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Identification and characterization of an enzyme involved in the biosynthesis of the 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(*2H*)-furanone in yeast

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4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) is considered a key flavor compound in soy sauce. The compound has a caramel-like aroma and several important physiological activities, such as strong antioxidant activity. Here, we report the identification and characterization of an enzyme involved in the biosynthesis of HEMF in yeast. We fractionated yeast cell-free extract from *Saccharomyces cerevisiae* using column chromatography and partially purified a fraction with HEMF-forming activity. Peptide mass fingerprinting analysis showed that the partially purified fraction contains aldehyde reductase encoded by *YNL134C*. This reductase shares low sequence identity with enone oxidoreductase, which is responsible for the formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and HEMF in plants. *YNL134C* was expressed heterologously in *Escherichia coli*, and the purified protein catalyzed the formation of HEMF from the mixture of Maillard reaction products, acetaldehydes, and NADPH. Multicopy expression in *S. cerevisiae* resulted in increased HEMF productivity, and gene knockout of *YNL134C* in *S. cerevisiae* resulted in decreased HEMF productivity. These data suggest that the translation product of *YNL134C* is the HEMF-producing enzyme in yeast. Detailed analyses of an intermediate in the enzymatic reaction mixture revealed that HEMF is synthesized from (2*E*)-2-ethylidene-4-hydroxy-5-methyl-3(2*H*)-furanone, which formed via Knoevenagel condensation between the acetaldehyde and 4-hydroxy-5-methyl-3(2*H*)-furanone derived from the Maillard reaction based on ribose and glycine, by YNL134Cp in an NADPH dependent manner. Overall, this study shed light on the molecular basis for the improvement of soy sauce flavor and the biotechnological production of HEMF.

[**Key words:** Soy sauce; 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone; Yeast; *YNL134C*; Furanone; Homofuraneol; *Saccharomyces cerevisiae*; *Zygosaccharomyces rouxii*]

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone [HEMF, also called homofuraneol, and sometimes described as 2 (or 5)-ethyl-4-hydroxy-5 (or 2)-methyl-3(2*H*)-furanone (EHMF)] is one of the most important characteristic compounds with a high flavor dilution factor in soy sauce (1,2). HEMF not only has a sweet caramel-like odor but also has strong antioxidant properties, such as anticarcinogenic effects (3) and anticataract effects (4). Therefore, the enrichment of HEMF in soy sauce would improve the functionalities of soy sauce. In this context, we have screened high-level-HEMF-producing mutants from a collection of gene deletion mutants of *Saccharomyces cerevisiae* and showed that the *ADH1* gene deletion mutant (Δ*adh1*) exhibited the maximum HEMF productivity (5). However, the precursor and enzyme involved in the biosynthesis of HEMF in yeast remain to be elucidated before the complete HEMF biosynthesis pathway in yeast can be understood.

To date, various HEMF synthesis pathways have been proposed (Fig. S1). For example, Sasaki et al. (6,7) proposed that the HEMF in soy sauce is biosynthesized by soy sauce yeasts from the sugar phosphates found in the pentose-phosphate cycle, such as D-xylulose 5-phosphate. Sugawara et al. (8,9) and Hayashida et al. (10) showed that the formation of HEMF is promoted by cultivating yeast in a medium including the Maillard reaction products based on ribose and amino acids. Further investigation using stable isotopes showed that the skeleton of the five-membered ring and the methyl group of the side chain of HEMF (C_5 precursor) originated from ribose, and the ethyl group (C_2 precursor) was derived from D-glucose (11). They also assumed that acetaldehyde derived from D-glucose is effective as the C_2 precursor and that the C_5 and C_2 precursors were most likely enzymatically combined by yeast (11). These assumptions can be partially supported by the two facts that HEMF was formed by a chemical reaction between the Maillard reaction products from pentose and acetaldehyde, which are the Strecker degradation products of alanine (12) and that Δ*adh1* mutant accompanying acetaldehyde accumulation exhibited the maximum HEMF productivity (5).

Recent studies in plants have demonstrated that enone oxidoreductase from strawberry (13), tomato (14), and mango (15) catalyzes the formation of 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF, also called furaneol and strawberry furanone) from D-fructose-1,6-diphosphate via 4-hydroxy-5-methyl-2-methylene-3(2*H*)-furanone (HMMF) by reducing α- and β-unsaturated bonds of HMMF. Interestingly, this enzyme also catalyzes the formation of HEMF from (2*E*)-2-ethylidene-4-hydroxy-5-methyl-3(2*H*)-furanone (EDHMF) in a manner similar to converting HMMF to HDMF (13,14,16,17). Along these lines, our attention was focused on whether the enone oxidoreductase in yeast also plays an important role in HEMF production.

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To gain insight into the biosynthesis of HEMF in yeast, we report the isolation and characterization of an enzyme involved in the transformation from Maillard reaction products based on ribose and glycine and acetaldehyde to HEMF in yeast. We also discuss the HEMF precursors and the physiological role of HEMF production.

MATERIALS AND METHODS

Strains, plasmid, media, and genetic method The *S. cerevisiae* BY4741 and BY4742, purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), was used to construct the *YNL134C* gene knockout mutant, and *S. cerevisiae* BY4743 was used as the wild type strain. The *Zygosaccharomyces rouxii* NBRC0506 and NBRC1130, purchased from National Bio Resource Center of Japan, were used to construct a gene knockout mutant and source of genomic DNA as a PCR template, respectively. The primers used in this study are listed in Table S1. The *Escherichia coli* DH5α and BL21 were used as the host for recombinant DNA manipulation and to express recombinant His-tagged YNL134Cp, respectively. The plasmid pYC250 was used to construct the multicopy plasmid for *YNL134C*. All yeast strains were routinely grown in YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) at 30°C unless otherwise stated. The gene knockout in yeast was performed by PCR-mediated gene replacement, and it was confirmed using PCR. pUG6, pUN6, and pUZ6 as PCR templates when deleting the genes. Each of these templates possesses G418-, nourseothricin-, and zeocin-resistant genes surrounded by two *loxP* sequences, respectively. The transformations of *Z. rouxii* and *S. cerevisiae* were performed using electroporation and lithium acetate procedures, respectively (18,19). The bacterial transformations and bacterial DNA manipulation were performed using standard methods (20). A YPD medium containing 100 mg/l G418, 100 mg/l nourseothricin, and/or 300 mg/l zeocin was used to select the *S. cerevisiae* transformants. A YPD medium containing 200 mg/l G418 and/or 5 mg/l nourseothricin was used to select the *Z. rouxii* transformants. The HEMF production medium (416 mM glucose, 200 mM glycine, 200 mM ribose, 7.3 mM KH_2PO_4, 20 mM $MgSO_4$, and 0.5% w/v yeast extract, pH 6.0) was prepared by heat sterilizing the mixture at 121°C for 15 min, as previously described (9). The HEMF production medium without glucose (HEMF-G medium) was prepared in the same way as the HEMF production medium except that the glucose was omitted; it was used as a substrate for enzymatic HEMF production. For the labeling experiment, soy sauce mash extract (SSME) medium was prepared as below. Soy sauce mash before alcoholic fermentation was filtered using No. 2 filter paper (Toyo Roshi, Tokyo, Japan). The filtrate was collected, supplemented with glucose or [U-^{13}C]-glucose (Cambridge Isotope Laboratories, Andover, MA, USA), and sterilized by filtration using a 0.2 μm filter (Toyo Roshi).

Sequence analysis The homology searches using BLASTP were performed against Saccharomyces genome database (SGD) (http://www.yeastgenome.org/). The amino acid sequences were obtained from SGD and National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The amino acid identities were colored according to the Clustal Omega color scheme.

Plasmid construction The *YNL134C* was amplified by PCR from *S. cerevisiae* BY4743 genomic DNA using the InFusion_134C-Fw and InFusion_134C-Rv primers. To construct pYC134C, the PCR products were ligated into linearized plasmids, amplified by PCR from pYC250 using pYC250_InversePCR-Fw and pYC250_InversePCR-Rv primers, using InFusion HD Cloning Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The *ZYRO0G05984g* was amplified by PCR from *Z. rouxii* NBRC1130 genomic DNA using the InFusion_05984g-Fw and InFusion_05984g-Rv primers. To construct pYC05984g, the PCR products were ligated into linearized plasmids in the same way as the pYC134C construction. For heterologous expression of YNL134Cp in *E. coli*, pColdI-YNL134C was constructed as below. The *YNL134C* was amplified by PCR from *S. cerevisiae* BY4743 genomic DNA using the InFusion_YNL134C-Fw and InFusion_YNL134C-Rv primers. Using InFusion HD Cloning Kit (Takara Bio), the PCR products were ligated into linearized plasmids, amplified by PCR from pColdI vector (Takara Bio) using pColdI_InversePCR-Fw and pColdI_InversePCR-Rv primers.

Culture conditions For the HEMF production assay, yeast cells were independently precultured with shaking in 10 ml of YPD medium for 63 h at 30°C, and afterward, a portion of the cells was transferred to 4 ml of the HEMF production medium in test tubes to reach an optical density at 600 nm (OD_{600}) of up to approximately 2.0, followed by incubation with shaking for 2 days at 30°C. For the labeling experiment, SSME medium was used instead of HEMF production medium, and the yeast cells were incubated with shaking for 4 days at 30°C. For the spot assays, early stationary phase cultures grown on YPD were harvested by centrifugation and washed in sterile water. Then, the cells were diluted with sterile water to reach OD_{600} of up to approximately 1.0. Five microliters of cell suspension that was serially diluted 10-fold was spotted aseptically on YPD or HEMF production medium supplemented with 0.5 M NaCl containing 10 mM of acetaldehyde or 0.0125 mM–0.4 mM of EDHMF, and then, the cells were cultured at 30°C for 3 and 7 days with YPD and HEMF production medium, respectively.

Protein purification *S. cerevisiae* BY4743 was precultured with shaking in 150 ml of 2×YPD medium for 36 h at 28°C. After cell culture was centrifuged and yeast cells were collected, they were transferred to 200 ml of HEMF production medium containing 100 mM acetaldehyde to reach an OD_{600} of up to approximately 5.0, followed by incubation with shaking for 6 h. Then, yeast cells were harvested and washed twice with ice-cold wash buffer (20 mM Tris–HCl, 20 mM NaCl, 0.25 mM $MgSO_4$, pH 7.4) and they were resuspended in 10 ml of breaking buffer (20 mM Tris–HCl, 2% glycerol, 5 μM dithiothreitol, pH 8.0). The cell suspension was divided into 1 ml aliquots and placed in 2 ml scale microtubes containing 0.3 mg glass beads. The tubes were vortexed at 4°C for 90 min and centrifuged at 4°C for 20 min, resulting in all of the supernatants being collected as a cell-free extract. Yeast cell-free extract was concentrated by ultrafiltration using Amicon (Merck Millipore, Darmstadt, Germany) with exclusion limits of 10 kDa, and the concentrated fraction was equilibrated with 50 mM Tris–HCl pH 9.0. The separated and concentrated sample above the molecular weight of 10 kDa was applied to an anion-exchange column (Toyopearl DEAE-650M, Tosoh Corporation, Tokyo, Japan) equilibrated with 50 mM Tris–HCl pH 8.0. Proteins bound to the anion-exchange columns were eluted with a linear 0–0.3 M NaCl gradient, and the eluted fractions were analyzed for HEMF-forming activity by the standard incubation assay described below. After the fractions with HEMF-forming activity were desalted and concentrated by ultrafiltration using Amicon with exclusion limits of 10 kDa, they were applied to a gel filtration column (Sephacryl S-300 HR, GE Healthcare, Buckinghamshire, UK) equilibrated with 50 mM Tris–HCl pH 9.0 containing 150 mM NaCl. Proteins were eluted with same buffer, and analyzed for HEMF-forming activity. After the fractions with HEMF-forming activity were desalted and concentrated by ultrafiltration using Amicon with exclusion limits of 10 kDa, they were applied to small-scale anion-exchange column (HiTrap Q-XL, GE Healthcare) equilibrated with 20 mM Tris–HCl pH 9.0. Binding proteins to anion-exchange column were eluted in a step-by-step manner with the same buffer containing 0.1 M, 0.2 M, 0.3 M, and 0.5 M NaCl, and the eluted fractions were analyzed for HEMF-forming activity and applied to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

E. coli BL21 was cotransformed with pColdI-YNL134C and chaperone plasmid pG-Tf2 and resulting transformants were selected on Luria–Bertani broth (LB) medium supplemented with 20 μg/ml chloramphenicol and 50 μg/ml ampicillin. The selected transformants were inoculated into 400 ml of LB medium supplemented with 5 ng/ml tetracycline as a chaperon inducer, 20 μg/ml chloramphenicol, and 50 μg/ml ampicillin. The expression of *YNL134C* was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase. After IPTG was added to cell culture, additional incubation was performed for 24 h at 15°C. Then, incubated cells were harvested by centrifugation, wash with 0.1 M Tris–HCl (pH 8.0) with 0.3 M NaCl and resuspended in same buffer. They were disrupted by sonication and the lysate was centrifuged at 8000 ×*g* for 30 min. The resulting supernatant was concentrated by ultrafiltration using Amicon with exclusion limits of 10 kDa and the recombinant YNL134Cp was purified with Ni Sepharose 6 Fast Flow (GE Healthcare) according to the manufacturer's instructions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis Samples were mixed with an equal amount of 2×sample buffer (0.125 M Tris–HCl pH 6.8, 100 mM dithiothreitol, 4% w/v sodium dodecyl sulfate, 10% w/v sucrose, 0.01% w/v bromophenol blue), heated in boiling water for 5 min, cooled on ice, and applied to Tris–Glycine gels (3% stacking gel and 12% resolving gel). The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Enzymatic assay for the measurement of HEMF-forming activity A standard incubation assay was carried out as below. Acetaldehyde and NADPH were added to HEMF-G medium up to reach 10 mM and 1.34 mM (final concentration), respectively, and then, the protein samples were added to 1 ml of the mixture. The reaction mixture was incubated at 30°C for 24 h, and then, the HEMF concentration was determined by high performance liquid chromatography (HPLC) analysis. An exact incubation assay was carried out as below. A substrate such as 2× HEMF-G medium or EDHMF solution was diluted with an equal amount of McIlvaine buffer pH 3–8 containing acetaldehyde and NADPH. The mixture was preincubated at 30°C for 10 min and then added 10 μg of purified enzyme. The final volume of the mixture was 600 μl, and the final concentration of acetaldehyde and NADPH was 10 mM and 1.34 mM, respectively. The reaction mixture was incubated at 30°C for 1.5 h and then mixed well with equal amount of ethyl acetate to stop the enzymatic reaction. The assay for the protein concentration was performed by the protein assay kit (Bio-Rad) using bovine serum albumin as a standard for quantification.

Protein identification Peptide mass fingerprinting analysis using nano liquid chromatography coupled with tandem mass spectrometry (nanoLC-MS/MS) was carried out thorough out-sourcing (Japan Bio Services, Saitama, Japan). The interested bands on the SDS-PAGE were extracted and sent to Japan Bio Services.

Assay for determining HEMF concentrations The determination of HEMF concentration using HPLC was carried out according to a previous study (5) with little modification. Briefly, 0.6 ml of either the supernatant of the cell culture or the reaction mixture of the enzymatic assay was mixed with an equal amount of

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