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Identification and Cloning of a Gene Encoding Tannase (Tannin Acylhydrolase) from *Lactobacillus plantarum* ATCC 14917^T

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Running title*: Cloning of Tannase Gene from Lactobacillus plantarum*

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List of non-standard abbreviations

TanLpl: tannase of *Lactobacillus plantarum*

tanLpl: gene encoding TanLpl

tanA: tannase gene of *Staphylococcus lugdunensis*

T-TBHIA: tannic acid treated brain heart infusion agar

HAP: hydroxylapatite

Km: Michaelis-Menten constant

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abstract

The TanLpl gene (*tanLpl*), encoding a novel tannase enzyme (TanLpl), has been cloned from *Lactobacillus plantarum* ATCC 14917^{T}. This is the first report of a tannase gene cloned from a bacterial source other than *Staphylococcus lugdunensis* reported elsewhere (Noguchi *et al.* in J. Gastroenterol. [2007] 42[5]):346-351). The open reading frame of *tanLpl,* spanning 1,410 bp, encodes a 469 -amino-acid protein that shows 28.8% identity to the tannase of *S. lugdunensis* with several commonly conserved sequences. These sequences could not be found in putative tannases reported for other bacteria and fungi. TanLpl was expressed in *Escherichia coli* DH5α from a pGEM-T expression system and purified. SDS-PAGE analysis indicated that purified TanLpl is a monomer polypeptide of approximately 50 kDa in size. Subsequent enzymological characterization revealed that TanLpl is most active in an alkaline pH range at 40^{o}C, quite different from that observed for a fungal tannase of *Asperigillus oryzae*. In addition, the Michaelis-Menten constant of TanLpl was markedly lower than that of *Asperigillus oryzae* tannase. The evidence suggests that TanLpl is classified into a novel family of tannases.

Key words: Tannase – Gene Cloning– *Lactobacillus plantarum* – *Staphylococcus lugdunensis* – *Aspergillus oryzae*

Introduction

Hydrolyzable tannins, such as gallotannin and ellagitannin, are polyphenolic compounds that occur widely in the plant kingdom [19]. These tannins are well known for their ability to bind readily with proteins to form indigestible complexes and chelate heavy metals [5, 9]. They are therefore considered as effective antinutritional [2] and antimicrobial compounds [21]. Tannase (tannin acylhydrolase), which specifically breaks the galloyl ester bonds of hydrolyzable tannins, was first reported for several fungal species (i.e. *Aspergillus oryzae* and *A. niger*) in which the enzymes were purified and their enzymatic and molecular properties were well characterized [1, 6]. Meanwhile, over the last two decades, many bacterial species have also been reported to have tannase activity. These include *Streptococcus gallolyticus* [15] and *Lonepinella koalarum* [16] isolated from animal feces, *B. cereus* from soil [11], and *Bacillus licheniformis* [4] and *Lactobacillus plantarum* isolated from various fermented plant materials [13]. However, unlike fungal tannases, little has been identified about genes encoding bacterial tannases or their protein properties. In this context, Noguchi et al. [14] recently reported cloning of a novel gene (*tanA*) of *Staphylococcus lugdunensis* that encodes a polypeptide of 613 amino acids with tannase activity while Kleerebezem et al. [7] reported the complete genome sequence *of L. plantarum* WCFS1, claiming that many of the genes may play important roles for the bacterium to adapt to its environment. Thus, we searched for nucleotide

sequences of *L. plantarum* WCFS1 similar to *tanA* using the BLAST program, and found that an ORF, designated as lp2956, encoding a “hypothetical protein,” had 46.7% similarity to *tanA*. We here describe cloning a gene (*tanLpl*) that encodes tannase of *L. plantarum* ATCC 14917^T on the basis of the nucleotide sequences of *tanA* and lp2956, characterization of the recombinant enzyme (TanLpl), and finding that the enzyme is a novel tannase with different properties from known tannases.

Materials and Methods

Bacterial strains and growth conditions. The various bacterial strains used in the study and their respective sources are listed in Table 1. *L. plantarum* ATCC 14917^T was used for cloning *tanLpl*. A total of 24 tannase-producing strains, consisting of 9 isolates of *L. plantarum* (inclusive of *L. plantarum* ATCC 14917^T), 6 isolates of *L. paraplantarum*, 6 isolates of *L. pentosus*, and 3 isolates of *S. gallolyticus* were used to study the specificity of the *tanLpl* taggeted primers used in PCR and subsequent Southern blotting assays targeting *tanLpl*-like genes. As negative controls, 2 strains of common species of the *Lactobacillaceae* without tannase activity were used. The bacterial cultures were propagated statically at 37˚C in MRS (Difco, Detroit, USA) or on MRS supplemented with 1.5% agar before the experiment. *Escherichia coli* DH5α (Promega, Madison, USA) was used for cloning and expression of the

tanLpl gene.

Preparation of genomic DNA. Genomic DNA from the bacterial strains was prepared essentially following the method of Marmur [10]. The purity and amount of DNA in each preparation was estimated colorimetrically, as described by Sambrook et al. [20], and stored at 4°C until use.

Cloning, sequencing, and expression of tanLpl in E. coli. Based on the *L. plantarum* WCFS1 sequence information available in the GenBank database, primers lp2956-f (5'-ATC ATT GGC ACA AGC CAT CA-3') and lp2956-r (5'-GGT CAC AAG ATG AGT AAC CG-3') were designed and synthesized (Integrated DNA Technologies, Coralville, Ia) to amplify a 1,410-bp DNA fragment, including lp2956 (positions 222,495 to 223,904, GenBank accession no. AL935260) from *L. plantarum* ATCC 14917^T. The PCR mixture (50 μl) consisted of 1x Easy-A reaction buffer (Stratagene, La Jolla, USA) containing 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM oligonucleotide primers (lp2956-f and lp2956-r), 2.5 U of Easy-A high-fidelity PCR cloning enzyme (Stratagene) and 50 ng of genomic DNA. PCR was carried out in a programmable thermocycler (PTC 100; MJ Research, Waltham, USA) under the following conditions. Initially, the reaction mixtures were heated at 92°C for 2 min, and then the PCR progressed through 30 cycles of melting at 94°C for 30 sec, annealing at 52°C for 1 min, and extension at 72°C for 2 min. A final extension for 10 min at 72°C was included at the end of the 30th cycle. Reaction mixtures were stored at 4°C until analysis by

electrophoresis on a 1% agarose gel in 1x Tris-acetate-EDTA (0.04 M Tris-acetate, 0.001 M EDTA) buffer at a field strength of 8 V/cm. Amplicons were detected by staining with ethidium bromide (0.5 μg/ml) and were photographed under a UV transilluminator.

The PCR product was purified with the QIAquick PCR purification kit (QIAGEN, Valencia, USA) and cloned into the pGEM-T Easy cloning vector (Promega, Madison, Wi), according to the manufacturer's protocol. Transformed *E. coli* DH5α (Promega) cells carrying the recombinant plasmid were selected by blue-white selection. The cloned product was sequenced by primer walking using a BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, USA) on ABI Prism 3100 Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol. Sequences of the cloned fragments were edited to remove the vector sequence and compared with those for lp2956 available in GenBank and EMBL using the BLAST program (www.ncb.nlm.nih.gov/blast/blast.cgi).

Tannase assays. E. coli DH5α strains carrying the recombinant plasmid (termed pGtanLpl) were innoculated in Luria-Bertani (LB) broth (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter) and incubated at 37°C for 24 h. After incubation, 20 μl of each culture was spotted onto a tannic acid-treated brain-heart infusion agar (Oxoid Ltd., Basingstoke, UK) plate (T-TBHIA), which had an opaque surface due to the formation of a tannin-protein complex. The

inoculated plates were incubated anaerobically in Anaero-Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C for 48 h. After incubation, well-isolated colonies with a clear zone extending beyond their edges on tannic acid-treated plates, indicating apparent bacterial degradation of tannic acid, were selected and their tannase activities were further confirmed qualitatively by the spectrophotometric method of Sharma et al. [21]. In this method, enzyme activity was calculated from the change in absorbance of gallic acid at 520 nm, i.e. $\Delta A_{520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$. One unit of the activity was defined as the amount of enzyme which liberated 1 μmole of gallic acid from methyl gallate formed per min at 30°C and pH 8.0.

Purification of L. plantarum tannase E. coli DH5α with the recombinant plasmid was inoculated into 2 L of LB broth plus ampicillin at 100 μg/ml, and incubated at 37°C for 24 h. After incubation, the cell-free culture supernatant was collected by centrifugation (12,000 x *g* for 10 min), and crude proteins were precipitated from the culture supernatant by adding solid ammonium sulfate to 60% saturation at 4°C. The precipitated materials were then collected by centrifugation (12,000 x *g*, 10 min), dissolved in 25 mM Tris-HCl buffer (pH 8.0), and extensively dialyzed against the same buffer for 24 h at 4°C. The dialyzed material was clarified by centrifugation (12,000 x *g*, 10 min) and applied to a Q-Sepharose Fast Flow column (ϕ2.6 x 12 cm, Amersham Biosciences, Piscataway, USA) equilibrated with 25 mM Tris-HCl buffer (pH 8.0). The proteins were eluted with a step-wise

increase of NaCl (0.1 M, 0.2 M, 0.3 M, and 0.5 M). The active fractions (0.2 M NaCl eluates) were dialyzed against 10 mM sodium phosphate buffer (pH 6.8) and loaded onto hydroxylapatite (HAP, Seikagakukogyo Co., Tokyo, Japan) column (ϕ1.0 x 10 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The adsorbed proteins were eluted with 30 mM, 60 mM, and 200 mM sodium phosphate buffer (pH 6.8). The active fractions (30 mM sodium phosphate eluates) were dialyzed against 25 mM Tris-HCl buffer (pH 8.0) and loaded onto a Mono-Q GL5/5 column (ϕ0.5 x 5 cm, Amersham Biosciences) equilibrated with 25 mM Tris-HCl buffer (pH 8.0). The adsorbed proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in 25 mM Tris-HCl buffer (pH 8.0). The active fractions eluted with 0.15 M NaCl in the buffer were collected and stored as final purified material. All purification steps were performed at 4°C using a fast protein liquid chromatography system (Amersham Biosciences). At each purification step, the activity of tannase was determined quantitavely by the spectrophotometric method [21] as described above.

Subsequent dependence of the activity on pH and temperature of the purlified TanLpl was determined in comparison with that of a commercially available *A. oryzae* tannase (Wako Pure Chemicals Industries, Tokyo, Japan). Enzymatic activity was measured by the spectrophotometric method [21] in 25 mM sodium citrate (pH 4.0, 4.5, 5.0, and 5.5), 25 mM sodium phosphate (pH 6.0, 6.5, and 7.0), and 25 mM Tris-HCl (pH 7.5, 8.0, and 8.5) at 30°C for 10 min, and at

various temperatures (20, 25, 30, 35, 40, 45, 50 and 55°C) in 25 mM Tris-HCl (pH 8.0) for TanLpl and in 25 mM sodium citrate (pH 5.5) for *A. oryzae* tannase (Wako), respectively. On calculation of Michaelis-Menten constant (K_m), the tannase activity of TanLpl enzyme was measured at 30°C and pH 8.0 using methyl gallate of different concentrations as a substrate. On the other hand, the activity of *Aspergillus oryzae* tannase was measured at 30°C and pH 5.5..

Protein determination and gel electrophoresis. Protein concentration of purified TanLpl was determined with a Bradford reagent kit (Bio-Rad Laboratories, Hercules, USA) by following the manufacturer's protocol. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system developed by Laemmli [8] was used to monitor homogeneity and determine the molecular mass of the purified protein. The subjected sample was pretreated with or without 30 mM dithiothreitol (DTT), which could cleave disulfide bonds. Proteins in gels were stained with a Quick-CBB kit (Wako).

*Amino acid sequencing.*The N-terminal amino acid sequence of TanLpl was determined by automated Edman degradation using a PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan).

tanLpl targeted PCR assay and Southern hybridization. PCRs were performed on purified DNA preparations of strains with or without tannase activity using a primer pair of lp2956-f and lp2956-r under the same PCR conditions as described above. Meanwhile, HincII-digested DNA fragments of the strains were

separated in 1.5% agarose gels and transferred to Hybond N+ nylon membranes (Amersham) using the capillary method. Hybridization probes were synthesized using A 418-bp internal *tanLpl* PCR product amplified with primers 5'-AAGTCTGGGCAACGGGTCG-3' and 5'- CCAACGAAAGGGCCTGTTCT-3'. Probe labeling, hybridization, washing and detection were performed with on AlkPhosDirect labeling kit (Amersham) and BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, USA) according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The nucleotide sequences of gene *tanLpl* of *L. plantarum* ATCC 14917^T have been deposited in the GenBank database under accession no. AB379685.

Results

Cloning of tanLpl. In order to confirm *tanLpl* as responsible for tannase activity in *L. plantarum* ATCC 14917^T, a PCR product containing *tanLpl* was inserted in pGtanLpl in *E. coli* DH5α cells. Eight positive clones were identified by the formation of colonies with a clear zone on T-TBHIA plates. PCR assay targeting the gene revealed that each clone contained the inserts (data not shown). Tannase elicited by the respective clone was further confirmed using the qualitative spectrophotometric method. One of the clones with the most apparent clear zone formation, designated *E. coli* A3-2, was then subjected to subsequent

nucleotide sequencing of the tanLpl gene, expression and purification of the recombinant enzyme.

The nucleotide and the deduced amino acid sequences of tanLpl. The nucleotide sequence of the *tanLpl* gene (1,410 bp) within pGtanLpl in *E. coli* A3-2 was determined. A homology search with the BLAST program revealed that the nucleotide sequence of the *tanLpl* gene was almost identical (99.6%) to lp2956 of *L. plantarum* WCFS1 with single base substitution at 4 positions, and was similar (46.7%) to *tanA* of *S. lugdunensis.* The gene encoded 469 amino acid residues and its molecular weight was evaluated as 50,747. The deduced amino acid sequence was highly similar (99.6%, 467/469 residues) to that encoded by *L. plantarum* WCFS1 lp2956, and relatively similar (28.8%, 135/469 residues) to that of TanA (Fig. 1). Alignment analysis also revealed the presence of highly conserved sequences among the three enzymes as follows: Val^{49}-Pro-Ala-Ala-Tyr^{53}, Val^{59}-Asn-Gly-Tyr^{62}, Thr^{66}-Ala-Pro-Ile^{69}, Lys^{137}-Ala-Ala-Ile-Arg-Tyr^{142}, Ala^{172}-Gly-Ala-Ser-Gly^{176}, Ala^{215}-Tyr-Glu-Trp-Gln^{219}, Ala^{334}-Tyr-Leu-Lys^{337}, and His^{410}-Trp-Arg-Ile-Arg^{414}, in which the numbers were based on the TanLpl sequence (Fig. 1).

Characterization of recombinant TanLpl expressed in E. coli. Attempts to purify TanLpl expressed in *E. coli* by using Q-Sepharose, HAP, and Mono-Q columns were successfully performed to yield 2.97 mg of tannase with a specific activity of 84.3 U/mg (Table 2). SDS-PAGE analysis of purified TanLpl revealed a single

protein band of approximately 50 kDa (Fig. 2), which is in close agreement with the molecular mass of 50.7 kDa deduced from the amino acid sequence of *tanLpl*. Amino acid sequencing confirmed that the N-terminal sequence (Met-Ser-Asn-Arg-Leu-Ile-Phe-Asp-Ala-Asp-) of purified TanLpl matched the corresponding sequence predicted from *tanLpl*. The molecular mass evaluated with SDS-PAGE and the N-terminal amino acid sequence of the recombinant enzyme showed that the recombinant enzyme was certainly derived from the *tanLpl* gene. Moreover, the migration profile of TanLpl was identical under both reducing and non-reducing conditions (Fig. 2), indicating that the enzyme contained no intermolecular disulfide bond and could function as a monomer.

The effects of pH and temperature on tannase activity were measured for purified TanLpl in comparison with *A. oryzae* tannase. The activity of TanLpl was found to be maximum at approximately pH 8.0 and at 40°C, in which the enzyme retained more than 50% activity between 25°C and 45°C but the activity dropped off markedly above 45°C (Fig. 3a). In contrast, the activity of *A. oryzae* tannase was maximum at approximately pH 5.5 and at 45–50°C, in which activity dropped sharply at a pH value above 5.5 or below 4.5 while more than 50% of activity was observed between 20°C and 60°C (Fig. 3b). The K_m value (0.62 mM) of purified TanLpl was significantly lower ($p < 0.01$) than that (2.94 mM) of *A. oryzae* tannase (Fig. 4).

Presence of tanLpl-like genes in other tannase-producing strains. The

tannase-producing strains listed in Table 1 were screened for *tanLpl* by the *tanLpl*-targeted PCR assay. PCR yielded amplicons of the expected size (approx. 1.4 kb) for all *L. plantarum* strains tested. Southern blotting assays revealed that the internal *tanLp* probe reacted with *Hinc*II-distested DNA fragments of not only all *L. plantarum* strains but also all *L. paraplantarum* strains tested at either or both approx. 20 kb and approx. 1kb (Fig. 5) but not with any other strains belonging to *L. pentosus* and *S. gallolyticus*. It would be worth noting that the *tanLpl* probe reached two different-sized fragments of *Hinc*II -digested DNA of *L. plantarum* 20A-3 (Fig. 5).

Discussion

In this work, we reported the identification and cloning of a novel tannase gene, *tanLpl* of *L. plantarum* ATCC 14917^T and characterized its polypeptide, TanLpl. Past studies [4, 13, 15, 16] reported that many bacterial species isolated from fermented foods and intestinal tracts of animals showed apparent tannase activity. Of these, *S. lugdunensis* was the only species, from which a gene (*tanA*) that encodes polypeptide with tannase activity, had been cloned [14], but its enzymatic characteristics have not been well described. Although the amino acid sequence of TanA (613 aa) is longer than that of TanLpl (469 aa) with limited homology, both enzymes shared several highly conserved sequences. These sequences are likely to include catalytic residues but show no significant

homology with any of the tannases of other bacteria and fungi deposited in the EMBL databank, including *Agrobacterium tumefaciens* str. C58, *Shewanella putrefaciens* CN-32, *Burkholderia multivorans* ATCC 17616, *Marinomonas* sp. MWYL1, *Actinobacillus succinogenes* 130Z, *Pseudomonas mendocina* ymp, *A. oryzae* and *A. niger*; therefore, TanLpl and TanA may have catalytic residues different from bacterial and fungal tannases, and are thus classified into a novel family of tannases.

Noguchi *et al.* [14] performed Southern blotting assay using a *tanA*-targeted probe on a wide range of bacterial species, including *S. lugdunensis, L. plantarum*, *L. pentosus*, and *S. gallolyticus*, and found that the probe reacted positively only with *S. lugdunensis*; however, our Southern blotting assay using the *tanLpl*-targeted probe revealed that homologous genes are possessed by *L. paraplantarum* strains. As *L. plantarum* and *L. paraplantarum* are known to share an almost identical 16 S rRNA sequence [3], tannase genes in these closely related lactobacilli might have been diversified along with their phylogeny. Additionally, observation of the *tanLpl* probe reacting with two different-sized fragments of *Hinc*II- digested DNA of one of the *L. plantarum strains* suggests that the strain has multiple copies of the *tanLpl*-like gene. Further work on cloning and sequencing possible tannase genes in *L. paraplantarum* is in progress to substantiate the above views.

The optimal pH for most fungal and bacterial tannase has been reported to be

acidic [1, 6]; for example, the optimum pH of tannase produced by *A. oryzae* was 5.5 [6], consistent with the present observation, and that of *B. licheniformis* was 5.8 [4]. Meanwhile, the optimal pH of TanLpl was more alkaline (approx. pH 8.0) and the enzyme was found to be susceptible to high temperature (> 40^{o}C). In this context, a contradictory observation has been recently reported by Rodríguez et al. [18] that cell-free extracts of the type strain *L. plantarum* CECT 748^{T} (= ATCC 14917^{T}) had tannase activity optimal at pH 5.0 and 30 °C. The discrepancy may be due to impurity in their tannase preparation, which was a simply filtered supernatant of disintegrated cell suspension. Alternatively, the strain may be producing another tannase that is quantitatively more significant than TanLpl. Further work will be necessary to evaluate the above explanations. The lower K_m value for TanLpl than *A. oryzae* tannase suggests that the substrate-binding site of TanLpl adopts a different conformation from that of *A. oryzae* tannase. Furthermore, the TanLpl enzyme can function as a monomer whereas *A. oryzae* tannase is known to consist of two subunits linked by a disulfide bond [6]. The difference in enzymatical and structural properties between TanLpl and *A. oryzae* tannase confirms that TanLpl belongs to a novel family of tannases, as with the results of the homology search mentioned above.

Tannins are widely distributed in the plant kingdom and bind readily with proteins or heavy metals to form insoluble complexes, thereby acting as a defense mechanism in plants against microbial attacks [12, 22]. Pulido et al. [17]

reported that the pH of plant materials during fermentation (or decomposition) was initially around neutral and decreased gradually along with the growth of microorganisms; thus, TanLpl will enable *L. plantarum* strains to utilize proteinous materials and heavy metals bound or chelated with tannins in plants and have an ecological advantage over not only tannase non-producing bacteria but also tannase-producing fungi at an early stage of plant fermentation or decomposition.

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Figure Legends

Fig. 1. Comparison of the deduced amino acid sequence of TanLpl with those of lp2956 of *L. plantarum* WCFS1 and TanA of *S. lugdunensis*. Identical residues among three enzymes are boxed. The two different residues (Ala^{154} and Gln^{406}) in TanLpl from lp2956 are indicated by arrowheads.

Fig. 2. SDS-PAGE of TanLpl. Enzymes purified from *E. coli* tanLpl-A3-2 with or without DTT treatment as described in Materials and Methods were analyzed by SDS-PAGE (12.5% separating and 2.5% stacking gel) and stained with Coomassie brilliant blue. Purified TanLpl without (lane 1) and with DTT pretreatment (lane 2) is shown. The band corresponding to TanLpl is indicated with an arrowhead. Molecular mass markers were loaded onto lanes labeled M, and their masses (in kilodaltons) are shown at the left of the gels.

Fig. 3. Effect of pH (a) and temperature (b) on activities of TanLpl (■)and *A. oryzae* tannase (○). The spectrophotometric method (21) was performed under various conditions for the hydrolysis of methyl gallate. pH experiments were performed at 30°C, and temperature experiments were performed at pH 8.0 and pH 5.5 for TanLpl and *A. oryzae* tannase, respectively. The values are shown as the relative activity, and the maximum relative activities for TanLpl (131 U/mg at its optimal pH, 122 U/mg at its optimal temperature) and *A. oryzae* tannase (44

U/mg at its optimal pH, 61 U/mg at its optimal temperature) are indicated as 100%. Each experiment was performed in triplicate.

Fig. 4. Lineweaver-Burk reciprocal plot of TanLpl (■) and A. oryzae tannase (○) with methyl gallate as a substrate. The results are plotted as 1/V versus 1/[S], where V and [S] are the reaction velocity and the substrate concentration, respectively. Apparent values for the K_m of of TanLpl and *A. oryzae* tannase were 0.62 mM and 2.94 mM, respectively.

Fig. 5. Southern hybridization of genomic DNA from tannase-producing strains. Genomic DNA from each strain was digested with *Hinc*II, separated on a 1.0% agarose gel, and transferred to a nylon membrane prior to hybridization with the *tanLpl* probe. Lanes: 1, *L. plantarum* ATCC 14917^T; 2, *L. plantarum* 18A-1; 3, *L. plantarum* 18A-2; 4, *L. plantarum* 19B-2; 5, *L. plantarum* 19B-4; 6, *L. plantarum* 20A-2; 7, *L. plantarum* 20A-3; 8, *L. plantarum* 20A-4; 9, *L. plantarum* 22A-4; 10, *L. paraplantarum* ATCC 700211^T; 11, *L. paraplantarum* 20B-1; 12, *L. paraplantarum* 37A-5; 13, *L. paraplantarum* 37B-1; 14, *L. paraplantarum* NOS120; and 15, *L. paraplantarum* NOS1461. Positive probe reaction was not observed in other tannase-producing strains belonging to *L. pentosus* and *S. gallolyticus* (data not shown)

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Table 1

TABLE 1. Bacterial strains used and their pheno- and genotypic tannase profiles

Strain	Isolated from:	Source	Tannase activity[a]	*tanLpl* gene[b]
L. plantarum ATCC 14917 [T]	Pickled cabbage	ATCC [c]	+	+
L. plantarum 16A-1	Kimchi	Our collection	+	+
L. plantarum 16A-2	Kimchi	Our collection	+	+
L. plantarum 19B-2	Turnip pickled with rice bran	Our collection	+	+
L. plantarum 19B-4	Turnip pickled with rice bran	Our collection	+	+
L. plantarum 20A-2	Turnip pickled with rice bran	Our collection	+	+
L. plantarum 20A-3	Turnip pickled with rice bran	Our collection	+	+
L. plantarum 20A-4	Turnip pickled with rice bran	Our collection	+	+
L. plantarum 22A-4	Eggplant pickled with rice bran	Our collection	+	+
L. paraplantarum ATCC 700211 [T]	Beer contaminant	ATCC	+	-
L. paraplantarum 20B-1	Turnip pickled with rice bran	Our collection	+	-
L. paraplantarum 37A-5	Takana pickles	Our collection	+	-
L. paraplantarum 37B-1	Takana pickles	Our collection	+	-
L. paraplantarum NOS120	Kimchi	Our collection	+	-
L. paraplantarum NOS146	Cucumber pickled with rice bran	Our collection	+	-
L. pentosus ATCC 8041 [T]	Silage	ATCC	+	-
L. pentosus 21A-1	Vegetable pickled with rice bran	Our collection	+	-
L. pentosus 21A-2	Vegetable pickled with rice bran	Our collection	+	-
L. pentosus 21A-3	Vegetable pickled with rice bran	Our collection	+	-
L. pentosus 21A-4	Vegetable pickled with rice bran	Our collection	+	-
L. pentosus 21A-5	Vegetable pickled with rice bran	Our collection	+	-
S. gallolitycus ATCC 3611 [T]	Koala feces	ATCC	+	-
S. gallolitycus RO-2	Koala feces	Our collection	+	-
S. gallolitycus RO-3	Koala feces	Our collection	+	-
L. gasseri JCM 1131	Human intestine	JCM [d]	-	-
L. casei JCM 1134	Cheese	JCM	-	-
E. coli DH5α	unknown	Promega	-	-

[a] determined by the qualitative method (20)

[b] determined by the PCR assay described in the present study

[c] ATCC, American Type Culture Collection

[d] JCM, Japan Collection of Microorganisms

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TABLE 2. Purification of tannase from *E. coli* tanLpl-A3-2

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Dissolved $(NH_4)_2SO_4$ precipitate	695.9	5218	7.5	1	100
Q-sepharose column chromatography	65.3	2208	33.81	4.51	42.32
HAP column chromatography	9.46	547	57.82	7.71	10.48
MonoQ column chromatography	2.97	250.5	84.34	11.25	4.8

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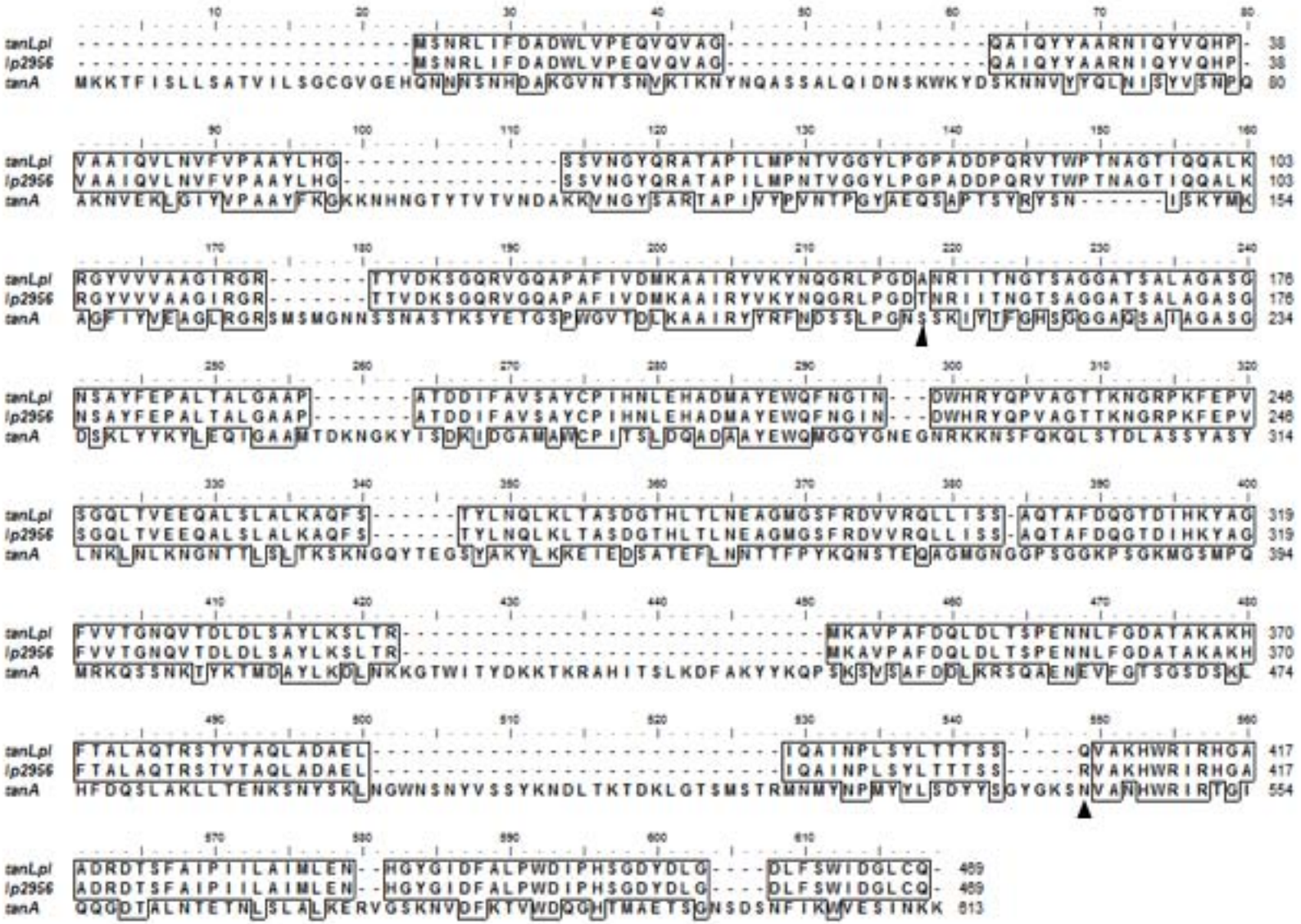
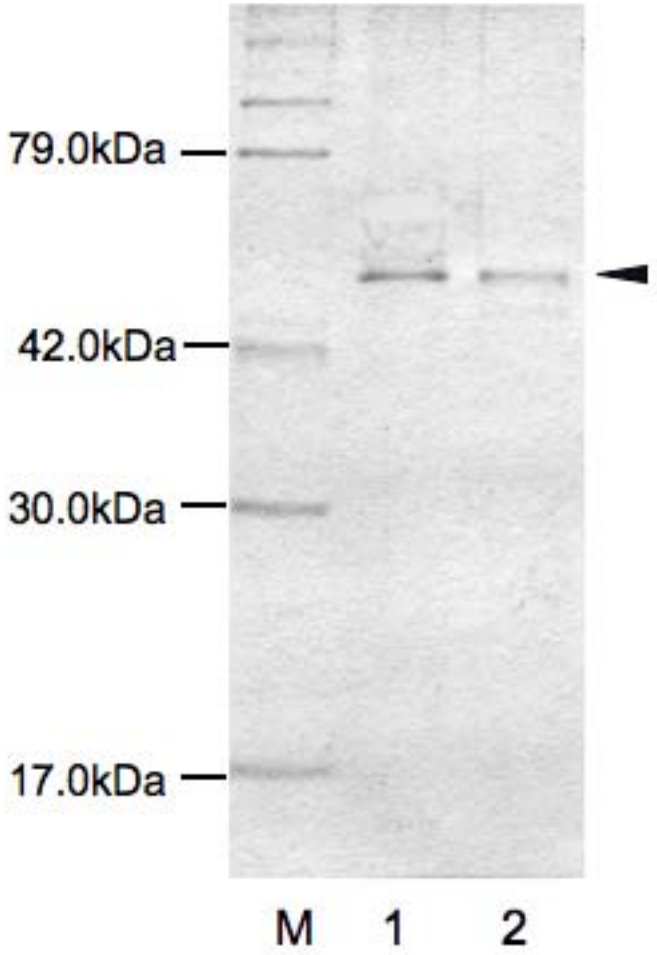
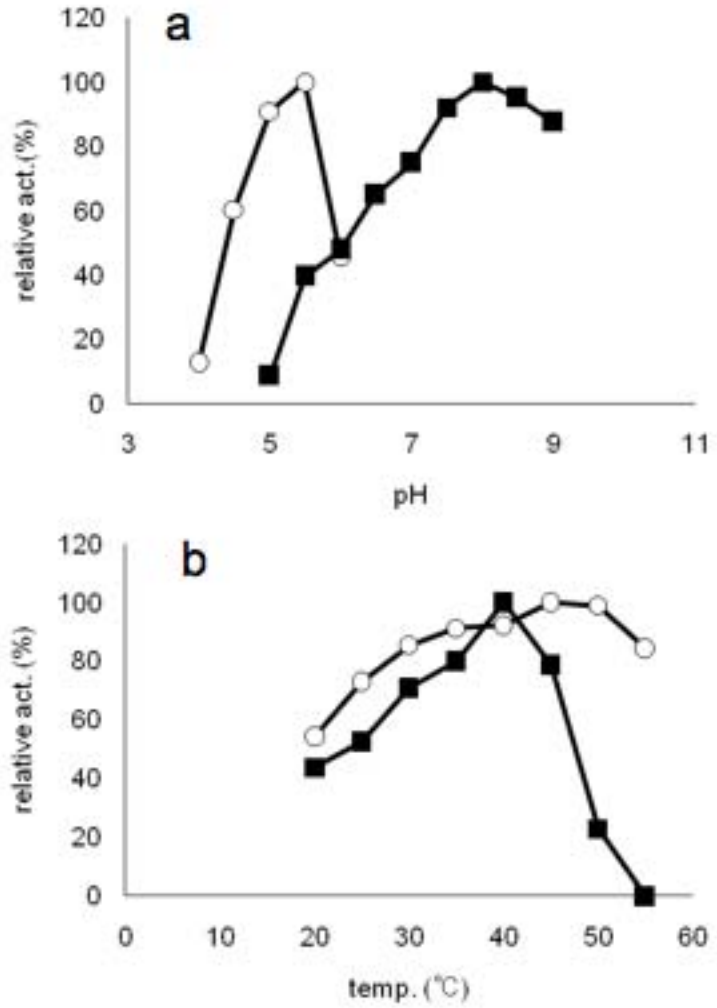


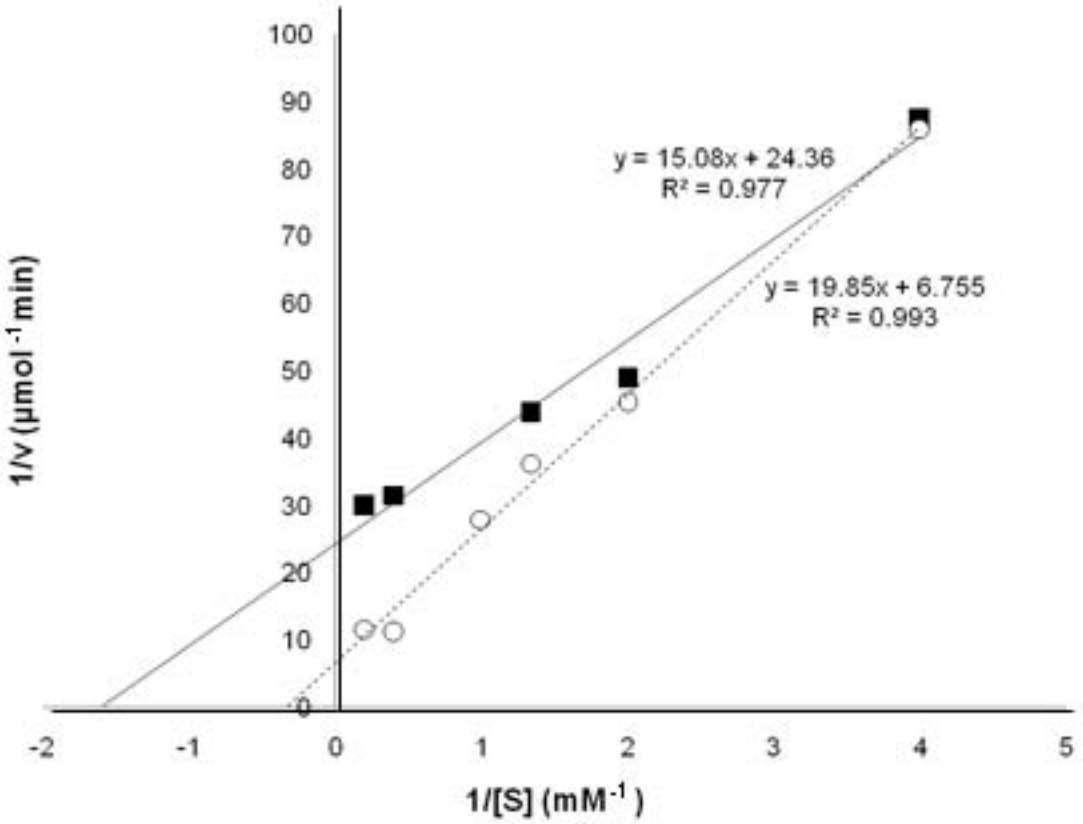
Iwamoto et al. Fig. 2



Iwamoto et al. Fig.3



Iwamoto et al. Fig.4



Iwamot et al. Fig. 5