Journal of Bioscience and Bioengineering
VOL. 118 No. 6, 640–645, 2014

www.elsevier.com/locate/jbiosc

Factors affecting phenolic acid liberation from rice grains in the sake brewing process

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Received 15 March 2014; accepted 19 May 2014
Available online 21 June 2014

Phenolic acid (ferulic and *p*-coumaric acid) liberation from rice grains was examined using rice samples containing phenolic acid at different levels, using two sake mash simulated digestion tests to elucidate influencing factors. Phenolic acid levels in a digest made from steamed rice using dialyzed rice *koji* enzymes were smaller than levels in a rice *koji* self-digest. Differences in phenolic acid levels among rice samples in the rice *koji* self-digest were larger than levels in a digest of steamed rice. In the rice *koji* self-digest, phenolic acid levels in the ingredient rice grains or in the formed digest related to feruloylesterase (FE) activity in the rice *koji*. Addition of exogenous FE to rice *koji* self-digestion increased phenolic acid levels, while addition of xylanase (Xyl) showed weak effects. A concerted effect of FE and Xyl was not clearly observed. Addition of ferulic acid to *koji* made from α-rice grains raised FE activity, but it did not increase the activity of other enzymes. A similar phenomenon was observed in an agar plate culture of *koji* mold. These results indicated that ferulic acid levels in ingredient rice grains correlate with FE activities of *koji*, as a resulut, they affect the phenolic acid levels in sake mash.

[**Key words:** Rice; *Koji*; Sake; Phenolic acid; Feruloylesterase]

Ferulic acid is a well-known antioxidant in sake (1), however, it is an unfavorable tasting compound with a potent bitter and/or astringent taste (2). Charcoal treatment significantly decreased ferulic acid levels in sake (2), while the levels of ferulic acid in several charcoal-untreated sake samples exceeded threshold values (3). *p*-Coumaric acid, also found in sake, has a similar taste to ferulic acid and has shown a lower threshold value than ferulic acid (4). Thus, control of phenolic acid (ferulic and *p*-coumaric acid) levels in sake is very important for the sensory quality. Charcoal treatment can cause a loss of favorable taste harmonies. In recent years, some sake products, such as *daiginjo*, have been commercialized without charcoal treatment, making it important to control phenolic acid levels in sake without such treatment.

Ferulic and *p*-coumaric acid are major phenolic acids in rice grains (5). In the plant cell wall ferulate, in polysaccharide esters, produces dehydrodimers which form strong polysaccharide–polysaccharide cross-linkages which may be involved in the mechanical properties and biodegradability of the plant (6,7). Phenolic acid is abundant in the outer layer of rice grains and is remarkably diminished by milling from brown rice to 90% polished rice (8,9). Further milling treatment from 90% to 70% is very effective in decreasing the phenolic acid levels, especially *p*-coumaric acid (10). Levels of phenolic acid in brown and 70% polished rice differed among cultivars and production areas (10). In the sake brewing process, phenolic acid is liberated from rice grains by rice *koji* enzymes in the mash, and the selected yeast strain (*Saccharomyces cerevisiae*) used in sake brewing for ethanol fermentation does not affect ferulic acid under the sake mash conditions (11). Therefore, the hydrolysis process of phenolic acid from rice grains in sake mash may be a crucial point in the control of phenolic acid levels in sake.

Biodegradation of plant cell wall xylan by *Aspergillus* enzymes has been well studied (12–14). For efficient degradation of plant xylan, an enzyme set including α-1,4-endoxylanase (Xyl), β-xylosidase, acethylxylanesterase, α-L-arabinofuranosidase, feruloylesterase (FE), and α-glucuronidase, have to concertedly participate. Many of the genes encoding *Aspergillus* enzymes are coordinately expressed under the control of a common transcriptional activator, XlnR (12,14). FE hydrolyzes phenolic acid from xylan (15), and enzyme activity is induced by addition of ferulic acid to the culture media of *Aspergillus niger* (16,17). The rice *koji* making process for sake brewing is a solid-state culture using steamed rice grains, under which conditions the *koji* mold, *Aspergillus oryzae*, preferentially produce solid-state specific enzymes (18,19). There is very little information regarding the production of FE by *A. oryzae* in the rice *koji* making process for sake brewing.

In 1967, a study reported the effect of polishing rate of ingredient rice grains on phenolic compounds in sake (20) and Uno et al. (21) researched the formation of ferulic acid in the sake brewing process, however, it is not clear how the different levels of phenolic acid in ingredient rice grains affect the levels in sake. Hence, we investigated factors affecting phenolic acid liberation from rice grains in the sake brewing process, especially focusing on the rice *koji* making process and *koji* enzymes, using polished rice grains at different polishing ratios.

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.05.013

TABLE 1. Ferulic and *p*-coumaric acid contents of material brown rice.

Cultivar	FA (mg/kg)	*p*-CA (mg/kg)
Omachi	572 ± 18[a]	105 ± 14
Miyamanishiki	483 ± 18	87 ± 2
Gohyakumanngoku	514 ± 15	121 ± 4
Yamadanishiki	384 ± 22	87 ± 13
Akitasakekomachi	387 ± 14	106 ± 11

[a] Mean ± SD of three determinations.

MATERIALS AND METHODS

Materials Brown rice from five cultivars (Omachi, Miyamanishiki, Gohakumangoku, Yamadanihsiki, and Akitasakekomachi) harvested in 2007 was used. They were milled using a vertical type rice-milling machine to polishing rates of 75%, 66%, 60%, or 50% by weight to the starting brown rice. Phenolic acid content in brown rice samples are shown in Table 1. Pregelatinized rice (α-rice) was prepared from 60% polished Akitasakekomachi steamed rice grains using an ethanol dehydration method (22). Moisture and ferulic acid content of the α-rice were 11.3% and 26.7 mg/kg, respectively. Dialyzed and lyophilized rice *koji* enzyme was prepared using rice *koji* for sake brewing, made from 60% polished *Yamadanishiki* rice grains and *A. oryzae* RIB128, in accordance with a previous report (11). FE and Xyl enzymes used in the digestion test was prepared from Hemicellulase Amano 90 (originated from *Aspergillus* sp., Amano Enzyme Co., Nagoya, Japan) using anion exchange chromatography, as reported previously (12).

Analysis of phenolic acid Total phenolic acids were extracted from flour samples using the NaOH hydrolyzing method (23) and determined by HPLC (10). Free phenolic acids in flour samples (100 mg) were extracted by 4 ml of 70% (v/v) ethanol and 0.1 M succinic acid aqueous solution in a capped glass tube with shaking at 180 rpm for 60 min. After centrifugation at 600 ×*g* for 5 min, obtained supernatant was analyzed by HPLC. The extraction procedure was conducted three times independently. The moisture content of sample flour was obtained from a loss of sample weight under 135°C for 3 h. Analytical results of the ferulic and *p*-coumaric acid content were expressed on a dry matter basis.

Rice *koji* making (solid-state culture of *A. oryzae* from the steamed rice grain) Rice *koji* for self-digestion tests was prepared from 50% or 75% polished rice grains of five cultivars. Polished rice grains of 100 g in each cultivar was steeped in water for 60 min, before being steamed for 50 min. The water absorption ratio after steaming (increase in weight to the original rice sample) was adjusted to 33 ± 0.5%, then the rice and 2 mg of conidia (spore) from *A. oryzae* RIB128 were mixed in a plastic container. *Koji* making was performed in the container using a temperature and humidity chamber PR-1KP (ESPEC CORP. Osaka, Japan). The conditions were as follows: (i) the mix was kept at 31°C for 22 h in the closed plastic container, then it was mixed once and the rice was covered using filter paper, (ii) temperature was then linearly raised to 40°C over 8 h under 90% humidity. In the third step, the temperature was raised to 42°C over 14 h under a constant humidity of 85%. The increase in weights of prepared rice *koji* to the ingredient polished rice ranged from 15% to 17% for 50% polished rice and from 12% to 14% for 75% polished rice. α-Rice *koji* making for the ferulic acid addition test was performed as follows: after adjustment of the moisture content of α-rice to 13.5%, α-rice (15 g), water (5.25 ml), *A. oryzae* RIB128 spores (1 mg), and a small amount of ferulic acid ethanol solution were mixed well before being placed into a plastic petri dish (90 mm × 15 mm) and cultivated under the above mentioned conditions for rice *koji* making. This test was conducted in triplicate. The increase in weights of rice *koji* to the ingredient α-rice ranged from 21% to 27%.

Agar plate culture One mg of *A. oryzae* RIB128 spores was inoculated on a modified Czapeck–Dox medium plate containing 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.2% KCl, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO4 \cdot 7H_2O$, 0.5% glucose, 2.0% starch, 3.0% agar and ferulic acid at 100 mg/L (or none for the control) and cultivated at 30°C for 3 days.

Digestion test Digestion test of rice *koji* (self-digestion) was conducted as follows: rice *koji* made from 1.0 g of ingredient rice grains was digested in 2.5 ml of 9.5% ethanol, 0.2 M succinate buffer (pH 4.3) at 30°C for 24 h, with shaking (120 rpm). The steamed rice digestion test was conducted as follows: steamed rice grains prepared from 2.0 g of polished ingredient rice grains was digested in 5.0 ml of 9.5% ethanol, 0.2 M succinate buffer (pH 4.3) containing dialyzed and lyophilized rice koji enzyme at 30°C for 24 h, with shaking (120 rpm). After digestion, it was centrifuged at 15,000 ×*g* for 10 min, the obtained supernatant was analyzed by HPLC and a digital refractometer, as reported previously (10). These digestion tests were conducted in triplicate.

Enzyme assay The *koji* extract solution was treated with a PD-10 column (GE Healthcare UK Ltd., Buckinghamshire, UK) before assay. FE activity was assayed according to a previously published method (11). Xyl activity was assayed using beech wood xylan (SERVA Electrophoresis GmbH, Heidelberg, Germany)

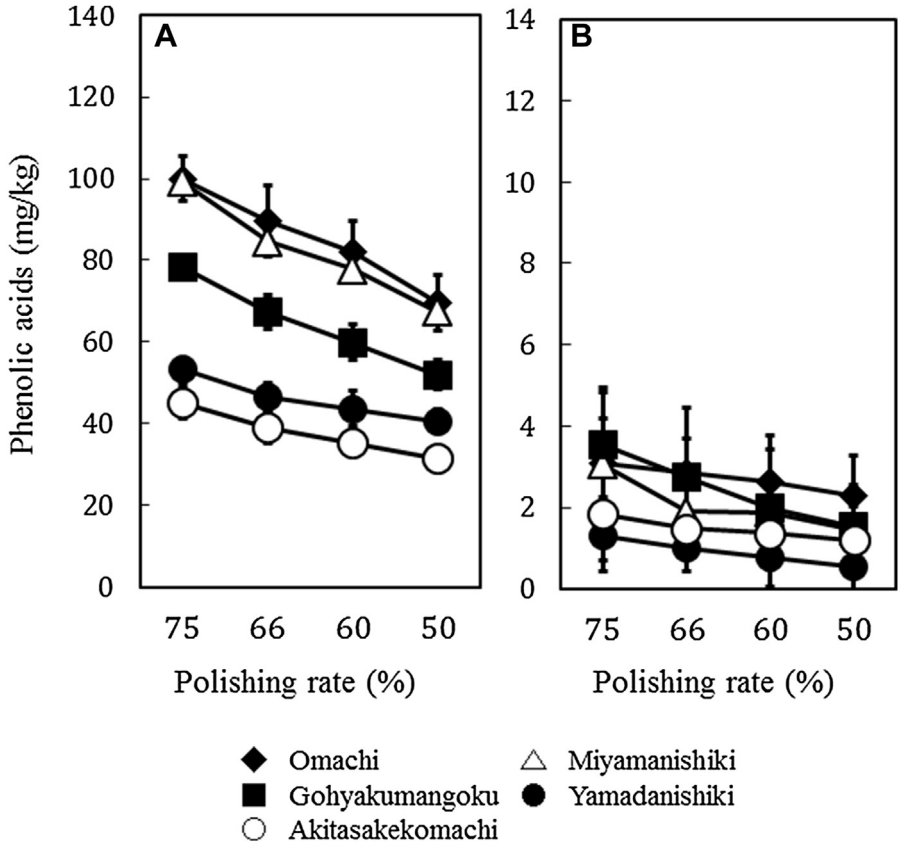

FIG. 1. Effects of polishing rate on levels of ferulic acid (A) and *p*-coumaric acid (B) in rice grains. Values are means ± SD of three determinations.