

Ferulic Acid Production in the Brewing of Rice Wine (Sake)

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ABSTRACT

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The traditional Japanese alcoholic beverage sake is produced by fermentation of rice by *Saccharomyces cerevisiae* and *Aspergillus oryzae*. *A. oryzae* releases ferulic acid, an antioxidant, from steamed rice during the fermentation process. The concentration of ferulic acid increased with time during fermentation and the production rate peaked 9–12 days post inoculation. Analysis of the fermentation cultures of *Aspergillus oryzae*, by high-performance liquid chromatography (HPLC), revealed that *p*-coumaric acid induced an 18.9-fold increase in the level of ferulic acid. Furthermore, SDS-PAGE analysis revealed an increase or decrease in the level of specific proteins after the addition of *p*-coumaric acid to fermentation cultures of *Aspergillus oryzae*. Ferulate esterase (FAE) activity was observed in the fermented sake ten days following the start of the fermentation process. These results suggest that the level of ferulic acid is regulated by the enzymes synthesized by *A. oryzae* during the sake brewing process.

Key words: *Aspergillus oryzae*, ferulic acid, koji, HPLC, sake.

INTRODUCTION

Aspergillus oryzae is used in Japanese fermentation industries to manufacture various products including sake, soy sauce and miso due to its ability to produce high levels of hydrolases such as amylases, phytase and phosphatase^{10,13}. Japanese sake, a traditional alcoholic beverage, is produced from steamed rice by the simultaneous utilization of two microorganisms, *A. oryzae* and *Saccharomyces cerevisiae*. During the sake manufacturing process, steamed rice is initially fermented with the fungus *A. oryzae* for 5–7 days. This fermented rice is then mixed with water and the yeast *S. cerevisiae* and incubated at approximately 4°C for 7 days. Preincubated mix-

tures of steamed rice (90 kg), fermented rice (90 kg) and water (440 L) are added three times over the next four days to the fermentation mixture. After incubation at 15–20°C for 2–3 weeks, sake is extracted from the fermentation mixture by a filtration process, heated at 60°C for 30 min and then stored at 15°C. Starch is digested into sugar by enzymes that are produced and secreted into the inoculate by *A. oryzae* during the fermentation process. The sugar is then converted into ethanol by yeast enzymes. Thus, the main role of *A. oryzae* is to provide the amylases, glucoamylases and proteases that hydrolyze the components of rice to produce the nutrients necessary for the growth of *S. cerevisiae*.

Together, *A. oryzae* and *S. cerevisiae* produce a distinct range of hydrolytic enzymes, including xylanases, amylases, tannases, esterases, and proteases, during the fermentation process^{10,13,14}. These enzymes produce compounds such as 3-methylbutanal and ethyl caproate from the saccharides, lipids, and peptides in rice that in addition to ethanol, impart a distinct flavour to sake^{2,16}. Other antioxidants, including ethyl alpha-D-glucoside and certain peptides found in sake, have health benefits and may help to prevent many lifestyle-based diseases such as diabetes and cancer^{13,17–19,26,27,29,30}. For example, ethyl alpha-D-glucoside is hepatoprotective and decreases both the toxicity of ethanol and the epidermal barrier disruption in murine skin caused by UV^{15,17}. Certain peptides found in sake have an antihypertensive effect through angiotensin I-converting enzyme inhibition³⁰.

Ferulic acid is an antioxidant initially bound to the hemicellulose of plant cell walls^{6–8}. Ferulic acid has many pharmacological attributes including anti-inflammatory, anticarcinogenic, and chemopreventive activity³¹.

The levels of ferulic acid production were examined under different fermentation conditions to investigate the mechanism underlying the production of ferulic acid during the fermentation process of sake brewing.

EXPERIMENTAL PROCEDURES

Materials

A. oryzae was purchased from Higuchi Moyashi Co. (Hyogo, Japan). Rice (*Oryza sativa*, Yamadanishiki) was purchased from JA Zennoh and polished to 50% (Tokyo, Japan). Acetonitrile, ferulic acid, methyl ferulate, *p*-coumaric acid, vanillin and silver staining kits were purchased from Wako Pure Chemicals (Tokyo, Japan).

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Assay for ferulic acid

The sample was fractionated at 40°C on a YMC-Pack ODS-A column (YMC Co., Ltd., Kyoto, Japan) using 15% aqueous acetonitrile and 0.05% trifluoroacetic acid (TFA) at a flow rate of 1.2 mL/min (LaChrom, Hitachi, Tokyo, Japan). Peaks were monitored at 280 nm and ferulic acid concentrations were determined relative to external standard solutions.

Fermentation process in the manufacture of sake

The powdered form (40 g) of *A. oryzae* was added to approximately 40 kg of steamed rice (*Oryza sativa*, Yamadanishiki) and incubated at about 35°C for five days. Subsequently, 40 kg of this fermented rice (koji) was mixed with water (210 L), yeast (10 g) and more steamed rice (80 kg). This stage is referred to as the first day of inoculation; the mixture was then incubated for a further seven days at approximately 4°C. A mixture of 90 kg each of steamed and fermented rice, as well as water (440 L), was added three times over the next four days to the above mixture. The entire mixture was then incubated at 15–20°C for an additional 2–3 weeks.

Ferulic acid production using *Aspergillus oryzae*

Fermented rice (3 g) was mixed with 10 mL of water and incubated for 24 h at 25, 37 or 54°C. After centrifugation at $3,000 \times g$ for 10 min, 25 μL of the supernatant was injected into the HPLC.

Addition of vanillin or *p*-coumaric acid to fermented rice

Fermented rice (3 g) was mixed with 10 mL of water and incubated at 37°C for 12 h. The mixture was incubated at 37°C for 12 h after the addition of *p*-coumaric acid or vanillin to a final concentration of 10 mM. After centrifugation at $3,000 \times g$ for 10 min, 25 μL of the supernatant was injected into the HPLC.

Ferulic acid production in the fermented mixture

Fermented rice (3 g) was mixed with 10 mL of water and incubated at 37°C for 12 h. After centrifugation at $3,000 \times g$ for 10 min, 10 mL of the supernatant was mixed with 10 g of steamed rice and incubated at 37°C for 2 h. After recentrifugation at $3,000 \times g$ for 10 min, 25 μL of the supernatant was injected into the HPLC. Sake fermented for 10 days after the initial inoculation was mixed with fresh steamed rice, incubated again, then centrifuged and analyzed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Typically, 20 μL of supernatant from a fermented mixture (produced as described above) was mixed with SDS-loading buffer, incubated at 80°C for 10 min and loaded onto a polyacrylamide gel. SDS-PAGE was performed according to the method of Laemmli²³, using a 4.5% stacking gel and a 15% separating gel at a constant cur-

rent of 16 mA. The separated proteins in the gel were visualized by silver staining. Protein concentrations of supernatants from the fermented mixtures were determined relative to bovine serum albumin (Fraction V, Sigma-Aldrich Japan, Tokyo, Japan) as the standard following the method of Lowry et al.²⁴

Ferulate esterase activity

Fermented rice (3 g) was mixed with 10 mL of water and incubated at 37°C for 12 h. Incubation was carried out for another 2 h after the addition of *p*-coumaric acid to a final concentration of 10 mM or 0.1 mL methanol (control). After centrifugation, the supernatant was mixed with methyl ferulate to a final concentration of 2 mg/mL and incubated at 37°C for 2 h. At the indicated times, 0.5 mL of 50% methanol was added to 0.2 mL of this mixture and a 25 μL sample of the resulting mixture was analyzed by HPLC. At 10 days post-inoculation 1 mL of supernatant from fermented sake was mixed with methyl ferulate to a final concentration of 1 mg/mL. The samples were analyzed following a further incubation at 37°C for 2 h. At 0 h, the ferulic acid concentration was calculated to be 0 $\mu\text{g}/\text{mL}$.

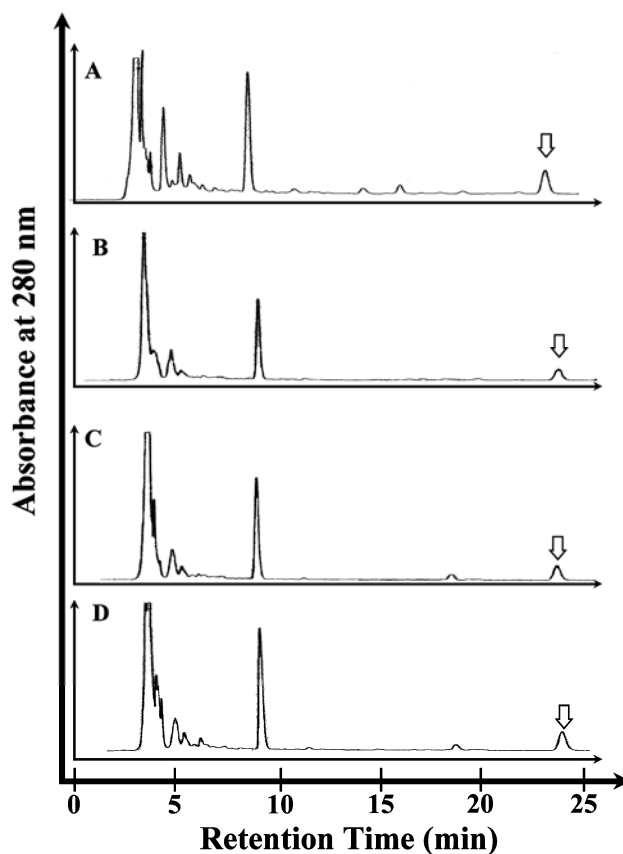


Fig. 1. HPLC chromatograms of sake product A (chromatogram A) and fermented culture (chromatograms B, C, and D). The materials in the fermented rice culture were analyzed by HPLC on day 5 (B), day 10 (C) and day 20 (D). Peaks were monitored at 280 nm. Open arrows indicate ferulic acid.

RESULTS

Ferulic acid in commercial sake

The concentrations of ferulic acid in sake manufactured by various companies (Fig. 1 and Table I) were examined by HPLC. Ferulic acid in a standard solution was eluted after 23.8 min. The ferulic acid concentration was the highest (6.6 $\mu\text{g/mL}$) in Sake A.

Ferulic acid production during fermentation

Having found the ferulic acid content to be the highest in Sake A, the concentrations of ferulic acid during fermentation of this kind of sake were determined. The concentration of ferulic acid was monitored during this process (Fig. 2) and was found to be almost constant for 7 days after inoculation. From 9–12 days post-inoculation, the level of ferulic acid increased dramatically and then only gradually from the 19th day post-inoculation. Apart from the ferulic acid peak, an additional peak eluted at 5.6 min on the 5th day post-inoculation (Fig. 1).

Ferulic acid production with *Aspergillus oryzae*

In the fermentation process described above, *A. oryzae* provides a range of enzymes including amylases, glucoamylases and proteases that hydrolyze the components of rice to produce the nutrients necessary for the growth of the yeast *S. cerevisiae*^{4,10,20,28}. It is likely that *A. oryzae*

Table I. The content of ferulic acid in commercially available sake from four companies^a.

Sake	Ferulic acid ($\mu\text{g/mL}$)
Sake A	6.6 \pm 0.2
Sake B	1.7 \pm 0.2
Sake C	0.11 \pm 0.01
Sake D	0.10 \pm 0.01

^aEach value is the mean of triplicate measurements. Samples A, B, C and D were from four companies. Each of these sakes is classified as junmai daiginjou, which is made from rice only.

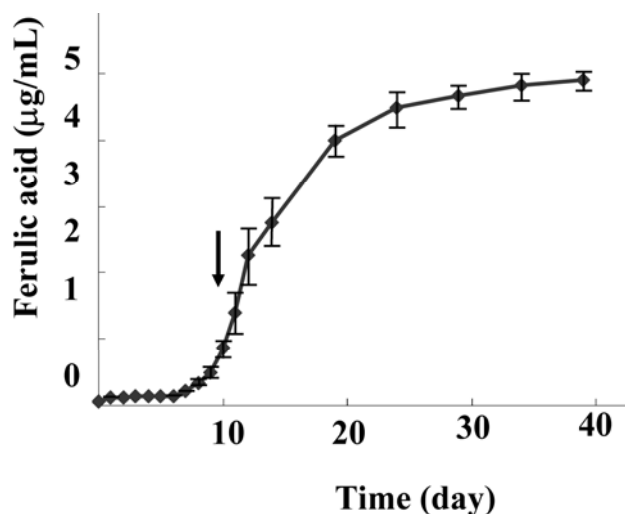


Fig. 2. Time course of ferulic acid production in the process of fermentation. The concentration of ferulic acid in fermented rice culture was determined by HPLC. The arrow indicates 10 days post-inoculation. The values were obtained by averaging three separate results.

also produces enzymes that catalyze the production and secretion of ferulic acid. To test this hypothesis, the ability of *A. oryzae* to produce ferulic acid was examined (Fig. 3). *A. oryzae* produced ferulic acid from steamed rice at 25, 37 and 54°C, with the level of ferulic acid increasing with increasing temperature.

Induction of ferulic acid by aromatic compounds

Aromatic compounds induce production of enzymes such as P450, xylase and ferulate esterase^{7,21,32}. Vanillin

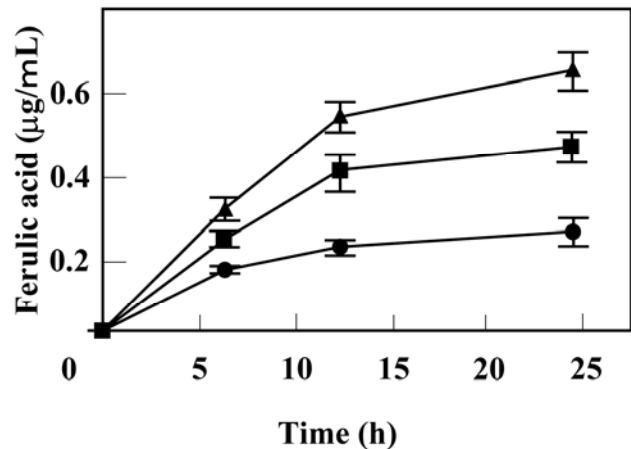


Fig. 3. Ferulic acid production by *A. oryzae*. Steamed rice was fermented with *A. oryzae* for 5–7 days. The fermented rice was then incubated at 25°C (●), 37°C (■) or 54°C (▲) in water. Control rice was incubated at 54°C. The values were obtained by averaging three separate results.

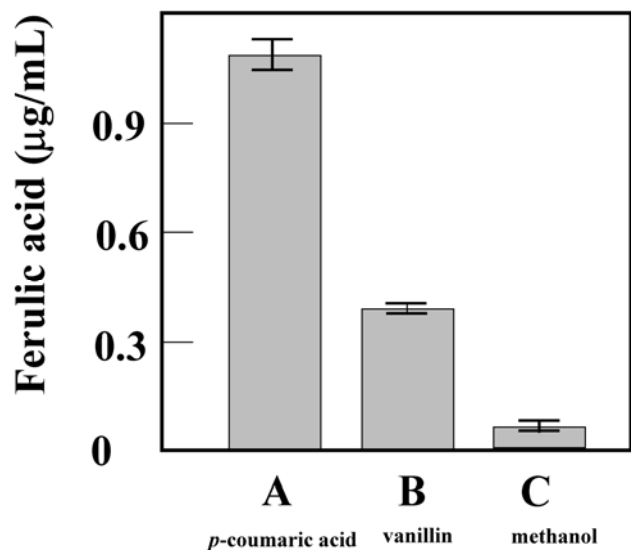


Fig. 4. Induction of ferulic acid by aromatic compounds. Steamed rice was fermented with *A. oryzae* for 5–7 days. The fermented rice was then incubated for an initial 12 h in water prior to the addition of *p*-coumaric acid (A), vanillin (B) and methanol (C) and incubation for a second 12 h. The level of induction of ferulic acid was determined by subtracting the concentration of ferulic acid determined after the first 12 h incubation from the concentration of ferulic acid determined after the total incubation time of 24 h. The values were obtained by averaging three separate results.

(formed from ferulic acid) and *p*-coumaric acid are two aromatic compounds that are structurally very similar to ferulic acid^{7,11}. The ferulic acid content in the inoculate after induction by vanillin and *p*-coumaric acid was measured (Fig. 4). The addition of vanillin and *p*-coumaric acid induced an increase in the concentration of ferulic acid in the culture supernatant by 6.6- and 18.9-fold, respectively.

Ferulic acid production in steamed rice by *A. oryzae*

A. oryzae supernatant was incubated with steamed rice to determine if the proteins produced and secreted by *A. oryzae* into the surrounding medium increase the concentration of ferulic acid. The ferulic acid concentration was 0.96 µg/mL ten days post-inoculation. These results confirm the hypothesis that ferulic acid is liberated from hemicellulose in steamed rice by protein secreted into the fermentation mixture by *A. oryzae*.

SDS-PAGE of *A. oryzae* and fermented rice

SDS-PAGE was performed on the inoculate treated with *p*-coumaric acid and on the fermented sake ten days post-inoculation to examine the expression pattern of the secreted proteins in the inoculate (Fig. 5). Four new bands were detected (17, 18, 44 and 52 kDa) and four other bands (21, 37, 46 and 82 kDa) had decreased in intensity in the inoculate treated with *p*-coumaric acid. Six new bands were detected (32, 36, 39, 59, 62 and 70 kDa) in the

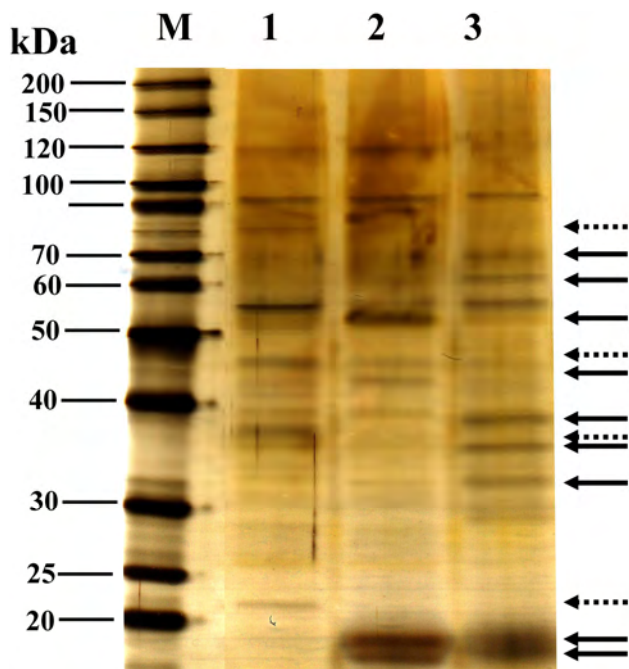


Fig. 5. SDS-PAGE of fermentation products during induction of ferulic acid. Steamed rice fermented with *A. oryzae* for 5–7 days then incubated in water for 12 h (Lane 1). Incubation of fermented rice for 2 h with *p*-coumaric acid (Lane 2). Incubation of the supernatant of fermented sake at day 10 for 2 h with *p*-coumaric acid (Lane 3). Solid arrows indicate the bands in Lanes 2 and/or 3 that had increased in intensity relative to the same bands in the control (lane 1). Dotted arrows indicate the bands in Lanes 2 and/or 3 that had decreased in intensity relative to the same bands in the control (lane 1). Lane M: markers

fermented sake. These results suggest that enzyme activity in the fermented sake mixture plays an important role in ferulic acid production.

Ferulate esterase activity in fermented rice

The activity of ferulate esterase, which liberates ferulic acid in sake using methyl ferulate as a substrate²², was examined (Fig. 6) and the ferulic acid concentration was 12.1 µg/mL, 2 h after substrate addition. The amount of ferulic acid produced as a result of the metabolism of methyl ferulate by the inoculate increased only slightly after induction with *p*-coumaric acid (to 14.9 µg/mL). By comparison, ferulic acid increased 2.2-fold (to 26.4 µg/mL) in the fermented sake 10 days post-inoculation. These results strongly suggest that the increase in ferulic acid content in the fermented sake was caused by the expression of ferulate esterase.

DISCUSSION

Japanese sake, a traditional alcoholic beverage, is produced from steamed rice by two microorganisms, *A. oryzae* and *S. cerevisiae*, and has many health benefits that include protecting the skin and preventing certain diseases^{1,5,9,12,17}. Ferulic acid is one such antioxidant and it has many pharmacological attributes including anti-inflammatory, anticarcinogenic and chemopreventive activity³¹.

HPLC analysis demonstrated the accumulation of ferulic acid during the fermentation of sake, and showed that the rate of ferulic acid production peaked around the 10th day of fermentation (Fig. 1 and 2). A similar pattern of ferulic acid accumulation, with rapid increases from the 10th day of fermentation onwards in another kind of sake was observed (data not shown). During the period from 8–11 days post-inoculation with yeast, steamed rice and water were overlaid on the fermented rice. These tech-

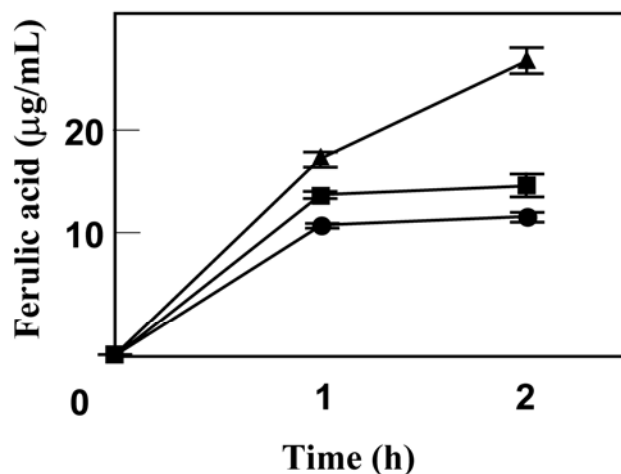


Fig. 6. Ferulate esterase activity in *A. oryzae*. Steamed rice was fermented with *A. oryzae* for 5–7 days. The fermented rice was then incubated for 12 h in water prior to the addition of *p*-coumaric acid (■) or methanol (●) and incubation for an additional 2 h. The fermented medium at day 10 was incubated with methyl ferulate for 2 h (▲). The control was fermented medium incubated without methyl ferulate.

niques may be responsible for the expression of many enzymes by *A. oryzae* and the resulting rapid accumulation of ferulic acid in the fermented rice. Our results also revealed that *A. oryzae* produces ferulic acid in the fermentation mixture, a process enhanced by the addition of aromatic compounds to the medium. The enzymes P450 (monooxygenase), ferulate esterase and xylanase, are also induced by aromatic compounds such as benzoic acid, vanillin and coumaric acid^{7,21,32}. In the present study, the level of induction of ferulic acid by other aromatic compounds, including vanillic acid and benzoic acid, was similar to the level induced by vanillin (data not shown). Thus, it is possible that aromatic compounds induce or repress enzymes responsible for ferulate production. This was confirmed by SDS-PAGE analysis, which revealed the presence of ferulic acid and certain proteins in the supernatant of the *A. oryzae*-containing fermentation mixture; the levels of these proteins increased or decreased after the addition of the aromatic compounds by the 10th day of the fermentation process (Fig. 5). Enzymes, including protease, glycosidases and phosphatases, have been purified and characterized from *A. oryzae*^{10,14,16,25}. Further investigation is required to determine if the proteins responsible for ferulic acid production in the fermentation mixture include any of the proteins already purified from *A. oryzae*.

Ferulate esterase liberates ferulic acid from hemicellulose^{6,8}. Our results show that ferulate esterase activity was elevated in the fermented sake mixture by the 10th day of the fermentation process (Fig. 6). FAE activity from sake was higher than that from *p*-coumaric acid-inducing culture.

Ferulate esterases from *Aspergillus* spp. exist mainly in two forms, one approximately 30 kDa (FAEA) and the other approximately 75 kDa (FAEB) in size³. It is possible that the bands at 32 and 70 kDa revealed by SDS-PAGE represent the two forms of ferulate esterase that remain unchanged in intensity following the addition of *p*-coumaric acid (Fig. 5). The mechanism underlying the increase in the level of ferulic acid during sake fermentation may involve the induction of ferulate esterase and other ferulic acid-forming enzymes and/or the repression of hydrolytic enzymes that catabolise ferulic acid. Mass spectroscopic studies are being conducted to further elucidate these mechanisms and to identify the proteins secreted during sake fermentation.

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