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Engineering the phenylacetaldehyde reductase mutant for improved substrate conversion in the presence of concentrated 2-propanol

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Abstract Phenylacetaldehyde reductase (PAR) from *Rhodococcus* sp. ST-10 is useful for chiral alcohol production because of its broad substrate specificity and high stereoselectivity. The conversion of ketones into alcohols by PAR requires the coenzyme NADH. PAR can regenerate NADH by oxidizing additional alcohols, especially 2-propanol. However, substrate conversion by wild-type PAR is suppressed in concentrated 2-propanol. Previously, we developed the Sar268 mutant of PAR, which can convert several substrates in the presence of concentrated 2-propanol. In this paper, further mutational engineering of Sar268 was performed to achieve higher process yield. Each of nine amino acid positions that had been examined for generating Sar268 was subjected to saturation mutagenesis. Two novel substitutions at the 42nd amino acid position increased *m*-chlorophenacyl chloride (*m*-CPC) conversion. Moreover, several nucleotide substitutions identified from libraries of random mutations around the start codon also improved the PAR activity. *E. coli* cells harboring plasmid pHAR1, which has the integrated sequence of the top clones from the above selections, provided greater conversion of *m*-CPC and ethyl 4-chloro-3-oxobutanoate than the Sar268 mutant, with very high optical purity of products. This mutant is a promising novel biocatalyst for efficient chiral alcohol production.

Keywords Asymmetric reduction · Phenylacetaldehyde reductase · 2-Propanol · Enzyme engineering · Chiral alcohols

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Introduction

The importance of biocatalysts in chemical industries is increasing (Patel 2003; Soetaert and Vandamme 2006; Pollard and Woodley 2007). Enzymes are used as biocatalysts with or without modifications such as immobilization or inclusion in resting cells. One advantage of enzymes is their catalytic activity in aqueous solutions under mild conditions of physiological temperature, pressure, and pH. This contributes to preventing the use of extra organic solvents or energy for the reactions. Another advantage of enzymes is their high selectivity, including substrate stereo-, regio-, and chemoselectivities. This improves the specificity of chemical reactions and contributes to a decrease in the need for chemical purification processes. Thus, the appropriate utilization of biocatalysts improves the efficiency of chemical processes. Currently, many pharmaceutical, food, and chemical industries have developed and introduced biocatalysts.

Alcohol dehydrogenase (ADH) is an enzyme that is a potentially valuable biocatalyst (Devaux-Basseguy et al. 1997; Nakamura et al. 2003; Goldberg et al. 2007a, b). ADH catalyzes the production of ketones or aldehydes from the corresponding alcohols. This reaction requires $NAD(P)^+$ as a coenzyme. In some cases, ADHs can catalyze reversible reactions. This property is used for the production of various chiral alcohols. For example, some ADHs of the medium-chain subclass that mainly catalyze the production of (*S*)-form chiral alcohols have been reported (e.g., Itoh et al. 2002; Kosjek et al. 2004). Another subgroup, i.e., the short-chain ADHs that mainly catalyze the production of (*R*)-form chiral alcohols, has also been reported (e.g., Inoue et al. 2005). Occasionally, the chiral alcohols produced using these ADHs have very high optical purity. Chiral alcohols with very high optical purity are

valuable as medicinal intermediates or chiral-building blocks in various fine chemicals. Additionally, these ADHs often accept multiple substrates, and this property facilitates their practical applications.

Some enzymes can be improved for practical use by introduction of mutation(s). Directed evolution is a powerful enzyme improvement technique (Farinas et al. 2001; Rubin-Pitel and Zhao 2006). Basically, this method comprises iterative cycles of amino acid mutation and screening. Ultimately, the mutant enzyme with accumulated amino acid substitution(s) provides the desired adaptive characteristics.

Previously, we reported the successful improvement of phenylacetaldehyde reductase (PAR) (Makino et al. 2005), one of the ADHs. PAR belongs to the subclass of medium-chain ADHs. It can catalyze various oxidation or reduction reactions with the coenzyme NAD^+ or NADH. It can also accept a variety of substrates and yield chiral alcohols (mainly (*S*)-form) with very high optical purity. The presence of 2-propanol was expected to be advantageous for the production of chiral alcohols because NADH could be regenerated in situ by the oxidation of 2-propanol by PAR itself (Itoh et al. 2002). 2-Propanol was also expected to facilitate the reaction by increasing the solubility of the substrates or products. When a whole-cell reaction is performed, cell-wall permeability can also be increased. However, we found that the conversion yield of the substrate dropped unexpectedly under high concentrations (20% [v/v] or more) of 2-propanol (Itoh et al. 2002). PAR was subjected to a directed evolution-like procedure, which comprised a single round of random library screening and the subsequent manual integration of advantageous amino acid substitutions. The conversion in the presence of concentrated 2-propanol by the resultant mutant enzyme Sar268 was successfully improved (Makino et al. 2005). Briefly, nine amino acid substitutions with improved *m*-chlorophenacyl chloride (*m*-CPC) conversion activity were found in four random mutants of PAR. Out of these nine mutations, six were identified as advantageous for *m*-CPC conversion. The Sar268 mutant has the integrated sequence of all these six mutations.

Although the Sar268 mutant was effective, there is scope for further improvement of the enzyme for increasing the conversion yield. First, the six amino acid substitutions introduced in the Sar268 mutant could be replaced with more effective amino acids. The three other previously examined mutation positions are also possible candidate positions. Previously, we employed the error-prone polymerase chain reaction (PCR) method for random amino acid substitutions for Sar268 development. It is known that a single round of error-prone PCR cannot accomplish substitutions with 20 amino acids at a specific site at a time (Miyazaki and Arnold 1999). Thus, the substitution of other amino acids by using another method might enable further improvement. In addition, a higher level of enzyme expression would also contribute to improved conversion yield. We used PAR for substrate conversion with resting cell suspensions. Increasing the expression level of PAR per *Escherichia coli* cell would increase the conversion yield. An improvement in conversion yield, even if relatively small, provides higher process yield and greater cost effectiveness, which increases the practical advantages of the enzyme.

In this paper, we attempted two approaches to further improve the Sar268 mutant. One approach was the scanning of all 20 amino acids introduced in the Sar268 mutant at each of the nine mutation sites. This methodology is also known as saturation mutagenesis (Hayashi et al. 1994). Several novel mutations were found to be effective at a particular mutation site. The other approach was mutation of the plasmid sequence around the N terminus of the Sar268 mutant. Using this approach, we aimed to increase the expression level of PAR by stabilizing the messenger ribonucleic acid (mRNA) and increasing the promoter and translation activities. Many mutant nucleotide sequences were found to increase the substrate conversion. Furthermore, combining the top mutant sequences by using the two abovementioned approaches further increased the conversion yield with several substrates.

Materials and methods

General materials and methods

All reagents were of analytical grade. Synthetic oligonucleotides were purchased from Espec Oligo Service (Ibaraki, Japan) and Nippon EGT (Toyama, Japan). Restriction enzymes and modification enzymes were purchased from Takara Bio (Shiga, Japan), Toyobo (Osaka, Japan), Fermentas International (Burlington, ON, Canada), and New England BioLabs (Beverly, MA, USA). *m*-CPC was provided by Sumitomo Chemical (Osaka, Japan). Deoxyribonucleic acid (DNA) manipulation was carried out according to standard protocols (Sambrook et al. 1989). Luria–Bertani (LB) medium that was used for the fermentation of *E. coli* cells contained 10 g/l bacto tryptone, 5 g/l bacto yeast extract (BD, Franklin Lakes, NJ), and 10 g/l NaCl. The culture medium was LB medium containing 100 μg/ml ampicillin. The induction medium was LB medium containing 100 μg/ml ampicillin, 0.4 mM isopropyl-β-D-thiogalactopyranoside, and 0.01% (w/v) $ZnCl_2$.

Polymerase chain reaction

KOD-plus DNA polymerase and reaction buffers were from Toyobo. The reaction mixture comprised 1× PCR buffer for

the KOD-plus DNA polymerase, 0.2 mM deoxyribonucleotide triphosphates, 1 mM $MgSO_4$, 0.3 μM sense primer, 0.3 μM antisense primer, 10 ng of plasmid DNA as the template, and 1 U of KOD-plus DNA polymerase. Thermal cycling was performed using a PTC-200 thermal cycler (MJ-research, Waltham, MA), and the cycling conditions were as follows: 2 min at 94°C; 30 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for extension; and 68°C for 5 min.

DNA sequencing

DNA sequence analysis was performed using an automated DNA sequencer (ABI Prism 310, Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. The sense primers were PARSQ-R0, PARSQ-R1, PARSQ-R2, and PARSQ-R3, and the antisense primers were PARSQ-F1, PARSQ-F2, and PARSQ-F3 (Table 1).

Small-scale *m*-CPC conversion reaction

Recombinant *E. coli* JM109 cells were transferred into 1 ml of induction medium in a 2-ml tube and incubated on a shaker (M-36; Taitec, Saitama, Japan) at 37°C for 22 h. The cells were pelleted by centrifugation, and the supernatant was removed. Next, 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.0) containing NAD^+ was added to each tube, followed by a solution of *m*-CPC in 2-propanol. The final volume of the reaction mixture was 500 μl, and the concentrations of MOPS buffer and NAD^+ were 50 and 1 mM, respectively. The lid of each tube was tightly closed, and the reaction mixture was incubated on a shaker at 1,200 rpm at 30°C for 22 h. The mixture was extracted once with 1 ml of ethyl acetate, and the organic phase was dried with anhydrous Na_2SO_4.

Gas chromatography analysis

Gas chromatography (GC) analysis was carried out on an HP6890 (Yokogawa Analytical Systems, Tokyo, Japan) equipped with a CP-cyclodextrin-β-236M-19 column (0.25 mm×25 m; Chromopack, Middelburg, The Netherlands). *m*-CPC conversion was analyzed under the following conditions: a column temperature of 160°C; injection and detection temperatures of 240 and 250°C, respectively, and a flow rate of 0.4 ml/min of He. The retention times of *m*-CPC, (*S*)-2-chloro-1-(3-chlorophenyl)ethanol ((*S*)-CCE), and (*R*)-CCE were 8.5, 12.7, and 13.1 min, respectively. Ethyl 4-chloro-3-oxobutanoate (ECOB) conversion was analyzed under the following conditions: a column temperature of 120°C and injection and detection temperatures and flow rate as mentioned above. The retention time of ECOB was 6.0 min. The retention time of (*S*)- or (*R*)-ethyl 4-chloro-3-hydroxybutanoate (ECHB) was 8.4 min.

Table 1 Oligonucleotide primers used in this study

Name	Sequence5′→3′[a]
PARSQ-R0	GAAAGCGGGCAGTGAGCGCAACGC
PARSQ-R1	CATTAGGCACCCCAGGCTTTACAC
PARSQ-R2	AACTGTTGGCACTGCTCACAAGG
PARSQ-R3	GCCGAGAACGTCCGCAAGATC
PARSQ-F1	CAGCTGGCGAAAGGGGGATG
PARSQ-F2	CGATGGTGGGCTGGTAGCCG
PARSQ-F3	GCACCGAGACCGGGAGGATTG
SatSar1	GAGAATCGGCGCGNNKCCCGAACTCACG
SatSar1-2	GAGAATCGGCGCGHDKCCCGAACTCACG
SatSar2	CACGGAGATTCCCNNKCCCGAGCCCGGTC
pos2_antisense	AGTTCGGGTCCCGCGCCGATTCTCG
SatSar3	CTGCCACTCGGACNNKTTCATCATGAGC
SatSar3–2	CTGCCACTCGGACMHYTTCATCATGAGC
SatSar4	GAAGGCGCAGGCNNKGTCGCCGCCGTC
SatSar4-2	GAAGGCGCAGGCHRKGTCGCCGCCGTC
SatSat4-Met	GAAGGCGCAGGCATGGTCGCCGCCGTC
SatSar4-Pro	GAAGGCGCAGGCCCGGTCGCCGCCGTC
SatSar4-His	GAAGGCGCAGGCCACGTCGCCGCCGTC
pos4_antisense	GTGGCCGAGCGTGAGCGGAAGGCCG
SatSar5	GCACCCGGCGCGNNKGCCGAGTTCATG
SatSar5-2	GCACCCGGCGCG442GCCGAGTTCATG
SatSar5-3	GCACCCGGCGCGRRKGCCGAGTTCATG
pos5_antisense	ACCGAGACCGGGAGGATTGATTCCGAG
SatSar6	GTATCACGCGATCNNKCGTTCTCTGCCG
pos6_antisense	GGCGTCAGACCGGCGTCGGTCAGCG
SatSar7	CGAAACTTCGCGGAGGCNNKTACGCGGTT GTC
SatSar7-2	CGAAACTTCGCGGAGGCSHTTACGCGGTTGTC
pos7_antisense	GCAGAGAACGCTTGATCGCGTGATAC
SatSar8	GATCGGGGACGGCNNKGCCCACGCCAAAG
pos8_antisense	CCGACGATCGTGACGTCTGATCCGAC
SatSar9	CTCGACAACGGTNNKGAAGCGTATCGAC
SatSar9-2	CTCGACAACGGT442GAAGCGTATCGAC
SatSar9-3	CTCGACAACGGTMHKGAAGCGTATCGAC
SatSat9-4	CTCGACAACGGTAAYGAAGCGTATCGAC
pos9_antisense	ACTGAAGGTCTCCACCGAGATGTCGAAG
−1to4rnd_as	NNNNGTTTCCTGTGTGAAATTGTTATCCGC
−5to8rnd_as	AGCTNNNNCCTGTGTGAAATTGTTATCCGC
−9to12rnd_as	AGCTGTTTNNNNTGTGAAATTGTTATCCGC
−13to16rnd_as	AGCTGTTTCCTGNNNNAAATTGTTATCCGC
PAR-ATG_s	ATGAAGGCCATCCAGTACACGAGAATCGGC
K2rnd_s	ATGNNNGCCATCCAGTACACGAGAATCGGC
A3rnd_s	ATGAAGNNNATCCAGTACACGAGAATCGGC
I4rnd_s	ATGAAGGCCNNNCAGTACACGAGAATCGGC
Q5rnd_s	ATGAAGGCCATCNNNTACACGAGAATCGGC
Y6rnd_s	ATGAAGGCCATCCAGNNNACGAGAATCGGC
pUC-lacSD_as	AGCTGTTTCCTGTGTGAAATTGTTATCCGC
PARHE-S	ATGAAATCATTACAATATACGAGAATCGGCG CG
PARHE-A	ATGAGACTCTCCAGTCAAATTGTTATCCGCT CAC

[a] The mixture of equal molar amounts of nucleotides at synthesis is shown according to the abbreviations of the IUB code (N, A/T/G/C; K, G/T; H, A/T/C; D, G/A/T; M, A/C; Y, C/T; R, A/G; and S, G/C); "2" and "4" correspond to the T/G and A/T/G/C mixtures at molar ratios of 0.74:0.26 and 0.19:0.28:0.18:0.35 at synthesis, respectively.

Color screening by plating

The mutant library stock was spread on an agar plate containing the induction medium and incubated for 24 h at 30°C. The colonies were transferred onto a nylon membrane (Biodyne A; Nihon Pall, Tokyo, Japan). The membrane was soaked for 30 min at room temperature in a reaction solution containing 50 mM MOPS buffer (pH 7.0), 1 mM NAD^+, 200 μM nitroblue tetrazolium, 10 μM 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS; Dojindo Laboratories, Kumamoto, Japan), and 20% (v/v) 2-propanol. The reaction was terminated by rinsing the membranes in distilled water, and colonies that developed a significant purple color were visually selected.

Saturation mutagenesis

The method of mutagenesis and the primers and restriction enzymes used are shown in Table 3. Megaprimer PCR (Sarkar and Sommer 1990) was performed using pSar268 as a template. The PCR product was digested, ligated into the same restriction sites of the vector (pSar268) (Ligation-Convenience Kit; Nippon Gene, Toyama, Japan), and electroporated into *E. coli* JM109. Inverse PCR was performed using phosphorylated primers and pSar268 plasmid as a template. The PCR product was ligated, treated with *Dpn*I restriction enzyme, and electroporated. For the six (12th, 42nd, 67th, 125th, 173rd, and 327th) amino acid mutation positions, colonies on the plate containing the culture medium were randomly picked up, and the nucleotide sequence of isolated plasmids was analyzed. For the 20th, 163rd, and 275th amino acid mutation positions, colonies on plates containing the induction medium were screened according to the color screening procedure. For each library, 24 colonies that developed a significant color were cultivated on master plates and stored until use.

Construction and selection of the random nucleotide mutants around the start codon

Mutagenesis was performed by the inverse PCR method described above with the primers (Table 4) and pSar268 as a template. After transformation, at least 2,000 colonies were collected using a small amount of LB liquid medium. Plasmids were isolated from the cell suspension and electroporated into *E. coli* JM109. The numerous colonies on the plate containing the culture medium were collected. The cell suspension was spread on a plate containing the induction medium and subjected to the color-screening procedure by plating. Twenty-four colonies, selected for each mutant library between the nucleotide position −1 to −16, were cultured and pelleted as the small-scale *m*-CPC conversion assay. After resuspension in 1 ml of 50 mM MOPS buffer (pH 7.0), small aliquots were transferred to a microtiter plate. Then, 50 μl of the color development mixture, containing 50 mM MOPS buffer (pH 7.0), 1 mM NAD^+, 200 μM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1; Dojindo Laboratories), 10 μM 1-methoxy PMS, and 20% (v/v) 2-propanol, was dispensed into each well. After incubation at 25°C for 30 min with shaking at 850 rpm, the absorbance at 450 nm was measured for each well using a microplate reader (Viento XS; Dainippon Pharmaceutical, Osaka, Japan). Eight colonies, selected for each mutant library between the second to sixth codons, were subjected to the small-scale *m*-CPC conversion assay.

Construction of combinatorial mutant plasmids

The integrated mutation around the start codon was introduced by the inverse PCR method, using the primers PARHE-S and PARHE-A (Table 1). The template plasmids were pUAR, pEAR2, pSar268, and pSar268 with a leucine or isoleucine substitution at the 42nd amino acid position. A control plasmid, pSar268D, in which the nucleotide sequence before the start codon of *lacZ* was directly connected to the start codon of PAR, was also constructed, using the primers pUC-lacSD_as and PAR-ATG_s (Table 1) and the template pSar268. The nucleotide sequence of the PAR-coding region and the sequence around the start codon were confirmed for all plasmids.

Conversion of substrates by using cells cultured on a large scale

One milliliter of recombinant *E. coli* culture stored in 15% (v/v) glycerol at −80°C was transferred into 100 ml of induction medium in a 500-ml Sakaguchi flask and incubated at 37°C with 121 rpm of reciprocal shaking for 22 h. The cells were harvested by centrifugation (5,000×*g* for 15 min, 4°C) and washed with ice-cold buffer containing 50 mM MOPS buffer (pH 7.0) and 50 mM NaCl. The cells were resuspended in the same buffer, and the suspension containing 30 mg of the cells was transferred to a 2-ml microtube. The cells were collected by centrifugation and stored at −80°C until use. For *m*-CPC conversion, 500 μl of 50 mM MOPS buffer (pH 7.0) containing 1 mM NAD^+ and 20% (v/v) 2-propanol (basic mixture) was added to each tube containing 30 mg of cells, followed by *m*-CPC solid powder. For ECOB conversion, 250 μl of the basic mixture, followed by 250 μl of *n*-octane containing ECOB, was added. For *N*-Boc-3-pyrrolidinone (Boc-PN) conversion, 500 μl of the basic mixture, followed

by Boc-PN solid powder, was added. After incubation on a shaker at 1,200 rpm at 30°C for 22 h, the reaction mixture was extracted with 1 ml of ethyl acetate and dried with anhydrous Na_2SO_4. The *m*-CPC conversion was analyzed as above. The ECOB conversion was quantitatively analyzed by GC. The optical purity of the product ((*S*)- or (*R*)-ECHB) was analyzed by high-performance liquid chromatography (HPLC; LC-10AT; Shimadzu, Kyoto, Japan) under the following conditions: column, Chiralcel OB-H (Daicel Chemical Industries, Osaka, Japan), mobile phase, hexane/2-propanol (9:1), flow rate, 1.0 ml/min, column temperature, 30°C, and detection wavelength, 220 nm. The retention time on the HPLC column was as follows: ECOB, 11.6 min; (*R*)-ECHB, 7.7 min; and (*S*)-ECHB, 8.3 min. For the Boc-PN conversion, the product ((*S*)- or (*R*)- *N*-Boc-3-pyrrolidinol (Boc-PL)) was analyzed by HPLC (LC-10AT) under the following conditions: column, Chiralcel OF (Daicel); mobile phase, hexane/2-propanol (4:1); flow rate, 0.5 ml/min; column temperature, 40°C; and detection wavelength, 220 nm. The retention time was as follows: (*S*)-Boc-PL, 11.9 min; (*R*)-Boc-PL, 13.4 min; and Boc-PN, 20.1 min.

Results

Saturation mutagenesis and selection of improved clones

To enhance the activity of Sar268, we employed two engineering approaches based on site-directed mutagenesis. The overall experimental flow is shown in Fig. 1b. One approach was saturation mutagenesis and screening of the mutants collected at all nine mutation positions previously examined during the development of the Sar268 mutant (Makino et al. 2005). The mutations in the Sar268 mutant are listed in Table 2, and the construction of pSar268 plasmid is schematically illustrated in Fig. 1a. Because six amino acid substitutions in the Sar268 mutant (Table 2) enhanced the activity, the further improvement by previously unassayed amino acid substitutions at these positions

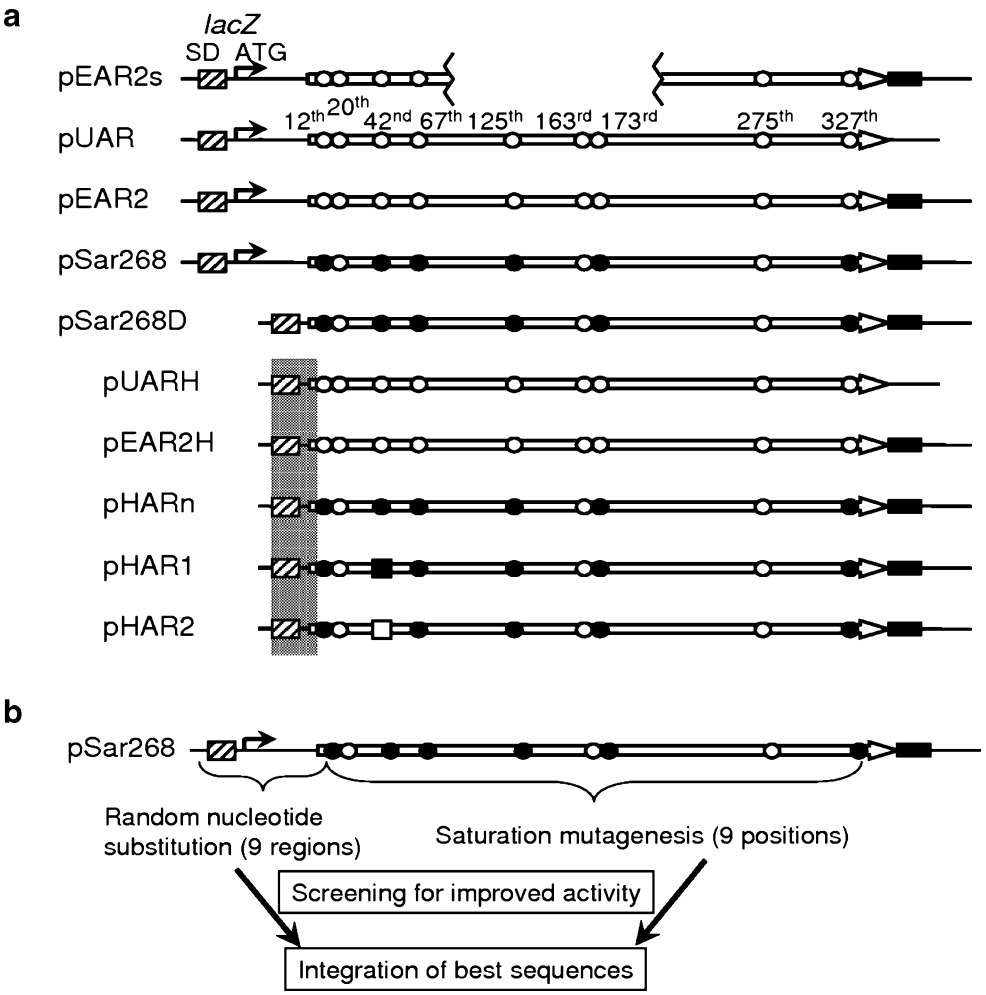


Fig. 1 **a** Schematic representation of the plasmids used in this study. The PAR coding region is represented by an *open arrow*. The polyhistidine tag is represented by a *closed rectangle*. The mutation positions examined during the construction of pSar268 are represented by *open* and *closed circles* that correspond to the wild-type and mutant amino acids, respectively. The *closed square* on pHAR1 and the *open square* on pHAR2 represent the leucine and isoleucine substitutions at the 42nd amino acid, respectively. The amino acid position numbers of the mutations are indicated on the pUAR construct. The Shine–Dalgarno (*SD*) sequence of *lacZ* and its start codon are indicated in the figure. The combination of the top nucleotide sequences around the start codon of PAR is represented by a *gray box*. **b** Schematic representation of overall experimental flow

Table 2 Amino acid substitutions in Sar268[a]

Position in the amino acid sequence	Amino acids in the wild-type PAR	Amino acids in Sar268
12th	Glu	Gly
20th	Lys	–[b]
42nd	Asp	Val
67th	Lys	Arg
125th	Leu	Met
163rd	Lys	–[b]
173rd	Ser	Pro
275th	Gln	–[b]
327th	Ala	Val

[a] Data from reference Makino et al. (2005) reproduced for the reader's convenience
[b] The same as wild type

seemed possible. To test this possibility, these six amino acid substitution positions were subjected to saturation mutagenesis. For each of the six amino acid substitution sites of Sar268, we obtained at least 19 amino acid substitution mutants by megaprimer and/or inverse PCR (Table 3), not including the amino acid substitutions present in the Sar268 mutant. Of the amino acid substitutions, 72.5% comprised multiple clones. The average number of clones that had the same amino acid substitution at the 12th, 42nd, 67th, 125th, 173rd, and 327th amino acid positions was 2.6, 2.4, 1.6, 2.1, 2.1, and 3.1, respectively. All clones were subjected to the small-scale conversion reaction of *m*-CPC to (*R*)-CCE (Fig. 2). The chemical structures of *m*-CPC and (*R*)-CCE are shown in Table 5. Although (*R*)-CCE peaks were observed in some GC charts, no noticeable (*S*)-CCE peaks were recorded. The yields obtained by almost all the clones were of the same level or lower than that obtained by Sar268; however, the yield was considerably enhanced when clones with two amino acid substitutions at the 42nd amino acid that differed from those in Sar268 were used. The valine (codon: GTT) of Sar268 was substituted with leucine (CTT) or isoleucine (ATT). The levels of enhancement were up to 1.17-fold for the leucine mutants and up to 1.15-fold for the isoleucine mutants. Second, the substitutions at three other positions (20th, 163rd, and 275th amino acids) were examined. During the development of Sar268, the amino acid substitutions at these three positions were rejected because the mutations were disadvantageous (Makino et al. 2005). To confirm that no substitution at these sites improved the conversion yield of the substrate, these sites were also subjected to saturation mutagenesis. In the 24 clones for each of the three libraries with NAD^+-reducing activity by a membrane assay, no clone with a significant improvement in 4% (w/v) *m*-CPC conversion activity of Sar268 was found (data not shown). Overall, two amino acid substitutions at the 42nd amino acid were identified as positive for improving *m*-CPC conversion activity and subjected to further combinatorial analysis as described below.

Mutation around the start codon and selection of improved clones

The other approach we used for the engineering of Sar268 was mutagenesis and screening of a mutant library that had randomized substitutions around the start codon of the PAR gene. The targets were two discrete regions. The first region was the nucleotide sequence from positions −16 to −1, numbered relative to the first "A" nucleotide of the PAR start codon. This region was divided into four subregions comprising four consecutive nucleotides (i.e., the nucleotide sequences from −16 to −13, −12 to −9, −8 to −5, and −4 to −1). The second region was the codons corresponding to the PAR amino acids positioned from the second to sixth. The nucleotide sequence corresponding to this region was also separated into five consecutive subregions of three nucleotides (i.e., corresponding to each codon). The "NNNN" and "NNN" nucleotide substitutions were introduced independently into these four and five subregions, respectively. For the color reaction assay, we evaluated at least 1,200 (for the "NNNN" substitution) and 300 (for the "NNN" substitution) colonies against 256 and 64 possible combinations, respectively. Thus, the coverage of diversity

Table 3 The conditions for the mutant library construction

Amino acid position	Method of mutagenesis	Primers used	Restriction sites for cloning
12th	Megaprimer PCR	SatSar1, SatSar1-2, PARSQ-F3, PARSQ-R1	*Eco*RI, *Xho*I
20th	Inverse PCR	SatSar2, pos2_antisense	–
42nd	Megaprimer PCR	SatSar3, SatSar3-2, PARSQ-F3, PARSQ-R1	*Eco*RI, *Xho*I
67th	Inverse PCR	SatSar4, SatSar4-2, SatSar4-Met, SatSar4-Pro, SatSar4-His, pos4_ansisense	–
125th	Megaprimer PCR	SatSar5, SatSar5-2, SatSar5-3, PARSQ-F2, PARSQ-R2	*Xho*I, *Kpn*I
	Inverse PCR	SatSar5-3, pos5_antisense	–
163rd	Inverse PCR	SatSar6, pos6_antisense	–
173rd	Inverse PCR	SatSar7, SatSar7–2, pos7_antisense	–
275th	Inverse PCR	SatSar8, pos8_antisense	–
327th	Inverse PCR	SatSar9, SatSar9-2, SatSar9-3, SatSar9-4, pos9_antisense	–

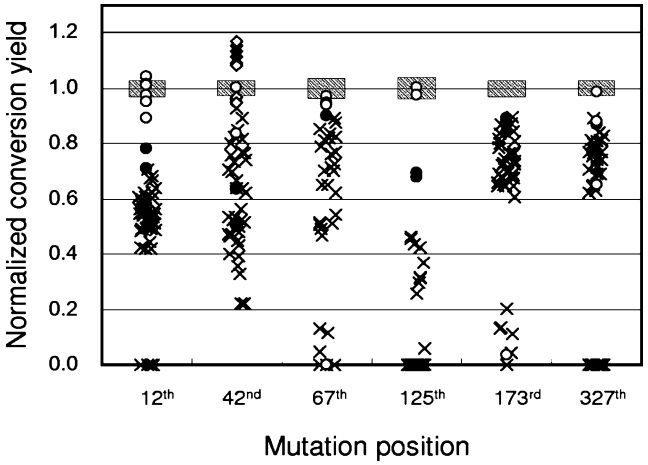


Fig. 2 Normalized conversion yields of *m*-CPC to (*R*)-CCE for different amino acid substitution mutants at the mutation positions indicated. The initial concentration of *m*-CPC and 2-propanol were 4 (w/v) and 17.4% (v/v), respectively. The conversion yield was calculated from GC charts as the (*R*)-CCE peak area divided by the sum of the areas of *m*-CPC, (*R*)-CCE, and (*S*)-CCE. Then, the values were normalized against the average of eight independent measurements using pSar268 (positive) and pEAR2s (negative). The standard deviation of pSar268 for each mutation position is shown by a gray box. The amino acids of the wild type, Sar268, and other mutations are shown with *closed circles*, *open circles*, and *crosses*, respectively. The *horizontal positions of crosses* are purposely wobbled in a random manner to avoid crowding. The mutants with leucine and isoleucine substitutions at the 42nd amino acid are indicated by *open diamonds* and *closed diamonds*, respectively

of the library was considered to be sufficient (about 4.7-fold). The screening of the four libraries with substitutions before the start codon was performed by the color reaction assay because alteration in enzymatic properties was not expected. A large number of mutant enzymes were successfully improved (Fig. 3). On the other hand, the screening flow of the five libraries with substitutions after the start codon included the *m*-CPC conversion assay because of the possibility of alteration of enzymatic properties. Several mutants showed improvement of the *m*-CPC conversion yields (Fig. 4a). Nucleotide substitutions were found in all of the top clones, and amino acid substitutions were found at the third and fourth positions (Table 4). Improvement of PAR expression from pSar268D was also observed for all of the top clones, at least in the soluble fractions (Fig. 4b). The sequences of these top clones were subjected to further combinatorial analysis as described below.

m-CPC conversion test of the combinatorial mutants

Various combinatorial mutants and control mutants were constructed for further analysis. If the amino acid substitutions and mutations around the start codon were additive, the conversion activity could be further enhanced by merely combining the mutations selected above. The construction of these combinatorial mutants is shown schematically in Fig. 1a. Briefly, at the 42nd amino acid, pHAR1 and pHAR2 have leucine and isoleucine substitutions, respectively. Additionally, pUARH, pEAR2H, pHARn, pHAR1, and pHAR2 have the same nucleotide sequence substitution around the start codon with an integrated sequence of the top clones selected from nine sublibraries (i.e., simply the connected sequence of the top clones in Table 4). The *m*-CPC conversion assay of the combinatorial mutants was performed (Fig. 5a). The clone harboring pEAR2s did not show any detectable conversion because of deletion of the PAR gene (data not shown). The clones harboring pUAR, pEAR2, pUARH, or pEAR2H also did not show any conversion (data not shown). The clone with pSar268 showed considerable conversion. The clone with pSar268D, in which the nucleotide sequence before the start codon of *lacZ* was directly connected to the PAR start codon, had an enhanced conversion yield as compared to pSar268. All clones harboring the pHAR-series plasmids (i.e., pHARn, pHAR1, and pHAR2) with integrated mutations around the start codon showed enhanced conversion yield as compared to pSar268D. Among all the clones tested, the highest conversion was obtained with pHAR1 (about threefold greater yield than with pSar268), followed by pHAR2. In other words, the mutant with the most advantageous mutations provided the best conversion yield. The change in the expression level was also analyzed for soluble and insoluble fractions (Fig. 5b). The significant enhancement of the expression level with the "HAR" series plasmids, especially in

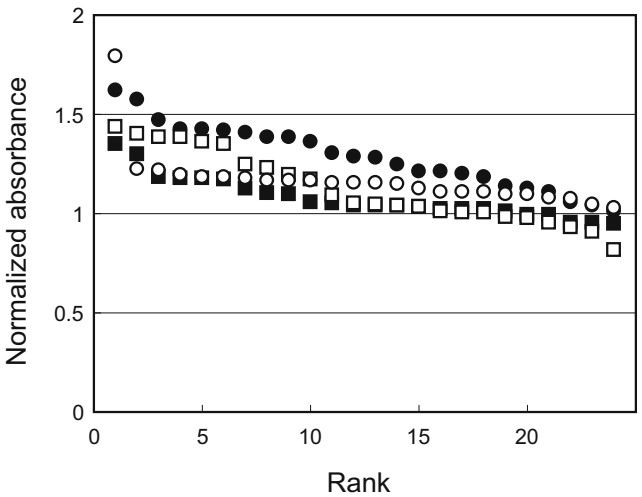


Fig. 3 The distribution of color development for the sublibraries with substitutions between the nucleotide positions −1 and −16. The absorbance recorded in the WST-1 assay was normalized against the pSar268D plasmid. The values of 24 selected clones from each sublibrary are shown in the descending order. The positions for random nucleotide substitutions are from −1 to −4 (*open circles*), −5 to −8 (*closed circles*), −9 to −12 (*open squares*), and −13 to −16 (*closed squares*)

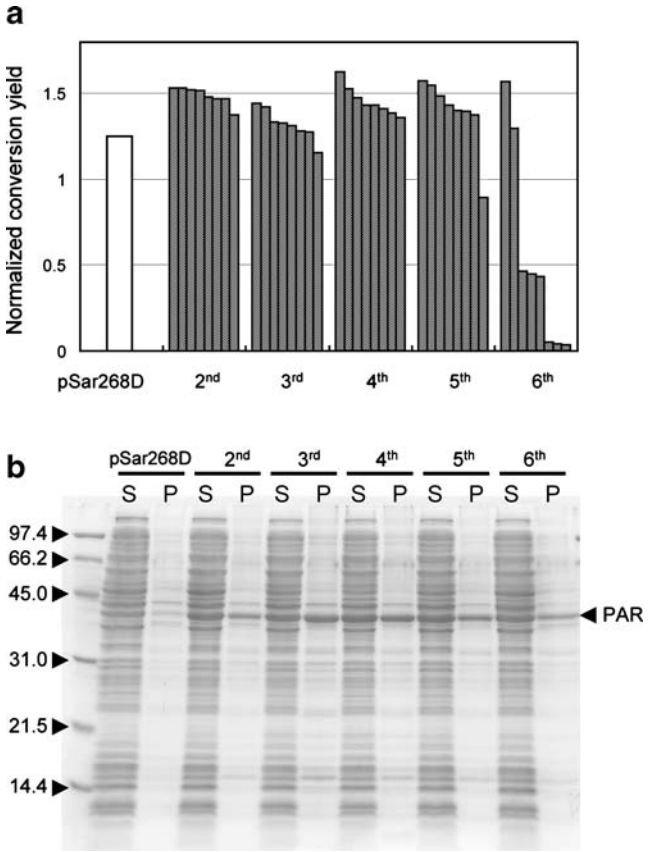


Fig. 4 **a** The distribution of *m*-CPC conversion for sublibraries with substitutions between the second and sixth codons. The initial concentrations of *m*-CPC and 2-propanol were as in Fig. 2. The conversion yield of *m*-CPC was normalized against the pSar268 plasmid. The normalized conversion yields of eight clones for each sublibrary are shown in the descending order. The value with the pSar268D plasmid is also shown. **b** 15% SDS-PAGE analysis of the top clones of second to sixth codon mutants. Cells from the 2-ml culture were resuspended in 200 μl of 50 mM MOPS buffer (pH 7.0) and disrupted by sonication (Ultrasonic Disruptor UD-200, Tomy, Tokyo, Japan). A 150-μl aliquot was withdrawn, and the supernatant was prepared by centrifugation. The pellet was washed twice with 500 μl of MOPS buffer and resuspended with 150 μl of the buffer. Twelve microliters of either the supernatant (*S*) or the pellet suspension (*P*) was mixed with sample buffer for SDS-PAGE, heated for 5 min at 98°C, and applied to a gel. The gel was stained with Quick-CBB (Wako Pure Chem. Indust., Osaka, Japan). Molecular weight standards (kDa; Bio-Rad Laboratories, Hercules, CA, USA) and PAR are indicated by *arrowheads*

insoluble fractions, suggests the strong induction of PAR expression by the integrated mutant sequence around the start codon. The highest level of expression in the soluble fraction was observed with pHAR1.

Conversion with the cells cultured on a large scale

The clone harboring pHAR1 was further subjected to the conversion test using multiple substrates of *m*-CPC, ECOB, and Boc-PN. The clone harboring pSar268 was used for comparison. The chemical structures of the substrates and products are shown in Table 5. The conversion yield of *m*-CPC is shown in Fig. 6a. The clone harboring pHAR1 showed a higher conversion yield than pSar268 for all the amounts of *m*-CPC tested. Complete conversion of 125 mg of *m*-CPC was observed with pHAR1, although the conversion yield was 90.7% with pSar268. Even with 200 mg of *m*-CPC, 87.4% conversion yield was observed with pHAR1, although the yield dropped to 71.5% with pSar268. The enantiomeric excess (e.e.) of the (*R*)-CCE produced was greater than 99% for pHAR1 and pSar268. Molar conversion from ECOB to (*R*)-ECHB was determined using GC and HPLC (Fig. 6b). The clone harboring pHAR1 also showed a higher molar conversion yield than pSar268 for all the amounts of ECOB tested. The molar conversion yields with pHAR1 (96.0%) and pSar268 (93.2%) were almost comparable for 50 μl of ECOB. However, the molar conversion yield with pHAR1 was higher by at least 22% under all other test conditions. (*R*)-ECHB was produced at an e.e. of greater than 99% with pHAR1 or pSar268. Almost complete conversion (molar yield greater than 98.8%) of Boc-PN was observed with pHAR1 or pSar268 under all the conditions tested, and (*S*)-Boc-PL was produced at an e.e. of greater than 99% (data not shown).

Discussion

The focus of our research was to improve the previously developed Sar268 mutant of PAR with regard to substrate conversion in the presence of concentrated 2-propanol because improving pSar268 was expected to provide a higher process yield and greater cost effectiveness. We attempted two different approaches for the further improvement of pSar268. First, by saturation mutagenesis of the mutation positions that had been examined for pSar268 construction, two advantageous amino acid substitutions at the 42nd amino acid were found. Moreover, considerable numbers of improved clones with mutant sequences were found by random nucleotide mutagenesis around the start codon of PAR. The mutant pHAR1, obtained by integrating all the top mutations identified by independent selection, showed improved conversion yields of *m*-CPC and ECOB, without any loss of stereoselectivity.

Although we confirmed the nucleotide sequence around the mutation site, there was a slight possibility that undesired mutation(s) would be introduced at some other site(s). To reduce this possibility, polymerase with the highest fidelity was used for all the experiments. Furthermore, at least 1.6 different clones (average) per amino acid substitution at a specific site were collected and analyzed to reduce the chance of errors. At the time of selection, the conversion yields of the mutants in which isoleucine or

Table 4 The mutation analysis around the start codon

Position of nucleotide[a]	Position of codon[b]	Primers	Nucleotide sequence in pSar268 (amino acid)	Nucleotide sequence of top clone (amino acid)
−16 to −13	–	PAR-ATG_s, −13to16rnd_as	CACA (–)	GACT (–)
−12 to −9	–	PAR-ATG_s, −9to12rnd_as	CAGG (–)	GGAG (–)
−8 to −5	–	PAR-ATG_s, −5to8rnd_as	AAAC (–)	AGTC (–)
−4 to −1	–	PAR-ATG_s, −1to4rnd_as	AGCT (–)	TCAT (–)
4 to 6	2	pUC-lacSD_as, K2rnd_s	AAG (Lys)	AAA (Lys)
7 to 9	3	pUC-lacSD_as, A3rnd_s	GCC (Ala)	TCA (Ser)
10 to 12	4	pUC-lacSD_as, I4rnd_s	ATC (Ile)	TTA (Leu)
13 to 15	5	pUC-lacSD_as, Q5rnd_s	CAG (Gln)	CAA (Gln)
16 to 18	6	pUC-lacSD_as, Y6rnd_s	TAC (Tyr)	TAT (Tyr)

[a] The nucleotide positions were counted from the first nucleotide of the start codon toward the C terminus of the amino acid sequence (note: the −1 position corresponds to the nucleotide just before the first nucleotide of the start codon).
[b] The codon positions were counted from the start codon at the first position toward the C terminus.

leucine was substituted at the 42nd amino acid varied somewhat (Fig. 2). However, the best substitution among the combinatorial clones was subsequently identified as leucine (pHAR1) and the next best as isoleucine (pHAR2) in the clones with confirmed sequences of the coding and surrounding regions of PAR. Thus, the possibility of the introduction of undesired mutations is considered to have been very low.

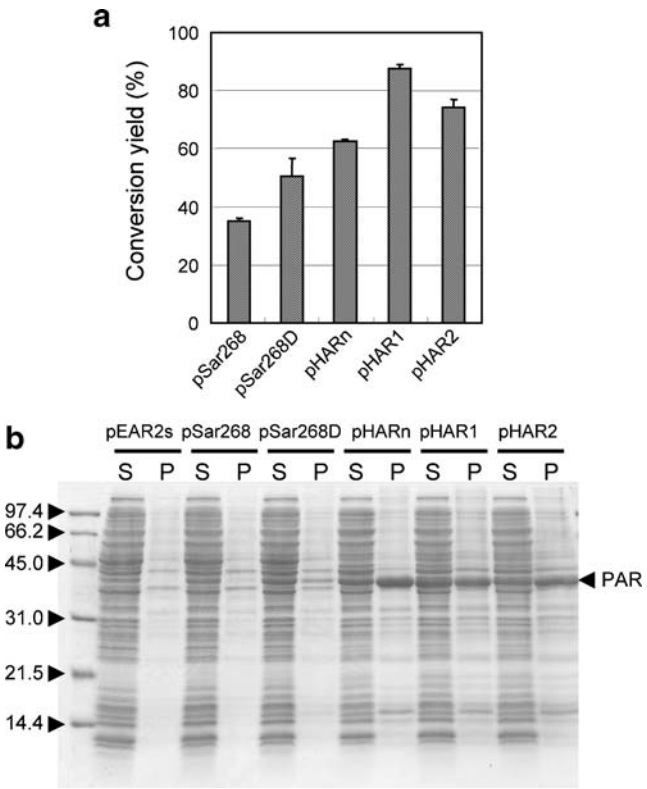
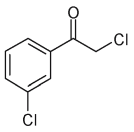
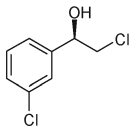
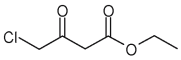
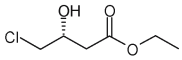
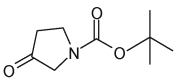
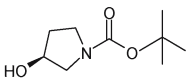


Fig. 5 The properties of combinatorial mutants from the small-scale culture. **a** *m*-CPC conversion. The initial concentration of *m*-CPC and 2-propanol were 5% (w/v) and 21.8% (v/v), respectively. The average and standard deviation of three independent measurements are shown for each clone harboring an indicated plasmid. **b** 15% SDS-PAGE analysis. Procedures were as described in the legend of Fig. 4b

We identified two beneficial amino acid substitutions, both at the 42nd amino acid. In our previous study, the greatest defect in *m*-CPC conversion was observed when the amino acid substitution (valine) at the 42nd amino acid in the combination mutant pSarA reverted to the wild-type amino acid (aspartic acid; Makino et al. 2005). Thus, the 42nd amino acid was considered to be the key position for improving conversion under our experimental conditions. Although the crystal structure of PAR is not available, the configuration of the 42nd amino acid in the PAR structure can be predicted from the known crystal structures in the protein data bank (PDB, http://www.pdb.org/) of the two most homologous ADHs (from *Pseudomonas aeruginosa* [PDB ID: 1LLU; Levin et al. 2004] and *Sulfolobus solfataricus* [PDB ID: 1R37; Esposito et al. 2003]) and the most homologous threonine dehydrogenase (from *Pyrococcus horikoshii* [PDB ID: 2DFV; Ishikawa et al. 2006]). Although the residues corresponding to the 42nd amino acid of PAR are located near the NADH molecule, the residues did not interact directly with NAD^+ (data not shown). Thus, the amino acid residue at the 42nd amino acid might modulate the interaction with NAD(H) and/or the substrate. Actually, we have preliminary results showing that both the K_m and k_{cat} values of Sar268 for the substrates 2-propanol or acetophenone were only about one third to one tenth those of the wild-type enzyme (unpublished), and the mutation at the 42nd amino acid might contribute to these properties to some extent. Additionally, the highest soluble expression was observed with pHAR1. The mutation at the 42nd amino acid in pHAR1 might contribute to an increase in the solubility of the enzyme and resultant improvement of substrate conversion. This is also supported by observation of a similar level of relative activity of purified Sar268 and HAR1 enzymes for the

Table 5 Chemical structure of the compounds used in this study

Substrate		Product	
m-CPC		(*R*)-CCE	
ECOB		(*R*)-ECHB	
Boc-PN		(*S*)-Boc-PL	

reduction of acetophenone (unpublished). However, the level of contribution and the detailed mechanism of these possible factors for the increase in the conversion yield remain unknown. Further analysis of these issues is now underway.

There is no evidence that the best sequence is obtained by merely combining locally optimal sequences. However, the combinatorial sequence obtained here increased the conversion to a certain extent. Morelle et al. (1991) described the relationship among the nucleotide sequences around the start codon, mRNA stability, and mRNA translation activity. They described the importance of the "T" residue preceding the "ATG" start codon, and this replacement was also present in our integrated sequence. The "A," "T," and "T" residues at the positions +15, +16, and +18, respectively, in our integrated nucleotide sequence coincided with the reported preference for "A" or "T." Thus, combinatorial engineering of the sequence around the start codon is considered to be a practically applicable technique for improving biocatalysts.

We have cloned the wild-type PAR in the plasmids pKK223-3 (tac promoter; Itoh et al. 2002) or pTrc99A (trc promoter; unpublished), expecting to thereby obtain improvement of the activity in the resting cell reaction under the strong promoters in these plasmids. However, no significant enhancement was observed. Therefore, as an alternative, we employed random mutagenesis around the start codon for activity improvement. We observed an improved expression by the mutations introduced at the second to sixth codons, and this is a reasonable cause of the enhancement of *m*-CPC conversion of each top clone. The introduction of integrated mutations of the most beneficial sequences around the start codon of PAR contributed to improving the conversion of several substrates. The level of expression was also improved by the integrated sequence. It was not obvious that the simple introduction of the top sequences around the start codon would improve the PAR activity for any coding sequence (e.g., pUARH or pEAR2H). The integrated sequence might be effective only for pSar268 and possibly related sequences.

The possibility of practical application of the mutants remains unclear. However, to the best of our knowledge, (*R*)-CCE is presently not commercially available, and it would be possible to develop a production process for (*R*)-CCE using mutant enzymes. (*R*)-ECHB is a valuable

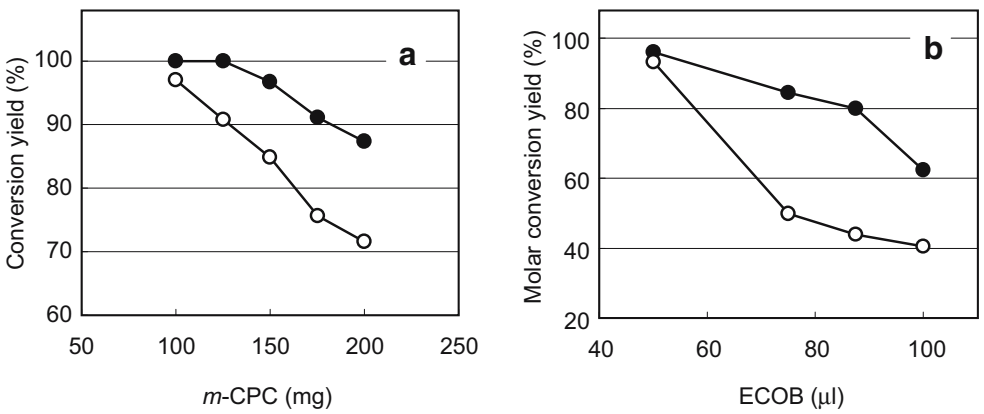


Fig. 6 Conversion of substrates by using cells cultured on a large scale. pSar268 and pHAR1 are shown by *open* and *closed circles*, respectively. **a** The conversion yield of 100, 125, 150, 175, or 200 mg of *m*-CPC. **b** The molar conversion yield of 50, 75, 87.5, or 100 µl of ECOB

compound for synthesis of, for example, pharmaceuticals. The best (*R*)-ECHB production level with pHAR1 (Fig. 6b) corresponds to 140 mg/ml from 87.5 μl of ECOB, much superior to the 93 mg/ml from 50 μl of ECOB with pSar268. This level is considerably higher than other high production systems reported (e.g., Yamamoto et al. 2002). One higher ECOB production result of 268 mg/ml with 91.7% e.e. has been reported (Kataoka et al. 1999). Although our production level was lower than this system, the optical purity of (*R*)-ECHB with our system was very high, and this is advantageous for excluding additional purification processes. Thus, our improved enzyme is a promising option for application in efficient (*R*)-ECHB production. The production of valuable compounds from various substrates by mutant enzymes is also possible.

In conclusion, we were able to develop the pHAR1 mutant, which showed improved substrate conversion, by performing two independent screening procedures of beneficial mutations followed by their integration. We demonstrated that each of these mutation procedures effectively improved PAR to a certain extent. These procedures might also work well for developing other efficient enzymes. It is expected that pHAR1 may also be successfully applied for the production of other valuable compounds. The relationship between beneficial mutations and resultant improvements is of interest. Analyses of the enzymatic properties of the mutants are in progress.

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