〈Brief Note〉

Improvement of thermal stability of *Leuconostoc pseudomesenteroides* glucose-6-phosphate dehydrogenase

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Summary *In vivo* random mutagenesis was used to enhance the thermal stability of *Leuconostoc pseudomesenteroides* glucose-6-phosphate dehydrogenase. Six thermostable mutants were isolated, and the following amino acid substitutions were identified: F65 to L, Y206 to F, N239 to S, D323 to G, S336 to A, and K352 to R. The half-life at 50℃ of the Y206F mutant enzyme was approximately 6-fold that of the wild type. Moreover, the multiple mutant constructed by site-directed mutagenesis was much more stable than the either the wild type or Y206F. These mutant enzymes are most likely to prove useful in clinical assays of glucose and creatine kinase.

Key words: *In vivo* random mutagenesis, Protein engineering, Stabilization, Glucose, Creatine kinase

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) from *Leuconostoc pseudomesenteroides* (the previously named: *Leuconostoc mesenteroides*) catalyzes the oxidation of glucose-6-phosphate in the presence of the coenzyme $NADP^+$ or NAD^+. The enzyme is one of the industrially important enzymes and is applied in clinical settings. It is mainly useful in determinations of glucose and creatine kinase by coupling with hexokinase (EC 2.7.1.1; ATP: D-hexose 6-phosphotransferase) or glucokinase (EC 2.7.1.2; ATP: D-glucose 6-phosphotransferase)[1, 2]. G6PDH has been cloned and expressed in *Escherichia coli*[3], and its tertiary structure has been determined in the presence of substrates, coenzymes, or both[4]. The catalytic function of G6PDH has been also investigated by site-directed mutagenesis[5].

The enhancement of enzyme thermostability is one of the important approaches in protein engineering. Using thermostable enzymes in clinical diagnostic reagents offers the benefits of longer reagent shelf-lives at normal storage temperatures. We have already described the enhancement of the thermal stability of *Saccharomyces pastorianus* hexokinase as well as the mutational effects of the amino

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acid substitutions discussed on the basis of the tertiary structure of S. *cerevisiae* hexokinase[6]. These provided information for further improvements in the functionality of the enzyme.

Here, we report on a successful enhancement of the thermal stability of G6PDH by means of *in vivo* mutagenesis techniques. The thermostable mutants thus constructed will be successfully applied to the assays of glucose and creatine kinase.

2. Materials and methods

1. Construction of plasmid and recombinant strains

L. pseudomesenteroides ATCC12291 was used as a G6PDH-producing strain[3], and its chromosomal DNA was prepared from harvested cells by a previous method[7]. The G6PDH gene and flanking regions were amplified from chromosomal DNA using the primer pair that was designed on the basis of a sequence of the G6PDH gene (DDBJ accession number; M64446). It conteins 107 bp of the 5'-flanking region including promoter and SD sequences, and 291 bp of the 3'-flanking region including a putative terminator. The PCR product was ligated with pBluescript-KS(+) expression vector, yielding the plasmid pG6DD9.

The transformation of *E. coli* and isolation of the plasmid were carried out as described previously[8]. The host strains used were *E. coli* JM109 and XL1-Red (Stratagene, La Jolla, CA, USA). Recombinant strains were grown in LB broth or on LB agar[7]; the antibiotic used was ampicillin.

2. Mutagenesis

In vivo random mutagenesis was performed using the *E. coli* XL1-Red mutator strain (Stratagene) according to the manufacturer's instructions. The mutagenized plasmid DNA was recovered from a saturated culture of XL1-Red (pG6DD9) and used to transform *E. coli* JM109. Site-directed mutagenesis was performed using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA).

3. Enzyme purification

Each recombinant strain was grown to the stationary phase at 30℃ in LB broth with agitation at 180 r.p.m. Cells were harvested by centrifugation, and a crude extract was prepared by sonication of the cells. Ammonium sulfate was added to the cell-free extract, and the precipitate collected by centrifugation was dissolved in 20 mM phosphate buffer (pH 7.5) and dialysed against that same buffer. The enzyme was purified from the dialysate by Q-Sepharose and Superdex 200 column chromatographies (GE Healthcare, Uppsala, Sweden). The protein was analysed by the standard method using SDS-PAGE.

4. Enzyme assay

The reaction of G6PDH is summarized below.

D-glucose-6-phosphate + NAD^+ → glucono-δ-lactone + NADH + H^+

The increase in NADH caused by the reaction of G6PDH was spectrophotometrically measured at 340 nm. Enzyme solutions (0.1-0.5 U/ml) were prepared by dilution with 50 mM Tris-HCl (pH 7.8) containing 0.1% of bovine serum albumin. The final assay mixture contained 3.3 mM D-glucose-6-phosphate, 2.0 mM NAD^+, 3.0 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.8). One unit of activity was defined as the formation of 1 micromole of glucono-δ-lactone per minute at 30℃ and pH 7.8. Reaction mixtures containing several concentrations of substrate solution were used to determine the K_m values.

3. Results and discussion

1. Random mutagenesis and isolation of thermostable mutants

Improvement in the thermal stability of G6PDH was achieved by means of phenotypic selection from recombinant cells carrying an *in vivo* mutagenized plasmid. As a result, four positive clones were found among about 5,000 colonies. After heat treatment (incubation at 50℃ for 60 min), the G6PDH activities of these clones were detected, although little activity was observed in the case of the wild-type producing strain, *E. coli* JM109(pG6DD9). Recombinant plasmids were extracted from these positive clones, and the DNA sequences were detected. Only one base change, resulting in the replacement of one amino acid residue, was found in each G6PDH gene.

The four G6PDH mutants with the mutations Phe65-Leu (codon: TTC→TTG), Tyr206-Phe (codon: TTC →TAC), Asp323-Gly (codon: GAT→GGT), or Ser336-Ala (codon: TCA→GCA) were designated F65L, Y206F, D323G, and S336A, respectively (Fig. 1).

2. Purification and comparison of wild type and mutants

Each recombinant strain producing the wild-type or a mutant G6PDH was cultured, and the G6PDH was purified to homogeneity. Upon SDS-PAGE analysis, each purified enzyme was seen to migrate as a single protein band (data not shown); the molecular mass of the subunit of each enzyme was determined to be ~54 kDa.

The thermal stabilities of the mutants were compared with that of the wild-type enzyme (Fig. 2A). All the mutants were more thermally stable than the wild type at 50℃. The increase in the half-life of each mutant was calculated. The half-lives of F65L, Y206F, D323G, and S336A were estimated to be 52, 65, 30, and 43 minutes, respectively, while that of the wild type was 10 minutes. Y206F was the most thermostable among the four, its half-life at 50℃ was approximately 6-fold that of the wild type. The K_m values for D-glucose-6-phosphate were also calculated for the purified wild type and mutants. Except for S336A, the values for the mutants were similar to that for the wild-type enzyme, which were calculated to be 0.23 mM. The value for S336A was about ten times higher (2.1 mM). As shown in Fig. 1, S336 is relatively close to the active site. Its low substrate affinity might be due to the relation of the side chain of S336 to the substrate binding.

3. Construction and comparison of multiple mutants

We also tried to re-mutagenize the Y206F-encoding gene, and obtained two clones showing further increased thermal stability. They were Asn239-Ser (codon: AAC→AGC) and Lys352-Arg (codon: AAG→AGG), respectively, as well as Tyr206-Phe, and the mutant enzymes were designated as Y206F+N239S and Y206F+K352R (Fig. 1).

In most cases, a combination of mutations affecting protein stability will exhibit simple additivity, with the exceptions being indicative of a strong interaction among mutations. To determine whether or not the mutational effects of these mutants were additive, a multiple mutant was constructed by site-directed mutagenesis. Because of its low substrate affinity, S336A was excluded from the mutational points. A five-point mutant was designated M5 (F65L+Y206F+N239S +D323G+K352R).

Each recombinant strain producing the multiple mutant G6PDH was cultured, and G6PDH was purified to homogeneity. The molecular mass of the subunit of each enzyme was the same as that of the wild type and single mutants as determined by SDS-PAGE analysis.

The thermal stabilities of multiple mutants were compared with that of the wild-type G6PDH (Fig. 2B), while those of Y206F+N239S and Y206F+K352R were elevated compared with those of the wild type and Y206F. Moreover, M5 was shown to be much more stable than all other mutants (Fig. 2B), confirming that the mutational effects of amino acid

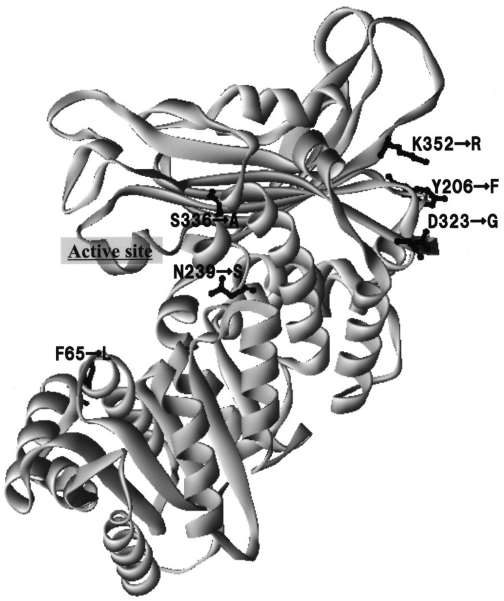

Fig. 1 Each mutational point on the tertiary structure of G6PDH. Amino acid residues that were mutated in thermostable mutants were represented by stick drawings.

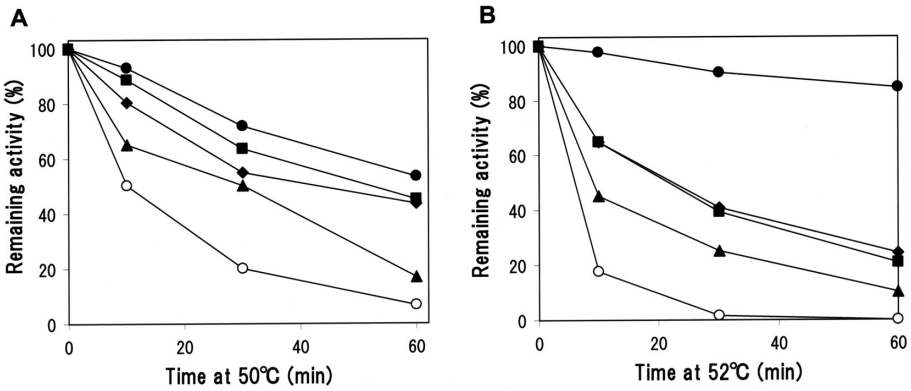

Fig. 2 Effect of temperature on the stability of wild-type and mutant G6PDHs. The enzyme solution (1 U/ml) was treated with 100 mM imidazole acetate buffer (pH 6.7) containing 2 mM EDTA and incubated for the indicated times. The remaining activity was measured as described in Materials and methods. A: the enzyme solutions were incubated at 50℃. Symbols: ○, wild-type enzyme; ●, Y206F; ■, F65L; ◆, S336A; ▲, D323G. B: the enzyme solutions were incubated at 52℃. Symbols: ○, wild-type enzyme; ●, M5 (F65L+Y206F+N239S+D323G+K352R); ■, Y206F+N239S; ◆, Y206F+K352R; ▲, Y206F.

positions 65, 206, 239, 323, and 352 were additive.

Thus, we could increase the thermal stability of G6PDH by random and site-directed mutagenesis. The thermostable mutant G6PDHs are most likely to be successfully applied to the assays of glucose and creatine kinase. For additional applications, further analysis of the enzymatic functions of thermostable mutants using protein engineering techniques is now underway.

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