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Effect of *pepA* deletion and overexpression in *Aspergillus luchuensis* on sweet potato *shochu* brewing

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The mash of sweet potato *shochu* (Japanese distilled spirit) has a low pH value because the *shochu* koji mold produces a large amount of citric acid, which prevents germ contamination. In this study, we examined acid protease PepA's role in *shochu* production. For this purpose, we constructed *pepA* deletion and overexpression strains, using a black koji mold *Aspergillus luchuensis* RIB 2604 (NBRC 4314), with the *Agrobacterium*-mediated transformation method. The rice koji, prepared using a *pepA* disruptant (Δ*pepA*) and *pepA*-overexpressing strain (OE*pepA*), demonstrated 1/2- and 24-fold acid protease activities compared to that prepared using the parental strain, respectively. A small-scale test of sweet potato *shochu* brewing indicated the mash of Δ*pepA* had a lower amino acid concentration, while the mash of OE*pepA* had a higher concentration than that produced by the parental strain. Therefore, the mash amino acid concentrations were proportional to these strains' acid proteases activities. After distilling these mashes, we examined each *shochu*'s aroma components. *Shochu* prepared using Δ*pepA* had relatively higher aroma components, such as alcohol and ester, compared to that prepared using parental strains. Meanwhile, *shochu* prepared using OE*pepA* had lower aroma components than that prepared using the parental strains. Based on these results, the amount of *shochu* aroma components showed an inverse correlation to the acid protease activity in the mash. Thus, the koji mold's acid protease content had a greater influence on the aroma qualities of sweet potato *shochu*. Accordingly, we have discussed the possibility of the breeding of *shochu* koji mold with acid protease as an indicator.

[**Key words:** Koji mold; *Aspergillus luchuensis*; Protease; *pepA*; Sweet potato *shochu*; Aroma components]

Sweet potato *shochu* is a traditional Japanese distilled spirit prepared from rice koji, yeast, and sweet potato. Two microorganisms, such as koji mold and yeast, are generally used to produce *shochu*, along with various types of yeast and koji mold strains. *Aspergillus luchuensis* (or black koji mold) (1) is an important microorganism used for rice koji preparation. Its main role is to decompose the starch content of rice and sweet potato into glucose and maltose by producing a high amount of saccharification enzymes. Moreover, black koji mold produces a large amount of citric acid, which prevents miscellaneous bacterial contamination during *shochu* production. In addition, koji mold is believed to affect the aroma of sweet potato *shochu*. For example, it has been reported that β-glucosidase, produced by koji mold, contributes to monoterpene alcohol production in sweet potato *shochu* (2). However, the relationship between enzymes produced by koji mold and *shochu* aroma components has rarely been investigated. On the other hand, there are several reports on yeast-related production of aroma components in several fermented foods, and yeast produces various aroma components via the amino acid synthesis pathway and/or the Ehrlich pathway (3–5). A few previous studies have reported that changing the amino acid concentration in the medium alters the amount of aroma components derived from yeast (6,7). In addition, it has been reported that the addition of protease and/or amino acids to the mash influences the aroma formation of sweet potato *shochu* (8,9). Various aldehydes are also produced by the amino acid metabolic pathway in yeast and/or by Strecker decomposition of amino acids (10). Therefore, increasing the amino acid concentration in *shochu* mash could produce different types of aroma components in *shochu*. We focused on the acid protease produced by koji mold as a means to control mash's amino acid concentration. Since this enzyme demonstrated activity under acidic conditions, the mash's amino acid concentration may be influenced by this enzyme. *pepA* encoding the major extracellular acid protease Aspergillopepsin A has been cloned in *Aspergillus awamori* (11), and studies on acid proteases similar to *pepA* have also been reported in *Aspergillus oryzae* and *Aspergillus niger* (12–14). Recently, some genomic information about *A. luchuensis* has been disclosed (15), and analysis of the gene functions in *A. luchuensis* is in progress. To investigate whether acid protease of black koji mold is involved in producing sweet potato *shochu* aroma components, deleting and overexpressing strains of *pepA* homologous gene were prepared in *A. luchuensis* RIB 2604 strain as *ΔpepA* and OE*pepA*, respectively. To investigate *pepA's* role in aroma production of *shochu*, we used WT, *ΔpepA*, and OE*pepA* strains to

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prepare and produce mash and *shochu*, respectively. Then we measured the amino acid and the aroma content. In this study, we demonstrated that *pepA* is greatly involved in aroma production during *shochu* production.

MATERIALS AND METHODS

Strains and media *A. luchuensis* RIB 2604 (NBRC 4314) strain and *A. luchuensis* RIB 2604 Δ*ligD* strain were used as the host for the transformation (16). *Escherichia coli* DH5α strain was used to amplify various plasmids. *Agrobacterium tumefaciens* C58C1 strain was used to transform *A. luchuensis* RIB 2604 strain and *A. luchuensis* RIB 2604 Δ*ligD* strain using the *Agrobacterium*-mediated transformation method (AMT). *Shochu* yeast *Saccharomyces cerevisiae* Kagoshima No. 5 strain was used for the small-scale fermentation test of sweet potato *shochu*. CD medium, supplemented with 100 μg/mL hygromycin B, was used as the selection medium for Δ*pepA* and OE*pepA* (CD-hyg). The PDA medium and CD-casein (CD-cas) medium, supplemented with 1% casein (from milk), was adjusted to pH 4.0 with lactic acid and used to compare acid protease activity by casein plate assay.

Construction of *pepA*-disrupted and overexpressing strains About 700 bp and 1.5 kbp fragment containing the 5′- and 3′-region of *pepA* were amplified by PCR with the primer pairs P1-f/P1-r and P2-f/P2-r, respectively. The *hphB*-expression cassette was obtained by *Kpn*I digestion from plasmid pB-Al-hph (O. Yamada, unpublished data) that carries an *hphB*-expression cassette, glyceraldehyde-3-phosphate dehydrogenase (ALgpdA) promoter, the *hphB* gene, and ALgpdA terminator. These three fragments were cloned into the pRIE *Eco*RI site by using the In-Fusion HD Cloning Kit (Takara Bio Inc., Otsu, Japan) to form pDpepA. *A. luchuensis* RIB 2604 Δ*ligD* strain was transformed with pDpepA by the AMT method (16,17), and the transformants were selected on CD-hyg medium containing 10 μg/mL cefotaxime and 10 μg/mL spectinomycin to kill the bacterial cells. The Δ*pepA* strains were confirmed by colony PCR with the primer pairs P4-f/P4-r and P5-f/P5-r. The *pepA* disruption was further confirmed by Southern blotting. The genomic DNA of *A. luchuensis* RIB 2604 Δ*ligD* strain and Δ*pepA* strain was digested with *Eco*RI, followed by agarose gel electrophoresis. After transferring to the nylon membrane, it was probed with DIG-labeled DNA amplified by PCR (PCR DIG Probe Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany), with a primer pair P7-f/P7-r. To construct a *pepA*-overexpressing strain, the *pepA*-coding region was PCR amplified with the primer pairs P3-f/P3-r, using genomic DNA of *A. luchuensis* RIB 2604 as a template. The amplified fragment was cloned into the *Sal*I site of pRglaA142 with the In-Fusion HD Cloning Kit to form pOEpepA, in which *pepA* was located under the control of the glaA142 promoter (18,19). *A. luchuensis* RIB 2604 strain was transformed with pOEpepA by the AMT method, and the transformants were selected on CD-hyg medium containing 10 μg/mL cefotaxime and 10 μg/mL spectinomycin. The OE*pepA* strains were confirmed by colony PCR with primer pairs P6-f/P6-r. Genomic DNAs of the *A. luchuensis* RIB 2604 and OE*pepA* strains were digested with *Hind*III and *Spe*I, followed by agarose gel electrophoresis. After transferring onto the nylon membrane, it was hybridized with DIG-labeled probe, amplified by PCR with the primer pairs P8-f/P8-r. The primers' nucleotide sequences are shown in Table 1.

Comparison of acid protease activity by casein plate assay The acid protease activity of transformants and parent strain was compared by the casein plate assay. The conidia suspension of Δ*pepA* strain, OE*pepA* strain, and parent strain were spotted onto the CD-cas plate. After incubation at 30°C for 48 h, the size of each halo was measured. Some of these strains were also cultured on PDA plate and used as control.

Preparation of rice koji Rice koji was prepared by adding 60 g of sterilized α-rice, 30 mL of sterilized water, and 1.5×10^7 spores into a 500-mL Erlenmeyer flask with a silicone cap. After stirring well, the flask was set in a constant temperature and humidity chamber for 48 h. During koji production, the temperature was sequentially controlled at 38°C (for 0–19 h) to 36°C (for 19–27 h) to 34°C (for 27–48 h). The humidity was maintained at 80% (for 0–27 h) and then not controlled (for 27–48 h).

Analysis of rice koji The acidity and acid protease activity were analyzed using the NTA official methods (20). The activity of α-amylase, α-glucosidase, glucoamylase, acid carboxypeptidase, and saccharification power were measured using a measuring kit (Kikkoman Biochemifa, Co., Ltd., Tokyo, Japan). The microbiomass in rice koji was calculated from the amount of glucosamine measured by the methods of Blix (21) and Sakurai et al. (22).

Small-scale fermentation test of sweet potato *shochu* The sweet potato *shochu* brewing test, with previously prepared rice koji from each strain, was performed as follows. The *shochu* yeast, Kagoshima No. 5, was cultured in 50 mL of YPD liquid medium at 30°C for 48 h to prepare the yeast culture. The primary mash was prepared by adding 60 mL of citric acid solution (pH 4.0) and 0.5 mL of yeast culture solution to 60 g of rice koji. The primary mash was incubated at 30°C for 5 days, and every 10 g were sampled on the 2nd and 5th days. Then, 200 g of steamed and crushed sweet potatoes (Koganesengan) and 120 mL of water were added to this primary mash in order to form the secondary mash, and the mixture was incubated at 30°C for 8 days, from which 10 g and 50 g samples were removed on days 2 and 8, respectively. Finally, 285 g of secondary mash was atmospherically distilled using a steam distillation apparatus (Miyamoto Riken Industry Co., Ltd., Osaka, Japan) and approximately 100 mL of sweet potato *shochu* was produced.

TABLE 1. Nucleotide sequences of primers used in this study.

Primer	Nucleotide sequence (5′→3′)
P1-f	CGTTTTTAATGAATTAAGACGCGATAGTCAAAGTCTGACTGTC
P1-r	TGGACCCCGAAGGCGCAAGCAAGAGAGTTCGGAGAAGAGATAG
P2-f	GAGCTCAATGGCCCGTCGTCGATGGGATACATCTGGACATATAG
P2-r	TAGTTTAAACTGAATTTAGGATGAGCATCAACTTGAGCTGATG
P3-f	GTCGAAGCAAGTCGAAAAATGGTCGTCTTCAGCAAAACCGCTGCC
P3-r	CCGCAAGCTTGTCGACTAAGCTTGAGCAGCGAAGCCCAGCTTAGG
P4-f	TTACATCATCTCATCTCGTCCGGTTCCTCG
P4-r	CAACATTGGGAGATATGGGGACTGGACAAAC
P5-f	CTGTCTTGGCAATTGCGAATATCCTCACGG
P5-r	CGCCCAACACGTTTCTACATGCAACCACTC
P6-f	CGCATCGTGGTTCATCGTCCCAGC
P6-r	GCTAATAGGATTCTGCCTTCCTGCTCAACG
P7-f	GGAAGGAATGAAGGTTGAGGGGGAATGGGCG
P7-r	CAATGGTGCCATCGCAGCGAAACGGACATG
P8-f	CGCAAGGGCTTCACCATCAACCAGATTGCC
P8-r	GGAGGGGTGGTCGAGCAGGAGAAAACGTAG

Analysis of mash and sweet potato *shochu* The harvested sample from the mash was centrifuged at 3000 rpm for 10 min, and the supernatant was used for analysis. The ethanol concentration was measured with a simple alcohol analyzer, AL-3 (Riken Keiki Co., Ltd., Tokyo, Japan), and the acidity and amino acidity were measured based on the NTA official methods (20). The mash's amino acid contents were also quantified as follows. An equal amount of 2% sulfosalicylic acid was added to appropriately diluted menthol supernatant, and the mixture was stirred and allowed to stand for 30 min, followed by filtration through 0.45-μm filter to remove the protein. The de-proteinized sample was subjected to quantitative determination using the fully automated amino acid analyzer JLC-500/V (JEOL Ltd., Tokyo, Japan). For analytical purposes, the obtained sweet potato *shochu* was adjusted with water to a 35% (v/v) ethanol concentration. The aroma components were analyzed under the following conditions. The low-boiling aroma components were measured by the internal standard method using gas chromatograph Shimadzu GC-2010 (Shimadzu Co., Kyoto, Japan). An analytical sample (total volume of 25 mL) was prepared by adding 1.25 mL of *n*-amyl alcohol to *shochu* as an internal standard. The temperature was maintained at 40°C for 5 min and then raised to 120°C at the rate of 5°C/min, from 120°C to 230°C. The temperature increase rate was changed to 20°C/min and held at 230°C for 10 min. The inlet temperature was 250°C, the FID temperature was 250°C, and the split ratio was 1/25. The medium and high boiling point aroma components were measured by an absolute calibration curve method using gas chromatograph Shimadzu GC-2025 (Shimadzu Co.) after extracting distilled spirit with hexane. Twenty milliliters of distilled spirit, 1 mL of hexane, and 4 g of common salt were added to a 25-mL co-stopper test tube. After shaking for 50 s, the mixture was allowed to stand for 2 min and then measured by the splitless method. The temperature was then maintained at 40°C for 5 min and then raised to 100°C at the rate of 10°C/min. The temperature increase rate was changed to 5°C/min from 100°C to 280°C and to 10°C/min from 280°C to 300°C, respectively, and finally held at 300°C for 2 min. The inlet temperature was 250°C, and the FID temperature was 310°C.

RESULTS

Constructions of *pepA*-disrupted strain and overexpression strain of *pepA* We searched for the ortholog of *A. niger* acid protease (AnPepA) in the *A. luchuensis* genome data base using the Uniprot Blast search site (http://www.uniprot.org/blast/) and detected AA1_SCon_0040250, which consists of 394 amino acid residues and shares 97.0% homology with the AnPepA. We designated this gene as *pepA*. To investigate whether *pepA* in *A. luchuensis* is involved in producing aroma components in sweet potato *shochu*, we constructed *pepA* disruptant (Δ*pepA*) and *pepA*-overexpressing (OE*pepA*) strains. From screening with a medium containing hygromycin, four candidates of Δ*pepA* strain and five candidates of OE*pepA* strain were obtained. The obtained strains

were confirmed by colony PCR and Southern blotting (Fig. 1A). Since no significant difference in the acid protease activities was observed among the four strains of Δ*pepA* and five strains of OE*pepA* (data not shown), one of each strain was selected and Southern blotting was performed. In addition, colony PCR was performed to confirm Δ*pepA* strains with the primer pairs P4-f/P4-r and P5-f/P5-r. When the P4-f/P4-r primer pairs were used, no amplified fragment was detected in the wild-type strain (WT), while a 4.0 kb amplified fragment was observed in the Δ*pepA* strain (Fig. 1B). The P5-f/P5-r primer pairs could amplify a 2.1 kb fragment in the WT strain and 3.2 kb fragment in the Δ*pepA* strain (Fig. 1C). Southern blotting revealed the expected hybridization signal at 2.9 and 4.8 kb in *ΔpepA* and WT strains, respectively (Fig. 1D). These results showed that gene replacement occurred successfully at the *pepA* locus. We confirmed OE*pepA* by colony PCR with the primer pairs P6-f/P6-r (Fig. 2A). The WT showed no amplified fragment; however, in the case of OE*pepA* strain, a 1.7 kb fragment was amplified (Fig. 2B). To confirm the *pepA* copy number in the OE*pepA* strain, Southern blotting was performed, and the insertion of a single copy of *pepA* with the *glaA* promoter was confirmed (Fig. 2C).

Evaluation of protease activity in Δ*pepA* and OE*pepA* strains by casein plate assay To examine the protease activity in the Δ*pepA* and OE*pepA* strains, we performed the halo assay using a casein plate (Fig. 3A). As a result, the Δ*pepA* strain's growth lagged behind the WT strain. Moreover, the halo size became smaller than the WT strain. On the other hand, the OE*pepA* strain grew faster than the WT strain, and the halo size increased. These results show the Δ*pepA* strain had lower acid protease activity than the WT strain, and the OE*pepA* strain had higher acid protease activity than the WT strain. However, the proliferation rate was similar in each strain when grown on the control PDA medium (Fig. 3B).

Analysis of rice koji To investigate the effect of *pepA* disruption and overexpression on the quality of rice koji, rice koji was prepared on the flask scale. Analysis of rice koji revealed that the acid protease activity was 1/2-fold lower in the Δ*pepA* strain than in the parent strain and that it was 24-times higher in the OE*pepA* strain than in the parent strain (Fig. 4A). Since the Δ*pepA* strain has extremely low microbiomass in rice koji compared to other strains (Fig. 5), the delayed proliferation may be attributed to the fact that the protein contained in rice could not be fully assimilated. In addition, Δ*pepA's* enzyme activity was generally lower because of the decreased microbiomass, except there was no significant difference in acid carboxypeptidase activity (Fig. 4C). In addition, when converted per microbiomass, the Δ*pepA* strain showed high enzyme activity and acidity, except α-amylase and acid protease, while those of the OE*pepA* strain were slightly lower (Fig. 4B, D).

Brewing test of sweet potato shochu of Δ*pepA* and OE*pepA* strains To investigate the effect of disruption and overexpression of *pepA* on sweet potato *shochu* fermentation, the sweet potato *shochu* brewing test was performed using rice koji prepared by the Δ*pepA* or OE*pepA* strain. We analyzed the alcohol concentration, pH, acidity, and amino acidity in the secondary mash (Table 2). The amino acidity was lower in the Δ*pepA* strain, and higher in the OE*pepA* strain, compared to the parent strain. Next, the amino acid concentration in the first and second mashes were measured by an amino acid analyzer, and the total amino acid concentration is shown in Fig. 6. The proportions of amino acids contained in both the first and second mashes were almost the same. When using the Δ*pepA* strain, the amino acid

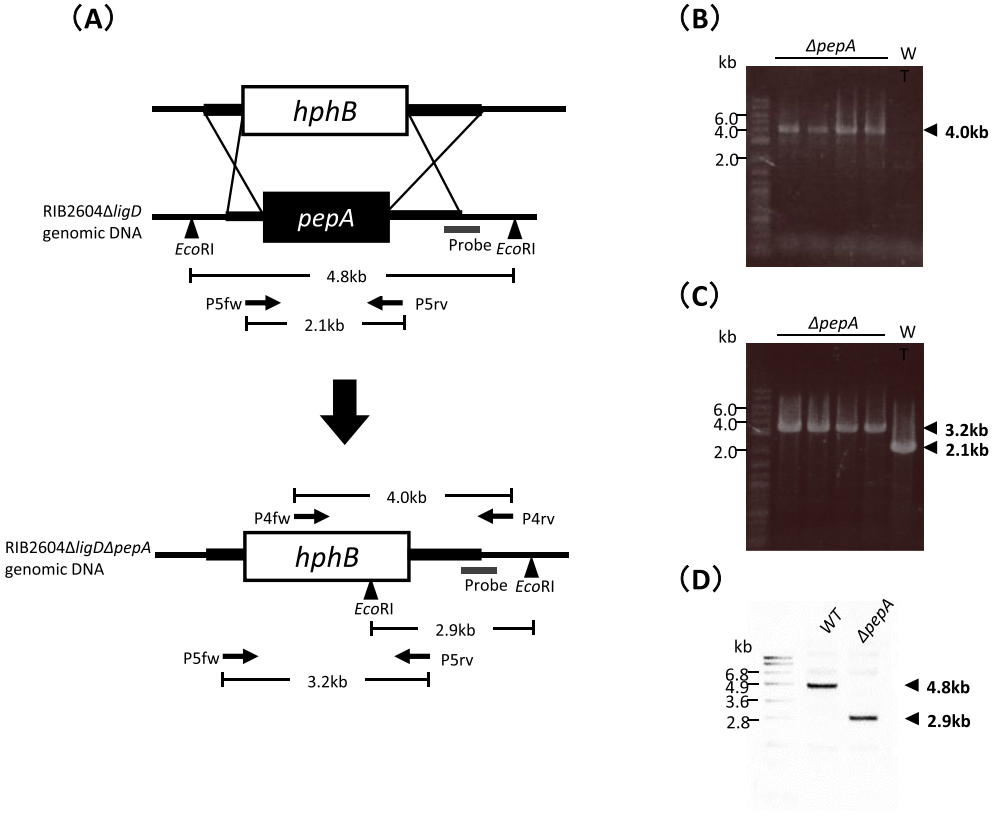

FIG. 1. The confirmation of Δ*pepA* strain by colony PCR and Southern blotting. (A) The outline of colony PCR and Southern blotting analysis. Colony PCR was performed with P4 (B) and P5 (C) primers. As a control, RIB 2604 Δ*ligD* strain was used. In Southern blotting, the total DNA isolated from Δ*pepA* strain and RIB 2604Δ*ligD* strain were digested with *Eco*R I and then hybridized with DIG-labeled probe (D).

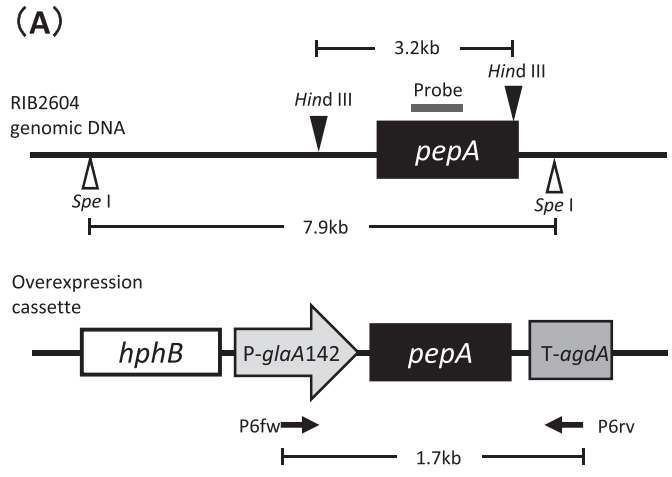

FIG. 2. Confirmation of OE*pepA* strain by colony PCR and Southern blotting. (A) The outline is shown. Colony PCR was performed with P6 primer (B) and the RIB 2604 strain was used as a control. In Southern blotting, total DNA, which was isolated from OE*pepA* strain and RIB 2604 strain, was digested with *Hin*dIII or *Spe*I and then hybridized with DIG-labeled probe specific for *pepA* ORF (C). Lane 1, RIB 2604/*Hin*dIII; lane 2, RIB 2604OE*pepA*/*Hin*dIII; lane 3, RIB 2604/*Spe*I; lane 4, RIB 2604 OE*pepA*/*Spe*I.

concentration at 48 h of secondary mash was extremely low, and some amino acids such as Met and Trp were not detected. Conversely, in the case of mash of OE*pepA* strain, all amino acids could be detected at higher concentrations. It was thus found that the amount of amino acid contained in the mash depended on the activity of the acid protease encoded by *pepA*.

Furthermore, to investigate whether the protease activity also affected the aroma components of sweet potato *shochu*, the aroma components of *shochu* were quantified by GC (Shimadzu GC-2010 and GC-2025). When the Δ*pepA* strain was used, higher alcohols, such as 1-propanol, isobutyl alcohol, isoamyl alcohol, and esters such as isoamyl acetate and β-phenylethyl acetate, were detected at higher concentrations (Table 3). In contrast, when the OE*pepA* strain was used, the concentrations of higher alcohols and esters reduced. From these results, the amino acid concentration was found to affect the aroma components of sweet potato *shochu*.

DISCUSSION

In this study, the effect of disruption or overexpression of *pepA* of *A. luchuensis* encoding acid protease on the growth, enzymatic activity, and brewing of sweet potato *shochu* was examined. A proliferation delay was observed in both casein plate adjusted to pH 4 (Fig. 3A) and steamed rice during koji production (Fig. 5) in the *pepA* strain rather than the WT strain. In addition, in the case of Δ*pepA* strain, a halo caused by casein decomposition was not observed in the casein plate (Fig. 3A). These results indicate that PepA is a major secretory acid protease under acidic conditions, and, since this acid protease is not secreted in Δ*pepA* strain, amino acids are not supplied by decomposition of proteins contained in the medium, which may result in delayed proliferation. On the other hand, the Δ*pepA* strain demonstrated high enzymatic activity, such as α-glucosidase, glucoamylase, and acid carboxypeptidase per microbiomass, and high medium acidity (Fig. 4D). Since the OE*pepA* strain conversely showed low enzyme activity and acidity per microbiomass, PepA may be involved in protein and organic acid secretion. In the production of heterologous proteins by fungi, the protein produced is sometimes degraded by the host proteases. Therefore, the breeding of fungi that do not produce proteases has also been reported (23–25). Since the Δ*pepA* strain also showed increased enzyme activity per microbiomass, it may be useful as a host for heterologous protein production.

In the brewing test of sweet potato *shochu*, using *pepA*-disrupted and overexpressing strains, the activity of PepA, an acid protease, affects the aroma component of sweet potato *shochu*. The strength of the acid protease activity can control the amino acid

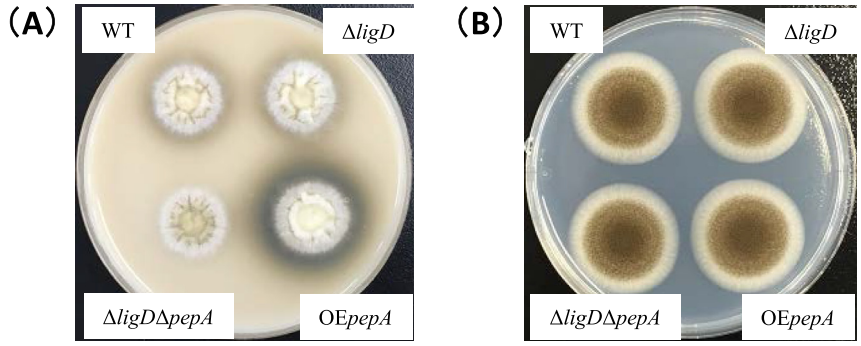

FIG. 3. Comparison of transformants by plate assay. The transformants and respective parent strains were cultured in a CD-cas plate (A) and a PDA plate (B) at 30°C for 72 h.

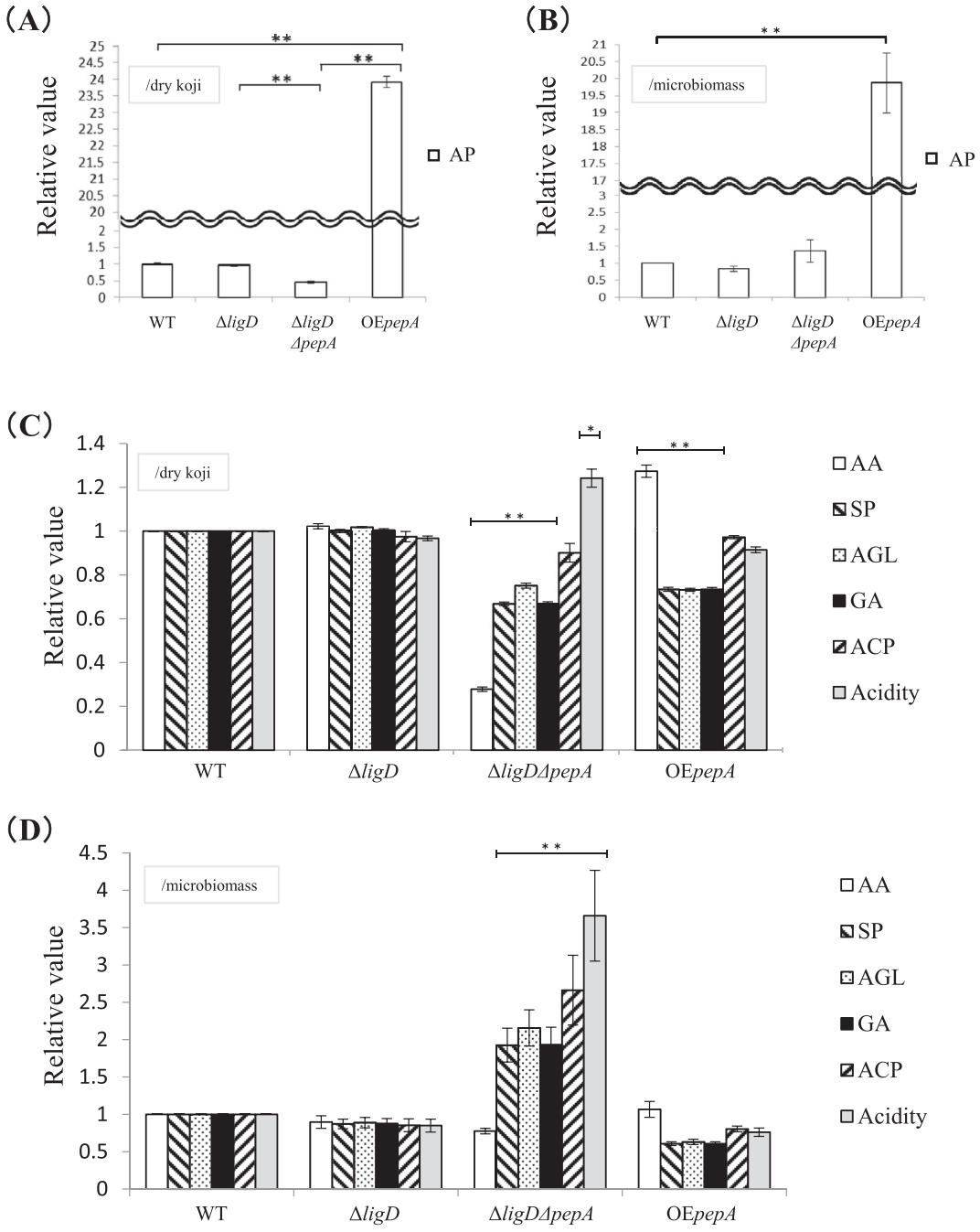

FIG. 4. Analysis of rice koji. The acid protease (AP) activity, per dried rice koji and per microbiomass, were compared for their relative values using RIB 2604 (WT) as a control (A, B). The other enzyme activities and acidity were also compared in their relative values using RIB 2604 (WT) as a control (C, D). AA, α-amylase (open bars); SP, saccharification power (right hatched bars); AGL, α-glucosidase (shaded bars); GA, glucoamylase (closed bars); ACP, acid carboxypeptidase (left hatched bars); acidity (gray bars). The error bars indicate the standard errors of four independent experiments. The asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$, unpaired Student's *t*-test) between the WT and three transformants.

concentration in the mash (Table 2); as a result, the contents of higher alcohols and esters derived from amino acids are also affected (Table 3). The higher alcohol concentrations were increased when the acid protease activity was low and, conversely, decreased as the acid protease activity was high (Table 3). These results were consistent with those of a previous report (8) that higher alcohols, such as 1-propyl alcohol, isobutyl alcohol, and isoamyl alcohol, are reduced by adding protease preparation during sweet potato *shochu* production. In addition, some esters, such as β-phenylethylalcohol and β-Phenylethylacetate, showed the same tendency. Since these aroma components are produced by the amino acid metabolism of yeast (3–5,26,27), the production amount changed due to the influence of the amino acid concentration in the mash. Indeed, according to the results of amino acid analysis of mash, the amino acid content decreased mostly in the secondary mash at 48 h of fermentation (Fig. 6). This phenomenon was common in the production of *shochu* from sweet potato which contained a small amount of protein. Since the amino acids were hardly detected, using the Δ*pepA* strain, especially in the mash, the strength of protease activity may have greatly affected the flavor of sweet potato *shochu*. In addition, we could not exclude the possibility that the sugar concentration in mash also affected the flavor

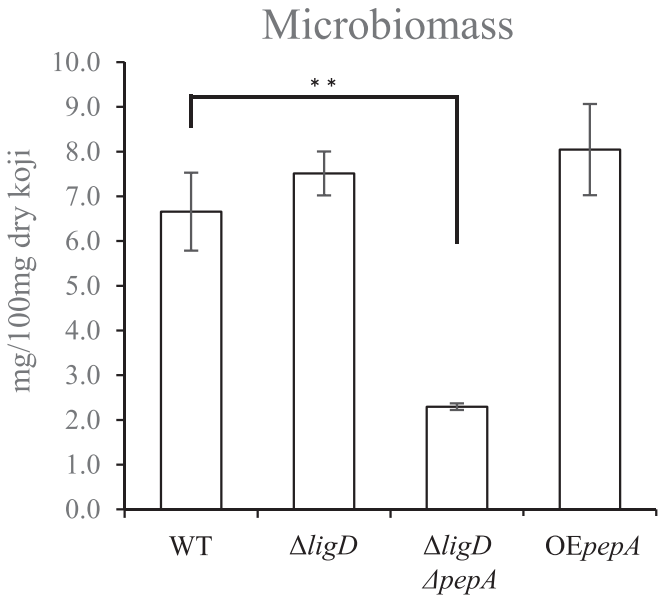

FIG. 5. Microbiomass in rice koji. The error bars indicate the standard errors of four independent experiments. The asterisks indicate significant differences (**$p < 0.01$, unpaired Student's *t*-test) between the WT and three transformants.

TABLE 2. Analysis result of secondary mash.

	Strain			
	WT	Δ*ligD*	Δ*ligD*Δ*pepA*	OE*pepA*
Alcohol concn. (%)	14.45	14.55	14.70	14.35
pH	4.43	4.42	4.36	4.42
Acidity	5.1	4.9	5.0	5.1
Amino acidity	2.64	2.68	1.64**	3.85**

Asterisks indicate significant differences (**$p < 0.01$, unpaired Student's *t*-test) compared to wild-type (WT) in four independent experiments.

of sweet potato *shochu* since the activity of α-glucosidase and glucoamylase of transformants was low in the *pepA*-overexpressing strain.

In conclusion, altering the activity of acid protease PepA of *A. luchuensis* may control the amino acid concentration in mash and thus affect the aroma component of sweet potato *shochu*. The

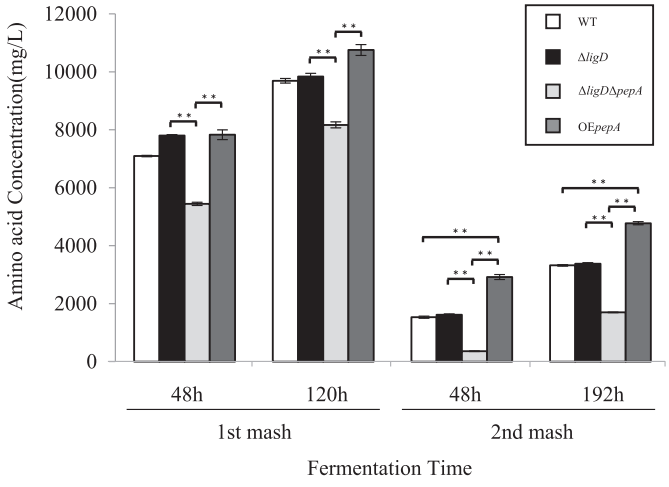

FIG. 6. Changes in total amino acid concentration during mash. Open bars, WT; closed bars, Δ*ligD*; gray bars, Δ*ligD*Δ*pepA*; dark gray bars, OE*pepA*. Error bars indicate the standard errors of four independent experiments. Double asterisk indicates significant differences (**$p < 0.01$, unpaired Student's *t*-test).

TABLE 3. Aroma component concentration in sweet potato *shochu* (mg/L).

	Strain			
	WT	Δ*ligD*	Δ*ligD*Δ*pepA*	OE*pepA*
Acetaldehyde	34.9	31.9**	26.5**	28.7**
Ethyl acetate	66.4	66.2	66.8	60.1*
Methanol	359.5	354.0	322.5**	322.4**
1-Propanol	91.2	84.2	119.7**	65.3**
Isobutanol	179.6	170.9	202.2**	163.4*
1-Butanol	2.2	1.8	2.4**	1.7*
Isoamylalcohol	295.1	284.9	363.5**	267.9**
Isoamyl acetate	1.5	1.6	2.9**	1.4
Ethyl caproate	0.10	0.10	0.10	0.09
β-Phenylethylalcohol	1.7	1.7	2.0s	1.4*
Ethyl caprylate	0.12	0.13	0.10	0.10
β-Phenylethyl acetate	1.0	0.9	1.4*	0.8*
Ethyl caprate	0.11	0.11	0.09	0.10

The asterisk shows significantly increased or decreased values (*$p < 0.05$, **$p < 0.01$, unpaired Student's *t*-test) compared to WT in four independent experiments.

activity of acid protease, including PepA, is useful as an indicator of breeding *Aspergillus* sp., which could be used to provide several types of *shochu* with various tastes and flavors.

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References

1. **Hong, S., Lee, M., Kim, D., Varga, J., Frisvad, C. J., Perrone, G., Gomi, K., Yamada, O., Machida, M., Houbraken, J., and Samson, A. R.:** *Aspergillus luchuensis*, an industrially important black *Aspergillus* in East Asia, PLoS One, **8**, e63769 (2013).
2. **Ohta, T., Shimojo, H., Hashimoto, K., Kondo, H., Samuta, T., and Ohba, T.:** β-Glucosidase activity in *Shiro-koji* and its contribution to sweet potato *shochu* flavor, J. Brew. Soc. Jpn., **86**, 536–539 (1991).
3. **Hazelwood, A. L., Daran, M. J., Maris, J. A. A., Pronk, T. J., and Dickinson, J. R.:** The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism, J. Appl. Environ. Microbiol., **74**, 2259–2266 (2008).
4. **Ouchi, K., Yamamoto, Y., Takagishi, M., and Akiyama, H.:** Effect of nitrogen source on higher alcohol formation by *Saccharomyces cerevisiae*, J. Ferment. Technol., **59**, 9–16 (1981).
5. **Ayrapaa, T.:** The formation of phenethyl alcohol from ^{14}C-labelled phenylalanine, J. Inst. Brew., **71**, 341–347 (1965).
6. **Ayrapaa, T.:** Biosynthetic formation of higher alcohols by yeast. Dependence on the nitorogenous nutrient level of the medium, J. Inst. Brew., **77**, 266–276 (1971).
7. **Akita, O., Hasuo, T., Oba, T., and Miyano, N.:** Effect of amino acid composition on higher alcohols and isoamyl acetate formation by a yeast, J. Ferment. Technol., **65**, 19–26 (1987).
8. **Shiraishi, Y., Ando, Y., Okutsu, K., Yoshizaki, Y., Futagami, T., Tamaki, H., Wagu, Y., and Takamine, K.:** Effects of the addition of a protease agent to imo-*shochu* moromi on the volatile compounds and sensory characteristics of imo-*shochu*, J. Brew. Soc. Jpn., **112**, 517–523 (2017).
9. **Shiraishi, Y., Ando, Y., Okutsu, K., Yoshizaki, Y., Futagami, T., Tamaki, H., Wagu, Y., and Takamine, K.:** Effects of amino acids on the flavor formation of imo-*shochu*, J. Brew. Soc. Jpn., **112**, 563–568 (2017).
10. **Okumura, J.:** The maillard reaction and flavor formation, J. Brew. Soc. Jpn., **88**, 178–187 (1993).
11. **Berka, M. R., Ward, M., Wilson, J. L., Hayenga, J. K., Kodama, H. K., Carlomagne, P. L., and Thompson, A. S.:** Molecular cloning of the gene encoding aspergillopepsin A from *Aspergillus awamori*, Gene, **86**, 153–162 (1990).
12. **Berka, M. R., Carmona, L. C., Hayenga, J. K., Thompson, A. S., and Ward, M.:** Isolation and characterization of the *Aspergillus oryzae* gene encoding aspergillopepsin O, Gene, **125**, 195–198 (1993).
13. **Gomi, K., Arikawa, k., Kamiya, N., Kitamoto, K., and Kumagai, C.:** Cloning and nucleotide sequence of the acid protease-encoding gene (*pepA*) from *Aspergillus oryzae*, Biosci. Biotechnol. Biochem., **57**, 1095–1100 (1993).
14. **Van Den Hombergh, J. P. T. W., Sollewijn Gelpke, M. D., Van De Vondervoort, P. J. I., Buxton, F. P., and Visser, J.:** Disruption of three acid

proteases in *Aspergillus niger* effects on protease spectrum, intracellular proteolysis, and degradation of target proteins, Eur. J. Biochem., **247**, 605–613 (1997).
15. **Yamada, O., Machida, M., Hosoyama, A., Goto, M., Takahashi, T., Futagami, T., Yamagata, Y., Takeuchi, M., Kobayashi, T., Koike, H., and other 17 authors:** Genome sequence of *Aspergillus luchuensis* NBRC 4314, DNA Res., **23**, 507–515 (2016).
16. **Takahashi, T., Mizutani, O., Shiraishi, Y., and Yamada, O.:** Development of an efficient gene-targeting system in *Aspergillus luchuensis* by deletion of the nonhomologous end joining system, J. Biosci. Bioeng., **112**, 529–534 (2011).
17. **Michielse, C. B., Hooykass, P. J., van den Hondel, C. A., and Ram, A. F.:** Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*, Nat. Protoc., **3**, 1671–1678 (2008).
18. **Minetoki, T.:** The expression system of heterologous genes in Aspergilli, Kagaku To Seibutsu, **38**, 831–838 (2000) (in Japanese).
19. **Hata, Y., Kitamoto, K., Gomi, K., Kumagai, C., and Tamura, G.:** Functional elements of the promoter region of the *Aspergillus oryzae glaA* gene encoding glucoamylase, Curr. Genet., **22**, 85–91 (1992).
20. **The Brewing Society of Japan:** NTA official methods of analysis, 4th ed., pp. 14–39. The Brewing Society of Japan, Tokyo (1993) (in Japanese).
21. **Blix, G.:** The determination of hexosamines according to Elson and Morgan, Acta Chem. Scand., **2**, 467–473 (1948).
22. **Sakurai, Y., Lee, H. T., and Shiota, H.:** On the convenient method for glucosamine estimation in koji, Agric. Biol. Chem., **41**, 619–624 (1977).
23. **Tsuchiya, K., Tada, S., Gomi, K., Kitamoto, K., Jigami, Y., and Tamura, G.:** High level expression of the synthetic human lysozyme gene in *Aspergillus oryzae*, Appl. Microbiol. Biotechnol., **38**, 109–114 (1992).
24. **Archer, D. B., MacKenzie, D. A., Jeenes, D. J., and Roberts, I. N.:** Proteolytic degradation of heterologous proteins expressed *Aspergillus niger*, Biotechnol. Lett., **14**, 357–362 (1992).
25. **Ward, M.:** Production of calf chymosin by *Aspergillus awamori*, pp. 288–294, in: Hershberger, C. L., Queener, S. W., and Hageman, G. (Eds.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, D.C. (1989).
26. **Koganemaru, K., Sumi, T., Kanda, K., Kato, F., Tasiro, K., and Kuhara, S.:** β-Phenyletyl alcohol production by a mutant of sake yeast and its highly productive mechanism, J. Brew. Soc. Jpn., **98**, 201–209 (2003).
27. **Akita, O., Hasuo, T., Miyano, N., and Yoshizawa, K.:** Formation of aroma components in fermented liquor made by the fermentation following after saccharification: studies on the brewing of alcoholic beverage by the system of fermentation following after saccharification (part 7), J. Brew. Soc. Jpn., **82**, 369–374 (1987).