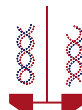

Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2018
www.elsevier.com/locate/jbiosc

Characterization and induction of phenolic acid decarboxylase from *Aspergillus luchuensis*

Maeda Mayumi,[1] Masashi Tokashiki,[2] Midori Tokashiki,[2] Keiko Uechi,[1] Susumu Ito,[1] and Toki Taira[1,*]

Department of Bioscience and Biotechnology, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan[1] *and Ishikawa Tanekouji, Chatan, Okinawa 904-0113, Japan*[2]

Received 17 December 2017; accepted 14 February 2018
Available online xxx

Awamori is a traditional distilled liquor in the Ryukyu Islands, made from steamed rice by the action of the black-koji mold *Aspergillus luchuensis* and awamori yeast *Saccharomyces cerevisiae*. One of the specific flavors in aged awamori *kusu* is vanillin, which is derived from ferulic acid (FA) in rice grains. FA is released from the cell wall material in the rice grain by ferulic acid esterase produced by *A. luchuensis*. Through decarboxylation of FA, 4-vinylguaiacol (4-VG) is produced, which is transferred to the distilled liquor, and converted to vanillin by natural oxidization during the aging process. However, the actual mechanism for conversion of FA to 4-VG in the awamori brewing process is unknown. A genetic sequence having homology to the phenolic acid decarboxylase (PAD)-encoding region from bacteria and the yeast *Candida guilliermondii* has been identified in *A. luchuensis* mut. *kawachii*. In the present study, recombinant PAD from *A. luchuensis*, designated as AlPAD, expressed as a homodimer, catalyzed the conversion of FA to 4-VG, displayed optimal catalytic activity at pH 5.7 and 40°C, and was stable up to 50°C. Both rice bran and FA could induce the bioconversion of FA to 4-VG and the expression of AlPAD in *A. luchuensis*. The amount of AlPAD determined using western blotting correlated with the level of FA decarboxylase activity during koji production. In awamori brewing process, AlPAD might be responsible for a part of the conversion of FA to 4-VG.

© 2018, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** *Aspergillus luchuensis*; Koji; Vanillin; Phenolic acid decarboxylase; Awamori; Ferulic acid; 4-Vinylguaiacol]

The black-koji mold *Aspergillus luchuensis* is used for making awamori, a traditional distilled liquor in the Ryukyu Islands (1). The awamori fermentation process comprises koji production and alcohol fermentation. First step of awamori brewing process is koji production, in which *A. luchuensis* is grown in solid-state culture (koji) on steamed rice to convert polysaccharides into smaller mono- or disaccharides and to produce citric acid. During alcohol fermentation, the resulting sugars from the koji are metabolized by yeast *Saccharomyces cerevisiae*. The *moromi* containing the koji, yeast, and water is cultured for approximately 2–3 weeks. After fermentation, the *moromi* is distilled, collected, and stored in a tank. Awamori aged in a tank for 3 years or longer is called *kusu*.

One of the specific flavors of *kusu* is vanillin, which is derived from ferulic acid (FA) in the rice grain. FA is released from the rice grain when ferulic acid esterase produced by *A. luchuensis* acts on the cell-wall materials. Decarboxylation of FA results in the production of 4-vinylguaiacol (4-VG) which is transferred into the distilled liquor and converted to vanillin by natural oxidization during the aging process in the tank. Koseki et al. (2) proposed that FA is converted to 4-VG during distillation by the combination of acid and heat. On the other hand, several studies specified that some strains of *S. cerevisiae* exhibit FA decarboxylase activity (3,4). There are also other studies showing that bacteria, such as the genera *Bacillus* (5–7) and *Lactobacillus* (8–10), have phenolic acid decarboxylase (PAD) which can catalyze the conversion of FA to 4-VG. Therefore, the conversion of FA to 4-VG during awamori *moromi* fermentation may be accomplished by PAD enzymes produced by yeast or co-existing bacteria (11).

Genomic analysis of *A. luchuensis* mut. *kawachii* (12) revealed sequence homology to PADs from bacteria and *Candida guilliermondii* (13). In the present study, the PAD gene from *A. luchuensis* (*alpad*) was cloned and expressed in *Escherichia coli*. The recombinant enzyme (AlPAD) was purified and characterized. We found that both rice bran and FA could induce the FA to 4-VG bioconversion and AlPAD expression in *A. luchuensis*. We successfully demonstrated that the amount of AlPAD determined using western blotting correlated with the level of FA decarboxylase activity during koji production. We discuss a contribution of AlPAD to the conversion of FA to 4-VG in awamori brewing.

MATERIAL AND METHODS

Materials FA, caffeic acid (CA), 4-VG, and 6-hydroxy-2-naphthoic acid (6H2N) were purchased from Wako Pure Chemical (Osaka, Japan). *p*-Coumaric acid (PCA) was purchased from MP Biomedicals (Solon, OH, USA), and 4-vinylphenol (4-VP) from Sigma–Aldrich (Steinheim, Germany). All other chemicals were analytical grade.

Organisms and culture media *A. luchuensis* var. *awamori* ISH1 (hereafter abbreviated as *A. luchuensis* ISH1), which is used in most awamori breweries (14), was generously supplied by Ishikawa Tanekoji. Potato dextrose with 1.5% agar (PDA) was used as subculture media. Rice bran medium was prepared using a previously described method (15). Basal medium containing 0.2% $(NH_4)_2SO_4$, 0.1%

* Corresponding author. Tel./fax: +81 98 895 8802.
E-mail address: tokey@agr.u-ryukyu.ac.jp (T. Taira).

1389-1723/$ – see front matter © 2018, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2018.02.009

Please cite this article in press as: Mayumi, M., et al., Characterization and induction of phenolic acid decarboxylase from *Aspergillus luchuensis*, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.02.009

KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.001% Tween 80, adjusted with 2 M H_2SO_4 to pH 4.0, was used to examine the inducibility of FA decarboxylase activity.

Cloning of the AlPAD gene Primers used are presented in Table S1. Total RNA was isolated from *A. luchuensis* ISH1 using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand complementary DNA (cDNA) synthesis was performed with 5 μg of total RNA using a SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with an oligo(dT) adaptor primer. The cDNA generated was used as a template for PCR amplification using specific primers designed from a sequence of the PAD candidate gene in *Aspergillus kawachii* IFO 4308, which was recently renamed *A. luchuensis* mut. *kawachii* IFO 4308 (16). PCR was performed using primers P1 and P2, which were specific for the 5′- and 3′-nucleotide sequences, respectively, of the candidate gene open reading frame (ORF). The PCR product was cloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced using the ABI Prism 3100 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA). The sequence analysis of inserts from three of individual colonies indicated that these inserts have the same sequence.

Expression and purification of recombinant AlPAD The corresponding cDNA region was amplified by PCR with forward primer P3 containing an *Nde*I restriction endonuclease site and reverse primer P4 containing a *Bam*HI restriction site (Table S1), using AlPAD cDNA as template. The amplified DNA fragment was digested with *Nde*I and *Bam*HI, and ligated into the expression vector pET 22b (Merck, Darmstadt, Germany), which had been digested with the same enzymes. The resulting recombinant plasmid (pET-AlPAD) was introduced into *E. coli* BL21(DE3). The transformed *E. coli* cells were grown in 100 mL of Luria−Bertani (LB) broth plus ampicillin (100 μg/mL) in 500 mL flasks at 37°C with shaking, to an optical density of 0.5 at an absorbance of 600 nm (A600). To induce protein expression of the recombinant AlPAD, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and further incubated for 24 h at 18°C. Cells were collected by centrifugation (6000 ×*g* for 15 min) at 4°C and the cell pellets obtained from the 100 mL cultures were used as starting material for AlPAD purification.

Purification of the AlPAD enzyme was performed at a temperature not exceeding 4°C. The *E. coli*-expressed recombinant AlPAD was purified by column chromatography using CM Toyopearl 650 M (Tosoh, Tokyo, Japan) and DEAE Sepharose FF (GE Healthcare & Bio Sciences, Uppsala, Sweden) columns successively. Specifically, the *E. coli* cell pellets were washed twice with saline and resuspended in seven volumes of MES extraction buffer (20 mM 2-morpholinoethanesulfonic acid), pH 6.0. The cells were disrupted by sonication and the cell debris removed by centrifugation (12,000 ×*g*, 20 min). The supernatant was directly applied to a CM Toyopearl 650 M column (3.0 cm × 20.0 cm) equilibrated with 20 mM MES buffer (pH 6.0). The flow-through fraction was applied to a DEAE Sepharose FF column (3.0 cm × 20.0 cm) equilibrated with 20 mM MES buffer (pH 6.0). The column was washed with 100 mL of 50 mM NaCl in MES buffer (pH 6.0), and proteins eluted with a 500 mL 50 mM to 0.4 M NaCl buffered linear gradient. The active fractions were equilibrated with 10 mM phosphate buffer (pH 7.0) and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA, USA). The concentrate was stored at −20°C until use. The purified recombinant enzymes were obtained 3.8-fold with a 48% yield.

***N*-terminal amino acid sequence analysis** *N*-terminal amino acid sequence analysis was done by Edman degradation with a protein sequencer (model PPSQ-23A; Shimadzu, Kyoto, Japan).

Estimation of molecular mass Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was done using a 15% (w/v) acrylamide gel for determination of the subunit molecular mass with a PageRuler Unstained Protein Ladder (Thermo Fisher Scientific, Rockville, MD, USA) as a standard marker. Proteins in the gel were stained with Coomassie Brilliant Blue R250.

The molecular mass of the recombinant AlPAD was also estimated by gel-filtration chromatography using a Superdex75 10/300 GL column (GE Healthcare & Bio Sciences) at a flow rate of 0.3 mL/min on a GE AKTA prime plus liquid chromatography system (GE Healthcare & Bio Sciences) with 50 mM phosphate buffer (pH 7.0) plus 0.15 M NaCl. The column was calibrated with the standard protein markers (Sigma−Aldrich) bovine serum albumin (66 kDa), chicken egg albumin (44 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

Assay of PAD activity The initial velocity of decarboxylation activity was measured at 40°C with phenolic acid as the substrate, unless stated otherwise. The reaction mixtures contained a suitable amount of diluted enzyme and 5 mM substrate (neutralized with 1.0 M NaOH) in 0.1 M MES buffer (pH 5.5) in a final volume of 1.0 mL. Reactions were terminated by adding 100 μL of 5.0 M NaOH and the reaction products were quantified by high-performance liquid chromatography (HPLC). Ten microliters of each sample was automatically injected on a Prominence HPLC system (Shimadzu) equipped with an SPD-M20A photodiode array detector. Analysis was done at 40°C on a Cosmosil $5C_{18}$-AR30 (Nacalai Tesque, Tokyo, Japan) packed column (4.6 mm × 150 mm) for reversed-phase chromatography with acetonitrile/0.05% phosphoric acid (7:3, v/v) as the mobile phase. The flow rate was 0.6 mL/min and the UV detector set at 260 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of 4-VG or 4-VP per min. Because the CA decarboxylation product 4-vinylcatechol was not commercially available, when CA was used as a substrate, the enzyme activity was expressed as the rate of CA degradation. Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific) using bovine serum albumin as a standard.

Resting cell reaction FA (1 mM), rice bran (1%), and white rice powder (1%) were used as inducer candidates. Glucose (2%) was used as a control. The fungus was grown at 30°C for 5 days with shaking, in 100 mL aliquots of media in 1 L flasks. Using a Buchner funnel, the cells were collected onto filter paper and washed with a saline solution. The cells were recovered from the filter paper following the removal of excess water, and used as resting cells for detecting baseline FA decarboxylase activity. Ten-fold saline solution (volume/wet weight of fungal cells) containing 4 mM FA was added to the cells, and the mixture was incubated at 30°C. At the 0, 3, 12, 24, and 36 h, the supernatant was collected and the amount of 4-VG determined as described above.

Quantification of ferulic acid in rice and rice bran Quantification of ferulic acid was done using a previously described method (17). White rice or rice bran (0.5 g each) was disrupted several times using a metal cone for 30 s at 3000 rpm in a Multi-Beads shocker (Yasui Kikai, Osaka, Japan). The resulting powder (0.1 g) was extracted with 0.5 M NaOH (5 mL) at 60°C for 90 min. 1.5 mL of 6 M HCl was added to the extract and then 0.8 mL of 1-butanol was added to the mixture. The mixture was vigorously vortexed and then the mixture was centrifuged (3000 ×*g*, 10 min, 25°C). The organic phase was collected and transferred to other tube. 1-Butanol (0.3 mL) was added to the retained aqueous phase, and then the mixture was vortexed and centrifuged by the same condition as above. The resulting organic phase was mixed with previously prepared the organic phase and stored until HPLC analysis.

Amount of FA in the sample solution was quantified using the same HPLC device and column as above, except the solvent and elution procedure. Elution was performed with a binary gradient of solvent A (50 mM sodium acetate buffer, pH 4) and solvent B (50 mM sodium acetate buffer, pH 4/methanol, 1:9, v/v) at a flow rate of 0.6 mL/min. The gradient was performed from 15 to 90% B for 35 min and then 15% B for 20 min to equilibrate the column before the next sample injection. Detection of FA was done at 322 nm.

Koji production One kilogram of 20% polished brown rice (Japonica) was soaked in water for 6 h. Excess water was drained and the swollen rice steamed at atmospheric pressure for 30 min. After cooling to 40°C, the rice was mixed with a starter inoculum of *A. luchuensis* ISH1. The inoculated rice was incubated at 40°C in a controlled environment. The koji was harvested after 30, 35, 43, 46, 56, and 68 h post inoculation. The koji was frozen with liquid nitrogen, then disrupted several times using a metal cone for 30 s at 2000 rpm in a Multi Beads shocker (Yasui Kikai, Osaka). At intervals during the disruption, the entire tube, including the koji and the metal cone, was cooled with liquid nitrogen to maintain a frozen state. The resulting koji powder was mixed with 10× (v/w) extraction buffer (0.1 M sodium phosphate buffer, pH 7; 1 mM EDTA; 1 mM PMSF) and incubated at 4°C for 1 h. The mixture was centrifuged (12,000 ×g, 10 min, 4°C) and the supernatant collected as a cell-free extract. The koji extract was assayed for FA decarboxylase activity, and AlPAD was quantified using western blotting.

Koji mycelium quantification Mycelia in the rice koji were quantified by quantification of *N*-acetylglucosamine (GlcNAc) (18) in the mycelial cell walls using a previously described method (19). Approximately 5 g of rice koji was dried in an oven at 100°C for 1 h, and crushed into a powder. The crushed koji (0.1 g) was washed three times with 1 mL of 50 mM sodium phosphate buffer (pH 7.0), and the suspension centrifuged at 10,000 ×*g* for 10 min. The pellet was resuspended in 1 mL of 50 mM sodium phosphate buffer containing 1 mg/mL Yatalase (Takara Bio, Shiga, Japan) and incubated at 37°C for 1 h. Following further centrifugation at 10,000 ×*g* for 10 min, the amount of GlcNAc in the supernatant was determined colorimetrically, as previously described (18).

Western blotting Specific polyclonal antisera were obtained by immunizing Japanese white rabbits with purified recombinant proteins. Rabbit was immunized subcutaneously with 1 mg of purified recombinant AlPAD mixed with equal volume of adjuvant on days 1, 15, 29, 45, and 60. Blood samples were collected from rabbits 1 week before immunization, and 2 weeks after the last immunization. For western blotting, protein samples were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane using the buffer system of Kyhse-Andersen (20). The membrane-bound protein was incubated with the polyclonal anti-AlPAD antibody, followed by a secondary HRP-conjugated anti-rabbit goat antibody. The bands were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) as the chemiluminescent reagent, and an ImageQuant LAS 4000 mini-luminescent image analyzer (GE Healthcare Japan, Tokyo, Japan). The chemiluminescence intensities of protein bands with western blotting were quantitatively analyzed using AlphaView Software (ProteinSimple, San Jose, CA, USA).

Accession number The nucleotide sequence encoding AlPAD has been deposited in the DDBJ/EMBL/GenBank nucleotide databases under accession number LC369499.

RESULTS

Cloning and sequence analysis of *A. luchuensis* phenolic acid decarboxylase PCR was performed using primers designed from the sequence of candidate PAD gene in *A. kawachii* IFO 4308, and

Download English Version:

https://daneshyari.com/en/article/6489626

Download Persian Version:

https://daneshyari.com/article/6489626

Daneshyari.com