Journal of Bioscience and Bioengineering
VOL. 121 No. 3, 281–285, 2016

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Formation of ethyl ferulate from feruloylated oligosaccharide by transesterification of rice *koji* enzyme under sake mash conditions

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Received 11 May 2015; accepted 24 June 2015
Available online 17 July 2015

Formation of ethyl ferulate (EF) and ferulic acid (FA) under sake mash conditions was studied using feruloylated oligosaccharide (FO), prepared from rice grains, as the substrate for rice *koji* enzyme. EF and FA were produced from FO over six times faster than from alkyl ferulates however, under the same ethanol concentration, only small differences were observed between the EF/FA ratios when either FO or methyl ferulate were used as substrates. Esterification and hydrolysis of FO or methyl ferulate showed similar pH dependencies and similar EF/FA ratios for each substrate in all of the pH ranges tested. Ethanol concentration clearly affected the EF/FA ratio; the ratio increased as ethanol concentration increased. Formation of EF and FA in the sake mash simulated rice digest was accelerated by addition of exogenous FO. These results indicated that supply of FO to sake mash is a crucial step for EF and FA formation, and ethanol is an influencing factor in the EF/FA ratio. The rice *koji* enzyme reaction suggested that EF and FA are formed through a common feruloylated enzyme intermediate complex by transesterification or hydrolysis, and these reactions occur competitively.

[**Key words:** Sake; Ethyl ferulate; Transesterification; Feruloylated oligosaccharide; Ferulic acid]

A previous study suggested ferulic acid (FA) has many favorable functional properties for human health (1). It was also found to be a potent antioxidant in sake by Ohta et al. (2) however, it can have a strong bitter and/or astringent taste. FA levels exceed the threshold value in charcoal untreated sake (3). Ethyl ferulate (EF), which is more hydrophobic than FA, is also contained in charcoal untreated sake and its levels also exceed the threshold value in sake (3). The antioxidizing powers of EF and FA are comparable (4,5), and EF also has favorable human health properties (6–9). The organoleptic characteristics of EF are advantageous to the sensory quality of sake, in contrast to FA. EF has been reported as a favorable flavor component of *mirin* (10), and it produces a rather sweet and moderate bitter and/or astringent taste in sake (3). EF also shows a masking effect to the unpleasant taste of FA in sake if EF exists at only one-fifth the concentration of FA (3). Sake components with a hydrophobic moiety, such as FA and EF, are removed by a charcoal treatment that is commonly applied to the sake manufacturing process (11,12). Charcoal treatment reduces the unpleasant taste caused by hydrophobic components; it also removes fragrance components and reduces the flavor harmony of sake. Non-charcoal treatment products are increased in high quality sake, for example *ginjyo-shu*, in which EF and FA will certainly affect the sensory quality (13).

FA in sake is derived from the rice grains. Polishing the rice grains remarkably decreases FA and *p*-coumaric acid levels (14), but the remaining phenolic acid is sufficient to affect the sensory quality of sake after brewing (15). FA exists as an esterified form in the polysaccharides within the rice endosperm cell wall; it is gradually liberated from feruloylated polysaccharide by the feruloylesterase of rice *koji* along with the enzymatic decomposition of rice grains in sake mash (15,16). We found that rice *koji* enzyme esterifies FA with ethanol under sake mash conditions. However, it hydrolyzes EF to FA far faster than the esterification reaction (17). The hydrolysis of EF is approximately 20 times faster than esterification (17). However, EF exists in sake at significant sensory levels. It is suggested that another factor maintains EF levels. Considering the importance of EF in the sensory quality of sake, the control of EF levels in sake, especially the ratio of EF to FA, is essential. A high EF/FA ratio in sake may contribute to both taste quality and functionality.

In this study, we prepared feruloylated oligosaccharide (FO) that is suggested to be an intermediate for FA formation from rice grains, and examined the rice *koji* enzymatic reaction using FO as a substrate, particularly focusing on the formation of EF and the ratio of EF/FA.

MATERIALS AND METHODS

Materials Ethyl and methyl ferulates were obtained from Wako Pure Chemical Industries (Osaka, Japan). *Jummai*-type sakes, entered in the Akita prefectural sake awards 2013, were used for sake analysis.

Analysis of sake samples FA and EF in sake were analyzed using a previously described method (3). pH values were measured using a Horiba F-52 pH meter (Horiba, Kyoto, Japan).

Preparation of rice *koji* enzyme Rice *koji* was made from 60% polished rice grains of *Miyamanishiki* using *Aspergillus oryzae* RIB 128. Rice *koji* enzyme was prepared according to a previous method (17). The lyophilized sample was

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.06.015

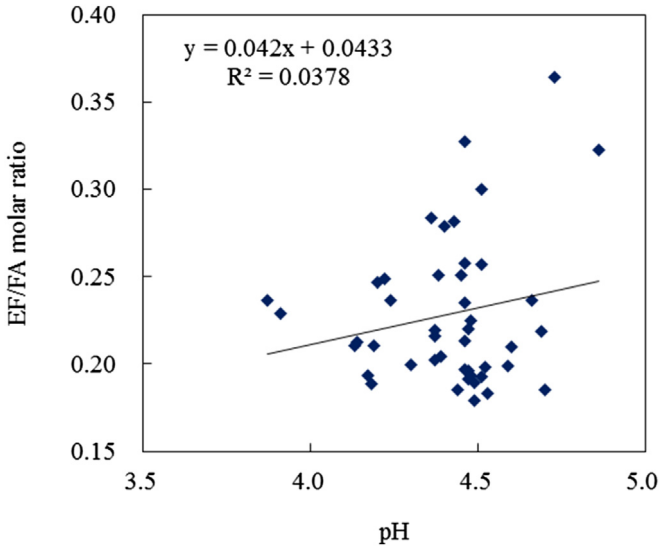

FIG. 1. Relationship between pH and EF/FA molar ratio in 45 *junmai*-type sake samples presented in the Akita prefectural sake award 2013.

re-dissolved with a small amount of distilled water and divided into small tubes (1.5 mL), and stored at −20°C until use. The enzyme solution (concentrated rice *koji* enzyme) had approximately 20 fold higher feruloylesterase activity (0.019 ± 0.003 unit/mL) than that of the original enzyme extract solution, and it was used in the enzyme assays after 10−100 times dilution with water.

Preparation of isopropyl ferulate Isopropyl ferulate was synthesized using sulfuric acid (0.5% (v/v)) in alcoholic solution. It was incubated at 80°C for 30min and the formed ester was purified by HPLC before use.

Preparation of FO from polished rice grains Steamed rice grains (400 g) of 60% polished *Miyamanishiki* was mixed with 800 mL of water containing 0.2 g of a saccharifying enzyme agent "Guluku-SB" (Amano Enzyme Inc., Nagoya, Japan) and 0.1 g of Cellulase T"Amano"4 agent (Amano Enzyme Inc.) before incubation at 55°C for 24 h. The digested solution was centrifuged at 12,000×*g* for 20 min, and the supernatant was filtered using filter paper. The solution was applied to 25 g of C18 Bond Elute (Varian, CA, USA) that had been successively pretreated with 100 mL of methanol and then 100 mL of water. After the column had been washed with water (100 mL), the trapped compounds were eluted with 80 mL of methanol and then 40 mL of water (FO-1), followed by 80 mL of ethanol (95%) and then 40 mL of water (FO-2). The solvent was removed from the eluted solution under vacuum at 50°C and then freeze-dried to give a dry sample. The weight of obtained samples was 1.7 g of FO-1 and 0.7 g of FO-2.

Analysis of FO samples The FA content of obtained FO was determined to be the same as that previously found in rice samples (14). The monosaccharide composition of FO was analyzed according to Malunga and Beta (18). The sample of 2.0 mg was suspended in 2 M sulfuric acid (0.3 mL) and hydrolyzed at 110°C for 1 h, before being neutralized with 4 M NaOH. The solution was diluted ten times with water, and 25 μL was applied to a Dionex HPLC system (Nippon Dionex K.K., Osaka, Japan) equipped with a Dionex CarboPac PA1 column. Solvent A was water, and solvent B was a 100 mM NaOH aqueous solution. A linear gradient was used from A:B = 95:5 to A:B = 85:15 in 25 min at a flow rate of 1.0 mL/min. The FO sample was digested with α-amylase (A9857) (Sigma−Aldrich Japan, Tokyo, Japan) and any formed sugar was analyzed using the same HPLC system. The ^{1}H NMR spectrum was measured in D_2O with a JNM ECS-400 spectrometer (Jeol, Tokyo, Japan).

Rice *koji* enzymatic reaction: EF and FA formation from FO or alkyl ferulates Reaction mixture (190 μL) consisted of 9.5% ethanol, 100 mM sodium succinate buffer (pH 4.3), and substrate. The amount of substrate in the reaction mixture was adjusted as the concentration of total FA was 0.1 mM. The assay was started by an addition of 100 times diluted enzyme solution (10 μL). In case of alkyl ferulate, 10 times diluted one was used for accurate HPLC analysis. After incubation at 30°C for 2 h, an equal volume of acetonitrile was added to the mixture. The reaction mixture was immediately frozen at −80°C before HPLC analysis. The pH profile of the reaction was examined using 100 mM sodium citrate buffer (pH 3.0−5.5). Ethanol concentration was varied from 4.75% to 19.0% as necessary. EF and FA formation was determined by HPLC, according to our previous method (17).

TABLE 1. Analysis of feruloylated oligosaccharide sample preparations.

	I	II
Ferulic acid (μg/g)	3405 ± 97[a]	4634 ± 133
Monosaccharide composition (mole%)		
Arabinose	4.1 ± 0.0	5.3 ± 0.0
Xylose	6.0 ± 0.0	7.8 ± 0.0
Glucose	89.9 ± 0.1	86.9 ± 0.0
Galactose	−	−

[a] Mean and SD of three determinations.

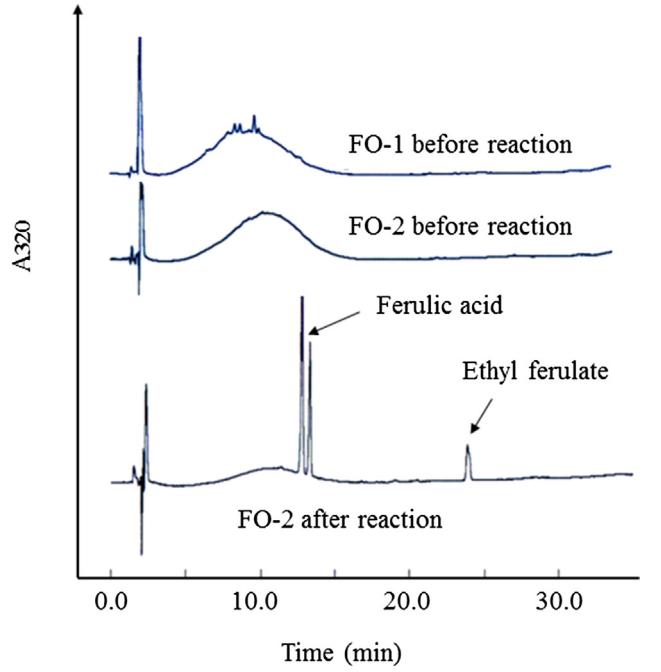

FIG. 2. HPLC chromatogram of FOs before and after the rice *koji* enzyme reaction.

EF and FA formation from FO under the model sake mash conditions The model sake mash solution was prepared as follows: 2.0 g of α-rice was digested in a 5 mL solution consisting of 14.25% ethanol, 100 mM sodium succinate buffer (pH4.3) and 0.2 mL of concentrated rice *koji* enzyme, at 30°C for 24 h, with shaking (120 rpm). Supernatant was obtained by centrifugation (15,000×*g* for 10min), and forming of EF and FA formation was examined using 400 μL of the digest solution and 100 μL of exogenous FO aqueous solution. The mixture was incubated at 30°C for

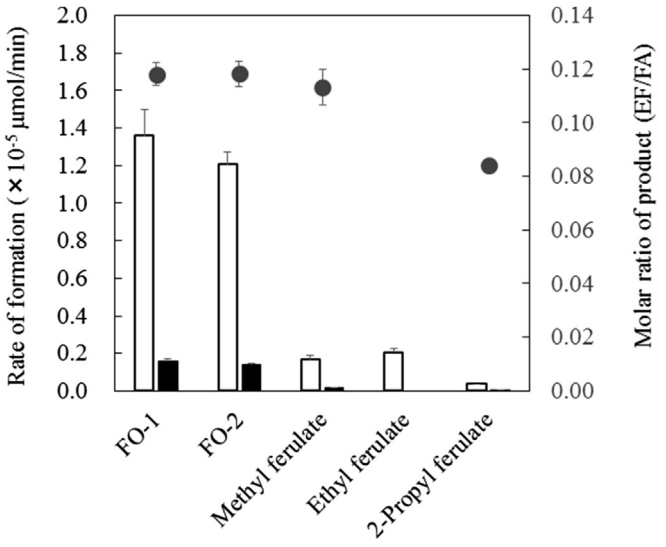

FIG. 3. Comparison between feruloylated oligosaccharide and alkyl ferulates in hydrolysis and ethyl esterification by rice *koji* enzyme. Forming rates of the equal enzyme unit are compared. Data are mean and SD of three determinations. Open square: Ferulic acid, black square: Ethyl ferulate, and dark gray circle: Molar ratio of product (EF/FA).