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Enantioselective synthesis of (*S*)-phenylephrine by recombinant *Escherichia coli* cells expressing the short-chain dehydrogenase/reductase gene from *Serratia quinivorans* BCRC 14811

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ABSTRACT

Background: An amino alcohol dehydrogenase gene (*RE_AADH*) from *Rhodococcus erythropolis* BCRC 10909 has been used for the conversion of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) to (*S*)-phenylephrine [(*S*)-PE]. However RE_AADH uses NADPH as cofactor, and only limited production of (*S*)-PE from HPMAE is achieved.
Methods: A short-chain dehydrogenase/reductase gene (*SQ_SDR*) from *Serratia quinivorans* BCRC 14811 was expressed in *Escherichia coli* BL21 (DE3) for the conversion of HPMAE to (*S*)-PE.
Results: The SQ_SDR enzyme was capable of converting HPMAE to (*S*)-PE in the presence of NADH and NADPH, with specific activities of 26.5 ± 2.3 U/mg protein and 0.24 ± 0.01 U/mg protein, respectively, at 30 °C and at a pH of 7.0. The *E. coli* BL21 (DE3), expressing NADH-preferring SQ_SDR, converted HPMAE to (*S*)-PE with more than 99% enantiomeric excess, a conversion yield of 86.6% and a productivity of 20.2 mmol/l h, which was much higher than our previous report using *E. coli* NovaBlue expressing NADPH-dependent RE_AADH as the biocatalyst.
Conclusion: The SQ_SDR enzyme with its high catalytic activity and strong preference for NADH as a cofactor provided a significant advantage in bioreduction.

1. Introduction

Phenylephrine (PE) is a sympathomimetic, which is widely used as a nasal decongestant in common cold and flu medicines [1]. Other sympathomimetics, including phenylpropanolamine, pseudoephedrine (PDE) and ephedrine, are also used in cough and cold medicines [2]. However, the over-the-counter (OTC) and prescription drug products containing phenylpropanolamine were no longer recommended for use by the Food and Drug Administration in the year 2000 because these drugs were associated with an increased risk of hemorrhagic stroke [3]. PDE and PE are now commonly used worldwide as systemic nasal decongestants. Because PDE is easily abused for the production of methamphetamine [4,5], the Combat Methamphetamine Epidemic Act in the USA banned OTC medicines containing PDE in 2005 to avoid the illicit conversion of PDE into methamphetamine [6]. This restriction imposed on PDE has led to a need for PE in common cold medicines.

PE contains one chiral carbon atom in the C_α of the side chain, and the *R* enantiomer of PE exhibits more potency than the *S* enantiomer for the activation of α_1-adrenergic receptors [7]. PE can be produced by various methods involving ring opening of 3-benzyloxystyrene epoxide [8], Curtius rearrangement of a β-hydroxy acid azide [9] and reduction of a mandelamide [10]. PE produced from these methods is a racemic mixture; therefore, asymmetric hydrogenation methods have been developed for the production of (*R*)-PE [11–14]. However, these methods require high pressure, high temperature and several environmentally unfriendly organic solvents.

We have cloned an amino alcohol dehydrogenase gene (*RE_AADH*) from *Rhodococcus erythropolis* BCRC 10909 into *Escherichia coli*, and the cells expressing RE_AADH were used for the conversion of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) to (*S*)-PE [15], which can be further converted to (*R*)-PE by the Walden inversion reaction. However, RE_AADH used NADPH as cofactor, and only limited production of PE from HPMAE was achieved. In this study, we have described the cloning of a short-chain dehydrogenase/reductase gene from *Serratia quinivorans* BCRC 14811 and expression in *E. coli* BL21 (DE3). The biochemical properties of recombinant SQ_SDR were characterized. We found that the SQ_SDR enzyme could utilize both NADH and NADPH as a cofactor for its catalytic activity. The yield and productivity of the conversion of HPMAE to PE by recombinant *E. coli* expressing SQ_SDR were also evaluated.

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2. Materials and methods

2.1. Materials

All solvents were LC grade and purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). DNA polymerase, ExTaq, and T4 DNA ligase were purchased from TaKaRa (Tokyo, Japan). Culture media were obtained from Becton, Dickinson and Company (Sparks, MD, USA).

2.2. Bacterial strains, plasmid and culture conditions

E. coli BL21 (DE3) was cultivated in Luria-Bertani (LB) medium at 37 °C. All bacterial strains were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultivated in LB medium at either 25 °C or 30 °C. The plasmids pET30a (Novagen, Inc., Madison, WI, USA) and pQE-30 (QIAGEN, Hilden, Germany) were used for the expression of the gene in *E. coli*.

2.3. Analysis of HPMAE and PE

HPMAE and PE were analyzed by high performance liquid chromatography (HPLC) with a reverse-phase INERTSIL 10 ODS column (3.2 mm × 250 mm) (VERCOPAK, Taipei, Taiwan). The mobile phase consisted of methanol and 0.5% sodium acetate (pH 5.5) at a ratio of 2:98, with a flow rate of 0.8 ml/min. The absorbance of the products was detected at a wavelength of 215 nm.

The chirality of PE was analyzed by HPLC with the chiral column CYCLOBOND I 2000 AC (4.6 mm × 250 mm) (Aztec, NJ, USA). The mobile phase consisted of methanol and 0.5% sodium acetate (pH 5.5) at a ratio of 5:95, with a flow rate of 0.4 ml/min. The absorbance of the products was detected at a wavelength of 215 nm. The retention times were 13.2 min for (*R*)-PE and 14.9 min for (*S*)-PE.

2.4. Screening of bacterial strains for the conversion of HPMAE to PE

Bacterial colonies selected from agar plates were inoculated into 5 ml of culture medium and incubated at the desired temperature with shaking at 150 rpm. One milliliter of overnight culture was inoculated into 100 ml of the same medium and incubated until the OD_{600} reached 2. Cells were collected by centrifugation at 9000 × *g* for 10 min and then washed with 10% glycerol. A cell pellet of 0.1 g (wet weight) was resuspended in 1 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM HPMAE and 2% glucose. After incubation at 30 °C for 24 h, the cells were removed by centrifugation. The supernatant was filtered through a 0.45-μm membrane and analyzed by HPLC.

2.5. Cloning of genes from S. quinivorans BCRC 14811

The aminoketone asymmetric reductase (*akr*), short-chain dehydrogenase/reductase (*sdr*) and alcohol dehydrogenase (*adh*) genes, which encode gene products with considerable identity to *R. erythropolis* BCRC 10909 RE_AADH protein, were amplified from the genomic DNA of *S. quinivorans* BCRC 14811 with PCR using gene-specific primers. The PCR products were digested with *Bam*HI and *Hin*dIII and cloned into pQE-30 or digested with *Nde*I and *Xho*I and cloned to pET30a. The recombinant plasmids were introduced into *E. coli* NovaBlue or *E. coli* BL21 (DE3).

2.6. Cell enzyme activities of the recombinant E. coli BL21 (DE3) harboring the akr, sdr or adh genes

E. coli BL21 (DE3) and *E. coli* NovaBlue harboring recombinant vectors containing either the *akr*, *sdr* or *adh* genes were cultivated in LB at 37 °C to an OD_{600} of 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture broth to a final concentration of 1 mM, and cultivation was continued at 28 °C for an additional 6 h for gene expression. Cells were collected by centrifugation at 9000 × *g* for 10 min. Cell pellets were washed with 10% glycerol and then resuspended in reaction mixture. The reaction mixture (15 ml), containing 1% (wet weight) cells, 100 mM sodium phosphate buffer (pH 7), 10 mM HPMAE and 2% glucose, was incubated at 30 °C with shaking. After the reaction, the cells were removed by centrifugation at 4 °C, and the supernatant was subjected to HPLC analysis. One unit (U) of cell enzyme activity was defined as the amount of recombinant cells required for production of 1 μmol PE from HPMAE per minute under assay conditions.

2.7. Expression and purification of recombinant SQ_SDR proteins

E. coli BL21 (DE3) harboring pET30a-*sdr*-39729 was cultivated in LB at 37 °C until an OD_{600} of 0.8 was reached. The expression of the *sdr* gene was induced by the addition of IPTG to a final concentration of 1 mM. The culture was incubated at 28 °C with shaking for 6 h. Cells were collected by centrifugation at 9000 × *g* and 4 °C, resuspended in sonication buffer (20 mM Tris–HCl and 100 mM NaCl, pH 8.0) and disrupted by sonication. The cell extract was clarified by centrifugation at 12,000 × *g* for 30 min at 4 °C, and the resulting supernatant was loaded onto a Ni-NTA column (QIAGEN, Hilden, Germany). After washing with wash buffer (20 mM Tris–HCl, 100 mM NaCl and 10 mM imidazole, pH 8.0), the adherent His-tagged SQ_SDR protein was eluted with a buffer containing 20 mM Tris-HCl, 100 mM NaCl and 100 mM imidazole (pH 8.0).

Table 1
Conversion of HPMAE to PE by microorganisms[a].

Microorganism	Concentration (mM)	
	Residual HPMAE	PE production
R. erythropolis BCRC 10909	3.38	0.15
R. erythropolis BCRC 13743	3.65	0
Streptomyces clavuligerus BCRC 11518	3.75	0
Streptomyces griseslus BCRC 13677	4.73	0
Streptomyces lividans M2	3.63	0
S. lividans TK24 BCRC 51705	3.53	0
Streptomyces spp. NCHU-1151	3.08	0
Streptomyces thermovvulgaris BCRC 12488	2.63	0.05
Agrobacterium tumefaciens LB4404	4.72	0
Serratia marcescens BCRC 10948	2.27	1.25
S. quinivorans BCRC 14811	2.12	1.84
Pseudomonas putida GB-1	4.95	0
Pseudomonas fluorescens BCRC 10304	4.80	0
P. fluorescens BCRC 11028	4.79	0

[a] Reaction was performed in 100 mM sodium phosphate buffer (pH 7.0) containing 10% (wet weight) cell, 2% glucose and 5 mM HPMAE at 30 °C for 24 h.

2.8. Enzyme activity assay of purified SQ_SDR

We found that HPMAE, PE and NAD(P)H have various absorbances at 340 nm the wavelength typically used for monitoring NAD(P)H concentration [16]. Therefore, in this study, the production of PE from HPMAE was determined by measuring the decrease in absorbance at 370 nm caused by oxidation of NAD(P)H at 30 °C.

A steady-state kinetics study of the conversion of HPMAE to PE by purified SQ_SDR enzyme was performed. The reaction mixture (1 ml) containing the appropriately diluted enzyme in 100 mM sodium phosphate buffer (pH 7.0), 0.1–5 mM NAD(P)H and 0.1–10 mM HPMAE was incubated at 30 °C. The K_m and k_{cat} values for HPMAE and K_m value for NAD(P)H were calculated by fitting the rates, as a function of substrate concentration, to the Michaelis–Menten equation.

The effect of pH on the specific activity of SQ_SDR was determined by measuring enzyme activity over a pH range from 4 to 9 using different buffers containing 1 mM HPMAE and 0.4 mM NADH at 30 °C. To determine the effect of temperature on the purified recombinant SQ_SDR activity, various assays were performed in sodium phosphate buffer (pH 7.0) at 25–60 °C. One unit (U) of enzyme activity was defined as the amount of enzyme required for production of 1 μmol PE from HPMAE per minute under assay conditions.

2.9. Toxicity of HPMAE and PE to E. coli BL21 (DE3)

E. coli BL21 (DE3) (pET30a) was cultivated in LB at 37 °C to an OD_{600} of 0.8. Cells were collected by centrifugation at 9000 × *g* for 10 min. To maintain the osmotic cell equilibrium [17,18] and stabilize the cell proteins [19], cell pellets were washed with 10% glycerol and then resuspended in reaction mixture. The reaction mixture (15 ml), containing 1% (wet weight) cells, 100 mM sodium phosphate buffer (pH 7.0), 2% glucose and 70 mM HPMAE or 70 mM PE, was incubated at 30 °C with shaking at 150 rpm. The cells were collected after incubating for 3, 6, 9, 12 and 15 h and plated on LB agar at an appropriate dilution. The agar plate was incubated at 37 °C for 24 h, and individual colonies were counted as viable cells.

3. Results and discussion

3.1. Screening of bacterial strains for the conversion of HPMAE to PE

In a previous report, we found that *R. erythropolis* BCRC 10909 converted just detectable levels of HPMAE to (*S*)-PE and that *E. coli* NovaBlue expressing RE_AADH from *R. erythropolis* BCRC 10909 converted HPMAE to PE with limited productivity [15]. Therefore, 13 strains of bacteria were screened for their ability to convert HPMAE to PE. As shown in Table 1, *S. quinivorans* BCRC 14811 was the most promising bacterium in this test, with a yield of 36.8%.

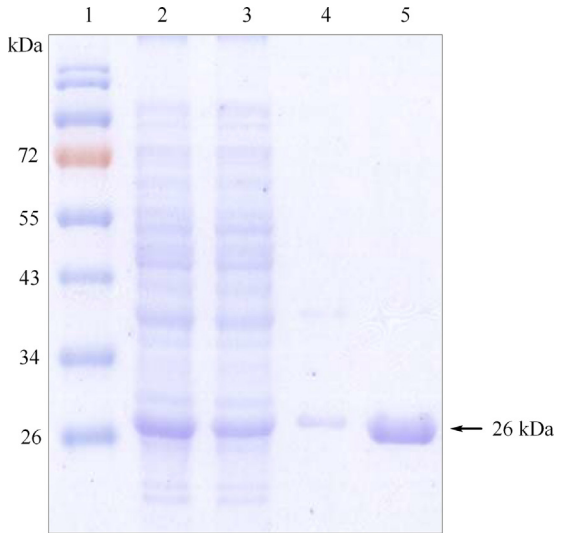

Fig. 1. SDS-PAGE analysis of SQ_SDR purified from *E. coli* BL21 (DE3) (pET30a-*sdr*-39729) by Ni-NTA column. Lane 1, protein size markers; lane 2, crude cell extract; lane 3, supernatant of cell lysate; lane 4, pellet of cell lysate; lane 5, purified SQ_SDR.

3.2. Cloning of the gene responsible for HPMAE reduction from *S. quinivorans* BCRC 14811

The genomic sequence of *S. quinivorans* (formerly *Serratia proteamaculans* subsp. *quinovora*) has been reported (GenBank: CP000826.1). To identify the enzyme responsible for the conversion of HPMAE to PE, the genes encoding proteins with amino acid sequences similar to *R. erythropolis* BCRC 10909 RE_AADH were searched using BLAST. Ten AKRs, 3 SDRs and 7 ADHs proteins exhibiting 35–49% sequence identities with RE_AADH were found. The genes encoding these proteins were amplified with PCR, cloned into pQE-30 or pET30a and expressed in the *E. coli* strains NovaBlue or BL21 (DE3). The ability to convert HPMAE to PE by these transformants was monitored in a reaction mixture (15 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 10% cells (wet weight), 2% glucose and 10 mM HPMAE. After incubating at 30 °C for 24 h, the supernatants obtained from the reaction mixtures were subjected to HPLC analysis, and only *E. coli* BL21 (DE3) harboring pET30a-*sdr*-39729 exhibited enzyme activity for the conversion of HPMAE to PE (3.23 U/g wet cells). The chirality of the resulting PE was analyzed by HPLC using a CYCLOBOND I 2000 AC chiral column, and a 99% enantiomeric excess of (*S*)-PE was obtained in this conversion (data not shown). The *sdr* gene (designated *SQ_SDR* with GenBank accession number ABV39729) responsible for this conversion contained a 783-bp ORF encoding the SQ_SDR protein with a calculated molecular mass of 26.61 kDa. The amino acid sequence of SQ_SDR showed 37.36% identity to the amino acid sequence of RE_AADH [15] from *R. erythropolis* BCRC 10909 (data not shown).

3.3. Biochemical characterization of SQ_SDR protein

The SQ_SDR protein was purified from the crude cell extract of *E. coli* BL21 (DE3) cells harboring the *SQ_SDR* gene by Ni-NTA affinity chromatography and then subjected to SDS-PAGE analysis. The molecular mass of recombinant SQ_SDR was approximately 26 kDa, which compared well with the calculated mass of the affinity-tag translated product from the corresponding gene. About 75% of SQ_SDR expressed in *E. coli* BL21 (DE3) was in a soluble form, which was desirable for biocatalysis. As shown in the lane 5 of Fig. 1, the purity of eluted SQ_SDR was more than 99%.

To investigate the coenzyme dependence and specificity of SQ_SDR, the conversion of HPMAE to PE by purified recombinant

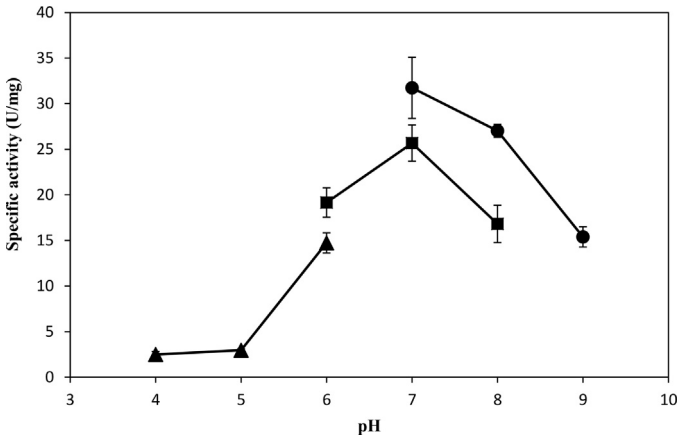

Fig. 2. Effect of pH on SQ_SDR activity. The reaction was performed by incubating the enzyme with 1 mM HPMAE in 100 mM sodium citrate buffer (▲, pH 4–6), sodium phosphate buffer (■, pH 6–8) or Tris–HCl buffer (●, pH 7–9) containing 0.4 mM NADH at 30 °C.

SQ_SDR was performed. In the absence of either cofactor NADH or NADPH, no enzyme activity was observed for SQ_SDR. When either NADH or NADPH was present in the reaction mixture, PE could be produced from HPMAE, indicating that SQ_SDR required either NADH or NADPH as a cofactor (data not shown). SQ_SDR was most active at pH 7.0 in 100 mM sodium phosphate buffer containing 0.4 mM NADH as cofactor. About 50% of the maximal activity was retained at pH 6 and pH 9. The activity was dramatically reduced at pHs below 6 (Fig. 2). The optimal temperature for SQ_SDR was 45 °C, and approximately 60% of maximal activity was observed at 30 °C (Fig. 3). The specific activities of SQ_SDR in the presence of NADH and NADPH were 26.5 ± 2.3 and 0.24 ± 0.01 U/mg protein, respectively, at 30 °C and pH 7.0. The specific activity (26.5 ± 2.3 U/mg protein) of SQ_SDR was 1.4×10^8-fold higher than that of RE_AADH (0.19 μU/mg protein) [15]. The kinetic parameters for HPMAE in the presence of these two cofactors were analyzed. The apparent k_{cat} and K_m of SQ_SDR for HPMAE were 74.7 ± 3.3 min^{-1} and 1.76 ± 0.08 mM, respectively, in the presence of 0.4 mM NADH, whereas only 0.85 ± 0.04 min^{-1} and 2.41 ± 0.09 mM were obtained in the presence of NADPH, respectively. The apparent K_m value for NAD(P)H was also determined. The apparent K_m values for NADH and NADPH were 0.32 ± 0.07 mM and 2.28 ± 0.03 mM, respectively. These results revealed that NADH was superior to NADPH in the conversion of HPMAE to PE.

The deduced amino acid sequence of SQ_SDR was compared with the sequence of NADPH-specific RE_AADH (with 37.36%

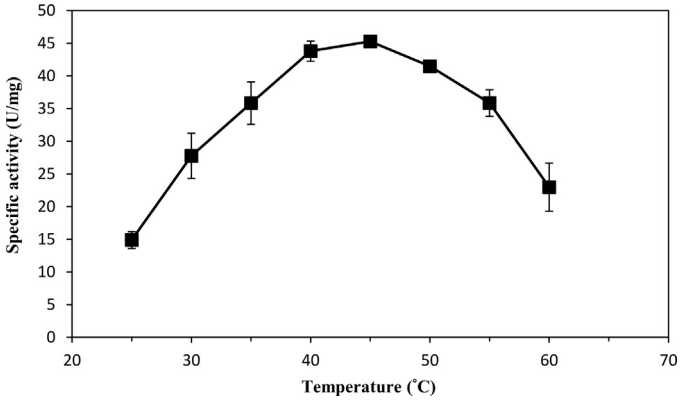

Fig. 3. Effect of temperature on SQ_SDR activity. The reaction was performed by incubating the enzyme with 1 mM HPMAE in 100 mM sodium phosphate buffer (pH 7.0) containing 0.4 mM NADH at different temperatures.

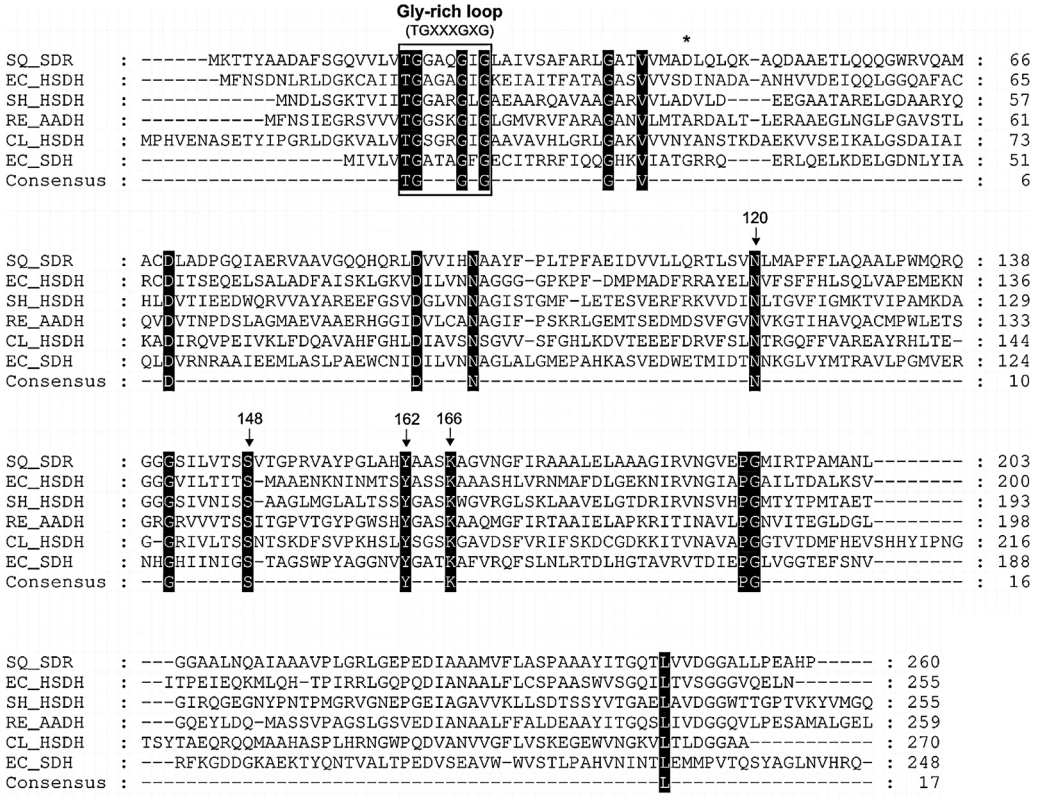

Fig. 4. Alignment of the amino acid sequences of SQ_SDR with RE_AADH and 3D structure available SDRs from *E. coli* (EC_HSDH), *Streptomyces hydrogenans* (SH_HSDH), *Cochliobolus lunatus* (CL_HSDH) and *E. coli* (EC_SDH). The conserved Gly-motif for cofactor binding is indicated with a box. The catalytic tetrad is indicated with an arrow. The cofactor-determined residue is marked with a star.

identity to SQ_SDR) and also with the sequences of SDRs for which 3D structures were available, such as NAD(H)-specific 7-alpha-hydroxysteroid dehydrogenase from *E. coli* (EC_HSDH, Protein Data Bank: 1FMC, with 23.08% identity to SQ_SDR) [20], NAD(H)-specific 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (SH_HSDH, Protein Data Bank: 2HSD, with 21.51% identity to SQ_SDR) [21], NADP(H)-specific serine dehydrogenase from *E. coli* (EC_SDH, Protein Data Bank: 3ASV, with 13.31% identity to SQ_SDR) [22] and NADP(H)-specific 17β-hydroxysteroid dehydrogenase from *Cochliobolus lunatus* (CL_HSDH, Protein Data Bank: 3QWF, with 11.11% identity to SQ_SDR) [23]. A conserved TGXXXGXG motif specific for NAD(P)H binding [15,24] was found in the N-terminal region of the protein (residues 18–25, all numbering refers to SQ_SDR). The catalytic tetrad Asn120-Ser148-Tyr162-Lys166, was also found in the protein. This tetrad, of which Tyr-162 is the most conserved residue within the SDR family, fits the conserved active site motif for the catalytic reaction (Fig. 4) [15,24–27].

SDR-family enzymes catalyze NAD(P)(H)-dependent oxidation/reduction reactions [25,28–30]. SQ_SDR belonged to the classical SDR family, which possesses a conserved Gly-motif, TGXXXGXG [15,24], for NAD(H) and NADP(H) binding in the coenzyme-binding fold (Rossmann fold). The results of our study demonstrated that SQ_SDR had dual coenzyme specificity, with a very strong preference for NADH over NADPH as a cofactor. NADH differs structurally from NADPH in the absence of a phosphate group esterified to the 2′-hydroxyl group of its AMP moiety, which suggests that residues other than TGXXXGXG are responsible for determining coenzyme specificity for SDRs. It has been reported that NAD(H)-preferring enzymes typically have a conserved aspartate residue present at the end of the second β-strand of the βαβ motif located at the beginning of the Rossmann fold [31–36]. Amino acid alignment of SQ_SDR with other NADH-preferring SDRs clearly showed that the corresponding position in SQ_SDR is occupied by Asp-43, indicating that SQ_SDR is a NAD(H)-preferring enzyme, in agreement with our experimental data.

3.4. Conversion of HPMAE to PE by E. coli BL21 (DE3) (pET30a-sdr-39729) expressing SQ_SDR

In a cell-free bioconversion system, NAD(P)H is a relatively unstable molecule and prohibitively expensive. Therefore, *E. coli* BL21 (DE3) (pET30a-*sdr*-39729) expressing SQ_SDR was used as a biocatalyst to evaluate the conditions for the conversion of HPMAE to PE. The effect of pH was investigated. The SQ_SDR activity of recombinant *E. coli* cells increased with pH up to a maximum of pH 7.0 and declined thereafter (data not shown). The effect of the carbon source on conversion was also determined. In the absence of a carbon source, a yield of only 1.2% was obtained in a 1-h reaction. Sucrose and xylose were ineffective carbon sources for bioconversion (Table 2). The addition of either glucose, fructose, maltose or lactose to the reaction mixture resulted in a great increase in the conversion yield of HPMAE. Of these, glucose was the most effective carbon source for the conversion (90.7% yield). The conversion yield of HPMAE decreased as the glucose concentration increased from 2% to 10%, and 2% glucose was determined to be the optimal concentration for conversion (Table 3). It has been reported that 2% glucose is better than higher glucose concentrations for *E. coli* growth and enzyme production [37]. In most cells the concentration of NAD^+ is significantly higher than NADH [38–41], indicating that efficient recycling of NADH is required to maintain the efficient reductive activity of NADH-preferring enzymes such as SQ_SDR during any bioconversion process. Our results indicated that NADH could be supplied effectively by the catabolic pathways of the *E. coli* cells using glucose, fructose, maltose and lactose as carbon sources.

Table 2
Effect of carbon source on the conversion of HPMAE to PE by *E. coli* BL21 (DE3) (pET30a-*sdr*-39729) expressing SQ_SDR.[a]

Carbon source (2%)	Concentration (mM)	
	Residual HPMAE	PE production
Glucose	0.74 ± 0.06	9.07 ± 0.82
Sucrose	7.83 ± 0.04	0.23 ± 0.03
Fructose	1.61 ± 0.17	4.56 ± 0.33
Xylose	7.59 ± 0.03	0.26 ± 0.02
Maltose	0.89 ± 0.16	7.31 ± 0.33
Lactose	0.16 ± 0.05	7.17 ± 0.60
None	6.69 ± 0.23	0.12 ± 0.01

[a] Reaction was performed in the reaction mixture (15 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1% (wet weight) cell mass, 2% carbon source and 10 mM HPMAE at 30 °C for 1 h.

Table 3
Effect of glucose concentration on the conversion of HPMAE to PE by *E. coli* BL21 (DE3) (pET30a-*sdr*-39729) expressing SQ_SDR.[a]

Glucose concentration (%)	Concentration (mM)	
	Residual HPMAE	PE production
0	6.84 ± 0.05	0.02 ± 0.02
2	0.74 ± 0.06	9.07 ± 0.82
4	1.29 ± 0.18	7.79 ± 1.02
6	2.18 ± 0.02	7.87 ± 0.22
8	3.33 ± 0.09	6.94 ± 0.13
10	4.37 ± 0.00	5.64 ± 0.01

[a] Reaction was performed in the reaction mixture (15 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1% (wet weight) cell mass, different concentrations of glucose and 10 mM HPMAE at 30 °C for 1 h.

Table 4
Effects of HPMAE concentration on the PE production.[a]

HPMAE (mM)	PE concentration (mM)	Yield (%)	Productivity (mmol/l, h)
10	9.67 ± 0.78	96.7	9.67
20	16.26 ± 1.07	81.3	13.01
40	32.52 ± 0.05	81.3	16.26
60	52.69 ± 0.12	87.8	17.56
70	60.60 ± 0.88	86.6	20.20

[a] Reaction was performed in the reaction mixture (15 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1% (wet weight) cell mass, 2% glucose and different concentrations of HPMAE at 30 °C.

Because conversion yield [42–44] and conversion rate [45–47] are usually affected by substrate concentration, the effects of HPMAE concentration on the PE production were examined (Fig. 5). The results showed that the productivity of PE was increased as substrate concentration increased, and the highest concentration of PE (60.6 mM) and level of productivity (20.2 mmol PE/l h) could be obtained from a reaction mixture containing 70 mM HPMAE (Table 4). HPMAE concentrations higher than 70 mM were difficult to achieve due to the limited solubility of HPMAE (18 g dissolves in 1 L of 100 mM sodium phosphate buffer, pH 7.0, at 30 °C). In the presence of 70 mM, a HPMAE conversion yield of 86.6% could be obtained in a 3-h reaction (Table 4). In the previous report, we used *E. coli* NovaBlue expressing NADPH-dependent RE_AADH from *R. erythropolis* BCRC 10909 as catalyst to produce PE from HPMAE [15]. The cell enzyme activity of *E. coli* NovaBlue expressing RE_AADH was only 0.42 U/g wet cells. In addition, a conversion yield of 78% and a productivity of 3.9 mmol PE/l h were obtained from a reaction mixture containing 60 mM HPMAE in a 12-h reaction [15]. In this study, *E. coli* BL21 (DE3)

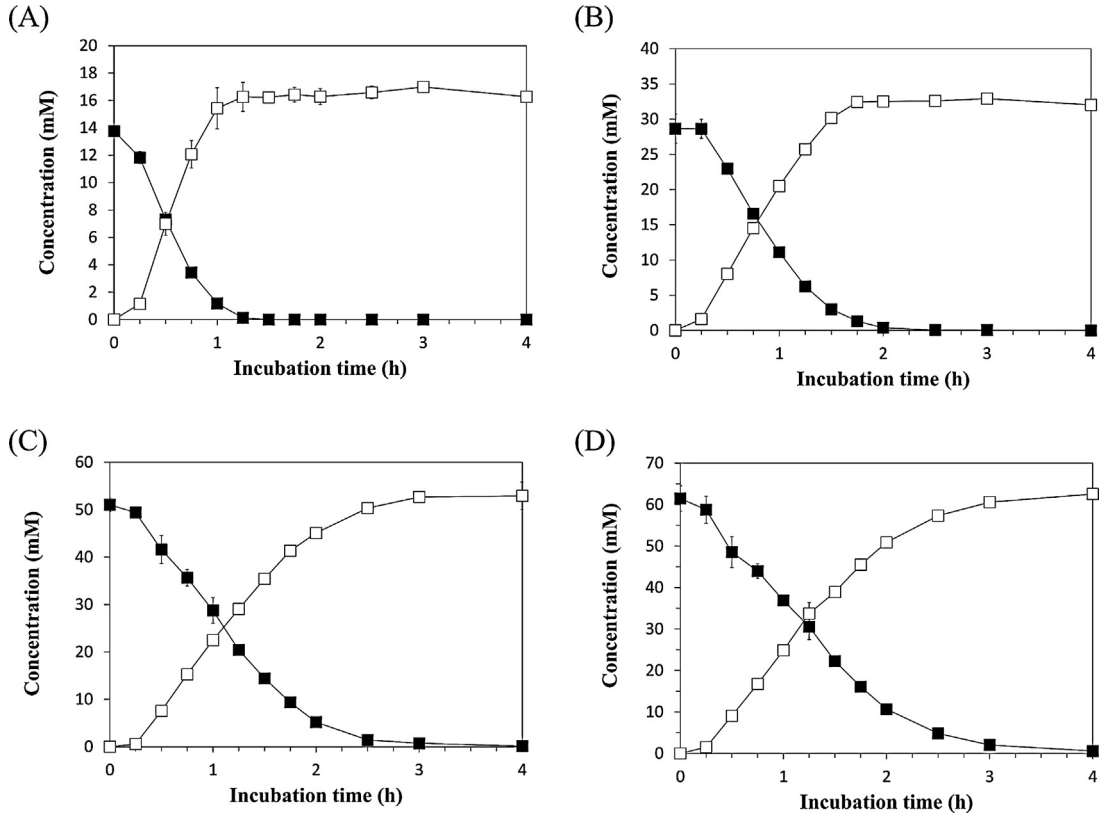

Fig. 5. Effect of substrate concentration on the conversion of HPMAE to PE by *E. coli* BL21 (DE3) (pET30a-*sdr*-39729) expressing SQ_SDR. The reaction was performed in 100 mM sodium phosphate buffer (pH 7.0) containing 1% cell mass, 2% glucose and various concentrations of HPMAE at 30 °C for 4 h. (A) 20 mM, (B) 40 mM, (C) 60 mM and (D) 70 mM. Symbols: ■, HPMAE consumption; □, (*S*)-PE production.

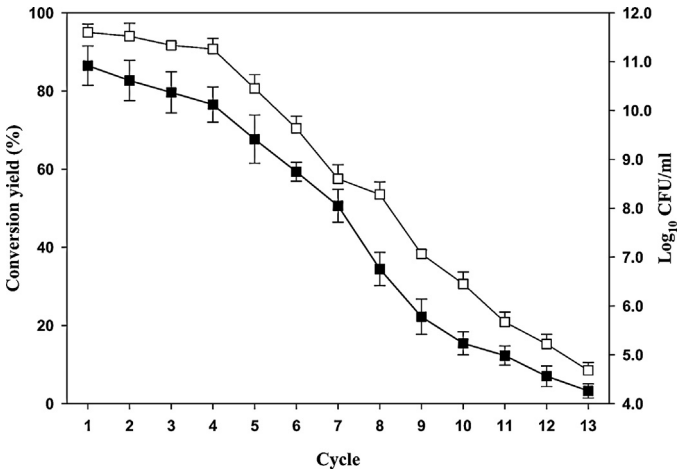

Fig. 6. Repeated use of *E. coli* cells expressing SQ_SDR for PE production. The reaction mixture (15 ml), containing 100 mM sodium phosphate buffer (pH 7.0), 1% (wet weight) *E. coli* BL21 (DE3) (pET30a-sdr-39729) expressing SQ_SDR cells, 70 mM HPMAE and 2% glucose, was incubated at 30 °C for 3 h in each cycle. Symbols: ■, conversion yield of HPMAE for PE; □, Log_{10} CFU/ml. Data are presented as mean ± SD (n = 3).

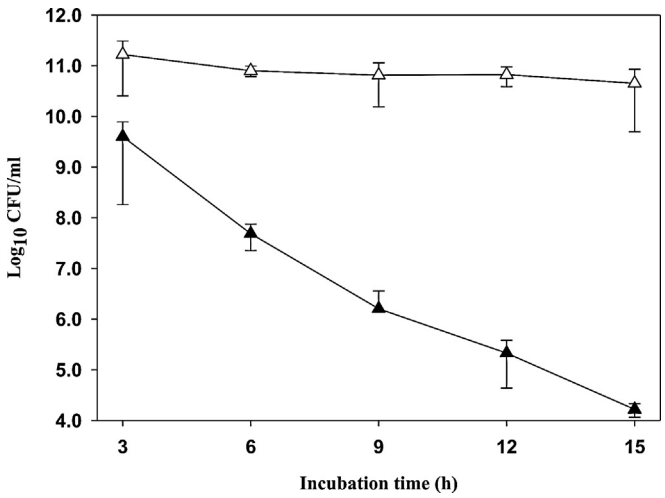

Fig. 7. The toxicity of HPMAE and PE to *E. coli* BL21 (DE3) (pET30a) cells. The reaction mixture (15 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1% (wet weight) cells, 70 mM HPMAE or PE and 2% glucose was incubated at 30 °C. Symbols: □, PE. ▲, HPMAE.

expressing SQ_SDR with a high HPMAE reduction activity (3.23 U/g wet cells) was used as biocatalyst. This strain exhibited a cell enzyme activity approximately 7.7-fold higher than that of *E. coli* cells expressing RE_AADH [15]. For the reaction mixture containing the highest substrate concentration (70 mM HPMAE), *E. coli* BL21 (DE3) expressing NADH-preferring SQ_SDR exhibited approximately 370%-higher PE formation (60.6 mM), a 63.2%-higher yield of HPMAE and a 367%-higher productivity than *E. coli* expressing NADPH-dependent RE_AADH [15] in a 3-h reaction.

Whole cell conversion of HPMAE to PE required a stoichiometric amount of the cofactor NAD(P)H, and thus practical applications would need efficient recycling of NAD(P)H for the reduction activity of SQ_SDR. The concentration of NADH in cells is usually higher than that of NADPH. Reiss et al. reported that the total amount of NAD^+ and NADH is approximately 10-fold higher than the concentration of $NADP^+$ and NADPH in rat liver [48]. In exponentially growing *E. coli* cell, the concentration of NAD(H) is roughly 3-fold higher than that of NADP(H) [49]. Apparently, NADH is superior to NADPH for its efficient recycling. Therefore, the specificity of the cofactor-binding pocket of NADPH-dependent 2,5-diketo-D-gluconic acid reductase has been modified to improve its ability to use NADH as a cofactor for vitamin C production [50]. Obviously, recombinant *E. coli* expressing SQ_SDR with a 80-fold preference for NADH over NADPH as a cofactor would be a valuable catalyst in industrial production of PE from HPMAE.

3.5. Recycling of E. coli BL21 (DE3) (pET30a-sdr-39729) cells in the conversion process

E. coli BL21 (DE3) (pET30a-*sdr*-39729) expressing SQ_SDR showed high activity in the conversion of HPMAE to PE. However, from an economical standpoint, the reusability of the recombinant cells in the conversion process should be determined. The reactions were performed with 70 mM HPMAE at 30 °C for 3 h for each cycle. After conversion, the cells were collected, washed and then used immediately in the next cycle. As shown in Fig. 6, the conversion yield remained at 77% in the fourth run. After 13 cycles, the conversion yield declined to 3%. The reduction of the conversion yield occurred simultaneously with the loss of viable cells in the reaction mixture (Fig. 6). To study the toxicity of HPMAE and PE to *E. coli* BL21 (DE3), cells were incubated with 70 mM of HPMAE or PE in 100 mM sodium phosphate buffer (pH 7.0) at 30 °C. Fig. 7 shows that PE caused only a slight reduction in the number of viable *E. coli* cells, whereas high concentrations of HPMAE were toxic to *E. coli*. Overall, these data indicated that HPMAE toxicity to *E. coli* cells was an important limiting factor for the cell-recycling process.

4. Conclusion

The *SQ_SDR* gene was cloned from *S. quinivorans* BCRC 14811 and functionally expressed in *E. coli* BL21 (DE3). The cell enzyme activity for the recombinant strain was 3.23 U/g wet cells. The recombinant *E. coli* strain expressing NADH-preferring SQ_SDR exhibited a much higher conversion yield and productivity for the conversion of HPMAE to (*S*)-PE than did our previous strain, *E. coli* NovaBlue expressing NADPH-dependent RE_AADH. We demonstrated that the SDR enzyme with its high catalytic activity and strong preference for NADH as a cofactor provided a significant advantage in bioreduction. Because (*S*)-PE must be further converted to (*R*)-PE by a Walden inversion reaction for medical use, a novel biocatalyst that is able to directly convert HPMAE to (*R*)-PE would be desirable for industrial production. An enzyme engineering approach to develop such a biocatalyst is currently in progress.

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