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Selection of stabilized 3-isopropylmalate dehydrogenase of *Saccharomyces cerevisiae* using the host-vector system of an extreme thermophile, *Thermus thermophilus*

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Abstract A *leuB* strain of *Thermus thermophilus,* TTY1, was transformed with a plasmid vector that directed expression of 3-isopropylmalate dehydrogenase (IPMDH) of *Saccharomyces cerevisiae* encoded by the *LEU2* gene. The original strain could not grow at 50°C without leucine, probably because of the low stability of *S. cerevisiae* IPMDH. The mutants that could grow without leucine were selected at 50°, 60°, 62°, 65°, 67°, and 70°C, step by step. All the mutant strains except for one isolated at 50°C accumulated mutations. Mutations were serially accumulated: Glu255Val, Asn43Tyr, Ala62Thr, Asn110Lys, and Ala112Val, respectively, at each step. The analyses of residual activity after heat treatment and the denaturation profile as monitored by circular dichroism showed that thermal stability was increased with accumulation of the mutations. The kinetic parameters of most mutant enzymes were similar to those of the wild type. However, some mutant enzymes showed a reverse correlation between stability and activity: the enzymes with a large increase in thermal stability showed lower activity. Although the wild-type enzyme is unstable in the absence of glycerol, the stabilizing effect of glycerol was not observed for all the mutant enzymes containing the Glu255Val substitution, which is assumed to be located at the hydrophobic interface between two subunits.

Key words Evolutionary engineering · 3-Isopropylmalate dehydrogenase · *Saccharomyces cerevisiae* · Thermostability · *Thermus thermophilus*

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Introduction

Enzymes have many useful features such as high substrate specificity and reaction specificity as catalysts under mild conditions. One of the major obstacles in their industrial use is their sensitivity to denaturants and heat. A general method of stabilizing proteins has been greatly needed. The evolutionary technique is a powerful method for creating robust enzymes. Several enzymes have been stabilized by directed evolution experiments (Giver et al. 1998; Hoseki et al. 1999; Miyazaki et al. 2000; Zhao and Arnold 1999), and detailed three-dimensional structures of the stabilized mutant enzymes have provided important insight for structure–stability relationships (Spiller et al. 1999).

3-Isopropylmalate dehydrogenase (IPMDH) is involved in leucine biosynthesis. Genes encoding IPMDH have been cloned from many species and significant sequence homology was found among them. Three-dimensional structures of IPMDHs from an extreme thermophile (Imada et al. 1991) and several mesophiles (Imada et al. 1998; Wallon et al. 1997) have been solved, and it has been shown that their structures are very similar. We have successfully screened thermostabilized IPMDHs from a chimeric enzyme composed of amino acid sequences from the thermophile IPMDH and *Bacillus subtilis* IPMDH (Kotsuka et al. 1996; Tamakoshi et al. 1995) with an evolutionary technique using a *Thermus thermophilus* gene cloning system. Similarly stabilized enzymes have been isolated from a carboxy-terminal truncated thermophile enzyme (Akanuma et al. 1996) and a mesophile enzyme from *B. subtilis* (Akanuma et al. 1998, 1999). Structural analyses of the mutant enzymes contributed to the understanding of the mechanisms for thermostabilization (Qu et al. 1997; Sakurai et al. 1995).

Many mutations were found using *B. subtilis* IPMDH as a starting material because the stability of the mesophile enzyme is considerably lower compared with the maximum growing temperature of the thermophile. The temperature at which 50% of the enzyme activity ($T_{1/2}$) or of the secondary structure (T_h) is lost during a fixed incubation period of the *B. subtilis* IPMDH containing three spontaneous

mutations was about 10°C higher than that of *B. subtilis* wild-type enzyme (Akanuma et al. 1998). In this report, we selected thermostabilized mutants of *Saccharomyces cerevisiae* IPMDH, whose amino acid sequence shows 50% identity and whose stability is comparable to that of *B. subtilis* IPMDH. The stability and mutation of the selected *S. cerevisiae* IPMDHs were compared with those of stabilized mutants of *B. subtilis* IPMDH.

Materials and methods

Strains

Thermus thermophilus TTY1 (Δ*leuB*Δ*pyrE*) (Tamakoshi et al. 1999) was used for expression of *S. cerevisiae* IPMDH and selection of stabilized enzymes. *Escherichia coli* MA153, which is a derivative of BL21(DE3) and has a deficiency in the *leuB* gene, was constructed in our laboratory and used for overexpression of *S. cerevisiae* IPMDHs.

DNA manipulations

All routine DNA manipulations, plasmid preparations, and subcloning in *E. coli* were done essentially as described by Sambrook et al. (1989). *T. thermophilus* was transformed as described previously (Koyama et al. 1986). Restriction endonucleases and DNA modification enzymes were used as recommended by the manufacturers.

pT8L2P, which was used for expression of *S. cerevisiae* IPMDH in *T. thermophilus* TTY1, was constructed as follows. An *Nde*I site was introduced at the initiation codon of the *LEU2* gene of YEp13 (Broach et al. 1979) by a PCR-based oligonucleotide-directed mutagenesis (Picard et al. 1994) with the primer Y2Nde (5′-CTTAGGGGCAGA-CATATGAATGGTATATCCTT-3′, the *Nde*I site is underlined). The *Nde*I-*Eco*RI fragment encoding the *pyrE* gene of pINV (Tamakoshi et al. 1997) was cloned in pT8*leuB* (Tamakoshi et al. 1999) in place of the *leuB* gene. The *LEU2* gene and the *pyrE* gene were cloned tandemly in pT8*leuB* in place of the *leuB* gene by a three-fragment ligation reaction involving linearized pT8*leuB* with *Nde*I and *Eco*RI restriction nucleases, the *Nde*I-*Acc*I blunt-ended *LEU2* gene, and the *Eco*RI-*Sal*I blunt-ended *pyrE* gene (pT8L2P). Both the *LEU2* gene and the *pyrE* gene in pT8L2P were expressed by the same promoter.

Overproduction and purification of *S. cerevisiae* IPMDH

The *Nde*I-*Sal*I fragment containing *S. cerevisiae* wild type or a mutant *LEU2* gene was cloned in the *Nde*I-*Sal*I site of pET21c (Novagen). With the resulting plasmids, *E. coli* MA153 was transformed. *S. cerevisiae* IPMDHs were overproduced by induction with 1 mM isopropyl thiogalactoside (IPTG). The cells were harvested by centrifugation, washed with buffer A (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, and 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstatin A, and 10 μg/ml leupeptin, and resuspended in the same buffer. The cells were disrupted by sonication, and after the cell debris was removed by centrifugation, the cell-free extract was applied to a DEAE-cellulose column (DE52; Whatman) equilibrated with buffer A. After the column was washed with buffer A, proteins were eluted with buffer A with 0.1 M NaCl. Active fractions were pooled, saturated with 25% ammonium sulfate, cleared by centrifugation, applied to a Butyl-Toyopearl 650S column (Toso, Tokyo, Japan), and eluted with a 25%–0% ammonium sulfate linear gradient. Active fractions were pooled, dialyzed against buffer A, applied to a HiLoad Q (Amersham-Pharmacia Biotech, Uppsala, Sweden) column, and eluted with a 0–0.1 M NaCl linear gradient. Finally, active fractions were pooled, dialyzed against buffer A, applied to a Resource Q column (Amersham-Pharmacia Biotech), and eluted with a 0–0.1 M NaCl linear gradient. All the enzymes used in this study were purified to homogeneity as judged by SDS-gel electrophoresis. Purified enzymes were stored in 60% saturated ammonium sulfate solution at 4°C. Protein concentration was determined with BCA protein assay reagents (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Thermal stability measurement

The remaining activity of purified enzymes after heat treatment was measured at 30°C (Akanuma et al. 1998). Thermal denaturation was monitored by circular dichroism as described previously (Akanuma et al. 1998).

Activity after dilution without glycerol

Enzymes dialyzed against 50 mM Tris-HCl (pH 8.3) containing 10% glycerol were diluted tenfold with 50 mM Tris-HCl (pH 8.3) without glycerol. After incubation for 20 min at 23°C, the enzyme activity was measured. The remaining activity was expressed as percentages of those enzymes diluted into 50 mM Tris-HCl (pH 8.3) containing 10% glycerol.

Enzyme assay

Enzymatic activity was measured in 50 mM sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES-Na, pH 7.6), containing 0.2 mM D-3-IPM, 100 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD in a total volume of 500 μl at 30°C by monitoring the absorption at 340 nm. Kinetic parameters, K_m for D-3-IPM and k_{cat}, were determined in steady-state experiments at 30°C in 50 mM HEPES-Na, pH 7.6, containing 100 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD with various concentrations of D-3-IPM, ranging from 2 to 32 μM. To determine K_m for

NAD, concentration of the coenzyme was varied from 50 to 3000 μM, with a fixed concentration of D-3-IPM (0.2 mM).

Results

Selection of thermostabilized mutant strains

Thermus thermophilus TTY1 was transformed with pT8L2P, which directs expression of *S. cerevisiae LEU2* gene as well as *T. thermophilus pyrE* gene encoding orotate phosphoribosyltransferase as a marker. All the transformed cells selected without uracil formed colonies on plates without leucine at 50°C after 10 days of incubation but could not grow at 60°C or higher. The transformant precultured at 70°C with leucine was transferred to medium without leucine and incubated at 60°C. After several days, the liquid culture was spread on plates without leucine and mutant strains were selected at 60°C. The isolated strain could grow at 60°C without leucine but not at 62°C. In this way *T. thermophilus* mutant strains growing at the temperature that was restrictive for the previous cycle were selected step by step. *T. thermophilus* mutant strains obtained in this study are summarized in Table 1.

Sequence analysis of the *LEU2* genes

The plasmid was recovered from the mutant strain selected at each temperature. The *LEU2* genes in the plasmids were subcloned in an *E. coli* cloning vector and the nucleotide sequences were analyzed. Table 1 shows the amino acid replacements deduced from the nucleotide sequence analyses. No mutation was found within the *LEU2* gene of the mutant selected at 50°C. It seemed that the mutation occurred within the chromosomal DNA instead of the plasmid DNA, because transformants with the plasmid recovered from the strain could not grow without leucine at 50°C. In the other mutant strains, mutations were found and accumulated within the *LEU2* gene with the increment of the selection temperature.

Thermal stability of mutant enzymes

Saccharomyces cerevisiae IPMDH was overproduced in *E. coli* cells and purified by a combination of anion-exchange and hydrophobic chromatography as described in Materials and methods. The specific activity of *S. cerevisiae* IPMDH in cell-free extract of the *E. coli* expression system (1.8 U/mg) was much higher than that of the *S. cerevisiae* expression system (0.36 U/mg). The purity (22 U/mg) was comparable to that reported previously (19 U/mg) (Hsu and Kohlhaw 1980; Kohlhaw 1988).

Thermal stability of the purified enzymes was estimated by measuring remaining activities at 30°C after heat treatment for 10 min at various temperatures ranging from 30° to 70°C. Thermal inactivation of the wild-type and mutant enzymes was irreversible under the conditions employed in the present study. Figure 1 shows that the half-inactivation temperatures, $T_{1/2}$, of the mutant enzymes selected at 60°, 62°, 65°, 67°, and 70°C (named as SCM60, -62, -65, -67, and

Table 1. Plasmids recovered from selected strains of *Thermus thermophilus* in this study

Plasmid	Selection temperature (°C)	Mutations found in *Saccharomyces cerevisiae* IPMDH[a]
pT8L2P	Wild type	
pT8L2P50	50	No mutation
pT8L2P60	60	E255V (GAA->GTA)
pT8L2P62	62	N43Y (AAT->TAT), E255V (GAA->GTA)
pT8L2P65	65	N43Y (AAT->TAT), A112V (GCA->GTA), E255V (GAA->GTA)
pT8L2P67	67	N43Y (AAT->TAT), N110K (AAC->AAG), A112V (GCA->GTA), E255V (GAA->GTA)
pT8L2P70	70	N43Y (AAT->TAT), A62T (GCG->ACG), N110K (AAC->AAG), A112V (GCA->GTA), E255V (GAA->GTA)

[a]Codons with mutation are shown in parentheses; the mutation that occurred additionally at each step is underlined

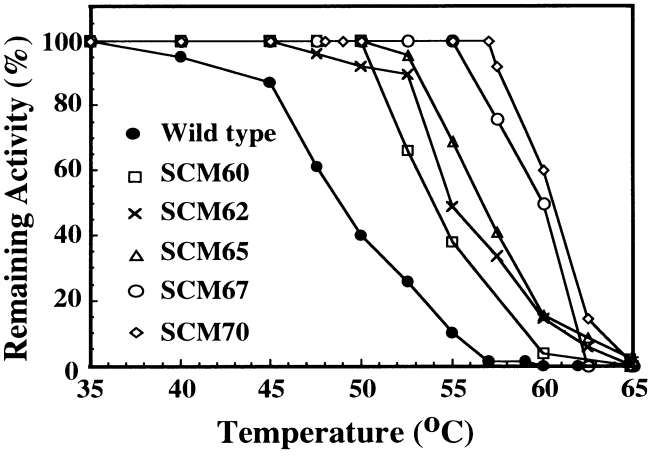

Fig. 1. Remaining activity of *Saccharomyces cerevisiae* IPMDHs after heat treatment. Each purified enzyme was diluted to 0.2 mg/ml with 30 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA and incubated at the indicated temperatures for 10 min. Remaining activity was measured at 30°C and is expressed as percentages of the original activity. Values are averages of three independent determinations

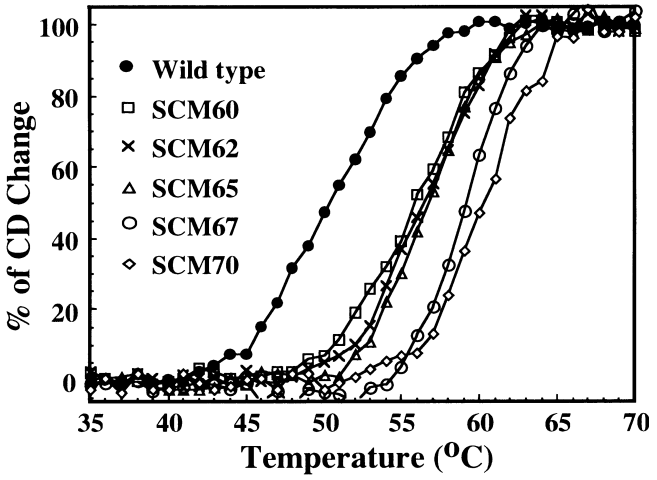

Fig. 2. Thermal melting profiles of *S. cerevisiae* IPMDHs at pH 7.6. Denaturation was monitored by measuring circular dichroism (*CD*) at 222 nm. The melting curves were normalized as described previously (Hayashi-Iwasaki et al. 1996)

-70, respectively) were improved from that of the wild-type enzyme by 5°, 6°, 7°, 11°, and 12°C, respectively.

Thermal denaturation processes of the wild-type and mutant enzymes were also analyzed by monitoring the change in ellipticity at 222 nm (Fig. 2). All the profiles showed a single-phase transition. The half-denaturation temperature of each enzyme (T_h) was similar to the corresponding $T_{1/2}$ estimated by the remaining activity measurement: T_h values were raised by 5.5°, 6°, 6.5°, 8.5°, and 10°C for SCM60, -62, -65, -67, and -70, respectively.

Enzymatic activity of mutant enzymes

Steady-state kinetic parameters of the wild-type and the mutant enzymes are listed in Table 2. Catalytic constants, k_{cat}, of the mutant enzymes were similar to that of the wild-type enzyme. The Michaelis constant, K_m, for D-3-isopropylmalate (IPM) of the SCM60 was twice of that of the wild-type enzyme. For the other mutant enzymes, K_m for IPM decreased as stability increased, and that of SCM67 and SCM70 was similar to that of the wild-type enzyme. K_m for NAD of all the mutant enzymes, especially that of SCM67 and SCM70, was higher than that of the wild-type enzyme. In spite of these differences, the catalytic efficiency of each mutant enzyme was of the same order as that of the wild-type enzyme.

Stability of the mutant enzymes without glycerol

It has been reported that glycerol is required for *S. cerevisiae* wild-type IPMDH to maintain the native structure and the catalytic activity. The enzyme is inactivated at pH 8.3 after dilution (Hsu and Kohlhaw 1980). It was confirmed that the enzymatic activity of the wild-type IPMDH was lowered to 40% when the enzyme was diluted with buffer without glycerol. In contrast, all the thermostabilized enzymes selected in this study retained their original activity completely after dilution without glycerol (data not shown).

Table 2. Kinetic constants of *Saccharomyces cerevisiae* IPMDH and its variants

Variant	K_m (μM)[a]		k_{cat} (s^{-1})[a, b]	k_{cat}/K_m (M^{-1} s^{-1})	
	D-3-IPM	NAD		D-3-IPM	NAD
Wild type	4.4 (±0.2)	230 (±13)	19.0 (±0.9)	4.8×10^6	8.2×10^4
SCM 60	10.1 (±0.5)	303 (±13)	23.0 (±0.2)	2.3×10^6	7.6×10^4
SCM 62	8.0 (±0.3)	375 (±13)	24.5 (±0.7)	3.1×10^6	6.5×10^4
SCM 65	7.4 (±0.6)	347 (±17)	25.3 (±0.9)	3.6×10^6	6.0×10^4
SCM 67	4.3 (±0.4)	714 (±20)	17.6 (±0.9)	4.4×10^6	2.4×10^4
SCM 70	5.9 (±0.5)	815 (±32)	22.3 (±0.9)	4.5×10^6	2.7×10^4

[a]Each value is the average of three independent determinations; SEM is shown in parentheses
[b]k_{cat} value is expressed as reaction per dimer

Discussion

In a previous work, we isolated stabilized enzymes of *B. subtilis* IPMDH. The stabilized mutant enzyme with three spontaneous mutations showed a $T_{1/2}$ 10°C higher than that of the wild-type enzyme (Akanuma et al. 1998). In this article, we isolated a mutant enzyme that has a $T_{1/2}$ at least 10°C higher than the wild-type *S. cerevisiae* IPMDH. *S. cerevisiae* IPMDH and *B. subtilis* IPMDH have 50% sequence identity. These enzymes could be stabilized to a similar extent by spontaneous mutations and selection in the thermophile. Extensive mutagenesis such as DNA shuffling (Stemmer 1994) may be necessary for further stabilization (Zhao and Arnold 1999). In fact, *B. subtilis* IPMDH has been further stabilized with error-prone PCR mutagenesis (Akanuma et al. 1999).

Three-dimensional structures of *T. thermophilus* IPMDH and several other mesophile IPMDHs have been reported (Imada et al. 1991, 1998; Wallon et al. 1997). IPMDH is composed of two identical subunits. Each subunit consists of two structural domains; one (domain 1) contains the N- and C-termini, and the other (domain 2) contains the subunit interface. Glu255 seems to be located in a hydrophobic region at the subunit–subunit interface. Strong hydrophobic interaction at the subunit–subunit interface of *T. thermophilus* IPMDH has been suggested from its crystal structure (Imada et al. 1991). Both *E. coli* IPMDH (Kirino et al. 1994) and *B. subtilis* IPMDH (Akanuma et al. 1999) were stabilized by substitution of the corresponding glutamate with leucine at the subunit interface. Similar substitution was found in *S. cerevisiae* IPMDH:

Table 3. Summary of mutations found in IPMDHs with the *Thermus thermophilus* host–vector system

Origin of IPMDH	Mutation	Corresponding residues in *T.t.*[a]	Secondary structure	Location	Possible mechanism	Reference
Saccharomyces cerevisiae	N43Y	V38	Beta	H. C.	H. I.	This work
	A62T	T57	Alpha	H. C.	H. B.	This work
	N110K	K107	Beta	D. I.	I. B.	This work
	A112V	F109	Beta	D. I.	H. I.	This work
	E255V	L246	Alpha	S. I.	H. I.	This work
Bacillus subtilis	T22K	L20	Alpha	M. S.	I. B. or S. H.	b
	I95L	L 93	Alpha	H. C.	R. S.	c
	M256V	V249	Alpha	S. I.	H. I.	b
	M292I	I 284	Loop	M. S.	H. I.	c
	T308I	H 300	Alpha	D. I.	H. I.	c
Chimera	I93L	L 93	Alpha	H. C.	R. S.	d, e
	A172V	A 172	Alpha	D. I.	H. I.	f

Beta, β-sheet; Alpha, α-helix; H. C., hydrophobic core; D. I., domain interface; S. I., subunit interface; M. S., molecular surface; H. I., hydrophobic interaction; H. B., hydrogen bonding; I. B., ionic bridge; S. H., stabilization of alpha-helix; R. S., removal of steric stress; *T.t.*, *Thermus thermophilus*
[a]Corresponding residues in *Thermus thermophilus*
[b]Akanuma et al. 1999
[c]Akanuma et al. 1998
[d]Tamakoshi et al. 1995
[e]Sakurai et al. 1995
[f]Kotsuka et al. 1996

Glu255 was replaced with a hydrophobic amino acid, valine, and the stability of the mutant increased to a large extent. The mutant is stable in the absence of glycerol. The wild-type IPMDH is rapidly inactivated without glycerol, and glycerol is expected to maintain the dimer structure (Hsu and Kohlhaw 1980). Priev et al. have suggested that glycerol decreases the specific volume and compressibility of the protein interior, which leads to enhancement of protein stability (Priev et al. 1996). Our result suggests that the glycerol requirement is related to the subunit–subunit interaction.

Some of the other mutations are expected to be located near the surface or domain interface. Asn110-Lys and Ala112-Val are located between two domains, probably near the hinge region. The replacement of Asn110 with lysine might lead to formation of a salt bridge. The corresponding residue Lys107 forms a salt bridge with Asp127 in *T. thermophilus* IPMDH. The counterpart of Asp127 is conserved in *S. cerevisiae* IPMDH. Ala112 in *S. cerevisiae* IPMDH corresponds to Phe109 in *T. thermophilus* IPMDH. It seems that hydrophobic interaction in this region may be important for stability.

Asn43Tyr and Ala62Thr are located within domain 1. Val38 in *T. thermophilus* IPMDH (Asn43 in *S. cerevisiae* IPMDH) is in a hydrophobic region composed of Val5, Pro7, and Tyr36. Because the corresponding amino acids in *S. cerevisiae* IPMDH are all hydrophobic (Val9, Pro11, and Phe41), the replacement of Asn43 with tyrosine would result in hydrophobic interaction. Ala62 in *S. cerevisiae* IPMDH corresponds to Thr57 in *T. thermophilus* IPMDH. The hydroxyl group of the threonine interacts with carbonyl oxygen of Pro40 through hydrogen bonding in *T. thermophilus* IPMDH. The same interaction may be formed in the mutation Ala62Thr. Asn43 and Ala62 are probably very close to each other in the tertiary structure, suggesting that this region also may be important for stability. No mutation in this region was found in the stabilized mutants of *B. subtilis* IPMDH or other IPMDHs.

Table 3 summarizes mutations found in IPMDHs by selection after random mutagenesis so far observed. Although mutations are found scattered in many regions of the molecule when mutations are classified as in the fifth column of Table 3, mutations found in the interfaces of domains and subunits dominated about half the mutations, suggesting the importance of interactions between structural units of a protein for the conformational stability. *T. thermophilus* IPMDH has about 40% alpha helices and 22% beta strands. The numbers of mutations found in alpha helices and in beta strands have a similar ratio to the content of the secondary structures in the IPMDH. Hydrophobic interaction seems to be the dominant mechanism found in stabilized IPMDHs.

k_{cat}/K_m values of mutant enzymes were of the same orders of magnitude as those of the wild-type *B. subtilis* IPMDH. However, a slight reverse correlation between activity and stability was seen in some mutant enzymes. A relatively large increase of stability was found in SCM60 and SCM67 compared with the mutants of the previous step, which was accompanied by decreased catalytic efficiency derived from increase of K_m for IPM and for NAD, respectively. Such correlation was not seen in the experiment with *B. subtilis* IPMDH (Akanuma et al. 1998). The difference may be related to the selection systems used. The stabilized *B. subtilis* IPMDHs were selected with an integration vector system in which one copy of the gene encoding IPMDH was integrated into the genome, whereas the mutant enzymes in this study were selected with a multicopy-plasmid-vector system. The transformant expressing

E. coli IPMDH with the plasmid vector system could grow at a higher temperature (60°C; Tamakoshi et al. 1999) than the transformant with the integration vector system (53°C) without leucine (Tamakoshi et al. 1998). The two stabilized enzymes in the present work might have been selected at the expense of their activity, which is possible because of the high-level expression of IPMDH with the plasmid vector system.

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