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Characterization of Lactobacilli with Tannase Activity Isolated from *Kimchi*

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Abstract Tannase catalyzes the hydrolysis of gallic acid esters and hydrolysable tannins. Twenty-two *Lactobacillus* strains with tannase activity were isolated from 7 types of *kimchi*. A polymerase chain reaction-based assay targeting the *recA* gene assigned all isolates to either *Lactobacillus plantarum* or *Lactobacillus pentosus*. The tannase activities of isolates measured in whole cells and cell-free extracts varied even within each species. The activities of the isolates varied with the assay method, but both methods indicated that isolate LT7 (identified as *L. pentosus*) showed the highest activity. The results of thin layer chromatography and high performance liquid chromatography, respectively, showed that tannic acid and gallic acid degraded to pyrogallol in resting *L. pentosus* LT7 cells. Therefore, the putative biochemical pathway for the degradation of tannic acid by *L. pentosus* implies that tannic acid is hydrolyzed to gallic acid and glucose, with the formed gallic acid being decarboxylated to pyrogallol. This study revealed the possible production of pyrogallol from tannic acid by the resting cell reaction with *L. pentosus* LT7.

Keywords: tannin, tannase, tannic acid, gallic acid, pyrogallol, *Lactobacillus plantarum*, *Lactobacillus pentosus*

Introduction

Tannins comprise a large group of polyphenolic compounds that are widely distributed in the plant kingdom. They can be broadly divided into two basic groups: hydrolysable tannins (HT) and condensed tannins. HT is polymers of gallic or ellagic acid esterified with a core molecule, commonly glucose or alcohols, whereas condensed tannins are flavonoid polymers. Both groups of tannins have the ability to bind proteins and form insoluble tannin-protein complexes that are not easily degraded by mammalian digestive enzymes, thus adversely affecting the digestibility of plant materials fed to animals (1).

Tannase (tannin acylhydrolase) catalyzes the breakdown of HT such as tannic acid, methyl gallate, ethyl gallate, *n*-propyl gallate, and isoamyl gallate, thereby inhibiting their protein-binding properties (2,3). The enzyme finds widespread applications in food and beverage processing, with one of its major applications being in the manufacture of instant tea. Tannase applications in food and beverage industrial products contribute to remove the undesirable effects of tannins (4). Another important application of tannase is the production of gallic acid and propyl gallate (5). Gallic acid is used in the pharmaceutical and food industries in the synthesis of antibacterial drugs and food preservatives (e.g., pyrogallol and gallates), respectively, whereas propyl gallate is a very important food antioxidant (6).

Whilst tannase can be obtained from plant, animal, and microbial sources, it is mainly obtained from a diverse group of microorganisms, including rumen bacteria (1-3,7,8), and its enzymatic activity was recently found in *Lactobacillus* strains isolated from human feces and fermented foods: *L. plantarum*, *L. paraplantarum*, and *L. pentosus* (9,10). These species reportedly play an important role in the production of many fermented foods, which has led to them being proposed as potential probiotics (11).

Kimchi is one group of traditionally fermented vegetables originating from Korea that is produced by the mixed fermentation of mainly naturally occurring lactic acid bacteria. Several types of vegetables are used for *kimchi*, and more than 30 *Leuconostoc*, *Lactobacillus*, and *Weissella* species are known to play an important role in its fermentation (12,13). Therefore, Korean researchers have considered *kimchi* useful for screening lactic acid bacteria with beneficial effects, such as bacteriocin producers (14).

In this study, the tannase activities of potentially probiotic tannic acid-degrading lactobacilli isolated from 7 types of *kimchi* were determined, for future potential applications in the food industry and animal feed additives. Additionally, the metabolic intermediates of tannic acid were identified to elucidate the microbial production of gallic acid and pyrogallol.

Materials and Methods

***Kimchi* samples** The following 7 types of *kimchi* were purchased from a market in Suwon, Korea: *oisobaki* (cucumber *kimchi*), *gochunipkimchi* (red pepper leaf *kimchi*), *godulpagi* (Korean wild lettuce *kimchi*), *kannipkimchi* (perilla leaf *kimchi*), *buchukimchi* (leek *kimchi*), *chonggakkimchi* (small radish *kimchi*), and *pakimchi* (green onion *kimchi*). The samples were filtered through sterilized gauze and appropriately diluted with saline.

Isolation of tannase-producing *Lactobacillus* strains Approximately 5 mL of filter-sterilized 2%(w/v) tannic acid (Sigma-Aldrich, St. Louis, MO, USA) solution was overlaid on a plate of brain-heart infusion (BHI) agar

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medium (Difco, Detroit, MI, USA) supplemented with 0.5%(w/v) yeast extract (Difco) for 20 min (15). This treatment made the surface of the medium opaque, indicating that the overlaid tannic acids were bound to a protein fraction available on the surface of the medium to form insoluble complexes. The overlaid tannic acid solution was then removed with an aspirator, with residual tannic acids removed by rinsing the exposed surface of the medium 3 times with sterile saline. About 0.1 mL of diluted *kimchi* samples was spread onto the tannin-treated BHI agar (T-TBHIA) and incubated facultative anaerobically at 37°C for 72 hr. After the incubation, colonies forming distinct, large, and clear zones just beyond their edges on the T-TBHIA were subcultured onto MRS agar (Difco) plates. The isolates considered to be pure on MRS agar plates were selected for further testing. The isolates with *L. plantarum* ATCC 14917^T, *L. paraplantarum* ATCC 700211^T, *L. pentosus* ATCC 8041^T, and *Lactococcus lactis* subsp. *lactis* ATCC 7962 as reference strains were compared.

Genotypic characterization of the isolates Isolates were identified using a polymerase chain reaction (PCR) assay designed to amplify species-specific sequences in the *recA* genes of *L. plantarum*, *L. paraplantarum*, and *L. pentosus* (16). The assay employed 3 forward primers: paraF (5'-GTC ACA GGC ATT ACG AAA AC-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), and planF (5'-CCG TTT ATG CGG AAC ACC TA-3'); and a reverse primer, pREV (5'-TCG GGA TTA CCA AAC ATC AC-3'). The PCR product was amplified in a UNOII Thermocycler (Biometra, Göttingen, Germany). The PCR reaction mixture consisted of template DNA, each primer at 0.5 μM, 1 U of *Taq* polymerase (Roche, Mannheim, Germany), 2.5 mM $MgCl_2$, and 100 mM dNTPs. The total DNAs from the isolates and reference strains were extracted with a DNeasy Tissue kit (Qiagen, Hilden, Germany). Samples were preheated for 5 min at 95°C, and then amplified using 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. PCR products were separated by electrophoresis in a 2%(w/v) agarose gel, stained with ethidium bromide, and then visualized by ultraviolet (UV) illumination to detect specifically amplified fragments (318 bp for *L. plantarum*, 218 bp for *L. pentosus*, and 107 bp for *L. paraplantarum*).

Tannase activity determination Whole cells and cell-free extracts were used for the enzyme reaction, and tannase activity was determined using the colorimetric method of Nishitani and Osawa (17). For whole cells, a 1 mL aliquot of an overnight culture in MRS broth was harvested by centrifugation, washed with 33 mM ammonium acetate buffer (pH 5.6), and resuspended in 1 mL of substrate solution containing 20 mM methyl gallate in the same buffer. The mixture was incubated at 37°C for 24 hr and cells were removed by centrifugation. The supernatant was alkalinized with an equal amount of saturated $NaHCO_3$ solution (pH 8.6) and exposed to the atmosphere at room temperature for 1 hr. The mixture was vortexed and centrifuged, and 200 μL of each supernatant was dispensed in a well of 96-well microplate (Becton Dickinson, Frankin Lakes, NJ, USA) and its absorbance at 450 nm was read by a microplate reader. For cell-free extracts, a 1 mL aliquot of an overnight culture in MRS broth was harvested, washed, and resuspended in 150 L of 33 mM ammonium acetate buffer (pH 5.6). The suspended cells were disrupted with glass beads (150-212 μm) and the cell-free extract was recovered by centrifugation. Twenty μL of cell-free extract instead of bacterial cells was mixed with 1 mL of substrate solution for the enzyme reaction. One unit of tannase hydrolyzes 1 μmol of tannic acid/min at pH 5.6 at 37°C. The specific activity was determined by counting the number of viable cells on MRS agar after serial dilution with saline, and the protein concentration of the cell-free extract determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). All assays were performed in triplicate.

Detection of tannic acid degradation by the resting cell reaction Cells cultured in 5 mL of MRS broth were harvested, washed, and suspended in 2 mL of 33 mM ammonium acetate buffer (pH 5.6) containing 1%(w/v) tannic acid. The reaction mixture was incubated aerobically at 37°C for 24 hr. The supernatant obtained by centrifugation was analyzed by thin layer chromatography (TLC) (18). Twenty μL of the reaction product was spotted on a silica gel plate (20×20 cm, Whatman, Florham Park, NJ, USA) and developed twice with a solvent system of formic acid-acetonitrile-toluene (1:40:20, v/v) in a TLC chamber. The plate was allowed to air-dry in a laboratory fume hood, and then sprayed with an iodine solution (0.5 g of iodine in 100 mL of 95% ethanol). The reaction product was visualized by heating at 110°C for 10 min.

Detection of gallic acid degradation by the resting cell reaction The degradation of gallic acid was activated in FT80 medium (19) with the following composition (in g/L): casein acid hydrolysate, 5.0; yeast extract, 4.0; glucose, 5.0; fructose, 3.5; DL-malic acid, 10.0; KH_2PO_4, 0.6; KCl, 0.45; $CaCl_2$, 0.13; $MgSO_4$, 0.13; $MnSO_4$, 0.003; and 1 mL of Tween 80 (pH 5.0). Filter sterilized gallic acid was added to the autoclaved medium at 100 mg/L concentration. Cells grown in FT80 medium supplemented with gallic acid were inoculated into 5 mL of the same medium and incubated at 37°C for 24 hr. After harvesting the cultured cells, the pellet was washed twice with 5 mL of 33 mM ammonium acetate buffer (pH 5.6) and suspended in 2 mL of the same buffer (pH 5.6) containing 0.1%(w/v) glucose and 1 mM gallic acid (20). The reaction mixtures taken at 0, 24, and 48 hr were centrifuged, and cell-free supernatants were analyzed by high performance liquid chromatography (HPLC).

HPLC was performed using a chromatograph (1100 Series; Agilent, Palo Alto, CA, USA) with a diode array detector working at 190-400 nm, and phenolic compounds were detected at 210 nm. The following gradient of solvent A (0.01% phosphoric acid, v/v) and solvent B (acetonitrile) was applied to a Zorbax C18 column (4.6×250 mm, 5 μm, Agilent): 0-7 min, 5% B, linear; 7-12 min, 17% B, linear; 12-15 min, 20% B, linear; 15-17 min, 5% B, linear; and 17-20 min, 5% B, isocratic. The flow rate was 1 mL/min and the column temperature was 25°C.

Results and Discussion

Identities of tannase-producing isolates Lactobacilli with tannase activity isolated from human feces and fermented

Table 1. Tannase activities of isolates and their taxonomic identities determined by a multiplex PCR-based assay targeting the *recA* gene

Strain	Source	Identification	Specific tannase activity[1)]	
			U/10^9 CFU	U/mg protein
7962	ATCC (American Type Culture Collection)	*Lc. lactis*	0.18	0.06
CK6	*Oisobaki* (cucumber *kimchi*)	*L. plantarum*	3.71	18.84
CK10		*L. plantarum*	19.29	9.47
CK18		*L. plantarum*	3.28	10.92
CK21		*L. plantarum*	3.70	32.87
CK23		*L. plantarum*	3.65	20.18
RK1	*Gochunipkimchi* (red pepper leaf *kimchi*)	*L. pentosus*	34.96	22.69
RK9		*L. pentosus*	24.69	32.55
RK11		*L. pentosus*	13.10	9.41
LT1	*Godulpagi* (Korean wild lettuce *kimchi*)	*L. plantarum*	3.43	30.14
LT3		*L. plantarum*	6.52	29.85
LT4		*L. pentosus*	31.56	30.75
LT5		*L. pentosus*	15.46	25.38
LT7		*L. pentosus*	60.61	63.31
PK5	*Kannipkimchi* (perilla leaf *kimchi*)	*L. plantarum*	3.63	5.63
PK9		*L. pentosus*	10.62	6.43
PK12		*L. pentosus*	8.82	15.72
LK2	*Buchukimchi* (leek *kimchi*)	*L. plantarum*	2.82	8.51
LK4		*L. plantarum*	2.98	18.15
SK1	*Chonggakkimchi* (small radish *kimchi*)	*L. plantarum*	4.86	12.32
GK17	*Pakimchi* (green onion *kimchi*)	*L. plantarum*	2.11	35.90
GK26		*L. plantarum*	1.64	24.15
GK35		*L. plantarum*	2.43	24.31

[1)]1 U of tannase hydrolyzes 1 μmol of tannic acid/min at pH 5.6 and 30°C.

vegetables were identified as *L. plantarum*, *L. paraplantarum*, and *L. pentosus* (9,10). These species have been frequently found in *kimchi*, and thus the isolates of the present study were likely to be one of these species (21,22). However, these species are genotypically closely related and show highly similar phenotypes, which makes their correct identification problematic due to the ambiguous results from traditional physiological tests and molecular methods. Torriani *et al.* (16) developed a multiplex PCR-based method that differentiated these 3 species based on differences in gene sequences encoding the RecA protein implicated in homologous DNA recombination.

In the present study, 22 tannase-producing bacterial strains were newly isolated from 7 types of *kimchi* (Table 1). These colonies exhibited distinct and clear zones that extended just beyond their edges (Fig. 1). Our use of this multiplex PCR assay identified 8 isolates as *L. pentosus* and the others as *L. plantarum* (Fig. 2, Table 1).

Variations in tannase activities Specific tannase activities of isolates were measured with whole cells and cell-free extracts. The measured activities of the isolates are summarized in Table 1, and varied even within each species. The activities of the isolates varied with the assay method, but both methods indicated that isolate LT7 showed the highest activity. For example, the enzymatic activity of cell-free extract was 11 times higher for LT7 than for PK5.

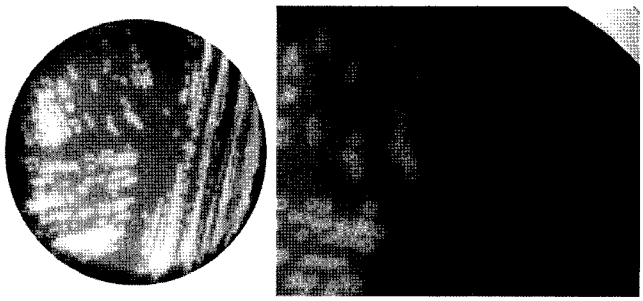

Fig. 1. Growth and clear zones of tannase-producing isolate LT7 on T-TBHIA after 72 hr of incubation at 37°C.

Interestingly LT7 was obtained from *godulpagi*, which shows the bitterest taste among *kimchi*. When whole cells were used, the activity was higher for *L. pentosus* strains than for *L. plantarum* strains, which is consistent with the observations of Nishitani *et al.* (10). Such an activity bias was not detected when cell-free extracts were used, and might be due to the growth rate differing between the 2 strains. When determining specific activities, the number of live cells in overnight cultures was higher for *L. plantarum* strains than for *L. pentosus* strains. Both methods showed that the tannase-negative reference strain *Lc. lactis* ATCC 7962 showed negligible activity.

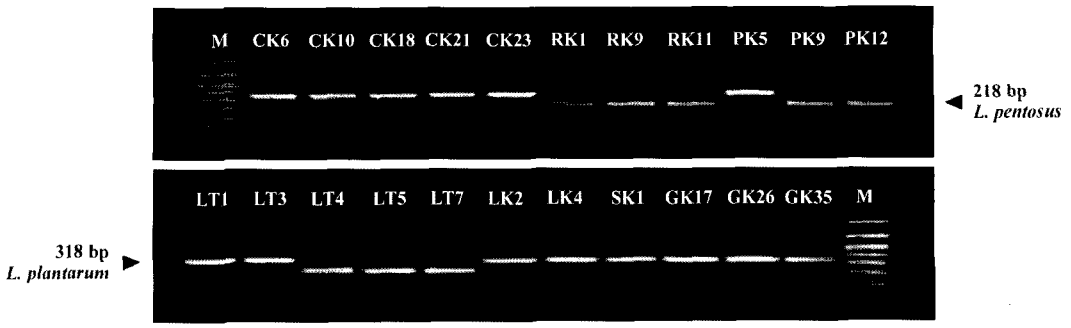

Fig. 2. PCR amplification products obtained from the species-specific multiplex PCR assay targeting the *recA* gene. Molecular markers (lane M) ranging from 50 to 1,000 bp were used to determine the sizes of amplicons. Each lane corresponds to the labeled isolate.

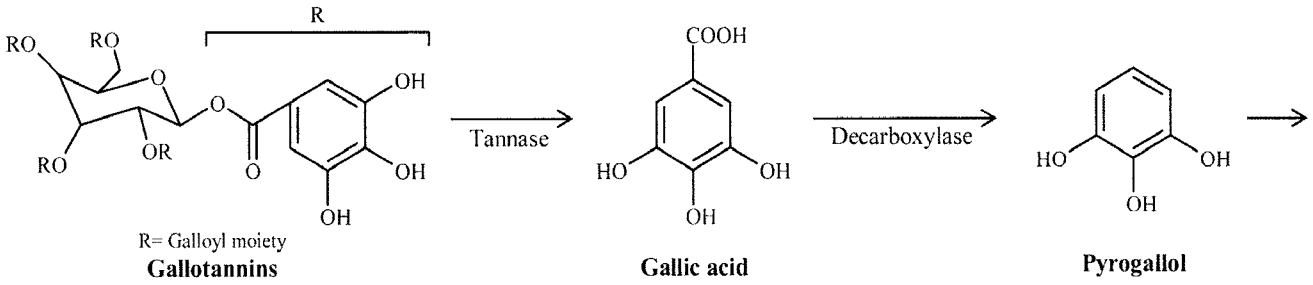

Fig. 3. Putative biodegradation pathway of tannic acid by *L. plantarum* and *L. pentosus*.

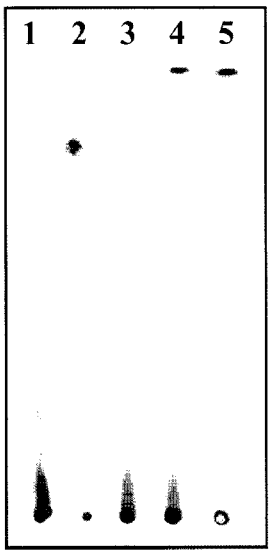

Fig. 4. TLC analysis of tannic acid biodegradation by *L. pentosus* LT7. Lanes: 1, tannic acid; 2, gallic acid; 3, reaction product for *Lc. lactis* ATCC 7962; 4, reaction product for *L. pentosus* LT7; 5, pyrogallol.

Degradation of tannic acid by *L. pentosus* LT7 Tannic acid, one of the most abundant reserve materials of plants, is a gallotannin consisting of esters of gallic acid and glucose, and contains galloyl groups esterified directly to the glucose molecules. Some bacteria, yeast, and filamentous fungi are known to develop the ability to degrade gallotannin, and a putative biochemical degradation pathway has been proposed (3,23). The biodegradation of tannic acid by the cell-free extract of *L. plantarum* was reported very recently (24). Commercial tannic acid contains several types of galloylated esters of glucose, including tetragalloyl glucose, pentagalloyl glucose, and hexagalloyl glucose. Depolymerization of high molecular weight gallotannins by tannase and gallic acid as well as pyrogallol has been detected by HPLC. Therefore, the proposed biochemical pathway for the degradation of tannic acid by *L. plantarum* implies that tannic acid is hydrolyzed to gallic acid and glucose, with the formed gallic acid being decarboxylated to pyrogallol (Fig. 3).

Isolate LT7 identified as *L. pentosus* showing the highest tannase activity was used to confirm the metabolic intermediates of tannic acid. Tannic acid biodegradation in resting LT7 cells was detected by TLC (Fig. 4). A spot coinciding with pyrogallol – known to be the last aromatic compound in the degradation of gallotannins – and not degraded tannic acid was detected, but a gallic acid spot was not detected. Therefore, gallic acid appears to be readily converted to pyrogallol.

In order to confirm the biochemical pathway involving the formation of pyrogallol, gallic acid was used as a substrate for the resting cell reaction, and the degraded products were detected by HPLC (Fig. 5). The gallic acid peak disappeared at 24 hr, whereas the pyrogallol peak was detected even at 48 hr. The peak area of pyrogallol at 48 hr was 85% of that at 24 hr, which implies that decarboxylation of gallic acid, occurs faster than the degradation of pyrogallol. The same observations were made for the conversion of tannic acid as the accumulation of pyrogallol at a TLC plate. The same pathway might underlie tannic acid degradation in *L. pentosus* and *L. plantarum* (Fig. 3).

The data presented here suggest that pyrogallol is produced from tannic acid by the resting cell reaction with *L. pentosus* LT7. Therefore, *L. pentosus* LT7 is an efficient and safe microorganism applicable to the reduction of tannins and the production of pyrogallol.

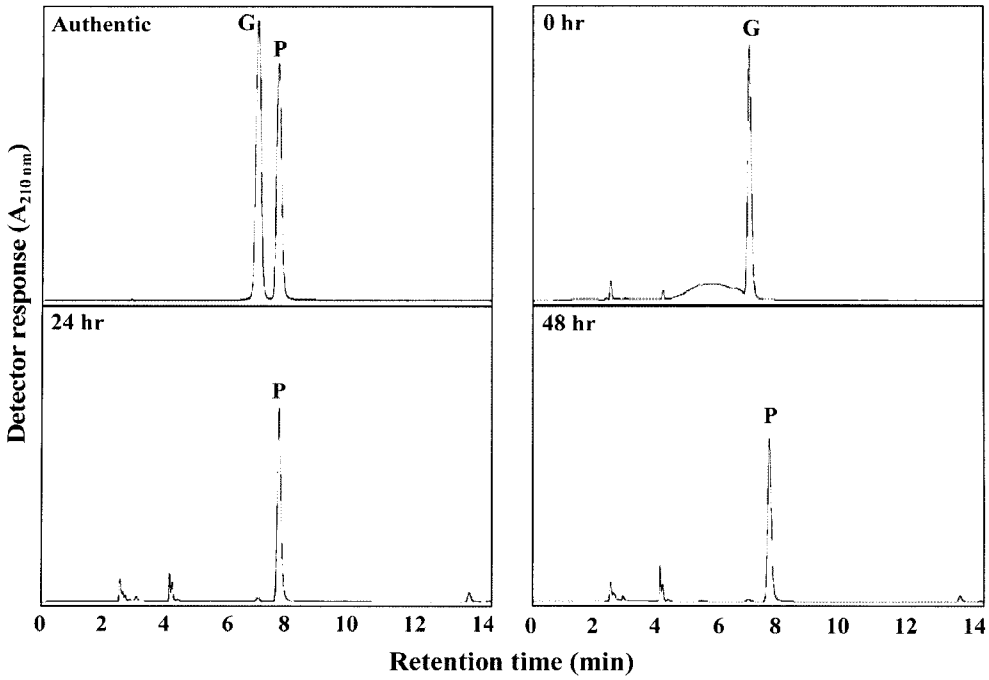

Fig. 5. HPLC analysis of gallic acid biodegradation by *L. pentosus* LT7 vs. the resting cell reaction time. The detected gallic acid (G) and pyrogallol (P) peaks are indicated.

Acknowledgments

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