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Molecular breeding of enzymes catalyzing asymmetric reactions: Aimed to provide a non-natural amino acid chemical library

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Research aims

Nonproteinous amino acids, which are not the building blocks of protein, are useful for the building blocks in medicines[1–4]. However, many optically active nonproteinous amino acids are expensive. Therefore, development of a low-cost and practical asymmetric-reaction-process of nonproteinous amino acids is expected. In this study, we aimed to improve thermostability and substrate specificity of the type II malate/lactate dehydrogenase family protein, DpkA which is useful for asymmetric synthesis by evolutionary molecular engineering. DpkA catalyzes the NADPH-dependent formation of *N*-methyl-L-amino acids from α-keto acids and methylamine and the NADPH-dependent asymmetric reduction of Δ^1-piperideine-2-carboxylate to form L-pipecolate[5,6]. Hence, DpkA is available for production of these optically active compounds, which are useful as building blocks in medicines and pesticides. We consider that the improved DpkA will contribute to the development of a practical and sustainable asymmetric-reaction-process.

Methods

A *dpkA* (*PP3591*) gene from *Pseudomonas putida* NBRC100650 (KT2440) was ligated into the *Nde*I-*Hin*dIII site of pET21a(+). The resultant plasmid was named pDpkA. Recombinant *Escherichia coli* BL21(DE3) cells harboring pDpkA were grown in LB medium containing 100 μg/ml ampicillin. After the induction of gene expression with 1 mM IPTG, the cells were harvested by centrifugation, disrupted by sonication on ice and centrifuged. The C-terminal six-histidine tagged DpkA was purified from the resultant crude extract by Ni-nitrilotriacetic acid column chromatography. Stabilities of DpkA against temperature, pH, NaCl, guanidine hydrochloride, SDS and ethanol were determined using the purified DpkA. Next, the optimal condition of error-prone PCR was examined to construct a mutant enzyme (DpkA) library. Moreover, a selection method for thermostabilized enzyme from the mutant enzyme library was examined.

Results

1) Stability of DpkA.

The stability of DpkA under various conditions is shown in Fig. 1. After incubation at 25°C for 30 min, DpkA did not retain its activity. The enzyme showed 59% activity after incubation at 30°C for 30 min and was inactivated by incubation at 45°C for 2.5 min (Fig. 1, A). Above pH 7, the activity was decreased as pH increased (Fig. 1, B). DpkA showed 63% activity in the reaction mixture containing 0.85% NaCl (Fig. 1, C). The enzyme showed 24% activity by treatment with 13 μM guanidine hydrochloride (Fig. 1, D). DpkA was inactivated by treatment with 20 μM SDS and 60% ethanol (Fig. 1, E and F).

2) Optimization of the conditions for error-prone PCR and selection method for thermostabilized enzyme from the mutant library.

Plasmid pDpkA was used as a template in mutagenesis for error-prone PCR. A 100 μl reaction mixture contained 10 μl 10×PCR buffer, 0.25 mM dGTP, 0.25 mM dCTP, 0.25 mM dTTP, 4 μM dATP, 33.5 pmol primers, 3.4 ng template DNA and 4.2 U of *Taq* polymerase (Takara). PCR was performed with an automatic thermal cycler for 10 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 25 cycles under the same conditions after the addition of 25 nmol of ATP only. Amplified mutant *dpkA* DNA fragments were ligated into pET21a(+) and *E. coli* BL21(DE3) was transformed by the resultant plasmids. Plasmid DNA harboring mutant *dpkA* was extracted from transformants and sequenced. Each extracted plasmid had from one to six substituted bases within the inserted genes. Therefore, we decided to prepare mutant *dpkA* by this PCR conditions in this study.

Next, the selection method for thermostabilized enzyme from the mutant library was examined. Crude extract from *E. coli* BL21(DE3) harboring pDpkA was incubated at 30, 45, and 50°C for 30 min. After incubation, *N*-methyl-L-amino acid dehydrogenase activity in the crude extract was assayed by a microplate reader. Crude extracts incubated at 30 and 45°C showed activity but crude extract incubated at 50°C was inert. Therefore, DpkA in the crude extract was denatured at 50°C. From these result we decided to select thermostabilized DpkA by comparing the activities of wild-

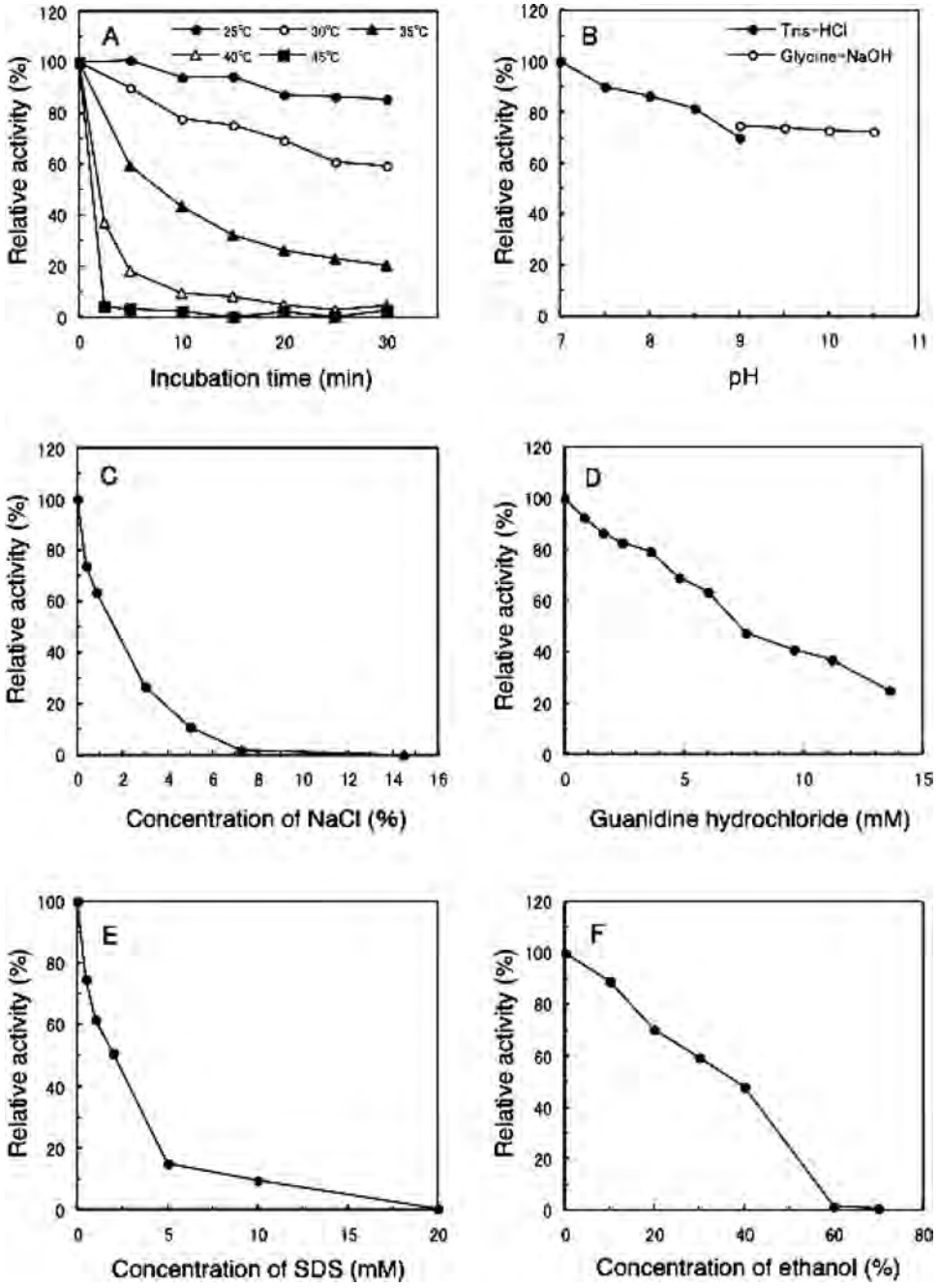

Fig. 1. Stability of DpkA. Effects of temperature (A), pH (B), concentration of NaCl (C), concentration of guanidine hydrochloride (D), concentration of SDS (E) and concentration of ethanol (F) on the stability of DpkA.
DpkA was incubated on ice for 30 min in experiments shown in (B) or for 10 min in experiments shown in (D), (E) and (F).

type DpkA incubated at 45°C and 50°C with those of mutant DpkAs incubated at the same temperatures.

We will attempt to obtain thermostabilized DpkA by the above-mentioned method. Moreover, we aim to obtain not only thermostabilized DpkA but also NADH-dependent DpkA by error-prone PCR.

Conclusion

This work demonstrated the stability of DpkA from *P. putida* NBRC100650 under various conditions. Moreover, we could construct a mutant enzyme library by optimized error-prone PCR. The selection method for thermostabilized enzyme from the library was also determined. Hereafter, we aim to obtain thermostable or NADH-dependent DpkA from the mutant enzyme library. We will attempt to synthesize chiral building blocks in medicines and pesticides such a *N*-methyl-L-amino acids and L-pipecolate by modified DpkA. The reaction process is expected to make long-term asymmetric synthesis by the enzyme possible and to increase productivity.

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