

<Original Article>

## Improvement of thermal stability of *Saccharomyces pastorianus* hexokinase by random mutagenesis and a structural interpretation

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**Summary** *In vivo* random mutagenesis was used to enhance the thermal stability of *Saccharomyces pastorianus* hexokinase. Four thermostable mutants were isolated, and the following amino acid substitutions were identified: Glu53 to Val, Ala161 to Val, Ser255 to Pro, and Gly408 to Asp. The wild-type and mutant enzymes were purified and characterized. The properties of mutants were similar to those of the wild type but they showed improved thermal activity. Analysis of multiple mutants constructed by site-directed mutagenesis showed that all the mutations brought an additive effect to the other mutations. These mutational effects were discussed in terms of the three-dimensional structure of *Saccharomyces cerevisiae* hexokinase.

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

### 1. Introduction

Hexokinase (EC 2.7.1.1; ATP:D-hexose 6-phosphotransferase) catalyses the ATP-dependent conversion of D-hexose to hexose-6-phosphate. This enzyme is industrially important and useful for the clinical determination of glucose and creatine kinase by coupling with glucose-6-phosphate dehydrogenase<sup>1,2)</sup>. Hexokinase from the genus *Saccharomyces* is produced commercially and used for application to diagnostic reagents.

The hexokinase of yeast *S. cerevisiae* has been studied by X-ray crystallography<sup>3,4)</sup>, which provided a

complete structural description of the enzyme. Substrate binding features of possible importance and their catalytic mechanisms have also been discussed.

Here, we report on a successful enhancement of the thermal stability of *Saccharomyces* hexokinase by means of *in vivo* mutagenesis techniques. We newly cloned a gene of hexokinase from *S. pastorianus* (*Hxk-Sp*), a species closely related to *S. cerevisiae*, and used it as the wild-type enzyme-encoding gene. We also describe the mutational effects of the amino acid substitutions discussed on the basis of the tertiary structure of *S. cerevisiae* hexokinase. These provide information for further improvements in the function-

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ality of the enzyme.

## 2. Materials and methods

### 1. Strain, plasmids, and culture conditions

*S. pastorianus* ATCC2366 was used as a hexokinase-producing strain. This yeast strain was grown in YPD broth (1% Polypepton, 1% yeast extract, 1% D-glucose (pH 5.3)) at 30°C for 16 hours, and the chromosomal DNA was prepared from harvested cells. The host strains used were *Escherichia coli* HB101 and XL1-Red (Stratagene, La Jolla, CA, USA). Plasmid pBluescript-KSN(+) was used for recombinant plasmid construction. Recombinant strains were grown in Terrific broth or on L agar<sup>5)</sup>; the antibiotic used was ampicillin.

### 2. Manipulations of DNA

The transformation of *E. coli*, isolation of the plasmid, cleavage of the DNA with restriction enzymes, and ligation with T4 DNA ligase were carried out as described previously<sup>6)</sup>.

### 3. Plasmid construction

The chromosomal DNA of *S. pastorianus* was purified by the previous method<sup>7)</sup>. Hexokinase (Hxk-*Sp*) gene was amplified using the following primer pair, that was designed on the basis of a sequence of the *S. cerevisiae* hexokinase gene (HXK2, DDBJ accession number; M14411): 5'-GGGGGCATAT-GGTTCAATTAGTCCAAAGAAACCACAG-3' (sense, *Nde*I site underlined) and 5'-GGGAGGGATCCTCATTAAGCGCCAATGAT-ACCAAGAGAC-3' (antisense, *Bam*HI site underlined). The PCR product was digested with *Nde*I and *Bam*HI, and ligated with pBluescript-KSN expression vector, yielding a pHXK10.

### 4. 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 (pHXK10) and used to transform *E. coli* HB101. Site-directed mutagenesis

was performed using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA).

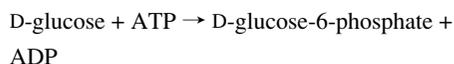
### 5. Enzyme purification

Each recombinant strain was grown to the stationary phase at 30°C in Terrific broth with agitation at 180 r.p.m. Cells were harvested by centrifugation, and 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 6.5) and dialysed against the same buffer. The enzyme was purified from the dialysate by DEAE-Sepharose CL-6B and Phenyl-Sepharose column chromatographies (GE Healthcare, Uppsala, Sweden). The protein was analysed by the standard method using SDS-PAGE.

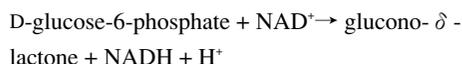
### 6. Enzyme assay

The reaction sequence of hexokinase assay is summarized below.

#### Step 1: hexokinase



#### Step 2: glucose-6-phosphate dehydrogenase



Hexokinase forms D-glucose-6-phosphate from D-glucose, and the increase in NADH caused by the following reaction of glucose-6-phosphate dehydrogenase was spectrophotometrically measured. Enzyme solutions (0.1-0.5 U/ml) were prepared by dilution with 20 mM potassium phosphate (pH 6.5). The final assay mixture contained 110 mM D-glucose, 0.55 mM ATP, 9.9 mM MgCl<sub>2</sub>, 0.23 mM NAD<sup>+</sup>, 38 mM Tris-HCl (pH 8.0), and 1 U/ml of glucose-6-phosphate dehydrogenase. One unit of activity was defined as the formation of 1 micromole of glucose-6-phosphate per minute at 30°C and pH 8.0. Reaction mixtures containing several concentrations of substrate solution were used to determine the K<sub>m</sub> values.

## 3. Results

### 1. Random mutagenesis and isolation of thermostable

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Sp 2' VHLGPKKQA RKGSMADVPK ELMQIQENFE KIFTVPTETL QAVTRHFISE LEKGLSKKGG NIPMIPGWVM DFPTGKESGD FLAIDLGGTN
*****
Sc 2' VHLGPKKQA RKGSMADVPK ELMQIQENFE KIFTVPTETL QAVTKHFISE LEKGLSKKGG NIPMIPGWVM DFPTGKESGD FLAIDLGGTN

92' LRVVLVKLGG DRTFDTTQSK YRLPDAMRTT QNPDELWEFI ADSLKAFIDE QFPQGISEPI PLGFTFSFPA SQNKINEGIL QRWTKGFDIP
*****
92' LRVVLVKLGG DRTFDTTQSK YRLPDAMRTT QNPDELWEFI ADSLKAFIDE QFPQGISEPI PLGFTFSFPA SQNKINEGIL QRWTKGFDIP

182' NIENHDVVPK LQKQITKRN IPIEVVALIND TTGTLVASYY TDPETKMGVI FGTGVNGAYY DVCSDIEKLQ GKLSDDIPPS APMAINCEYG
*****
182' NIENHDVVPK LQKQITKRN IPIEVVALIND TTGTLVASYY TDPETKMGVI FGTGVNGAYY DVCSDIEKLQ GKLSDDIPPS APMAINCEYG

272' SFDNEHVLP RTRVDITIDE ESPRPGQQT EKMSSGYLGE IILRLALMDM YKQGFIFKNQ DLSKFDKPFV MDTSPARIE EDPFENLEDT
*****
272' SFDNEHVLP RTRVDITIDE ESPRPGQQT EKMSSGYLGE IILRLALMDM YKQGFIFKNQ DLSKFDKPFV MDTSPARIE EDPFENLEDT

362' DDLFQNEFGI NTTVQERKLI RRLSELIGAR AARLSVCGIA AVCCQKRGYKT GHIAADGSVY NRYPGFKEKA ANALKDIIYGV TQTSLLDDYPI
*****
362' DDLFQNEFGI NTTVQERKLI RRLSELIGAR AARLSVCGIA AVCCQKRGYKT GHIAADGSVY NRYPGFKEKA ANALKDIIYGV TQTSLLDDYPI

452' KIVPAEDGSG AGAAVIAALA QKRIAEGKSL GIIGA
*****
452' KIVPAEDGSG AGAAVIAALA QKRIAEGKSV GIIGA

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Fig. 1 Comparison of amino acid sequences between hexokinases from *S. cerevisiae* (Sc) and *S. pastorianus* (Sp). Amino acids of both hexokinases are numbered from the first Met, which is posttranslationally processed. Substituted amino acid residues are enclosed by boxes.

## mutants

The Hxk-*Sp* gene on pHXK10 was sequenced (DDBJ accession number; E16728), and the deduced amino acid sequence of Hxk-*Sp* exhibited 99.2% identity with that of the *S. cerevisiae* hexokinase. Among all the 485 amino acid residues, only four were replaced with other amino acids: Lys45 of *S. cerevisiae* hexokinase to Arg, Lys283 to Arg, Ile402 to Val, and Val480 to Leu (Fig. 1).

Improvement in the thermal stability of Hxk-*Sp* 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 4,000 colonies. After heat treatment (incubation at 45°C for 1 hour), the hexokinase activities of these clones were detected, although little activity was observed in the case of the wild-type producing strain, *E. coli* HB101 (pHXK10). 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 Hxk-*Sp* gene. The four Hxk-*Sp* mutants with the Glu53-Val (codon: TCT→CCT), Ala161-Val (codon: GCT→GTT), Ser255-Pro (codon: TCT→CCT), or Gly408-Asp (codon: GGT→GAT) mutations were designated E53V, A161V, S255P, and G408D,

respectively.

## 2. Purification and characterization of wild type and mutants

Each recombinant strain producing the wild-type or a mutant Hxk-*Sp* was cultured, and the hexokinase 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 specific activities (with 110 mM D-glucose as the substrate at 30°C and pH 8.0) of E53V, A161V, S255P, and G408D were found to be 750, 670, 750, and 620 U/mg, respectively, while that of the wild type was 770 U/mg. The mutants thus exhibited specific activities similar to that of the wild-type enzyme.

The thermal stabilities of the mutants were compared with that of the wild-type enzyme (Fig. 2). All the mutants were more thermally stable than the wild type at 45°C. The increase in the half-life of each mutant was calculated. The half-lives of E53V, A161V, S255P, and G408D were estimated to be 35.7, 65.7, 73.0, and 33.4 minutes, respectively, while that of the wild type was 17.5 minutes. The increased thermal stabilities of A161V and S255P were particularly marked. The mutants had the same optimum

temperature (50°C) and optimum pH (8.5-9.0) for their activity as those of the wild-type enzyme. The  $K_m$  values for D-glucose and ATP were calculated for the purified wild type and mutants. The values for the mutants were similar to those for the wild-type enzyme, which were calculated to be 0.17 and 0.28 mM, respectively.

### 3. Construction, purification, and characterization of multiple mutants

In most cases, a combination of mutations affecting protein stability will exhibit simple additivity, with exceptions being indicative of a strong interaction among mutations. To determine whether or not the mutational effects of these four mutants were additive, multiple mutants were constructed by site-directed mutagenesis. Two-, three-, and four-point mutants were designated M2 (E53V and S255P), M3 (E53V, S255P, and A161V), and M4 (E53V, S255P, A161V, and G408D), respectively.

Each recombinant strain producing the mutant Hxk-*Sp* was cultured, and hexokinase 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 specific activities of M2, M3, and M4 were found to be 760, 590, and 680 U/mg, respectively.

The thermal stabilities of multiple mutants were compared with that of the wild-type Hxk-*Sp* (Fig. 3). The stabilities of M2, M3, and M4 were elevated compared with those of the wild type and E53V, showing that the mutational effects of amino acid positions 53, 161, 255, and 408 were additive.

All mutants showed the same optimum temperature and optimum pH as those of the wild-type enzyme. The  $K_m$  values of M2, M3, and M4 for D-glucose were calculated to be 0.17, 0.57, and 0.58 mM, respectively. The affinities of M3 and M4 to D-glucose were reduced although they were practical levels. On the other hand,  $K_m$  of M2, M3, and M4 for ATP were 0.16, 0.17, 0.17 mM, respectively, which were similar or slightly lower than that of the wild-type enzyme.

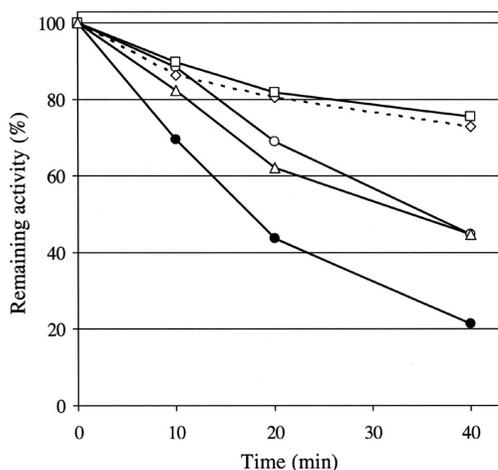


Fig. 2 Effect of temperature on stability of wild-type and mutant Hxk-Sps. The enzyme solution (5 U/ml) was treated with 100 mM imidazole acetate buffer (pH 6.6) and incubated for the indicated times at 45°C. Symbols: ●, wild-type enzyme; ○, E53V; ◇, A161V; □, S255P; △, G408D. The remaining activity was measured as described in Materials and methods.

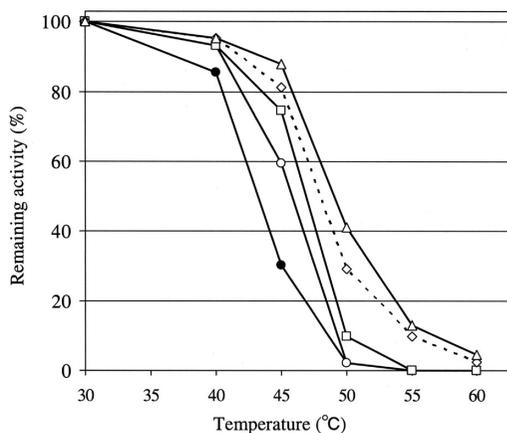


Fig. 3 Effect of temperature on stability of multiple mutant Hxk-Sps. The enzyme solution (5 U/ml) was treated with 100 mM imidazole acetate buffer (pH 6.6) and incubated for 30 min at the indicated temperatures. Symbols: ●, wild-type enzyme; ○, E53V; □, M2; ◇, M3; △, M4. The remaining activity was measured as described in Materials and methods.

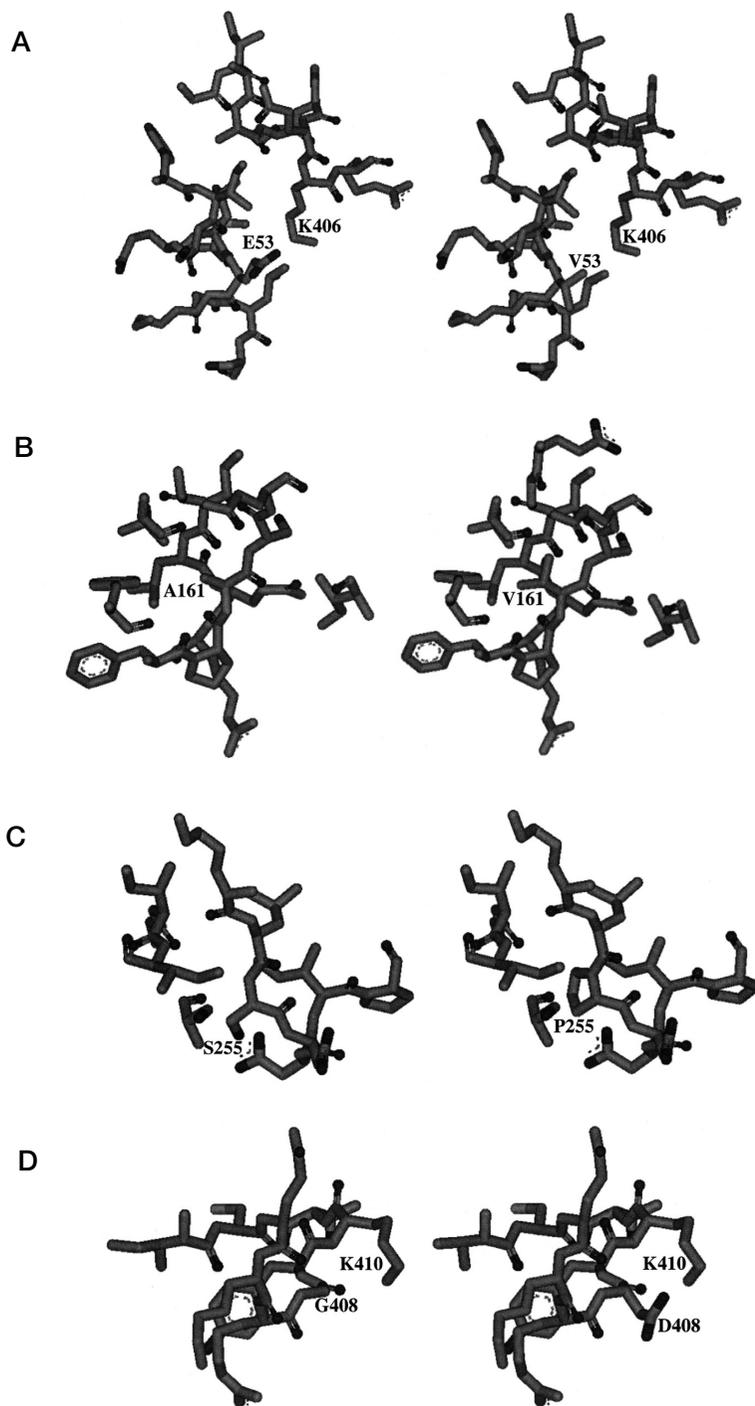


Fig. 4 Localized hexokinase structure around each mutation.  
(A) View of residues around E53 and V53 in E53V with K406.  
(B) View of residues around A161 and V161 in A161V.  
(C) View of residues around S255 and P255 in S255P.  
(D) View of residues around G408 and D408 in G408D with K410.

#### 4. Discussion

We succeeded in creating thermostable hexokinase by random and site-directed mutagenesis. The thermostable mutants, especially M3 and M4, were successfully applied to the assays of glucose and creatine kinase with glucose-6-phosphate dehydrogenase (data not shown).

Four mutations contributing to the stability of the enzyme were identified. The structural model helps to enhance our understanding of the stabilization of the enzyme. Hxk-*Sp* exhibited almost the same amino acid sequence as that of the *S. cerevisiae* hexokinase. Therefore, the three-dimensional structure of the *S. cerevisiae* hexokinase<sup>9</sup> provided a reasonable starting point for analysing the mutational effects.

The side chain of E53 consists of an  $\alpha$ -helix, and is able to form an ion pair with that of K406 consisting of another helix. The two helices were located near each other. The E53V substitution, therefore, was likely to reduce the steric interference between these neighboring helices (Fig. 4A). There was a relatively large cavity around the side chain of A161, and the side chain of V161 in A161V can be accommodated to strengthen the atomic packing there. The increased thermal stability of A161V was likely due to this additional hydrophobic interaction (Fig. 4B). The S255 residue lay on a loop structure. The S255P substitution stabilizes the loop, since a proline residue mediates a tight structure (Fig. 4C). On the other hand, the side chain of D408 in G408D is possible to form an ion pair with that of K410 (Fig.

4D). This attractive interaction is able to contribute to the stabilization of the conformation.

Thus, we could increase the thermal stability of Hxk-*Sp* by stabilizing the local structures of the enzyme through protein engineering. For additional applications, further analysis of the enzymatic functions of thermostable mutant Hxk-*Sps* using protein engineering techniques is now underway.

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