-aconitic acid is decarboxylated to itaconic acid by the second key enzyme, cis-aconitate decarboxylase (CAD). Recently, various heterologous hosts have been metabolically engineered to produce itaconic acid, including Escherichia coli (Harder et al., 2016; Jeon et al., 2015; Okamoto et al., 2014; Vuoristo et al., 2015), Saccharomyces cerevisiae (Blazeck et al., 2014), Yarrowia lipolytica (Blazeck et al., 2013), Aspergillus niger (Blumhoff et al., 2013; Van der Straat et al., 2014), and so on. Van der Straat et al. (2014) overexpressed a codon-optimized CAD and two putative transporters in A. niger, resulting in 25-fold higher yields of itaconic acid compared to the strain only expressed CAD gene. E. coli has been proposed as an excellent host to produce itaconic acid (Yu et al., 2011) because of its several unique advantages in biotechnological processes, such as high tolerance to organic acids. Wild-type E. coli cannot produce itaconic acid because it lacks CAD. Vuoristo et al. (2015) developed a metabolic strategy to accumulate itaconic acid up to 690 mg/L in E. coli by overexpressing CAD gene from A. terreus and citrate synthase and aconitase from Corynebacterium glutamicum, and by deleting the genes encoding phosphate acetyltransferase and lactate dehydrogenase.

Many researchers reported that substrate channeling, the direct transfer of a reactant from one enzyme to another without diffusing to the bulk environment, is leading to a high local concentration of intermediate along a pathway, which also overcome unfavorable thermodynamics in the bulk environment (Wheeldon et al., 2016). Structuring non-natural cascade catalysis by protein, nucleic acid, and polymer scaffolds to assemble and co-localize of multi enzymes of a cascade has achieved considerable success. Dueber et al. (2009) used an artificial scaffold to assemble three mevalonate biosynthesis enzymes, leading to a significant increase in production. Dennis et al. constructed porous enzyme-containing
polymersomes to perform one-pot multi-step reactions, converting the substrate GA4 into glucose, which was then used by GOx and HRP to generate ABTS*+ (Vriezema et al., 2007). Wang et al. (2014) constructed an efficient multi-enzyme cascade system based on ultrathin, hybrid microcapsules by combining the unique functions of catechol and gelatin, which maintained a methanol yield of 52.6% even after nine recycling steps. All these examples showed that assembly and co-localization of multi-enzymes into controlled compartments can enhance cascade reactions, leading to high selectivities and yields.

Multi-protein assembly via protein–peptide interactions is a novel and efficient tool to facilitate multi-enzyme biocatalysis (Liu et al., 2013). The self-assembly strategy of fusing a PDZ domain and its corresponding ligand (PDZlig) (Dueber et al., 2009) to the self-assembly strategy is based on fusing a PDZ domain and its corresponding ligand (PDZlig) (Dueber et al., 2009) to the C-termini of ACO and CAD, yielding ACO-PDZ domain (APd) and CAD-PDZlig (CPl), respectively (Fig. S1). Via the interactional force of protein–peptide, APd was able to connect with CPl to self-assemble in strain APd-CPl (sPP), greatly enhancing production of itaconate (Shanghai, China), and CAD was reverse transcribed and amplified.

In this work, we developed a novel strategy for the production of itaconic acid by introducing and assembling aconitase (ACO) and cis-aconitate decarboxylase (CAD) from A. terreus in E. coli. The self-assembly strategy is based on fusing a PDZ domain and its corresponding ligand (PDZlig) (Dueber et al., 2009) to the C-termini of ACO and CAD, yielding ACO-PDZ domain (APd) and CAD-PDZlig (CPl), respectively (Fig. S1). Via the interactional force of protein–peptide, APd was able to connect with CPl to self-assemble in strain APd-CPl (sPP), greatly enhancing production of itaconate.

The codon-optimized ACO gene was synthesized by Generay (Shanghai, China), and CAD was reverse transcribed and amplified using cDNA derived from mRNA extracted from A. terreus NH 2624 (Rainesalo et al., 2005). The experimental plasmids (Fig. S1) pAPd-pCPl and control plasmids pACO–pCAD were both co-transformed into E. coli Rosetta Competent (DE3) Cell to express strains self-assembled APd-CPl (sPP) and unassembled ACO–CAD (uCA). To achieve the highest level of protein heterologous expression, various conditions, such as the concentration of isopropyl-β-thiogalactopyranoside (IPTG) and induction temperature were screened (Fig. S2). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed that, at high concentrations of IPTG, almost all CAD protein was expressed in the form of inclusion bodies, and ACO showed similar result. When sPP was induced at lower concentrations of IPTG, the amount of soluble CAD and ACO was increased slightly (Fig. S2B). The effect of induction temperature on protein expression was shown in Figure S2A. The expression of soluble ACO appeared to increase with decreasing temperature, with induction temperatures from 30 to 18°C, while CAD was barely expressed in soluble form under higher induction temperatures (Fig. S2A). These results indicated that the best expression of these two soluble proteins occurred with 0.2 mM of IPTG and at 18°C.

To examine the enzymatic activities of APd and CPl, purified enzymes (Fig. S3) were used, with equal amounts of purified ACO and CAD as control. The reactions were performed at 37°C in several minutes, and the specific activities were summarized in Figure 1. The specific activity of APd was mostly equal to the original protein ACO at the same condition, while the specific activity of CPl decreased lightly. These results suggested that the fusion did not significantly influence enzymatic conformations and activities. Additionally, to further confirm the self-assembly form, the molecular masses of the purified enzymes APd and CPl were determined by gel filtration chromatography and evaluated using the original standard curve (Fig. S4). The results (see Table S2) showed that, compared to the theoretical molecular weights of single subunit of APd and CPl, which were 101 and 54 kDa, respectively, the calculated molecular weights were consistent with one subunit of APd and two subunits of CPl. Furthermore, the molecular weights of the mixture of APd and CPl (2:1 molar ratio after 30 min) are shown in Table S2 according to the results of gel filtration chromatography, which was close to a theoretical self-assembled molecular weight of 310 kDa, suggesting that APd and CPl might assemble in a molar ratio of 2:1 (Fig. 2). Dynamic light scattering (DLS) measurements (Fig. S5) also showed that most of the APd and CPl assembled in vitro after 30 min of mixing when the molar ratio of APd to CPl was 2:1 (1 μM) comparing to other molar ratios, while still retaining a small amount of residuals might be due to the low concentrations.

Before the self-assembly experiments were performed, fusion expression of ACO and CAD was carried out as a control strategy to co-locate ACO and CAD. Fused ACO and CAD cannot be expressed in soluble form (data not shown), showing that the fusion strategy was not suitable for the expression of these two enzymes. Then, the self-assembly of ACO and CAD was performed by co-expression of APd-CPl in E. coli, with co-expression of ACO–CAD as a control. Itaconate production by sPP and uCA was respectively monitored in 500 mL baffled shake flasks containing 100 mL of M9 minimal media at 30°C after induced by the addition of 0.2 mM IPTG at 18°C for 24 h (Fig. S6). The results showed that the self-assembly of APd and CPl significantly increased itaconate production during in vivo fermentation experiments, resulting in a higher itaconate titer of up to 222.15 mg/L for sPP, while the production titer of uCA was 117.25 mg/L under the same condition.

We also harvested cells to catalyze sodium citrate into itaconate in vitro. The itaconic acid yields at various pH and substrate concentrations at 37°C by sPP and uCA were monitored. The results are shown in Figure 3. The conversion rate of the substrate was higher in acidic reaction buffer than at alkaline reaction buffer for both sPP and uCA (Fig. 3A and B). Indeed, itaconate production of sPP was strongly enhanced at pH 6.5 (in a pH-controlled bioreactor) to nearly 2.9 g/L after sodium citrate was transformed for 8 h, while other higher or lower pH values decreased itaconate production levels at the same condition. Production by uCA was similar in the same conditions. To test the effect of different concentrations of substrate, the sPP- and uCA-catalyzed reactions were conducted in Erlenmeyer flasks at 37°C (pH was maintained at 6.5 with 1 M HCl). The itaconate production by uCA increased with increasing concentrations of sodium citrate, and at 0.5 M sodium citrate, the reaction reached the point of substrate saturation (Fig. 3C and D). Strain sPP showed similar results. However, the itaconate production by sPP was approximately twofold greater than that of uCA (Fig. 3C). In this case, sPP reached the maximum itaconate production with a substrate concentration of 0.5 M, which was selected as the final substrate concentration for all future whole-cell catalysis experiments in vitro.

To further confirm the effect of self-assembly, we performed reactions catalyzed by sPP and uCA in a 30 mL pH-controlled bioreactor to sustain catalysis with 0.5 M sodium citrate and at a pH
of 6.5. As shown in Figure 4, at the beginning of reactions, the catalysis efficiency of sPP was nearly threefold, and 2.6-fold higher than that of uCA after 2 and 4 h, respectively, of the pH-controlled reaction. Finally, the reaction catalyzed by sPP resulted in a titer of 8.7 g/L itaconate, which was 1.7-fold higher than that of uCA (5 g/L). The itaconate yields of sPP and uCA from sodium citrate were 13.4% and 7.7%, respectively, under substrate-excess condition after 30-h reaction. These results suggested that the high local flux of metabolic intermediate cis-aconitic acid in sPP could be converted into itaconate by CPl more efficiently compared to uCA, resulting in high production titers. Self-assembly of APd and CPl leaded to steric hindrance between enzymes in a cascade in cells which increased yields from reactions by improving the local concentration of metabolic intermediate. Notably, production by the self-assembled enzymes diminished with increasing reaction, which might be due to the reduced activity of enzymes in the later stages of the reaction and the inhibition of enzyme CPl/CAD by itaconate. We found that CAD and ACO proteins were rather unstable in vitro (data not shown), and its stability might be also low in vivo, which agreed with the other research (Blumhoff et al., 2013). To test another possible explanation for this phenomenon, an itaconate inhibition assay was conducted according to a previously described method. For this assay, we added various amounts of itaconic acid into the bioreactor at the beginning of the catalysis reaction (Fig. S7). The initial high concentration of itaconic acid led to a decrease in itaconate production, showing the product inhibition of this reaction, which could be caused by the product inhibition of enzyme CAD (Fig. S8B). After 30 h, all reaction groups reached nearly the same level of production, indicating that the capacity of the itaconate pathway was limited by CAD enzyme activity in E. coli. These results suggested that, with the formation of itaconate, the inhibiting effect on production increased throughout the catalytic reaction, thereby reducing the efficiency of the assemblies.

In conclusion, this study presents a novel strategy for the production of itaconic acid by the self-assembly of APd and CPl in E. coli via interacting protein PDZ domain and PDZ ligand. Strain uCA resulted in low levels of itaconate (117.25 mg/L) after 48 h fermentation. However, the itaconate titre was significantly improved up to 222.15 mg/L by strain sPP under the same conditions, which was 1.9-fold higher than that of uCA. Enzymes APd and CPl self-assembled in the molar ratio of 2:1 in vitro which was determined by gel filtration chromatography and Dynamic light scattering analysis. To further confirm the effect of self-assembly, we performed the reactions catalyzed by sPP and uCA. Compared to uCA, sPP was more efficient in the early catalytic period, showing an approximately threefold increasement in itaconate yields relative to the control. Furthermore, the itaconate production by sPP

![Figure 1](image1.png)  
**Figure 1.** The specific activities of purified ACO, APd, CAD, and CPl. (A) Gray bar showed the enzyme activity of ACO, and the blank one APd; (B) gray bar showed the enzyme activity of CAD, and the blank one CPl. Activity values [U/μmol proteins] are the arithmetic mean of at least three different measurements. Error bars show the standard deviation of three measurements.

![Figure 2](image2.png)  
**Figure 2.** Strategy for the self-assembly of monomeric ACO and dimeric CAD. The scheme at right shows the putative reaction mechanism for the conversion of sodium citrate (CIT) into itaconate by assembled ACO and CAD. Cis-ACO, cis-aconitic acid.
increased from 5 to 8.7 g/L compared to uCA. This study demonstrates a multi-enzyme assembly strategy in a heterologous production pathway for itaconic acid synthesis in *E. coli* cells and provides a promising stepping-stone toward the development of an efficient itaconic acid production method for industrial application.

Materials and Methods

Construction of Fusion Genes

Plasmids were constructed using a normal enzymatic assembly method (Gao et al., 2014). Target ACO gene was synthesized with codon optimization (Generay Biotech Co., Ltd. Shanghai, China), while CAD gene was amplified from the cDNA which was derived from mRNA extracted from *A. terreus* NIH 2624. Oligonucleotide sequences of the PDZ domain were previously constructed in our laboratory. The ACO gene and PDZ gene were inserted in sequence into the formal modified pET-21a plasmid (Fig. S1A), constructing the plasmid pET21a-ACO-PDZ (pAPd; Fig. S1B). ER/K, a 5 nm α-helical linker segment, was inserted between the ACO and PDZ fragments. The PDZ-ligand was fused to the reverse primer of CAD to construct the plasmid pET28a-CAD-PDZlig (pCPl; Fig. S1C). The ACO gene and CAD gene with 5' NdeI and 3' Xhol (Thermo Fisher Scientific, Rockford, IL) restriction sites were amplified by PCR and then the genes were sequentially inserted into the plasmids pET-21a and pET-28a to form pET21a-ACO and pET28a-CAD, respectively. The two control plasmids were constructed to express the two enzymes without fusion with PDZ domain or its ligand.

All plasmids were transformed or co-transformed into *E. coli* Rosetta Competent (DE3) Cells for heterologous protein expression and whole-cell catalysis.

Protein Expression and Purification

Recombinant *E. coli* Rosetta (DE3) strains were cultured in Luria-Bertani (LB) medium or M9 minimal media with antibiotics at 37°C. When the optical density at 600 nm (OD600) of the LB liquid medium reached approximately 0.6–0.8, protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and then *E. coli* were cultivated at 18°C for 24 h.

After induction of recombinant *E. coli* Rosetta (DE3), the harvested cells were washed twice with phosphate-buffered saline (PBS; pH 7.0) and resuspended in 20 mM PBS. The resuspended cells were disrupted by sonication with the addition of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF). After centrifugation at 10,000g for 30 min, the supernatant was purified by an AKTA Prime system equipped with a 5-mL HisTrap FF column (GE Healthcare, Waukesha, WI). The protein concentration was determined by the Bradford protein assay kit.

Enzymatic Activity Assays

The activity of cis-aconitate decarboxylase (CAD; CPl) was measured according to Blumhoff et al. (2013). The freshly prepared stock solution of cis-aconitic acid contained 160 mM cis-aconitic acid (98%, Aladdin Chemistry Co. Ltd., Shanghai, China), 200 mM sodium phosphate buffer (pH 7.0), and the working solution was then diluted twice in which the pH was maintained at 6.2 by adding 2 M NaOH. The standard assay mixture contained 80 mM freshly prepared cis-aconitic acid in 100 mM sodium phosphate buffer (pH 6.2) and 5 μL of purified enzyme. The reaction was carried out at 37°C for 2 min and then inactivated at 80°C for 30 min. The enzymatic product itaconic acid was analyzed by high-pressure liquid chromatography (HPLC) and detected by a UV–VIS detector (Spectrasystem UV1000, λ = 210 nm). One CAD unit was defined
Itaconic Acid (g/L) vs. time (h) for cells harvested in pH-controlled bioreactors. The activity of aconitase (ACO; APd) was measured by the formation of isocitrate detected by the commercial D-Isocitric Acid Assay Kit (Megazyme) following the manufacturer's instructions. The reaction, containing 100 mM sodium citrate in 100 mM sodium phosphate buffer (pH 7.0) and 100 μL of purified enzyme, was carried out at 37°C for 10 min and then inactivated at 80°C for 30 min. One ACO unit was defined as the amount of enzyme that catalyzed the formation of 1 mM isocitrate/min at 37°C.

The purified proteins ACO and CAD were used to determine the subunit number using a Gel Filtration Marker Kit for Protein Molecular Weights 29,000–700,000 Da (Sigma–Aldrich, Catalog Number MWGF 1000). The method was described in the product information. The molecular masses determined by gel filtration chromatography were compared with the standard proteins in the kit, with molecular masses ranging 29–443 kDa in this study. The elution buffer used for gel filtration chromatography contained 100 mM Na₂SO₄ in 100 mM phosphate buffer (pH 7.4).

Freshly purified APd and CPI were mixed in 2-mL epoxy epoxide tubes and incubated at 4°C for 30 min to induce the self-assembly, which was evaluated by gel filtration chromatography and dynamic light scattering (DLS). A DynaPro NanoStar instrument (Wyatt Technology, Santa Barbara, CA) was used to analyze APd, CPI, and APd–CPI samples at 2:1 molar ratio of API (1 μM) to CPId (1 μM). Samples were filtered using a 0.22 μm syringe filter (MILLEX-GP) prior to analysis and each measurement was made three times at 25°C.

In Vivo Fermentation
Cells were cultivated in 100 mL of M9 minimal media (containing 100 μg/mL ampicillin, 50 μg/mL kanamycin, and 34 μg/mL chloromycetin) at 30°C after induced by the addition of 0.2 mM IPTG at 18°C for 24 h. Samples were regularly taken to measure the concentrations of itaconate. Detection methods were described above.

In Vitro Whole-Cell Catalysis
Cells were cultivated at 18°C on a shaker (200 rpm) in 1-L baffled shake flasks containing 200 mL of LB medium after being induced by adding 0.2 mM IPTG for 24 h. Co-transformation strains sPP and uCA were harvested to catalyze sodium citrate into itaconate in vitro to compare the self-assembled strain with the unassembled one.

The pH and concentration of sodium citrate were regularly changed to achieve high efficiency in the production of itaconic acid. The catalytic reaction containing 30 g/L of wet cells in 50 mM sodium phosphate buffer (pH 6.5) and 0.5 M sodium citrate was carried out at 37°C for 30 h, and the pH was maintained at 6.5 by the automated addition of 1 M HCl. Samples were regularly taken to measure the concentrations of substrate and product. Detection methods were described above.

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References


Supporting Information

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