Effect of L-Proline on Sake Brewing and Ethanol Stress in Saccharomyces cerevisiae

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During the fermentation of sake, cells of Saccharomyces cerevisiae are exposed to high concentrations of ethanol, thereby damaging the cell membrane and functional proteins. L-Proline protects yeast cells from damage caused by freezing or oxidative stress. In this study, we evaluated the role of intracellular L-proline in cells of S. cerevisiae grown under ethanol stress. An L-proline-accumulating laboratory strain carries a mutant allele of PRO1, pro1D154N, which encodes the Asp154Asn mutant γ-glutamyl kinase. This mutation increases the activity of γ-glutamyl kinase and γ-glutamyl phosphate reductase, which catalyze the first two steps of L-proline synthesis and which together may form a complex in vivo. When cultured in liquid medium in the presence of 9% and 18% ethanol under static conditions, the cell viability of the L-proline-accumulating laboratory strain is greater than the cell viability of the parent strain. This result suggests that intracellular accumulation of L-proline may confer tolerance to ethanol stress. We constructed a novel sake yeast strain by disrupting the PUT1 gene, which is required for L-proline utilization, and replacing the wild-type PRO1 allele with the pro1D154N allele. The resultant strain accumulated L-proline and was more tolerant to ethanol stress than was the control strain. We used the strain that could accumulate L-proline to brew sake containing five times more L-proline than what is found in sake brewed with the control strain, without affecting the fermentation profiles.

Sake is a traditional Japanese alcoholic beverage made from steamed rice by multiple parallel fermentations of the fungus Aspergillus oryzae and the yeast Saccharomyces cerevisiae, which produce saccharification enzymes and ethanol from glucose, respectively. During sake fermentation, yeast cells are exposed to various stresses under anaerobic conditions, including high concentrations of ethanol (−20% [vol/vol]) and low temperature (−15°C). Ethanol is toxic. It damages the cell membrane and functional proteins (22), gradually reducing cell viability and leading to cell death during fermentation. Therefore, the use of ethanol-resistant yeast strains should make it possible to reduce the fermentation time.

Amino acids and organic acids produced by yeast during fermentation influence the taste of sake. The effects of organic acids such as malic, fumaric, and succinic acids have been evaluated by disrupting the genes encoding the appropriate metabolic enzymes in sake yeast strains (2, 21, 23). Little attention has been paid to the effects of amino acids during sake fermentation. By breeding yeasts with various amino acid composition profiles, it may be possible to expand the diversity of sake tastes.

L-Proline is an osmoprotectant (10, 12) and a sweet amino acid that helps protect yeast cells from damage by freezing, desiccation, or oxidative stress (26, 27, 36–38). L-Proline enhances the stability of proteins and membranes in environments with low water activity or high temperature (30) and inhibits aggregation during protein refolding (32). These observations suggest that intracellular L-proline could play a crucial role in reducing ethanol stress by preventing protein denaturation and membrane disorder during sake fermentation. S. cerevisiae synthesizes L-proline from glutamate via a pathway consisting of three enzymes: γ-glutamyl kinase (γ-GK) (the PRO1 gene product), γ-glutamyl phosphate reductase (the PRO2 gene product), and Δ1-pyrroline-5-carboxylate (P5C) reductase (the PRO3 gene product) (Fig. 1) (6, 39). L-Proline is converted to L-glutamate within mitochondria in two steps by the enzymes proline oxidase (the PUT1 gene product) and P5C dehydrogenase (the PUT2 gene product) (Fig. 1) (5, 40). We previously isolated a mutant of S. cerevisiae that was resistant to the L-proline analog 1-azetidine-2-carboxylic acid (AZC), accumulated L-proline, and tolerated freezing (36). This mutant has a single mutation in PRO1 that results in an Asp154Asn amino acid substitution in γ-GK and increased activity of both γ-GK and γ-glutamyl phosphate reductase, which may be part of a single protein complex in vivo (27, 38).

Our objectives in this study were (i) to determine if L-proline reduces ethanol stress in yeast cells and (ii) to determine if cells that accumulate L-proline alter the sake fermentation process or product. We report here the protective effect of L-proline on ethanol stress in yeast cells. In addition, to test the hypothesis that the improved ethanol tolerance is due to L-proline accumulation, we constructed the L-proline-accumulating sake yeast and analyzed its fermentation profiles during sake brewing.

MATERIALS AND METHODS

Strains and plasmids. The S. cerevisiae strains used in this study are listed in Table 1. Escherichia coli strain DH5α [F− λ− deoR recA1 endA1 hsdR17(rK− mK−) supE44 thi-1 gvrA96 relA1] and the plasmid

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and oligonucleotide primers based on the available nucleotide sequences. The forward primer was 5'-GAG GAT CGG AAC ACA AAC TCC A-A'-s, and the reverse primer was 5'-GCG GTA CCC CAA AAT CCT TAC A'-s (the underlined sequences indicate the positions of the BamHI and KpnI restriction sites, respectively). A unique amplified band of 1.9 kb was digested with BamHI and KpnI and then ligated into the BamHI and KpnI sites of pBluescript II SK(+) to construct pBlue-PUT1. Plasmid pBlueDput1-TRP1 was constructed by deleting the 0.9-kb Ball-Aad fragment in PUT1 from pBlue-PUT1 and inserting the 2.6-kb SacI-NcoI fragment containing TRP1 of plasmid pRS414 by blunt-end ligation. The 3.6-kb BamHI-KpnI fragment containing put1::TRP1 of pBlueDput1-TRP1 was integrated into the PUT1 locus in strain XUW-14 to construct strain XDput1 by transformation. The Trp⁺ phenotype was selected, and the correct disruption was verified by chromosomal PCR analysis. To remove the influence of tryptophan auxotrophy, pRS404 was cut with MfeI in TRP1, and the linearized plasmid was introduced to integrate TRP1 into the control strain (XUW-TRP).

Assay of proline oxidase activity. Proline oxidase (EC 1.4.3.2) activity was assayed by monitoring P5C-aminobenzaldehyde as previously described (7, 26). The enzyme is very unstable, so whole-cell extracts were prepared as previously described (7). Yeast cells were grown in 50 ml of SD medium or SD medium plus l-proline at 30°C for 48 h with shaking, collected on 0.8-μm nitrocellulose filter (Whatman, Clifton, N.J.), and immediately immersed in liquid N2 for 10 s. Each filter was then placed in a small test tube containing 0.5 ml of 0.1 M HEPES buffer (pH 7.5) with 3 mM MgCl₂ and kept on ice. Each tube was vortexed vigorously to transfer the cells from the filter to the buffer. A 0.4-ml portion of 10% l-proline was added to each tube and incubated without shaking at 30°C for 15 min. One hundred microliters of o-aminobenzaldehyde (6 mg/ml in 20% ethanol: Sigma Chemical) was added, followed by 0.5 ml of 10% trichloroacetic acid to stop the reaction. The tube was mixed, and the color was allowed to develop for 30 min. The A₅₃₀ was recorded against a blank identical to the one described above but lacking l-proline. The millimolar extinction coefficient of the P5C-aminobenzaldehyde complex was 2.71. One unit of activity was defined as the amount of enzyme required to produce 1 nmol of P5C per min. Protein concentrations were determined by using a Bio-Rad protein assay kit (Hercules, Calif.) with bovine serum albumin as the standard (4).

Replacement of the wild-type PRO1 gene with the proD154N gene. A two-step method was used to replace the wild-type PRO1 sequence with proD154N at the native chromosomal location. The 1.8-kb HindIII-SacI fragment from pRS-D154NPRO1 was ligated into the large fragment of pRS406 digested with HindIII and SacI to yield pRS406-D154NPRO1. Plasmid pRS406-D154NPRO1 was cut with XbaI in the proD154N allele, and the linearized DNA was integrated into the PRO1 locus of strain XDput1 by transformation. The Ura⁻ phenotype was selected as a single-crossover transformant that duplicates the PRO1 locus (one copy is the wild type and the other is the mutant) with plasmid sequences in between. The transformant was cultured in 1 ml of YPD medium at 30°C for 24 h with shaking to obtain 5-fluoroorotic acid-resistant strains that have excised the plasmid and lost one of the two copies of the duplicated region by homologous crossover. Depending on the location of the crossover, the PRO1 allele that remains may be either the mutant or the wild type. The proD154N sak yeast strain XUDput1-MT was selected as an AZC-resistant phenotype due to the overproduction of l-proline (27, 38). The construct was checked by direct sequencing of PCR products amplified from the chromosomal DNA.

Intracellular contents of l-proline. For the determination of intracellular l-proline, yeast cells were grown to the stationary phase in 10 ml of SD medium, SD medium plus l-proline, or YPD medium at 30°C for 48 h under either shaking (120 rpm) or static conditions. Five milliliters of cell suspension was removed, and the cells were washed twice with 0.9% NaCl and suspended in 0.5 ml of distilled water. The 1.5-ml microcentrifuge tube containing cells was transferred to a boiling-water bath, and intracellular amino acids were extracted by boiling for 10 min. After centrifugation (15,000 × g, 5 min, 4°C), each supernatant was analyzed quantitatively with an amino acid analyzer (model L-8500; Hitachi, Tokyo, Japan).

vector pBluescript II SK(+) (Toyobo Biochemicals, Osaka, Japan) were used to subclone the PUT1 gene. Plasmid pRS414 (Stratagene, La Jolla, Calif.), which carries the TRP1 gene, was used to disrupt the PUT1 gene. Plasmid pRS404 (Stratagene), which contains the TRP1 gene, was used for the integration of the TRP1 gene into strain XUW-14. Two yeast plasmids, pRS-D154NPRO1, carrying the proD154N allele of PRO1 (27), and pRS406 (Stratagene), carrying the URA3 gene, were used to integrate the proD154N allele into strain XDput1. Yeast episomal plasmids pUV2, carrying the URA3 gene, and pTV3, carrying the TRP1 gene, were used to complement the auxotrophic marker (26).

Culture media. The medium used for growth of S. cerevisiae was SD medium (20 g/liter glucose, 6.7 g/liter Bacto yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.] and YPD medium (20 g/liter glucose, 10 g/liter Bacto extract, 20 g/liter Bacto peptone). The SD medium contains 1 g/liter ammonium sulfate as the nitrogen source. When the put1 mutant was grown, 1 g/liter monosodium l-glutamate or l-proline was used instead of ammonium sulfate as the sole source of nitrogen. Required supplements were added to the media for auxotrophic strains as necessary. Yeast strains were also cultured on SD agar plates with 100 μg/ml of the l-proline analogue AZC (Sigma Chemical, St. Louis, Mo.) or with 1 mg/ml of 5-fluoroorotic acid (35). E. coli strains were grown in Luria-Bertani medium (31) supplemented with 50 μg/ml of the l-proline-accumulating sake yeast strain XUDput1-MT was selected as an AZC-resistant phenotype due to the overproduction of l-proline (27, 38). The construct was checked by direct sequencing of PCR products amplified from the chromosomal DNA.

Intracellular contents of l-proline. For the determination of intracellular l-proline, yeast cells were grown to the stationary phase in 10 ml of SD medium, SD medium plus l-proline, or YPD medium at 30°C for 48 h under either shaking (120 rpm) or static conditions. Five milliliters of cell suspension was removed, and the cells were washed twice with 0.9% NaCl and suspended in 0.5 ml of distilled water. The 1.5-ml microcentrifuge tube containing cells was transferred to a boiling-water bath, and intracellular amino acids were extracted by boiling for 10 min. After centrifugation (15,000 × g, 5 min, 4°C), each supernatant was analyzed quantitatively with an amino acid analyzer (model L-8500; Hitachi, Tokyo, Japan).

TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Background and/or description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB329-17C</td>
<td>α trp1 ura3-52 put1-54</td>
<td>S288C and 21278b; put1 mutant</td>
</tr>
<tr>
<td>FHS15</td>
<td>α trp1 ura3-52 put1-54 proD154N</td>
<td>MB329-17C; pro1 mutant; the l-proline-accumulating laboratory strain</td>
</tr>
<tr>
<td>XUW-14</td>
<td>α trp1 put1-54</td>
<td>Sak yeast strain Kyokai no. 14 (K-14)</td>
</tr>
<tr>
<td>XUW-TRP</td>
<td>α trp1 put1-54</td>
<td>XUW-14; TRP1 revertant; the control sake yeast strain</td>
</tr>
<tr>
<td>XUDput1</td>
<td>α trp1 put1-54</td>
<td>XUW-14; put1 disruptant</td>
</tr>
<tr>
<td>XUDput1-MT</td>
<td>α trp1 put1-54</td>
<td>XUDput1; pro1 mutant; the l-proline-accumulating sake yeast strain</td>
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</table>
Sake brewing. Laboratory-scale sake was brewed (20) with a sake mash consisting of 160 g of steamed rice (α-rice), 40 g of koji rice (a culture of A. oryzae on steamed rice), and 260 ml of water added in three steps (at 4, 6, and 7 days). One-third of the amount specified was added each time. Each strain was grown in YPD medium at 30°C for 2 days under static conditions and inoculated into the mash to yield 1 × 10⁷ cells per g of mash. Fermentation profiles were monitored by weight loss in conjunction with CO₂ evolution. When about 65 g of mass had been lost (after ~24 days of fermentation at 15°C), the sake mash was centrifuged (175 × g, 10 min, 4°C), and the supernatant was obtained as sake. Yeast cells in sake mash were isolated by centrifugation (175 × g, 10 min, 4°C) (25), and the number of viable cells and their intracellular L-proline content were determined.

General components of the sake, such as ethanol, glucose, amino acids, organic acids, and aroma compounds, were analyzed by the standard method established by the Japanese National Tax Administration Agency (8). The ethanol concentration in the sake was measured with a gas chromatograph (model GC-15A; Shimadzu, Kyoto, Japan). The amino acid and organic acid compositions were analyzed with an amino acid analyzer and a high-performance liquid chromatograph (model LC-6A; Shimadzu) equipped with a conductivity detector and an SCR-10H2 column (Shimadzu), respectively. The glucose concentration was determined using a Glucose CII-Test (Wako Pure Chemical Industries, Osaka, Japan). In general, the sake meter indicates the apparent specific gravity of sake and is basically a Baume à meter, which works on the principle that alcohol is lighter than water while glucose is heavier. The lower the sake meter value, the heavier the gravity (20). Water is given a value of 0 at 15°C and a Baume value of 1 corresponding to a sake meter value of ~10.

Nucleotide sequence accession numbers. The GenBank accession numbers for the PRO1 and PUT1 genes are M85293 and M18107, respectively.

RESULTS

L-Proline accumulation and tolerance to ethanol stress. We compared the number of viable cells (Fig. 2) and the intracellular L-proline content (Fig. 3) of laboratory strains in the presence of 9% or 18% ethanol. When pregrown in liquid YPD medium with shaking (120 rpm), the mutant strain FH515 accumulated higher levels of l-proline (3.0% of the dry weight) than did the parent strain, MB329-17C (1.3%). A significant amount of L-proline was detected intracellularly even in strain MB329-17C, possibly due to the uptake of L-proline derived from YPD medium. The growth pattern in the absence of ethanol was virtually the same in both strains. However, the number of viable cells at 2, 5, and 8 days after cultivation in SD medium containing ethanol gradually decreased. The ethanol tolerance of strain FH515 was much higher than that of strain MB329-17C (Fig. 2), even though a significant decrease in L-proline content occurred during growth (Fig. 3).

We also evaluated the intracellular content of trehalose (Fig. 4) and total amino acid content (Table 2) of both strains during cultivation. Trehalose content increased transiently in response to ethanol stress, as previously reported (1). Total amino acid content, except for L-proline, decreased in the presence of ethanol. However, no difference in the contents of the two laboratory strains was detected. These results are consistent with the hypothesis that the accumulation of high levels of L-proline helps protect yeast cells against ethanol stress.

Construction of an L-proline-accumulating sake yeast. We constructed a novel sake strain that accumulates L-proline and is disrupted in put1. The put1-disrupted strain XUDput1-MT grew on SD medium plus L-glutamate but not on SD medium plus L-proline. The put1 disruptant also had no detectable proline.
oxidase activity in cell extracts, although such activity was readily detected when wild-type strain XUW-TRP was grown in a similar manner (0.82 ± 0.10 U/mg of protein). Both the wild-type strain and the put1 disruptant contain trace amounts of intracellular L-proline after cultivation in liquid SD medium with shaking. When strain XUDput1 was grown in SD medium plus L-proline, L-proline accumulated (0.72% ± 0.10% of dry weight), as expected from previous studies (37).

If the wild-type PRO1 allele was replaced with the pro1D154N allele, AZC resistance resulted. Overproduction of L-proline dilutes AZC, which competes with L-proline for incorporation into proteins (13, 27, 36). The PRO1 allele in strain XUDput1-MT has a single base change from G to A at position 460 that results in the replacement of aspartate with asparagine at position 154 in the γ-GK enzyme for pro1D154N.

Ethanol resistance of the L-proline-accumulating sake yeast. When strain XUDput1-MT was cultured in liquid SD medium under shaking conditions (120 rpm), it accumulated L-proline (0.59% ± 0.12% of dry weight), but strain XUW-TRP did not. When grown in liquid YPD medium under shaking conditions, there was three times as much intracellular L-proline in cells of strain XUDput1-MT (3.38% ± 0.57%) as there was in cells of strain XUW-TRP (1.03% ± 0.15%).

Strain XUDput1-MT was also more tolerant to ethanol (Fig. 2) than was strain XUW-TRP. The growth of neither strain was inhibited for the first 5 days in the presence of 9% ethanol. We also determined the intracellular contents of each strain during cultivation. There was no significant difference between the two sake strains in the intracellular content of trehalose (Fig. 4) or total amino acids (Table 2). These results are consistent with the hypothesis that the improved ethanol tolerance is due to L-proline accumulation by the sake strains.

Sake brewing with the L-proline-accumulating strain. Under laboratory-scale sake brewing conditions, strain XUDput1-MT accumulated more L-proline (0.31% ± 0.03%) and had higher cell viability (69% ± 9.5%) than did strain XUW-TRP (0.20% ± 0.01% L-proline content and 46% ± 3.8% cell viability). This result is consistent with the conclusion that intracellular L-proline accumulation confers ethanol tolerance under sake brewing conditions. Throughout the fermentation, strain XUDput1-MT evolved CO2 (66 ± 1.6 g), produced ethanol (20% ± 1.2%), and consumed glucose (0.6% ± 0.1%) at the same rate as strain XUW-TRP (69 ± 3.9 g, 20%, and 0.4% ± 0.2%, respec-
Intracellular total amino acid content of laboratory and sake strains grown in SD medium without or with 9% ethanol and incubated under static conditions

<table>
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<tr>
<th>Strain</th>
<th>Culture time (days)</th>
<th>Total amino acid content (% dry weight)</th>
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<td></td>
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<td>Without ethanol</td>
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<tr>
<td>Laboratory strains</td>
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<tr>
<td>MB329-17C</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
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</tr>
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<tr>
<td></td>
<td>8</td>
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</tr>
<tr>
<td>FH515</td>
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</tr>
<tr>
<td></td>
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<td>18</td>
</tr>
<tr>
<td></td>
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<td>Sake strains</td>
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</tr>
<tr>
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* α-L-proline content is not included in total amino acids. Yeast cells were pregrown in YPD medium at 30°C for 2 days with shaking (120 rpm). Values are means of results from five independent experiments. The standard deviations for these values were <10% of the value of the point.


duces stress responses such as the expression of heat shock proteins and the accumulation of trehalose (1). The trehalose content increased transiently in response to ethanol stress (Fig. 4). However, there was no significant difference in the content between the α-L-proline-accumulating and control strains. With respect to the intracellular total amino acid content, there was no significant difference between two sake strains (Table 2). Despite these findings, the mechanism(s) of ethanol toxicity and tolerance remains unclear. Sake yeasts produce more than 20% ethanol in sake brewing, and this high tolerance for ethanol, as inferred from the increased viability of yeast cells in the presence of ethanol, is one of the most important parameters in brewing sake. Increasing ethanol tolerance should improve fermentation performance and product quality while simultaneously simplifying the brewing process.

Intracellular α-L-proline protects yeast cells from damage by freezing, desiccation, and oxidative stress (26, 27, 36–38). In many plants, osmotic stress induces the rapid accumulation of α-L-proline resulting from the simultaneous activation of its biosynthesis and the inactivation of its degradation (11). Overproduction of α-L-proline may lead to increased osmotolerance in transgenic plants (19). In these cases, the α-L-proline concentration required to increase stress resistance is 10 to 200 μmol per g fresh or dry weight (10, 18, 25, 38). At higher concentrations (>1.5 M), α-L-proline effectively prevents aggregation during protein refolding (32) and can preserve membrane structure and function during freezing (30). Somewhat lower levels of α-L-proline (>1.0 M) can increase the stability and the solubility of hydrophobic macromolecules and sparingly soluble proteins (33, 34). In vivo, α-L-proline in the cell could reduce or prevent ethanol-induced membrane disorder and protein denaturation.

We confirmed that l-proline increases cell viability in the presence of ethanol during static incubations of both laboratory and sake yeast strains. The unusually high l-proline content does not appear to stress the cells such that the levels of trehalose or other amino acids increase. Further research to identify the mechanism(s) by which l-proline interacts with membrane phospholipids and proteins is needed to understand how l-proline increases ethanol tolerance. Thus, l-proline might serve as a protective agent for industrial microorganisms and enzymes.

**DISCUSSION**

Ethanol toxicity affects the growth and fermentation rates of yeast cells as well as their viability (16, 22). The major effect of ethanol seems to increase the permeability of the yeast cell membrane, which is equated with an increase in membrane fluidity (3, 17, 18). The incorporation of certain sterols and long-chain fatty acyl residues, such as ergosterol and palmitoyl-coenzyme A, into cell membranes is important for overcoming changes in fluidity (16, 28). Intracellular ethanol levels can be high enough to denature enzymes and functional proteins. Heat shock treatment increases ethanol tolerance, which in-
The present study is the first to report the construction of an L-proline-accumulating sake yeast strain. We expected the L-proline-accumulating strain XUDput1-MT to have a shorter fermentation time than the wild-type control strain. However, the sake mash of the two strains showed no significant differences in sake fermentation ability, such as CO₂ evolution or ethanol productivity. There are at least two explanations for these observations. First, the degree of ethanol tolerance might depend on cultural conditions and the stress responses, and cell metabolism under experimental static and sake brewing conditions could be quite different. Alternatively, ethanol tolerance could vary by strain. The sake yeast strains have a different genetic background than do the laboratory strains. Our results could also reflect the influence of factors other than L-proline on ethanol tolerance. A combination of ethanol protectants, e.g., trehalose (24), ergosterol (16), inositol (14), and L-proline, might result in even higher resistance to ethanol stress.

The large amount of L-proline in sake made by fermenting S. cerevisiae is of particular interest. The L-proline biosynthetic pathway would affect the whole metabolism of strains that can produce specific or various amino acids could enable the production of sake with distinctive tastes.

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