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N-terminal truncation of a maleate *cis–trans* isomerase from *Rhodococcus jostii* RHA1 results in a highly active enzyme for the biocatalytic production of fumaric acid

Xiangtao Liu, Qing Zhao, Jie Ren, Wenyue Dong, Qiaqing Wu*, Dunming Zhu*

National Engineering Laboratory for Industrial Enzymes, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 Xi Qi Dao, Tianjin Airport Economic Area, Tianjin 300308, China

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ABSTRACT

As part of the project to develop an efficient biocatalytic process for the production of fumaric acid, a full-length putative maleate *cis–trans* isomerase gene from *Rhodococcus jostii* RHA1 was synthesized and expressed in *Escherichia coli* Rosetta2 (DE3) pLysS, but the protein was not soluble and showed no catalytic activity. Bioinformatics analysis of the protein sequence indicated that there were two hydrophilic and two hydrophobic amino acid clusters in an alternate arrangement at the N-terminus, and 50 extra amino acid residues at the N-terminus were not present in the known maleate *cis–trans* isomerases. The alternate hydrophilic and hydrophobic clusters at the N-terminus were thus truncated one by one to evaluate their effect on the gene expression and enzyme activity. Three mutants (MaiR-D41/42-304AA, MaiR-D48/49-304AA and MaiR-D52/53-304AA) without the hydrophilic and hydrophobic clusters were expressed as soluble protein with maleate *cis–trans* isomerase activity. Among them, MaiR-D48 was purified and its properties were studied. The purified enzyme had a temperature optimum of 40 °C and a wide pH range (5.0–9.0) with the optimum pH being 8.0. The whole cells of *E. coli* expressing MaiR-D48 catalyzed the isomerization of maleic acid to fumaric acid at 1 M substrate concentration, showing its potential for industrial use.

1. Introduction

The multiple functional groups of fumaric acid offer it many potential applications in the chemical synthesis. The most important one is its use as the starting material for the industrial production of aspartic acid by aspartase (L-aspartate ammonia-lyase [EC 4.3.1.1]) [1,2]. Fumaric acid can also be converted to many other useful chemicals or materials, such as L-malic acid [2–4], polyester resins and other biodegradable polymers [5–7]. Fumaric acid has been widely used as a food acidulent in the bakery and beverage industries.

Currently, industrial production of fumaric acid is based on catalytic isomerization of maleic acid in aqueous solutions under strong acidic conditions. Maleic acid is obtained in large volumes by hydrolysis of maleic anhydride, which is produced via catalytic oxidation of benzene or butane. Maleate *cis–trans* isomerase [EC 5.2.1.1] catalyzes the isomerization of maleate to its geometric isomer fumarate with a high equilibrium constant in a broad pH range. As such, it should serve as a promising enzyme for the production of fumarate from maleate. Despite its potential industrial application, this enzyme has been barely studied since several reports by Hatakeyama et al. around 2000 [8–10]. The growing need for replacing the current production process of fumaric acid with a greener one has stimulated our interest in the discovery of new maleate isomerases.

In recent years, the growing microbial genomic data offers a tremendous opportunity for discovery of new enzyme catalysts with industrial application potential [11,12]. A genome mining approach has been successfully used to identify target enzymes [13]. As part of our project aiming to develop an efficient process for the production of fumaric acid from maleic acid catalyzed by a maleate isomerase to replace the acid-catalyzed one, an in silico screening of DNA sequence database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) for the putative maleate *cis–trans* isomerase genes in microorganisms was performed [14]. *R. jostii* RHA1 was isolated from lindane contaminated soil and utilizes a wide range of aromatic compounds, carbohydrates, nitriles, steroids, and other compounds as sole sources of carbon and energy [15]. It is known to catabolize a diverse range of plant derived compounds in an O_2-rich environment [16]. A putative maleate *cis–trans* isomerase gene (accession number YP_700472) from *R. jostii* RHA1 was then selected as the target gene

* Corresponding authors. Tel.: +86 22 84861962; fax: +86 22 84861996.
E-mail addresses: wu_qq@tib.cas.cn (Q. Wu), zhu_dm@tib.cas.cn (D. Zhu).

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Table 1
The oligonucleotides used for PCR to obtain the truncated genes of maleate *cis–trans* isomerase from *R. jostii*.

Amino acid residue	Nde I-forward primer	BamH I-reverse primer
MaiR-D20 (21-304AA)	5′-GGAATTCCATATGCTCGCGCCACTCGGTG-3′	5′-CGGGATCCTACTGAACAGTGGTGGC-3′
MaiR-D27 (28-304AA)	5′-GGAATTCCATATGACGAATCA-3′	
MaiR-D34 (35-304AA)	5′-GGAATTCCATATGATACTCGTCAT-3′	
MaiR-D41 (42-304AA)	5′-GGAATTCCATATGCTACGTCCG-3′	
MaiR-D48 (49-304AA)	5′-GAATTCCATATGAACCGGCATGC-3′	
MaiR-D52 (53-304AA)	5′-GAATTCCATATGGGCATTCATCG-3′	

The restriction sites are underlined.

[16]. However, the full-length protein was obtained in an inactive aggregate form. Based on the bioinformatics analysis of the protein sequence, truncations of the different number of amino acid residues at the N-terminus were performed, resulting three active mutant maleate *cis–trans* isomerases. Herein we report the characterization of one of these active mutant enzymes and its application in the biocatalytic synthesis of fumaric acid.

2. Materials and methods

2.1. Materials and strains

Bacto™ Peptone, yeast extract were purchased from BD, USA. Unless otherwise stated, all chemicals were of analytical grade and purchased from Sigma-Aldrich or other commercial sources. *Escherichia coli* Rosetta 2 (DE3) pLysS was purchased from Merck (Bad Soden, USA).

2.2. Construction of expression vectors

The putative maleate *cis–trans* isomerase gene (accession number YP_700472) from *R. jostii* RHA1 was synthesized by Xuguan Biotechnology company (Shanghai, China), and cloned into pET32a with NdeI and BamHI sites, giving plasmid pET32a-MaiR. The plasmids of the truncated genes of *MaiR* were obtained as follows: The truncated genes were amplified by PCR with *PrimerStar HS* DNA polymerase (TAKARA, Japan) using primers in Table 1 and the full-length gene as template. The PCR products were cloned into pET32a with NdeI and BamHI sites to get the recombined plasmids, which were verified by restriction analysis and sequencing of the plasmid DNA. The profile computation and representation of full-length protein sequence coded by maleate *cis–trans* isomerase gene was predicted by Protein Identification and Analysis Tools on the ExPASy Server (http://web.expasy.org/protscale/)

2.3. Expression of MaiR and the truncated MaiRs

The pET32a-MaiR or the truncated pET32a-MaiR vector (e.g. pET32a-MaiR-D48) was transformed into *E. coli* strain Rosetta2 (DE3) pLysS, containing a chromosomal copy of the T7 RNA polymerase gene under the control of the *lac*UV5 promoter. *E. coli* Rosetta2 (DE3) cells carrying pET32a-MaiR or truncated pET32a-MaiR genes were grown for up to 3 h in 50 mL of LB medium containing 100 μg mL^{-1} of ampicillin and 34 μg mL^{-1} of chloramphenicol at 37 °C and shaken at 220 rpm. When OD_{600} reached approximately 0.8, cells were induced at 25 °C for up to 8 h by the addition of IPTG (0.5 mM). The cells were harvested by centrifugation (9000 × *g* for 15 min). After being re-suspended in Buffer A (20 mM Potassium phosphate, 5 mM β-mercaptoethanol [β-ME], pH 7.2), cells were lysed by sonication on ice. Centrifugation (15,000 × *g* for 5 min) was performed at 4 °C, and SDS-PAGE analysis was performed to monitor the soluble expression of the genes.

2.4. Purification of MaiR-D48

E. coli Rosetta2 (DE3) pLysS cells carrying pET32a-MaiR-D48 were cultivated at 1 L scale and 5 g of wet cells were harvested (9000 × *g* for 15 min). The cells were re-suspended in 100 mL of Buffer A and lysed by High Pressure Homogenizer. Cell debris was discarded after centrifugation at 30,000 *g* for 20 min. The supernatant was precipitated by 45% ammonium sulfate and the precipitate was removed by centrifugation. The resulting solution was further precipitated by continually adding ammonium sulfate up to 55%, and the precipitate was collected and re-suspended in buffer A. The resulting solution was desalted and then loaded onto a DEAE Sepharose Fast Flow column (GE Healthcare). The target protein in the column was eluted with Buffer A containing 0.25 M NaCl. The active fractions were collected and concentrated using Amicon Ultra-4 (10 kDa cutoff, Millipore). The concentrated sample was then applied onto a sephacryl S-200 HR column (GE Healthcare), which was equilibrated with Buffer A containing NaCl (0.15 M). The entire purification procedure was followed by SDS-PAGE analysis. The concentrations of protein solution were determined by the Bradford method using BSA as a standard, and the enzyme activity was assayed as described in the following section. The purified enzymes were concentrated to about 1 mg/mL and stored at −80 °C for use.

2.5. Activity assay

Ammonium maleate solution (1 M) was prepared by dissolving maleate in H_2O and then adjusting pH with ammonia to 8.0. The reaction mixture, obtained by mixing 10 μL of MaiR-D48 enzyme solution (1 mg/mL) and 20 μL of ammonium maleate (1 M, pH 8.0) and then adding Buffer B (20 mM potassium phosphate, 5 mM of β-ME, pH 8.0) to 1 mL, was shaken at desired temperature for 10 min. A sample of 500 μL reaction mixture was withdrawn, and the reaction was terminated by adding 500 μL of HCl solution (pH 1.37), the contents of maleic acid and fumaric acid were measured by HPLC analysis. One unit of maleate *cis–trans* isomerase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fumaric acid from maleic acid in 1 min.

2.6. Determining the temperature dependence and pH profile of MaiR-D48

To study the effect of temperature on the activity of MaiR-D48, the activity assays were carried out as described above at pH 8.0 and temperatures ranging from 25 to 60 °C. The activity assays were also performed as described above at 40 °C and different pH to determine the pH profile of MaiR-D48.

2.7. The oxidation stability of MaiR-D48

The oxidative stability of MaiR-D48 enzyme toward H_2O_2 was assessed by treating a solution of the enzyme in buffer A with 0–20 mM of H_2O_2 (final concentration) at room temperature for

60 min. The activity of the H_2O_2-treated enzyme was assayed as described above. To evaluate the oxidative stability of MaiR-D48 enzyme toward air, the purified protein was loaded into glass-tubes which were 6–7 bed volume of the tested MaiR-D48. All tubes were shaken in 40 °C for 0, 24, 48, 72, 96, 120, 144 h, respectively. The remaining activity was assayed as described above.

2.8. Measurement of kinetic parameters of MaiR-D48

To determine the kinetic parameters of MaiR-D48, the activity assay was performed with different substrate concentrations in Buffer B at 40 °C in the presence of certain amount of purified enzyme. The maleate ammonium solutions of different

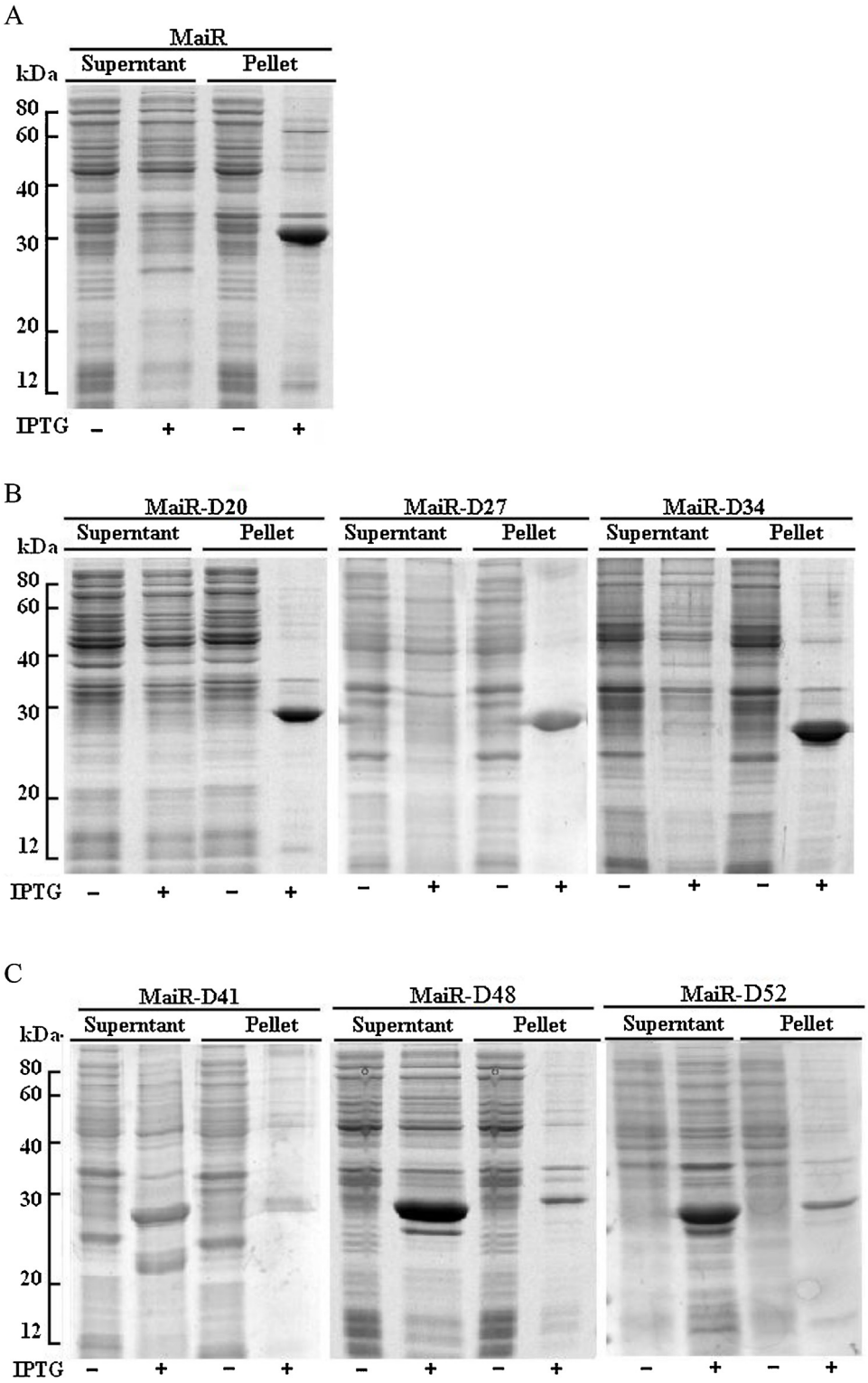

Fig. 1. SDS-PAGE analysis for the expression of the full-length (A) and truncated *MaiR* genes (B and C) in *E. coli*. "–" and "+" mean un-induced and induced by IPTG, pellet and supernatant mean the pellet and supernatant fractions of the lysates, respectively.

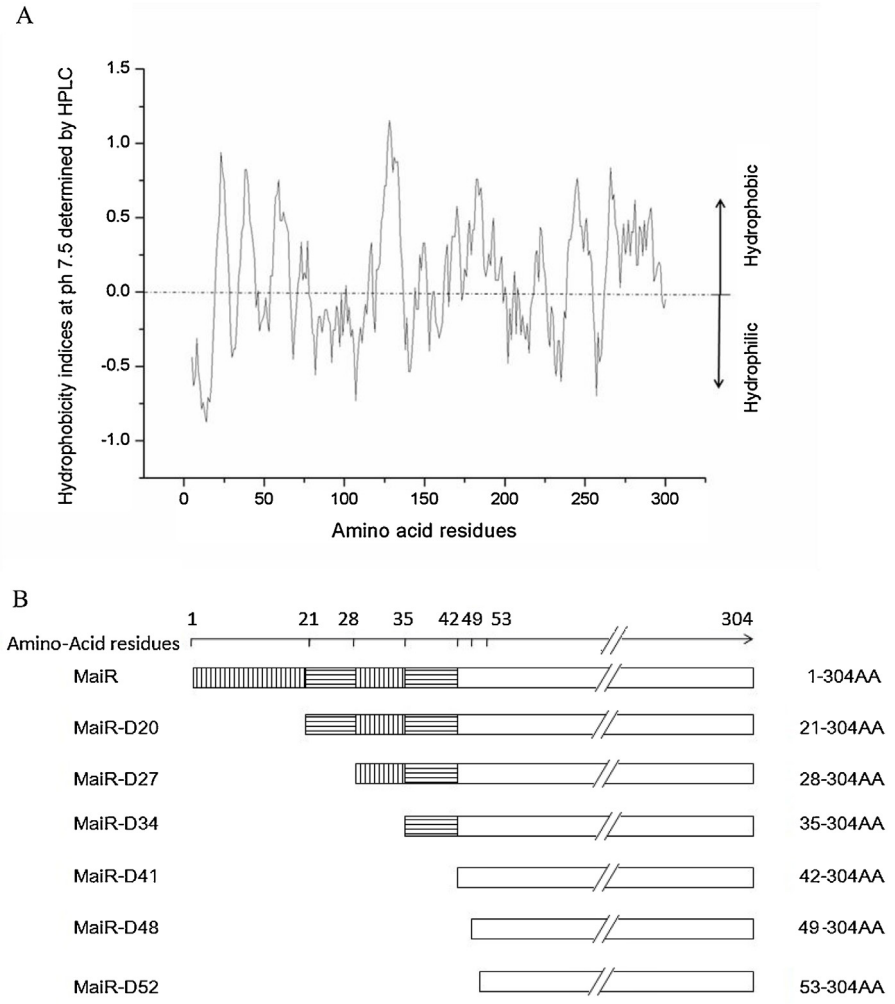

Fig. 2. (A) Hydrophobicity prediction of full-length MaiR with Protein Identification and Analysis Tools on the ExPASy Server (http://web.expasy.org/protscale/). (B) Schematic domain structures of full-length MaiR and its deletion mutants.

concentrations (10, 20, 60, 100, 200, 300, 400 μM) were prepared by diluting a mother solution (1 M, pH 8.0) with Buffer B. A solution of MaiR-D48 (500 μL, 57.2 ng/mL) and a maleate ammonium solution (500 μL) were mixed and the reaction was carried out at 40 °C for 10 min. The amount of product fumaric acid was determined as described above.

2.9. Preparative scale conversion of maleate to fumarate catalyzed by MaiR-D48

The *E. coli* cells from 40 mL of fermentation broth were collected by centrifugation (9000 × *g* for 15 min), and added into a maleate ammonium solution (40 mL, 1 M, pH 8.0). The reaction mixture was stirred at 30 or 40 °C, respectively. The reaction was monitored by TLC analysis (methanol/1,2-dichloroethane = 20/80, v/v). After the maleate disappeared, the biomass was removed by centrifugation. The pH of the resulting solution was adjusted to 2.0 by adding HCl solution (10 M), the product was precipitated and separated by filtration. 3.83 g (83% yield) and 1.76 g (38% yield) of fumaric acid were obtained for the reactions at 30 and 40 °C, respectively.

The cells (10 g wet weight) from 2 L of fermentation broth were harvested by centrifugation (9000 × *g* for 15 min), and added into a maleate ammonium solution (2 L, 1 M, pH 8.0). The reaction mixture was stirred at 30 °C, and monitored by TLC analysis. Fumaric acid (167 g, 72% yield) was obtained by the similar work-up procedure as described above.

3. Results and discussion

3.1. Expression of the putative maleate cis–trans isomerase gene and its N-terminus deletion mutants

A number of attempts were made to express the full length of putative maleate *cis–trans* isomerase gene ro00478 (accession number YP_700472) from *R. jostii* RHA1 in Rosetta2 (DE3) pLysS, but the encoded protein was expressed mainly as insoluble protein (Fig. 1A) and no maleate *cis–trans* isomerase activity was detected. The full-length maleate *cis–trans* isomerase gene ro00478 was then analyzed by Protein Identification and Analysis Tools on the ExPASy Server (http://web.expasy.org/protscale/) [17]. The results indicated that there were two hydrophilic and two hydrophobic amino acid clusters in an alternate arrangement at the N-terminus (Fig. 2A). When the protein sequence (*MaiR*) was aligned with the known maleate *cis–trans* isomerases from *Geobacillus stearothermophilus* (MI101) [9], *Serratia marcescens* (IFO3736) [8], *Nocardia farcinica* (Q5YXQ1) [18] and *Alcaligenes faecalis* (O24766) [10], it was found that the first 50 amino acid residues at the N-terminus were not present in the known counterparts (Fig. 3). As such, the hydrophilic (1-20AA)–hydrophobic (21-27AA)–hydrophilic (28-34AA)–hydrophobic (35-41AA) alternate clusters were deleted one by one to evaluate their effect on the gene expression and enzyme activity (Fig. 2B). The SDS-PAGE analysis (Fig. 1B and C) showed that the protein was in soluble form only when two

Fig. 3. Alignment of amino acid sequences of MaiR with the known maleate *cis–trans* isomerases from *G. stearothermophilus* (MI101) [9], *S. marcescens* (IFO3736) [8] and *N. farcinica* (Q5YXQ1) [18] and *A. faecalis* (O24766) [10]. Result from EMBL-EBI online tools, ClustalW2 of multiple sequence alignments (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

hydrophilic and two hydrophobic clusters were all removed (MaiR-D41, MaiR-D48 and MaiR-D52), and these soluble proteins had maleate *cis–trans* isomerase activity. The specific activities of both MaiR-D41 and MaiR-D52 were about 95% of that of MaiR-D48, and the later showed higher expression level under the same conditions. As such, MaiR-D48 was chosen for the further studies. The hydrophilic and hydrophobic clusters inducing protein aggregation is similar to that by the amphipathic peptide originating from the Zuotin protein sequence, EAK16 (AEAEAKAKAEAEAKAK, or (AEAEAKAK) [19], where the alternate hydrophilic and hydrophobic clusters were substituted with hydrophilic and hydrophobic residues.

3.2. Biochemical characterization of MaiR-D48

The mutant enzyme MaiR-D48 was purified by ammonium sulfate precipitation, DEAE Sepharose Fast Flow column and sephacryl S-200 HR column. The SDS-PAGE analysis for each step of the purification procedure is shown in Fig. 4 and the yields of protein during each step of purification are summarized in Table 2. The specific activity of the purified MaiR-D48 reached 106.8 U/mg.

The dependence of the enzyme activity on temperature and pH were investigated for MaiR-D48, and the results are shown in Figs. 5 and 6, respectively. The mutant MaiR-D48 enzyme had an optimal reaction temperature of 40 °C (Fig. 5) and showed high activity in a wide range of pH from 5 to 9, with the optimum pH being 8.0 (Fig. 6), which is a little higher than that of maleate isomerase from *N. farcinica* (pH 7.5) [18]. Maleate *cis–trans* isomerases from *A. faecalis* and *S. marcescens* also had optimal reaction temperatures around 40 °C, while the activity of the enzyme from *A. stearothermophilus* MI-102 increased as the temperature increased up to 70 °C [8]. The MaiR-D48 enzyme was incubated at specific temperature for 30 min, and the residue activity was determined by the activity assay procedure described in Experimental section. As shown in Fig. 7, the enzyme retained greater than 95% activity at 55 °C, while the remaining activity decreased to about 50% at 60 °C. These results suggested that MaiR-D48 enzyme exhibited relatively high thermo-stability, which was compatible with that for the maleate *cis–trans* isomerases from *S. marcescens* and *B. stearothermophilus* MI-102, but much higher than that for the enzyme from *A. faecalis* [8–10].

When the purified MaiR-D48 enzyme was exposed to 5 mM of H_2O_2 for 60 min, the remaining activity was about 60% of that of the untreated enzyme. As the concentration of H_2O_2 increased

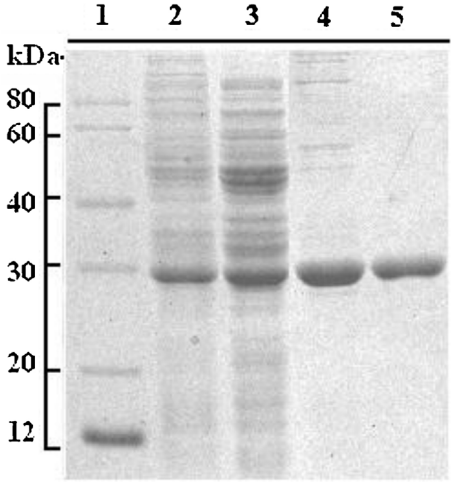

Fig. 4. SDS-PAGE analysis for the purification of MaiR-D48. Lane 1, protein markers; lane 2, crude cell-free extract; lane 3, 45–55% ammonium sulfate precipitation; lane 4, elution from DEAE column; lane 5, elution from sephacryl S-200 HR column.

Table 2
The purification of MaiR-D48.

Purification steps	Protein concentration (mg/mL)	Total activity (U)	Specific activity (U/mg)	Yield of activity (%)	Purification factor
Crude extract	8.6	34,076.2	39.44	100	1
Precipitation with $(NH_4)_2SO_4$	9.7	10,321.7	35.4	30.5	0.9
DEAE sepharose	6.0	4912.3	68.0	14.5	1.7
Sephacryl S-200	3.8	1972.7	106.8	5.8	2.7

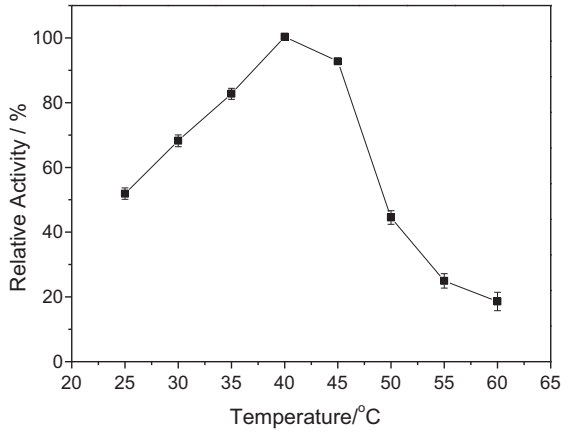

Fig. 5. Effect of temperature on the activity of MaiR-D48. The experiment is representative of 3 repeats.

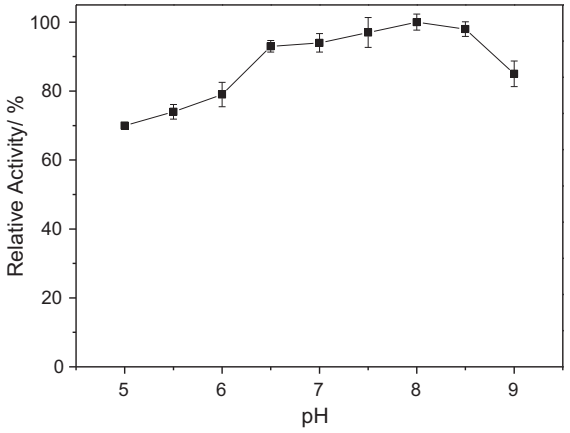

Fig. 6. Effect of pH on the activity of MaiR-D48. The experiment is representative of 3 repeats.

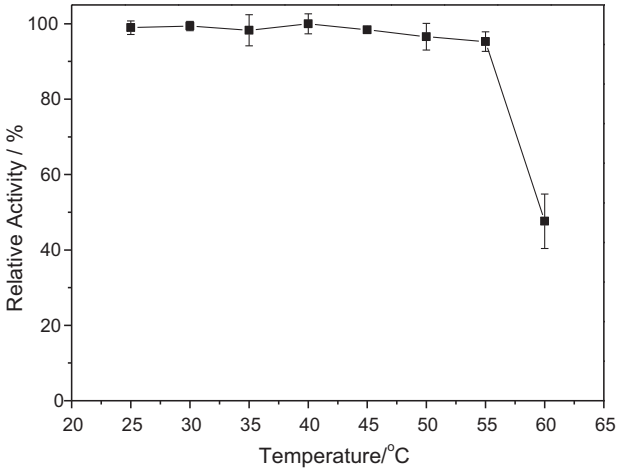

Fig. 7. Thermal stability of MaiR-D48. The experiment is representative of 3 repeats.

to 15 mM, its residue activity leveled off below 5% of the initial activity (Fig. 8A). This is different from the observations for the known maleate *cis–trans* isomerases from *A. faecalis*, *S. marcescens* and *B. stearothermophilus* MI-102, which were completely inhibited by 1 mM H_2O_2 [8,9]. When the purified MaiR-D48 enzyme was exposed to air for 60 h, the remaining activity was about 50% of the untreated enzyme (Fig. 8B).

The kinetic parameters K_m and k_{cat} of MaiR-D48 for maleate were 149.6 ± 9.2 μM and $96.7 \pm 1.3\ s^{-1}$, respectively, with catalytic efficiency k_{cat}/K_m being $6.5 \times 10^5\ M^{-1}\ s^{-1}$. The K_m value was in the range of reported data for known maleate isomerases from *A. faecalis*, *N. farcinica* and *P. fluorescens* [10,18,20]. Although the K_m value of MaiR-D48 was much higher than that of *N. farcinica* maleate isomerase, they had similar catalytic efficiency k_{cat}/K_m, due to a much higher k_{cat} of MaiR-D48 (Table 3) [18].

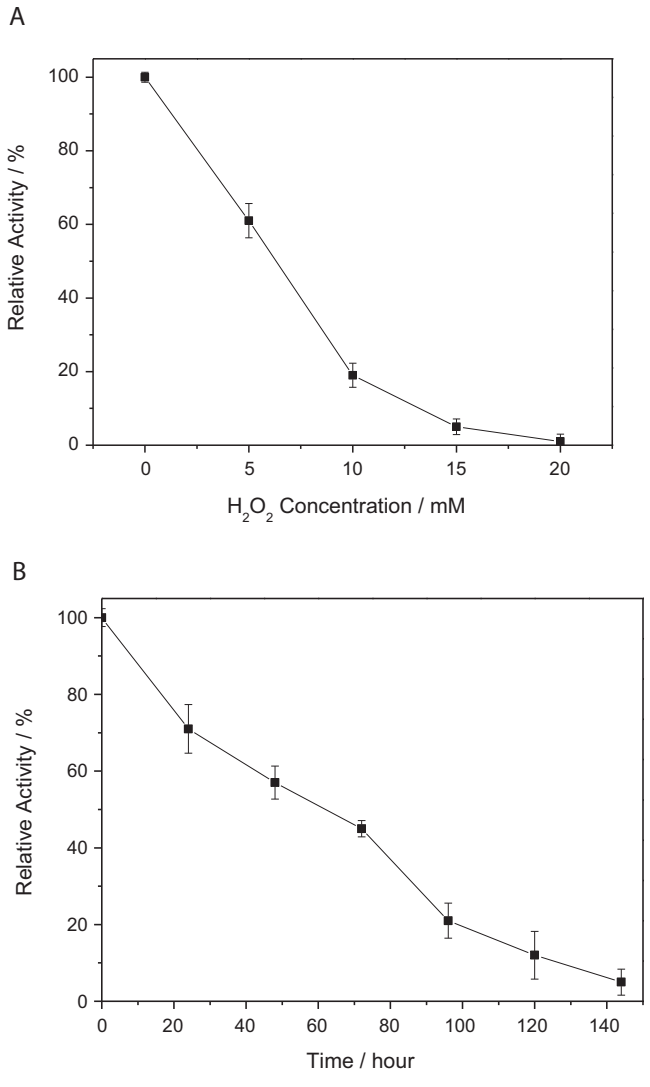

Fig. 8. Effect of hydrogen peroxide (A) and air (B) on the activity of MaiR-D48. The experiment is representative of 3 repeats.

Table 3
The kinetic parameters of different maleate *cis–trans* isomerases.

Enzyme[a]	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	References
MaiR-D48	96.7 ± 1.3	149.6 ± 9.2	6.5×10^5	This study
O24766	NR	40.0	NR	[10]
PfMI	NR	300	NR	[20]
Q5YXQ1	2.8 ± 0.7	4.6 ± 0.5	6.1×10^5	[18]

NR, not reported.
[a] Maleate isomerases from *A. faecalis* (O24766), *P. fluorescens* (PfMI) and *N. farcinica* (Q5YXQ1), respectively.

3.3. Biocatalytic synthesis of fumaric acid by MaiR-D48

The *E. coli* cells expressing MaiR-D48 were tested as the catalyst for the isomerization of maleate to fumarate at 1 M substrate concentration. It has been found that the yield of fumaric acid was very low when the reaction was carried out at the optimal temperature (40 °C). It was due to the metabolism of fumarate by the whole cell catalyst, as demonstrated by the observation that fumarate disappeared when the ammonium fumarate solution (1 M, pH 8.0) was treated with the *E. coli* cells without pET-32a-MaiR-D48 plasmid for 25 h at 40 °C. Under the same conditions, ammonium maleate was not obviously metabolized. Fumaric acid was isolated in 83% yield when the isomerization was performed at 30 °C, more than two times of that at 40 °C, suggesting that the rate of fumarate metabolism was reduced at lower reaction temperature.

In order to explore the potential application of MaiR-D48 in the biocatalytic production of fumaric acid, the isomerization was scaled up to 2 L. The reaction was carried out at 30 °C with the final concentration of ammonium maleate being 1 M. After 28 h, fumaric acid was obtained in 72% isolated yield.

4. Conclusions

A putative maleate *cis–trans* isomerase gene ro00478 from *R. jostii* RHA1 was expressed as insoluble protein with no activity in *E. coli*. The truncation of the alternate hydrophilic and hydrophobic clusters at the N-terminus resulted in three N-terminus truncated mutants which were expressed as soluble protein with maleate *cis–trans* isomerase activity in *E. coli*. These results indicate that two hydrophilic and two hydrophobic amino acid clusters at the N-terminus of this annotated maleate *cis–trans* isomerase prevented it from proper folding for the catalytic activity. Further studies showed that MaiR-D48 possessed relatively high thermo-stability and a broad reaction pH range. The *E. coli* cells expressing MaiR-D48 catalyzed the isomerization of maleate to fumarate at the substrate concentration of 1 M, suggesting its application potential for the biocatalytic production of fumaric acid in the replacement of the acid-catalyzed process.

Acknowledgements

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