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Identification of enzymes from genus *Trichoderma* that can accelerate formation of ferulic acid and ethyl ferulate in collaboration with rice *koji* enzyme in sake mash

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The enzymes responsible for acceleration of ferulic acid and ethyl ferulate formation in sake mash were studied. Ferulic acid and ethyl ferulate are formed during the sake brewing process from feruloylated glucuronoarabinoxylan. Cellulase reagent from genus *Trichoderma* was used instead of rice *koji*, because rice *koji* for sake brewing produces extremely low levels of xylan-degrading enzymes. A combination of the reagent with rice *koji* enzymes accelerated the formation of ferulic acid from α-rice powder. Addition of the reagent to sake mash increased ferulic acid and ethyl ferulate formation. The enzyme responsible for the accelerated formation was purified using a newly developed assay method and α-rice powder as a substrate. During the assay procedure, feruloylated oligosaccharide was converted to ferulic acid by feruloylesterase for HPLC analysis. Analysis of the N-terminal amino acid sequence of the purified samples was successfully conducted after pyroglutamyl aminopeptidase de-blocking. Purified enzymes were identified as members of the glycoside hydrolase family 10 (GH10) and family 11 (GH11) xylanases by BLASTP database research. The GH10 xylanase showed higher specific activity for α-rice powder and insoluble wheat arabinoxylan compared with GH11 xylanase; the GH11 xylanase showed higher specific activity for the other xylan substrates, especially glucuronoarabinoxylan. The GH10 xylanase showed higher accelerating activity than the GH11 xylanase in the sake mash. The results of this study provides useful knowledge on ferulic acid and ethyl ferulate formation in sake mash, the relative levels of these compounds and their influence on the sensory quality of sake.

[**Key words:** Sake; Rice; *Koji*; Ferulic acid; GH10 xylanase; GH11 xylanase]

Ferulic acid, a ubiquitous phenolic acid in the plant kingdom, has been reported to have many pharmaceutical functions (1,2). Ethyl ferulate, a naturally occurring product in Japanese alcoholic beverages, is reported to have antioxidant activity and various physiological functions, similar to ferulic acid (3–5). Ferulic acid has sour, bitter, and astringent flavors and contributes to the taste characteristics of grain flour products (6,7). Previous reports suggested the sensory role of ferulic acid in beer and red wine was very low because the concentration did not reach a significant level (8–10), whereas it is reported to have a bitter or astringent taste in sake, contributing to the sensory quality, especially in charcoal-untreated products (11,12). Ethyl ferulate has a moderate bitter and sweet taste in sake and can mask the unpleasant bitter or astringent taste of ferulic acid (12), therefore it is important to control ferulic acid and ethyl ferulate levels in the sake manufacturing process.

Ferulic acid and ethyl ferulate are formed during the sake brewing process by rice *koji* enzymes from feruloylated glucuronoarabinoxylan in the endosperm cell wall of rice grains (13,14). Ferulic acid is hydrolyzed by type A feruloylesterase from *Aspergillus oryzae* (15) during the final forming step. Ethyl ferulate is formed by transesterification using the same enzyme but under sake mash conditions where the ethanol concentration often reaches up to 20 %. Both hydrolyzation and esterification proceed rapidly when the substrate is a feruloylated oligosaccharide (16). It is reported that sake *koji* mold (*A. oryzae*) produces extremely low levels of xylan-degrading enzymes when grown on polished steaming rice for sake brewing (17). In the common sake brewing process, less than 20 % of the ferulic acid in the rice grains is transferred to sake: the low transfer ratio may be due to the very low levels of xylan-degrading enzymes in rice *koji*.

The object of this study was to investigate enzymes responsible for an acceleration in the formation of ferulic acid and ethyl ferulate in sake mash. We used a commercial cellulase reagent from genus *Trichoderma* instead of rice *koji* in the sake brewing process. The cellulase reagent has high xylan-degrading activity but little feruloylesterase activity which is in contrast to the rice *koji* which contains low levels of xylan-degrading enzymes. We developed a new assay method in which α-rice powder was used as the substrate. The formation of ferulic acid precursor was determined as the amount of ferulic acid measured after the feruloylesterase reaction. Using this method, we purified target enzymes and

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identified them by analysis of their N-terminal amino acid sequence and BLASTP database searches. Proteins purified were examined for their enzymatic properties. The results of this study may provide useful knowledge of ferulic acid and ethyl ferulate formation in sake mash, and may help in controlling the levels of these compounds in sake.

MATERIALS AND METHODS

Chemicals and materials Cellulase T "Amano" 4 enzyme reagent was gifted by Amano Enzyme Inc. (Nagoya, Japan). α-Rice powder was prepared from 60 % polished *Akitasakekomachi* α-rice grains by milling for 2 min using an Iwatani Millser IFM-77G (Osaka, Japan). Feruloylesterase was prepared from a Hemicellulase Amano 90 enzyme reagent (Amano Enzyme Inc.) using anion exchange chromatography, as reported previously (15). Ferulic acid and ethyl ferulate were purchased from Fujifilm Wako Pure Chemical Industries (Osaka, Japan). Beech wood xylan, Remazol Brilliant Blue R-D-xylan (RBB xylan), and glucuronoarabinoxylan (wheat bran) were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany), Sigma-Aldrich Japan Inc. (Tokyo, Japan), and OligoTech (Crolles, France), respectively. AZCL-arabinoxylan (wheat) and insoluble wheat arabinoxylan were purchased from Megazyme Inc. (Chicago, IL, USA). Sequi-Blot PVDF membrane and Coomassie G-250 stain (CBB) were obtained from Bio-Rad Laboratories, Inc. (CA, USA). Ponceau-S staining solution was purchased from Beacle, Inc. (Kyoto, Japan). *Pfu* pyroglutamate aminopeptidase was obtained from Takara Bio Inc. (Shiga, Japan). All HPLC columns for enzyme purification and Toyopearl DEAE-650 were purchased from Tosoh Co. (Tokyo, Japan).

Preparation of rice *koji* enzyme and its extract Rice *koji* was prepared from 40 % *Akitasakekomachi* or 70 % polished *Ginsan* steamed rice using *A. oryzae* RIB128 according to the standard sake *koji* making process (14). Rice *koji* extract was prepared as reported previously (14). Protein concentration was measured using the Bradford method, and BSA as the standard (18).

Formation test of ferulic acid from α-rice powder by rice *koji* enzymes and the cellulase reagent Reaction mixture consisted of 20 mg α-rice powder, 40 μL of 1 M sodium citrate buffer (pH 5.0), 60 μL of enzyme solution, and 180 μL of water. The concentration of the cellulase reagent was 1 mg/mL. The reaction was conducted for 15 h at 30°C with reciprocal shaking at 120 rpm. After the addition of 200 μL of acetonitrile, the reaction mix was centrifuged (15,000 ×g for 15 min), before the formation of ferulic acid in the supernatant was measured using HPLC, as described previously (12).

Enzyme assay Ferulic acid precursor forming activity was assayed using α-rice powder as a substrate. The reaction mixture consisted of 20 mg α-rice powder and 180 μL of 100 mM sodium citrate buffer (pH 5.0) and enzyme solution. After the reaction at 30°C for 60–180 min with shaking at 1000 rpm, the reaction mixture was centrifuged (15,000 ×g for 15 min) before 20 μL of the supernatant was mixed with 20 μL of the feruloylesterase solution. The enzyme reaction was incubated at 30°C for 60 min and the formation of ferulic acid was measured using HPLC, as described previously (12). A blank reaction without feruloylesterase was measured as the control. Ferulic acid precursor forming activity using insoluble wheat arabinoxylan as the substrate was assayed as described for α-rice powder with the exception that the substrate amount was 1 mg. One unit of enzyme activity was defined as the amount of enzyme required to form ferulic acid after the feruloylesterase reaction, at the rate of 1 μmol min^{-1} at 30°C. Feruloylesterase activity was determined using ethyl ferulate as a substrate (15). Xylanase activity using beech wood xylan or glucuronoarabinoxylan was assayed at 30°C for 60 min in 0.2 M sodium succinate buffer (pH 4.3). The formation of reducing sugar was assayed using the Somogyi–Nelson method, as reported previously (14). RBB-xylanase activity was assayed at 30°C in 0.2 M sodium succinate buffer (pH 4.3) (19). AZCL-arabinoxylan degrading activity was assayed at 30°C in 0.2 M sodium succinate buffer (pH 4.3) with shaking at 1300 rpm (20).

Small-scale sake brewing and enzyme addition test Small-scale sake brewing was conducted to examine the effects of the cellulase reagent on the levels of ferulic acid and ethyl ferulate in sake mash. Two kinds of rice *koji* made from 40 % polished *Akitasakekomachi* and 70 % polished *Ginsan* were used for this test. All steamed rice (*kakemai*) was made from 70 % polished *Ginsan*. The sake brewing process used a total of 500 g of rice (steamed rice plus *koji* rice) and *Saccharomyces cerevisiae* K-1501 yeast, added in a three step addition protocol: first addition (*Soe*) 100 g, second addition (*Naka*) 150 g, and third addition (*Tome*) 250 g. The mashing temperature of *Soe*, *Naka*, and *Tome* was 17°C, 10°C, and 8°C, respectively. The ratio of *koji* rice to *kakemai* rice was 20 % and the ratio of water to mashing rice was 130 %. After the third addition step (*Tome*), the mash temperature was raised 0.5°C per day to a maximum temperature of 12°C and then decreased 0.5°C per day to 8°C before being maintained at 8°C for 10 days. The cellulase reagent (0.5 g) was added 15 days after *Tome*. The sake was separated from the solids using centrifugation (1200 ×g for 10 min) 25 days after *Tome*. Purified enzyme was added to a sake mash made from 20 g of 40 % polished *Akitasakekomachi* rice *koji* and 80 g of steaming rice (*kakemai*). The mashing method was as described above in the 500 g sake brewing method. The purified enzyme samples were added to the mash when the ethanol concentration reached 8.5 %. Enzyme sample (100 μl) was added to 0.9 g of the mash and kept at 10°C for 3 days with shaking (1000 rpm).

Purification of a ferulic acid precursor forming enzyme Cellulase T Amano 4 enzyme reagent (80 g) was dissolved in 400 mL of 50 mM sodium phosphate buffer (pH 6.8), before purification by 80 % saturation of ammonium sulfate precipitation. The precipitate was dialyzed against 10 mM sodium phosphate buffer (pH 6.8) and treated with Toyopearl DEAE-650 (75 mL). The enzyme solution was re-precipitated by 80 % saturation of $(NH_4)_2SO_4$ and the precipitant was dissolved in a small volume of 10 mM sodium phosphate buffer (pH 6.8) before it was applied to a TSKgel Ether-5PW column (7.5 × 75 mm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 1.2 M $(NH_4)_2SO_4$. The column was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.2–0.0 M). Two active fractions of the hydrophobic chromatography were pooled and individually applied to a TSKgel SQ-5PW column (7.5 × 75 mm) after dialysis against Tris-phosphate buffer (pH 9.15). The column was eluted with 10 mM Tris-phosphate buffer (pH 9.15) and the active fraction was lyophilized. The active fraction was then dissolved in a small volume of 10 mM Tris-phosphate buffer (pH 9.15), and applied to a TSKgel DEAE-5PW column (2.0 × 75 mm). The column was eluted with Tris-phosphate buffer (pH 9.15) and the active fraction was lyophilized. After dialysis against 10 mM sodium phosphate buffer (pH 4.6) the active fraction was applied to a TSKgel CM-5PW column (7.5 × 75 mm) equilibrated with the same buffer. The enzyme was eluted using a NaCl gradient (0–0.6 M). The active fraction was concentrated by Amicon Centriprep, then applied to the same column for re-chromatography. Part of the active fraction from the second CM-5PW column chromatography was applied to a TSKgel SuperSW3000 column (4.6 × 300 mm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 0.2 M NaCl. The column was eluted with the same buffer. Protein concentration was estimated using an absorbance of 280 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and N-terminal amino acid sequencing Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (21). Protein was blotted onto PVDF membrane and stained using CBB or Ponceau-S staining solution. The CBB stained band was analyzed using an Applied Biosystems 491 protein sequencer. The Ponceau-S stained band was treated with *Pfu* pyroglutamate aminopeptidase according to Hirano et al. (22), before analysis using a Shimadzu PPSQ-31A protein sequencer.

RESULTS AND DISCUSSION

Formation of ferulic acid from α-rice powder by rice *koji* enzymes and the cellulase reagent Ferulic acid forming activity from α-rice powder by rice *koji* enzymes or the cellulase reagent and the effects of combined addition of rice *koji* enzyme and cellulase reagent were examined (Fig. 1). The cellulase reagent, which has little feruloylesterase activity but high feruloyl oligosaccharide forming activity (Okamoto et al., Japanese patent application, Tokukai2007-202504, 2007), produced greater amounts of ferulic acid than the rice *koji* enzyme. Combined use of rice *koji* enzymes and the cellulase reagent produced even larger amounts of ferulic acid (Fig. 1). Ferulic acid forming activity was not dependent on feruloylesterase activity. It was suggested that ferulic acid formation from α-rice powder by *koji* enzymes is restricted at the precursor forming step. The precursor is suggested to be feruloyl oligo-arabinoxylan. The reaction may be similar to the synergistic effects between xylanase and feruloylesterase in the release of phenolic acid from cereal arabinoxylan (23,24).

Addition test of the cellulase reagent to the sake mash Amylase and protease activity within the cellulase reagent is very low and the major components among the brewed sake samples were similar. Ethanol concentration ranged from 18.40 to 18.95 %, and extract content ranged from 6.5 to 7.8 %. Titratable acidity ranged from 1.55 to 1.60, and amino acidity ranged from 1.55 to 1.60. Addition of the cellulase reagent increased ferulic acid and ethyl ferulate (Fig. 2A and B), even under low temperature (8°C) and high ethanol conditions (16–18 %). The ratio of ethyl ferulate to ferulic acid, an important indicator of sake quality (12), was clearly increased in the 40 % *Akitasakekomachi koji* mash, but not in the 70 % *Ginsan koji* mash (Fig. 2C and D). High feruloylesterase activity was observed in the

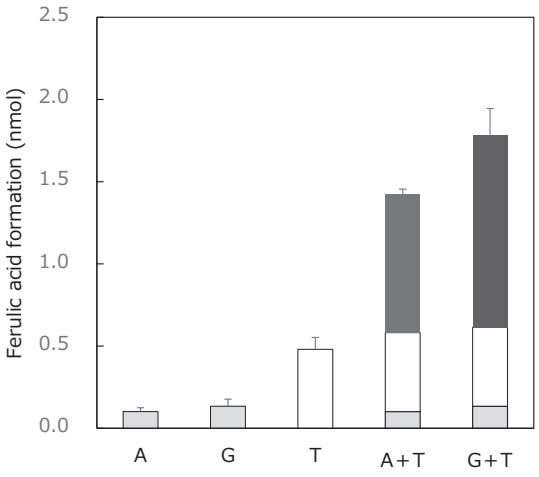

FIG. 1. Ferulic acid forming activity from α-rice powder by rice *koji* enzymes or the Cellulase T Amano 4 reagent and the effects of combined addition of rice *koji* enzyme and cellulase reagent. A, G, and T indicate 40 % *Akitasakekomachi koji* extract, 70 % *Ginsan koji* extract, and Cellulase T Amano 4 agent solution, respectively. A + T indicate combined addition of A and T, G + T indicate combined addition of G and T, respectively. The protein concentrations of A, G, and T were 0.063, 0.089, and 0.029 mg/mL, respectively. The feruloylesterase activities of A, G, and T were 0.049 ± 0.008, 0.125 ± 0.009, and 0.014 ± 0.002 nmol/min/reaction mixture, respectively. Bars indicate the amount of ferulic acid formed; shaded bars, rice *koji* enzyme; open bars, cellulase reagent; closed bars, synergistic effects of rice koji enzyme and the cellulase reagent.

70 % *Ginsan koji* which may lead to rapid hydrolyzation of ethyl ferulate, leading to an equilibrium state between ferulic acid and ethyl ferulate, which is governed by the pH and ethanol concentrations (18).

Purification of the enzyme in the cellulase agent which accelerated the release of ferulic acid from α-rice powder A newly developed assay method was used to assess enzyme activity in which the natural substrate, α-rice powder, simulated the sake brewing process; detection of the ferulic acid precursor in the feruloylesterase reaction, which occurs after the main reaction, focused on the activity of the target enzyme. The purification procedure is summarized in Table 1. During hydrophobic chromatography, the active fraction was divided into two pools (I and II) (Fig. S1) which were purified separately, as described in the following steps: absorbance was used to monitor which pooled fraction matched the enzyme activity measured in the 2nd cation exchange chromatography (Figs. S2A and B). The specific activity measured in fraction II matched the required enzyme activity whereas enzyme activity in fraction I did not. It was estimated that a lot of protein of low specific activity was contained in the sample I. After the final size exclusion chromatography, the fold purification of fraction II was 97 and that of fraction I was 17. Through the purification procedure, the recovery ratio was very low which might be due to inactivation by freeze thawing cycles of both samples. In addition, fast degradation might have occurred, especially in sample I. Possible inactivation by freezing and thawing was prevented by addition of 50 % glycerol at the final purification step before storage at −20°C.

SDS-PAGE analysis, N-terminal amino acid sequencing, and identification SDS-PAGE analysis of the active fractions from the second cation exchange chromatography revealed that fraction I had 3 major bands of 21, 12, and 9.5 kDa and fraction II had a single major band of 33.5 kDa (Fig. 3). The N-terminus of the protein in fraction II (Fig. 3) was analyzed using a protein sequencer but could not be determined. All four detected bands were de-

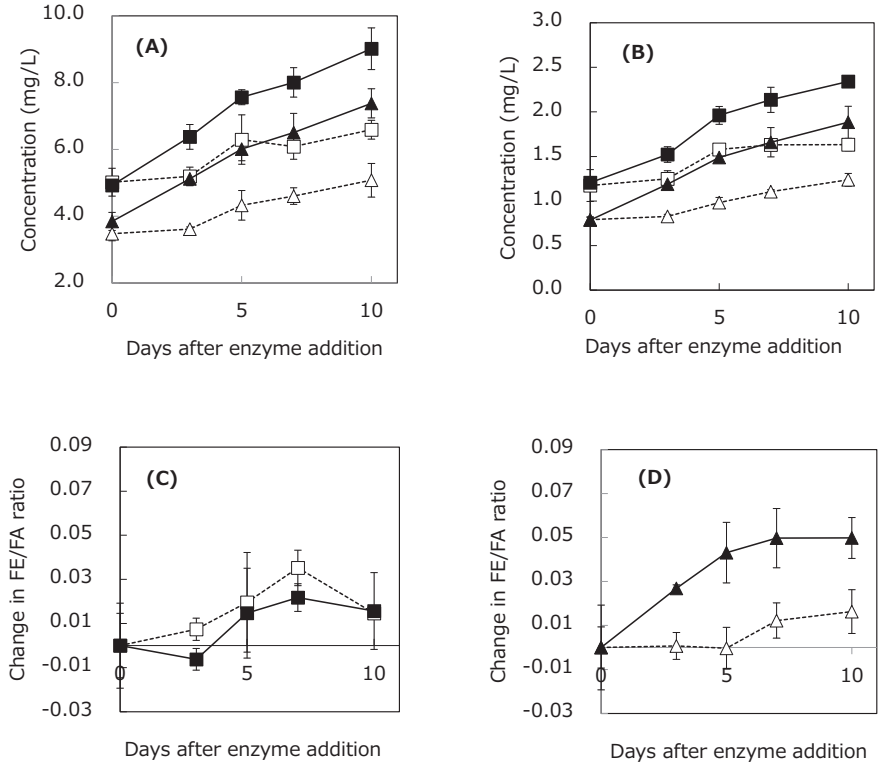

FIG. 2. Effects of the cellulase reagent addition on ferulic acid and ethyl ferulate levels in two sake mashes. (A) Variation of ferulic acid concentration. (B) Variation of ethyl ferulate concentration. (C) Change of ethyl ferulate (FE)/ferulic acid (FA) ratio in 70 % *Ginjan koji* mash. (D) Change of FE/FA ratio in 40 % *Akitasakekomachi koji* mash. The ferulic acid precursor forming activity of the added cellulase reagent was 5.5×10^{-5} U/g of the sake mash. Data are the mean and SD of three determinations. Symbols are as follows: closed squares, 70 % *Ginjan koji* and the enzyme reagent; open squares, 70 % *Ginjan koji* blank; closed triangles, 40 % *Akitasakekomachi koji* and the enzyme reagent; open triangles, 40 % *Akitasakekomachi koji* blank.

TABLE 1. Purification of the enzyme which accelerated the release of ferulic acid from α-rice powder.

Step	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude solution	1752	5.25	0.003	100.0	1.0
$(NH_4)_2SO_4$ precipitation	1128	2.42	0.002	46.0	0.7
Hydrophobic chromatography					
Pool I	210	0.84	0.004	16.0	1.3
SQ-5PW chromatography	81	0.65	0.008	12.3	2.6
DEAE-5PW chromatography	73	0.51	0.007	9.7	2.3
CM-5PW chromatography (twice)	0.43	0.002	0.004	0.1	1.3
Super SW3000 chromatography[a]	6.8×10^{-4}	3.5×10^{-6}	0.052	6.7×10^{-7}	17.3
Pool II	141	0.56	0.004	10.6	1.3
SQ-5PW chromatography	41	0.37	0.009	7.0	3.0
DEAE-5PW chromatography	23	0.37	0.016	7.0	5.3
CM-5PW chromatography (twice)	0.12	0.004	0.031	0.6	10.3
Super SW3000 chromatography[a]	7.8×10^{-4}	2.3×10^{-4}	0.292	4.3×10^{-5}	97.3

[a] Part of the sample from the previous purification step was applied.

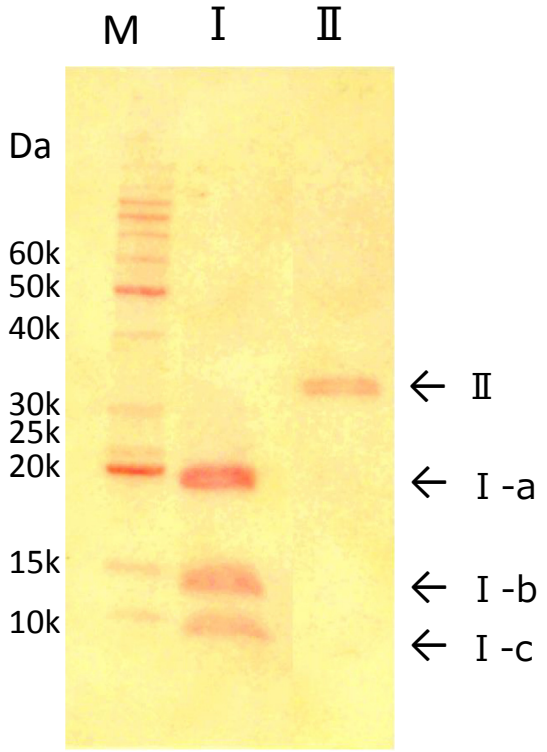

FIG. 3. SDS-PAGE analysis of the active fractions of the second cation exchange chromatography. Proteins were stained with Ponceau-S staining solution. Lane M, molecular mass marker; lane I, fraction I; lane II, fraction II. Three bands of I-a, I-b, and I-c from fraction I and a single band of II from fraction II were analyzed using a protein sequencer.

blocked by *Pfu* pyroglutamate aminopeptidase: three bands (I-a, I-c and II) could be analyzed, but I-b could not. According to BLASTP database searches, band I-a (21 kDa) matched the N-terminus of glycoside hydrolase family 11 xylanase (GH11 xylanase) of *Trichoderma citrinoviride* (accession no. XP_024751773.1; top score 55.8) (Fig. 4). Band I-c (9.5 kDa) matched an internal sequence of the same protein (top score 52.8). It was suggested that bands I-b (12 kDa) and I-c might be formed by degradation of band 1-a. Band II (33.5 kDa) matched the GH10 xylanase of *T. citrinoviride* (accession no. XP_024754233.1; top score 59.6). Thus, fractions I and II were identified as GH11 and GH10 xylanase (25), respectively. The observed characteristics of the GH11 xylanase were well matched with the major xylanase of high pI value (9.0) of *Trichoderma ressei* (26), and the GH10 xylanase was well matched to the xylanase III of *T. ressei* PC-3-7 (27). The molecular mass, estimated by size exclusion chromatography, showed far smaller values: 8 kDa for sample I and 9 kDa for sample II (Figs. S3A and B). The differences might be due to the affinity between the sample protein and the gel filtration chromatography carrier. In this analysis, fast degradation of sample protein was observed: the peak fraction of sample I showed a smeared broad band at 16–20 kDa and sample II showed a sharp single band at 35 kDa and a lot of smaller molecular weight constituents (Figs. S3A and B).

Properties of identified enzymes Substrate specificity of the identified enzymes is shown in Table 2. The GH10 xylanase showed 5.6 and 3.6 times higher specific activity for α-rice powder and insoluble wheat arabinoxylan than the GH11 xylanase, respectively. While the GH11 xylanase showed higher specific activity for the other xylan substrates, especially for glucuronoarabinoxylan. The optimum temperature for the reaction with α-rice powder was 30°C for the GH11 xylanase and 40°C for the GH10 xylanase (Fig. 5A). The observed temperature optimums were lower than those of *Trichoderma reesei* GH11 and GH10 xylanases (26,27), and *A. oryzae* GH11 and GH10 xylanases (28,29). The optimum pH of both enzymes was 4.5–5.0, and there was little difference in the pH profiles (Fig. 5B). The pH optimum for GH11 xylanase was similar to that of *T. reesei* GH11 xylanase (26), but rather lower than that of *A. oryzae* GH11 xylanase (28). The pH optimum for GH10 xylanase was similar to those of other reported GH10 xylanases (27,29). Both enzymes were inhibited by 10–20 % ethanol: the GH10 xylanase was more susceptible than the GH11 xylanase (Fig. 5C). Addition of the two xylanases obtained in the active fractions of the second cation exchange chromatography accelerated the formation of ferulic acid and ethyl ferulate in the sake mash (Fig. 6A and B). The

FIG. 4. Amino acid alignments of the analyzed protein bands and the matched proteins. I-a, I-c and II means analyzed protein bands. TC GH11 and TC GH10 means glycoside hydrolase family 11 protein of *T. citrinoviride* (XP_024751773.1) and glycoside hydrolase family 10 proteins of *T. citrinoviride* (XP_024754233.1), respectively. These proteins had the highest scores (55.8, 52.8, and 59.6, respectively) in the BLASTP database searches.

TABLE 2. Substrate specificity of crude and purified enzyme samples.

Substrate	Activity unit	Crude enzyme	I	II
α-Rice powder	U/mg protein	0.003 ± 0.000	0.052 ± 0.029	0.292 ± 0.022
Insoluble wheat arabinoxylan	U/mg protein	0.35 ± 0.01	1.49 ± 0.09	5.17 ± 0.54
Beech wood xylan	μmol/min/mg protein[a]	23.9 ± 5.4	253.4 ± 25.6	117.8 ± 4.8
Glucuronoarabinoxylan	μmol/min/mg protein[a]	33.8 ± 9.3	346.2 ± 30.9	45.6 ± 20.9
RBBxylan	ΔA_{595}/min/mg protein	0.5 ± 0.1	58.2 ± 5.5	20.8 ± 4.2
AZCLxylan	ΔA_{590}/min/mg protein	10.9 ± 2.9	757.3 ± 43.3	387.2 ± 41.1

[a] Formed sugar was assayed as xylose.

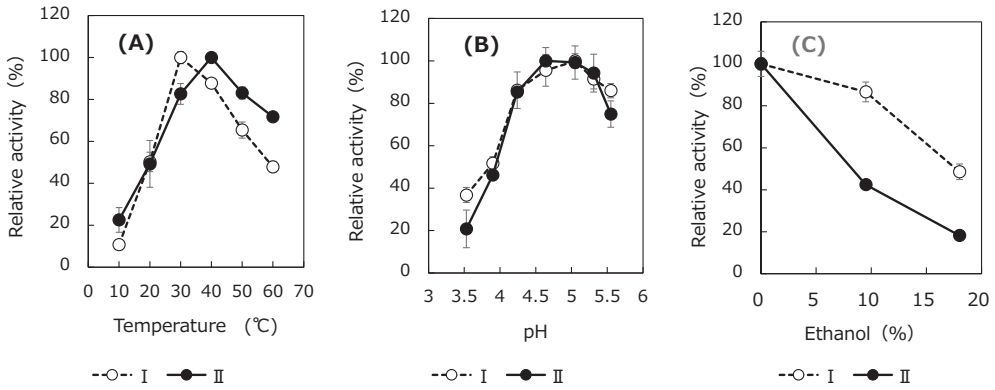

FIG. 5. Effects of temperature, pH and ethanol on enzyme activities using α-rice powder as the substrate. (A) Temperature. (B) pH. (C) Ethanol concentration.

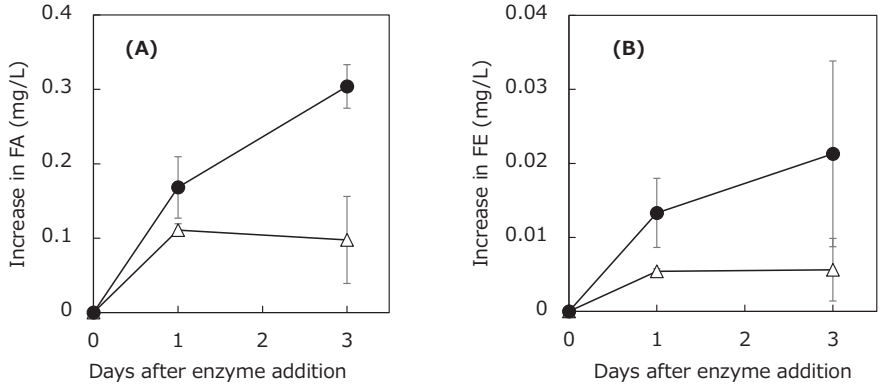

FIG. 6. Addition of purified GH10 and GH11 xylanases to the sake mash and its effect on the levels of ferulic acid and ethyl ferulate. (A) Variation of ferulic acid concentration. (B) Variation of ethyl ferulate concentration. Amount of added precursor forming activity of sample I or II enzyme to the 1 g of the sake mash was 1.7×10^{-5} U or 0.85×10^{-5} U, respectively. Data are the mean and SD of triplicated experiments. Closed circles, addition of fraction II; open triangles, addition of fraction I.

amount of GH11 unit added to the reaction was two times greater than that of GH10, however acceleration by the GH10 xylanase was clearly higher than that of the GH11, which may be due to the instability of the GH11 xylanase. The properties of both enzymes indicated that almost all of the effects of the cellulase reagent shown in Fig. 2 were due to the identified GH10 and GH11 xylanases, where GH10 xylanase might predominate. Differences in the accelerating effects of the two xylanases might be due to the difference in reaction products (30,31), where small feruloylated oligo-arabinoxylan might be the preferable substrate for feruloylesterase. The concentration of ferulic acid and ethyl ferulate produced by the purified enzymes was reduced after 3 days; however, their levels were continuously raised over 10 days by the cellulase reagent (Fig. 2). The continuous activity of the cellulase reagent might be partially due to the degradation of cellulose in the rice cell wall because arabinoxylan binds cellulose fiber and is crosslinked by ferulic acid esters in the plant cell wall (32). The genus *Aspergillus*, including *A. oryzae*, produce both GH10 and GH11 xylanases (33–39), however *A. oryzae* produces extremely small amounts of xylanase in rice sake *koji* (17). The low productivity might lead to the low formation of feruloylated oligo-arabinoxylan, and consequently lead to low ferulic acid levels in sake. The low levels of ferulic acid in sake may contribute to a taste harmony in sake, because excess ferulic acid produces negative effects on the sensory quality of sake (12). In the future, xylanases from *A. oryzae* that accelerate the formation of ferulic acid precursor should be elucidated, and the newly developed enzyme assay method and knowledge obtained in this study may be useful.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.014.

ACKNOWLEDGMENTS

The authors declare no conflict of interest.

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