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Note A novel colorimetric method to quantify tannase activity of viable bacteria

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Abstract

A novel colorimetric method to quantify tannase activity of viable tannase-producing bacterial strains was developed through application of a visual reading method that was to detect the activity qualitatively. The novel method was sensitive enough to quantify the marginal tannase activity of strains that could not be otherwise measured by conventional spectrophotometric or colorimetric methods.

Keywords: Tannase quantitaive assay; *Lactobacillus plantarum*; *Lonepinella koalarum*; *Streptococcus gallolyticus*

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(R. Osawa).

Hydrolyzable tannins such as gallotannin and ellagitannin are widely distributed in the plant kingdom (Salunkhe et al., 1989). These tannins bind readily with proteins and other macromolecules to form indigestible or insoluble complexes, thereby not only reducing nutritional value of the animal feed (McLeod, 1974; Rhoades and Cates, 1976) but also presenting undesirable taste and coloration in tea (Sanderson et al., 1974), wine (Chae and Yu, 1983), and beer (Lekha and Lonsane, 1997). The tannins have a polyol (e.g. D-glucose) as a central core that is partially or totally esterified with phenolic acids (e.g. gallic acid, ellagic acid) (Salunkhe et al., 1989). Tannase (tannin acylhydrolase) breaks specifically the ester bonds to diminish the tannins' binding property (Bhat et al., 1998). It has long been known that several fungi species such as *Aspergillus* spp. (Banerjee and Mondal, 2001) and *Penicillium* spp. (Rajakumar and Nandy, 1983) are capable of producing a large amount of this enzyme. For industrial use, the enzyme is invariably of fungal origin (Lekha and Lonsane, 1997).

Over the past decade, many bacterial species have also been reported to produce tannase. These include *Streptococcus gallolyticus* (Osawa et al., 1995a), *Lonepinella koalarum* (Osawa et al., 1995b), *Bacillus licheniformis* (Mondal and Pati, 2000), and several lactobacilli species (Osawa et al., 2000). In the course of further characterizing tannase-producing bacteria, we have noticed appreciable variations in size of the clear zone among the isolates grown on tannin-treated plates (Osawa, 1990; Osawa et al., 2000), implying variability in the tannase activities of the isolates. Meanwhile, Osawa and Walsh (1993) developed a novel visual reading method to confirm tannase activity of the isolates that formed clear zones on the tannin-treated plate medium. This used methyl gallate (MG), a simple galloylester of methanol as a substrate to be hydrolyzed by the bacterial tannase, and then gallic acid (GA)

released from MG was oxidized to give a green to brown color, sufficient to be recognized visually. If the intensity of coloration corresponds to the amount of gallic acid released after hydrolysis, this colorimetric method could be a valuable way to quantify the enzymatic activity. Although several spectrophotometric (Iibuchi et al., 1967) and colorimetric methods (Haslam and Tanner, 1970; Sharma et al., 2000; Mondal et al., 2001) have been developed elsewhere for the quantitative assay of tannase, mainly of fungal origin, here we evaluate an applied use of the visual reading method to quantify bacterial tannase activity.

Firstly, we prepared mixtures of 5mM methyl gallate (Aldrich Chemical Co. Inc., Milwaukee, WI) solution and 5 mM gallic acid (Wako Pure Chemical Ltd., Osaka, Japan) solution at various proportions (0:10 to 4:6) in 33 mM NaH_2PO_4 (pH 5.0). One hundred μl of each mixture was dispensed in a microtube, and then alkalinized with an equal amount of saturated NaHCO_3 solution (pH 8.6). The alkalinized mixtures were incubated aerobically at 37°C for up to 2 h. After 15 min, 30min, 1h and 2 h of incubation, 200 μl of each mixture was taken, vortexed and centrifuged ($8,000 \times g \cdot 20 \text{ sec}$), and 100 μl of each supernatant was dispensed in a well of 96-well microplate (CFM-100; Iwaki, Tokyo, Japan) and its absorbance at 450 nm was read on a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA). Triplicate tests of the above assay revealed that the absorbance read after 2 h incubation was well correlated ($r = 0.98$, $p < 0.01$) with an increase in GA concentrations of up to 2 mM (Fig. 1).

Subsequently, enzyme solutions containing purified tannase made from *Aspergillus oryzae* (Wako) at a range of concentrations from 0.63 mU to 1,000 mU (or 1 U) per ml were subjected to quantification by three methods as follows: (a), A conventional spectrophotometric quantification used in the

present study followed the methodology described by libuchi *et al.* (5). Briefly, the enzyme solutions containing tannase at the above concentration range were made in 50 mM citrate buffer (pH 5.5). One hundred μ l of each dilution thus prepared was mixed with 400 μ l of a substrate solution containing tannic acid (Wako) at concentration of 0.35% (w/v) in the citrate buffer. The mixtures were incubated at 30°C for up to 3 h. After 3 h of incubation, 10 μ l of each mixture was added to 1 ml of ethanol, and subsequently its absorbance at 310 nm was read by a spectrophotometer (UV-1600; Shimadzu Co., Kyoto, Japan). The difference in the absorbance at 310 nm between samples immediately prior to the incubation and those incubated for 3 h were subsequently calculated; (b), A conventional colorimetric quantification followed the method described by Sharma *et al.* (20). Briefly, the enzyme solutions were made in 50 mM citrate buffer (pH 5.0) and 250 μ l of each dilution was mixed with an equal volume of a substrate solution containing 10 mM MG (Wako) in the citrate buffer. The mixtures were incubated at 30°C for 5 min. After incubation, 300 μ l of methanol containing rhodamine (2-thioxo-4-thiazolidion; Wako) at a concentration of 0.667 % was added to each mixture, and incubated at 30°C for 5 min. After incubation, 200 μ l of 0.5 N KOH solution was added to the mixture, and incubated for a further 5 min at 30°C, and the mixture was then diluted with 4 ml distilled water, and incubated for 10 min more at 30°. After incubation, the absorbance at 520 nm was read by a spectrophotometer (UV-1600; Shimadzu); and (c), For the new colorimetric method, the enzyme solutions were made in 33 mM NaH₂PO₄ buffer (pH 5.0) and 50 μ l of each dilution was mixed with 5 ml of a substrate solution containing 5 mM MG in the phosphate buffer. The mixtures were incubated at 37° C for 24 h. After incubation, 100 μ l of each mixture was then mixed with an equal volume of saturated NaHCO₃, and incubated for a further 2h at 37°. The mixture was

vortexed and centrifuged ($8,000 \times g \cdot 20 \text{ sec}$) and 100 μl of each supernatant was dispensed in a well of 96-well microplate (CFM-100; Iwaki, Tokyo, Japan) and its absorbance at 450 nm was read by a microplate reader (Model 550; Bio-Rad).

Triplicate test results from these three methods showed that the novel method successfully calibrated a range of tannase activities (ca. 0.63 to 10.0 mU/ml. The equation of the regression line was; $y = 0.032x + 0.014$, $r^2 = 0.99$ [$p < 0.01$]) of the test solutions but the other conventional methods failed to calibrate the activities (Fig. 2a). In contrast, the two conventional methods successfully estimated tannase activity at much higher concentrations (ca. 100 to 1,000 mU/ml) whereas our novel method failed to do so (Fig. 2b).

Finally, the novel colorimetric method was used to quantify tannase activity of viable tannase-producing bacterial cells. The bacterial strains used were: *Lactobacillus plantarum* ATCC 14917^T; *L. plantarum* CNRZ 184; *L. paraplantarum* 61D (kindly provided from F. Bringle, Institut de Botanique, Centre National de la Recherche Scientifique, Strasbourg, France); *S. gallolyticus* ACM 3611^T; and *Lonpinella. koalarum* ACM 3666^T. Fresh cultures of the lactobacilli strains grown on MRS agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), *S. gallolyticus* ACM 3611T on brain heart infusion agar (BHIA; Oxoid), and *L. koalarum* 3666 on Wilkins-Chalgren anaerobe agar (Oxoid) were each harvested with sterile cotton swabs and suspended in 1ml of substrate medium (pH 5.0) containing 33 mM NaH_2PO_4 and 5 mM MG at final concentration to prepare a dense suspension whose absorbance at 660 nm was adjusted to 0.4 for the three lactobacilli strains and *S. gallolyticus* ACM 3611^T and to 0.05 for *L. koalarum* ACM 3666^T. This resulted in populations of colony-forming units per 1 ml of the bacterial suspensions of approximately 1.4 to 1.6×10^8 for the lactobacilli, 1.3×10^8 for

S. gallolyticus ACM 3611^T, and 2.8×10^8 for *L. koalarum* 3666^T. The substrate medium was incubated aerobically at 37°C for 24 h. After incubation, 1 ml of each bacterial suspension was dispensed in a microtube and centrifuged (8,000 x g, 5 min). Then 100 µl of supernatant was mixed with an equal volume of the saturated NaHCO₃ solution (pH 8.6) and incubated at 37°C for 2 h. The mixture was vortexed and centrifuged (8,000 x g, 20 sec). Next, 100 µl of each supernatant was dispensed in a well of 96-well microplate (CFM-100; Iwaki) and its absorbance at 450 nm was read on a microplate reader (Model 550; Bio-Rad). The results of triplicate assays revealed that *L. koalarum* 3666^T had markedly strong tannase activity for a set number of viable cells (ca. 3.0×10^8) among the strains tested, that the lactobacilli strains had an appreciable difference in their tannase activities (Table 1).

Since many tannase-producing bacteria strains have been isolated from intestinal microflora of animals and human, it is likely that they might play an important role in digestion of dietary tannins. In this context, the novel colorimetric method is useful to reveal variation in their ability to produce tannase, which could not be otherwise detected by the conventional quantitative methods. Further characterization of tannase producing bacterial strains is in progress with use of the novel method.

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Table 1. Tannase activities of bacterial strains of established culture collections, measured by the novel colorimetric method developed in the present study.

Strain	Tannase activity ^a (mU/ml) \pm SD	CFU/ml ^b	Estimated tannase activity (mU) per 3.0 x 10 ⁸ CFU
<i>L. plantarum</i> ATCC ^c 14917 ^T	5.7 \pm 0.2	1.6 x 10 ⁸	10.7
<i>L. plantarum</i> CNRZ ^d 184	0.8 \pm 0.1	1.4 x 10 ⁸	1.7
<i>L. paraplantarum</i> 61D	<0.1	1.5 x 10 ⁸	<0.2
<i>S. gallolyticus</i> ACM ^e 3611 ^T	0.7 \pm 0.2	1.3 x 10 ⁸	1.6
<i>L. koalarum</i> ACM 3666 ^T	2.8 \pm 0.2	2.7 x 10 ⁸	3.1

^a Tannase activity was measured in triplicate tests

^b CFU, colony forming units, were obtained from plate counts of 10-fold serial dilutions of the bacterial suspensions.

^c ATCC, American Type Culture Collection, Manassas, Va.

^d CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France.

^e ACM, Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, St. Lucia, Queensland, Australia.

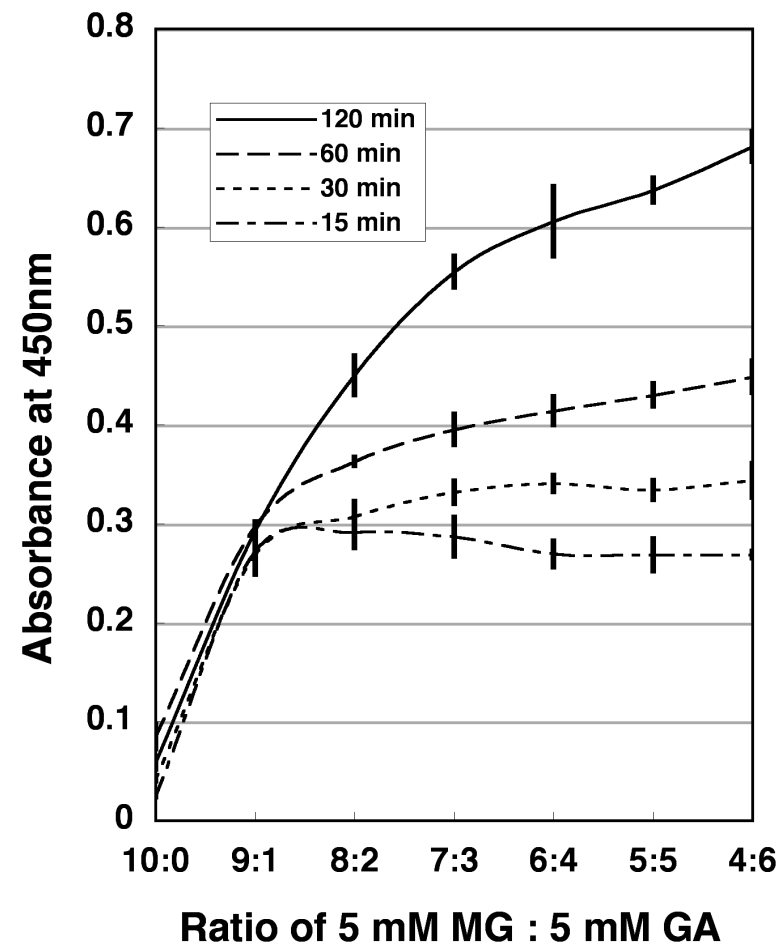


Fig. 1

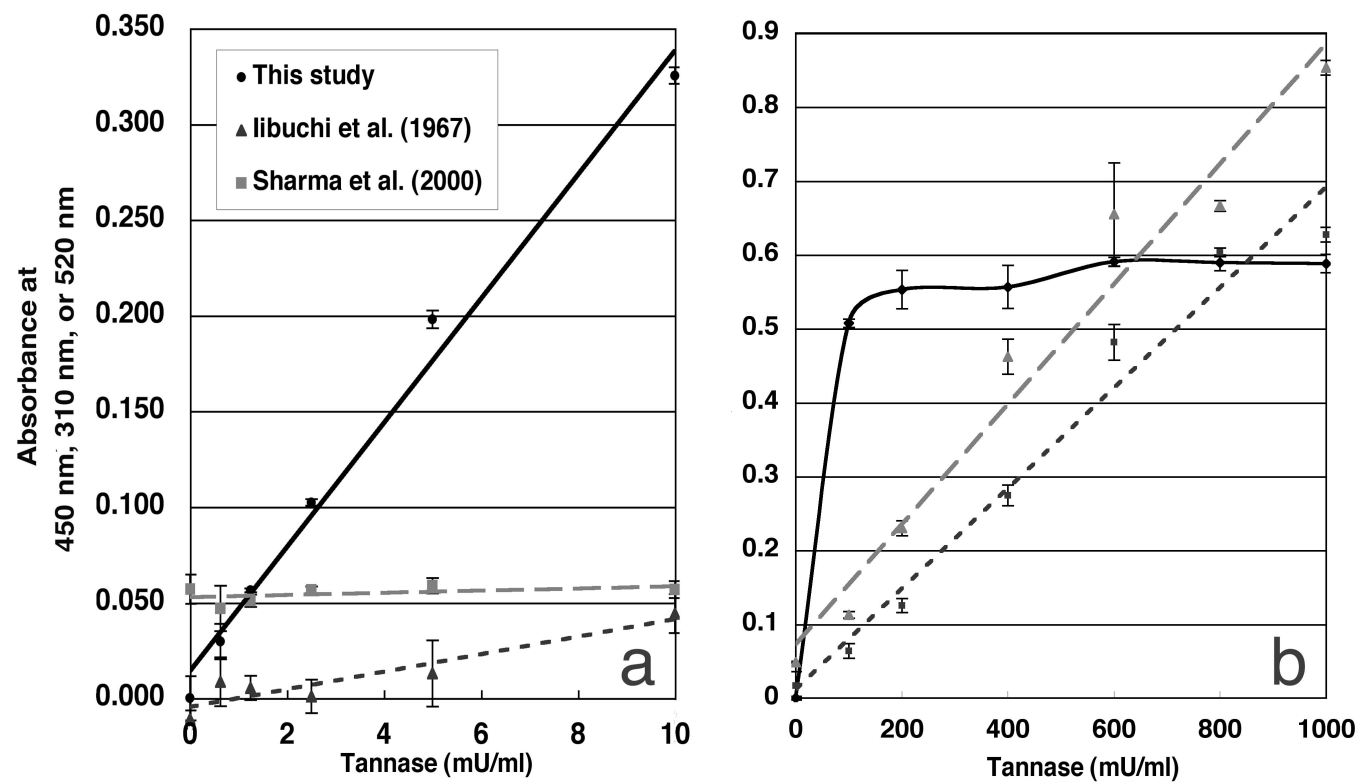


Fig. 2