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The Formation Mechanism by Yeast of 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone in Miso

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The mechanism of the formation of 4-hydroxy-2(or 5)-ethyl-5-(or 2)-methyl-3(2*H*)-furanone (HEMF) with yeast under caltivation in a medium containing amino-carbonyl reactants of ribose and glycine was investigated using stable isotopes of the corresponding compounds. It was confirmed that the skeleton of the five-membered ring and the methyl group of the side chain of HEMF was formed from ribose, and that the ethyl group was derived from the glucose metabolite by yeast. The formation of HEMF was confirmed when acetaldehyde as the glucose metabolite and a cell-free extract from yeast were added to the medium containing amino-carbonyl reactants. These results suggest that the role of yeast in HEMF formation is not only to provide the glucose metabolite, but also in combining the amino-carbonyl reactants with the glucose metabolite.

Key words: 4-hydroxy-2(or 5)-ethyl-5-(or 2)-methyl-3(2*H*)-furanone; yeast; miso

More than 200 flavor components have been confirmed in miso.[1,2] 4-Hydroxy-2(or 5)-ethyl-5-(or 2)-methyl-3(2*H*)-furanone (HEMF) has a strong, sweet, and caramel-like aroma and is very important as the characteristic component of soy sauce and miso, with a very low threshold value of less than 20 ppb in water.[3] Although a small amount of HEMF has been detected in cheese[4] and beer[5] in recent years, it was first found in soy sauce,[6–8] and then in miso,[9] their contents of HEMF being much higher than in other fermented foods.

Blank and Fay have reported a formation mechanism for HEMF by the amino-carbonyl reaction.[10] According to their report, HEMF was formed by combining the C_5-1-deoxydiketose from pentose with acetaldehyde generated by the Strecker degradation of alanine when pentose and alanine were heated together, but the amount of HEMF formed was very small and did not reach the content levels detected in miso or soy sauce.

Sugawara and Sakurai have reported on a formation mechanism by yeast for HEMF in miso,[11] whereby HEMF formation was promoted by cultivating yeast (*Zygosaccharomyces rouxii* 061) in a heat-sterilized medium that contained glucose, ribose, and an amino acid (glutamic acid, threonine, serine, or alanine). It was also found that much more HEMF formed when yeast was cultivated in a model medium containing glycine instead of alanine or serine. In addition, the amount of HEMF formed by the yeast was influenced by the glucose content in the medium (Sugawara, E. *et al.*, unpublished results). We assumed that the precursor of HEMF, containing 7 carbons, is formed from the amino-carbonyl reaction products of 5 carbons (the C_5 precursor) formed by ribose and glycine, and from the chemical compounds of 2 carbons (the C_2 precursor), which perhaps form by active glucose metabolism of the yeast, as in the case of acetaldehyde, reported by Blank and Fay.[10]

The main purpose of the present study was to clarify the hypothetical pathway of HEMF formation by yeast. Stable isotopes were used to investigate how the sugar moiety and amino acid contribute to HEMF formation by yeast. Yeast was incubated in a medium containing the stable isotopes [U-^{13}C]-ribose, [U-^{13}C]-glycine, [U-^{13}C]-glucose, and [6-^{13}C]-glucose, and the incorporation of ^{13}C atoms into the skeleton of HEMF was investigated to confirm the roles of ribose, glycine, and glucose, which were in the medium. It is also investigated whether the C_2 precursor would be provided by yeast. We considered acetaldehyde to be the C_2 precursor because it is one of the glucose metabolites,

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Abbreviations: HEMF, 4-hydroxy-2(or 5)-ethyl-5-(or 2)-methyl-3(2*H*)-furanone; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; FID, flame ionization detector

and acetaldehyde generated by the Strecker degradation of alanine and C_5-1-deoxydiketose from pentose has been shown to lead to HEMF by Blank and Fay.[10] We also investigated whether the C_5 precursor and acetaldehyde would be combined by an enzymatic reaction using a cell-free extract prepared from yeast, in order to clarify the role of yeast in HEMF formation, besides providing the C_2 precursor.

Materials and Methods

Reagents. D-glucose, sodium chloride, glycine, acetaldehyde, potassium dihydrogenphosphate, magnesium sulfate heptahydrate, anhydrous sodium sulfate, glycerol, dithiothreitol, diethyl ether, and hydrochloric acid were obtained from Kanto Chemical Company (Tokyo). D-ribose was obtained from Nacalai Tesque (Kyoto), and the yeast extract was obtained from Becton, Dickinson (New Jersey). Kiage soy sauce was purchased from a local market in Morioka, Japan, and tris(hydroxymethyl)aminomethane and dichloromethane were purchased from Aldrich (Tokyo). [6-^{13}C]-Glucose, [U-^{13}C]-glucose, and [U-^{13}C]-ribose were obtained from Cambridge Isotope Laboratories (MA), and [U-^{13}C]-glycine was obtained from Isotec (Tokyo). The standard for HEMF was obtained from Tokyo Kasei Kogyo (Tokyo).

Microorganisms. The yeast strain used in this study was *Zygosaccharomyces rouxii* 061, a halophilic yeast commonly used in producing Sendai miso, due to its ability to form a large amount of HEMF in miso. This strain was provided by the Experimental Station of the Miyagi Miso-Shoyu Industry Cooperative (Miyagi, Japan).

Medium for the starter culture. The medium for the starter culture was prepared from 10.0 ml of Kiage soy sauce, 5.0 g of D-glucose, and 10.0 g of sodium chloride dissolved in 100 ml of distilled water. The medium was adjusted to pH 5.2 with sodium hydroxide and then sterilized by autoclaving (1 kg/cm^2 at 121 °C for 15 min). The amino-carbonyl reaction appeared to proceed during sterilization.

Experimental media. Table 1 shows the compositions of the experimental media. The basic medium used in the experiments[11] was prepared from 7.5 g of D-glucose, 2.5 g of D-ribose, 1.0 g of glycine, 10.0 g of sodium chloride, 1.0 g of potassium dihydrogenphosphate, 0.5 g of magnesium sulfate heptahydrate, and 0.5 g of yeast extract dissolved in 100 ml of distilled water. The medium was adjusted to pH 5.2 with sodium hydroxide, and then sterilized. Four additional media were prepared: medium A-I, the basic medium without glucose; medium A-II, the basic medium without glucose but with 0.05 g of acetaldehyde; medium B-I, the basic medium without glucose or sodium chloride; and medium B-II, the basic medium without glucose or sodium chloride, but with 0.05 g of acetaldehyde. The total volume of the experimental media with the stable isotope was 3.0 ml of the basic medium. Each medium was sterilized after preparation.

Table 1. Composition of Media Used in HEMF Production

	Basic medium	Medium A-I	Medium A-II	Medium B-I	Medium B-II
Glucose (g)	7.5	—[b]	—	—	—
Ribose (g)	2.5	2.5	2.5	2.5	2.5
Glycine (g)	1.0	1.0	1.0	1.0	1.0
NaCl (g)	10.0	10.0	10.0	—	—
KH_2PO_4 (g)	1.0	1.0	1.0	1.0	1.0
$MgSO_4 \cdot 7H_2O$ (g)	0.5	0.5	0.5	0.5	0.5
Yeast extract (g)	0.5	0.5	0.5	0.5	0.5
Acetaldehyde (g)[a]	—	—	0.05	—	0.05
Water (ml)	100.0	100.0	100.0	100.0	100.0

[a]Acetaldehyde was added after sterilization.
[b]No addition.

Cultivation. A loopful of *Z. rouxii* 061 from a slant culture was incubated in the medium for the starter culture (50 ml) at 27 °C for 2 d, before the medium was centrifuged (7,000 rpm for 10 min at 4 °C) and the yeast cells were collected. The collected cells were washed with a washing buffer containing 10 ml of 1 M Tris–hydrochloric acid (pH 7.4), 2.48 g of sodium chloride, and 0.123 g of magnesium sulfate heptahydrate dissolved in distilled water, and this solution was made up to 500 ml with distilled water, and sterilized. The supernatant was separated by centrifuging it twice under the same conditions to obtain intact cells. The intact cells were added to each experimental medium and the final concentration was adjusted to 10^8 cells/ml, before the medium was incubated at 27 °C. Acetaldehyde and the intact cells were added to the medium in the experiments with acetaldehyde.

Preparation of a cell-free extract from Z. rouxii *061.* The starter culture, incubated for 2 d, was centrifuged (7,000 rpm for 10 min at 4 °C), and the yeast cells were collected. The collected cells were washed with the washing buffer, and the supernatant was separated by centrifuging it twice under the same conditions to obtain washed cells. Ten grams of sterilized glass beads and 10 ml of a breaking buffer containing 10 ml of 1 M Tris–hydrochloric acid (pH 8.0), 20 g of glycerol, and 1.5 mg of dithiothreitol dissolved in distilled water was made up to 500 ml with distilled water, before it was sterilized. The washed cells were added to the breaking buffer, and then the buffer solution was frozen at −20 °C for 1 h. The cells were then disrupted in a vortex mixer (Vortex-Genie 2, Scientific Industries, Tokyo) at 0 °C for a total of 30 min. The solution of disrupted cells was centrifuged (7,000 rpm for 10 min at 4 °C), and the supernatant obtained was recentrifuged (12,000 rpm for 10 min at 4 °C) to obtain cell-free extract.

Application of cell-free extract. Ten ml of cell-free extract was added to media B-I and B-II adjusted to pH 7.5, and the solution incubated at 27 °C for 6–24 h.

Measurement of quantities of acetaldehyde and protein. After this incubation, the quantity of acetaldehyde in the centrifuged supernatant was measured using the F-KIT (Roche, Mannheim), while the quantity of protein in the prepared cell-free extract was measured according to the method of Bradford.[12)]

Determination of HEMF. Aroma concentrates were prepared by the Tenax TA resin adsorption method[9,11)] from the supernatant that had been centrifuged to remove yeast cells. These aroma concentrates were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS); the experimental and analytical conditions were described in detail in previous reports,[9,11)] except for the GC–MS model. The GC–MS model used here was a Shimadzu QP-2010 gas chromatograph-mass spectrometer (GC part: a Shimadzu GC-2010 gas chromatograph). All data were processed according to Wiley database 338323 compounds.

Experiments using stable isotopes were conducted by extracting the supernatant (3 ml) of the yeast cells removed by centrifugation after incubation with two successive additions of 3 ml of dichloromethane in a 20-ml vial under magnetic stirring 5 min each time. The two organic phases were collected, dried over anhydrous sodium sulfate, and concentrated under nitrogen. The aroma concentrate obtained was analyzed by GC–MS under the analysis conditions already indicated.

Results and Discussion

Contribution of ribose and glycine to HEMF structure

We have explained that pentose, amino acid, and glucose have important roles in the formation of HEMF by yeast in the model medium,[11)] although it has not been clarified how each compound transformed into the structure of HEMF. A control experiment indicated that HEMF was effectively formed when yeast was incubated using normal glucose, normal ribose, and normal glycine in the basic medium. The structure was confirmed by GC–MS. The mass spectrum is shown in Fig. 1A. The main fragment ions of HEMF were assigned as follows: m/z 142, M^+; 127, $(M - CH_3)^+$; 114, $(M - CH_3CH_2)^+$; 99, $(M - COCH_3)^+$; 85, $(M - COCH_2CH_3)^+$; 71, $(M - CH_3COCHO)^+$; 57, $(CH_3CH_2CO)^+$ and 43, $(CH_3CO)^+$. [U-^{13}C]-ribose instead of normal ribose was then incubated in the same medium. The MS data for the HEMF obtained are shown in Fig. 1B. A fragment of the molecular ion was found at m/z 147, indicating that the HEMF formed contained five ^{13}C atoms. These ^{13}C atoms were not present in the ethyl group of the side chain, because they were found in the fragments $(M - CH_3)^+$ at m/z 132 and $(M - CH_3CH_2)^+$ at m/z 119. The MS result that all carbons included in the fragments of $(^{13}CH_3{}^{13}CO)^+$ at m/z 45 and $(M - {}^{13}COCH_2CH_3)^+$ at m/z 89 were ^{13}C atoms confirmed that all carbons of the five-ring and the methyl group of the side chain were ^{13}C atoms. In further experiment, [U-^{13}C]-glycine was added to the medium instead of normal glycine and the same incubation was conducted. The MS data for the HEMF obtained are shown in Fig. 1C. In this case, the MS data for HEMF were the same as those in the control experiment. This result indicates that no carbons of glycine contributed to the structure of HEMF. These results reveal that the five-ring and the methyl group of the side chain of HEMF were formed from ribose, and that the ethyl group of the side chain was not formed from glycine. We assumed that the formation mechanism of HEMF by yeast is based on the formation of the amino-carbonyl products of ribose and glycine, and that its subsequent decomposition (the elimination of the moiety from glycine) forms the C_5-1-deoxydiketose (the C_5 precursor), reported to be formed from pentose by Blank and Fay.[10)] Further enolization of this compound can lead to the intermediate, which reacts with the C_2 precursor formed by active metabolism of yeast. Accordingly, the carbons of glycine did not contribute to the structure of the C_5 precursor, and were not included in HEMF.

Contribution of glucose to the ethyl group of HEMF

No HEMF was detected when the control experiment was done without glucose. It appears that glucose had an important role in the formation of HEMF by yeast. To determine the contribution of glucose, [U-^{13}C]-glucose was added to the medium instead of common glucose. The MS data for the HEMF obtained are shown in Fig. 2A. After incubation, the fragment of the molecular ion at m/z 144 indicates that two ^{13}C atoms from glucose were incorporated into the structure of HEMF. The incorporation of two ^{13}C atoms is also indicated by fragments $(^{13}CH_3{}^{13}CH_2CO)^+$, $(M - CH_3COCHO)^+$, and $(M - COCH_3)^+$, at m/z 59, 73 and 101 respectively. Fragments $(CH_3CO)^+$ at m/z 43 and $(M - CO^{13}CH_2{}^{13}CH_3)^+$ at m/z 85 were the same as those obtained using common glucose. In addition, the daughter ions at m/z 128 $(M - {}^{13}CH_3)^+$ and m/z 114 $(M - {}^{13}CH_3{}^{13}CH_2)^+$ showed the presence of two ^{13}C atoms in the ethyl group of the side chain.

These results suggest that the C_2 precursor, in which all carbons were labeled, was initially generated from [U-^{13}C]-glucose by metabolism of yeast. This C_2 precursor and the C_5 precursor from ribose then combined before cyclization of the compound of 7 carbons was obtained, resulting in HEMF finally being formed.

To investigate the formation pathway of the ethyl group from glucose, a similar incubation was conducted using [6-^{13}C]-glucose instead of [U-^{13}C]-glucose. The MS data for the HEMF obtained are shown in Fig. 2B. The intensity of the parent molecular ions at m/z 142

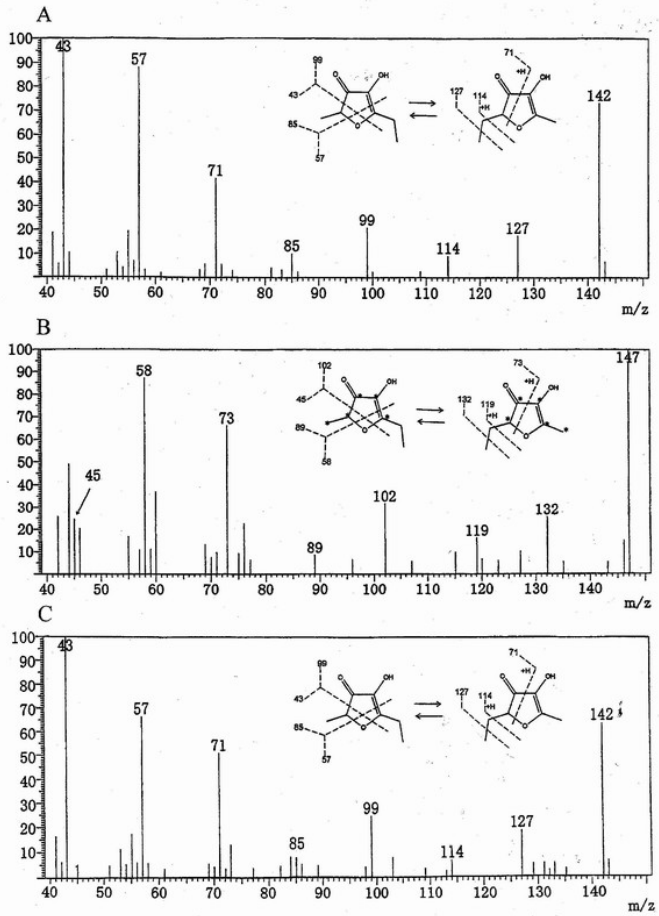

Fig. 1. GC–MS Data for HEMF Obtained by Incubation of Yeast with Normal Glucose, [U-^{13}C]-Ribose, or [U-^{13}C]-Glycine Instead of the Corresponding Unlabeled Compound in the Basic Medium.

A, normal glucose; B, [U-^{13}C]-ribose; C, [U-^{13}C]-glycine

*, ^{13}C atom

and 143 was about one to one, indicating that monolabeled HEMF and unlabeled HEMF formed in approximately a ratio of one to one. The daughter ions at m/z 58, 72, and 100 respectively produced fragments at m/z 57, 71, and 99 by the fragmentation of monolabeled and unlabeled HEMF. These ions were of nearly equal intensity. The fragments at m/z 43 and m/z 85 were the same as those of unlabeled HEMF, while the daughter ions at m/z 127 $(M - {}^{13}CH_3)^+$ and m/z 114 $(M - {}^{13}CH_3CH_2)^+$ showed the presence of one ^{13}C atom in the methyl moiety of the ethyl group. These results indicate that the same amounts of the C_2 precursors, one of which was labeled and the other unlabeled, were formed from one molecule of glucose, and reacted equally with the C_5 precursor. Hence we were able to confirm that the C_2 precursor was formed by the glucose metabolism of yeast, and that this formed C_2 precursor constituted the ethyl group of HEMF.

Effectiveness of acetaldehyde as the C_2 precursor

It was apparent that the C_2 precursor was formed by glucose metabolism of yeast, although the chemical structure of this C_2 precursor could not be determined. Acetaldehyde was used as the C_2 precursor in this experiment because it is the intermediate in the glucose metabolism of yeast. In the glycolysis system (glucose metabolism) of yeast, two molecules of pyruvic acid, which is a very important intermediate in biosynthesis, are generated by the anaerobic degradation of one molecule of glucose. The pyruvic acid generated is degraded to acetaldehyde by alcoholic fermentation of the yeast. In addition, as described by Blank and Fay,[10] the C_2 precursor was acetaldehyde generated by the degradation of alanine in the formative pathway of HEMF in the absence of yeast. To investigate the contribution of acetaldehyde as the C_2 precursor to HEMF formation, the effects of medium A-II with acetaldehyde instead of glucose, and of medium A-I without acetaldehyde or glucose, were compared with that of the basic medium.

HEMF was generated in basic medium containing 7.5% glucose at a concentration of 5.8 ppm after 6 h,

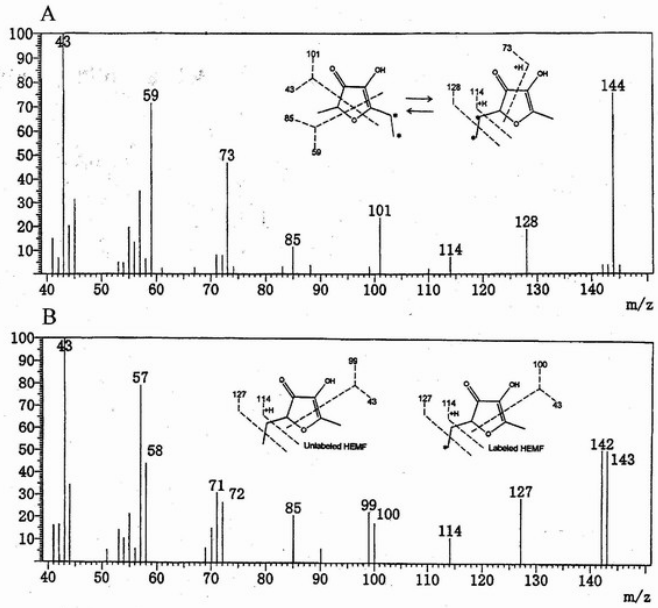

Fig. 2. GC–MS Data for HEMF Obtained by Incubation of Yeast with [U-^{13}C]-Glucose or [6-^{13}C]-Glucose Instead of the Corresponding Unlabeled Compound in the Basic Medium.
A, [U-^{13}C]-glucose; B, [6-^{13}C]-glucose
*, ^{13}C atom

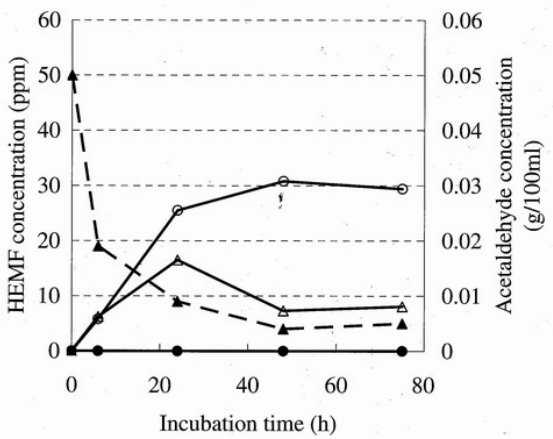

Fig. 3. Formation of HEMF by Yeast Using Acetaldehyde as the C_2 Precursor, and Concentration of Acetaldehyde in Medium A-II.
—○—, HEMF concentration in the basic medium (containing glucose); —●—, HEMF concentration in medium A-I (containing neither glucose nor acetaldehyde); —△—, HEMF concentration in medium A-II (containing acetaldehyde but no glucose); --▲--, acetaldehyde concentration in medium A-II.

25.5 ppm after 24 h, 30.8 ppm after 48 h, and 29.4 ppm after 72 h, as shown in Fig. 3. HEMF was not detected when no yeast was added to the both medium. The concentration of HEMF in medium A-I (containing no glucose or acetaldehyde) was below the detection limit of GC analysis during the period of incubation, while the concentration of HEMF in medium A-II (containing acetaldehyde but no glucose) was 6.2 ppm after 6 h and 16.5 ppm after 24 h. These results show the effectiveness of acetaldehyde as a C_2 precursor. But the amounts of HEMF formed declined with incubation at more than 30 h, because HEMF is an unstable compound.[7] It was concluded that the amount of decomposed HEMF was greater than the amount of formed HEMF. Moreover, as shown in Fig. 3, acetaldehyde was almost absent in medium A-II at 30 h, because this compound was used for the formation of HEMF and/or vaporized. Therefore, HEMF was thought to be formed by the addition of acetaldehyde in the medium intermittently. In this study, the concentration of acetaldehyde generated by the glucose metabolism of yeast was not determined in the basic medium. The concentration of acetaldehyde in the basic medium must be determined.

Combination of the C_5 precursor with the C_2 precursor through the action of yeast

Yeast was probably involved in the combination of the C_5 precursor and acetaldehyde, although the combination of these compounds was not confirmed to be due to the enzymatic reaction caused by yeast. To investigate whether it involved the enzymatic reaction caused by yeast, a cell-free extract was obtained by disrupting the cells of *Z. rouxii* 061. The quantity of protein in the cell-free extract prepared in this study was 1.22 mg/ml. Medium B-I, not containing acetaldehyde as the C_2 precursor, and medium B-II, containing acetaldehyde, were incubated with prepared cell-free extract to investigate whether HEMF would form. The HEMF concentrations after incubation are shown in Table 2.

Table 2. Formation of HEMF by Reaction with a Cell-Free Extract of *Z. rouxii* 061

Incubation time (h)	HEMF concentration (ppm)	
	B-I[a]	B-II[b]
6	—[c]	0.5
24	—	3.8

[a]Medium contained no acetaldehyde.
[b]Medium contained acetaldehyde.
[c]Not detected.

No HEMF was detected in medium B-I (no acetaldehyde), but a significant concentration of 0.5 ppm after 6 h and 3.8 ppm after 24 h, formed in medium B-II. In addition, no HEMF was detected in the case of an added cell-free extract that had been inactivated by heating at 70 °C for 10 min or in the case of no cell-free extract. These results suggest that the C_5 precursor and acetaldehyde were enzymatically combined by the *Z. rouxii* 061 yeast.

The results of this study confirm that the five-ring and the methyl group of the side chain of HEMF were formed from the chemical compound of 5 carbons generated by the amino-carbonyl reaction of ribose and glycine (the C_5 precursor), and that the ethyl group of the side chain of HEMF was formed from the chemical compound of 2 carbons generated by the glucose metabolism of yeast (the C_2 precursor). Acetaldehyde appears to have been effective as the C_2 precursor, and the C_5 and C_2 precursors were probably enzymatically combined by yeast. Consequently, the formation mechanism of HEMF in Fig. 4 was inferred from the results obtained in this study. In miso and soy sauce, HEMF was probably formed by the combination of the compound of 5 carbons generated by the amino-carbonyl reaction under mild conditions of fermentation and the 2 carbon compound of the glucose metabolite formed by yeast, enzymatically.

Acknowledgments

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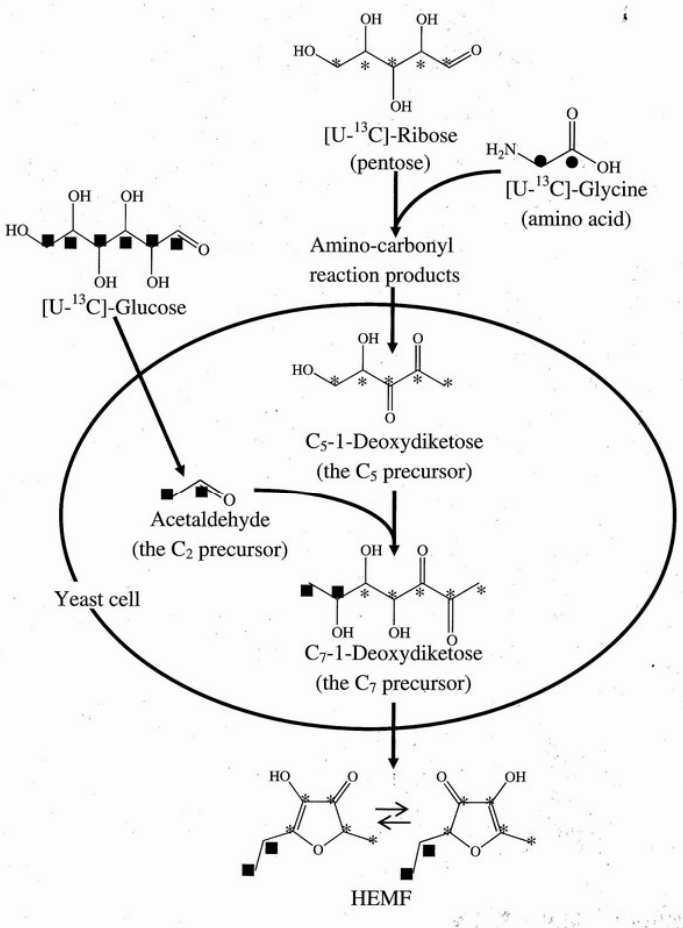

Fig. 4. Hypothetical Formation Pathway of HEMF by Yeast.
*, ●, or ■, ^{13}C atom

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