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Research Article 研究报告

Enzymatic characterization of TbrA, the first identified aconitate isomerase from *Bacillus thuringiensis*

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Abstract: [Objective] Aconitate isomerase (AI) can mediate the biosynthesis of *trans*-aconitic acid (TAA), a multi-bioactive small molecule with many application potentials. Although the first AI gene (*tbrA*) was just identified in the bacterium *Bacillus thuringiensis*, the property of AI remains unclear. Here we characterized TbrA to understand the AI catalysis. **[Methods]** We expressed His_6-tagged TbrA protein in *Escherichia coli* Rosetta strain and purified it with Ni^{2+} affinity chromatography. We characterized TbrA *in vitro* and applied HPLC to determine product formation and enzyme activity. **[Results]** The optimal pH, temperature and NaCl ionic strength for TbrA activity were determined to be 8.0, 37 °C and 25 mmol/L, respectively. TbrA exhibited a cold tolerance, retaining nearly 60% of its enzymatic activity at 10 °C. Notably, TbrA activity was significantly enhanced by Mg^{2+}, Ca^{2+} and DTT, but strongly inhibited by Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}. K_m, V_{max}, k_{cat} and k_{cat}/K_m for TbrA forward reaction [from *cis*-aconitic acid (CAA) to TAA] were 6.25 mmol/L, 1.39 μmol/(L·s), 4.08 1/s and 0.65 L/(mmol·s), and for TbrA reverse reaction (from TAA to CAA) were 71.50 mmol/L, 4.17 μmol/(L·s), 12.25 1/s and 0.17 L/(mmol·s), respectively. **[Conclusion]** The AI enzyme TbrA can achieve maximum activity under mild conditions, and favors for TAA formation. This study quantitatively described the catalytic properties of TbrA protein, which provided the basis for its potential application in TAA industrial bioproduction.

Keywords: aconitate isomerase, *Bacillus thuringiensis*, *trans*-aconitic acid, *cis*-aconitic acid

Aconitate isomerase (AI; EC 5.3.3.7) catalyzes the interconversion between *cis*-aconitic acid (CAA) and *trans*-aconitic acid (TAA)[1–3], a metabolic branch of the central tricarboxylic acid (TCA) cycle (Figure 1). Rao and Altekar first reported the existence of AI in 1961 in some *Pseudomonas* bacteria, that could grow on a synthetic medium with TAA as the sole carbon source (later called the ACO medium[4–5]); they observed that their cell-free extracts could mediate the formation of citric acid

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from TAA as well as TAA from CAA *in vitro*[6]. In addition, many plants such as maize, wheat, barnyard grass and sugar cane were also found to produce AI[7–9], which generally exists at high concentrations in the leaf and root tissues[7]. Although its existence has been proposed for more than half a century, the first AI-encoding gene, *tbrA* (TAA biosynthesis-related gene *A*), was just identified recently in our research on *Bacillus thuringiensis*, one of the most excellent nematode pathogens[10–13], by both *in vivo* and *in vitro* experiments[1]. However, AI genes of plant origin continue to remain unknown. The *tbrA* gene (1074 bp) is located on the *B. thuringiensis* plasmid pCT281 and encodes TbrA (357 aa), a cytoplasmic protein that belongs to the PrpF isomerase superfamily. The sequence of TbrA shares 27% identify and 97% coverage with that of PrpF (397 aa) in *Shewanella oneidensis* strain MR-1[1,14–15]. Another gene, *tbrB*, which is located 111 bp downstream of *tbrA*, encodes a TAA extracellular-transporter protein and comprises a *tbr* operon with *tbrA*. However, the physiological function mediated by TbrA is different from the function of TAA assimilation mediated by most of the other AI proteins[4,6], TbrA is a seemingly new and distinct type of AI protein because it cannot support the growth of *B. thuringiensis* on an ACO medium[16], however, it could specifically enable the cell to produce considerable amounts of TAA. The significance of high TAA production was later demonstrated as that TAA is a natural nematicidal factor, which is specifically biosynthesized by the *B. thuringiensis* pathogen to intoxicate nematode hosts[1]. These findings revealed the first biological sequence of AI protein, and may facilitate the progress of identifying the AI gene in plant species.

TAA has the potential for application in many fields. On one hand, TAA has long been used as industrial chemicals like antioxidant, plasticizer, and lubricant and as a substrate of functional polymers[17–19]. On the other hand, TAA shows attractive potential for applications in agriculture

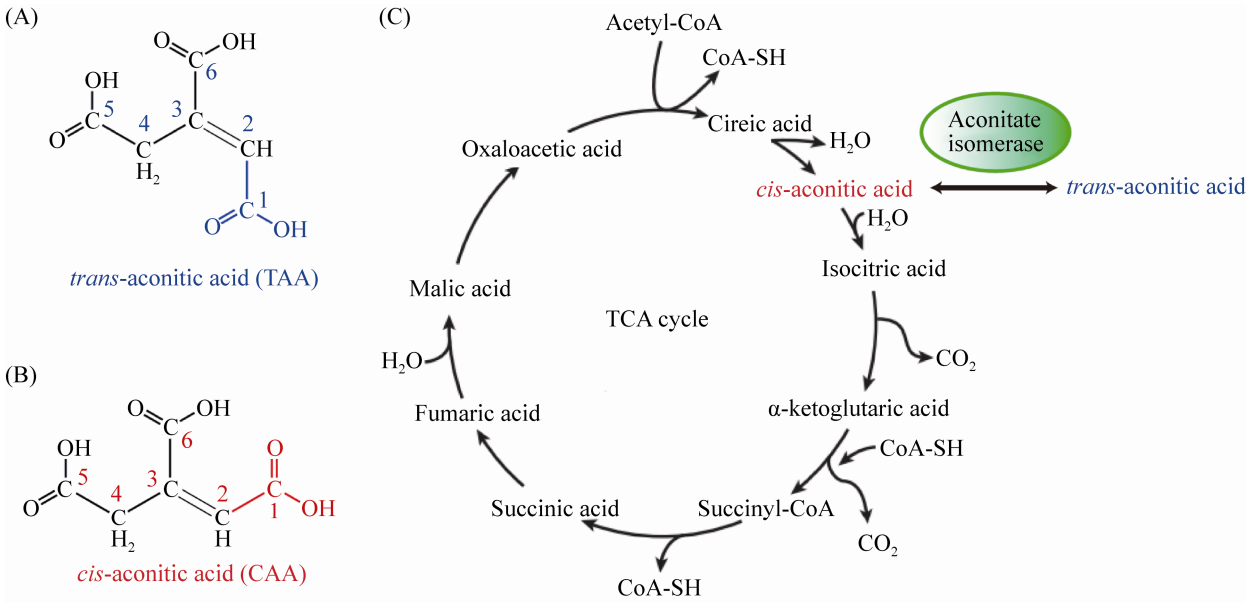

Figure 1. Aconitate isomerase mediates a specific branch of the TCA cycle. A: Chemical structural formula of TAA; B: Chemical structural formula of CAA. The conformational differences between the 1-carboxyl groups of TAA and CAA molecules are highlighted in blue and red, respectively; C: The relationship between the AI-mediated pathway and the TCA cycle.

and medicine. In addition to intoxicate nematodes[16], for example killing the most damaging root-knot and cyst nematodes[20–22], TAA is also active against the brown planthopper (the most notorious pest of rice)[8,23–26], the *Leishmania* parasite (the pathogen of kala-azar disease)[27–28], and mammalian inflammation[29–30]. However, presently, TAA is mainly produced through industrial chemosynthesis[31], a process that is accompanied by the release of numerous byproducts. Hence, an effective, selective, and eco-friendly enzymatic bioprocess needs to be developed for TAA production, and one possible improvement includes inclusion of a TbrA-based biocatalyst. Therefore, the catalytic properties of this functional enzyme should be elucidated.

In this study, the enzymatic characteristics of TbrA, the sole AI protein that is specific in TAA biosynthesis, were quantitatively determined under optimal reaction conditions including cofactor effects, optimal pH, temperature as well as ionic strength parameters, and kinetic constants of K_m, V_{max}, k_{cat}, and k_{cat}/K_m. This work provides a more detailed understanding of AI catalysis and may serve as a foundation for utilizing enzymatic engineering for the industrial bioproduction of TAA in the future.

1 Materials and methods

1.1 Bacterial strains and culture conditions

The *tbrA* gene (1074 bp) is located on the largest plasmid, namely the pCT281 (281231 bp) plasmid of the *B. thuringiensis* CT-43 strain[32]. Thus, the CT-43 strain was used as a *tbrA* gene donor. *Escherichia coli* DH5α and Rosetta strains were used for *tbrA* cloning and TbrA expression, respectively. All *B. thuringiensis* and *E. coli* strains were cultured in Luria-Bertani (LB) medium at 28 °C for both CT-43 and Rosetta cells, and at 37 °C for DH5α cells. When appropriate, antibiotics were added at the following final concentrations: 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 50 μg/mL kanamycin.

1.2 Construction of a TbrA-expression plasmid and transformation of bacterial cells

DNA fragment harboring the open reading frame (ORF) region of *tbrA* (stop codon-excluded; 1071 bp) was amplified from the genomic DNA of *B. thuringiensis* CT-43 strain and cloned into *Nde* I and *Xho* I sites of the pET28a expression vector to generate a recombinant pET28a-*tbrA* plasmid. Thereafter, the plasmid was transformed firstly into *E. coli* DH5α cells for sequence verification, and finally into *E. coli* Rosetta cells, to yield recombinant Rosetta-TbrA for subsequent TbrA-inducible expression. The primer sequences for *tbrA* ORF amplification were as follows: *tbrA-F-Nde* I, GG AATTCCATATGATGAAAATACCTTGTTTTGTT, and *tbrA-R-Xho* I, GCCCTCGAGAGGTATTATT AATTCGCCTTT. *Nde* I and *Xho* I restriction sites are underlined.

1.3 Purification of recombinant TbrA

A single colony of Rosetta-TbrA cells was cultured in 5 mL of LB medium with kanamycin and chloramphenicol at 37 °C for 4 h. Subsequently, the culture was transferred into 100 mL of LB medium with an appropriate amount of antibiotics in a ratio of 1:100, and was cultured at 37 °C to an OD_{600} of 0.8. Following the addition of IPTG (final concentration of 0.1 mmol/L), the TbrA protein was induced at 28 °C for 6 h with vigorous shaking. Cells were harvested and subjected to high-pressure homogenization at 4 °C. Thereafter, the generated cell-free extract was loaded onto a Ni^{2+} nitrilotriacetic acid column, on which the C-terminal His_6-tagged TbrA was remained. The column was washed and the His-tagged TbrA was eluted using imidazole at concentrations of 60 and 500 mmol/L, respectively. The collected fractions were dialyzed against 20 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 1 mmol/L EDTA, 0.1 mmol/L DTT, and subsequently stored at –80 °C.

1.4 HPLC quantification of CAA and TAA production

The HPLC system used for determining the amount of CAA and TAA synthesized in the enzymatic reaction systems was composed of a HITACHI Primaide 1110 pump, a HITACHI Primaide 1430 diode array detector, and a TC-C_{18} column (250 mm×4.6 mm, 5 μm; Agilent). To quantify the CAA content, a standard curve of CAA concentration against the CAA peak area was first plotted and the external standard method was used. Thereafter, a 10-μL volume of reaction sample was delivered at a flow rate of 1.0 mL/min by the mobile phase containing 10% methanol with 0.1% formic acid, and was monitored at 260 nm and at 25 °C for 15 min. The CAA and TAA peaks were efficiently separated under the conditions described above. The TAA content was quantified by adopting the same aforementioned procedure; however, a standard curve with TAA concentration against TAA peak area was plotted. CAA and TAA commercial standards were purchased from Sigma-Aldrich and Tokyo Chemical Industry, respectively.

1.5 Characterization of the AI activity of TbrA

1.5.1 Influence of pH and temperature on enzyme activity: All enzymatic activity assays were performed using 3.4 μmol/L purified TbrA (to a final concentration of 0.34 μmol/L), which was dissolved in the dialysate buffer containing 20 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 1 mmol/L EDTA, and 0.1 mmol/L DTT. The TbrA enzymatic assay was performed by firstly adjusting the pH of CAA or TAA substrate samples to 7.0 by adding 5 mol/L NaOH prior to their addition to the reaction system. Along with each of the characterization assays, a control reaction, in which the purified TbrA enzyme was excluded, was also assayed, so that the non-enzymatic isomerization between CAA and TAA could be deducted from the total product formation of each reaction. All reactions were conducted at 250 μL test volume at a relevant designated temperature for 30 min. The reactions were terminated by the addition of 10 μL of 6 mol/L HCl, and the samples were subsequently analyzed by HPLC as mentioned before. In this study, all experiments were preformed in triplicates, and the error bars represented the standard deviation of the three replicates.

The optimum pH of TbrA catalysis was determined by performing an enzymatic assay using different buffers (as described in the legend of Figure 2) at 37 °C. Additionally, the optimal temperature was determined by incubating each of the samples at various temperatures including 10, 20, 28, 37, 50, 60, 70 °C at pH 8.0.

1.5.2 Effect of metal ions and DTT: To evaluate the effect of metal ions and DTT on the isomeric activity of TbrA, the enzymatic systems containing CAA as the substrate were treated with a 10 mmol/L final concentration of $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $FeCl_2$, $CuCl_2$, $NiSO_4$, $CoCl_2$, and DTT solution in 50 mmol/L HEPES buffer (pH 8.0), at 37 °C for 30 min. TbrA activity in the control tube that contained no additives was set to 100%; thereafter, the relative activity of each additive-containing tube was calculated. To examine the optimal concentration of metal ion that has the maximum stimulation effect on enzymatic activity, the ion samples with final concentrations ranging from 10 mmol/L to 250 mmol/L were used.

1.5.3 Effect of ionic strength: The effect of ionic strength on the activity of TbrA was evaluated by incubating the reaction supernatant in 50 mmol/L HEPES buffer (pH 8.0), containing NaCl at concentrations ranging from 0 to 500 mmol/L, at 37 °C for 30 min. The highest isomeric activity was set to 100%.

1.5.4 Kinetic parameters of TbrA enzyme: The K_m and V_{max} values of TbrA were measured in reaction samples that comprised of varying final concentrations of CAA (1, 2, 5, 10, 20, 50 mmol/L) or TAA (1, 2.5, 5.0, 10.0, 25.0, 50.0 mmol/L) in

50 mmol/L HEPES buffer (pH 8.0) at 37 °C for 30 min. The K_m and V_{max} kinetic parameters were determined using the Lineweaver-Burk plot. The concentration of TbrA enzyme used for k_{cat} and k_{cat}/K_m calculation was determined by the Bradford method[33], for which bovine serum albumin was used as the standard.

2 Results and discussion

2.1 Cloning, expression, and purification of TbrA

The DNA fragment of *tbrA* ORF (1071 bp, the stop-codon 'TAA' was excluded), which was amplified from the genomic DNA of the *B. thuringiensis* CT-43 strain, was cloned into the pET28a expression vector and introduced into *E. coli* Rosetta cells for TbrA-inducible expression. After inducing the cells with 0.1 mmol/L IPTG for 6 h at 28 °C, the C-terminal His_6-tagged TbrA was released into the cell supernatant by a high-pressure homogenizer, and subsequently purified using Ni^{2+} affinity chromatography. SDS-PAGE analysis of the eluted fraction revealed a band of approximately 40 kDa (Figure 2-A), which corresponds to the theoretical molecular weight of the His_6-tagged TbrA (41487 Da), as predicted by the Compute pI/M_w tool available from ExPASy. Consequently, about 1 mg of TbrA protein was obtained from 200 mL of cell culture in LB medium.

2.2 Effect of pH and temperature on TbrA activity

Generally, AI catalyzes both the forward (from CAA to TAA) and the reverse (from TAA to CAA) isomerization reactions. In this study, we chose the forward reaction, which is responsible for TAA biosynthesis, to determine the optimal conditions for TbrA catalysis.

To detect the effect of pH, TbrA activity was measured at pH values ranging from 5.5 to 10.0, using 50 mmol/L MES buffer (pH 5.5–6.5), 50 mmol/L HEPES buffer (pH 7.0–8.0), and 50 mmol/L CHES buffer (pH 8.5–10.0). The reactions were performed at 37 °C for 30 min using 10 mmol/L CAA as the substrate. As shown in Figure 2-B, TbrA could efficiently synthesize TAA in weakly alkaline conditions (pH 7.5–8.0); the highest relative TbrA activity was observed at pH 8.0, but which rapidly decreased at the strongly alkaline pH range. Therefore, unless otherwise specified, 50 mmol/L HEPES at pH 8.0 was used for TbrA reactions.

The effect of temperature was analyzed from 10 to 70 °C. The optimal temperature for TbrA activity was observed to be 37 °C (Figure 2-C). Clearly, TbrA has a relatively wide suitable temperature range and is more tolerant to low temperatures, as nearly 60% of the relative activity was stored at 10 °C, whereas at 70 °C, only 30% of the activity remained. This characteristic may allow for higher product output with lower industrial energy input, which may be advantageous for possible future industrial applications of TbrA. Therefore, the reaction temperature was controlled at 37 °C in the following characterization experiments.

2.3 Effect of chemical additives on TbrA activity

The effect of eight metal ions and DTT chemical on TbrA activity was assessed. As shown in Figure 3-A, Mg^{2+}, followed by Ca^{2+}, exhibits the strongest activating effect. Figure 3-B indicates that the optimal Mg^{2+} concentration is 75 mmol/L. Furthermore, DTT did not inhibit, but rather promoted TbrA activity (Figure 3-A), suggesting the absence of disulfide bonds in TbrA, despite the presence of five Cys residues in its amino acid sequence. On the contrary, most divalent metal cations including Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} inhibited TbrA activity to different extents, thus, indicating that chemical contamination of these metal cations should be avoided in TbrA applications. A previous study showed that an AI

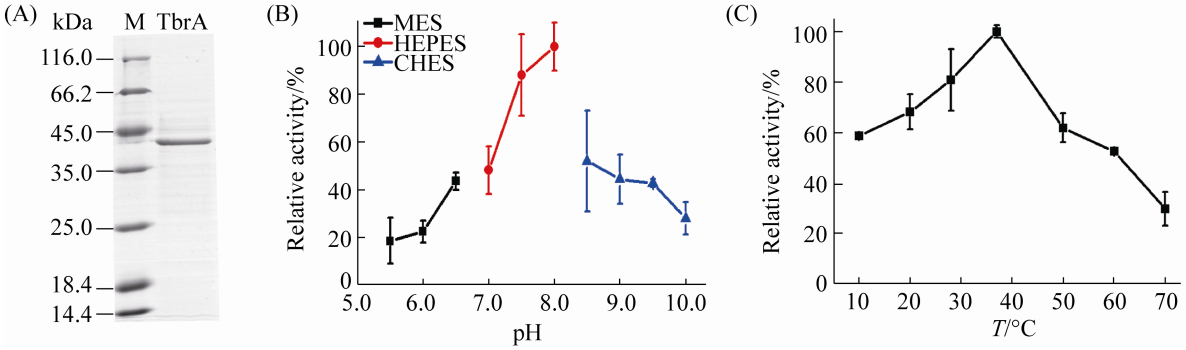

Figure 2. Biochemical characterization of TbrA at various pH levels and temperatures. A: SDS-PAGE of the purified TbrA. To purify TbrA, the recombinant protein with a His_6-tag was overexpressed in *E. coli* Rosetta-TbrA cells and purified using Ni^{2+} affinity chromatography and dialysis. The protein marker size has been indicated. B: The effect of pH on TbrA activity. The activity of TbrA was measured at 37 °C under various pH conditions: black squares, 5.5–6.5 (50 mmol/L MES); red circles, 7.0–8.0 (50 mmol/L HEPES); blue triangles, 8.5–10.0 (50 mmol/L CHES). C: The effect of temperature on TbrA activity. The enzyme activity of TbrA was measured at temperatures ranging from 10 °C to 70 °C in 50 mmol/L HEPES buffer (pH 8.0).

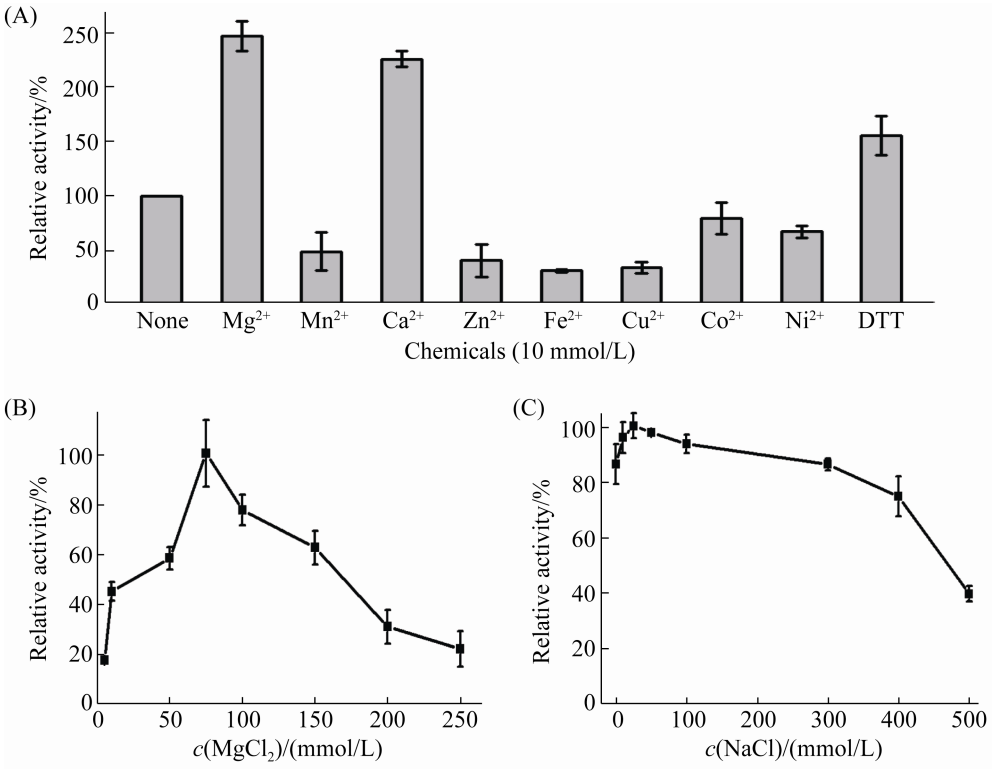

Figure 3. Biochemical characterization of TbrA regarding inclusion of chemical additives and ionic strength. A: The effect of metal ions and DTT on TbrA activity. This effect was determined at 37 °C in 50 mmol/L HEPES buffer (pH 8.0), containing 10 mmol/L final concentration of either DTT or various metal ions ($MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $FeCl_2$, $CuCl_2$, $NiSO_4$, $CoCl_2$). B: The optimal concentration of Mg^{2+} on TbrA activity. The activity of TbrA was measured at 37 °C under various final concentrations of $MgCl_2$, ranging from 10 to 250 mmol/L in 50 mmol/L HEPES buffer (pH 8.0). C: The effect of ionic strength on TbrA activity. The enzyme activity of TbrA was measured at 37 °C under various final concentrations of NaCl, ranging from 0 to 500 mmol/L, in 50 mmol/L HEPES buffer (pH 8.0).

protein, which was non-specifically isolated from the cell-free extract of the *Pseudomonas putida* A 3.12 strain, required Fe^{2+} for its activity[2]. In contrast, our finding shows TbrA activity to be independent of any metal cofactors, with Fe^{2+} even showing a significant inhibitory effect (Figure 3-A). It is possible that AI from different bacterial species evolved independently and specifically, and thus exhibits distinct cofactor preferences. However, this speculation requires confirmation through more efforts in isolation of AI enzymes from different bacterial species in the future.

2.4 Effect of ionic strength on TbrA activity

A series of tests were carried out by adding increasing amount of NaCl to the reaction samples (0–500 mmol/L). The activity of TbrA was found to be initially activated at low NaCl concentration, but attained maximum activity at 25 mmol/L; thereafter, TbrA activity was inversely correlated to the increase in NaCl concentration, ranging between 100 mmol/L to 500 mmol/L. This result suggests that maintaining appropriate ionic strength would be beneficial for TbrA reaction.

2.5 Kinetic characterization of the AI protein TbrA

The K_m, V_{max}, k_{cat}, and k_{cat}/K_m of TbrA enzyme for both its forward and reverse reactions were calculated (Figure 4) and summarized in Table 1. It can be concluded that although TbrA mediates the interconversion between aconitic acid isomers, the affinity (K_m) and the catalytic efficiency (k_{cat}/K_m) of TbrA were much higher when CAA, rather than TAA, was used as the substrate, suggesting that TbrA favors the equilibrium for TAA formation. This is consistent with the conventional knowledge of AI protein[2]. Hence, minimizing CAA formation, resulting from the reverse reaction, by directed mutagenesis of *tbrA* may be worth considering to increase the TAA yield.

Consistent with the increased CAA consumption to form TAA that was observed *in vitro*, TbrA also specifically contributes to the net production of TAA in *B. thuringiensis* cells *in vivo*. Notably, the majority of reported AI proteins (primarily in *Pseudomonas* spp.) display similar preference for TAA formation as TbrA; however, they specifically mediate the net consumption and not the net production of TAA[4,6]. The reason behind the distinct physiological functions of similarly-behaved AI proteins has not been studied thus far. Here, we proposed two hypotheses. First, the intracellular biochemical environments of *B. thuringiensis* and *Pseudomonas* are different and comprise some distinct active substance(s), which favor opposite directions of AI catalysis, thus resulting in distinct physiological functions. Second, the bacterial cells

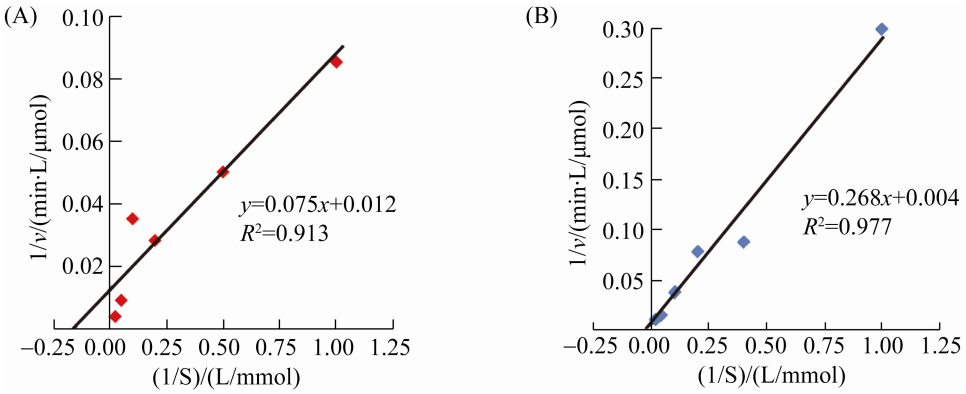

Figure 4. Lineweaver-Burk plots for determining the kinetic parameters of TbrA. A: Forward reaction with CAA as the substrate; B: Reverse reaction with TAA as the substrate. Data are expressed as the mean of triplicates.

Table 1. Kinetic parameters of TbrA with CAA and TAA as the substrates

Substrates	K_m/ (mmol/L)	V_{max}/ (μmol/L·s)	k_{cat}/(1/s)	(k_{cat}/K_m)/ (L/mmol·s)
CAA	6.25	1.39	4.08	0.65
TAA	71.50	4.17	12.25	0.17

may evolve two types of TAA transporters, one for intracellular-transport and the other for extracellular-transport, and these two functions are highly independent. Notably, in *B. thuringiensis* CT-43 strain, we found the TAA extracellular transporter TbrB to be essential for TAA production[1]. Therefore, deletion of *tbrB* abolished TAA biosynthesis to prevent the accumulation of TAA molecules so that its toxic effect on the important enzymes involved in central metabolisms can be reduced[34–35]. Meanwhile, with the presence of TbrA, the CT-43 cell could not assimilate TAA, indicating that although anchored on the cell surface, TbrB cannot transport TAA intracellularly. As for the TAA-assimilating bacteria, they may employ an intracellular transporter to avail TAA as a substrate for the AI enzymes, so that the carbon source can be incorporated into the TCA cycle through the CAA metabolite. Whether the TAA-assimilating bacteria are further able to produce TAA depends on their ability to encode the extracellular TAA transporter. To date, no relevant gene or protein sequence has been reported; however, our group is currently conducting research to identify extracellular transporter genes in TAA-assimilating bacteria.

3 Conclusion

The aconitate isomerase TbrA from *B. thuringiensis* prefers CAA as substrate to catalyze TAA production. The highest enzymatic activity of TbrA was achieved at moderate pH and temperature conditions and was enhanced by 75 mmol/L Mg^{2+} and 25 mmol/L NaCl. The biosafe and multi-active TAA molecule has potential for use in crop pest control, with wider application in the polymer industry and even disease treatment in the future. This work is the first to determine the catalytic characteristics of the TAA-biosynthesizing enzyme and may serve as the basis for future use of the enzyme in the industrial bioproduction of TAA.

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分离自苏云金芽胞杆菌的首例细菌乌头酸异构酶 TbrA 的酶学性质研究

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摘要：【目的】乌头酸异构酶(aconitate isomerase，AI)可介导具有多重生物学活性及应用潜力的小分子物质反式乌头酸(*trans*-aconitic acid，TAA)的合成。本文通过表征来自苏云金芽胞杆菌的生物体首条 AI 基因(*tbrA*)的产物——TbrA 蛋白的催化性质，填补人们对于 AI 酶学特性的认识。【方法】我们利用大肠杆菌 Rosetta 菌株和 Ni^{2+}柱亲和纯化获得了 His_6-TbrA 蛋白，并在体外通过 HPLC 检测了产物生成及对应酶活。【结果】TbrA 蛋白的最适 pH、温度与离子强度分别为 8.0，37 °C 和 25 mmol/L。TbrA 在 10 °C 时仍保留约 60%的活性，展现了较好的耐低温特性。金属离子 Mg^{2+}、Ca^{2+}与还原剂 DTT 可显著增强 TbrA 活性，而 Fe^{2+}、Cu^{2+}、Zn^{2+}、Mn^{2+}则强烈抑制 TbrA 活性。TbrA 将顺式乌头酸(*cis*-aconitic acid，CAA)异构为 TAA 的正反应 K_m、V_{max}、k_{cat}、k_{cat}/K_m值分别为 6.25 mmol/L、1.39 μmol/(L·s)、4.08 1/s、0.65 L/(mmol·s)，将 TAA 异构为 CAA 的逆反应的上述数值分别为 71.50 mmol/L、4.17 μmol/(L·s)、12.25 1/s、0.17 L/(mmol·s)。【结论】AI 酶蛋白 TbrA 可以在温和的理化条件下表现出最高活性，且更倾向于合成 TAA。本研究定量描述了 TbrA 催化特性，为其今后应用于 TAA 工业生物合成奠定基础。

关键词：乌头酸异构酶，苏云金芽胞杆菌，反式乌头酸，顺式乌头酸

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