Asp578 in LEU4p Is One of the Key Residues for Leucine Feedback Inhibition Release in Sake Yeast

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We identified a new mutation, Asp578Tyr, in α-isopropylmalate synthase (a LEU4 gene product) that releases leucine feedback inhibition and causes hyperproduction of isoamyl alcohol (i-AmOH) in sake yeast. Spontaneous sake yeast mutants that express resistance to 5,5,5-trifluoro-DL-leucine (TFL) were isolated, and a mutant strain, TFL20, was characterized at the genetic and biochemical levels. An enzyme assay for α-isopropylmalate synthase showed that strain TFL20 was released from feedback inhibition by L-leucine. Furthermore, DNA sequencing of the LEU4 gene for a haploid of the mutant TFL20 revealed that aspartic acid in position 578 changes to tyrosine. A comparison of the three-dimensional structures of wild-type LEU4p and mutant LEU4D578Yp by the homology modeling method showed that Asp578 is important for leucine feedback inhibition. We conclude that the mutation from Asp to Tyr in 578 is a novel change causing release from leucine feedback inhibition.

Key words: Saccharomyces cerevisiae; 5,5,5-trifluoroor- DL-leucine; α-isopropyl malate synthase; leucine feedback inhibition

Dominant resistance to the leucine analog 5,5,5-trifluoroor-DL-leucine (TFL) in yeast is associated with a mutation that causes α-isopropylmalate (α-IPM) synthase (EC 4.1.3.12), the first enzyme in the leucine biosynthesis pathway, to become resistant to feedback inhibition by L-leucine. Baichwal et al. 3 showed that TFL resistance is due to a mutation in the LEU4 gene, and that the mutant allele conferring resistance to TFL, LEU4Δ578, cosegregates with the resistance of α-IPM synthase to leucine feedback inhibition. Isoamyl acetate (i-AmOAc), a major ginjo-flavor component, plays a significant role in determining the quality of sake (Japanese rice wine). The formation of this ester depends highly on the leucine biosynthetic pathway in the yeast S. cerevisiae. Isoamyl alcohol (i-AmOH), the alcoholic part of i-AmOAc, is converted from α-ketoisocaproate through two reduction steps, the reductions of α-ketoisocaproate and the resulting isovaleraldehyde by aldehyde dehydrogenase and alcohol dehydrogenase respectively. Ashida et al. 4 reported that a TFL-resistant mutant overproduced i-AmOH and i-AmOAc. The LEU4 genes of seven spontaneous mutants resistant to TFL have been characterized at the molecular level by Cavalieri et al. 5 Their DNA sequences showed that one mutation is a codon deletion localized close to the 3′ end of the LEU4 ORF, and the other mutations involve four transitions and two transversions. The seven mutations defined the R-region, which is involved both in leucine feedback inhibition and in Zn2+-mediated inactivation by CoA. 5 Recently, the crystal structure of α-IPM synthase (a leuA gene product) from Mycobacterium tuberculosis was determined by multilength wavelength anomalous dispersion methods, 6 and the atomic coordinates and structure factors have been deposited in the Protein Data Bank (pdb code: 1sr9). Moreover, the co-crystal structure of α-IPM synthase with leucine reveals a binding between the three-dimensional structures of the R-region and leucine.

In this paper, we show the mechanism by which the mutation from Asp to Tyr in 578 causes release from leucine feedback inhibition by the homology modeling method with the three-dimensional structures of the wild-type LEU4p and mutant LEU4Δ578Yp.

Materials and Methods

Strain and media. S. cerevisiae K30 was isolated
from sake mash using an industrial diploid yeast, Kyokai No. 9 (K-9). The TFL medium was M medium (0.67% yeast nitrogen base without amino acids [Difco, U.S.A.] and 2% glucose) containing 400 ppm of TFL. The YM medium was 0.3% yeast extract, 0.5% malt extract, 1% polypeptone, and 1% glucose; the YM-5 medium had the same composition as the YM medium except that glucose was at 5%. The sporulation medium was 0.4% sodium acetate and 2% agar.

Isolation of TFL resistant mutant. K30 was cultured overnight, inoculated into TFL medium at 5 x 10^2 cells/plate, and cultivated at 30°C for 5 d.

Production of flavor. Wild-type and mutant strains were cultured in YM-5 liquid medium for 3 d at 30°C, and the production of flavor was analyzed by headspace gas chromatography.

Measurement of α-IPM synthase activity. α-IPM synthase activity was assayed according to the method of Ulm et al.7)

DNA sequencing. DNA sequencing was performed by the method of Sanger et al.8) using an ABI Prism™ DYE Terminator Cycle Sequencing Ready Reaction Kit and a DNA Sequencer 373 (Applied Biosystems, U.S.A.). DNA sequencing of the wild-type strain, TFL-resistant mutant (diploid), and haploid of the TFL-resistant mutant was performed on the full-length LEU4 gene, which was obtained by amplification via PCR using the primers FOR (5'-CTG TAGACTTTCCTACTAAGAAAC-3') for upstream and REV (5'-GAAGCGGAATAAGTCCTGAATACAGAGG-3') for downstream. PCR reactions were carried out in a 100 µl volume containing 200 ng of template, with each primer at 0.5 mM and each dNTP at 200 mM, 2 mM MgCl₂, 1 U of Vent DNA polymerase (New England Biolabs, U.S.A.) and 10 µl of 10 x Thermo Pol buffer. Amplifications were performed using a Perkin Elmer PI2000 Thermocycler and the following program: 3 min at 95°C for one cycle, 1 min at 95°C, then 1 min at 55°C and 2 min at 72°C for 30 cycles. PCR products for sequencing were electrophoresed on 1.2% agarose gels and purified with the Geneclean II kit (BIO 101, U.S.A.). Analysis of nucleotide and protein sequences were done with DNASIS-Mac™ ver. 3.6 (Hitachi Software Engineering).

Isolation of haploid from TFL20. Using the Fowell method,9) we cultured K30 on YM medium overnight, collected the overnight culture, washed it three times with sterile distilled water, and sporulated it by cultivating it in sporulation medium at 30°C for 3 d. By random spore plating,9) we isolated haploids from TFL20 and determined the mating type of these by mass mating to NBRC10175 (MATa) and NBRC10176 (MATα).

### Results and Discussion

Twenty-four colonies of TFL-resistant mutants were isolated. All the selected strains produced much higher concentrations of i-AmOH than the wild-type strain, which produced 58.0 ppm of i-AmOH (Table 1). Although the wild-type strain K30 was isolated from sake mash using an industrial diploid yeast, K-9, the two strains have equal brewery properties, such as alcohol, flavor, and organic acid. Strain TFL20 was used for the subsequent experiment because it produced the highest concentrations of i-AmOH, 115.9 ppm, among the mutants obtained.

<table>
<thead>
<tr>
<th>Strain</th>
<th>i-BuOH (ppm)</th>
<th>i-AmOH (ppm)</th>
<th>i-AmOAc (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K30 (Wt)</td>
<td>16.9</td>
<td>58.0</td>
<td>0.09</td>
</tr>
<tr>
<td>TFL2</td>
<td>12.8</td>
<td>105.7</td>
<td>0.17</td>
</tr>
<tr>
<td>TFL6</td>
<td>12.7</td>
<td>106.1</td>
<td>0.24</td>
</tr>
<tr>
<td>TFL15</td>
<td>12.1</td>
<td>115.6</td>
<td>0.20</td>
</tr>
<tr>
<td>TFL18</td>
<td>13.1</td>
<td>107.4</td>
<td>0.21</td>
</tr>
<tr>
<td>TFL20</td>
<td>13.3</td>
<td>115.9</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Wt, wild-type
Five strains that produced much i-AmOH among the 24 strains are represented.

TFL resistance. The strain tested was inoculated into TFL medium and cultivated at 30°C for 5 d.

Homology modeling. Three-dimensional structures of wild-type LEU4p (referring to pdb code: 1sr9) and mutant LEU4p were constructed by comparative modeling method using SWISS-MODEL (http://swissmodel.expasy.org).

### Table 1. Production of Higher Alcohols and Ester in Mutant Strains
mediated inactivation by CoA. We conclude that the TFL-resistance of mutant TFL20 is due to its release from the feedback inhibition by L-leucine of \( \text{C}11 \)-IPM synthase.

To determine the nature of the mutation in mutant strain TFL20, PCR amplification and sequencing was performed as described in “Materials and Methods”. The result of direct PCR DNA sequencing of the LEU4 gene in K30 and TFL20 shows that the sequence of the LEU4 gene in K30 is identical to that of the laboratory strain (DNA sequence data from SGD), whereas the sequence of the LEU4 gene in TFL20 is a mixture of G and T at 1732. Therefore, we assume that the LEU4 gene in TFL20 is heteroallelic. We isolated two haploids from TFL20, TFL-H-2(\( \text{MAT}/C11 \)) and TFL-H-73(\( \text{MATa} \)). Direct PCR DNA sequencing of the LEU4 gene in TFL-H-2 and TFL-H-73 showed that the LEU4 gene in TFL-H-2 is a mutant type due to the T at 1732, and in TFL-H-73 it is a wild type due to the G at 1732. This result shows that TFL-H-2 has only one point mutation, which changed G to T at 1732 and caused alteration of a polar residue Asp578 to an aromatic tyrosine in the LEU4 gene. Table 2 summarizes i-AmOH production and growth on a TFL plate for base at 1732, amino acid at 578. Since TFL-H-2 exhibits TFL resistance and produces higher concentrations of i-AmOH than TFL-H-73, we conclude that the substitution of Asp578 for tyrosine is responsible for the TFL resistance and hyperproduction of i-AmOH.

Recently, the X-ray crystal structure of \( \alpha \)-IPM synthase (a leuA gene product) from Mycobacterium tuberculosis was determined (pdb code: 1sr9). Koon et al.\(^6\) found that Gly514, Gly516, and Ser519 in \( \text{C}11 \)-IPM synthase of \( \text{S. cerevisiae} \) correspond to Gly531, Gly533, and Ala536 in \( \text{M. tuberculosis} \), which are part of the leucine binding site, by cocrystallization of \( \text{C}11 \)-IPM synthase with leucine. Ser547 and Ala552 in LEU4p in \( \text{S. cerevisiae} \), which are related to release from leucine feedback inhibition, are located in the leucine binding pocket. The identity between the amino acid sequences of LEU4p in \( \text{S. cerevisiae} \) and \( \text{C}11 \)-IPM synthase in \( \text{M. tuberculosis} \) was about 45%. We then constructed the wild-type and mutant LEU4p by homology modeling using the X-ray structure of \( \text{C}11 \)-IPM synthase (pdb code: 1sr9) as a template. When the residues related to the leucine binding site in wild-type and mutant LEU4p were estimated by alignment, Ile518, Ser519, Val535, Tyr538, Ser542, Thr549, and Ala551 in \( \text{S. cerevisiae} \) corresponded to Leu535, Ala536, Val551, Tyr554, Ala558, Ala565, and Ala567 in \( \text{M. tuberculosis} \), which were defined as the leucine binding sites in 1sr9, respectively. Figure 2 shows the R-regions (colored green) including the leucine binding site in wild-type LEU4p and mutant LEU4D578Yp. Asp578 in wild-type LEU4p and Tyr578 in mutant LEU4D578Yp were located near the leucine binding site and close to the five mutated residues according to a report of Cavalieri et al.\(^5\) Asp578 and Tyr578 are oriented toward the

Table 2. Characteristics of Wild-Type, TFL, Mutant, and Haploid from TFL20

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ploidy</th>
<th>Base at 1732</th>
<th>Amino acid at 578</th>
<th>i-AmOH (ppm)</th>
<th>Growth on TFL plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>K30</td>
<td>diploid</td>
<td>G</td>
<td>Asp</td>
<td>107.1(1.0)</td>
<td>–</td>
</tr>
<tr>
<td>TFL20</td>
<td>diploid</td>
<td>G/T</td>
<td>Asp/Tyr</td>
<td>161.0(1.5)</td>
<td>++</td>
</tr>
<tr>
<td>TFL-H-2</td>
<td>haploid</td>
<td>T</td>
<td>Tyr</td>
<td>72.2(1.6)</td>
<td>+</td>
</tr>
<tr>
<td>TFL-H-73</td>
<td>haploid</td>
<td>G</td>
<td>Asp</td>
<td>46.3(1.0)</td>
<td>–</td>
</tr>
</tbody>
</table>

Methods of DNA sequencing, sensitivity for TFL, and i-AmOH productivity are described in “Materials and Methods”.

Fig. 1. Determination of \( \alpha \)-IPM Synthase Sensitivity to Leucine Feedback Inhibition and to Zn\(^{2+}\)-CoA Inactivation in \( \text{S. cerevisiae} \) Wild-Type Strain K30 and Mutant Strain TFL20.

A, \( \alpha \)-IPM synthase activity in the absence or presence of L-leucine. B, \( \alpha \)-IPM synthase activity in the absence or presence of CoA. The \( \alpha \)-IPM synthase activity in the control is taken as 100%.

Table 2. Characteristics of Wild-Type, TFL, Mutant, and Haploid from TFL20
leucine binding site in the wild-type LEU4p and mutant LEU4D578Yp. To investigate the effect of the mutation on the leucine binding site, we calculated the distance between residues 578 (Asp578 Oᵣ and Tyr578 Oᵣ) and the residues related to the leucine binding site in wild-type LEU4p and mutant LEU4D578Yp (Ile518 Cᵣ, Cᵢ, Ser519 Oᵣ, Val535 Cᵣ, Cᵢ, Tyr538 Oᵣ, Ser542 Oᵣ, Thr549 Cᵣ, Oᵣ, and Ala551 Cᵢ). The distances between Tyr578 and Ile518, Ser519, Val535, and Tyr538 in mutant LEU4D578Yp were shorter than those in wild-type LEU4p, but the distances between Tyr578 and Ser542, Thr549, and Ala551 were longer than those in wild-type LEU4p. The size difference of the side-chain between Tyr578 and Asp578 might cause the pocket to become narrow in mutant LEU4D578Yp. According to the steric constraint caused by the mutation from Asp to Tyr in 578, we conclude that leucine is inhibited from binding to the leucine binding site in mutant LEU4D578Yp.

References